Investigating IFN-γ, IL-21 and TNF-α as a novel cytokine therapy in glioblastoma

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Thesis Abstract:

Gliomas are the most common primary malignant brain tumours found in humans, in which grade IV glioblastoma accounts for the vast majority of all glioma diagnoses. Current glioblastoma treatments are associated with poor survival and high rates of tumour recurrence, thus alternative therapies urgently need to be developed. Cancer immunotherapies have significant therapeutic potential, as they can alter the immune system to promote anti-tumour immunity. IL-21 is an emerging cytokine that is rapidly gaining popularity as a novel therapeutic agent for various cancer types, though very little is known about the impact of IL-21 in glioblastoma. On the other hand, both IFN-γ and TNF-α have shown significant potential as individual cancer immunotherapies, but very few studies have explored their combined effects in glioblastoma.

Although IFN-γ, IL-21 and TNF-α each demonstrated independent anti-tumour effects on two glioblastoma cell lines (U251 and U373), tumour rejection was potently enhanced when the cytokines were administered together. In particular, the combination of IFN-γ, IL-21 and TNF-α significantly induced cell death and inhibited proliferation in both glioblastoma cell lines. Furthermore, results showed that IFN-γ, IL-21 and TNF-α either induced or enhanced the expression of a range of co-stimulatory immunological ligands on U251 and U373 cells. In addition, IFN-γ, IL-21 and TNF-α impaired tumour-mediated immunosuppression by downregulating Foxp3 expression in CD4+ and CD8+ T cells, even when co-cultured with the glioblastoma secretome. Altogether, these results established that IFN-γ, IL-21 and TNF-α could have significant therapeutic value as a novel cytokine therapy in glioblastoma through direct inhibitory anti-tumour effects and modulation of tumour-associated immunosuppression, thus they have the potential to simultaneously promote tumour rejection and restore immune function in glioblastoma patients, but this requires substantial further investigation.

Key words: IFN-γ, IL-21, TNF-α, glioblastoma, cytokine, immunotherapy, immunosuppression, T cells

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41BB	Tumour necrosis factor receptor superfamily member 9
41BB-L	Tumour necrosis factor ligand superfamily member 9
7-AAD	7-Aminoactinomycin D
ADP	Adenosine diphosphate
AKT	Protein kinase B
AP-1	Activator protein 1
Apaf-1	Apoptotic protease activating factor-1
Ang	Angiopoietin
APC	Antigen-presenting cell
ARF	ADP ribosylation factor
ATP	Adenosine triphosphate
BBB	Blood-brain barrier
Bcl-2	Bcl-2 apoptosis regulator
Bcl-6	B cell lymphoma 6
Bcl-xL	B-cell lymphoma-extra large
Blimp-1	B lymphocyte-induced maturation protein-1
c-Maf	Proto-oncogene c-Maf
c-Myc	MYC proto-oncogene, bHLH transcription factor
CAD	Caspase-activated DNase
cAMP	Cyclic adenosine monophosphate
CAR	Chimeric antigen receptor
CBP	CREB-bindng protein
CCL	Chemokine ligand
CCR	Chemokine receptor
CD	Cluster of differentiation
Cdc42	Cell division control 42 protein homolog
CDK	Cyclic-dependent kinase
CIITA	Class II major histocompatibility complex transactivator
CKI	Cyclic-dependent kinase inhibitor
CNS	Central nervous system
CPC	Cetylpyridinium chloride
CpG	Cytosine-phosphate-guanine
CRTAM	Cytotoxic and regulatory T cell molecule
CRTH2	Prostaglandin D_2 receptor 2
CSF-1R	IL-34 receptor
CTLA-4	Cytotoxic T-lymphocyte associated protein 4
CXCL	CXC chemokine ligand
CXCR	CXC chemokine receptor
DAMP	Danger-associated molecular pattern
DC	Dendritic cell

DED	Death-effector domain
DISC	Death-inducing signalling complex
DNA	Deoxyribonucleic acid
DP	Double positive
DR	Death receptor
ECM	Extracellular matrix
EGFR	Epidermal growth factor
endoG	Endonuclease G
EV	Extracellular vesicle
FADD	Fas-associated death domain
FCS	Foetal calf serum
Foxp3	Forkhead box protein 3
GABRA1	Gamma-aminobutyric acid receptor subunit α-1
GATA3	GATA Binding Protein 3
GBM	Glioblastoma multiforme
GLUT1	Glucose transporter 1
HA	Hyaluronic acid
HEB/E2A	Transcription factor 12/Transcription factor 3
HIF-1α	Hypoxia-inducible factor-1α
HIV	Human immunodeficiency virus
HK	Hexokinase 2
HRE	Hypoxia-responsive elements
HVEM	Tumour necrosis factor receptor superfamily member 14
ICAD	Inhibitor of caspase-activated DNase
ICAM-1	Intercellular adhesion molecule 1
ICER	Inducible cAMP early repressor
ICOS	Inducible T cell costimulator
ICOS-L	Inducible T cell costimulator ligand
Id2	Inhibitor of DNA binding 2
IDH	Isocitrate dehydrogenase
IDO	Indoleamine 2,3-dioxygenase
IFN-γ	Interferon gamma
IFNGR	IFN-γ receptor
IFNGR1	IFN-γ receptor 1
IFNGR2	IFN-γ receptor 2
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IL	Interleukin
IL-13Rα2	Interleukin-13 receptor subunit alpha-2
ILT	Immunoglobulin-like transcript
IRF	Interferon regulatory factor

iTreg	Inducible Treg
JAK	Janus kinase
KLF2	Krüppel-like factor 2
LAG-3	Lymphocyte-activation gene 3
LAP	Latency-associated peptide
LDHA	Lactate dehydrogenase A
LIGHT	Tumour necrosis factor ligand superfamily member 14
mAb	Monoclonal antibody
MAP	Mitogen-activated protein
MAZR	POZ/BTB and AT-Hook containing zinc finger 1
MDM2	Mouse double minute 2
MDSC	Myeloid-derived suppressor cell
MGMT	O6-methylguanine DNA methyltransferase
MHC	Major histocompatibility complex
MICA/B	MHC class I chain-related proteins A or B
MMPs	Matrix metalloproteinases
MRI	Magnetic resonance imaging
mTOR	Mechanistic target of rapamycin
NADP+	Nicotinamide adenine dinucleotide phosphate
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NEFL	Neurofilament light polypeptide
NF-κB	Nuclear factor kappa B
NF1	Neurofibrin 1
NFAT	Nuclear factor of activated T cells
NHSBT	National health service blood and transplant
NK	Natural killer
NKG2D	Natural killer group 2D
Nrp1	Neuropilin-1
nTreg	Natural Treg
OX40	Tumour necrosis factor receptor superfamily member 4
OX40L	Tumour necrosis factor ligand superfamily member 4
p53	Tumour protein 53
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PD-1	Progammed cell death protein 1
PDK1	Pyruvate dehydrogenase kinase 1
PD-L1	Programmed death-ligand 1
PDGFR	Platelet derived growth factor
PFA	Paraformaldehyde
PHD	Prolyl-hydroxylase

PI3K	Phosphoinositide 3-kinase
PI	Propidium iodide
PIP_2	Phosphatidylinositol-4,5-biphosphate
PIP_3	Phosphatidylinositol-3,4,5-triphosphate
PMA	Phorbol myristate acetate
PRR	Pattern recognition receptor
PS	Phosphatidylserine
PTEN	Phosphatase and tensin homolog
R132	Arginine 132
Rac1	Ras-related C3 botulinum toxin
RB	Retinoblastoma protein 1
ROG	Repressor of GATA3
RoRγT	Retinoic acid-related orphan receptor gamma t
Runx3	RUNX family transcription factor 3
S1PR1	Spingosine-1-phosphate receptor 1
SAP	Signalling lymphocyte activation molecule-associated protein
SLAM	Signalling lymphocyte activation molecule
SLC12A5	Potassium-chloride transporter member 5
SOCS1	Suppressor of cytokine signalling 1
SP	Single positive
ST2	IL-33 receptor
STAT	Signal transducer and activator of transcription
SYT1	Synaptotagmin-1
T-bet	T-box transcription factor
TAM	Tumour-associated monocyte/macrophage
Tc	Cytotoxic T cell
TCA	Tricarboxylic acid
TCF-1	T cell factor 1
T_{CM}	Central memory T cells
TCR	T cell receptor
T_{EM}	Effector memory T cells
T_{EMRA}	Terminally differentiated effector memory cells
T_{EX}	Exhausted T cells
Tfc	T follicular cytotoxic cell
Tfh	T follicular helper
Tfr	T follicular regulatory cell
TGF-β	Transforming growth factor beta
Th	T helper
ThPOK	T-helper-inducing POZ/Krüppel-like factor
TIM-3	T-cell immunologlobulin and mucin-domain containing 3
TLR	Toll-like receptor

TME	Tumour microenvironment
TNF-α	Tumour necrosis factor alpha
TNFR1	TNF-α receptor 1
TNFR2	TNF-α receptor 2
T_{PEX}	Precursor exhausted T cells
TRA	Tissue-restricted antigen
TRAIL	Tumour necrosis factor-related apoptosis-inducing ligand
Treg	Regulatory T cell
VCAM-1	Vascular cell adhesion molecule 1
VEGF	Vascular endothelial growth factor
VEGFR	VEGF receptor
VHL	Von Hippel-Lindau
WHO	World Health Organisation

1. Introduction

1.1. Cancer

1.1.1. Gliomas

According to Public Health England and the Office for National Statistics (2019), there were over 305,000 new cancer diagnoses in 2017, of which 1.5% were cancers that affect the brain. Whilst there are a range of different brain tumours, gliomas are the most common. These tumours originate from glial cells, which can be sub-divided into three cell types: microglia, astrocytes and oligodendrocytes (Von Bartheld et al., 2016). In brief, oligodendrocytes are responsible for the formation of the myelin sheath around axons to support neuronal signalling, whilst microglia are largely viewed as CNS-resident macrophages, due to the fact that they have demonstrated phagocytotic abilities and have also been shown to express toll-like receptors (TLRs), which recognise pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) (R. Fu et al., 2014; Kigerl et al., 2014; Simons & Nave, 2016). On the other hand, astrocytes have been implicated in a wide range of vital functions within the brain, including: neuronal support, regulation of synaptic transmission, modulation of cerebral blood flow, and maintenance of the blood-brain barrier (Abbott et al., 2006; Gordon et al., 2007; Halassa et al., 2007; Khakh & Sofroniew, 2015). Altogether, these studies highlight the significant role glial cells play in the maintenance of the healthy CNS and provide some insight into the profound neurological dysfunction seen in individuals affected by gliomas.

The most frequently diagnosed gliomas are astrocytomas, which are derived from astrocytes and graded by the World Health Organisation (WHO) from I (lowest grade) to IV (highest grade), based upon how quickly the tumour cells divide and how localised (focal/diffuse) they remain (Louis et al., 2007). Recent advances in glioma research led to alterations of the classification system by the WHO in 2016 and 2021, as shown in Table 1.1 below. The updated classification system reflects improvements in the understanding of molecular markers, which have been shown to correlate better with tumour biology and behaviour than the previous histology-based system (Louis et al., 2016, 2021).

Grade	2007 WHO	2016 WHO	2021 WHO
I	Pilocytic astrocytoma	Pilocytic astrocytoma	Pilocytic astrocytoma
II	Diffuse astrocytoma	Diffuse astrocytoma, IDH-mutant	Astrocytoma, IDH-mutant
			Diffuse astrocytoma, IDH-wildtype
III	Anaplastic astrocytoma	Anaplastic astrocytoma, IDH-mutant	Astrocytoma, IDH-mutant
			Diffuse astrocytoma, IDH-wildtype
IV	Glioblastoma	Glioblastoma, IDH-mutant	Astrocytoma, IDH-mutant
		Glioblastoma, IDH-wildtype	Glioblastoma, IDH-wildtype

Table 1.1. WHO Glioma Classification

1.1.2. Glioblastoma Subtypes

Over 80% of all glioma diagnoses are glioblastoma (also known as glioblastoma multiforme – GBM), which have an extremely poor survival rate, with patients surviving for an average of 10 to 14 months post-diagnosis. This is largely attributed to late diagnoses, poor treatments and frequent tumour recurrence (Wilson et al., 2014). Glioblastoma can be defined as a primary or secondary tumour, in which the former has no known precursor, whilst the latter develops from a lower grade tumour (generally, either grade II or III astrocytomas). Unfortunately, the vast majority (approximately 90%) of patients are diagnosed with primary glioblastoma, which has a significantly poorer prognosis than those with secondary glioblastoma (Ohgaki & Kleihues, 2013).

Based on the genetic analysis of glioblastoma patient samples, Verhaak et al. (2010) proposed that there were four glioblastoma subtypes: classical, proneural, mesenchymal, and neural, as shown in Table 1.2 below. In brief, classical glioblastoma had mutations in phosphatase and tensin homolog (PTEN) with overexpression of epidermal growth factor receptor (EGFR), but no mutations in tumour protein 53 (TP53 or p53). The proneural subtype had mutations in p53, platelet-derived growth factor receptor A (PDGFRA) and isocitrate dehydrogenase (IDH) 1 (IDH1) – this subtype is generally associated with younger patients that have secondary glioblastoma, thus proneural glioblastoma patients often have a better prognosis (Phillips et al., 2006). Mesenchymal glioblastoma was predominantly found in older patients with lower EGFR expression than other subtypes, alongside mutations in neurofibromin 1 (NF1), p53 and PTEN. Research has shown that roughly 30% of proneural glioblastoma transition to mesenchymal glioblastoma upon recurrence, which leaves these patients with a significantly worse prognosis, as the mesenchymal subtype responds poorly to radiotherapy (Bhat et al., 2013; Phillips et al., 2006).

Gene	Proneural	Neural	Classical	Mesenchymal
TP53	54%	21%	0%	32%
PTEN	16%	21%	23%	32%
EGFR	16%	26%	32%	5%
PDGFRA	11%	0%	0%	0%
IDH1	30%	5%	0%	0%
NF1	5%	16%	5%	37%

Table 1.2. Distribution of Gene Mutations in Glioblastoma Subtypes

Finally, neural glioblastoma was defined by the expression of a number of neuronal markers including: neurofilament light polypeptide (NEFL), gamma-aminobutyric acid receptor subunit α-1 (GABRA1), synaptotagmin-1 (SYT1) and potassium-chloride transporter member 5 (SLC12A5) (Verhaak et al., 2010). However, the legitimacy of the neural subtype has been questioned in recent years, due to the lack of characteristic genetic abnormalities seen in other glioblastoma subtypes. Thus, several studies have suggested that samples which were previously considered to belong to the neural

subtype had actually been contaminated by healthy brain tissue at the tumour margin (Behnan et al., 2017; Gill et al., 2014; Sturm et al., 2012). Subsequent analyses have since concluded that there are only three glioblastoma subtypes: classical, mesenchymal and proneural, which have a median survival of 14.7, 11.5, and 17 months, respectively (Brennan et al., 2009; Shen et al., 2012; Wang et al., 2017).

1.1.3. Glioblastoma Signalling Mutations

Prior to the identification of these glioblastoma subtypes, the Cancer Genome Atlas Research Network identified a number of genetic aberrations when they analysed 206 glioblastoma patient samples (McLendon et al., 2008). Aside from those mentioned previously, this also included mutations in the retinoblastoma protein 1 (RB1)/E2F, ADP ribosylation factor (ARF)/mouse double minute 2 (MDM2)/p53, and phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT)/mechanistic target of rapamycin (mTOR) pathways, which were also found in other genetic analyses completed by Parsons et al. (2008) and Brennan et al. (2013), as summarised in Table 1.3 below.

Gene	Full Name	Function	% Patients Affected
CDKN2A	Cyclin-dependent kinase inhibitor 2A	Cell signalling – proliferation, invasion and survival	50-55
EGFR	Epidermal growth factor receptor	Cell signalling – proliferation, invasion and survival	32-45
IDH1	Isocitrate dehydrogenase 1	Metabolism	5-11
MDM2	Mouse double minute 2	Cell signalling – proliferation, invasion and survival	11-14
PIK3CA	Phosphoinositide-3-kinase catalytic α polypeptide	Cell signalling – proliferation, invasion and survival	7-10
PIK3R1	Phosphoinositide-3-kinase regulatory subunit 1 α	Cell signalling – proliferation, invasion and survival	8-11
PTEN	Phosphatase and tensin homolog	Cell signalling – proliferation, invasion and survival	30-36
RB1	Retinoblastoma 1	Cell cycle	9-12
TP53	Tumour protein 53	Cell signalling – proliferation, invasion and survival	34-40

Table 1.3. Frequent Gene Alterations Found in Glioblastoma Patients

RB1 plays a crucial role in regulating the cell cycle, acting as a G1 checkpoint by binding to E2F, preventing downstream transcription of genes involved in proliferation. In order to facilitate the G1/S transition, RB1 is phosphorylated by CDK-cyclin complexes, enabling E2F release and the activation of genes required for the S-phase of the cell cycle (Furnari et al., 2007). This pathway was found to be dysregulated in almost 80% of glioblastoma patient samples, with more than half of the patients expressing altered CDK-cyclin complexes and around 10% having RB1 gene deletion/mutation,

allowing glioblastoma cells to rapidly proliferate by evading this vital cell cycle checkpoint (McLendon et al., 2008).

Within the ARF/MDM2/p53 pathway, p53 regulates the expression of over 3,500 genes – hence, it is often referred to as the 'guardian of the genome' (Lane, 1992; Li et al., 2012). In particular, p53 acts as a tumour suppressor by promoting DNA repair, cell cycle arrest, apoptosis and senescence, in response to cellular stress and/or DNA damage (Riley et al., 2008). This signalling pathway is tightly regulated through a negative feedback loop, in which p53 controls MDM2 expression and MDM2 inactivates p53 by either degrading or directly binding to p53, preventing downstream gene expression (Honda et al., 1997; Oliner et al., 1993). MDM2 activity is also controlled by ARF (encoded by CDKN2A) which binds to MDM2, thereby preventing p53 degradation (Weber et al., 1999). Nevertheless, the genetic analyses revealed that the ARF/MDM2/p53 pathway was altered in 85% of the glioblastoma patients studied, featuring CDK2NA deletion, p53 mutation, as well as MDM2 amplification (McLendon et al., 2008). Collectively, these genetic aberrations inactivate p53, compromising a vital tumour suppression mechanism, leading to increased glioblastoma tumourigenesis and invasion (Djuzenova et al., 2015; Y. Zhang, Dube, et al., 2018).

The PI3K/AKT/mTOR pathway begins with PI3K, a complex consisting of a catalytic protein (P110α, encoded by PIK3CA) and a regulatory protein (P85α, encoded by PIK3R1). Once activated, PI3K phosphorylates phosphatidylinositol-4,5-biphosphate (PIP_2) into phosphatidylinositol-3,4,5-triphosphate (PIP_3) which activates AKT, initiating a variety of downstream signals, including mTOR activation (P. Liu et al., 2009). In healthy cells, PTEN regulates this pathway by converting PIP_3 into PIP_2 to inhibit AKT activation and signalling (Cantley & Neel, 1999). The PI3K/AKT/mTOR pathway was found to be constitutively active in over 70% of glioblastoma patients, due to upregulation of growth factors that activate PI3K (e.g. EGFR and PDGFR), mutations in PI3K itself, and/or deletion/mutation of PTEN (McLendon et al., 2008; Mizoguchi et al., 2004). Each of these have been shown to support glioblastoma pathogenesis by promoting angiogenesis, invasion, survival and proliferation (Chakravarti et al., 2004; Karar & Maity, 2011).

1.1.4. Glioblastoma IDH1 Mutation

In the updated WHO classification system, glioblastoma was classified into two groups based upon their IDH mutation status, as shown in Table 1.1 (Louis et al., 2007, 2016, 2021). There are three IDH isoforms in humans, known as IDH1, IDH2 and IDH3. IDH1 is an enzyme involved in glucose metabolism during the Krebs cycle, where it catalyses the oxidative decarboxylation of isocitrate to produce α-ketoglutarate, resulting in the reduction of nicotinamide adenine dinucleotide phosphate (NADP+) to reduced nicotinamide adenine dinucleotide phosphate (NADPH) (Bleeker et al., 2010). All IDH1 mutations in glioblastoma patients involve arginine 132 (R132), which is an amino acid residue located in the IDH active site (Parsons et al., 2008; X. Xu et al., 2004). When R132 becomes mutated, isocitrate can no longer bind to the active site, leading to reduced production of α-ketoglutarate and

NADPH (Balss et al., 2008). In addition, mutated IDH1 can convert α-ketoglutarate into 2-hydroxyglutarate, oxidising NADPH back to NADP+ in the process (L. Dang et al., 2009). 2-hydroxyglutarate exists in two distinct forms: L-2-hydroxyglutarate and D-2-hydroxyglutarate, with the latter being considered an oncometabolite. Research has associated high levels of accumulated D-2-hydroxyglutarate with upregulation of vascular endothelial growth factor (VEGF) and hypoxia-inducible factor-1α (HIF-1α) (Sasaki et al., 2012; S. Zhao et al., 2009). Altogether, these factors promote the formation of the tumour microenvironment and encourage tumour cell invasion, leading to glioblastoma development (J. Huang et al., 2019).

The IDH1 mutation was first discovered in glioblastoma by Parsons et al. (2008), who conducted gene expression analyses in 22 glioblastoma patient samples, identifying that 12% had IDH1 mutations. Interestingly, when they explored the data further, they made three important observations. First, they found that IDH1 mutations occurred in younger glioblastoma patients, with a mean age of 33 vs 53 years old for those with wild-type IDH1. Second, they discovered that almost all of the IDH1 mutations were found in patients with secondary glioblastoma and, finally, those with IDH1 mutations had a significantly better prognosis, with a median overall survival of 3.8 years, compared to 1.1 years for IDH1 wild-type patients. It has since been shown that 90% of all glioblastoma diagnoses are primary IDH1 wild-type tumours, with the remaining 10% being IDH1-mutant secondary glioblastoma (Yan et al., 2009). In addition, research has identified that the loss of NADPH reduces the ability to neutralise oxygen radicals, thereby increasing tumour sensitivity to both radiotherapy and chemotherapy, which could explain the increase in overall survival for patients with IDH1-mutant glioblastoma (Baldewpersad Tewarie et al., 2013).

As wild-type IDH1 has a crucial role in the Krebs cycle, this IDH1 mutation has a profound impact on cellular metabolism, driving glioblastoma cells towards glutamine or glutamate as energy sources. In order to explore this, studies have focused on the impact of inhibiting glutaminase, an enzyme responsible for converting glutamine into glutamate. Ohka et al. (2014) demonstrated that proliferation was reduced in IDH1-mutated glioblastoma cells when glutaminase was inhibited, but this could be restored by administering α-ketoglutarate. Further analyses highlighted that 2-hydroxyglutarate levels were significantly elevated in IDH1-mutated cells, whilst glutamine and glutamate levels were considerably reduced, compared to IDH1 wild-type. Collectively, this implied that there was a dependency on glutamine and glutamate to generate α-ketoglutarate via glutaminolysis, in order to promote glioblastoma proliferation (Seltzer et al., 2010; Wise & Thompson, 2010).

This dependency on glutamine and glutamate in IDH-mutant glioblastoma is particularly interesting as glutamate is found in high concentrations in the brain, specifically in synaptic clefts, due to its role as a neurotransmitter (Bak et al., 2006; Daikhin & Yudkoff, 2000). It is also present in the white matter of the brain: an area that glioblastoma cells frequently utilise for tumour invasion (Ziskin et al., 2007). The relationship between IDH1 mutation status and invasiveness was explored by Baldock et al. (2014) using mathematical modelling of magnetic resonance imaging (MRI) data from 158

glioblastoma patients. Although IDH1 mutant and wild-type glioblastoma had similar proliferation rates, IDH1-mutated glioblastoma was significantly more invasive, as shown in Figure 1.1. In response to this, van Lith et al. (2014) proposed that glioblastoma cells use glutamate from the tumour microenvironment as a chemotactic signal for invasion, but this has yet to be proven.

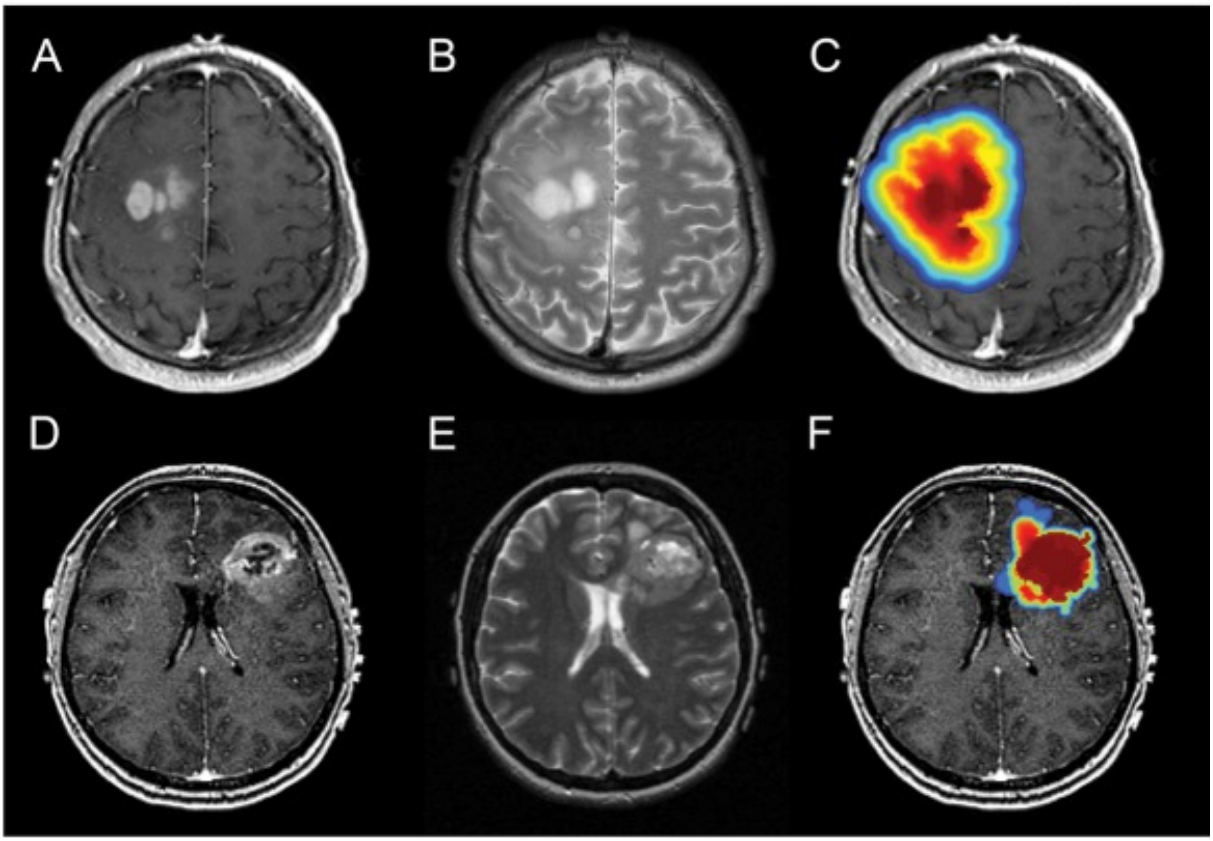


Figure 1.1. Glioblastoma Invasion
MRI scans of two glioblastoma patients: IDH1-mutant (top row) and IDH1 wild-type (bottom row). All 6 images were taken pre-treatment: C and F are false-colour images showing tumour cell density (highest cell density in red; lowest cell density in blue) illustrating the invasive nature of IDH-mutant glioblastoma (Baldock et al., 2014).

1.1.5. Glioblastoma Migration

Research has shown that only 0.4-2% of glioblastoma metastasise beyond the brain, with the vast majority of tumour cells preferring to invade the surrounding tissues (Beauchesne, 2011; Hamilton et al., 2014; Lun et al., 2011). In fact, up to 20% of glioblastoma patients have several lesions, as a result of invasion across brain lobes and hemispheres, thereby increasing both surgical complexity and patient mortality (Shieh et al., 2020; R. P. Thomas et al., 2013). Despite advances in surgical techniques to provide clearer distinctions between tumour cells and healthy brain tissue (e.g. fluorescent-guided resection), it is still incredibly difficult to complete a gross total tumour resection without compromising patient neurological function (Gulati et al., 2011; Perry et al., 2012; Rahman et al., 2017; Stummer et al., 2006). Unsurprisingly, sub-total surgical resections substantially reduce survival rates compared to gross total surgical resections, likely due to the increased risk of glioblastoma recurrence (Brown et al.,

2016). Altogether, this highlights the importance of developing treatments that prevent glioblastoma migration and invasion.

Whilst the complex mechanisms of glioblastoma migration are yet to be fully understood, they are believed to be centred around the extracellular matrix (ECM): a three-dimensional network that provides structural and biochemical support to cells in the extracellular space, in order to regulate their behaviour and maintain homeostasis. However. it has proven difficult to study the ECM in the past, due to a lack of technology that could accurately replicate the complexity of the ECM and the surrounding cellular environment (Abdeen et al., 2016). Nevertheless, studies have shown that tumour cells interact with components of the ECM in order to promote cell proliferation, angiogenesis, survival, motility, and invasion. This occurs when the ECM binds to receptors expressed on the cancer cell surface, activating signalling pathways that modulate cell behaviour (Lukashev & Werb, 1998). In the brain, the ECM predominantly consists of glycosaminoglycans, proteoglycans (e.g. lecticans) and glycoproteins (e.g. tenascin), but some fibrous proteins (e.g. collagen IV) are also present. In particular, the glycosaminoglycan hyaluronic acid (HA) is abundantly expressed, acting as a central backbone and binding site for other ECM components in the brain ECM (Ruoslahti, 1996). For example, perineuronal nets are ECM complexes that surround neurons to assist with synaptic stabilisation, which consist of lecticans (e.g. brevican and aggrecan) bound to HA, alongside tenascin-R (Reichelt et al., 2019).

Although research is limited, it has been suggested that HA phosphorylates a cell-surface receptor (CD44) upon binding, leading to interactions between the CD44 intracellular domain and cytoskeletal proteins (e.g. ankyrin), as well as activation of downstream signalling pathways, which promote both cell adhesion and migration (Aruffo et al., 1990; Ponta et al., 2003; Zöller, 2011). Examples of these downstream targets include Rac1 and Cdc42, which have been implicated in the development of lamellipodia and filopodia to drive breast cancer invasion (Bourguignon et al., 2000; Coso et al., 1995; Minden et al., 1995; Nobes & Hall, 1995; Ridley et al., 1992). As HA is highly expressed in the brain ECM, researchers questioned whether glioblastoma cells used this HA-CD44 mechanism to facilitate migration and invasion. In support of this, early research identified that glioblastoma cells secreted almost three times more HA than normal brain tissues via HA-synthases (Delpech et al., 1993). Additionally, proteomic analysis revealed that Rac1 was highly expressed in glioblastoma, which directly correlated with poor patient survival (Salhia et al., 2008).

Interestingly, Koochekpour et al. (1995) found that pre-coating cell culture plates with HA increased glioblastoma cell line (IPRM-5) adherence *in vitro*, but this effect was lost when cells were cultured with a CD44 monoclonal antibody (mAb). In line with this, Knupfer et al. (1999) identified high CD44 expression in a number of glioblastoma cell lines (T98G, A172 and 86HG39), which correlated with their adhesion to HA. Research has also shown that glioblastoma cells alter the brain ECM to generate a more dense and rigid ECM structure, in order to enhance cell adhesion and migration, as shown in Figure 1.2 (Mahesparan et al., 2003; Nakada et al., 2007; Paszek et al., 2005; Ulrich et al., 2009). This is reflected in studies which have shown that CD44-expressing glioblastoma cells can

secrete matrix metalloproteinases (MMPs), in order to degrade the ECM and increase HA production, thereby facilitating tumour infiltration into healthy brain tissues (Bellail et al., 2004; Sameshima et al., 2000; Toole & Slomiany, 2008). Subsequent studies have identified a correlation between increasing CD44 expression and astrocytoma grading, in which glioblastoma patients had the highest levels of expression (Ranuncolo et al., 2002; Wei et al., 2010). In particular, research has identified that mesenchymal glioblastoma has the highest levels of CD44 expression, compared to other glioblastoma subtypes, which correlates with their more invasive phenotype (Pietras et al., 2014). Collectively, these studies highlight a critical need for future glioblastoma treatments to reduce CD44 expression and restrict invasion into surrounding tissues, as this could enable surgeons to remove more of the tumour, thereby decreasing the risk of recurrence and improving patient survival rates.

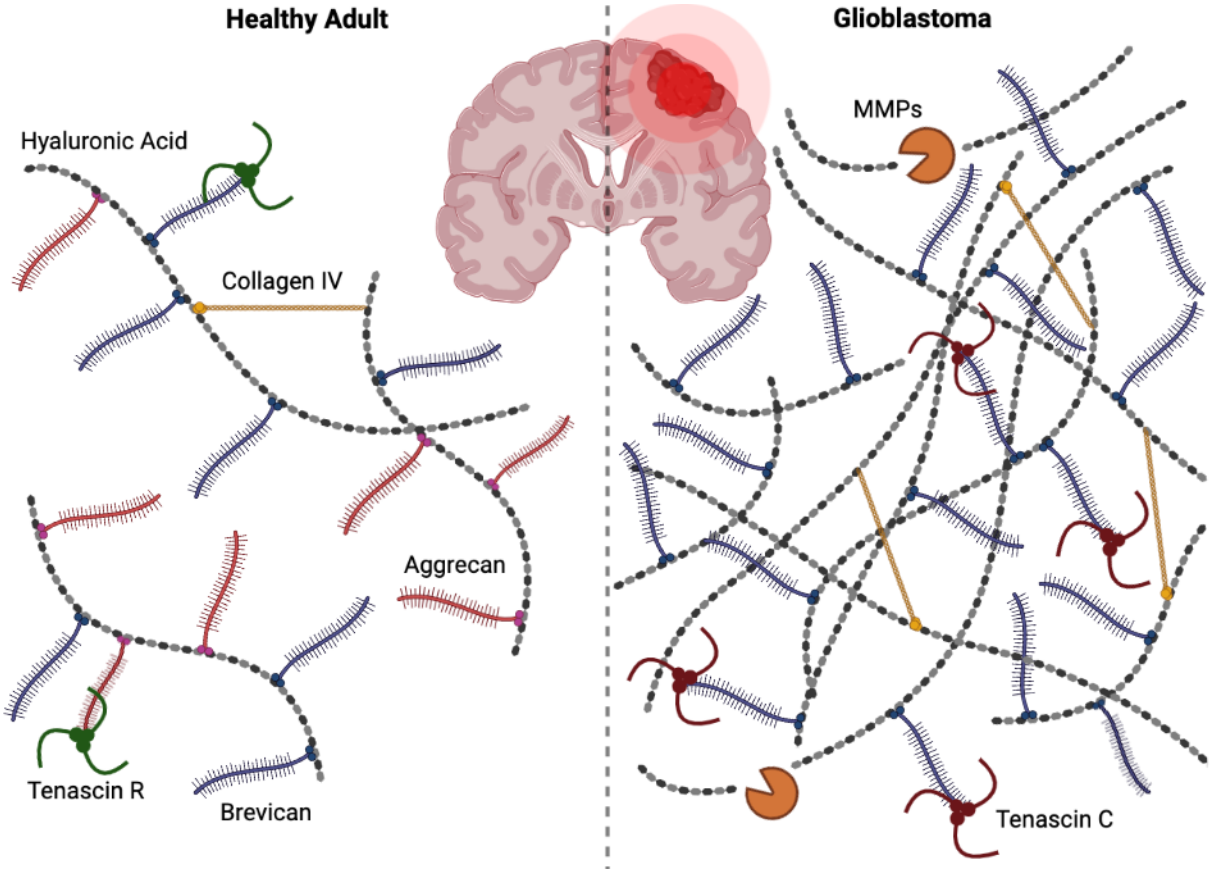


Figure 1.2. Tumourigenic Extracellular Matrix
In the healthy adult brain (left), the ECM consists of proteoglycans (e.g. aggrecan and brevican), tenascin R and collagen IV attached to a HA backbone. Glioblastoma cells (right) alter the ECM to lose aggrecan and tenascin R expression, whilst producing large amounts of HA and MMPs to increase the density and stiffness of the surrounding ECM, thereby enhancing tumour migration. Figure generated using BioRender.com.

1.1.6. Glioblastoma Treatments

Glioblastoma is notoriously difficult to treat, as the tumour cells are largely resistant to current therapeutics. The gold-standard treatment for glioblastoma is surgical resection, followed by a course of whole brain irradiation and/or chemotherapy. This treatment schedule was designed by Stupp et al.

(2005) during a clinical trial that investigated the post-surgical benefits of administering radiotherapy in combination with chemotherapy in patients with newly diagnosed glioblastoma, compared to radiotherapy alone. Results showed that the median (and overall survival) rate two years after treatment was 12.1 months (10.4%) when glioblastoma patients were prescribed radiotherapy alone, which increased up to 14.6 months (26.5%) when combined with Temozolomide. Follow up analyses revealed that the overall survival five years post-treatment was 1.9% with radiotherapy alone, compared to 9.8% of glioblastoma patients treated with both Temozolomide and radiotherapy (Stupp et al., 2009). Whilst this multifaceted approach did improve the survival rate of glioblastoma patients, new treatments need to be developed to improve patient survival even further.

Temozolomide is the most frequent chemotherapeutic agent prescribed to glioblastoma patients, as it is able to initiate glioblastoma cell death by affecting DNA-repair mechanisms, including DNA methylation at the N7 and O6 positions of guanine residues, in addition to the N3 position of adenine residues. Altogether, this disrupts the mismatch repair system that is designed to repair DNA damage, as it cannot integrate the complementary base pairs to the affected residues. As the DNA damage is irreparable, lesions form along the DNA strand, inhibiting DNA replication and trigging apoptosis of the glioblastoma cells (Denny et al., 1994; Newlands et al., 1997). But, retrospective analysis of the Stupp clinical trial revealed that glioblastoma cells can be resistant to Temozolomide if they express the O6-methylguanine DNA methyltransferase (MGMT) gene, which encodes a DNA repair enzyme that demethylates the affected residues – this restricts the downstream effects of Temozolomide thereby preventing glioblastoma apoptosis (Hegi et al., 2005; Stupp et al., 2005, 2009). As a result of this, the efficacy of Temozolomide is defined by the methylation status of the CpG islands of the MGMT promoter region. If the CpG islands are methylated, MGMT gene expression is prevented and Temozolomide can function effectively to induce apoptosis in glioblastoma cells. The epigenetic silencing of MGMT has been found in approximately half of all glioblastoma patients and research has shown that preventing MGMT expression increases the effectiveness of Temozolomide (Esteller & Herman, 2004; Rivera et al., 2010). Conversely, glioblastoma patients with unmethylated CpG islands are considerably less responsive to Temozolomide, thus they have a significantly poorer prognosis (Hegi et al., 2005; Stupp et al., 2009).

An alternative treatment for glioblastoma patients is Bevacizumab, a mAb that restricts angiogenesis: the development of new blood vessels from pre-existing vessels – this is particularly crucial for glioblastoma survival as it facilitates tumour growth (Ferrara et al., 2004). Bevacizumab is able to inhibit angiogenesis by binding to circulating VEGF-A, preventing interaction between VEGF-A and VEGF receptors (VEGFRs), which is a key rate-limiting step in blood vessel growth (Gerber & Ferrara, 2005; Zondor & Medina, 2004). As glioblastoma is one of the most angiogenic tumours found in humans, there were high expectations for Bevacizumab in treating glioblastoma (Brem et al., 1972; Plate et al., 1992). However, a recent review of 11 clinical trials, which all used anti-angiogenic therapies in glioblastoma patients, showed that Bevacizumab alone did not improve overall

survival in patients with either newly diagnosed or recurrent glioblastoma, but it did slow tumour growth, providing limited progression-free survival (Ameratunga et al., 2018). Similar results were found when Bevacizumab was administered in combination with Temozolomide; thus, the review concluded that there was insufficient evidence to support the use of Bevacizumab in glioblastoma patients, leading researchers to pursue other areas for future glioblastoma therapeutics.

1.2. Glioblastoma Immunology

1.2.1. CNS Immunoprivilege

One of the most exciting areas in glioblastoma research is the development of immunotherapies, which utilise the immune system to specifically target tumour cells. In particular, active immunotherapy involves boosting the patients' own immune system to detect tumour-specific antigens, in order to provide both short-term and long-term anti-tumour immunity through the development of immunological memory – for example, checkpoint inhibitors and vaccines. Conversely, passive immunotherapy employs external influences that are chronically administered to the patient in order to generate an anti-tumour immune response, including: mAbs, cytokine therapies, and chimeric antigen receptor (CAR) T cells (Farkona et al., 2016).

Due to the long-standing belief that the brain was an immunoprivileged site, there is a significant lack of understanding of immune reactions that occur in the CNS. This immunoprivilege theory was first proposed by Shirai (1921) when a rat sarcoma grew after transplantation into the brain of a mouse, but it did not grow when implanted subcutaneously or intramuscularly. Subsequent research by Medawar (1948) showed that rabbit skin was rejected at a much lower rate when grafted into the brain, compared to a similar graft on the chest. Both of these studies suggested that their results were due to a lack of an immune response in the brain. The notion of CNS immune privilege was further supported by the discovery of the blood-brain barrier (BBB) and subsequent research detailing how it provides the brain with physical and biochemical protection from toxins (Cardoso et al., 2010).

Altogether, researchers believed that the BBB would prevent T cells from entering and encountering antigens in the brain, whilst the absence of a lymphatic draining system would prevent T cells in the cervical lymph nodes from being exposed to antigens from the brain, plus it was suggested that antigen presentation couldn't occur in the brain, due to a lack of antigen-presenting cells (APCs) (M. J. Carson et al., 2006). However, research exploring various CNS disorders have challenged this theory of immune privilege. For example, Minagar and Alexander (2003) discovered that T cells and monocytes were able to cross the BBB to cause inflammation in patients with multiple sclerosis. In addition, Louveau et al. (2015) crucially identified a lymphatic drainage system between the CNS and deep cervical lymph nodes in the neck. Furthermore, numerous studies have determined that the BBB is disrupted in glioblastoma patients, due to their highly vascularised nature (Long, 1970; Schaffenrath et al., 2021; Van Tellingen et al., 2015). Collectively, these findings led to a change in perception, providing researchers with the exciting opportunity to develop brain tumour immunotherapies.

1.2.2. Tumour and Anti-Tumour Immunity

Typically, the immune system does not react to 'self' antigens – however, these antigens can be mutated by cancer cells, creating novel tumour-specific antigens that can be recognised by T cells as 'non-self', leading to an anti-tumour immune response that is often a combination of the innate and adaptive immune systems (Boon et al., 1994). In the adaptive system, cytotoxic T cells are activated and primed via the recognition of these tumour-specific antigens, which are presented alongside a costimulatory signal by the APC to the T cell (Inaba et al., 1987; van der Merwe et al., 1997). These T cells can then rapidly proliferate and selectively target cells expressing that tumour-specific antigen, inducing cell death via perforin-granzyme and/or Fas-FasL signalling pathways, alongside NK (natural killer) cells from the innate system (Diefenbach et al., 2003; Trapani & Smyth, 2002; Waring & Müllbacher, 1999).

CAR T cells are T cells that have been isolated from cancer patients and genetically altered to specifically target tumour-specific antigens before they are re-infused into patients to initiate an anti-tumour immune response. For example, CD19 is highly expressed in various haematological malignancies, thus CAR T cells that have been designed to selectively target CD19 have achieved notable success (Davila & Brentjens, 2016). However, this appears to be less straightforward in glioblastoma, due to intratumoural heterogeneity and low mutational burden, which makes it challenging to identify suitable tumour-specific antigens. Nevertheless, CAR T cells have been developed to target EGFRvIII and IL-13Rα2, as these are both are frequently overexpressed in glioblastoma, with limited expression in normal brain tissues (Thaci et al., 2014; Wikstrand et al., 1998). Whilst these CAR T cells have demonstrated efficacy in some glioblastoma patients, they were not curative and patients often experienced tumour recurrence several months later. The poor clinical benefits of these CAR T cells in glioblastoma has largely been attributed to intratumoural heterogeneity, which limits the amount of the overall tissue that could be selectively targeted by these T cells, whilst studies have also shown that targeted tumour cells were able to adapt to these treatments by either downregulating or eliminating their expression of the targeted antigen to prevent immune recognition (C. E. Brown et al., 2016; O'Rourke et al., 2017).

The ability of cancer cells to adapt to avoid immune destruction is one of the ten hallmarks of cancer, as defined by Hanahan & Weinberg (2011). This process begins in the early stages of tumour development, when both the innate and adaptive arms of the immune system are able to recognise and eliminate cancer cells through their expression of tumour-associated antigens. Surviving tumour cells then enter a period of equilibrium, in which the anti-tumour immune response can no longer clear all of the cancer cells, but it can restrict tumour growth. Both of these stages are considered a form of immunosurveillance, which can lead to immune exhaustion and enable tumour cells to develop mutations that ultimately facilitate their escape from the immune system (Dunn et al., 2004).

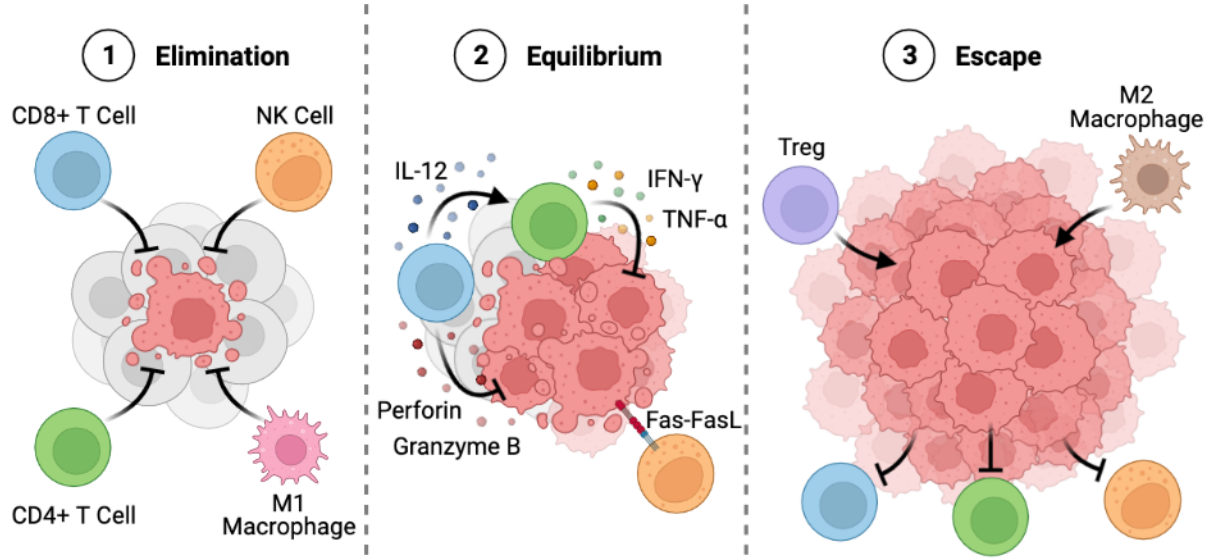


Figure 1.3. Cancer Immunoediting
Initially, the immune system can eliminate cancer cells through recognition of tumour-associated antigens. However, cancer cells can exhaust the immune response and develop mutations to facilitate their escape from immune recognition, whilst creating a highly immunosuppressive tumour microenvironment. Figure generated using BioRender.com.

1.2.3. Glioblastoma Tumour Microenvironment (TME)

Glioblastoma cells utilise a variety of methods to escape the immune system, most significantly through the generation of an immunosuppressive tumour microenvironment (TME). Emerging evidence has shown that the glioblastoma TME appears to be highly heterogeneous, consisting of both cellular components (e.g. neurons, glial cells, endothelial cells, and immune cells) and non-cellular components (e.g. extracellular matrix), alongside a complex signalling network that includes extracellular vesicles (EVs) and soluble factors (e.g. chemokines, cytokines and growth factors) (DeCordova et al., 2020; Perrin et al., 2019). Collectively, the dynamic nature of the TME ensures that it is able fulfil its two key responsibilities: promoting tumour growth and restricting anti-tumour immunity.

1.2.3.1. Hypoxia

The ability of glioblastoma cells to adapt to changes in the surrounding environment is reflected as distinct histological features. which include a necrotic centre, surrounded by actively migrating cells (pseudopalisades) and new blood vessels generated to support tumour growth (microvascular hyperplasia) – all of which are associated with hypoxia and the transcription factor, HIF-1 (Brat & Van Meir, 2004; Zagzag et al., 2000). In normal oxygen conditions (normoxia), HIF-1 expression is suppressed through hydroxylation of HIF-1α by prolyl-hydroxylases (PHD1-3), followed by von Hippel-Lindau (VHL) E3 ubiquitin ligase-mediated ubiquitination and subsequent degradation via proteasomes. However, the lack of oxygen in hypoxic conditions dysregulates PHD1-3 activity, leading to sustained HIF-1α expression (Maxwell et al., 1999; Semenza, 2004). In glioblastoma, HIF-1 expression is also upregulated by the PI3K/AKT/mTOR signalling pathway, which is constitutively

active in most glioblastoma patients, due to EGFR upregulation and loss of PTEN expression, as discussed previously (Kaur et al., 2005; Zundel et al., 2000). Furthermore, research has suggested that the loss of p53 expression in glioblastoma may help to stabilise HIF-1 expression by restricting MDM2-mediated degradation of HIF-1α (Ravi et al., 2000). Once activated, HIF-1α co-operates with HIF-1β and p300/CBP to bind to hypoxia-responsive elements (HREs) within the promoter region of various genes to initiate their transcription and activate multiple signalling pathways involved in angiogenesis, migration, and tumour survival (Berra et al., 2003; Semenza, 2001; G. L. Wang et al., 1995).

Numerous studies have suggested that initial glioblastoma growth is supported through co-option of existing blood vessels in the brain to gain access to a sufficient supply of oxygen and nutrients (Holash et al., 1999; Seano & Jain, 2020). As the tumour infiltrates the white matter tracts, glioblastoma cells compress the vasculature and produce Ang-2, which binds to its receptor (Tie-2) on endothelial cells to further destabilise these blood vessels (Padera et al., 2004; Stratmann et al., 1998). The subsequent malperfusion leads to hypoxia in the surrounding environment and increased HIF-1 expression in glioblastoma cells. Research has shown that HIF-1 is responsible for increasing the production of angiogenic factors (e.g. VEGF, Ang-1 and Ang-2) in glioblastoma cells, which bind to their receptors (VEGF-R, Tie-1 and Tie-2, respectively) on endothelial cells and initiate angiogenic activity (Ahir et al., 2020; Kaur et al., 2005). During this time, glioblastoma cells also secrete MMP-2 to degrade the ECM and facilitate endothelial cell migration, so that they can begin to form new blood vessels (Du et al., 2008).

However, it is important to note that tumour vasculature has markedly abnormal structural and functional properties caused by dysfunctional basement membranes and a lack of pericyte coverage to stabilise the blood vessels (Barlow et al., 2013; Carmeliet & Jain, 2000). Various studies have identified that the highly permeable nature of the glioblastoma vasculature increases interstitial pressure and impairs blood flow to the tumour site (R. K. Jain et al., 2007; Noell et al., 2012). Altogether, tumour-associated angiogenesis can lead to the generation of two forms of hypoxia: perfusion-restricted or diffusion-restricted (Vaupel & Harrison, 2004). Perfusion-restricted hypoxia is associated with cycling between hypoxia and re-oxygenation in the TME, due to fluctuations in blood flow from these aberrant blood vessels. On the other hand, diffusion-restricted hypoxia occurs when the tumour size exceeds the available blood supply; therefore, tumour cells become increasingly necrotic as the distance between the tumour and the vasculature grows (Emami Nejad et al., 2021; Mendichovszky & Jackson, 2011).

In order to facilitate tumour survival, it is imperative that glioblastoma cells migrate from hypoxic areas and become less reliant on oxygen, demonstrating a need for altered metabolic programming. Normal cells typically meet their energy demands by breaking down glucose into pyruvate, which then undergoes oxidative phosphorylation in the mitochondria to generate ATP. However, cancer cells increase the rate of glycolysis to generate more pyruvate, which is then converted into lactate through lactic acid fermentation in the cytosol – this phenomenon is known as the Warburg effect (Warburg, 1956). Research has shown that this mechanism is elevated in hypoxia as HIF-1

independently upregulates GLUT1 (glucose transporter 1) and LDHA (lactate dehydrogenase A), whilst it co-operates with the oncogene c-Myc to increase HK2 (hexokinase 2) and PDK1 (pyruvate dehydrogenase kinase 1) expression. Altogether, this means that HIF-1 activity increases glucose uptake through GLUT1 and the generation of pyruvate by HK2, which then cannot be converted into acetyl Co-A, due to the loss of pyruvate dehydrogenase via PDK, thus pyruvate cannot enter the TCA cycle, so it is converted into lactate by LDHA instead (C. Chen et al., 2001, p. 1; C. V. Dang et al., 2008; J. Kim et al., 2006; Papandreou et al., 2006). Although it has already been established that glioblastoma cells upregulate HIF-1 expression, studies have also identified increased GLUT1, LDHA, HK2 and PDK expression in both glioblastoma patient samples and cell lines (J. Kim et al., 2015; Komaki et al., 2019; Larrieu et al., 2022; Wolf et al., 2011). Collectively, this highlights how HIF-1 activation supports glioblastoma metabolism in hypoxia to promote tumour growth.

1.2.3.2. Extracellular Vesicles

Recently, it has been proposed that glioblastoma cells produce EVs to promote tumorigenesis. These are membranous structures that can be secreted as exosomes, microvesicles, or apoptotic bodies, which provide a key mechanism of intercellular communication, as they can carry a wide range of cargo, including proteins, lipids, and RNA (Théry et al., 2018; van Niel et al., 2018). Exosomes are released into the extracellular space when specialised endosomes (multivesicular bodies) fuse with the plasma membrane, whilst microvesicles and apoptotic bodies are released by outward membrane budding (Doyle & Wang, 2019). Target cells can receive these EV-mediated signals through a range of techniques, including: endocytosis, phagocytosis, membrane fusion, and receptor-ligand interaction (Mulcahy et al., 2014).

Glioblastoma-derived EVs have only recently been explored within the last couple of decades, thus the complexity of this signalling network and all of the mechanisms involved are still yet to be fully understood. Nevertheless, Skog et al. (2008) were crucially able to isolate glioblastoma-derived EVs from both tumour and blood samples taken from glioblastoma patients. Subsequent research has supported this by demonstrating the glioblastoma-derived EVs can cross the BBB and enter the circulation, thus they can facilitate both localised and systemic immunosuppression (Morad et al., 2019; Osti et al., 2019; Shao et al., 2012; Simon et al., 2020; Simpson et al., 2009). In particular, Hellwinkel et al. (2015) established that EVs isolated from both glioblastoma patients and cell lines suppressed peripheral blood mononuclear cells (PBMCs) from healthy donors in a dose-dependent manner.

Within the glioblastoma TME, patient-derived EVs have been shown to downregulate p53, whilst inducing c-Myc expression and transforming growth factor beta (TGF-β) signalling in astrocytes, so that they can either support tumour growth or even transform into tumour cells themselves (Hallal et al., 2019; Oushy et al., 2018). Research has also indicated that glioblastoma cell line-derived EVs induce angiogenin and VEGF expression in endothelial cells to promote angiogenesis (C. C. Li et al., 2013; Skog et al., 2008). Furthermore, glioblastoma cell lines have been shown to release EVs containing

ECM-binding proteins, including CD44, to promote tumour migration (D. Choi et al., 2018; R. Lane et al., 2019). Additionally, research has shown that glioblastoma patient-derived EVs polarise monocytes and macrophages towards an immunosuppressive phenotype *in vitro*, which inhibit T cell proliferation in co-culture experiments (de Vrij et al., 2015; Domenis et al., 2017). Other factors in the glioblastoma secretome include IDO, interleukin-6 (IL-6), IL-10 and TGF-β, which induce an immunosuppressive phenotype in tumour-infiltrating immune cells (Avril et al., 2010; Hishii et al., 1995; Ikushima et al., 2009; See et al., 2015; Wainwright et al., 2012; Zou et al., 1999). Altogether, this highlights that targeting the TME could have significant therapeutic potential in glioblastoma, as it is plausible that altering the dynamics of the TME could restore anti-tumour immunity. In order to develop an effective immunotherapy to overcome glioblastoma-mediated immunosuppression, it is important to have a comprehensive understanding of the mechanisms involved in immune cell development and the roles that these cells have in tumour immunology, so as to identify key therapeutic targets.

1.2.4. T Cell Signalling

The generation of T cells that can mediate an immune response is crucially reliant on their activation, which begins with antigen presentation by APCs via MHC (major histocompatibility complex) class I or II molecules that interact with the TCR on the T cell (Call et al., 2002). Original models of T cell activation stated that only two signals were required from APCs: first, MHC class I/II molecule binding to the TCR, followed by interactions between APC ligands and co-stimulatory receptors on the T cell (Lafferty & Cunningham, 1975). In the absence of the second co-stimulatory signal, T cells either undergo apoptosis or become anergic; therefore, co-stimulation is crucial for T cell survival and function (Linsley & Ledbetter, 1993). One of the most characterised co-stimulatory pathways in T cells involves CD28, which binds to either CD80 or CD86 on APCs, leading to T cell activation and proliferation, alongside IL-2 production and upregulation of anti-apoptotic genes (Azuma et al., 1993; Jenkins et al., 1991; June et al., 1994; Linsley et al., 1990). The activation of T cells via CD28 has also been shown to further drive T cell activation through the induction of ICOS, 41BB and OX40 expression, which bind to APCs via ICOS-L, 41BB-L, and OX40L, respectively. This leads to the generation of a third signal in the form of inflammatory cytokine production by the APC – this includes IL-2, which creates an autocrine loop through the upregulation of IL-2Rα (CD25) on the T cell, to further promote T cell expansion, survival and differentiation (Beier et al., 2000; Cannons et al., 2001; Croft et al., 2009; Curtsinger et al., 1999; Gramaglia et al., 1998; McAdam et al., 2000; Vinay & Kwon, 1998).

However, a fourth signal has also been proposed, which involves the activation of innate immunity through maturation of APCs – this is often referred to as ‘Signal 0’. Immature DCs monitor the periphery, looking for signs of any pathogens and damaged cells that are releasing PAMPs and DAMPs, respectively. DC maturation is initiated by antigen capture via phagocytosis and PRR signalling in the presence of inflammatory cytokines, including IL-1β and tumour necrosis factor alpha

(TNF-α) (Cella, Sallusto, et al., 1997; Jonuleit et al., 1997; Sallusto & Lanzavecchia, 1994; Sozzani et al., 1998). During the maturation process, MHC expression, as well as adhesion and co-stimulatory molecules are upregulated, alongside cytokine and chemokine production – these are all key components of T cell activation (P. Jin et al., 2010; Reis e Sousa, 2006; Thaiss et al., 2011). Interestingly, the DC microenvironment can influence their polarisation abilities: for example, exposure to IFN-γ during DC maturation is vital for the induction of IL-12 secretion upon CD40/CD40L binding, which helps to skew T cell differentiation and upregulate MHC class I molecules to promote CD8+ T cell activation (Cella et al., 1996; n F. Koch et al., 1996; Vieira et al., 2000). Typically, DC maturation results in the loss of CCR1 and CCR5 expression with concomitant CCR7 upregulation, which attracts them towards CCL19 and CCL21-rich secondary lymphoid organs, where they can then present their antigen-MHC complexes to naïve T cells and initiate T cell activation (Dieu et al., 1998; Gunn et al., 1998; Ngo et al., 1998; Sallusto et al., 1998). However, exposure to hypoxia during DC maturation results in reduced expression of MHC class II, CD40, CD80 and CD86, whilst the upregulation of CCR7 is impaired, thus DC migration and T cell activation is profoundly inhibited in the TME (Mancino et al., 2008).

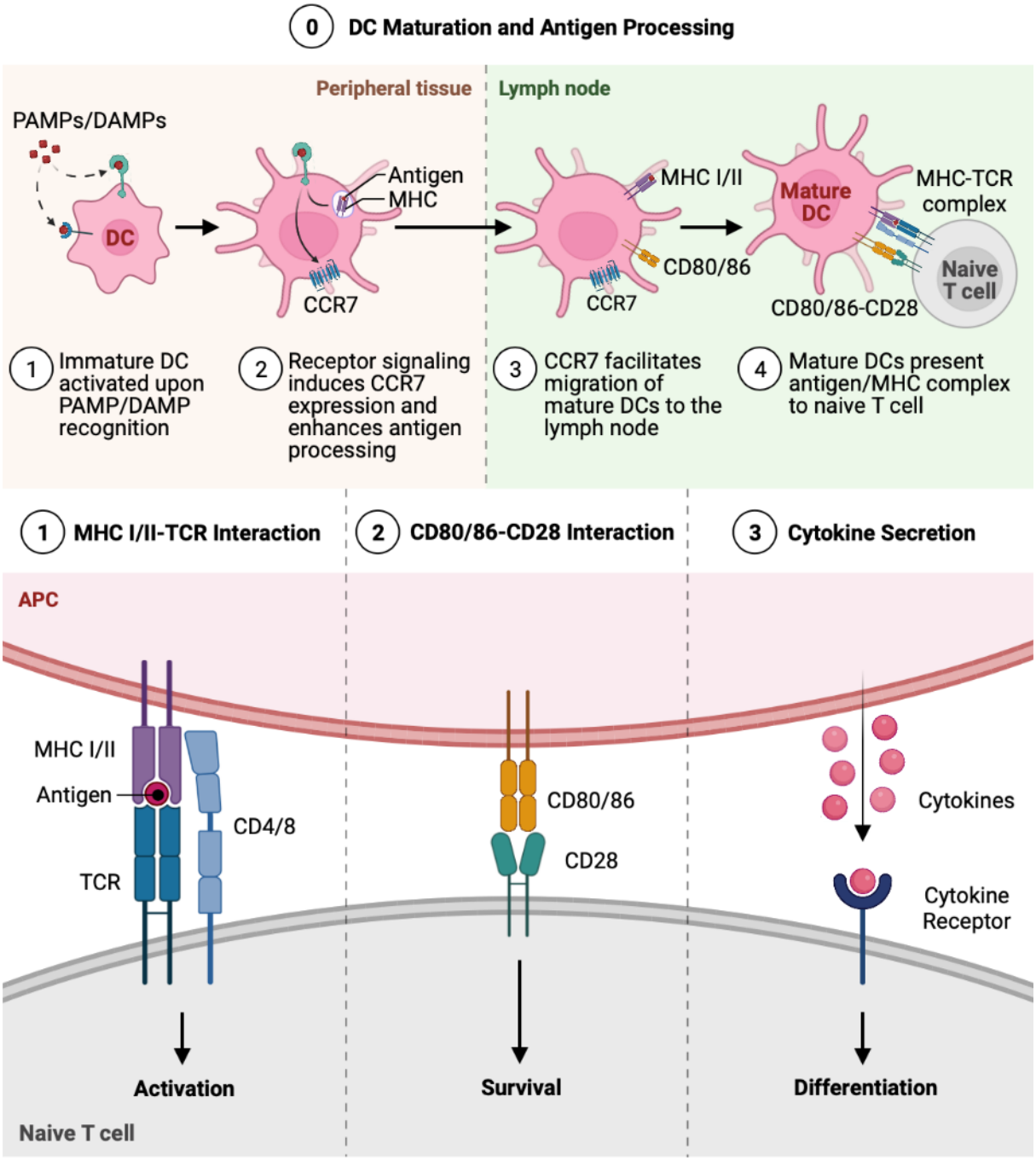


Figure 1.4. Signals Required for T Cell Activation.

Four signals have been shown to be required for naïve T cell activation, including: Signal 0 – DC maturation; Signal 1 – MHC-TCR interaction; Signal 2 – CD80/86-CD28 interaction; Signal 3 – Cytokine secretion. Figure generated using BioRender.com.

T cell activity is also tightly regulated through a range of co-inhibitory T cell receptors, including CTLA-4. Interestingly, research has shown that CTLA-4 binds to CD80 and CD86 at a higher affinity than CD28, which raises questions about how CD28 and CTLA-4 expression/activity is controlled. Current models suggest that CD28 is constitutively and stably expressed on the T cell surface, whereas CTLA-4 expression is regulated by TCR signal strength and subsequently delayed as it is trafficked to the immunological synapse during membrane reorganisation (Egen & Allison, 2002; Kupfer et al., 1987; Walunas et al., 1994). Once bound to CD80/CD86, CTLA-4 inhibits T cell cycle progression and IL-2 production, whilst inducing T cell tolerance (Collins et al., 2002; Eagar et al., 2002; Krummel & Allison, 1995; Linsley et al., 1994). This inhibitory signalling is exacerbated by the downregulation of CD28 via endocytosis in response to the induction of CTLA-4 expression (Rudd et al., 2009). Other examples of inhibitory mechanisms include the APC ligands PD-L1 and Galectin-9, in which PD-L1 restricts T cell proliferation and promotes T cell apoptosis by binding to PD-1 on T cells, whilst Galectin-9 induces T cell exhaustion and apoptosis by binding to T cells via TIM-3 (Ishida et al., 1992; C. Zhu et al., 2005). The balance between stimulatory and inhibitory signalling is managed through the induction of key T cell receptors upon T cell activation, in order to provide multiple layers of immunomodulation, as summarised in Table 1.4 below (Beier et al., 2007).

TCR Expression Level	Signal Type	Receptor (T Cell)	Ligand (APC)
Constitutive	Stimulatory	CD28	CD80, CD86
Constitutive	Stimulatory	CD27	CD70
Constitutive	Stimulatory	HVEM	LIGHT
Inducible	Stimulatory	ICOS	ICOS-L
Inducible	Stimulatory	OX40	OX40L
Inducible	Stimulatory	41BB	41BB-L
Inducible	Inhibitory	CTLA-4	CD80, CD86
Inducible	Inhibitory	PD-1	PD-L1, PD-L2
Inducible	Inhibitory	TIM-3	Galectin-9

Table 1.4. Co-Stimulatory/Inhibitory T Cell Receptors and APC Ligands

Whilst the glioblastoma TME plays a significant role in modulating immune responses, it is also important to consider that the glioblastoma cells could directly interact with T cells to suppress an anti-tumour immune response through these co-stimulatory and co-inhibitory signalling pathways. For example, research has shown that MHC class I expression is downregulated in glioblastoma, which compromises the ability of CD8+ T cells to recognise tumour cells and induce apoptosis (Facoetti et al., 2005; Lehtipuro et al., 2019; Zagzag et al., 2005). Reduced MHC expression via MHC class I chain-related proteins A and B (MICA/B) cleavage also enables glioblastoma cells to evade NK cells, due to the loss of NKG2D ligands (Castro et al., 2020; Eisele et al., 2006). Although research is limited, it appears that glioblastoma cells do not express CD80 or CD86, so they are unable to directly activate T

cells via CD28 (R. C. Anderson et al., 2007). Similarly, low levels of 41BB-L expression have been identified in glioblastoma patient samples, though it remains to be seen whether glioblastoma cells directly interact with T cells via 41BB/41BB-L binding (Blank et al., 2015).

On the other hand, Parsa et al. (2007) discovered that the lack of PTEN expression frequently seen in glioblastoma patients correlates with an increase in PD-L1 expression, thus glioblastoma cells can directly induce T cell death via PD-1. PD-L1 expression has also been identified on the surface of glioblastoma patient-derived EVs, thus these EVs were able to directly inhibit T cell activation and proliferation, alongside the tumour cells (Himes et al., 2020; Ricklefs et al., 2018). Furthermore, glioblastoma cells express the Fas death receptor, thus they can also induce apoptosis in T cells via Fas-FasL signalling (Weller et al., 1997). Similarly, Z. Liu et al. (2016) identified high Galectin-9 expression in glioblastoma patient samples, which correlated with TIM-3 expression on tumour-infiltrating T cells, indicating that glioblastoma cells were utilising Galectin-9 to directly induce T cell apoptosis in the TME. Altogether, these studies highlight that inducing or enhancing the expression of co-stimulatory ligands, whilst downregulating co-inhibitory ligands could alter how glioblastoma cells directly interact with T cells in the TME to restore anti-tumour immunity.

1.2.5. CD4+ T Cell Differentiation

Aside from co-stimulatory signalling, the surrounding cytokine milieu also plays a critical role in T cell activity as it directs the differentiation of naïve T cells towards specific T cell subsets, in order to ensure that an appropriate immune response is initiated. This is particularly important in glioblastoma, as improving our understanding of how the TME influences naïve T cell differentiation and moderates the behaviour of these T cell subsets to promote immunosuppression could highlight potential therapeutic targets.

The first two subsets of T cells were identified by Tada et al. (1978), which they termed T helper 1 (Th1) and T helper 2 (Th2) cells. It was later discovered by Mosmann et al. (1986) that Th1 and Th2 cells could be characterised by their expression of signature cytokines. Subsequent research has shown that based upon MHC-TCR binding, co-stimulatory signalling and localised cytokine exposure, naïve T cells can differentiate into a range of effector cells, including Th1, Th2, Th17, T follicular helper (Tfh), and Treg cells, as shown in Figure 1.5 below. Each of these T cell subsets can be defined by their expression of specific cell surface markers and/or transcription factors, as well as the production of particular cytokines, which allow them to play a variety of roles in the immune system.

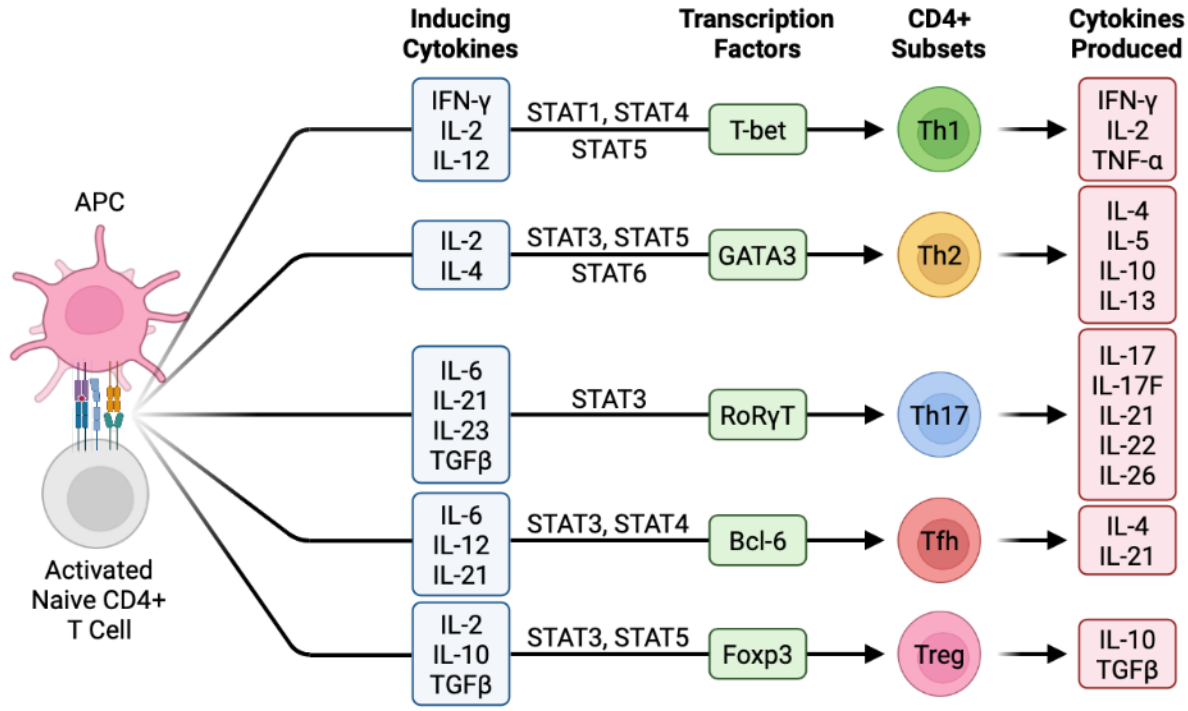


Figure 1.5. CD4+ T Cell Differentiation
Key cytokines and transcription factors involved in the differentiation of naïve CD4+ T cells into various CD4+ T cell subsets, which produce a range of cytokines. Figure generated using BioRender.com.

1.2.6. Th1 and Th2 Cells

Both interferon gamma (IFN-γ) and IL-12 are vital for the differentiation of activated naïve CD4+ T (Th0) cells into Th1 cells. In particular, exposure to IFN-γ induces the expression of the transcription factor T-bet via STAT1 signalling (Afkarian et al., 2002; Szabo et al., 2000). T-bet upregulates expression of the transcription factor Runx3, before both independently bind to the promoter region of the IFN-γ gene in order to induce IFN-γ production, thereby creating a feedback loop to sustain IFN-γ expression (Djuretic et al., 2007; Yagi et al., 2010). In addition, research has shown that TCR signalling activates the transcription factors NFAT and AP-1, which co-operate with T-bet to induce TNF-α expression in Th1 cells (Jenner et al., 2009; Rao et al., 1997; Tsai et al., 1996). On the other hand, Runx3 and T-bet bind directly to a silencer element on the IL-4 gene to prevent its transcription and subsequent Th2 polarisation (Jenner et al., 2009; Naoe et al., 2007). Induction of T-bet also results in upregulation of the IL-12 receptor (IL-12R), thereby enabling exogenous IL-12 to activate STAT4, leading to downstream signalling that stabilises the Th1 phenotype by promoting Th1 cell proliferation and survival, as well as enhancing IFN-γ production (Afkarian et al., 2002; Mullen et al., 2001; Szabo et al., 1997; Thierfelder et al., 1996). IL-27 has also been shown to provide an important role in the early induction of Th1 differentiation, as it is able to induce T-bet and IFN-γ expression via STAT1 signalling (Hibbert et al., 2003; Lucas et al., 2003; Owaki et al., 2005; Takeda et al., 2003). Similarly, IL-2 activation of STAT5 leads to upregulation of IL-12R, IFN-γ, IL-2, and CD25, creating an autocrine loop to support Th1 differentiation (Kasahara et al., 1983; W. Liao et al., 2011). In addition, IL-2 upregulates expression of Blimp-1 via STAT4, which subsequently represses expression of Bcl-6 and Tfh differentiation (Oestreich et al., 2012). Furthermore, Runx3, T-bet and IL-12/STAT4 signalling also actively skew Th0 cells away from Th2 differentiation by suppressing expression of IL-4, IL-5, and the transcription factor GATA3 (Djuretic et al., 2007; Hwang et al., 2005; Naoe et al., 2007; Ouyang et al., 1998).

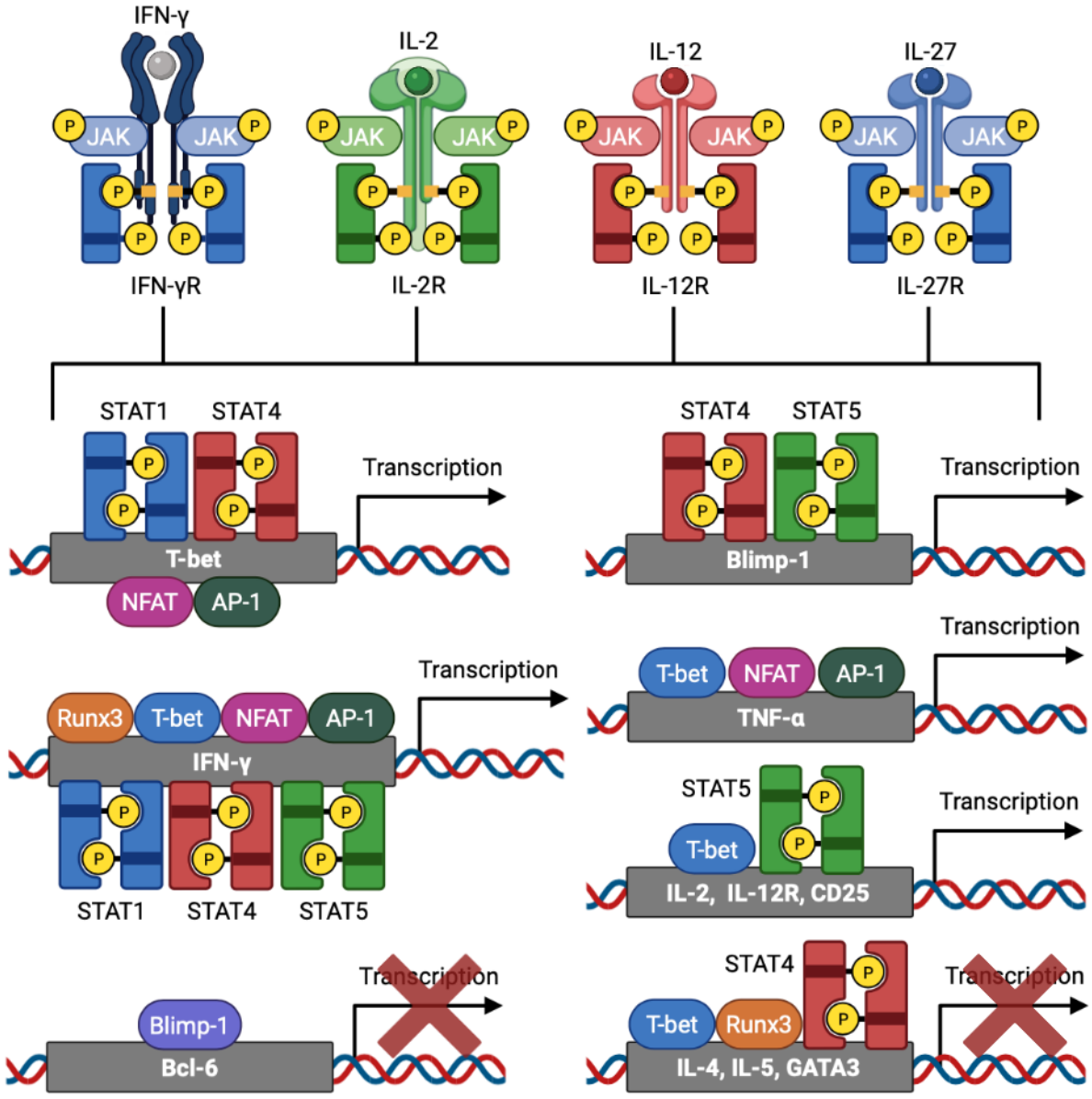


Figure 1.6. Th1 Transcriptional Regulation

Alongside MHC-TCR and co-stimulatory signalling, IFN-γ, IL-2, IL-12, and IL-27 facilitate naïve CD4+ T cell differentiation into Th1 cells. Figure generated using BioRender.com.

Aside from IFN-γ, TNF-α and IL-2, Th1 cells also produce TNF-β (also known as lymphotoxin-alpha – LT-α), but the mechanisms that regulate TNF-β expression are not known. Nevertheless, each of these cytokines are considered pro-inflammatory, thus Th1 cells have been implicated in autoimmune diseases (e.g. rheumatoid arthritis), as well as cell-mediated immunity against intracellular pathogens including bacteria, viruses and protozoa (Berger, 2000; Hsieh et al., 1993; Schulze-Koops & Kalden, 2001). In particular, Th1 cells upregulate expression of CCR5 and CXCR3 upon activation, which facilitates their localisation to these sites of inflammation (C. H. Kim et al., 2001; Loetscher et al., 1998; J. H. Xie et al., 2003). Activated Th1 cells also express CD40L which can subsequently activate CD8+ T cells either through direct CD40/CD40L binding or indirectly through CD40/CD40L-mediated activation of APCs to enhance antigen presentation and co-stimulatory signalling, as well as chemokine and cytokine secretion (Bennett et al., 1998; B. O. Lee et al., 2002; Ridge et al., 1998; Schoenberger et al., 1998). Furthermore, IFN-γ has been shown to upregulate MHC class I and II expression to enhance T cell recognition of both immune and non-immune cells, including glioblastoma cells (Früh & Yang, 1999; I. Yang et al., 2004). In addition, IFN-γ and TNF-α promote differentiation of tumour-associated macrophages (TAMs) to a pro-inflammatory M1 phenotype – these M1 TAMs subsequently secrete CXCL9 and CXCL10 to attract Th1 and cytotoxic cells to the site of inflammation, thus increased expression of CXCL9/10 correlates with improved glioblastoma patient survival (Gjorgjevski et al., 2019). The activation of Th1 cells, as well as cytotoxic CD4+ and CD8+ T cells, also provides mechanisms for inducing apoptosis in target cells, which will be discussed later.

Unlike Th1 cells, Th2 cell differentiation is dependent upon exposure to IL-4, as it leads to activation of STAT6, which subsequently upregulates GATA3 and IRF4 expression (Hou, Schindler, et al., 1994; M. H. Kaplan et al., 1996; W. Zheng & Flavell, 1997). GATA3 facilitates signature Th2 cytokine production by binding directly to the promoter regions of IL-4, IL-5 and IL-13, whilst IRF4 co-operates with NFAT to induce IL-10 expression (Ahyi et al., 2009; C.-M. Hu et al., 2002; Jenner et al., 2009). In addition, STAT6 and GATA3 induce expression of c-Maf, which provides additional signalling to promote IL-4 transcription (Ho et al., 1998; Kurata et al., 1999; Ouyang et al., 2000). IL-4 also activates STAT3, which subsequently upregulates expression of the IL-4 receptor (IL-4Rα) and STAT6 to enhance Th2 polarisation (Deimel et al., 2021). On the other hand, GATA3 suppresses expression of the IL-12 receptor, thereby limiting STAT4 activation and IFN-γ production, driving Th0 cells away from Th1 differentiation (Ouyang et al., 1998; Usui et al., 2003). Research has shown that GATA3 also represses IFN-γ expression at the transcriptional level by binding directly to the IFN-γ gene (S. Chang & Aune, 2007). However, GATA3 expression is also reliant on activation of STAT5 via IL-2, in order to induce IL-4 production and commit cells to the Th2 lineage (Zhu et al., 2003). Furthermore, it has been suggested that IL-2 primes naïve T cells for Th2 differentiation, as STAT5 upregulates IL-4Rα expression and increase accessibility of the IL-4 promoter region, thereby enhancing Th2 polarisation (Cote-Sierra et al., 2004; W. Liao et al., 2008).

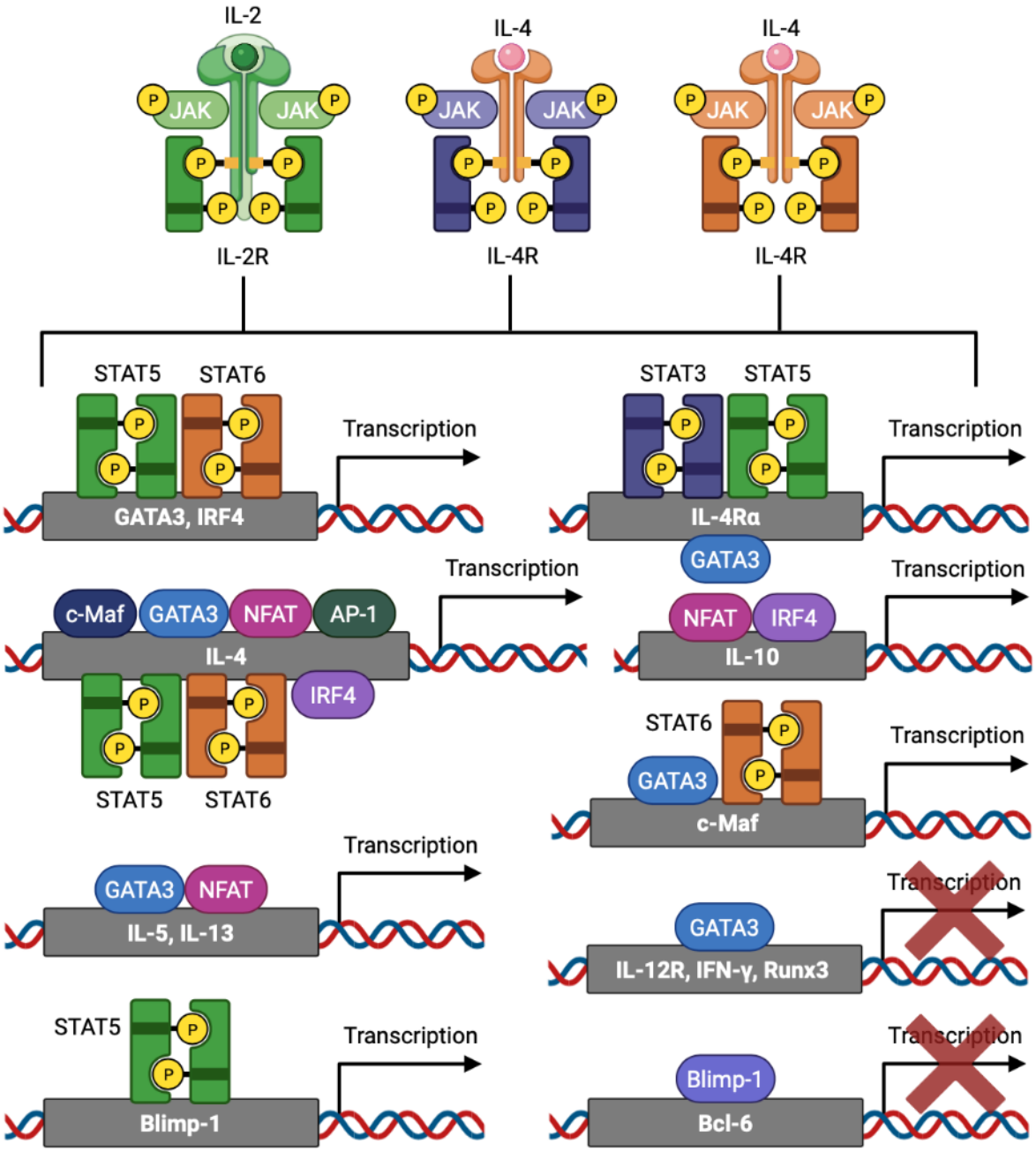


Figure 1.7. Th2 Transcriptional Regulation

Alongside MHC-TCR and co-stimulatory signalling, IL-2 and IL-4 facilitate naïve CD4+ T cell differentiation into Th2 cells. Figure generated using BioRender.com.

In particular, Th2 cells have been implicated in allergic disorders (e.g. asthma and atopic dermatitis), as well as antibody-mediated immunity against extracellular pathogens (e.g. helminth infections) (Anthony et al., 2007; Brandt & Sivaprasad, 2011; Lambrecht & Hammad, 2015). Migration of Th2 cells towards these sites of inflammation is facilitated by their upregulation of the chemokine receptors CCR4, CCR8 and CRTH2 upon activation (Cosmi et al., 2000; D'Ambrosio et al., 1998). In addition, Th2 cells exclusively express the IL-33 receptor (ST2), thus IL-33 has been shown to act as a chemoattractant for Th2 cells in models of skin inflammation, whilst also enhancing expression of IL-5 and IL-13 (Hueber et al., 2011; Komai-Koma et al., 2007; Schmitz et al., 2005). Unfortunately, ST2 and IL-33 expression also correlates with tumourigenesis and poor prognoses for patients with renal cell carcinoma, breast cancer or glioblastoma (Gramatzki et al., 2016; C. Wu et al., 2018; Z.-P. Yang et al., 2015; J. Zhang et al., 2017). Regarding glioblastoma, research has shown that tumour cells also express high levels of IL-13Rα2, in which binding of exogenous IL-13 increases resistance to apoptosis, thus IL-13Rα2 expression is associated with poor patient survival (C. E. Brown et al., 2013; Hsi et al., 2011). This could be explained through inhibition of STAT6 by IL-13Rα2, leading to aberrant activation of STAT3 by IL-4, which subsequently transactivates Bcl-2 and Bcl-xL (Deimel et al., 2021; Rahaman et al., 2005). In addition, IL-4, IL-10 and IL-13 polarise TAMs to an immunosuppressive M2 phenotype, which infiltrate glioblastoma tissues to enhance angiogenesis, growth and invasion. Whilst IFN-γ and TNF-α can drive differentiation to a pro-inflammatory M1 phenotype, this is suppressed by Th2 cells and M2 macrophages to prevent an anti-tumour immune response (J. Liu et al., 2021; B.-Z. Qian & Pollard, 2010; N. Wang et al., 2014).

Early studies hypothesised that homeostasis was maintained through tight regulation of Th1 and Th2 cell differentiation: if this balance became skewed, it could cause inappropriate immune responses and lead to the development of diseases (e.g. asthma or multiple sclerosis) (Abbas et al., 1996; Dardalhon, Korn, et al., 2008; Romagnani, 1994). In the context of glioblastoma, Kumar et al. (2006) discovered that serum levels of IL-12 were reduced, whilst IL-10 expression was higher in newly diagnosed glioblastoma patients, compared to healthy controls. This was supported by Shimato et al. (2012) who found that PBMCs from glioblastoma patients (especially those with recurrent glioblastoma) produced less IFN-γ, but more IL-5 *in vitro*, than PBMCs taken from healthy controls. Similarly, Zisakis et al. (2007) examined cytokine secretion from PBMCs and primary glioblastoma cell cultures, finding that both produced significantly less IFN-γ and TNF-α, yet considerably more IL-4 and IL-10, compared to healthy controls, reflecting a noticeable shift towards Th2 differentiation. Likewise, Waziri et al. (2008) identified glioblastoma-infiltrating Th2 cells that produced high levels of IL-4, IL-10 and IL-13.

Interestingly, Harshyne et al. (2015) proposed that tumour-derived EVs were responsible for the enrichment of Th2 cells and M2 TAMs in glioblastoma patient sera. In line with this, Vidyarthi et al. (2019) identified a significant population of M2 TAMs in glioblastoma patient samples, which were associated with poorer overall survival. This was reflected in a study completed by Takashima et al. (2018) which explored the Th1/Th2 ratio in glioblastoma patients, finding that the expression of either

$Th1^{low}Th2^{low}$ and $Th1^{high}Th2^{low}$ ratios resulted in a better prognosis than a $Th1^{high}Th2^{high}$ immunological profile. Using adoptively transferred *in vitro* polarised T cells, Hoepner et al. (2013) discovered that Th1 cells infiltrated brain tumours more efficiently than Th2 cells, whilst actively recruiting Tc1 cells to the tumour site, thereby enhancing overall survival. Altogether, these studies highlight that Th2 cells play a crucial role in glioblastoma pathogenesis, thus depletion of Th2 and/or enrichment of Th1 cells through modifying the dynamics of the TME represents a potential area for therapeutic development.

1.2.7. Th17 Cells

The discovery of a novel cytokine similar to IL-12, now known as IL-23, led to the identification of a new population of T cells, which produced IL-17 and appeared to be dependent on IL-23 for expansion (Langrish et al., 2005; Oppmann et al., 2000). Further investigations concluded that they were distinct from Th1 and Th2 cells; thus, they were termed Th17 cells (Harrington et al., 2005; H. Park et al., 2005). Whilst IL-23 was initially believed to be involved in Th17 differentiation, this theory was disproven when it was discovered that IL-23R was only expressed by activated T cells, thus IL-23 could not induce Th17 differentiation in naïve T cells (S. Aggarwal et al., 2003; Parham et al., 2002). Instead, Bettelli et al. (2006) identified IL-6 and TGF-β as the key cytokines required for Th17 induction, suggesting that IL-23 was responsible for Th17 cell maintenance. Subsequent research has shown IL-23 stabilises Th17 differentiation by inducing expression of the transcription factor Blimp-1, which suppresses Bcl-6 expression, whilst promoting IL-17 and IL-17F expression (R. Jain et al., 2016). Exposure to IL-21 has also been shown to induce Th17 differentiation and sustain IL-17 production, although this also requires the presence of TGF-β (Korn et al., 2007). Interestingly, IL-6 can induce IL-21 production, leading to upregulation of the IL-21R and IL-23R via STAT3, creating an autocrine loop for IL-21 and IL-23 to support IL-17 production and Th17 differentiation (Korn et al., 2007; Zhou et al., 2007). Similarly, ICOS has been shown to induce c-Maf, which subsequently upregulates IL-21 expression to enhance Th17 polarisation (Bauquet et al., 2009).

In particular, TGF-β plays a central role in Th17 differentiation by driving Th0 cells away from the Th1 and Th2 lineages through downregulation of IFN-γ and T-bet, as well as IL-4 and GATA3 expression, likely via MAP kinase signalling (Gorelik et al., 2000, 2002; I.-K. Park et al., 2007). On the other hand, the classical TGF-β signalling pathway involves phosphorylation of Smad2 and Smad3, which then form complexes with Smad4 and translocate to the nucleus to contribute the activation of key Th17 genes (D. Chang et al., 2020; Derynck et al., 1998). However, naïve CD4+ T cell exposure to TGF-β alone results in upregulation of Foxp3 (a signature markers of Tregs, which will be discussed later), thus other factors must be involved in Th17 polarisation (Chen et al., 2003). An example of this is IL-1β, which is required for early commitment to the Th17 lineage as it regulates the expression of the transcription factor IRF4.

Research has shown that IRF4-deficient Th0 cells could not differentiate into Th17 cells, due to increased expression of Foxp3 and reduced expression of RoRγT, a crucial transcription factor in

Th17 cells (Brüstle et al., 2007; Chung et al., 2009). This reflects a vital mechanism in determining Th17 cell fate, as Foxp3 prevents Th17 polarisation by binding directly to RoRγT to disrupt downstream signalling (Yang et al., 2008; Zhou et al., 2008). However, Foxp3 can be downregulated by IL-6 and IL-21, which drive naïve CD4+ T cells towards Th17 commitment rather than Treg differentiation (Bettelli et al., 2006; Korn et al., 2007; Nurieva et al., 2007). In addition, IL-6, IL-21 and IL-23 activate STAT3, initiating downstream signals to induce the expression of RoRγT and subsequently control the production of signature Th17 cytokines: IL-17, IL-17F, IL-21, IL-22, and IL-26 (Ivanov et al., 2006; Wei et al., 2007; Wilson et al., 2007; Yang et al., 2007). Likewise, TCR signalling activates NFAT, which works alongside c-Maf and AP-1 to enhance Th17 polarisation (Hermann-Kleiter & Baier, 2010; H.-P. Kim et al., 2005; Yahia-Cherbal et al., 2019). However, some Th17 cells have been known to co-express cytokines that are associated with different Th lineages. In particular, Th17 cells have been shown to produce IFN-γ and IL-10, although the function of these cells is not clear, nor is it known whether they represent a stable cell type or if they are simply transitioning between Th phenotypes (Tesmer et al., 2008; Wilson et al., 2007; Zielinski et al., 2012).

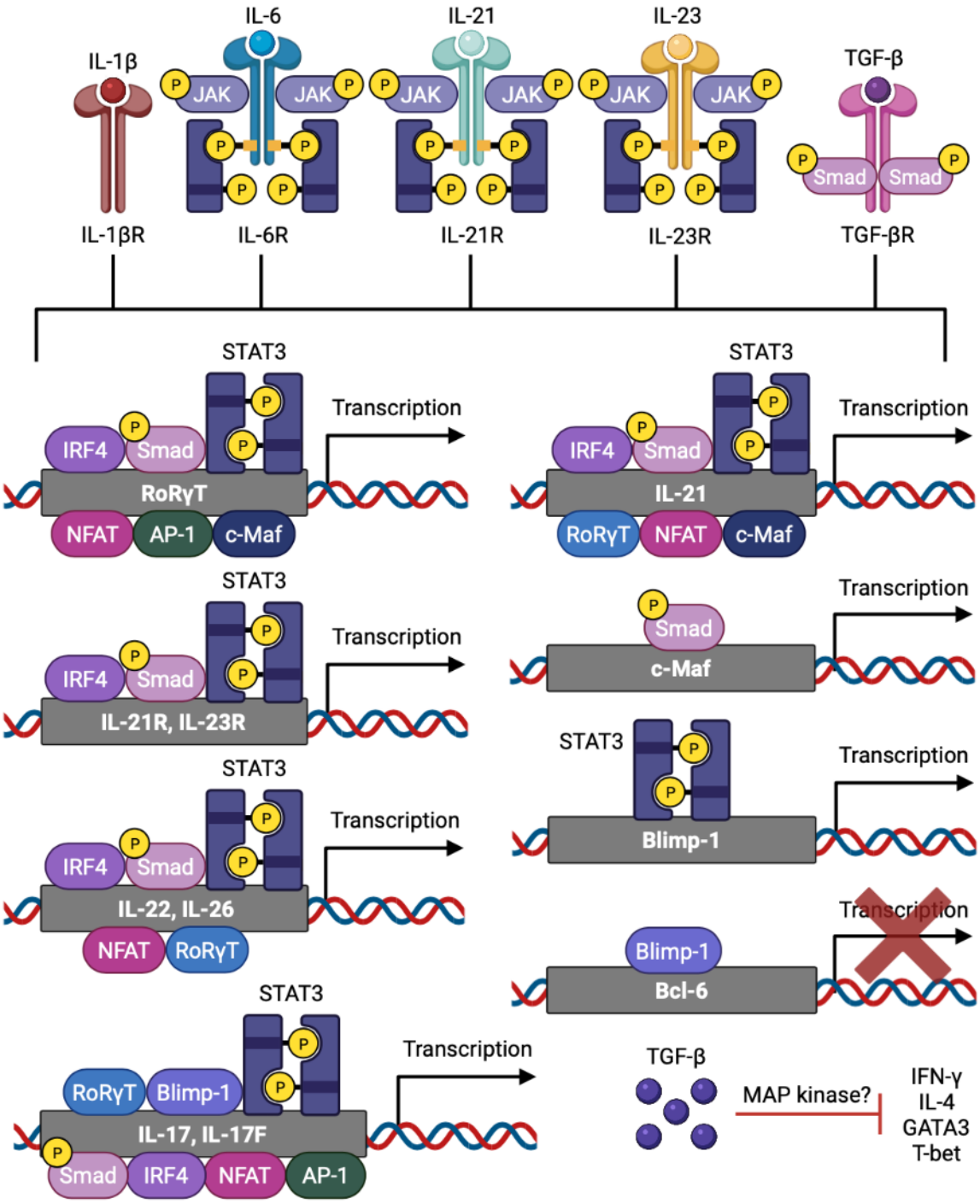


Figure 1.8. Th17 Transcriptional Regulation

Alongside MHC-TCR and co-stimulatory signalling, IL-1β, IL-6, IL-21, IL-23, and TGF-β facilitate naïve CD4+ T cell differentiation into Th17 cells. Figure generated using BioRender.com.

Functionally, Th17 cells are involved in the clearance of extracellular bacteria and fungi, but they have also been implicated in a range of conditions, including: asthma, rheumatoid arthritis and Crohn's disease (Brand, 2009; Cosmi et al., 2011; Leipe et al., 2010). Wainwright et al. (2010) were the first to identify that both Th17 cells and IL-17 were enriched in glioblastoma patient samples, compared to healthy brain tissue. Initial data suggested that these high levels of IL-17 were associated with increased survival in glioblastoma patients, but this was later questioned by large-scale experiments, which correlated high levels of IL-17-producing T cells in glioblastoma patient samples with poor overall survival (Bronsart et al., 2016; Cui et al., 2013; Madkouri et al., 2017). In addition, Madkouri et al. (2017) showed that IL-17 induced the release of VEGF-A in both glioblastoma cell lines and patient-derived cells, whilst Th17 cells in glioblastoma patient samples were surrounded by blood vessels, thus it appeared that glioblastoma cells used IL-17 to promote tumour survival and growth via neoangiogenesis. Furthermore, Zheng et al. (2019) discovered that IL-17 enhanced glioblastoma migration and invasion via PI3K/Akt signalling in two glioblastoma cell lines (U87MG and U251). Crucially, Paladugu et al. (2013) identified that U87 cells secreted TGF-β and IL-6 into the surrounding media, which could then induce low levels of Th17 differentiation when cultured with naïve T cells. Collectively, these results suggested that glioblastoma cells utilised Th17 cells and/or IL-17 to facilitate tumour pathogenesis, indicating that these both represent targets for future glioblastoma therapeutics.

1.2.8. CD4+ Tfh Cells

T cells are also involved with the activation of B cells and the development of germinal centres, where they assist with high-affinity B cell selection, whilst promoting B cell differentiation into long-lived plasma cells and memory B cells. The CD4+ T cells involved in this vital adaptive immunity process are known as Tfh cells, which were first identified in tonsils, but have since been found in other secondary lymphoid organs, such as the spleen and lymph nodes (Breitfeld et al., 2000; Schaerli et al., 2000). The differentiation of Tfh cells from naïve CD4+ T cells is a complex process, beginning with early modulation of the following T cell homing markers: CCR7, CD62L, S1PR1, and CXCR5 – this process appears to be controlled by the transcription factor KLF2, which is highly expressed in naïve CD4+ T cells (Bai et al., 2007; Carlson et al., 2006; Haynes et al., 2007). Typically, KLF2 promotes expression of Blimp-1, T-bet and GATA3, whilst repressing CXCR5 expression. However, co-stimulatory CD28 and ICOS-mediated signalling induced by follicular B cells results in KLF2 suppression, leading to reduced CCR7, CD62L and S1PR1 expression, subsequently upregulating CXCR5 (Lee et al., 2015; Weber et al., 2015). This is incredibly important as CXCR5 expression facilitates the migration of Tfh and B cells into lymphoid follicles, due to its chemoattraction to CXCL13 (Ansel et al., 2000; Förster et al., 1996; Kim et al., 2004). Tfh cells then begin to secrete IL-21, whilst rapidly upregulating CD40L and ICOS (which bind to B cells via CD40 and ICOS-L, respectively), leading to B cell activation and maturation, as well as germinal centre formation (Banchereau et al., 1994; Elgueta et al., 2009; Vogelzang et al., 2008). Aside from these co-stimulatory pathways, Tfh cells

can also inhibit B cell function through CTLA-4 and PD-1, demonstrating their ability to closely maintain activity within the germinal centre (Haynes et al., 2007; Sage et al., 2014; J. Shi et al., 2018).

In addition to KLF2, Bcl-6 and Blimp-1 appear to strongly influence the fate of differentiating naïve T cells. In particular, Bcl-6 is exclusively expressed by Tfh cells, whilst Blimp-1 is expressed by all other T helper lineages. Studies have shown that Blimp-1 expression can directly inhibit Bcl-6, whilst expression of Bcl-6 can in turn repress Blimp-1; thus, they are mutually antagonistic (Johnston et al., 2009; Shaffer et al., 2000). Remarkably, the decision between whether Bcl-6 or Blimp-1 should be expressed appears to be made within the first two divisions of the differentiating cell, representing a crucial point in determining cell fate (Choi et al., 2011, 2013). TCF-1 has been implicated in regulating the early stages Tfh differentiation by directly repressing Blimp-1 transcription, whilst upregulating ICOS, IL-6R and Bcl-6 expression (T. Wu et al., 2015; L. Xu et al., 2015). Bcl-6 facilitates Tfh lineage commitment by binding directly to the promoter regions of T-bet, GATA3, and RoRγT, in order to disrupt their transcriptional activity and inhibit Th1, Th2, and Th17 differentiation, respectively (Kusam et al., 2003; Nurieva et al., 2007; Yu et al., 2009). In addition, research has shown that mice with Bcl-6 deficiency were unable to form germinal centres, thus Bcl-6 expression is vital for Tfh function and differentiation (Dent et al., 1997; B. Ye H. et al., 1997).

In line with other T helper lineages, the cytokine milieu is crucial for the development of Tfh cells: however, there are contrasting differences in human and mice Tfh differentiation. In mice, the most significant cytokines involved in Tfh differentiation are IL-6, IL-21 and IL-27, all of which activate STAT3 – although, IL-12 has been shown to play a role in early Tfh development (Batten et al., 2010; Nakayamada et al., 2011). On the other hand, research has suggested that IL-12, which primarily activates STAT4, is more essential for human Tfh differentiation than IL-6, IL-21 and IL-27 (Schmitt et al., 2009, 2013). In particular, IL-12 has a stronger influence on the induction of IL-21, ICOS, CXCR5 and Bcl-6 in human naïve CD4+ T cells, compared to IL-6, IL-21 and IL-27 (C. S. Ma et al., 2009, 2012). Nevertheless, STAT3 signalling is vital for human Tfh cells as STAT3-deficiency inhibits IL-6, IL-21 and IL-12 production, as well as Tfh generation and germinal centre development (Ma et al., 2012; Nurieva et al., 2008). In addition, activation of STAT3 by IL-6 leads to downregulation of IL-2R expression, which is important as IL-2 acts as a potent inhibitor of Tfh development by upregulating Blimp-1 and suppressing CXCR5, c-Maf, Bcl-6 and IL-21 expression via STAT5 (Choi et al., 2013; Nurieva et al., 2012).

Whilst IL-4 is primarily associated with Th2 cells, it can also be produced by Tfh cells, especially those within the germinal centre (GC Tfh cells), which have the highest expression levels of Bcl-6, CXCR5 and ICOS in humans (Rasheed et al., 2006; Reinhardt et al., 2009). Unlike Th2 cells, this process does not rely on GATA3 signalling, instead IL-4 production is controlled by the transcription factors SAP and SLAM (Yusuf et al., 2010). In addition, BATF co-operates with c-Maf, IRF4, NFAT, STAT3 and STAT6 to transactivate IL-4 in Tfh cells (J. I. Kim et al., 1999; Rengarajan et al., 2002; Sahoo et al., 2015). IL-4 is essential for B cell development, as it plays a crucial role in

promoting B cell survival in germinal centres by upregulating expression of Bcl-xL via STAT6 (Nelms et al., 1999; Wurster et al., 2002). Alongside its involvement in IL-4 production, c-Maf also binds to the promoter region of IL-21 to induce IL-21 secretion in Tfh cells (Hiramatsu et al., 2010). In the early stages of Tfh development, ICOS activation leads to c-Maf upregulation, driving an increase in IL-21 production and the expression of IL-21R, creating an autocrine loop to induce Bcl-6 expression and sustain Tfh differentiation (Bauquet et al., 2009). This is incredibly important as research has shown that IL-21 is vital for germinal centre formation, Tfh generation and B cell proliferation (Nurieva et al., 2008; Vogelzang et al., 2008). In addition, ICOS signalling induced by B cells is crucial for the maintenance of Bcl-6 expression in Tfh cells (Y. S. Choi et al., 2011).

Activation of STAT3 by IL-6 leads to upregulation of BATF and subsequently induces expression of Bcl-6 and c-Maf, which then co-operate to promote Tfh function and differentiation (Y. S. Choi, Eto, et al., 2013; Ise et al., 2011; Kroenke et al., 2012; X. O. Yang et al., 2008). In particular, Kroenke et al. (2012) found that Bcl-6 controlled the expression of CXCR5, ICOS, CD40-L and PD-1, whilst c-Maf induced IL-4 and IL-21 expression. In line with this, the amount of IL-4 and IL-21 produced did not increase when Bcl-6 was co-expressed with c-Maf, demonstrating that their expression was solely dependent on c-Maf. Conversely, PD-1 and ICOS were both upregulated when c-Maf was co-expressed with Bcl-6, compared to when Bcl-6 was expressed alone. Altogether, Kroenke et al. (2012) concluded that Bcl-6 was vital for Tfh differentiation, promoting Tfh migration and enabling direct interactions with B cells, whilst c-Maf was responsible for cytokine production, enhancing Tfh function and maintaining Tfh differentiation. These findings were supported by Andris et al. (2017) who also suggested that the early phase of Tfh differentiation driven by c-Maf helped to stabilise Bcl-6 expression in fully differentiated Tfh cells.

Surprisingly, TGF-β signalling via STAT3 and STAT4 has also been implicated in human Tfh differentiation. Schmitt et al. (2014) showed that administering TGF-β alongside IL-12 or IL-23 increased expression levels of CXCR5, ICOS, IL-21, Bcl-6 and c-Maf in differentiating Tfh cells, compared to IL-12 or IL-23 alone. In addition, they discovered that supplementing these polarising conditions with IL-1β and IL-6 resulted in a further rise in Bcl-6 and c-Maf expression, whilst suppressing Blimp-1. Remarkably, Schmitt et al. (2014) also identified that developing Tfh cells also expressed RoRγT, likely due to the fact that these cytokines are also associated with the induction of Th17 differentiation. Altogether, this study suggested that there was significant overlap in the signalling pathways responsible for the generation of Th17 and Tfh cells – however, the exact point that these differentiating cells diverge into their separate lineages is not currently known.

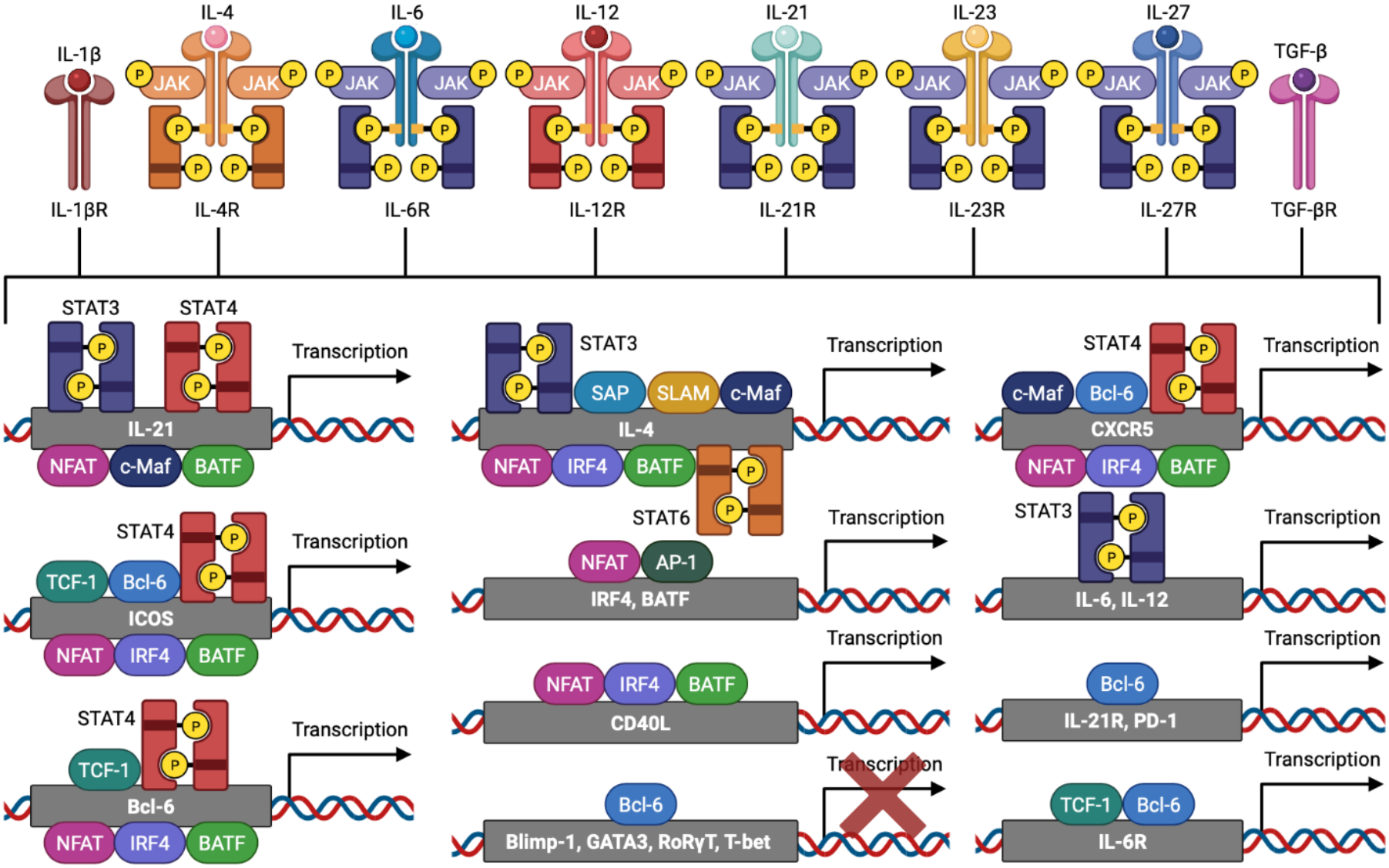


Figure 1.9. CD4+ Tfh Transcriptional Regulation

Alongside MHC-TCR and co-stimulatory signalling, IL-1β, IL-4, IL-6, IL-12, IL-21, IL-23, IL-27, and TGF-β facilitate naïve CD4+ T cell differentiation into CD4+ Tfh cells. Figure generated using BioRender.com.

The role of Tfh cells in glioblastoma is unclear. Research has linked the production of IL-4 by Tfh cells with inhibition of anti-tumour immunity, thus Tfh cells have been identified alongside IL-4R expression in glioblastoma (B. H. Joshi et al., 2001; J. Lu et al., 2019; Shirota et al., 2017). However, this has since been questioned, as Qiu et al. (2020) conducted large-scale genetic analyses of tumour-infiltrating immune cells in glioma patients, which indicated that downregulation of Tfh cells in glioblastoma patients correlated with a poor survival. Likewise, increased Tfh cell infiltration was associated with improved prognosis in glioblastoma patients (P. Liang et al., 2020). Furthermore, Tfh cells were found at reduced levels in the peripheral blood of patients with IDH-wildtype glioblastoma, compared to IDH-mutant glioblastoma patients and healthy controls (Mohme et al., 2021). Collectively, these studies largely indicated that glioblastoma cells suppressed Tfh cells to prevent an anti-tumour immune response. It is possible that this is due to the effect IL-21 has on promoting intratumoural Tc1 production of IFN-γ, TNF-α and granzyme B to restrict tumour growth, as seen in murine models of melanoma and colorectal cancer, but this requires substantial further investigation (Niogret et al., 2021; R. I. Nurieva et al., 2019; Singh et al., 2020).

1.2.9. CD4+ Treg Cells

Tregs were first discovered by Sakaguchi et al. (1995) when they identified a highly suppressive subset of T cells that expressed CD25. They found that these cells provided immunological tolerance against both non-self and self-antigens, whilst depletion of these Tregs increased immune reactions to allogeneic skin transplantation and induced the development of a number of autoimmune diseases in mice. As CD25 is upregulated by T cells upon activation, alternative Treg markers needed to be identified – this led to the discovery of Foxp3 as a transcription factor uniquely expressed by Tregs generated in both the thymus (nTregs) and the periphery (iTregs) (Fontenot et al., 2003; Hori et al., 2003; Khattri et al., 2003). These two subsets are functionally similar and express the same characteristic markers, except for the transcription factors Neuropilin-1 (Nrp1) and Helios, which are believed to be primarily expressed by nTregs (Thornton et al., 2010; Yadav et al., 2012).

Miyara et al. (2009) proposed that nTregs were a heterogenous population that could be separated into three subsets: resting/naive nTregs were CD45RA+CD25^{low}Foxp3^{low}, activated/effector nTregs were CD45RA-CD25^{high}Foxp3^{high}, and non-Tregs were CD45RA-CD25^{low}Foxp3^{low}. In particular, they suggested that naïve nTregs upregulated CD25, CTLA-4 and Foxp3 expression upon activation, thereby facilitating their differentiation into activated/effector nTregs, which work alongside iTregs to promote immunosuppression. An additional nTreg marker is CD15s, which is exclusively expressed by activated/effector nTregs (Miyara et al., 2015). Unfortunately, Foxp3 expression in non-Tregs appeared to be unstable, thus they failed to demonstrate suppressive activity, instead secreting moderate amounts of IL-2, IFN-γ and IL-17 (Miyara et al., 2009). Research has shown that loss of Foxp3 expression in Tregs profoundly affects their suppressive ability, whilst promoting the acquisition of IL-2, IL-17, IFN-γ, and TNF-α production (Hoffmann et al., 2009; Williams & Rudensky, 2007). This

process is referred to as Treg plasticity, but it is not known whether it is reversible – this highlights how vital Foxp3 expression is for maintaining Treg differentiation, stability and function (Junius et al., 2021; Kleinewietfeld & Hafler, 2013).

Early work suggested that high-affinity interactions between TCRs and self-antigen/MHC complexes in the thymus were responsible for driving cells towards nTreg development (M. S. Jordan et al., 2001). Subsequent research found that co-stimulatory signalling via CD28 facilitated the generation of a population of Treg precursor cells that required IL-2 in order to undergo maturation (Burchill et al., 2008; Lio et al., 2010). As a result of this, a two-step model for Treg development in the thymus was proposed, in which TCR affinity testing against self-antigens upregulated CD25 expression, thereby increasing cell sensitivity to IL-2 and STAT5 activation, leading to the induction of Foxp3 expression (Lio & Hsieh, 2008). However, TGF-β has been also been implicated in nTreg differentiation, as it is able to activate Foxp3 gene expression through Smad3 and NFAT (Tone et al., 2008). In addition, Ouyang et al. (2010) discovered that TGF-β protected autoreactive naïve T cells from undergoing apoptosis, thereby increasing the pool of Treg precursor cells in the thymus.

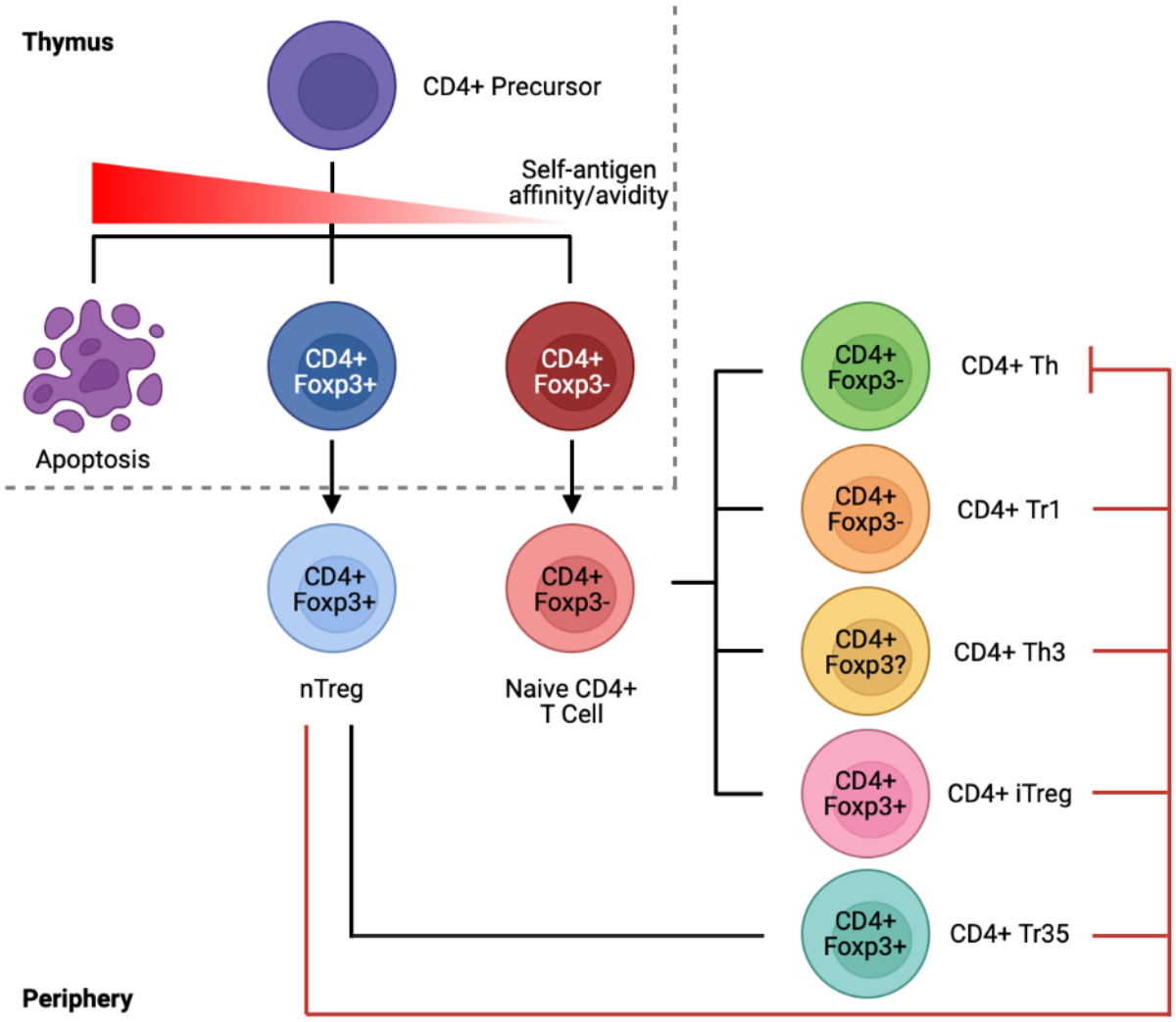


Figure 1.10. CD4+ Treg Thymic Development.
Foxp3 expression is determined in the thymus, thereby generating CD4+ nTregs and naïve CD4+ T cells, which can subsequently differentiate into CD4+ iTregs in the periphery. Figure generated using BioRender.com.

TGF-β has also been shown to be vital in Treg differentiation in the periphery by inducing the expression of CD25 and Foxp3 in naïve CD4+ T cells (Chen et al., 2003; Yamagiwa et al., 2001). In particular, Smad3, NFAT, AP-1 and STAT5 bind to the promoter region of the Foxp3 gene to drive Foxp3 transcription (Tone et al., 2008; Y. Wu et al., 2006). This represents a key stage in Treg differentiation, as Foxp3 binds directly to GATA3 and RoRγT to inhibit Th2 and Th17 polarisation, respectively (Bettelli et al., 2006; Dardalhon, Awasthi, et al., 2008; L. Zhou et al., 2008). In addition, TGF-β has a vital role in preventing an anti-tumour immune response through downregulation of IFN-γ, perforin, granzyme B and Fas-L expression in cytotoxic T cells (D. A. Thomas & Massagué, 2005). Alongside TGF-β, research has shown that IL-10 is important for both Treg differentiation and function, by enhancing the expression of CTLA-4 and Foxp3 via STAT3 and Foxo1 (Groux et al., 1997; P. Hsu et al., 2015). Foxo1 also co-operates with TGF-β to restrict Th1 polarisation in Tregs by downregulating T-bet (Kerdiles et al., 2010). In addition, STAT5 activation by IL-2 leads to upregulation of IRF4 and Blimp-1, which in turn promote IL-10 production (Cretney et al., 2011; Tsuji-Takayama et al., 2008). Altogether, increased IL-2 consumption by Tregs stabilises their immunosuppressive phenotype through Foxp3 and IL-10 expression, whilst simultaneously inducing IL-2 deprivation in surrounding T cells to limit their proliferation and function (Busse et al., 2010).

The mechanisms which control TGF-β production are still under investigation, but it has been proposed that the transcription factor GLI2 transactivates TGF-β in CD4+ Tregs (Furler & Uittenbogaart, 2012). As CTLA-4 is constitutively expressed by Tregs, it has been suggested that there is a feedback loop between TGF-β and CTLA-4, in which CTLA-4 signalling is required for TGF-β to induce Foxp3 expression, whilst activation of CTLA-4 by Foxp3 and NFAT promotes TGF-β production (Chen et al., 1998; Salomon & Bluestone, 2001; Zheng et al., 2006). In addition, CTLA-4 has been shown to restrict co-stimulatory signalling from DCs by capturing and internally degrading CD80 and CD86 through trans-endocytosis, thereby suppressing effector T cell activation (Qureshi et al., 2011; Wing et al., 2008). Furthermore, CTLA-4 signalling enhances IDO expression in DCs and effector T cells, thus increasing catabolism of tryptophan into kynurenine, which subsequently inhibits effector T cell proliferation and induces apoptosis, whilst promoting Treg differentiation (Fallarino et al., 2002, 2003, 2006; Grohmann et al., 2002; Mellor et al., 2003). Likewise, Tregs transfer high levels of cAMP into CD4+ T cells and APCs in a contact-dependent manner, leading to upregulation of ICER, which increases expression of CTLA-4 and inhibits NFAT-mediated transcription of IL-2 (Bodor et al., 2012). Tregs can also suppress activation of effector T cells by removing antigen-MHC complexes from DCs through trogocytosis (Akkaya et al., 2019). Alternatively, Tregs can induce apoptosis in target cells directly by secreting perforin and granzyme B or through TNF-related apoptosis-inducing ligand (TRAIL) and Fas-mediated signalling (X. Cao et al., 2007; Ren et al., 2007; Strauss et al., 2009). Similarly, Tregs express both Galectin-1 and Galectin-9 upon activation, which have been shown to induce effector T cell apoptosis (Garín et al., 2006; Kashio et al., 2003; C. Zhu et al., 2005).

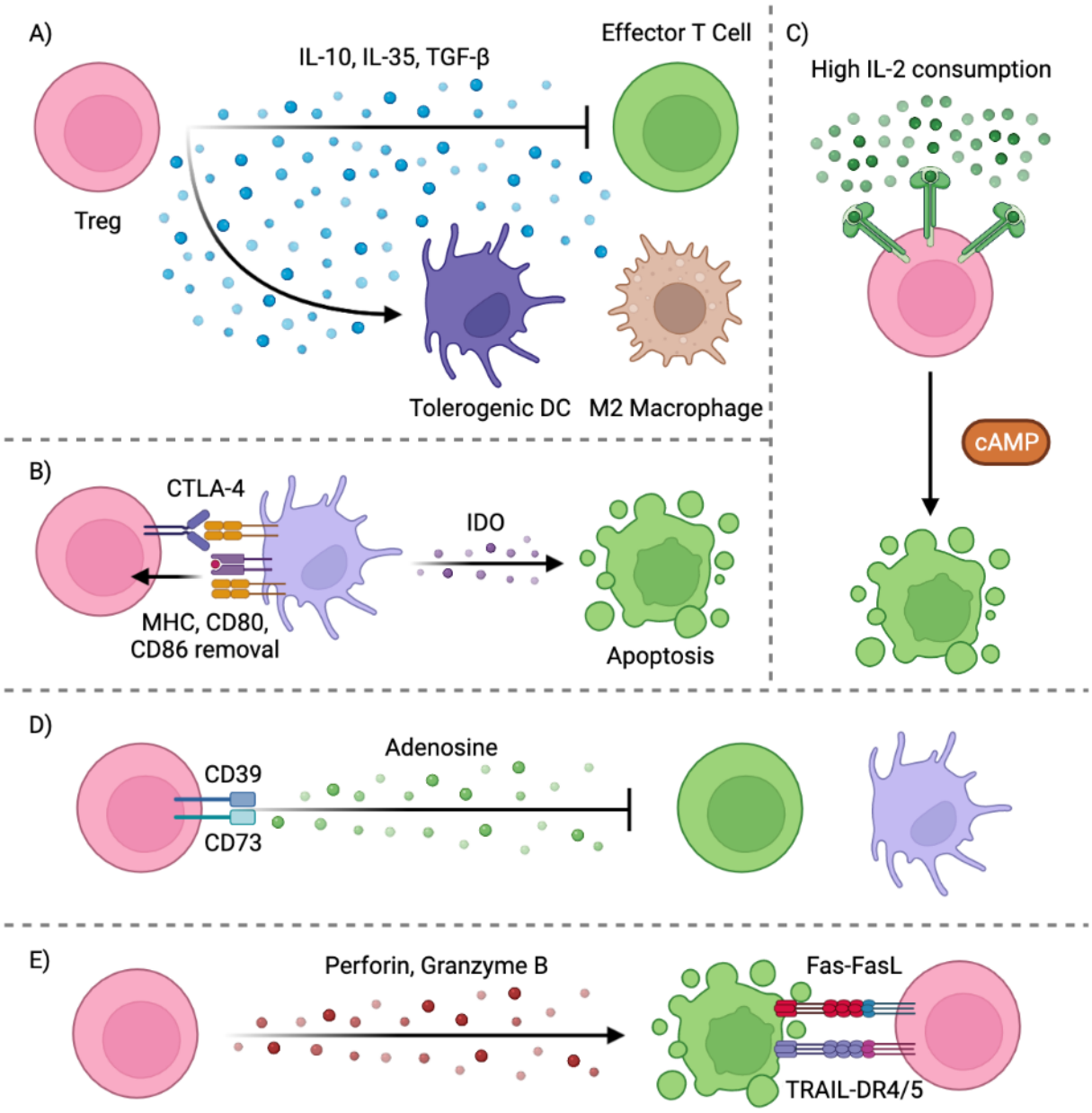


Figure 1.11. Treg-mediated Immunosuppression

Tregs can induce immunosuppression, using a variety of mechanisms: **A)** IL-10, IL-35 and TGF-β secretion by Tregs suppresses effector T cells, whilst tolerising DCs and polarising TAMs to an M2 phenotype; **B)** Tregs can remove MHC, CD80 and CD86 expression from DCs, whilst CTLA-4 signalling upregulates IDO production by DCs, which induces effector T cell apoptosis; **C)** cAMP transfer reduces IL-2 signalling in effector T cells, whilst high IL-2 consumption by Tregs leads to IL-2 deprivation and apoptosis; **D)** Adenosine generated by CD39 and CD73 potently inhibits effector T cells and DCs; **E)** Tregs can directly induce apoptosis in effector T cells through the secretion of perforin and granzyme B or in a cell contact manner via Fas-FasL or TRAIL-DR4/5 signalling. Figure generated using BioRender.com

Research has indicated that some nTregs secrete IL-10 and IL-35, which can polarise naïve CD4+ T cells in the periphery to differentiate into Tr35 cells that produce high levels of IL-35 and subsequently suppress effector T cell proliferation and function (Collison et al., 2007, 2010). In particular, IL-35 expression by intratumoural Tr35 cells has been found in several murine cancer models, in which IL-35 blockade promoted tumour rejection (Turnis et al., 2016). Similarly, breast cancer and colorectal cancer cells have been shown to produce IL-35 to enhance the immunosuppressive tumour microenvironment, thus elevated levels of IL-35 in patient serum correlated with poor patient survival (S. Hao et al., 2018; Y. Ma et al., 2016). Researchers have suggested that there are adaptive populations of Tregs which fluctuate between producing IL-10 or IL-35, in order to upregulate effector T cell expression of LAG-3, PD-1 and TIM-3 to limit their activity (Sawant et al., 2019).

A fourth Treg subset (Tr1) has also been proposed, which lack constitutive Foxp3 expression, but uniquely co-express CD49b and LAG-3, alongside a signature cytokine profile: high production of IL-10 and IFN-γ, with low/no expression of IL-2 and IL-4 (Gagliani et al., 2013; Groux et al., 1997; Häringer et al., 2009; Levings & Roncarolo, 2000). Despite the loss of Foxp3, Groux et al. (1997) showed that Tr1 cells could be induced from precursor cells in response to IL-10 exposure. Subsequent research discovered that IL-27 can also promote Tr1 differentiation by inducing c-Maf and ICOS expression, which subsequently enhance IL-27 production, generating a positive feedback loop that sustains c-Maf and IL-10 expression (Pot et al., 2009). In addition, IL-27 has been shown to induce expression of the endonucleotidase CD39 – this facilitates a key immunosuppressive mechanism, as CD39 is involved in the conversion of extracellular ATP into adenosine, which induces a range of downstream effects via the A2A receptor to inhibit DC function, as well as effector T cell activation and proliferation (Borsellino et al., 2007; Mandapathil et al., 2010; Mascanfroni et al., 2015; Ohta & Sitkovsky, 2014; Panther et al., 2003). Tr1 cells have also been shown to express granzyme B and perforin, thus they can directly induce target cell death (Grossman et al., 2004). Interestingly, Tr1 cells have been identified in the peripheral blood and tumour tissues from glioblastoma patients, which demonstrated highly suppressive activity in co-culture experiments by inhibiting IFN-γ and TNF-α secretion in CD4+ and CD8+ T cells, as well as impairing their cytotoxic activity (Z. Li et al., 2016).

Th3 cells were identified by Chen et al. (1994) when they were investigating oral tolerance, which is the suppression of local and systemic immune responses to antigens consumed orally (e.g. food) (Chase, 1946). Subsequent research by Weiner (2001a) found that these immunosuppressive cells secreted TGF-β and could be induced from precursor cells by exposure to IL-4, IL-10 and TGF-β. Whilst the CD25 and Foxp3 expression status of Th3 cells remains unclear, other unique markers have been identified, including the membrane-bound form of TGF-β (LAP) and CD69 (Carrier et al., 2007; Gandhi et al., 2010; Y. Han et al., 2009; Oida et al., 2003). In particular, the gut has high concentrations of TGF-β, as well as tolerogenic DCs that can induce Th3 differentiation upon encounter with oral antigen; thus, it is unsurprising that Th3 cells have been strongly implicated in colorectal cancer (Scurr et al., 2014; Weiner, 2001b).

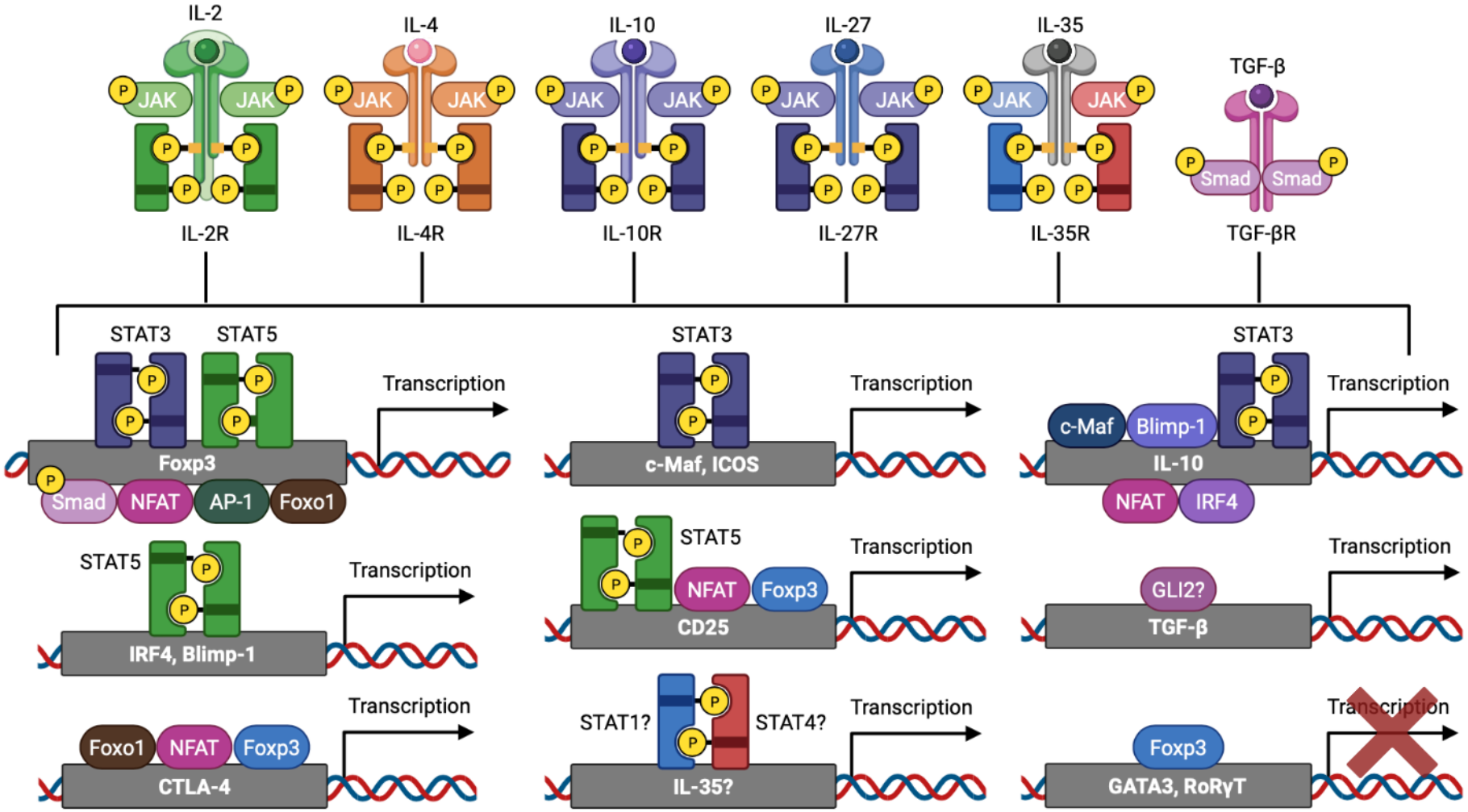


Figure 1.12. CD4+ Treg Transcriptional Regulation

Alongside MHC-TCR and co-stimulatory signalling, IL-2, IL-4, IL-10, IL-27, IL-35, and TGF-β facilitate naïve CD4+ T cell differentiation into CD4+ Tregs. Figure generated using BioRender.com.

Several research groups have identified a small population of Treg cells that express Blimp-1, CD25, CTLA-4 and Foxp3, alongside some characteristic Tfh markers (CXCR5, ICOS, PD-1, Bcl-6), which they termed follicular regulatory (Tfr) cells. In particular, they identified that Tfr were generated from Tregs in the periphery; hence, they produce IL-10, but do not express all of the signature Tfh markers (e.g. CD40L, IL-4 or IL-21) (Chung et al., 2011; Linterman et al., 2011; Wollenberg et al., 2011). The production of IL-10 by Tfr cells appears to regulate activity of both Tfh and B cells to prevent the generation of self-reactive antibodies (Dominguez-Sola et al., 2015; Laidlaw et al., 2017). On the other hand, secretion of IL-21 by Tfh cells leads to downregulation of CD25 via Bcl-6, thereby limiting IL-2 signalling and Tfr proliferation (Jandl et al., 2017). As discussed previously, Blimp-1 and Bcl-6 have an antagonistic relationship, thus it is unusual for them to be co-expressed. However, knockout studies revealed that they both have an important role in Tfr cells – in particular, Bcl-6 is vital for enabling Tfr differentiation, whilst Blimp-1 controls IL-10 production (Cretney et al., 2011; Linterman et al., 2011). Furthermore, Tfh cells acquired cytotoxic function in Tfr-deficient mice, thus they induced apoptosis in B cells and restricted antibody production in the germinal centre, demonstrating how vital Tfr cells are for controlling germinal centre activity (M. M. Xie et al., 2019).

Similar research by Duhen et al. (2012) identified regulatory subsets of Th1, Th2, Th17 and Th22 cells, which maintained expression of some of their signature Th features, whilst acquiring Foxp3 and IL-10 expression. These findings led to the proposal of two models of Treg plasticity, known as the multiple subset and multipotent plasticity models (Tian et al., 2012). In the multiple subset model, the upregulation of characteristic Th markers in Tregs facilitates their differentiation into effector-like subsets which have specialised regulatory control over individual Th lineages, as seen with Tfr and Tfh cells. However, questions have been raised about the stability of these Th/Treg subsets, leading to the development of the multipotent plasticity model – this proposes that each individual Treg cell has the capacity to transiently express effector T cell markers to enable Treg migration and localised immunosuppression (Tian et al., 2012). For example, exposure to IFN-γ results in the upregulation of CXCR3 and T-bet by CD4+Foxp3+ Tregs, which facilitates their co-localisation and subsequent suppression of Th1 cells (M. A. Koch et al., 2009). It is plausible that low levels of transient T-bet expression in Tregs regulated by SOCS1 could facilitate CXCR3 expression, whilst restricting high IFN-γ production and Th1 polarisation by reducing STAT1 signalling (Takahashi et al., 2011). Likewise, upregulation of IRF4 in Tregs can inhibit Th2 cells through enhanced CTLA-4 signalling, whilst STAT3-mediated IL-10 production by Tregs has been shown to suppress Th17 cells in the gut (Alegre et al., 1998; Chaudhry et al., 2011; Rubtsov et al., 2008; Tian et al., 2011).

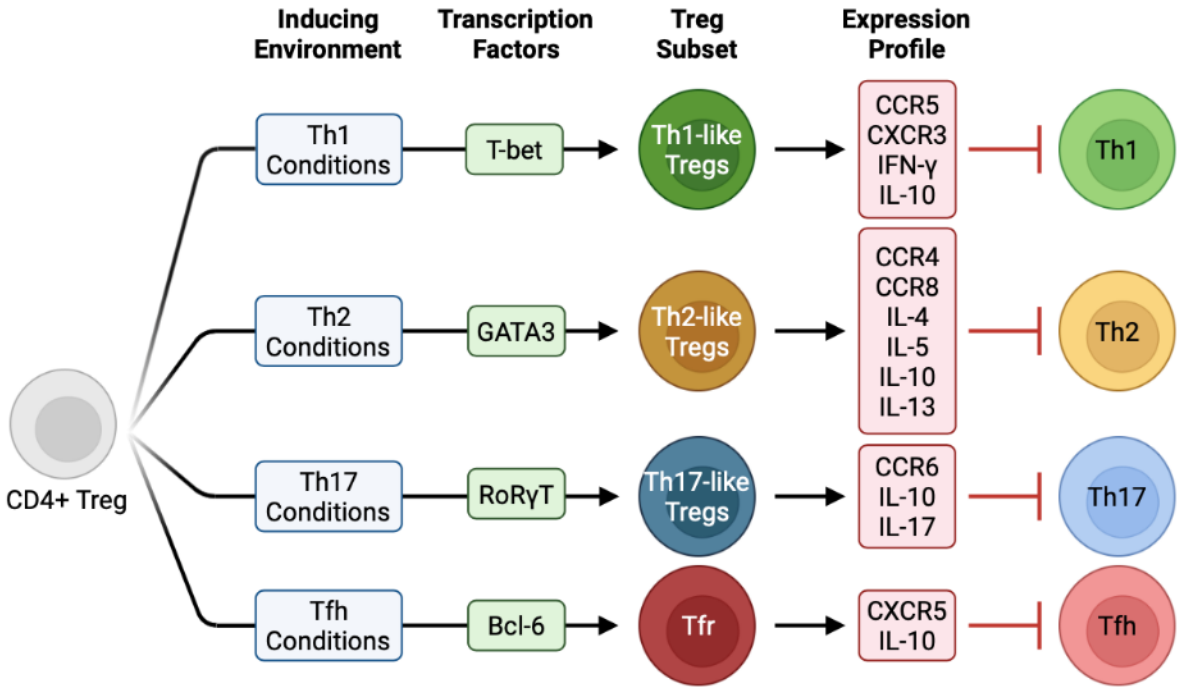


Figure 1.13. Effector-like Treg Subsets

Expression profiles of Tregs which have acquired characteristics of CD4+ T cell subsets, in order to facilitate localised immunosuppression. It not currently known whether these Th-like Tregs are stable lineages or simply populations of Tregs with transient expression of effector T cell features. Figure generated using BioRender.com.

As both TGF-β and its receptors are highly expressed in glioblastoma, it had been suggested that Tregs cells play a crucial role in glioblastoma immunosuppression and tumourigenesis (C. Hao et al., 2002; Shimizu et al., 1999). This has been supported by research which showed that high levels of TGF-β/Smad signalling correlated with enhanced tumour proliferation and poor prognosis in glioblastoma patients (Bruna et al., 2007). Similarly, high Foxp3 expression correlated with increased astrocytoma grading, whilst Treg infiltration was identified in glioblastoma patient samples (El Andaloussi & Lesniak, 2006, 2007; Heimberger et al., 2008; Hussain et al., 2006). In particular, Fecci et al. (2006) found that the proportion of Tregs in blood samples taken from glioblastoma patients was almost three times higher than healthy controls, leading to reduced proliferation of effector T cells *in vitro*: however, this could be restored by reducing the Treg population. Furthermore, El Andaloussi et al. (2006) found that depleting Tregs could prolong survival in a glioblastoma mouse model – although, they used an anti-CD25 mAb, which would have had considerable off-target effects, as CD25 is upregulated on activated T cells. Nevertheless, this collectively highlights that Tregs are a significant therapeutic target for the treatment of glioblastoma as it indicates that altering the glioblastoma TME could diminish Treg activity and restore anti-tumour immunity.

1.2.10. Cytotoxic T Cells

The ability of cytotoxic T cells to directly induce cell death in target cells represents a vital aspect of our immune system. Initial studies identified exocytosis of granules into the extracellular space between cytotoxic T cells and the target cell, in which calcium-dependent perforin created a pore in the target cell membrane, in order to provide a route for serine protease granzyme B to enter the cell and initiate apoptosis (Dennert & Podack, 1983; Dourmashkin et al., 1980; Peters et al., 1989; Stinchcombe et al., 2001). However, subsequent research revealed that perforin was not always required for granzyme B entry into target cells (Froelich et al., 1996; Shi et al., 1997). This led to the discovery of a receptor for granzyme B, which provided a mechanism in which granzyme B could enter target cells through receptor-mediated endocytosis – although, perforin assists with the release of granzyme B from the endosomes into the cytoplasm of target cells (Motyka et al., 2000; Pinkoski et al., 1998). Once inside the target cell, granzyme B triggers apoptosis by activating members of the caspase family, including caspase-3 and caspase-8 (Atkinson et al., 1998; Darmon et al., 1995; Medema et al., 1997). In addition, granzyme B cleaves Bid into a truncated form, which translocates to the mitochondria to activate Bax and Bak, leading to cytochrome c release and formation of the apoptosome – this drives the activation of caspase-9 via Apaf-1 to further promote caspase-3 activity (Alimonti et al., 2001; Heibein et al., 2000; Li et al., 1998; Sutton et al., 2000). Granzyme B also activates CAD through cleavage of its inhibitor (ICAD), enabling CAD release, alongside endoG, through the disrupted outer mitochondrial membrane, in order to induce apoptosis via DNA fragmentation (Enari et al., 1998; Li et al., 2001).

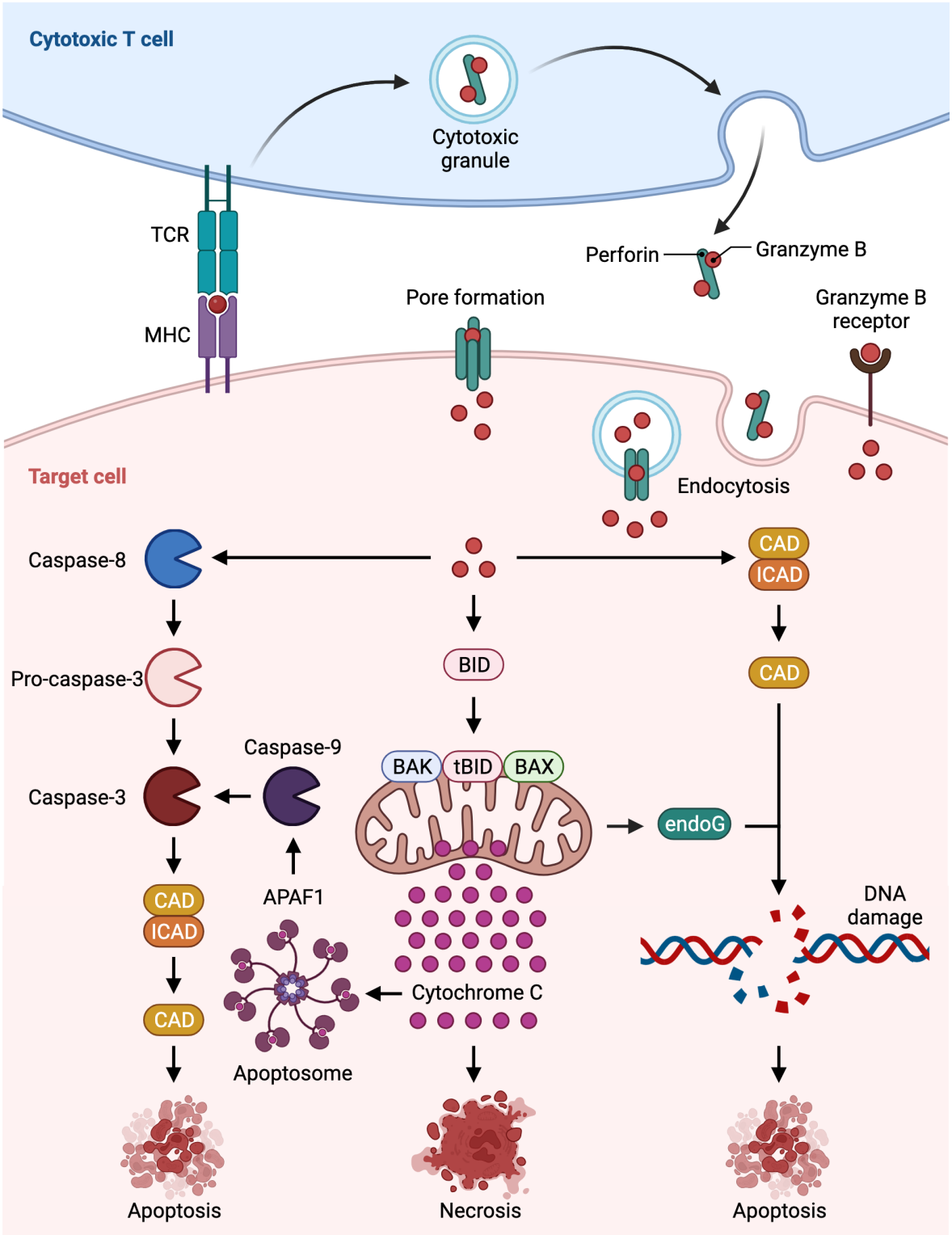


Figure 1.14. Perforin and Granzyme B-mediated Cell Death.
Granzyme B induces cell death in target cells by initiating a caspase cascade and cytochrome c release, as well as DNA damage. Figure generated using BioRender.com.

Another method of inducing apoptosis in target cells involves cell-to-cell contact via Fas on the target cell and Fas-L on the cytotoxic T cell surface, which is rapidly upregulated by cytotoxic T cells upon activation (Klas et al., 1993; Leithäuser et al., 1993; Rouvier et al., 1993). Fas/Fas-L binding results in the formation of a death-inducing signalling complex (DISC) in the target cell, consisting of Fas-associated death domain (FADD) and its death-effector domain (DED), which recruits the dormant form of caspase-8 (procaspase-8) (Kischkel et al., 1995; Muzio et al., 1996). The activation and subsequent release of caspase-8 from the DISC leads to either the direct activation of caspase-3 to induce apoptosis or indirect activation of caspase-3 and caspase-9 via Bid-mediated cytochrome c release, utilising the same pathway as granzyme B to drive cell death (Li et al., 1998; Luo et al., 1998). Similar apoptotic mechanisms are also activated by binding of TRAIL to DR4/5, as well as exogenous TNF-α and IFN-γ binding to TNFR1 and IFNGR on the target cell, respectively (Brincks et al., 2008; Chawla-Sarkar et al., 2003; H. Hsu et al., 1996; Jorgovanovic et al., 2020; Kayagaki et al., 1999; Micheau & Tschopp, 2003; Ossina et al., 1997). Whilst the process of inducing cell death using these pathways is much slower than the alternative perforin/granzyme mechanism, the ability to use multiple routes to induce apoptosis is crucial for survival as it ensures efficient pathogen clearance (Hassin et al., 2011; Lynch et al., 1995).

An additional weapon in the cytotoxic T cell arsenal is the secretion of pro-inflammatory cytokines, including IFN-γ and TNF-α – these cytokines also play a central role in the differentiation of effector CD4+ and CD8+ T cells (Haring et al., 2006; N. S. Joshi et al., 2007; N. S. Joshi & Kaech, 2008). In particular, research has shown that genes that encode IFN-γ, perforin and granzyme B are significantly upregulated in effector and memory CD8+ T cells, compared to naïve CD8+ T cells (Bachmann et al., 1999; Grayson et al., 2001; Teague et al., 1999; Veiga-Fernandes et al., 2000). Pro-inflammatory cytokines have been shown to enhance the cytotoxicity of effector and memory CD8+ cells by increasing their signalling capacity and reducing the threshold of antigen required for activation (Richer et al., 2013). In particular, IFN-γ has been implicated in the upregulation of MHC class I via STAT1 signalling in APCs, thereby enhancing antigen processing and presentation to CD8+ T cells, whilst also enhancing TRAIL-mediated apoptosis (Früh & Yang, 1999; S.-Y. Park et al., 2004; F. Zhou, 2009). Similarly, IFN-γ and TNF-α can directly induce apoptosis in various cancer cell lines, including glioblastoma, by upregulating Fas and concurrently downregulating Bcl-2 expression (Weller et al., 1994, 1995; X. Xu et al., 1998). Furthermore, IFN-γ and TNF-α upregulate the expression of a range of adhesion molecules and chemokines to facilitate the migration of monocytes and macrophages, as well as CD4+ and CD8+ T cells to the site of inflammation, including: ICAM-1, VCAM-1, CXCL9 (MIG), CXCL10 (IP-10), CCL2 (MCP-1), CCL3 (MIP-1α), CCL4 (MIP-1β), and CCL5 (RANTES) (Gil et al., 2001; Hou, Baichwal, et al., 1994; F. Liao et al., 1995; Pober, 2002; Roebuck & Finnegan, 1999; Taub et al., 1993).

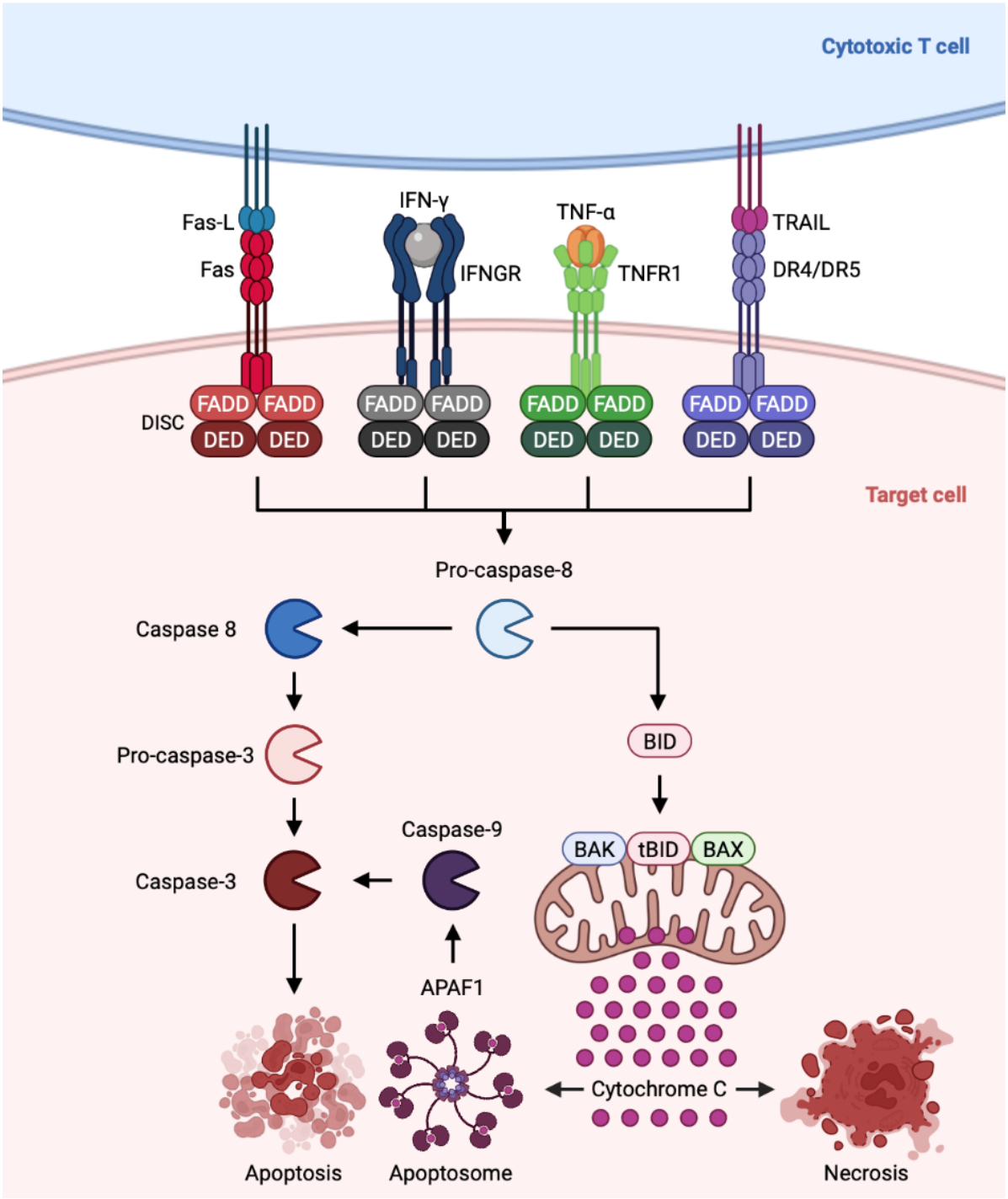


Figure 1.15. Fas-L/IFN-γ/TNF-α/TRAIL-mediated Cell Death.

Target cell death mediated by Fas-L/Fas, IFN-γ/IFNGR, TNF-α/TNFR1, and TRAIL/DR4/5, which induce a caspase cascade and cytochrome c release, leading to apoptosis or necrosis. Figure generated using BioRender.com.

1.2.11. Cytotoxic CD4+ T Cells

For many years, CD8+ T cells were considered to be the sole cytotoxic T cell population, but this has since been challenged with the identification of cytotoxic CD4+ T cells. Early phenotypic analysis identified perforin expression in a subset of CD4+ T cells that were CD45RO+CD27-CD28-CD57+, thus they reflected mature T cells (Appay et al., 2002; Casazza et al., 2006; Globerson & Effros, 2000; Hintzen et al., 1993). On the other hand, genetic analyses revealed that an IL-7Rα^{high} subset of CD45RA+CCR7- T_{EMRA} cells had significantly higher expression of perforin and granzyme B, compared to CD45RA-CCR7+ T_{CM} and CD45RA-CCR7- T_{EM} subsets, indicating that cytotoxic CD4+ T cells could be terminally differentiated (Patil et al., 2018). Whilst cytotoxic CD4+ T cells could be identified in the blood of healthy controls, they were expressed at significantly higher levels in patients with chronic viral infections, rheumatoid arthritis or multiple sclerosis, suggesting that persistent antigen exposure could drive cytotoxic CD4+ T cell differentiation (Appay et al., 2002; Markovic-Plese et al., 2001; D. Schmidt et al., 1996). Alongside perforin and granzyme B, cytotoxic CD4+ T cells have also been shown to produce IL-2, IFN-γ and TNF-α, whilst expressing FasL (Appay et al., 2002; Duftner et al., 2003; N Porakishvili et al., 2004; Stalder et al., 1994; Zaunders et al., 2004).

Recent studies have indicated that cytotoxic CD4+ T cells are distinct from CD4+ Th cells, as they do not express the signature CD4+ Th transcription factor ThPOK (Mucida et al., 2013; Reis et al., 2013; Vacchio & Bosselut, 2016). During T cell development in the thymus, the expression of ThPOK in DP cells directs cells towards CD4+ lineage commitment by suppressing CD8 and Runx3 expression, whilst Runx3 facilitates CD8+ T cell differentiation by repressing expression of CD4 and ThPOK (Egawa et al., 2007; Egawa & Littman, 2008). Interestingly, experiments have revealed that cytotoxic CD4+ T cell expression of ThPOK is lost post-thymically in response to chronic antigen stimulation and the subsequent upregulation of Runx3 appears to relieve the suppression of cytotoxic gene expression, thereby facilitating their differentiation into cytotoxic CD4+ T cells (Mucida et al., 2013; Reis et al., 2013; Vacchio & Bosselut, 2016). Similarly, the loss of ThPOK enables expression of the transcription factor Eomes, which has been shown to promote expression of perforin and granzyme B in cytotoxic CD4+ T cells (Gruarin et al., 2019). As enhanced Runx3 expression also leads to upregulation of CD8, other factors are required to suppress CD8 expression and/or stabilise CD4 expression in cytotoxic CD4+ T cells, including MAZR and HEB/E2A (Bilic et al., 2006; Sawada & Littman, 1993). However, Th1 cells can acquire cytotoxic function, whilst maintaining ThPOK expression, through gradual upregulation of T-bet and Runx3 – although, ThPOK knockdown in Th1 cells significantly enhances perforin and granzyme B expression (Serroukh et al., 2018).

Serroukh et al. (2018) also found that naïve CD4+ T cells cultured in Th1 polarising conditions upregulated perforin and granzyme B expression, but this effect was not seen when they were cultured in Th2 or Th17 polarising conditions. Similarly, D. M. Brown et al. (2009) demonstrated that Th2-polarised naïve CD4+ T cells showed low levels of cytotoxicity, whilst those cultured in Th1 polarising conditions had high levels of cytotoxic activity. Interestingly, naïve CD4+ T cells that were cultured

with IL-2 (Th0 cells) appeared to be twice as cytotoxic compared to Th1 cells, which was reflected in their increased expression of granzyme B – however, both Th0 and Th1 cells expressed similar levels of IFN-γ and TNF-α (D. M. Brown et al., 2009). Takeuchi et al. (2015) identified that these Th0 cells exclusively expressed the marker CRTAM and, despite comparable expression of T-bet and Runx3 in CRTAM- and CRTAM+ CD4+ T cells, Eomes, IFN-γ and granzyme B were considerably upregulated in Th0 cells, indicating that CRTAM has a central role in the acquisition of cytotoxic function. Furthermore, Takeuchi et al. (2015) demonstrated that the cytotoxic activity of these Th0 cells was comparable to cytotoxic CD8+ T cells. Interestingly, they also showed that naïve CRTAM+ CD4+ T cells retained the ability to differentiate into Th1, Th2, Th17 or Treg cells, when cultured in the relevant polarising conditions – although, cytotoxic gene expression was only retained in CRTAM+ Th0, Th1 and Th2 cells. In addition, expression of CRTAM was upregulated upon activation, with the highest levels of expression induced by co-culture of naïve CD4+ T cells with DCs (Takeuchi et al., 2015). This was also seen in a previous study, which used neutralising antibodies in co-culture experiments to reveal that APCs were vital for the acquisition of cytotoxic abilities, whereas exposure to IL-12, IFN-γ or TNF-α was not (D. M. Brown et al., 2009). In particular, studies have identified that OX40 and 41BB co-stimulatory signalling is important for cytotoxic CD4+ T cell differentiation and expansion (Hirschhorn-Cymerman et al., 2012; Qui et al., 2011). Additionally, IL-2 controls STAT5-mediated upregulation of Blimp1, which subsequently induces T-bet expression in cytotoxic CD4+ T cells, enabling transactivation of perforin and granzyme B by both T-bet and STAT5 (D. M. Brown et al., 2009; Hua et al., 2013; Workman et al., 2014). Expression of T-bet is also associated with upregulation of Runx3, IFN-γ and TNF-α, thus highlighting the importance of T-bet expression in cytotoxic CD4+ T cell differentiation (Djuretic et al., 2007; Jenner et al., 2009; Reis et al., 2013; Yagi et al., 2010).

Intratumoural cytotoxic CD4+ T cells that co-expressed granzyme B, perforin, IFN-γ, TNF-α, PD-1, LAG-3 and TIM-3 have been identified in bladder cancer patients. Subsequent *ex vivo* co-culture experiments revealed that cytotoxic CD4+ T cells could induce MHC class II-dependent bladder cancer cell death, but their activity was profoundly inhibited by the presence of Tregs, thus depletion of the Treg population enhanced tumour killing (Oh et al., 2020). Similar results were seen in an adoptive mouse model of melanoma, in which cytotoxic CD4+ T cells promoted tumour rejection, but this could be enhanced by blockade of CTLA-4, thereby promoting cytotoxic CD4+ T cell proliferation and diminishing the Treg population (Quezada et al., 2010). Likewise, inhibition of tumour growth correlated with increased cytotoxic CD4+ T cell infiltration, which was enhanced by PD-1 blockade in a mouse model of Hodgkin lymphoma (Nagasaki et al., 2020). Furthermore, expression of circulating and liver-infiltrating cytotoxic CD4+ T cells decreased in line with Treg expansion, as the tumour advanced in hepatocellular carcinoma patients; thus, loss of cytotoxic CD4+ T cells correlated with poorer patient survival (Fu et al., 2013). Unfortunately, research exploring cytotoxic CD4+ T cells in glioblastoma is extremely limited, although one study did identify infiltration of granzyme B-producing CD4+ T cells in glioblastoma patient samples (Barcia et al., 2009).

1.2.12. CD8+ T Cell Differentiation

The cytotoxic activity of effector CD8+ T cells depends on the activation and differentiation of naïve CD8+ T cells into their effector subtypes. Naïve CD8+ T cell activation, expansion and differentiation reflects the same four-step process as CD4+ T cells, starting with DC maturation and antigen processing/presentation to naïve CD8+ T cells by MHC class I, followed by co-stimulatory signalling via CD28, 41BB and OX40. The subsequent proliferative phase enables the differentiation of naïve CD8+ T cells, driven by their exposure to a range of cytokines produced by APCs, leading to the acquisition of effector function (Curtsinger et al., 1999, 2005; C. S. Schmidt & Mescher, 2002). Effector CD4+ T cells have also been implicated in CD8+ T cell priming through the secretion of chemokines and cytokines, as well as CD40-CD40L signalling with APCs, in order to enhance antigen presentation and co-stimulatory signalling (Bennett et al., 1998; Castellino et al., 2006; Ridge et al., 1998; Schoenberger et al., 1998). For many years, effector CD8+ T cells were believed to be a homogenous population of cytotoxic cells; however, this has recently been challenged. Early studies believed that there were two subsets of effector CD8+ T cells (Tc1 and Tc2), but it has since been proposed that more subsets exist, as shown in Figure 1.16 below (Croft et al., 1994; Sad et al., 1995).

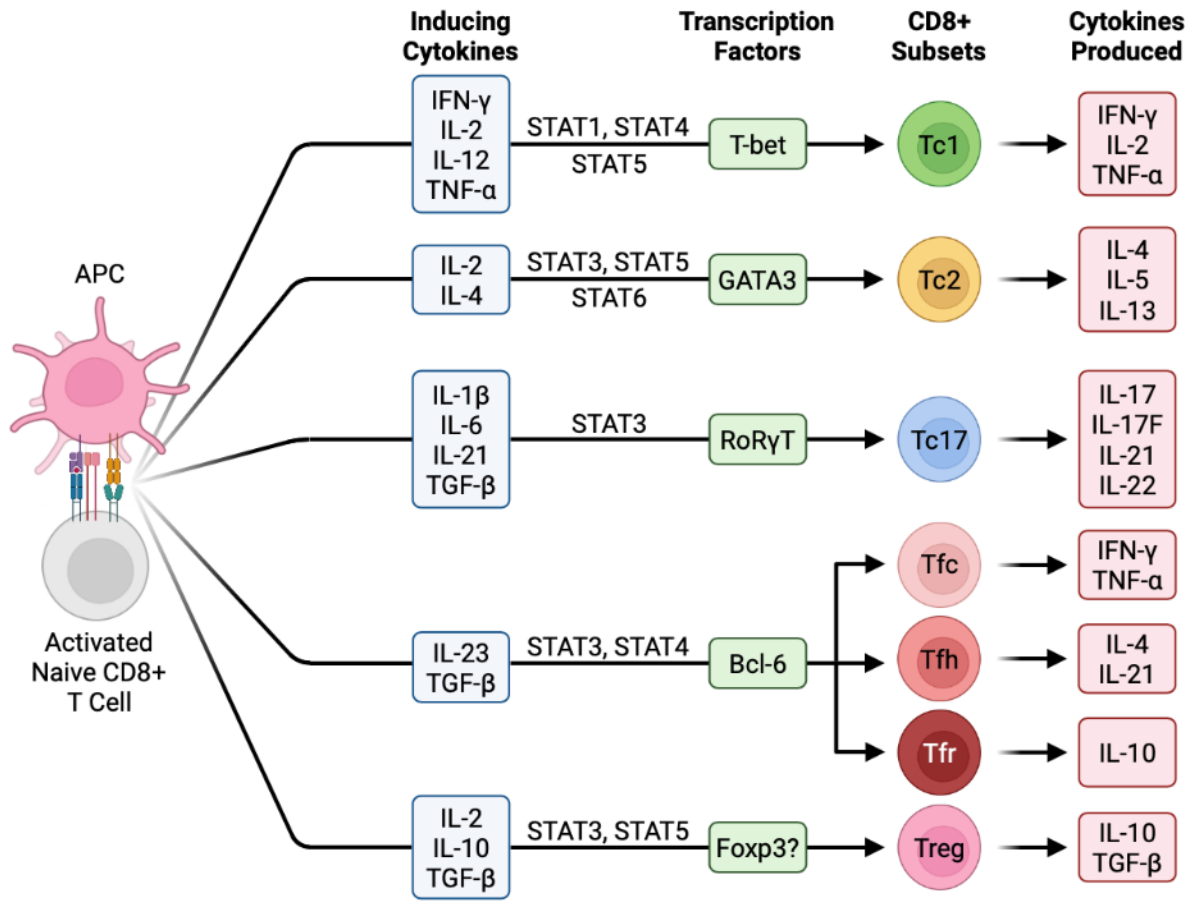


Figure 1.16. CD8+ T Cell Differentiation
Key cytokines and transcription factors involved in the differentiation of naïve CD8+ T cells into various CD8+ T cell subsets, which produce a range of cytokines. Figure generated using BioRender.com.

1.2.13. Tc1 and Tc2 Cells

Tc1 cell differentiation is similar to Th1 generation in that it is induced by exposure to IL-12, which activates BATF and STAT4, leading to upregulation of T-bet and production of IFN-γ (Croft et al., 1994; Kuroda et al., 2011; Szabo et al., 2002; Y. Yang et al., 2007). IFN-γ secretion also leads to the generation of an autocrine loop, in which IFN-γ helps to drive Tc1 lineage commitment by enhancing T-bet expression and promoting IFN-γ production (Lighvani et al., 2001). Tc1 cell differentiation is also maintained by Runx3, which co-operates with TCF-1 to induce Eomes expression – both Eomes and Runx3 promote the expression of perforin, whilst Runx3 works with T-bet to transactivate IFN-γ, perforin and granzyme B (Cruz-Guilloty et al., 2009; Intlekofer et al., 2005; McLane et al., 2013; Pearce et al., 2003; X. Zhou et al., 2010). CTLA-4 has been implicated in restricting Tc1 cell differentiation, as it is able to downregulate Eomes expression, leading to reduced production of IFN-γ, perforin and granzyme B – however, the cytotoxicity of these Tc1 cells is not fully abrogated as T-bet expression is unaffected by CTLA-4 signalling (Hegel et al., 2009; Pandiyan et al., 2007). As seen in Th1 cells, the transcription factor NFAT plays a central role in Tc1 cell differentiation by facilitating the expression of signature Tc1 cell features, including: Eomes, IFN-γ, IL-2, IL-12R, IRF4, TNF-α, T-bet, perforin and granzyme B (Klein-Hessling et al., 2017; Teixeira et al., 2005; Trifari et al., 2006; T. Xu et al., 2019). IRF4 expression directly correlates with TCR signalling strength, with stronger signalling inducing higher levels of IRF4 expression (Man et al., 2013). Research has shown that IRF4 promotes expression of Blimp-1 and T-bet, which enhance IFN-γ and granzyme B production, whilst suppressing pro-apoptotic and anti-proliferative signalling pathways; thus, not only is IRF4 vital for Tc1 cell differentiation, but it supports the expansion and survival of Tc1 cells (Yao et al., 2013).

Similar survival mechanisms are also controlled by Id2, which is upregulated in response to IL-12 and IL-2. Studies have shown that loss of Id2 results in impaired Tc1 differentiation and function, as well as increased Tc1 apoptosis, due to upregulation of Bim and loss of Bcl-2 (Cannarile et al., 2006; Knell et al., 2013; Yang et al., 2011). In addition, IL-2 signalling via STAT5 plays a crucial role in Tc1 proliferation and expansion, as well as IFN-γ production (Grange et al., 2013; Kasahara et al., 1983; Ross & Cantrell, 2018). Research has showed that the Blimp-1/Bcl-6 axis shifts in favour of Blimp-1 expression in response to IL-2, which also activates Eomes and STAT5, leading to upregulation of perforin and granzyme B (Janas et al., 2005; Pipkin et al., 2010). The expression of Blimp-1 is also supported by IL-12 activation of STAT4, which binds to the promoter region of the gene responsible for Blimp-1, in order to drive its transcription (Xin et al., 2016). On the other hand, activation of TNFR2 by TNF-α provides co-stimulatory signalling, whilst simultaneously lowering the threshold required for Tc1 activation. It has also been suggested that TNF-α is crucial for CD8+ T cell survival, as TNFR2 signalling leads to upregulation of Bcl-2, Bcl-$_X$L and survivin (Kim et al., 2006; Kim & Teh, 2001, 2004). Exposure to TNF-α also induces TNF-α secretion in Tc1 cells, albeit at lower concentrations than IFN-γ (Brehm et al., 2005; Hamann et al., 1997; Slifka & Whitton, 2000).

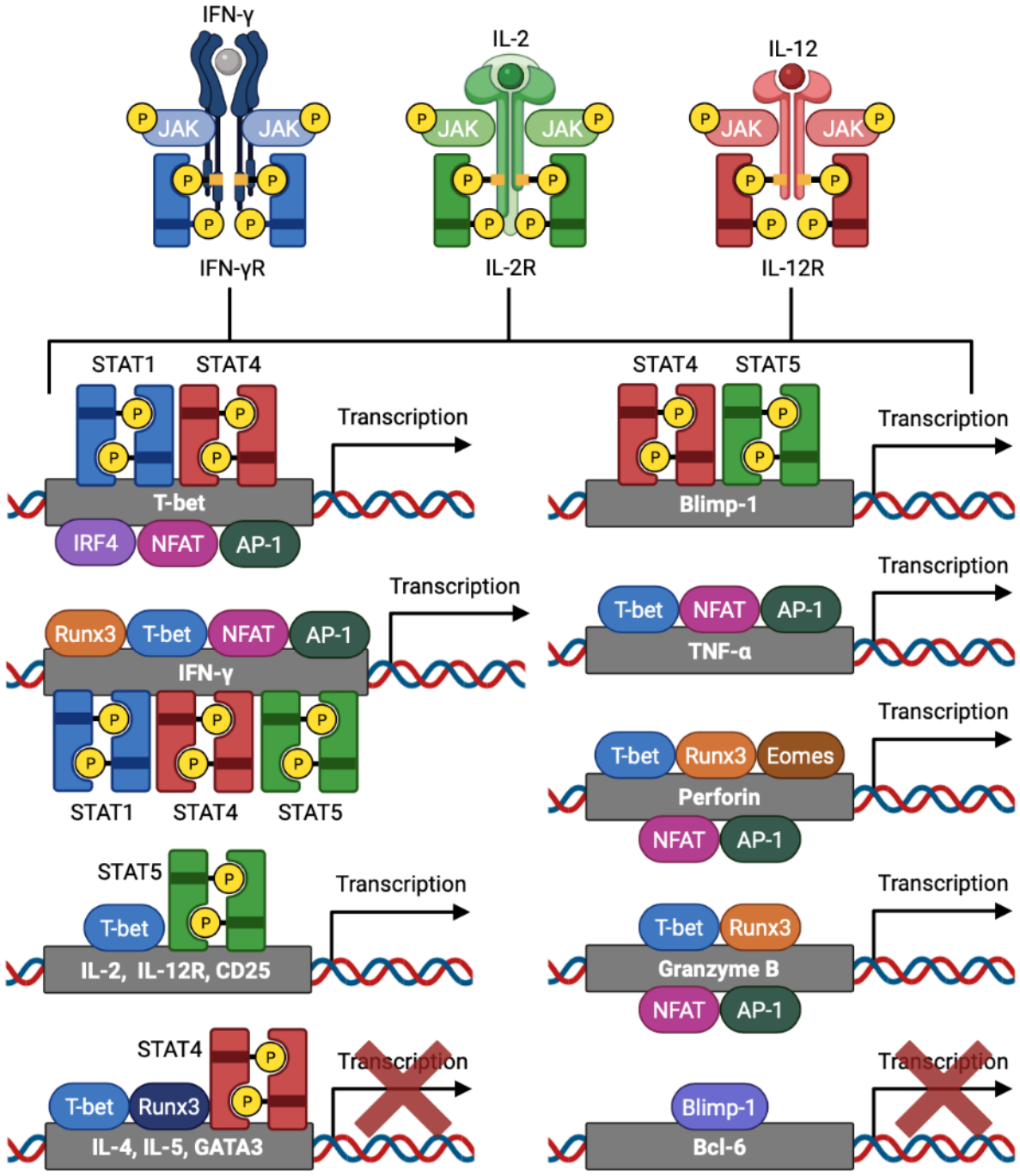


Figure 1.17. Tc1 Transcriptional Regulation

Alongside MHC-TCR and co-stimulatory signalling, IFN-γ, IL-2, IL-12, and IL-27 facilitate naïve CD8+ T cell differentiation into Tc1 cells. Figure generated using BioRender.com.

Tc1 cells highly express the chemokine receptors, CCR5 and CXCR3, which facilitate their chemoattraction to sites of inflammation. Glioblastoma cells have been shown to highly express both CCR5 and its ligand CCL5 – however, they use this to create an autocrine loop that enhances tumour growth and invasion, as well as M2 TAM polarisation, thus CCR5/CCL5 expression correlates with poor patient prognosis (Kouno et al., 2004; Laudati et al., 2017; Y. Pan et al., 2017; L. Zhao et al., 2015). Tregs also highly express CCR5 and have been shown to migrate towards CCL5-producing tumour cells, which could explain the infiltration of Tregs in glioblastoma patient samples (S. Han et al., 2014; Tan et al., 2009). Altogether, these studies have indicated that abrogating the immunosuppressive nature of the glioblastoma microenvironment could enhance infiltration of Tc1 cells and promote tumour rejection. This is reflected in studies which correlated enhanced intratumoural Tc1 cell infiltration with increased long-term survival in glioblastoma patients (Mauldin et al., 2021; I. Yang et al., 2010). Similarly, several studies have shown that enhancing DC production of the CXCR3 ligand CXCL10 increases Tc1 infiltration and subsequently reduces tumour growth and angiogenesis, thereby improving overall survival in glioblastoma mouse models (Fujita et al., 2008; X. Jiang et al., 2009; Nishimura et al., 2006; Ohkuri et al., 2021).

Whilst the extent of their cytotoxic abilities is largely debated, researchers unanimously agree than Tc2 cells are significantly less cytotoxic than Tc1 cells (Fox et al., 2018; Halverson et al., 1997; Kemp & Ronchese, 2001; Vukmanovic-Stejic et al., 2000). Tc2 cells can also be distinguished from Tc1 cells by their expression of the chemokine receptors CCR4 and CRTH2 (Cosmi et al., 2000; D'Ambrosio et al., 1998; Loyal et al., 2020). Differentiation of Tc2 cells largely reflects Th2 cells in that it is induced by IL-4, which leads to the secretion of IL-4, IL-5 and IL-13 (Croft et al., 1994; Salgame et al., 1991; Seder et al., 1992). Exposure to IL-4 also creates a positive feedback loop by activating STAT3 and subsequently increasing IL-4Rα expression (Deimel et al., 2021). In addition, IL-4 activates STAT6, which transactivates GATA3 to drive Tc2 differentiation (Maier et al., 2012). Research has shown that loss of STAT6 in CD8+ T cells reduces their ability to differentiate into Tc2 cells, likely due to diminished GATA3 expression (M. H. Kaplan et al., 1999).

Interestingly, studies have shown that Tc2 cells have lower GATA3 expression and produce less IL-4 than Th2 cells – this is believed to be due to the expression of repressor of GATA3 (ROG) in Tc2 cells, which limits GATA3 activation and the subsequent transcription of IL-4 (Omori et al., 2003; Pai et al., 2004). Nonetheless, GATA3 is still crucial for Tc2 polarisation and key cytokine secretion: loss of GATA3 in Tc2 cells leads to reduced production of IL-4 and IL-5, whilst overexpression of GATA3 in CD8+ T cells activated in non-polarising conditions results in an increase in IL-4 and IL-13 expression (Lavenu-Bombled et al., 2002; Pai et al., 2004). The production of Tc2 cytokines is driven by GATA3 binding to the promoter region of IL-4, IL-5 and IL-13 genes to induce their transcription (Lavenu-Bombled et al., 2002; G. Wei et al., 2011). Furthermore, GATA3 can skew cells away from Tc1 polarisation by suppressing expression of Runx3, which promotes IFN-γ, perforin and granzyme B expression (Cruz-Guilloty et al., 2009; Loyal et al., 2020; Yagi et al., 2010). However, T-bet also

influences Tc1 and Tc2 lineage commitment, as seen in Th1 and Th2 cell polarisation. Research has shown that overexpression of T-bet in CD8+ T cells activated in Tc2 polarising conditions led to dramatic increase in IFN-γ production and significant decrease in IL-4 and IL-5 secretion, indicating that T-bet skewed cells towards a Tc1 phenotype (Szabo et al., 2000). These findings were also reflected in T-bet deficient CD8+ T cells, which showed an increase in IL-4 and IL-5 production, whilst IFN-γ secretion was lost, despite activation occurring in non-polarising conditions (Sullivan et al., 2003).

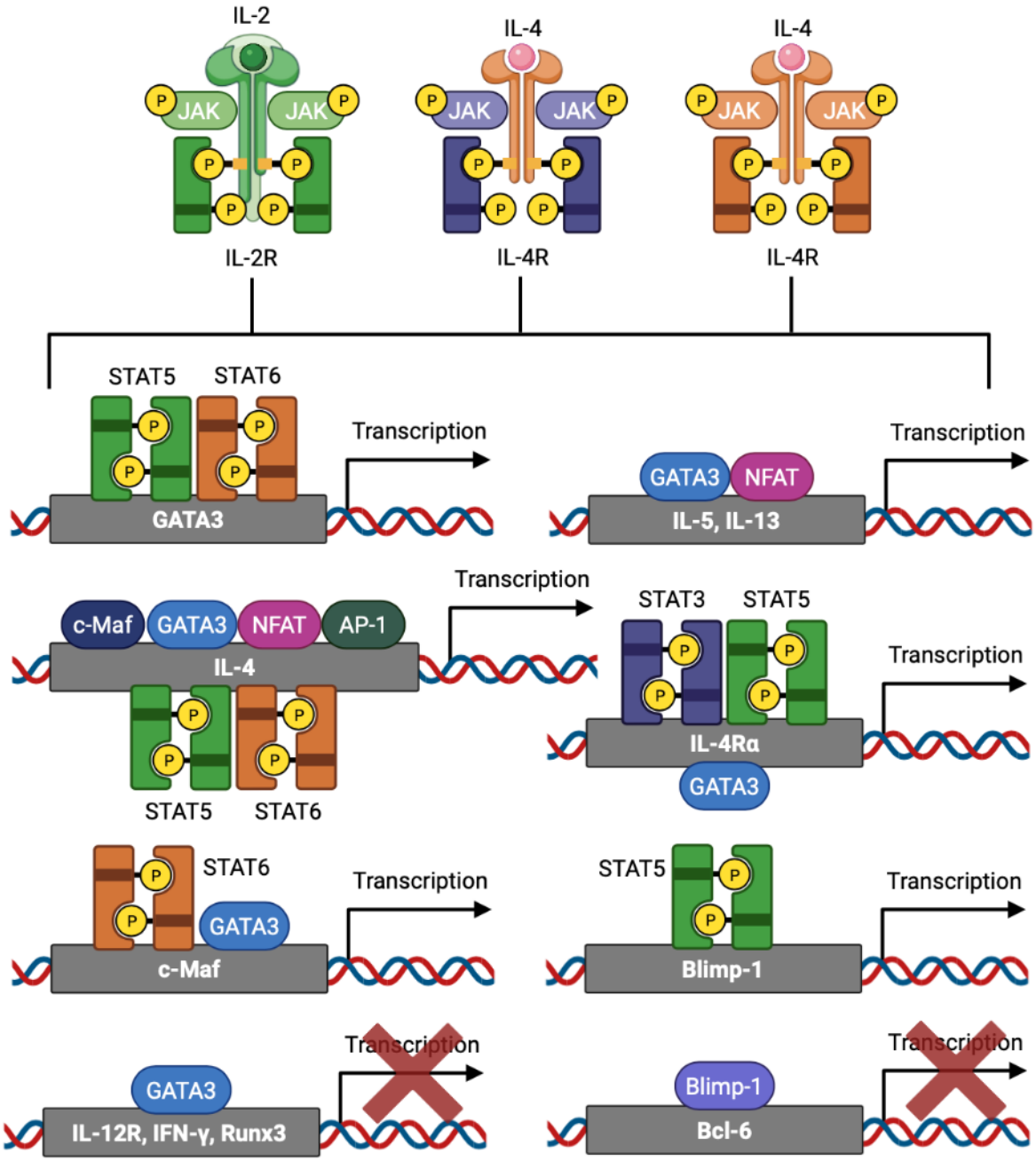


Figure 1.18. Tc2 Transcriptional Regulation

Alongside MHC-TCR and co-stimulatory signalling, IL-2 and IL-4 facilitate naïve CD8+ T cell differentiation into Tc2 cells. Figure generated using BioRender.com.

In line with Th1 and Th2 cells, the ratio between Tc1 and Tc2 cells also appears to be important for anti-tumour immunity as skewing the Tc1/Tc2 ratio towards Tc2 cells has been associated with disease progression in patients with salivary gland tumours, B-cell chronic lymphocytic leukaemia, and lung carcinoma (Haghshenas et al., 2016; Ito et al., 1999; Podhorecka et al., 2002). Whilst data exploring Tc2 cells in glioblastoma is limited, genetic analyses revealed downregulation of genes involved in MHC class I binding and cytotoxicity, plus reduced production of IL-2, IFN-γ and TNF-α production in CD8+ T cells isolated from glioblastoma patients (Huff et al., 2021; Woroniecka et al., 2018). Previous research linked the decline of Th1 cytokines with an increase in Th2 cytokine expression and generation of an immunosuppressive microenvironment, as discussed previously, thus it is plausible that Th2 and Tc2 cells co-operate to suppress Th1 and Tc2 cell polarisation and function in glioblastoma patients (Harshyne et al., 2015; Hoepner et al., 2013; Kumar et al., 2006; Shimato et al., 2012; Takashima et al., 2018; Zisakis et al., 2007). Collectively, previous studies have indicated that correcting the ratio of Th1/Th2 and Tc1/Tc2 cells by reducing immunosuppression and subsequently increasing the 'Type 1' immune response could evoke a potent anti-tumour immune response in glioblastoma.

1.2.14. Tc17 Cells

Tc17 cells are a distinct subset of CD8+ T cells that express RoRγT and produce a number of cytokines, including: IL-17, IL-17F, IL-21 and IL-22 (Intlekofer et al., 2008; Kryczek et al., 2007; Ortega et al., 2009; Yen et al., 2009). Similar to Th17 cells, their differentiation is primarily driven by the combination of IL-6 and TGF-β, but it can be enhanced by the addition of IL-1β and IL-21 (He et al., 2006; Huber et al., 2009; Intlekofer et al., 2008; Kondo et al., 2009; Yen et al., 2009). IL-23 was originally implicated in Tc17 differentiation but, as research revealed that IL-23R is only expressed by activated T cells, it appears that IL-23 only promotes Tc17 proliferation (S. Aggarwal et al., 2003; Ciric et al., 2009; Curtis et al., 2009; Kuang et al., 2010; Parham et al., 2002). Research has demonstrated that TGF-β activates both Smad2 and Smad3 in CD8+ T cells, which facilitate expression of c-Maf and RoRγT to polarise cells towards Tc17 lineage commitment (Carrasco Hope, 2021). TGF-β also appears to limit the cytotoxic activity of Tc17 cells by suppressing IFN-γ, perforin and granzyme B expression (Huber et al., 2009; S.-J. Liu et al., 2007; Yen et al., 2009). However, Dwivedi et al. (2012) identified Tc17-mediated intestinal inflammation in mice with a dominant negative form of the TGF-β receptor, suggesting that the loss of TGF-β did not affect *in vivo* Tc17 differentiation, but this requires further investigation in humans.

Nevertheless, Tc17 differentiation is driven by IL-6 activation of STAT3, in order to promote RoRγT expression, which subsequently transactivates IL-17, IL-17F, IL-21 and IL-22 (Arra et al., 2017a; Durant et al., 2010). It is also believed that the production of IL-21 induced by STAT3, as well as the expression of IL-21R and IL-23R, creates a feedback loop for STAT3 to drive Tc17 polarisation, as seen in Th17 cells (Huber et al., 2009; Korn et al., 2007; Zhou et al., 2007). Loss of either STAT3 or RoRγT in CD8+ T cells significantly reduces IL-17 production and Tc17 differentiation, skewing cells

towards a Tc1 phenotype by upregulating IFN-γ, T-bet and granzyme B (Ciucci et al., 2017; El-Behi et al., 2014; Huber et al., 2009). IFN-γ has also been shown to reduce STAT3 phosphorylation and induce both T-bet and Eomes in CD8+ T cells, leading to inhibition of RoRγT activity and subsequently repressing Tc17 differentiation (Ciucci et al., 2017; Intlekofer et al., 2008; Zha et al., 2017). Whilst loss of T-bet alone in CD8+ T cells can promote Tc17 differentiation, this effect can be enhanced by eliminating Blimp-1 expression as well, as Xin et al. (2016) showed that T-bet and Blimp-1 double-deficient CD8+ T cells expressed IL-17 and RoRγT, whilst expression of IFN-γ, granzyme B, perforin and Fas-L were significantly reduced.

Recently, two IRF family members have also been implicated in Tc17 differentiation. In particular, the transcription factor IRF3 has been shown to suppress Tc17 differentiation by binding directly to RoRγT to restrict IL-17 production (Ysebrant de Lendonck et al., 2013). On the other hand, Huber et al. (2013) showed that Tc17 differentiation was compromised in IRF4$^{-/-}$ CD8+ T cells due to upregulation of Eomes and Foxp3, alongside reduced RoRγT and IL-17 expression, despite being cultured in Tc17 polarising conditions. Whilst inducing overexpression of RoRγT in these IRF4-deficient CD8+ T cells did stimulate production of IL-17, it was significantly lower than control cells; thus, IRF4 plays a vital role in promoting Tc17 differentiation. However, Mielke et al. (2019) discovered that IRF4 expression is reduced in Tc17 cells compared to other CD8+ effector subsets. Interestingly, they also identified that the transcription factor TCF-1 binds directly to the regulatory regions of genes responsible for c-Maf and RoRγT to restrict their transcription, resulting in a significant reduction in Tc17 polarisation. Therefore, in the absence of TCF-1 expression, CD8+ T cells significantly upregulate RoRγT and IL-17, as well as IL-1R1, IL-23R, CCR4, CCR6 and CCR9 (Mielke et al., 2019). Other chemokine receptors expressed by Tc17 cells include CXCR3, CXCR6, CCR5, and CD161 – research has suggested that the variety of chemokine receptors is linked to the substantial presence of Tc17 cells in tissues with chronic inflammation (Billerbeck et al., 2010; Kondo et al., 2009; Oo et al., 2012).

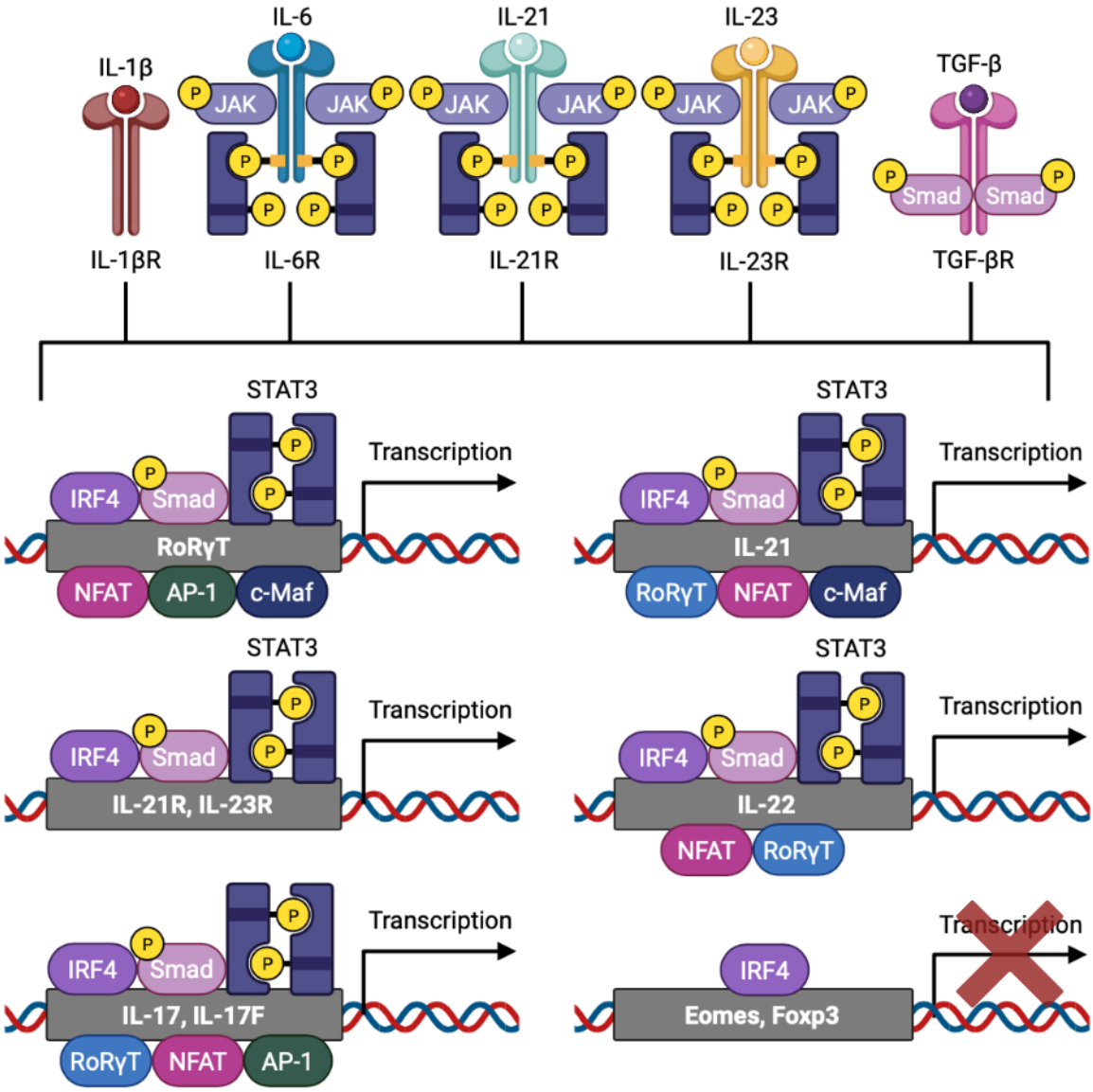


Figure 1.19. Tc17 Transcriptional Regulation

Alongside MHC-TCR and co-stimulatory signalling, IL-1β, IL-6, IL-21, IL-23, and TGF-β facilitate naïve CD8+ T cell differentiation into Tc17 cells. Figure generated using BioRender.com.

Using adoptive transfer in a mouse model of pulmonary inflammation, Yen et al. (2009) discovered that Tc17 cells have functional plasticity, showing that Tc17 cells transitioned to a Tc1/Tc17 hybrid phenotype, with higher IFN-γ and TNF-α production than Tc1 cells, whilst maintaining IL-17 secretion. In addition, macrophages express IL-17R and begin secreting IL-12 in response to IL-17 exposure, creating a cytokine milieu that could polarise Tc17 cells towards Tc1 lineage development (Barin et al., 2012). On the other hand, Chen et al. (2016) showed that Tc1 cells could be converted to this Tc1/Tc17 phenotype, producing both IFN-γ and IL-17, when cultured with TGF-β and IL-21. This Tc1/Tc17 plasticity has been seen in a range of experimental models, including: protection against viral infection (e.g. influenza) and autoimmune disorders (e.g. Type 1 diabetes), whilst promoting anti-tumour immunity against melanoma (Hamada et al., 2009; Saxena et al., 2012; Yeh et al., 2010; Yu et al., 2013). However, Tc1/Tc17 cells were less cytotoxic than Tc1 cells in an adoptive transfer mouse model of glioblastoma, whilst IL-17 production was also significantly reduced in Tc1/Tc17 cells after transfer (Ohkuri et al., 2021). As Tc1/Tc17 and Tc1 cells expressed similar levels of IFN-γ, it was suggested that Tc1 cells were more effective in restricting tumour growth, due to their high expression of VLA-4, which enables them to cross the BBB – this was further supported by the increased presence of Tc1/Tc17 cells in peripheral blood compared to Tc1 cells, demonstrating their exclusion from the brain (Ohkuri et al., 2021; Sasaki et al., 2012).

Interestingly, Pick et al. (2014) linked CTLA-4 signalling with the plasticity of Tc17 cells, as CTLA-4$^{-/-}$ Tc17 cells downregulated IL-23R, RoRγT, IRF4, IL-17 and IL-21 expression, whilst acquiring significant production of IFN-γ and TNF-α. In addition, the loss of IL-17 production in these CTLA-4$^{-/-}$ Tc17 cells could not be restored with overexpression of RoRγT or IRF4. In a follow-up study, Arra et al. (2017) reported that CTLA-4 enhanced STAT3 activation and IL-17 production, but this effect was lost in Tc17 cells that lacked CTLA-4 expression, as STAT5 bound to the promoter region of the IL-17 gene instead of STAT3, thereby inhibiting its transcription. Furthermore, RoRγT and IL-23R expression were significantly reduced in CTLA-4 deficient Tc17 cells; thus, STAT3 activity mediated by CTLA-4 activation is vital for Tc17 differentiation and function (Arra et al., 2017b). Previous research by other groups identified that CTLA-4 downregulated expression of Eomes in Tc1 cells, thereby reducing the expression of IFN-γ, perforin and granzyme B (Hegel et al., 2009; Pandiyan et al., 2007). This signalling pathway also appears to be present in Tc17 cells, as loss of CTLA-4 in Tc17 cells resulted in enhanced Eomes expression and IFN-γ production (Arra et al., 2017a). Altogether, these studies have indicated that CTLA-4 has a crucial role in Tc17 differentiation and stability, as the absence of this signalling leads to the development of a Tc1 phenotype.

1.2.15. CD8+ Tfh/Tfc/Tfr Cells

Despite several studies suggesting that CD8+ T cells were excluded from entry into B cell follicles, Förster et al. (1996) identified a distinct population of CD8+ T cells that expressed CXCR5 in the peripheral blood of healthy human donors (Connick et al., 2007; Fukazawa et al., 2015; Hong et al., 2012). In support of this, Quigley et al. (2007) found CD8+ T cells in human tonsil B cell follicles, which were further characterised by their expression of CXCR5 and lack of CCR7 expression, providing a mechanism for their migration into germinal centres. Unlike Tfh cells, these CD8+CXCR5+ T cells expressed CCR5, indicating that these cells had the capacity to exit B cell follicles, in order to migrate to peripheral sites of inflammation, as seen with the presence of a small population of CD8+CXCR5+ T cells in peripheral blood. However, follicular CD8+CXCR5+ T cells had different markers to those found in tonsils, which expressed CD45RA and CD62L, but had reduced CCR5 and CD69 expression, indicating that these could be T_{EMRA} cells rather than CD8+CXCR5+ T cells that have recently left the follicle, but this has not yet been clarified (Quigley et al., 2007).

Research has shown that the expression of CXCR5 in murine CD8+ T cells is induced by IL-6, but it appears that TGF-β and IL-23 are responsible for the induction and expansion of human CD8+CXCR5+ T cells (Chu et al., 2019; Mylvaganam et al., 2017; Perdomo-Celis et al., 2019; H.-G. Yang et al., 2020). In line with CD4+ Tfh cells, the development of these CD8+CXCR5+ cells is supported by a range of transcription factors, including: BATF, Bcl-6, c-Maf, IRF4 and NFAT (Y. Chen et al., 2019). Using *in vitro* co-culture experiments, CD8+CXCR5+ T cells have been shown to promote B cell survival and IgG production more effectively than CD8+CXCR5- T cells (Quigley et al., 2007). Interestingly, B cell proliferation, plus IgG and IgA secretion, were enhanced when B cells were cultured with both CD8+CXCR5+ T cells and CD4+ Tfh cells, compared to co-cultures with CD4+ Tfh cells alone, demonstrating the importance of CD8+CXCR5+ T cells in supporting germinal centre activity (H. Jiang et al., 2017; Le et al., 2018). Other studies have identified that CD8+CXCR5+ T cells express a range of cell surface markers including CD40L, CD70, OX40, and/or ICOS, whilst also producing IL-4 and IL-21, thus it is appears that they act as CD8+ Tfh cells to directly enhance the activity of both CD4+ Tfh and B cells (Im et al., 2016; H. Jiang et al., 2017; Leong et al., 2016; Quigley et al., 2007; Wagner et al., 1998).

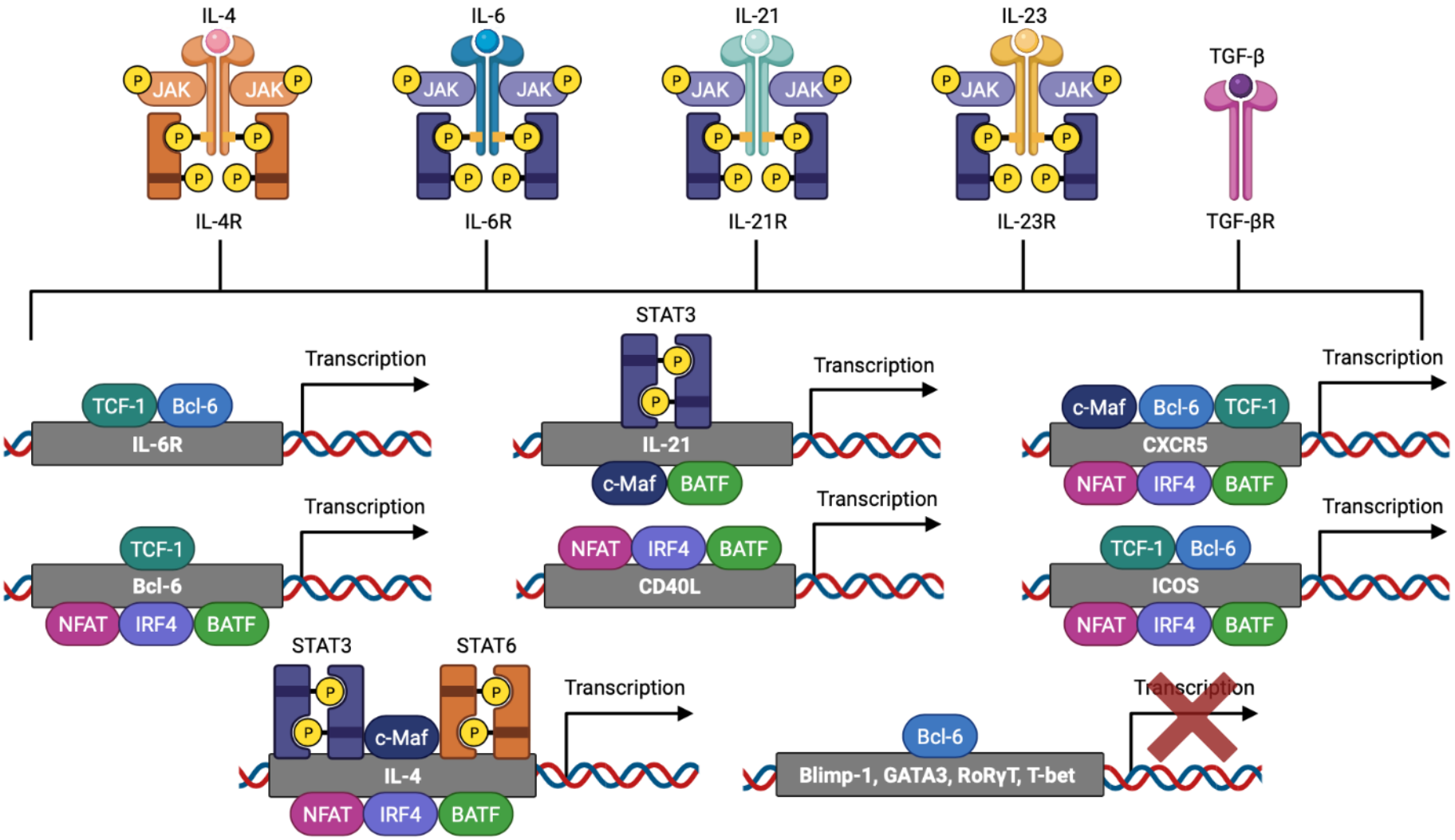


Figure 1.20. CD8+ Tfh Transcriptional Regulation

Alongside MHC-TCR and co-stimulatory signalling, IL-1β, IL-4, IL-6, IL-12, IL-21, IL-23, IL-27, and TGF-β facilitate naïve CD8+ T cell differentiation into CD8+ Tfh cells. Figure generated using BioRender.com.

Conversely, studies exploring the role of CD8+CXCR5+ T cells in chronic viral infections have found that these cells also express highly variable cytotoxic activity, acting as follicular cytotoxic (Tfc) cells. In particular, CD8+CXCR5+ T cells from HIV-infected patients have been shown to produce both IFN-γ and TNF-α, whilst upregulating CD107 and perforin, thus they can migrate into B cell follicles to eliminate virus-infected CD4+ Tfh cells and B cells (R. He et al., 2016; Leong et al., 2016). Likewise, Y. Chen et al. (2019) identified a population of CD8+CXCR5+PD-1- T cells in mice, which highly expressed IFN-γ, granzyme B and Fas-L, indicating highly cytotoxic abilities. In addition, increasing infiltration of CD8+CXCR5+ T cells in hepatocellular carcinoma patients was shown to correlate with increased survival and reduced risk of recurrence (L. Ye et al., 2019). On the other hand, B. Miles et al. (2016) identified a population of CD8+CXCR5+ T cells in *ex vivo* HIV-infected human tonsils, which lacked CCR7 expression, but had enhanced expression of TIM-3 and IL-10, compared to CD8+CXCR5- T cells, which they termed CD8+ Tfr cells. Results showed that CD8+ Tfr cells suppressed the production of IL-2 and IL-21 by Tfh cells via TIM-3, whilst co-culture experiments showed that Tfr cells inhibited IgG production by B cells in the germinal centre. In addition, CD8+ Tfr provided resistance against HIV by reducing HIV replication and inducing apoptosis in HIV-infected CD4+ Tfh cells, thus CD8+ Tfc and CD8+ Tfr play a central role in impairing germinal centre activity during HIV infection (Miles et al., 2016). The range of functions demonstrated by CD8+CXCR5+ T cells has led to the suggestion that CD8+ Tfc and CD8+ Tfr cells are either transitioning to/from a follicular phenotype, or that the CD8+CXCR5+ T cell population is quite heterogenous, with various subsets that have not yet been fully characterised (Perdomo-Celis et al., 2017).

Research has shown that TCF-1 is vital for CXCR5 expression in CD8+ T cells, as downregulation of TCF-1 leads to upregulation of Blimp-1, TIM-3, CCR5, CCR2, CXCR6, IFN-γ and TNF-α, reflecting the genetic signature of Tc1 cells. This is due to the fact that TCF-1 regulates the transcriptional activity of Bcl-6 and Blimp-1, thus CD8+TCF-1^{high} T cells express high levels of CXCR5, Bcl-6 and OX40, whilst downregulating TIM-3 and Blimp-1, reflecting a CD8+ Tfh cell phenotype (Danilo et al., 2018; Teijaro et al., 2013; T. Wu et al., 2016). It recently been proposed by numerous groups that these CD8+TCF-1^{high}TIM-3^{low} T cells are precursor exhausted T (T_{PEX}) cells that sustain long-term T cell responses during chronic viral infection through their self-renewal and differentiation into terminally exhausted CD8+ T (T_{EX}) cells. In particular, it has been shown that T_{PEX} cells differentiate into intermediary CD8+CD101-TIM-3+ effector T cells, which upregulate expression of CX3CR1 to facilitate migration to sites of inflammation, as well as the expression of genes associated with cytotoxic function, including: T-bet, Blimp-1, granzyme B, perforin, IFN-γ, TNF-α, Fas-L and TRAIL. However, persistent antigen exposure results in the generation of CD8+CD101+TIM-3+ T_{EX} cells with limited effector capacity and increased expression of PD-1, CD244, CD160 and LAG-3 (Hudson et al., 2019; Im et al., 2016; Im & Ha, 2020; Kallies et al., 2020; Paley et al., 2012; Utzschneider et al., 2016). In response to PD-1 blockade, several studies have shown that T_{PEX} enhanced CD28-mediated signalling to increase their proliferation and differentiation into intermediary and terminally differentiated T_{EX} cells, thereby

improving viral/tumour clearance through enhanced cytotoxic activity (Brummelman et al., 2018; Hui et al., 2017; Im et al., 2016; Kamphorst et al., 2017; Miller et al., 2019; Pauken et al., 2016; Sade-Feldman et al., 2018; Siddiqui et al., 2019). However, research has shown that CD4+ Tfr and CD4+ Tregs are enriched in glioblastoma tissues, which were highly effective in suppressing the proliferation and cytotoxic activity of CD8+CXCR5+ T cells in co-culture experiments, thereby preventing an anti-tumour immune response (L. Lu et al., 2021).

1.2.16. CD8+ Treg Cells

The existence of regulatory CD8+ T cells was first proposed many years ago by Gershon & Kondo (1970), but they have largely been neglected as researchers struggled to identify unique markers. However, interest in CD8+ Tregs has steadily been increasing, after they were shown to express CD25 and implicated in resistance against a murine model of multiple sclerosis by inhibiting autoreactive CD4+ T cells (H. Jiang et al., 1992, 1998; Sakaguchi et al., 1995). As the majority of CD8+ Treg studies have been completed using mice, key markers for human CD8+ Tregs are still under investigation, thus numerous phenotypes have been proposed. Nevertheless, both CD25 and Foxp3 have been identified on a subset of human CD8+ T cells, which expand in response to IL-2, IL-7, and IL-15, whilst suppressing effector T cell proliferation via CTLA-4, TGF-β and IL-10, demonstrating functional similarities with CD4+ Tregs (Churlaud et al., 2015; Cosmi et al., 2003; Imamichi et al., 2008). In addition, some human CD8+CD25+Foxp3+CTLA-4+ T cells express Lag3 and secrete CCL4, providing a mechanism in which they can attract CCR5+ T cells, in order to inhibit their activation and proliferation (Joosten et al., 2007). In particular, CD8+CD25+Foxp3+ T cells have been identified as an immunosuppressive population in several cancer types, but their presence and function in glioblastoma is largely unknown (Chaput et al., 2009; Kiniwa et al., 2007).

Ordonez et al. (2009, 2013) identified an immunosuppressive CD8+CD45RC^{low} T cell population that secreted IL-4, IL-5 and IL-10. However, a subset of these CD8+CD45RC^{low} T cells also express Foxp3 and expand in response to IL-2 and IL-15, whilst producing IL-10, IL-34 and TGF-β. In particular, IL-34 has been shown to induce the differentiation of M2 macrophages, which enhance the immunosuppressive microenvironment by potently producing IL-10 and promoting CD8+CD45RC^{low} Treg proliferation (Bézie et al., 2018; Foucher et al., 2013). It has also been suggested that the regulatory effects of CD8+CD45RC^{low} T cells are enhanced by signals provided by APCs that have become tolerogenic due to exposure to IFN-γ and IDO (Bézie et al., 2015; Guillonneau et al., 2007; X. L. Li et al., 2010). Research has shown that IL-34 receptor (CSF-1R) inhibition prevents polarisation of M2 TAMs and their subsequent recruitment to the tumour site, thereby restricting glioblastoma growth and angiogenesis (Achyut et al., 2015; Stafford et al., 2016).

Another subset of CD8+Foxp3+ T cells are CD8+CD28- T cells, which have been shown to directly inhibit CD40-mediated upregulation of CD80 and CD86 on DCs, thus they upregulate ILT3/ILT4 and become tolerogenic, thereby inducing effector T cell anergy (Cella, Döhring, et al., 1997; C. C. Chang

et al., 2002; Correale & Villa, 2010; J. Li et al., 1999; Manavalan et al., 2003; J. Wu & Horuzsko, 2009). In addition, allogeneic CD40L-activated plasmacytoid DCs, which express high levels of ILT3 and ILT4, have been shown to promote naïve CD8+ T cell differentiation into CD8+CD28- T cells, suggesting that there is a positive feedback loop in which CD8+CD28- T cells generate tolerogenic APCs that then promote CD8+CD28- differentiation, leading to inhibition of effector T cell proliferation via IL-10 secretion (Gilliet & Liu, 2002). Furthermore, *in vitro* co-culture experiments revealed that APC-mediated differentiation of CD8+CD28- T cells could be supported by exposure to IL-2, but this was further enhanced with the addition of IL-7 and IL-15 (Y. Yu et al., 2011; L. Zhang et al., 2009). Nevertheless, tumour-infiltrating CD8+CD28-Foxp3+ Tregs have been identified in glioblastoma patients, alongside ILT3 and ILT4-expressing APCs. The tolerisation of APCs was reflected in the fact that only a small percentage of APCs expressed CD40 or CD27, whilst all of the tumour-infiltrating T cells lacked CD40L and CD70 expression, thus the lack of co-stimulatory signalling helped to promote immunosuppression in glioblastoma (Kmiecik et al., 2013).

However, the regulatory activity of CD8+ T cells does not appear to be depend on Foxp3 expression, as several populations of CD8+Foxp3- T cells have demonstrated immunosuppressive effects. For example, naïve CD8+ T cells upregulate CD103 expression in response to IL-4, IL-10 or TGF-β – research has shown that these CD8+Foxp3-CD103+ T cells secrete high levels of IL-10 and TGF-β. Although the exact mechanisms are unknown, they are believed to mediate immunosuppressive effects on both T and B cells through cell-to-cell contact (Y. Liu et al., 2014; Uss et al., 2006; Zhong et al., 2018). Both CD8+CD28- and CD8+CD103+ T cells express CD39 and CD73, which are ectonucleotidases that generate adenosine to moderate T cell activity: CD39 hydrolyses ATP and ADP into AMP, which is then converted into adenosine by CD73 (Deaglio et al., 2007; Kmiecik et al., 2013; Robson et al., 2006; Yegutkin, 2008; X. Zhang et al., 2019). Extracellular adenosine binds to the A2A receptor on effector T cells, initiating a variety of downstream signals that restrict their activation, proliferation and cytotoxic activity (Cekic & Linden, 2014; S. Huang et al., 1997; Lappas et al., 2005; Sorrentino et al., 2019). However, tumour-infiltrating CD8+CD39+CD103+ T cells have also been shown to be able to induce tumour cell death through their expression of PD-1, CTLA-4, TIM-3 and granzyme B (Duhen et al., 2018; Kortekaas et al., 2020). This indicates that there are two unique populations of CD8+Foxp3-CD103+ T cells that mediate immunosuppression either directly or indirectly, but the factors that regulate their activity are not fully known – although, research has shown that upregulation of CD73 expression on T cells can be facilitated by glioblastoma-derived EVs (M. Wang et al., 2021).

Interestingly, a subset of regulatory CD8+ T cells were identified in mice that did not express CD25 or expand in response to IL-2, instead they overexpressed CD122 and required IL-15 for expansion, whilst relying on PD-1/PD-L1 signalling for IL-10 production (H. Dai et al., 2010; Z. Dai et al., 2014; X. Zhang et al., 1998). Despite their ability to suppress T cell proliferation and IFN-γ production by secreting IL-10, it also appeared that they maintained immune homeostasis by inducing cell death in effector T cells via Fas-FasL signalling (Akane et al., 2016; Endharti et al., 2005; H. Liu et al., 2017). Genetic

analyses revealed that the human equivalent of murine CD8+CD122+PD-1+ T cells were CD8+CXCR3+ T cells, which utilise CXCR3 to facilitate their migration alongside other CXCR3-expressing cells, thus they can accumulate and efficiently suppress Th1 and Tc1 cells by secreting IL-10 (Rifa'i et al., 2004; Z. Shi et al., 2009). Remarkably, another subset of human CD8+ T cells that is reliant on IL-15 for expansion has been identified, though these CD8+ T cells express Foxp3 and retain a naïve phenotype through the expression of CD45RA and CCR7. Research has shown that CD8+CD45RA+CCR7+ T cells do not require IL-10, TGF-β, CTLA-4 or adenosine to mediate their immunosuppressive effects, instead they appear to interfere with TCR signalling in CD4+ T cells, leading to downregulation of CD40L and CD25 expression, thereby reducing IL-2 uptake and T cell activation (Suzuki et al., 2012).

Whilst the cytotoxicity of regulatory CD8+ T cells is largely unknown, a subset of CD8+ T cells have been shown to directly induce apoptosis in CD4+ T cells. In particular, these CD8+ T cells highly express CD56, which is more commonly associated with NK cells – however, these CD8+CD56+ T cells do not co-express CD161, Vα24 or Vβ11, which are other characteristic markers of NK cells (Bendelac et al., 2007; D. Hu et al., 2013; Kronenberg, 2005). Research has linked IL-15 exposure with the acquisition of CD56 in CD8+ T cells, whilst identifying that CD8+CD56+ T cells have high expression levels of CCL5, IFN-γ, TNF-α, granzyme B and perforin (Cookson & Reen, 2003; Correia et al., 2009, 2011; D. Hu et al., 2013; Kelly-Rogers et al., 2006; Pittet et al., 2000). Although these CD8+CD56+ T cells have functional similarities with cytotoxic T cells, CD8+CD56+ T cells can be identified by their high expression of CD94 and Bcl-2, alongside low expression of CD62L and CD27 upon activation (Correia et al., 2011; D. Hu et al., 2013).

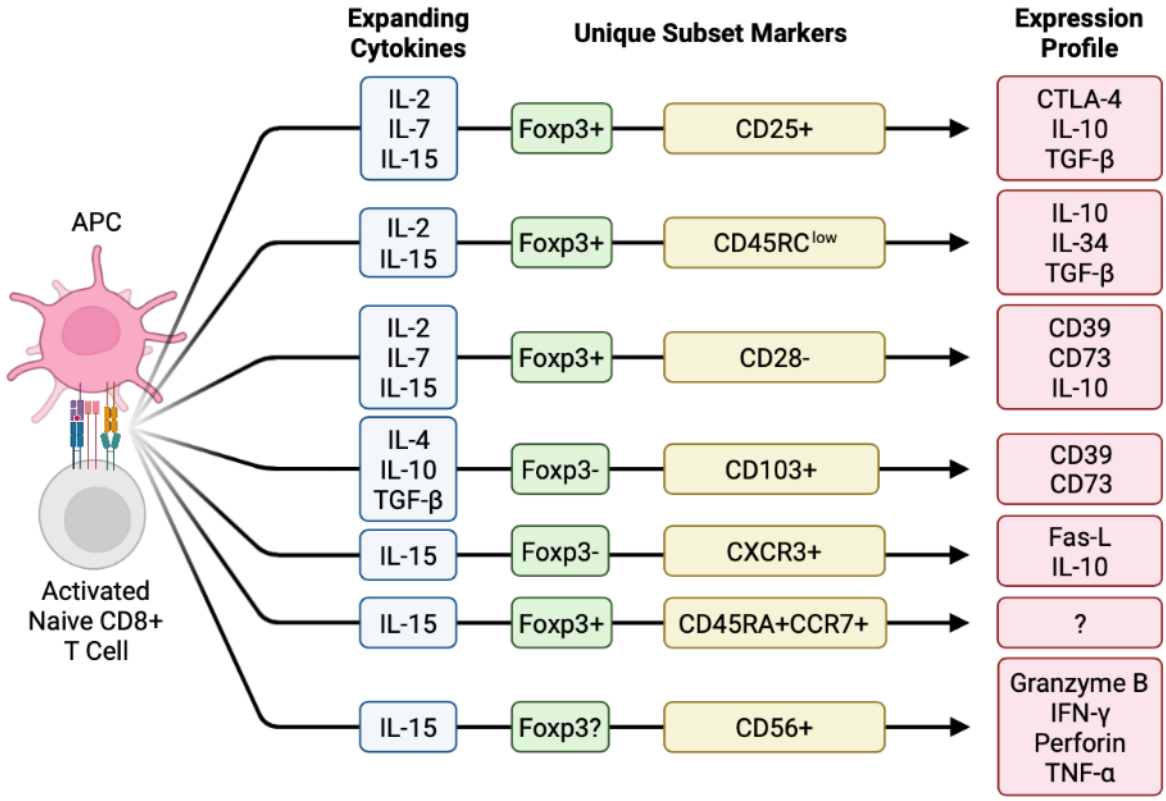


Figure 1.21. CD8+ Treg Subsets

Several CD8+ T cell subsets have been identified with varying Foxp3 expression and mechanisms of inducing immunosuppression. Figure generated using BioRender.com.

1.3. Cytokine Therapies

1.3.1. Therapeutic Targets

Based upon our current knowledge, it appears that there are several mechanisms to target therapeutically in glioblastoma patients. In particular, it is important to alter the glioblastoma TME to shift Th1/Th2 and Tc1/Tc2 ratios in favour of Th1 and Tc1 cells, in order to promote an anti-tumour immune response, as seen in studies which have correlated this pro-inflammatory profile with improved glioblastoma patient survival (Harshyne et al., 2015; Hoepner et al., 2013; Kumar et al., 2006; Shimato et al., 2012; Takashima et al., 2018; Zisakis et al., 2007). Conversely, it is arguably more vital to eliminate the immunosuppressive control CD4+ and CD8+ Tregs have on the glioblastoma TME, so as to elicit an organic anti-tumour immune response (Achyut et al., 2015; Bruna et al., 2007; El Andaloussi & Lesniak, 2006; Fecci et al., 2006; C. Hao et al., 2002; Heimberger et al., 2008; Hussain et al., 2006; Shimizu et al., 1999; Stafford et al., 2016). It would be particularly useful to support this by enhancing the expression of co-stimulatory ligands (e.g. CD80/86) on glioblastoma cells, whilst reducing co-inhibitory ligand expression (e.g. CTLA-4) to promote T cell activation (R. C. Anderson et al., 2007; Fallarino et al., 2003; J. Li et al., 1999; Parsa et al., 2007; Qureshi et al., 2011; Wing et al., 2008). Additionally, inhibition of the HA-CD44 axis in glioblastoma is crucial to restrict invasion and enable gross surgical resection, thereby reducing tumour recurrence (T. J. Brown et al., 2016; Knupfer et al., 1999; Koochekpour et al., 1995; Mahesparan et al., 2003; Nakada et al., 2007; Paszek et al., 2005; Pietras et al., 2014; Ranuncolo et al., 2002; Ulrich et al., 2009; K.-C. Wei et al., 2010). In an ideal scenario, novel therapeutics would be able to achieve all of these goals to prevent glioblastoma invasion and enhance anti-tumour immunity. In order to achieve this, three cytokines have been identified that hold significant potential as a cytokine therapy for glioblastoma.

1.3.2. IFN-γ

Wheelock (1965) first identified IFN-γ many years ago, when they were exploring the anti-viral activity of PHA-stimulated human leukocytes. Whilst IFN-γ is predominantly associated with Th1 cells, it can also be produced by CD8+ T cells, NK cells and APCs to facilitate an inflammatory immune response, as a result of IL-12/STAT4-mediated signalling (Schoenborn & Wilson, 2007; Thierfelder et al., 1996). The IFN-γ receptor consists of two subunits, IFNGR1 and IFNGR2, which signal via JAK1 and JAK2 to phosphorylate STAT1 and subsequently initiate a range of downstream effects (Bach et al., 1997; Schindler & Darnell Jr, 1995). As IFNGR1 is constitutively expressed by T cells, the immunomodulatory effects of IFN-γ are solely determined by IFNGR2 – in particular, high IFNGR2 expression induces rapid STAT1 signalling to trigger apoptotic pathways, whilst reduced IFNGR2 expression leads to slower activation of STAT1 that subsequently promotes proliferation (Bernabei et al., 2001). For example, IFNGR2 expression is downregulated during Th1 differentiation, in order to enhance

the proliferative effects of IFN-γ, whilst Th2 cells maintain IFNGR2 expression, thus they are particularly sensitive to the inhibitory effects of IFN-γ exposure (Bach et al., 1995; Pernis et al., 1995).

Early studies identified that IFN-γ signalling was associated with profound upregulation of MHC class I and II complexes by increasing expression of IRF1 and CIITA, thereby enhancing antigen processing and presentation to CD4+ and CD8+ T cells (C.-H. Chang & Flavell, 1995; Mach et al., 1996; Pestka et al., 1987; Wallach et al., 1982). Additionally, IFN-γ is involved in DC maturation by upregulating expression of co-stimulatory ligands (e.g. CD40, CD80 and CD86), as well as IL-12 production to promote an inflammatory immune response (J. Pan et al., 2004). IFN-γ has also been implicated in macrophage priming via STAT1 signalling to upregulate TLR expression, in order to enhance their sensitivity to PAMPs/DAMPs and subsequently promote M1 polarisation (Schroder et al., 2006). Alongside this, IFN-γ re-programs IL-10 to signal via STAT1, rather than STAT3, to mediate upregulation of inflammatory genes (Herrero et al., 2003). Furthermore, IFN-γ has been shown to induce expression of Smad7, which prevents both the activation of Smad3 by TGF-β and the transcription of TGF-β target genes – this is particularly important as it enables IFN-γ to inhibit Treg differentiation and function (Caretto et al., 2010; Ulloa et al., 1999).

Previous research by Delgoffe et al. (2013) determined that expression of Nrp1 was critical for maintaining Treg survival, stability and function in the tumour microenvironment, as Nrp1-deficient Tregs lost their ability to reduce tumour growth or suppress intratumoural immune infiltration in various murine cancer models. Follow up experiments in a murine melanoma model revealed that Nrp1-deficient Tregs maintained Foxp3 expression, although they acquired IFN-γ production, which subsequently impaired conventional Tregs and promoted an anti-tumour immune response. Interestingly, they also showed that intratumoural Tregs were particularly susceptible to Nrp1 expression loss, especially if they were exposed to IFN-γ (Overacre-Delgoffe et al., 2017). Most recently, Gocher et al. (2020) discovered that IFN-γ production by Tregs neutralised the immunosuppressive nature of the tumour microenvironment and subsequently enhanced tumour rejection, using various murine cancer models.

Dighe et al. (1994) were the first to implicate IFN-γ in anti-tumour immunity, demonstrating that IFNGR1-deficient cells grew much faster and were rejected much slower than control cells in a fibrosarcoma mouse model. Subsequent research showed that both IFNGR1-deficient and STAT1-deficient mice developed significantly more tumours compared to wild-type mice, in response to treatment with carcinogens (D. H. Kaplan et al., 1998). On the other hand, Beatty & Paterson (2001) found that Th1 cells infiltrated murine colon cancer tissues quicker than Tc1 cells, whilst their combined production of IFN-γ significantly inhibited tumour growth and angiogenesis. Similarly, Nakajima et al. (2001) observed tumour rejection, as well as CD4+ and CD8+ T cell infiltration in wild-type mice with fibrosarcoma, but neither intratumoural infiltration or tumour rejection were seen IFN-γ-deficient mice. This reflects the ability of IFN-γ to promote T cell and NK cell tumour infiltration by upregulating the expression of the adhesion molecules ICAM-1 and VCAM-1, as well as several chemokines, including: CCL3, CCL4, CCL5, CXCL9, CXCL10 and CXCL11 (Banks et al., 1993; Groom & Luster, 2011).

Interestingly, aside from promoting T cell migration, CXCL10 can also enhance tumour rejection by restricting tumour angiogenesis via CXCR3 (Bodnar et al., 2009; Luster et al., 1998). In particular, Dobrzanski et al. (2001) noted that Tc1-derived IFN-γ induced CCL5 and CXCL10 expression, thereby increasing tumour infiltration of CD4 and CD8+ T cells to provide an anti-tumour immune response in a murine lung cancer model.

As discussed previously, IFN-γ has a central role in promoting the cytotoxic activity of T cells by either directly inducing apoptosis via IFNGR or indirectly through upregulation of various caspases, perforin, granzyme B, TRAIL and Fas/Fas-L (Chin et al., 1997; Fulda & Debatin, 2002; S.-Y. Park et al., 2004; X. Xu et al., 1998). Combined with the ability of IFN-γ to upregulate MHC expression, this provides a mechanism to facilitate the recognition and clearance of cancer cells by the immune system. In the context of glioblastoma, IFN-γ has been shown to significantly upregulate both MHC class I and II expression *in vitro* on glioblastoma cell lines and primary human glioblastoma cells, whilst intratumoural IFN-γ administration enhanced infiltration of CD4+ and CD8+ T cells in a rat glioblastoma model (Frewert et al., 2004; Z. Liu et al., 2017; Soos et al., 2001; I. Yang et al., 2004). Similarly, IFN-γ also upregulated MHC class I and II expression on TAMs in both rat and murine glioblastoma models to increase antigen presentation and T cell activation (Dutta et al., 2003; Schartner et al., 2005).

Research has also shown that IFN-γ can directly reduce cell viability in two glioblastoma cell lines (T98G and U87MG) through increased Bax and CAD expression, alongside concomitant cytochrome c release and loss of Bcl-2 (R. Zhang et al., 2007). Additionally, Das et al. (2009) identified that IFN-γ also activated caspase-8 and truncated Bid, in order to induce apoptosis in both T98G and U87MG cell lines. On the other hand, IFN-γ can inhibit proliferation in target cells via STAT1-mediated upregulation of the cyclic-dependent kinase inhibitors (CKIs) p21 and p27, leading to cell cycle arrest at the G1/S phase transition through inhibition of CDK2 and CDK4, respectively (Bromberg et al., 1996; Chin et al., 1996; Mandal et al., 1998). The anti-proliferative effect of IFN-γ has been seen in several glioblastoma cell lines (T98G, SNB-19 and U373), in which the strength of inhibition strongly correlated with increased expression levels of p21 and reduced CDK2 activity (S. Kominsky et al., 1998). Interestingly, they later determined that IFN-γ did not affect the proliferation of normal human astrocytes *in vitro*, indicating that there could be minimal off-target neurological effects in clinical testing of IFN-γ in glioblastoma patients (S. L. Kominsky et al., 2000).

In bladder carcinoma patients, IFN-γ was well tolerated and significantly improved tumour rejection through upregulation of both CD4+ and CD8+ T cells, as well as NK cells (Giannopoulos et al., 2003). Furthermore, pre and post-treatment biopsies showed that IFN-γ enhanced CD4+ and CD8+ T cell infiltration and MHC class I expression on tumour cells, whilst increasing serum levels of CCL2 and CXCL10 in patients with synovial sarcoma (S. Zhang et al., 2019). Similarly, IFN-γ demonstrated clinical responses in patients with ovarian cancer, T-cell lymphoma or B-cell lymphoma (Dummer et al., 2010; Pujade-Lauraine et al., 1996). On the other hand, IFN-γ had mixed results in patients with metastatic melanoma or renal cell carcinoma, whilst it had either no impact or negative effects on pancreatic

carcinoma, hepatocellular carcinoma, small-cell lung cancer or colon cancer patients (Aulitzky et al., 1989; Creagan et al., 1987; Ernstoff et al., 1987; Forbes et al., 1985; Gleave et al., 1998; Jett et al., 1994; Von Hoff et al., 1990; Wiesenfeld et al., 1995). Furthermore, administering intratumoural IFN-γ after radiotherapy did not influence tumour growth or patient survival in glioblastoma patients (Färkkilä et al., 1994).

Song et al. (2019) recently correlated low IFN-γ expression in the tumour microenvironment with increased tumour grading, chemo-resistance and poor overall survival in non-small cell lung cancer patients. Interestingly, they found that IFN-γ could only reduce proliferation and induce apoptosis in non-small cell lung cancer cell lines at relatively high doses (e.g. 100ng/ml) through STAT1-mediated upregulation of caspase-3. Conversely, low doses of IFN-γ (e.g. 0.1ng/ml) promoted tumour growth, thus it is possible that some of the aforementioned clinical trials did not use a strong enough dose to mediate an anti-tumour response. Alternatively, it might be beneficial to explore the adjuvant effects of IFN-γ with other therapeutic agents to develop novel cancer immunotherapies.

1.3.3. TNF-α

TNF-α was first identified nearly 50 years ago, although it was only named 10 years later, after its ability to induce necrosis in various tumours implanted in mice (Carswell et al., 1975; Old, 1985). In particular, TNF-α is largely produced by macrophages, but it is also associated with Th1 and Th2 cells, as well as NK cells (Parameswaran & Patial, 2010; Ware et al., 1992). Interestingly, TNF-α has two distinct receptors, which differ both in terms of their expression and function. TNFR1 is almost ubiquitously expressed and is involved in the induction of apoptosis, as discussed previously, whereas TNFR2 expression is largely restricted to immune cells, in which it promotes cell survival and proliferation through downstream activation of NF-κB and AP-1 target genes (Cabal-Hierro & Lazo, 2012; Rothe et al., 1995). In addition, researchers have identified a superfamily of proteins that are similar to TNF-α, both in terms of structure and function, including CD27, CD40, Fas-L, OX40L, TRAIL and 41BB-L (B. B. Aggarwal, 2003).

With regard to the immune system, the effects of TNF-α on Tregs have been debated for a number of years. Initial experiments showed that TNFR2 was highly expressed by both murine and human CD4+ Tregs, thus they suggested that TNF-α enhanced CD4+ Treg proliferation, as well as CD25 and Foxp3 expression (X. Chen et al., 2007, 2010, 2013). In response to this, Torrey et al. (2017) developed TNFR2 antagonists to show that inactivating TNFR2 promoted effector CD4+ T cell proliferation and inhibited CD4+ Treg expansion in ovarian cancer patients. Conversely, research has also shown that TNFR2 blockade promoted CD4+ Treg polarisation and proliferation (Boks et al., 2014; Zhong et al., 2015). Several studies have since demonstrated that TNF-α can alleviate CD4+ Treg-mediated immunosuppression on effector CD4+ T cells (Nagar et al., 2010; Nie et al., 2013; Valencia et al., 2006; Zanin-Zhorov et al., 2010). In order to provide clarity, Zaragoza et al. (2016) completed a series of comprehensive experiments, which concluded that TNF-α alone could not upregulate CD25 or Foxp3

expression on human CD4+ Tregs, nor did it enhance their suppressive activity, but it could increase effector T cell proliferation.

As one of the first cancer cytokine therapies, early studies were promising, as they identified that TNF-α was cytotoxic in various murine and human cancer cell lines, including breast cancer and hepatocellular carcinoma, both *in vitro* and *in vivo* (Balkwill et al., 1986; Brouckaert et al., 1986; Dealtry et al., 1987; Fransen et al., 1986; Motoo et al., 1986). In addition, *in vivo* TNF-α administration was shown to induce tumour necrosis by damaging the tumour vasculature, indicating that it could have clinical relevance in glioblastoma (Palladino et al., 1987; Shimomura et al., 1988). In fact, TNF-α inhibited tumour-driven angiogenesis in a rat glioblastoma model – however, this effect could be dose-dependent, as low-dose TNF-α promoted angiogenesis and increased tumour blood flow in other rodent cancer models (Fajardo et al., 1992; Naredi et al., 1993; Niida et al., 1995; Watanabe et al., 1988). In line with this, researchers suggested that increased tumour vascularisation correlated with enhanced tumour response to high-dose TNF-α treatment (Olieman et al., 1997; van Etten et al., 2003). Yet, some tumours appeared to be resistant to TNF-α, with subsequent analyses revealing that only 28% of cancer cell lines were responsive to TNF-α treatment (Salmon et al., 1987; Sugarman et al., 1985).

Clinical testing revealed that systemic TNF-α administration was not well tolerated and patients responses were mixed in a range of clinical trials – although, localised administration appeared more effective (Blick et al., 1987; T. D. Brown et al., 1991; Creaven et al., 1987; De Wilt et al., 2000; Eggermont, Koops, et al., 1996; Eggermont, Schraffordt Koops, et al., 1996; Kimura et al., 1987; Kuei et al., 1989; Selby et al., 1987). Additionally, both ovarian cancer and colorectal adenocarcinoma cells were shown to secrete TNF-α, thus questions were raised questions as to whether TNF-α was involved in promoting tumour growth (Beissert et al., 1989; Naylor et al., 1990). Research has shown that TNF-α exposure results in reduced TCR signalling and the induction of an exhausted phenotype in CD4+ T cells, during chronic viral infection (Beyer et al., 2016). In addition, TNFR1 signalling in macrophages has been shown to inhibit IL-12 secretion, thereby limiting Th1 and Tc1 cell differentiation and function (Notley et al., 2008). Consequently, TNF-α antagonists were developed, which provided clinical benefits to patients with metastatic breast cancer, ovarian cancer or renal cell carcinoma (Harrison et al., 2007; Madhusudan et al., 2004, 2005). Altogether, these results suggest that TNF-α can have therapeutic value, but it is largely dependent on the dose, cancer type, and method of administration.

Due to the independent limitations of IFN-γ and TNF-α, researchers have also explored whether they could provide clinical benefits when administered together. In particular, IFN-γ and TNF-α have been shown to reduce proliferation and/or induce apoptosis in a range of cell lines, including pancreatic, cervical, and colon cancer (Schmiegel et al., 1988; Suk et al., 2001; Wright et al., 1999). Using a murine melanoma model, Dobrzanski et al. (2004) identified that IFN-γ rapidly upregulated Fas, TNFR1, and caspase-8 expression on tumour cells, but the addition of TNF-α enhanced tumour apoptosis and significantly reduced tumour growth – altogether, this indicated that IFN-γ increased cell sensitivity to the pro-apoptotic effects of TNF-α. Several clinical trials found that IFN-γ and TNF-α synergised to

stabilise disease progression in cancer patients, but responses were limited and particularly poor in patients with colorectal cancer (Demetri et al., 1989; Fiedler et al., 1991; Kurzrock et al., 1989; Schiller et al., 1992; Smith II et al., 1991).

In the context of glioblastoma, two studies have identified that both glioblastoma patient samples and a wide range of glioblastoma cell lines consistently express high levels of IFNGR1, IFNGR2 and TNFR1, whilst TNFR2 expression was either low or absent (C. Hao et al., 2002; M. Tada et al., 1994). In particular, research has shown that TNF-α inhibited proliferation and invasion in several glioblastoma cell lines (CRT, STT and WIT), but this was significantly enhanced when IFN-γ was co-administered (Iwasaki et al., 1993). Recently, Braumüller et al. (2013) discovered that the combination of IFN-γ and TNF-α induced senescence in murine cancer models by mediating stable cell cycle arrest in G1/G0, but the exact mechanism in glioblastoma has not been identified. Only one study has explored the combined effects of IFN-γ and TNF-α in a glioblastoma murine model, which showed that they significantly reduced tumour growth and improved survival by inducing apoptosis. In addition, IFN-γ and TNF-α upregulated expression of both MHC class I and II on glioblastoma cells, whilst facilitating enhanced tumour infiltration of CD4+ and CD8+ T cells (Ehtesham et al., 2002). Interestingly, IFN-γ can convert astrocytes to APCs by inducing MHC class II expression, which can subsequently be boosted by TNF-α to enhance antigen presentation and T cell activation (Benveniste et al., 1989). Altogether, these studies have indicated that IFN-γ and TNF-α have significant potential value as a combined therapy for glioblastoma, but they require significant further investigation.

1.3.4. IL-21

Aside from IFN-γ and TNF-α, IL-21 has also been proposed as a potential cytokine therapy for various cancers. IL-21 belongs to a family of cytokines that includes IL-2, IL-4, IL-7, IL-9 and IL-15, which each have a common γ_c-chain in their receptors. Both IL-2R and IL-15R are heterotrimeric, consisting of distinct α-chains, alongside common β-chains and γ-chains (D. M. Anderson et al., 1995; X. Wang et al., 2005). In contrast, the IL-21R is heterodimeric, composed of a unique α-chain and the common γ_c-chain – this receptor has been identified on a range of human cells, including: CD4+ and CD8+ T cells, B cells and NK cells (Parrish-Novak et al., 2002). Although IL-21 is largely associated with CD4+ T cells, CD8+ T cells and some APCs have been shown to produce low levels of IL-21 (Elsaesser et al., 2009; Parrish-Novak et al., 2000). Regulation of IL-21 production is controlled by c-Maf and IRF4, in response to TCR and/or co-stimulatory signalling, as well as exposure to a range of cytokines including IL-1β, IL-6, IL-21 and IL-27 (Bauquet et al., 2009; H.-P. Kim et al., 2005; Pot et al., 2009; Suto et al., 2008; Végran et al., 2014). In particular, IL-21R primarily signals via JAK1 and JAK3, which phosphorylate and subsequently activate several STATs, leading to a range of downstream effects (Levy & Darnell, 2002). Although researchers believe that STAT1 and STAT3 are the primary transcription factors activated by IL-21, there is some evidence for IL-21-mediated activation of STAT5

(Leonard & Spolski, 2005). Examples of some IL-21 target genes include those responsible for the expression of Blimp-1, Bcl-6, c-Maf, Eomes, granzyme B, and RoRγT (Spolski & Leonard, 2014).

With regard to Bcl-6 and c-Maf, these are upregulated by IL-21 during Tfh differentiation, as discussed previously. Research has shown that IL-21 works in concert with IL-4, plus TCR/MHC and CD40/CD40L-signalling in B cells to enhance Blimp-1 expression and subsequently promote the differentiation of B cells into memory B cells and antibody-producing plasma cells – although, IL-21 can also upregulate Bim to induce apoptosis in autoreactive B cells (Boudigou et al., 2021; H. Jin et al., 2004; Mehta et al., 2003). In addition. Attridge et al. (2014) found that IL-21 upregulated CD86 expression on B cells through a STAT3/PI3K-dependent pathway, thereby promoting CD86/CD28 binding and co-stimulatory signalling in T cells, leading to enhanced CD4+ T cell activation and proliferation. The effect of IL-21 on other APC ligands is not currently known but, if IL-21 could upregulate other co-stimulatory ligands (e.g. CD80, 41BB-L, ICOS-L), whilst concurrently preventing co-inhibitory ligand expression (e.g. PD-L1 and Galectin-9), this could provide a mechanism for the restoration of anti-tumour immunity in cancer patients.

IL-2 was the first cytokine therapy developed for cancer patients, based upon the theory that it would enhance effector T cell activation, differentiation and proliferation to promote an anti-tumour immune response – however, both metastatic renal cancer and melanoma patients had limited responses to IL-2 (Rosenberg et al., 1994). This was later discovered to be due to the fact that IL-2 administration significantly increased the frequency of immunosuppressive CD4+CD25+Foxp3+ Tregs in cancer patients, which aligns with findings that IL-2 has a central role in both inducing Treg differentiation in the thymus, as well as maintaining Treg populations in the periphery (Ahmadzadeh & Rosenberg, 2006; Lio & Hsieh, 2008; Tsuji-Takayama et al., 2008). As IL-21 is structurally very similar to IL-2, questions were raised about whether IL-21 would also promote Treg expansion and immunosuppression when administered to cancer patients. Subsequent research showed that IL-21 enhanced the proliferation of both CD4+ and CD8+ T cells both *in vitro* and *in vivo*, but it could only relieve CD4+ T cells from CD4+ Treg-mediated immunosuppression (Clough et al., 2008; Peluso et al., 2007). Using IL-21R deficient mice, Attridge et al. (2012) identified that IL-21 was able to indirectly counteract the immunosuppressive effects of CD4+ Tregs by significantly inhibiting the amount of IL-2 secreted by conventional CD4+ T cells. Interestingly, the loss of exogenous IL-2 did not have a negative impact on these CD4+ T cells, as IL-21 effectively replaced IL-2 as a T cell growth factor – but, this effect was not seen in Tregs, which failed to maintain their CD25+Foxp3+ phenotype in the absence of IL-2. In addition, IL-21 also restricts TGF-β-mediated Foxp3 induction in naïve CD4+ T cells, thereby preventing Treg polarisation (Kannappan et al., 2017; Korn et al., 2007; Y. Li & Yee, 2008; R. Nurieva et al., 2007). Whilst these studies largely focused on CD4+ T cells, Khattar et al. (2014) showed IL-2 deprivation induced apoptosis in CD8+ T cells, but this could be abrogated in co-culture experiments through IL-21 production by CD4+ T cells.

On the other hand, Spolski et al. (2009) showed that IL-21 induced IL-10 expression in freshly isolated naïve CD4+ and CD8+ T cells, as well as naïve T cells cultured in Th1, Th17, Tc1 and Tc17-polarising conditions. In addition, they found that IL-21 could cause lineage-committed Th1, Tc1 and Tc17 cells to express IL-10, whilst co-culture experiments revealed that Th1 cells used the acquisition of IL-10 production to inhibit naïve CD8+ T cell activation (Spolski et al., 2009). Similarly, the polarisation of Tr1 cells by IL-27 has been shown to induce c-Maf, which subsequently transactivates both IL-21 and IL-21R, thus it has been suggested that IL-21 acts as an autocrine growth factor to facilitate Tr1 expansion and IL-10 production (Pot et al., 2009). However, it is important to note than both of these studies were completed in mice, thus their relevance to human immune cells should be questioned – especially as Doganci et al. (2013) failed to identify IL-10 production when freshly isolated human CD4+ T cells, as well as Th1, Th2 and Th17 cells were cultured with IL-21. Furthermore, studies have found that culturing naïve CD8+ T cells with IL-27 generates a population of CD8+ T cells that express IL-21, IFN-γ and T-bet, in which IL-21 production enhances CD8+ T cell cytotoxicity by promoting perforin and granzyme B expression (Mittal et al., 2012; Sutherland et al., 2013). Similarly, IL-21 can enhance NK cell and Tc1 cell cytotoxicity by upregulating IFN-γ, TNF-α, perforin and granzyme B expression, both independently or in combination with IL-7/IL-15 (Kasaian et al., 2002; S. Liu et al., 2007; Parmigiani et al., 2011; Skak et al., 2008; Strengell et al., 2003; Zeng et al., 2005). Altogether, these findings suggest that IL-21 has the potential to inhibit Treg-mediated immunosuppression and facilitate a potent anti-tumour immune response, thus making it an ideal candidate for cancer therapies.

Initial studies revealed that IL-21 could inhibit tumour growth and improve survival in murine models of melanoma and fibrosarcoma, which they believed was due to upregulation of perforin in CD8+ T cells and NK cells (H.-L. Ma et al., 2003; G. Wang et al., 2003). Subsequent researched showed that intratumoural delivery was more effective than intravenous IL-21 administration, as it correlated with increased tumour-infiltration of CD8+ T cells producing high levels of IFN-γ and granzyme B (Søndergaard et al., 2010). Similarly, C. Chen et al. (2018) found that IL-21 enhanced IFN-γ production by CD4+ and CD8+ T cells, as well as NK cells, whilst significantly reducing tumour size and improving survival in a murine colon cancer model. In particular, Topchyan et al. (2021) used a murine melanoma model to determine that IL-21 increased expression of BATF via STAT3 in CD8+ T cells to enhance their infiltration, differentiation and anti-tumour effects. On the other hand, several research groups induced IL-21 expression in colon, renal and bladder cancer cells, prior to subcutaneous transplantation to demonstrate that IL-21 promoted tumour rejection in mice through enhanced Th1, Tc1 and NK cell-mediated immunity (Furukawa et al., 2006; Kumano et al., 2007; Ugai et al., 2003). Similarly, Kim-Schulze et al. (2009) showed that inducing IL-21 secretion in murine melanoma cells both inhibited tumour growth, increased CD8+ T cell activation and proliferation, whilst preventing accumulation of CD4+CD25+Foxp3+ Tregs within the tumour microenvironment.

These findings were supported by human clinical studies, which demonstrated that IL-21 enhanced the cytotoxic activity of CD8+ T cells and NK cells by upregulating IFN-γ, perforin and

granzyme B expression, whilst also boosting the activation, proliferation, migration of CD8+ T cells in patients with advanced metastatic melanoma and renal cell carcinoma (Frederiksen et al., 2008). Furthermore, intratumoural IL-21 also skewed polarisation of TAMs away from the immunosuppressive M2 phenotype towards a potent anti-tumour M1 profile in patients with breast cancer (M. Xu et al., 2015). Clinical trials demonstrated that intravenous IL-21 was largely well tolerated and able to produce an anti-tumour response in nearly 60% of patients with metastatic melanoma, whilst the overall response rate was 89% for renal cancer carcinoma patients (Petrella et al., 2012; Thompson et al., 2008). Serum analyses from these patients revealed that IL-21 enhanced expression of IL-15, IL-18 and TNF-α, thus they suggested that IL-18 increased the differentiation of Th1/Tc1 cells, which could then be subsequently maintained by macrophage-derived IL-15 to sustain TNF-α production (Dodds et al., 2009). IL-15 has also been implicated in NK cell activation, migration and survival, providing a mechanism for the indirect induction of NK cell-mediated anti-tumour immunity by IL-21 (W. E. Carson et al., 1997).

Although IL-21 appears to have significant clinical benefits, there also needs to be confidence that IL-21 does not have any direct effects on cancer cells to undermine an anti-tumour immune response. Xue et al. (2019) showed that IL-21 upregulated IL-21R expression and directly inhibited the proliferation, migration and invasion of a non-small cell lung cancer cell line. Conversely, L.-N. Wang et al. (2015) identified variable expression of IL-21R on several breast cancer cell lines, in which higher IL-21R expression correlated with increased proliferation, migration and invasion, in response to IL-21 treatment. Alternatively, the IL-21R was not found on two colorectal cancer cell lines, thus IL-21 had no impact on STAT3 activation, cell proliferation or survival (De Simone et al., 2015). Unfortunately, only one study has explored the effects of IL-21 on glioblastoma: Daga et al. (2007) induced IL-21 secretion in glioblastoma cells prior to transplantation in mice – remarkably, 100% of these tumours were subsequently rejected. In addition, intratumoural IL-21 administration induced tumour rejection in established glioblastoma tissues. Collectively, this indicated that IL-21 induced a potent anti-tumour immune response in glioblastoma, but significantly more research is required as the direct effects of IL-21 on glioblastoma cells are still not known.

1.4. Thesis Aims

The first aim of the present thesis was to evaluate the therapeutic potential of IFN-γ, IL-21 and TNF-α as a triple cytokine therapy in glioblastoma, with a particular focus on the direct impact on tumour proliferation, survival, and migration. However, the second aim of the present thesis was to explore whether IFN-γ, IL-21 and TNF-α could also demonstrate therapeutic value through altering the expression of various co-stimulatory and co-inhibitory ligands to potentially impact the direct interactions between tumour cells and immune cells. Similarly, the final aim of the present thesis was to assess whether IFN-γ, IL-21 and TNF-α could overcome the immunosuppressive nature of the glioblastoma secretome by skewing the differentiation of naïve T cells and altering the characteristics of mature T cells towards a phenotype that could possibly facilitate an anti-tumour immune response.

The specific objectives of the research project were to:

- Investigate the direct anti-proliferative and pro-apoptotic effects of IFN-γ and TNF-α, whilst exploring the potential therapeutic benefits of IL-21, on two glioblastoma cell lines (U251 and U373).
- Explore IL-21R expression on two glioblastoma cell lines (U251 and U373), whilst establishing whether STAT1, STAT3 and/or STAT5 were activated and phosphorylated, in response to treatment with IFN-γ, IL-21 and/or TNF-α.
- Examine the effects of IFN-γ, TNF-α and/or IL-21 on the tumour migration of two glioblastoma cell lines (U251 and U373).
- Determine the expression of several immunological co-stimulatory and co-inhibitory ligands on the surface of two glioblastoma cell lines (U251 and U373), in order to improve our understanding of how these tumour cells interact with the immune system, whilst evaluating whether IFN-γ, TNF-α and/or IL-21 can influence the expression of these ligands to theoretically promote an anti-tumour immune response.
- Study the effects of the supernatant from two glioblastoma cell lines (U251 and U373) on CD4+ and CD8+ T cells, in order to establish whether IFN-γ, TNF-α and/or IL-21 can overcome the immunosuppressive glioblastoma secretome to alter the expression profile of these T cells towards more inflammatory phenotypes.

2. Materials and Methods

2.1.1. Cell Lines

Human glioblastoma U251 and U373 cell lines (courtesy of Prof. Tracy Warr, University of Wolverhampton) were incubated at 37°C, 5% CO_2 in RPMI 1640 medium (R0883 – Sigma-Aldrich, UK), which contained 1% L-glutamine (G7513 – Sigma-Aldrich, UK) and 1% Penicillin Streptomycin (15140122 – Gibco, UK), supplemented with 10% Foetal Calf Serum (FCS) (F0804 – Sigma-Aldrich, UK). Glioblastoma patient-derived 367 cells (courtesy of Prof. Tracy Warr, University of Wolverhampton) were incubated at 37°C, 5% CO_2 in Ham's F-10 Nutrient Mix (22390058 – Gibco, UK), supplemented with 10% FCS (10091148 – Gibco, UK). These untreated media will be referred to as either the RPMI Control or Control for all experiments in the present thesis. All T-75 culture flasks, 12/24/96 flat-bottom well plates, 15/25ml falcons, and 12x75mm FACS tubes were purchased from VWR International (USA).

2.1.2. Cell Counts

$2.5x10^4$ U251 and U373 cells were seeded onto a 24 well plate in either fresh medium or medium containing a titration (50-200ng/ml) of recombinant human IFN-γ, IL-21 and/or TNF-α (all from Peprotech, UK), before being placed into an incubator. After 24/48/72 hours of treatment, the media was removed from each well, the cells were washed with PBS (Gibco, UK) and harvested using 1X Trypsin (Sigma-Aldrich, UK), which was then neutralised using fresh medium, before the cell suspensions were transferred into individual FACS tubes. These were then centrifugated at 400g for 5 minutes, before the supernatant was removed and the cells were re-suspended in 100µL medium, prior to analysis using the Beckman Coulter CytoFLEX S flow cytometer, FlowJo and GraphPad Prism software.

2.1.3. Cell Density Imaging

$2.5x10^4$ U251 and U373 cells were seeded onto a 24 well plate in either fresh medium or medium containing recombinant human 100ng/ml IFN-γ, IL-21 and/or TNF-α, before being placed into an incubator. After 72 hours, the plate was removed from the incubator and images were taken of the cells using an Invitrogen EVOS FL Cell Imaging System at 10x magnification (Thermo Fisher Scientific, UK).

2.1.4. Patient-derived Glioblastoma Cell Counts and Density Imaging

$2.5x10^4$ patient-derived 367 cells were seeded onto a 24 well plate in either fresh medium or medium containing recombinant human 100ng/ml IFN-γ, IL-21 and/or TNF-α, before being placed into an incubator. After 72 hours, the plate was removed from the incubator and images were taken of the cells using an Invitrogen EVOS FL Cell Imaging System at 10x magnification (Thermo Fisher Scientific, UK). Following this, the media was removed from each well, the cells were washed with PBS and harvested using 1X Trypsin, which was then neutralised using fresh medium, before the cell suspensions

were transferred into individual FACS tubes. These were then centrifugated at 400g for 5 minutes, before the supernatant was removed and the cells were re-suspended in 100μL medium, prior to analysis using the Beckman Coulter CytoFLEX S flow cytometer, FlowJo and GraphPad Prism software.

2.1.5. Annexin V and 7-AAD Staining

$5x10^4$ U251 and U373 cells were seeded onto a 12 well plate in either fresh medium or medium containing recombinant human 100ng/ml IFN-γ, IL-21 and/or TNF-α, before being placed into an incubator. After 72 hours, the media was removed from each well, then the cells were washed with PBS and harvested using 1X Trypsin, which was then neutralised using fresh medium, before the relevant cell suspensions were transferred into individual FACS tubes. These were then centrifugated at 400g for 5 minutes and the supernatant was removed, before the cells were re-suspended in 500μL PBS and centrifugated at 400g for 5 minutes again. Apoptosis and necrosis were measured using an Annexin V Apoptosis Detection Kit (88-8102 – eBioscience, UK). In brief, upon removal of the cell supernatant, cells were re-suspended in 1X Annexin V Binding Buffer, before 1μL PE Annexin V and 2μL 7-AAD were added to each FACS tube for incubation at room temperature in the dark for 10 minutes, prior to flow cytometric analysis. Each condition also had an Unstained Control, which was used as a reference point for gating, during analysis using the Beckman Coulter CytoFLEX S flow cytometer, FlowJo and GraphPad Prism software.

2.1.6. Ki67 Staining

$5x10^4$ U251 and U373 cells were seeded onto a 12 well plate in either fresh medium or medium containing recombinant human 100ng/ml IFN-γ, IL-21 and/or TNF-α, before being placed into an incubator. After 72 hours, the media was removed from each well, then the cells were washed with PBS and harvested using 1X Trypsin, which was then neutralised using fresh C medium, before the relevant cell suspensions were transferred into individual FACS tubes. These were then centrifugated at 400g for 5 minutes and the supernatant was removed, before the cells were re-suspended in 500μL PBS and centrifugated at 400g for 5 minutes again. Ki67 expression was measured using a Foxp3/Transcription Factor Staining Buffer Set (00-5523-00 – eBioscience, UK). In brief, upon removal of the cell supernatant, 500μL cold Fixation Buffer was added to each FACS tube and they were briefly vortexed, before incubation at 4°C for 30 minutes. Following incubation, the cells were centrifugated at 400g for 5 minutes, before the supernatant was removed and the cells were re-suspended in 1ml PBS, in order to remove traces of the Fixation Buffer, during centrifugation at 400g for 5 minutes. After the supernatant had been removed, 2ml room temperature Permeabilisation Buffer was added to each FACS tube and they were briefly vortexed, prior to centrifugation at 400g for 5 minutes – this process was repeated once, so the cells endured two rounds of permeabilisation. Disposal of the supernatant was followed by the addition of 5μL Alexa Fluor® 488 mouse anti-Ki-67 (561165 – BD Biosciences, USA), before cells were

briefly vortexed and incubated at room temperature for 30 minutes in the dark. Following incubation, 1X Permeabilisation Buffer was added to each FACS tube and they were briefly vortexed, prior to centrifugation at 400g for 5 minutes. The supernatant was removed from each tube and the cells were re-suspended in 300μL Permeabilisation Buffer, prior to flow cytometric analysis. Each condition also had an Unstained Control, which was used as a reference point for gating, during analysis using the Beckman Coulter CytoFLEX S flow cytometer, FlowJo and GraphPad Prism software.

2.1.7. STAT1/STAT3/STAT5 Phosphorylation

$1x10^5$ U251 and U373 cells were seeded onto a 24 well plate in fresh medium and incubated for 24 hours to ensure cell attachment to the plate. The following day, the media was removed, before being replaced with either fresh medium or medium containing 100ng/ml IFN-γ, IL-21 and/or TNF-α for 30 minutes (0.5 hour), 1 hour, 2 hours, and 4 hours incubation. After this, the media was removed from each well and the cells were washed with PBS, harvested using 1X Trypsin, neutralised with fresh medium, and transferred into FACS tubes, which were then centrifugated at 400g for 5 minutes. The supernatant was discarded and the cells were re-suspended in 1ml PBS, prior to centrifugation at 400g for 5 minutes again. After the supernatant had been removed, the cells were fixed using 200μL 4% Paraformaldehyde (PFA – Sigma-Aldrich, UK), which was added to each FACS tube for 10 minutes at room temperature. The cells were then washed in 1ml PBS and centrifugated at 400g for 5 minutes. Once the supernatant had been discarded, 500μL ice cold Methanol (CEAC, UK) was added to each FACS tube, before incubation at 4°C for 30 minutes, followed by centrifugation at 400g for 5 minutes. The supernatant was then removed and the cells were re-suspended in 1ml PBS, before being centrifugated again at 400g for 5 minutes – this process was repeated twice. After the final supernatant had been discarded, 20μL Alexa Fluor® 647 anti-STAT1 (pY701; 612597), 20μL PE anti-STAT3 (pS727; 558557), and 20μL Alexa Fluor® 488 anti-STAT5 (pY694; 612598) antibodies were added to each FACS tube (BD Biosciences, USA). These were then briefly vortexed and incubated at 4°C for 30 minutes. Following incubation, 1ml PBS was added to each FACS tube and they were centrifugated at 400g for 5 minutes. The supernatant was then removed from each FACS tube and the cells were re-suspended in 300μL PBS, prior to flow cytometric analysis. Each condition also had an Unstained Control, which was used as a reference point for gating, during analysis using the Beckman Coulter CytoFLEX S flow cytometer, FlowJo and GraphPad Prism software.

2.1.8. IL-21R, CD44 and Co-stimulatory/Inhibitory Ligand Expression

$2.5x10^4$ U251 and U373 cells were seeded onto a 24 well plate in either fresh medium or medium containing recombinant human 100ng/ml IFN-γ, IL-21 and/or TNF-α, before being placed into an incubator. After 72 hours of treatment, the media was removed from each well, the cells were washed in PBS and harvested using 1X Trypsin, which was then neutralised using fresh medium, prior to the cell suspension being transferred into FACS tubes. These were then centrifugated at 400g for 5 minutes,

before the supernatant was removed and the cells were re-suspended in 100μL P2 (98% PBS and 2% FCS). As each condition was completed in triplicate, this meant that there were 3 three separate FACS tubes available for subsequent antibody staining at 4°C for 30 minutes. In particular, cells in the first FACS tube were stained with 5μL PE mouse anti-human Galectin-9 (565890), 5μL V450 mouse anti-human CD44 (561292) and 20μL FITC mouse anti-human CD80 (557226). Those in the second FACS tube were stained with 20μL PE anti-human 41BB ligand (559446), 20μL FITC mouse anti-human PD-L1 (558065), and 5μL V450 mouse anti-human CD86 (560357). Finally, cells in the third FACS tube were stained with 20μL PE mouse anti-human IL-21R (560264) and 5μL Alexa Fluor® 647 mouse anti-human ICOS-L (564278) (all from BD Biosciences, USA). Following incubation, 500μL P2 was added to each FACS tube and they were centrifugated at 400g for 5 minutes, before the supernatant was removed and the cells were re-suspended in 300μL P2, prior to flow cytometric analysis. Each condition also had an Unstained Control, which was used as a reference point for gating, during analysis using the Beckman Coulter CytoFLEX S flow cytometer, FlowJo and GraphPad Prism software.

2.1.9. Cell Migration

2 Well Culture-inserts (Ibidi, Germany) were placed inside a 24 well plate, into which fresh medium or medium containing recombinant human 100ng/ml IFN-γ, IL-21 and/or TNF-α was added. Next, $1.5x10^4$ U251 and U373 cells were seeded into the Culture-inserts to create two cell populations separated by a 500μm cell-free gap, prior to incubation for 24 hours to facilitate cell attachment to the plate. Culture-inserts were then removed and the cells were washed with PBS to remove any debris, before fresh medium or medium containing recombinant human 100ng/ml IFN-γ, IL-21 and/or TNF-α was added to each well. The plate was then inserted into a Cell IQ SLF machine (Chip-Man Technologies, Finland) for 72 hours, with pictures taken every hour at three different points of the area between cell populations. Each treatment was completed in triplicate; thus, images were taken of 3 wells for each condition. Cell migration was then analysed using Cell IQ Analysis and GraphPad Prism software.

2.1.10. HA Plate Coating and CPC Precipitation

HA (H5388 – Sigma-Aldrich, UK) was diluted in distilled water using a titration of doses ranging from 0 to 20mg/ml, which were then seeded in triplicate onto a 24-well plate for 24-hour incubation at room temperature. The following day, the wells were briefly washed with PBS to remove any excess HA, before 300μL 10% Cetylpyridinium chloride (CPC – Sigma Aldrich, UK) was added to each well – CPC precipitates HA, thus it provides a direct measurement of HA coating efficacy. 100uL of precipitate was then transferred into a 96-well plate in triplicate, before optical density was measured at an absorbance of 560nm using a Multiskan GO microplate reader (Thermo Fisher Scientific, UK) for subsequent analysis using GraphPad Prism software.

2.1.11. CD44 mAb

$2.5x10^4$ U251 and U373 cells were seeded onto a 24 well plate and placed into an incubator for 24 hours to ensure cell attachment to the plate. The following day, the media was removed from each well and the cells were washed in PBS, before being treated with a titration of CD44 mAb (MA4400 – Invitrogen, UK) that had been diluted in medium. In the first experiment, cells were treated with 0-4μg/ml CD44 mAb for 30 minutes (0.5 hour), whilst they were treated with 0-20μg/ml CD44 mAb for 1 hour in the second experiment. At the end of these incubations, the media was removed, before the cells were washed with PBS and harvested using 1X Trypsin, which was then neutralised using fresh medium, before the cell suspensions were transferred into FACS tubes. These were then centrifugated at 400g for 5 minutes, the supernatant was removed and the cells were re-suspended in 100μL P2, before 5μL V450 mouse anti-human CD44 (561292 – BD Biosciences, USA) was added for incubation at 4°C for 30 minutes. Following this, 500μL P2 was added to each FACS tube and they were centrifugated at 400g for 5 minutes. The supernatant was removed and the cells were re-suspended in 300μL P2, prior to analysis using the Beckman Coulter CytoFLEX S flow cytometer, FlowJo and GraphPad Prism software.

2.1.12. Blood Cells

The following T cell isolations were completed using healthy adult blood leukocyte cones (NC24), obtained from the NHS Blood and Transplant (NHSBT, UK) site located on Vincent Drive, Birmingham. In line with this, no identifiable donor information was provided – this included their age and sex; thus, it was not possible to control for these parameters or any other extraneous variables.

2.1.12.1. Bulk CD4+ T Cell Isolation

Bulk CD4+ T cells were isolated using 2ml of blood, which was transferred into a 5ml falcon, before 100μL RosetteSep™ Human CD4+ T Cell Enrichment Cocktail (15022 – Stemcell, Canada) was added and the cells were incubated at room temperature for 20 minutes. The enriched blood was then diluted in 2ml P2, which was layered over 3ml Histopaque (10771 – Sigma-Aldrich, UK), prior to centrifugation at 1200g for 20 minutes. Purified CD4+ T cells were then extracted, before they underwent two rounds of centrifugation at 300g for 10 minutes in 5ml P2. Upon removal of the final supernatant, bulk CD4+ T cells were re-suspended in P2 for counting, before subsequent dilution in medium for seeding.

2.1.12.2. Bulk CD8+ T Cell Isolation

Bulk CD8+ T cells were isolated using 2ml of blood, which was transferred into a 5ml falcon, before 100μL RosetteSep™ Human CD8+ T Cell Enrichment Cocktail (15023 – Stemcell, Canada) was added and the cells were incubated at room temperature for 20 minutes. The enriched blood was then diluted in 2ml P2, which was layered over 3ml Histopaque, prior to centrifugation at 1200g for 20

minutes. Purified CD8+ T cells were then extracted, before they underwent two rounds of centrifugation at 300g for 10 minutes in 5ml P2. Upon removal of the final supernatant, bulk CD8+ T cells were re-suspended in P2 for counting, before subsequent dilution in medium for seeding.

2.1.12.3. Naïve CD4+CD45RA+ T Cell Isolation

Blood was diluted in 20ml PBS, before it was layered over 15ml Histopaque and underwent centrifugation at 400g for 30 minutes with the brakes off. PBMCs were then extracted and diluted in 20ml PBS, before they underwent centrifugation at 250g for 10 minutes. Following disposal of the supernatant, cells were re-suspended in 10ml ACK Lysing Buffer (A1049201 – Thermo Fisher Scientific, UK) for incubation at room temperature for 5 minutes, prior to centrifugation at 400g for 5 minutes. Upon removal of the supernatant, PBMCs were suspended in P2 for counting, in which $1x10^9$ PBMCs were diluted in 2ml P2 for naïve CD4+CD45RA+ T cell isolation, using the EasySep™ Human Naïve CD4+ T Cell Isolation Kit II (17555 – Stemcell, Canada). In brief, 100μL isolation cocktail was added for incubation at room temperature for 5 minutes, before 100μL Rapidspheres™ were added. Next, the cells were topped up to 5ml with PBS and placed inside a Big EasySep™ magnet (10771 – Stemcell, Canada) for 3 minutes. The enriched cell suspension was transferred into a fresh 15ml falcon and subsequently placed back inside the magnet for 3 minutes again. Upon transfer of the enriched cell suspension to another fresh 15ml falcon, naïve CD4+CD45RA+ T cells were counted, before centrifugation at 400g for 5 minutes, followed by removal of the supernatant and re-suspension in medium for seeding.

2.1.12.4. T Cell Seeding

Each of these isolated T cells were seeded into a 96-well plate at a density of $2.5x10^4$, alongside $2.5x10^4$ CD3/CD28 Human T-Activator Dynabeads™ (Gibco, UK). There were three main media conditions: fresh medium, medium taken from 3-day old U251 cell cultures that had reached 70% confluency in a T-75 flask, and medium taken from 3-day old U373 cell cultures that had reached 70% confluency in a T-75 flask. As both the cells (50μL) and CD3/CD28 Dynabeads™ (50μL) were seeded in fresh medium, this meant that the glioblastoma cell line supernatants (100μL) were effectively diluted 1:1. Nonetheless, within these media conditions, cells were either treated with media alone or media supplemented with recombinant human 100ng/ml IFN-γ, IL-21 and/or TNF-α, before being placed into an incubator for 5 days. Each condition was completed in triplicate, but these wells were pooled when the T cells were harvested and transferred into FACS tubes.

2.1.13. T Cell Staining for Transcription Factor Expression

After 5 days of culture, T cells were harvested into FACS tubes, which then underwent centrifugation at 400g for 5 minutes, before the supernatant was removed and the cells were re-suspended in 500μL PBS, prior to centrifugation at 400g for 5 minutes again. Transcription factor expression was

measured using a Foxp3/Transcription Factor Staining Buffer Set, as used previously. In brief, upon removal of the cell supernatant, 500µL cold Fixation Buffer was added to each FACS tube and they were briefly vortexed, before incubation at 4°C for 30 minutes. Following incubation, the cells were centrifugated at 400g for 5 minutes, before the supernatant was removed and the cells were re-suspended in 1ml PBS, in order to remove traces of the Fixation Buffer, during centrifugation at 400g for 5 minutes. After the supernatant had been removed, 2ml room temperature Permeabilisation Buffer was added to each FACS tube and they were briefly vortexed, prior to centrifugation at 400g for 5 minutes – this process was repeated once, so the cells endured two rounds of permeabilisation. Disposal of the supernatant was followed by the addition of the following antibodies: 5µL BV650 mouse anti-T-bet (564142), 20µL Alexa Fluor® 488 mouse anti-GATA3 (560163), 5µL PE mouse anti-human RoRγT (563081), 5µL PE-Cy™7 mouse anti-Bcl-6 (563582), and 5µL V450 mouse anti-human Foxp3 (561182). In addition, both naïve CD4+CD45RA+ T cells and bulk CD4+ T cells were stained with PE-Cy™5 mouse anti-human 5µL CD4 (555348), whilst bulk CD8+ T cells were stained with 5µL PE-Cy™5 mouse anti-human CD8 (555368) (all from BD Biosciences, USA), before cells were briefly vortexed and incubated at room temperature for 30 minutes in the dark. Following incubation, 1X Permeabilisation Buffer was added to each FACS tube and they were briefly vortexed, prior to centrifugation at 400g for 5 minutes, before being re-suspended in 300µL Permeabilisation Buffer, prior to flow cytometric analysis. Each condition also had an Unstained Control, which was used as a reference point for gating, during analysis using the Beckman Coulter CytoFLEX S flow cytometer, FlowJo and GraphPad Prism software.

2.1.14. T Cell Staining for Cytokine Expression

Cell Stimulation Cocktail with Protein Transport Inhibitors (500X) (00-4975-03 – Thermo Fisher Scientific, UK) was added to each well at a final concentration of 1X – this treatment ensures intracellular retention of cytokines stimulated by phorbol 12-myristate 13-acetate (PMA) and ionomycin. After 5 hours of re-stimulation, T cells were harvested into FACS tubes, which then underwent centrifugation at 400g for 5 minutes, before the supernatant was removed and the cells were re-suspended in 500µL PBS, prior to centrifugation at 400g for 5 minutes again. Cytokine expression was measured using a Foxp3/Transcription Factor Staining Buffer Set, as used previously. In brief, upon removal of the cell supernatant, 500µL cold Fixation Buffer was added to each FACS tube and they were briefly vortexed, before incubation at 4°C for 30 minutes. Following incubation, the cells were centrifugated at 400g for 5 minutes, before the supernatant was removed and the cells were re-suspended in 1ml PBS, in order to remove traces of the Fixation Buffer, during centrifugation at 400g for 5 minutes. After the supernatant had been removed, 2ml room temperature Permeabilisation Buffer was added to each FACS tube and they were briefly vortexed, prior to centrifugation at 400g for 5 minutes – this process was repeated once, so the cells endured two rounds of permeabilisation. Disposal of the supernatant was followed by the addition of the following antibodies: 5µL V450 mouse anti-human IFN-γ (560371), 5µL PE-Cy™7

mouse anti-human TNF-α (560678), 5µL BV711 rat anti-human IL-13 (564288), 5µL BV510 mouse anti-human IL-17A (563295), 20µL PE mouse anti-human IL-21 (560463), and 5µL Alexa Fluor® 488 mouse anti-human TGF-β1 (562545). In addition, both naïve CD4+CD45RA+ T cells and bulk CD4+ T cells were stained with 5µL PE-Cy™5 mouse anti-human CD4 (555348), whilst bulk CD8+ T cells were stained with 5µL PE-Cy™5 mouse anti-human CD8 (555368) (all from BD Biosciences, USA), before cells were briefly vortexed and incubated at room temperature for 30 minutes in the dark. Following incubation, 1X Permeabilisation Buffer was added to each FACS tube and they were briefly vortexed, prior to centrifugation at 400g for 5 minutes, before being re-suspended in 300µL Permeabilisation Buffer, prior to flow cytometric analysis. Each condition also had an Unstained Control, which was used as a reference point for gating, during analysis using the Beckman Coulter CytoFLEX S flow cytometer, FlowJo and GraphPad Prism software.

2.1.15. Statistical Analyses

Using GraphPad Prism (Version 9) software, statistical analyses were performed using an unpaired non-parametric Kruskal-Wallis test, followed by an Uncorrected Dunn's test for all experiments with 3 or more variables. For direct comparisons between two variables, unpaired non-parametric Mann-Whitney's tests were used, which were two tailed with a 95% confidence interval. P Values: * = <0.05; ** = <0.01; *** = <0.001; **** = <0.0001.

3. Identification of a novel multi-cytokine therapy for glioblastoma

3.1. Introduction

Gliomas are the most common primary malignant brain tumours found in humans, with glioblastoma accounting for 80% of all glioma diagnoses. Current glioblastoma treatments are associated with poor survival and tumour recurrence, thus alternative therapies urgently need to be developed (T. A. Wilson et al., 2014). Cancer immunotherapies have significant promise as they can alter the immune system to promote anti-tumour immunity. In particular, IFN-γ and TNF-α have each shown significant potential as individual cytokine therapies in glioblastoma. Unfortunately, the direct effects of IL-21 on glioblastoma cells have not been explored, nor it is not currently known whether each of these cytokines are effective when administered together. In order to improve our understanding of the clinical benefits of these cytokines in glioblastoma, the direct effects of IFN-γ, IL-21 and TNF-α, both individually and combined, on two glioblastoma cell lines (U251 and U373) were investigated, with a particular focus on proliferation and cell death.

3.2. Results

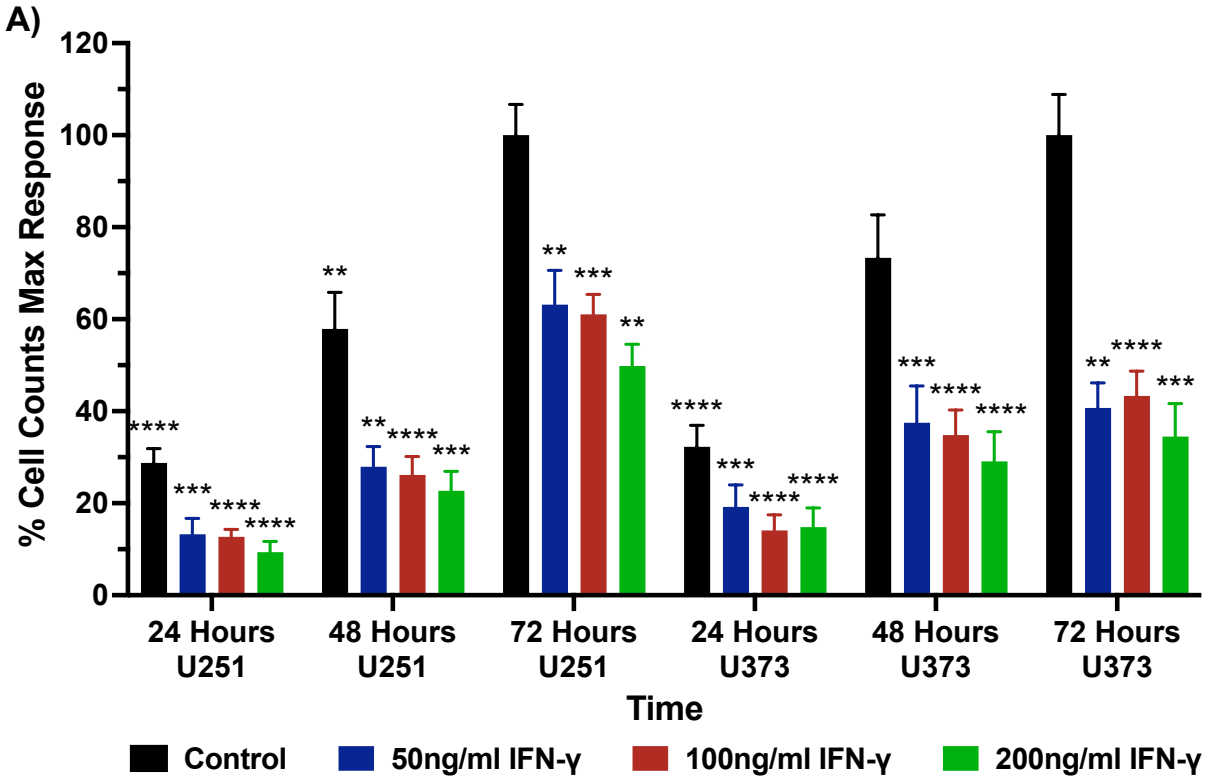
3.2.1. IFN-γ, IL-21 and TNF-α independently reduced glioblastoma cell counts

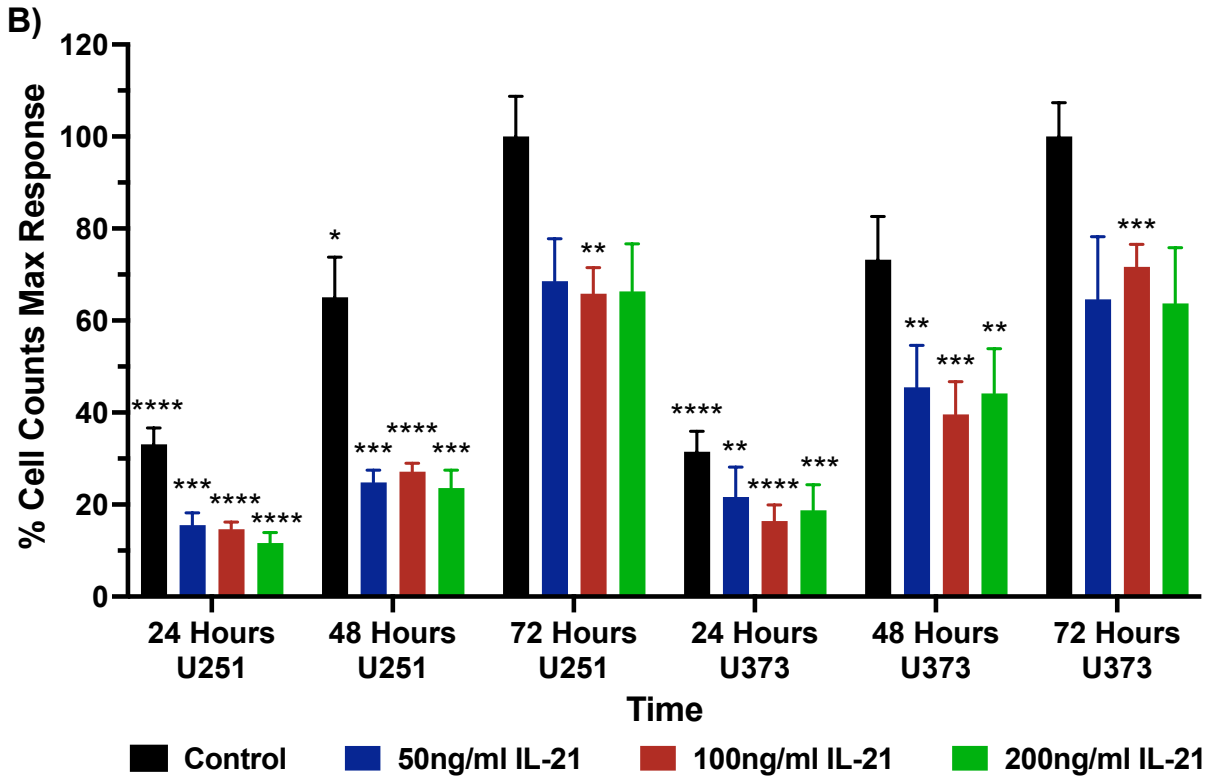
Based upon previous literature that explored the direct effects of IFN-γ, IL-21 or TNF-α on other cancer cell lines, three doses were selected (50ng/ml, 100ng/ml and 200ng/ml) to determine their impact on the cell counts of two glioblastoma cell lines (U251 and U373), over the course of 72 hours. Every 24 hours, the cells were harvested and counts were generated using flow cytometry, with the 72h Control result set as the 100% maximum response.

An immediate inhibitory effect of IFN-γ on both cell lines was visible after 24 hours: this was subsequently maintained across each of the doses throughout the time course, with the most significant reduction apparent after 72 hours, highlighting a possible titratable effect between doses. After 72 hours, 200ng/ml IFN-γ potently reduced cell counts (50% - $p = 0.0001$), with 100ng/ml IFN-γ providing a moderate response (61% - $p = 0.0001$), whilst the weakest response seen in U251 cells was with 50ng/ml IFN-γ (63% - $p = 0.0565$) (Figure 3.1A). On the other hand, all three doses of IFN-γ had a much stronger impact on U373 cells after 72 hours, reducing the maximum response much further to 41% (50ng/ml – $p = 0.0020$), 43% (100ng/ml – $p < 0.0001$), and 35%, respectively (200ng/ml – $p = 0.0002$) (Figure 3.1A).

IL-21 administration produced similar results in both cell lines after 24 hours, although there was minimal indication of a titratable effect by the end of the time course. After 72 hours, all three doses reduced the maximum response to a comparable level, with 50ng/ml at 69% ($p = 0.2487$), 100ng/ml at 66% ($p = 0.0019$), and 200ng/ml IL-21 restricting U251 cell counts to 66% ($p = 0.1573$) of the maximum response (Figure 3.1B). Similar results were also found in U373 cells, as only 100ng/ml IL-21 significantly reduced cell counts after 72 hours of treatment (72% - $p = 0.0004$), compared to the Control (Figure 3.1B).

Along with IFN-γ and IL-21, TNF-α was also effective in reducing the number of cells in both cell lines after 24 hours. At the end of the time course, 50ng/ml TNF-α proved to have no impact on U251 cell counts, whilst 100ng/ml TNF-α had quite a substantial effect (73% - $p = 0.0121$), relative to the Control (Figure 3.1C). These findings were also reflected in U373 cells, in which 50ng/ml TNF-α had a highly variable response (82% - $p = 0.3915$), whilst 100ng/ml TNF-α (65% - $p = 0.0023$) and 200ng/ml TNF-α (46% - $p = 0.0029$) demonstrated potent inhibitory effects (Figure 3.1C). Nonetheless, these findings established that IFN-γ, IL-21 and TNF-α could each independently reduce U251 and U373 cell counts.


A)
% Cell Counts Max Response
120
100
80
60
40
20
0
24 Hours U251
48 Hours U251
72 Hours U251
24 Hours U373
48 Hours U373
72 Hours U373
Time
Control
50ng/ml IFN-γ
100ng/ml IFN-γ
200ng/ml IFN-γ


B)
% Cell Counts Max Response
120
100
80
60
40
20
0
24 Hours U251
48 Hours U251
72 Hours U251
24 Hours U373
48 Hours U373
72 Hours U373
Time
Control
50ng/ml IL-21
100ng/ml IL-21
200ng/ml IL-21

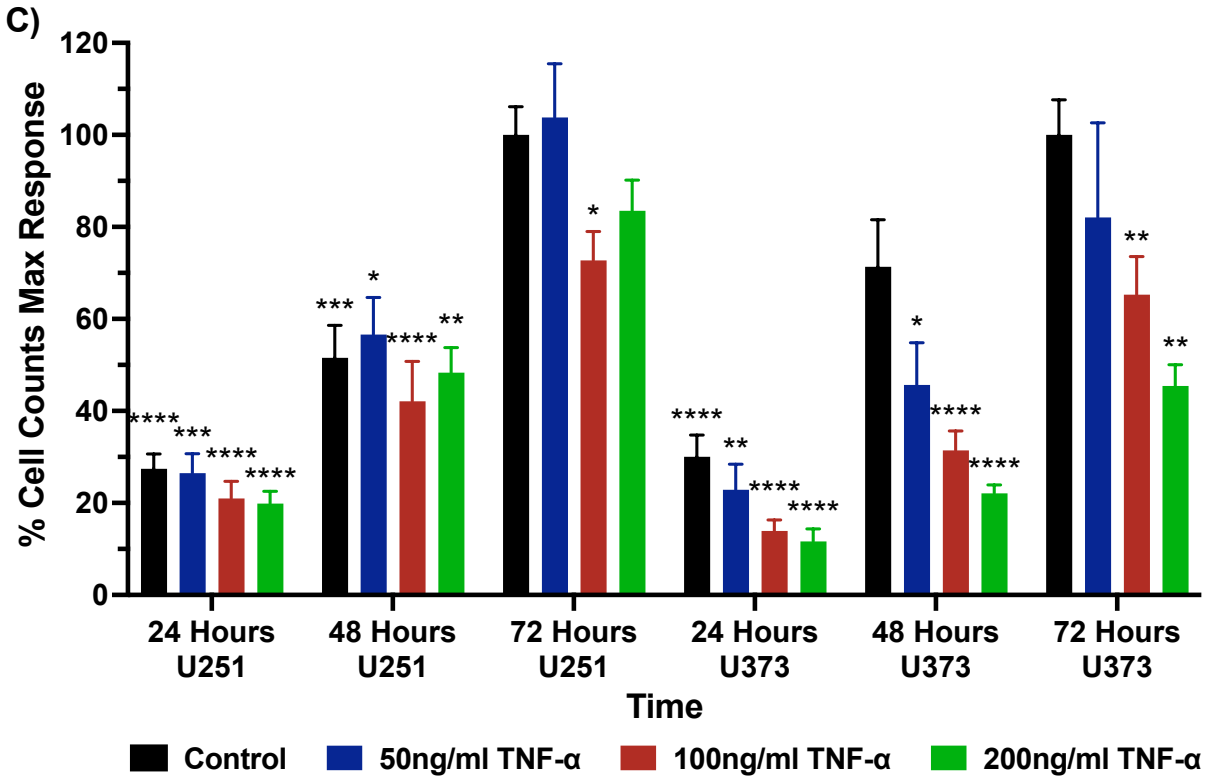


Figure 3.1. IFN-γ, IL-21 and TNF-α independently reduced U251 and U373 cell counts

$2.5x10^4$ U251 cells and $2.5x10^4$ U373 cells were seeded with **A)** an IFN-γ titration (50, 100 and 200ng/ml), **B)** an IL-21 titration (50, 100 and 200ng/ml) and **C)** a TNF-α titration (50, 100 and 200ng/ml) for 72 hours. Counts were acquired using flow cytometry every 24 hours. Bars represent means compared to the 72 hour Control and SEM is shown for > 3 independent experiments. P Values: * = <0.05; ** = <0.01; *** = <0.001; **** = <0.0001.

3.2.2. Combination of IFN-γ, IL-21 and TNF-α reduced glioblastoma cell counts

The results from the initial experiments demonstrated that each of the cytokines at all of the selected doses were effective in reducing cell counts in both glioblastoma cell lines. Next, it was necessary to explore the combinatorial effects of IFN-γ, IL-21 and TNF-α to see whether it was possible to reduce the cell counts further. Based upon initial titration results, it appeared that a 100ng/ml dose of each cytokine provided the most consistent and sufficient results in both cell lines; therefore, this was the dose selected to use in all future experiments. In line with this, U251 and U373 cell lines were seeded with 100ng/ml IFN-γ, IL-21 and TNF-α, both independently and combined, over the course of 72 hours. Every 24 hours, the cells were harvested and counts generated using flow cytometry, with the 72h Control result set as the 100% maximum response.

Supporting the initial experiments, 100ng/ml IFN-γ, IL-21 and TNF-α each independently provided a significant reduction in U251 cell counts after 24 hours and 48 hours, though the significance of this effect for IL-21 was lost by the end of the time course, likely due to experimental variability. In particular, IFN-γ treatment diminished U251 cell counts to 66% ($p = 0.0074$) of the maximum response, with comparable results seen with IL-21 (76% - $p = 0.0878$) and TNF-α (75% - $p = 0.0383$), after 72 hours (Figure 3.2). When the cytokines were combined as dual treatments, only IFN-γ and TNF-α further enhanced their inhibitory effects on U251 cell counts (45% - $p < 0.0001$). However, the most potent response was seen when all three of the cytokines were combined together, providing just 44% of the U251 cell count maximum response, after 72 hours ($p < 0.0001$).

Likewise, each of the cytokines also significantly reduced U373 cell counts (Figure 3.2). As shown in the previous experiments, IFN-γ (48% - $p < 0.0001$) had the strongest independent effect in U373 cells, followed by an intermediate TNF-α effect (63% - $p = 0.0049$) and the weakest impact was from IL-21 treatment (74% - $p = 0.1033$), after 72 hours. Unlike the results found with U251 cells, all of the dual therapies were more powerful in their abilities to diminish U373 cell counts (45% - $p < 0.0001$; 41% - $p < 0.0001$; 49% – $p = 0.0002$, respectively), which was further enhanced when all three of the cytokines were combined together (33% - $p < 0.0001$). Altogether, these results demonstrated that combining IFN-γ, IL-21 and TNF-α together potently enhanced their individual abilities to reduce U251 and U373 cell counts.

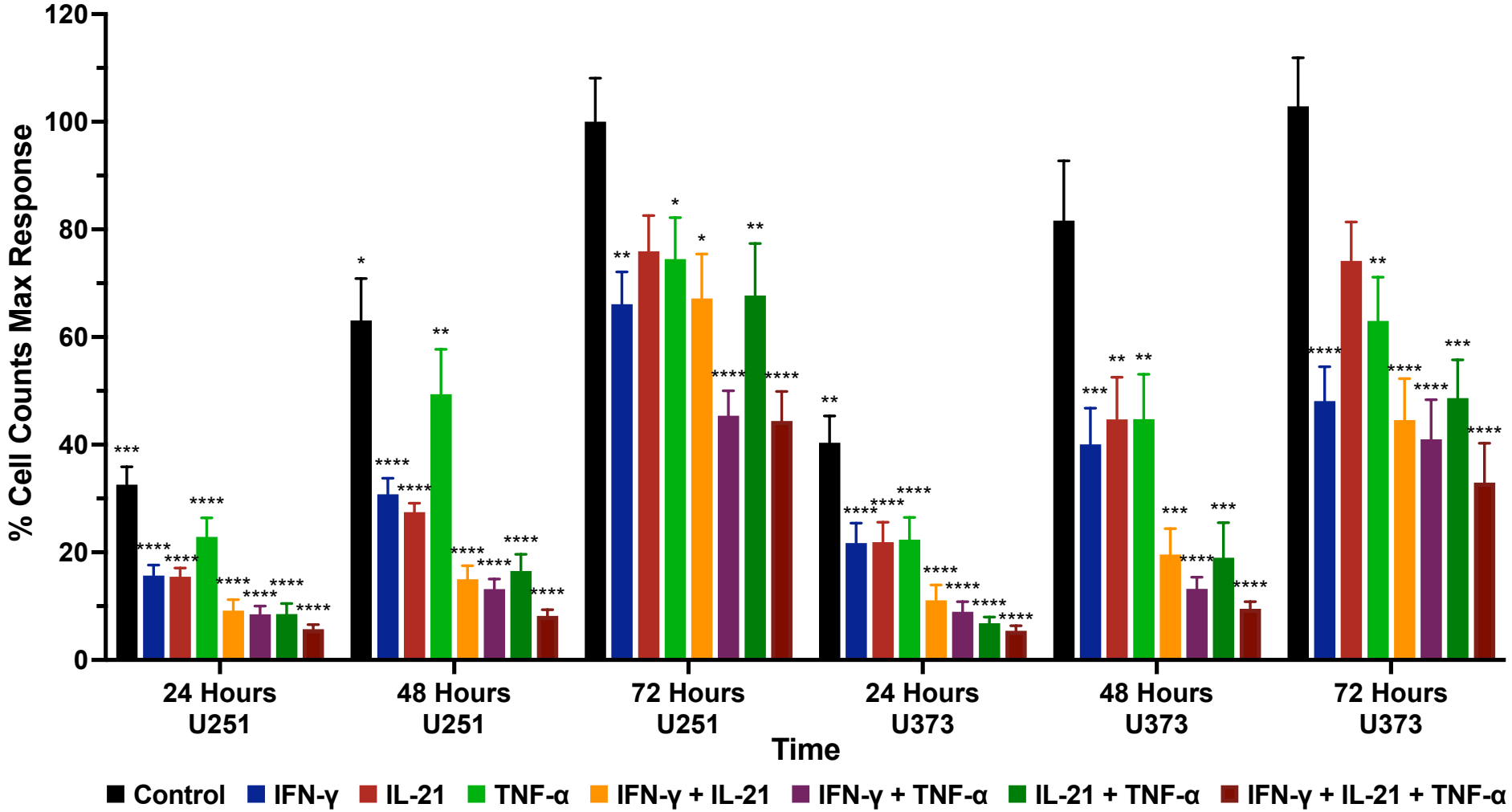


Figure 3.2. Combination of IFN-γ, IL-21 and TNF-α reduced U251 and U373 cell counts

$2.5x10^4$ U251 cells and $2.5x10^4$ U373 cells were independently seeded with 100ng/ml IFN-γ, IL-21 and TNF-α, both individually and combined, for 72 hours. Counts were acquired using flow cytometry every 24 hours. Bars represent means compared to the 72 hour Control for each cell line and SEM is shown for > 3 independent experiments. P Values: * = <0.05; ** = <0.01; *** = <0.001; **** = <0.0001.

3.2.3. IFN-γ, IL-21 and TNF-α profoundly affected glioblastoma morphology and density

Having established that IFN-γ, IL-21 and TNF-α had direct anti-tumour effects on both cell lines, it was important to investigate this further by exploring their visual impact on glioblastoma cells. In order to achieve this, U251 and U373 cells were cultured for 72 hours with 100ng/ml IFN-γ, IL-21 and TNF-α, both independently and combined, before images were taken to compare against cells cultured in media alone (Control) to evaluate any changes in cell shape and/or density.

Consistent with previous findings, there were notably fewer U251 cells across all of the cytokine conditions (Figure 3.3B-H), compared to the Control (Figure 3.3A). In addition, when U251 cells were cultured with IFN-γ alone (Figure 3.3B) or IFN-γ plus TNF-α (Figure 3.3F), a substantial amount of the cells displayed a more rounded morphology – a key characteristic of cell death. On the other hand, U251 cells became thinner and more elongated in cultures with IFN-γ plus IL-21 (Figure 3.3E), IL-21 plus TNF-α (Figure 3.3G) or all three cytokines together (Figure 3.3H), perhaps due to the decline in cell density and subsequent loss of cell-to-cell contact.

Similar results were identified in U373 cells as well, with a noticeable decrease in cell density and rounded morphology in each of the cytokine conditions (Figure 3.4B-H), compared to the Control (Figure 3.4A). However, the presence of cellular extensions in U373 cells only appeared when treated with IFN-γ plus TNF-α (Figure 3.4F), as well as when all three cytokines were administered together (Figure 3.4H). Altogether, these images supported previous findings that IFN-γ, IL-21 and TNF-α inhibited the growth of both glioblastoma cell lines, whilst identifying that these cytokines profoundly affected their morphology, perhaps by disrupting their proliferation or inducing cell death.

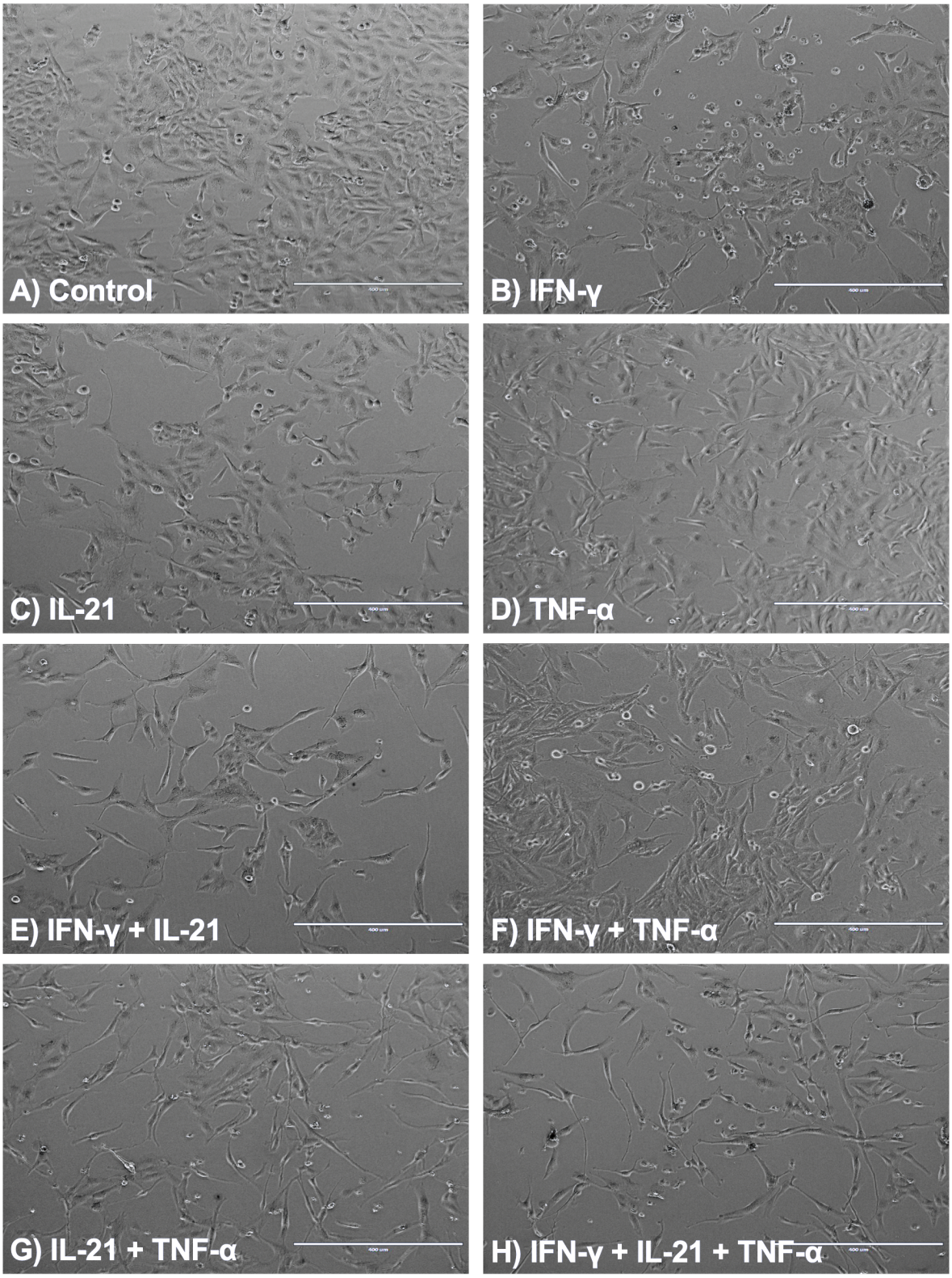


Figure 3.3. IFN-γ, IL-21 and TNF-α affected U251 cell morphology and density

$2.5x10^4$ U251 cells were seeded with **A)** RPMI Control; **B)** 100ng/ml IFN-γ; **C)** 100ng/ml IL-21; **D)** 100ng/ml TNF-α; **E)** 100ng/ml IFN-γ and IL-21; **F)** 100ng/ml IFN-γ and TNF-α; **G)** 100ng/ml IL-21 and TNF-α; **H)** 100ng/ml IFN-γ, IL-21 and TNF-α. Bar is equivalent to 400µm. Images taken after 72 hours, at 10X resolution and are representative of > 3 independent experiments

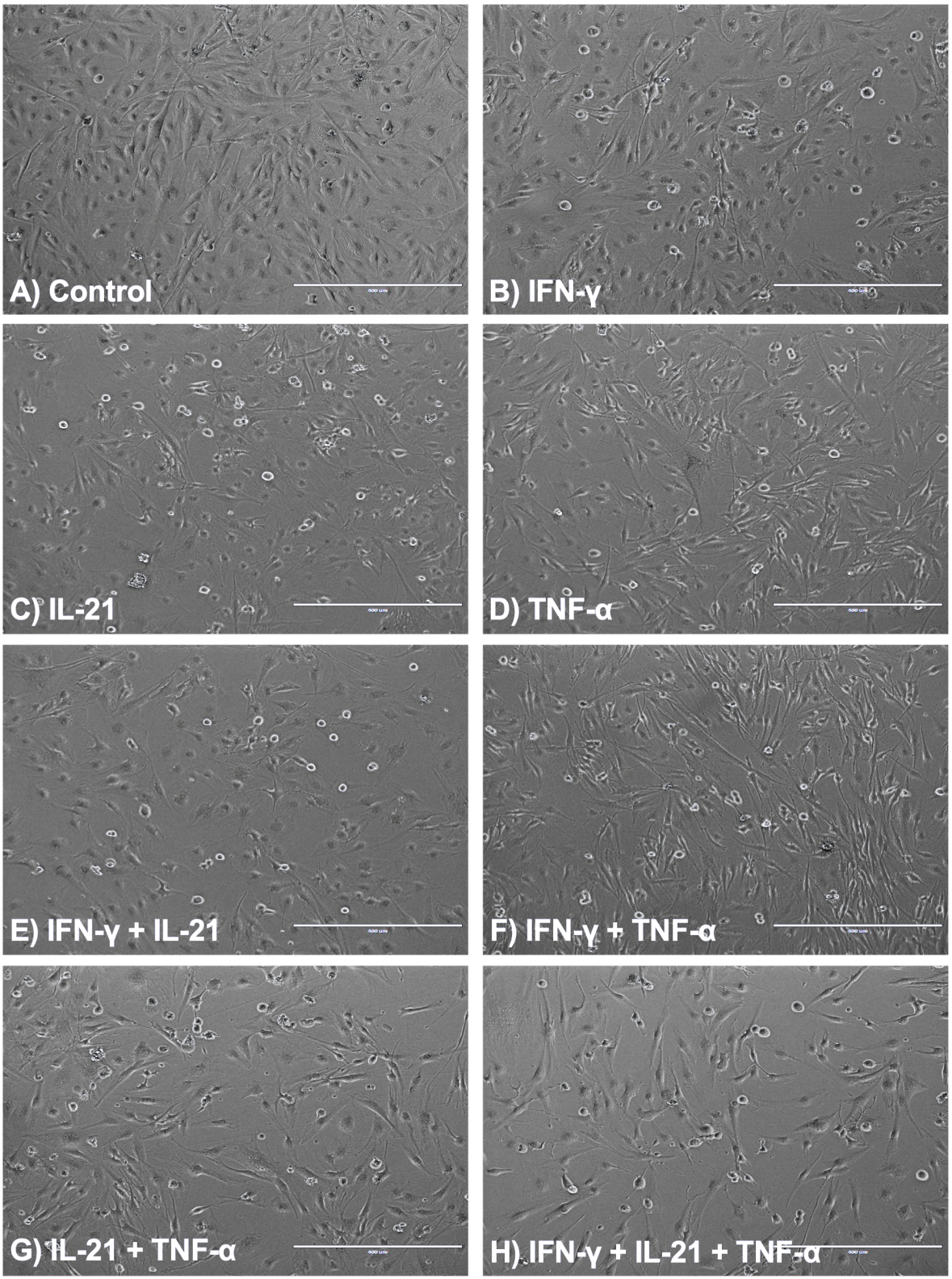


Figure 3.4. IFN-γ, IL-21 and TNF-α affected U373 cell morphology and density

$2.5x10^4$ U373 cells were seeded with **A)** RPMI Control; **B)** 100ng/ml IFN-γ; **C)** 100ng/ml IL-21; **D)** 100ng/ml TNF-α; **E)** 100ng/ml IFN-γ and IL-21; **F)** 100ng/ml IFN-γ and TNF-α; **G)** 100ng/ml IL-21 and TNF-α; **H)** 100ng/ml IFN-γ, IL-21 and TNF-α. Bar is equivalent to 400µm. Images taken after 72 hours, at 10X resolution and are representative of > 3 independent experiments.

3.2.4. IFN-γ, IL-21 and TNF-α reduced patient-derived glioblastoma cell counts

In order to validate these experiments, it was necessary to repeat the same processes to explore the impact of IFN-γ, IL-21 and TNF-α on patient-derived glioblastoma cells. Unfortunately, these cells were particularly sensitive and difficult to maintain *in vitro*, thus most of the cell lines rapidly deteriorated after arrival and were not viable for subsequent experimentation. However, a small number of experiments were completed with one of the patient-derived cell lines (367). In particular, 367 cells were seeded with 100ng/ml IFN-γ, IL-21 and TNF-α, both independently and combined, before images were taken after 72 hours to compare against Control cells, in order to evaluate any changes in cell shape and/or density. Immediately after this, the cells were harvested and counts were generated using flow cytometry, with the Control result set as the 100% maximum response.

Results largely aligned with data from U251 and U373 cells, in that IFN-γ, IL-21 and TNF-α each individually reduced the 367 cell counts, but the independent effects were not statistically significant – this is likely due to the variability in the growth of the Control cells. Nevertheless, there was a potent inhibition of 367 cell growth when IFN-γ, IL-21 and TNF-α were administered together, producing just 41% of the maximum response ($p = 0.0016$) (Figure 3.5). These findings were further supported by the clear loss in cell density across each of the individual treatments (Figure 3.6B-D), with the largest impact seen in the triple cytokine condition (Figure 3.6E), compared to the Control (Figure 3.6A). Additionally, 367 cells became noticeably longer and thinner, particularly when treated with the combination of IFN-γ, IL-21 and TNF-α – this was also seen in both U251 and U373 cell lines, potentially as a consequence of the decrease in cell density and subsequent loss of cell-to-cell contact.

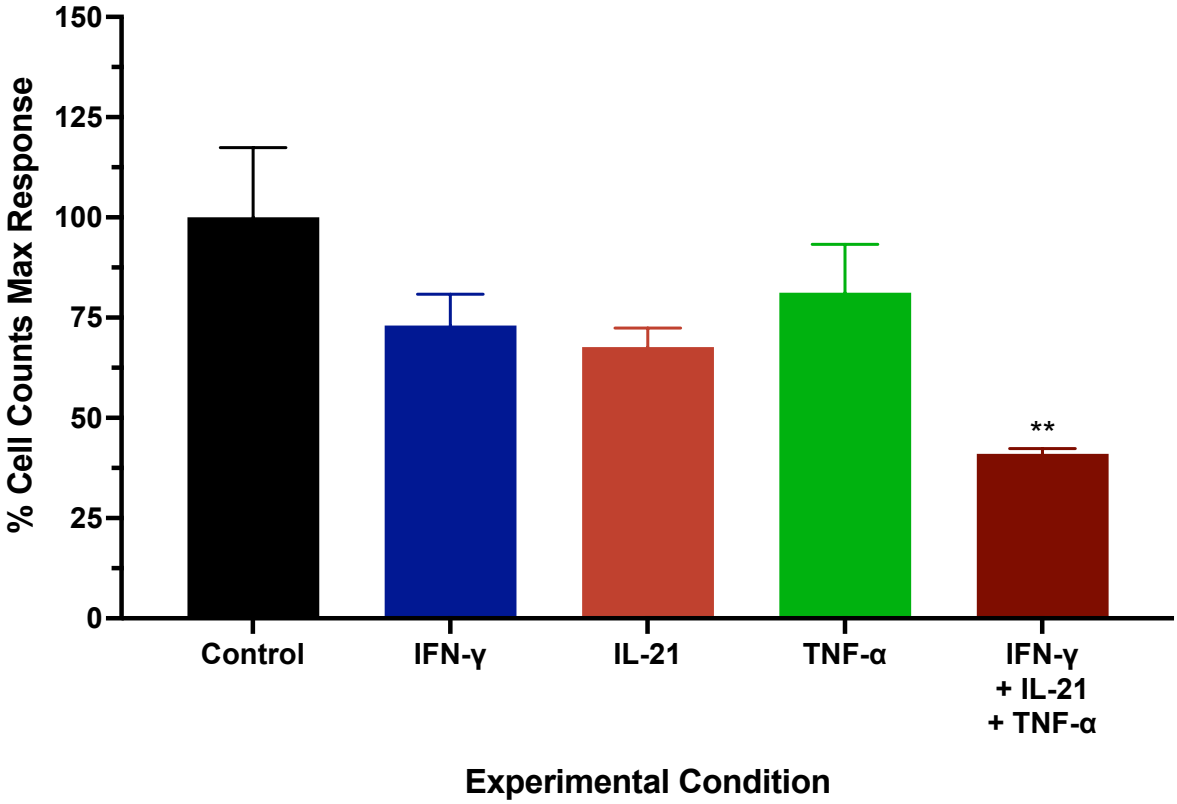


Figure 3.5. IFN-γ, IL-21 and TNF-α reduced patient-derived glioblastoma cell counts

$2.5x10^4$ 367 cells were seeded with 100ng/ml IFN-γ, IL-21 and TNF-α, both individually and combined. Counts were acquired using flow cytometry after 72 hours. Bars represent means compared to the Control and SEM is shown for 3 independent experiments. P Values: * = <0.05; ** = <0.01; *** = <0.001; **** = <0.0001.

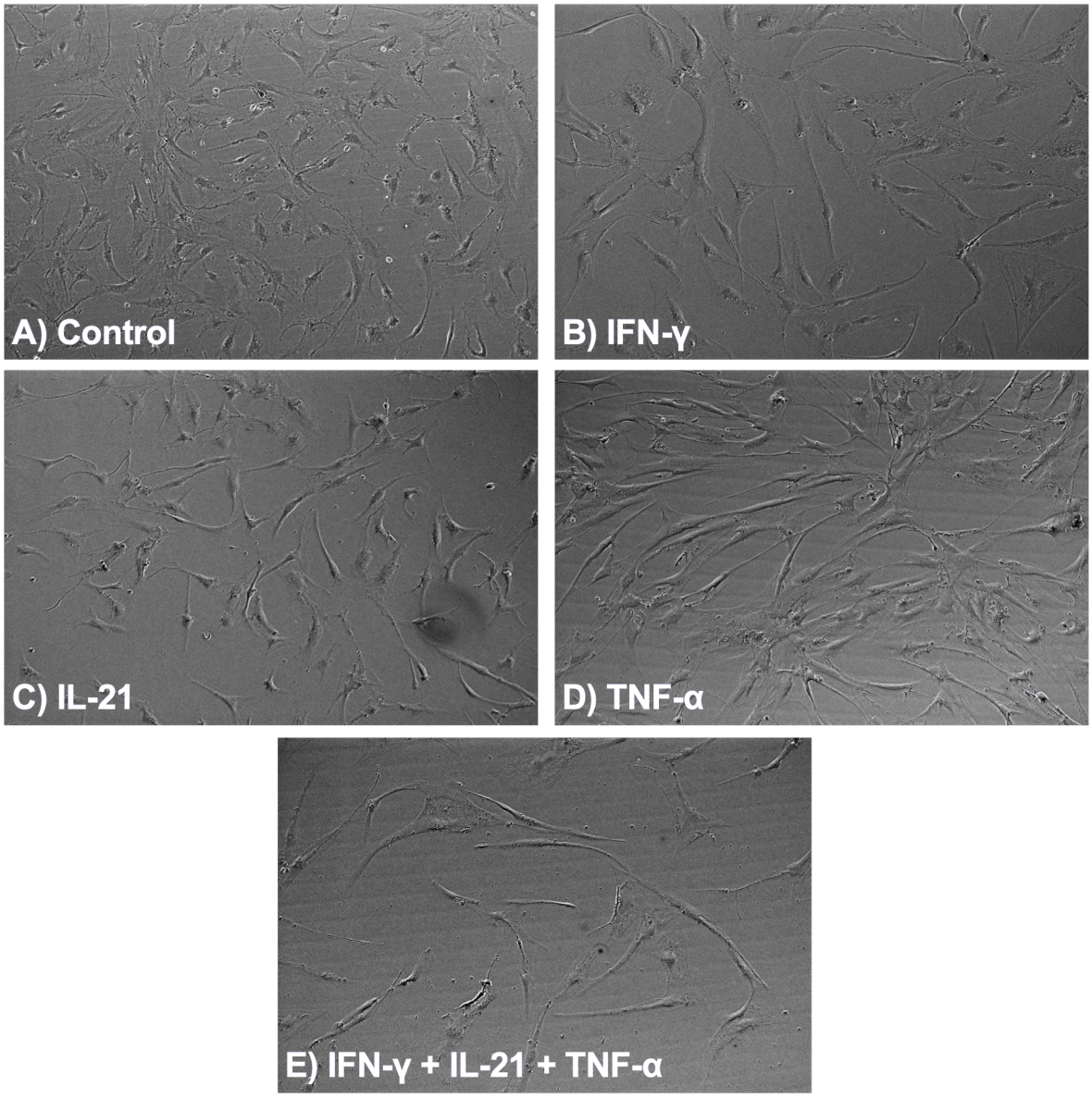


Figure 3.6. IFN-γ, IL-21 and TNF-α reduced patient-derived glioblastoma cell density

$2.5x10^4$ 376 cells were seeded with **A)** RPMI Control; **B)** 100ng/ml IFN-γ; **C)** 100ng/ml IL-21; **D)** 100ng/ml TNF-α; **E)**100ng/ml IFN-γ, IL-21 and TNF-α. Images were taken after 72 hours, at 10X resolution and are representative of 3 independent experiments.

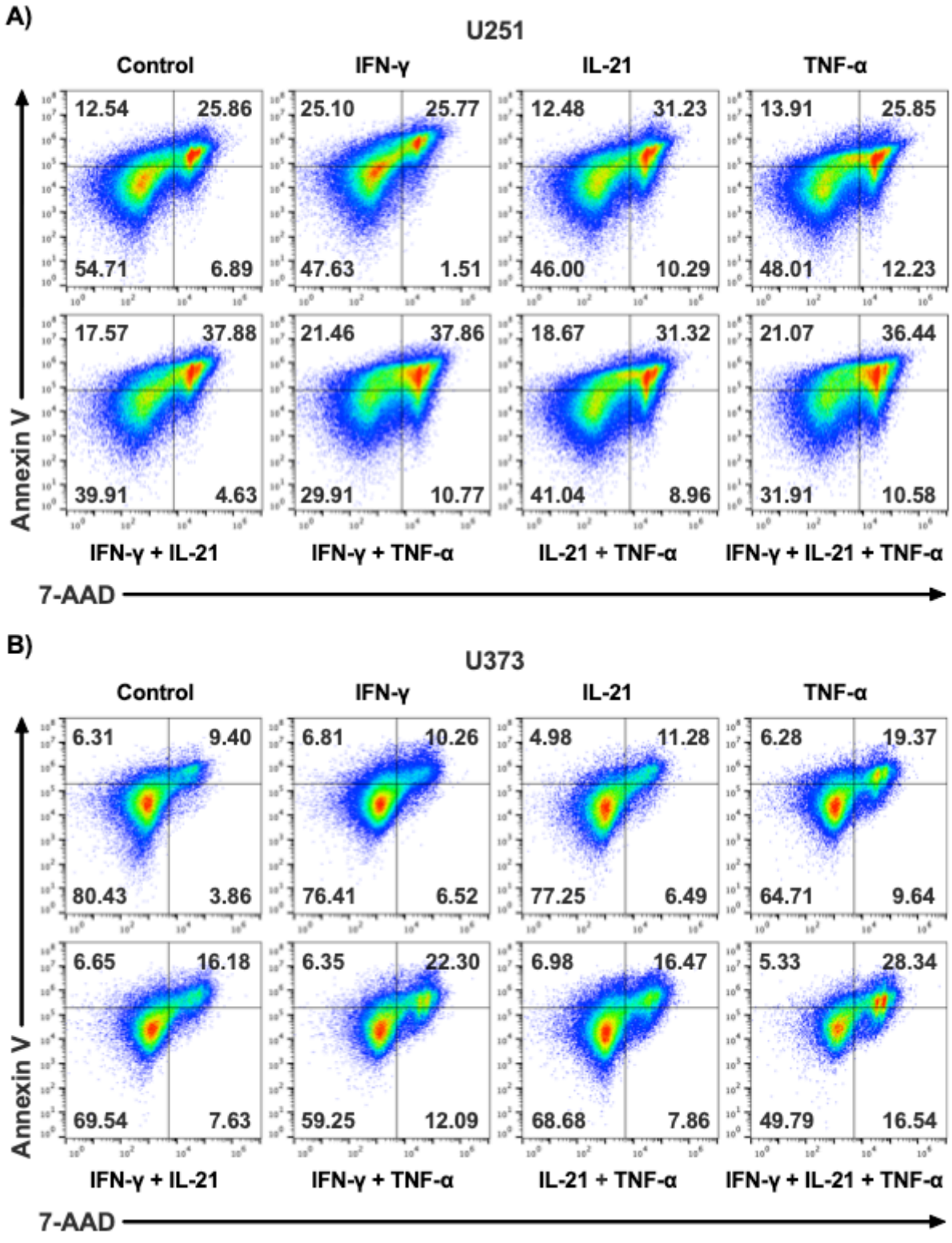
3.2.5. IFN-γ, IL-21 and TNF-α induced glioblastoma cell death

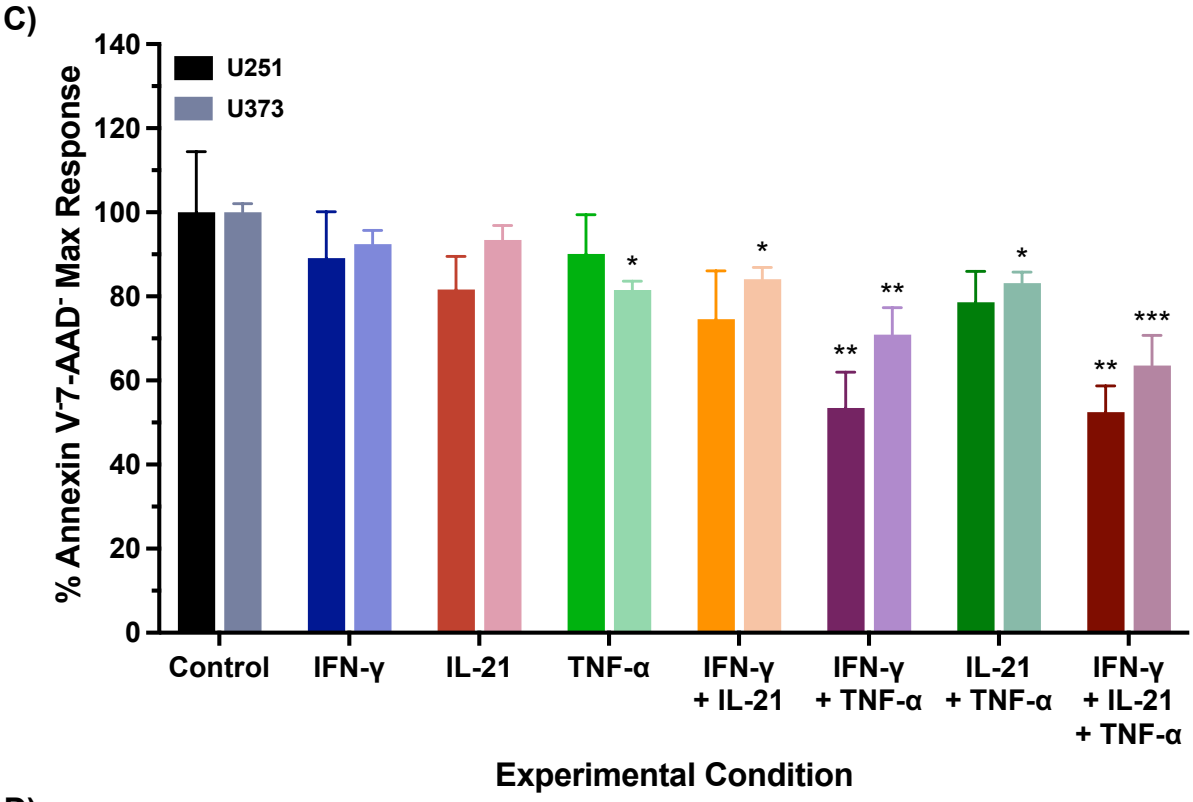
The decline in cell counts and presence of cell rounding indicated that IFN-γ, IL-21 and TNF-α could have been inducing glioblastoma cell death. In the early stages of apoptosis, phosphatidylserine (PS) is translocated to the outer layer of the cell membrane to enable recognition and clearance by phagocytes (Fadok et al., 1992). Annexin V has a high affinity for PS, thereby allowing its expression to be utilised as an accurate method of measuring PS translocation. During apoptosis, the cell membrane becomes disrupted by the generation of apoptotic bodies, thus Annexin V can also enter these late apoptotic cells and bind to PS on the inner layer (Vermes et al., 1995). In order to establish membrane integrity, Annexin V expression must be measured in conjunction with a dye-exclusion test (e.g. Propidium Iodide or 7-AAD), which bind to DNA to provide insight into the balance between apoptosis and necrosis. As these dyes are excluded from cells with intact membranes, both of these markers enable the identification of healthy cells (Annexin $V^-7\text{-}AAD^-$), early apoptotic cells (Annexin $V^+7\text{-}AAD^-$), late apoptotic cells (Annexin $V^+7\text{-}AAD^+$), or necrotic cells (Annexin $V^-7\text{-}AAD^+$) (Baskić et al., 2006; Kepp et al., 2011). In line with this, U251 and U373 cells were seeded with 100ng/ml IFN-γ, IL-21 and TNF-α, both independently and in combination with each other for 72 hours. The cells were then harvested and stained for both Annexin V and 7-AAD for analysis via flow cytometry, with the 72h Control result set as the 100% maximum response.

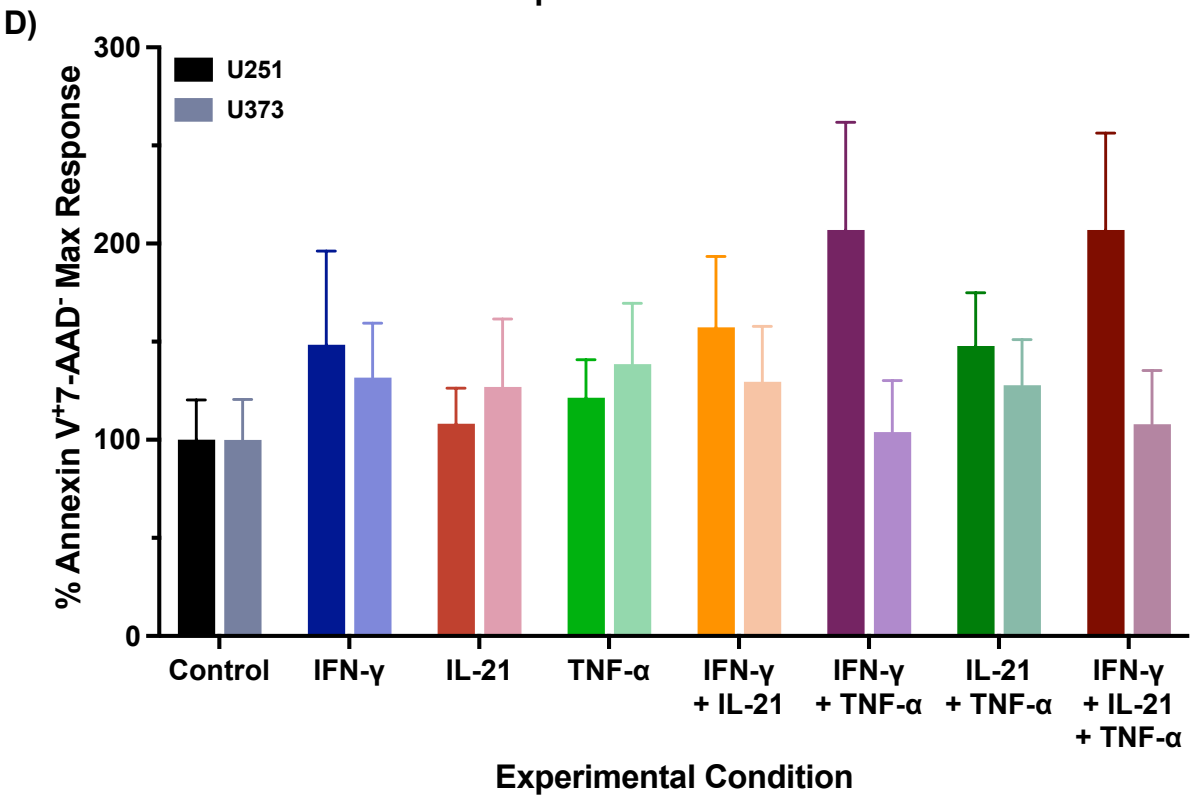
In terms of the healthy U251 cell population, IFN-γ plus TNF-α (54% - $p = 0.0074$) produced almost identical results to the triple cytokine treatment (53% - $p = 0.0027$), indicating that IFN-γ and TNF-α were likely the driving forces behind the induction of U251 cell death (Figure 3.7C). Although the results were highly variable and not statistically significant, the data somewhat suggested that IFN-γ alone (150% - $p = 0.4818$) could induce early apoptosis, but this was sustained by the addition of IL-21 (157% - $p = 0.3169$) or augmented in combination with TNF-α (207% - $p = 0.1333$), as reflected in the triple cytokine condition (207% - $p = 0.1105$) (Figure 3.7D). Similar results were found when investigating the induction of necrosis in U251 cells, as the relative number of necrotic cells was elevated when IFN-γ, IL-21 and TNF-α were administered together, but this was not statistically significant (178% - $p = 0.1941$) (Figure 3.7E). Following this pattern of results, there was a slight trend which indicated that both of the dual treatments containing IFN-γ (135% - $p = 0.4171$ and 149% - $p = 0.2448$, respectively) and the triple cytokine condition (149% - $p = 0.2134$) could have been involved in increasing the proportion of cells in the latter stages of apoptosis, but none of these results were statistically significant (Figure 3.7F).

Consistent with the findings in U251 cells, IFN-γ plus TNF-α (71% - $p = 0.0012$) significantly diminished the healthy U373 cell population, but this was enhanced with the addition of IL-21 (64% - $p = 0.0003$) (Figure 3.7C). However, none of the cytokine treatments induced early apoptosis in U373 cells, even when administered all together (108% - $p = 0.8802$) (Figure 3.7D). Conversely, the dual treatment of IFN-γ and TNF-α alone notably induced necrosis (350% - $p = 0.0025$), which was boosted by the addition of IL-21 (421% - $p = 0.0009$). Given that the independent administration of

IFN-γ and IL-21 produced comparable results to the Control (97% - p = 0.9304 and 107% – p = 0.8351, respectively), it is plausible that this effect was mediated by TNF-α, despite the lack of statistical significance (148% - p = 0.1101) (Figure 3.7E). The influence of TNF-α is further reflected in the proportion of cells in the latter stages of apoptosis, with a moderate induction caused by TNF-α alone (185% - p = 0.0053), which was enhanced in combination with IFN-γ (233% - p = 0.0015) and boosted even further when administered alongside both IFN-γ and IL-21 (262% - p = 0.0005) (Figure 3.7F). Collectively, these results demonstrated that IFN-γ, IL-21 and TNF-α induced apoptosis and necrosis in U373 cells, whilst suggesting that IFN-γ, IL-21 and TNF-α could induce apoptosis in U251 cells.


A)
U251
Control
IFN-γ
IL-21
TNF-α
12.54
25.86
54.71
6.89
25.10
25.77
47.63
1.51
12.48
31.23
46.00
10.29
13.91
25.85
48.01
12.23
17.57
37.88
39.91
4.63
21.46
37.86
29.91
10.77
18.67
31.32
41.04
8.96
21.07
36.44
31.91
10.58
Annexin V
IFN-γ + IL-21
IFN-γ + TNF-α
IL-21 + TNF-α
IFN-γ + IL-21 + TNF-α
7-AAD
B)
U373
Control
IFN-γ
IL-21
TNF-α
6.31
9.40
80.43
3.86
6.81
10.26
76.41
6.52
4.98
11.28
77.25
6.49
6.28
19.37
64.71
9.64
6.65
16.18
69.54
7.63
6.35
22.30
59.25
12.09
6.98
16.47
68.68
7.86
5.33
28.34
49.79
16.54
Annexin V
IFN-γ + IL-21
IFN-γ + TNF-α
IL-21 + TNF-α
IFN-γ + IL-21 + TNF-α
7-AAD


C)
U251
U373
% Annexin V-7-AAD- Max Response
140
120
100
80
60
40
20
0
*
*
**
**
*
**
***
Control
IFN-γ
IL-21
TNF-α
IFN-γ + IL-21
IFN-γ + TNF-α
IL-21 + TNF-α
IFN-γ + IL-21 + TNF-α
Experimental Condition


D)
U251
U373
% Annexin V+7-AAD- Max Response
300
200
100
0
Control
IFN-γ
IL-21
TNF-α
IFN-γ + IL-21
IFN-γ + TNF-α
IL-21 + TNF-α
IFN-γ + IL-21 + TNF-α
Experimental Condition

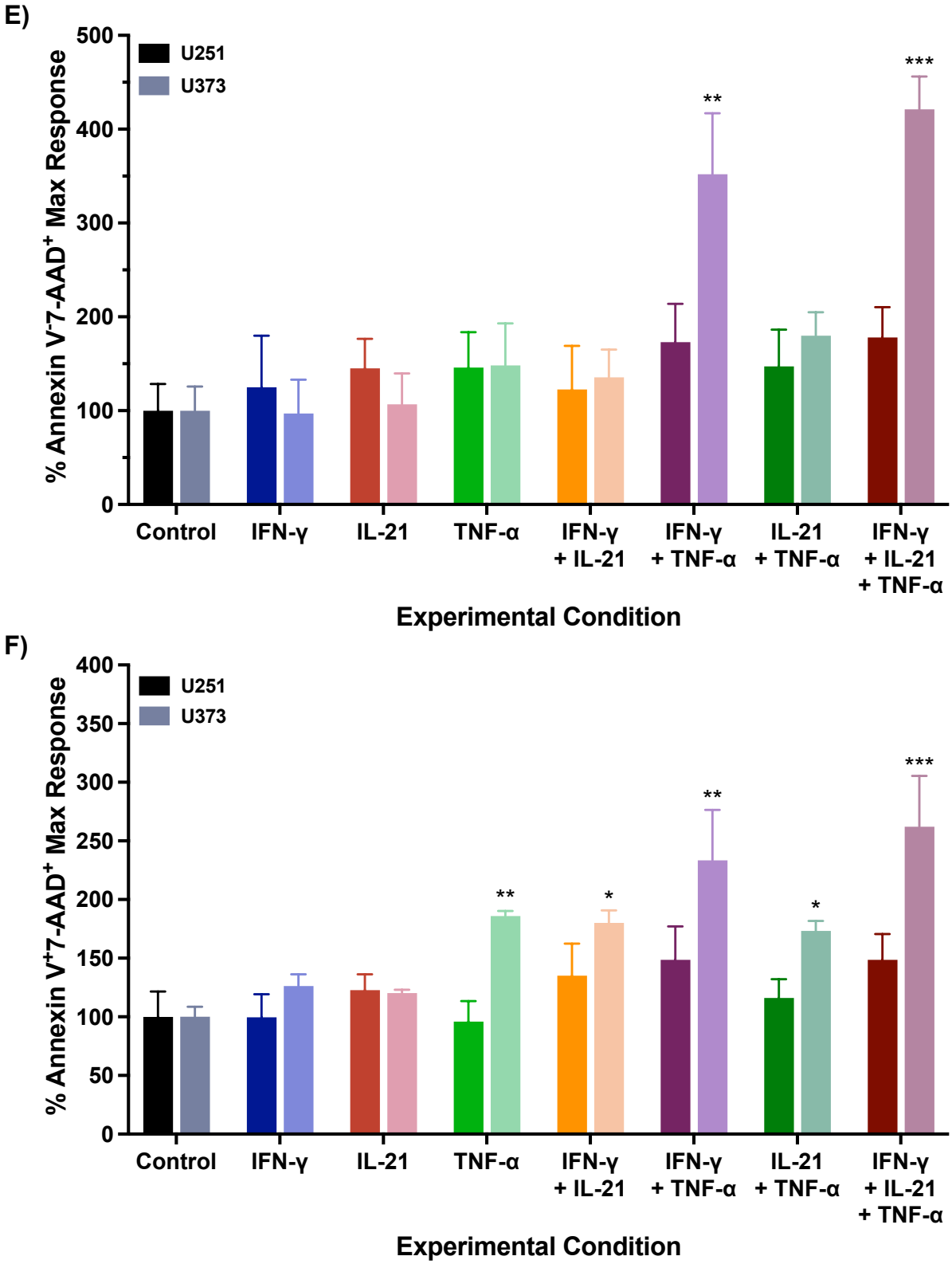


Figure 3.7. IFN-γ, IL-21 and TNF-α induced apoptosis and necrosis in U373 cells

$5x10^4$ U251 cells **A)** and $5x10^4$ U373 cells **B)** were seeded with an RPMI Control or 100ng/ml IFN-γ, 100ng/ml IL-21, 100ng/ml TNF-α, both individually and combined. Cells were harvested after 72 hours and stained for Annexin V and 7-AAD for analysis via flow cytometry, generating **C)** healthy Annexin V^-7AAD^-; **D)** early apoptotic Annexin $V^+7\text{-}AAD^-$**; E)** necrotic Annexin $V^-7\text{-}AAD^+$; and **F)** late apoptotic Annexin $V^+7\text{-}AAD^+$ cell populations. Bars represent means compared to the Control and SEM is shown for > 3 independent experiments. P Values: * = <0.05; ** = <0.01; *** = <0.001; **** = <0.0001.

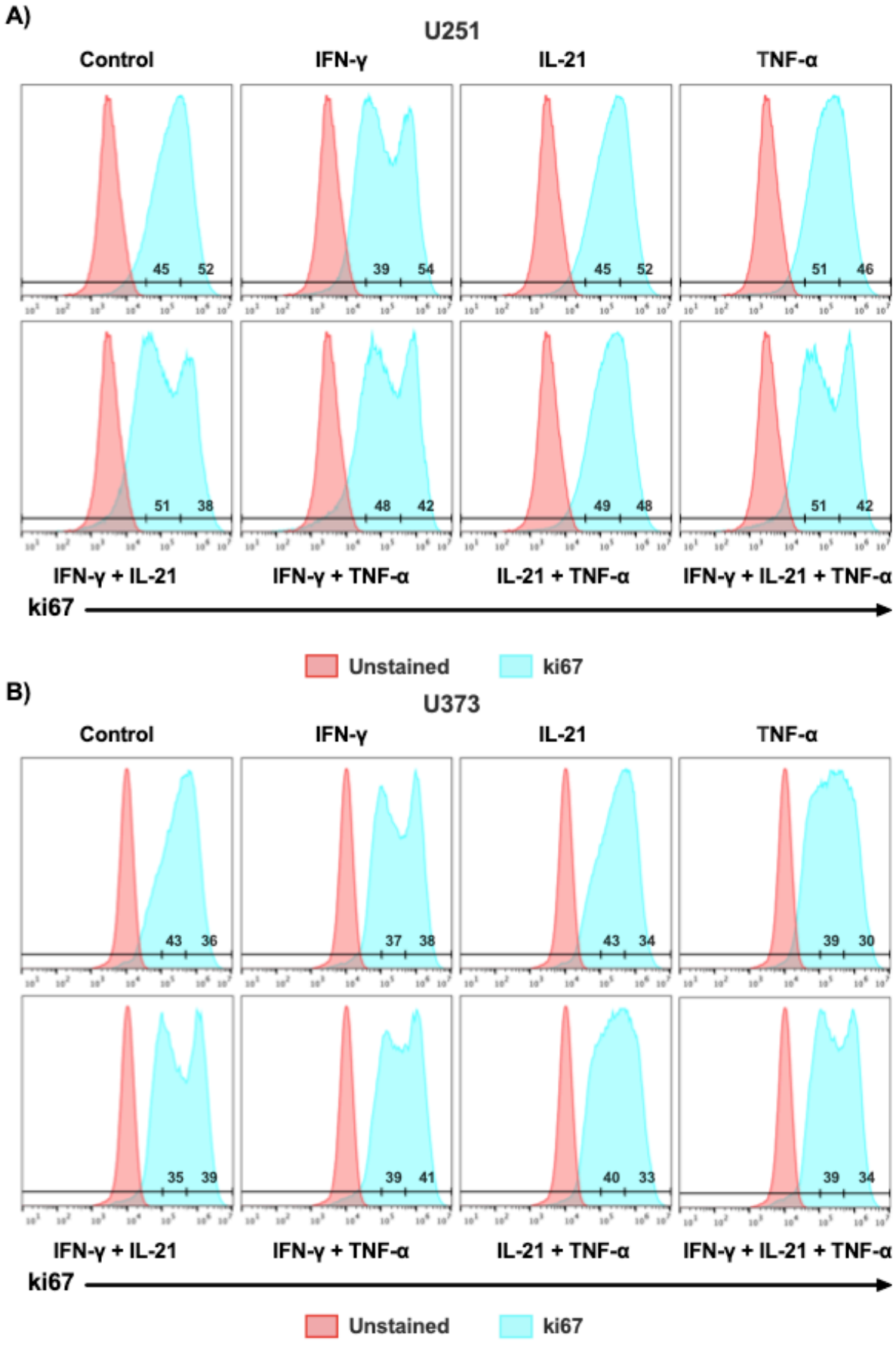
3.2.6. IFN-γ, IL-21 and TNF-α negatively affected glioblastoma cell proliferation

The decrease in U251 and U373 cell counts also raised the possibility that IFN-γ, IL-21 and TNF-α were having an impact on cell proliferation. An accurate method of measuring proliferation is to examine ki67 expression, which can only be detected during the active phases of the cell cycle, thus it is absent from cells in G0, as they are in a resting state (Scholzen & Gerdes, 2000). As the cell begins pass through the active phases of the cell cycle, ki67 expression gradually increases, reaching peak expression levels in the M-phase before the cell divides (Bruno & Darzynkiewicz, 1992). In line with this, U251 and U373 cells were seeded with 100ng/ml IFN-γ, IL-21 and TNF-α for 72 hours, both independently and combined, before they were harvested and their expression of ki67 was measured using flow cytometry, with the 72h Control result set as the 100% maximum response.

As shown in Figures 3.8A and B, there is a noticeable biphasic ki67 expression profile in each of the conditions containing IFN-γ for both cell lines, which was not visible in culture conditions containing IL-21 and/or TNF-α. This indicates that IFN-γ treatment split cells into two distinct populations, defined by their level of ki67 expression ($ki67^{lo}$ and $ki67^{hi}$), suggesting that there was a particular shift towards inhibiting peak ki67 expression, thereby restricting both U251 and U373 cell division.

In U251 cells, IFN-γ reduced the $ki67^{hi}$ maximum response to 88% ($p = 0.2674$), but this was marginally enhanced with the addition of IL-21 or TNF-α (81% - $p = 0.0326$ and 82% - $p = 0.0583$, respectively), whilst the triple cytokine treatment had the strongest effect on significantly suppressing U251 $ki67^{hi}$ expression (76% - $p = 0.0110$), relative to the Control (Figure 3.8D). The same trend was present when considering the broader picture, with the combination of IFN-γ, IL-21 and TNF-α significantly depleting the pool of ki67+ cells, relative to the Control (94% - $p = 0.0041$) (Figure 3.8C).

Similar results were seen in U373 cells, although none of the cytokine treatments demonstrated a statistically significant effect. Nevertheless, the biphasic histogram expression profile remained in each of the conditions containing IFN-γ, suggesting that IFN-γ had a similar impact on $ki67^{hi}$ expression in U373 cells. In particular, IFN-γ alone (93% - $p = 0.5443$) had the strongest independent influence on reducing the proportion of $ki67^{hi}$ U373 cells, but this was enhanced when combined with IL-21 (79% - $p = 0.1275$). However, this effect was largely sustained when IFN-γ, IL-21 and TNF-α were administered together (81% - $p = 0.1687$) (Figure 3.8D). The same trend was apparent when examining total ki67+ expression in U373 cells with the largest reduction induced by IFN-γ and IL-21 treatment (83% - $p = 0.1738$), but this was not sustained when combined with TNF-α (91% - $p = 0.5335$) (Figure 3.8C). Collectively, these results demonstrated that the triple cytokine treatment was only effective in reducing ki67 expression in U251 cells, but it is possible that IFN-γ was disrupting ki67 expression in both cell lines.


A)
U251
Control
IFN-γ
IL-21
TNF-α
45 52
39 54
45 52
51 46
51 38
48 42
49 48
51 42
IFN-γ + IL-21
IFN-γ + TNF-α
IL-21 + TNF-α
IFN-γ + IL-21 + TNF-α
ki67
Unstained
ki67
B)
U373
Control
IFN-γ
IL-21
TNF-α
43 36
37 38
43 34
39 30
35 39
39 41
40 33
39 34
IFN-γ + IL-21
IFN-γ + TNF-α
IL-21 + TNF-α
IFN-γ + IL-21 + TNF-α
ki67
Unstained
ki67

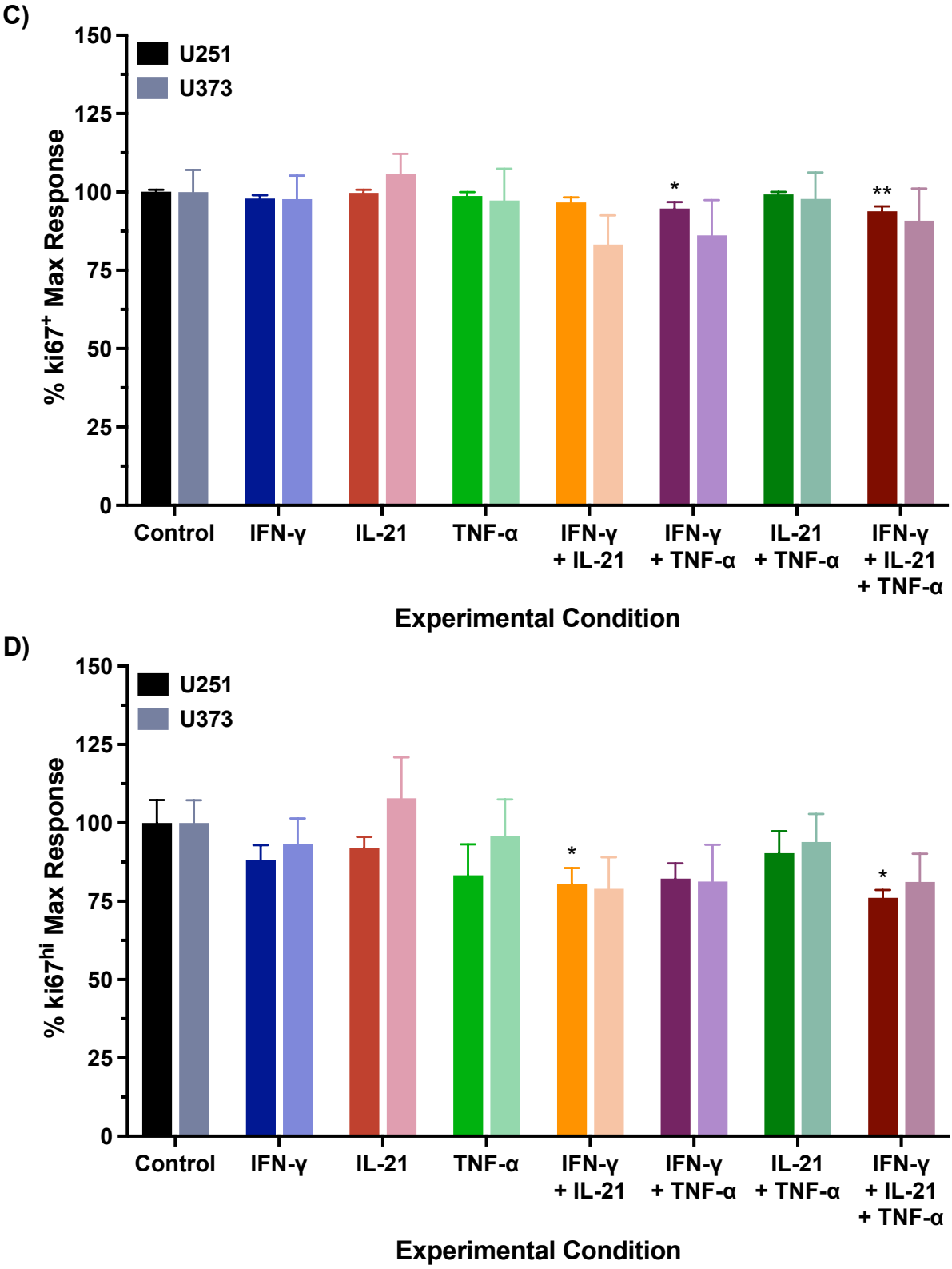


Figure 3.8. IFN-γ, IL-21 and TNF-α reduced ki67 expression in U251 cells

$5x10^4$ U251 cells **A)** and $5x10^4$ U373 cells **B)** were seeded with an RPMI Control or 100ng/ml IFN-γ, 100ng/ml IL-21, 100ng/ml TNF-α, both individually and combined. Cells were harvested after 72 hours and stained for ki67 for analysis via flow cytometry – U251 **A)** and U373 **B).** Bars represent means compared to the Control for all ki67+ cells **C)** and the fraction of cells that were $ki67^{hi}$ **D)** with SEM shown in both for > 3 independent experiments. P Values: * = <0.05; ** = <0.01; *** = <0.001; **** = <0.0001.

3.2.7. Glioblastoma cells highly expressed IL-21R, but this was not affected by IFN-γ, IL-21 and TNF-α treatment

Previous research has determined that both U251 and U373 cells highly express IFNGR1, IFNGR2 and TNFR1; however, the expression of IL-21R by glioblastoma cells is not known (C. Hao et al., 2002; M. Tada et al., 1994). As earlier experiments have demonstrated that IL-21 can reduce U251 and U373 cell counts, it was important to establish the expression levels of IL-21R by both cell lines, whilst exploring whether IFN-γ, IL-21 and/or TNF-α affected IL-21R expression. In line with this, U251 and U373 cells were stained for IL-21R expression, which was compared against an Unstained Control for each cell line, during subsequent flow cytometric analysis. Results showed that both U251 ($p = 0.0043$) and U373 cells ($p = 0.0286$) highly expressed IL-21R, but there was no significant difference in terms of expression levels between both cell lines (Figure 3.9).

In order to determine whether the cytokine treatments influenced IL-21R expression in U251 and U373 cells, both cell lines were cultured for 72 hours with 100ng/ml IFN-γ, IL-21 and TNF-α, both independently and combined, before they were harvested and stained for IL-21R for analysis via flow cytometry, with the 72h Control result set as the 100% maximum response. Interestingly, IFN-γ alone (95% - $p < 0.9999$) marginally reduced the mean fluorescent intensity (MFI) of the IL-21R staining in U251 cells, which was maintained in combination with IL-21 (95% - $p < 0.9999$). Furthermore, the IFN-γ and TNF-α dual treatment (86% - $p < 0.9999$) proved to be slightly more effective in reducing IL-21R expression in U251 cells, which was sustained in the triple cytokine condition (81% - $p = 0.6090$) (Figure 3.10B). Although the results were not statistically significant, there is a slight trend in the data to indicate that IFN-γ could have been mediating a minor reduction in IL-21R expression in U251 cells, which was enhanced with the addition of IL-21 and TNF-α – this is visible as a slight shift in the histogram expression profile as well (Figure 3.10A).

Conversely, each of the cytokines appeared to increase the IL-21R MFI in U373 cells, with the largest individual impact caused by independent treatment with TNF-α (150% - $p = 0.2017$). Similar levels of IL-21R expression were seen when U373 cells were treated with the IFN-γ and TNF-α dual treatment (143% - $p = 0.9912$), as well as the triple cytokine combination (141% - $p < 0.9999$) (Figure 3.10B). The impact of IFN-γ, IL-21 and TNF-α treatment on IL-21R expression in U373 cells is also apparent in the histogram expression profile, as there is a minor shift to demonstrate an increase in IL-21R expression, relative to the Control (Figure 3.10A). Altogether, these results demonstrated that both U251 and U373 cells expressed high levels of IL-21R, but this was not significantly affected by IFN-γ, IL-21 and TNF-α treatment.

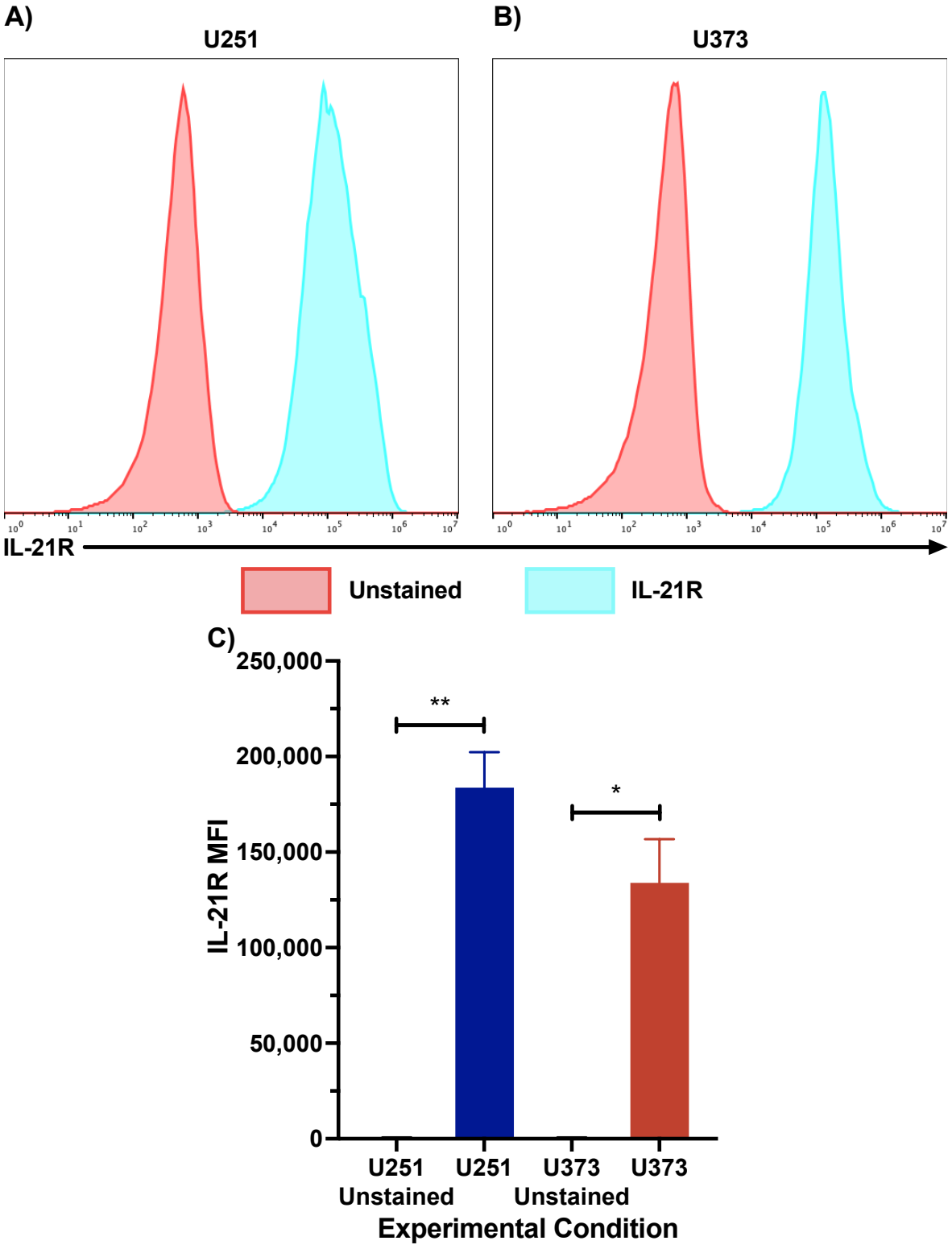


Figure 3.9. U251 and U373 cells highly expressed IL-21R

U251 cells **A)** and U373 cells **B)** were either stained for IL-21R (as shown in light blue) or left unstained (as shown in red) for analysis via flow cytometry. Flow plots are representative of > 3 independent experiments. Bars represent IL-21R MFI **C)** as means with SEM shown for > 3 independent experiments. P Values: * = <0.05; ** = <0.01; *** = <0.001; **** = <0.0001.

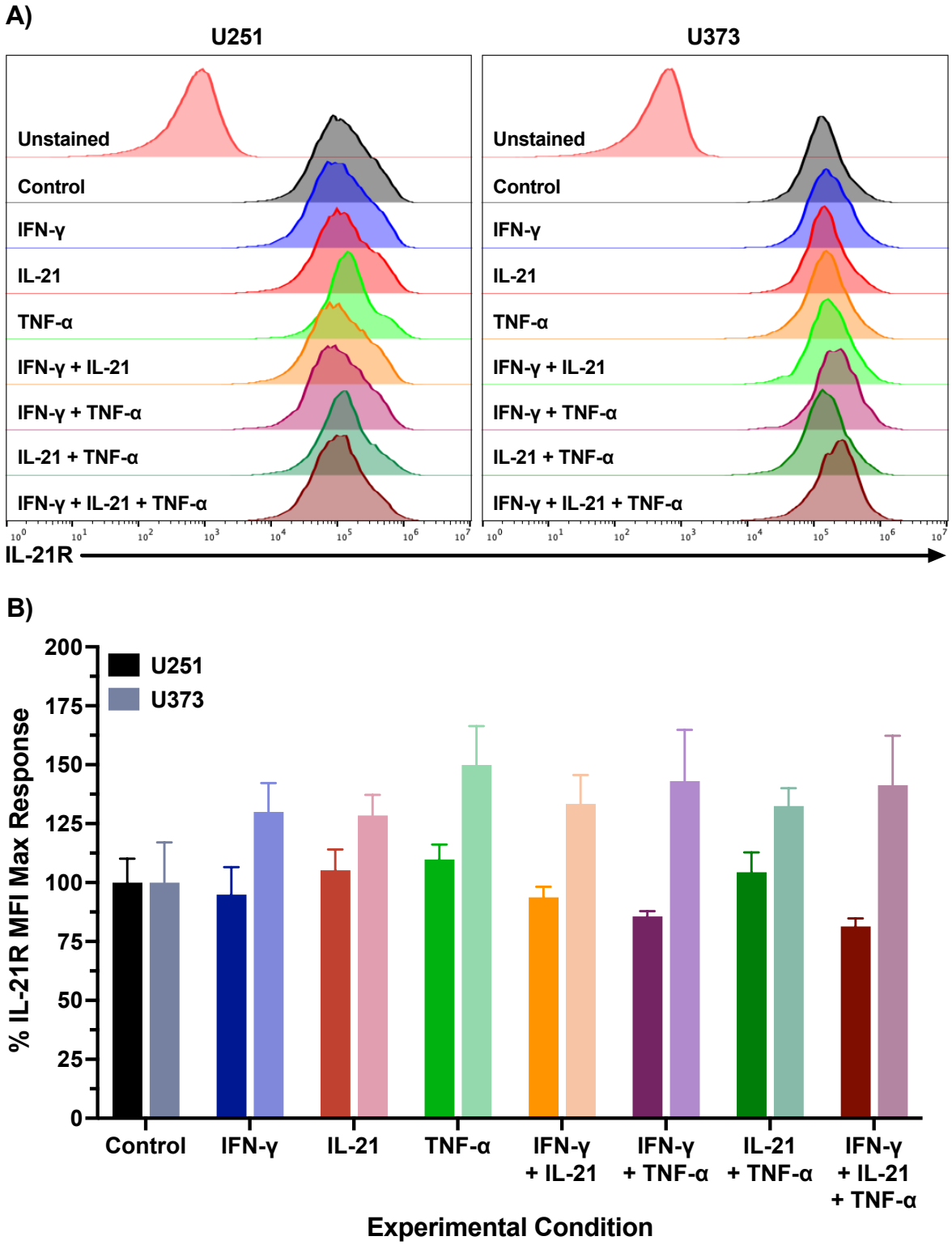


Figure 3.10. IFN-γ, IL-21 and TNF-α did not affect IL-21R expression in U251 and U373 cells

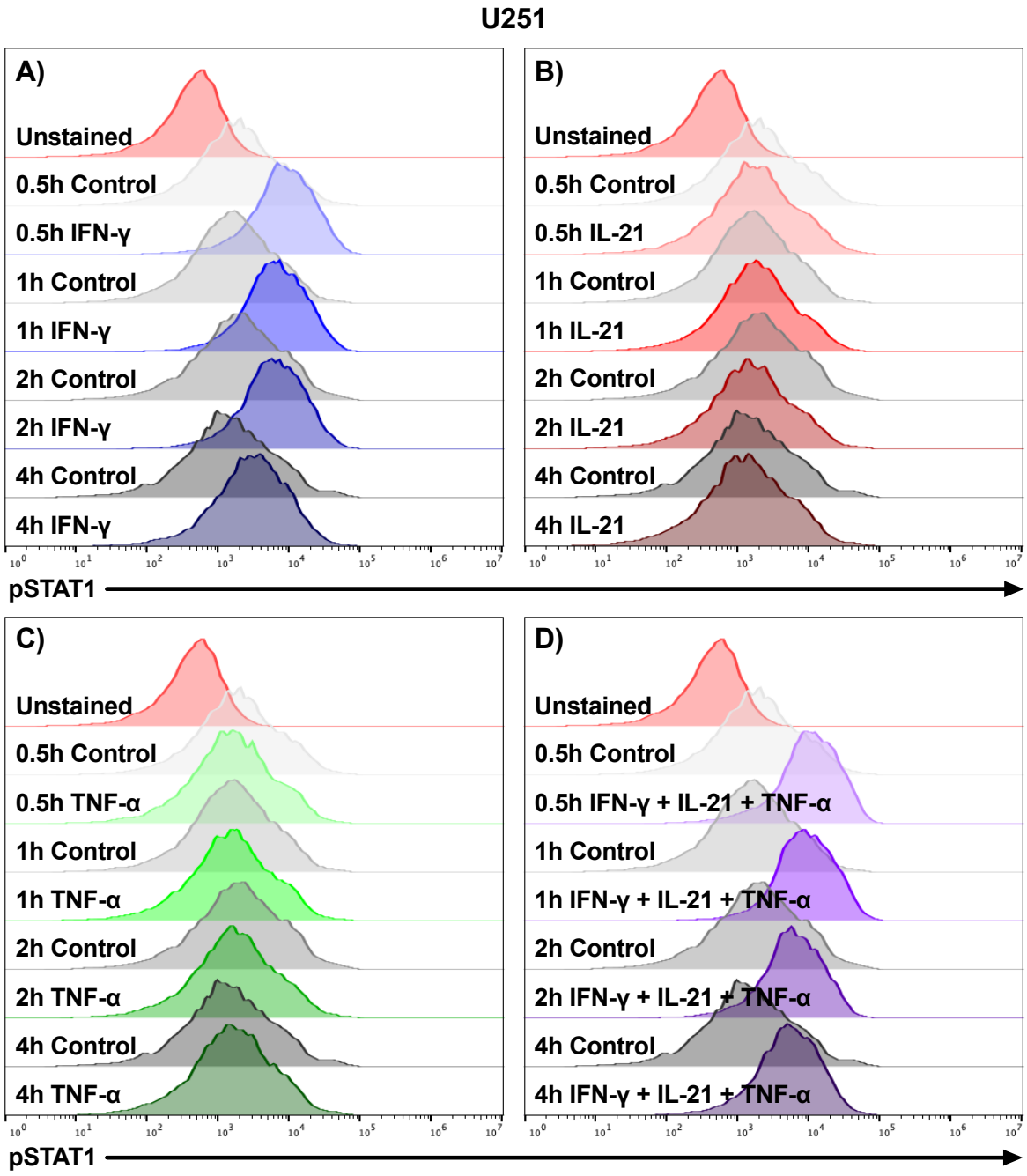
2.5x10^4 U251 cells and 2.5x10^4 U373 cells were seeded with an RPMI Control or 100ng/ml IFN-γ, 100ng/ml IL-21, 100ng/ml TNF-α, both individually and combined, before they were stained for IL-21R for analysis via flow cytometry. Flow plots **A)** are representative of > 3 independent experiments. Bars represent IL-21R MFI **B)** as means compared to the Control, with SEM shown for > 3 independent experiments. P Values: * = <0.05; ** = <0.01; *** = <0.001; **** = <0.0001.

3.2.8. IFN-γ, IL-21 and TNF-α increased pSTAT1 expression in glioblastoma cells

As both glioblastoma cell lines expressed high levels of IL-21R, the next logical step was to explore the impact of IFN-γ, IL-21 and TNF-α on IL-21R signalling – this is predominantly mediated through the phosphorylation and activation of STAT1, STAT3, and STAT5, which subsequently translocate into the nucleus to regulate the transcription of target genes. The process of STAT activation is incredibly quick, with numerous studies demonstrating that phosphorylated STATs (pSTATs) can be detected in various cell types after just 5 minutes of IFN-γ, IL-21 or TNF-α exposure (Deenick et al., 2013; Guo et al., 1998; Sadzak et al., 2008). In order to capture the impact of these cytokines on pSTAT expression in glioblastoma cells, it was important to consider a shorter time course than the 72 hours used in previous experiments.

In line with this, U251 and U373 cells were cultured with IFN-γ, IL-21 and TNF-α, both individually and combined, with pSTAT1 expression measured via flow cytometry at regular intervals over a 4-hour period. Results showed an immediate increase in pSTAT1 expression in U251 cells after 0.5 hours of culture with IFN-γ (242% - $p = 0.0114$), but this effect gradually decreased as time progressed, settling at 140% ($p = 0.1059$) after 4 hours, compared to the Control (Figure 3.11). On the other hand, IL-21 and TNF-α had no effect on pSTAT1 expression in U251 cells, indicating that the immediate and sustained profound increase in STAT1 phosphorylation when U251 cells were cultured with the triple cytokine combination (0.5 hour: 240% - $p = 0.0078$ and 4 hour: 170% - $p = 0.0233$) must have been driven by IFN-γ (Figure 3.11).

Despite an increase in variability, the same trend was seen in U373 cells, with an immediate rise in pSTAT1 expression after 0.5 hours of culture with IFN-γ alone (268% - $p = 0.0102$) (Figure 3.12). The impact on pSTAT1 expression independently mediated by IFN-γ was still visible at later timepoints, but the significance of the effect was lost by the end of the time course, due to experimental variability. Conversely, neither IL-21 or TNF-α had a significant impact on pSTAT1 expression in U373 cells. In line with the results found in U251 cells, this suggests that IFN-γ was responsible for the significant increase in pSTAT1 expression when U373 cells were cultured with the combination of IFN-γ, IL-21 and TNF-α, producing an immediate increase (242% - $p = 0.0270$) after 0.5 hours that was still present at the end of the time course (237% - $p = 0.0943$) (Figure 3.12). Altogether, these results demonstrated that IFN-γ induced an increase in STAT1 phosphorylation in glioblastoma cells, which was largely sustained by the addition of IL-21 and TNF-α.


U251
A)
Unstained
0.5h Control
0.5h IFN-γ
1h Control
1h IFN-γ
2h Control
2h IFN-γ
4h Control
4h IFN-γ
B)
Unstained
0.5h Control
0.5h IL-21
1h Control
1h IL-21
2h Control
2h IL-21
4h Control
4h IL-21
pSTAT1
C)
Unstained
0.5h Control
0.5h TNF-α
1h Control
1h TNF-α
2h Control
2h TNF-α
4h Control
4h TNF-α
D)
Unstained
0.5h Control
0.5h IFN-γ + IL-21 + TNF-α
1h Control
1h IFN-γ + IL-21 + TNF-α
2h Control
2h IFN-γ + IL-21 + TNF-α
4h Control
4h IFN-γ + IL-21 + TNF-α
pSTAT1

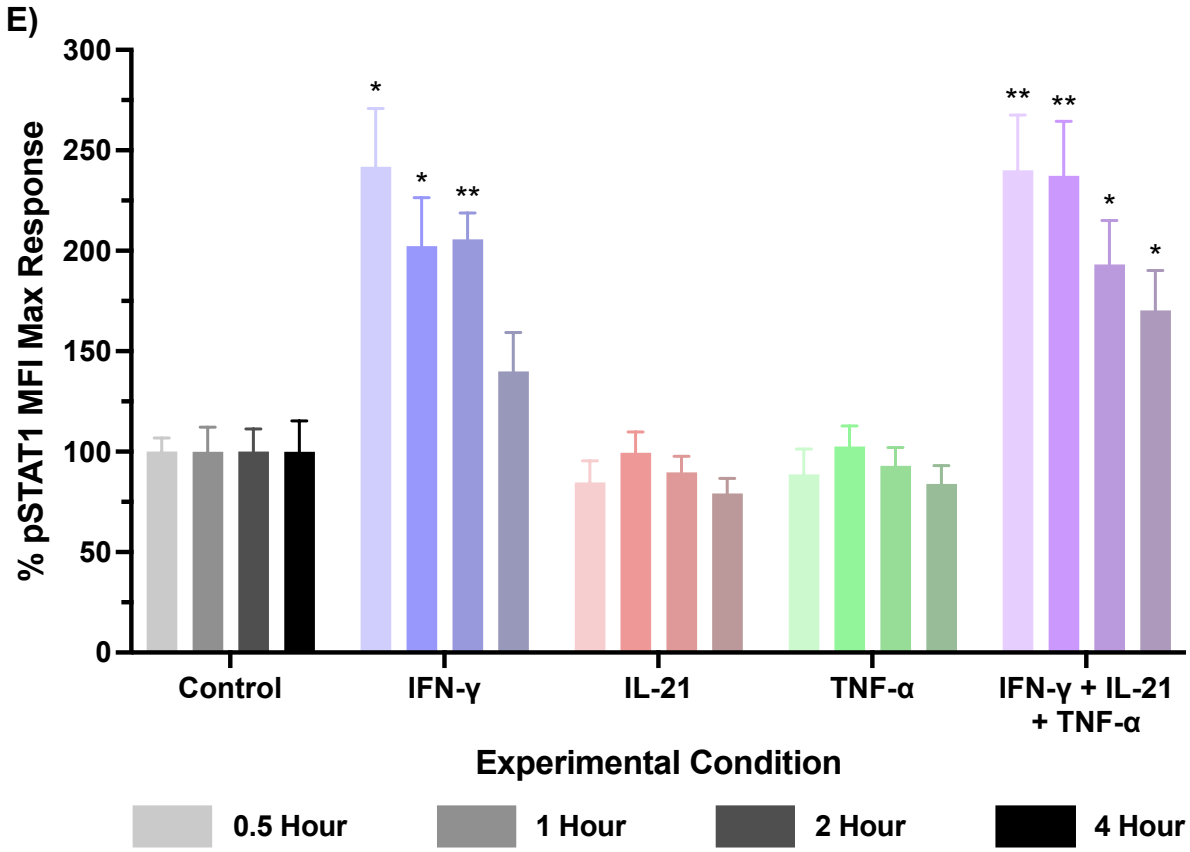
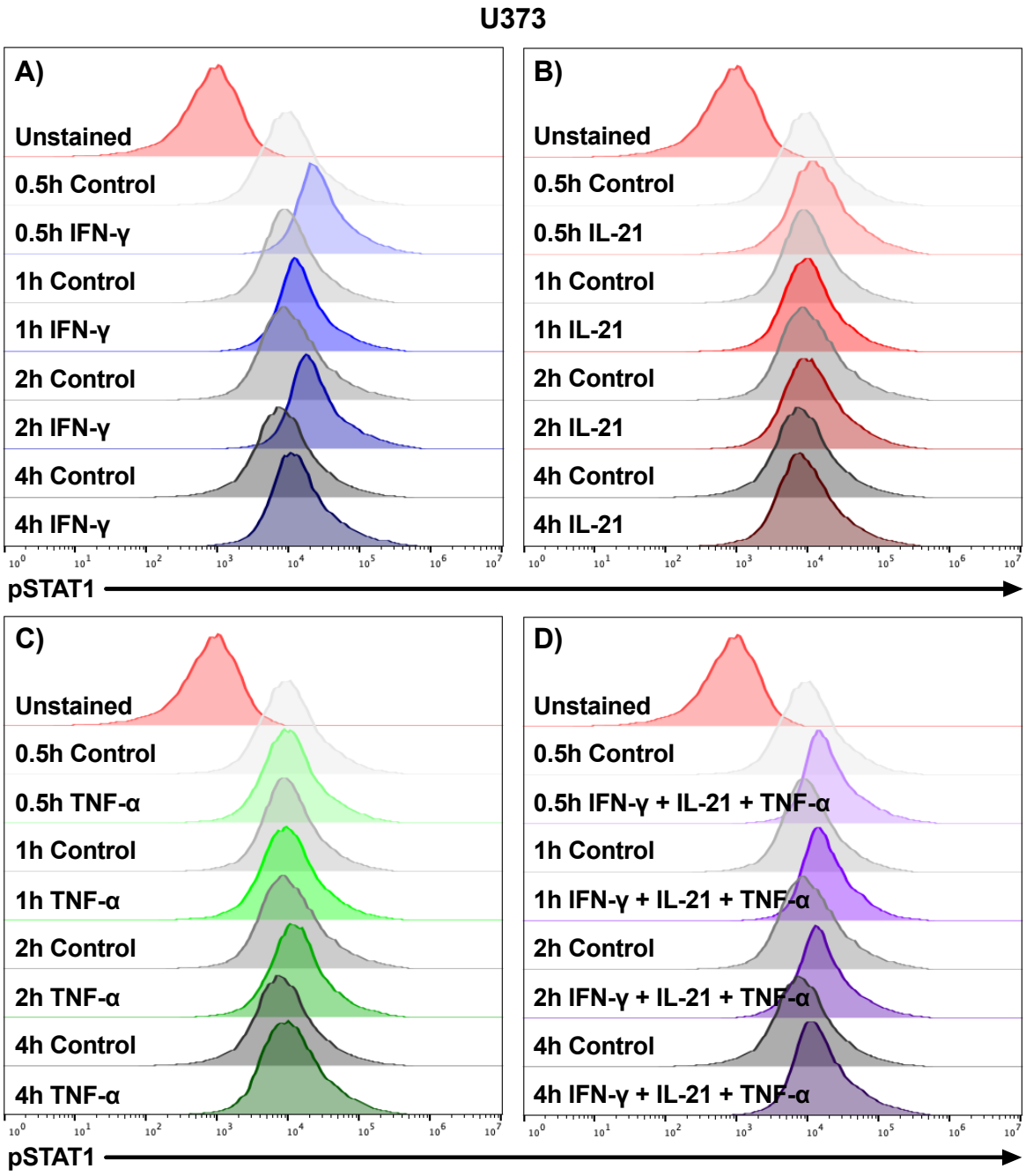


Figure 3.11. IFN-γ, IL-21 and TNF-α increased pSTAT1 expression in U251 cells

$1x10^5$ U251 cells were seeded with an RPMI Control or 100ng/ml IFN-γ, 100ng/ml IL-21, 100ng/ml TNF-α, both individually and combined, before they were stained for pSTAT1 for analysis via flow cytometry after 0.5 hours, 1 hour, 2 hours and 4 hours. Flow plots **A-D)** are representative of > 3 independent experiments. Bars represent pSTAT1 MFI **E)** as means compared to the relevant timepoint Control, with SEM shown for > 3 independent experiments. P Values: * = <0.05; ** = <0.01; *** = <0.001; **** = <0.0001.


U373
A)
Unstained
0.5h Control
0.5h IFN-γ
1h Control
1h IFN-γ
2h Control
2h IFN-γ
4h Control
4h IFN-γ
B)
Unstained
0.5h Control
0.5h IL-21
1h Control
1h IL-21
2h Control
2h IL-21
4h Control
4h IL-21
pSTAT1
C)
Unstained
0.5h Control
0.5h TNF-α
1h Control
1h TNF-α
2h Control
2h TNF-α
4h Control
4h TNF-α
D)
Unstained
0.5h Control
0.5h IFN-γ + IL-21 + TNF-α
1h Control
1h IFN-γ + IL-21 + TNF-α
2h Control
2h IFN-γ + IL-21 + TNF-α
4h Control
4h IFN-γ + IL-21 + TNF-α
pSTAT1

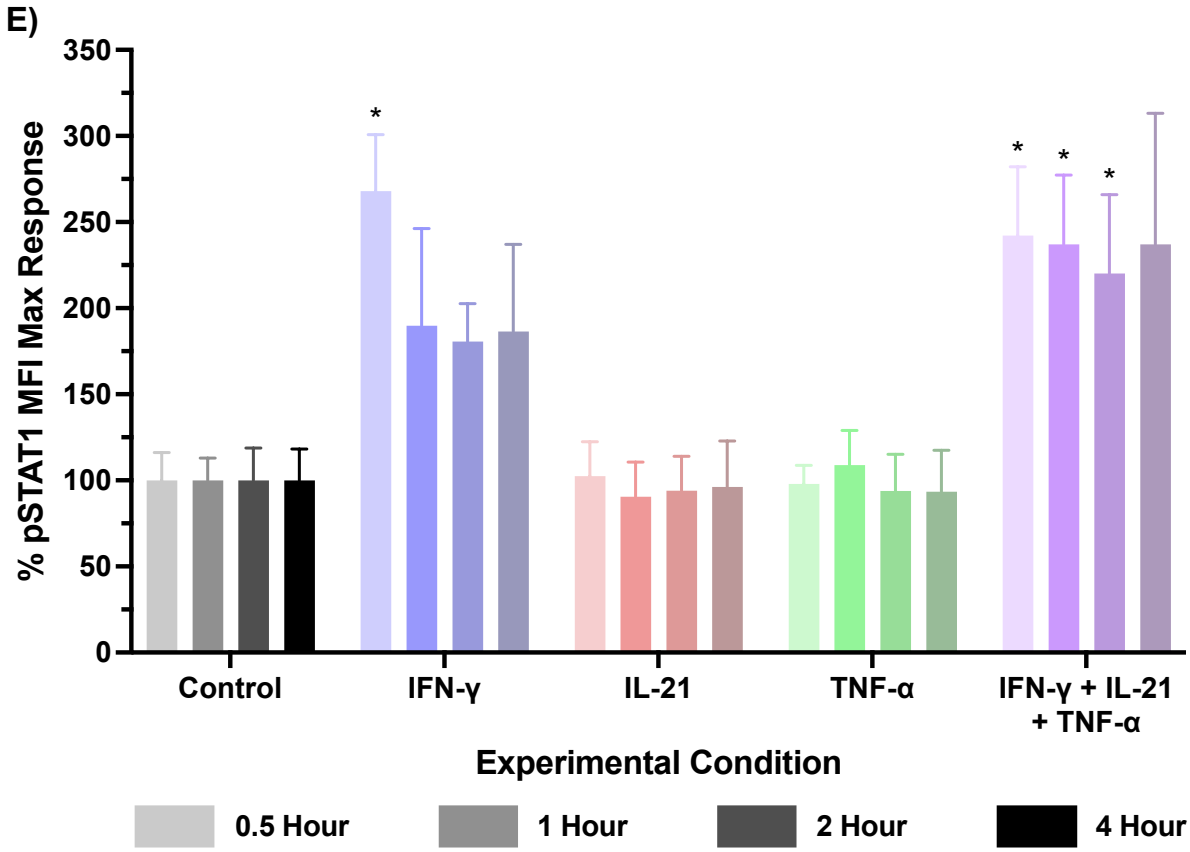
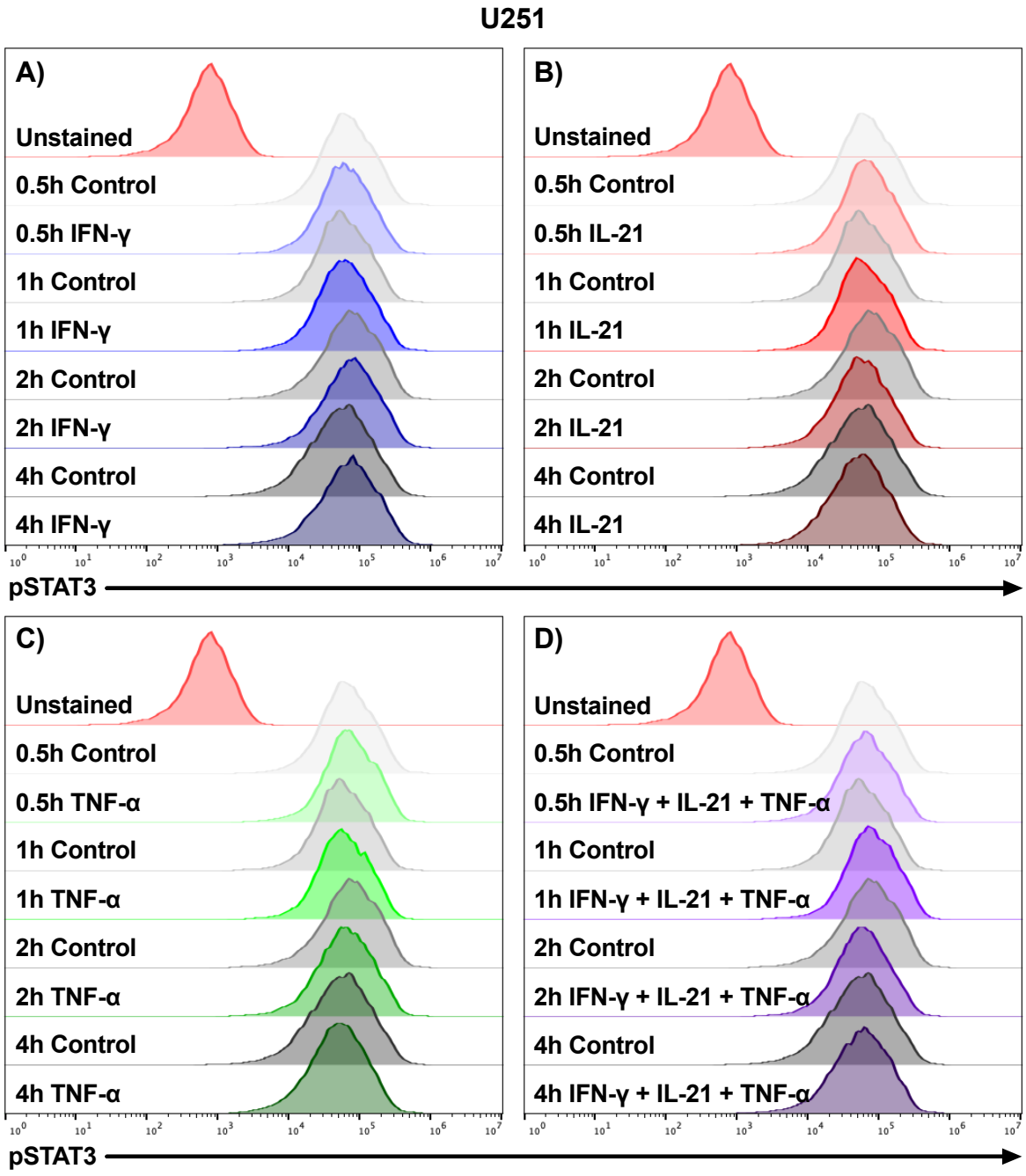


Figure 3.12. IFN-γ, IL-21 and TNF-α increased pSTAT1 expression in U373 cells

$1x10^5$ U373 cells were seeded with an RPMI Control or 100ng/ml IFN-γ, 100ng/ml IL-21, 100ng/ml TNF-α, both individually and combined, before they were stained for pSTAT1 for analysis via flow cytometry after 0.5 hours, 1 hour, 2 hours and 4 hours. Flow plots **A-D)** are representative of > 3 independent experiments. Bars represent pSTAT1 MFI **E)** as means compared to the relevant timepoint Control with SEM shown for > 3 independent experiments. P Values: * = <0.05; ** = <0.01; *** = <0.001; **** = <0.0001.

3.2.9. IFN-γ, IL-21 and TNF-α did not affect pSTAT3 expression in glioblastoma cells

Using the same experimental conditions, the impact of IFN-γ, IL-21 and TNF-α on STAT3 phosphorylation in U251 and U373 cells was also explored. The results produced largely demonstrated that IFN-γ, IL-21 and TNF-α did not have a significant impact on pSTAT3 expression in U251 cells. However, there was an interesting drop in pSTAT3 expression 2 hours after treatment with IL-21 (76% - $p = 0.0285$), which was also visible when combined with IFN-γ and TNF-α (81% - $p = 0.1238$). Although, it is important to note that this effect was not sustained after 4 hours of treatment in either experimental condition (Figure 3.13). Unfortunately, there was considerable experimental variability across each of the experimental conditions involving U373 cells, so it was not possible to determine whether these cytokine treatments had any impact on STAT3 phosphorylation (Figure 3.14). Altogether, these findings collectively indicated that IFN-γ, IL-21 and TNF-α had no impact on pSTAT3 expression in U251 or U373 cells.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A)
Unstained
0.5h Control
0.5h IFN-γ
1h Control
1h IFN-γ
2h Control
2h IFN-γ
4h Control
4h IFN-γ
B)
Unstained
0.5h Control
0.5h IL-21
1h Control
1h IL-21
2h Control
2h IL-21
4h Control
4h IL-21
pSTAT3
C)
Unstained
0.5h Control
0.5h TNF-α
1h Control
1h TNF-α
2h Control
2h TNF-α
4h Control
4h TNF-α
D)
Unstained
0.5h Control
0.5h IFN-γ + IL-21 + TNF-α
1h Control
1h IFN-γ + IL-21 + TNF-α
2h Control
2h IFN-γ + IL-21 + TNF-α
4h Control
4h IFN-γ + IL-21 + TNF-α
pSTAT3

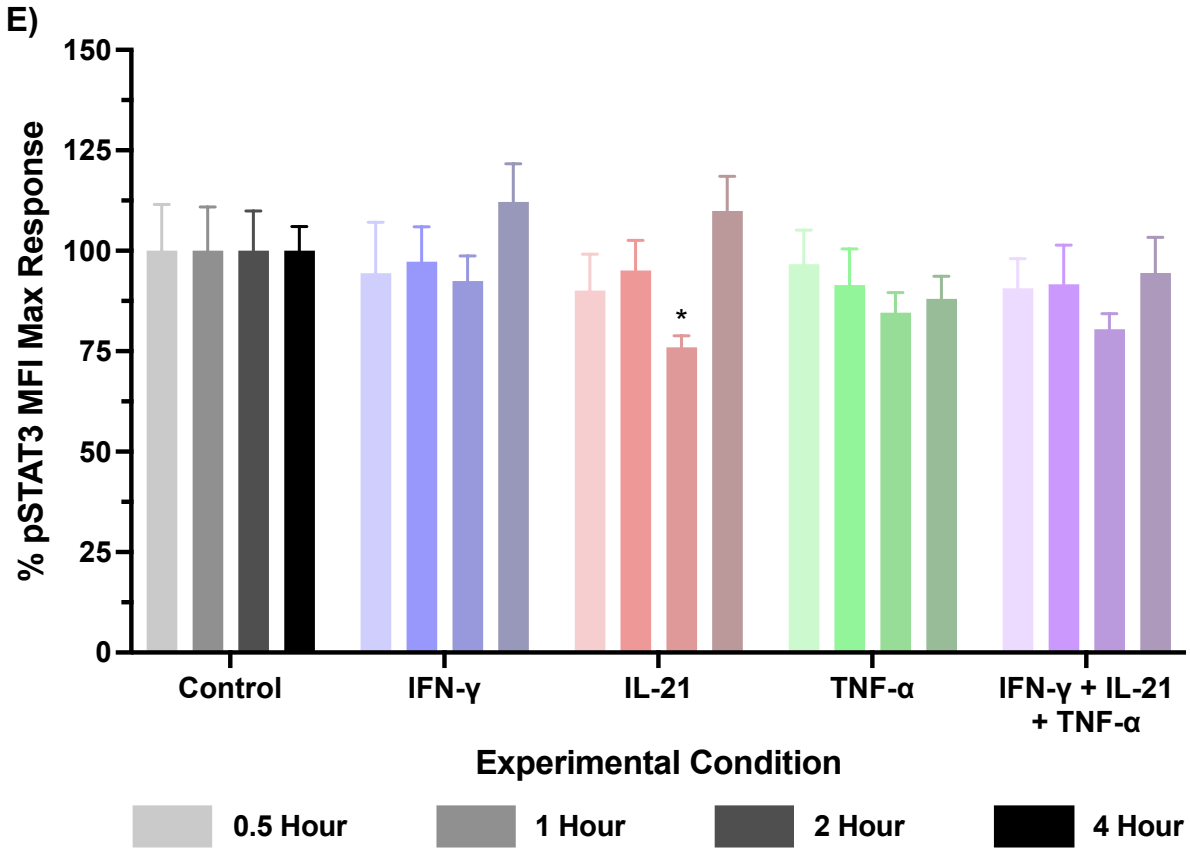
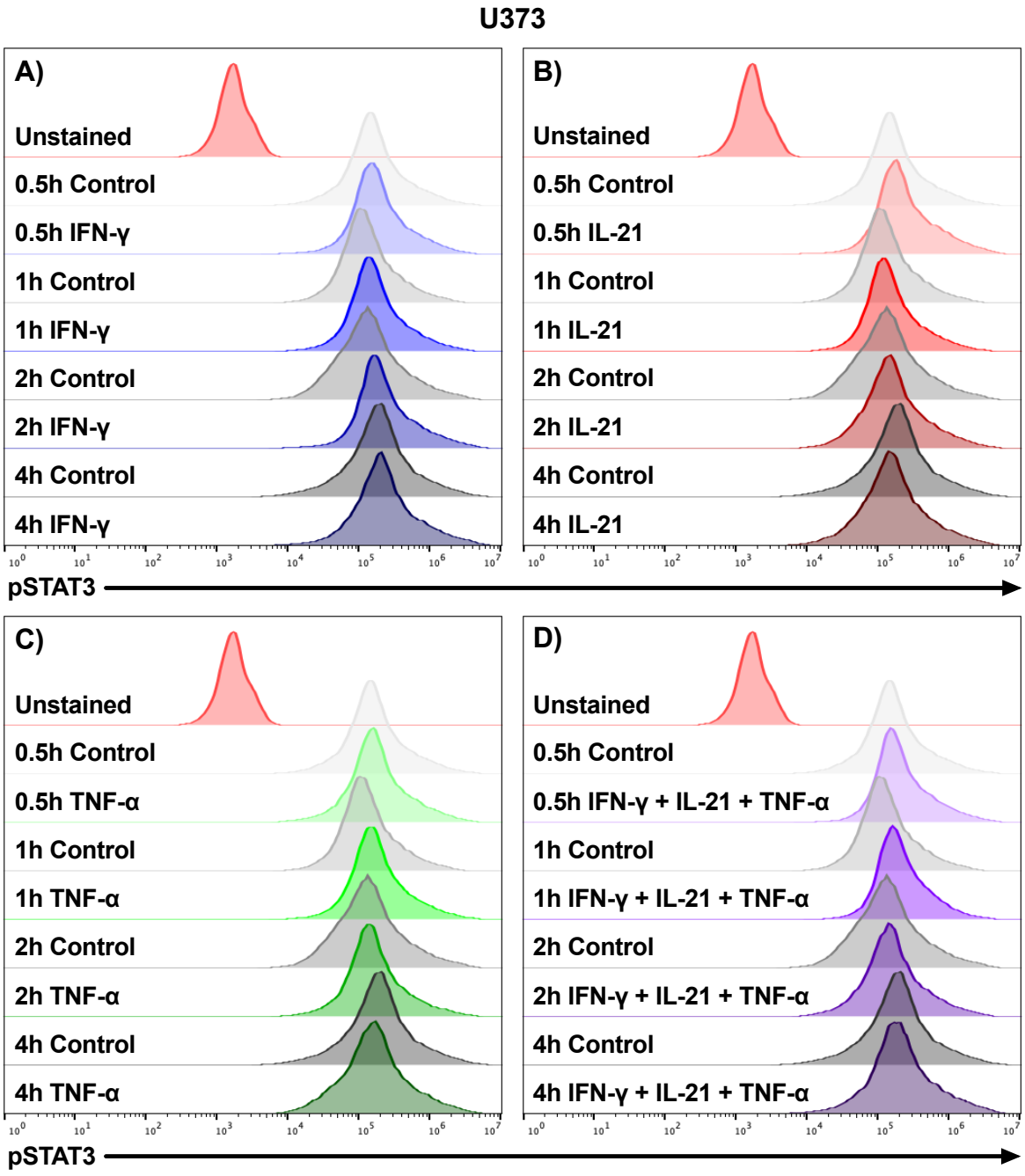


Figure 3.13. IFN-γ, IL-21 and TNF-α did not affect pSTAT3 expression in U251 cells

$1x10^5$ U251 cells were seeded with an RPMI Control or 100ng/ml IFN-γ, 100ng/ml IL-21, 100ng/ml TNF-α, both individually and combined, before they were stained for pSTAT3 for analysis via flow cytometry after 0.5 hours, 1 hour, 2 hours and 4 hours. Flow plots **A-D)** are representative of > 3 independent experiments. Bars represent pSTAT3 MFI **E)** as means compared to the relevant timepoint Control with SEM shown for > 3 independent experiments. P Values: * = <0.05; ** = <0.01; *** = <0.001; **** = <0.0001.


U373
A)
Unstained
0.5h Control
0.5h IFN-γ
1h Control
1h IFN-γ
2h Control
2h IFN-γ
4h Control
4h IFN-γ
B)
Unstained
0.5h Control
0.5h IL-21
1h Control
1h IL-21
2h Control
2h IL-21
4h Control
4h IL-21
pSTAT3
C)
Unstained
0.5h Control
0.5h TNF-α
1h Control
1h TNF-α
2h Control
2h TNF-α
4h Control
4h TNF-α
D)
Unstained
0.5h Control
0.5h IFN-γ + IL-21 + TNF-α
1h Control
1h IFN-γ + IL-21 + TNF-α
2h Control
2h IFN-γ + IL-21 + TNF-α
4h Control
4h IFN-γ + IL-21 + TNF-α
pSTAT3

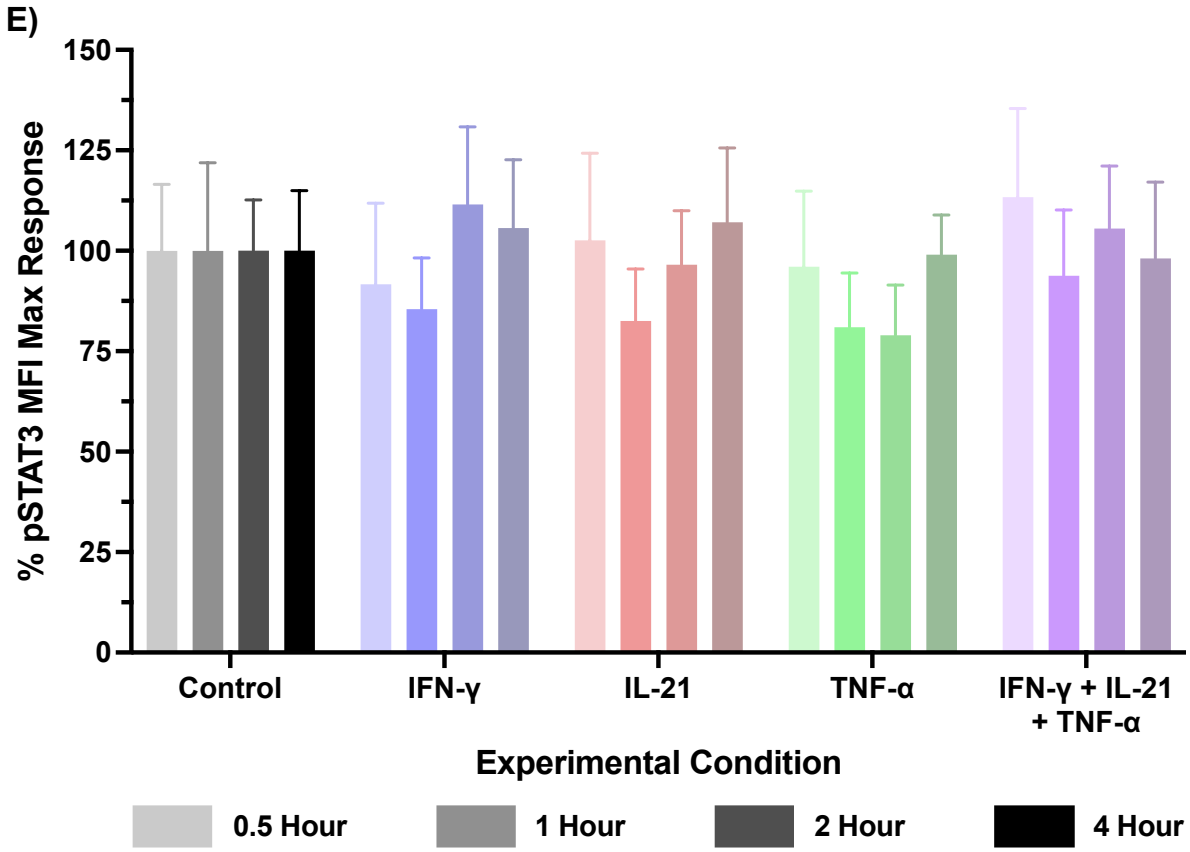
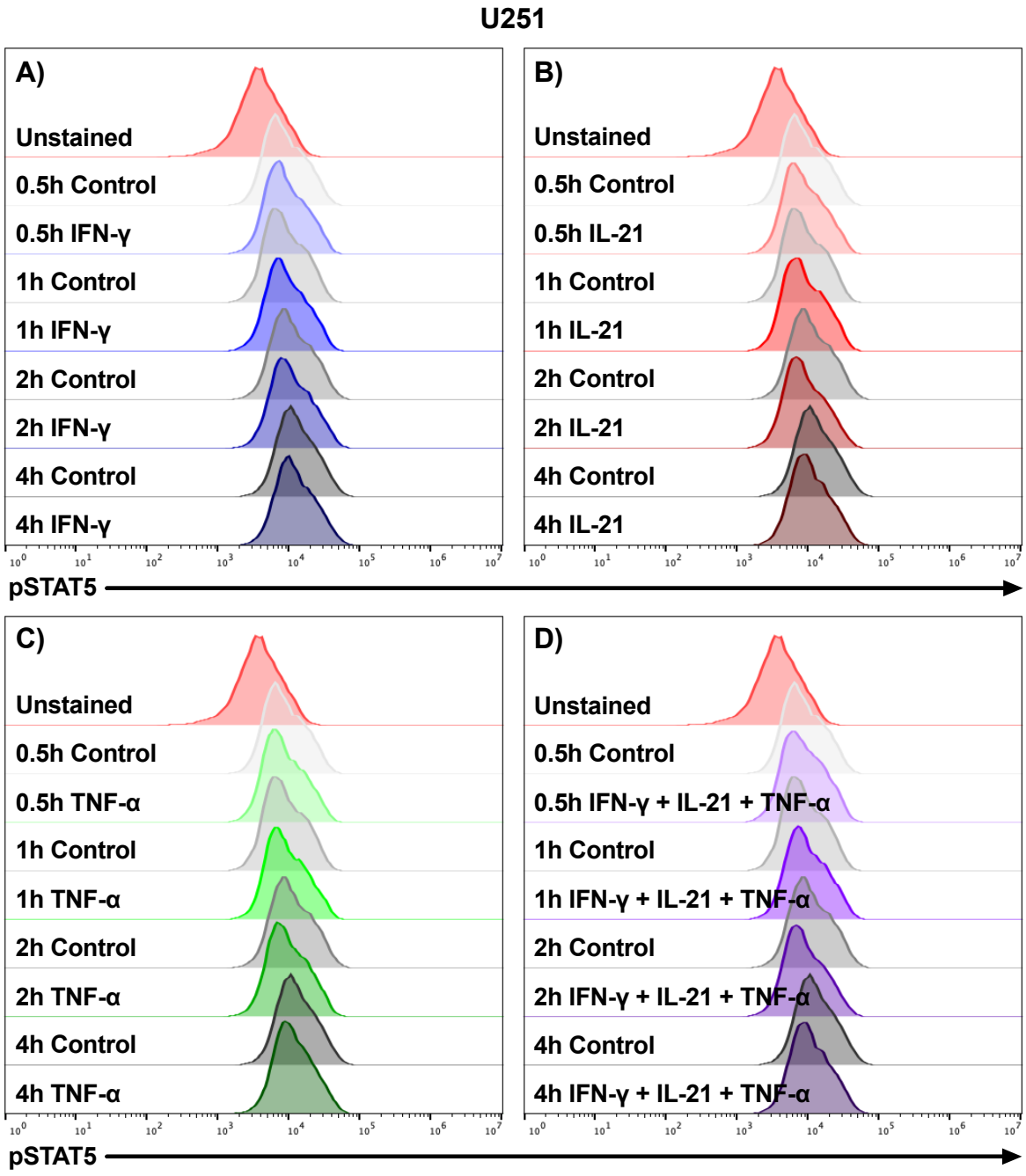


Figure 3.14. IFN-γ, IL-21 and TNF-α did not affect pSTAT3 expression in U373 cells

$1x10^5$ U373 cells were seeded with an RPMI Control or 100ng/ml IFN-γ, 100ng/ml IL-21, 100ng/ml TNF-α, both individually and combined, before they were stained for pSTAT3 for analysis via flow cytometry after 0.5 hours, 1 hour, 2 hours and 4 hours. Flow plots **A-D)** are representative of > 3 independent experiments. Bars represent pSTAT3 MFI **E)** as means compared to the relevant timepoint Control, with SEM shown for > 3 independent experiments. P Values: * = <0.05; ** = <0.01; *** = <0.001; **** = <0.0001.

3.2.10. IFN-γ, IL-21 and TNF-α did not affect pSTAT5 expression in glioblastoma cells

Finally, the impact of IFN-γ, IL-21 and TNF-α treatment on pSTAT5 expression in U251 and U373 cells was evaluated, over the same 4-hour time period. Interestingly, the results showed that there was no impact on STAT5 phosphorylation in U251 cells across any of the cytokine conditions with a firmly consistent level of expression present at all timepoints, equally comparable to the untreated Control (Figure 3.15). On the other hand, there was considerable experimental variability when exploring pSTAT5 expression in U373 cells, thus it was not possible to ascertain whether any of the cytokine treatments had a clear impact on STAT5 phosphorylation (Figure 3.16). Altogether, these findings illustrated that IFN-γ, IL-21 and TNF-α did not impact STAT5 phosphorylation in U251 or U373 cells.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A)
Unstained
0.5h Control
0.5h IFN-γ
1h Control
1h IFN-γ
2h Control
2h IFN-γ
4h Control
4h IFN-γ
B)
Unstained
0.5h Control
0.5h IL-21
1h Control
1h IL-21
2h Control
2h IL-21
4h Control
4h IL-21
pSTAT5
C)
Unstained
0.5h Control
0.5h TNF-α
1h Control
1h TNF-α
2h Control
2h TNF-α
4h Control
4h TNF-α
D)
Unstained
0.5h Control
0.5h IFN-γ + IL-21 + TNF-α
1h Control
1h IFN-γ + IL-21 + TNF-α
2h Control
2h IFN-γ + IL-21 + TNF-α
4h Control
4h IFN-γ + IL-21 + TNF-α
pSTAT5

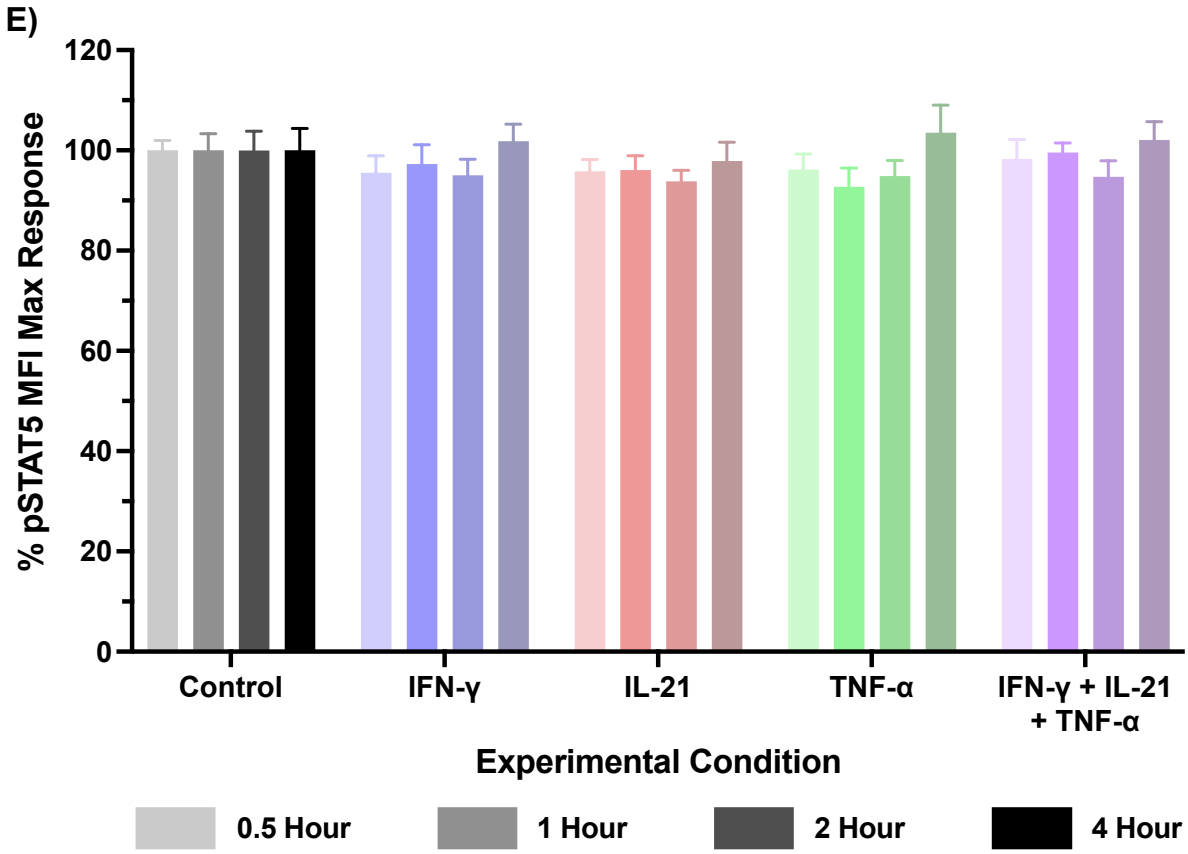
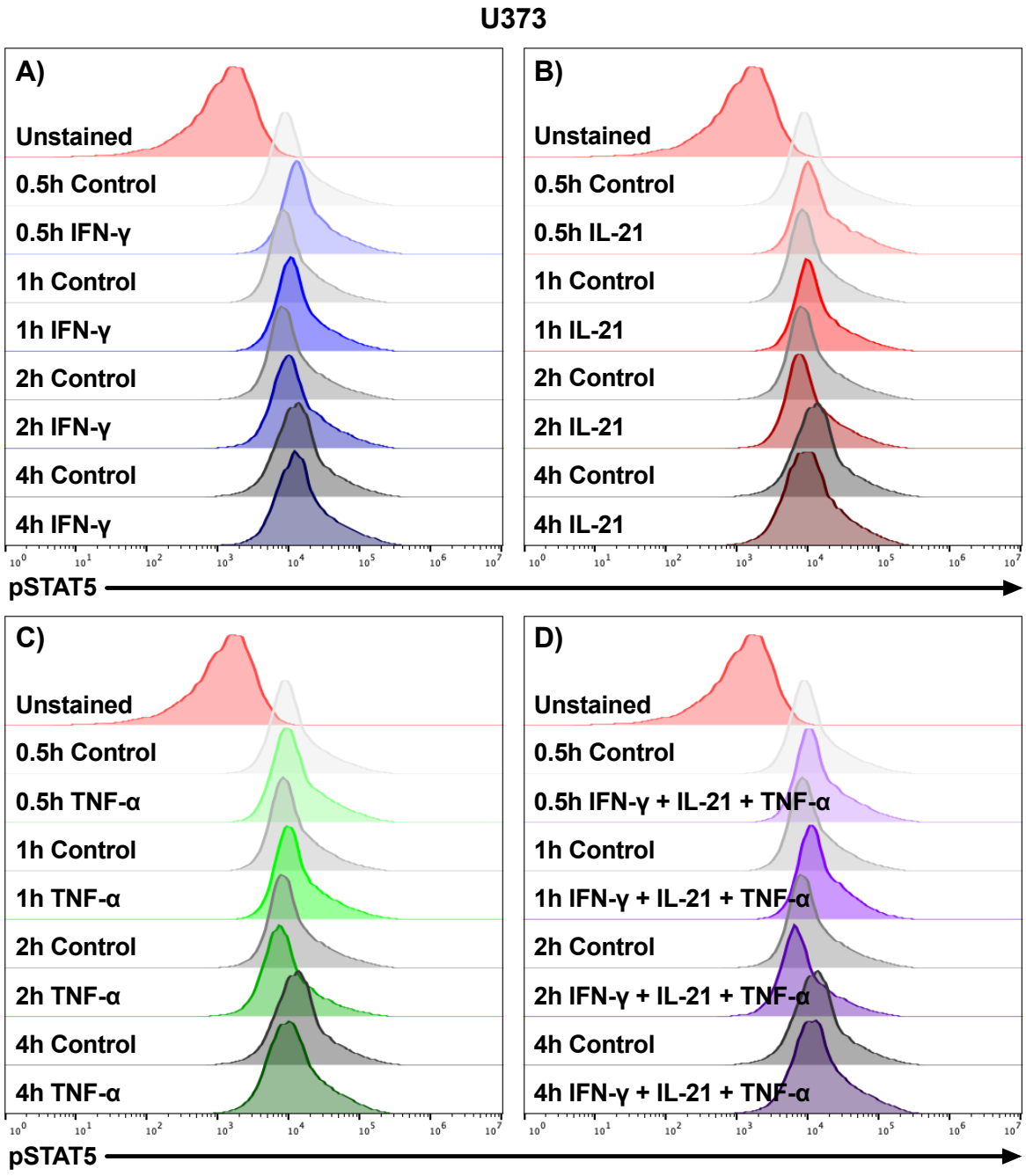


Figure 3.15. IFN-γ, IL-21 and TNF-α did not affect pSTAT5 expression in U251 cells

$1x10^5$ U251 cells were seeded with an RPMI Control or 100ng/ml IFN-γ, 100ng/ml IL-21, 100ng/ml TNF-α, both individually and combined, before they were stained for pSTAT5 for analysis via flow cytometry after 0.5 hours, 1 hour, 2 hours and 4 hours. Flow plots **A-D)** are representative of > 3 independent experiments. Bars represent pSTAT5 MFI **E)** as means compared to the relevant timepoint Control with SEM shown for > 3 independent experiments. P Values: * = <0.05; ** = <0.01; *** = <0.001; **** = <0.0001.


U373
A)
Unstained
0.5h Control
0.5h IFN-γ
1h Control
1h IFN-γ
2h Control
2h IFN-γ
4h Control
4h IFN-γ
B)
Unstained
0.5h Control
0.5h IL-21
1h Control
1h IL-21
2h Control
2h IL-21
4h Control
4h IL-21
pSTAT5
C)
Unstained
0.5h Control
0.5h TNF-α
1h Control
1h TNF-α
2h Control
2h TNF-α
4h Control
4h TNF-α
D)
Unstained
0.5h Control
0.5h IFN-γ + IL-21 + TNF-α
1h Control
1h IFN-γ + IL-21 + TNF-α
2h Control
2h IFN-γ + IL-21 + TNF-α
4h Control
4h IFN-γ + IL-21 + TNF-α
pSTAT5

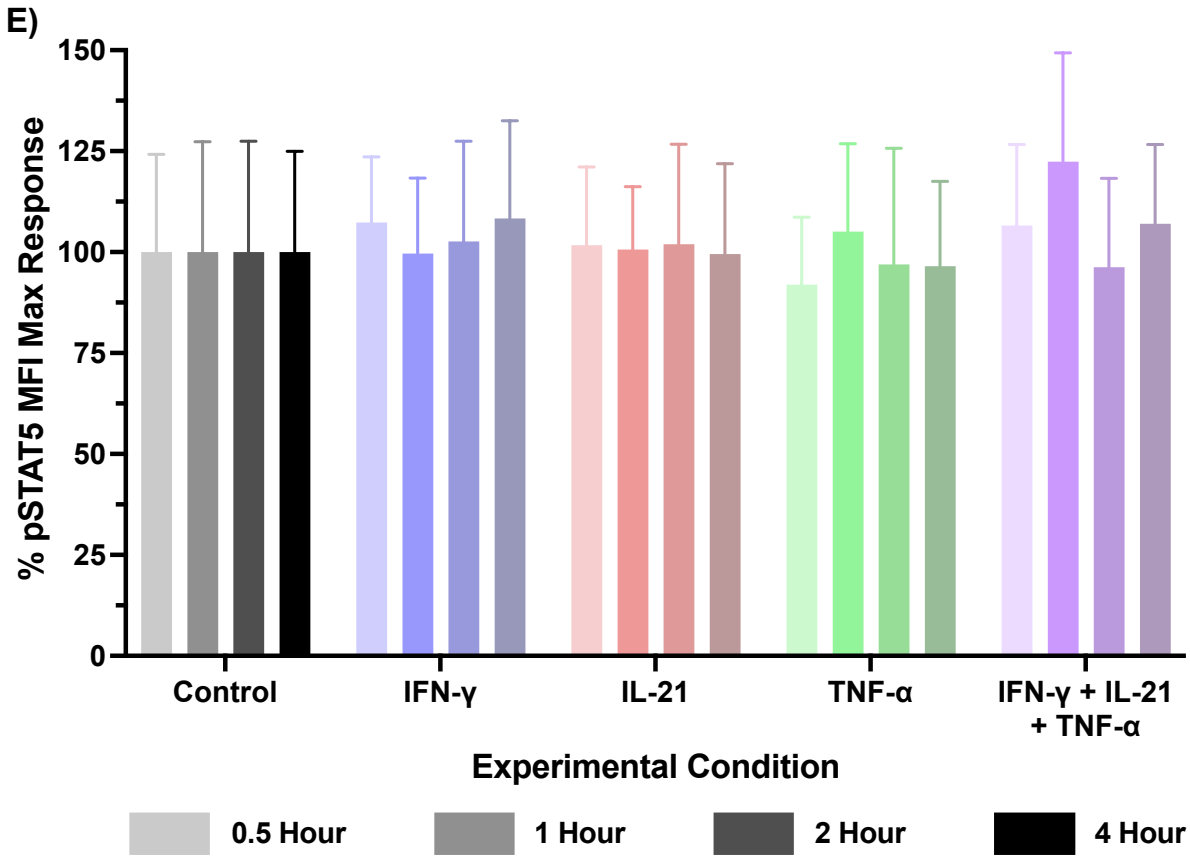


Figure 3.16. IFN-γ, IL-21 and TNF-α did not affect pSTAT5 expression in U373 cells

$1x10^5$ U373 cells were seeded with an RPMI Control or 100ng/ml IFN-γ, 100ng/ml IL-21, 100ng/ml TNF-α, both individually and combined, before they were stained for pSTAT5 for analysis via flow cytometry after 0.5 hours, 1 hour, 2 hours and 4 hours. Flow plots **A-D)** are representative of > 3 independent experiments. Bars represent pSTAT5 MFI values **E)** means compared to the relevant timepoint Control with SEM shown for > 3 independent experiments. P Values: * = <0.05; ** = <0.01; *** = <0.001; **** = <0.0001.

3.3. Discussion

Glioblastoma is an aggressive form of brain cancer, with an extremely limited number of treatments options available; therefore, alternative therapies desperately need to be developed. As with various forms of cancer, immunotherapies have shown significant therapeutic value, particularly cytokine therapies. Specifically, IFN-γ has been explored in various cancer types, due to its strong anti-tumour effects: for example, IFN-γ can upregulate chemokine and MHC expression to enhance intratumoural T cell infiltration, whilst downregulating the secretion of pro-angiogenic factors (e.g. VEGF) by M2 TAMs (Bodnar et al., 2009; Luster et al., 1998; Sun et al., 2014). Furthermore, IFN-γ and TNF-α can both induce apoptosis in cancer cells – although, TNF-α can also enhance tumour proliferation and survival through NF-κB-mediated signalling (Cabal-Hierro & Lazo, 2012; S.-Y. Park et al., 2004; Rothe et al., 1995; X. Xu et al., 1998). Conversely, the direct effects of IL-21 on cancer cells have not been thoroughly investigated, thus they are largely unknown. Whilst some research has been conducted to explore the effects of IFN-γ, IL-21 or TNF-α in various glioblastoma models, the results are limited and have rarely considered utilising a multi-cytokine approach. As a result of this, the present thesis investigated the direct effects of IFN-γ, IL-21 and TNF-α, both individually and combined, on two glioblastoma cell lines (U251 and U373).

In particular, the results in this chapter demonstrate that IFN-γ potently affected U251 and U373 cell counts, with 200ng/ml IFN-γ almost halving the number of U373 cells present after 72 hours (Figure 3.1). The inhibition of glioblastoma proliferation was reflected in the exclusive biphasic form of ki67 expression seen in conditions containing IFN-γ (Figure 3.8). As ki67 is not expressed by cells in G0, the reduction in the proportion of ki67+ cells supports previous research, which showed that IFN-γ disrupted the proliferation of various glioblastoma cell lines (T98G, SNB-19 and U373) by inducing cell cycle arrest at the G1/S-phase transition via p21 upregulation (S. Kominsky et al., 1998). Aside from this, the most profound changes in cell morphology were seen in the experimental conditions containing IFN-γ, with a large number of cells in both cell lines becoming noticeably more rounded, reflecting cells undergoing apoptosis (Figures 3.3 and 3.4).

When exploring the impact of IFN-γ on A172 cells, Knüpfer et al. (2001) measured Propidium Iodide expression to determine that IFN-γ induced necrosis in A172 cells, but they did not explore whether IFN-γ also induced apoptosis. On the other hand, Haque et al. (2007) identified that IFN-γ was capable of inducing apoptosis in both T98 and U87 glioblastoma cell lines through the use of a colourimetric assay to assess caspase-3 activity. Furthermore, several studies have demonstrated that IFN-γ induced apoptosis in glioblastoma cells through activation of a caspase cascade and cytochrome c release (C. Choi et al., 2004; Das et al., 2009; Fulda & Debatin, 2002). The findings contrast with the results in the present chapter, which failed to demonstrate an independent role of IFN-γ in the induction of apoptosis or necrosis in either cell line. However, there was a slight trend to suggest that IFN-γ alone could be involved in the induction of early apoptosis in U251 cells, but there was considerable experimental variability, so the result was not statistically significant (Figure 3.7).

Unfortunately, experiments exploring the impact of TNF-α on glioblastoma cells have produced variable results. For example, Chen et al. (1993) found that TNF-α increased ki67 expression in A172 cells, whilst U87 cells had reduced ki67 expression – however, they only measured the effect of TNF-α treatment after 48 hours. Regarding the present study, results showed that TNF-α alone was effective in reducing cell counts and density in both cell lines (Figures 3.1, 3.3 and 3.4), but there was no impact on ki67 expression in either U251 or U373 cells (Figure 3.8), indicating that the inhibitory effects of TNF-α were not associated with cell proliferation.

Previous research has suggested that several glioblastoma cell lines (U87, A172 and T98G) were resistant to TNF-α-mediated apoptosis through activation of NF-κB, which promoted the transcription of several anti-apoptotic genes (e.g. Bcl-2 and Bcl-xL) (Roesler et al., 2016; Sharma et al., 2008). In terms of the cell lines used in this study, Duan et al. (2001) found that U373 cells were resistant to TNF-α-mediated apoptosis, but this was only measured after 24 hours of treatment. Similarly, Koul et al. (2006) discovered that U251 cells were impervious to TNF-α treatment, but this could be restored by inducing PTEN expression to inhibit the transcriptional activity of NF-κB, thereby increasing their sensitivity to TNF-α. These findings are somewhat consistent with the results in this chapter, as TNF-α did not significantly induce apoptosis or necrosis in U251 cells, but there was clear expression of late apoptosis associated with independent TNF-α treatment in U373 cells, suggesting that perhaps this cell line is susceptible to TNF-α-mediated cell death over a longer time course (Figure 3.7).

As a relatively new therapeutic agent, there isn't a significant amount of research available that has explored the direct effects of IL-21 on cancer cells. Critically, the results in this chapter prove that IL-21 itself has direct anti-tumour effects on both U251 and U373 glioblastoma cell lines. In particular, results showed that IL-21 reduced glioblastoma growth through dose-independent decreases in cell counts (Figure 3.1) and a clear visual decline in cell density in both cell lines (Figures 3.3 and 3.4). However, IL-21 alone did not prove to have a significant impact on reducing the proportion of ki67+ cells, indicating that these results were not caused by inhibition of glioblastoma cell proliferation (Figure 3.8). Furthermore, independent IL-21 treatment did not induce necrosis in U251 or U373 cells, but there was a slight (albeit non-significant) trend to suggest that IL-21 could have been involved in the induction of apoptosis in both cell lines (Figure 3.7). Previous research in other cancer cell lines have found that IL-21 induced apoptosis through upregulation of Bim and Bax, plus increased activation of caspase-3 and caspase-8, whilst downregulating Bcl-2 and Bcl-xL, thus it is plausible that IL-21 could mediate the same signalling pathways in glioblastoma cells (Gelebart et al., 2009; Gowda et al., 2008; Sarosiek et al., 2006).

In terms of combining the cytokine conditions, IFN-γ and TNF-α have been explored as a dual treatment in various cancer cell lines, although research is limited in glioblastoma. In particular, Iwasaki et al. (1993) showed that IFN-γ synergised with TNF-α to reduce the proliferation of three glioblastoma cell lines (CRT, STT and WIT). This somewhat aligns with the results produced in this chapter, which showed that the combination of IFN-γ and TNF-α potently reduced the number of U251 and U373 cells,

both in terms of cell counts and density (Figures 3.2, 3.3 and 3.4). However, TNF-α did not enhance the effects of IFN-γ on reducing ki67+ expression in either U251 or U373 cells (Figure 3.8). Conversely, the IFN-γ and TNF-α dual treatment significantly increased the proportion of apoptotic and necrotic cells in both cell lines (Figure 3.7) – it is possible that IFN-γ upregulated TNFR1 expression to increase cell sensitivity to TNF-α, as this has previously been seen in a murine melanoma model, but this was beyond the scope of the present study (Dobrzanski et al., 2004).

Nevertheless, the addition of IL-21 to either IFN-γ or TNF-α enhanced their inhibitory effects on U251 and U373 cell counts, compared to their independent administration (Figure 3.2). In particular, combining IL-21 with either IFN-γ or TNF-α increased the proportion of apoptotic U373 cells, but there was no obvious synergy in U251 cells (Figure 3.7). On the other hand, IL-21 enhanced the anti-proliferative effects of IFN-γ, but did not appear to alter the impact of TNF-α on ki67 expression in U251 and U373 cells (Figure 3.8). In fact, the most significant benefits of IL-21 treatment were seen when IL-21 was combined with IFN-γ and TNF-α together, which had remarkably potent effects as a triple cytokine treatment on the cell counts and density of both U251 and U373 cell lines (Figures 3.2, 3.3 and 3.4). The addition of IL-21 clearly increased the proportion of U373 cells in the latter stages of either apoptosis or necrosis, compared to the IFN-γ and TNF-α dual treatment (Figure 3.7). In contrast, the triple cytokine condition did not appear to affect U373 cell proliferation, but there was a notable reduction in ki67+ expression in U251 cells (Figure 3.8). Altogether, these findings suggest that the combination of IFN-γ, IL-21 and TNF-α affected the glioblastoma cell lines in different ways, with significant anti-proliferative effects in U251 cells, whilst there was particular focus on cell death in U373 cells – however, these multifaceted abilities could prove to be quite useful, considering the high levels of intratumoural heterogeneity seen in glioblastoma patients. Nonetheless, the results in the present chapter have demonstrated a novel impact of IL-21 on enhancing the direct anti-tumour effects of IFN-γ and TNF-α on glioblastoma cells.

In particular, the inhibitory effects of IL-21 on glioblastoma cells appears to be facilitated by IL-21R, as this was expressed at high levels in both U251 and U373 cells (Figure 3.9). However, treatment with IFN-γ, IL-21 and/or TNF-α did not profoundly affect IL-21R expression in either cell line (Figure 3.10). Interestingly, Brenne et al. (2002) found that culturing myeloma cells with a low dose of TNF-α (10ng/ml) for 18 hours significantly upregulated IL-21R expression, but this also correlated with an increase in cell proliferation. On the other hand, Xue et al. (2019) identified that IL-21 upregulated IL-21R expression in a non-small cell lung cancer cell line, as well as cancer patient samples. Furthermore, they determined that the inhibitory effects of IL-21 on tumour proliferation and migration were mediated by IL-21R signalling (D. Xue et al., 2019). Although both U251 and U373 cells highly expressed IL-21R at a baseline level, this was not compromised by IFN-γ, IL-21 and TNF-α treatment, thus it is plausible that IL-21R signalling could be involved in the inhibition of glioblastoma proliferation and survival.

IL-21R signalling primarily involves JAK1 and JAK3, which subsequently phosphorylate and activate STATs, particularly STAT1, STAT3 and STAT5 (Leonard & Spolski, 2005; Levy & Darnell, 2002). Constitutive STAT3 expression in glioblastoma (including U251 and U373 cells) has been linked with tumour pathogenesis through the upregulation of anti-apoptotic proteins, thus loss of STAT3 expression in U251 cells has been linked with the induction of apoptosis, due to the downregulation of Bcl-2 and Bcl-xL (Rahaman et al., 2002). In addition, research has implicated STAT3 in glioblastoma proliferation, as treatment with various STAT3 inhibitors induced U251 cell cycle arrest (Rahaman et al., 2002; Su et al., 2008). Similarly, Liu et al. (2010) discovered that STAT3 facilitated U251 migration and invasion through upregulation of MMP-9, whilst downregulation of STAT3 restricted tumour angiogenesis in a murine glioblastoma model (Y. Xu et al., 2012).

In the present study, both U251 and U373 cells demonstrated high levels of STAT3 phosphorylation, but IFN-γ, IL-21 and TNF-α treatment did not alter pSTAT3 expression in either cell line (Figures 3.13 and 3.14). Nevertheless, it is plausible that this pSTAT3 expression could be reduced at the end of the 72-hour time course used for assessing cell proliferation and death in the present chapter, thus it is possible that a reduction in STAT3-mediated signalling could be responsible for the inhibition of proliferation in response to IFN-γ, IL-21 and TNF-α treatment in U251 cells. Furthermore, previous research has proven that loss of pSTAT3 expression in U373 cells can lead to a reduction in cell survival, which would align with the results seen in previous experiments – thus it is possible that inhibitory effects of IFN-γ, IL-21 and TNF-α on U373 pSTAT3 expression could be found, but this would require additional testing to diminish experimental variability (Iwamaru et al., 2007). On the other hand, it is plausible that the inhibitory effects of these cytokines on U251 and U373 cells was mediated through alternative signalling pathways. This is reflected in the variable expression of pSTAT1 and pSTAT5 in both cell lines at a baseline level, with U251 cells weakly expressing pSTAT1 and pSTAT5, whilst U373 cells demonstrated moderate expression of both (Figures 3.11, 3.12, 3.15 and 3.16). In line with this, pSTAT1 expression has also been shown to be inconsistent across glioblastoma patient samples, though the majority of them (68%) exhibited intermediate to high levels of pSTAT1 expression (Haybaeck et al., 2007).

With regard to STAT1, IFN-γ signalling via IFNGR2 has been implicated in STAT1 phosphorylation, leading to the induction of apoptosis in various immune cells (Bernabei et al., 2001). It is feasible that the same mechanisms are active in glioblastoma cells as IFN-γ significantly upregulated pSTAT1 expression in both U251 and U373 cells, which somewhat aligns with the slight induction of apoptosis by IFN-γ through increased Annexin V expression. Furthermore, neither IL-21 or TNF-α had any impact on pSTAT1 expression in either cell line, indicating that the upregulation of pSTAT1 expression in the triple cytokine condition was solely driven by IFN-γ (Figures 3.11 and 3.12). In particular, research has shown that upregulation of pSTAT1 induces apoptosis in both U251 and U87 cells, due to the upregulation of Bax and caspase-3, alongside concurrent loss of Bcl-2 expression (P. Huang et al., 2019; Ju et al., 2013). Furthermore, they discovered that pSTAT1 affected the cell cycle

of both glioblastoma cell lines through upregulation of p21, thereby increasing the percentage of cells in G0 – this correlates with data in the present study, which showed that treating U251 cells with the combination of IFN-γ, IL-21 and TNF-α resulted in a significant reduction in the proportion of actively proliferating ki67+ cells. Similarly, Zhang et al. (2018) found that overexpression of pSTAT1 reduced U251 and U373 cell proliferation, migration and invasion – but, it is important to note that these experiments were completed under hypoxic conditions, thus they are not wholly comparable to the present study.

Unfortunately, research exploring the role of STAT5 in glioblastoma is extremely limited, although there is some evidence that STAT5 controls glioblastoma proliferation and survival through expression of Bcl-2, p21 and p27, whilst loss of STAT5 expression correlated with a significant reduction in U87 and U251 invasion (S. Cao et al., 2011; C. Feng & Cao, 2014; Q.-C. Liang et al., 2009). Due to the low expression of pSTAT5 in U251 cells, it proved difficult to identify any changes in expression as a result of IFN-γ, IL-21 and TNF-α treatment. On the other hand, U373 cells had higher baseline pSTAT5 expression, but there was considerable experimental variability (Figures 3.15 and 3.16). Altogether, these findings fail to provide any clarity regarding the functional role of pSTAT5 in glioblastoma – alas, this requires substantial investigation in future research. Nonetheless, IFN-γ, IL-21 and TNF-α strongly upregulated pSTAT1 and pSTAT3 in U251 cells, whilst exclusively enhancing pSTAT1 expression in U373 cells. Collectively, this indicated that pSTAT1 and pSTAT3 were downstream targets of IFN-γ, IL-21 and TNF-α treatment, thus they are likely to be involved in the inhibition of glioblastoma proliferation and/or induction of apoptosis.

A key limitation of these experiments is that they have been completed using immortal cell lines, which are not representative of cells found in patients. Attempts were made to explore IFN-γ, IL-21 and TNF-α treatment in patient-derived cultures, but these cells proved incredibly difficult to maintain *in vitro*. Nonetheless, IFN-γ, IL-21 and TNF-α significantly reduced the cell counts of a patient-derived glioblastoma cell line (367), when administered both independently and as a triple cytokine treatment (Figures 3.5). These findings were further supported by the profound visual losses of cell density, particularly when 367 cells were cultured with the combination of IFN-γ, IL-21 and TNF-α (Figure 3.6). Altogether, results largely reflected those seen in U251 and U373 cells, apart from the lack of 367 cell rounding, suggesting that the cytokines may not have been inducing cell death. However, further investigation is required to validate these experiments in other patient-derived cell lines, whilst also exploring the expression of Annexin V, 7-AAD, ki67, IL-21R and STAT phosphorylation to determine the impact of IFN-γ, IL-21 and TNF-α on proliferation and cell death. Nevertheless, the data produced in this chapter demonstrated that IFN-γ, IL-21 and TNF-α each had individual anti-tumour effects in glioblastoma cells, but this was substantially improved when all three of the cytokines are administered together, indicating that they have significant potential as a novel cytokine therapy for the treatment of glioblastoma.

4. Examining the impact of IFN-γ, IL-21 and TNF-α on glioblastoma migration

4.1. Introduction

The high levels of tumour migration seen in glioblastoma patients is frequently associated with increased surgical complexity, due to the fact that patients can have multiple tumours in different areas of the brain – plus, the highly invasive nature of glioblastoma cells makes it incredibly difficult to distinguish between tumour cells and healthy brain tissue (Gulati et al., 2011; Rahman et al., 2017; Shieh et al., 2020; R. P. Thomas et al., 2013). As a result of this, surgeons often fail to complete a gross total tumour resection, which significantly increases the risk of tumour recurrence in glioblastoma patients, leading to reduced survival rates (T. J. Brown et al., 2016). Although research is limited, hyaluronic acid (HA) and its receptor (CD44) have been implicated in glioblastoma migration, thus they have both been identified as significant potential therapeutic targets (Delpech et al., 1993; Ranuncolo et al., 2002; K.-C. Wei et al., 2010). In line with this, the impact of IFN-γ, IL-21 and TNF-α on U251 and U373 cell migration was examined, which was followed by initial experiments that explored whether these cytokines had any impact on the CD44-HA signalling axis.

4.2. Results

4.2.1. IFN-γ, IL-21 and TNF-α variably affected glioblastoma cell migration

Having established that IFN-γ, IL-21 and TNF-α induced cell death and inhibit proliferation in U251 and U373 cells, it was also important to consider whether the cytokines had any impact on glioblastoma migration. In order to examine this, U251 and U373 cells were seeded into two-well inserts within a 24-well plate to generate two cell populations that were separated by a defined area. After 24 hours, the inserts were removed and the cells were washed, before subsequent treatment with IFN-γ, IL-21 and TNF-α, both individually and combined. The 24-well plate containing U251 and U373 cells was then placed into a Cell IQ machine, in which images were taken of the area between the cell populations at three separate points, in order to quantify the level of migration of both cell lines over a 72-hour period. For the sake of brevity and clarity, only the images from the 0-hour and 72-hour time points will be presented in this chapter, but the images from the 24-hour and 48-hour time points are available in the Appendix (Figures 9.1-9.4).

Results showed that none of the cytokines had a significant impact on U251 cell migration after 24 hours of treatment (Figure 4.2). However, the images taken at this time point illustrate the rapid migration of U251 cells treated with IL-21 and TNF-α, both independently and as a dual treatment, as well as the Control, whilst U251 cell migration in each of the remaining conditions appeared to be delayed (Figures 4.2 and 9.1). These findings were confirmed at the 48-hour time point, as almost all of the cytokine conditions surpassed 70% area closure, except for U251 cells treated with IFN-γ alone (56% - $p = 0.0489$) and the combination of IFN-γ and IL-21 (63% - $p = 0.0796$), which had both migrated into the area between cell populations at a significant lower rate than the Control (82%) (Figures 4.2 and 9.1). The inhibitory effects of IFN-γ alone (67% - $p = 0.0027$) and the dual treatment of IFN-γ and IL-21 (81% - $p = 0.0239$) were still present at the end of the time course (Figure 4.2). Unfortunately, TNF-α appeared to neutralise the combination of IFN-γ and IL-21, as the triple cytokine condition failed to demonstrate a significant impact on U251 cell migration at any time point.

In contrast, the combination of IFN-γ and IL-21 (27% - $p = 0.0173$) substantially reduced U373 cell migration 24 hours post-insert removal, compared to the Control (65%), which was sustained at the 48-hour time point (47% - $p = 0.0043$) (Figures 4.4 and 9.3). However, there was also a noticeable decrease in U373 migration mediated by all of the other experimental conditions that contained IFN-γ after 48 hours: IFN-γ alone (54% - $p = 0.0371$), IFN-γ and TNF-α (63% - $p = 0.0397$), and IFN-γ, IL-21 and TNF-α (60% - $p = 0.0314$) (Figures 4.4 and 9.4). The inhibitory effects of these cytokine conditions on U373 cell migration were still present at the end of the time course, with the most potent effect induced by the IFN-γ and IL-21 dual treatment (58% - $p = 0.0013$). In addition, the combination of IFN-γ and TNF-α (71% - $p = 0.0137$) and the triple cytokine cocktail (75% - $p = 0.0436$) notably restricted U373 cell migration, compared to the Control (98%) (Figure 4.4). Collectively, these findings demonstrated that IFN-γ both alone and in combination with IL-21 significantly reduced U251 and U373 cell migration.

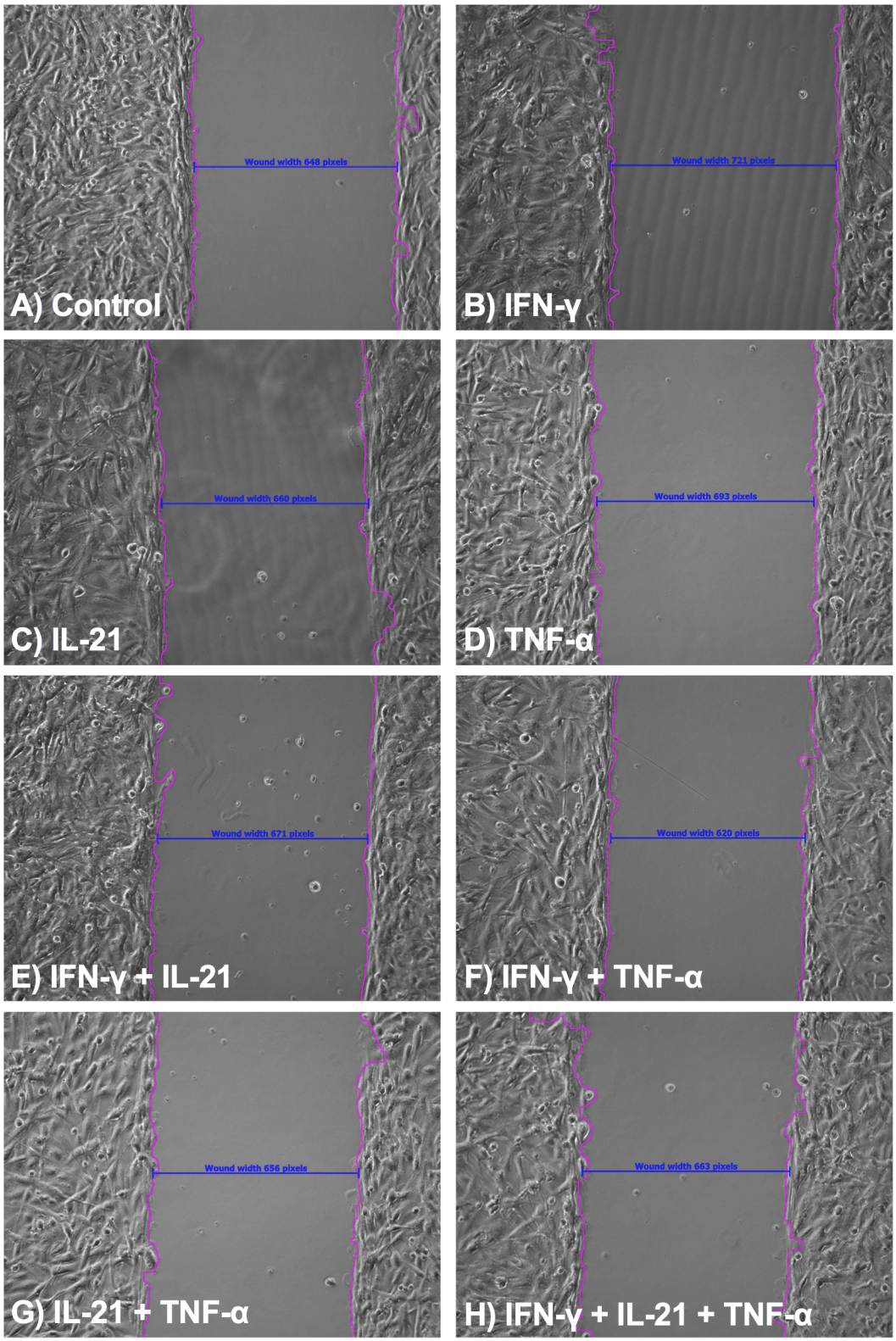
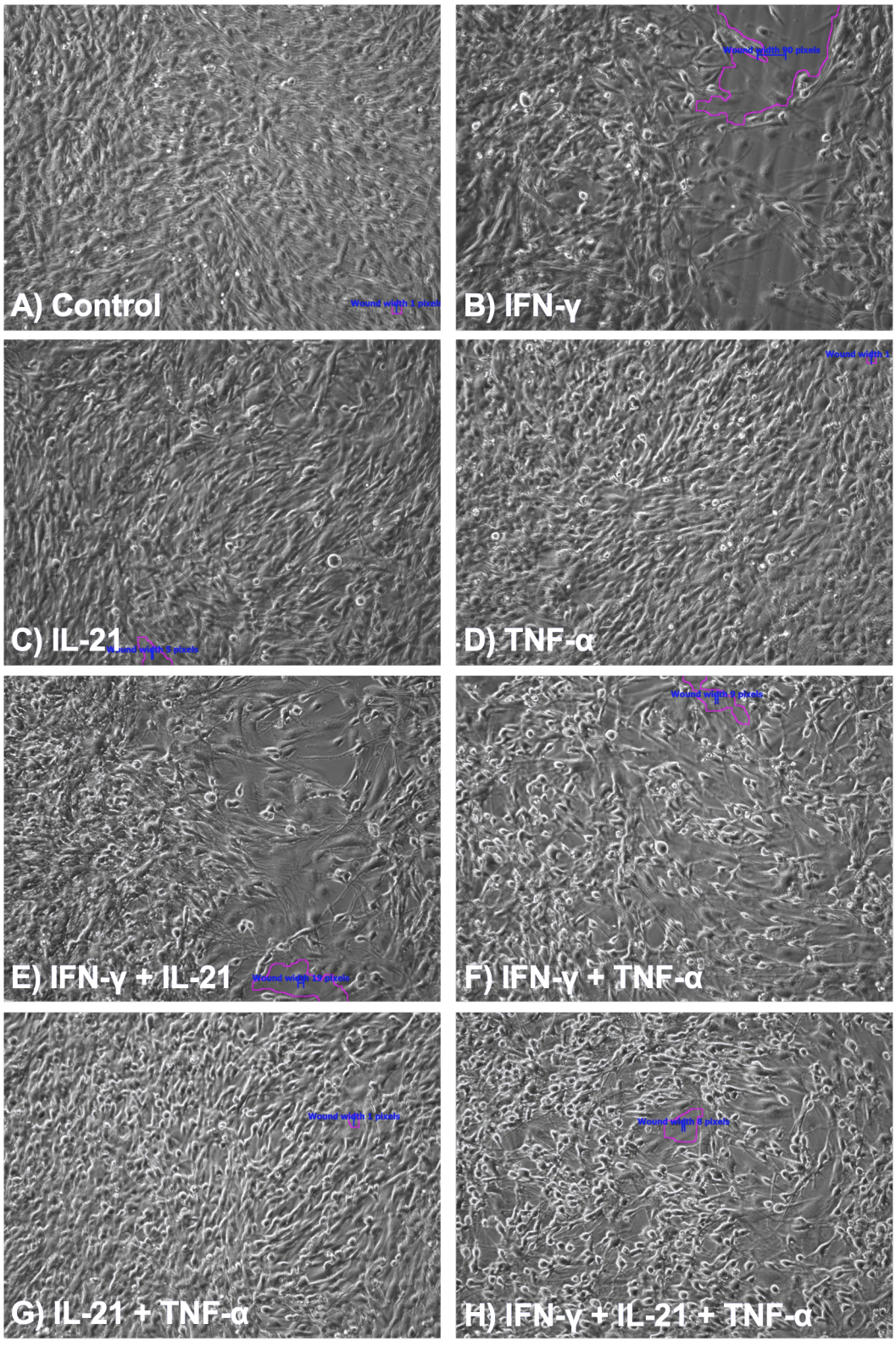


Figure 4.1. Baseline measurement of the area between U251 cell populations

$1.5x10^4$ U251 cells were seeded into two-well culture inserts inside a 24-well plate for 24 hours before the inserts were removed and U251 cells were freshly treated with **A)** RPMI Control; **B)** 100ng/ml IFN-γ; **C)** 100ng/ml IL-21; **D)** 100ng/ml TNF-α; **E)** 100ng/ml IFN-γ and IL-21; **F)** 100ng/ml IFN-γ and TNF-α; **G)** 100ng/ml IL-21 and TNF-α; **H)** 100ng/ml IFN-γ, IL-21 and TNF-α. Images were taken every hour over a 72-hour period, whilst the size of the area between cell populations was determined using Cell IQ analysis software. These images were taken immediately post-insert removal and are representative of >3 independent experiments.


A) Control
B) IFN-γ
C) IL-21
D) TNF-α
E) IFN-γ + IL-21
F) IFN-γ + TNF-α
G) IL-21 + TNF-α
H) IFN-γ + IL-21 + TNF-α

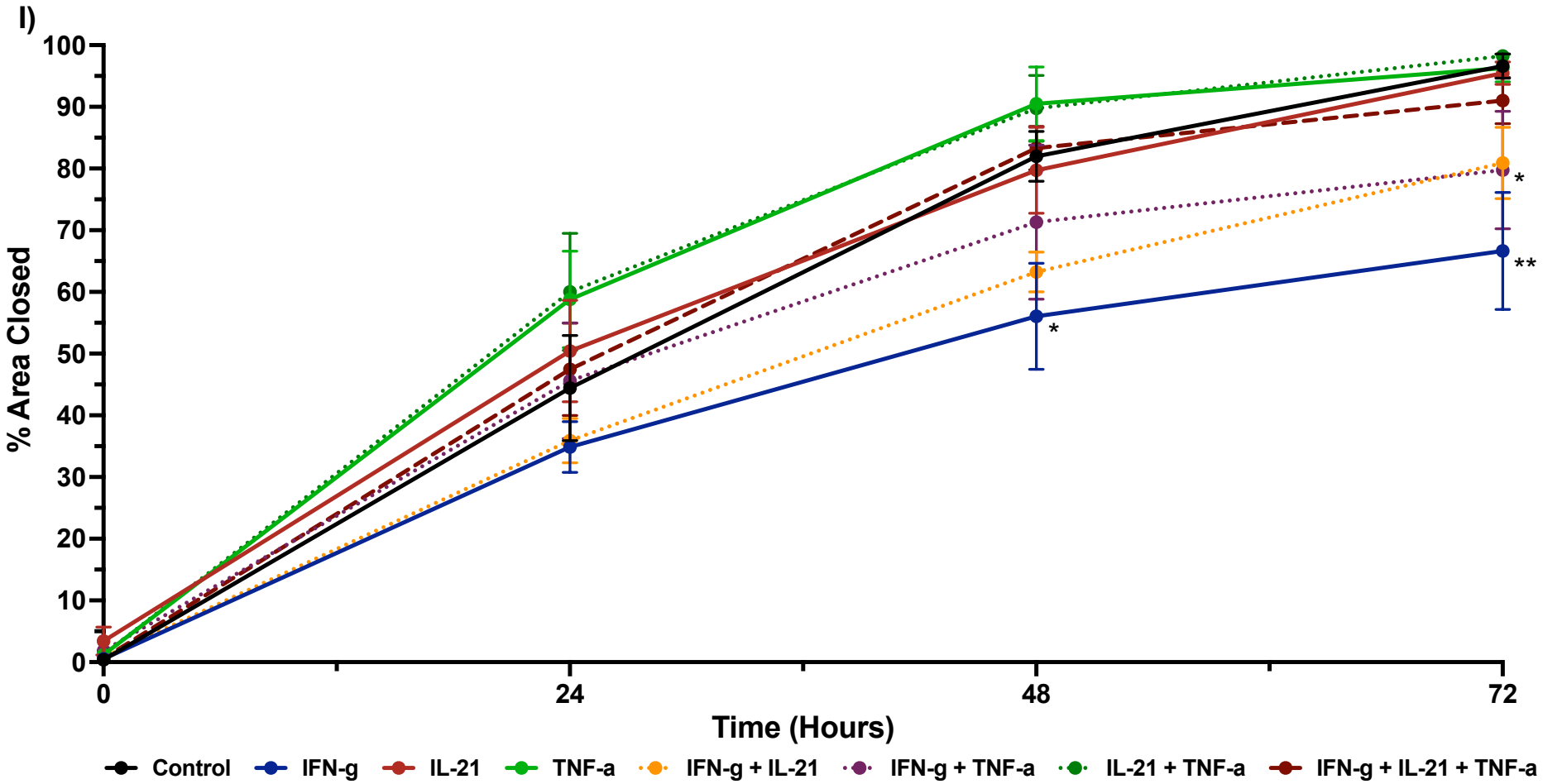


Figure 4.2. IFN-γ alone and in combination with IL-21 reduced U251 cell migration 72 hours post-insert removal

$1.5x10^4$ U251 cells were seeded into two-well culture inserts inside a 24-well plate for 24 hours before the inserts were removed and the media was changed, so that U251 cells could subsequently be treated with **A)** RPMI Control**; B)** 100ng/ml IFN-γ; **C)** 100ng/ml IL-21; **D)** 100ng/l TNF-α; **E)** 100ng/ml IFN-γ and IL-21; **F)** 100ng/ml IFN-γ and TNF-α; **G)** 100ng/ml IL-21 and TNF-α; **H)** 100ng/ml IFN-γ, IL-21 and TNF-α. Images were taken every hour over a 72-hour period, with these particular images taken 72 hours post-insert removal. **I)** The size of the area between cell populations was determined using Cell IQ analysis software. Bars represent means of % area closed and SEM is shown for > 3 independent experiments. P Values: * = <0.05; ** = <0.01; *** = <0.001; **** = <0.0001.

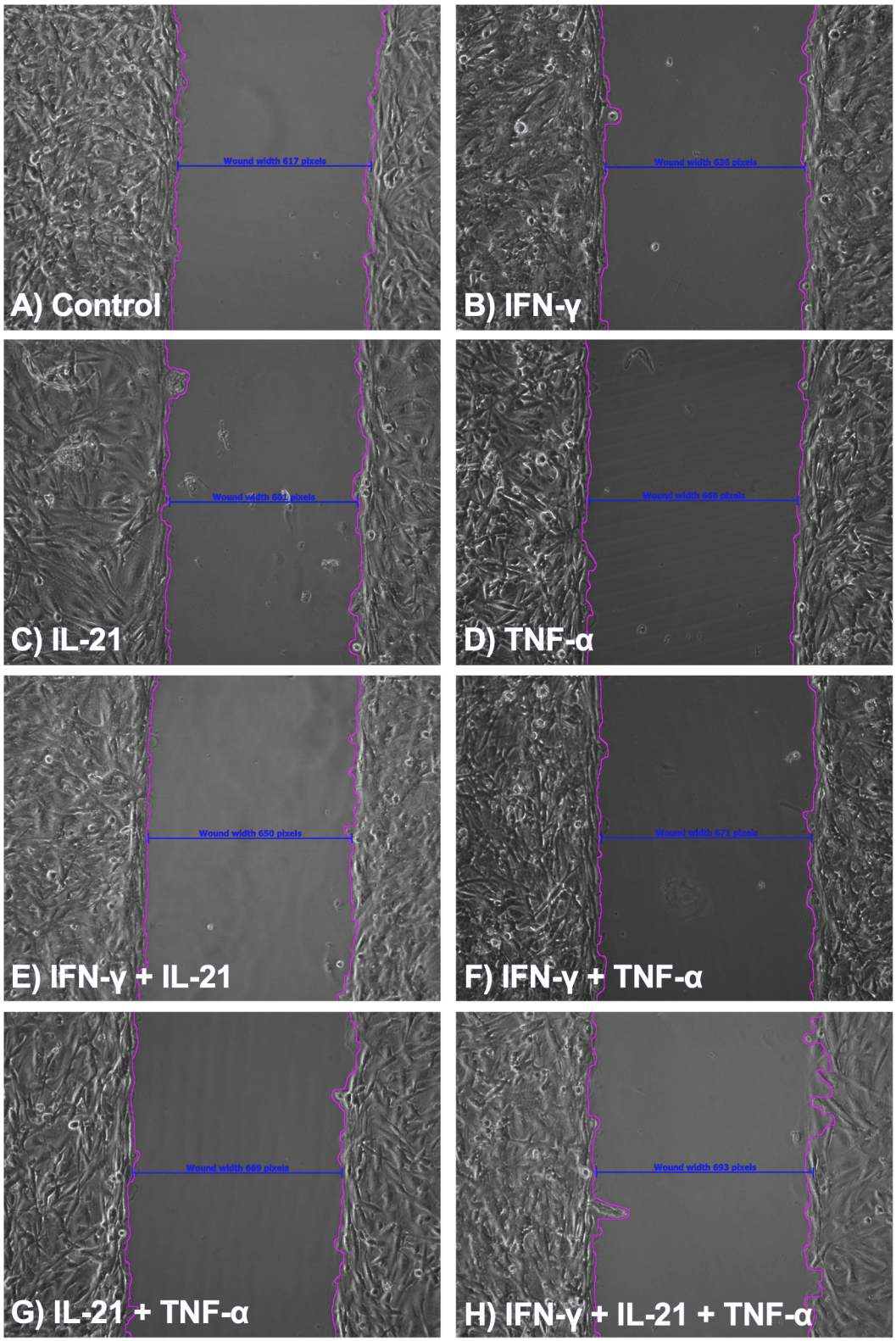
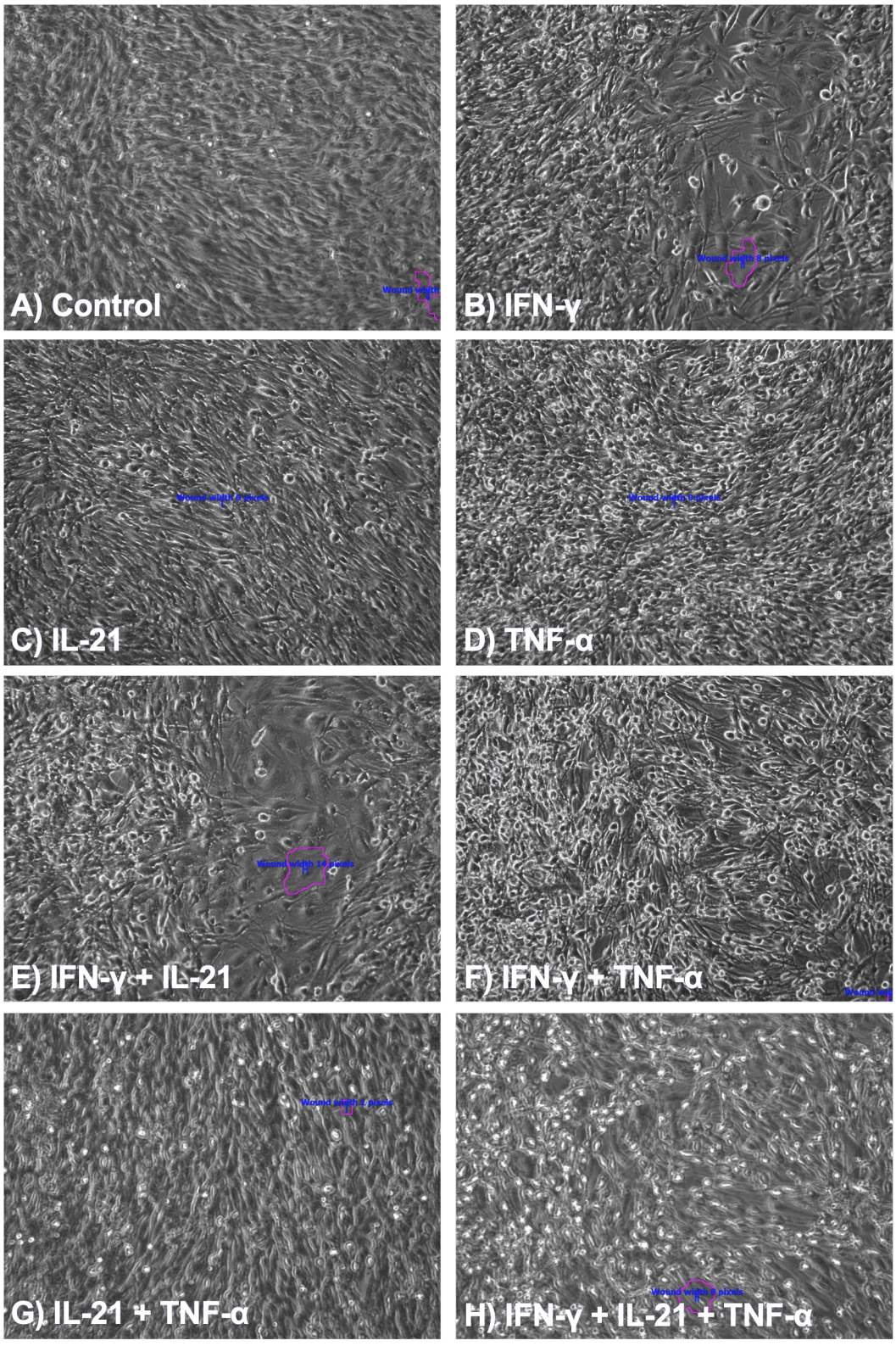


Figure 4.3. Baseline measurement of the area between U373 cell populations

$1.5x10^4$ U373 cells were seeded into two-well culture inserts inside a 24-well plate for 24 hours before the inserts were removed and U373 cells were freshly treated with **A)** RPMI Control; **B)** 100ng/ml IFN-γ; **C)** 100ng/ml IL-21; **D)** 100ng/ml TNF-α; **E)** 100ng/ml IFN-γ and IL-21; **F)** 100ng/ml IFN-γ and TNF-α; **G)** 100ng/ml IL-21 and TNF-α; **H)** 100ng/ml IFN-γ, IL-21 and TNF-α. Images were taken every hour over a 72-hour period, whilst the size of the area between cell populations was determined using Cell IQ analysis software. These images were taken immediately post-insert removal and are representative of >3 independent experiments.


A) Control
B) IFN-γ
C) IL-21
D) TNF-α
E) IFN-γ + IL-21
F) IFN-γ + TNF-α
G) IL-21 + TNF-α
H) IFN-γ + IL-21 + TNF-α

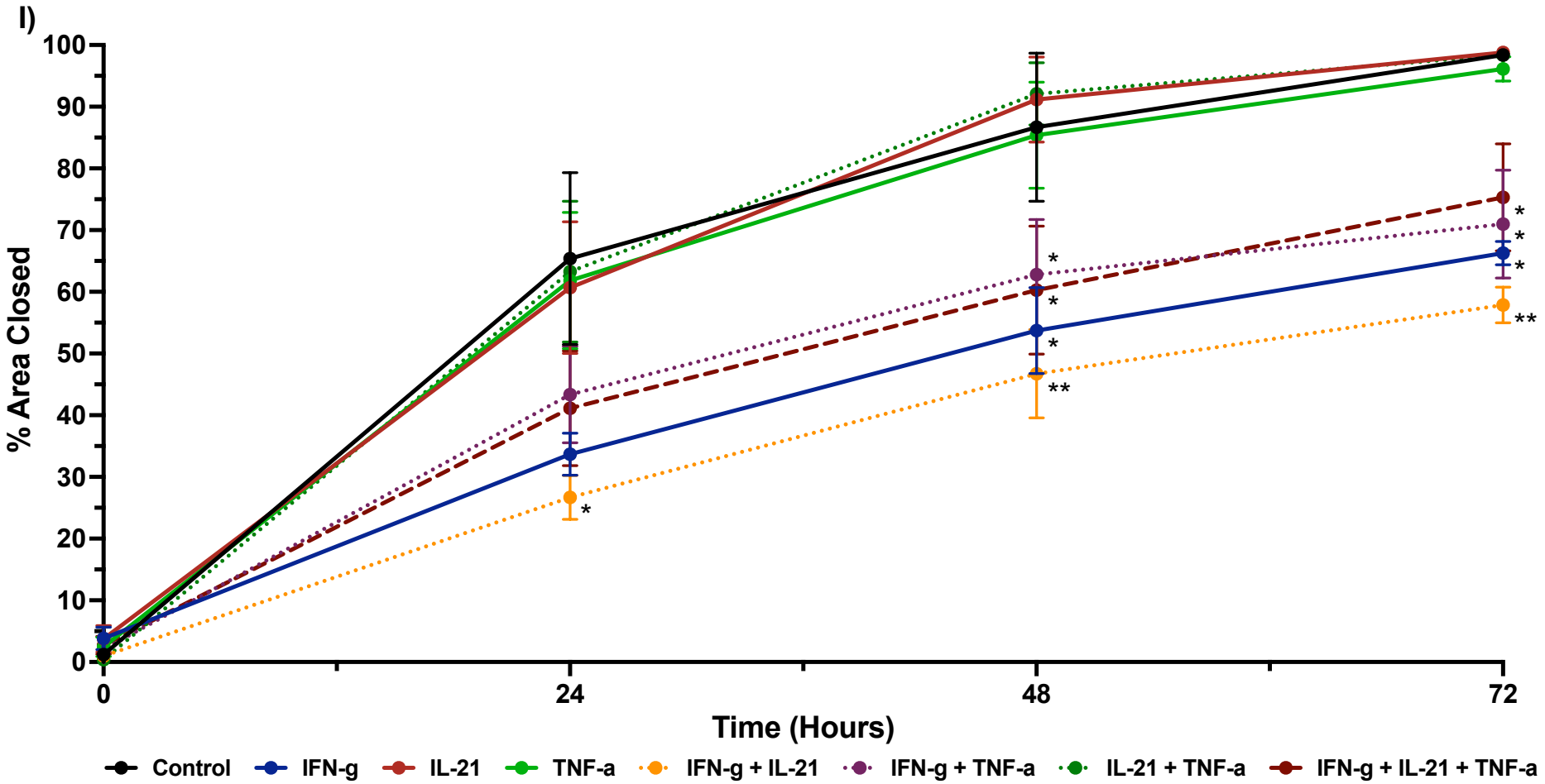


Figure 4.4. IFN-γ alone and in combination with IL-21 and/or TNF-α reduced U373 cell migration 72 hours post-insert removal

$1.5x10^4$ U373 cells were seeded into two-well culture inserts inside a 24-well plate for 24 hours before the inserts were removed and the media was changed, so that U373 cells could subsequently be treated with **A)** RPMI Control; **B)** 100ng/ml IFN-γ; **C)** 100ng/ml IL-21; **D)** 100ng/l TNF-α; **E)** 100ng/ml IFN-γ and IL-21; **F)** 100ng/ml IFN-γ and TNF-α; **G)** 100ng/ml IL-21 and TNF-α; **H)** 100ng/ml IFN-γ, IL-21 and TNF-α. Images were taken every hour over a 72-hour period, with these particular images taken 72 hours post-insert removal. **I)** The size of the area between cell populations was determined using Cell IQ analysis software. Bars represent means of % area closed and SEM is shown for > 3 independent experiments. P Values: * = <0.05; ** = <0.01; *** = <0.001; **** = <0.0001.

4.2.2. IFN-γ, IL-21 and TNF-α reduced CD44 expression in glioblastoma cells

Previous research has suggested that glioblastoma cells used CD44 to bind to the HA-rich brain ECM to facilitate their migration and invasion into healthy brain tissues (Bellail et al., 2004; Mahesparan et al., 2003; Nakada et al., 2007). Furthermore, Delpech et al. (1993) discovered that glioblastoma cells also secreted high levels of HA to further enhance their motility. In line with this, it was logical to explore whether the inhibition of U251 and U373 cell migration by IFN-γ, IL-21 and/or TNF-α was mediated by suppression of this CD44-HA signalling axis. The first step in this process was to determine whether CD44 was expressed by U251 and U373 cells – therefore, both cell lines were stained for CD44 expression, which was compared against an Unstained Control for each cell line, during subsequent flow cytometric analysis. Results showed that both U251 ($p = 0.0008$) and U373 cells ($p = 0.0048$) highly expressed CD44, but there was no significant different in terms of expression levels between each cell line (Figure 4.5).

As shown in Figure 4.6, there is a clear biphasic CD44 expression profile when both cell lines were exposed to the IFN-γ and TNF-α dual treatment. As this was also visible in the culture condition containing the triple cytokine combination, this indicates that IFN-γ and TNF-α synergised to split cells into two distinct populations, defined by their level of CD44 expression ($CD44^{lo}$ and $CD44^{hi}$), suggesting that there was a particular shift towards inhibiting peak CD44 expression.

In U251 cells, IFN-γ and TNF-α partially reduced $CD44^{hi}$ expression to 85% of the maximum response ($p = 0.1195$), but this was notably enhanced with the addition of IL-21 (77% - $p = 0.0288$), as only the triple cytokine treatment demonstrated as significant effect on suppressing U251 $CD44^{hi}$ expression (Figure 4.6D). The same trend was present when examining the broader picture, with the combination of IFN-γ, IL-21 and TNF-α also significantly depleting the pool of CD44+ cells, when compared against the Control (79% - $p = 0.0001$) (Figure 4.6C).

Similar results were seen in U373 cells, with the biphasic histogram expression profile present when U373 cells were treated with either IFN-γ and TNF-α or the combination of IFN-γ, IL-21 and TNF-α. Whilst the IFN-γ and TNF-α dual treatment (71% - $p = 0.0288$) was effective in reducing $CD44^{hi}$ expression, relative to the Control, the addition of IL-21 potently enhanced this suppressive effect (64% - $p = 0.0075$) (Figure 4.6D). The same trend was apparent when examining total CD44+ expression in U373 cells, with the largest reduction induced by IFN-γ, IL-21 and TNF-α (72% - $p = 0.0009$) (Figure 4.6C). Collectively, these results highlighted that IFN-γ and TNF-α reduced CD44 expression in U251 and U373 cells, but this suppressive effect was enhanced when combined with IL-21 as a triple cytokine treatment.

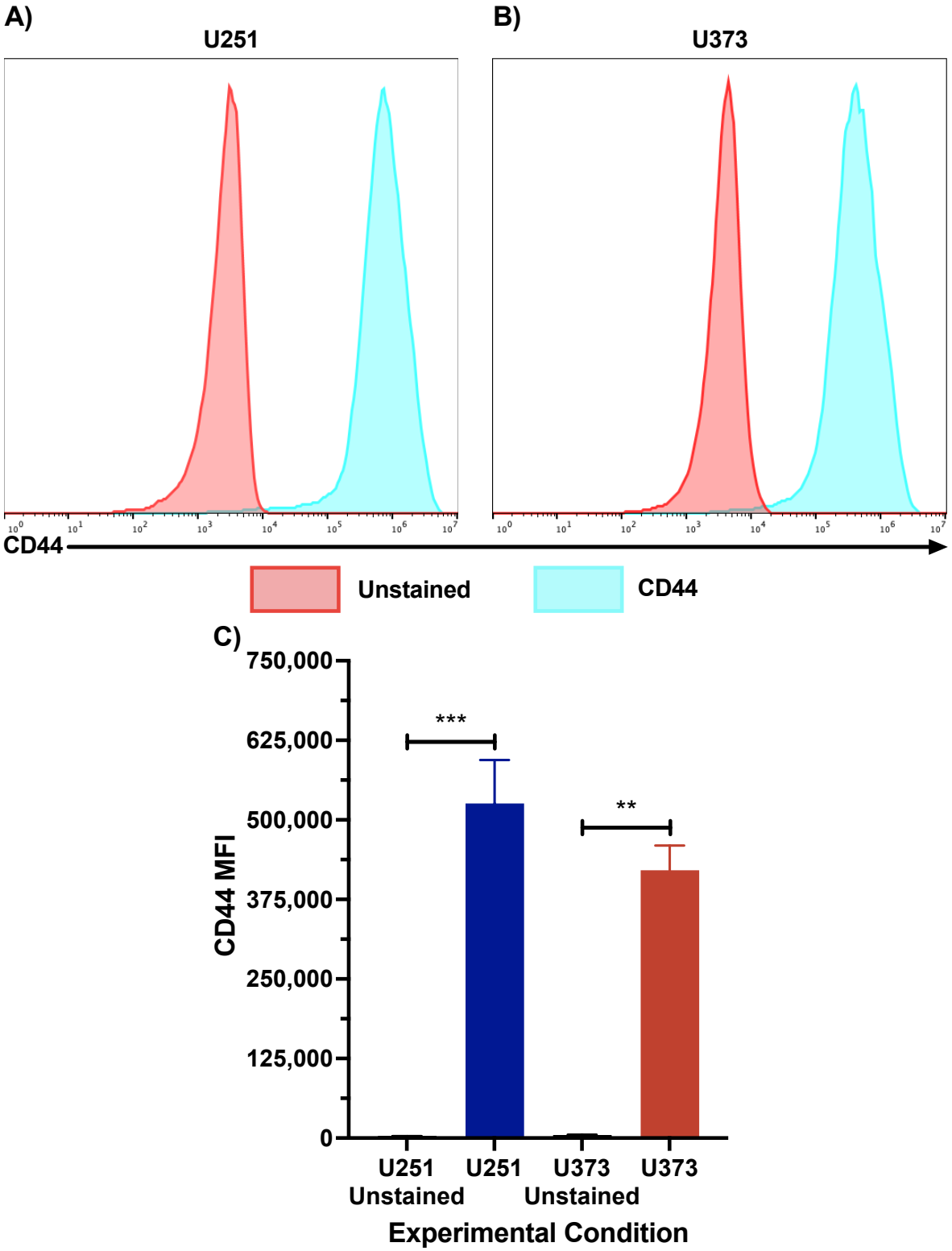
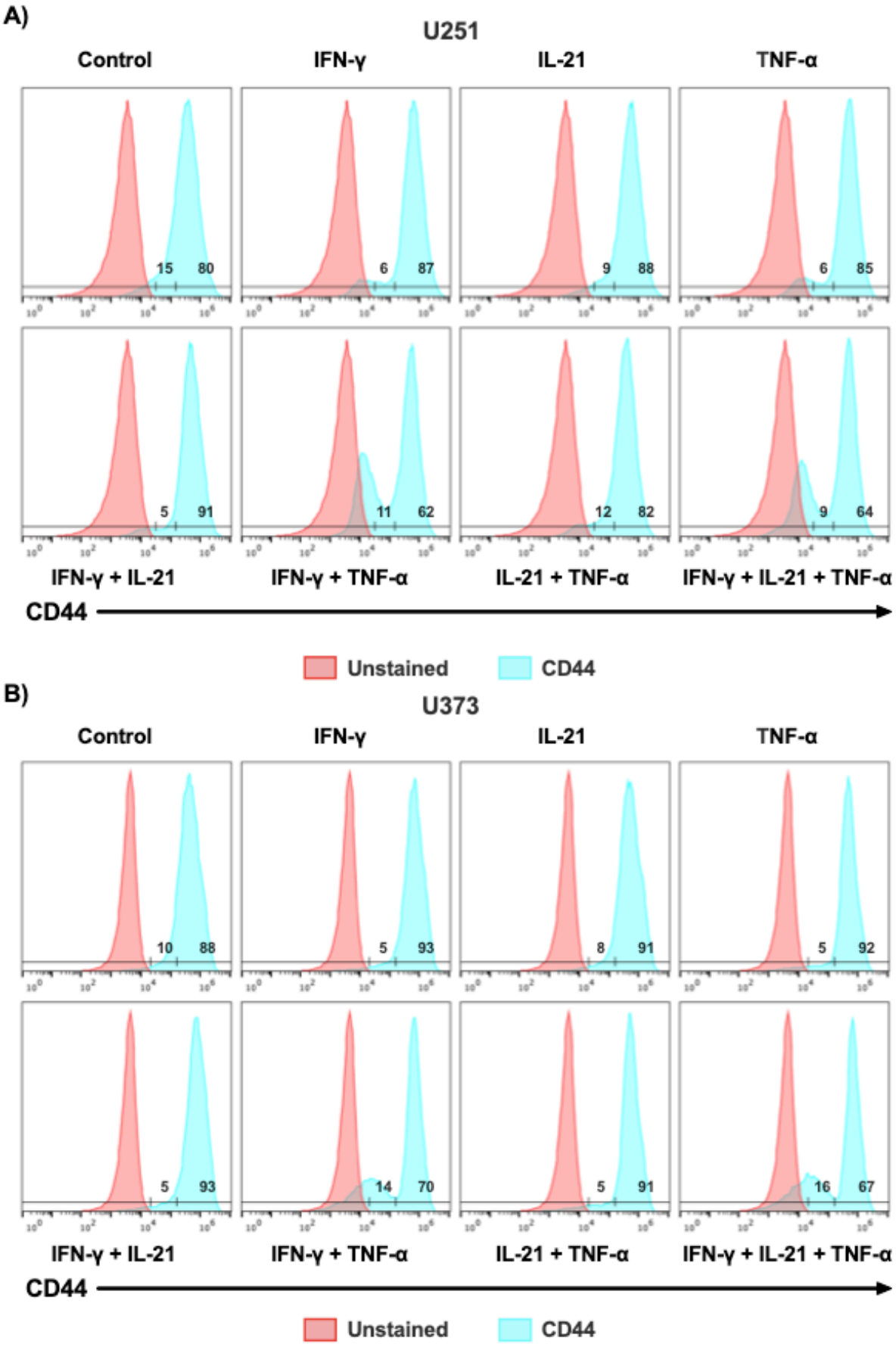


Figure 4.5. U251 and U373 cells highly expressed CD44

U251 cells **A)** and U373 cells **B)** were either stained for CD44 or left unstained for analysis via flow cytometry. Flow plots are representative of > 3 independent experiments. Bars represent CD44 MFI C) as means with SEM shown for > 3 independent experiments. P Values: * = <0.05; ** = <0.01; *** = <0.001; **** = <0.0001.


A)
U251
Control
IFN-γ
IL-21
TNF-α
15 80
6 87
9 88
6 85
5 91
11 62
12 82
9 64
IFN-γ + IL-21
IFN-γ + TNF-α
IL-21 + TNF-α
IFN-γ + IL-21 + TNF-α
CD44
Unstained
CD44
B)
U373
Control
IFN-γ
IL-21
TNF-α
10 88
5 93
8 91
5 92
5 93
14 70
5 91
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IFN-γ + IL-21
IFN-γ + TNF-α
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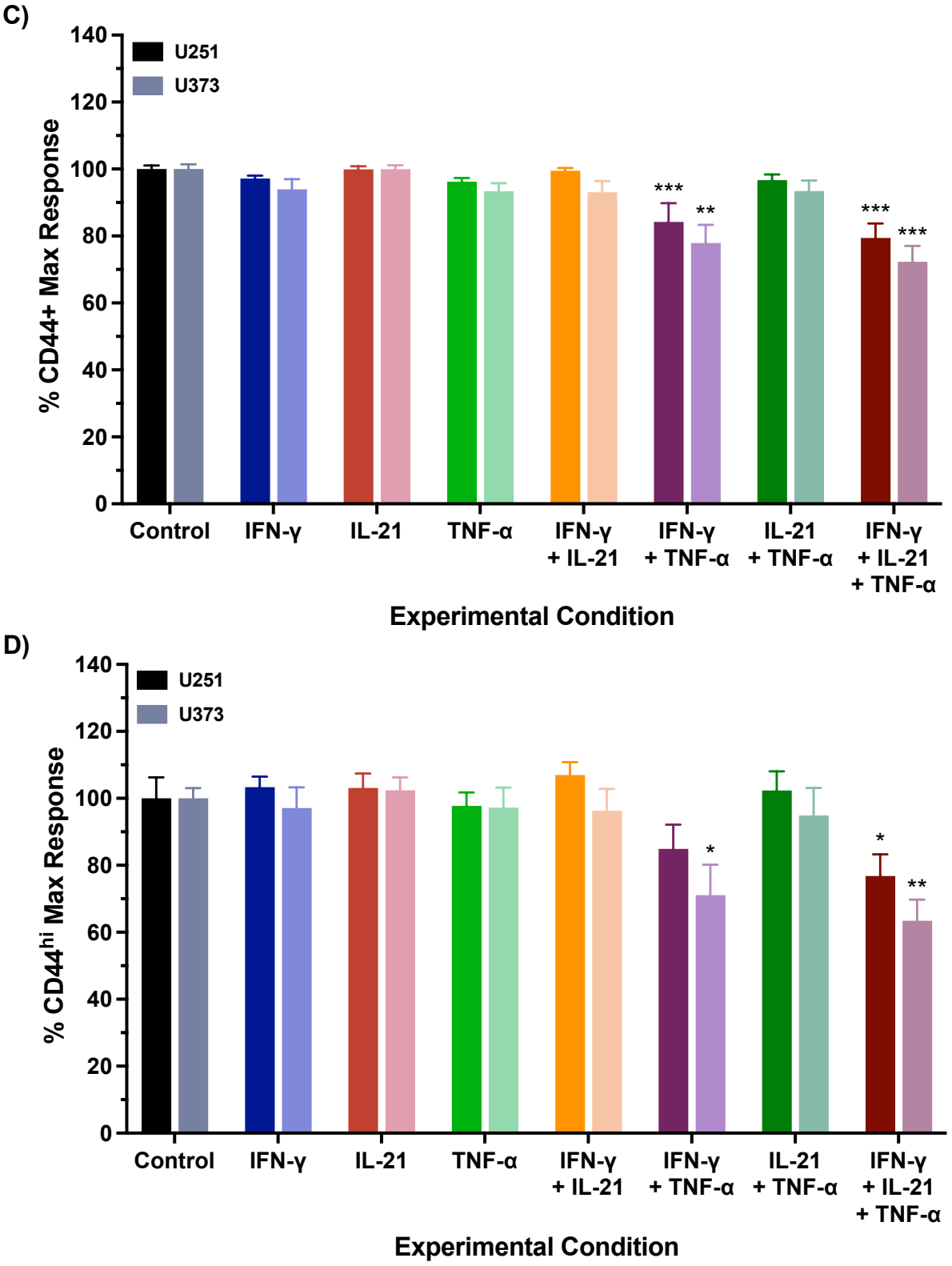


Figure 4.6. IFN-γ, IL-21 and TNF-α negatively affected CD44 expression in U251 and U373 cells
$2.5x10^4$ U251 cells **A)** and $2.5x10^4$ U373 cells **B)** were seeded with an RPMI Control or 100ng/ml IFN-γ, 100ng/ml IL-21, 100ng/ml TNF-α, both individually and combined, before they were stained for CD44 for analysis via flow cytometry – U251 **A)** and U373 **B)**. Bars represent means compared to the Control for all CD44+ cells **C)** and the fraction of cells that were $CD44^{hi}$ **D)** with SEM shown in both for > 3 independent experiments. P Values: * = <0.05; ** = <0.01; *** = <0.001; **** = <0.0001.

4.3. Discussion

Alongside reducing tumour growth, restricting tumour migration is also an incredibly important factor to consider, when evaluating the therapeutic potential of a novel cancer treatment. This is particularly vital for brain tumours, as the capacity for tumour invasion is profoundly limited, due to the compact nature of the tissues contained within the skull. Furthermore, the risk of surgical error is far more significant, as it can profoundly compromise patient neurological function – this is particularly relevant in glioblastoma, as their highly invasive nature substantially increases the difficulty in distinguishing between tumour cells and healthy brain tissue (Gulati et al., 2011; Rahman et al., 2017). As a result of this, it is often not possible for gross total tumour resections to be completed, despite the fact that research has shown that leaving tumour tissue intact significantly increases the risk of glioblastoma recurrence (T. J. Brown et al., 2016).

Of the three cytokines used in the present thesis, only IFN-γ has been previously shown to inhibit glioblastoma migration. In particular, Knüpfer et al. (2001) utilised a transwell experimental system to demonstrate that A172 cells treated with a low dose of IFN-γ (10ng/ml) migrated at almost half the rate of the untreated Control, over a 24-hour period. Although the extent of these findings was not identified in either U251 and U373 cells in the present study, it is possible that this is due to the differences in models of migration, glioblastoma cell lines, and/or IFN-γ dose. Nonetheless, IFN-γ alone did significantly reduce the migration of both cell lines after 48 hours, which was maintained at the 72-hour time point (Figures 4.2 and 4.4). Interestingly, the decrease in glioblastoma migration mediated by IFN-γ was not compromised by the addition of TNF-α in U373 cells or IL-21 in either cell line. As neither of these cytokines had an independent impact on the rate of migration in U251 or U373 cells at any time point, the results suggest that that IL-21 and TNF-α were supporting IFN-γ signalling, but the exact mechanisms are not clear.

As HA is highly expressed in the brain ECM, the expression of CD44 and its adhesion to HA have strongly been implicated in facilitating glioblastoma migration (Merzak et al., 1994; Radotra & McCormick, 1997). Knupfer et al. (1999) found that multiple glioblastoma cell lines highly expressed CD44, whilst demonstrating that they each used the CD44-HA signalling axis to drive cell motility *in vitro* – although, they did not explore these effects in U251 or U373 cells. Results from the present study established that both U251 and U373 cells highly expressed CD44 (Figure 4.5). However, CD44 expression was not affected by treatment with IFN-γ alone in either cell line, indicating that the inhibition of glioblastoma migration mediated by IFN-γ was not regulated through CD44 signalling (Figure 4.6). It is possible that the inhibitory effects of IFN-γ on U251 and U373 cells were mediated through increased STAT1 signalling, as Zhang et al. (2018) found that enhanced pSTAT1 expression significantly reduced U251 and U373 cell migration and invasion. This theory is further supported by the findings in Chapter Three, which demonstrated an increase in pSTAT1 expression in both cell lines, in response to treatment with IFN-γ (Figures 3.13 and 3.14).

On the other hand, the combination of IFN-γ, IL-21 and TNF-α significantly reduced CD44 expression in U251 and U373 cells, both in terms of the proportion of CD44+ and $CD44^{hi}$ cells (Figure 4.6). As IFN-γ alone had no direct impact on CD44 expression, these results suggested that IL-21 or TNF-α could be responsible for the inhibition of migration in the triple cytokine condition through downregulation of CD44. Unlike IL-21, several studies have highlighted that TNF-α can reduce CD44 expression and subsequently restrict tumour invasion in breast and ovarian cancer cells (J. Li et al., 2012; Muthukumaran et al., 2006). Interestingly, both of these studies found that CD44 expression was regulated by a c-Jun N-terminal kinase (JNK) pathway, but the exact mechanisms controlled by TNF-α were not identified. In particular, research has shown that JNK signalling can lead to the activation and phosphorylation of STAT3, thus it is possible that building upon our understanding of how these cytokines affect pSTAT3, as discussed in Chapter Three, could also uncover a direct link to modulation of CD44 expression (Gkouveris et al., 2016; J.-H. Kim et al., 2010; Lim & Cao, 1999). This theory is supported by studies showing that blockade of STAT3 signalling reduced CD44 expression in prostate cancer and nasopharyngeal carcinoma cells (Lin et al., 2017; C.-T. Wu et al., 2019). Furthermore, Liu et al. (2010) identified that pSTAT3 upregulated MMP-9 expression in U251 cells, in order to augment their migration and invasion, providing another mechanism in which downregulation of pSTAT3 could lead to reduced cell motility. Altogether, the results in the present chapter demonstrated that IFN-γ, IL-21 and TNF-α reduced glioblastoma migration, which could be facilitated through pSTAT3, CD44 and/or HA, thus they should be explored in future research.

5. Investigating co-stimulatory and co-inhibitory ligand expression in glioblastoma

5.1. Introduction

Co-stimulatory and co-inhibitory ligands represent a crucial part of the mechanisms involved in the regulation of the immune response. Due to the highly immunosuppressive nature of the glioblastoma microenvironment, it is important to consider that there may be direct interactions between immune cells and glioblastoma cells through these signalling pathways, in order to restrict T cell activation or induce T cell exhaustion. Unfortunately, very few studies have evaluated the expression of key co-stimulatory and co-inhibitory ligands on glioblastoma cells, thus our knowledge of the direct relationship between these tumour cells and T cells are not known. As a result of this, the present chapter investigated the baseline expression of various key co-stimulatory (CD80, CD86, ICOS-L, 41BB-L) and co-inhibitory (PD-L1 and Galectin-9) ligands on U251 and U373 cells, in an attempt to establish whether it was theoretically possible for glioblastoma cells to bind directly to T cells and modulate their activity. Furthermore, the impact of IFN-γ, IL-21 and TNF-α treatment on the expression of these co-stimulatory and co-inhibitory ligands in U251 and U373 cells was also assessed to evaluate whether these cytokines could direct glioblastoma cells towards promoting T cell function and/or restore anti-tumour immunity.

5.2. Results

5.2.1. IFN-γ, IL-21 and TNF-α upregulated CD80 expression in glioblastoma cells

As one of the most vital receptors involved in T cell activation, the co-stimulatory ligands of CD28 (CD80 and CD86) are of particular importance, as they promote T cell proliferation, survival, and IL-2 production (Azuma et al., 1993; Jenkins et al., 1991; June et al., 1994; Linsley et al., 1990). Previous research has suggested that neither U251 or U373 cell lines expressed CD80 or CD86, so as to restrict tumour-mediated T cell activation (Wintterle et al., 2003). In order to confirm this, U251 and U373 cells were stained for CD80 expression, which was compared against an Unstained Control for each cell line, during subsequent flow cytometric analysis. Supporting the findings by Wintterle et al, (2003), U251 cells demonstrated no CD80 expression, but, in contrast, U373 cells showed marginal CD80 expression, when compared against the Unstained Control (Figure 5.1).

Although neither cell line highly expressed CD80, it was still important to consider the possibility that each of the cytokine treatment conditions could induce CD80 expression in either glioblastoma cell line. In order to explore this, both U251 and U373 cells were cultured for 72 hours with 100ng/ml IFN-γ, IL-21 and TNF-α, independently and combined, before they were harvested and stained for CD80 for analysis via flow cytometry, with the 72h Control result set as the 100% maximum response. Remarkably, it appeared that IFN-γ could independently induce CD80 expression in U251 cells (174% - $p = 0.0129$), which was largely sustained when combined with IL-21 (165% - $p = 0.0262$). However, the dual combination of IFN-γ and TNF-α (196% - $p = 0.0018$) had a stronger impact on inducing CD80 expression in U251 cells, though this was further enhanced when combined with IL-21 (231% - $p = 0.0003$), as detailed by the noticeable shift in the CD80 expression profile (Figure 5.2).

Similar results were found when exploring the impact of IFN-γ, IL-21 and/or TNF-α treatment in U373 cells. In particular, IFN-γ alone upregulated CD80 expression (163% - $p = 0.0087$), but this was unaffected by the addition of IL-21 (165% - $p = 0.0074$) or TNF-α (167% - $p = 0.0071$). Likewise, administering all three cytokines together did not appear to further enhance CD80 expression, which remained stable (167% - $p = 0.0080$), as reflected in the consistency of the CD80 expression profile compared to the Control (Figure 5.2). Overall, these results showed that IFN-γ, IL-21 and TNF-α treatment could either induce or upregulate CD80 expression in glioblastoma cells.

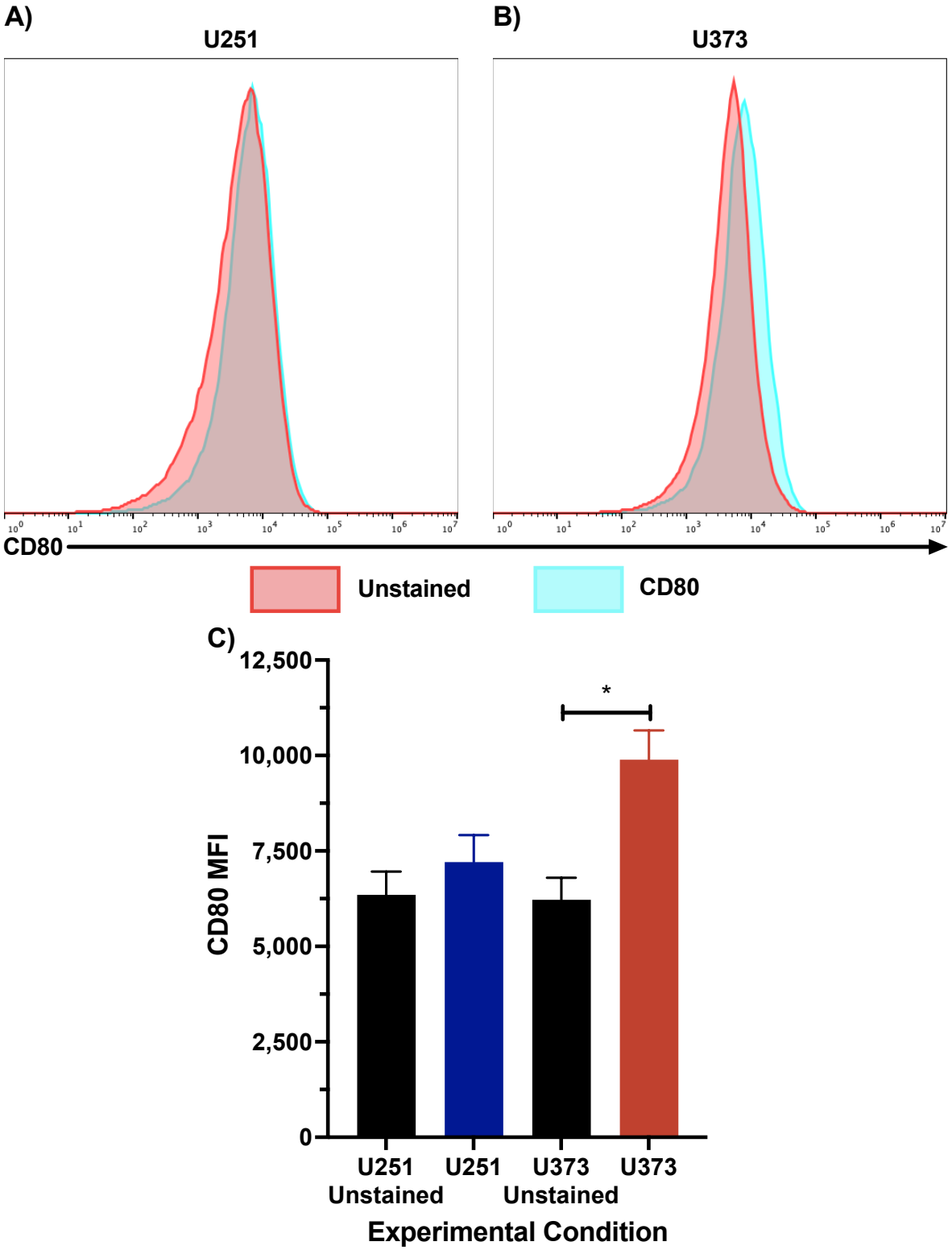


Figure 5.1. U373 cells marginally expressed CD80

U251 cells **A)** and U373 cells **B)** were either stained for CD80 or left unstained for analysis via flow cytometry. Flow plots are representative of > 3 independent experiments. Bars represent CD80 MFI **C)** with means and SEM shown for > 3 independent experiments. P Values: * = <0.05; ** = <0.01; *** = <0.001; **** = <0.0001.

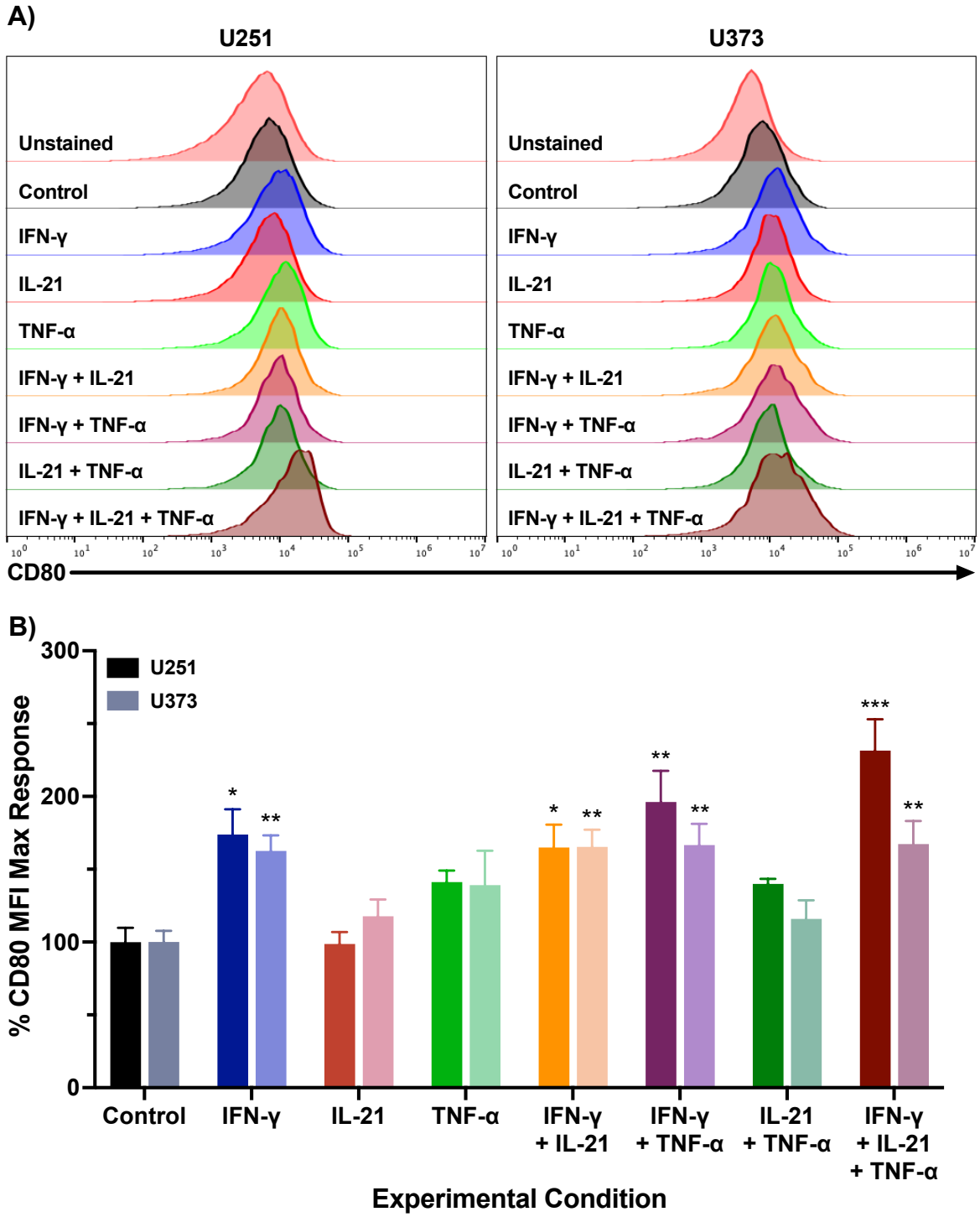


Figure 5.2. IFN-γ, IL-21 and TNF-α upregulated CD80 expression in U251 and U373 cells

2.5x10^4 U251 cells and 2.5x104 U373 cells were seeded with an RPMI Control or 100ng/ml IFN-γ, 100ng/ml IL-21, 100ng/ml TNF-α, both individually and combined, for 72 hours before they were stained for CD80 for analysis via flow cytometry. Flow plots **A)** are representative of > 3 independent experiments. Bars represent CD80 MFI **B)** as means compared to the Control, with SEM shown for > 3 independent experiments.
P Values: * = <0.05; ** = <0.01; *** = <0.001; **** = <0.0001.

5.2.2. IFN-γ, IL-21 and TNF-α upregulated CD86 expression in glioblastoma cells

Alongside CD80, CD86 also provides co-stimulatory signalling when bound to the CD28 receptor on the T cell surface, in order to support T cell activation, proliferation and survival – however, previous research has indicated that glioblastoma cells did not express CD86 (Wintterle et al., 2003). In order to verify this, U251 and U373 cells were stained for CD86 expression, which was compared against an Unstained Control for each cell line, during subsequent flow cytometric analysis. Interestingly, results showed that U251 expressed CD86 at low levels ($p = 0.0159$), whilst no CD86 expression was detected in U373 cells (Figure 5.3).

In line with the results seen with CD80 expression, both IFN-γ alone (152% - $p = 0.0458$) and the IFN-γ and TNF-α dual treatment increased CD86 expression in U251 cells (188% - $p = 0.0416$) and U373 cells (154% - $p = 0.0349$ and 217% - $p = 0.0012$, respectively) (Figure 5.4B). Furthermore, administration of IFN-γ, IL-21 and TNF-α together led to the development of a shoulder-like peak in the CD86 expression profile of both cell lines (Figure 5.4A). The resulting changes to the CD86 MFI indicated that the triple cytokine treatment had the largest impact on increasing CD86 expression in U251 cells (195% - $p = 0.0023$) and inducing CD86 expression in U373 cells (215% - $p = 0.0010$) (Figure 5.4B). Altogether, these results demonstrated that IFN-γ, IL-21 and TNF-α treatment could induce CD86 expression in glioblastoma cells.

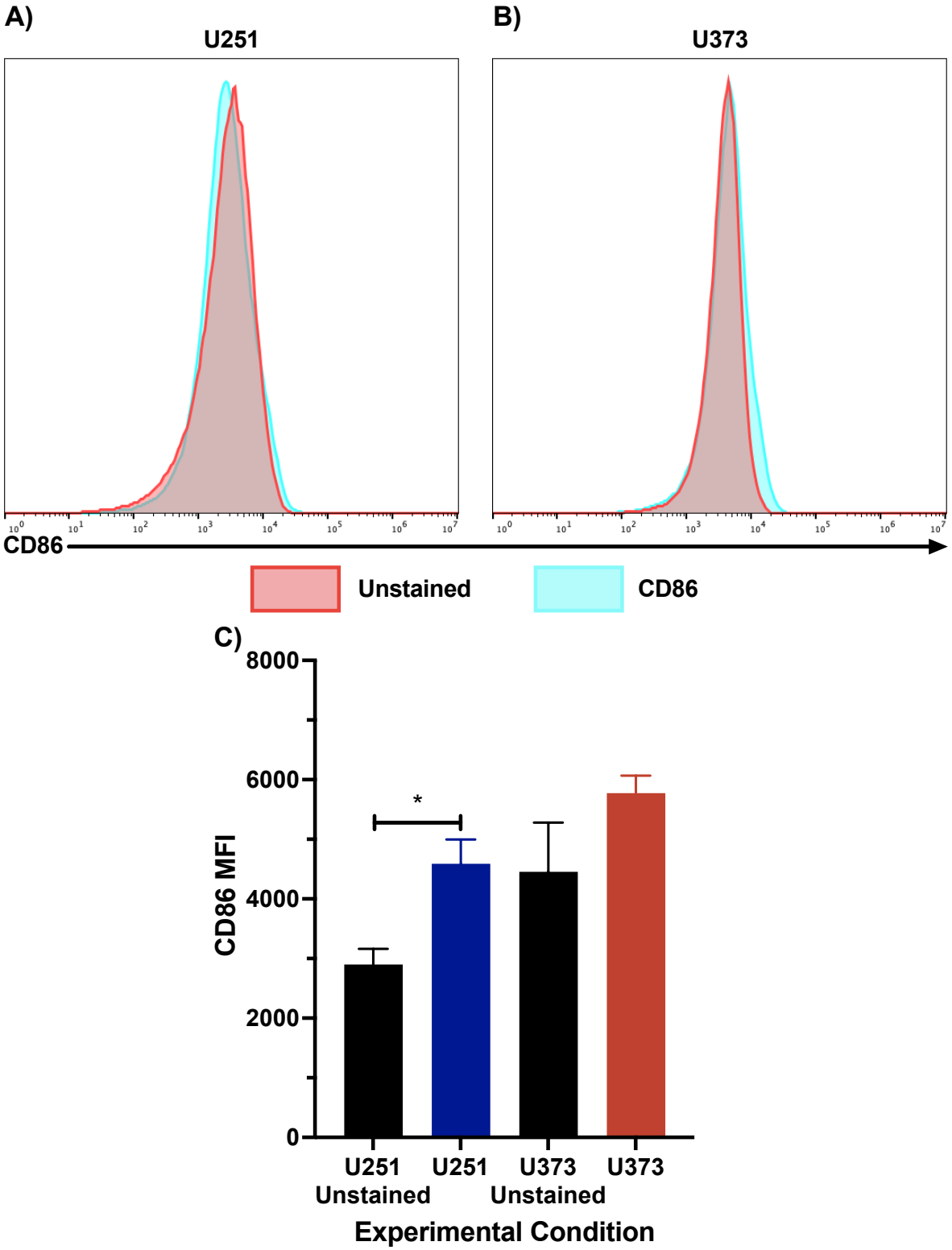


Figure 5.3. U251 cells exclusively expressed CD86

U251 cells **A)** and U373 cells **B)** were either stained for CD86 or left unstained for analysis via flow cytometry. Flow plots are representative of > 3 independent experiments. Bars represent CD86 MFI **C)** with means and SEM shown for > 3 independent experiments. P Values: * = <0.05; ** = <0.01; *** = <0.001; **** = <0.0001.

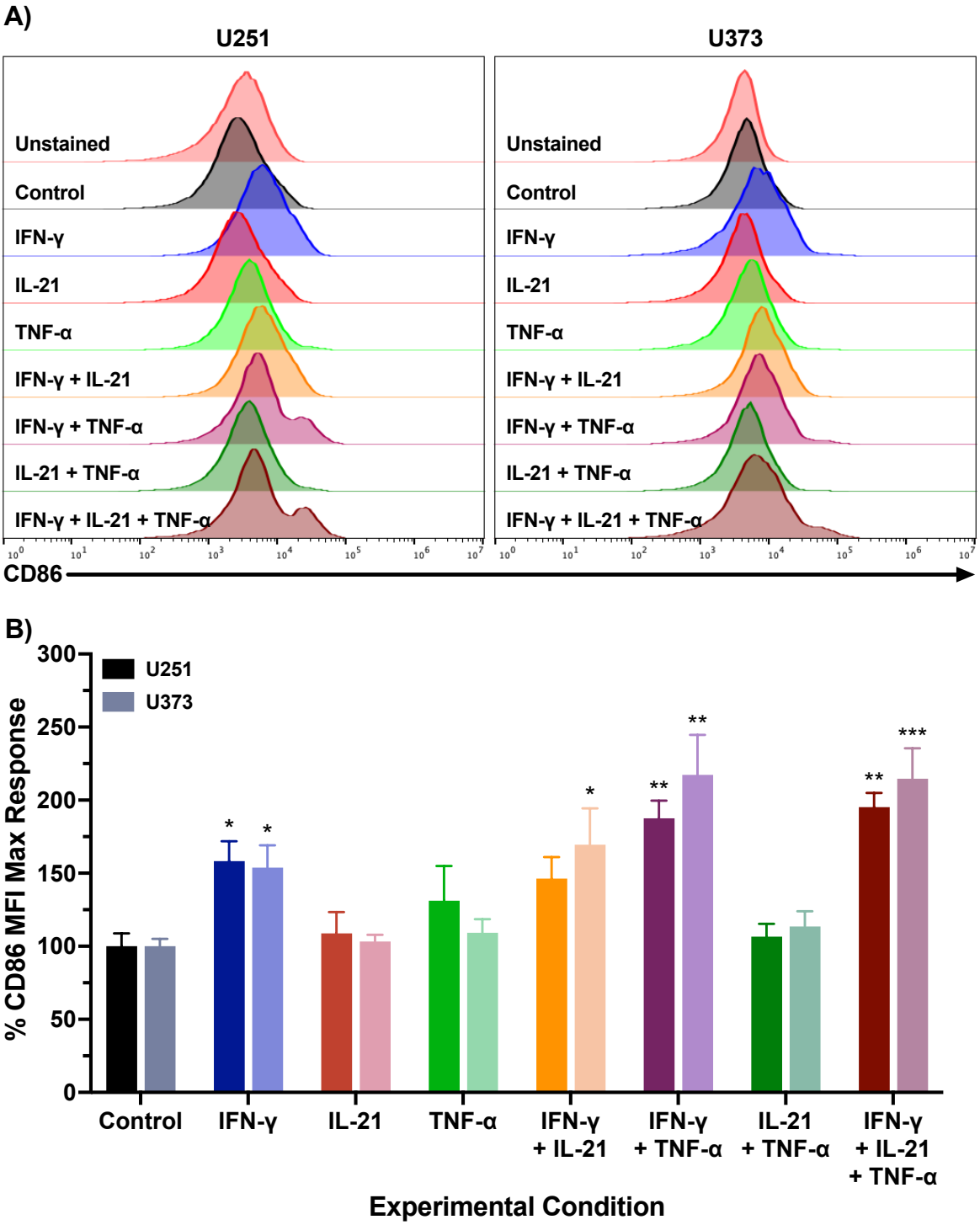


Figure 5.4. IFN-γ, IL-21 and TNF-α upregulated CD86 expression in U251 and U373 cells

$2.5x10^4$ U251 cells and $2.5x10^4$ U373 cells were seeded with an RPMI Control or 100ng/ml IFN-γ, 100ng/ml IL-21, 100ng/ml TNF-α, both individually and combined, for 72 hours before they were stained for CD86 for analysis via flow cytometry. Flow plots **A)** are representative of > 3 independent experiments. Bars represent CD86 MFI **B)** as means compared to the Control, with SEM shown for > 3 independent experiments.
P Values: * = <0.05; ** = <0.01; *** = <0.001; **** = <0.0001.

5.2.3. IFN-γ, IL-21 and TNF-α upregulated ICOS-L expression in glioblastoma cells

Downstream CD28 signalling has also been shown to enhance T cell activation though the upregulation of the co-stimulatory receptor ICOS, which binds to ICOS-L to promote T cell proliferation and function (Dong et al., 2001). Interestingly, Schreiner et al. (2003) measured mRNA expression and used flow cytometric analyses to determine that U251 cells highly expressed ICOS-L, whilst U373 cells showed moderate levels of ICOS-L expression. In order to validate this, U251 and U373 cells were stained for ICOS-L expression, which was compared against an Unstained Control for each cell line, during subsequent flow cytometric analysis. Consistent with the findings produced by Schreiner et al. (2003), results showed that both U251 cells ($p = 0.0079$) and U373 cells expressed ICOS-L ($p = 0.0286$) (Figure 5.5).

Curiously, the combination of IFN-γ and TNF-α induced a biphasic ICOS-L expression profile in both cell lines, suggesting that that a fraction of these cells was expressing ICOS-L at a very high level (Figure 5.6A). As a result of this, there was a clear rise in ICOS-L expression in U251 cells (145% - $p = 0.0326$) and U373 cells (201% - $p = 0.0007$) compared to the Control, when treated with IFN-γ and TNF-α. Similar results were seen in both cell lines with the triple cytokine treatment as well, though the impact on U373 cells was more profound (224% - $p = 0.0002$) than U251 cells (157% - $p = 0.0094$) (Figure 5.6B). Overall, these results indicated that glioblastoma cells expressed high levels of ICOS-L, but this could be enhanced by exposure to IFN-γ, IL-21 and TNF-α.

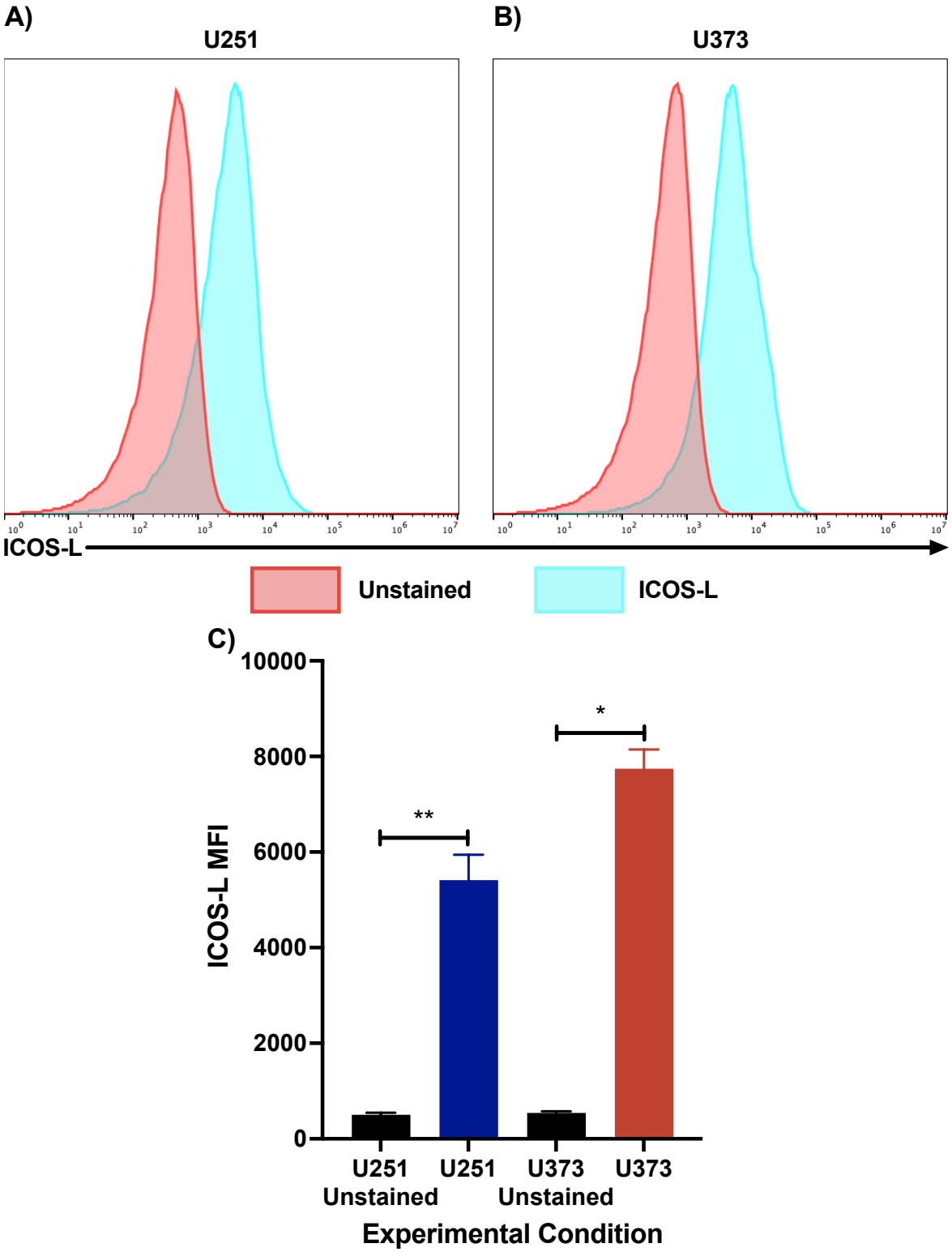


Figure 5.5. U251 and U373 cells expressed ICOS-L

U251 cells **A)** and U373 cells **B)** were either stained for ICOS-L or left unstained for analysis via flow cytometry. Flow plots are representative of > 3 independent experiments. Bars represent ICOS-L MFI **C)** with means and SEM shown for > 3 independent experiments. P Values: * = <0.05; ** = <0.01; *** = <0.001; **** <0.0001.

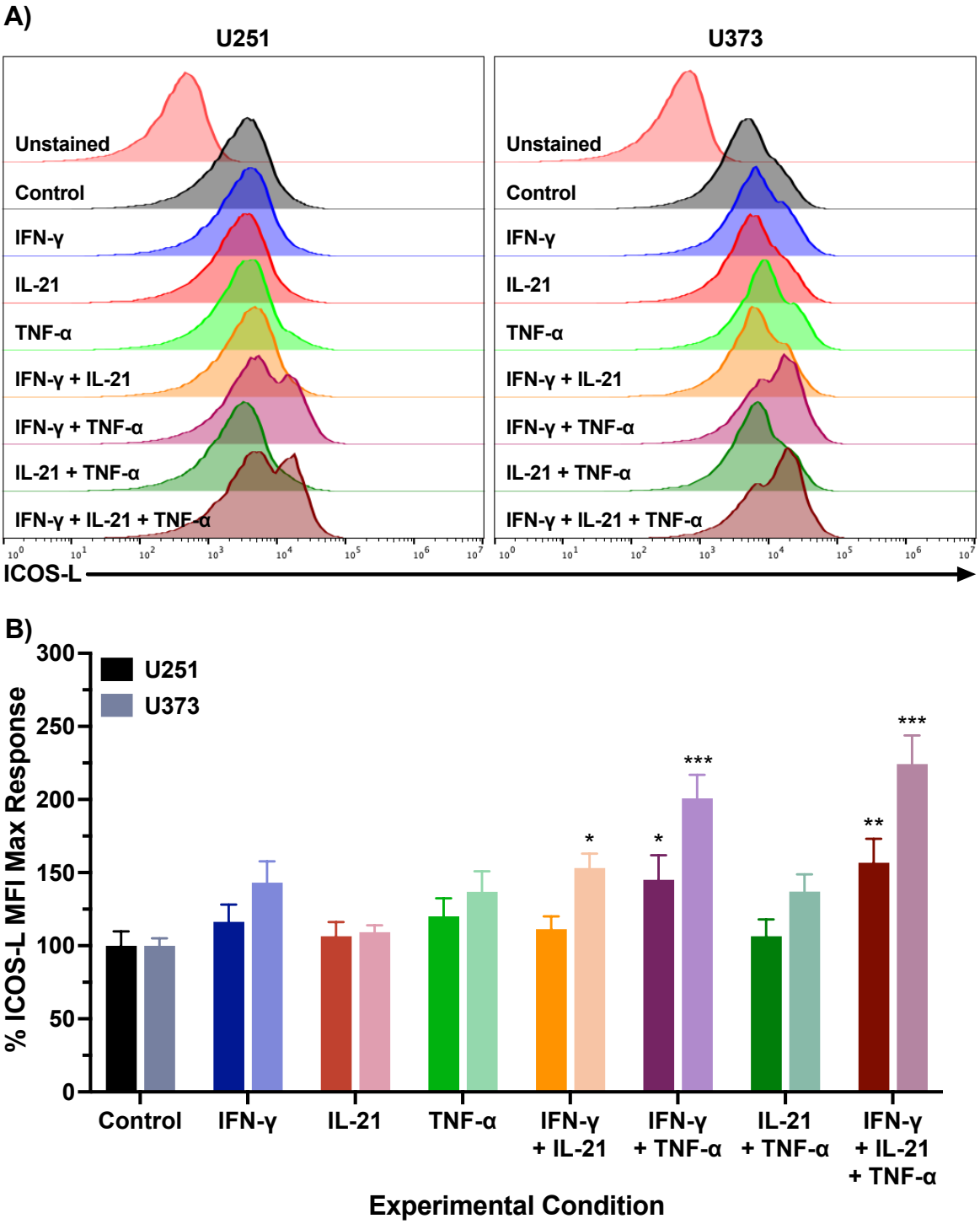


Figure 5.6. IFN-γ, IL-21 and TNF-α upregulated ICOS-L expression in U251 cells

$2.5x10^4$ U251 cells and $2.5x10^4$ U373 cells were seeded with an RPMI Control or 100ng/ml IFN-γ, 100ng/ml IL-21, 100ng/ml TNF-α, both individually and combined, for 72 hours before they were stained for ICOS-L for analysis via flow cytometry. Flow plots **A)** are representative of > 3 independent experiments. Bars represent ICOS-L MFI **B)** as means compared to the Control, with SEM shown for > 3 independent experiments. P Values: * = <0.05; ** = <0.01; *** = <0.001; **** = <0.0001.

5.2.4. IFN-γ, IL-21 and TNF-α variably affected 41BB-L expression in glioblastoma cells

Another downstream target of CD28-mediated signalling is 41BB, which binds to the co-stimulatory ligand 41BB-L, in order to promote T cell proliferation, survival and cytokine production (Cannons et al., 2001). Although, 41BB-L has been identified at low levels on some glioblastoma cell lines, the level of expression in U251 or U373 cells had not previously been established (Mu et al., 2006). In order to explore this, U251 and U373 cells were stained for 41BB-L expression, which was compared against an Unstained Control for each cell line, during subsequent flow cytometric analysis. Interestingly, results showed that both U251 ($p = 0.0079$) and U373 cells ($p = 0.0159$) highly expressed 41BB-L (Figure 5.7).

Unfortunately, IFN-γ appeared to mediated downregulation of 41BB-L in U251 cells, due to a clear visible shift in the 41BB-L expression profile – although this effect was not statistically significant (81% - $p = 0.1231$). Nonetheless, the inhibitory effects of IFN-γ on 41BB-L expression in U251 cells were clearly exacerbated with the addition of either IL-21 (71% - $p = 0.0186$) or TNF-α (61% - $p = 0.0020$), which was sustained when the cytokines were all administered together (63% - $p = 0.0032$) (Figure 5.8B). However, given the high baseline expression of 41BB-L in U251 cells, it is important to note that the level of reduction with IFN-γ, IL-21 and TNF-α treatment was relatively minor, as 41BB-L expression was still maintained at a high level, when compared against the Unstained Control (Figure 5.8A). Interestingly, the same effects were not seen in U373 cells, as none of the cytokine treatments had any significant impact on 41BB-L expression. Altogether, these results indicated that glioblastoma cells expressed high levels of 41BB-L, but this was largely not compromised by treatment with IFN-γ, IL-21 and TNF-α.

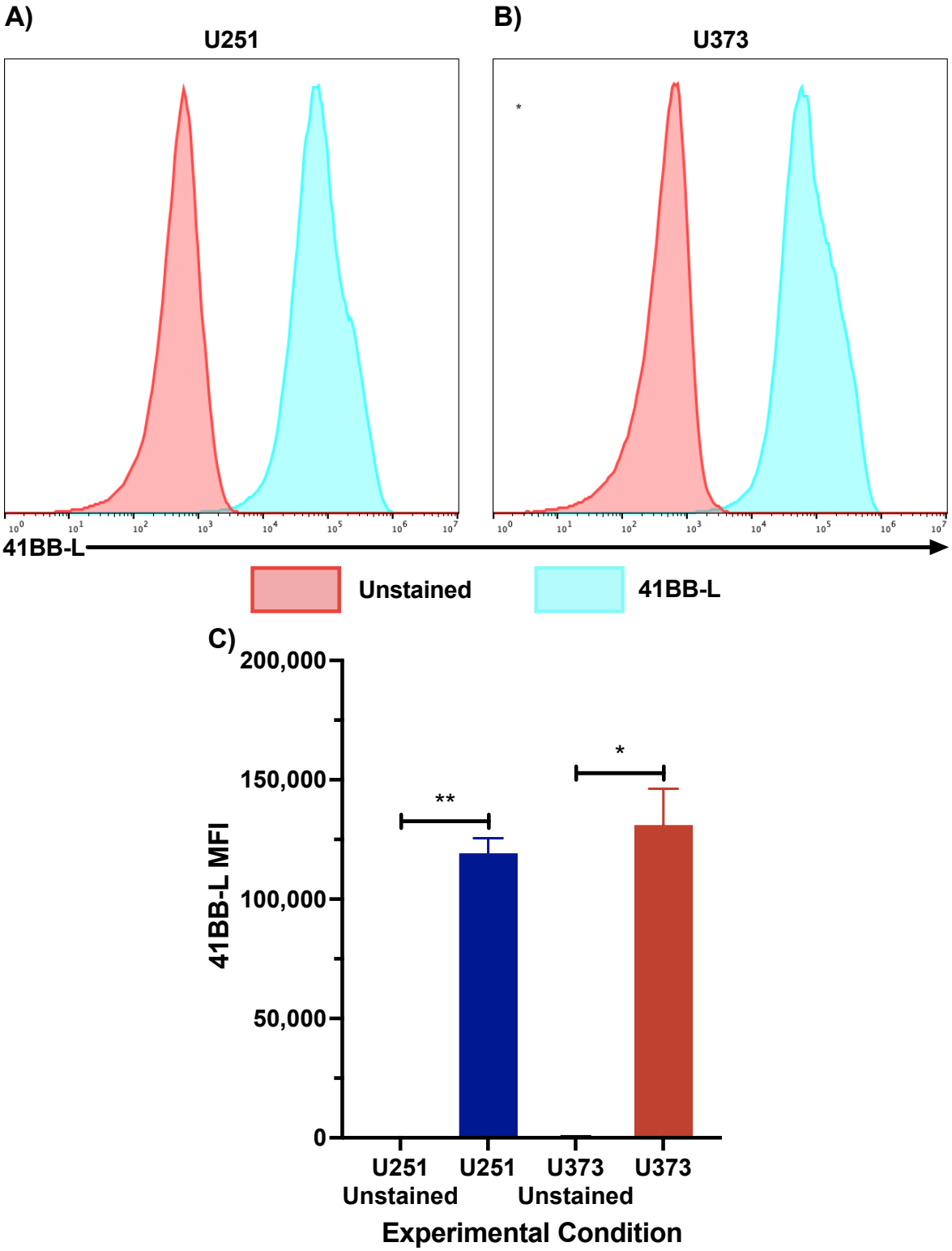


Figure 5.7. U251 and U373 cells highly expressed 41BB-L

U251 cells **A)** and U373 cells **B)** were either stained for 41BB-L or left unstained for analysis via flow cytometry. Flow plots are representative of > 3 independent experiments. Bars represent 41BB-L MFI **C)** with means and SEM shown for > 3 independent experiments. P Values: * = <0.05; ** = <0.01; *** = <0.001; **** <0.0001.

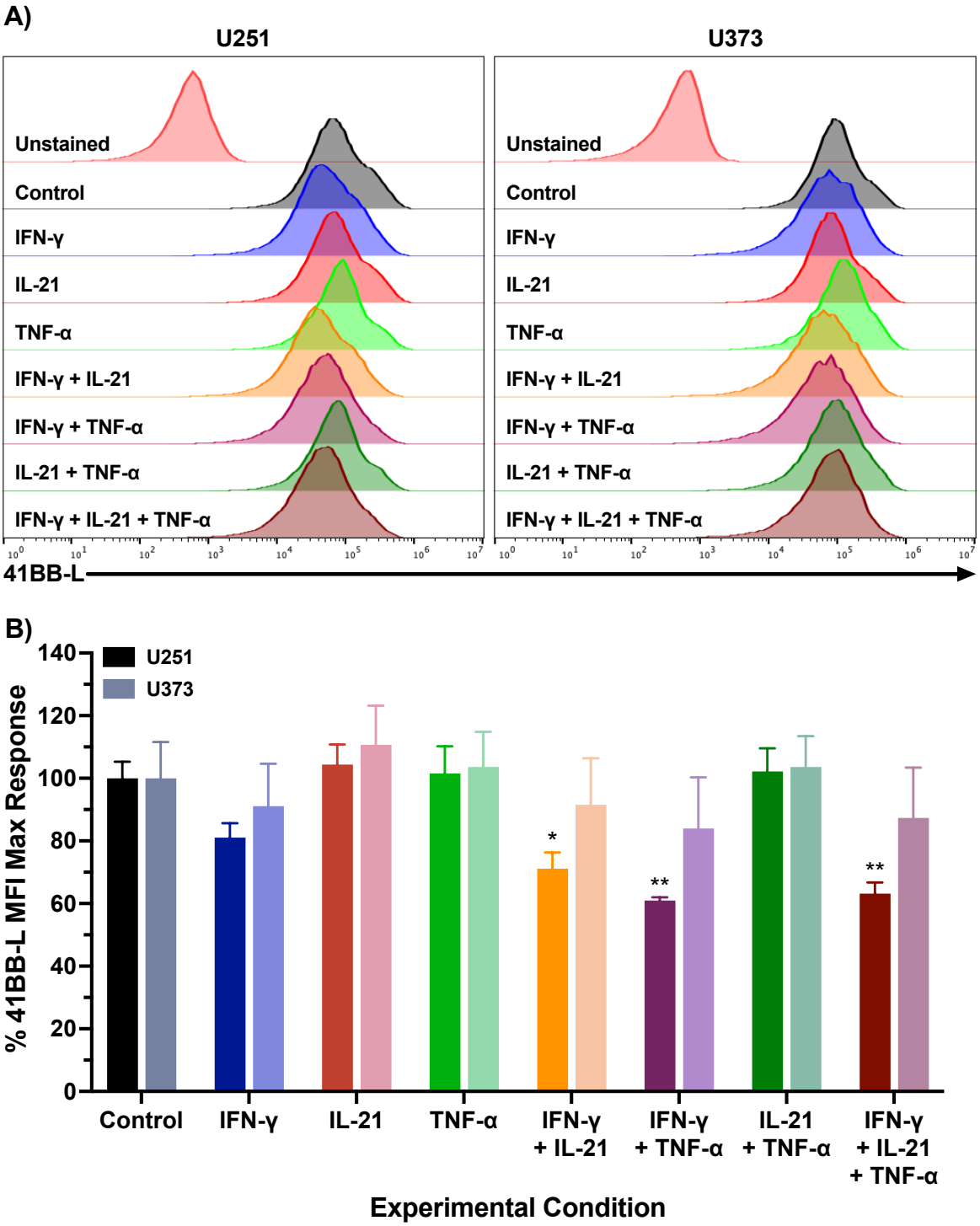


Figure 5.8. IFN-γ, IL-21 and TNF-α downregulated 41BB-L expression in U251 cells

2.5x10^4 U251 cells and 2.5x10^4 U373 cells were seeded with an RPMI Control or 100ng/ml IFN-γ, 100ng/ml IL-21, 100ng/ml TNF-α, both individually and combined, for 72 hours before they were stained for 41BB-L for analysis via flow cytometry. Flow plots **A)** are representative of > 3 independent experiments. Bars represent 41BB-L MFI **B)** as means compared to the Control, with SEM shown for > 3 independent experiments. P Values: * = <0.05; ** = <0.01; *** = <0.001; **** = <0.0001.

5.2.5. IFN-γ, IL-21 and TNF-α did not affect Galectin-9 expression in glioblastoma cells

Alongside co-stimulatory signalling, co-inhibitory signals are also required to ensure that the immune response is appropriately controlled – for example, the co-inhibitory ligand Galectin-9 induces T cell exhaustion and apoptosis when bound to TIM-3 (C. Zhu et al., 2005). Using large-scale genetic analyses, Yuan et al. (2020) determined that Galectin-9 was significantly upregulated in glioblastoma, compared to both lower-grade gliomas and healthy brain tissues, but this also correlated with poorer survival rates. Similarly, P. Liang et al. (2020) identified that Galectin-9 played a key role in promoting immunosuppression in glioblastoma patients, suggesting that Galectin-9 was a promising therapeutic target.

As a relatively new area of research, the expression levels of Galectin-9 in U251 and U373 cells had not previously been identified – therefore, both U251 and U373 cells were stained for Galectin-9 expression in the present study, which was compared against an Unstained Control for each cell line, during subsequent flow cytometric analysis. Results showed that both U251 ($p = 0.0079$) and U373 cells ($p = 0.0095$) highly expressed Galectin-9 (Figure 5.9A). Across each of the cytokine treatments, there was a slight trend to suggest that IFN-γ could have marginally reduced Galectin-9 expression in U251 cells, with the largest effect seen in the IFN-γ and TNF-α condition (80% - $p = 0.1941$), but none of the results were statistically significant (Figure 5.10). Collectively, these findings indicated that U251 and U373 cells highly expressed Galectin-9, but this was unaffected by IFN-γ, IL-21 and TNF-α treatment.

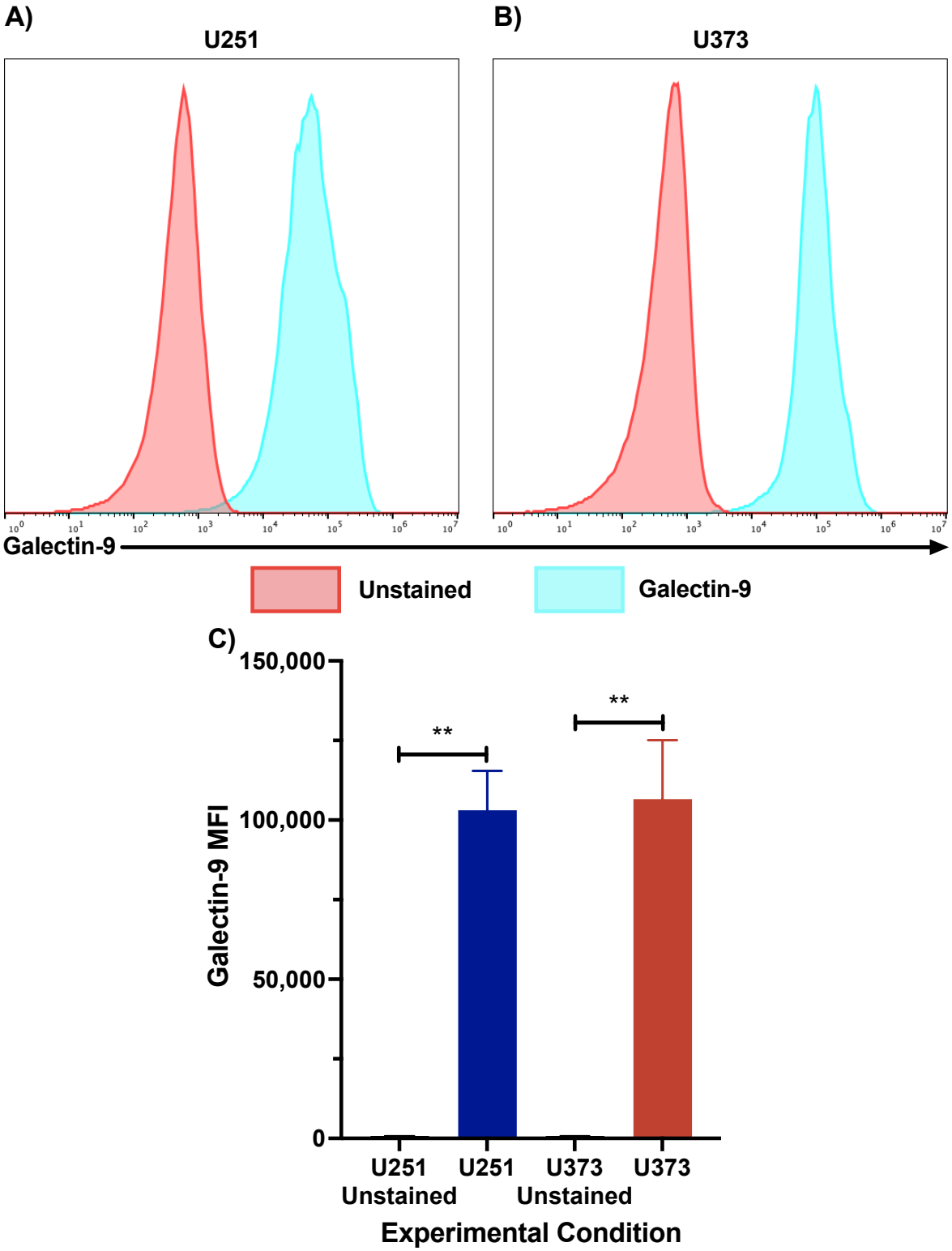


Figure 5.9. U251 and U373 cells highly expressed Galectin-9

U251 cells **A)** and U373 cells **B)** were either stained for Galectin-9 or left unstained for analysis via flow cytometry. Flow plots are representative of > 3 independent experiments. Bars represent Galectin-9 MFI **C)** with means and SEM shown for > 3 independent experiments. P Values: * = <0.05; ** = <0.01; *** = <0.001; **** <0.0001.

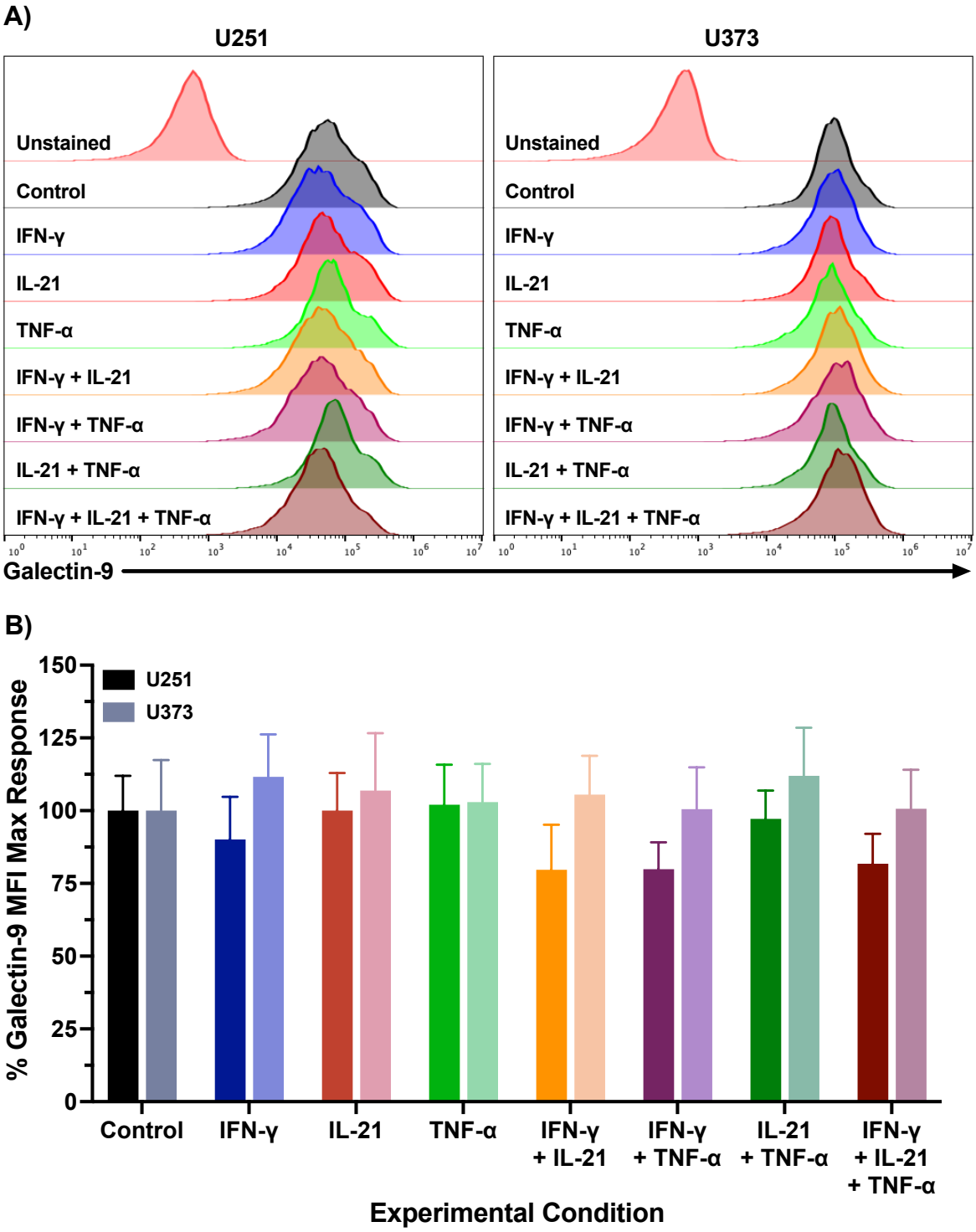


Figure 5.10. IFN-γ, IL-21 and TNF-α did not affect Galectin-9 expression in U251 and U373 cells

$2.5x10^4$ U251 cells and $2.5x10^4$ U373 cells were seeded with an RPMI Control or 100ng/ml IFN-γ, 100ng/ml IL-21, 100ng/ml TNF-α, both individually and combined, for 72 hours before they were stained for Galectin-9 for analysis via flow cytometry. Flow plots **A)** are representative of > 3 independent experiments. Bars represent Galectin-9 MFI **B)** as means compared to the Control, with SEM shown for > 3 independent experiments. P Values: * = <0.05; ** = <0.01; *** = <0.001; **** = <0.0001

5.2.6. IFN-γ, IL-21 and TNF-α increased PD-L1 expression in glioblastoma cells

One of the most significant co-inhibitory pathways in the immune response involves the ligand PD-L1, which binds to PD-1 on T cells, in order to inhibit their proliferation and induce apoptosis, thus it represents a key mechanism used by tumour cells to mediate immunosuppression (Iwai et al., 2002). Previous research has found that several glioblastoma cell lines express PD-L1, including U251 and U373 cells (Parsa et al., 2007). In order to validate this, U251 and U373 cells were stained for PD-L1 expression, which was compared against an Unstained Control for each cell line, during subsequent flow cytometric analysis. In line with previous research, results showed that both U251 ($p = 0.0043$) and U373 cells ($p = 0.0159$) expressed PD-L1 (Figure 5.11).

Upon examining the PD-L1 expression profile of U251 cells, it was quite clear that IFN-γ caused a visible shift in the level of PD-L1 expression (Figure 5.12A) – quantifying this in U251 cells revealed that IFN-γ alone significantly increased the PD-L1 MFI by almost three-fold (293% - $p = 0.0004$), relative to the Control. Interestingly, treating U251 cells with IFN-γ alongside either IL-21 (285% - $p = 0.0008$) or TNF-α (290% - $p = 0.0006$) as dual treatments did not compromise the increase of the PD-L1 MFI, which was further enhanced when all of the cytokines were administered together (331% - $p < 0.0001$) (Figure 5.12B).

Unfortunately, the effects of IFN-γ, IL-21 and TNF-α were even more potent in U373 cells, with the dual treatment of IFN-γ and TNF-α almost increasing the PD-L1 MFI by four-fold, when normalised to the Control (380% - $p = 0.0003$). Similar results were also seen when U373 cells were treated with the combination of IFN-γ, IL-21 and TNF-α (375% - $p = 0.0001$) (Figure 5.12B). Alas, these findings collectively demonstrated that IFN-γ, IL-21 and TNF-α significantly increased PD-L1 expression in glioblastoma cells.

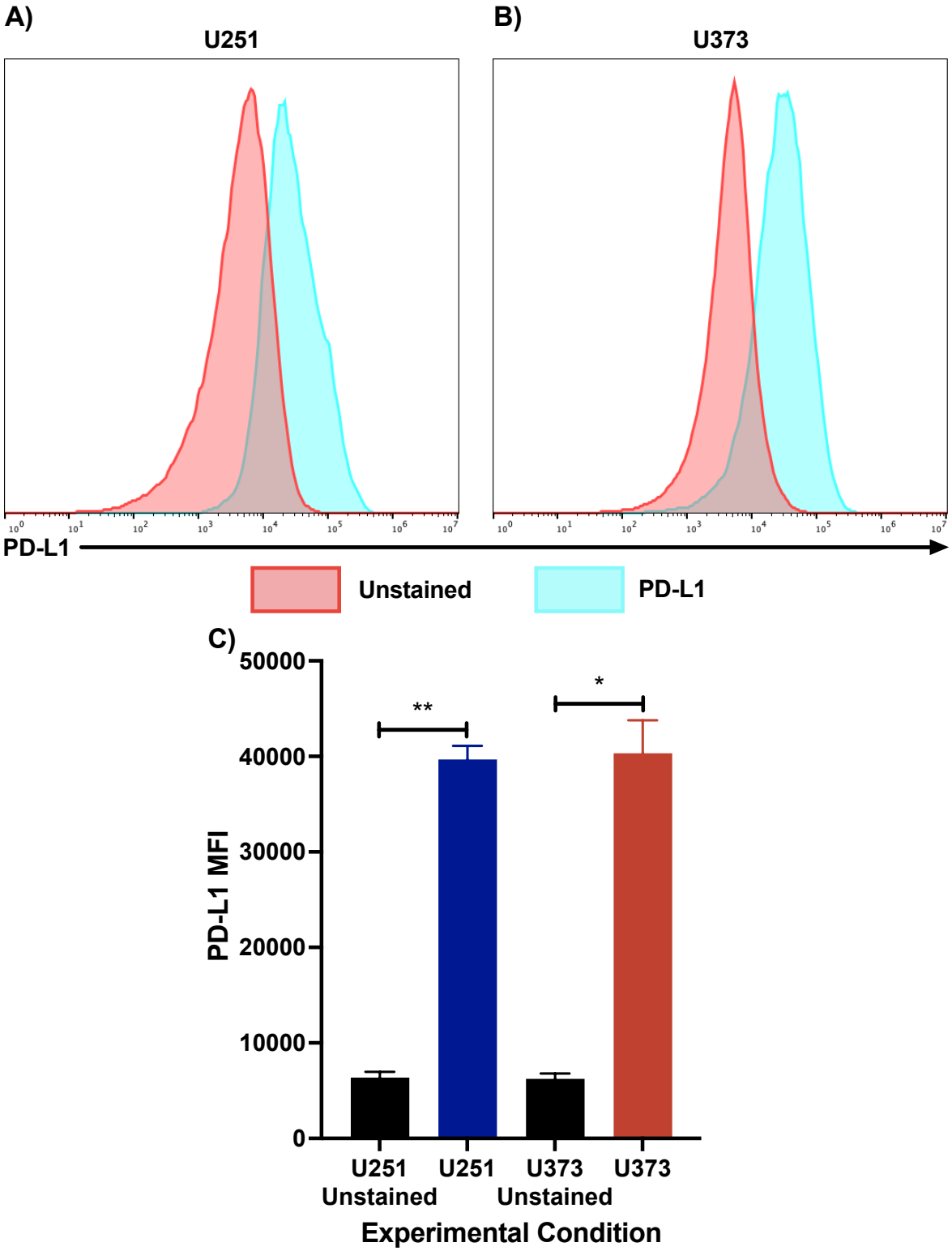


Figure 5.11. U251 and U373 cells highly expressed PD-L1

U251 cells **A)** and U373 cells **B)** were either stained for PD-L1 or left unstained for analysis via flow cytometry. Flow plots are representative of > 3 independent experiments. Bars represent PD-L1 MFI **C)** with means and SEM shown for > 3 independent experiments. P Values: * = <0.05; ** = <0.01; *** = <0.001; **** <0.0001.

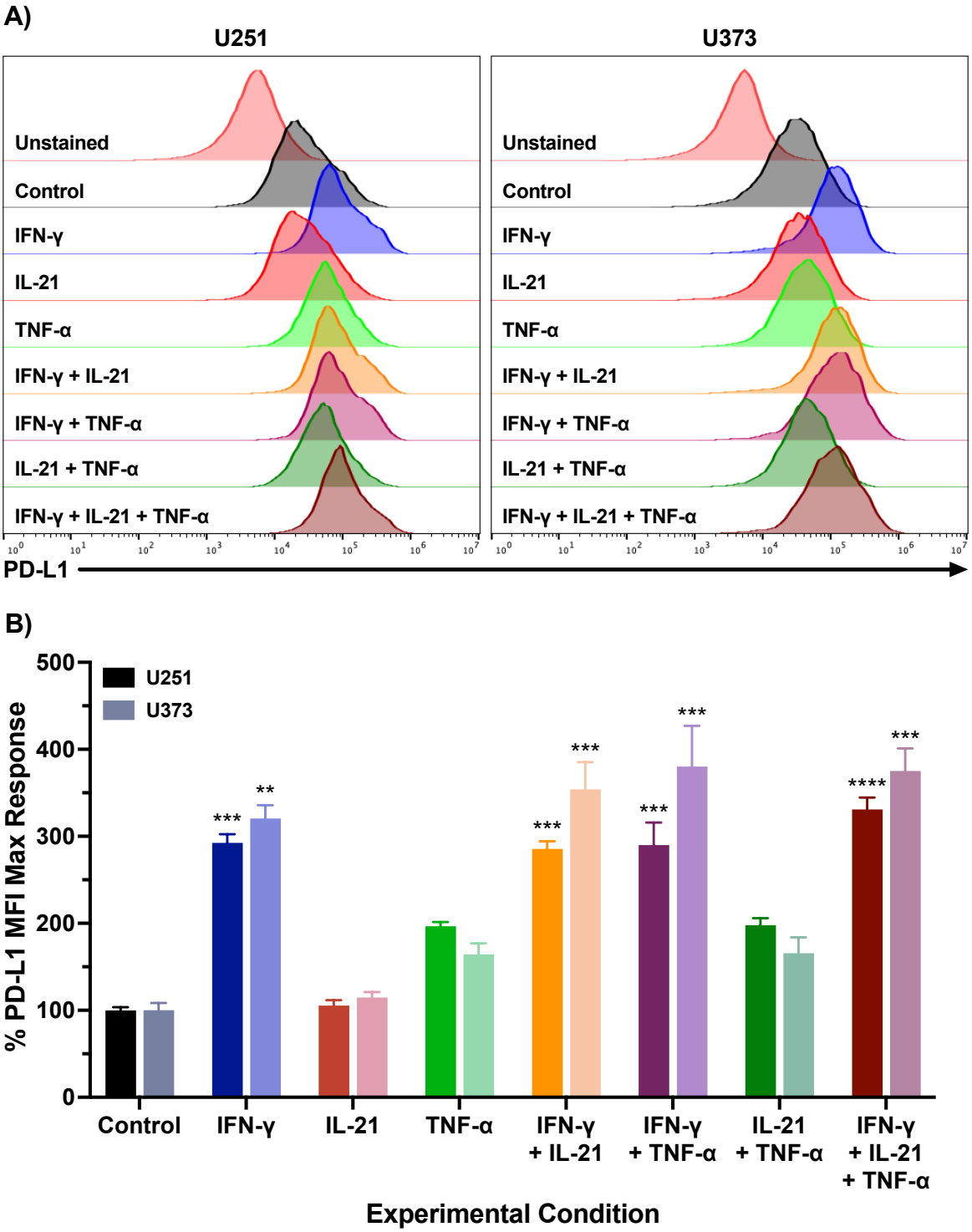


Figure 5.12. IFN-γ, IL-21 and TNF-α upregulated PD-L1 expression in U251 and U373 cells

$2.5x10^4$ U251 cells and $2.5x10^4$ U373 cells were seeded with an RPMI Control or 100ng/ml IFN-γ, 100ng/ml IL-21, 100ng/ml TNF-α, both individually and combined, for 72 hours before they were stained for PD-L1 for analysis via flow cytometry. Flow plots **A)** are representative of > 3 independent experiments. Bars represent PD-L1 MFI **B)** as means compared to the Control, with SEM shown for > 3 independent experiments. P Values: * = <0.05; ** = <0.01; *** = <0.001; **** = <0.0001

5.2.7. Summary Heat Maps

In order to summarise the impact of IFN-γ, IL-21 and TNF-α on the expression of these co-stimulatory and co-inhibitory ligands in U251 and U373 cells, the average MFI values for CD80, CD86, ICOS-L, 41BB-L, Galectin-9 and PD-L1 expression were used to generate Heat Maps for each cell line, as shown in Figure 5.13 below.

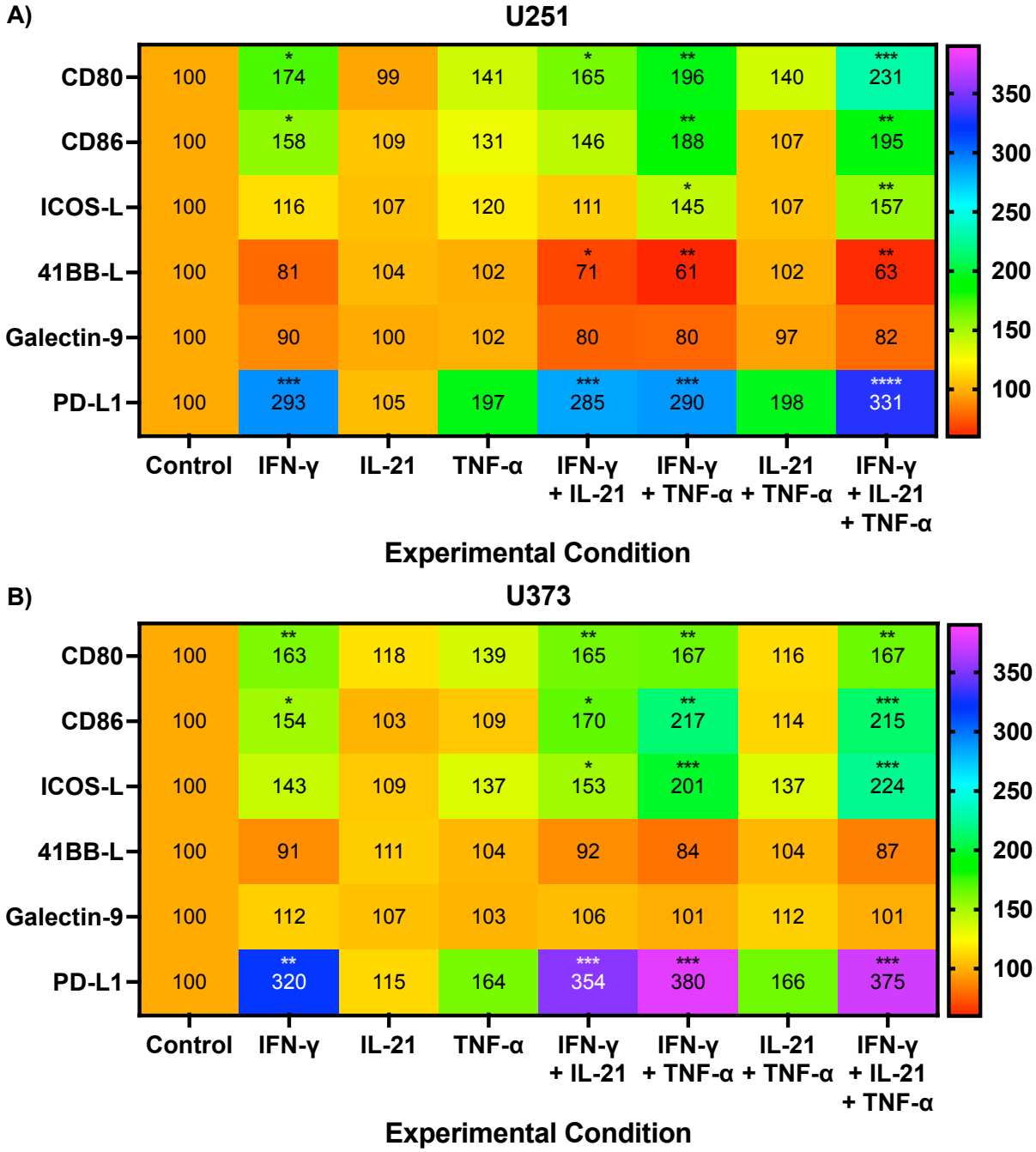


Figure 5.13. Heat Maps summarising the impact of IFN-γ, IL-21 and TNF-α on CD80, CD86, ICOS-L, 41BB-L, Galectin-9, and PD-L1 expression in U251 and U373 cells

$2.5x10^4$ U251 cells and $2.5x10^4$ U373 cells were seeded with an RPMI Control or 100ng/ml IFN-γ, 100ng/ml IL-21, 100ng/ml TNF-α, both individually and combined, for 72 hours before they were stained for CD80, CD86, ICOS-L, 41BB-L, Galectin-9 or PD-L1 for analysis via flow cytometry. Average MFI values for these ligands in both U251 **A)** and U373 **B)** cells were used to generate Heat Maps from > 3 independent experiments. The variation in colour of the boxes within both Heat Maps represent changes in expression for each cell line, relative to the untreated Controls. P Values: * = <0.05; ** = <0.01; *** = <0.001; **** = <0.0001.

5.3. Discussion

Multiple signals are required to initiate T cell activation, leading to the proliferation and differentiation of various effector T cell subtypes. Alongside TCR-MHC interactions, a critical stage in this process involves binding between specific receptors on the T cell surface and their relevant ligands on APCs, activating T cell co-stimulatory signalling pathways – in the absence of these signals, T cells either become anergic or apoptotic. Tumour cells utilise a range of immunosuppressive mechanisms to restrict anti-tumour immune responses, including downregulation of co-stimulatory ligands and upregulation of co-inhibitory ligands on the tumour cell surface to limit T cell activation. Altering the expression levels of both of these ligand subgroups on tumour cells represents a key mechanism for the restoration of immune cell function and subsequent induction of anti-tumour immunity.

As one of the first co-stimulatory ligands discovered in humans, CD80 is a particularly important therapeutic target, as it potently enhances T cell activation, proliferation, survival and cytokine secretion when bound to CD28 (Gimmi et al., 1991; Linsley et al., 1991). In particular, research has shown that the scale of CD80 downregulation on gastric adenocarcinoma cells, relative to the surrounding healthy tissues, correlated with increased tumour grading and poorer patient survival (X. Feng et al., 2019). In addition, the reduction in CD28-mediated co-stimulatory signalling, due to the loss of CD80 expression, has been shown to enhance Treg differentiation and stabilise the Treg phenotype, thereby facilitating the generation of an immunosuppressive tumour microenvironment (Mikami et al., 2020). However, numerous studies have shown that enhancing CD80 expression on tumour cells promotes anti-tumour immunity, leading to tumour rejection in various murine cancer models (Baskar et al., 1993; Y. Li et al., 1994; Tirapu et al., 2006; Townsend & Allison, 1993).

Although research is limited, it has been suggested that glioblastoma cells did not express CD80, which largely aligns with the findings produced in the present study, as only low level expression was identified in U373 cells (Schreiner et al., 2003; Tietze et al., 2021). However, results showed that the combination of IFN-γ, IL-21 and TNF-α significantly induced CD80 expression in both U251 and U373 cell lines. Whilst this effect was largely mediated by IFN-γ with minor support from TNF-α, the addition of IL-21 further enhanced CD80 expression in both cell lines, suggesting that the triple cytokine treatment had the potential to restore anti-tumour immune function in glioblastoma patients, though this requires further investigation (Figure 5.2). Previous research has shown that IFN-γ upregulated CD80 expression in monocytes via IRF1, whilst an NF-κB binding site has been found in the enhancer region of the CD80 gene in B cells, providing a mechanisms in which TNF-α could activate NF-κB and subsequently upregulate CD80 expression (Bauvois et al., 2009; J. Zhao et al., 1996). Together, these represent two mechanisms of CD80 regulatory control that should be considered in future studies exploring IFN-γ, IL-21 and TNF-α treatment in glioblastoma.

Alongside CD80, CD86 also binds to CD28 to activate T cell co-stimulatory signalling pathways. In contrast to previous research, which found that glioblastoma cells did not express CD86, the present study identified low level CD86 expression in U251 cells, which was subsequently enhanced

by IFN-γ, IL-21 and TNF-α treatment (Wintterle et al., 2003) (Figures 5.3 and 5.4). Despite the fact that CD86 was not initially detected in U373 cells, this was significantly induced by the combination of IFN-γ, IL-21 and TNF-α (Figures 5.3 and 5.4). Interestingly, the upregulation of CD86 in both glioblastoma cell lines appeared to be largely mediated by IFN-γ – these findings have also been corroborated in various colorectal, gastric, hepatocellular, and oesophageal carcinoma cell lines, as well as metastatic renal cell carcinoma patient samples (Flörcken et al., 2017; J. Li et al., 1996; Tatsumi et al., 1997). Although the exact mechanisms have not been established, research has shown that upregulation of CD86 by IFN-γ in monocytes was facilitated by the activity of pSTAT1 at two binding sites in the promoter region of the CD86 gene (J. Li et al., 2000). Furthermore, upregulation of CD86 promoted tumour rejection and enhanced survival in multiple murine cancer models, due to the induction of a potent anti-tumour immune response mediated by CD8+ T cells (Wintterle et al., 2003). These findings suggested that IFN-γ, IL-21 and TNF-α treatment could have significant potential in promoting anti-tumour immunity in glioblastoma patients through upregulation of CD86, but this needs to be explored in future research.

Unlike CD80 and CD86, ICOS is rapidly upregulated as a downstream target of TCR and/or CD28 co-stimulatory signalling. The subsequent interaction between ICOS and ICOS-L critically enhances T cell activation, proliferation and differentiation, including the production of characteristic cytokines by CD4+ Tfh cells (Hutloff et al., 1999). As human Tfh cells constitutively express ICOS, it is possible that Tfh cells could interact with ICOS-L expressed on the glioblastoma cell surface, leading to the induction of localised IL-21 production to evoke an anti-tumour immune response (Crotty, 2014). The relevance of ICOS-L as a therapeutic target is also supported by murine models of other cancers, which have shown that increased ICOS-L expression enhanced anti-tumour immune responses mediated by T cells, thereby promoting tumour rejection (X. Liu et al., 2001; Wallin et al., 2001).

Unfortunately, only one study has explored the expression of ICOS-L on glioblastoma cells, which found that U251 cells expressed higher levels of ICOS-L than U373 cells (Schreiner et al., 2003). These findings were not validated in the present study, as results showed that U373 cells had marginally higher levels of ICOS-L expression than U251 cells, but there was no significant difference between the two cell lines (Figure 5.5). Furthermore, Schreiner et al. (2003) showed that TNF-α upregulated ICOS-L expression on U251 and U373 cells, whilst IFN-γ had no effect. Although neither IFN-γ or TNF-α had an independent impact on ICOS-L expression in the present study, they did synergise as a dual treatment to upregulate ICOS-L expression in both cell lines (Figure 5.6). Previous research has identified that TNF-α upregulates ICOS-L expression on various immune cells through NF-κB signalling, whilst modulation of ICOS-L by IFN-γ has not previously been identified, thus the mechanisms of regulation are not currently known (R. I. Nurieva, 2005; Swallow et al., 1999). Despite the fact that IL-21 alone showed minimal benefits in terms of ICOS-L expression, there was a slight trend to suggest that IL-21 enhanced the proportion of ICOS-L+ cells in both cell lines when it was

combined with IFN-γ and TNF-α, highlighting the importance of its inclusion in this multi-cytokine therapy for the treatment of glioblastoma.

Beside ICOS, 41BB is also upregulated by T cells following the initial stages of activation, thus 41BB/41BB-L signalling helps to sustain T cell function by promoting T cell survival, expansion and cytokine production (Croft, 2003; Shuford et al., 1997; Watts, 2005). The ability to enhance T cell function, whilst increasing localised secretion of IFN-γ and TNF-α, highlights the significant potential 41BB-L has as a cancer therapeutic target. In line with this, Innamarato et al. (2020) showed that increasing 41BB-41BB-L interactions enhanced T cell co-stimulatory signalling and promoted tumour rejection, using various murine cancer models and *in vitro* T cell co-culture experiments. Although 41BB-L expression has been detected in glioblastoma patient samples, the level of expression was significantly lower compared to other glioma subtypes (Blank et al., 2015). However, they also discovered that IDH1-mutant glioblastoma had increased 41BB-L expression, compared to IDH1-wildtype – this is particularly interesting as it suggests that enhanced immunoreactivity could contribute to the improved survival of IDH1-mutant glioblastoma patients.

Recently, Lee-Chang et al. (2020) used a B cell-based vaccine to demonstrate that high levels of 41BB-L expression on B cells was associated with potent tumour rejection in a murine glioblastoma model, which also prevented the growth of new tumours upon subsequent transplantation, due to the development of immunological memory. Despite the fact that U251 and U373 cells highly expressed 41BB-L in the present study (Figure 5.7), IFN-γ significantly reduced the 41BB-L MFI in U251 cells, when administered in combination with IL-21 and/or TNF-α (Figure 5.8). However, due to the incredibly high baseline 41BB-L expression in U251 cells, it is possible that this may not compromise any interactions between glioblastoma cells and T cells, following IFN-γ, IL-21 and TNF-α treatment.

The upregulation of CD80, CD86 and ICOS-L in U251 and U373 cells by IFN-γ, IL-21 and TNF-α indicate that this novel cytokine-based therapy has significant promise in restoring anti-tumour immunity in glioblastoma patients. However, it is important to consider that tumour cells also frequently express co-inhibitory ligands to induce immunosuppression in the tumour microenvironment, thus the impact of IFN-γ, IL-21 and TNF-α on co-inhibitory ligand expression also needed to be investigated, including Galectin-9 and PD-L1.

The regulatory functions of Galectin-9 in immune responses are well established: Galectin-9 induces T cell apoptosis via TIM-3, whilst enhancing the differentiation and function of Tregs (Kashio et al., 2003; Lv et al., 2013; C. Zhu et al., 2005). On the other hand, the expression of Galectin-9 by cancer cells and its role in tumour development are still under investigation. In particular, Galectin-9 expression has been identified in hepatocellular carcinoma cells, in which loss of Galectin-9 expression resulted in reduced tumour migration, as well as the induction of apoptosis through upregulation of caspase-3 and Bax, alongside downregulation of Bcl-2 (S. Chen et al., 2018). In addition, Yasinska et al. (2019) completed co-culture experiments to show that breast cancer cells used Galectin-9 expression to induce apoptosis in cytotoxic CD8+ T cells, thereby providing a mechanism of tumour-mediated

immunosuppression. In line with this, Z. Liu et al. (2016) identified co-localisation of high Galectin-9 expression in glioblastoma tissues with TIM-3+ tumour-infiltrating T cells, suggesting that the Galectin-9/TIM-3 pathway was a critical mechanism used in tumour-mediated immunosuppression. The strong expression of Galectin-9 by glioblastoma cells is corroborated by the present study, as results showed that both U251 and U373 cells highly expressed Galectin-9 (Figure 5.9). However, IFN-γ, IL-21 and TNF-α had no individual or combined effects on Galectin-9 expression in U251 or U373 cells, relative to the Control (Figure 5.10).

Another crucial co-inhibitory ligand is PD-L1, which has been identified in various glioblastoma cell lines, whilst co-culture experiments have revealed that PD-L1 was involved in the potent inhibition of CD4+ and CD8+ T cell activation and cytokine production, demonstrating a key mechanism in glioblastoma-mediated immune evasion (Wintterle et al., 2003). In addition, high PD-L1 expression has been linked with poor overall survival in patients with either newly diagnosed or recurrent glioblastoma (Berghoff et al., 2015; S. Xue et al., 2017). Interestingly, numerous studies have shown that IFN-γ exposure induces PD-L1 expression in various cancer cell lines via IRF1 and STAT1, regardless of their baseline level of expression (S. Chen et al., 2019; S.-J. Lee et al., 2006; Mimura et al., 2018; T. Zhao et al., 2020).

In the context of glioblastoma, IFN-γ has been shown to upregulate PD-L1 expression in multiple cell lines, including U251 cells (Lou et al., 2017; J. Qian et al., 2018). These findings align with the results produced in the present study, as IFN-γ alone significantly upregulated expression of both pSTAT1 and PD-L1 in U251 and U373 cells (Figures 3.11, 3.12 and 5.12). However, there was a slight trend to suggest that TNF-α could increase PD-L1 expression, both independently and in combination with IFN-γ (Figure 5.12). Although there is emerging evidence that activation of NF-κB by TNF-α can mediate upregulation of PD-L1 in various cancer cell lines, Li et al. (2018) found that the synergistic effects of IFN-γ and TNF-α on hepatocellular carcinoma cells was caused by TNF-α indirectly increasing STAT1 signalling through upregulation of IFN-γ receptors (Gowrishankar et al., 2015; Ritprajak & Azuma, 2015; X. Wang et al., 2017). Despite the increase in PD-L1 expression caused by IFN-γ, IL-21 and TNF-α treatment, it is important to consider that the baseline level of PD-L1 expression was already high, therefore the relative upregulation of PD-L1 expression may actually be quite minor, compared to the Control. In line with this, it is possible that the upregulation of PD-L1 may not compromise the changes in the tumour microenvironment mediated by the significant increase in CD80, CD86, and ICOS-L expression.

Collectively, the results in the present chapter demonstrated that IFN-γ, IL-21 and TNF-α exposure enhanced expression of CD80, CD86 and ICOS-L in U251 and U373 cells, but the triple cytokine treatment had no impact on Galectin-9 expression, whilst marginally upregulating PD-L1. As a result of this, it is plausible that IFN-γ, IL-21 and TNF-α could promote an anti-tumour immune response in glioblastoma, but this requires further investigation.

6. Exploring the impact of IFN-γ, IL-21 and TNF-α on glioblastoma-mediated immunosuppression

6.1. Introduction

Once activated, naïve T cells are strongly influenced by their surrounding environment to inform their differentiation and function, so that they are able to produce an appropriate immune response. In line with this. previous studies have suggested that glioblastoma cells release EVs and/or secrete other factors to induce both localised and systemic immunosuppression in patients to restrict anti-tumour immunity (de Vrij et al., 2015; Hellwinkel et al., 2015; Morad et al., 2019; Simon et al., 2020). This can be assessed through monitoring how the glioblastoma secretome skews the differentiation of naïve T cells and mature T cells to direct them towards immunosuppressive T cell phenotypes. As a result of this, it is particularly crucial for novel immunotherapies to evaluate whether they have the ability to overcome the immunosuppressive nature of the glioblastoma secretome, in order to restore immune function and promote anti-tumour immunity in patients. In particular, various studies have associated Th2, Th17, and Treg cells with glioblastoma pathogenesis, whilst intratumoural Tfh and Th1 cells have been shown to correlate with increased patient survival, thus it important to evaluate whether IFN-γ, IL-21 and TNF-α can alter the expression profile of T cells in favour of Th1 and Tfh phenotypes with concomitant loss of Th2, Th17 and Treg features in the presence of the glioblastoma secretome (Waziri et al., 2008; Madkouri et al., 2017; P. Liang et al., 2020; El Andaloussi et al., 2006; El Andaloussi & Lesniak, 2006, 2007; Heimberger et al., 2008).

In order to explore this in the present study, two T cell populations (bulk CD4+ T cells and naïve CD4+CD45RA+ T cells) were isolated from human leukocyte cones and subsequently cultured with either fresh RPMI or conditioned RPMI (cell supernatant) taken from U251 and U373 cell cultures, in which the latter was used to model the glioblastoma secretome in the TME. Within these media conditions, both CD4+ T cell populations were also cultured with CD3/CD28 beads plus IFN-γ, IL-21 and/or TNF-α for 5 days, before cells were harvested and the impact of the glioblastoma secretome on various T cell subsets was assessed through the expression of a range of transcription factors and cytokines. In particular, T-bet, IFN-γ, and TNF-α expression were used to define Th1 cells, whilst Th2 cells were categorised by the expression of GATA3. On the other hand, Th17 cells were classified by RoRγT and IL-17 expression, whereas Tfh cells were characterised through Bcl-6 and IL-21 expression, with Tregs identified through the expression of Foxp3. For the sake of brevity, the results in the present chapter will focus solely on these expression of these subset markers in CD4+ T cells, but these were also explored in CD8+ T cells, as shown in the Appendix (Figures 9.8-9.17).

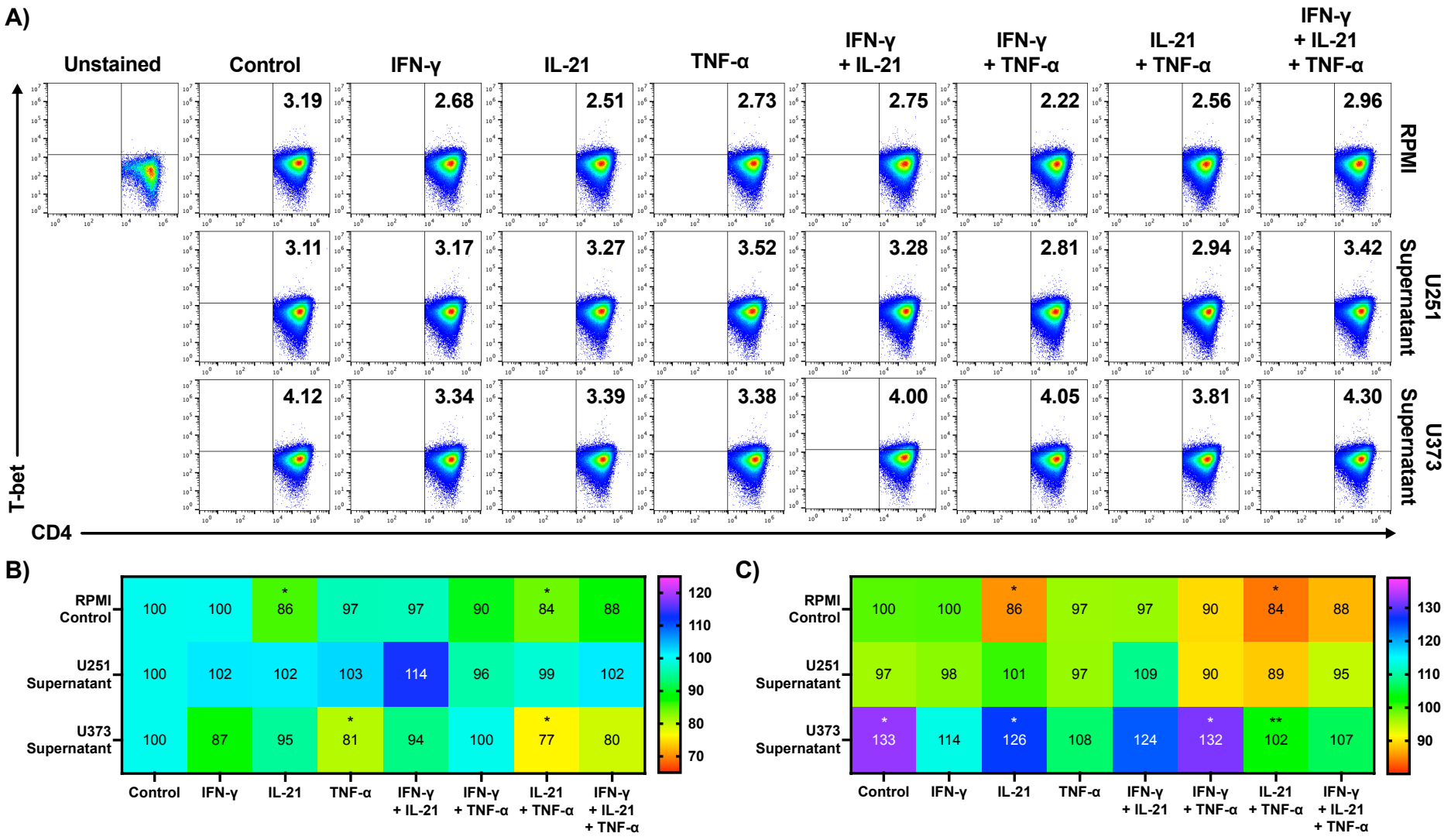
6.2. Results

6.2.1. IFN-γ, IL-21 and TNF-α variably affected Th1-associated T-bet expression in bulk CD4+ and naïve CD4+CD45RA+ T cells cultured with glioblastoma supernatant

Th1 cells represent a key component of the anti-tumour immune response, particularly through the production of IFN-γ and TNF-α (Gjorgjevski et al., 2019). As the expression of both of these cytokines is mediated by T-bet, it was important to consider whether IFN-γ, IL-21 and TNF-α could overcome the immunosuppressive nature of the glioblastoma secretome and restore Th1 function by promoting T-bet expression. In order to explore this, bulk CD4+ and naïve CD4+CD45RA+ T cells were isolated from healthy human leukocyte cones, before they were subsequently cultured with CD3/CD28 beads for 5 days. Within these culture conditions, both CD4+ T cell subsets were exposed to U251 or U373 cell supernatant, alongside treatment with IFN-γ, IL-21 and/or TNF-α, before they were harvested and stained for CD4 and T-bet for flow cytometric analysis. The data produced was initially normalised against the untreated Controls for each media condition, but the results from experiments cultured with U251 or U373 cell supernatant were also compared against the RPMI Control, in order to fully capture the influence of the glioblastoma secretome and/or cytokine treatment on T-bet expression in bulk CD4+ or naïve CD4+CD45RA+ T cells.

IL-21 (86% - $p = 0.0148$) was the only cytokine to independently influence T-bet expression in bulk CD4+ T cells, which was maintained when combined with TNF-α (84% - $p = 0.0166$). The downregulation of T-bet in bulk CD4+ T cells was also present when treated with IFN-γ, IL-21 and TNF-α, though the effect was not statistically significant (88% - $p = 0.0757$). Curiously, the combination of IFN-γ and TNF-α produced a minor reduction in T-bet expression in bulk CD4+ T cells (90% - $p = 0.1214$), despite the fact that neither cytokine had an independent impact (Figures 6.1B and D). Although none of the cytokine treatments significantly affected T-bet expression when bulk CD4+ T cells were cultured with U251 cell supernatant, there was a slight trend across the cytokine conditions, with the largest response seen with IFN-γ and IL-21 treatment (114% - $p = 0.2335$). However, normalising the data against the RPMI Control showed that neither the U251 cell supernatant alone (97% - $p = 0.3686$) or in combination with any of the cytokine treatments had a significant effect on T-bet expression in bulk CD4+ T cells (Figures 6.1C and E).

On the other hand, TNF-α alone (81% - $p = 0.0303$) or with IL-21 (73% - $p = 0.0109$) had a similar impact on reducing T-bet expression as the triple cytokine treatment (77% - $p = 0.0581$), when bulk CD4+ T cells were cultured with U373 cell supernatant (Figures 6.1B and D). When the data was compared against the RPMI Control, results showed that U373 cell supernatant (133% - $p = 0.0107$) independently upregulated T-bet expression in bulk CD4+ T cells, but this was neutralised with the addition of IFN-γ, IL-21 and TNF-α (107% - $p = 0.2346$) (Figures 6.1C and E). Overall, these results demonstrated that IFN-γ, IL-21 and TNF-α largely did not affect T-bet expression in bulk CD4+ T cells, when they were exposed to the glioblastoma secretome.


A)
Unstained
Control
IFN-γ
IL-21
TNF-α
IFN-γ + IL-21
IFN-γ + TNF-α
IL-21 + TNF-α
IFN-γ + IL-21 + TNF-α
RPMI
U251 Supernatant
U373 Supernatant
T-bet
CD4
3.19 2.68 2.51 2.73 2.75 2.22 2.56 2.96
3.11 3.17 3.27 3.52 3.28 2.81 2.94 3.42
4.12 3.34 3.39 3.38 4.00 4.05 3.81 4.30
B)
RPMI Control
U251 Supernatant
U373 Supernatant
100 100 86 97 97 90 84 88
100 102 102 103 114 96 99 102
100 87 95 81 94 100 77 80
Control
IFN-γ
IL-21
TNF-α
IFN-γ + IL-21
IFN-γ + TNF-α
IL-21 + TNF-α
IFN-γ + IL-21 + TNF-α
120
110
100
90
80
70
C)
RPMI Control
U251 Supernatant
U373 Supernatant
100 100 86 97 97 90 84 88
97 98 101 97 109 90 89 95
133 114 126 108 124 132 102 107
130
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110
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90

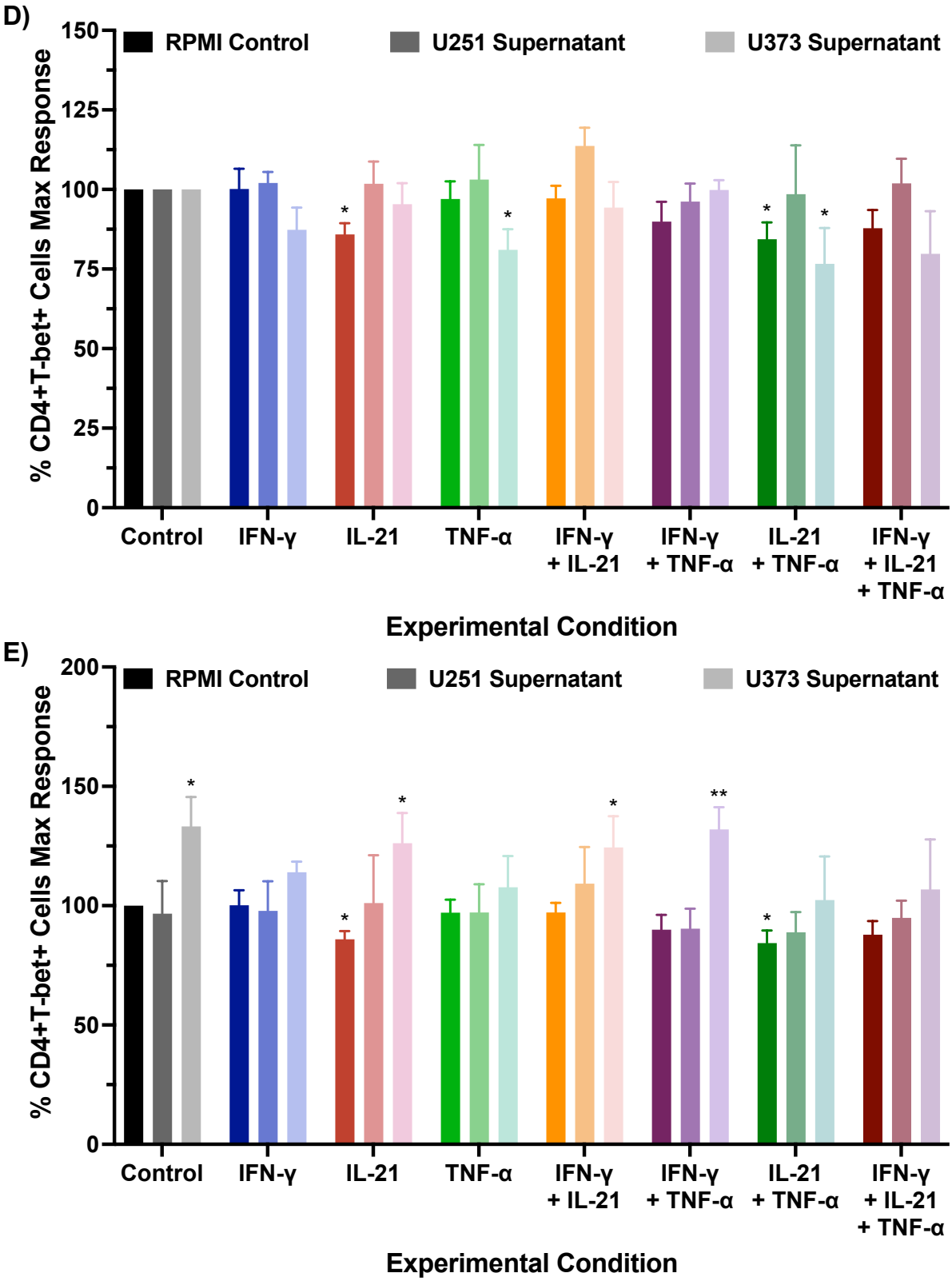
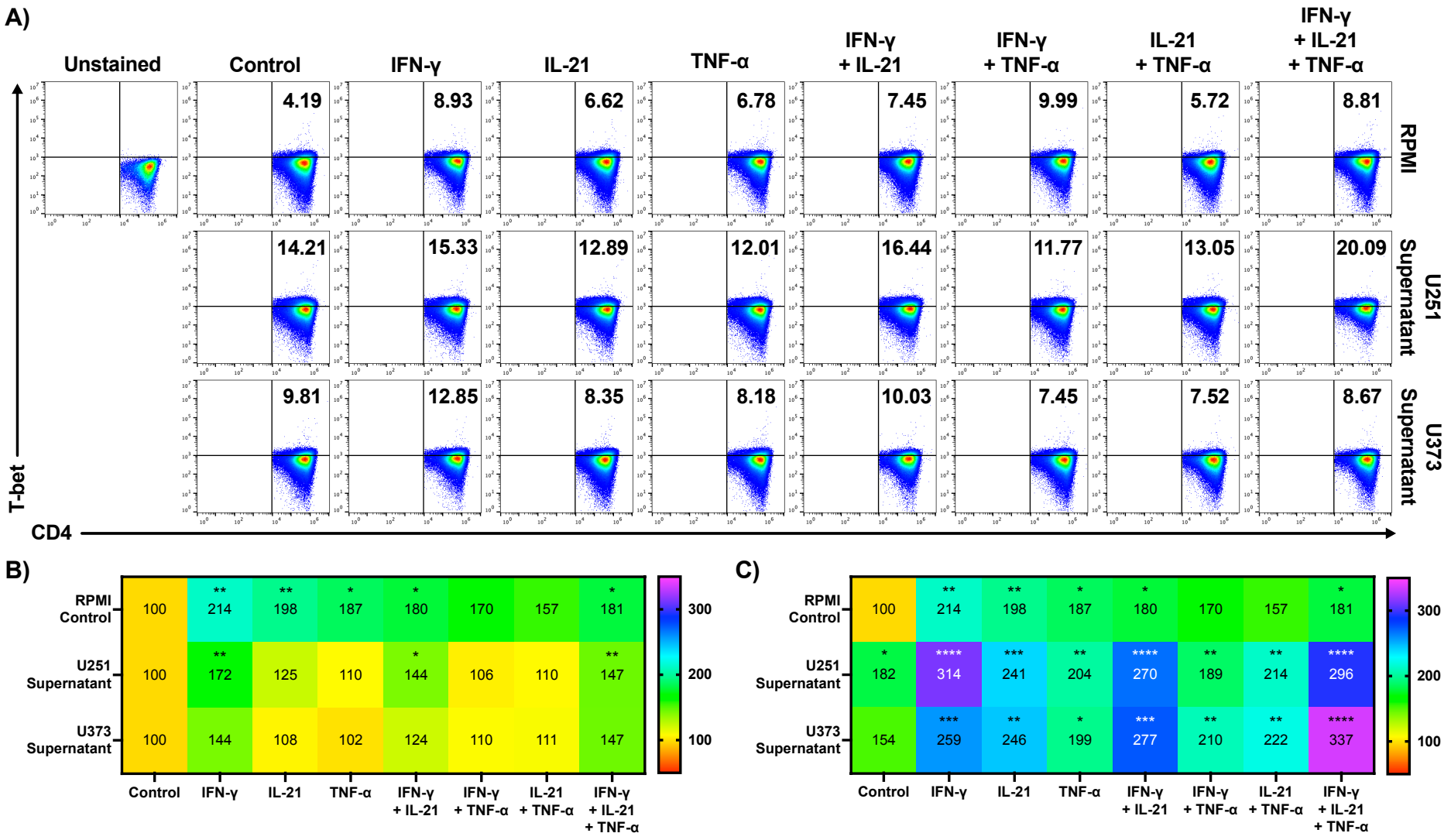


Figure 6.1. IFN-γ, IL-21 and TNF-α did not affect T-bet expression in bulk CD4+ T cells

$2.5x10^4$ bulk CD4+ T cells were cultured 1:1 with CD3/CD28 beads in RPMI, U251 cell supernatant or U373 cell supernatant, plus treatment with 100ng/ml IFN-γ, IL-21 and TNF-α, both individually and combined for 5 days. Cells were then harvested and stained for CD4 and T-bet for analysis via flow cytometry. Flow plots **A)** are representative of > 3 independent experiments. Heat Maps and Bars represent % CD4+T-bet+ cells as means compared to the relevant Control **B)** and **D)**, plus % CD4+T-bet+ cells as means compared to the RPMI Control **C)** and **E)** with SEM shown for > 3 independent experiments. P Values: * = <0.05; ** = <0.01; *** = <0.001; **** = <0.0001.

In contrast to the results seen in bulk CD4+T cells, IFN-γ (214% - p = 0.0024), IL-21 (198% - p = 0.0051) and TNF-α (187% - p = 0.0420) each independently upregulated T-bet expression in naïve CD4+CD45RA+ T cells (Figures 6.2B and D). Despite this, only the IFN-γ and IL-21 dual treatment (180% - p = 0.0166) and triple cytokine combination (181% - p = 0.0353) were able to sustain T-bet upregulation. Similar results were seen when naïve CD4+CD45RA+ T cells were cultured with U251 cell supernatant, in which IFN-γ alone (172% - p = 0.0016) and in combination with IL-21 (144% - p = 0.0146) also increased T-bet expression to a similar extent as IFN-γ, IL-21 and TNF-α (147% - p = 0.0087). However, as U251 cell supernatant (182% - p = 0.0161) independently upregulated T-bet expression, the addition of the triple cytokine treatment almost tripled the level of T-bet expression, relative to the RPMI Control (296% - p < 0.0001) (Figures 6.2C and E).

Although the data was not statistically significant, there was a slight trend to suggest that IFN-γ alone (144% - p = 0.3686) could have been involved in increasing T-bet expression in naïve CD4+CD45RA+ T cells, when cultured with U373 cell supernatant, which was largely maintained when combined with IL-21 and TNF-α (147% - p = 0.3305) (Figures 6.2B and D). Normalising these findings against the RPMI Control revealed that U373 cell supernatant (154% - p = 0.1194) did not independently impact T-bet expression, but the addition of IFN-γ, IL-21 and TNF-α clearly had a profound impact on elevating T-bet expression in naïve CD4+CD45RA+ T cells (337% - p < 0.0001) (Figures 6.2C and E). Collectively, these findings indicated that IFN-γ, IL-21 and TNF-α upregulated T-bet expression in naïve CD4+CD45RA+ T cells, when they were exposed to the glioblastoma secretome.


A)
Unstained
Control
IFN-γ
IL-21
TNF-α
IFN-γ + IL-21
IFN-γ + TNF-α
IL-21 + TNF-α
IFN-γ + IL-21 + TNF-α
RPMI
U251 Supernatant
U373 Supernatant
4.19
8.93
6.62
6.78
7.45
9.99
5.72
8.81
14.21
15.33
12.89
12.01
16.44
11.77
13.05
20.09
9.81
12.85
8.35
8.18
10.03
7.45
7.52
8.67
T-bet
CD4
B)
RPMI Control
U251 Supernatant
U373 Supernatant
100
214
198
187
180
170
157
181
100
172
125
110
144
106
110
147
100
144
108
102
124
110
111
147
Control
IFN-γ
IL-21
TNF-α
IFN-γ + IL-21
IFN-γ + TNF-α
IL-21 + TNF-α
IFN-γ + IL-21 + TNF-α
300
200
100
C)
RPMI Control
U251 Supernatant
U373 Supernatant
100
214
198
187
180
170
157
181
182
314
241
204
270
189
214
296
154
259
246
199
277
210
222
337
Control
IFN-γ
IL-21
TNF-α
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IFN-γ + TNF-α
IL-21 + TNF-α
IFN-γ + IL-21 + TNF-α
300
200
100

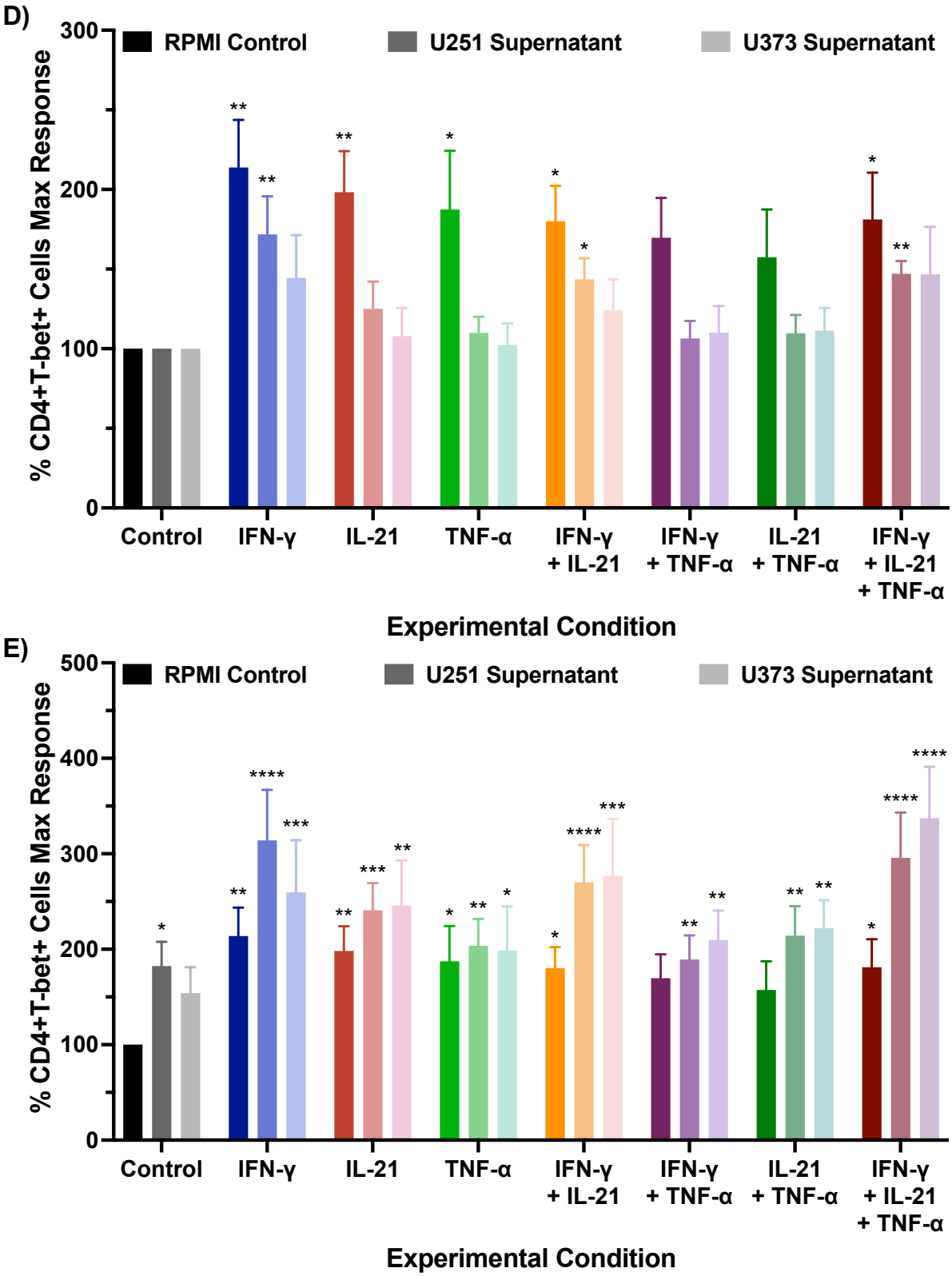


Figure 6.2. IFN-γ, IL-21 and TNF-α upregulated T-bet expression in naïve CD4+CD45RA+ T cells

$2.5x10^4$ naïve CD4+CD45RA+ T cells were cultured 1:1 with CD3/CD28 beads in RPMI, U251 cell supernatant or U373 cell supernatant, plus treatment with 100ng/ml IFN-γ, IL-21 and TNF-α, both individually and combined for 5 days. Cells were then harvested and stained for CD4 and T-bet for analysis via flow cytometry. Flow plots **A)** are representative of > 3 independent experiments. Heat Maps and Bars represent % CD4+T-bet+ cells as means compared to the relevant Control **B)** and **D)**, plus % CD4+T-bet+ cells as means compared to the RPMI Control **C)** and **E)** with SEM shown for > 3 independent experiments. P Values: * = <0.05; ** = <0.01; *** = <0.001; **** = <0.0001.

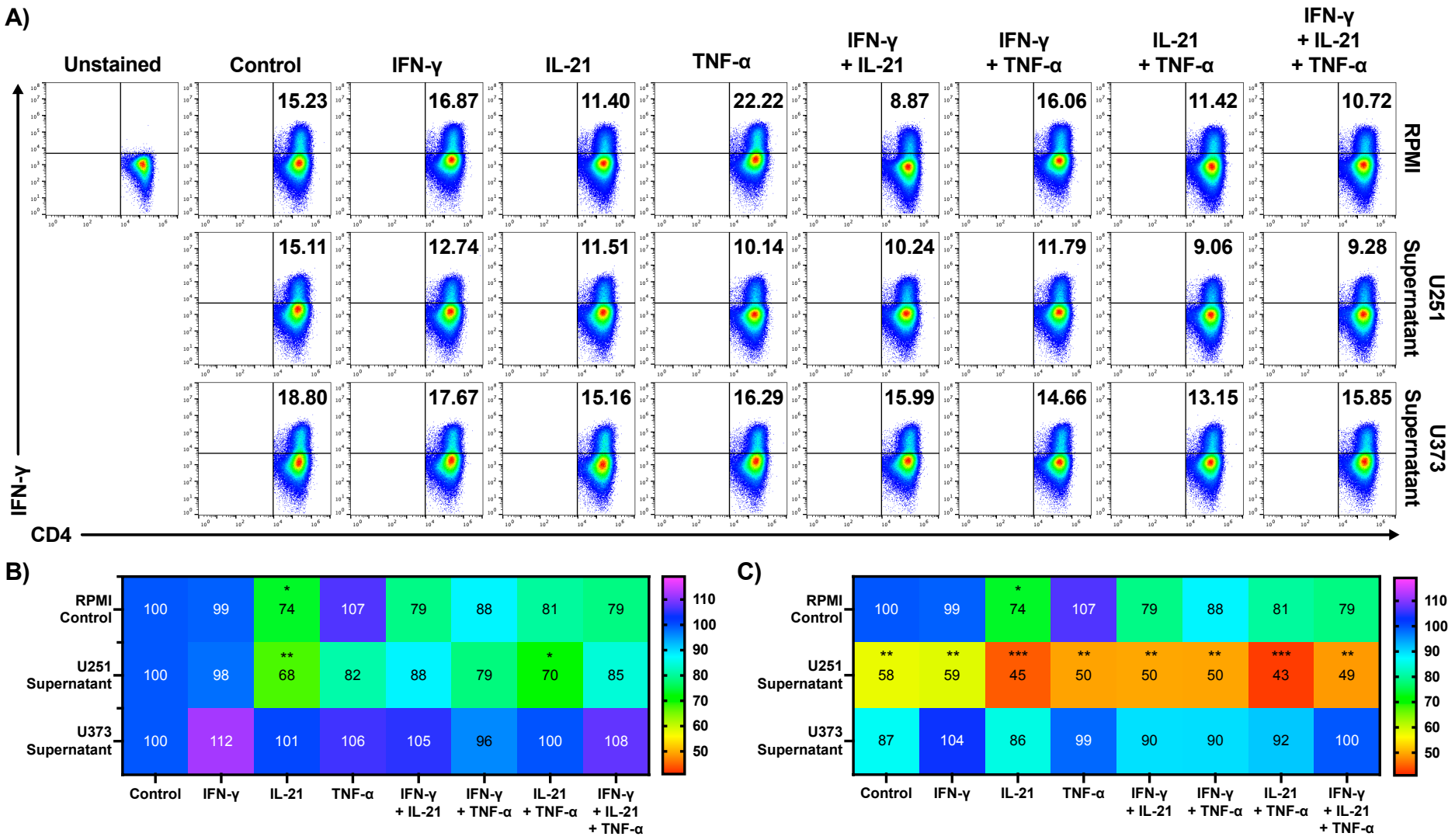
6.2.2. IFN-γ, IL-21 and TNF-α variably affected Th1-associated IFN-γ expression in bulk CD4+ and naïve CD4+CD45RA+ T cells cultured with glioblastoma supernatant

Th1 cells secrete high levels of IFN-γ to either promote an inflammatory immune response or directly induce target cell death, therefore it was important to evaluate whether IFN-γ, IL-21 and TNF-α could overcome the immunosuppressive nature of the glioblastoma secretome and restore IFN-γ production in CD4+ T cells. In order to explore this, bulk CD4+ and naïve CD4+CD45RA+ T cells were isolated from healthy human leukocyte cones, before they were subsequently cultured with CD3/CD28 beads for 5 days. Within these culture conditions, both T cell subsets were exposed to U251 or U373 cell supernatant, alongside treatment with IFN-γ, IL-21 and/or TNF-α, before they were harvested and stained for CD4 and IFN-γ for flow cytometric analysis. The data produced was normalised against the untreated Controls for each media condition, though the results from experiments cultured with U251 or U373 cell supernatant were also compared against the RPMI Control, in order to fully capture the influence of the glioblastoma secretome and/or cytokine treatment on IFN-γ expression in bulk CD4+ or naïve CD4+CD45RA+ T cells.

Results from the Control condition showed that independent IL-21 administration reduced IFN-γ expression in bulk CD4+ T cells (74% - $p = 0.0296$), whilst IFN-γ (99% - $p > 0.9999$) and TNF-α alone (107% - $p = 0.6649$) had no effect. There was a trend to suggest that IL-21 was mediating downregulation of IFN-γ in each of the remaining conditions, including when IFN-γ, IL-21 and TNF-α were administered together (79% - $p = 0.0772$), but this was not statistically significant (Figures 6.3B and D).

Nevertheless, these inhibitory effects were still present when bulk CD4+ T cells were cultured with U251 cell supernatant, as IL-21 alone (68% - $p = 0.0050$) and in combination with TNF-α (70% - $p = 0.0129$) significantly reduced IFN-γ expression, but this was not sustained in the IFN-γ, IL-21 and TNF-α condition (85% - $p = 0.1034$) (Figures 6.3B and D). Normalising the data against the RPMI Control revealed that the U251 cell supernatant (58% - $p = 0.0062$) independently diminished IFN-γ expression in bulk CD4+ T cells, thus each of the cytokine conditions significantly downregulated IFN-γ, including the combination of IFN-γ, IL-21 and TNF-α (48% - $p = 0.0018$) (Figures 6.3C and E).

In contrast, no significant differences in IFN-γ expression were identified when bulk CD4+ T cells were cultured with U373 cell supernatant either alone or with any of the cytokine treatments, even when normalised against the RPMI Control (Figure 6.3). Altogether, these results demonstrated that IFN-γ, IL-21 and TNF-α treatment did not affect IFN-γ expression in bulk CD4+ T cells, when they were exposed to the glioblastoma secretome.


A)
Unstained
Control
IFN-γ
IL-21
TNF-α
IFN-γ + IL-21
IFN-γ + TNF-α
IL-21 + TNF-α
IFN-γ + IL-21 + TNF-α
15.23
16.87
11.40
22.22
8.87
16.06
11.42
10.72
RPMI
15.11
12.74
11.51
10.14
10.24
11.79
9.06
9.28
U251 Supernatant
18.80
17.67
15.16
16.29
15.99
14.66
13.15
15.85
U373 Supernatant
IFN-γ
CD4
B)
RPMI Control
U251 Supernatant
U373 Supernatant
100 99 74 107 79 88 81 79
100 98 68 82 88 79 70 85
100 112 101 106 105 96 100 108
Control
IFN-γ
IL-21
TNF-α
IFN-γ + IL-21
IFN-γ + TNF-α
IL-21 + TNF-α
IFN-γ + IL-21 + TNF-α
110
100
90
80
70
60
50
C)
RPMI Control
U251 Supernatant
U373 Supernatant
100 99 74 107 79 88 81 79
58 59 45 50 50 50 43 49
87 104 86 99 90 90 92 100
Control
IFN-γ
IL-21
TNF-α
IFN-γ + IL-21
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IFN-γ + IL-21 + TNF-α
110
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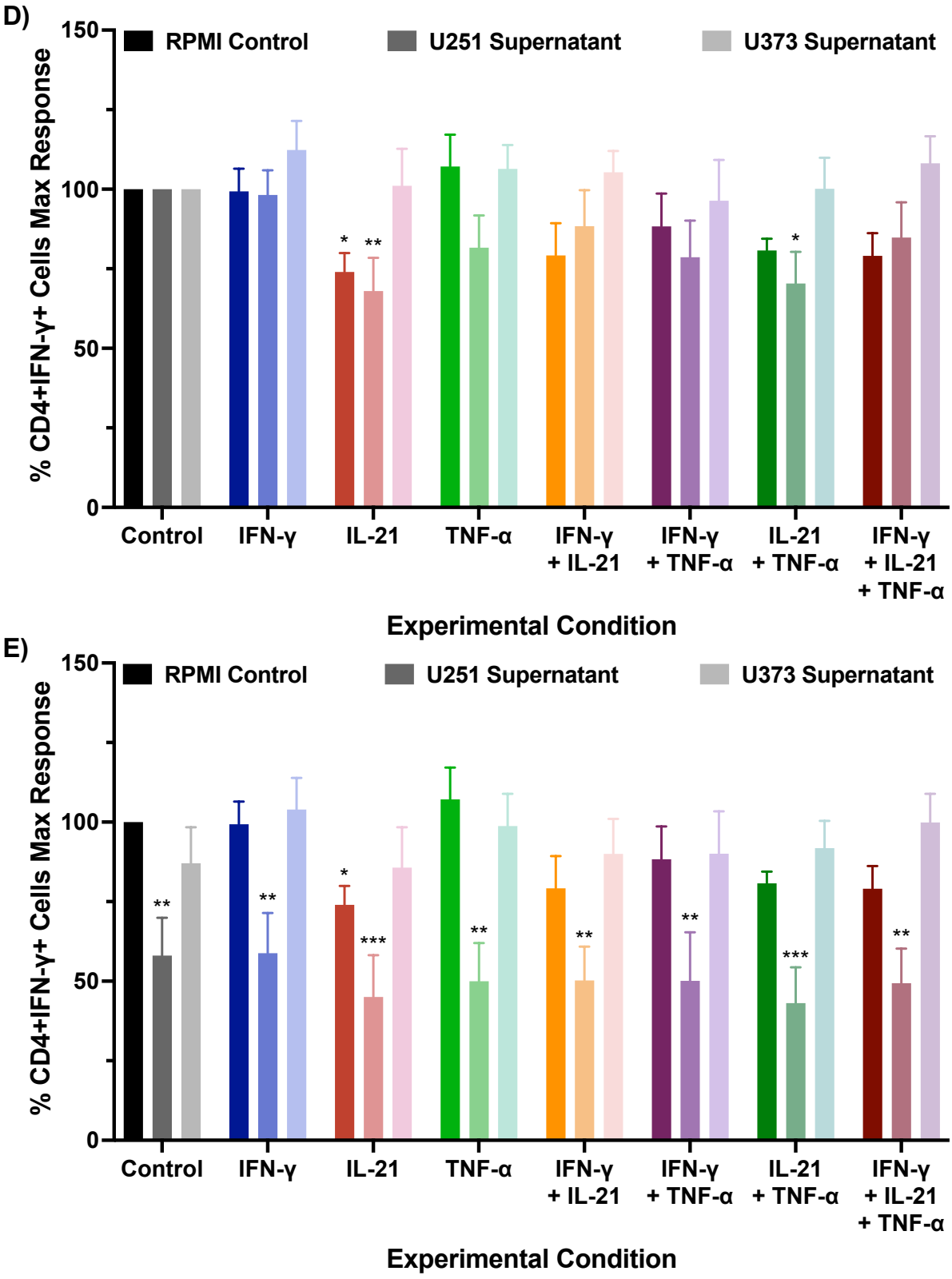
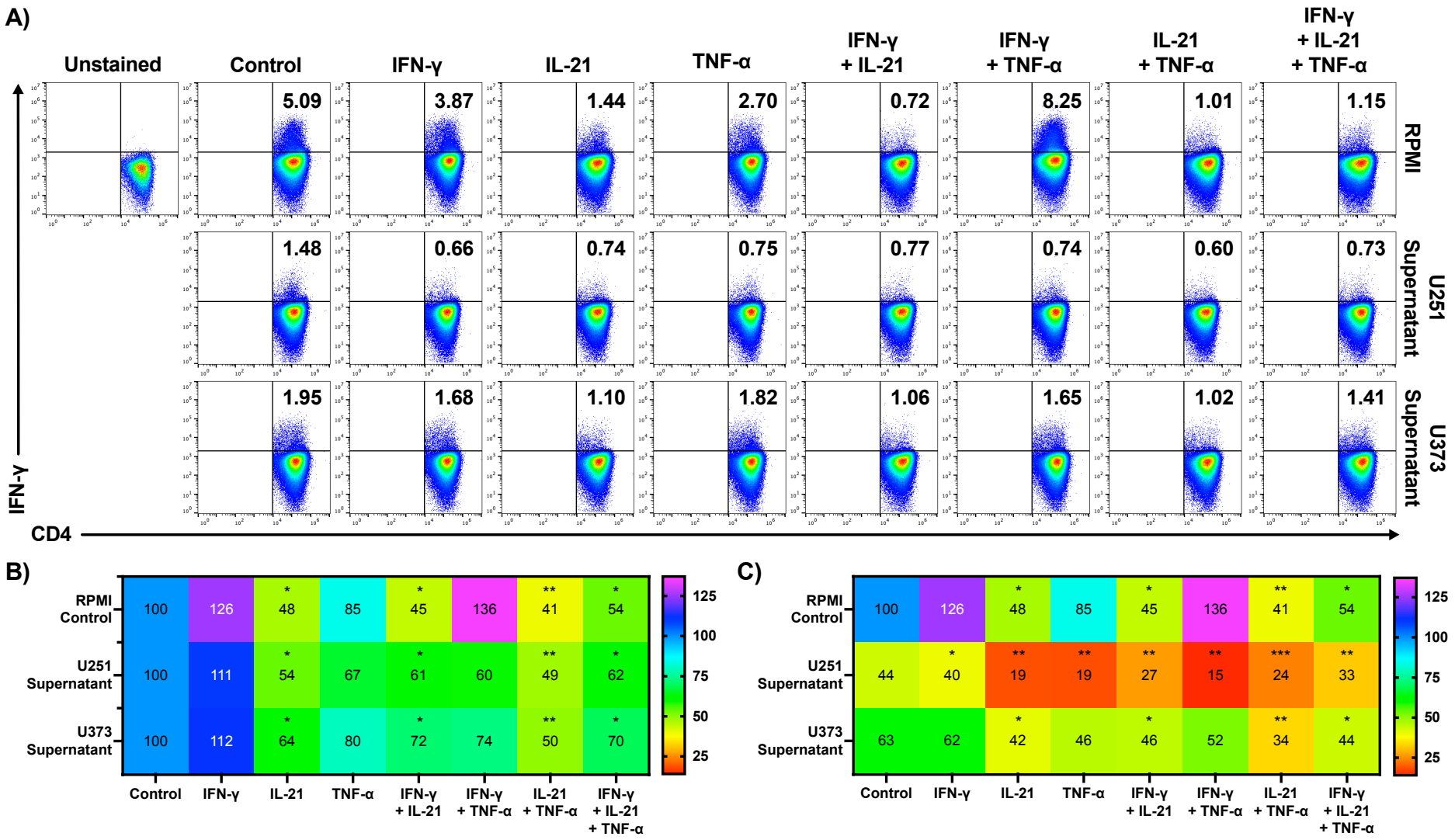


Figure 6.3. IFN-γ, IL-21 and TNF-α did not affect IFN-γ expression in bulk CD4+ T cells

$2.5x10^4$ bulk CD4+ T cells were cultured 1:1 with CD3/CD28 beads in RPMI, U251 cell supernatant or U373 cell supernatant, plus treatment with 100ng/ml IFN-γ, IL-21 and TNF-α, both individually and combined for 5 days. Cells were then harvested and stained for CD4 and IFN-γ for analysis via flow cytometry. Flow plots **A)** are representative of > 3 independent experiments. Heat Maps and Bars represent % CD4+IFN-γ+ cells as means compared to the relevant Control **B)** and **D)**, plus % CD4+IFN-γ+ cells as means compared to the RPMI Control **C)** and **E)** with SEM shown for > 3 independent experiments. P Values: * = <0.05; ** = <0.01; *** = <0.001; **** = <0.0001.

Results from the Control condition revealed that IL-21 potently reduced IFN-γ expression in naïve CD4+CD45RA+ T cells, regardless of whether IL-21 was administered alone (48% p = 0.0193), in conjunction with IFN-γ (45% - p = 0.0297) or TNF-α (41% - p = 0.0091), or as a triple cytokine combination (54% - p = 0.0477) (Figures 6.4B and D). The impact of IL-21 on IFN-γ expression in naïve CD4+CD45RA+ T cells was sustained with the addition of U251 cell supernatant with notable downregulation seen with the combination of IFN-γ, IL-21 and TNF-α (62% - p = 0.0375) (Figures 6.4B and D). Normalising the data against the RPMI Control suggested that U251 cell supernatant could independently reduce IFN-γ expression in naïve CD4+CD45RA+ T cells, but the effect was not statistically significant (44% - p = 0.0783). Nevertheless, combining U251 cell supernatant with IFN-γ, IL-21 and TNF-α potently reduced IFN-γ expression (33% - p = 0.0022) in naïve CD4+CD45RA+ T cells (Figures 6.4C and E).

Similar results were found when naïve CD4+CD45RA+ T cells were cultured with U373 cell supernatant, as IFN-γ was downregulated by the combination of IFN-γ, IL-21 and TNF-α (70% - p = 0.0427) (Figures 6.4B and D). Furthermore, in line with the results seen with U251 cell supernatant, the data appeared to suggest that that U373 cell supernatant could potentially reduce IFN-γ expression in naïve CD4+CD45RA+ T cells (63% - p = 0.3320), but this was significantly augmented when administered in combination with IFN-γ, IL-21 and TNF-α (44% - p = 0.0232), relative to the RPMI Control (Figures 6.4C and E). Overall, these findings suggested that the glioblastoma secretome could downregulate IFN-γ expression in naïve CD4+CD45RA+ T cells, but this was potently enhanced with the addition of IFN-γ, IL-21 and TNF-α.


A)
Unstained
Control
IFN-γ
IL-21
TNF-α
IFN-γ + IL-21
IFN-γ + TNF-α
IL-21 + TNF-α
IFN-γ + IL-21 + TNF-α
5.09
3.87
1.44
2.70
0.72
8.25
1.01
1.15
1.48
0.66
0.74
0.75
0.77
0.74
0.60
0.73
1.95
1.68
1.10
1.82
1.06
1.65
1.02
1.41
RPMI
U251 Supernatant
U373 Supernatant
IFN-γ
CD4
B)
RPMI Control
U251 Supernatant
U373 Supernatant
100 126 * 48 85 * 45 136 ** 41 * 54
100 111 * 54 67 * 61 60 ** 49 * 62
100 112 * 64 80 * 72 74 ** 50 * 70
Control
IFN-γ
IL-21
TNF-α
IFN-γ + IL-21
IFN-γ + TNF-α
IL-21 + TNF-α
IFN-γ + IL-21 + TNF-α
125
100
75
50
25
C)
RPMI Control
U251 Supernatant
U373 Supernatant
100 126 * 48 85 * 45 136 ** 41 * 54
44 * 40 ** 19 ** 19 ** 27 ** 15 *** 24 ** 33
63 62 * 42 46 * 46 52 ** 34 * 44
Control
IFN-γ
IL-21
TNF-α
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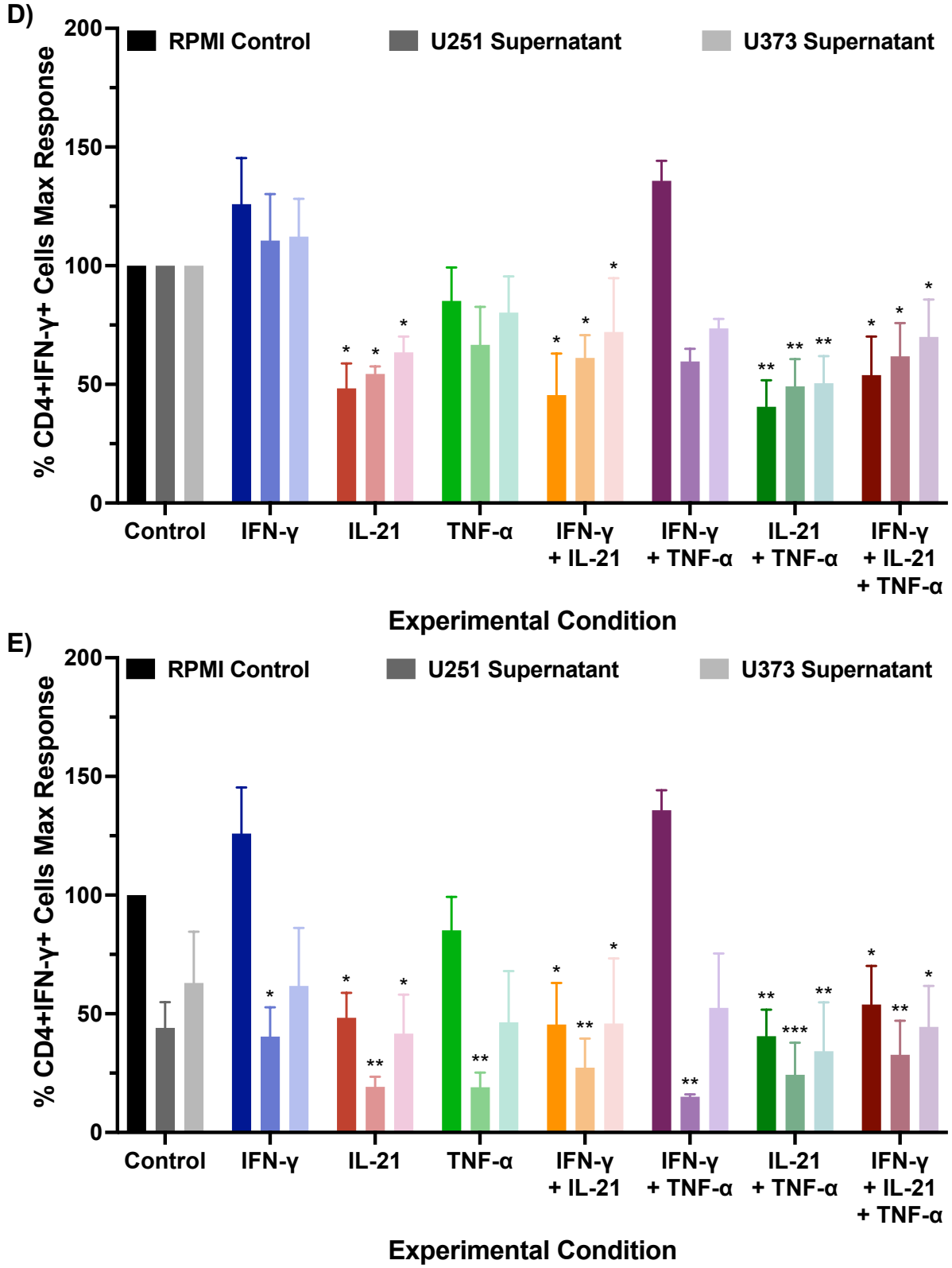


Figure 6.4. IFN-γ, IL-21 and TNF-α downregulated IFN-γ expression in naïve CD4+CD45RA+ T cells

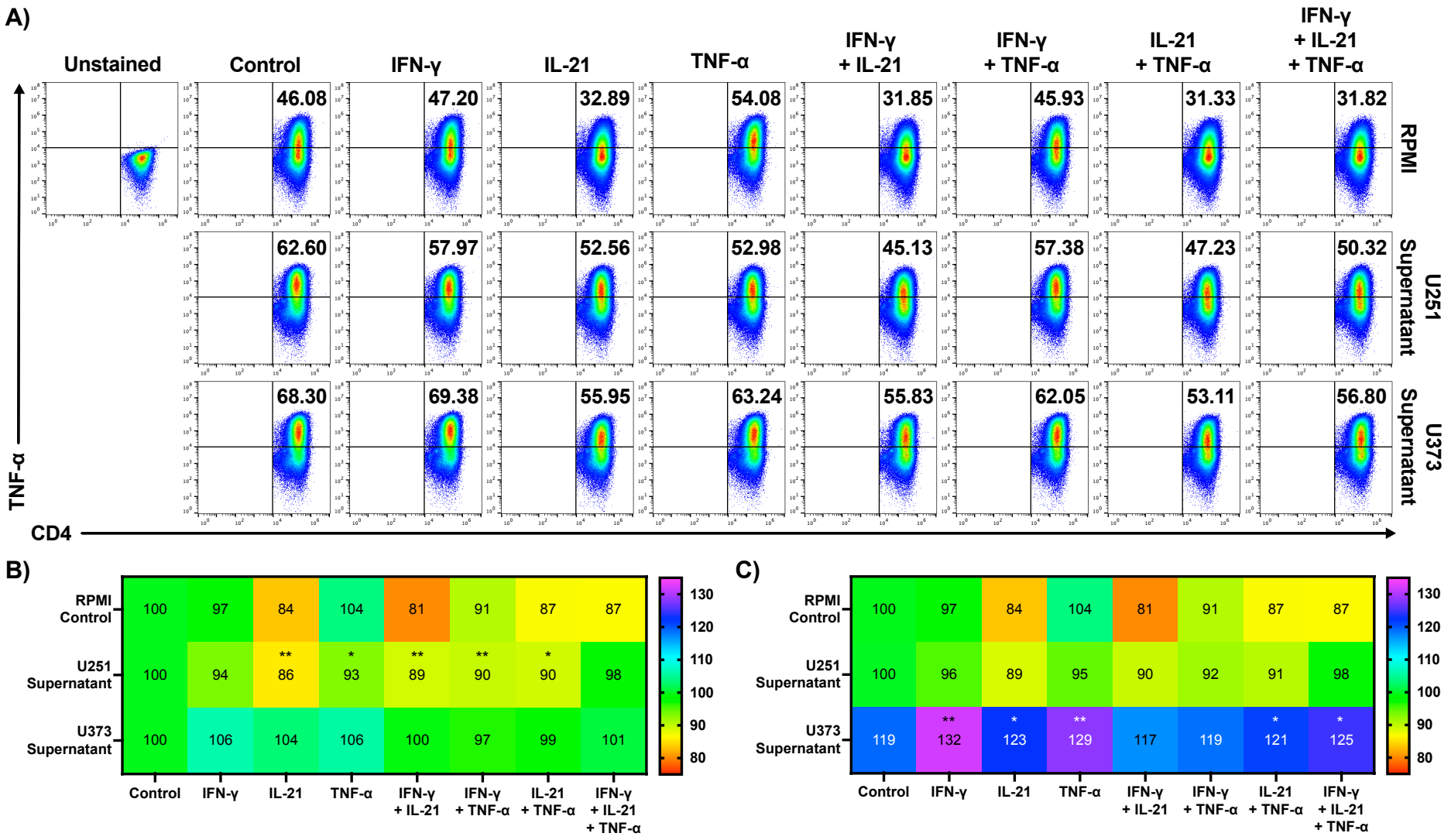
$2.5x10^4$ naïve CD4+CD45RA+ T cells were cultured 1:1 with CD3/CD28 beads in RPMI, U251 cell supernatant or U373 cell supernatant, plus treatment with 100ng/ml IFN-γ, IL-21 and TNF-α, both individually and combined for 5 days. Cells were then harvested and stained for CD4 and IFN-γ for analysis via flow cytometry. Flow plots **A)** are representative of > 3 independent experiments. Heat Maps and Bars represent % CD4+IFN-γ+ cells as means compared to the relevant Control **B)** and **D)**, plus % CD4+IFN-γ+ cells as means compared to the RPMI Control **C)** and **E)** with SEM shown for > 3 independent experiments. P Values: * = <0.05; ** = <0.01; *** = <0.001; **** = <0.0001.

6.2.3. IFN-γ, IL-21 and TNF-α variably affected Th1-associated TNF-α expression in bulk CD4+ and naïve CD4+CD45RA+ T cells cultured with glioblastoma supernatant

Alongside IFN-γ, Th1 cells also produce high levels of TNF-α to either facilitate an inflammatory immune response or directly induce target cell death, therefore it was important to evaluate whether IFN-γ, IL-21 and TNF-α could overcome the immunosuppressive nature of the glioblastoma secretome and restore TNF-α production in CD4+ T cells. In order to explore this, bulk CD4+ and naïve CD4+CD45RA+ T cells were isolated from healthy human leukocyte cones, before they were subsequently cultured with CD3/CD28 beads for 5 days. Within these culture conditions, both T cell subsets were exposed to U251 or U373 cell supernatant, alongside treatment with IFN-γ, IL-21 and/or TNF-α, before they were harvested and stained for CD4 and TNF-α for flow cytometric analysis. The data produced was normalised against the untreated Controls for each media condition – however, the results from experiments cultured with U251 or U373 cell supernatant were also compared against the RPMI Control, in order to fully capture the influence of the glioblastoma secretome and/or cytokine treatment TNF-α expression in bulk CD4+ and naïve CD4+CD45RA+ T cells.

Although none of the results were statistically significant, the data suggested that IL-21 was able to marginally suppress TNF-α expression in bulk CD4+ T cells to a similar extent, either as an independent agent (84% - $p = 0.0940$), in combination with IFN-γ (81% - $p = 0.1022$) or TNF-α (87% - $p = 0.1770$), or as a triple cytokine treatment (87% - $p = 0.2722$) (Figures 6.5B and D). Introducing U251 cell supernatant to these experimental conditions produced some interesting results. In particular, IL-21 (86% - $p = 0.0036$) and TNF-α (93% - $p = 0.0218$) demonstrated independent effects on reducing TNF-α expression in bulk CD4+ T cells, which was sustained throughout all of the dual treatments, but the effect was lost when IFN-γ, IL-21 and TNF-α were administered together (98% - $p = 0.2061$) (Figures 6.5B and D). Conversely, no significant differences in TNF-α expression were identified when bulk CD4+ T cells were cultured with U251 cell supernatant and the various cytokine treatments, relative to the RPMI Control (Figures 6.5C and E).

Similarly, none of the experimental conditions with U373 cell supernatant had any significant impact on TNF-α expression in bulk CD4+ T cells (Figures 6.5B and D). Unlike U251 cell supernatant, results suggested that U373 cell supernatant could independently slightly increase TNF-α expression (119% - $p = 0.1407$), thus the addition of IFN-γ, IL-21 and TNF-α could significantly upregulate TNF-α (125% - $p = 0.0220$), when compared against the RPMI Control (Figures 6.5C and E). Collectively, the lack of statistical significance suggested that IFN-γ, IL-21 and TNF-α did not affect TNF-α expression in bulk CD4+ T cells, when exposed to the glioblastoma secretome.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IFN-γ
IL-21
TNF-α
IFN-γ + IL-21
IFN-γ + TNF-α
IL-21 + TNF-α
IFN-γ + IL-21 + TNF-α
RPMI
U251 Supernatant
U373 Supernatant
TNF-α
CD4
46.08
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32.89
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31.82
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57.97
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45.13
57.38
47.23
50.32
68.30
69.38
55.95
63.24
55.83
62.05
53.11
56.80
B)
RPMI Control
U251 Supernatant
U373 Supernatant
100 97 84 104 81 91 87 87
100 94 ** 86 * 93 ** 89 ** 90 * 90 98
100 106 104 106 100 97 99 101
Control
IFN-γ
IL-21
TNF-α
IFN-γ + IL-21
IFN-γ + TNF-α
IL-21 + TNF-α
IFN-γ + IL-21 + TNF-α
130
120
110
100
90
80
C)
RPMI Control
U251 Supernatant
U373 Supernatant
100 97 84 104 81 91 87 87
100 96 89 95 90 92 91 98
119 ** 132 * 123 ** 129 117 119 * 121 * 125
Control
IFN-γ
IL-21
TNF-α
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120
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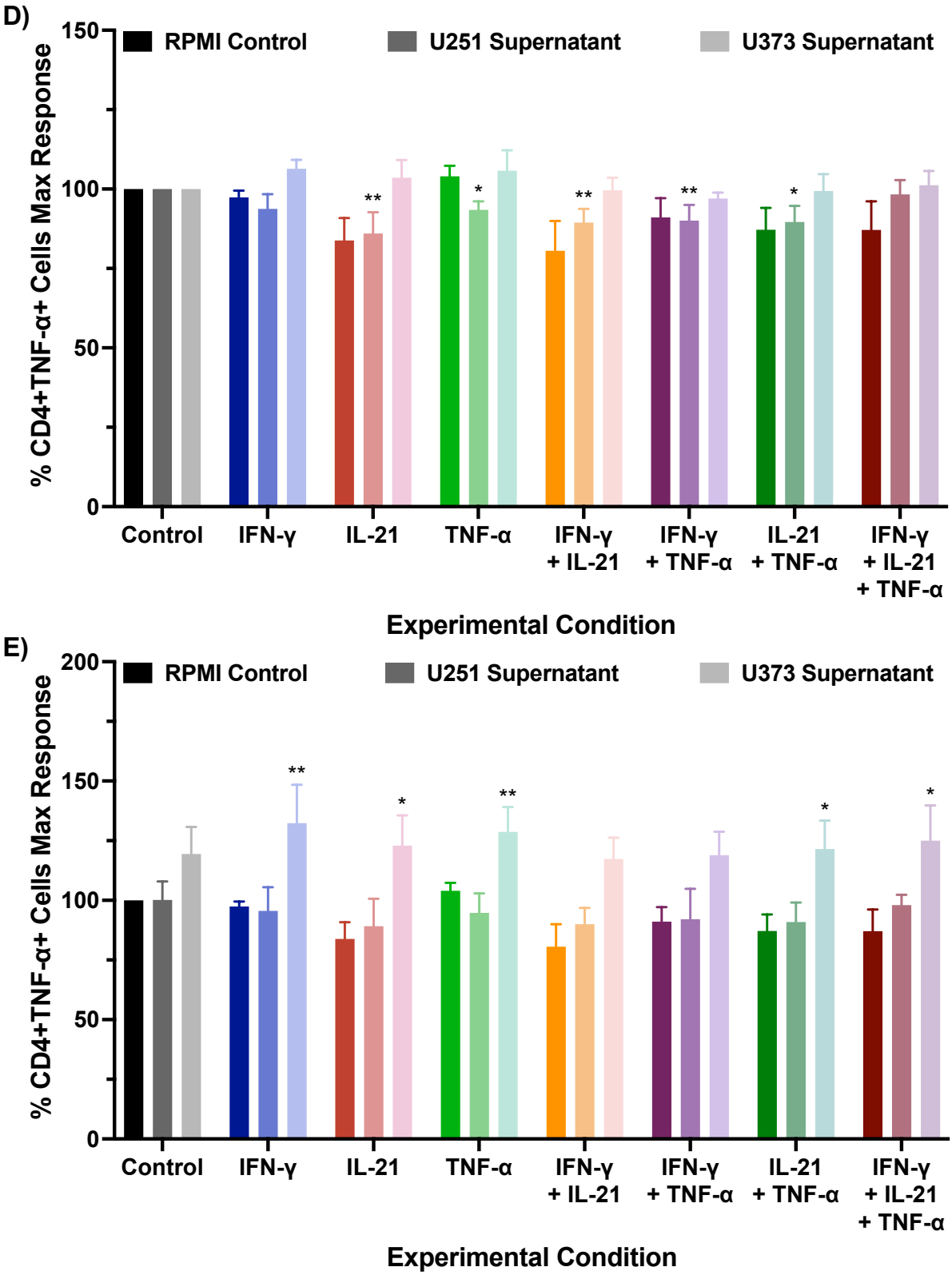
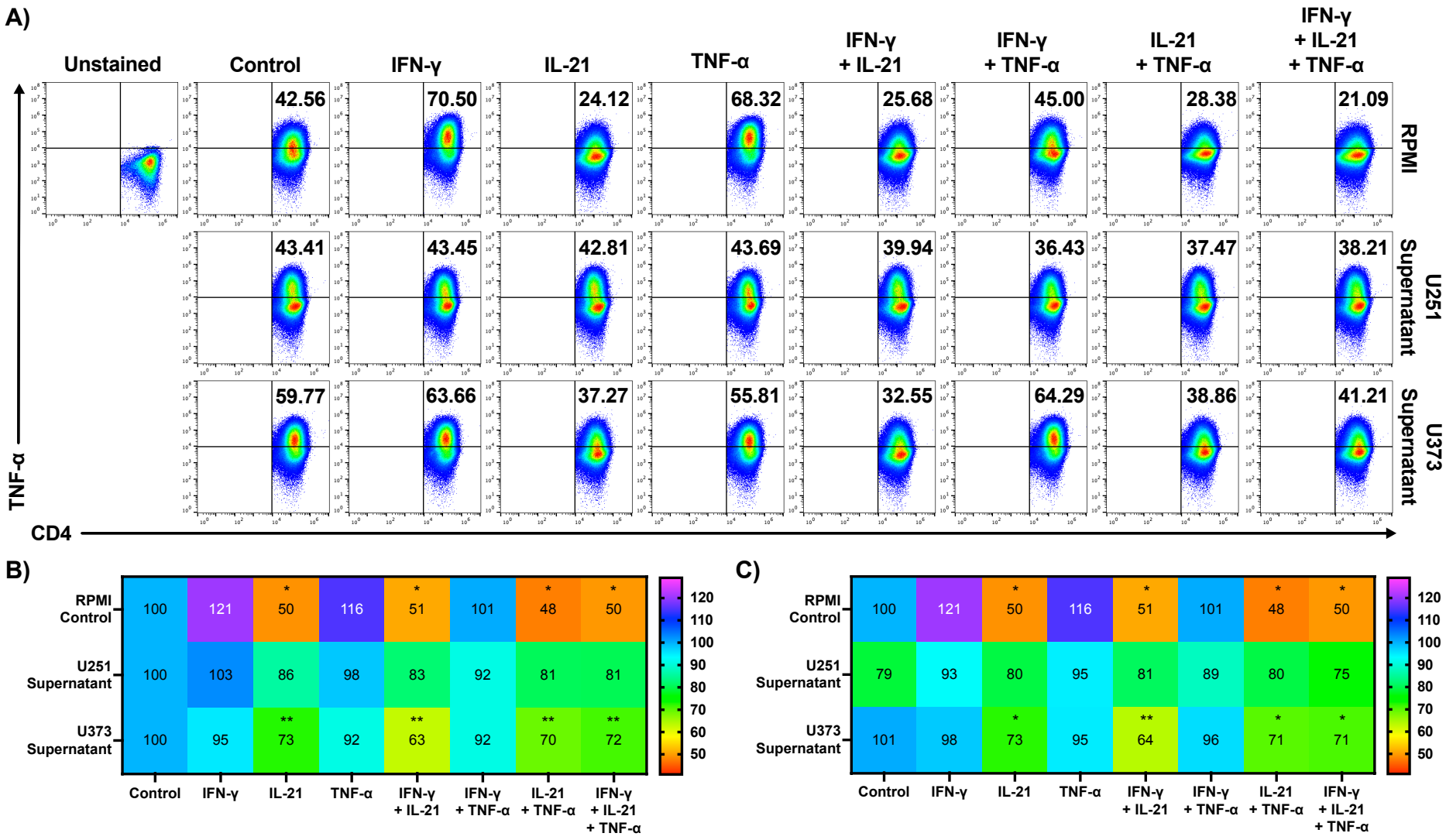


Figure 6.5. IFN-γ, IL-21 and TNF-α did not affect TNF-α expression in bulk CD4+ T cells

$2.5x10^4$ bulk CD4+ T cells were cultured 1:1 with CD3/CD28 beads in RPMI, U251 cell supernatant or U373 cell supernatant, plus treatment with 100ng/ml IFN-γ, IL-21 and TNF-α, both individually and combined for 5 days. Cells were then harvested and stained for CD4 and TNF-α for analysis via flow cytometry. Flow plots **A)** are representative of > 3 independent experiments. Heat Maps and Bars represent % CD4+TNF-α+ cells as means compared to the relevant Control **B)** and **D)**, plus % CD4+TNF-α+ cells as means compared to the RPMI Control **C)** and **E)** with SEM shown for > 3 independent experiments. P Values: * = <0.05; ** = <0.01; *** = <0.001; **** = <0.0001.

The results from the Control condition showed that IL-21 potently suppressed TNF-α expression in naïve CD4+CD45RA+ T cells, regardless of whether IL-21 was administered alone (50% - p = 0.0185), in combination with IFN-γ (51% - p = 0.0260) or TNF-α (48% - p = 0.0227), or as a triple cytokine combination (50% - p = 0.0161) (Figures 6.6B and D). However, the strength and significance of this effect was lost when naïve CD4+CD45RA+ T cells were cultured with U251 cell supernatant, even when combined with IFN-γ, IL-21 and TNF-α (81% - p = 0.2156). Normalising the data against the RPMI Control revealed that U251 cell supernatant had a slight (albeit non-significant) effect on reducing TNF-α expression (79% - p = 0.2055), thus the addition of the triple cytokine treatment (75% - p = 0.1234) proved to have no impact on TNF-α expression in naïve CD4+CD45RA+ T cells (Figures 6.6C and E).

In contrast, in the experiments with U373 cell supernatant, IL-21 independently downregulated TNF-α in naïve CD4+CD45RA+ T cells (73% - p = 0.0074), which was sustained across each of the remaining cytokine conditions, including the combination of IFN-γ, IL-21 and TNF-α (72% - p = 0.0065) (Figures 6.6B and D). Interestingly, U373 cell supernatant produced comparable results to the RPMI Control (101% - p = 0.6918), thus the efficacy of each of these cytokine conditions largely stayed the same, with the triple cytokine condition reducing TNF-α expression to 71% (p = 0.0194), compared to the RPMI Control (Figures 6.6C and E). Altogether, these findings demonstrated that the glioblastoma secretome did not affect TNF-α expression in naïve CD4+CD45RA+ T cells, but this was downregulated by IFN-γ, IL-21 and TNF-α treatment.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Unstained
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IL-21
TNF-α
IFN-γ + IL-21
IFN-γ + TNF-α
IL-21 + TNF-α
IFN-γ + IL-21 + TNF-α
42.56
70.50
24.12
68.32
25.68
45.00
28.38
21.09
RPMI
43.41
43.45
42.81
43.69
39.94
36.43
37.47
38.21
U251 Supernatant
59.77
63.66
37.27
55.81
32.55
64.29
38.86
41.21
U373 Supernatant
TNF-α
CD4
B)
RPMI Control
U251 Supernatant
U373 Supernatant
100 121 * 50 116 * 51 101 * 48 * 50
100 103 86 98 83 92 81 81
100 95 ** 73 92 ** 63 92 ** 70 ** 72
Control
IFN-γ
IL-21
TNF-α
IFN-γ + IL-21
IFN-γ + TNF-α
IL-21 + TNF-α
IFN-γ + IL-21 + TNF-α
120
110
100
90
80
70
60
50
C)
RPMI Control
U251 Supernatant
U373 Supernatant
100 121 * 50 116 * 51 101 * 48 * 50
79 93 80 95 81 89 80 75
101 98 * 73 95 ** 64 96 * 71 * 71
Control
IFN-γ
IL-21
TNF-α
IFN-γ + IL-21
IFN-γ + TNF-α
IL-21 + TNF-α
IFN-γ + IL-21 + TNF-α
120
110
100
90
80
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60
50

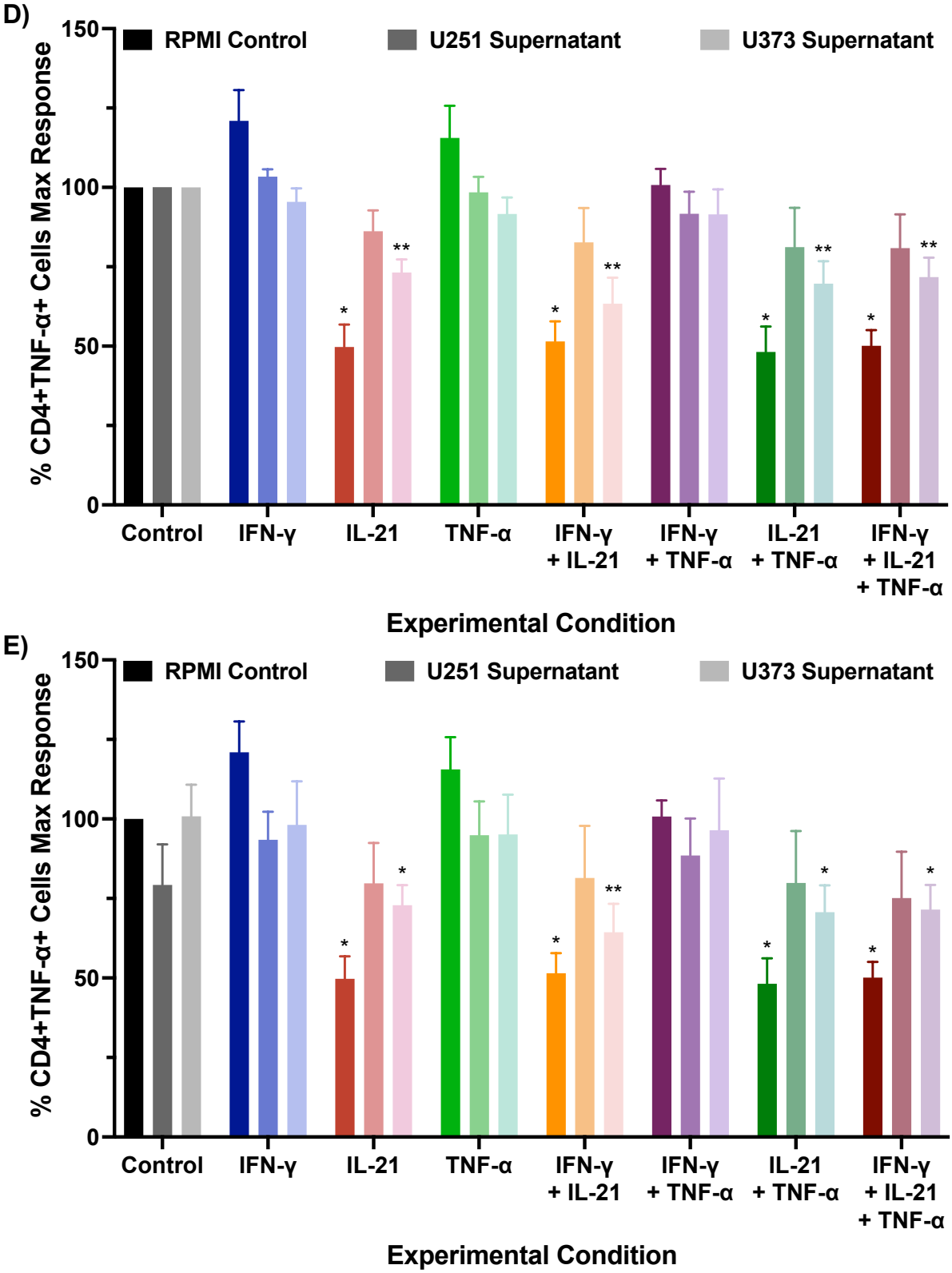


Figure 6.6. IFN-γ, IL-21 and TNF-α downregulated TNF-α expression in naïve CD4+CD45RA+ T cells

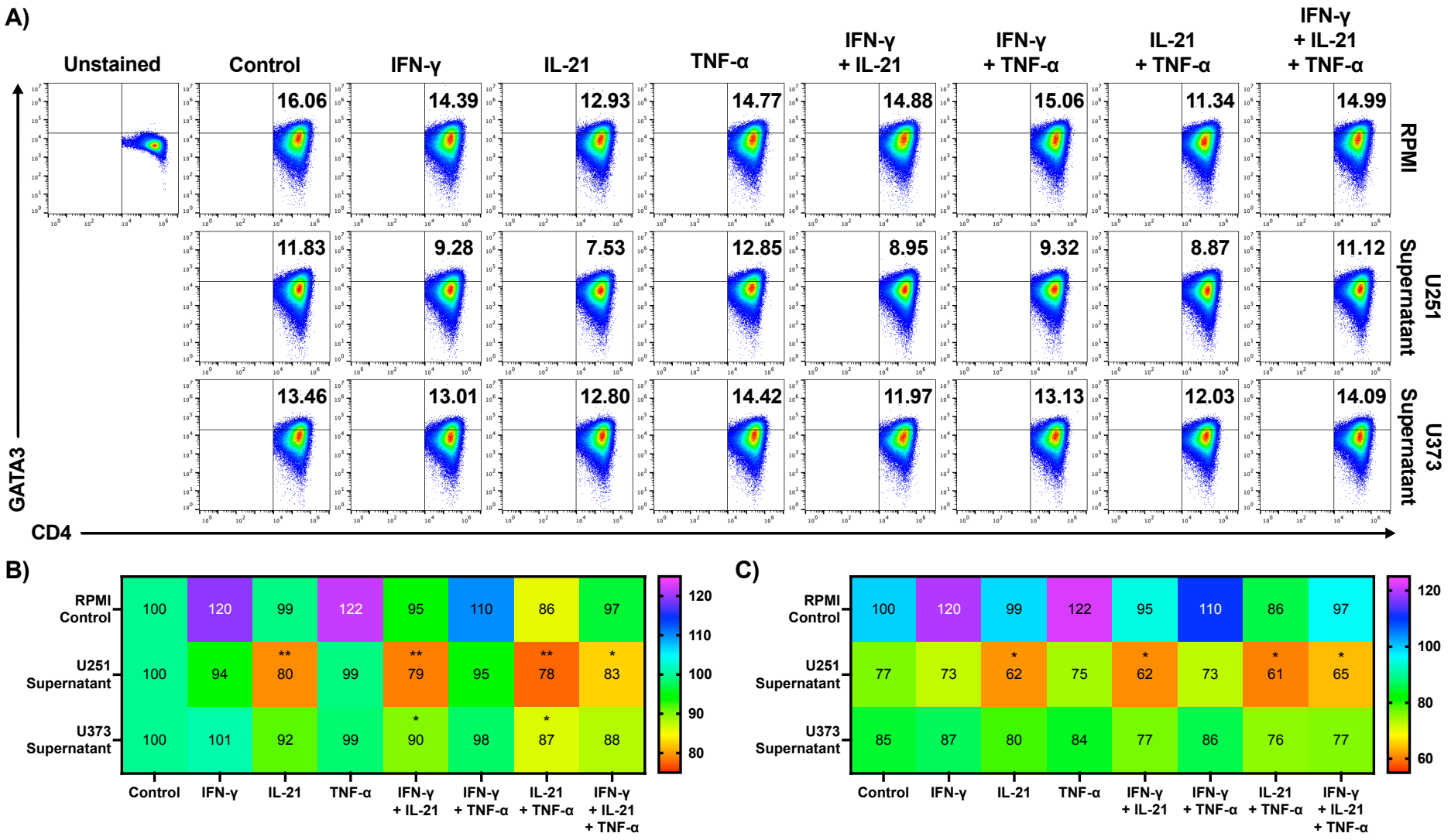
2.5x10^4 naïve CD4+CD45RA+ T cells were cultured 1:1 with CD3/CD28 beads in RPMI, U251 cell supernatant or U373 cell supernatant, plus treatment with 100ng/ml IFN-γ, IL-21 and TNF-α, both individually and combined for 5 days. Cells were then harvested and stained for CD4 and TNF-α for analysis via flow cytometry. Flow plots **A)** are representative of > 3 independent experiments. Heat Maps and Bars represent % CD4+TNF-α+ cells as means compared to the relevant Control **B)** and **D)**, plus % CD4+TNF-α+ cells as means compared to the RPMI Control **C)** and **E)** with SEM shown for > 3 independent experiments. P Values: * = <0.05; ** = <0.01; *** = <0.001; **** = <0.0001.

6.2.4. IFN-γ, IL-21 and TNF-α downregulated Th2-associated GATA3 expression in bulk CD4+ and naïve CD4+CD45RA+ T cells cultured with glioblastoma supernatant

Previous research has shown that the expression of Th2-associated markers were upregulated in glioblastoma patients (Shimato et al., 2012; Takashima et al., 2018; Waziri et al., 2008; Zisakis et al., 2007). As a result of this, it was important to explore whether IFN-γ, IL-21 and TNF-α could overcome the influence of the glioblastoma secretome on GATA3 expression, as this is a key transcription factor involved in facilitating Th2 cell differentiation and function. In order to explore this, bulk CD4+ and naïve CD4+CD45RA+ T cells were isolated from healthy human leukocyte cones, before they were subsequently cultured with CD3/CD28 beads for 5 days. Within these culture conditions, both T cell subsets were exposed to U251 or U373 cell supernatant, alongside treatment with IFN-γ, IL-21 and/or TNF-α, before they were harvested and stained for CD4 and GATA3 for flow cytometric analysis. The data produced was normalised against the untreated Controls for each media condition, but the results from experiments cultured with U251 or U373 cell supernatant were also compared against the RPMI Control, in order to fully capture the influence of the glioblastoma secretome and/or cytokine treatment on GATA3 expression in bulk CD4+ and naïve CD4+CD45RA+ T cells.

Despite the fact that none of the cytokine treatments significantly affected GATA3 expression in bulk CD4+ T cells, it was curious to see that both IFN-γ (120% - $p = 0.3583$) and TNF-α (122% - $p = 0.1355$) independently appeared to increase GATA3 expression, with a slight reduction in GATA3 expression mediated by the IL-21 and TNF-α dual treatment (86% - $p = 0.1231$) (Figures 6.7B and D). On the other hand, each of the treatment conditions containing IL-21 significantly reduced GATA3 expression, when bulk CD4+ T cells were cultured with U251 cell supernatant, with the most potent effect facilitated by the combination of IL-21 and TNF-α (78% - $p = 0.0021$) (Figures 6.7B and D). Normalising the data against the RPMI Control revealed that the U251 cell supernatant alone (77% - $p = 0.2383$) slightly influenced GATA3 expression, but the effect was not statistically significant. Nevertheless, combining exposure to U251 cell supernatant with IFN-γ, IL-21 and TNF-α treatment clearly resulted in downregulation of GATA3 expression in bulk CD4+ T cells (65% - $p = 0.0299$) (Figures 6.7C and E).

Conversely, IL-21 in combination with IFN-γ (90% - $p = 0.0416$) or TNF-α (87% - $p = 0.0456$) were the only conditions that significantly reduced GATA3 expression, when bulk CD4+ T cells were cultured with U373 cell supernatant (Figures 6.7B and D). However, U373 cell supernatant alone (85% - $p = 0.3282$) did not significantly influence GATA3 expression, when compared against the RPMI Control, thus the impact of these cytokine treatments was lost (Figures 6.7C and E). Overall, the results indicated that IFN-γ, IL-21 and TNF-α either maintained GATA3 expression in bulk CD4+ T cells or enhanced the inhibitory effects of the glioblastoma secretome.


A)
Unstained
Control
IFN-γ
IL-21
TNF-α
IFN-γ + IL-21
IFN-γ + TNF-α
IL-21 + TNF-α
IFN-γ + IL-21 + TNF-α
RPMI
U251 Supernatant
U373 Supernatant
GATA3
CD4
16.06
14.39
12.93
14.77
14.88
15.06
11.34
14.99
11.83
9.28
7.53
12.85
8.95
9.32
8.87
11.12
13.46
13.01
12.80
14.42
11.97
13.13
12.03
14.09
B)
RPMI Control
U251 Supernatant
U373 Supernatant
100 120 99 122 95 110 86 97
100 94 80 99 79 95 78 83
100 101 92 99 90 98 87 88
** ** * ** * *
Control
IFN-γ
IL-21
TNF-α
IFN-γ + IL-21
IFN-γ + TNF-α
IL-21 + TNF-α
IFN-γ + IL-21 + TNF-α
120
110
100
90
80
C)
RPMI Control
U251 Supernatant
U373 Supernatant
100 120 99 122 95 110 86 97
77 73 62 75 62 73 61 65
85 87 80 84 77 86 76 77
* * * *
Control
IFN-γ
IL-21
TNF-α
IFN-γ + IL-21
IFN-γ + TNF-α
IL-21 + TNF-α
IFN-γ + IL-21 + TNF-α
120
100
80
60

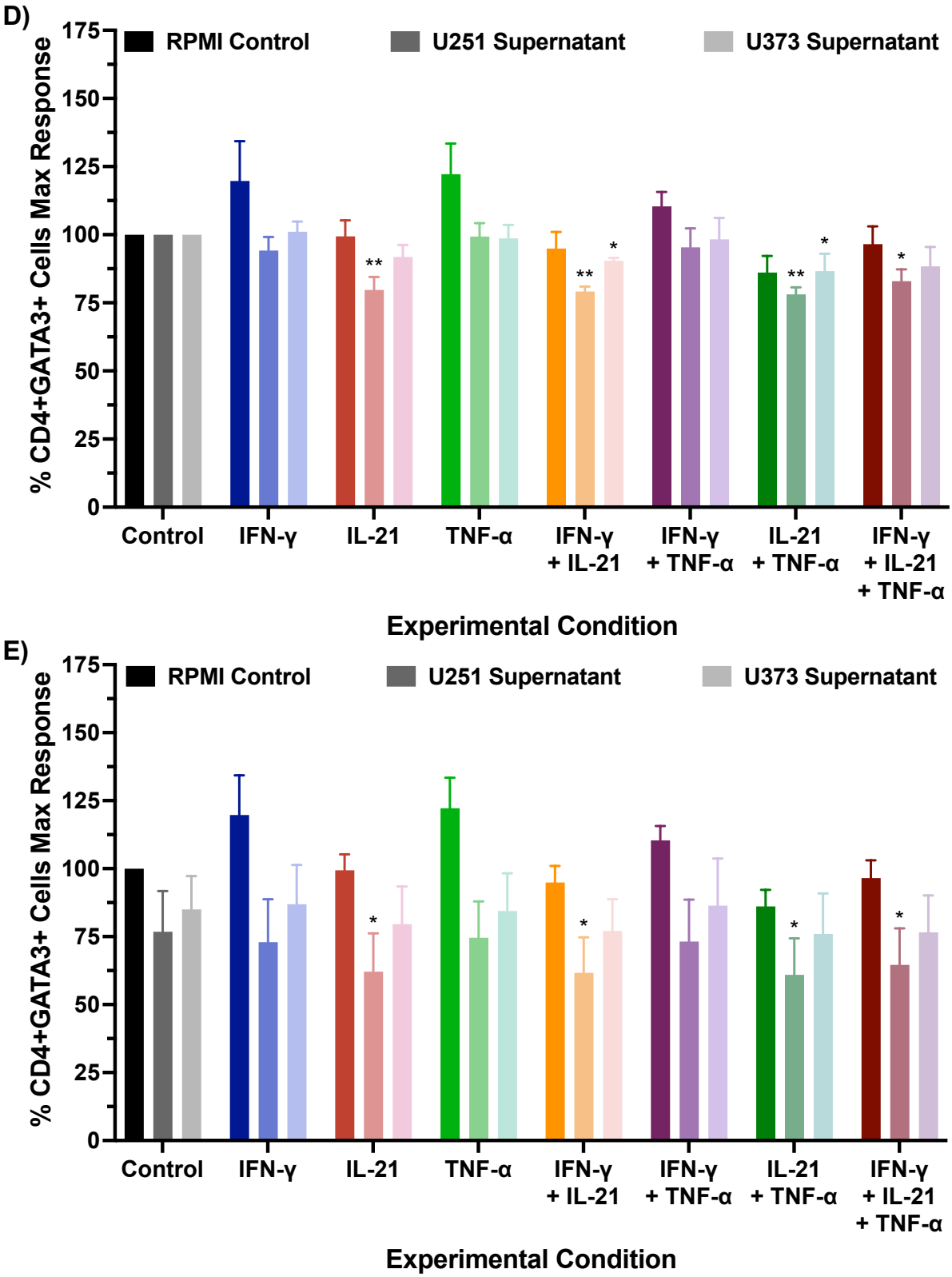


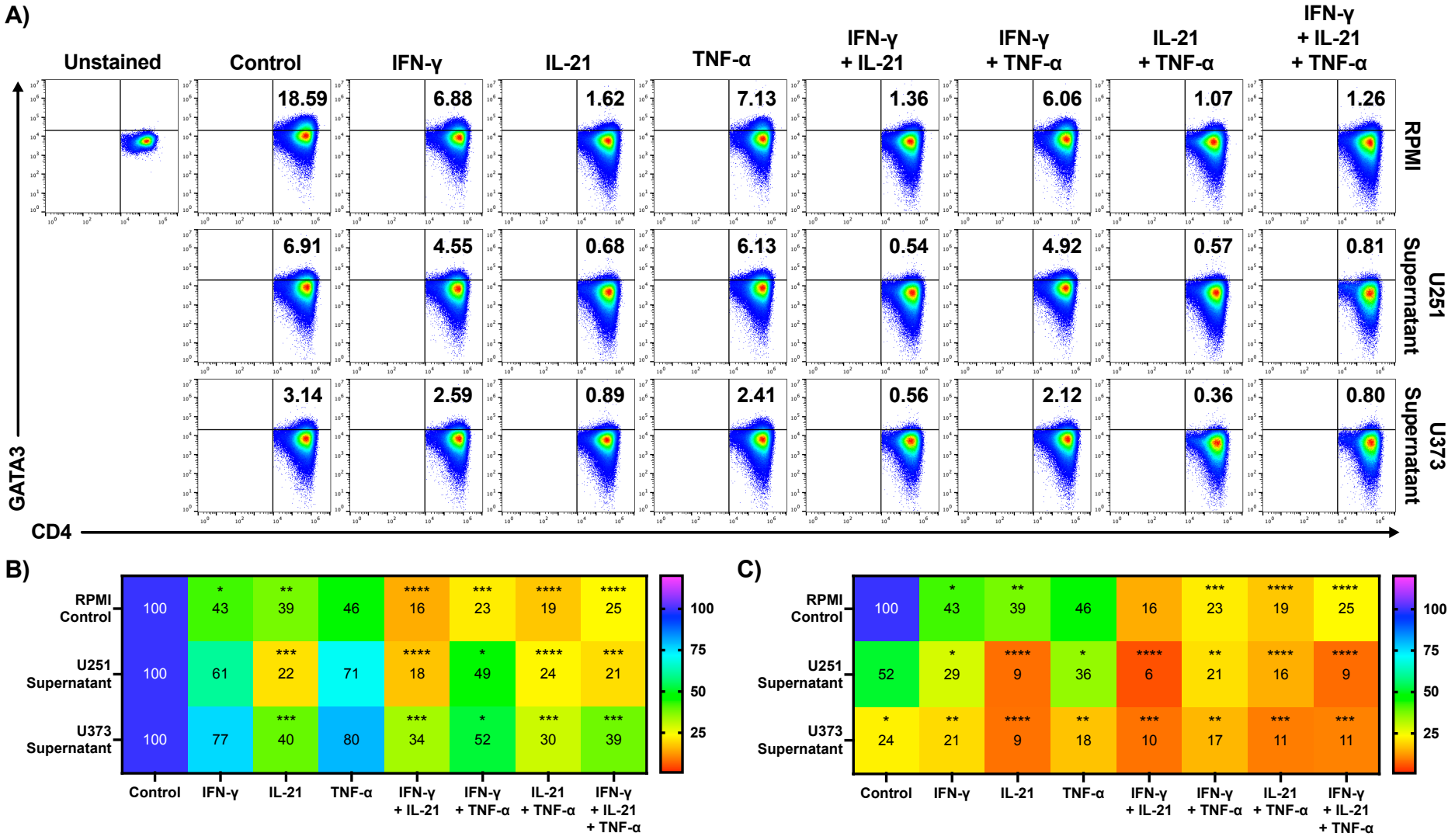
Figure 6.7. IFN-γ, IL-21 and TNF-α variably affected GATA3 expression in bulk CD4+ T cells

$2.5x10^4$ bulk CD4+ T cells were cultured 1:1 with CD3/CD28 beads in RPMI, U251 cell supernatant or U373 cell supernatant, plus treatment with 100ng/ml IFN-γ, IL-21 and TNF-α, both individually and combined for 5 days. Cells were then harvested and stained for CD4 and GATA3 for analysis via flow cytometry. Flow plots **A)** are representative of > 3 independent experiments. Heat Maps and Bars represent % CD4+GATA3+ cells as means compared to the relevant Control **B)** and **D)**, plus % CD4+GATA3+ cells as means compared to the RPMI Control **C)** and **E)** with SEM shown for > 3 independent experiments. P Values: * = <0.05; ** = <0.01; *** = <0.001; **** = <0.0001.

In contrast with the results seen in bulk CD4+ T cells, both IFN-γ (43% - p = 0.0489) and IL-21 (39% - p = 0.0025) were able to independently downregulate GATA3 expression in naïve CD4+CD45RA+ T cells. A similar effect was seen when naïve CD4+CD45RA+ T cells were treated with TNF-α alone, but this was not statistically significant (46% - p = 0.0557) (Figures 6.8B and D). In line with this, each of the dual treatments significantly reduced GATA3 expression, with a remarkably potent response visible when naïve CD4+CD45RA+ T cells were treated with IFN-γ, IL-21 and TNF-α (25% - p < 0.0001) (Figures 6.8B and D).

On the other hand, only IL-21 (22% - p = 0.0001) was able to independently inhibit GATA3 expression in naïve CD4+CD45RA+ T cells, when cultured with U251 cell supernatant (Figures 6.8B and D). The impact of IL-21 is reflected in the fact that each of the conditions containing IL-21 had the strongest effect on reducing GATA3 expression in these cells, including the triple cytokine treatment (21% - p = 0.0001). Although the U251 cell supernatant alone (52% - p = 0.1244) did not significantly alter GATA3 expression in naïve CD4+CD45RA+ T cells, the addition of IFN-γ, IL-21 and TNF-α (9% - p < 0.0001) clearly diminished GATA3 expression far below the levels seen in the RPMI Control (Figures 6.8C and E).

The same trend was evident when naïve CD4+CD45RA+ T cells were cultured with U373 cell supernatant, with the combination of IFN-γ, IL-21 and TNF-α (39% - p = 0.0009) having a notable impact, though this appeared to be primarily facilitated by IL-21 alone (40% - p = 0.0006) (Figures 6.8B and D). Normalising the data against the RPMI Control revealed that the U373 cell supernatant alone (24% - p = 0.0184) significantly reduced GATA3 expression in naïve CD4+CD45RA+ T cells, which was further enhanced with the addition of IFN-γ, IL-21 and TNF-α (11% - p = 0.0002) (Figures 6.8C and E). Collectively, these findings suggested that the glioblastoma secretome significantly reduced GATA3 expression in naïve CD4+CD45RA+ T cells, but this was enhanced with the addition of IFN-γ, IL-21 and TNF-α.


A)
Unstained
Control
IFN-γ
IL-21
TNF-α
IFN-γ + IL-21
IFN-γ + TNF-α
IL-21 + TNF-α
IFN-γ + IL-21 + TNF-α
18.59
6.88
1.62
7.13
1.36
6.06
1.07
1.26
RPMI
6.91
4.55
0.68
6.13
0.54
4.92
0.57
0.81
U251 Supernatant
3.14
2.59
0.89
2.41
0.56
2.12
0.36
0.80
U373 Supernatant
GATA3
CD4
B)
RPMI Control
U251 Supernatant
U373 Supernatant
100 * 43 ** 39 46 **** 16 *** 23 **** 19 **** 25
100 *** 61 22 71 **** 18 * 49 **** 24 *** 21
100 *** 77 40 80 *** 34 * 52 *** 30 *** 39
Control
IFN-γ
IL-21
TNF-α
IFN-γ + IL-21
IFN-γ + TNF-α
IL-21 + TNF-α
IFN-γ + IL-21 + TNF-α
100
75
50
25
C)
RPMI Control
U251 Supernatant
U373 Supernatant
100 * 43 ** 39 46 16 *** 23 **** 19 **** 25
52 * 29 **** 9 * 36 **** 6 ** 21 **** 16 **** 9
* 24 ** 21 **** 9 ** 18 *** 10 ** 17 *** 11 *** 11
Control
IFN-γ
IL-21
TNF-α
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IL-21 + TNF-α
IFN-γ + IL-21 + TNF-α
100
75
50
25

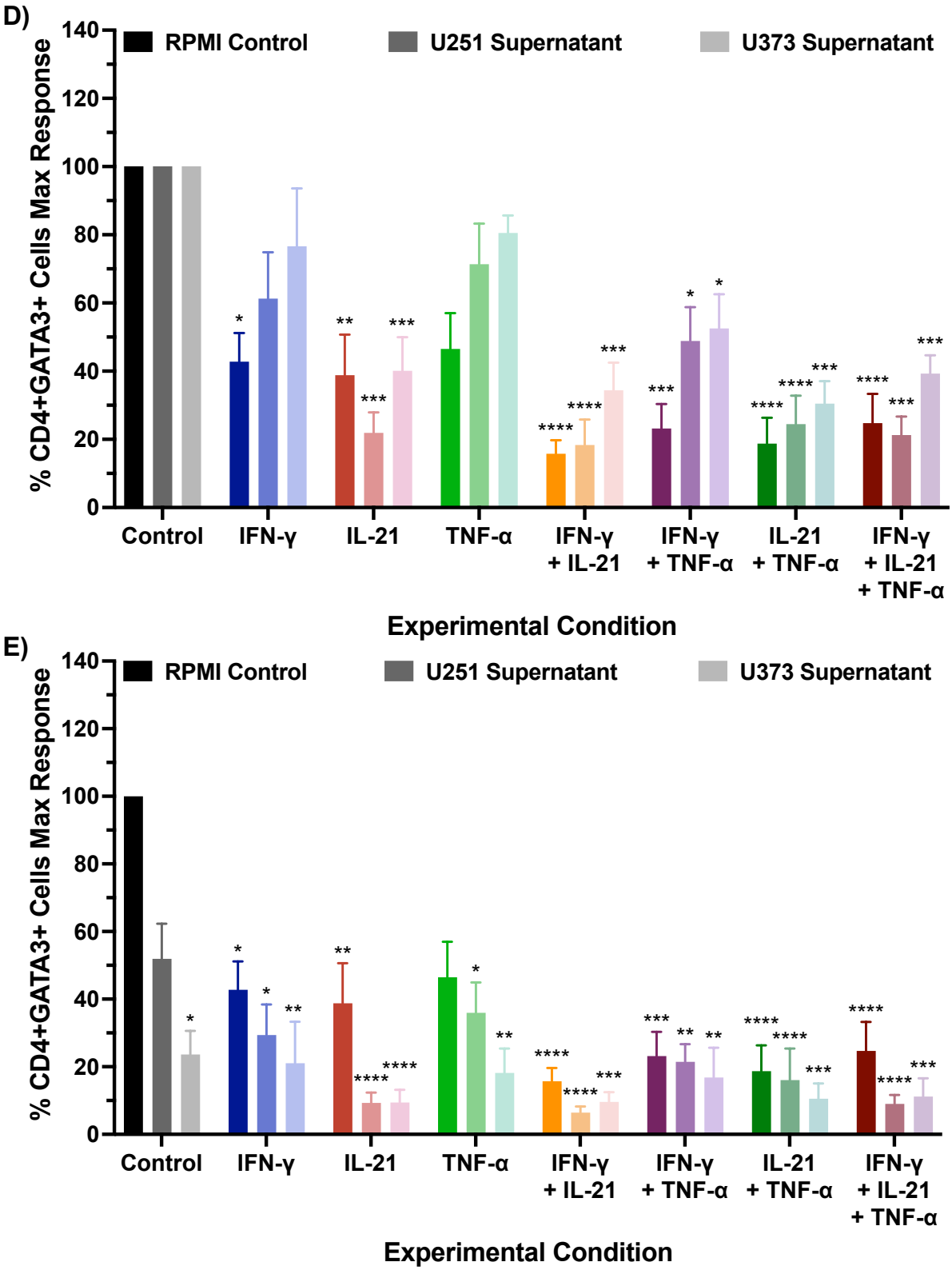


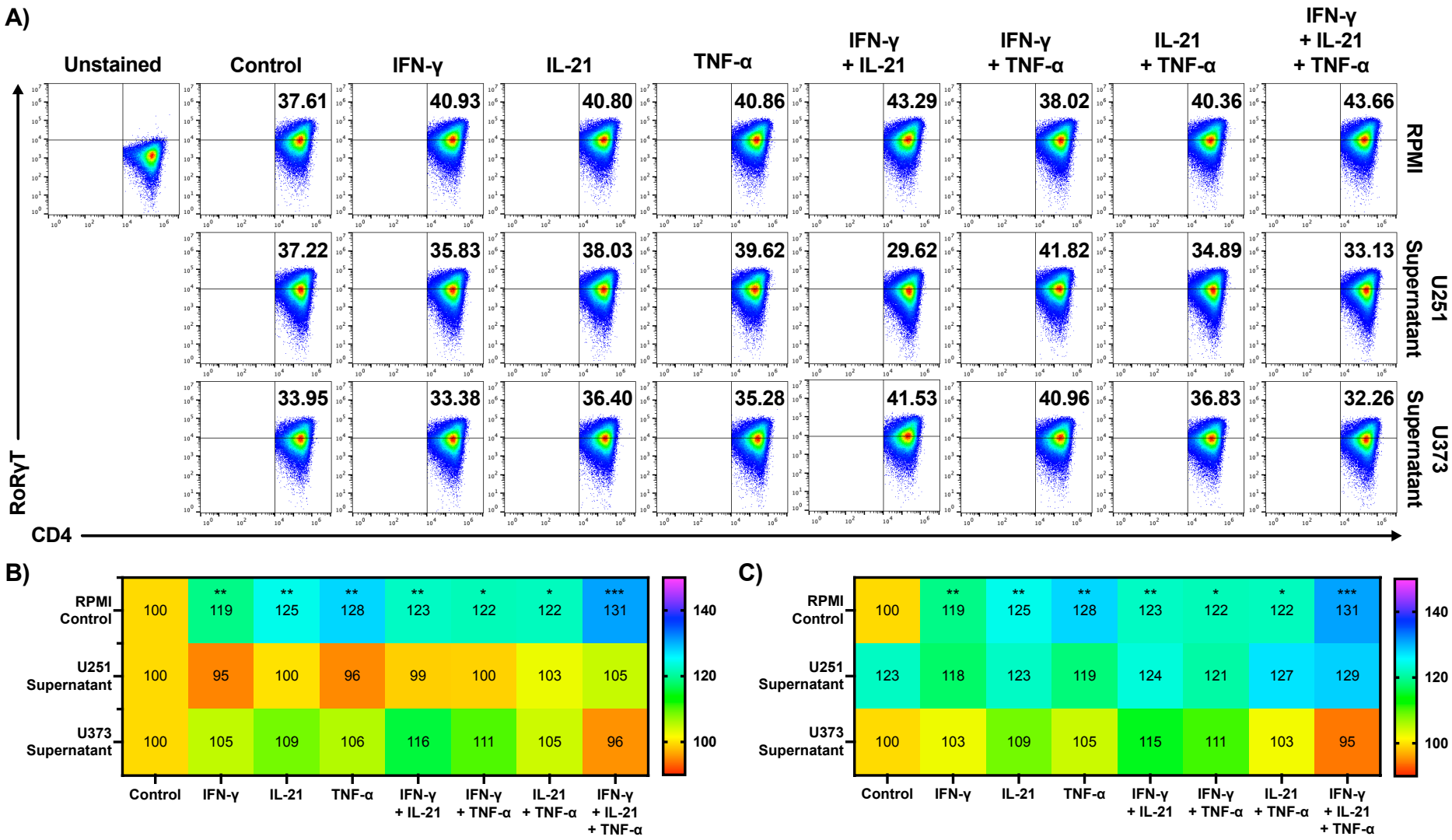
Figure 6.8. IFN-γ, IL-21 and TNF-α downregulated GATA3 expression in naïve CD4+CD45RA+ T cells

$2.5x10^4$ naïve CD4+CD45RA+ T cells were cultured 1:1 with CD3/CD28 beads in RPMI, U251 cell supernatant or U373 cell supernatant, plus treatment with 100ng/ml IFN-γ, IL-21 and TNF-α, both individually and combined for 5 days. Cells were then harvested and stained for CD4 and GATA3 for analysis via flow cytometry. Flow plots **A)** are representative of > 3 independent experiments. Heat Maps and Bars represent % CD4+GATA3+ cells as means compared to the relevant Control **B)** and **D)**, plus % CD4+GATA3+ cells as means compared to the RPMI Control **C)** and **E)** with SEM shown for > 3 independent experiments. P Values: * = <0.05; ** = <0.01; *** = <0.001; **** = <0.0001.

6.2.5. IFN-γ, IL-21 and TNF-α maintained Th17-associated RoRγT expression in bulk CD4+ and naïve CD4+CD45RA+ T cells cultured with glioblastoma supernatant

As Madkouri et al. (2017) correlated Th17 cells with poor survival in glioblastoma patients, it was important to consider the impact of the glioblastoma secretome on RoRγT expression in CD4+ T cells and explore whether this could be influenced by IFN-γ, IL-21 and TNF-α. In order to explore this, bulk CD4+ and naïve CD4+CD45RA+ T cells were isolated from healthy human leukocyte cones, before they were subsequently cultured with CD3/CD28 beads for 5 days. Within these culture conditions, both T cell subsets were exposed to U251 or U373 cell supernatant, alongside treatment with IFN-γ, IL-21 and/or TNF-α, before they were harvested and stained for CD4 and RoRγT for flow cytometric analysis. The data produced was normalised against the untreated Controls for each media condition, though the results from experiments cultured with U251 or U373 cell supernatant were also compared against the RPMI Control, in order to fully capture the influence of the glioblastoma secretome and/or cytokine treatment on RoRγT expression in bulk CD4+ and naïve CD4+CD45RA+ T cells.

Interestingly, all of the cytokine conditions upregulated RoRγT expression in bulk CD4+ T cells, with the largest response seen with IFN-γ, IL-21 and TNF-α treatment (131% - $p = 0.0001$). In contrast, none of the cytokines significantly affected RoRγT expression, when bulk CD4+ T cells were cultured with U251 cell supernatant (Figures 6.9B and D). Furthermore, when the data was compared against the RPMI Control, this revealed that the U251 cell supernatant alone did not influence RoRγT expression in bulk CD4+ T cells (123% - $p = 0.4568$), thus this was maintained with IFN-γ, IL-21 and TNF-α treatment (129% - $p = 0.4837$) (Figures 6.9C and E). Similarly, neither the U373 cell supernatant alone (100% - $p = 0.5744$) or in combination with any of the cytokine conditions had an impact on RoRγT expression in bulk CD4+ T cells, relative to the RPMI Control (Figures 6.9C and E). Altogether, these results showed that the glioblastomas secretome did not affect RoRγT expression in bulk CD4+ T cells, nor was this influenced by IFN-γ, IL-21 and TNF-α treatment.


A)
Unstained
Control
IFN-γ
IL-21
TNF-α
IFN-γ + IL-21
IFN-γ + TNF-α
IL-21 + TNF-α
IFN-γ + IL-21 + TNF-α
RPMI
U251 Supernatant
U373 Supernatant
RoRγT
CD4
37.61
40.93
40.80
40.86
43.29
38.02
40.36
43.66
37.22
35.83
38.03
39.62
29.62
41.82
34.89
33.13
33.95
33.38
36.40
35.28
41.53
40.96
36.83
32.26
B)
RPMI Control
U251 Supernatant
U373 Supernatant
100 119 125 128 123 122 122 131
100 95 100 96 99 100 103 105
100 105 109 106 116 111 105 96
** ** ** ** * * ***
Control
IFN-γ
IL-21
TNF-α
IFN-γ + IL-21
IFN-γ + TNF-α
IL-21 + TNF-α
IFN-γ + IL-21 + TNF-α
140
120
100
C)
RPMI Control
U251 Supernatant
U373 Supernatant
100 119 125 128 123 122 122 131
123 118 123 119 124 121 127 129
100 103 109 105 115 111 103 95
** ** ** ** * * ***
Control
IFN-γ
IL-21
TNF-α
IFN-γ + IL-21
IFN-γ + TNF-α
IL-21 + TNF-α
IFN-γ + IL-21 + TNF-α
140
120
100

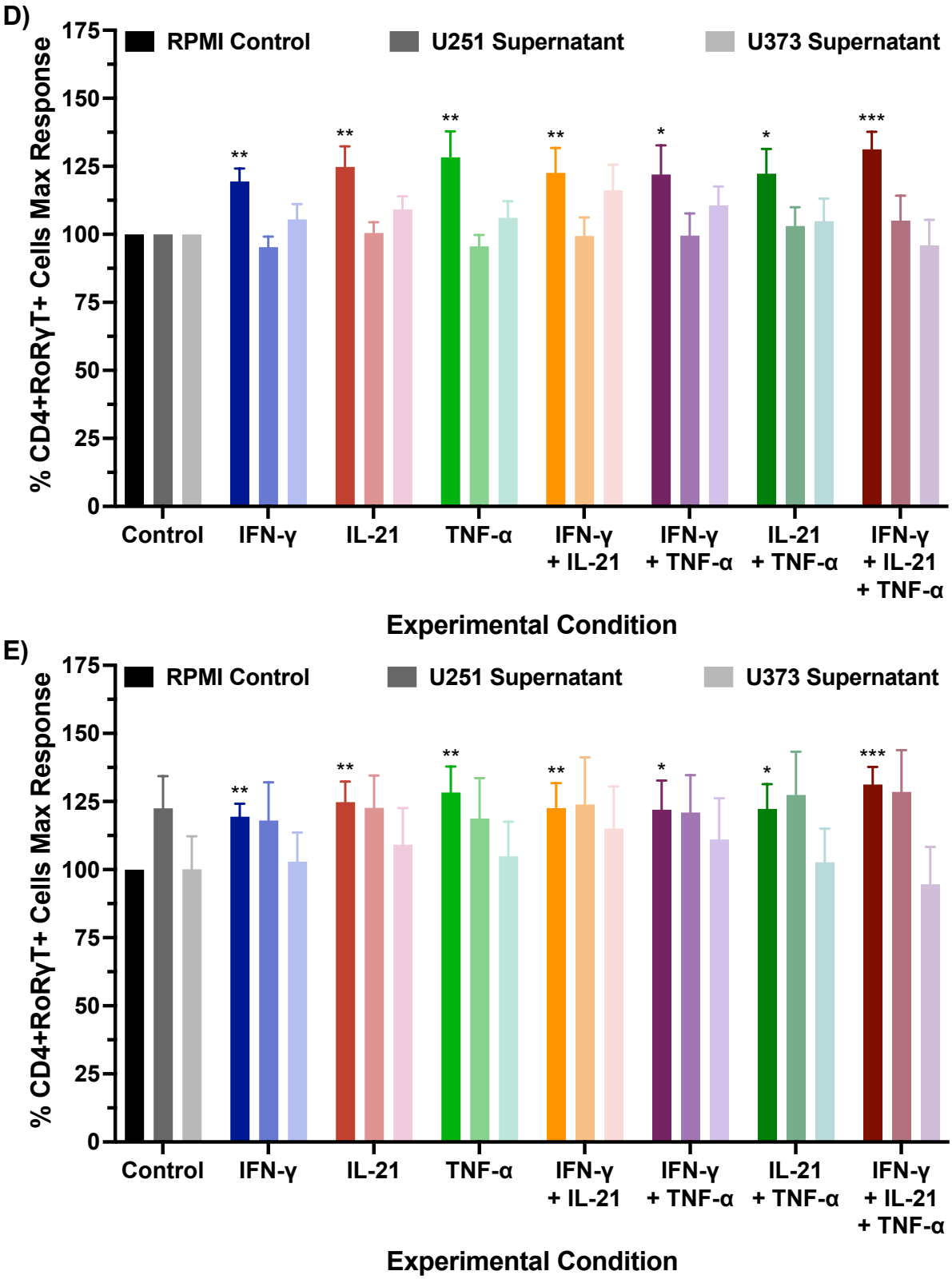
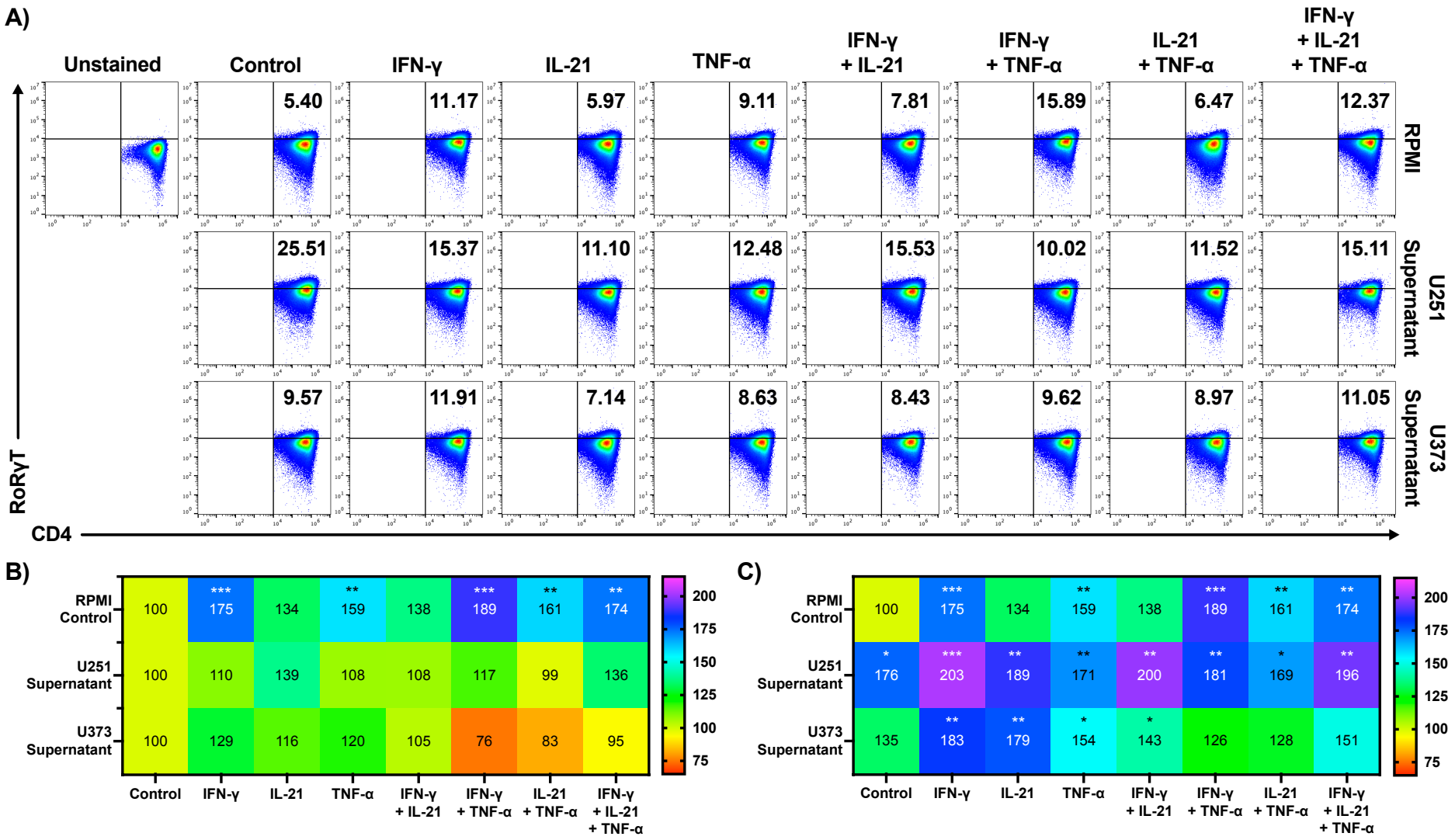


Figure 6.9. IFN-γ, IL-21 and TNF-α upregulated RoRγT expression in bulk CD4+ T cells

$2.5x10^4$ bulk CD4+ T cells were cultured 1:1 with CD3/CD28 beads in RPMI, U251 cell supernatant or U373 cell supernatant, plus treatment with 100ng/ml IFN-γ, IL-21 and TNF-α, both individually and combined for 5 days. Cells were then harvested and stained for CD4 and RoRγT for analysis via flow cytometry. Flow plots **A)** are representative of > 3 independent experiments. Heat Maps and Bars represent % CD4+RoRγT+ cells as means compared to the relevant Control **B)** and **D)**, plus % CD4+RoRγT + cells as means compared to the RPMI Control **C)** and **E)** with SEM shown for > 3 independent experiments. P Values: * = <0.05; ** = <0.01; *** = <0.001; **** = <0.0001.

In line with the results seen in bulk CD4+ T cells, IFN-γ (175% - p = 0.0004) and TNF-α (159% - p = 0.0079) independently upregulated RoRγT expression in naïve CD4+CD45RA+ T cells. Although IL-21 alone (134% - p = 0.0832) did not produce a statistically significant result, this did not compromise the efficacy of the triple cytokine condition (174% - p = 0.0043) in increasing RoRγT expression (Figures 6.10B and D). Conversely, none of the cytokines demonstrated any impact on RoRγT expression when the naïve CD4+CD45RA+ T cells were cultured with either U251 or U373 cell supernatant. Normalising the data against the RPMI Control revealed that the U251 cell supernatant alone (176% - p = 0.0316) upregulated RoRγT expression, thus the level of expression was maintained with the combination of IFN-γ, IL-21 and TNF-α (196% - p = 0.0027). While a similar trend was present when naïve CD4+CD45RA+ T cells were cultured with U373 cell supernatant (135% - p = 0.0892), this was not statistically significant, thus administering the triple cytokine treatment proved to have no effect on RoRγT expression, relative to the RPMI Control (151% - p = 0.0535) (Figures 6.10C and E). Overall, these results suggested that the glioblastoma secretome could upregulate RoRγT expression in naïve CD4+CD45RA+ T cells, but this was maintained with IFN-γ, IL-21 and TNF-α treatment.


A)
Unstained
Control
IFN-γ
IL-21
TNF-α
IFN-γ + IL-21
IFN-γ + TNF-α
IL-21 + TNF-α
IFN-γ + IL-21 + TNF-α
RPMI
U251 Supernatant
U373 Supernatant
RoRγT
CD4
5.40
11.17
5.97
9.11
7.81
15.89
6.47
12.37
25.51
15.37
11.10
12.48
15.53
10.02
11.52
15.11
9.57
11.91
7.14
8.63
8.43
9.62
8.97
11.05
B)
RPMI Control
U251 Supernatant
U373 Supernatant
100 *** 175 134 ** 159 138 *** 189 ** 161 ** 174
100 110 139 108 108 117 99 136
100 129 116 120 105 76 83 95
Control
IFN-γ
IL-21
TNF-α
IFN-γ + IL-21
IFN-γ + TNF-α
IL-21 + TNF-α
IFN-γ + IL-21 + TNF-α
200
175
150
125
100
75
C)
RPMI Control
U251 Supernatant
U373 Supernatant
100 *** 175 134 ** 159 138 *** 189 ** 161 ** 174
* 176 *** 203 ** 189 ** 171 ** 200 ** 181 * 169 ** 196
135 ** 183 ** 179 * 154 * 143 126 128 151
Control
IFN-γ
IL-21
TNF-α
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175
150
125
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75

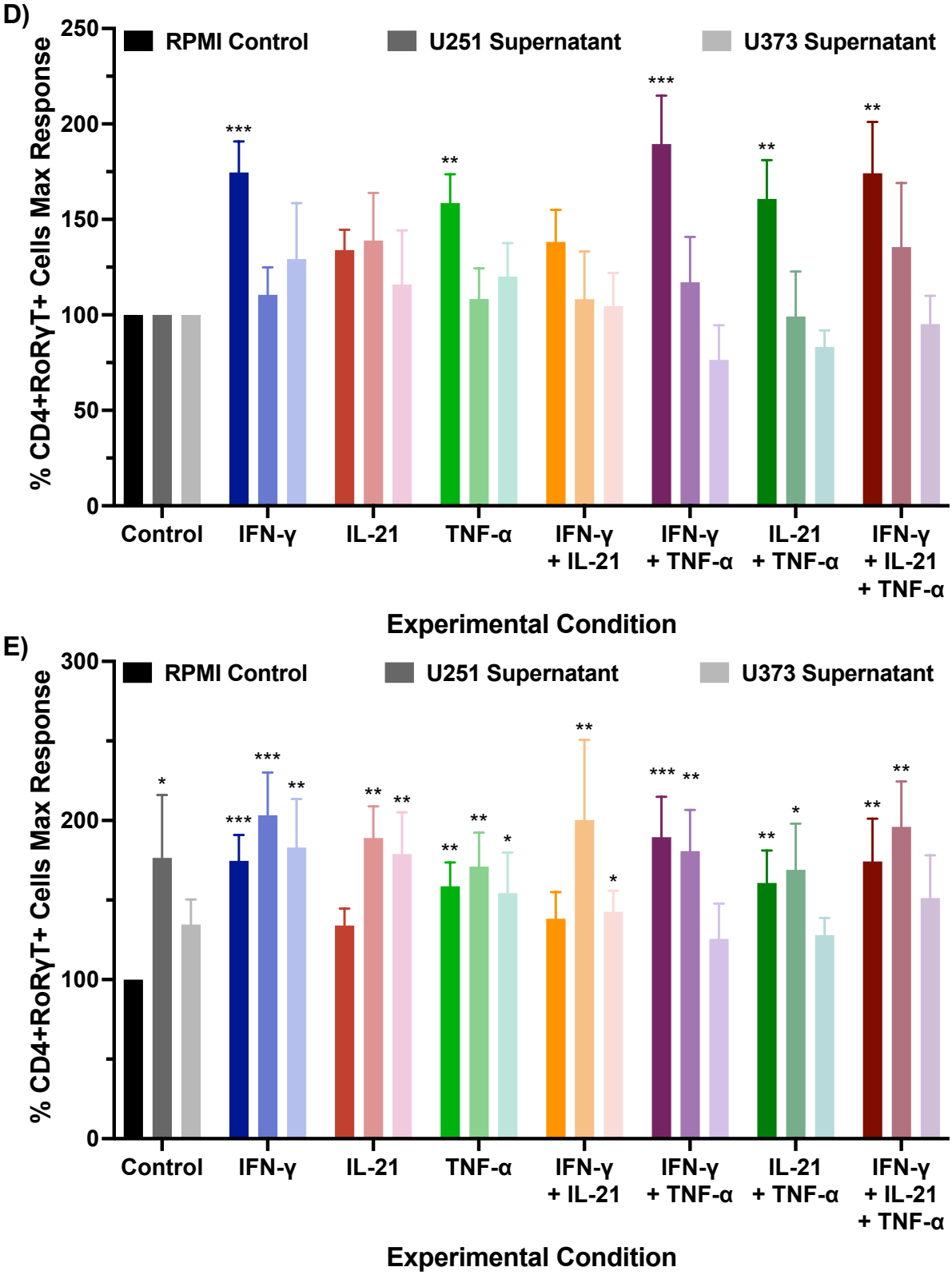


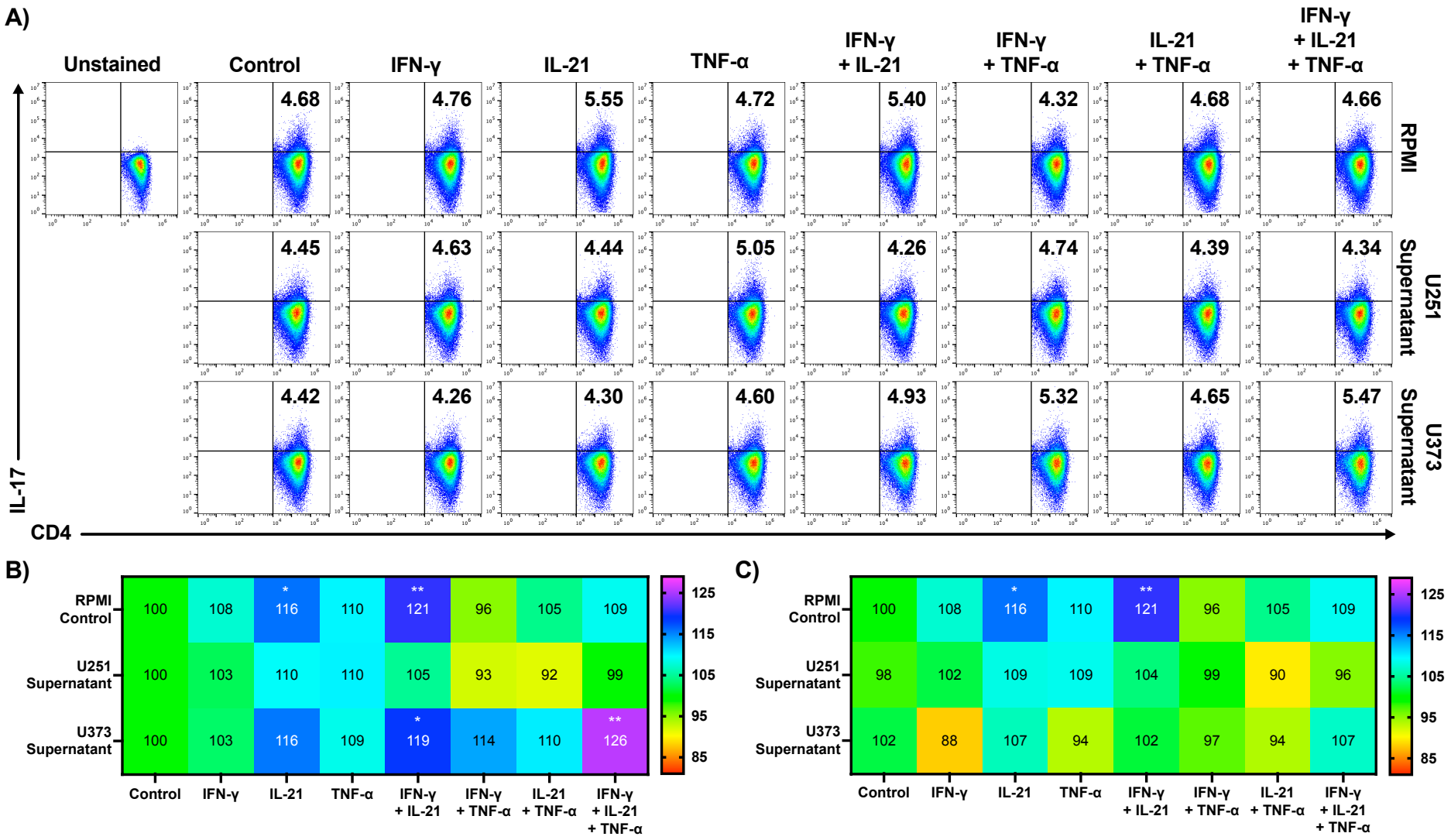
Figure 6.10. IFN-γ, IL-21 and TNF-α upregulated RoRγT expression in naïve CD4+CD45RA+ T cells

$2.5x10^4$ naïve CD4+CD45RA+ T cells were cultured 1:1 with CD3/CD28 beads in RPMI, U251 cell supernatant or U373 cell supernatant, plus treatment with 100ng/ml IFN-γ, IL-21 and TNF-α, both individually and combined for 5 days. Cells were then harvested and stained for CD4 and RoRγT for analysis via flow cytometry. Flow plots **A)** are representative of > 3 independent experiments. Heat Maps and Bars represent % CD4+RoRγT+ cells as means compared to the relevant Control **B)** and **D)**, plus % CD4+RoRγT+ cells as means compared to the RPMI Control **C)** and **E)** with SEM shown for > 3 independent experiments. P Values: * = <0.05; ** = <0.01; *** = <0.001; **** = <0.0001.

6.2.6. IFN-γ, IL-21 and TNF-α largely did not affect Th17-associated IL-17 expression in bulk CD4+ and naïve CD45RA+ T cells cultured with glioblastoma supernatant

Previous research has linked high IL-17 expression with an increase in glioblastoma angiogenesis, migration and invasion (Zheng et al., 2019). As a result of this, it was important to determine the influence of the glioblastoma secretome and explore the impact of IFN-γ, IL-21 and TNF-α on IL-17 expression in CD4+ T cells. In order to explore this, bulk CD4+ and naïve CD4+CD45RA+ T cells were isolated from healthy human leukocyte cones, before they were subsequently cultured with CD3/CD28 beads for 5 days. Within these culture conditions, both T cell subsets were exposed to U251 or U373 cell supernatant, alongside treatment with IFN-γ, IL-21 and/or TNF-α, before they were harvested and stained for CD4 and IL-17 for flow cytometric analysis. The data produced was normalised against the untreated Controls for each media condition – however, the results from experiments cultured with U251 or U373 cell supernatant were also compared against the RPMI Control, in order to fully capture the influence of the glioblastoma secretome and/or cytokine treatment on IL-17 expression in bulk CD4+ and naïve CD4+CD45RA+ T cells.

Results from the Control condition revealed that IL-21 alone (116% - $p = 0.0139$) and in combination with IFN-γ (121% - $p = 0.0040$) significantly upregulated IL-17 expression in bulk CD4+ T cells (Figures 6.11B and D). Although, IL-21 did not independently impact IL-17 expression when bulk CD4+ T cells were cultured with U373 cell supernatant (116% - $p = 0.0744$), IFN-γ and IL-21 (119% - $p = 0.0407$), as well as the triple cytokine condition (126% - $p = 0.0095$) both increased IL-17 expression in bulk CD4+ T cells. However, the influence of these cytokines was lost when the data was normalised against the RPMI control – instead this highlighted that IFN-γ alone (88% - $p = 0.2098$) could slightly reduce IL-17 expression when bulk CD4+ T cells were cultured with U373 cell supernatant, though the effect was not statistically significant (Figures 6.11C and E). On the other hand, U251 cell supernatant with/without IFN-γ, IL-21 and TNF-α had no impact on IL-17 expression in bulk CD4+ T cells (Figure 6.11). Collectively, these findings suggested that neither the glioblastoma secretome nor IFN-γ, IL-21 and TNF-α treatment affected IL-17 expression in bulk CD4+ T cells.


A)
Unstained
Control
IFN-γ
IL-21
TNF-α
IFN-γ + IL-21
IFN-γ + TNF-α
IL-21 + TNF-α
IFN-γ + IL-21 + TNF-α
RPMI
U251 Supernatant
U373 Supernatant
IL-17
CD4
4.68 4.76 5.55 4.72 5.40 4.32 4.68 4.66
4.45 4.63 4.44 5.05 4.26 4.74 4.39 4.34
4.42 4.26 4.30 4.60 4.93 5.32 4.65 5.47
B)
RPMI Control 100 108 * 116 110 ** 121 96 105 109
U251 Supernatant 100 103 110 110 105 93 92 99
U373 Supernatant 100 103 116 109 * 119 114 110 ** 126
Control IFN-γ IL-21 TNF-α IFN-γ + IL-21 IFN-γ + TNF-α IL-21 + TNF-α IFN-γ + IL-21 + TNF-α
125 115 105 95 85
C)
RPMI Control 100 108 * 116 110 ** 121 96 105 109
U251 Supernatant 98 102 109 109 104 99 90 96
U373 Supernatant 102 88 107 94 102 97 94 107
Control IFN-γ IL-21 TNF-α IFN-γ + IL-21 IFN-γ + TNF-α IL-21 + TNF-α IFN-γ + IL-21 + TNF-α
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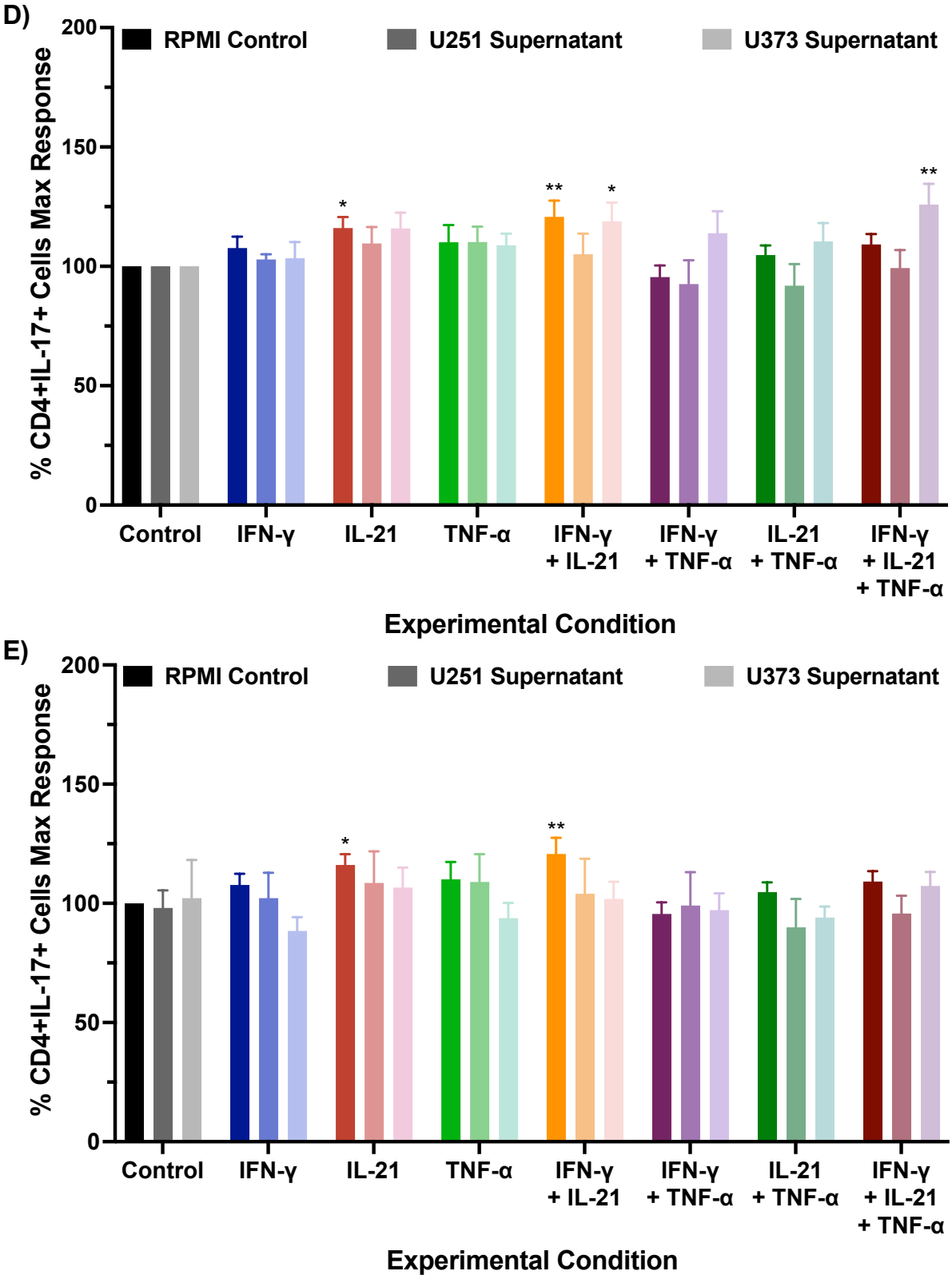
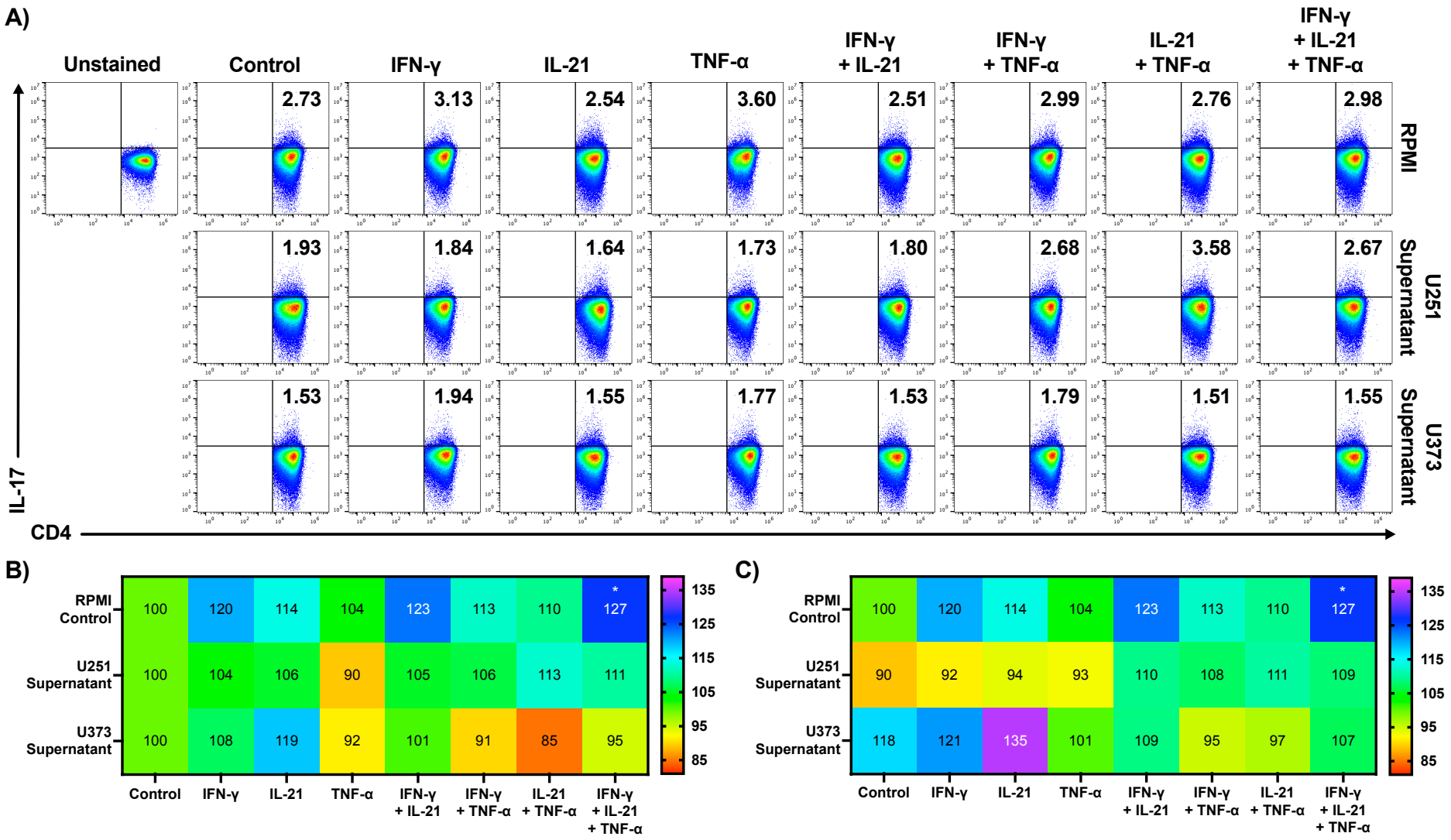


Figure 6.11. IFN-γ, IL-21 and TNF-α did not affect IL-17 expression in bulk CD4+ T cells

$2.5x10^4$ bulk CD4+ T cells were cultured 1:1 with CD3/CD28 beads in RPMI, U251 cell supernatant or U373 cell supernatant, plus treatment with 100ng/ml IFN-γ, IL-21 and TNF-α, both individually and combined for 5 days. Cells were then harvested and stained for CD4 and IL-17 for analysis via flow cytometry. Flow plots **A)** are representative of > 3 independent experiments. Heat Maps and Bars represent % CD4+IL-17+ cells as means compared to the relevant Control **B)** and **D)**, plus % CD4+IL-17 + cells as means compared to the RPMI Control **C)** and **E)** with SEM shown for > 3 independent experiments. P Values: * = <0.05; ** = <0.01; *** = <0.001; **** = <0.0001.

In the Control condition, there was a slight trend to suggest that IFN-γ could independently upregulate IL-17 in naïve CD4+CD45RA+ T cells (120% - p = 0.1864), which was maintained in the IFN-γ and IL-21 dual treatment (123% - p = 0.0819) and marginally enhanced in the triple cytokine condition (127% - p = 0.0437) (Figures 6.12B and D). However, the same results were not present when naïve CD4+CD45RA+ T cells were cultured with U251 cell supernatant – instead, the data suggested that TNF-α alone (90% - p = 0.3283) could possibly downregulate IL-17 expression, though this effect was not statistically significant (Figures 6.12B and D). A similar trend was present when naïve CD4+CD45RA+ T cells were cultured with U373 cell supernatant, as TNF-α independently reduced IL-17 expression (92% - p = 0.8512), which was sustained across the remaining cytokine conditions, including IFN-γ, IL-21 and TNF-α (95% - p = 0.8073) (Figures 6.12B and D).

Compared to the RPMI Control, results showed that U251 cell supernatant could slightly reduce IL-17 expression (90% - p = 0.8376), but this was restored to similar levels as the RPMI Control with IFN-γ, IL-21 and TNF-α treatment (109% - p = 0.2379) (Figures 6.12C and E). Similarly, normalising the data from the naïve CD4+CD45RA+ T cells cultured with U373 cell supernatant against the RPMI Control revealed that the U373 cell supernatant alone could marginally increase IL-17 expression (118% - p = 0.3219), but this was neutralised with the addition of IFN-γ, IL-21 and TNF-α (107% - p = 0.5175) (Figures 6.12C and E). Altogether, these results indicated that neither the glioblastoma secretome nor IFN-γ, IL-21 and TNF-α had a significant impact on IL-17 expression in naïve CD4+CD45RA+ T cells.


A)
Unstained
Control
IFN-γ
IL-21
TNF-α
IFN-γ + IL-21
IFN-γ + TNF-α
IL-21 + TNF-α
IFN-γ + IL-21 + TNF-α
2.73
3.13
2.54
3.60
2.51
2.99
2.76
2.98
1.93
1.84
1.64
1.73
1.80
2.68
3.58
2.67
1.53
1.94
1.55
1.77
1.53
1.79
1.51
1.55
RPMI
U251 Supernatant
U373 Supernatant
IL-17
CD4
B)
RPMI Control
U251 Supernatant
U373 Supernatant
100 120 114 104 123 113 110 * 127
100 104 106 90 105 106 113 111
100 108 119 92 101 91 85 95
Control
IFN-γ
IL-21
TNF-α
IFN-γ + IL-21
IFN-γ + TNF-α
IL-21 + TNF-α
IFN-γ + IL-21 + TNF-α
135
125
115
105
95
85
C)
RPMI Control
U251 Supernatant
U373 Supernatant
100 120 114 104 123 113 110 * 127
90 92 94 93 110 108 111 109
118 121 135 101 109 95 97 107
Control
IFN-γ
IL-21
TNF-α
IFN-γ + IL-21
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IFN-γ + IL-21 + TNF-α
135
125
115
105
95
85

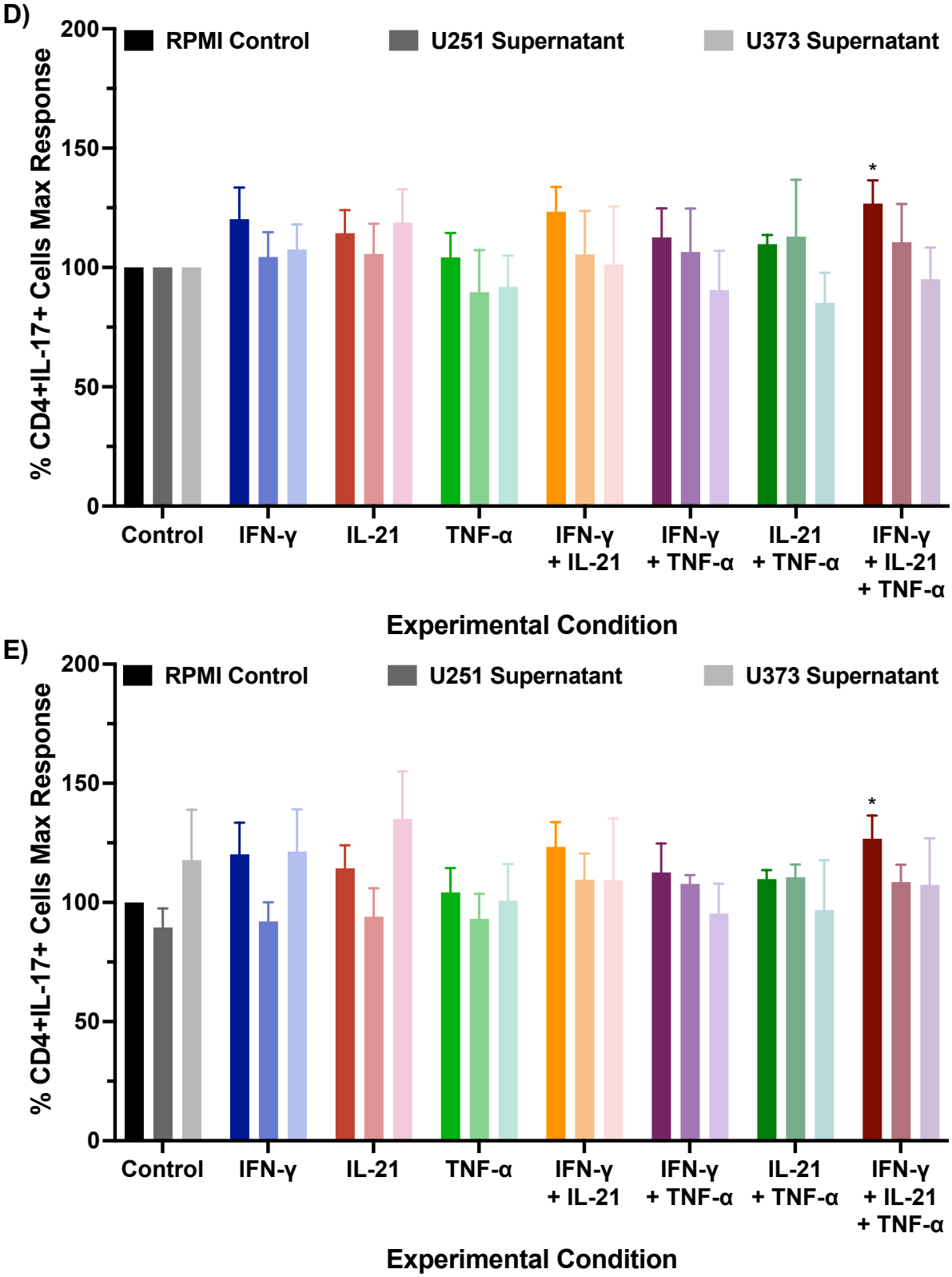


Figure 6.12. IFN-γ, IL-21 and TNF-α largely did not affect IL-17 expression in naïve CD4+CD45RA+ T cells

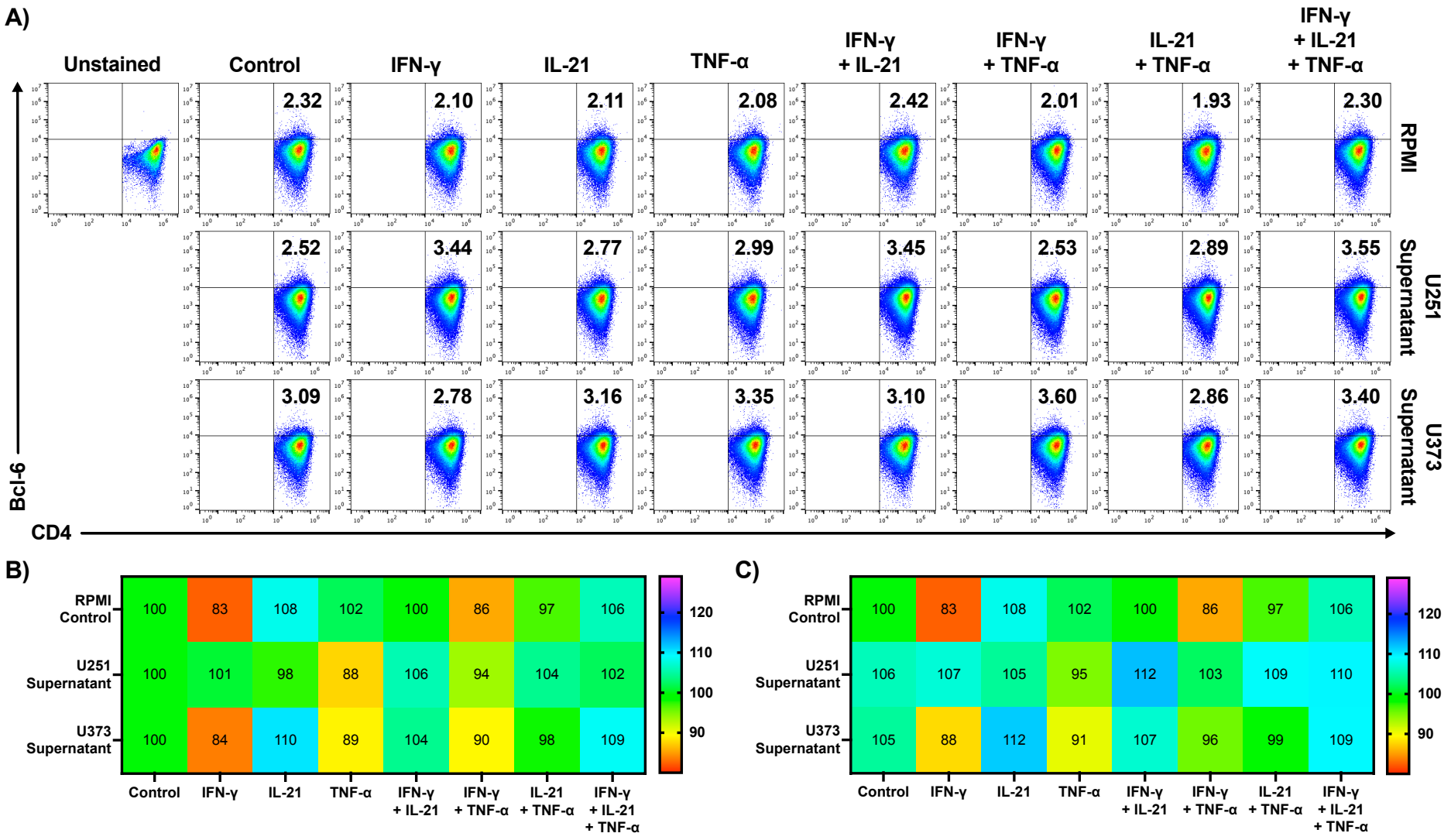
$2.5x10^4$ naïve CD4+CD45RA+ T cells were cultured 1:1 with CD3/CD28 beads in RPMI, U251 cell supernatant or U373 cell supernatant, plus treatment with 100ng/ml IFN-γ, IL-21 and TNF-α, both individually and combined for 5 days. Cells were then harvested and stained for CD4 and IL-17 for analysis via flow cytometry. Flow plots **A)** are representative of > 3 independent experiments. Heat Maps and Bars represent % CD4+IL-17+ cells as means compared to the relevant Control **B)** and **D)**, plus % CD4+IL-17 + cells as means compared to the RPMI Control **C)** and **E)** with SEM shown for > 3 independent experiments. P Values: * = <0.05; ** = <0.01; *** = <0.001; **** = <0.0001.

6.2.7. IFN-γ, IL-21 and TNF-α did not affect Tfh-associated Bcl-6 expression in bulk CD4+ and naïve CD4+CD45RA+ T cells cultured with glioblastoma supernatant

Previous research has correlated high levels of Tfh cells in glioblastoma patient samples with improved overall survival (P. Liang et al., 2020; Qiu et al., 2020). As a key marker for Tfh cells, it was important to explore whether IFN-γ, IL-21 and TNF-α could overcome the immunosuppressive nature of the glioblastoma secretome and restore Bcl-6 expression in CD4+ T cells. In order to explore this, bulk CD4+ and naïve CD4+CD45RA+ T cells were isolated from healthy human leukocyte cones, before they were subsequently cultured with CD3/CD28 beads for 5 days. Within these culture conditions, both T cell subsets were exposed to U251 or U373 cell supernatant, alongside treatment with IFN-γ, IL-21 and/or TNF-α, before they were harvested and stained for CD4 and Bcl-6 for flow cytometric analysis. The data produced was normalised against the untreated Controls for each media condition, but the results from experiments cultured with U251 or U373 cell supernatant were also compared against the RPMI Control, in order to fully capture the influence of the glioblastoma secretome and/or cytokine treatment on Bcl-6 expression in bulk CD4+ and naïve CD4+CD45RA+ T cells.

Despite the fact that none of the results were statistically significant, IFN-γ (83% - $p = 0.0762$) appeared to restrict Bcl-6 expression in bulk CD4+ T cells, which was somewhat maintained when administered alongside TNF-α (86% - $p = 0.4735$). However, the influence of IL-21 alone (108% - $p = 0.7628$) on possible Bcl-6 upregulation was further reflected in the neutralisation of IFN-γ and TNF-α in the triple cytokine condition (106% - $p = 0.8801$) (Figures 6.13B and D). In contrast, there was not a clear trend or any significant influence on Bcl-6 expression mediated by IFN-γ, IL-21 and/or TNF-α, when bulk CD4+ T cells were cultured with U251 cell supernatant (Figures 6.13B and D). Normalising the data against the RPMI Control revealed that neither the U251 cell supernatant alone (106% - $p = 0.6507$) nor in combination with any of the cytokine treatments had any impact on Bcl-6 expression in bulk CD4+ T cells (Figures 6.13C and E).

On the other hand, culturing bulk CD4+ T cells with U373 cell supernatant largely followed the same trends seen when cultured with the RPMI Control, as IFN-γ (84% - $p = 0.2156$) appeared to downregulate Bcl-6 expression, which was sustained when combined with TNF-α (90% - $p = 0.3860$). Conversely, IL-21 alone (110% - $p = 0.4329$) possibly enhanced Bcl-6 expression, thereby eliminating the inhibitory impact of IFN-γ and TNF-α in the triple cytokine condition (109% - $p = 0.8044$) (Figures 6.13B and D). However, as the U373 cell supernatant (105% - $p = 0.8781$) did not independently affect Bcl-6 expression, the influence of the cytokine treatments was ultimately comparable to the RPMI Control (Figures 6.13C and E). Overall, due to the lack of statistical significance, these findings demonstrated that the glioblastoma secretome did not affect Bcl-6 expression in bulk CD4+ T cells, nor was this influenced by IFN-γ, IL-21 and TNF-α treatment.


A)
Unstained
Control
IFN-γ
IL-21
TNF-α
IFN-γ + IL-21
IFN-γ + TNF-α
IL-21 + TNF-α
IFN-γ + IL-21 + TNF-α
RPMI
U251 Supernatant
U373 Supernatant
2.32
2.10
2.11
2.08
2.42
2.01
1.93
2.30
2.52
3.44
2.77
2.99
3.45
2.53
2.89
3.55
3.09
2.78
3.16
3.35
3.10
3.60
2.86
3.40
Bcl-6
CD4
B)
RPMI Control
U251 Supernatant
U373 Supernatant
100 83 108 102 100 86 97 106
100 101 98 88 106 94 104 102
100 84 110 89 104 90 98 109
Control
IFN-γ
IL-21
TNF-α
IFN-γ + IL-21
IFN-γ + TNF-α
IL-21 + TNF-α
IFN-γ + IL-21 + TNF-α
120
110
100
90
C)
RPMI Control
U251 Supernatant
U373 Supernatant
100 83 108 102 100 86 97 106
106 107 105 95 112 103 109 110
105 88 112 91 107 96 99 109
Control
IFN-γ
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IFN-γ + IL-21 + TNF-α
120
110
100
90

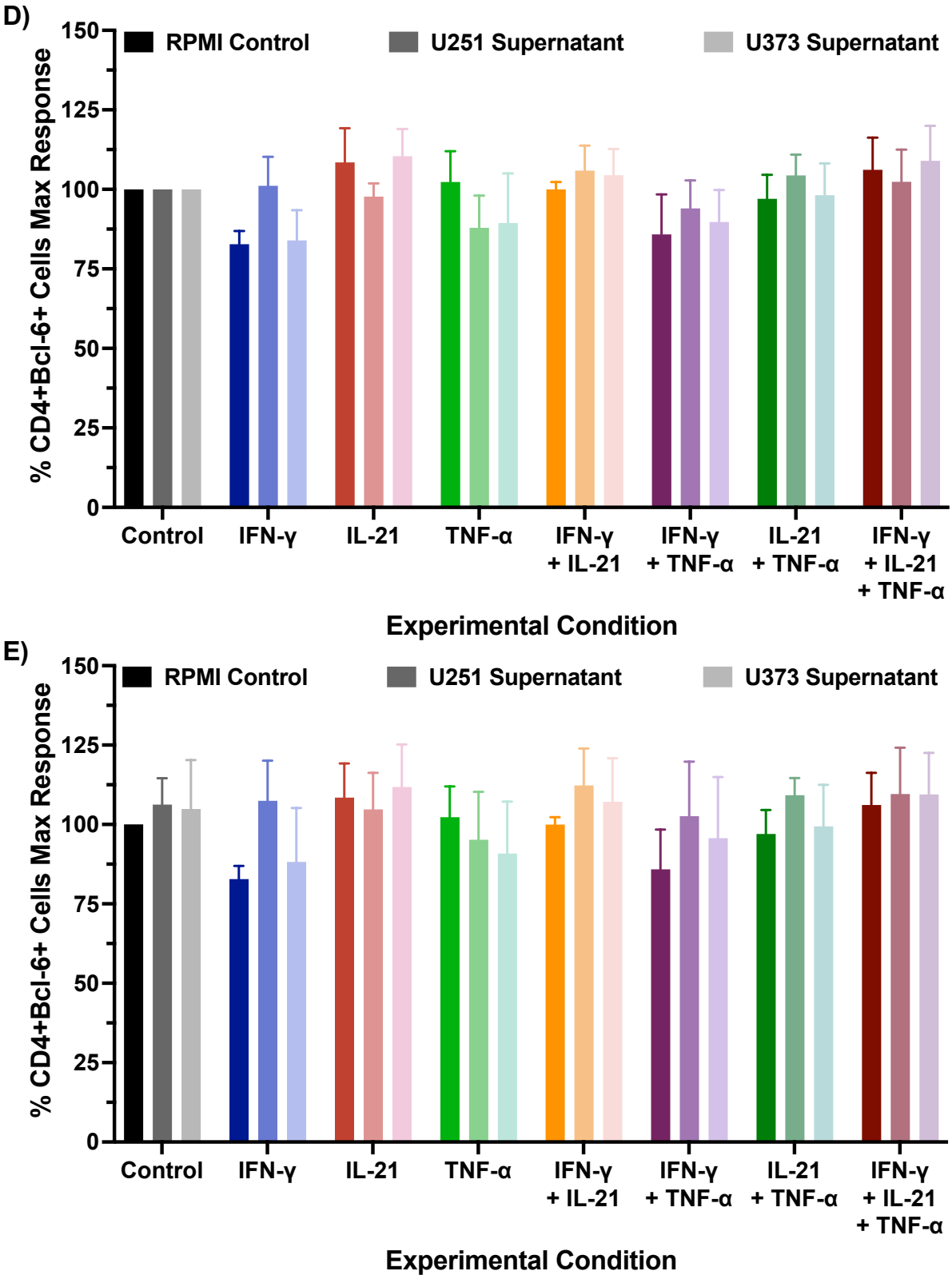
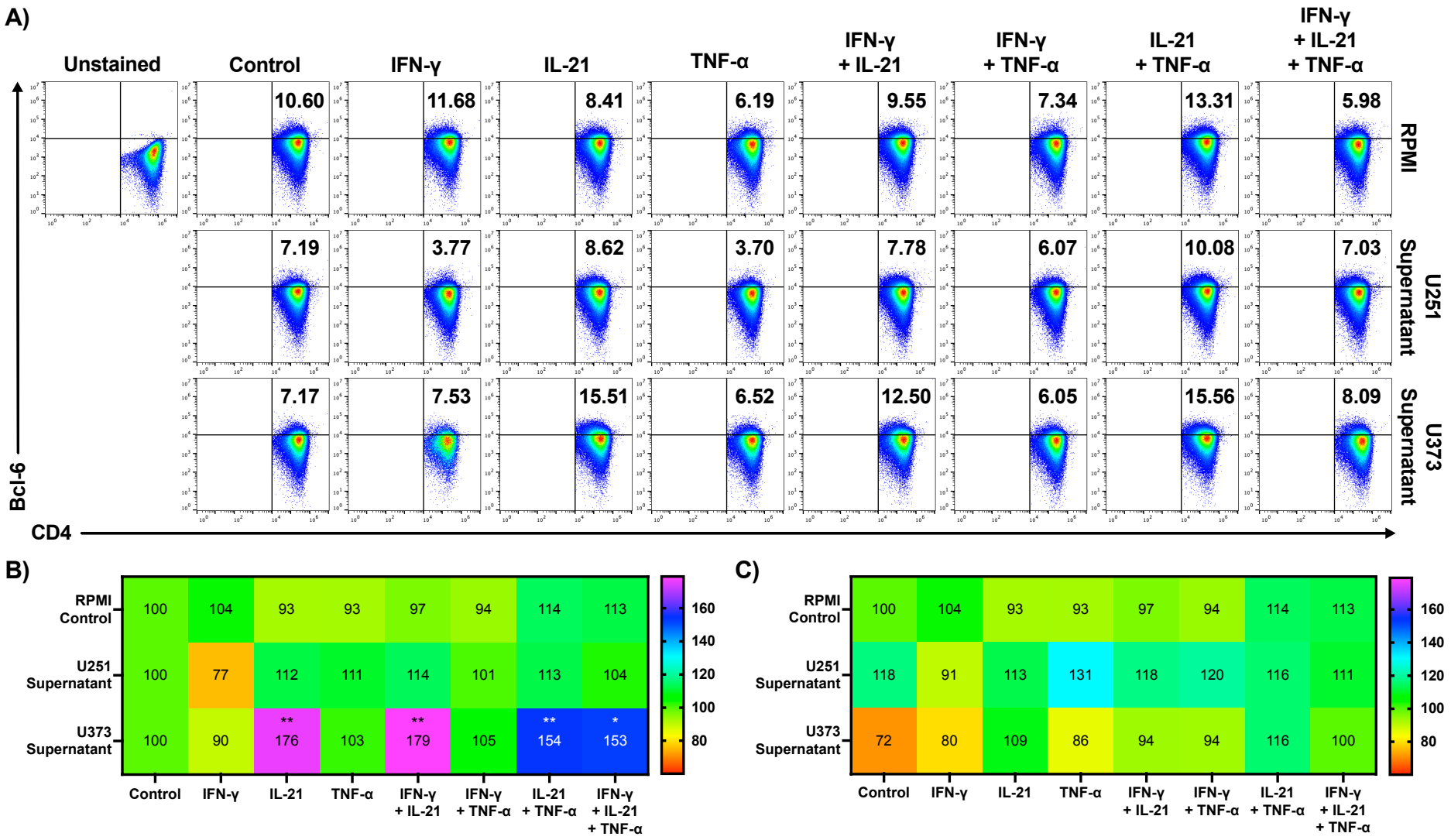


Figure 6.13. IFN-γ, IL-21 and TNF-α did not affect Bcl-6 expression in bulk CD4+ T cells

$2.5x10^4$ bulk CD4+ T cells were cultured 1:1 with CD3/CD28 beads in RPMI, U251 cell supernatant or U373 cell supernatant, plus treatment with 100ng/ml IFN-γ, IL-21 and TNF-α, both individually and combined for 5 days. Cells were then harvested and stained for CD4 and Bcl-6 for analysis via flow cytometry. Flow plots **A)** are representative of > 3 independent experiments. Heat Maps and Bars represent % CD4+Bcl-6+ cells as means compared to the relevant Control **B)** and **D)**, plus % CD4+Bcl-6+ cells as means compared to the RPMI Control **C)** and **E)** with SEM shown for > 3 independent experiments. P Values: * = <0.05; ** = <0.01; *** = <0.001; **** = <0.0001.

In line with the results seen in bulk CD4+ T cells, none of the cytokine treatments demonstrated a significant impact on Bcl-6 expression in naïve CD4+CD45RA+ T cells cultured with RPMI. Although the possible inhibitory effects of IFN-γ (77% - p = 0.3630) on Bcl-6 expression were visible when naïve CD4+CD45RA+ T cells were cultured with U251 cell supernatant, this was not statistically significant nor was the effect maintained when combined with IL-21 and/or TNF-α (Figures 6.14B and D). As U251 cell supernatant (118% - p = 0.2525) did not independently alter Bcl-6 expression in naïve CD4+CD45RA+ T cells, the addition of IFN-γ, IL-21 and TNF-α proved to have no impact on Bcl-6 expression, relative to the RPMI Control (111% - p = 0.4312) (Figures 6.14C and E).

On the other hand, each of the conditions containing IL-21 significantly upregulated Bcl-6 expression in naïve CD4+CD45RA+ T cells cultured with U373 cell supernatant, including the triple cytokine treatment (153% - p = 0.0114) (Figures 6.14B and D). Normalising the data against the RPMI Control revealed that the U373 cell supernatant (72% - p = 0.2256) could somewhat independently influence Bcl-6 expression, thus the addition of IFN-γ, IL-21 and TNF-α (100% p = 0.9161) effectively restored Bcl-6 expression to the exact same level as the RPMI Control (Figures 6.14C and E). Collectively, these results indicated that the glioblastoma secretome did not significantly affect Bcl-6 expression in naïve CD4+CD45RA+ T cells, thus this was maintained by IFN-γ, IL-21 and TNF-α treatment.


A)
Unstained
Control
IFN-γ
IL-21
TNF-α
IFN-γ + IL-21
IFN-γ + TNF-α
IL-21 + TNF-α
IFN-γ + IL-21 + TNF-α
10.60
11.68
8.41
6.19
9.55
7.34
13.31
5.98
7.19
3.77
8.62
3.70
7.78
6.07
10.08
7.03
7.17
7.53
15.51
6.52
12.50
6.05
15.56
8.09
RPMI
U251 Supernatant
U373 Supernatant
Bcl-6
CD4
B)
RPMI Control
U251 Supernatant
U373 Supernatant
100 104 93 93 97 94 114 113
100 77 112 111 114 101 113 104
100 90 ** 176 103 ** 179 105 ** 154 * 153
Control
IFN-γ
IL-21
TNF-α
IFN-γ + IL-21
IFN-γ + TNF-α
IL-21 + TNF-α
IFN-γ + IL-21 + TNF-α
160
140
120
100
80
C)
RPMI Control
U251 Supernatant
U373 Supernatant
100 104 93 93 97 94 114 113
118 91 113 131 118 120 116 111
72 80 109 86 94 94 116 100
Control
IFN-γ
IL-21
TNF-α
IFN-γ + IL-21
IFN-γ + TNF-α
IL-21 + TNF-α
IFN-γ + IL-21 + TNF-α
160
140
120
100
80

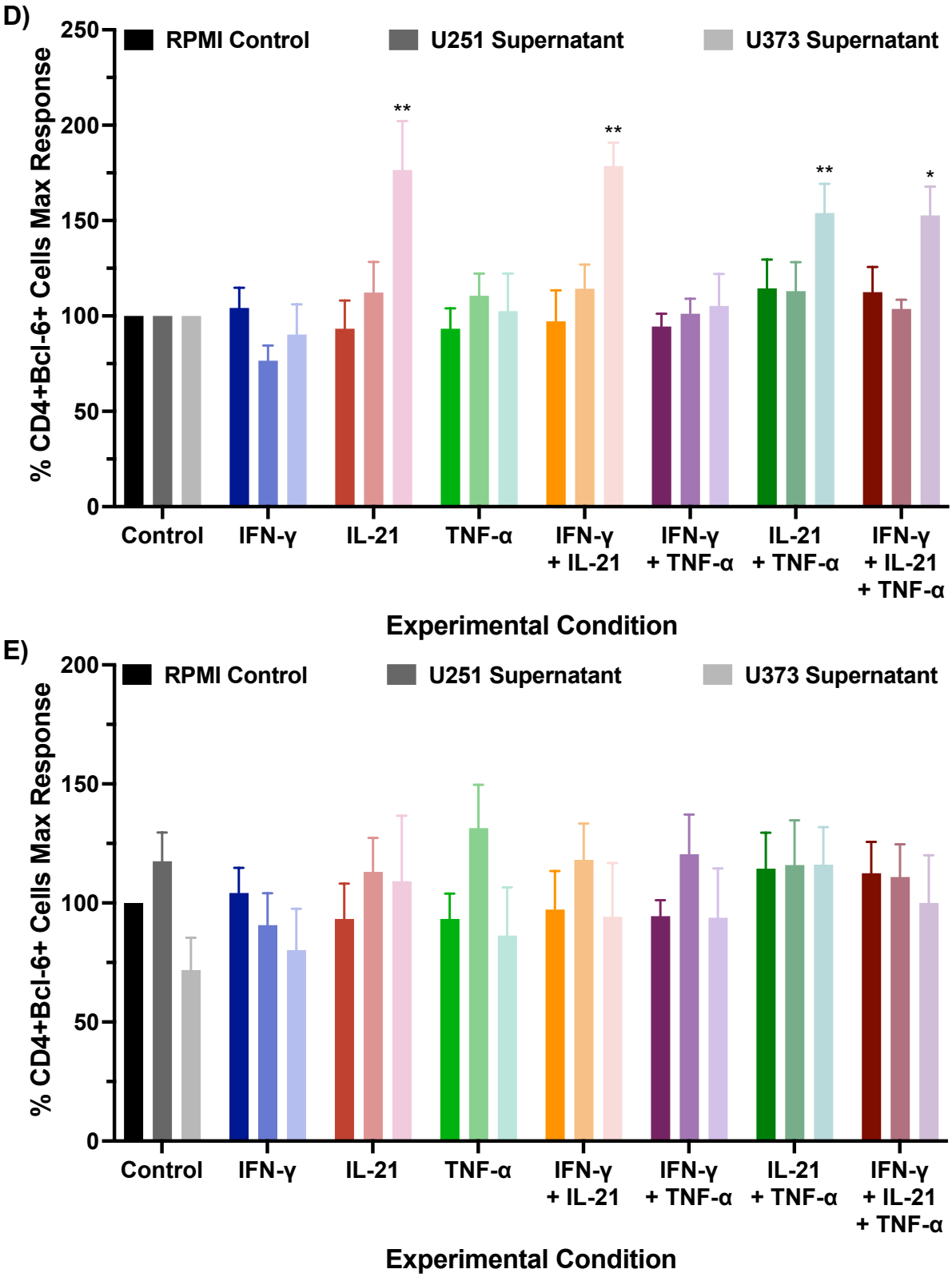


Figure 6.14. IFN-γ, IL-21 and TNF-α variably affected Bcl-6 expression in naïve CD4+CD45RA+ T cells

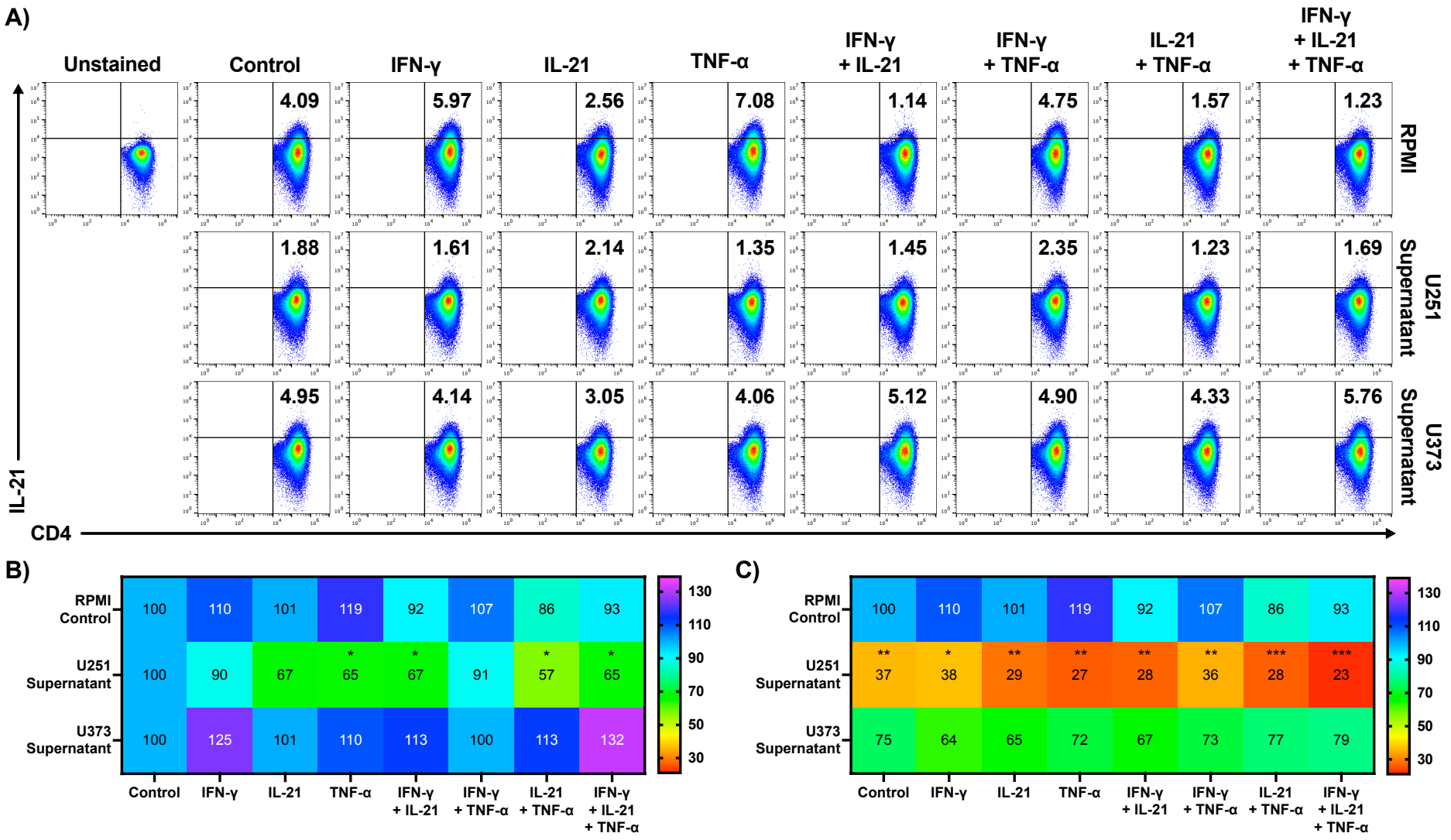
$2.5x10^4$ naïve CD4+CD45RA+ T cells were cultured 1:1 with CD3/CD28 beads in RPMI, U251 cell supernatant or U373 cell supernatant, plus treatment with 100ng/ml IFN-γ, IL-21 and TNF-α, both individually and combined for 5 days. Cells were then harvested and stained for CD4 and Bcl-6 for analysis via flow cytometry. Flow plots **A)** are representative of > 3 independent experiments. Heat Maps and Bars represent % CD4+Bcl-6+ cells as means compared to the relevant Control **B)** and **D)**, plus % CD4+Bcl-6+ cells as means compared to the RPMI Control **C)** and **E)** with SEM shown for > 3 independent experiments. P Values: * = <0.05; ** = <0.01; *** = <0.001; **** = <0.0001.

6.2.8. IFN-γ, IL-21 and TNF-α variably affected Tfh-associated IL-21 expression in bulk CD4+ and naïve CD4+CD45RA+ T cells cultured with glioblastoma supernatant

In line with the results in the present thesis, previous research has shown that IL-21 can reduce glioblastoma growth (Daga et al., 2007) – therefore, it was important to consider the influence of the glioblastoma secretome and explore the impact of IFN-γ, IL-21 and TNF-α on IL-21 expression in CD4+ T cells. In order to explore this, bulk CD4+ and naïve CD4+CD45RA+ T cells were isolated from healthy human leukocyte cones, before they were subsequently cultured with CD3/CD28 beads for 5 days. Within these culture conditions, both T cell subsets were exposed to U251 or U373 cell supernatant, alongside treatment with IFN-γ, IL-21 and/or TNF-α, before they were harvested and stained for CD4 and IL-21 for flow cytometric analysis. The data produced was normalised against the untreated Controls for each media condition, though the results from experiments cultured with U251 or U373 cell supernatant were also compared against the RPMI Control, in order to fully capture the influence of the glioblastoma secretome and/or cytokine treatment on IL-21 expression in bulk CD4+ and naïve CD4+CD45RA+ T cells.

Results from the Control condition were particularly interesting, as the data suggested that IFN-γ (118% - $p = 0.4203$) and TNF-α alone (119% - $p = 0.2855$) could possibly increase IL-21 expression in bulk CD4+ T cells, but these results were not statistically significant. Although the same effect was apparent when IFN-γ and TNF-α were administered together, the influence of these cytokines was lost when combined with IL-21 (93% - $p = 0.7160$) (Figures 6.15B and D). In contrast, culturing bulk CD4+ T cells with U251 cell supernatant and TNF-α significantly reduced IL-21 expression (65% - $p = 0.0364$), which was maintained in the triple cytokine condition (65% - $p = 0.0332$) (Figures 6.15B and D). Normalising the data against the RPMI Control revealed that U251 cell supernatant alone (37% - $p = 0.0078$) potently suppressed IL-21 expression in bulk CD4+ T cells, therefore this was enhanced with the addition of each of the cytokine conditions, including IFN-γ, IL-21 and TNF-α (23% - $p = 0.0009$) (Figures 6.15C and E).

On the other hand, no significant differences in IL-21 expression were identified in any of the experimental conditions, when bulk CD4+ T cells were cultured with U373 cell supernatant. However, there was a slight trend to suggest that IFN-γ could independently upregulate IL-21 expression in bulk CD4+ T cells (125% - $p = 0.2712$), which was somewhat sustained in the triple cytokine condition (132% - $p = 0.0945$) (Figures 6.15B and D). Comparing the data to the RPMI Control showed that the U373 cell supernatant had no impact on IL-21 expression in bulk CD4+ T cells (75% - $p = 0.1462$), even when combined with each of the cytokine treatments (Figures 6.15C and E). Altogether. these findings failed to demonstrate a consistent impact of the glioblastoma secretome with/without IFN-γ, IL-21 and TNF-α treatment on IL-21 expression in bulk CD4+ T cells.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Control
IFN-γ
IL-21
TNF-α
IFN-γ + IL-21
IFN-γ + TNF-α
IL-21 + TNF-α
IFN-γ + IL-21 + TNF-α
RPMI
U251 Supernatant
U373 Supernatant
IL-21
CD4
4.09
5.97
2.56
7.08
1.14
4.75
1.57
1.23
1.88
1.61
2.14
1.35
1.45
2.35
1.23
1.69
4.95
4.14
3.05
4.06
5.12
4.90
4.33
5.76
B)
RPMI Control
U251 Supernatant
U373 Supernatant
100 110 101 119 92 107 86 93
100 90 67 * 65 * 67 91 * 57 * 65
100 125 101 110 113 100 113 132
Control
IFN-γ
IL-21
TNF-α
IFN-γ + IL-21
IFN-γ + TNF-α
IL-21 + TNF-α
IFN-γ + IL-21 + TNF-α
130
110
90
70
50
30
C)
RPMI Control
U251 Supernatant
U373 Supernatant
100 110 101 119 92 107 86 93
** 37 * 38 ** 29 ** 27 ** 28 ** 36 *** 28 *** 23
75 64 65 72 67 73 77 79
Control
IFN-γ
IL-21
TNF-α
IFN-γ + IL-21
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110
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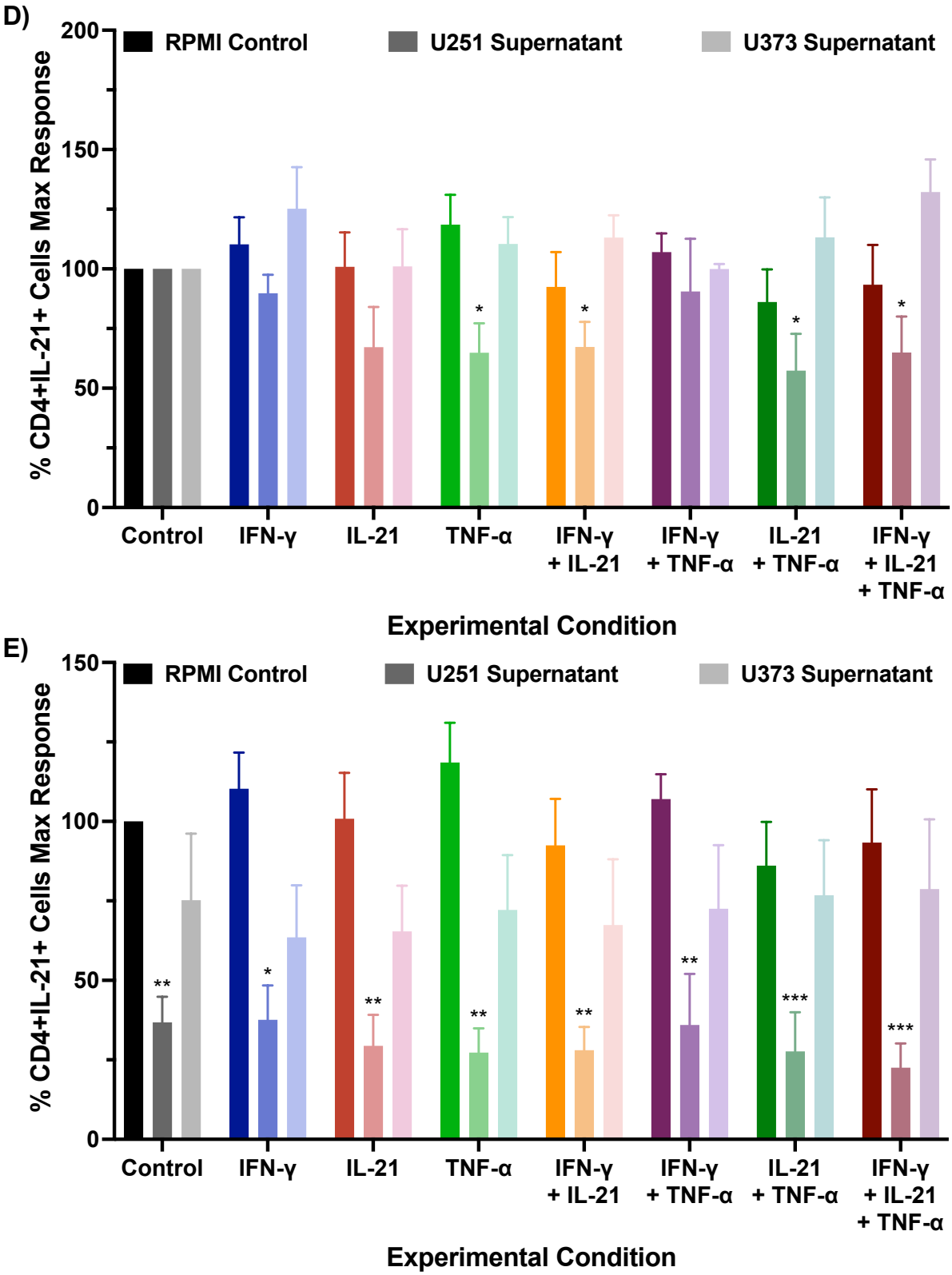
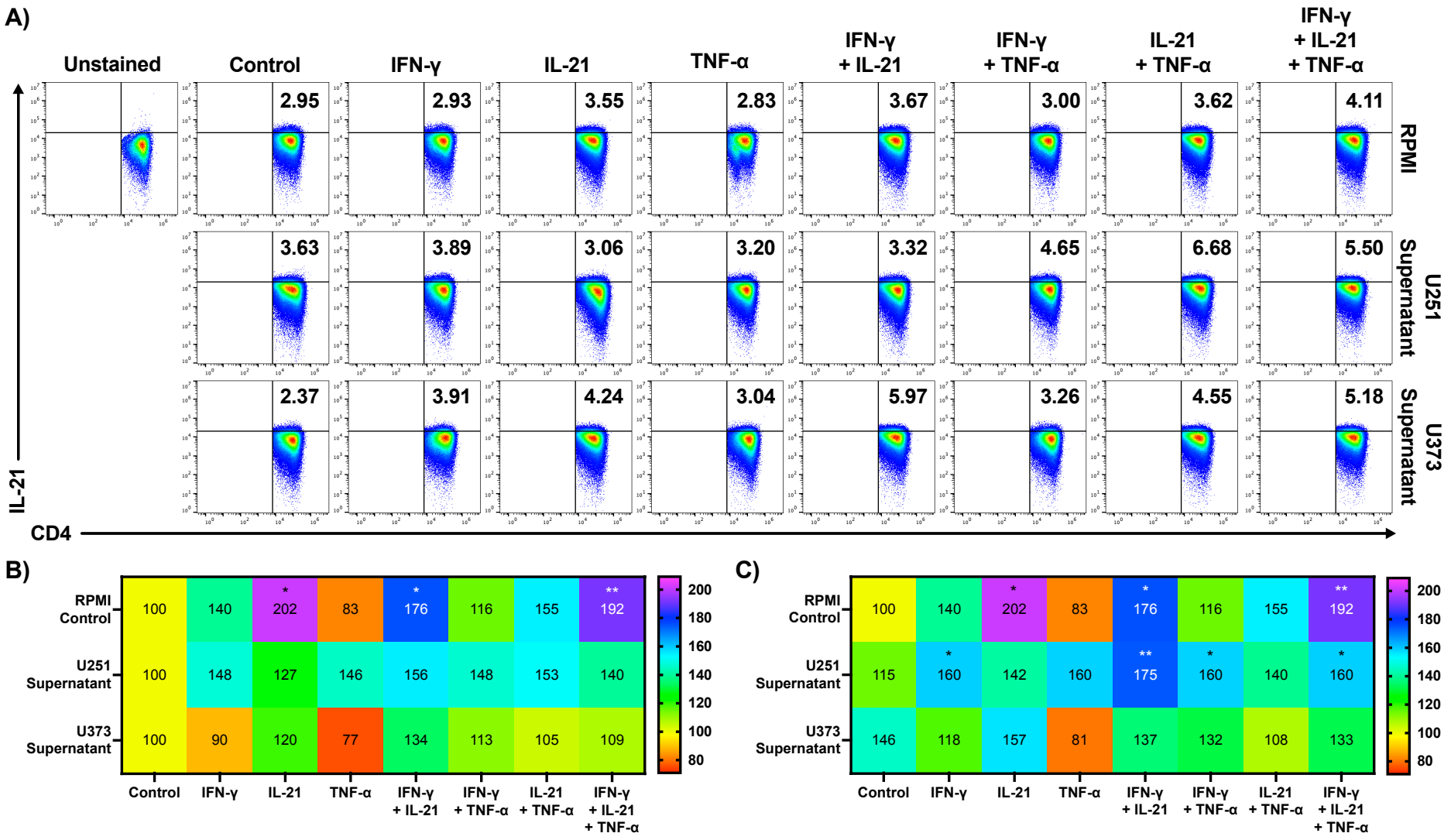


Figure 6.15. IFN-γ, IL-21 and TNF-α variably affected IL-21 expression in bulk CD4+ T cells
$2.5x10^4$ bulk CD4+ T cells were cultured 1:1 with CD3/CD28 beads in RPMI, U251 cell supernatant or U373 cell supernatant, plus treatment with 100ng/ml IFN-γ, IL-21 and TNF-α, both individually and combined for 5 days. Cells were then harvested and stained for CD4 and IL-21 for analysis via flow cytometry. Flow plots **A)** are representative of > 3 independent experiments. Heat Maps and Bars represent % CD4+IL-21+ cells as means compared to the relevant Control **B)** and **D)**, plus % CD4+IL-21+ cells as means compared to the RPMI Control **C)** and **E)** with SEM shown for > 3 independent experiments. P Values: * = <0.05; ** = <0.01; *** = <0.001; **** = <0.0001.

Results from the Control condition were particularly interesting as IL-21 alone (202% - p = 0.0165) and in combination with IFN-γ (176% - p = 0.0248) potently upregulated IL-21 expression in naïve CD4+CD45RA+ T cells, which was maintained when IFN-γ, IL-21 and TNF-α were administered together (192% - p = 0.0080). The strength of this self-mediated IL-21 upregulation is reflected in the IL-21 and TNF-α dual treatment (155% - p = 0.0512), as TNF-α alone (83% - p = 0.3457) appeared to reduce IL-21 expression in naïve CD4+CD45RA+ T cells (Figures 6.16B and D).

Despite the fact that none of the results were statistically significant, it was surprising to see that all of the cytokine treatments increased IL-21 expression when naïve CD4+CD45RA+ T cells were cultured with U251 cell supernatant, including the triple cytokine condition (140% - p = 0.2282) (Figures 6.16B and D). Normalising the data against the RPMI Control revealed that each of the conditions that contained IFN-γ upregulated IL-21 expression with a notable increase mediated by the IFN-γ and IL-21 dual treatment (175% - p = 0.0066) (Figures 6.16C and E).

On the other hand, the data suggested that TNF-α alone (77% - p = 0.9055) could mediate IL-21 downregulation in naïve CD4+CD45RA+ T cells cultured with U373 cell supernatant, but this was not maintained when combined with IFN-γ and IL-21 (109% - p = 0.5077) (Figures 6.16B and D). The same trend was present when the combination of U373 cell supernatant with TNF-α (81% - p = 0.9055) was compared against the RPMI Control, but this was not statistically significant either (Figures 6.16C and E). As the U373 cell supernatant itself (146% - p = 0.2581) did not alter IL-21 expression in naïve CD4+CD45RA+ T cells, the triple cytokine treatment failed to demonstrate any significant impact on IL-21 expression as well, relative to the RPMI Control (133% - p = 0.2267) (Figures 6.16C and E). Overall, these results indicated that the glioblastoma secretome did not affect IL-21 expression in naïve CD4+CD45RA+ T cells, but this was either maintained or upregulated with IFN-γ, IL-21 and TNF-α treatment.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Control
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IL-21
TNF-α
IFN-γ + IL-21
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IL-21 + TNF-α
IFN-γ + IL-21 + TNF-α
RPMI
U251 Supernatant
U373 Supernatant
IL-21
CD4
2.95 2.93 3.55 2.83 3.67 3.00 3.62 4.11
3.63 3.89 3.06 3.20 3.32 4.65 6.68 5.50
2.37 3.91 4.24 3.04 5.97 3.26 4.55 5.18
B)
RPMI Control 100 140 * 202 83 * 176 116 155 ** 192
U251 Supernatant 100 148 127 146 156 148 153 140
U373 Supernatant 100 90 120 77 134 113 105 109
Control IFN-γ IL-21 TNF-α IFN-γ + IL-21 IFN-γ + TNF-α IL-21 + TNF-α IFN-γ + IL-21 + TNF-α
200 180 160 140 120 100 80
C)
RPMI Control 100 140 * 202 83 * 176 116 155 ** 192
U251 Supernatant 115 * 160 142 160 ** 175 * 160 140 * 160
U373 Supernatant 146 118 157 81 137 132 108 133
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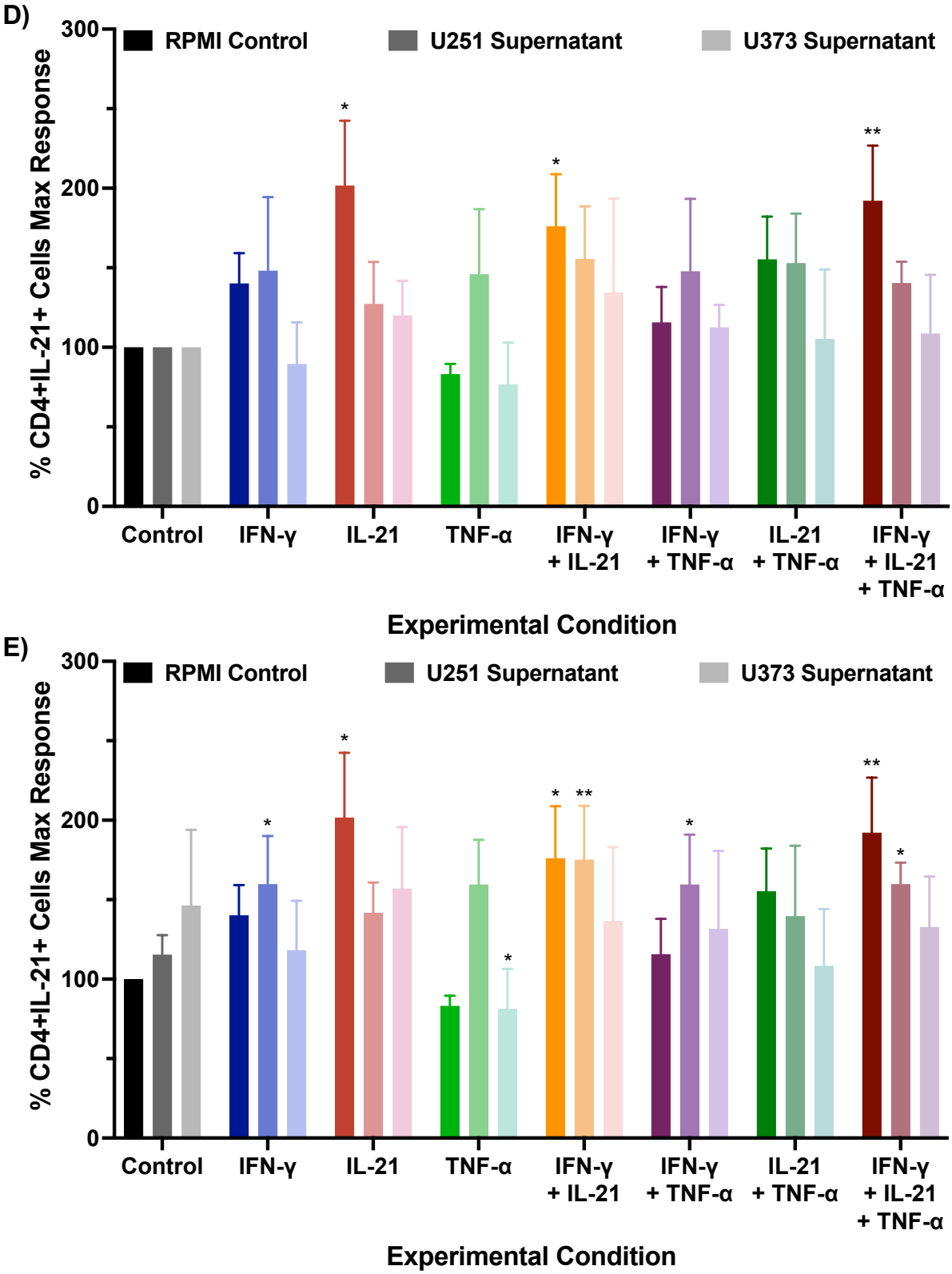


Figure 6.16. IFN-γ, IL-21 and TNF-α variably affected IL-21 expression in naïve CD4+CD45RA+ T cells

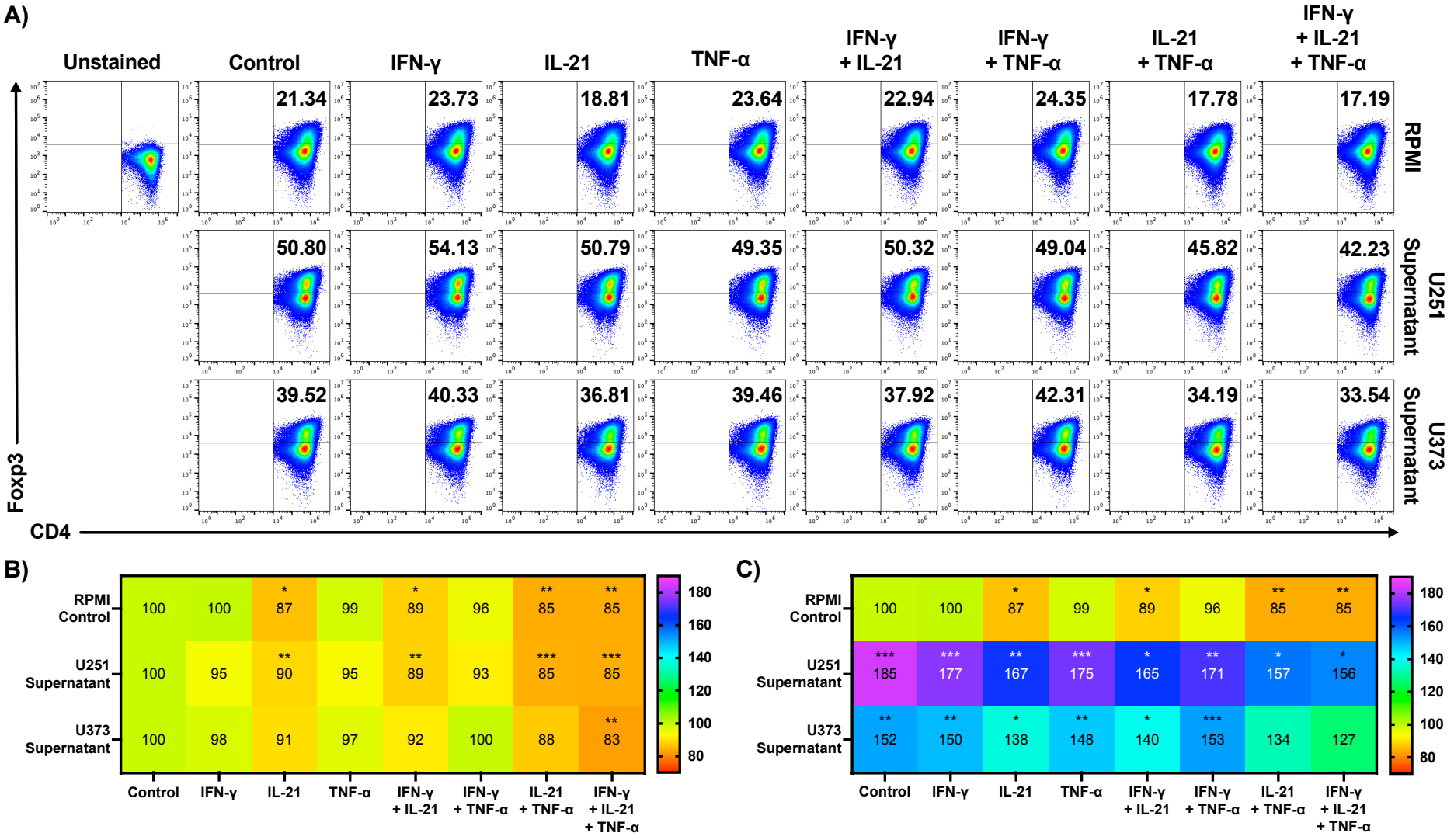
$2.5x10^4$ naïve CD4+CD45RA+ T cells were cultured 1:1 with CD3/CD28 beads in RPMI, U251 cell supernatant or U373 cell supernatant, plus treatment with 100ng/ml IFN-γ, IL-21 and TNF-α, both individually and combined for 5 days. Cells were then harvested and stained for CD4 and IL-21 for analysis via flow cytometry. Flow plots **A)** are representative of > 3 independent experiments. Heat Maps and Bars represent % CD4+IL-21+ cells as means compared to the relevant Control **B)** and **D)**, plus % CD4+IL-21+ cells as means compared to the RPMI Control **C)** and **E)** with SEM shown for > 3 independent experiments. P Values: * = <0.05; ** = <0.01; *** = <0.001; **** = <0.0001.

6.2.9. IFN-γ, IL-21 and TNF-α impaired Treg-associated Foxp3 expression in bulk CD4+ and naïve CD4+CD45RA+ T cells cultured with glioblastoma supernatant

Foxp3 expression is a key marker for CD4+ Tregs, which have been strongly implicated in mediating immunosuppression in glioblastoma to prevent anti-tumour immunity (El Andaloussi et al., 2006; El Andaloussi & Lesniak, 2006, 2007; Heimberger et al., 2008; Hussain et al., 2006; Kmiecik et al., 2013). As a result of this, it was vital to explore whether IFN-γ, IL-21 and TNF-α could overcome the immunosuppressive nature of the glioblastoma secretome to restrict the induction of Foxp3 expression in CD4+ T cells. In order to explore this, bulk CD4+ and naïve CD4+CD45RA+ T cells were isolated from healthy human leukocyte cones, before they were subsequently cultured with CD3/CD28 beads for 5 days. Within these culture conditions, both T cell subsets were exposed to U251 or U373 cell supernatant, alongside treatment with IFN-γ, IL-21 and/or TNF-α, before they were harvested and stained for CD4 and Foxp3 for flow cytometric analysis. The data produced was normalised against the untreated Controls for each media condition – however, the results from experiments cultured with U251 or U373 cell supernatant were also compared against the RPMI Control, in order to fully capture the influence of the glioblastoma secretome and/or cytokine treatment on Foxp3 expression in bulk CD4+ and naïve CD4+CD45RA+ T cells.

IL-21 alone (87% - $p = 0.0199$) appeared to mediate downregulation of Foxp3 expression in bulk CD4+ T cells, with similar results seen across each of the cytokine conditions that included IL-21. However, the most potent effect was seen when IL-21 was combined with IFN-γ and TNF-α (85% - $p = 0.0068$) (Figures 6.17B and D). The influence of IL-21 was also apparent when bulk CD4+ T cells were cultured with U251 cell supernatant, with the strongest level of downregulation visible in the triple cytokine condition (85% - $p = 0.0008$). Normalising the data against the RPMI Control demonstrated the extent to which U251 cell supernatant alone (185% - $p = 0.0002$) notably upregulated Foxp3 expression in bulk CD4+ T cells, but this was not completely neutralised by IFN-γ, IL-21 and TNF-α treatment, unfortunately (156% - $p = 0.0162$) (Figures 6.17C and E).

Conversely, only the triple cytokine treatment (83% - $p = 0.0077$) showed an inhibitory effect on Foxp3 expression, when bulk CD4+ T cells were cultured with U373 cell supernatant (Figures 6.17B and D). As U373 cell supernatant (152% - $p = 0.0014$) independently upregulated Foxp3 expression in bulk CD4+ T cells to a lesser extent than U251 cell supernatant alone, this was ultimately neutralised by the addition of IFN-γ, IL-21 and TNF-α (127% - $p = 0.1321$) to a similar level of expression as seen when bulk CD4+ T cells were cultured with the RPMI Control (Figures 6.17C and E). Collectively, these findings showed that the glioblastoma secretome potently upregulated Foxp3 expression in bulk CD4+ T cells, but this was reduced by IFN-γ, IL-21 and TNF-α treatment.


A)
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Control
IFN-γ
IL-21
TNF-α
IFN-γ + IL-21
IFN-γ + TNF-α
IL-21 + TNF-α
IFN-γ + IL-21 + TNF-α
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49.35
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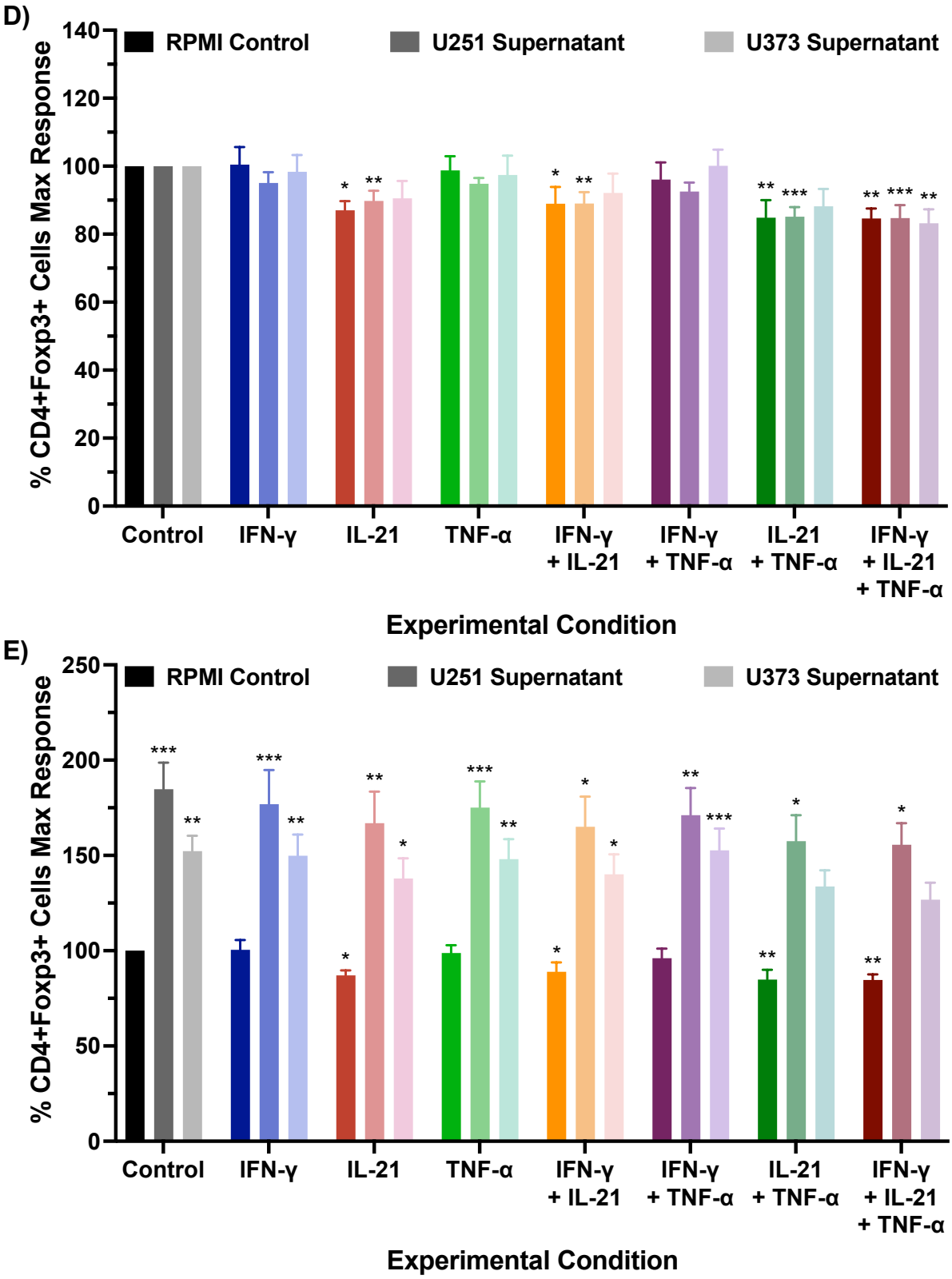
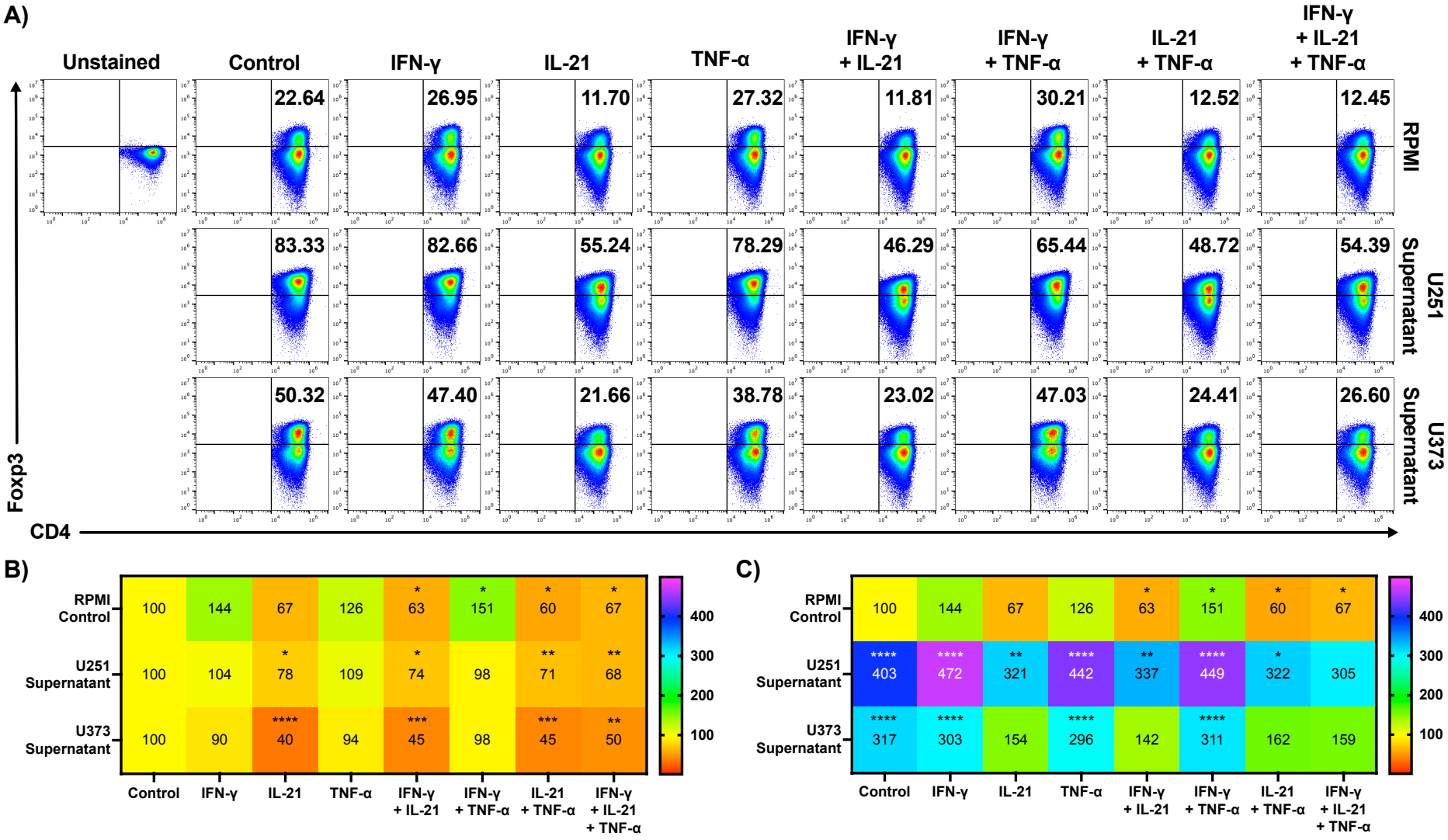


Figure 6.17. IFN-γ, IL-21 and TNF-α downregulated Foxp3 expression in bulk CD4+ T cells

$2.5x10^4$ bulk CD4+ T cells were cultured 1:1 with CD3/CD28 beads in RPMI, U251 cell supernatant or U373 cell supernatant, plus treatment with 100ng/ml IFN-γ, IL-21 and TNF-α, both individually and combined for 5 days. Cells were then harvested and stained for CD4 and Foxp3 for analysis via flow cytometry. Flow plots **A)** are representative of > 3 independent experiments. Heat Maps and Bars represent % CD4+Foxp3+ cells as means compared to the relevant Control **B)** and **D)**, plus % CD4+Foxp3+ cells as means compared to the RPMI Control **C)** and **E)** with SEM shown for > 3 independent experiments. P Values: * = <0.05; ** = <0.01; *** = <0.001; **** = <0.0001.

In contrast to the results seen in bulk CD4+ T cells, IL-21 alone (67% - $p = 0.0530$) did not significantly downregulate Foxp3 expression in naïve CD4+CD45RA+ T cells. However, it was clear that IL-21 was the main influence in reducing Foxp3 expression in each of the dual treatments, as well as the triple cytokine condition (67% - $p = 0.0424$). The same trend was present when naïve CD4+CD45RA+ T cells were cultured with U251 cell supernatant with comparable downregulation seen across each of the conditions that contained IL-21, though the largest reduction was seen when IFN-γ, IL-21 and TNF-α were administered together (68% - $p = 0.0048$) (Figures 6.18B and D). Normalising the data revealed the potent upregulation of Foxp3 in naïve CD4+CD45RA+ T cells by U251 cell supernatant alone, reaching expression levels over four times higher than the RPMI Control (403% - $p < 0.0001$). Although IFN-γ, IL-21 and TNF-α treatment reduced the extent of Foxp3 induction caused by U251 cell supernatant, the level of expression was still considerably higher than the RPMI Control, unfortunately (305% - $p = 0.0521$) (Figures 6.19C and E).

Similarly, IL-21 alone (40% - $p < 0.0001$) induced downregulation of Foxp3 expression in naïve CD4+CD45RA+ T cells cultured with U373 cell supernatant (Figures 6.19B and D). Nonetheless, the rise in Foxp3 expression mediated by U373 cell supernatant alone (317% - $p < 0.0001$) was much lower than U251 cell supernatant, thus the addition of IFN-γ, IL-21 and TNF-α almost completely neutralised Foxp3 expression to the same level as the RPMI Control (159% - $p = 0.0886$) (Figures 6.19C and E). Altogether, these findings showed that the glioblastoma secretome potently upregulated Foxp3 expression in naïve CD4+CD45RA+ T cells, but this was reduced with IFN-γ, IL-21 and TNF-α treatment.


A)
Unstained
Control
IFN-γ
IL-21
TNF-α
IFN-γ + IL-21
IFN-γ + TNF-α
IL-21 + TNF-α
IFN-γ + IL-21 + TNF-α
22.64
26.95
11.70
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78.29
46.29
65.44
48.72
54.39
50.32
47.40
21.66
38.78
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47.03
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RPMI
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U373 Supernatant
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U251 Supernatant
U373 Supernatant
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100 104 78 109 74 98 71 68
100 90 40 94 45 98 45 50
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U251 Supernatant
U373 Supernatant
100 144 67 126 63 151 60 67
403 472 321 442 337 449 322 305
317 303 154 296 142 311 162 159
Control
IFN-γ
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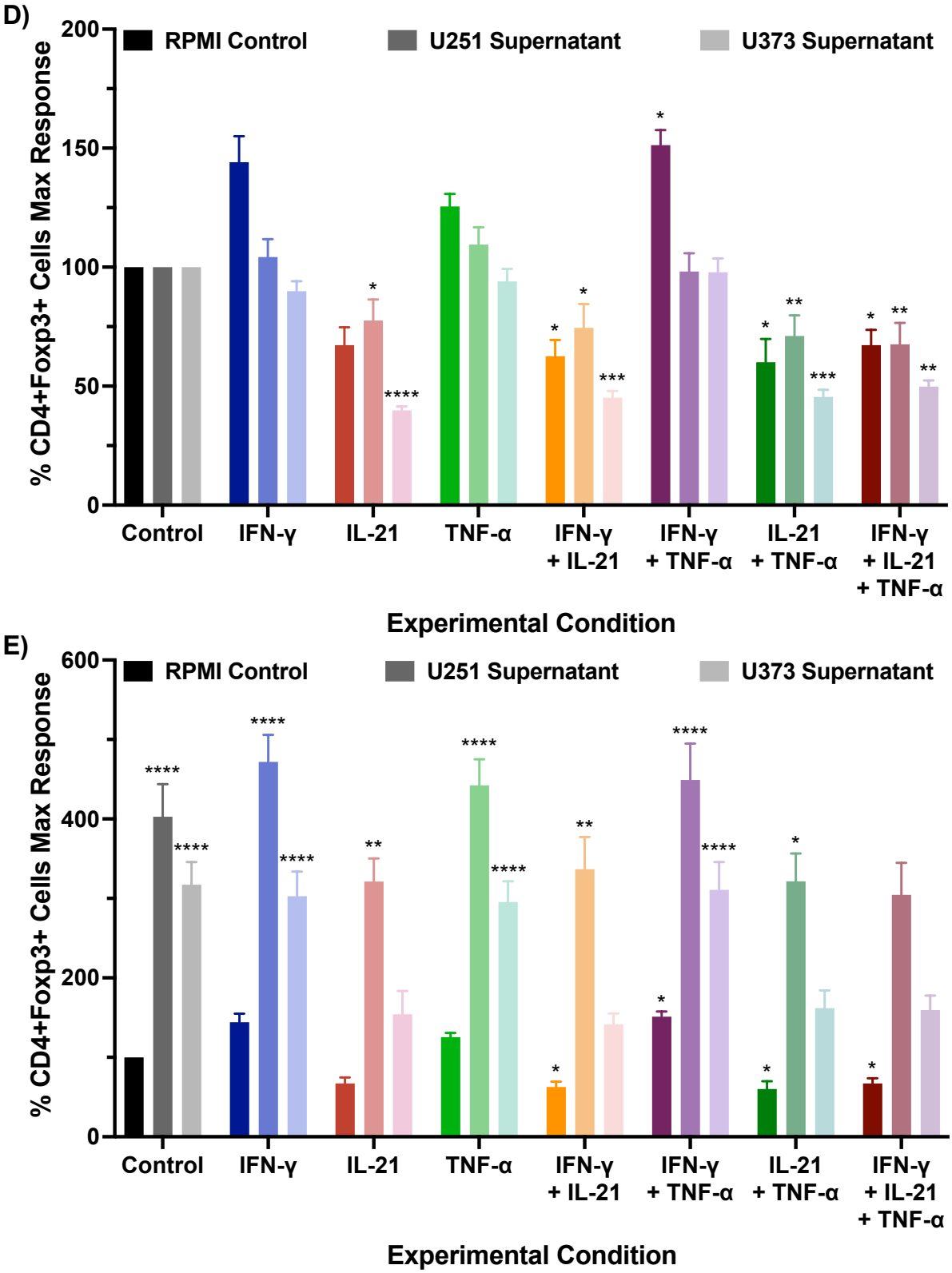

Figure 6.18. IFN-γ, IL-21 and TNF-α downregulated Foxp3 expression in naïve CD4+CD45RA+ T cells

$2.5x10^4$ naïve CD4+CD45RA+ CD4+ T cells were cultured 1:1 with CD3/CD28 beads in RPMI, U251 cell supernatant or U373 cell supernatant, plus treatment with 100ng/ml IFN-γ, IL-21 and TNF-α, both individually and combined for 5 days. Cells were then harvested and stained for CD4 and Foxp3 for analysis via flow cytometry. Flow plots **A)** are representative of > 3 independent experiments. Heat Maps and Bars represent % CD4+Foxp3+ cells as means compared to the relevant Control **B)** and **D)**, plus % CD4+Foxp3+ cells as means compared to the RPMI Control **C)** and **E)** with SEM shown for > 3 independent experiments. P Values: * = <0.05; ** = <0.01; *** = <0.001; **** = <0.0001.

6.2.10. Summary Heat Maps

In order to summarise the impact of U251 and U373 cell supernatant with/without IFN-γ, IL-21 and TNF-α treatment on the expression of key T cell subset markers in bulk CD4+ T cells and naïve CD4+CD45RA+ T cells, the percentage of cells that were CD4+T-bet+, CD4+IFN-γ+, CD4+TNF-α+, CD4+GATA3+, CD4+RoRγT+, CD4+IL-17+, CD4+Bcl-6+, CD4+IL-21+, and CD4+Foxp3+ were normalised against the RPMI Control and used to generate Heat Maps, as shown in Figure 6.19 below.

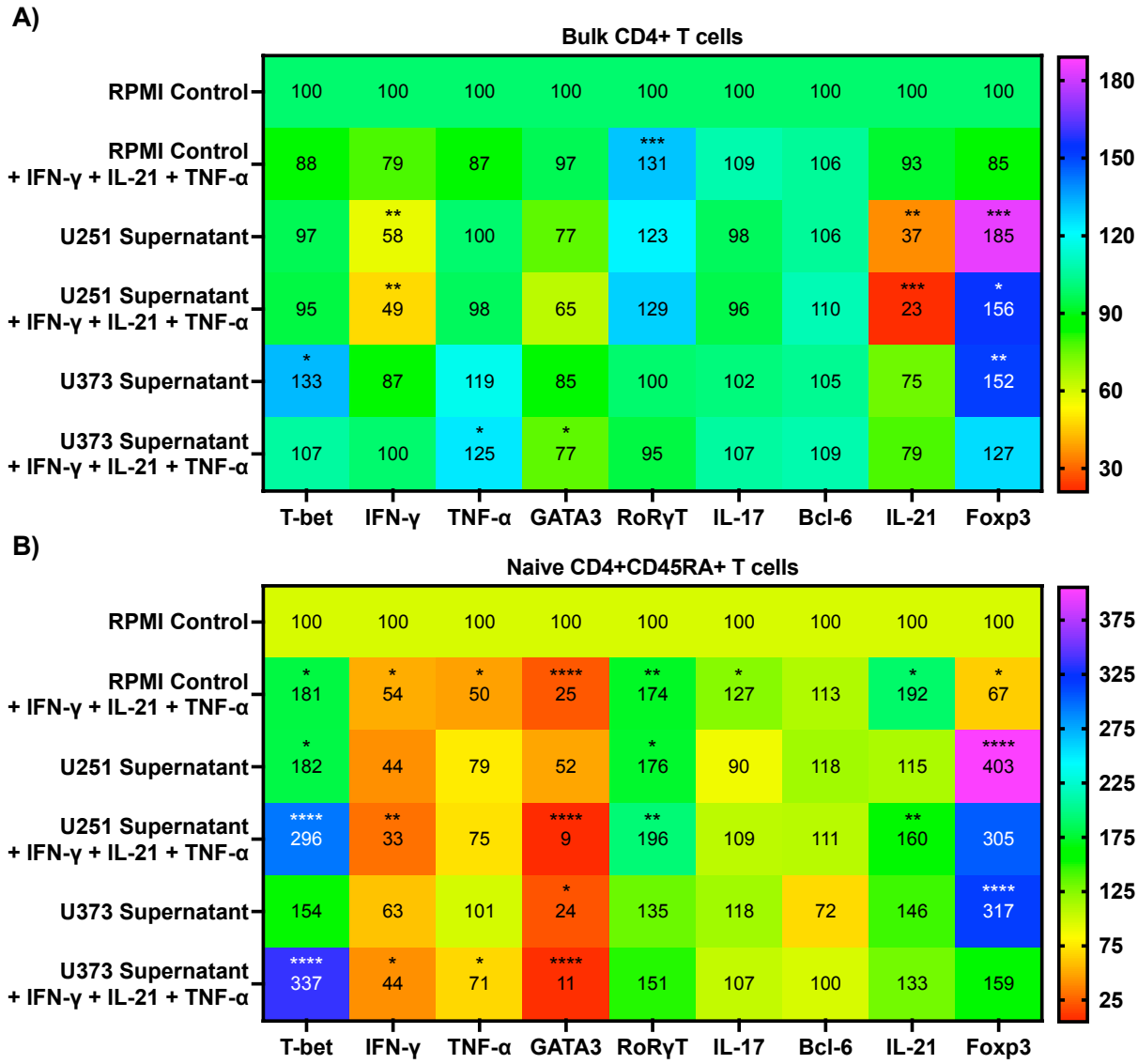

Figure 6.19. Heat Maps summarising the impact of U251 and U373 cell supernatant with IFN-γ, IL-21 and TNF-α treatment on T-bet, IFN-γ, IL-21, TNF-α, GATA3, RoRγT, IL-17, Bcl-6, IL-17, and Foxp3 expression in bulk CD4+ and naïve CD4+ T cells

$2.5x10^4$ bulk CD4+ T cells and $2.5x10^4$ naïve CD4+CD45RA+ CD4+ T cells were cultured 1:1 with CD3/CD28 beads in RPMI, U251 cell supernatant or U373 cell supernatant, plus treatment with 100ng/ml IFN-γ, IL-21 and TNF-α, both individually and combined for 5 days. Cells were then harvested and stained for CD4 plus T-bet, IFN-γ, TNF-α, GATA3, RoRγT, IL-17, Bcl-6, IL-21, or Foxp3 for analysis via flow cytometry. Heat Maps represent % CD4+ positive expression for each of these markers in bulk CD4+ T cells **A)** and naïve CD4+CD45RA+ T cells **B)** as means compared to the RPMI Control for > 3 independent experiments. P Values: * = <0.05; ** = <0.01; *** = <0.001; **** = <0.0001.

6.3. Discussion

Researchers have suggested that glioblastoma cells use the release of EVs and secretory factors as key mechanisms to promote both localised and systemic immunosuppression (de Vrij et al., 2015; Hellwinkel et al., 2015; Hishii et al., 1995; Morad et al., 2019; Simon et al., 2020; Zou et al., 1999). As a result of this, it is particularly crucial for novel immunotherapies to evaluate whether they have the ability to overcome the glioblastoma secretome, in order to restore anti-tumour immunity in patients. Although the finer details of the glioblastoma secretome are still under investigation, some studies have identified that glioblastoma cell lines secrete IL-6, IL-10, VEGF, TGF-β, though very few have investigated the direct impact of the glioblastoma secretome on T cell function and/or differentiation (Albulescu et al., 2013; Polisetty et al., 2011; J. Wei et al., 2010). In order to explore this in the present study, three T cell subsets (bulk CD4+ and CD8+ T cells, as well as naïve CD4+CD45RA+ T cells) were isolated from human leukocyte cones and subsequently cultured with either fresh RPMI or conditioned RPMI (cell supernatant) taken from U251 and U373 cell cultures, in which the latter were used to model the glioblastoma microenvironment. Within these media conditions, each of the T cell subsets were also cultured with CD3/CD28 beads plus IFN-γ, IL-21 and/or TNF-α for 5 days, before cells were harvested and the expression of a range of transcription factors and cytokines were measured.

Defined by their expression of T-bet, IFN-γ, and TNF-α, Th1 cells represent a key component of the anti-tumour immune response, as IFN-γ has been shown to enhance tumour recognition by immune cells through upregulation of MHC class I and II complexes on glioblastoma cells (Ehtesham et al., 2002; Frewert et al., 2004; Soos et al., 2001). In addition, IFN-γ and TNF-α have been shown to induce an M1 phenotype in macrophages, which subsequently attract Th1 and Tc1 cells through the secretion of CXCL9 and CXCL10 – high expression of both of these chemokines has been associated with improved survival in glioblastoma patients (Gjorgjevski et al., 2019). Furthermore, previous research has shown that CD4+ T cells taken from glioblastoma patients produced less IFN-γ and TNF-α than healthy controls, indicating that glioblastoma cells impair Th1 cell differentiation and/or function, likely through their secretome (Zisakis et al., 2007).

Results from the present chapter showed that the glioblastoma secretome variably affected the expression levels of these Th1 markers across all CD4+ and CD8+ T cell populations. Whilst U251 and U373 cell supernatant independently produced variable results in bulk CD4+ and CD8+ T cells, there was clear upregulation of T-bet in naïve CD4+CD45RA+ T cells, following the addition of IFN-γ, IL-21 and TNF-α (Figure 6.2). However, this did not correlate with an increase in IFN-γ or TNF-α expression – in fact, the combination of tumour supernatant with IFN-γ, IL-21 and TNF-α resulted in downregulation of IFN-γ in naïve CD4+CD45RA+ T cells, with similar effects seen in bulk CD8+ T cells (Figures 6.4 and 9.9). Together, these results indicated that there was a disconnect between T-bet and IFN-γ expression, thus it is possible that the glioblastoma secretome and/or cytokines could have been disrupting T-bet downstream signalling, perhaps through inhibition of Runx3 or STAT4 to restrict IFN-γ transactivation, but this requires further investigation.

Conversely, TNF-α expression in bulk CD4+ T cell was either unaffected or upregulated by exposure to the glioblastoma secretome and/or IFN-γ, IL-21 and TNF-α treatment (Figure 6.5). As results showed that T-bet expression was maintained in bulk CD4+ T cells and TCR signalling would have been sustained by the CD3/CD28 beads, it is likely that NFAT and AP-1 were still able to co-operate with T-bet to induce TNF-α expression in bulk CD4+ T cells across each of the experimental conditions, but this should be confirmed in future research. On the other hand, IFN-γ, IL-21 and TNF-α downregulated TNF-α expression in bulk CD8+ T cells when they were cultured with the glioblastoma secretome, though this appeared to be largely driven by IL-21 (Figure 9.10) – nevertheless, the regulatory pathways involved in this should be explored in future research.

Although the ratio between Th1 and Th2 cells is tightly controlled in healthy humans, various studies have shown that Th2 cells were enriched in glioblastoma patients (Shimato et al., 2012; Takashima et al., 2018; Waziri et al., 2008; Zisakis et al., 2007). Furthermore, Harshyne et al. (2015) proposed that Th2-mediated suppression of both Th1 cell and M1 macrophage activity was influenced by the release of glioblastoma-derived EVs. However, these findings were not reflected in the present study, as the glioblastoma secretome appeared to downregulate GATA3 expression, particularly in naïve CD4+CD45RA+ T cells (Figures 6.7, 6.8 and 9.11). Nonetheless, the reduction in GATA3 expression was further enhanced by the addition of IFN-γ, IL-21 and TNF-α, suggesting that this cytokine treatment suppressed Th2 and Tc2 differentiation and/or function. In order to provide further clarity of the impact of the glioblastoma secretome and/or IFN-γ, IL-21 and TNF-α treatment on Th2 and Tc2 cells, attempts were made to explore IL-13 expression in each of the T cell subsets but, unfortunately, this was not detected in any of the experimental conditions (data not shown). Although the reduction in GATA3 expression mediated by IFN-γ, IL-21 and TNF-α highlights the therapeutic value of this as a potential treatment for glioblastoma, the discrepancies between the impact of the glioblastoma secretome on GATA3 expression in T cells in the present study and previous research suggests that these experiments require further validation in future research.

A crucial aspect of Th17 and Tc17 cell differentiation involves downregulation of both IFN-γ and GATA3, thus it was plausible that the glioblastoma secretome and/or IFN-γ, IL-21 and TNF-α could have been facilitating the development of these phenotypes in CD4+ and CD8+ T cells through upregulation of RoRγT and IL-17 (Gorelik et al., 2000, 2002; Huber et al., 2009; I.-K. Park et al., 2007). This theory is supported by previous research, which have correlated high levels of IL-17 expression in glioblastoma patient samples with poor overall survival, as IL-17 supports tumour growth by promoting neoangiogenesis (Madkouri et al., 2017). Similarly, U87 cells have been shown to secrete TGF-β and IL-6, thus they induced Th17 differentiation when co-cultured with naïve CD4+ T cells (Paladugu et al., 2013). However, the results in the present study indicated that U251 cell supernatant could increase RoRγT expression in bulk CD4+ and naïve CD4+CD45RA+ T cells, which was marginally enhanced with IFN-γ, IL-21 and TNF-α treatment, but this did not correlate with any changes in IL-17 expression (Figures 6.9-6.12). Conversely, the glioblastoma secretome did not impact RoRγT expression in bulk

CD8+ T cells, but there was a clear trend to suggest that adding IFN-γ, IL-21 and TNF-α to the culture conditions resulted in reduced IL-17 expression (Figures 9.13). Altogether, it appeared that the changes in Th1/Tc1 and/or Th2/Tc2 markers in T cells were not caused by an increase in Th17/Tc17 differentiation.

Each of the aforementioned CD4+ T cell subsets share common expression of Blimp-1, but this can be dysregulated by the induction of Bcl-6 expression, which is a unique characteristic of Tfh cells. In line with this, it is possible that the changes mediated by the glioblastoma secretome and/or IFN-γ, IL-21 and TNF-α could have been facilitated by the development of a Tfh phenotype in each of the T cell subsets. Recent research has identified Tfh markers in glioblastoma patient samples, in which increased expression was associated with improved patient survival (P. Liang et al., 2020; Qiu et al., 2020). In particular, results from the present study showed that the glioblastoma secretome largely did not affect Bcl-6 or IL-21 expression in most of the experimental conditions with each of the T cell populations. Curiously, bulk CD4+ T cells potently downregulated IL-21 expression in response to exposure to U251 cell supernatant, which was sustained with the addition of IFN-γ, IL-21 and TNF-α (Figure 6.15). However, opposing results were seen in naïve CD4+CD45RA+ T cells with the combination of U251 cell supernatant plus IFN-γ, IL-21 and TNF-α significantly increasing IL-21 expression, relative to the RPMI Control (Figure 6.16). Given that these results did not correlate with any changes in Bcl-6 expression to provide a clear indication that these conditions were directing these cells towards alternate T cell phenotypes, the regulatory controls involved in this disparity between the two CD4+ T cell populations remains to be seen. Nonetheless, the results largely suggested that IFN-γ, IL-21 and TNF-α helped to maintain the Tfh phenotype in both CD4+ and CD8+ T cells, thus they could support any anti-tumour immune responses mediated by these cells in glioblastoma patients.

Although it would have been beneficial for IFN-γ, IL-21 and TNF-α to promote their own expression in T cells to enhance their direct anti-tumour effects through in situ production, this was not an essential therapeutic requirement, as the larger objective was to disrupt the immunosuppressive nature of the glioblastoma secretome. In order to achieve this, it was imperative that IFN-γ, IL-21 and TNF-α reduced the differentiation and/or function of CD4+ and CD8+ Tregs by downregulating Foxp3 and/or TGF-β expression. These Tregs are able to facilitate tumour development by potently inhibiting anti-tumour immunity using a variety of mechanisms, as discussed previously. Previous research has identified both CD4+Foxp3+ and CD8+Foxp3+ T cell infiltration in glioblastoma patient samples, whilst depletion of Tregs increased survival in a murine glioblastoma model (El Andaloussi et al., 2006; El Andaloussi & Lesniak, 2006, 2007; Heimberger et al., 2008; Hussain et al., 2006; Kmiecik et al., 2013). Furthermore, glioblastoma-derived EVs have been shown to upregulate CD73 expression in T cells: CD73 is involved in the conversion of extracellular ATP to adenosine, which subsequently inhibits T cell activation, proliferation and cytotoxic activity – this demonstrates how the glioblastoma secretome can promote immunosuppression (Deaglio et al., 2007; M. Wang et al., 2021).

Additionally, previous research has identified expression of both TGF-β and its receptors in glioblastoma patients, whilst increased TGF-β/Smad signalling has been shown to correlate with increased tumour proliferation and poor patient prognosis (C. Hao et al., 2002; Shimizu et al., 1999). Unfortunately, TGF-β expression was not detected in any of the experimental conditions with each of the T cell populations (data not shown). Nevertheless, potent upregulation of Foxp3 by the glioblastoma secretome was identified in all of the T cell subsets, but this was significantly reduced by the addition of IFN-γ, IL-21 and TNF-α. In fact, Foxp3 expression was completely neutralised in both naïve CD4+CD4RA+ T cells and CD8+ T cells cultured with U251 or U373 cell supernatant plus the triple cytokine treatment (Figures 6.18 and 9.16). The same effect was seen when bulk CD4+ T cells were cultured with U373 cell supernatant plus IFN-γ, IL-21 and TNF-α, but the significance of this Foxp3 downregulation was lost when IFN-γ, IL-21 and TNF-α were administered alongside U251 cell supernatant (Figure 6.17). As Foxp3 expression is vital for maintaining the Treg phenotype by promoting Treg differentiation, stability and function, these findings are extremely promising because they demonstrate the therapeutic potential that the combination of IFN-γ, IL-21 and TNF-α has as a novel cytokine therapy that could restore anti-tumour immunity in glioblastoma patients (Junius et al., 2021; Kleinewietfeld & Hafler, 2013).

Despite the fact that the results from the present chapter were varied when exploring the influence of IFN-γ, IL-21 and TNF-α on skewing CD4+ and CD8+ T cells towards phenotypes that can directly promote anti-tumour immunity, the data consistently demonstrated that this cytokine combination impaired the immunosuppressive nature of the glioblastoma secretome by reducing Foxp3 expression in both CD4+ and CD8+ T cells. This provides the foundation for future research to build upon as they evaluate the significant potential IFN-γ, IL-21 and TNF-α has as a novel cytokine-based therapy in restoring anti-tumour immunity in glioblastoma patients.

7. General Discussion

The overall aim of the present thesis was to evaluate the therapeutic potential of IFN-γ, IL-21 and TNF-α as a novel immunotherapy in glioblastoma. Initially, this was assessed by exploring the direct impact of the triple cytokine combination on glioblastoma proliferation, survival, and migration. In particular, results showed that IFN-γ, IL-21 and TNF-α had potent anti-tumour effects in glioblastoma, which were mediated through the induction of cell death and inhibition of cell proliferation. However, it was also important to consider how these cytokines influenced immunological aspects of the glioblastoma TME, as tumour-mediated immunosuppression has been shown to be a key mechanism in supporting glioblastoma pathogenesis. Unfortunately, very little research has been conducted to establish whether glioblastoma cells can directly bind to T cells to modulate their activity. In line with this, it was necessary to first establish whether glioblastoma cells expressed several key co-stimulatory ligands that are required for T cell activation, along with any co-inhibitory ligands that can inhibit T cell activity. The findings in the present study demonstrated that glioblastoma cells lacked expression of two vital co-stimulatory ligands (CD80 and CD86), but these could both be upregulated through IFN-γ, IL-21 and TNF-α treatment. Conversely, PD-L1 was highly expressed in both cell lines and this was enhanced in response to IFN-γ, IL-21 and TNF-α exposure, indicating that this could facilitate increased T cell death, thus the impact of these cytokines on direct glioblastoma-T cell interactions needs to be explored further. However, results in the present study also demonstrated that the glioblastoma secretome potently upregulated Foxp3 expression in various T cell subsets, but this was neutralised by IFN-γ, IL-21 and TNF-α treatment, demonstrating that this cytokine treatment has significant potential to restore anti-tumour immunity in glioblastoma. Altogether, these findings have provided the foundation for future research to build upon, as they look to explore the potential therapeutic value of IFN-γ, IL-21 and TNF-α as a novel cytokine-based immunotherapy in glioblastoma patients.

Unfortunately, there are several experimental limitations within the present thesis that should be considered in future research. The most prominent issue is that the U251 and U373 cell lines are not wholly representative of glioblastoma cells that are found in patients. Although attempts were made, it proved incredibly difficult to maintain sufficient glioblastoma patient-derived cultures, in order to improve the reliability and validity of the data in the present study. Similarly, the use of animal glioblastoma models would further improve our understanding of the potential therapeutic benefits of IFN-γ, IL-21 and TNF-α in glioblastoma. In line with this, exploring the impact of these cytokines on healthy brain cells would also help to assess any off-target effects, thereby determining the feasibility of their use in human glioblastoma patients.

Another major limitation of the present study is the fact that all of the experiments were completed in normoxia, when it has previously been established that hypoxia is a key component of the glioblastoma TME. In particular, hypoxia directly results in the upregulation of the HIF-1 transcription factor in glioblastoma cells, which has a wide variety of downstream effects. This is particularly relevant

when considering the impact of IFN-γ, IL-21 and TNF-α on glioblastoma migration, as HIF-1 upregulation has been linked with enhanced MMP-2 secretion, which in turn increases ECM degradation to facilitate migration (Du et al., 2008). On the other hand, alterations to the metabolic profile of glioblastoma cells as a result of increased HIF-1 expression in hypoxia could also drive tumour proliferation (J. Kim et al., 2015; Komaki et al., 2019; Larrieu et al., 2022; Wolf et al., 2011). As a result of this, it is possible that glioblastoma cells could grow and migrate faster in hypoxic conditions, thus it is imperative for future research to establish whether IFN-γ, IL-21 and TNF-α can inhibit tumour migration and proliferation in these circumstances.

The reliance on flow cytometry as the primary method of analysis also represents a key limitation in the present study, particularly in experiments exploring expression of specific markers. Specifically, the expression of Annexin V and 7-AAD as measurements of apoptosis and necrosis should be confirmed by exploring the expression of other key markers involved in cell death, including caspases and cytochrome c. Furthermore, the expression of immunological markers should be validated using methods that provide clearer outputs for quantifying changes in expression – e.g. ELISA, qPCR and western blotting. In addition, the use of an Unstained Control rather than an Isotype Control also compromises the reliability of the results in the present thesis, therefore Isotype Controls should be used in future research to ensure that flow cytometric data is appropriately gated and analysed.

Aside from this, a number of tests would have benefited from additional experimental repeats, as changes were made throughout data collection to optimise experimental conditions. In particular, several scratch assays were completed using a 200μL pipette tip to divide the cell populations, but it eventually became apparent that this was not an appropriate method to measure cell migration, as it was impossible to ensure that the space between cell populations was a consistent size. This is quite important when attempting to measure inhibition of a cell migration, as experiments with smaller gaps closed before it was possible to see an impact of cytokine treatment. As a result of this, it was necessary to switch to the 2-well culture insert system, which provide a fixed 500μm gap, but more experimental repeats could have improved the statistical significance of several key results. In addition, the Cell IQ analysis software would erroneously define the edges of the cell populations, thus it would fail to accurately analyse closure of the gap. As a result of these issues, it would perhaps be beneficial to explore alternative mechanisms of measuring cell migrations, perhaps through the use of Transwell plates or gel-based models that are more representative of brain parenchyma.

In order to explore the relationship between CD44 and HA in glioblastoma cells further, initial optimisation experiments were completed, so that the migration experiments could be completed with the addition of pre-coating the cell culture wells with HA and/or adding a CD44 mAb to the treatment conditions (Figures 9.5-9.7). Pre-coating the wells with HA would increase the validity of these experiments by creating an environment that is more representative of the HA-rich brain ECM, whilst increasing the ability to visualise the impact that the loss of CD44 expression had on the migration of U251 and U373 cells, due to treatment with IFN-γ, IL-21 and TNF-α. Similarly, the scale of the reliance

on CD44 signalling to facilitate tumour migration and invasion could be accurately quantified through the use of a CD44 blocking mAb. Unfortunately, these experiments could not be pursued further, due to the time constraints of the project, therefore the exact mechanisms controlled by IFN-γ, IL-21 and TNF-α to reduce glioblastoma migration remain unclear and should be evaluated in future research.

A recurring theme throughout the present thesis is the lack of knowledge surrounding glioblastoma, particularly regarding the interactions these tumour cells have with immune cells and other components of the TME – however, this is largely a result of the long-standing belief that the brain was an immunoprivileged site. Whilst T cells form an essential fraction of the anti-tumour immune response, they are not the only immune cells that are found within the tumour microenvironment; therefore, future research should consider exploring the impact of IFN-γ, IL-21 and TNF-α on NK cells, DCs, myeloid derived suppressor cells (MDSCs) and macrophages in glioblastoma. For example, research has shown that the glioblastoma TME has a profound impact on DCs and macrophages, specifically impairing their ability to activate T cells and promote anti-tumour immune responses. In particular, various studies have demonstrated that the glioblastoma TME skews macrophages towards an immunosuppressive M2 phenotype, thus it would be interesting to see whether IFN-γ, IL-21 and TNF-α could re-direct these macrophages towards an M1 phenotype to facilitate an anti-tumour immune response (de Vrij et al., 2015; Domenis et al., 2017). This is somewhat supported by previous research, which showed that IFN-γ and TNF-α could induce an M1 phenotype in macrophages, which subsequently secreted CXCL9 and CXCL10 to increase Th1 and Tc1 cell migration – high expression of both of these chemokines has been associated with improved glioblastoma patient survival (Gjorgjevski et al., 2019).

It would also be particularly interesting for future research to explore whether the loss of Foxp3 expression in CD4+ and CD8+ T cells as a result of IFN-γ, IL-21 and TNF-α treatment affects their abilities to restrict anti-tumour immunity. Aside from polarising various immune cell population towards immunosuppressive phenotypes, Tregs also directly interact with these cells to impair their ability to promote anti-tumour immune responses. For example, Tregs utilise CD39/CD73 expression to generate adenosine, which in turn restricts the activation and function of DCs and effector T cells, therefore it would be useful to explore whether IFN-γ, IL-21 and TNF-α could downregulate CD39/CD73 expression in Tregs to restore anti-tumour immunity (Borsellino et al., 2007; Mandapathil et al., 2010; Mascanfroni et al., 2015; Ohta & Sitkovsky, 2014; Panther et al., 2003). Similarly, Tregs utilise various mechanisms to induce cell death in effector T cell populations, so it would be particularly interesting to see whether IFN-γ, IL-21 and TNF-α could downregulate the cytotoxic activity of Tregs to support effector T cell populations in promoting an anti-tumour immune response (X. Cao et al., 2007; Ren et al., 2007; Strauss et al., 2009).

Furthermore, it would be useful for future research to also explore how IFN-γ, IL-21 and TNF-α directly affect the secretome of treated glioblastoma cells, before subsequently evaluating how this influences various immune cells. For example, alongside promoting Treg function and

differentiation, glioblastoma cells have been shown to promote Treg migration through the secretion of CCL22 (Crane et al., 2012; Jacobs et al., 2010; J. T. Jordan et al., 2008). If treatment with IFN-γ, IL-21 and TNF-α could prevent CCL22 expression by glioblastoma cells, whilst reducing Foxp3 expression in T cells within the tumour microenvironment, this could further support the restoration of anti-tumour immunity in glioblastoma patients.

Early studies have begun to explore the role of EVs in the glioblastoma TME, but more needs to be done to establish what cargo these EVs are transporting and explore how these affect immune cells both locally and systemically. Various studies have showed that EVs can directly interact with T cells through PD-L1/PD-1 and CD73/CD39, but it is plausible that there could be other markers on the surface of EVs that inhibit T cell activity to suppress anti-tumour immunity (e.g. Fas or Galectin-9) (Deaglio et al., 2007; Himes et al., 2020; Ricklefs et al., 2018; M. Wang et al., 2021). Furthermore, proteomic analyses could be utilised to identify factors of the glioblastoma secretome that could be targeted therapeutically – it is possible that IFN-γ, IL-21 and TNF-α treatment could alter the expression of these components to change the dynamics of the TME in favour of an anti-tumour immune response. In particular, using conditioned medium from glioblastoma patient-derived cells could help to provide clarity on the impact of the glioblastoma secretome on T cells, as there were numerous instances of variability between the impact of U251 and U373 cell supernatant on the expression on several T cell markers. It would also be beneficial to compare the effect of IFN-γ, IL-21 and TNF-α on immune cells taken from glioblastoma patients against healthy controls to improve our understanding of how glioblastoma cells impair the immune response and evaluate whether immune function can be restored with IFN-γ, IL-21 and TNF-α treatment.

Similarly, it would be worthwhile for future research to evaluate how the glioblastoma secretome and subsequent treatment with IFN-γ, IL-21 and TNF-α affects T cell activation, proliferation, exhaustion, and cell death to fully assess T cell function. Furthermore, it is important to also consider that IFN-γ, IL-21 and TNF-α treatment impaired IFN-γ production in both CD4+ and CD8+ T cells, which may compromise the development of an anti-tumour immune response by restricting Th1, Tc1 and/or M1 macrophage differentiation in the glioblastoma microenvironment. In line with this, studies should consider adding IFN-γ, IL-21 and TNF-α to glioblastoma and T cell co-cultures to explore whether the combination of their direct anti-tumour effects and impairment of the glioblastoma secretome can co-operate to potently promote tumour rejection. This would also build upon results found in Chapter Five, in which IFN-γ, IL-21 and TNF-α treatment facilitated changes in the immunological ligand expression profile on both U251 and U373 cells, thereby increasing their potential to enhance co-stimulatory T cell signalling. Likewise, it would be interesting to explore the impact of the glioblastoma secretome and/or IFN-γ, IL-21 and TNF-α treatment on APCs to establish whether the disruption on T cell activation and antigen processing (e.g. removal of CD80, CD86 and MHC-antigen complexes) mediated by Tregs can also be restored to further promote anti-tumour immunity.

It is also particularly important for future research to determine how IFN-γ, IL-21 and TNF-α affected signalling pathways within each of these T cell subsets, perhaps through disruption to the activity of other transcription factors, including: AP-1, c-Maf, IRF4, NFAT, pSTATs, Runx3, and/or TCF-1. In addition, it would be useful to expand the antibody panel to include unique T cell surface markers to improve our understanding of how the glioblastoma secretome and/or IFN-γ, IL-21 and TNF-α influence each of these T cell subsets – for example, changes in chemokine ligand expression could affect their migration to the tumour site. Similarly, future studies should also consider widening the panel of immune ligands on glioblastoma cells to include MHC class I/II, CD40-L and CTLA-4, as these are also important signalling molecules involved in regulating the anti-tumour immune response. Likewise, it would be fascinating to evaluate the impact of IFN-γ, IL-21 and TNF-α on the cytotoxic activity of CD4+ and CD8+ T cells, as upregulation of perforin, granzyme B and Fas-L could provide another mechanism of enhancing anti-tumour immunity in glioblastoma. Furthermore, as previous research has identified there is considerable plasticity among T cell subsets, it is important for future studies to also explore co-expression of each of the markers used in the present thesis to improve our understanding of how the glioblastoma secretome affects these T cell subsets and explore the impact of IFN-γ, IL-21 and TNF-α treatment in further detail.

Overall, the present thesis has highlighted significant therapeutic potential in IFN-γ, IL-21 and TNF-α as a novel immunotherapy for glioblastoma. In particular, results demonstrated that this triple cytokine combination could directly inhibit tumour growth and induce cell death in glioblastoma cells. Furthermore, IFN-γ, IL-21 and TNF-α could alter the expression of various immunological co-stimulatory ligands on glioblastoma cells, whilst impairing the immunosuppressive nature of the glioblastoma secretome, thus these cytokines have the significant potential to restore anti-tumour immunity in glioblastoma patients. Altogether, these findings have provided a solid foundation for future research to build upon, in order to explore the therapeutic value of IFN-γ, IL-21 and TNF-α as a novel cytokine-based immunotherapy in glioblastoma patients.

Total Word Count: 54,300

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9. Appendix

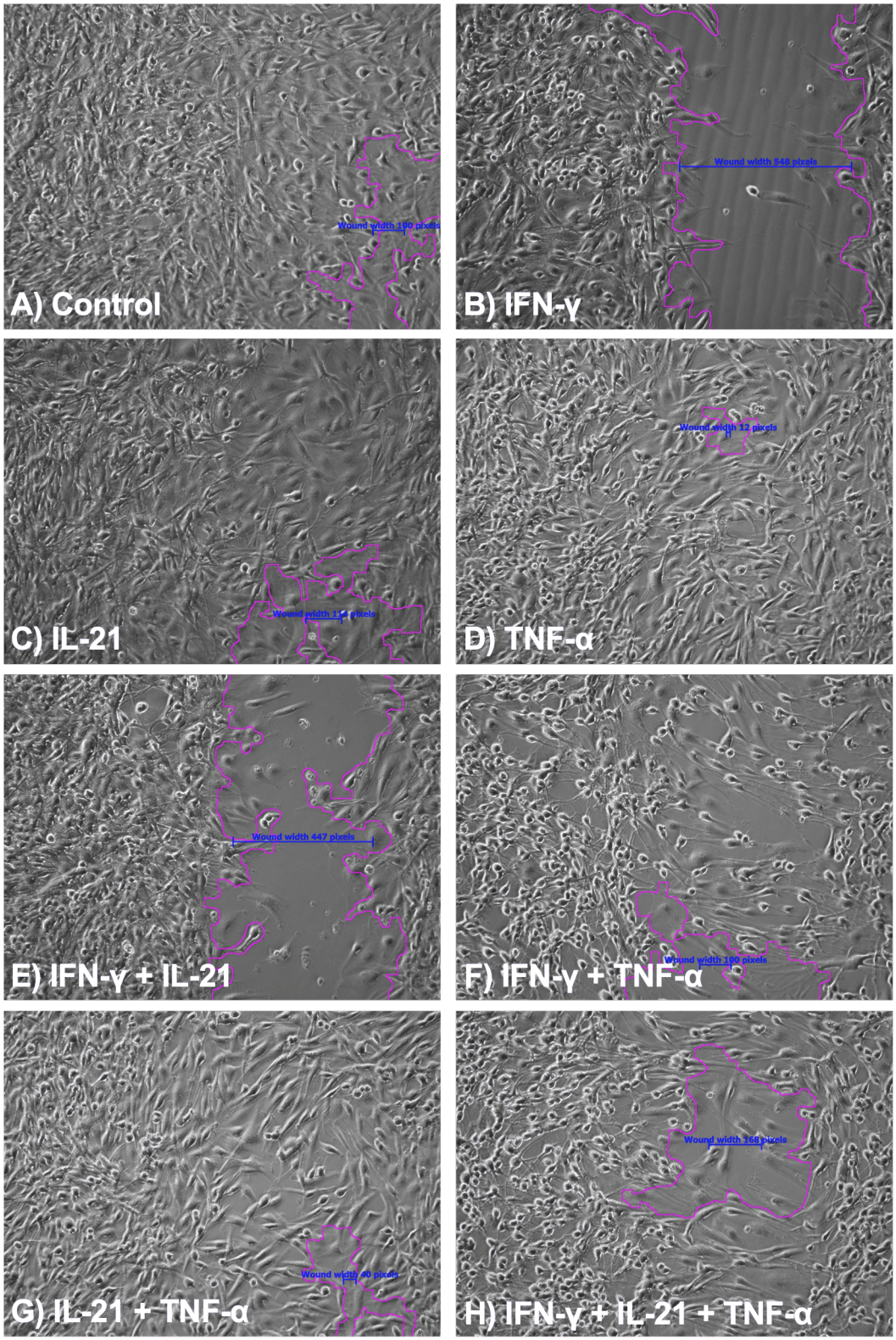


Figure 9.1. IFN-γ, IL-21 and TNF-α had no impact on U251 cell migration after 24 hours

$1.5x10^4$ U251 cells were seeded into two-well culture inserts inside a 24-well plate for 24 hours before the inserts were removed and U251 cells were freshly treated with **A)** RPMI Control; **B)** 100ng/ml IFN-γ; **C)** 100ng/ml IL-21; **D)** 100ng/ml TNF-α; **E)** 100ng/ml IFN-γ and IL-21; **F)** 100ng/ml IFN-γ and TNF-α; **G)** 100ng/ml IL-21 and TNF-α; **H)** 100ng/ml IFN-γ, IL-21 and TNF-α. Images were taken every hour over a 72-hour period, with these particular images taken 24 hours post-insert removal. The size of the area between cell populations was determined using Cell IQ analysis software. Images are representative of > 3 independent experiments.

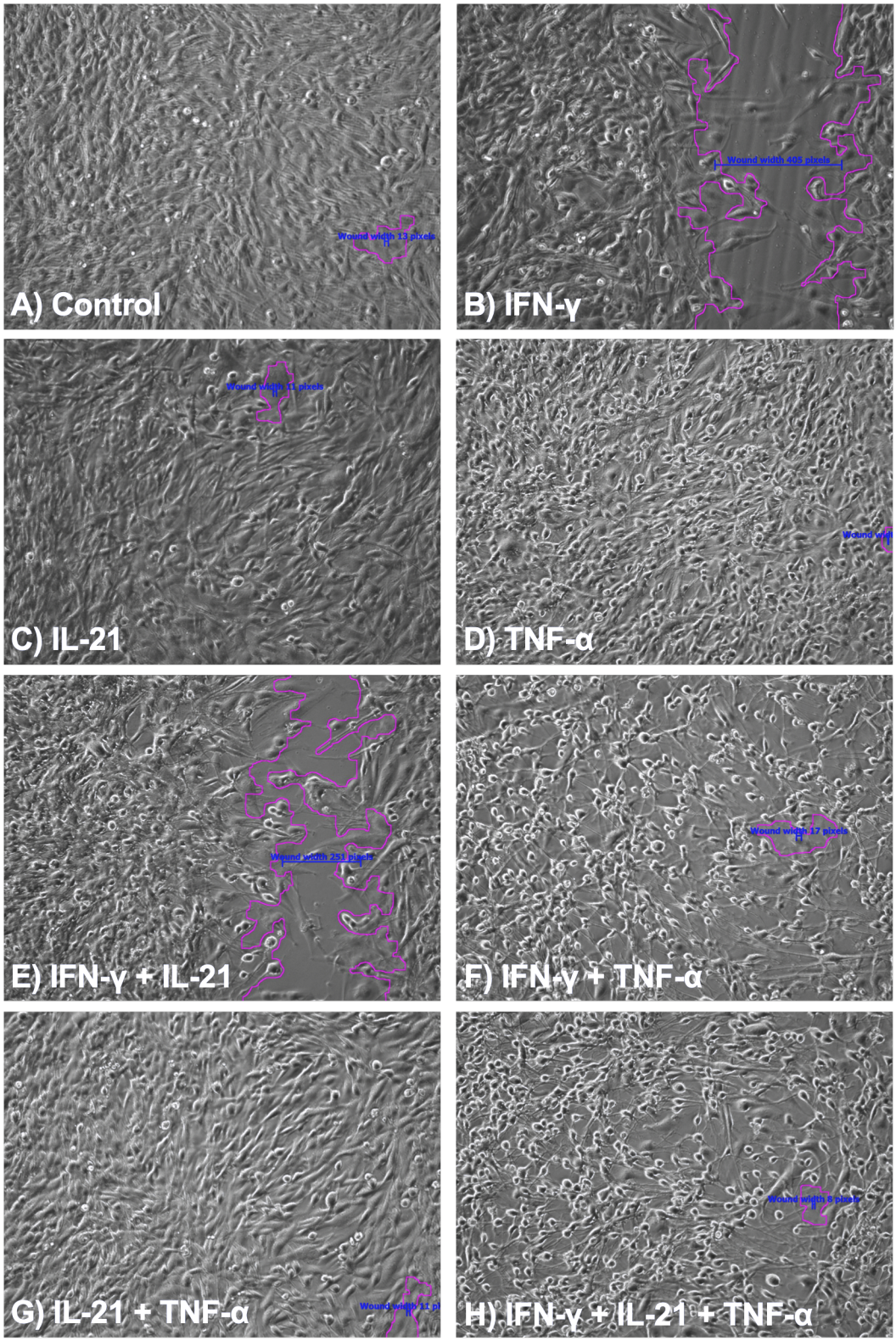


Figure 9.2. IFN-γ and IL-21 reduced U251 cell migration after 48 hours

$1.5x10^4$ U251 cells were seeded into two-well culture inserts inside a 24-well plate for 24 hours before the inserts were removed and U251 cells were freshly treated with **A)** RPMI Control; **B)** 100ng/ml IFN-γ; **C)** 100ng/ml IL-21; **D)** 100ng/ml TNF-α; **E)** 100ng/ml IFN-γ and IL-21; **F)** 100ng/ml IFN-γ and TNF-α; **G)** 100ng/ml IL-21 and TNF-α; **H)** 100ng/ml IFN-γ, IL-21 and TNF-α. Images were taken every hour over a 72-hour period, with these particular images taken 48 hours post-insert removal. The size of the area between cell populations was determined using Cell IQ analysis software. Images are representative of > 3 independent experiments.

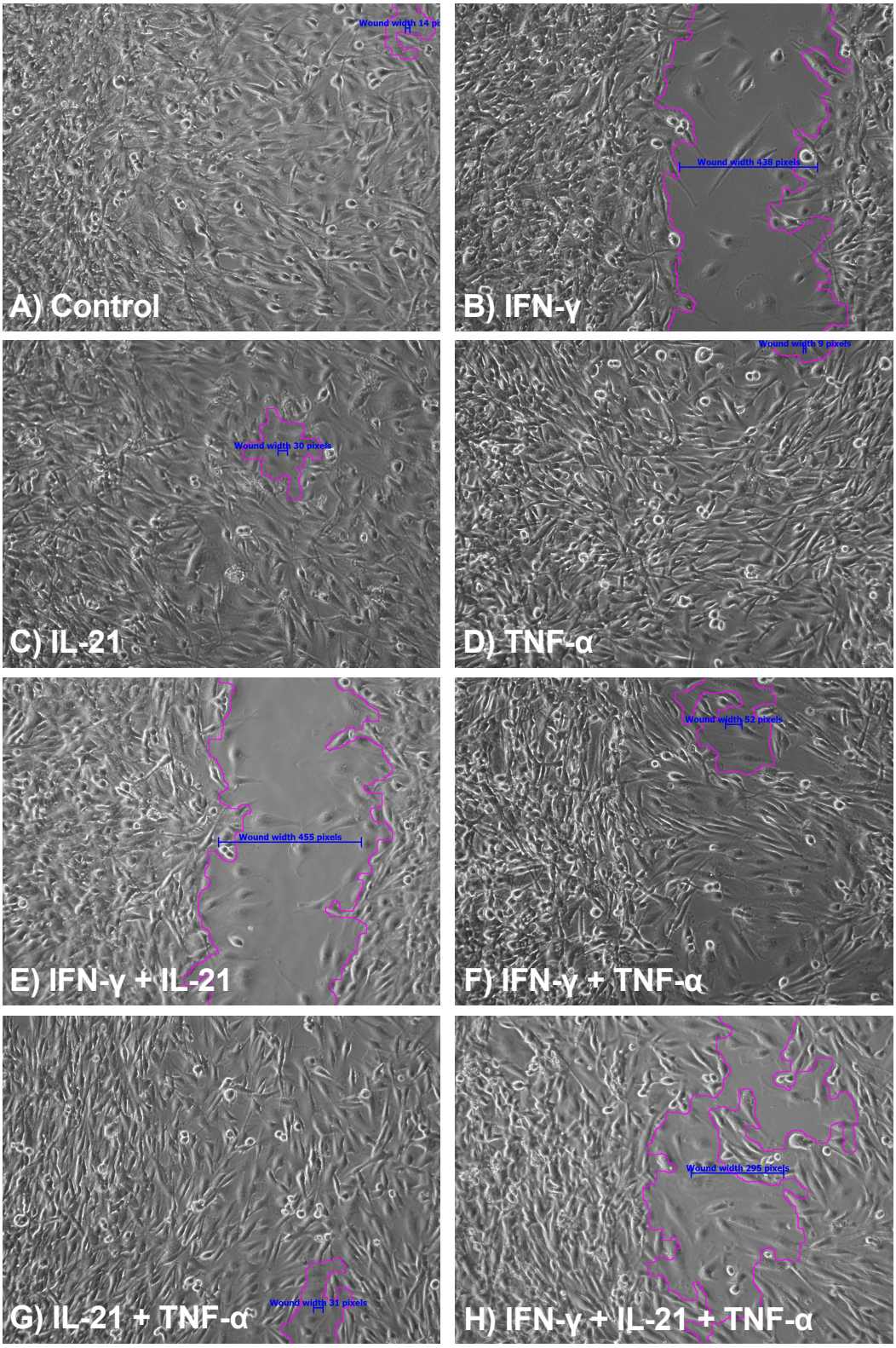


Figure 9.3. IFN-γ and IL-21 reduced U373 cell migration after 24 hours

$1.5x10^4$ U373 cells were seeded into two-well culture inserts inside a 24-well plate for 24 hours before the inserts were removed and U373 cells were freshly treated with **A)** RPMI Control; **B)** 100ng/ml IFN-γ; **C)** 100ng/ml IL-21; **D)** 100ng/ml TNF-α; **E)** 100ng/ml IFN-γ and IL-21; **F)** 100ng/ml IFN-γ and TNF-α; **G)** 100ng/ml IL-21 and TNF-α; **H)** 100ng/ml IFN-γ, IL-21 and TNF-α. Images were taken every hour over a 72-hour period, with these particular images taken 24 hours post-insert removal. The size of the area between cell populations was determined using Cell IQ analysis software. Images are representative of > 3 independent experiments.

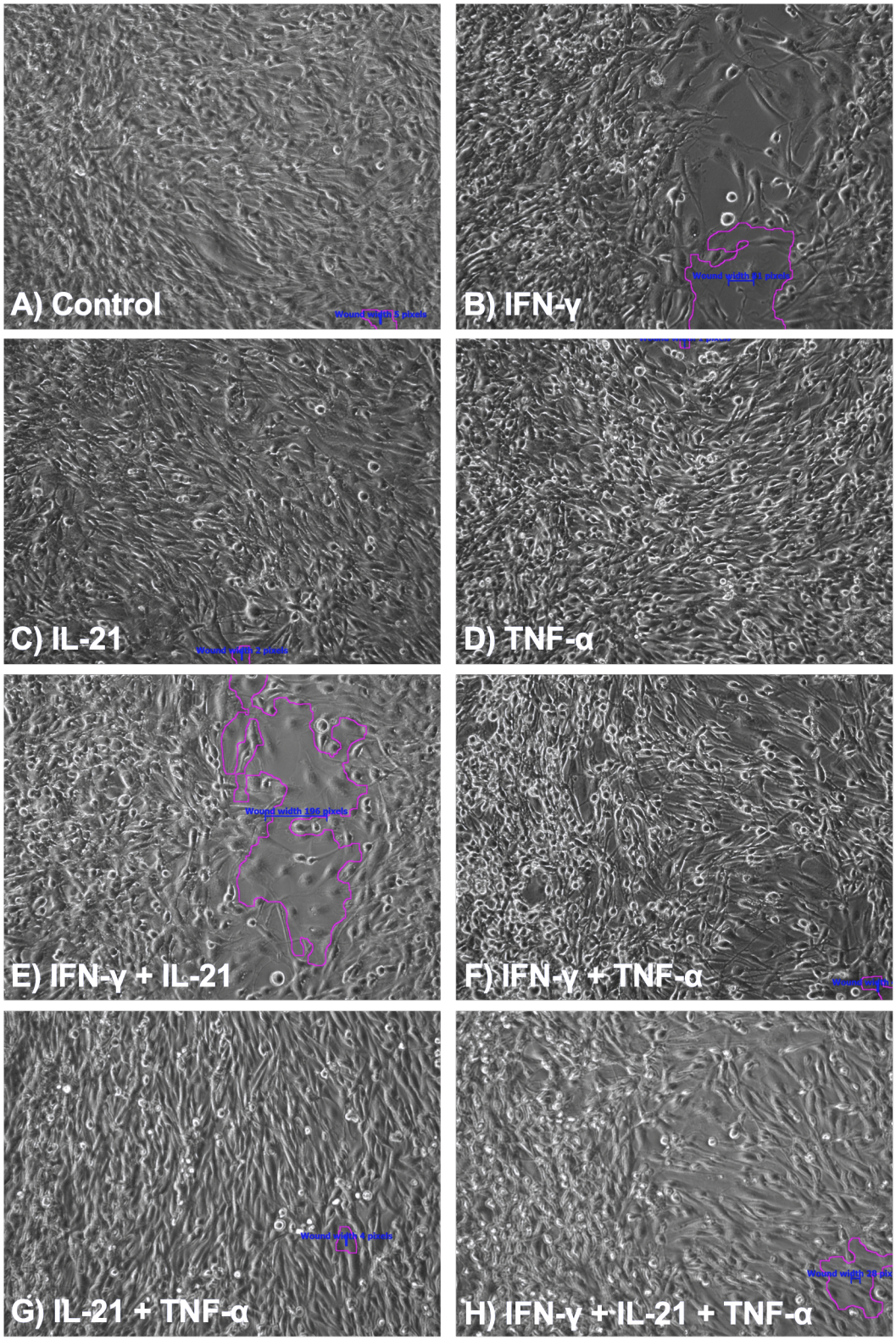


Figure 9.4. IFN-γ and IL-21 reduced U373 cell migration after 48 hours

$1.5x10^4$ U373 cells were seeded into two-well culture inserts inside a 24-well plate for 24 hours before the inserts were removed and U373 cells were freshly treated with **A)** RPMI Control; **B)** 100ng/ml IFN-γ; **C)** 100ng/ml IL-21; **D)** 100ng/ml TNF-α; **E)** 100ng/ml IFN-γ and IL-21; **F)** 100ng/ml IFN-γ and TNF-α; **G)** 100ng/ml IL-21 and TNF-α; **H)** 100ng/ml IFN-γ, IL-21 and TNF-α. Images were taken every hour over a 72-hour period, with these particular images taken 48 hours post-insert removal. The size of the area between cell populations was determined using Cell IQ analysis software. Images are representative of > 3 independent experiments.

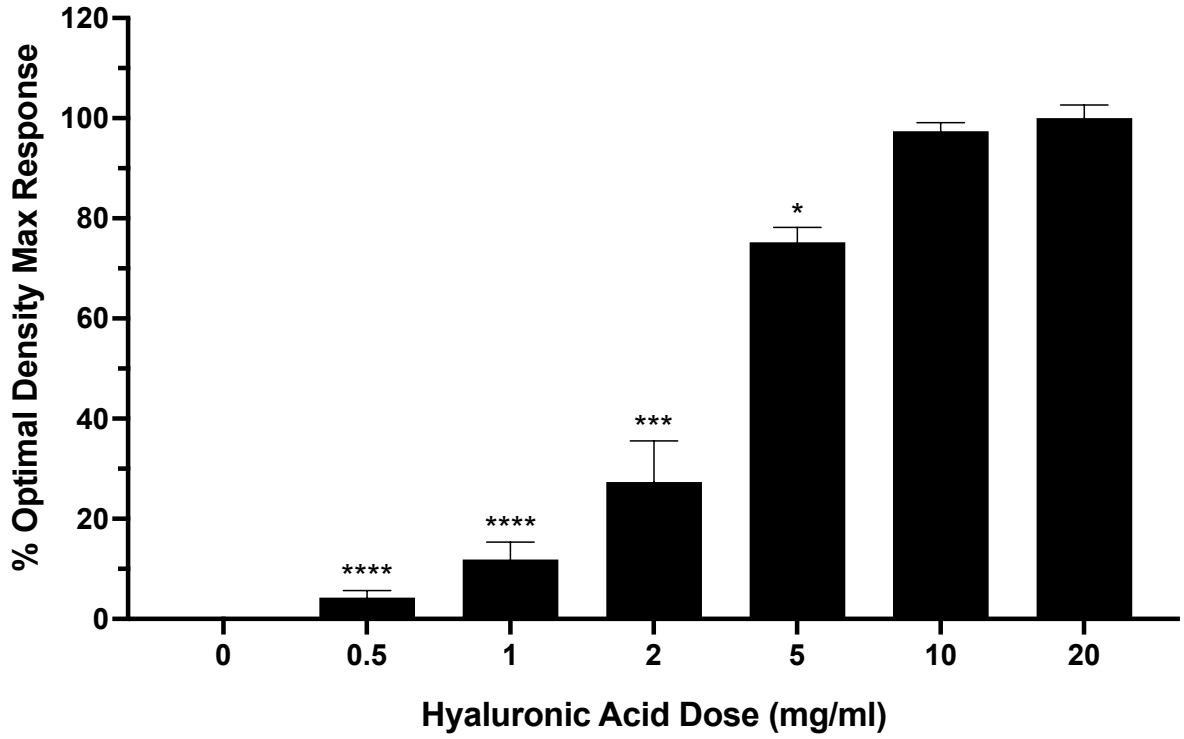


Figure 9.5. Optimisation of HA coating

24-well plates were coated for 24 hours with a titration of HA, ranging from 0.5mg/ml to 20mg/ml, before the wells were washed with CPC to induce HA precipitation, which was subsequently analysed at 595nm using a plate reader to generate a measurement of optical density. Bars represent means compared to 20ng/ml HA with SEM shown for > 3 independent experiments. P Values: * = <0.05; ** = <0.01; *** = <0.001; **** = <0.0001.

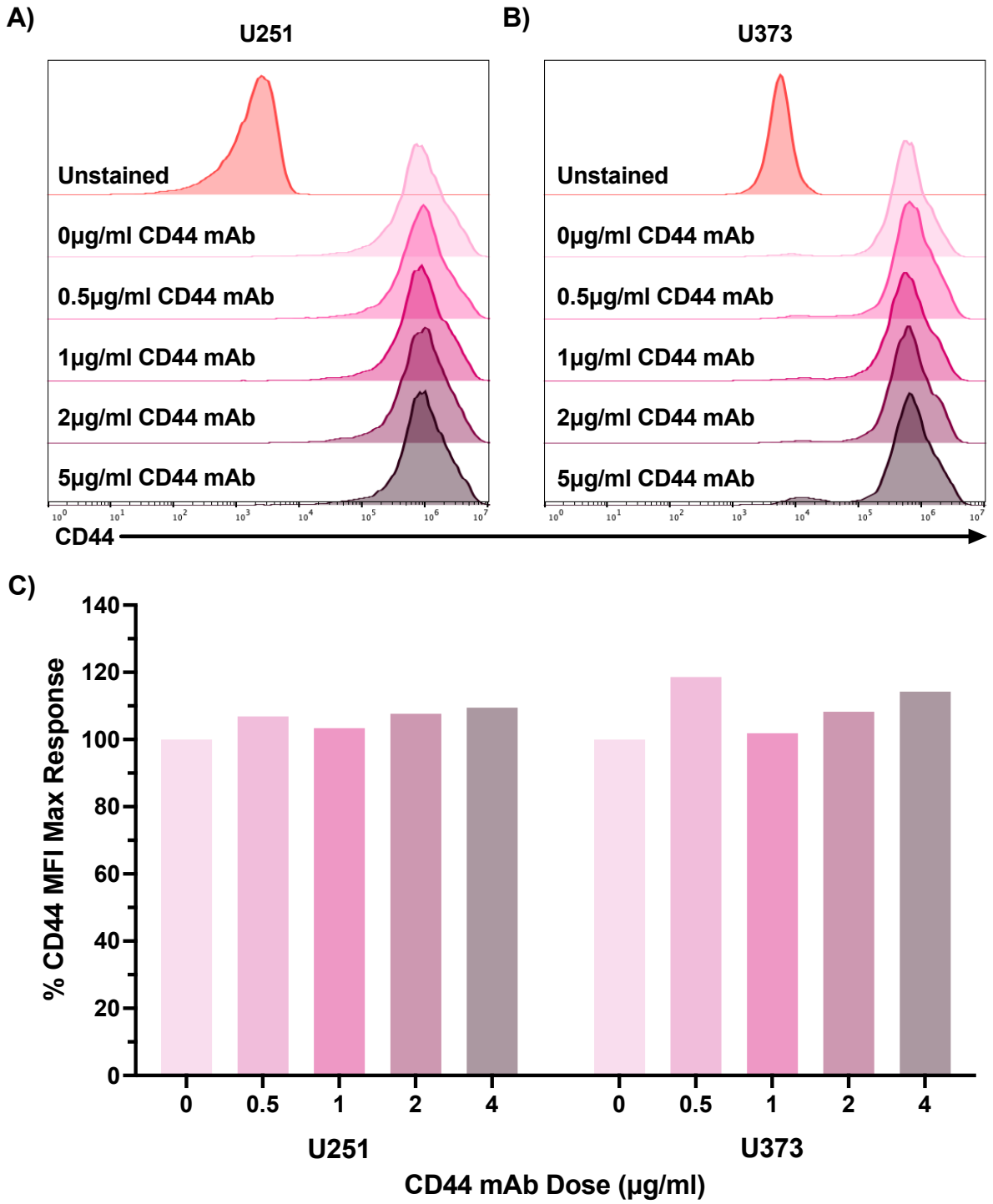


Figure 9.6. Optimisation of CD44 mAb after 30 minutes of treatment

$2.5x10^4$ U251 **A)** and U373 cells **B)** were treated for 30 minutes with a titration of CD44 mAb, ranging from 0.5µg/ml to 4µg/ml, before they were stained for CD44 for analysis via flow cytometry. Flow plots **A)** are representative of 1 independent experiment. Bars represent CD44 MFI **C)** and as raw values normalised to the 0µg/ml CD44 mAb Control.

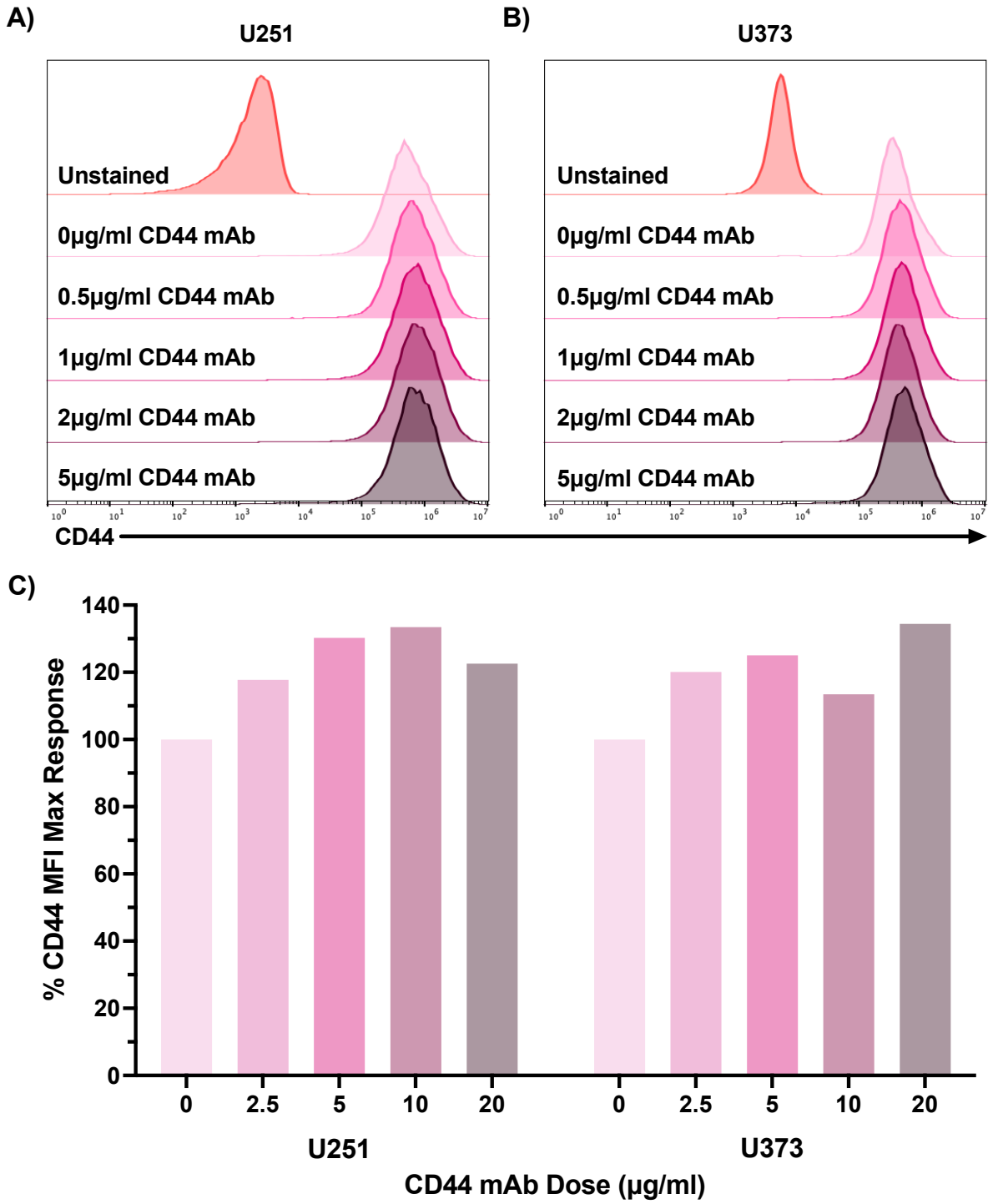
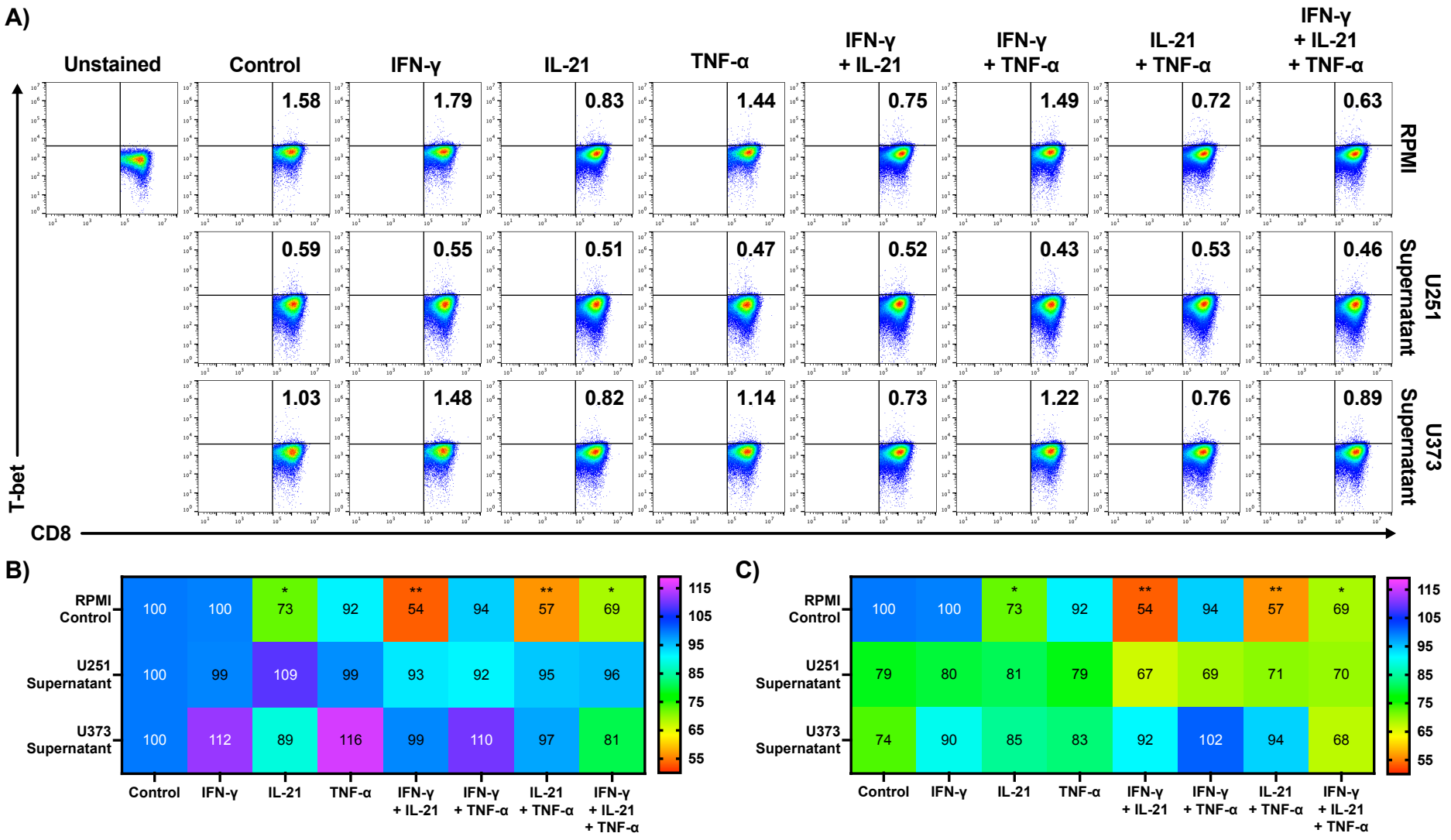


Figure 9.7. Optimisation of CD44 mAb after 1 hour of treatment

$2.5x10^4$ U251 **A)** and U373 cells **B)** were treated for 1 hour with a titration of CD44 mAb, ranging from 0.5µg/ml to 4µg/ml, before they were stained for CD44 for analysis via flow cytometry. Flow plots **A)** are representative of 1 independent experiment. Bars represent CD44 MFI **C)** and as raw values normalised to the 0µg/ml CD44 mAb Control.


A)
Unstained
Control
IFN-γ
IL-21
TNF-α
IFN-γ + IL-21
IFN-γ + TNF-α
IL-21 + TNF-α
IFN-γ + IL-21 + TNF-α
RPMI
U251 Supernatant
U373 Supernatant
T-bet
CD8
1.58
1.79
0.83
1.44
0.75
1.49
0.72
0.63
0.59
0.55
0.51
0.47
0.52
0.43
0.53
0.46
1.03
1.48
0.82
1.14
0.73
1.22
0.76
0.89
B)
RPMI Control
U251 Supernatant
U373 Supernatant
100 100 * 73 92 ** 54 94 ** 57 * 69
100 99 109 99 93 92 95 96
100 112 89 116 99 110 97 81
Control
IFN-γ
IL-21
TNF-α
IFN-γ + IL-21
IFN-γ + TNF-α
IL-21 + TNF-α
IFN-γ + IL-21 + TNF-α
115
105
95
85
75
65
55
C)
RPMI Control
U251 Supernatant
U373 Supernatant
100 100 * 73 92 ** 54 94 ** 57 * 69
79 80 81 79 67 69 71 70
74 90 85 83 92 102 94 68
Control
IFN-γ
IL-21
TNF-α
IFN-γ + IL-21
IFN-γ + TNF-α
IL-21 + TNF-α
IFN-γ + IL-21 + TNF-α
115
105
95
85
75
65
55

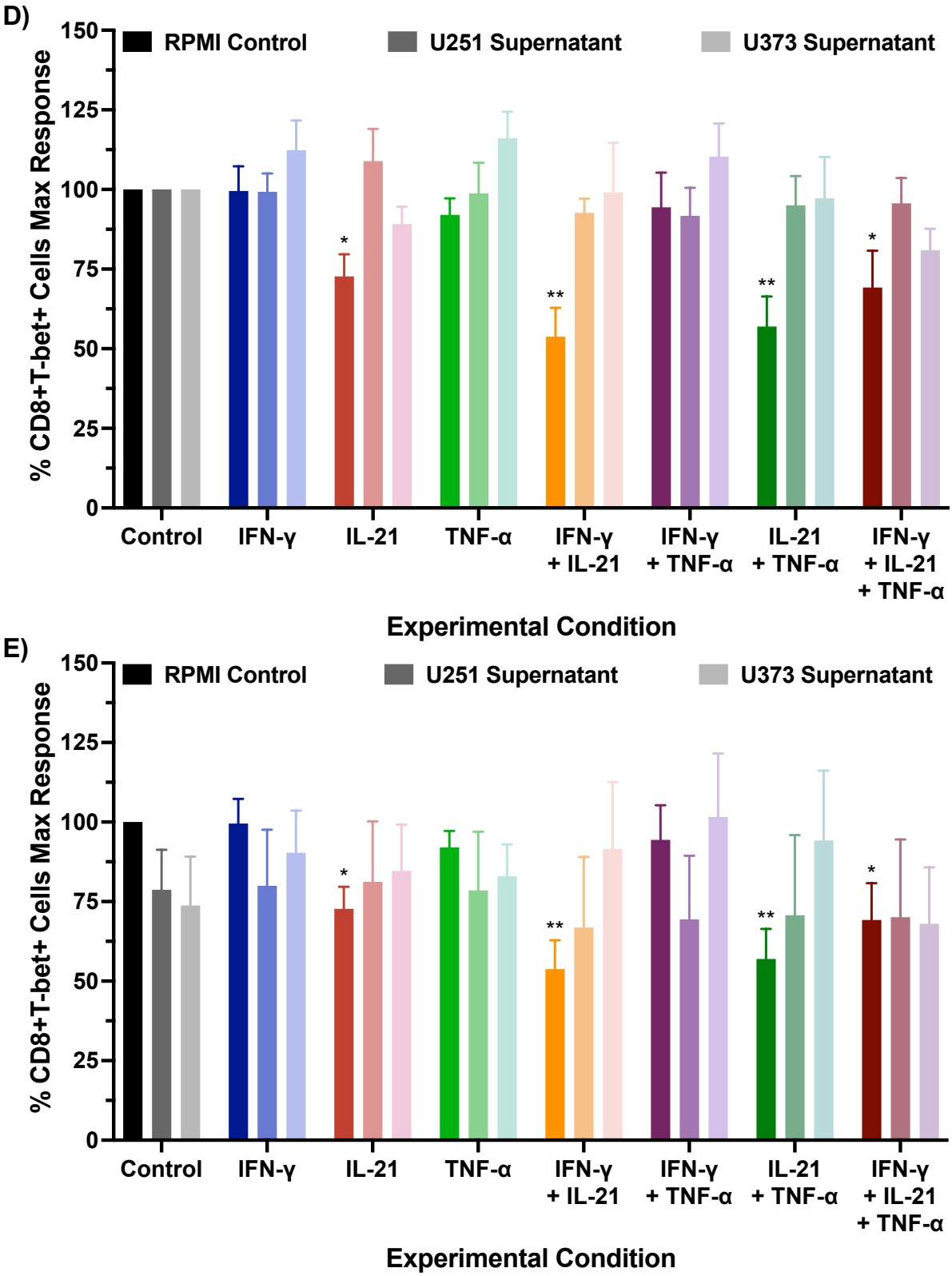
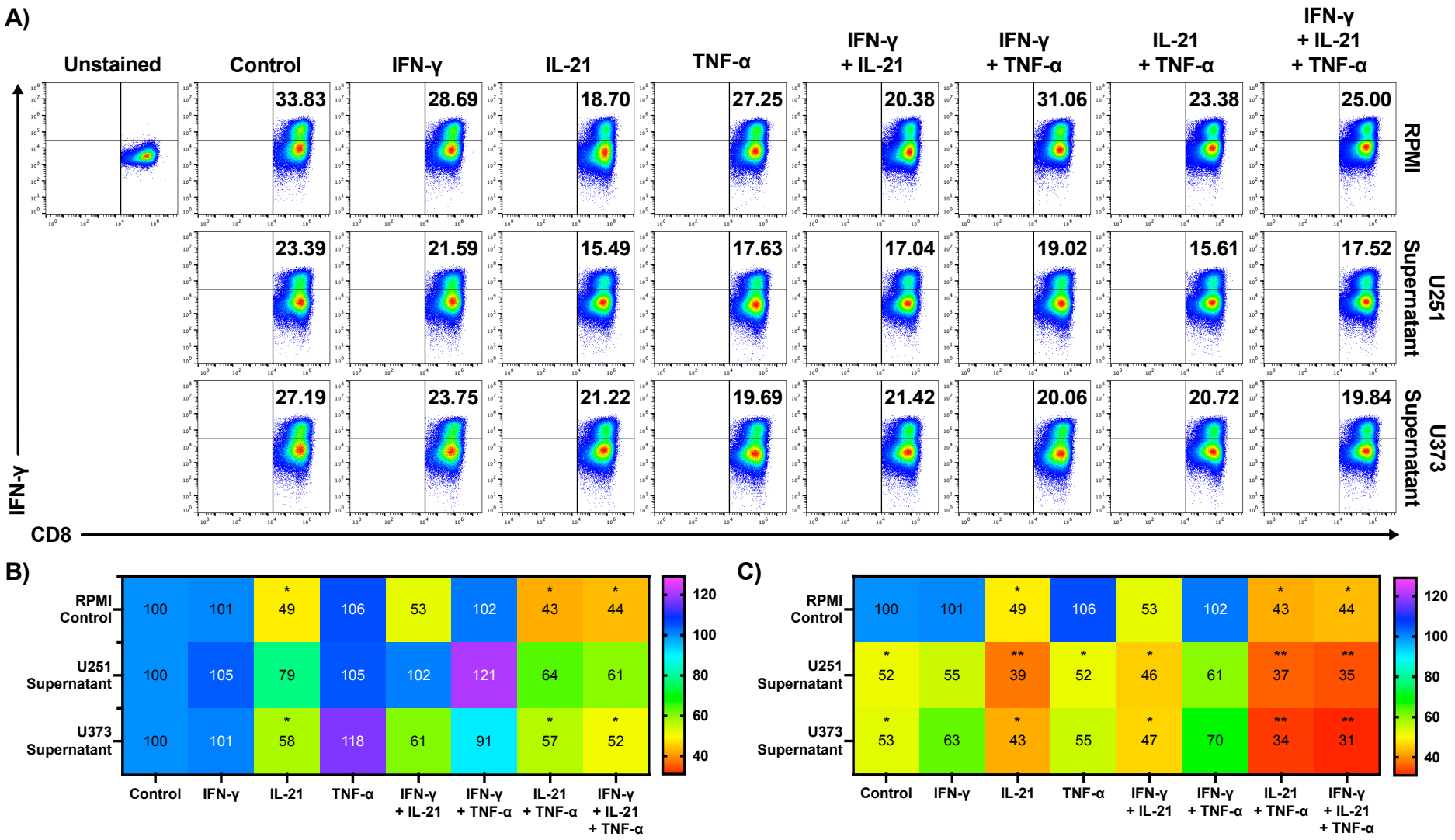


Figure 9.8. IFN-γ, IL-21 and TNF-α largely did not affect T-bet expression in bulk CD8+ T cells
$2.5x10^4$ bulk CD8+ T cells were cultured 1:1 with CD3/CD28 beads in RPMI, U251 cell supernatant or U373 cell supernatant, plus treatment with 100ng/ml IFN-γ, IL-21 and TNF-α, both individually and combined for 5 days. Cells were then harvested and stained for CD8 and T-bet for analysis via flow cytometry. Flow plots **A)** are representative of > 3 independent experiments. Heat Maps and Bars represent % CD8+T-bet+ cells as means compared to the relevant Control **B)** and **D)**, plus % CD8+T-bet+ cells as means compared to the RPMI Control **C)** and **E)** with SEM shown for > 3 independent experiments. P Values: * = <0.05; ** = <0.01; *** = <0.001; **** = <0.0001.


A)
Unstained
Control
IFN-γ
IL-21
TNF-α
IFN-γ + IL-21
IFN-γ + TNF-α
IL-21 + TNF-α
IFN-γ + IL-21 + TNF-α
RPMI
U251 Supernatant
U373 Supernatant
33.83
28.69
18.70
27.25
20.38
31.06
23.38
25.00
23.39
21.59
15.49
17.63
17.04
19.02
15.61
17.52
27.19
23.75
21.22
19.69
21.42
20.06
20.72
19.84
IFN-γ
CD8
B)
RPMI Control
U251 Supernatant
U373 Supernatant
100 101 49 106 53 102 43 44
100 105 79 105 102 121 64 61
100 101 58 118 61 91 57 52
Control
IFN-γ
IL-21
TNF-α
IFN-γ + IL-21
IFN-γ + TNF-α
IL-21 + TNF-α
IFN-γ + IL-21 + TNF-α
120
100
80
60
40
C)
RPMI Control
U251 Supernatant
U373 Supernatant
100 101 49 106 53 102 43 44
52 55 39 52 46 61 37 35
53 63 43 55 47 70 34 31
Control
IFN-γ
IL-21
TNF-α
IFN-γ + IL-21
IFN-γ + TNF-α
IL-21 + TNF-α
IFN-γ + IL-21 + TNF-α
120
100
80
60
40

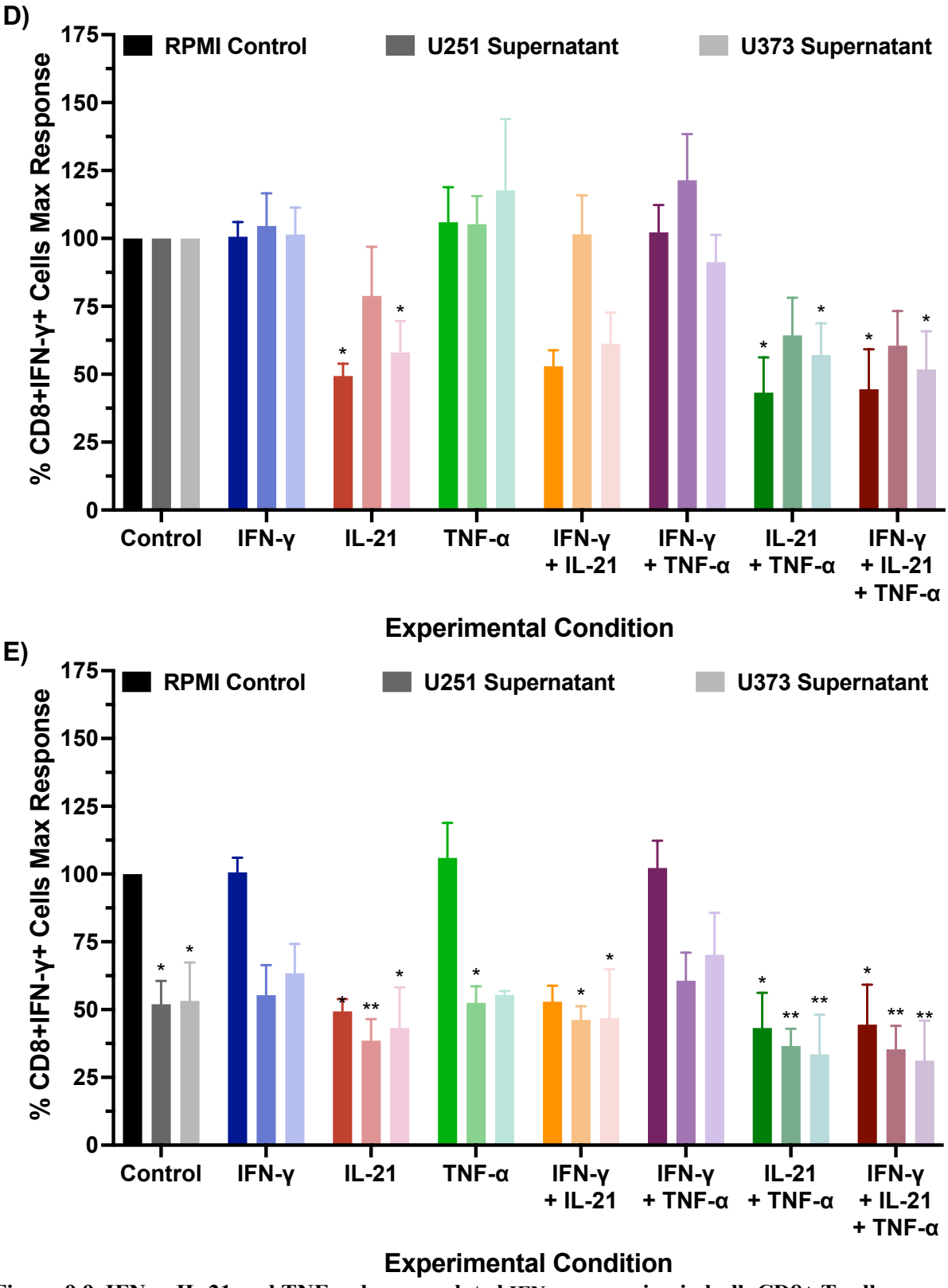
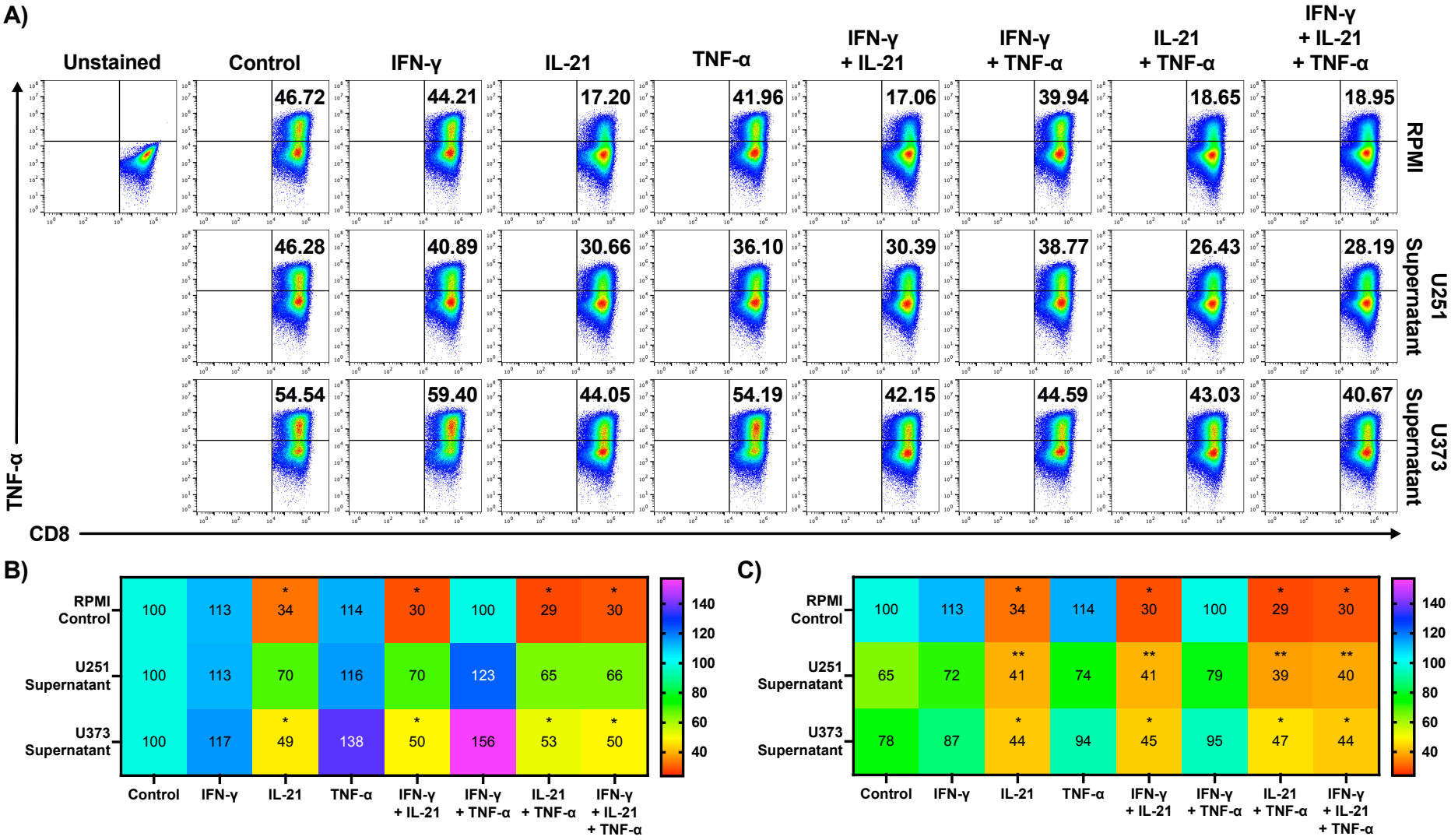


Figure 9.9. IFN-γ, IL-21 and TNF-α downregulated IFN-γ expression in bulk CD8+ T cells
$2.5x10^4$ bulk CD8+ T cells were cultured 1:1 with CD3/CD28 beads in RPMI, U251 cell supernatant or U373 cell supernatant, plus treatment with 100ng/ml IFN-γ, IL-21 and TNF-α, both individually and combined for 5 days. Cells were then harvested and stained for CD8 and IFN-γ for analysis via flow cytometry. Flow plots **A)** are representative of > 3 independent experiments. Heat Maps and Bars represent % CD8+IFN-γ+ cells as means compared to the relevant Control **B)** and **D)**, plus % CD8+IFN-γ+ cells as means compared to the RPMI Control **C)** and **E)** with SEM shown for > 3 independent experiments. P Values: * = <0.05; ** = <0.01; *** = <0.001; **** = <0.0001.


A)
Unstained
Control
IFN-γ
IL-21
TNF-α
IFN-γ + IL-21
IFN-γ + TNF-α
IL-21 + TNF-α
IFN-γ + IL-21 + TNF-α
46.72
44.21
17.20
41.96
17.06
39.94
18.65
18.95
46.28
40.89
30.66
36.10
30.39
38.77
26.43
28.19
54.54
59.40
44.05
54.19
42.15
44.59
43.03
40.67
RPMI
U251 Supernatant
U373 Supernatant
TNF-α
CD8
B)
RPMI Control
U251 Supernatant
U373 Supernatant
100 113 * 34 114 * 30 100 * 29 * 30
100 113 70 116 70 123 65 66
100 117 * 49 138 * 50 156 * 53 * 50
Control
IFN-γ
IL-21
TNF-α
IFN-γ + IL-21
IFN-γ + TNF-α
IL-21 + TNF-α
IFN-γ + IL-21 + TNF-α
140
120
100
80
60
40
C)
RPMI Control
U251 Supernatant
U373 Supernatant
100 113 * 34 114 * 30 100 * 29 * 30
65 72 ** 41 74 ** 41 79 ** 39 ** 40
78 87 * 44 94 * 45 95 * 47 * 44
Control
IFN-γ
IL-21
TNF-α
IFN-γ + IL-21
IFN-γ + TNF-α
IL-21 + TNF-α
IFN-γ + IL-21 + TNF-α
140
120
100
80
60
40

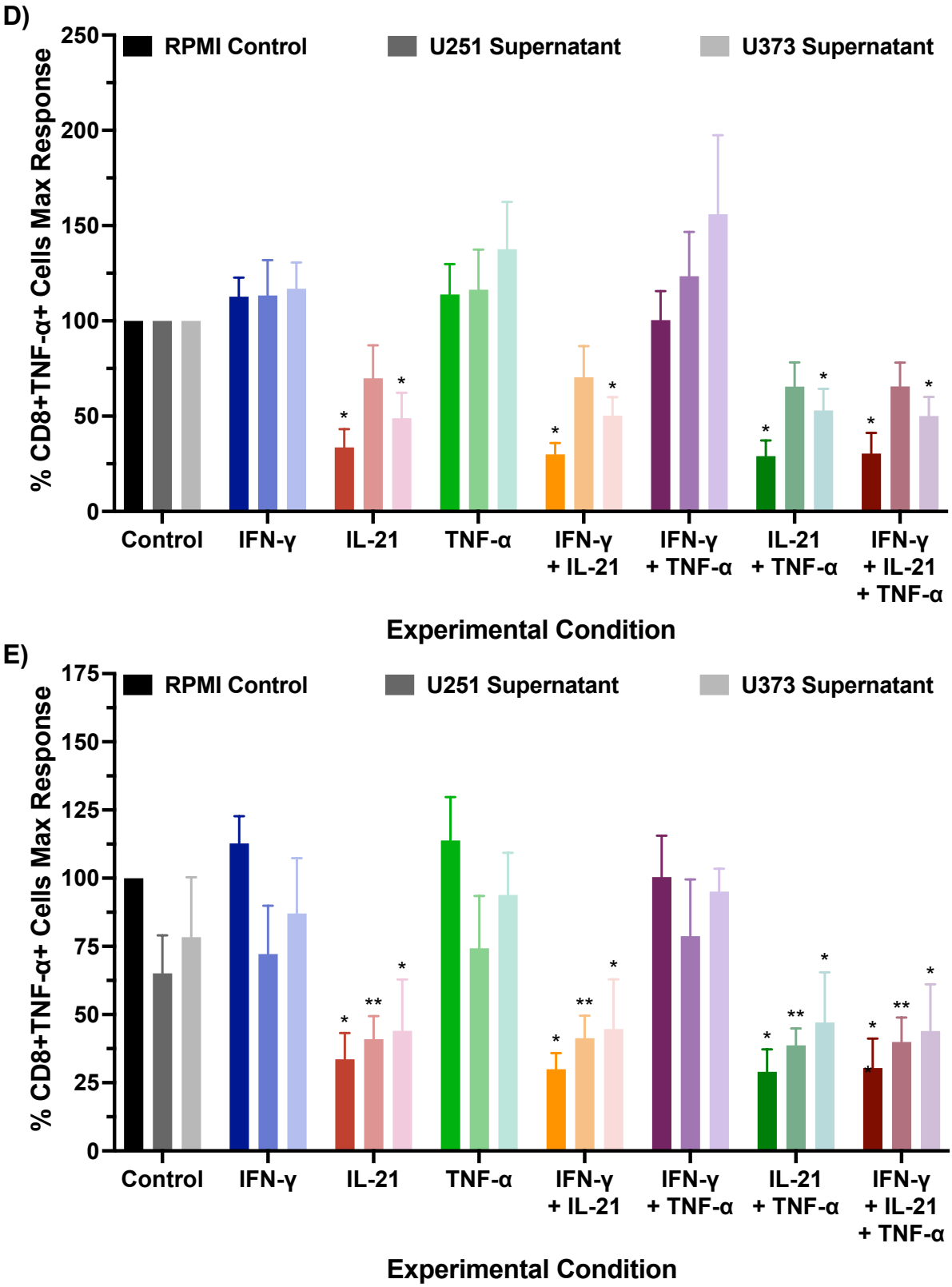
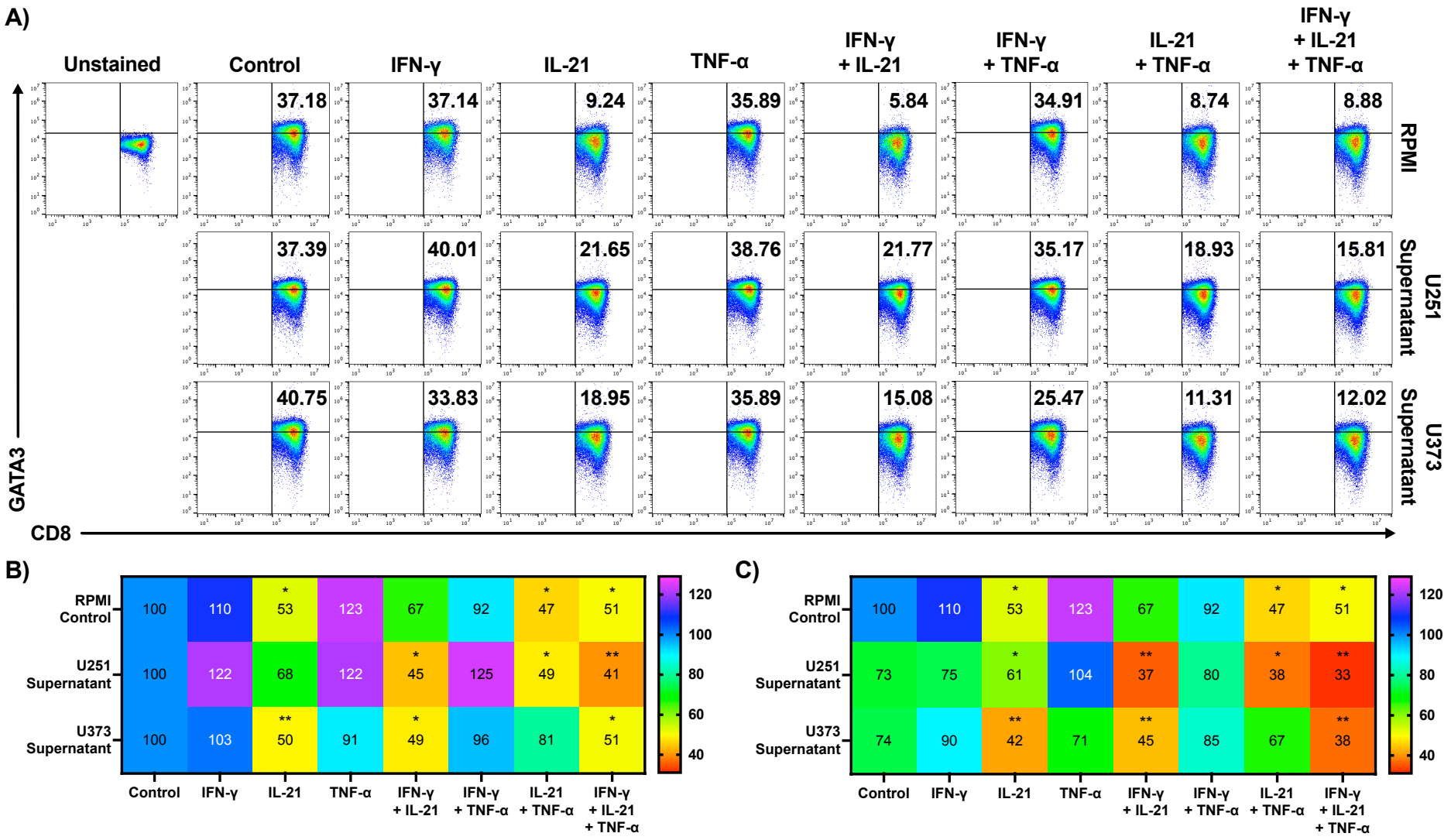


Figure 9.10. IFN-γ, IL-21 and TNF-α downregulated TNF-α expression in bulk CD8+ T cells
$2.5x10^4$ bulk CD8+ T cells were cultured 1:1 with CD3/CD28 beads in RPMI, U251 cell supernatant or U373 cell supernatant, plus treatment with 100ng/ml IFN-γ, IL-21 and TNF-α, both individually and combined for 5 days. Cells were then harvested and stained for CD8 and TNF-α for analysis via flow cytometry. Flow plots **A)** are representative of > 3 independent experiments. Heat Maps and Bars represent % CD8+TNF-α+ cells as means compared to the relevant Control **B)** and **D)**, plus % CD8+TNF-α+ cells as means compared to the RPMI Control **C)** and **E)** with SEM shown for > 3 independent experiments. P Values: * = <0.05; ** = <0.01; *** = <0.001; **** = <0.0001.


A)
Unstained
Control
IFN-γ
IL-21
TNF-α
IFN-γ + IL-21
IFN-γ + TNF-α
IL-21 + TNF-α
IFN-γ + IL-21 + TNF-α
RPMI
U251 Supernatant
U373 Supernatant
GATA3
CD8
37.18
37.14
9.24
35.89
5.84
34.91
8.74
8.88
37.39
40.01
21.65
38.76
21.77
35.17
18.93
15.81
40.75
33.83
18.95
35.89
15.08
25.47
11.31
12.02
B)
RPMI Control
U251 Supernatant
U373 Supernatant
100 110 * 53 123 67 92 * 47 * 51
100 122 68 122 * 45 125 * 49 ** 41
100 103 ** 50 91 * 49 96 81 * 51
Control
IFN-γ
IL-21
TNF-α
IFN-γ + IL-21
IFN-γ + TNF-α
IL-21 + TNF-α
IFN-γ + IL-21 + TNF-α
120
100
80
60
40
C)
RPMI Control
U251 Supernatant
U373 Supernatant
100 110 * 53 123 67 92 * 47 * 51
73 75 * 61 104 ** 37 80 * 38 ** 33
74 90 ** 42 71 ** 45 85 67 ** 38
Control
IFN-γ
IL-21
TNF-α
IFN-γ + IL-21
IFN-γ + TNF-α
IL-21 + TNF-α
IFN-γ + IL-21 + TNF-α
120
100
80
60
40

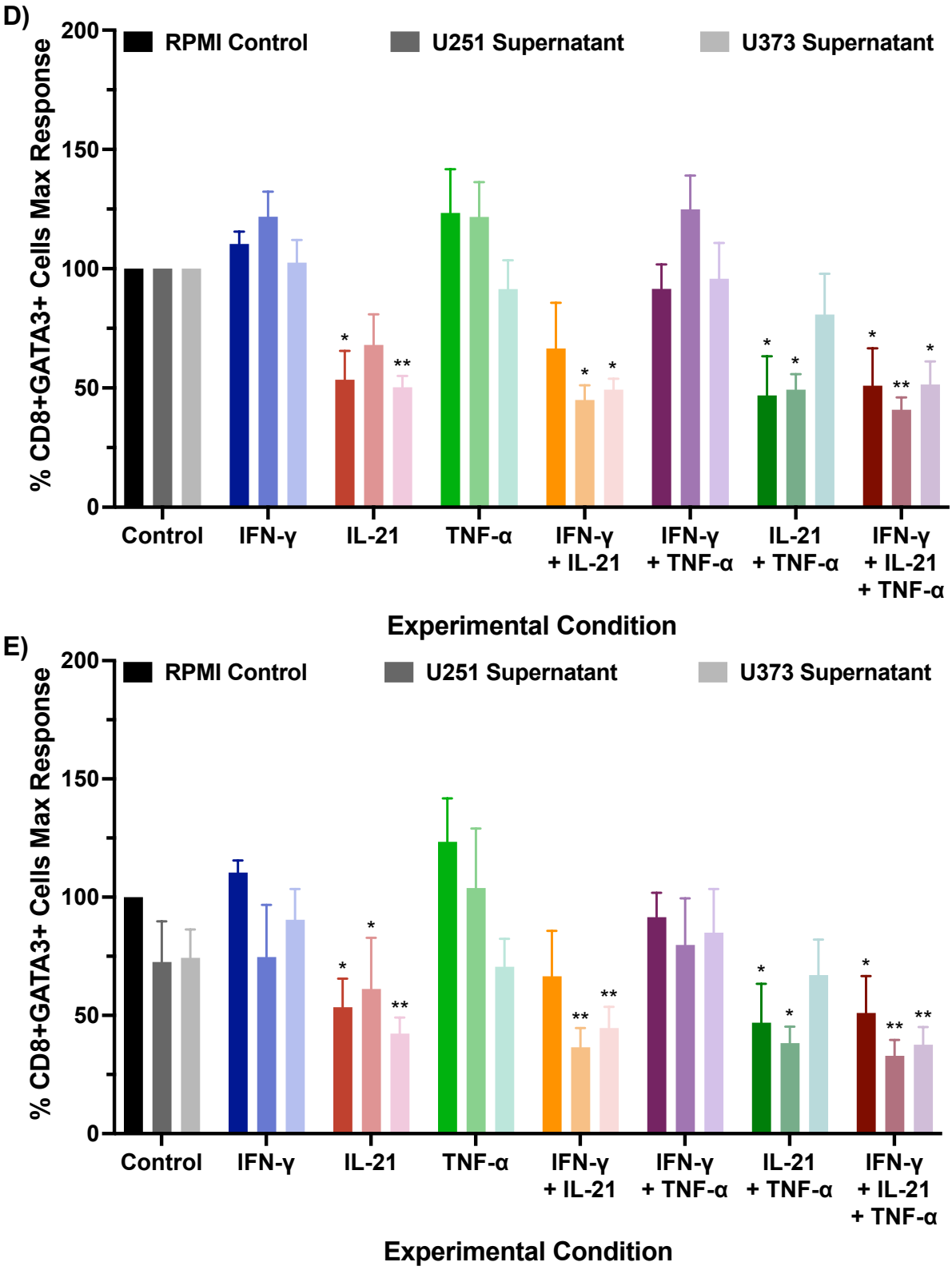
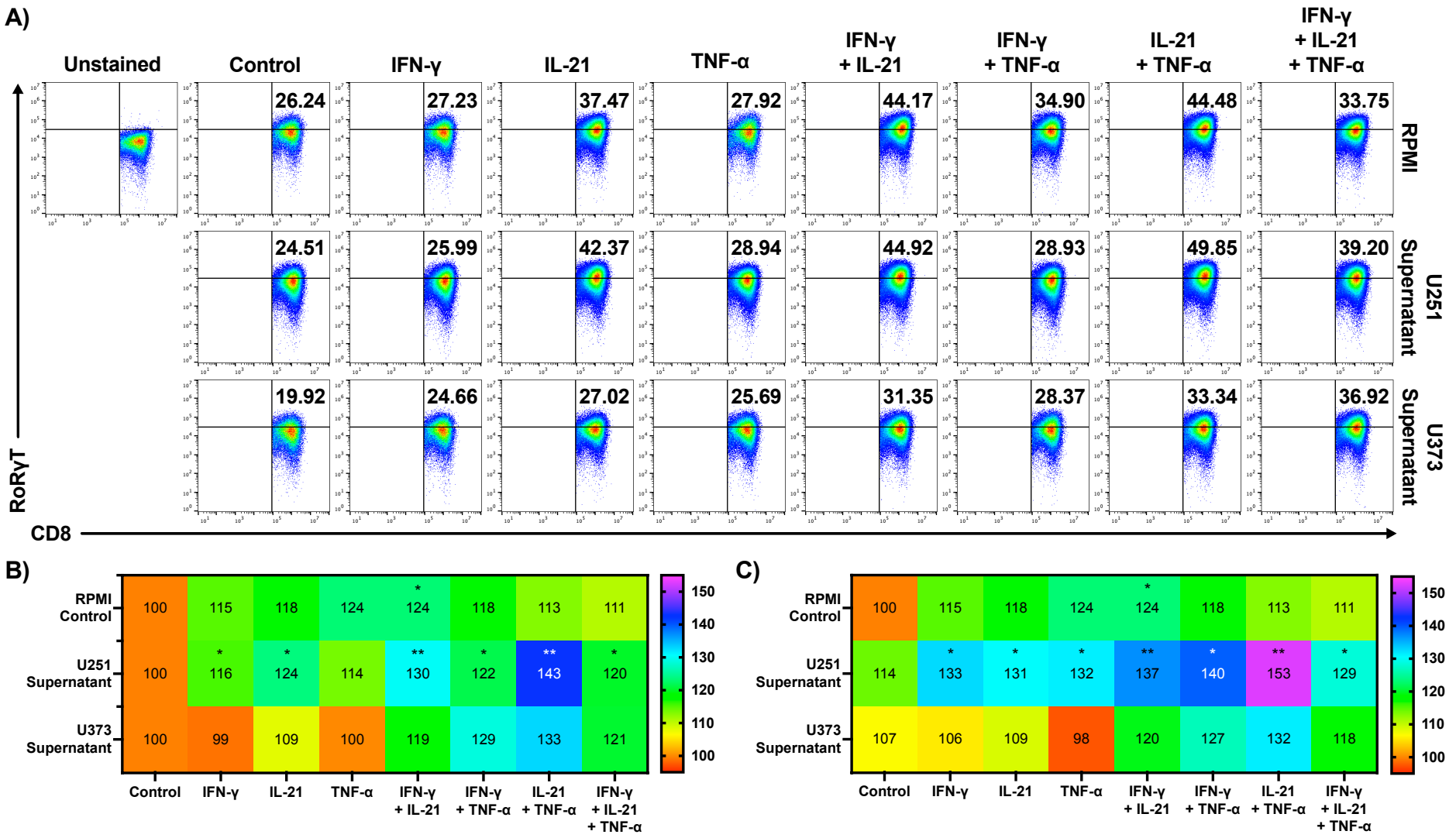


Figure 9.11. IFN-γ, IL-21 and TNF-α downregulated GATA3 expression in bulk CD8+ T cells
$2.5x10^4$ bulk CD8+ T cells were cultured 1:1 with CD3/CD28 beads in RPMI, U251 cell supernatant or U373 cell supernatant, plus treatment with 100ng/ml IFN-γ, IL-21 and TNF-α, both individually and combined for 5 days. Cells were then harvested and stained for CD8 and GATA3 for analysis via flow cytometry. Flow plots **A)** are representative of > 3 independent experiments. Heat Maps and Bars represent % CD8+GATA3+ cells as means compared to the relevant Control **B)** and **D)**, plus % CD8+GATA3+ cells as means compared to the RPMI Control **C)** and **E)** with SEM shown for > 3 independent experiments. P Values: * = <0.05; ** = <0.01; *** = <0.001; **** = <0.0001.


A)
Unstained
Control
IFN-γ
IL-21
TNF-α
IFN-γ + IL-21
IFN-γ + TNF-α
IL-21 + TNF-α
IFN-γ + IL-21 + TNF-α
RPMI
U251 Supernatant
U373 Supernatant
RoRγT
CD8
26.24
27.23
37.47
27.92
44.17
34.90
44.48
33.75
24.51
25.99
42.37
28.94
44.92
28.93
49.85
39.20
19.92
24.66
27.02
25.69
31.35
28.37
33.34
36.92
B)
RPMI Control
U251 Supernatant
U373 Supernatant
100 115 118 124 124 118 113 111
100 116 124 114 130 122 143 120
100 99 109 100 119 129 133 121
Control
IFN-γ
IL-21
TNF-α
IFN-γ + IL-21
IFN-γ + TNF-α
IL-21 + TNF-α
IFN-γ + IL-21 + TNF-α
150
140
130
120
110
100
C)
RPMI Control
U251 Supernatant
U373 Supernatant
100 115 118 124 124 118 113 111
114 133 131 132 137 140 153 129
107 106 109 98 120 127 132 118
Control
IFN-γ
IL-21
TNF-α
IFN-γ + IL-21
IFN-γ + TNF-α
IL-21 + TNF-α
IFN-γ + IL-21 + TNF-α
150
140
130
120
110
100

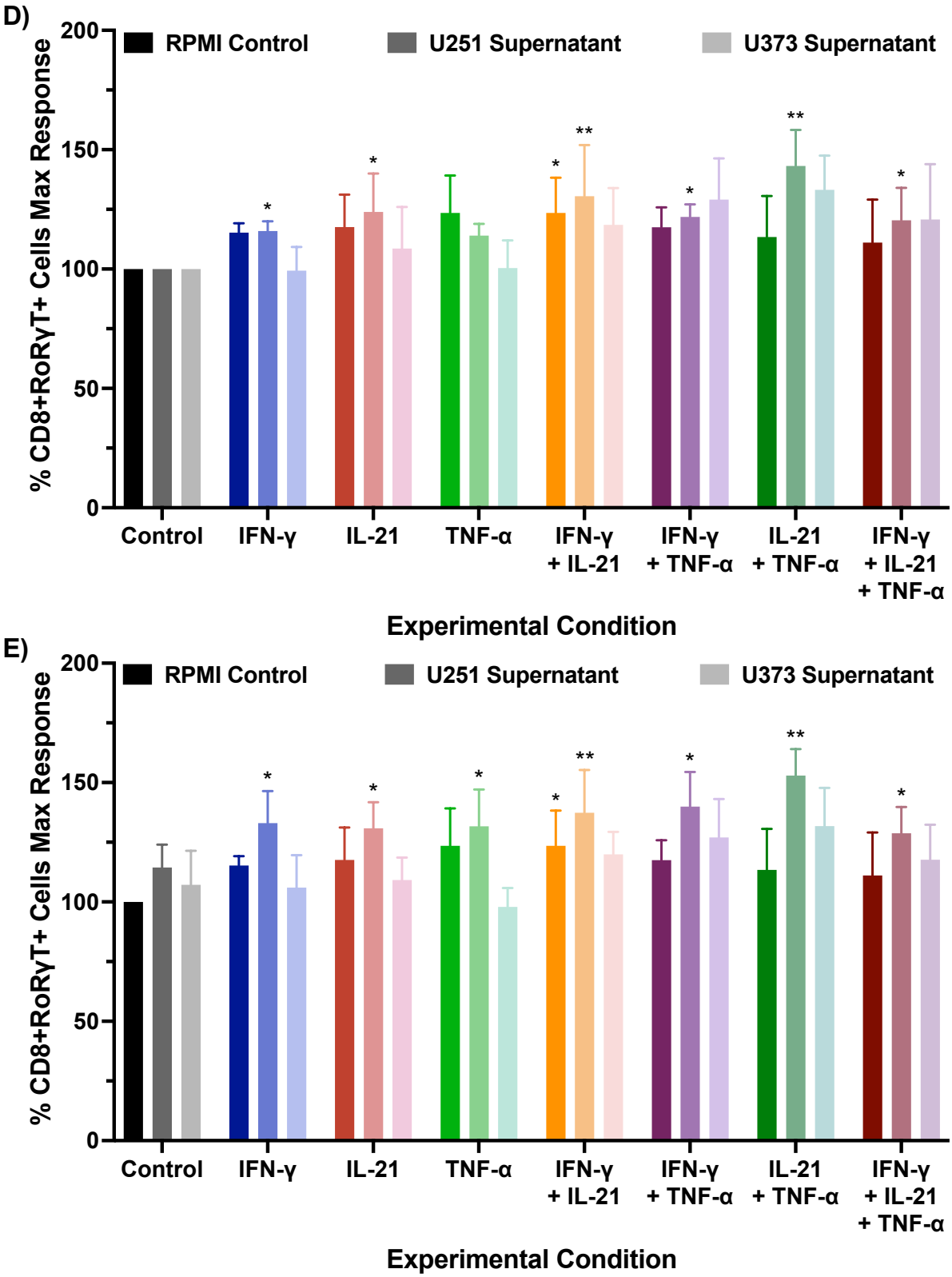
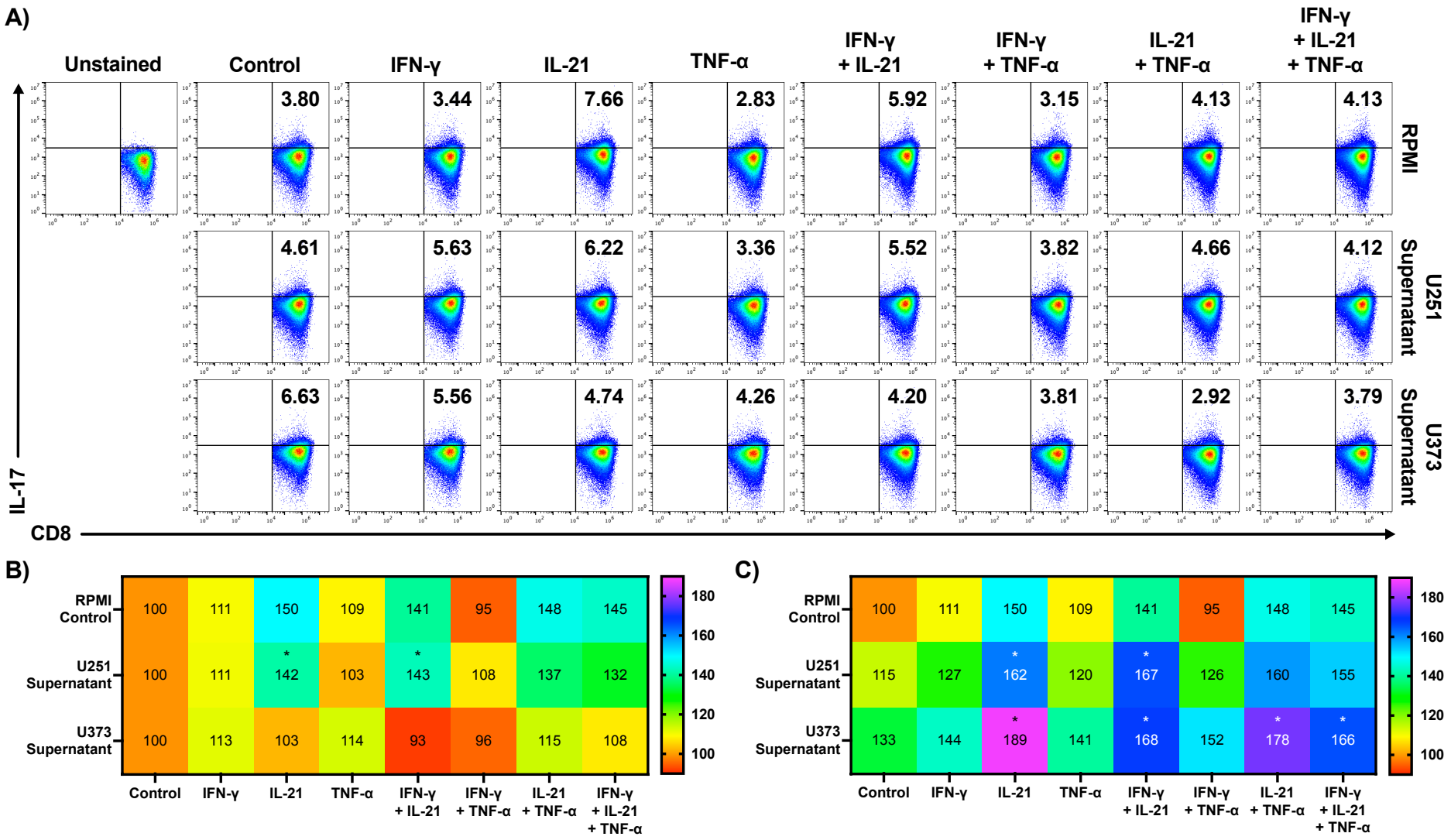


Figure 9.12. IFN-γ, IL-21 and TNF-α upregulated RoRγT expression in bulk CD8+ T cells cultured with U251 cell supernatant

$2.5x10^4$ bulk CD8+ T cells were cultured 1:1 with CD3/CD28 beads in RPMI, U251 cell supernatant or U373 cell supernatant, plus treatment with 100ng/ml IFN-γ, IL-21 and TNF-α, both individually and combined for 5 days. Cells were then harvested and stained for CD8 and RoRγT for analysis via flow cytometry. Flow plots **A)** are representative of > 3 independent experiments. Heat Maps and Bars represent % CD8+RoRγT+ cells as means compared to the relevant Control **B)** and **D)**, plus % CD8+RoRγT+ cells as means compared to the RPMI Control **C)** and **E)** with SEM shown for > 3 independent experiments. P Values: * = <0.05; ** = <0.01; *** = <0.001; **** = <0.0001.


A)
Unstained
Control
IFN-γ
IL-21
TNF-α
IFN-γ + IL-21
IFN-γ + TNF-α
IL-21 + TNF-α
IFN-γ + IL-21 + TNF-α
3.80
3.44
7.66
2.83
5.92
3.15
4.13
4.13
4.61
5.63
6.22
3.36
5.52
3.82
4.66
4.12
6.63
5.56
4.74
4.26
4.20
3.81
2.92
3.79
RPMI
U251 Supernatant
U373 Supernatant
IL-17
CD8
B)
RPMI Control
U251 Supernatant
U373 Supernatant
100 111 150 109 141 95 148 145
100 111 142 103 143 108 137 132
100 113 103 114 93 96 115 108
Control
IFN-γ
IL-21
TNF-α
IFN-γ + IL-21
IFN-γ + TNF-α
IL-21 + TNF-α
IFN-γ + IL-21 + TNF-α
180
160
140
120
100
C)
RPMI Control
U251 Supernatant
U373 Supernatant
100 111 150 109 141 95 148 145
115 127 162 120 167 126 160 155
133 144 189 141 168 152 178 166
Control
IFN-γ
IL-21
TNF-α
IFN-γ + IL-21
IFN-γ + TNF-α
IL-21 + TNF-α
IFN-γ + IL-21 + TNF-α
180
160
140
120
100

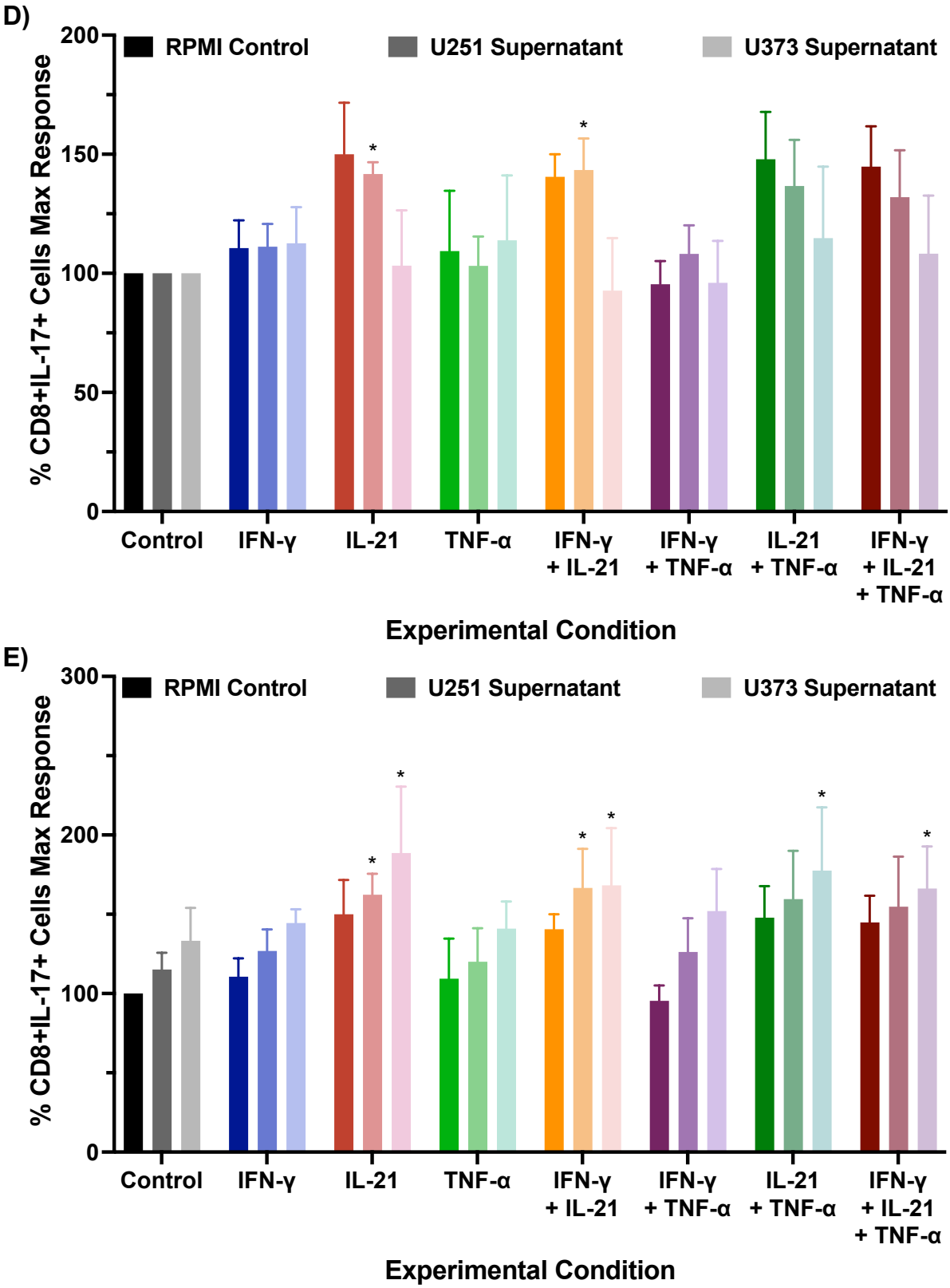
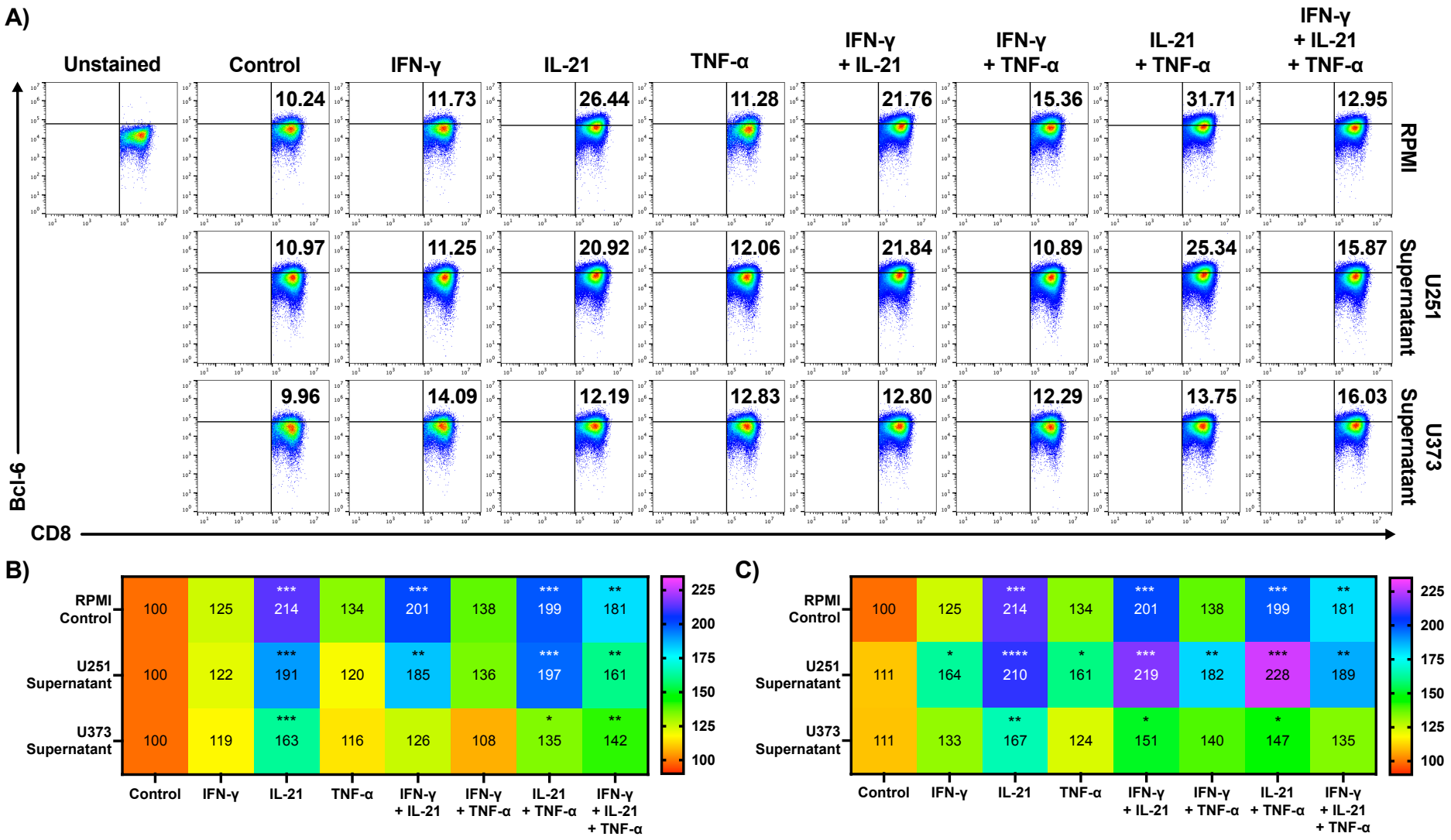


Figure 9.13. IFN-γ, IL-21 and TNF-α largely did not affect IL-17 expression in bulk CD8+ T cells
$2.5x10^4$ bulk CD8+ T cells were cultured 1:1 with CD3/CD28 beads in RPMI, U251 cell supernatant or U373 cell supernatant, plus treatment with 100ng/ml IFN-γ, IL-21 and TNF-α, both individually and combined for 5 days. Cells were then harvested and stained for CD8 and IL-17 for analysis via flow cytometry. Flow plots **A)** are representative of > 3 independent experiments. Heat Maps and Bars represent % CD8+IL-17+ cells as means compared to the relevant Control **B)** and **D)**, plus % CD8+IL-17+ cells as means compared to the RPMI Control **C)** and **E)** with SEM shown for > 3 independent experiments. P Values: * = <0.05; ** = <0.01; *** = <0.001; **** = <0.0001.


A)
Unstained
Control
IFN-γ
IL-21
TNF-α
IFN-γ + IL-21
IFN-γ + TNF-α
IL-21 + TNF-α
IFN-γ + IL-21 + TNF-α
10.24
11.73
26.44
11.28
21.76
15.36
31.71
12.95
RPMI
10.97
11.25
20.92
12.06
21.84
10.89
25.34
15.87
U251 Supernatant
9.96
14.09
12.19
12.83
12.80
12.29
13.75
16.03
U373 Supernatant
Bcl-6
CD8
B)
RPMI Control
U251 Supernatant
U373 Supernatant
100 125 214 134 201 138 199 181
100 122 191 120 185 136 197 161
100 119 163 116 126 108 135 142
*** *** *** ** *** ** *** **
Control
IFN-γ
IL-21
TNF-α
IFN-γ + IL-21
IFN-γ + TNF-α
IL-21 + TNF-α
IFN-γ + IL-21 + TNF-α
225
200
175
150
125
100
C)
RPMI Control
U251 Supernatant
U373 Supernatant
100 125 214 134 201 138 199 181
111 164 210 161 219 182 228 189
111 133 167 124 151 140 147 135
*** * **** * *** ** *** **
Control
IFN-γ
IL-21
TNF-α
IFN-γ + IL-21
IFN-γ + TNF-α
IL-21 + TNF-α
IFN-γ + IL-21 + TNF-α
225
200
175
150
125
100

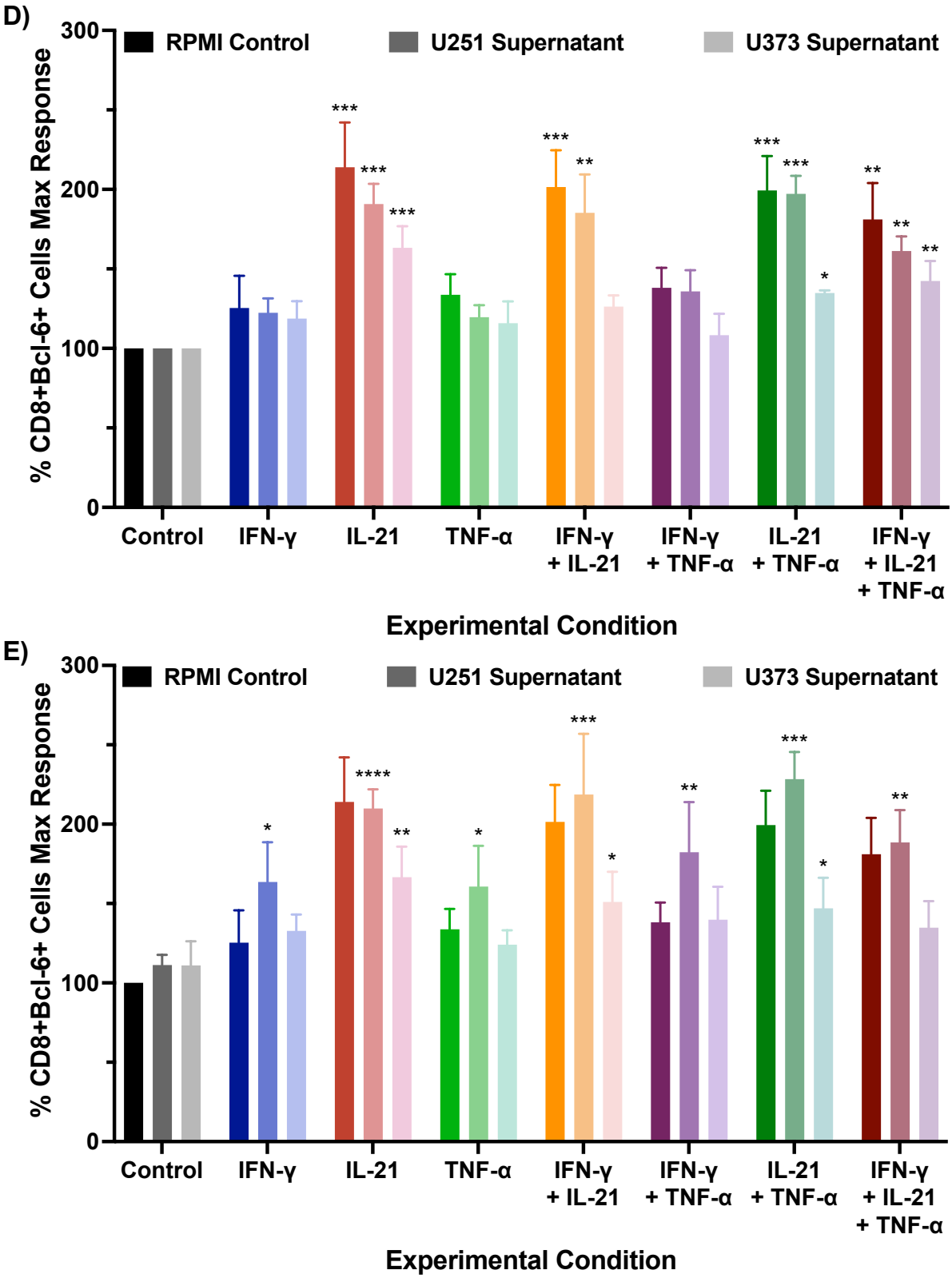
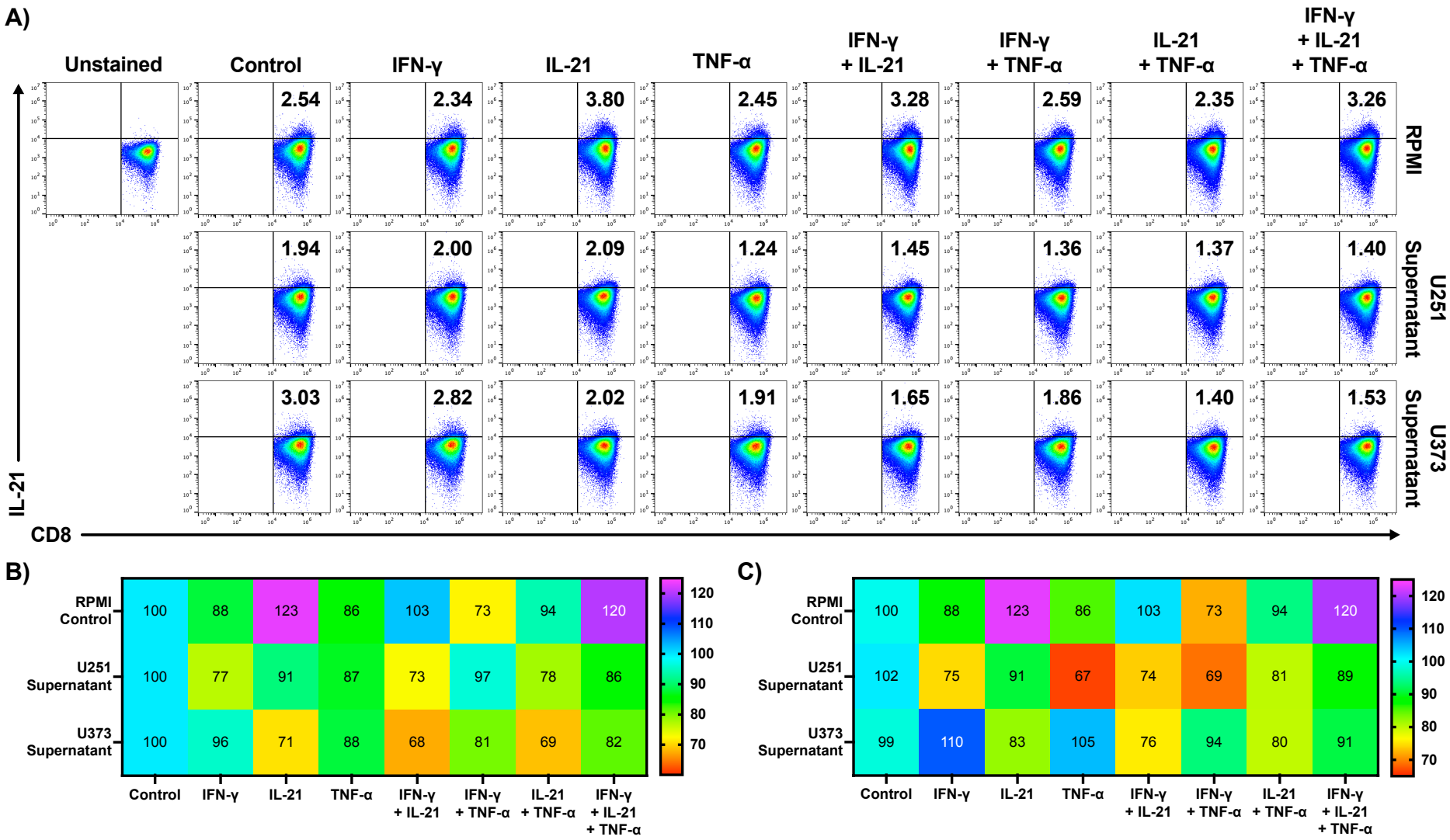


Figure 9.14. IFN-γ, IL-21 and TNF-α upregulated Bcl-6 expression in bulk CD8+ T cells
$2.5x10^4$ bulk CD8+ T cells were cultured 1:1 with CD3/CD28 beads in RPMI, U251 cell supernatant or U373 cell supernatant, plus treatment with 100ng/ml IFN-γ, IL-21 and TNF-α, both individually and combined for 5 days. Cells were then harvested and stained for CD8 and Bcl-6 for analysis via flow cytometry. Flow plots **A)** are representative of > 3 independent experiments. Heat Maps and Bars represent % CD8+Bcl-6+ cells as means compared to the relevant Control **B)** and **D)**, plus % CD8+Bcl-6+ cells as means compared to the RPMI Control **C)** and **E)** with SEM shown for > 3 independent experiments. P Values: * = <0.05; ** = <0.01; *** = <0.001; **** = <0.0001.


A)
Unstained
Control
IFN-γ
IL-21
TNF-α
IFN-γ + IL-21
IFN-γ + TNF-α
IL-21 + TNF-α
IFN-γ + IL-21 + TNF-α
RPMI
U251 Supernatant
U373 Supernatant
2.54
2.34
3.80
2.45
3.28
2.59
2.35
3.26
1.94
2.00
2.09
1.24
1.45
1.36
1.37
1.40
3.03
2.82
2.02
1.91
1.65
1.86
1.40
1.53
IL-21
CD8
B)
RPMI Control
U251 Supernatant
U373 Supernatant
100 88 123 86 103 73 94 120
100 77 91 87 73 97 78 86
100 96 71 88 68 81 69 82
Control
IFN-γ
IL-21
TNF-α
IFN-γ + IL-21
IFN-γ + TNF-α
IL-21 + TNF-α
IFN-γ + IL-21 + TNF-α
120
110
100
90
80
70
C)
RPMI Control
U251 Supernatant
U373 Supernatant
100 88 123 86 103 73 94 120
102 75 91 67 74 69 81 89
99 110 83 105 76 94 80 91
Control
IFN-γ
IL-21
TNF-α
IFN-γ + IL-21
IFN-γ + TNF-α
IL-21 + TNF-α
IFN-γ + IL-21 + TNF-α
120
110
100
90
80
70

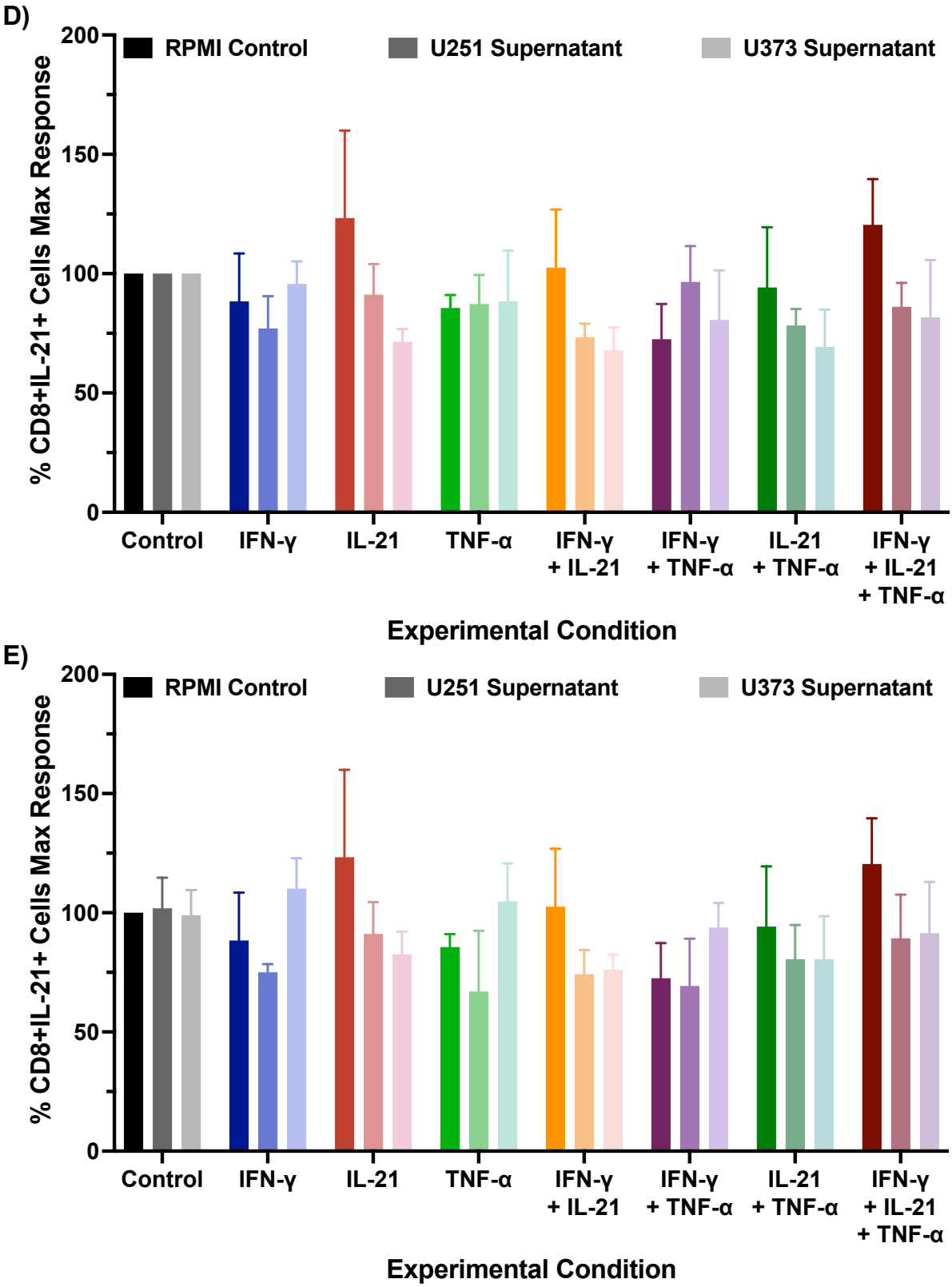
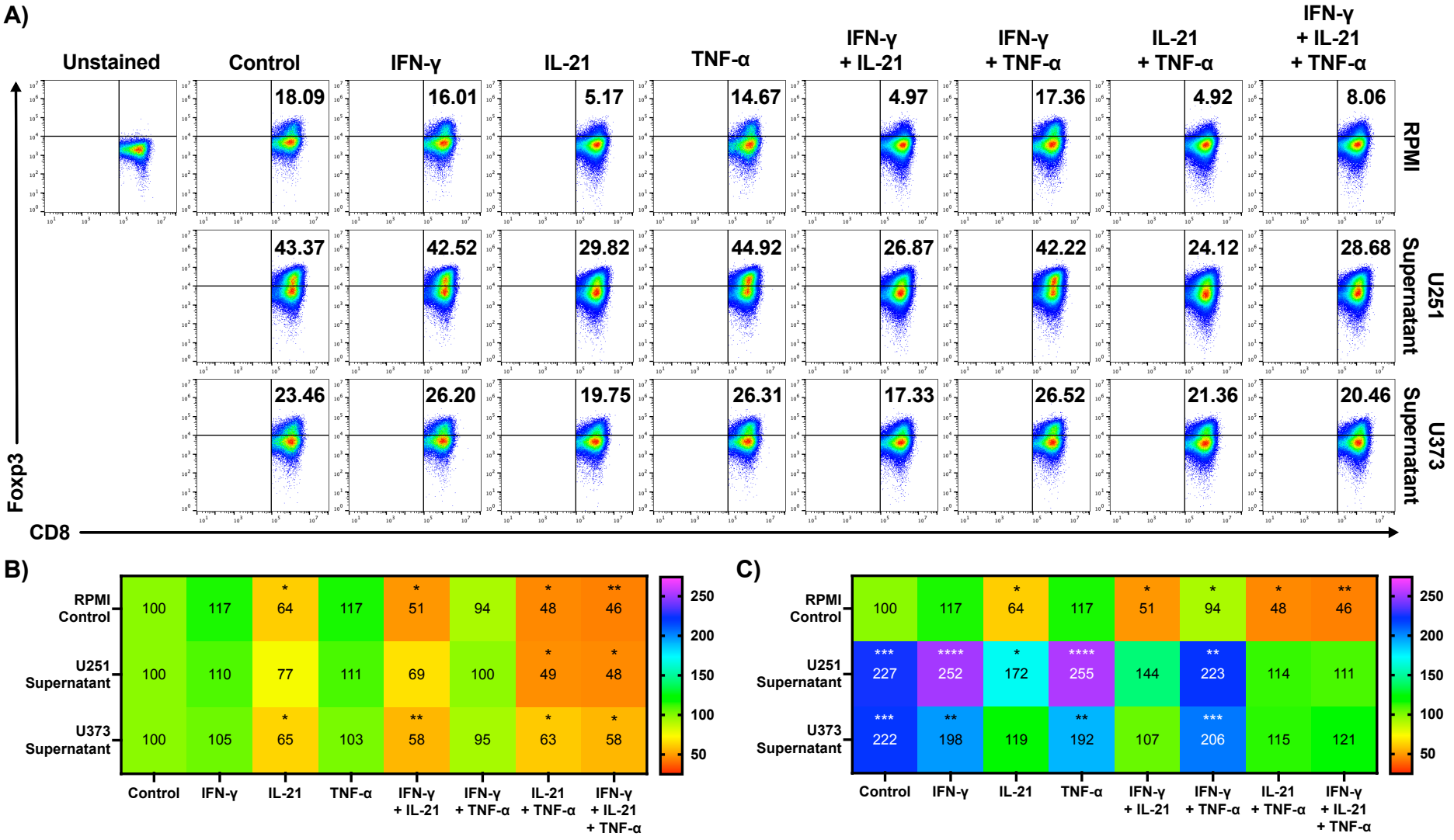


Figure 9.15. IFN-γ, IL-21 and TNF-α did not affect IL-21 expression in bulk CD8+ T cells
$2.5x10^4$ bulk CD8+ T cells were cultured 1:1 with CD3/CD28 beads in RPMI, U251 cell supernatant or U373 cell supernatant, plus treatment with 100ng/ml IFN-γ, IL-21 and TNF-α, both individually and combined for 5 days. Cells were then harvested and stained for CD8 and IL-21 for analysis via flow cytometry. Flow plots **A)** are representative of > 3 independent experiments. Heat Maps and Bars represent % CD8+IL-21+ cells as means compared to the relevant Control **B)** and **D)**, plus % CD8+IL-21+ cells as means compared to the RPMI Control **C)** and **E)** with SEM shown for > 3 independent experiments. P Values: * = <0.05; ** = <0.01; *** = <0.001; **** = <0.0001.


A)
Unstained
Control
IFN-γ
IL-21
TNF-α
IFN-γ + IL-21
IFN-γ + TNF-α
IL-21 + TNF-α
IFN-γ + IL-21 + TNF-α
18.09
16.01
5.17
14.67
4.97
17.36
4.92
8.06
RPMI
43.37
42.52
29.82
44.92
26.87
42.22
24.12
28.68
U251 Supernatant
23.46
26.20
19.75
26.31
17.33
26.52
21.36
20.46
U373 Supernatant
Foxp3
CD8
B)
RPMI Control 100 117 64 117 51 94 48 46
U251 Supernatant 100 110 77 111 69 100 49 48
U373 Supernatant 100 105 65 103 58 95 63 58
Control
IFN-γ
IL-21
TNF-α
IFN-γ + IL-21
IFN-γ + TNF-α
IL-21 + TNF-α
IFN-γ + IL-21 + TNF-α
250
200
150
100
50
C)
RPMI Control 100 117 64 117 51 94 48 46
U251 Supernatant 227 252 172 255 144 223 114 111
U373 Supernatant 222 198 119 192 107 206 115 121
Control
IFN-γ
IL-21
TNF-α
IFN-γ + IL-21
IFN-γ + TNF-α
IL-21 + TNF-α
IFN-γ + IL-21 + TNF-α
250
200
150
100
50

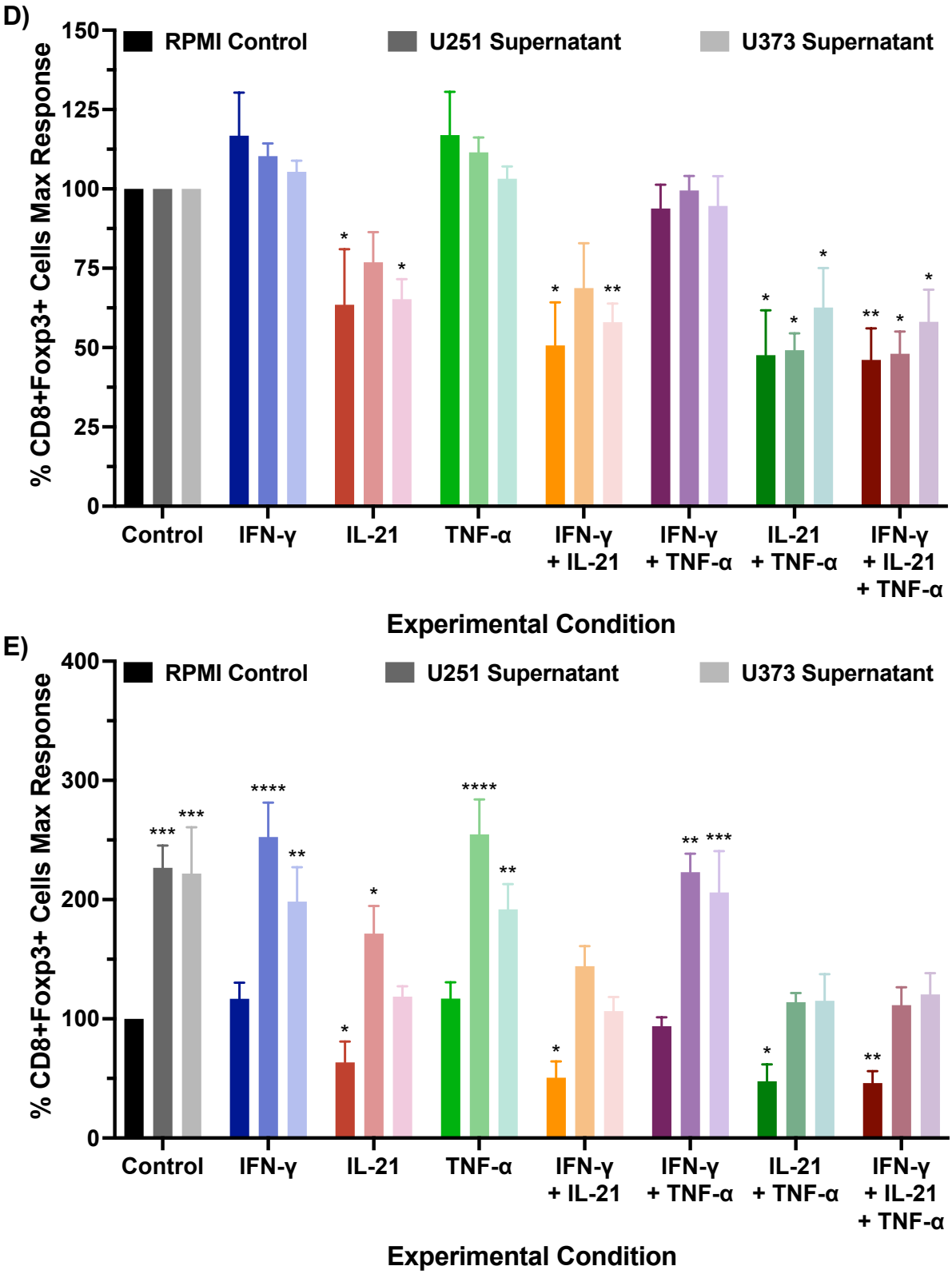


Figure 9.16. IFN-γ, IL-21 and TNF-α downregulated Foxp3 expression in bulk CD8+ T cells
$2.5x10^4$ bulk CD8+ T cells were cultured 1:1 with CD3/CD28 beads in RPMI, U251 cell supernatant or U373 cell supernatant, plus treatment with 100ng/ml IFN-γ, IL-21 and TNF-α, both individually and combined for 5 days. Cells were then harvested and stained for CD8 and Foxp3 for analysis via flow cytometry. Flow plots **A)** are representative of > 3 independent experiments. Heat Maps and Bars represent % CD8+Foxp3+ cells as means compared to the relevant Control **B)** and **D)**, plus % CD8+Foxp3+ cells as means compared to the RPMI Control **C)** and **E)** with SEM shown for > 3 independent experiments. P Values: * = <0.05; ** = <0.01; *** = <0.001; **** = <0.0001.

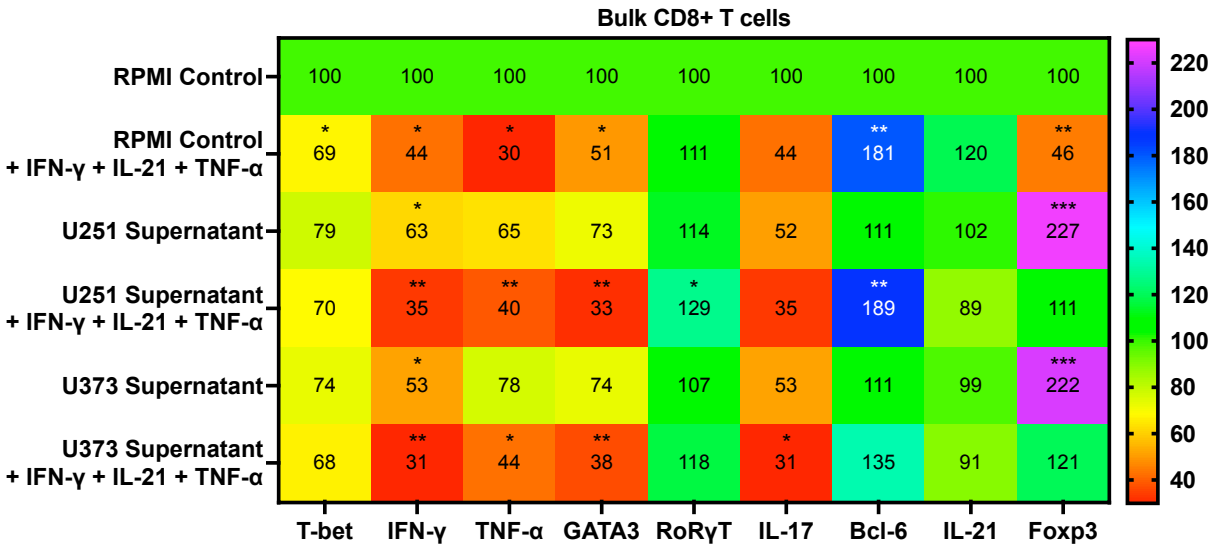


Figure 9.17. Heat Maps summarising the impact of U251 and U373 cell supernatant with IFN-γ, IL-21 and TNF-α treatment on T-bet, IFN-γ, IL-21, TNF-α, GATA3, RoRγT, IL-17, Bcl-6, IL-17, and Foxp3 expression in bulk CD8+ T cells

$2.5x10^4$ bulk CD8+ T cells were cultured 1:1 with CD3/CD28 beads in RPMI, U251 cell supernatant or U373 cell supernatant, plus treatment with 100ng/ml IFN-γ, IL-21 and TNF-α, both individually and combined for 5 days. Cells were then harvested and stained for CD8, T-bet, IFN-γ, TNF-α, GATA3, RoRγT, IL-17, Bcl-6, IL-21, and Foxp3 for analysis via flow cytometry. Heat Maps represent % CD8+ positive expression for each of these markers in bulk CD8+ T cells as means compared to the RPMI Control for > 3 independent experiments. P Values: * = <0.05; ** = <0.01; *** = <0.001; **** = <0.0001.