Transglutaminase 2 interacts with syndecan 4 and CD44 at the surface of human macrophages to promote removal of apoptotic cells

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Abbreviations: AC – apoptotic cell; MØ – macrophage; HMDMØ – Human monocyte-derived macrophage; TG2 – Transglutaminase-2; SDC4 – Syndecan-4; PRR – pattern-recognition receptor, MDC – monodansylcadaverine, HSPG – Heparan sulphate proteoglycan, HS – Heparan sulphate, PMA – phorbol 13-myristate 12-acetate, VD3 – dihydroxyvitamin D3, gpITG – guinea pig liver transglutaminase, HRP - Horseradish peroxidise, ECM – Extracellular matrix, BTC – Biotin-cadaverine

Abstract

Tissue transglutaminase (TG2) is a multifunctional protein cross-linking enzyme that has been implicated in apoptotic cell clearance but is also important in many other cell functions including cell adhesion, migration and monocyte to macrophage differentiation. Cell surface-associated TG2 regulates cell adhesion and migration, via its association with receptors such as syndecan-4 and β1 and β3 integrins. Whilst defective apoptotic cell clearance has been described in TG2-deficient mice, the precise role of TG2 in apoptotic cell clearance remains ill-defined. Our work addresses the role of macrophage extracellular TG2 in apoptotic cell corpse clearance. Here we reveal TG2 expression and activity (cytosolic and cell surface) in human macrophages and demonstrate that inhibitors of protein crosslinking activity reduce macrophage clearance of dying cells. We show also that cell-impermeable TG2 inhibitors significantly inhibit the ability of macrophages to migrate and clear apoptotic cells through reduced macrophage recruitment to, and binding of, apoptotic cells. Association studies reveal TG2-syndecan-4 interaction through heparan sulphate side chains, and knockdown of syndecan-4 reduces cell surface TG2 activity and apoptotic cell clearance. Furthermore, inhibition of TG2 activity reduces crosslinking of CD44, reported to augment AC clearance. Thus our data define a role for TG2 activity at the surface of human macrophages in multiple stages of AC clearance and we propose TG2, in association with heparan sulphates, may exert its effect on AC clearance via a mechanism involving the crosslinking of CD44.

Keywords: Macrophages; Transglutaminase 2; syndecan 4; CD44; apoptotic cell clearance; heparan sulphate proteoglycan

Introduction

Transglutaminases are a family of enzymes that catalyse covalent bond formation between lysine and glutamine-residues in target proteins leading to their posttranslational modification[1]. TG2 is the most widely expressed family member[2] and is found both in the cytoplasm and extracellularly at the cell surface[3]. When externalized, TG2 is found associated with integrins[4] and heparan sulphates (HS)[5-8]. Following translocation to the cell surface, it is deposited into the extracellular matrix[3]. Whilst protein cross-linking is the most studied function of TG2, it also participates in other biological processes unrelated to its transamidase activity[9]- e.g. cell adhesion through cell surface association with integrins[4] and syndecans[5, 6, 8, 10, 11]. However recent work has shown that the extracellular crosslinking activity of TG2 is involved in S100A4-related cell migration involving a syndecan-4 and β1 integrin co-signalling pathway[12].

Unwanted cells within the body are removed by apoptosis, a process which culminates in apoptotic cell (AC) removal by professional phagocytes (macrophages) and accompanying anti-inflammatory responses to prevent inflammatory and autoimmune conditions[13]. Removal of AC is an integrated, multistep process that in vivo involves recruitment of macrophages. This is followed by recognition and binding of cell corpses prior to engulfment (phagocytosis) through the use of a range of receptors and soluble bridging molecules to bind dying cell ligands[14-17]. Tissue transglutaminase (TG2) has been shown to play an important role in this process.

Important previous work utilised $TG2^{-/-}$ mice to reveal defective clearance of AC and reduced TGF-β1 with strong association with inflammation and autoimmunity[18]. Further detailed study defined a role for cell surface TG2 in engulfment of AC through phagocytic portal stabilisation[19]. In this context TG2 was shown to function through the interaction with β3 integrin and the AC opsonin MFG-E8. Despite this significant previous work, the molecular associations and function of TG2 at the surface of human macrophages has not been fully defined.

The aim of this study was to characterise TG2 in the context of human macrophages. Using a panel of TG2 inhibitors, including both known cell-permeable

and cell impermeable inhibitors, TG2 targeted siRNA and an inhibitory antibody; we reveal a role of TG2 in the different phases of AC clearance (phagocyte recruitment and AC tethering) and also define the cellular site of action of TG2. Furthermore we demonstrate for the first time the importance of syndecan-4 in the macrophage cell surface localisation of TG2 which is required for efficient clearance of apoptotic cells. Recent studies have highlighted a role for cross-linked macrophage CD44 in augmenting AC clearance[20]. Here we further address the involvement of TG2 in interacting with CD44 to promote AC clearance.

Materials and Methods

General Reagents

The general reagents were purchased from Sigma-Aldrich (Poole, UK), unless stated otherwise. Purified guinea pig liver transglutaminase (gplTG), biotinylated-cadaverine (BTC) and the cell permeable TG2 inhibitor Z-DON (*Z-DON-Val-Pro-Leu-OMe*) were purchased from Zedira (Darmstadt, Germany). The monoclonal TG2 activity-blocking antibody D11D12 (GB patent filing 1209096.5) was a kind gift of Dr. Tim Johnson (Sheffield University, UK). The TG2 inhibitor 1, 3-dimethyl-2-imidazolium derivative R283[21] and the peptidic TG2 inhibitors R294 and R281 were synthesized at Aston University[22,23] . Inhibitor R281 is a membrane-impermeable, irreversible TG2 inhibitor[22, 23]. R294 shows greater specificity for TG2 over other TG family members[24] and is more water-soluble than R281 and thus is considered to be similarly membrane impermeable[22]. Cell permeability of R283 has not been formally demonstrated and Z-DON is reported to be cell permeable (Zedira). R281, R283 and R294 were used at 500μM unless otherwise stated. Monodansylcadaverine (MDC) was used at 100μM.

The commercial antibodies used in this study are listed in Supplementary Table 1. All blots were undertaken using the indicated mouse monoclonal antibodies. Immunofluorescence studies utilised either mouse monoclonal or rabbit polyclonal antibodies as indicated.

Cell lines, Cell isolation and culture

THP-1 (human myelomonocytic cell lines; ATCC) and Mutu I BL[25] were cultured in RPMI-1640 medium (PAA, Yeovil, UK) containing 2mM L-glutamine supplemented with 10% FCS (PAA, Yeovil, UK) and 100IU/ml penicillin and 100μg/ml streptomycin. Cells were cultured at 37°C in a humidified environment at 5% CO_2.

Primary blood mononuclear cells were isolated from citrated blood of healthy volunteers, following informed consent, by dextran sedimentation and Percoll fractionation[26]. Monocytes were allowed to adhere to tissue culture wells (24 well plates) for 1 hour prior to removal of other cells by washing with serum-free RPMI. Isolated monocytes were cultured for 7-8 days in macrophage-SFM medium (Invitrogen Corp., Paisley, UK) at 37°C in 5% CO_2 incubator [27].

THP-1 differentiation to macrophage-like cells

Differentiation of THP-1 into macrophage-like cells was induced by treating THP-1 cells with 250nM phorbol ester (PMA; Sigma, UK), 100nM dihydroxyvitamin D3 (VD3; Enzo Life Sciences, UK) or both (DS:double-stimulated) for 48-72h at 37°C in 5% CO_2 incubator in growth medium. Differentiation was confirmed by light microscopy[28].

Apoptosis induction and quantification

Mutu I (Burkitt's lymphoma cells) were exposed to 100mJ/cm^2 UV-B irradiation, using a Chromata-vue C71 light box and UVX radiometer (UV-P Inc., Upland, CA, USA) and incubated for 16h to allow apoptosis to proceed[29]. For analysis of apoptotic nuclear morphology, cells were fixed in 1% (w/v) formaldehyde in PBS, stained with 4,6-diamidino-2-phenylinole (DAPI, Sigma, 250ng/ml in PBS) for 5 min and observed using inverted epifluorescence microscopy. For quantitative analyses, cells stained for 5 min with acridine orange (Sigma, 10µg/ml in PBS) were enumerated as a percentage of apoptotic cells per total number of cells counted per sample using Zeiss Axiovert 200M fluorescence microscope (Carl Zeiss Ltd, Welwyn Garden City, UK) and Hamamatsu Orca camera driven by Volocity (Perkin-Elmer, UK).

Annexin V labelling and flow cytometry

UV irradiated Mutu BL were stained with annexin V-FITC (eBiosciences Ltd, UK). Briefly, cells were washed once by centrifugation (200x*g*, 5 min) and resuspended in binding buffer (10mM HEPES pH 7.4, 150mM NaCl, 2.5mM $CaCl_2$) containing annexin V-FITC (1µl per 2x10^5 cells) for 15min on ice. Following washing once with binding buffer, cells were resuspended in 1ml binding buffer containing propidium iodide at a final concentration of 20µg/ml. Samples were analysed immediately on a Quanta SC flow cytometer (Beckman Coulter, High Wycombe, UK). A minimum of 5000 events was collected.

Assays of Phagocyte interaction with apoptotic cells

Interaction (defined as tethering plus phagocytosis) of apoptotic Mutu by phagocytes (THP-1 derived macrophages, HMDMØ) was carried out in 24-well plates[30]. Briefly, phagocytes and AC at a ratio of 1:100 were co-cultured for 1h at 37°C (for

interaction) or 20°C (for tethering alone) in RPMI containing 0.2% (w/v) bovine serum albumin (Sigma). In assays using inhibitors (e.g. TG2 inhibitors, P1 peptide), macrophages were treated with appropriate concentrations of inhibitors in serum-free RPMI for 1h before and throughout the co-culturing with apoptotic cells (2h total). Unbound apoptotic cells were removed by extensive washing with PBS and cells fixed with 1% (w/v) formaldehyde in PBS. Cells were stained with DiffQuick II (Medion Diagnostics GmbH, Switzerland) for 2 min, the stain replaced with PBS and cells scored by light microscopy for the percentage of macrophages interacting with apoptotic cells. At least 200 macrophages were scored in each of triplicate wells per experiment and at least three independent replicates were undertaken. For the assay of only tethering of AC to macrophages (i.e. excluding phagocytosis), co-culturing was carried at room temperature (20°C), a temperature non-permissive for phagocytosis [29].

Western blotting

Cells were washed with ice-cold PBS, pH 7.4 and lysed with cell lysis buffer (50mM Tris-HCl, pH 7.4, containing 1% (v/v) Nonidet, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 1mM benzamidine, 1mM NaF, 1mM Na_3VO_4, 0.1mM phenylmethylsulphonyl fluoride (PMSF) and 1% (v/v) protein inhibitor cocktail (Sigma)). Protein quantity in samples was estimated by the DC^{TM}-protein assay (detergent-compatible, Biorad). Following protein quantification, lysates containing 50µg of protein were dissolved with an equal volume of 2X Laemmli buffer (Sigma), heated to 95^{o}C for 5min and resolved by standard SDS-polyacrylamide gel electrophoresis. Following electro-blotting to nitrocellulose (0.45µm; GE Healthcare, UK), membranes were blocked with 5% w/v non-fat milk in TBS-Tween (50mM Tris, 150mM NaCl, 0.05% v/v Tween 20; pH 7.4) and probed using specific primary mouse monoclonal antibodies (1:1000 dilution; Table 1). Anti-mouse secondary antibody (1:2000 dilution; GE Healthcare) conjugated with horseradish peroxidise (HRP) was used to detect primary antibodies. Detection was carried out by using ECL chemiluminescence (GE Healthcare, UK). Equal loading was verified by using mouse anti-*α*-tubulin/β-actin antibodies (1:2000 dilution) and normalisation undertaken using densitometry where appropriate.

Heparinase-treatment of cell surface proteins

Heparan sulphate side chains were removed from the surface of cells by treatment of cells with heparinase II or, as a control, chondroitinase. Briefly, THP-1-derived macrophages were treated with heparinase II (5 units/ml) or chondroitinase (5 units/ml) in serum-free RPMI (1h, 37°C). Following incubation cells were washed three times with complete culture medium (RPMI supplemented with serum) prior to use in further experiments (e.g. assays of macrophage function for AC interaction).

Isolation of cell surface proteins using biotinylation

Biotinylation of cell surface proteins was carried out, to permit purification and assessment of cell surface proteins in isolation from other cell components, as described previously[6]. Briefly, cells were rinsed with ice-cold PBS (pH 8.0) and were labelled with 0.8μM sulfo-NHS-LC-biotin (Pierce) dissolved in PBS (pH 8.0) at 4°C for 20 min. Following washing with 50mM Tris–HCl (pH 8.0) cells were lysed in 1% (w/v) SDS in PBS with benzonase (1:1000 of 250U/μl) for 30 min on ice. Cell lysates containing 600μg of protein, following centrifugation at 13,000 x*g* for 20 min to remove non-broken cells, were added to 50μl Neutr-Avidin-Agarose resin beads (Pierce) which were initially washed with PBS supplemented with 1% (w/v) BSA and incubated at 4°C overnight on a rotating platform. After washing with PBS, resin beads bound biotin-labelled cell surface proteins were extracted by boiling with 2X Laemmli buffer and analysed by western blotting. A non-biotinylated cell sample was also run to ensure that the pulldown was specific only for cell surface biotinylated TG2 (supplementary figure 1A).

Co-immunoprecipitation to study protein association

Co-immunoprecipitation to detect interaction between proteins was carried out as described previously[11]. Briefly, cell lysates (equivalent of 150μg of protein) were pre-cleared by the addition of 50μl of washed protein-G-Sepharose beads (GE Healthcare) for 1 hour prior to bead removal by centrifugation (13000x*g*, 10 min). Approximately 0.5μg of appropriate mouse monoclonal antibody was added to pre-cleared cell lysates and incubated for 90min at 4^{o}C on a rocking platform to form immunocomplexes with respective protein of interest. Immunocomplexes were pulled down by incubating with protein-G-Sepharose beads (50μl), the beads washed in PBS three times prior to harvest by centrifugation. Immunocomplexes were subsequently collected via boiling in 30μl of 2x Laemmli buffer. Immunocomplexes

with an isotype control antibody (mouse IgG1/kappa: MoPC21) were also pulled down as a known negative control to exclude the possibility that the protein of interest is binding non-specifically to beads or antibody. Immunoprecipitated proteins were separated using SDS-PAGE and detected via western blotting by using specific antibodies.

Assessment of cell surface TG2 activity via biotin cadaverine incorporation

Measurement of TG2 activity via biotin cadaverine incorporation into fibronectin was carried as described previously[5, 11] with minor modifications. Briefly, a 96-well plate was coated with 5 µg/ml of fibronectin in wash buffer (50mM Tris-HCl, pH 7.4) overnight at 4°C. After rinsing wells with 50mM Tris-HCl (pH 7.4) and blocking with 3% (w/v) BSA in 50mM Tris-HCl, pH 7.4 (30min, 37°C), cells were seeded into the wells at $2x10^4$ per well in serum-free RPMI medium containing 0.132mM biotin-cadaverine. Wells with 100ng of gpITG with 10mM Ca^{2+} or 10mM EDTA are used as positive and negative controls, respectively. Following incubation for 2h at 37°C (in the presence of inhibitors as appropriate to the individual experiment), the reaction was terminated by addition of 2mM EDTA in PBS, pH 7.4 and the cells were removed by 0.1% (w/v) deoxycholate in 2mM EDTA in PBS, pH 7.4. After rinsing wells with wash buffer and blocking with 3% (w/v) BSA in 50mM Tris-HCl, pH 7.4 for 30min at 37°C, biotin-cadaverine incorporated into fibronectin was detected by incubating at 37°C for 1h with blocking buffer conjugated with HRP-conjugated Extr-avidin (1:1000; Sigma-Aldrich, UK). HRP was detected using OPD (Sigma) and colour development was terminated by addition of 50µl of 2.5M H_2SO_4. The absorbance was measured 492nm.

Similarly, biotin cadaverine incorporation into TG2-CD44 cross-linking was also carried out, where $1X10^5$ THP-1/DS MØ were pre-incubated with 8µM biotin-cadaverine in the presence or absence of non cell-permeable TG2 inhibitor R281 in serum-free RPMI medium at 37°C for 2h. Where R281 was used, MØ were pre-treated with inhibitor for 1h prior to the addition of BTC. Following BTC incorporation, cell lysates equivalent to 150µg protein were subject to pull down of biotin-containing molecules by Neutr-Avidin-Agarose resin beads. The pull downs were probed by western blotting for CD44 to reveal TG2-mediated BTC incorporation into CD44.

Chemotaxis and migration studies

MØ chemotaxis towards AC was studied using a Dunn chemotaxis chamber (Hawksley, Sussex) in conjugation with time-lapse microscopy[31]. This horizontal migration chamber establishes a true gradient of attractant rather than the 'step gradient' associated with use of vertical transwell-based systems. THP-1/DS cells, treated as appropriate with cell-impermeable TG2 inhibitor R281 (1h in serum-free RPMI medium), were detached into 5mM EDTA in PBS at 37^{o}C and reseeded to plastic cover slips. Following incubation at 37^{o}C for 20min in RPMI, the coverslip was inverted on a chemotaxis chamber and sealed in position using wax with chemoattractants in the outer well. Cell migration was recorded using time-lapse microscopy (Zeiss Axiovert 200M controlled by Improvision Volocity software). Quantitative assessment of collected sequential images to allow mapping of individual cells was carried out using Image J and the Ibidi Chemotaxis and Migration tool (version 2.0, Ibidi), which provides measures of distance migrated (Euclidian) and directness.

Immunofluorescence staining

To detect cell surface proteins by indirect immunofluorescence, cells at 5x10^{4} per well on multi-well glass slides (Hendley, Essex, UK) were blocked with 3% (w/v) BSA in PBS (pH 7.4) for 30min on ice. Following washes with PBS, cells were incubated with primary mouse monoclonal antibodies (1:100 dilution) or rabbit polyclonal antibodies (1:100 dilution), in serum-free RPMI for 2h on ice. Cell washing with PBS, pH 7.4 was followed by incubation with specific secondary antibody reagents (anti-mouse or anti-rabbit as appropriate) conjugated with either FITC or PE (1:100 dilution) for 2h on ice. Stained cells were washed twice with PBS, fixed with 1% w/v formaldehyde in PBS and mounted with Vectashield containing DAPI (Vector Laboratories). Cells were examined using confocal microscopy (Leica Microsystems).

siRNA silencing of individual cellular components

Four different siRNAs targeting human syndecan-4 and another four different siRNAs targeting TG2 were used to inhibit/knockdown the expression of both the proteins, while the non-silencing control siRNA was used as the negative control. All siRNA were supplied at 10μM (Qiagen). Transfection was carried out according to

manufacturer's protocol with slight modifications. Briefly, $5x10^5$ THP-1 cells were seeded (1.5ml) to 6-well plates and were stimulated with PMA and VD3 to differentiate to macrophage-like cells. Following 24h of differentiation, cells were transfected by the dropwise addition of 100µl of transfection mix (1µl siRNA, 9µl HiPerfect transfection reagent (Qiagen), 90µl serum-free RPMI). Following 48h of incubation with the siRNA, whole cell lysates were used to analyse the level of protein expression by western blotting. Additionally cells at this same time point were used for additional functional studies as appropropriate.

Statistical Analysis

Data are expressed as the mean ± S.E. for at least three independent replicate experiments (n ≥3). Statistical tests were undertaken using InStat (GraphPad, La Jolla, CA, USA). Statistical analysis of results was undertaken using one-way analysis of variance (ANOVA) using a post-test depending on the requirement.

Results

TG2 inhibitors reduce MØ-AC interaction through actions on macrophages

Initial studies sought to address whether THP-1 cells, stimulated to differentiate to MØ, express TG2 as this cell model provides a valuable tool for undertaking many experiments in a controllable system. Thus we undertook western blot analysis for TG2 on cell lysates of THP-1 cells (stimulated with dihydroxyvitamin D3, PMA or PMA/VD3) and analysed for the presence of MØ cell surface TG2 following cell surface biotinylation, We show stimulated THP-1 cells (PMA or PMA/VD3) express TG2 strongly and this expression includes cell surface expression (Figure 1*A* and Supplementary Figure 1*A*). Cell surface expression was also clearly revealed with immunofluorescence microscopy (Figure 1*A*) in PMA and PMA/VD3 cells but not in THP-1 cells or VD3 cells. The lack of detectable cell surface TG2 in THP-1 cells or those stimulated solely with VD3 is consistent with the lack of TG2 expression noted in whole cell lysates.

To confirm a role for TG2 in human MØ clearance of AC, THP-1 MØ (THP-1/PMA, THP-1/VD3 and THP-1/DS) were treated initially with TG2 inhibitors R283 & Z-DON (the latter is claimed to be cell permeable by the manufacturer Zedira) or R281 and R294 (R281 is cell-impermeable[22, 23] and R294 more water soluble and likely therefore to be similarly cell-impermeable[22]), irreversible TG2 site-directed inhibitors at a concentration of 500μM[22]. Following a 1h inhibitor treatment, macrophages were assessed for their ability to interact with apoptotic B cells, where interaction is defined as the sum of both tethering (binding) and phagocytosis (engulfment) of AC. All four TG2 inhibitors including the cell-impermeable inhibitors 281 and 294 reduced significantly MØ-AC interaction in those MØ that expressed TG2 (THP-1/PMA, THP-1/DS; Supplementary Figure 2*A*), suggesting that TG2 is mediating its actions at the cell surface. Further studies revealed the inhibitor effects to be dose dependent (Figure 2*B* and Supplementary Figure 2*B*) with no cellular toxicity (Supplementary Figure 3). AC clearance by phagocytes is a multistage process and to assess the role of TG2 in the tethering of AC to MØ, TG2 inhibitors were added to MØ-AC co-cultures at 20^{o}C, a temperature non-permissive for phagocytosis[29]. Inhibition of cell tethering was shown by all the TG2 inhibitors tested (Figure 1*C*). Similarly, the

inhibitors reduced AC interaction with primary human monocyte-derived macrophages (Figure 1*D*).

In order to identify the cellular target of TG2 inhibition MØ or AC were individually treated with TG2 inhibitors prior to co-culture. Treatment of MØ led to the most profound reduction in MØ-AC interaction (Figure 1*E*), suggesting that the principal TG2 inhibition effect is on the MØ not the AC, and that MØ TG2 is required for MØ-AC interaction. This is further supported by TG2 expression studies (Supplementary Figure 3) since in leucocyte cells used as apoptotic targets, TG2 was not detectable by western blot. These data support a prominent role for TG2 in MØ-AC interaction in those highly differentiated human MØ cells (HMDMØ, THP-1/PMA, THP-1/DS) but not in the monocyte-like models (THP-1 and THP-1/VD3).

TG2 inhibitors reduce MØ migration towards AC-derived microparticles

Macrophage migration to dying cells is an important event in vivo for phagocytic removal of AC and MØ have been shown to migrate towards AC along a gradient of AC-derived microparticles[29]. To determine the role of TG2 on MØ migration to AC, THP-1 cells double-stimulated with PMA/VD3 (DS) were assessed for their migration towards AC microparticles in the presence or absence of the TG2 cell-impermeable inhibitor R281. Inhibition of TG2 resulted in a dramatic loss of MØ migration towards AC (Figure 2*A*), with a parallel effect on MØ directionality (Figure 2*A*). Direct (Euclidean) distance migrated between start and end points and velocity of migration had reduced significantly with TG2 inhibitor treatment (Figure 2*B*).

TG2 knockdown in MØ showed limited ability to interact with AC

To further confirm a role for TG2 in MØ-AC interaction, THP-1 cells were transfected with four different siRNA targeting human TG2, while the global non-silencing (NS) siRNA was used as the control. After stimulating THP-1 with PMA and VD3 for 24h, cells were transfected with siRNA for a further 48h before whole cell lysates were analysed by western blotting for TG2 expression (Figure 3*A*). TG2 siRNA1 caused a reduction (approximately 70%) in TG2 expression, in comparison to NS siRNA, and this TG2 knockdown reduced interaction between siRNA1-treated macrophages and AC by around 50% when compared to the inactive control siRNA transfected cells (Figure 3*B*). Importantly, the reduced MØ-AC interaction noted with either the cell-

impermeable TG2 inhibitor (R281) or siRNA knockdown of TG2, acting either independently or in combination, could be partially rescued by the addition of exogenous active TG2 (Figure 3*C*) indicating the specificity of action of the TG2 effects shown here and confirming that active TG2 at the MØ cell surface is important for apoptotic cell clearance.

Cell surface TG2 protein cross-linking activity mediates MØ-AC interaction

Site-directed TG2 irreversible inhibitors can block both the transamidating activity and alter TG2 conformation by holding it in its open conformation after reaction[32]. Such changes to TG2 conformation could affect binding of TG2 to its high affinity ligands such as heparan sulphates[6]. To address the involvement of TG2 activity and/or conformation in MØ-AC interactions, the use of the competitive primary amine substrate monodansylcadaverine (MDC) at a concentration of 100μM, which also blocks protein cross-linking was used. MDC reduced MØ-AC interaction to the same degree as the irreversible cell-impermeable inhibitor R281; suggesting alterations to TG2 activity, not conformation were responsible for altered AC clearance (Figure 4*A*).

Given that our results suggest the activity of cell surface TG2 is important in AC clearance, we next demonstrated the presence of *in situ* cell surface TG2 activity in live cells (Figure 4*B*) using established techniques[5, 11]. This activity was inhibited using our panel of TG-2 inhibitors. Add-back of purified exogenous active TG2 to the inhibitor treated MØ resulted in partial rescue of TG2 activity (Figure 4*B*). Primary human MØ showed a similar pattern of cell surface TG2 activity (Supplementary Figure 5). Additionally, TG2 knockdown by siRNA resulted in loss of cell surface TG2 activity (Figure 4*C*) consistent with reduced TG2 expression (Figure 3*A*). Furthermore, a specifically targeted TG2 activity-blocking antibody[33] inhibited MØ-AC interaction in a dose-dependent manner (Figure 4*D*). Taken together these data demonstrate an important role for TG2 cross-linking activity at the surface of human MØ in mediating AC removal.

TG2 associates with Syndecan-4

We next investigated the involvement of TG2 binding partners at the human MØ cell surface. In other cells, TG2 has been shown to have a strong binding affinity for

heparan sulphates (HS) of the cell surface receptor syndecan-4 that is important in the translocation of TG2 to the cell surface and extracellular matrix (ECM)[5, 11]. Thus an attractive candidate partner was syndecan-4, a heparan sulphate proteoglycan (HSPG) that acts as a receptor for TG2 via its HS chains thus mediating an RGD-independent cell adhesion via a TG2-FN complex[6]. Through the use of western blotting and immunofluorescence staining (Figure 5*A*) we demonstrate clear expression of syndecan-4 in THP-1/DS cells that also show strong TG2 expression. To confirm a possible interaction between cell surface TG2 and syndecan-4, we performed co-immunoprecipitation assays and demonstrate TG2-syndecan-4 interaction in MØ cells (Figure 5*B* and Supplementary Figure 1*B*).

Loss of cell surface heparan sulphates or syndecan-4 reduces cell surface TG2 expression and activity resulting in reduced MØ-AC interaction

HS on HSPGs (e.g. syndecan-4) mediate TG2 binding at cell surfaces[5, 11]. In order to test the importance of HS and syndecan-4 in presenting TG2 at the human macrophage cell surface where it mediates AC clearance, we studied the effect of HS removal, syndecan-4 knockdown and blocking TG2-syndecan-4 binding on MØ-AC interaction. Loss of HS, via heparinase II treatment, resulted in loss of cell surface TG2 activity (Figure 6*A*) and reduced MØ-AC interaction (Figure 6*B*). Using syndecan-4 specific siRNAs, a maximal reduction in SDC4 expression (approximately 60%) was obtained (Supplementary Figure 6*A*) resulting in reduced presence (Figure 6*C*) and activity (Figure 6*D*) of cell surface TG2 though this was only evident with the most robust knockdown of SDC4 provided by siRNA construct 2. This strongly suggests that cell surface TG2 is anchored, at least in part, to syndecan-4, the loss of which results in loss of cell surface TG2 activity. No loss of syndecan-4 expression is found in TG2 knockdown THP-1/DS (Supplementary Figure 6*B*) and total TG2 expression was unaffected by loss of syndecan-4 expression.

Syndecan-4 knockdown reduces MØ-AC interaction and when syndecan-4 knockdown THP-1/DS cells were also treated with R281, no further reduction in MØ-AC interaction occurs (Figure 6*E*) suggesting no additive effect and supporting the notion that the involvement of syndecan-4 or TG2 in AC clearance are functionally

related. These data confirm HS as prime receptors for TG2 binding on the MØ cell surface and syndecan-4 as one HSPG important for TG2 binding.

To further strengthen our understanding of TG2-syndecan-4 binding via HS, we used the P1 peptide (^{200}NPKFLKNAGRDCSRRSS^{216}), which mimics the heparin binding domain within TG2 and competes for its binding to syndecan-4[8]. Cells were treated with P1 peptide or a scrambled control P1 peptide for 30min and here we demonstrate that P1 peptide (but not the scrambled control peptide) reduces MØ-AC interaction (Figure 6*F*) in a dose-dependent manner (supplementary Figure 6*C*). However, upon exogenous addition of active TG2, the loss of MØ-AC interaction was found to be partially compensated (Figure 6*F*), suggesting that TG-2 bound to syndecan-4 via HS is crucial in mediating MØ-AC interaction.

TG2 interacts with CD44 at MØ cell surface: A possible TG2 mediated CD44 cross-linking.

CD44, an HSPG and established receptor for hyaluronan, is important in immune cells' proliferation and differentiation, cell adhesion, migration, and inflammation[34-36]. CD44 undergoes complex alternative mRNA splicing and posttranslational modifications resulting in a family of isoforms with distinct biological functions[37]. CD44 is a known phagocytic receptor[38] that, when cross-linked by antibody, augments AC clearance[20]. We therefore investigated whether TG2 may mediate CD44 crosslinking to facilitate AC clearance.

Initially we examined CD44 expression and found it paralleled TG2 expression (Figure 7*A*) in THP-1/DS, THP-1/PMA and HMDMØ. Interestingly, two major distinct molecular weight bands for CD44 were expressed. To address the possibility that the high molecular weight CD44 is a TG2 cross-linked form (cross-linked to itself or other molecules), CD44 expression in TG2 inhibitor-treated cells was examined. Inhibition of TG2 was associated with a loss in the intensity of the high molecular CD44 (~150kDa) and an increase in the intensity of low molecular weight CD44 (~85kDa) consistent with the notion that the high molecular weight CD44 species arises from TG2 activity (Figure 7B). Precipitation of TG2 resulted in the co-precipitation of CD44 (Figure 7*C*) suggesting a possible TG2-CD44 interaction. Importantly, in support of the small molecular CD44 species being a substrate of TG2 following prior treatment

with the non-cell-permeable inhibitor R281 the presence of the high molecular weight CD44 species was barely detectable in primary human macrophages (Figure 7*D*).

To further confirm that CD44 is a potential substrate of macrophage cell surface TG2, THP-1/DS were incubated with the TG2 primary amine substrate biotin-cadaverine (BTC). Following cell lysis, proteins with TG2 incorporated BTC were affinity purified with Neutr-Avidin-Agarose resin beads. Western blot analysis for CD44 revealed both high and low molecular weight bands for CD44 were present (Figure 8*A*). However, treatment with the TG2 irreversible inhibitor R281 prior to BTC incorporation resulted in a significant loss in high molecular weight CD44 (Figure 8*A*) confirming that CD44 is a potential substrate for cell surface TG2. Whilst R281 is an irreversible inhibitor of TG2, the inhibition is incomplete (figure 4B) and this provides an explanation of why there is BTC incorporation remaining even in the presence of TG2 inhibitors. The cell surface interaction of TG2 with CD44 was confirmed by immunofluorescence analysis of TG2 and CD44 which showed co-localisation of CD44 with TG2, although not all TG2 showed co-localisation with CD44 (Figure 8*B*). This provides further evidence that TG2 interacts with CD44 supporting both the co-immunoprecipitation analysis and BTC incorporation studies.

To study the effect of TG2-CD44 interaction on MØ ability to interaction with AC, MØ-AC interaction assay was performed following CD44 knockdown in MØ. CD44 siRNA-9 caused a significant reduction in CD44 expression in comparison to NS siRNA (Supplementary Figure 7*A*), and loss of CD44 resulted in reduced MØ-AC interaction by around 40% when compared to the NS siRNA transfected cells (Figure 8*C*). There was no effect on cell surface TG2 expression (Supplementary Figure 7*B)*. Interestingly, treating MØ with TG2 inhibitor R281 or treating CD44 siRNA-9 transfected MØ with R281 showed a further reduction in the MØ-AC interaction, suggesting that TG2 can act independently of CD44, possibly through syndecan 4.

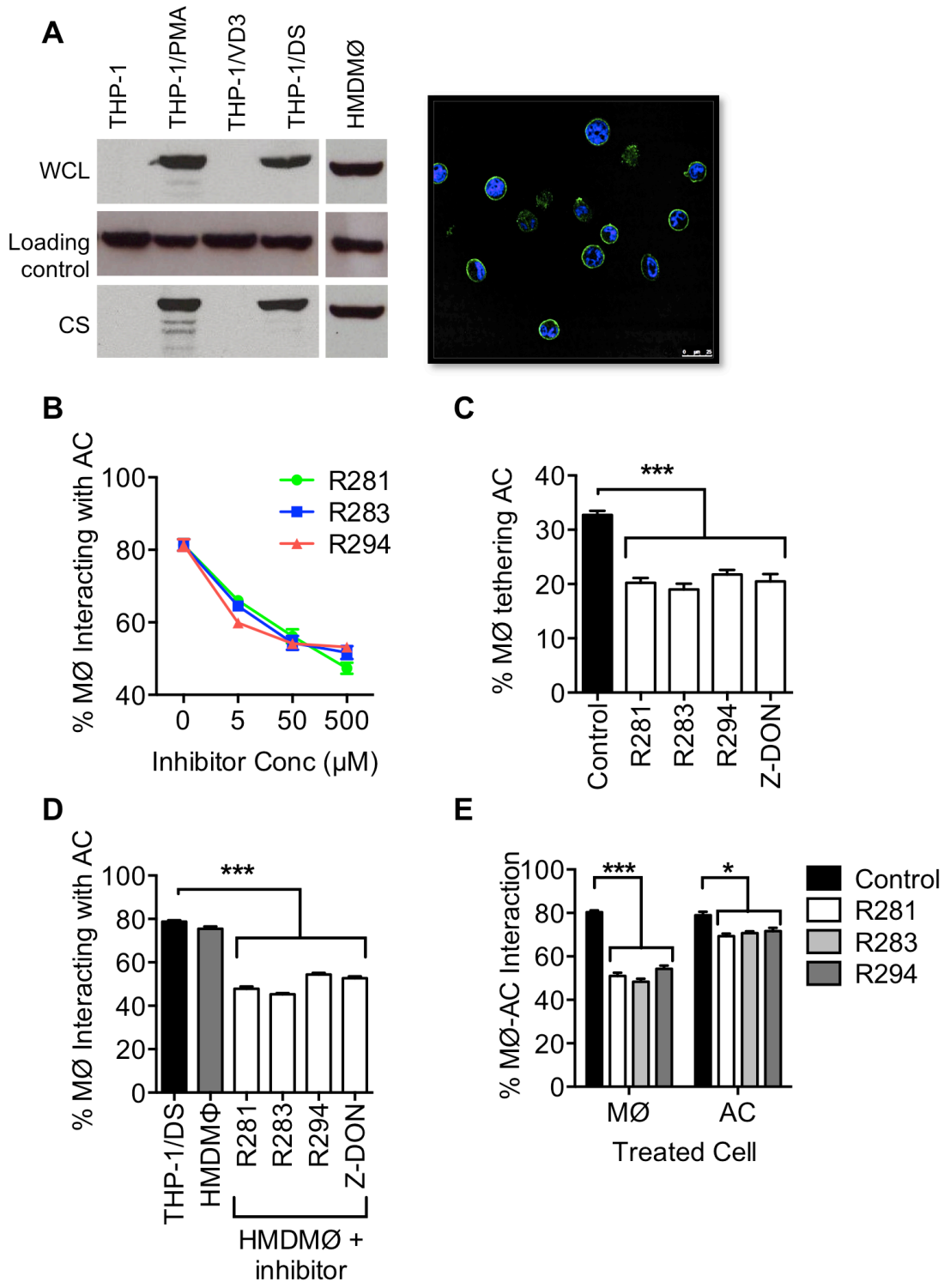


FIGURE 1. TG2 inhibitors reduce apoptotic cell clearance by macrophages.

(A) Western blot analysis revealing both whole cell lysate (WCL) and cell surface (CS) TG2 expression in THP-1 cells-derived MØ (through stimulation with VD3, PMA or both (DS)) and primary HMDMØ. THP-1/PMA, THP-1/DS and HMDMØ but not undifferentiated THP-1 and THP-1/VD3 showed detectable TG2 expression by western blotting (left panel). Cell surface TG2 was detected spefically following cell

surface protein biotinylation, prior to isoation of biotinylated proteins using Neutr-avidin as described in the materials and methods. Equal loading was verified by probing with mouse anti-tubulin antibody on stripped membranes. Similarly, surface expression of TG2 in THP-1/DS was revealed upon indirect immunofluorescence staining of live (i.e. unpermeabilised) cells with mouse monoclonal anti-TG2 antibody (Cub7402) in conjugation with FITC-congugated anti-mouse secondary antibody (right panel). Nuclei were stained with DAPI. The bar corresponds to 25μm. (B) Dose-dependent effect of three site-directed irreversible TG2 inhibitors on MØ (THP-1/DS) interaction with AC. Inhibitors were applied to MØ for 1h prior to and throughout the co-culture with AC. 'Interaction' is defined as the combination of those AC tethered (bound) to the surface of MØ and those internalised (phagocytosed). (C) TG2 inhibitors (including cell-impermeable R281 and its more water soluble counterpart R294) inhibited MØ (THP-1/DS) tethering of AC (i.e. where only surface binding of AC to MØ is assayed without contribution from phagocytic events) and, (D) a similar effect of TG2 inhibitors is seen in HMDMØ interacting with AC. (E) Treatment of either MØ alone or AC alone with TG2 inhibitors showed significant reduction in MØ-AC interaction following inhibitor treatment of MØ. Data shown are mean ±S.E. for n≥3 independent replicates. Statistical analysis was conducted using ANOVA followed by Bonferroni post-test (*P<0.05; *** P<0.001).

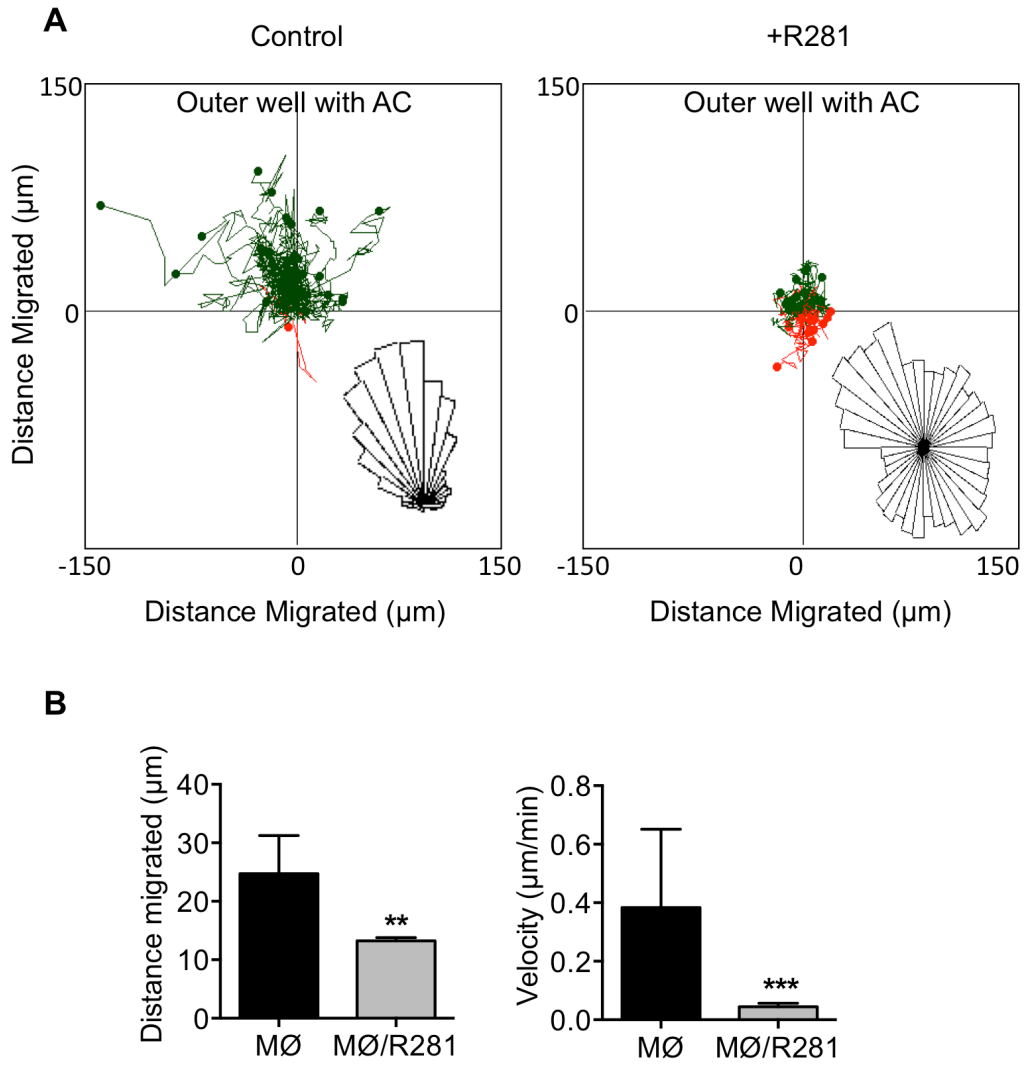


FIGURE 2. TG2 inhibition reduces macrophage migration to AC

(A) The effect of TG2 inhibitor R281 on MØ (THP-1/DS) migration towards AC was tested using a Dunn chemotaxis chamber coupled with time-lapse imaging phase contrast microscopy over 20h. MØ were pre-treated for 1h with the inhibitor prior to exposure to AC in a Dunn chamber. Cell migration was tracked using ImageJ software and tracks were analysed using Chemotaxis and Migration tool 2.0 (IBIDI), and were plotted as distance migrated (µm). The track of each cell is shown, starting at the cross hairs and finishing at a dot, with the position of the attractant at the top of each plot. Representative plots are shown. R281, a cell-impermeable TG2 inhibitor induced a dramatic reduction in MØ migration. A loss of directionality is shown in the rose diagram (insets). (B) The distance migrated and the velocity at which the cells migrate in the presence and absence of TG2 inhibitor (R281) is

presented as mean ±S.E. for n≥3 independent replicates. Statistical analysis was conducted using ANOVA followed by Bonferroni post-test (**P<0.01; *** P<0.001).

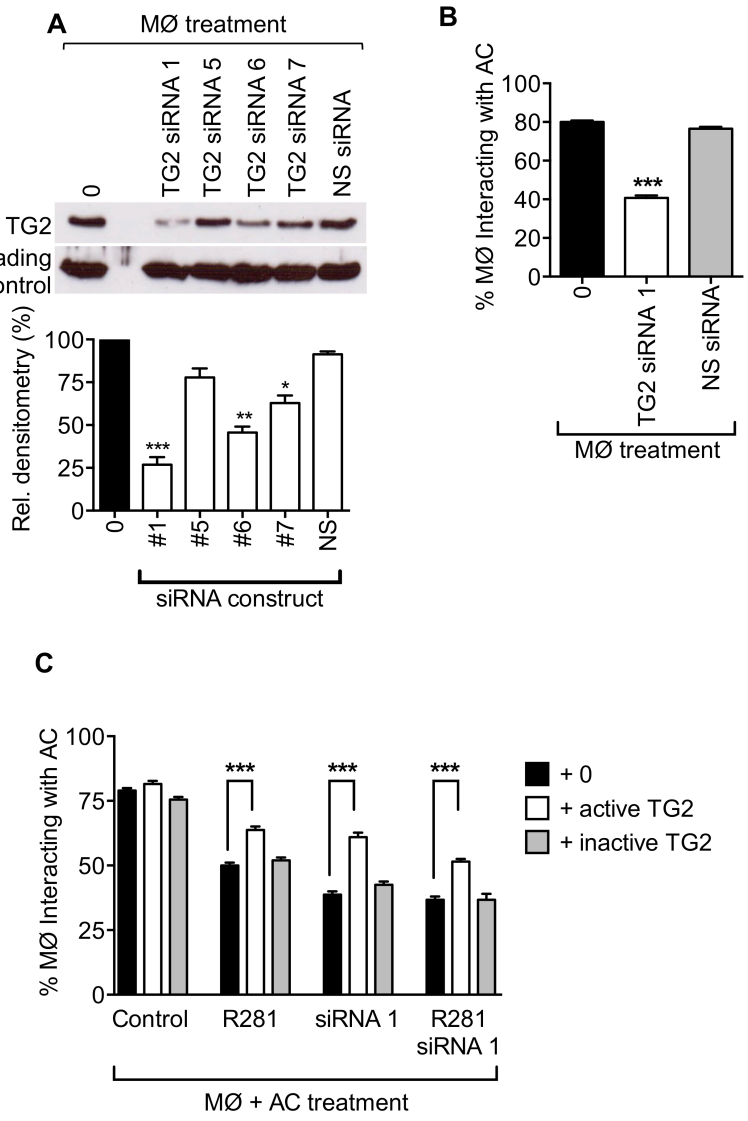


FIGURE 3. Addition of exogenous active TG2 can compensate for the impaired macrophage-apoptotic cell interaction seen with TG2 knockdown or inhibition

(A) Western blot analysis of TG2 expression using mouse anti-TG2 in THP-1/DS following transfection with the indicated TG2-targeted siRNA constructs or a non-specific control (NS). A representative blot is shown along with densitometric analysis from three independent experiments. Statistics compare to NS control construct. (B) TG2 knockdown with siRNA 1 reduces MØ (THP-1/DS) interaction with AC compared to cells similarly transfected with a control siRNA (NS siRNA). (C)

Reduced MØ-AC interaction as a result of TG2 knock down (siRNA 1) or TG2 inhibition (R281) is rescued by the addition of exogenous active, but not inactive TG2. Where appropriate, inhibitors were applied to MØ for 1h prior to and throughout the co-culture with AC. Data shown are mean ±S.E. for n≥3 independent replicates. Statistical analysis was conducted using ANOVA followed by Bonferroni post-test (*** $P<0.001$).

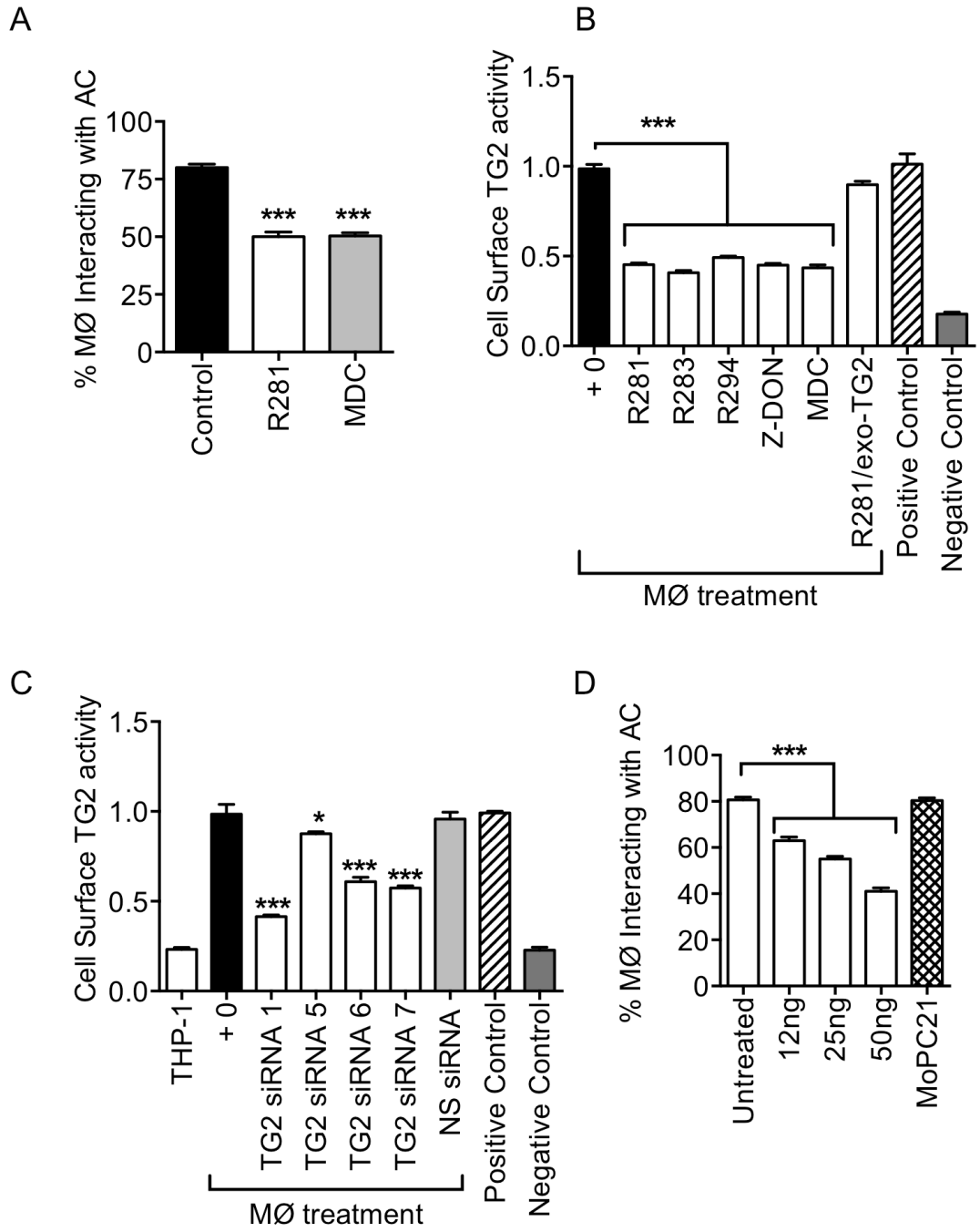


FIGURE 4. TG2 activity promotes macrophage-apoptotic cell interaction.

(A) Monodansylcadaverine (MDC: inhibits crosslinking in the presence of Ca^{2+}, as used here, without changing the conformation of TG2) reduces MØ (THP-1/DS) interaction with AC. Similar levels of inhibition are noted with irreversible TG2 inhibitor R281. All inhibitors were applied to MØ for 1h prior to and throughout the co-culture with AC. (B) MØ (THP-1/DS) cell surface TG activity was assessed (via biotin-cadaverine incorporation into fibronectin) following MØ treatment with the indicated TG2 inhibitor and adding active TG2 exogenously following R281

treatment. The positive control is gplTG. The negative control comprises the addition of 10mM EDTA. (C) Cell surface transglutaminase activity was similarly assessed in MØ transfected with the TG2-specific siRNA or a non-specific control (NS siRNA). (D) TG2 binding antibody (D11D12) inhibits MØ-AC interaction in a dose-dependent manner compared to the isotype control mAb MoPC21. Data shown are mean ±S.E. for n≥3 independent replicates. Statistical analysis was conducted using ANOVA followed by Bonferroni post-test (*** $P<0.001$).

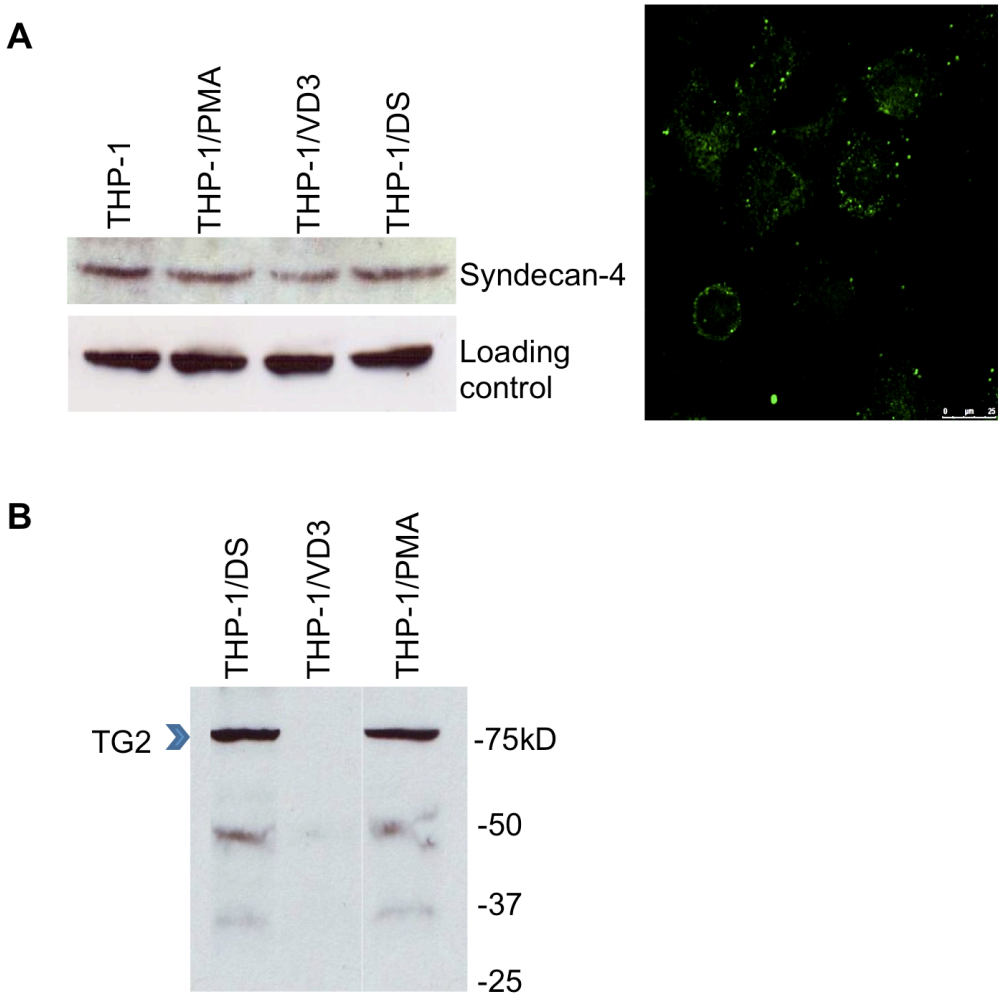


FIGURE 5. Macrophage cell surface TG2 associates with syndecan-4.

(A) Western blot analysis (left panel) reveals the expression of syndecan-4 (detected with mouse anti-SDC4 mAb) in undifferentiated and a panel of differentiated THP-1 MØ cells. Stripped membranes were re-probed with mouse anti-α-tubulin antibody as a loading control. Similarly, syndecan 4 expression at the cell surface of THP-1/DS MØ was detected using indirect immunofluorescence (mAb to syndecan-4; goat anti-mouse FITC) and confocal imaging. (B) Interaction between TG2 and syndecan-4 in THP-1/DS MØ is revealed by co-immunoprecipitation analyses. Syndecan-4 (SDC4) was precipitated using an anti-SDC4 antibody. Precipitated material was separated by PAGE and co-precipitation of TG2 assessed by anti-TG2 western blotting (using mouse anti-TG2 mAb; anti-mouse-HRP).

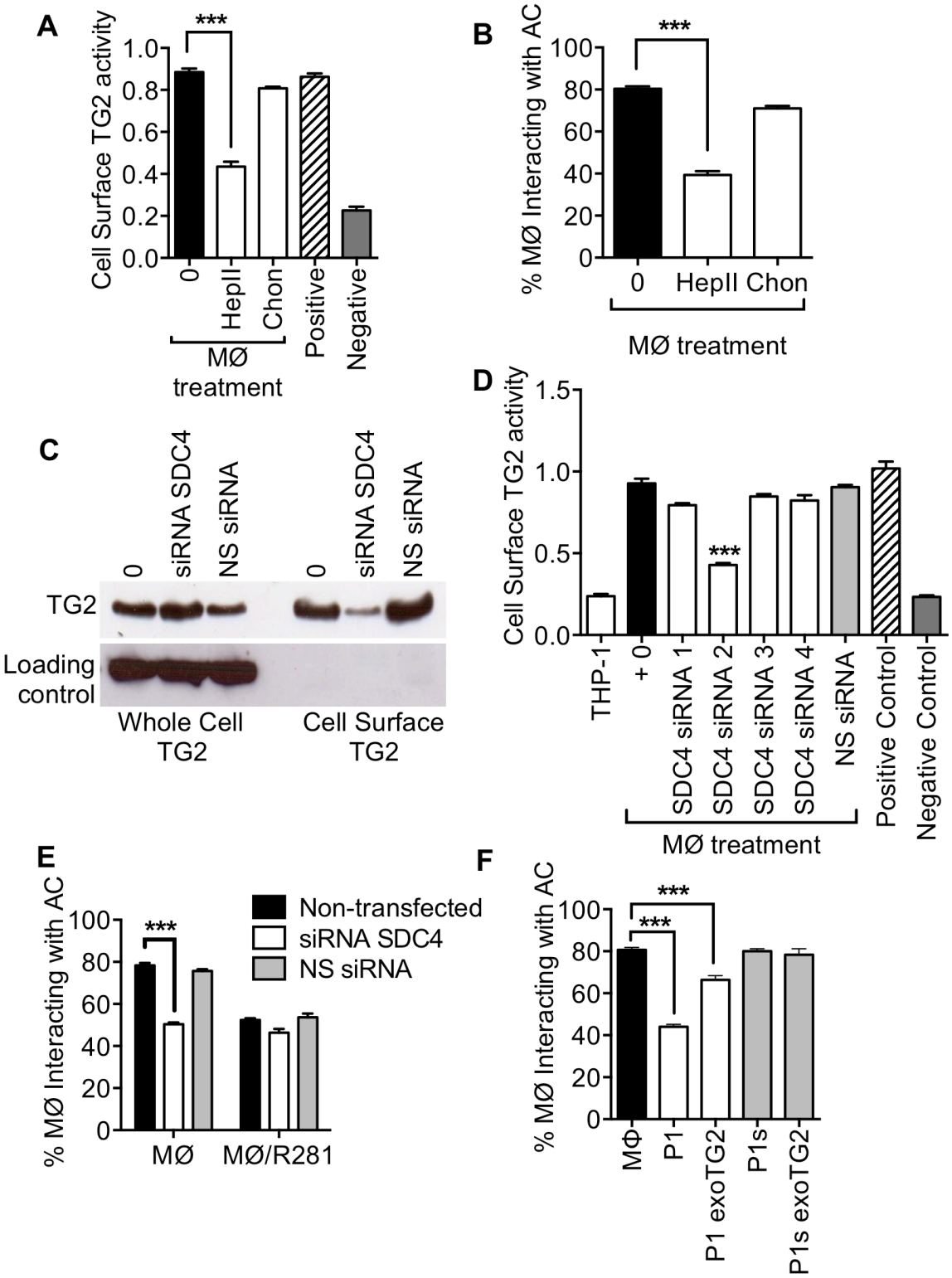


Figure 6. TG2 associates with syndecan-4 heparan sulphate side chains and mediates macrophage-apoptotic cell interaction.

Cell surface activity of TG2, and MØ-AC interaction was assessed following treatment of MØ (THP-1/DS) with heparinase II (HepII) or chondroitinase (Chon) as

described in the Methods section. (A) TG activity is reduced following treatment with heparinase II to remove heparan sulphate chains. (B) Treatment of MØ with heparinase II also resulted in significant loss of MØ interaction with AC. (C) Western blot analysis of TG2 (whole cell TG2 or cell surface TG2 isolated following cell surface biotinylation and Neutr-avidin isolation of cell surface components) in MØ (THP/DS) cells transfected with syndecan-4 specific siRNA or a non-specific (NS) siRNA. This reveals a loss of cell surface (but not whole cell) TG2 when syndecan-4 expression is reduced. (D) Following siRNA-mediated syndecan-4 knockdown in MØ (THP/DS) with the indicated siRNA constructs, cell surface TG2 activity was assessed and shows a loss of cell surface TG activity. (E) Syndecan-4 knockdown with siRNA (construct siRNA 2) in MØ (THP/DS) was undertaken prior to assessing MØ-AC interaction. Addition of R281 was also included (for 1h prior to co-culture and throughout the co-culture period) to assess additive effects. (F) MØ (THP-1/DS) treated with P1 peptide for 1h prior to co-culture and throughout the co-culture period (P1; which competes the heparin binding domain within the TG2) but not a scrambled P1 peptide (P1s) show reduced interaction with AC, that is rescued when 200ng purified exogenous TG2 (exoTG2) is added. Data shown are mean ±S.E. for n≥3 independent replicates. Statistical analysis was conducted using ANOVA followed by Bonferroni post-test (*** $P<0.001$).

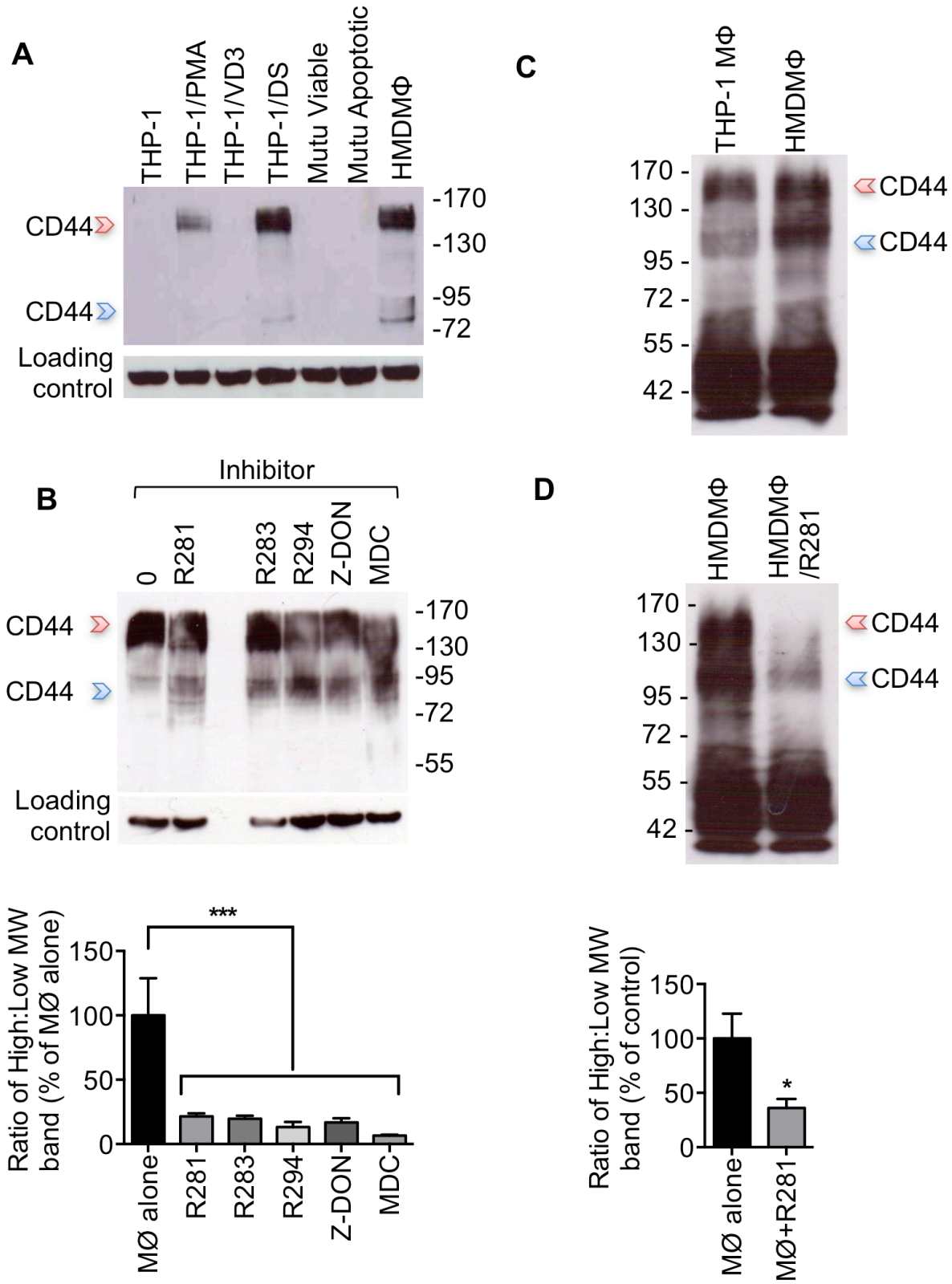


Figure 7. TG2 interacts with CD44.

(A) Western blot analysis of CD44 in whole cell lysates of THP-1/PMA MØ, THP-1/DS MØ and HMDMØ reveals two distinct molecular weight bands for CD44.

Arrowheads indicate high and low molecular weight bands for CD44. Stripped membranes were re-probed with mouse anti-β-actin antibody as a loading control. (B) MØ (THP/DS) were treated with the indicated TG2 inhibitors for 1h prior to cell lysis and western blot analysis for CD44. Upper panel: A representative blot showing significant loss in high molecular CD44 (~150-160kDa) and an increase in the intensity of low molecular weight CD44 (~85-95kDa) in comparison to untreated MØ. Stripped membranes were re-probed with anti-β-actin antibody as a loading control. Lower panel shows the ratio of the high:low molecular weight bands assessed by densitometry from three independent experiments (mean±S.E.). Data shown were normalised to the signal from MØ alone. (C) Immunoprecipitation of TG2 from membrane lysates of THP-1/DS MØ and primary HMDMØ cells was undertaken and probed on western blot for CD44. This reveals interaction between TG2 and CD44 proteins. (D) Upper panel: Western blot analysis of CD44 from immunoprecipitates of TG2 following inhibition of TG2 with R281 in HMDMØ (representative blot); Lower panel shows the ratio of the high:low molecular weight bands assessed by densitometry from three independent experiments (mean±S.E.). Arrowheads indicate high and low molecular weight bands for CD44. HC indicates heavy chain and LC indicates light chain. Statistical analysis was conducted using ANOVA followed by Dunnett's post-test (* $P<0.05$; ** $P<0.01$, *** $P<0.001$).

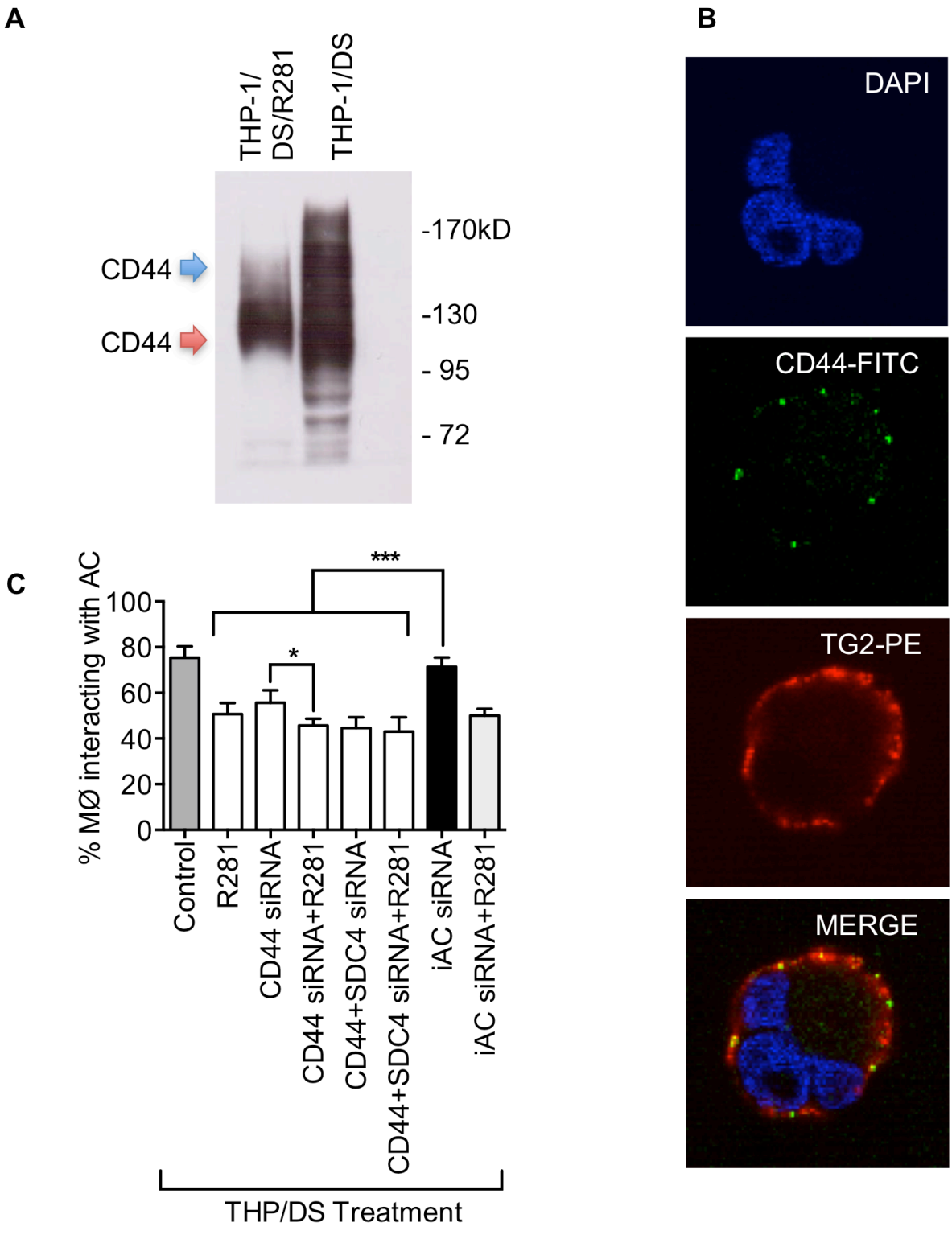


Figure 8. CD44 acts as a substrate for TG2 in mediating apoptotic cell clearance.

(A) THP-1/DS MØ were exposed, in the presence or absence of TG2 inhibitor R281, to biotin-cadaverine (BTC) as a measure of cell surface TG2 activity. Where R281 was used, MØ were pre-treated with inhibitor for 1h prior to the addition of BTC. Following BTC incorporation, cell lysates of THP-1/DS MØ were subject to pull down of biotin-containing molecules by Neutr-Avidin-Agarose resin beads. The pull downs were probed by western blotting for CD44 to reveal TG2-mediated BTC incorporation into CD44. This reveals incorporation of biotin-cadaverine into CD44 proteins in TG2 expressing cells and reduced incorporation upon treatment with non-permeable TG2 inhibitor R281. Arrowheads indicate high and low molecular weight bands for CD44. A significant loss in high molecular CD44 (~150-160kDa) in comparison to untreated MØ is shown. (B) Cell surface TG2 (Red) and CD44 (Green) on the MØ cell surface were stained by indirect immunofluorescence, incubating live cells with rabbit anti-TG2 and mouse anti-CD44 primary antibodies. Cells were further stained with secondary reagent (anti-rabbit-PE; anti-mouse-FITC). TG2 co-localizes with CD44 on the MØ cell surface (MERGE/Yellow). Staining is visualized by confocal microscopy. (C) CD44 and/or SDC4 knockdown with siRNA in MØ (THP/DS) was undertaken prior to assessing MØ-AC interaction. Addition of R281 was also included to assess additive effects. Data shown are mean ±S.E. for n≥3 independent replicates. Statistical analysis was conducted using ANOVA followed by Bonferroni post-test (*** $P<0.001$).

Discussion

Phagocytic removal of apoptotic cells involves an array of phagocyte receptors which, directly or indirectly via soluble bridging molecules, facilitate recognition and binding of AC associated ligands ultimately leading to corpse uptake[16, 17]. As evident from previous studies using TG2 deficient mice, TG2 plays a prominent role in AC engulfment by promoting phagocytic portal formation[18,19]. It is also crucially important in immune modulation to prevent inflammation and autoimmunity[18]. Here we extend those studies to characterise TG2 in AC clearance and address its location and function in human macrophages. Using a TG2-inhibitor-based approach, rather than TG2-deficient animals, we address TG2 in human cells that have developed in a TG2-replete status before specifically inhibiting TG-2 activity or expression. Thus taking our results together with those existing, we provide a comprehensive view of TG2 function in AC clearance.

Our initial western blot studies addressed whole cell and cell surface TG2 expression in a panel of THP-1 cell-derived macrophage models and HMDMØ. These cells expressed TG2 at the cell surface[39] with the exception of THP-1 parental cells and THP-1/VD3 cells where TG2 was not detectable. Given the monocyte-like (relatively immature) morphology of these cells[28], these data suggest TG2 expression is linked to monocyte/macrophage maturity[40, 41].

Building on the important previous work in mice, we assessed TG2 in human MØ through the use of irreversible site-directed TG2 inhibitors[22] including proven cell-permeable (Z-DON, Zedira) or cell-impermeable (R281) inhibitors. All inhibitors tested strongly reduced MØ-AC interaction (i.e. tethering and phagocytosis combined) and tethering (binding alone) by those MØ that expressed TG2. Importantly, similar results were noted for primary human MØ. Detection of TG-2 expression at the MØ surface coupled with the ability of cell-impermeable inhibitors and a TG2-specific inactivating antibody to reduce MØ-AC interaction suggests that MØ cell surface TG2 plays a prominent role in MØ-AC interaction. To address the issue of inhibitor-induced conformational changes of TG2, as an explanation for reduced MØ-AC interaction, the competitive TG2-substrate MDC was employed since it blocks TG2-induced protein crosslinking in the presence of Ca^{2+}without any irreversible change in TG2 conformation[32]. Again we demonstrated a significant

reduction in MØ-AC interaction confirming TG2 crosslinking activity as required for AC clearance by human macrophages. This conclusion is further supported by our use of siRNA to knockdown TG2 that resulted in reduced expression and cell surface activity of TG2. Taken together, with the ability of the TG2 activity-blocking antibody to reduce MØ-AC interaction, these data demonstrate for the first time that TG2 on the surface of human MØ is important in the tethering and removal of apoptotic cells. Importantly we show a requirement for TG2 crosslinking activity in this function. In addition to TG2, macrophages are known to express Factor XIIIa (another TG family members)[42], [40]. To rule out the involvement of this enzyme in macrophage–AC interactions we used a TG2-specific inactivating mAb. Additionally we used the irreversible TG2 inhibitors R294 (that has an IC_{50} for Factor XIIIa of greater than 200μM but has a IC_{50} for TG2 of 8μM) and R281 (which also has an IC_{50} for FactorXIIIa of greater than 100μM but an IC_{50} for TG2 of 10μM)[24]. In addition, we also used TG2 targeted siRNA. The use of the inactivating TG2 mAb and the use of TG2 targeted siRNA will also rule out the involvement of other possible isoforms of TG that might be present.

Removal of AC *in vivo* is preceded by MØ migration to sites of cell death[30]. Since TG2 cell surface activity has also been reported to be important in cell migration[12, 43] we investigated the ability of TG2 inhibitors to modulate MØ migration towards AC using a horizontal migration chamber. We demonstrate that inhibition of TG2 reduces both the velocity and distance migrated by MØ in response to AC and results in a complete loss of directionality. These data suggest that TG2 is involved in MØ migration. As syndecan-4 is known to be a key signalling receptor determining directional migration[44], loss of TG2 which is known to be a strong binding partner would have resulted in loss of directional migration. However, it is unclear if this effect is mediated through reduced cell adhesion or reduced detection of 'find me' signals released from apoptotic cells and this requires further study.

In light of the importance of cell surface TG2 in our studies, we addressed the mechanism by which TG2 may arrive at the MØ surface. Syndecan-4, a HSPG, has been shown to act, in non-MØ, as a receptor for TG2 via its HS chains where it can mediate TG2 translocation to the cell surface and ECM[5, 11]. Here we demonstrate

syndecan-4 expression in all our THP-1 cell-derived models, irrespective of their TG2 expression. Through co-immunoprecipitation studies we demonstrated syndecan-4 interacts with TG2 (where expressed), consistent with earlier studies[5, 6, 8, 10]. Loss of syndecan-4 or cell surface HS resulted in significant loss of cell surface TG2 expression and activity, affecting MØ-AC interaction. Interestingly, whilst two different siRNA constructs for syndecan-4 resulted in reduced syndecan-4 expression, only the most robust blockade of syndecan-4 impacted on surface TG2 expression and activity, possibly suggesting that a threshold of syndecan 4 is required on the cell surface for TG2 function. Taken together with the inhibitory effect of the P1 peptide, which mimics the syndecan-4 binding domain within the TG2 molecule[8], and which can be rescued through the addition of exogenous TG2, these data support the notion that cell surface TG2, in association with HS of syndecan-4 mediates MØ-AC interaction. Sequential knockdown of syndecan-4 expression and inhibition of TG2 activity together showed no further reduction in AC clearance suggesting TG2 exerts most, if not all of its cell surface effects on AC clearance through its association with syndecan-4.

Interaction of cell surface adhesion molecules either with ECM components or neighbouring cells are known to influence cell behaviour including phagocytosis. CD44, a principal surface receptor for the ECM molecule hyaluronan (a well-known cell-cell and cell-matrix interaction mediator), is known for recruiting monocytes to sites of inflammation[45] and is implicated in AC clearance[46]. Furthermore CD44, an HSPG, is a known phagocytic receptor[38] that, when cross-linked experimentally by antibody augments AC clearance by an, as yet, incompletely defined mechanism[20]. In light of these pieces of information, we hypothesised that TG2 might modulate AC clearance by mediating CD44 cross-linking to promote AC clearance. Co-immunoprecipitation studies revealed an interaction between TG2 and CD44 but this may be a direct or indirect interaction. CD44 was also detected at the MØ cell surface and was detected in TG2-expressing MØ (THP-derived or HMDMØ) as two distinct molecular weight bands. CD44 exists in variant isoforms as a result of alternative splicing or variable N- and O- linked glycosylation[36, 47, 48]. CD44s is the most prevalent form (40kDa) and with extensive post-translation glycosylation can increase in mass (80kDa)[49, 50]. Notably, TG2 inhibition with a cell-impermeable inhibitor resulted in reduced high molecular CD44 and an increased intensity of low

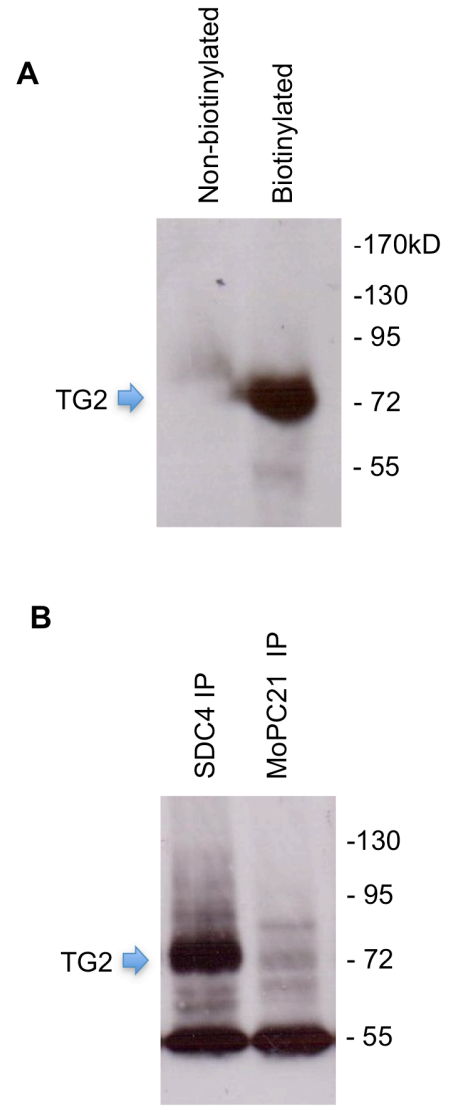
molecular weight CD44. Biotin cadaverine incorporation by cell surface TG2 and subsequent pulling down of labelled substrates with Neutr-Avidin and probing for CD44 on western blot showed high and low molecular weight CD44 but a loss of high molecular weight CD44 was evident upon treatment with TG2 inhibitor R281 prior to biotin-cadaverine incorporation. This confirms that *in situ* CD44 is a potential substrate of MØ-cell surface TG2. These data suggest that CD44 is a substrate of TG2 and can be cross-linked by the enzyme via a mechanism requiring its binding to cell surface HS. This may provide a physiological equivalent of antibody cross-linking to augment AC clearance by MØ[20]. Taken together, our data support the hypothesis that TG2 exerts its effect on AC clearance through the cell surface association with its high affinity binding partner syndecan-4 and that this may be due, at least in part, through CD44 cross-linking. However, further work is required to assess the details of this interaction and the subsequent downstream signalling that results.

MØ-CD44 ligation mediated augmented AC intake is evident in both *in vitro* and *in vivo*[20, 51] conditions. Cuff *et al.* demonstrated that CD44 promotes MØ recruitment to atherosclerotic lesions (sites of extensive cell death)[52], while MØ from CD44$^{-/-}$ mice showed delayed migration into areas of inflammation[53] and a defective clearance of AC[46]. Interestingly, our MØ migration studies revealed reduced migration and directionality following TG2 inhibition. Taken together these suggest TG2, syndecan-4 and CD44 may work together to mediate migration to and clearance of AC. Such a mechanism for TG2 involvement in cell migration is not without precedent, since TG2 binding to syndecan-4 has also been implicated in the crosslinking of cell surface S100A4 resulting in the increased motility of mammary cancer cells[12].

Overall, it seems likely that the binding of TG2 to cell surface heparan sulphates (e.g. on syndecan 4, CD44 and potentially other molecules), which are required for its translocation to the MØ cell surface, may provide the platform for TG2's interaction with a range of other cell surface molecules to mediate a range of TG2-mediated functions. Such functions may include those where the enzyme is brought into contact with substrates (such as CD44, S100A4 or the large latent TGF-beta binding proteins) and also other high-affinity binding proteins such as fibronectin and integrins (β1 and β3), the latter of which are needed for cell adhesion, migration[40] and phagocytosis[19]. Indeed this may help to explain the compensatory increase in

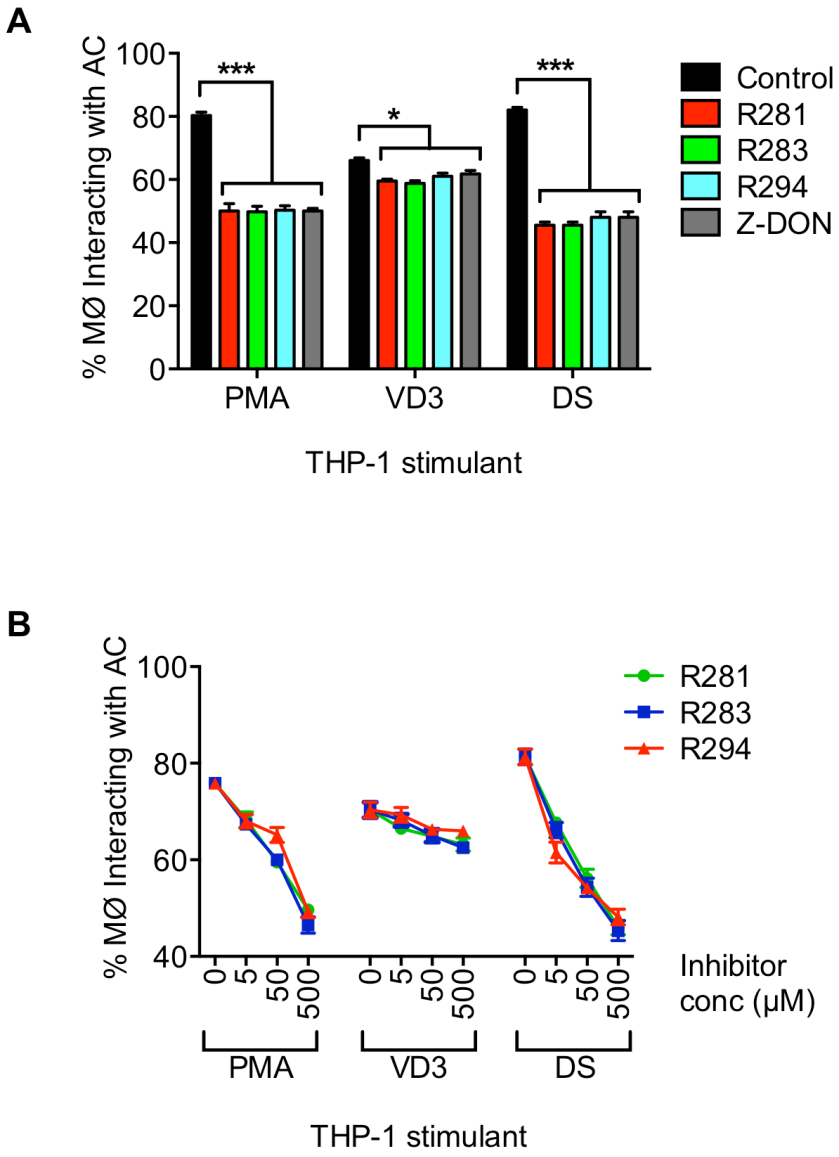
β3 integrin expression seen macrophages from $TG2^{-/-}$ animals. To date the molecular mechanism by which CD44-augmented phagocytosis occurs remains to be elucidated but it has been shown unequivocally to be independent of serum opsonins and glucocorticoid-induced Mer-tk/protein S pathway[20]. CD44 crosslinking has been suggested to stabilise AC-MØ interactions through an ill-defined mechanism but the proposal that CD44 is a substrate for TG2 raises the possibility of a functional link between CD44 and the established role of TG2 in the development of phagocytic portals[19].

In conclusion, we demonstrate that TG2 associates with HS during its translocation to the cell surface of human macrophages. At this site, in association with syndecan-4, its crosslinking activity is central for it to exert its effect on AC removal, at least in part, by promoting tethering of AC. However TG2 is also involved in human MØ migration to dying cells, raising the possibility that TG2 inhibitors may be used to modulate MØ migration for therapeutic gain, in those situations where MØ migration to dying cells is not beneficial. We further reveal a novel TG2-CD44 interaction and demonstrate that CD44 is a potential substrate of TG2 and that inhibition of TG2 activity reduces high molecular weight CD44 complexes. Such complexes may play a role in augmenting AC clearance at least in part through CD44 cross-linking. This is entirely compatible with established ability of TG2 to function with MFG-E8 and β3 integrin to remove AC and mediate its uptake via signalling the recruitment of CrkII-DOCK180-Rac1 complex and thus activating GTPase Rac1[21]. Further work is now needed to assess, in detail, the importance of CD44-TG2 interactions to AC clearance and to define the nature of the TG2-mediated high molecular mass complexes that result.



Supplementary Figure 1: control blots

(A) Surface biotinylation control: THP-1/DS cells were surface biotinylated or non-biotinylated as described in the methods section. Surface proteins were isolated by Neutr-Avidin beads and isolated proteins separated by SDS-PAGE and subject to western blot analysis with mouse anti-TG-2. TG2 was only detected from biotinylated cell surfaces. (B) Syndecan 4 (SDC4) immunoprecipitation control: Interaction between TG2 and syndecan-4 in THP-1/DS MØ is revealed by co-immunoprecipitation analysis. THP-1/DS cell lysates were immunoprecipitated either by mouse anti-SDC4 mAb or by the IgG1 isotype control antibody (MoPC21). Immunoprecipitates were separated by SDS-PAGE and co-precipitation of TG2 assessed by anti-TG2 western blotting TG2 was only detected following specific IP of SDC4.



Supplementary Figure 2. TG2 inhibitors reduce AC clearance by MØ.

(A) THP-1 monocyte (THP-1) cells were stimulated to differentiate to MØ in the presence of dihydroxyvitamin D3 (VD3), phorbol ester (PMA) or both (VD3/PMA) for 48h. Differentiated THP-1 were treated with site-directed irreversible TG2 inhibitors R283, Z-DON (cell-permeable), R281 (cell-impermeable) and R294 for 1h and co-cultured with AC and inhibitors for a further 1h. All four TG2 inhibitors reduced THP-1/DS and THP-1/PMA interaction with AC whilst a smaller degree of inhibition was noted with THP-1/VD3. (B) The dose-dependence of the effect for all three TG2 inhibitors is shown with three THP-1 MØ models. Data shown is mean ±S.E. for n≥3

1 independent replicates. Statistical analysis was conducted using ANOVA followed by
2 Bonferroni post-test (*P<0.05; *** P<0.001).

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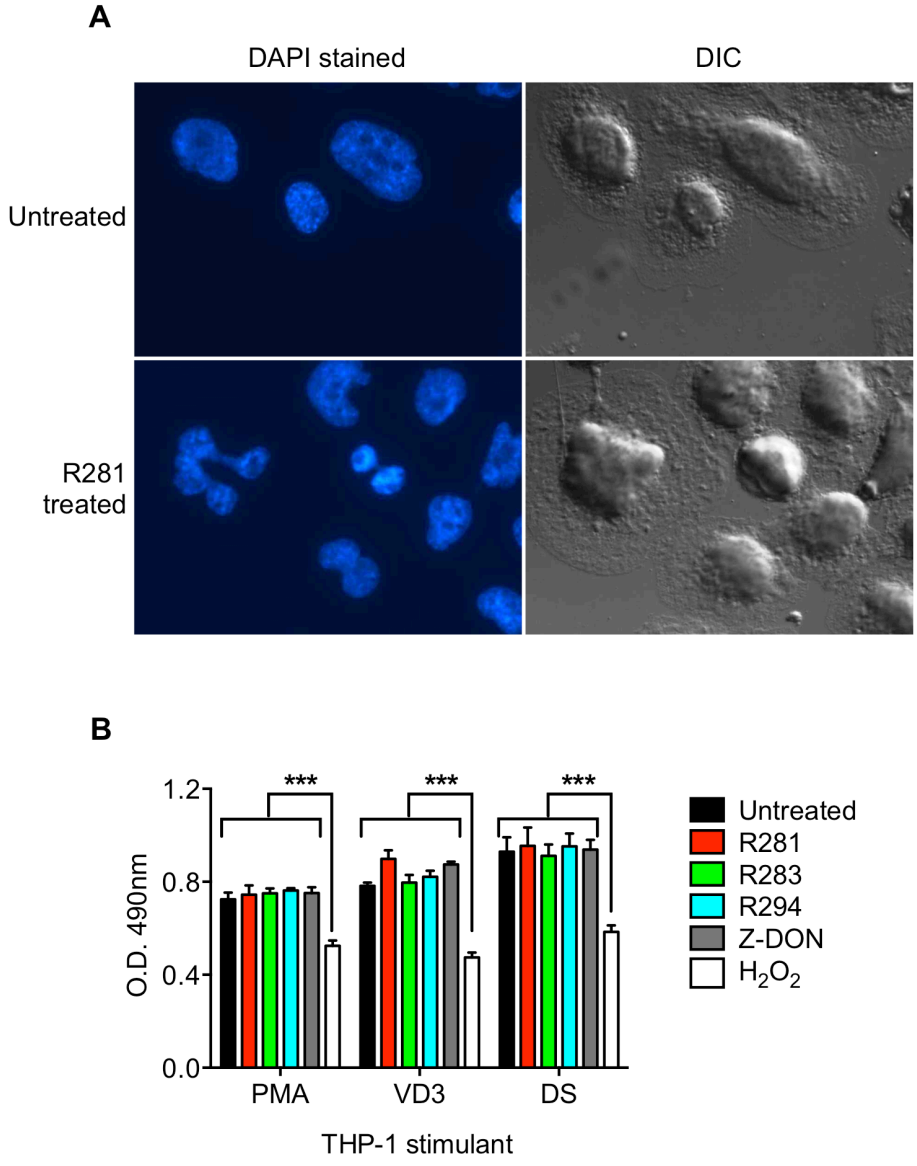
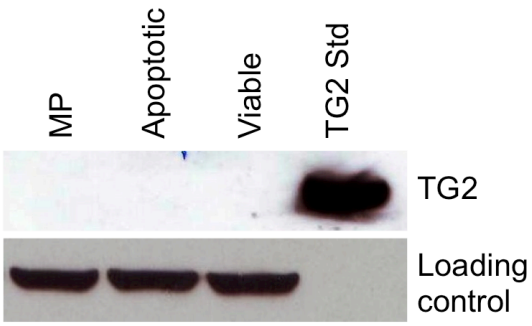


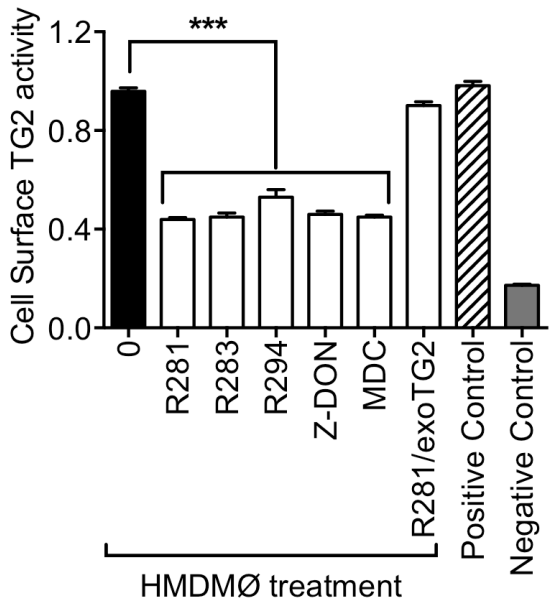
Supplementary Figure 3. TG2 inhibitors exert no toxic effect on THP-1/MØ.

(A) THP-1/DS were treated with or without cell-impermeable TG2 inhibitor R281 at 500μM final concentration for 1hr. Following washing, cells were fixed with 1% w/v formaldehyde, stained with DAPI and imaged using fluorescence microscopy. (B) THP-1-derived MØ cells were treated with TG2 inhibitors (R281, R294, R283 or Z-DON) for 1h and subsequently incubated with XTT reagent for 4h, as per the manufacturer's instructions. Colour development, a measure of cell viability, was read at 490nm. MØ treated with H_2O_2 was used as positive control. Data shown is mean ±S.E. for n≥3 independent replicates. Statistical analysis was conducted using ANOVA followed by Bonferroni post-test (*** P<0.001).



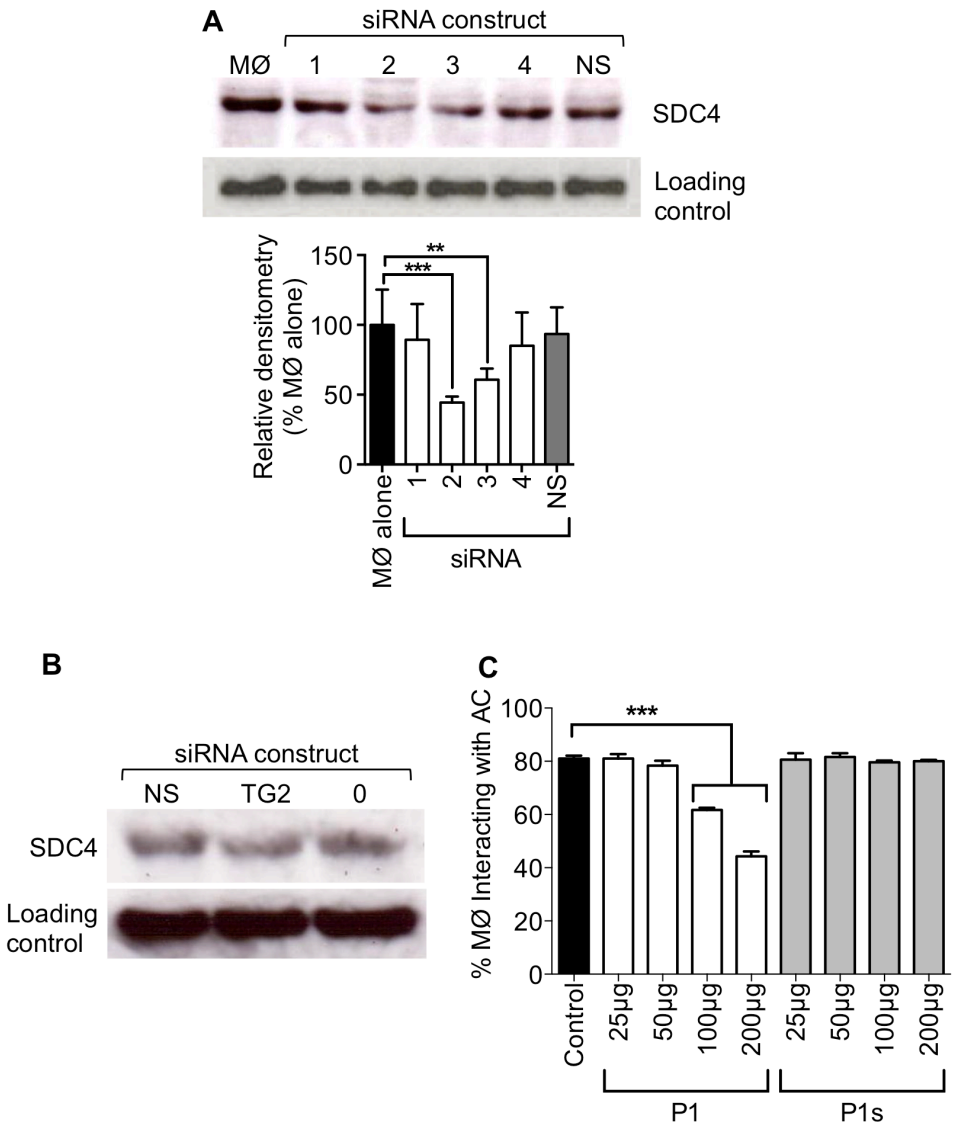
Supplementary Figure 4. TG2 expression in Mutu B cells.

Mutu B cells (viable, UV-induced apoptotic cells or apoptotic cell-derived microparticles 'MP') were prepared for western blot analysis. The anti-TG2 immunoblot is shown and reveals no detectable TG2 expression in viable B cells, apoptotic B cells, or apoptotic B cell-derived microparticles. Pure TG2 (TG2 Std; gplTG) is included as a positive control. Stripped membranes were re-probed with anti-β-actin antibody to confirm equal loading.



Supplementary Figure 5. TG2 inhibitors reduce cell surface TG2 activity in HMDMØ.

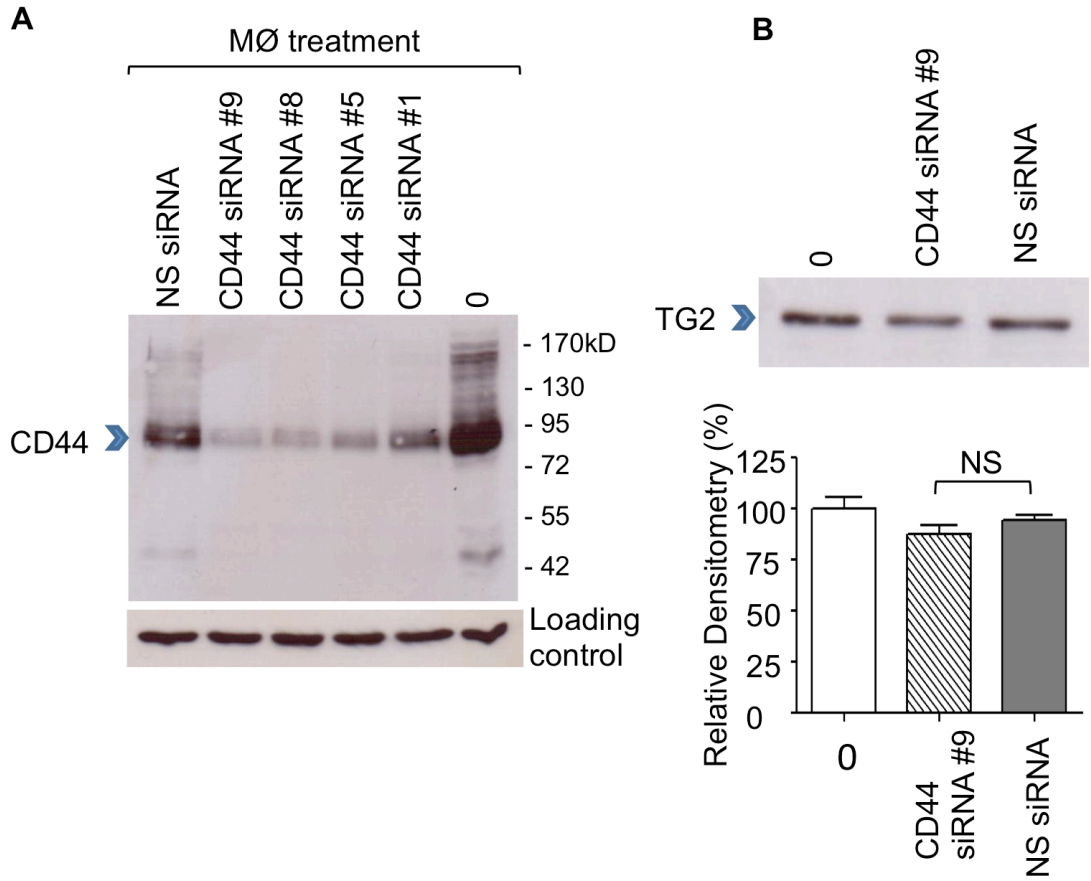
Cell surface TG activity of primary human monocyte-derived MØ (HMDMØ) was tested, via biotin-cadaverine incorporation into fibronectin as described in the Methods section, in the presence of the indicated TG2 inhibitors. Cell surface TG activity is reduced in the presence of irreversible and amine competitive TG2 inhibitors. Addition of 100ng exogenous TG2 (exo-TG2) in the presence of 10mM Ca^{2+} can rescue the inhibition. The positive control was guinea pig liver TG. The negative control was 10mM EDTA to chelate Ca^{2+} and inactivate TG2. Data shown is mean ±S.E. for n≥3 independent replicates. Statistical analysis was conducted using ANOVA followed by Bonferroni post-test (*** P<0.001).



Supplementary figure 6.

(A) Western blot analysis revealing syndecan-4 expression in MØ (THP-1/DS) following transfection, as described in the methods, with human syndecan-4-specific siRNA constructs and a non-specific siRNA control (NS). Stripped membranes were re-probed with mouse anti-β-actin antibody to ensure equal loading. A representative blot is shown along with densitometric analysis of SDC4 expression (relative to the loading control) from 3 independent replicate knockdown experiments. (B) MØ (THP-1/DS) cells transfected with TG2-specific siRNA and a non-specific siRNA control (NS) to knockdown TG2 were analysed by western blotting for syndecan-4 expression. Stripped membranes were re-probed with mouse anti-β-actin antibody to ensure equal loading (C) MØ (THP-1/DS) were treated with the P1 peptide or its scrambled counterpart (P1s) for 1h and co-cultured with AC and inhibitor for a further

1h. Dose-dependent effects of P1 peptide and P1 scrambled (P1s) control peptide on MØ-AC interaction are shown. Data shown is mean ±S.E. for n≥3 independent replicates. Statistical analysis was conducted using ANOVA followed by Bonferroni post-test (*** *P*<0.001).



Supplementary figure 7.

(A) Western blot analysis revealing CD44 expression (detected with mouse anto-CD44) in MØ (THP-1/DS) following transfection with a panel of human CD44-specific siRNA constructs or a non-specific siRNA control (NS siRNA). Stripped membranes were re-probed with mouse anti-β-actin antibody to ensure equal loading. (B) MØ (THP-1/DS) cells transfected with CD44-specific siRNA (construct #9) to knockdown CD44, were analysed by western blotting for cell surface TG2 expression. A representative blot is shown along with the relative densitometric values of the band intensities from independent replicates. Data shown are mean ±S.E. for n≥3 independent replicates. Statistical analysis was conducted using ANOVA followed by Bonferroni post-test (NS: Not significant).

Supplementary Table 1. List of Antibodies

Primary Antibodies			
Antigen	**Host Species**	**Clone**	**Company**
TG2 (CUB 7402)	Mouse	Monoclonal	Neomarks, Pierce, UK
TG2 (TG100)	Mouse	Monoclonal	Neomarks, Pierce, UK
TG2	Rabbit	Polyclonal	Neomarks, Pierce, UK
Isotype control IgG1/κ (MOPC21)	Mouse	Monoclonal	Sigma-Aldrich, UK
Syndecan-4	Mouse	Monoclonal	Santa Cruz Biotechnology, UK
Syndecan-4	Rabbit	Polyclonal	Invitrogen, UK
CD44	Mouse	Monoclonal	Cell Signalling, UK
α-Tubulin	Mouse	Monoclonal	Sigma-Aldrich, UK
β-Actin	Rabbit	Polyclonal	Abcam, UK
Secondary Antibodies			
	Host Species		**Company**
Goat anti-mouse	FITC		Sigma-Aldrich, UK
Goat anti-mouse	PE		Sigma-Aldrich, UK
Goat anti-mouse	HRP		Sigma-Aldrich, UK
Goat anti-rabbit	FITC		Sigma-Aldrich, UK
Goat anti-rabbit	PE		Sigma-Aldrich, UK
Goat anti-rabbit	HRP		Sigma-Aldrich, UK

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