Increased severity of respiratory infections associated with elevated anti-LPS IgG2 which inhibits serum bactericidal killing

Timothy J. Wells,[1,2] Deborah Whitters,[3,4] Yanina R. Sevastsyanovich,[1,2] Jennifer N. Heath,[1,2] John Pravin,[1,2] Margaret Goodall,[2] Douglas F. Browning,[1,2] Matthew K. O'Shea,[5] Amy Cranston,[6] Anthony De Soyza,[7] Adam F. Cunningham,[1,2] Calman A. MacLennan,[1,2] Ian R. Henderson,[1,2] and Robert A. Stockley[4]

[1]Institute of Microbiology and Infection, [2]School of Immunity and Infection, [3]School Clinical and Experimental Medicine, University of Birmingham, Birmingham B15 2TT, England, UK
[4]Lung Investigation Unit, Queen Elizabeth Hospital, Birmingham B15 2TH, England, UK
[5]The University of Oxford, The Jenner Institute, Oxford OX3 7DQ, England, UK
[6]Sir William Leech Centre for Respiratory Research Newcastle upon Tyne Hospitals Trust, Newcastle NE7 7DN, England, UK
[7]Institute of Cellular Medicine, Newcastle University and Adult Bronchiectasis service Freeman Hospital, Newcastle NE7 7DN, England, UK

Although specific antibody induced by pathogens or vaccines is a key component of protection against infectious threats, some viruses, such as dengue, induce antibody that enhances the development of infection. In contrast, antibody-dependent enhancement of bacterial infection is largely unrecognized. Here, we demonstrate that in a significant portion of patients with bronchiectasis and *Pseudomonas aeruginosa* lung infection, antibody can protect the bacterium from complement-mediated killing. Strains that resist antibody-induced, complement-mediated killing produce lipopolysaccharide containing O-antigen. The inhibition of antibody-mediated killing is caused by excess production of O-antigen–specific IgG2 antibodies. Depletion of IgG2 to O-antigen restores the ability of sera to kill strains with long-chain O-antigen. Patients with impaired serum-mediated killing of *P. aeruginosa* by IgG2 have poorer respiratory function than infected patients who do not produce inhibitory antibody. We suggest that excessive binding of IgG2 to O-antigen shields the bacterium from other antibodies that can induce complement-mediated killing of bacteria. As there is significant sharing of O-antigen structure between different Gram-negative bacteria, this IgG2-mediated impairment of killing may operate in other Gram-negative infections. These findings have marked implications for our understanding of protection generated by natural infection and for the design of vaccines, which should avoid inducing such blocking antibodies.

CORRESPONDENCE
Ian R. Henderson:
I.R.Henderson@bham.ac.uk

Abbreviations used: CF, cystic fibrosis; FEV1, forced expiratory volume in one second; HCS, healthy control serum; MAC, membrane attack complex.

Non-cystic fibrosis (non-CF) bronchiectasis is a pathological condition characterized by inflamed, dilated, and thick-walled bronchi. Conditions predisposing to bronchiectasis include host immune defects, post-infective sequelae, and defects in mucociliary clearance (Pasteur et al., 2000), although in most cases no cause can be found. Bronchiectasis is notable for chronic sputum production, recurrent lower respiratory tract infections, and persistent bacterial colonization. Such patients frequently undergo a vicious cycle of events: failure to clear bacterial infections followed by inflammatory responses that further impair host defenses and mucociliary clearance, resulting in chronic inflammation that in turn leads to persistent bacterial colonization (Whitters and Stockley, 2012). *Pseudomonas aeruginosa* is isolated in up to 30% of adult patients

T.J. Wells, D. Whitters, I.R. Henderson, and R.A. Stockley contributed equally to this paper.
C.A. MacLennan's present address is Novartis Vaccines Institute for Global Health, 53100 Siena, Italy.

The Rockefeller University Press $30.00
J. Exp. Med. 2014 Vol. 211 No. 9 1893-1904
www.jem.org/cgi/doi/10.1084/jem.20132444

Table 1. Disease severity of bronchiectasis patients, with or without IgG2 blocking factor

Patient ID	Colonized with *P. aeruginosa?*	Serum ID	Does serum mixed with HCS kill B1	FEV1% predicted
Impaired serum killing, *P. aeruginosa* colonized				
P1	Yes- B1	S1	No	28
P2	Yes- B2	S2	No	66
P3	Yes- B3	S3	No	26
PN1	Yes- BN1	SN1	No	17
PN2	Yes- BN2	SN2	No	37
PN3	Yes- BN3	SN3	No	31
Normal killing, *P. aeruginosa* colonized				
P4	Yes- B4	S4	Yes	105
P5	Yes- B5	S5	Yes	78
P6	Yes- B6	S6	Yes	97
P7	Yes- B7	S7	Yes	43
P8	Yes- B8	S8	Yes	59
P9	Yes- B9	S9	Yes	64
P10	Yes- B10	S10	Yes	90
P11	Yes- B11	S11	Yes	68
PN4	Yes- BN4	SN4	Yes	61
PN5	Yes- BN5	SN5	Yes	52
PN6	Yes- BN6	SN6	Yes	67
PN7	Yes- BN7	SN7	Yes	85
PN8	Yes- BN8	SN8	Yes	42
PN9	Yes- BN9	SN9	Yes	93
PN10	Yes- BN10	SN10	Yes	65
PN11	Yes- BN11	SN11	Yes	54
PN12	Yes- BN12	SN12	Yes	43
PN13	Yes- BN13	SN13	Yes	48
PN14	Yes- BN14	SN14	Yes	75
PN15	Yes- BN15	SN15	Yes	101
PN16	Yes- BN16	SN16	Yes	58
PN17	Yes- BN17	SN17	Yes	32
Normal killing, Not colonized by *P. aeruginosa*				
PN18	No	SN18	Yes	46
PN19	No	SN19	Yes	70
PN20	No	SN20	Yes	35
PN21	No	SN21	Yes	46
PN22	No	SN22	Yes	52
PN23	No	SN23	Yes	78
PN24	No	SN24	Yes	22
PN25	No	SN25	Yes	54
PN26	No	SN26	Yes	63
PN27	No	SN27	Yes	69
PN28	No	SN28	Yes	62
PN29	No	SN29	Yes	23
PN30	No	SN30	Yes	58
Patients with cystic fibrosis and *P. aeruginosa*				
CF1	Yes	SCF1	Yes	ND[a]
CF2	Yes	SCF2	No	ND
CF3	Yes	SCF3	Yes	ND
CF4	Yes	SCF4	Yes	ND
CF5	Yes	SCF5	Yes	ND
CF6	Yes	SCF6	No	ND
CF7	Yes	SCF7	Yes	ND
CF8	Yes	SCF8	Yes	ND

[a]Not determined.

with bronchiectasis (Pasteur et al., 2010) and is a risk factor for declining lung function (Martínez-García et al., 2007); it is also associated with reduced quality of life and a poorer prognosis (Wilson et al., 1997; Martínez-García et al., 2005; Bilton, 2008). Once established, it is difficult to eradicate and is often resistant to numerous antibiotics, making routine management less effective. Therefore, understanding both the infecting bacterium and the response to the infection is vital to combat this disease.

Examination of the literature reveals a single report of a patient with bronchiectasis with impaired serum-killing of *P. aeruginosa* who died despite treatment (Waisbren and Brown, 1966). We hypothesized that similar impaired serum-killing also exists for other bronchiectasis patients with chronic *Pseudomonas* infections and that this contributes to disease severity. Indeed, IgG antibody to *P. aeruginosa* and complement components are readily detectable in the serum and sputum of patients with bronchiectasis (Hill et al., 1998), and these factors are known to opsonize colonizing *P. aeruginosa* in the lung (Hann and Holsclaw, 1976; Hill et al., 1998). Thus, it is highly likely that antibody-mediated killing is involved in the host defense against bacterial lung infection. Here, we sought to establish if impaired serum killing is a common phenomenon in patients with bronchiectasis and to elucidate the mechanism underpinning a lack of serum bactericidal activity. In addition, we sought to identify if the lack of serum bactericidal activity in patients had a correlation with disease severity.

RESULTS

Impaired serum killing in bronchiectasis patients

Historical data associated impaired serum-killing of *P. aeruginosa* with poor outcome in a patient with bronchiectasis (Waisbren and Brown, 1966). To explore if this is an isolated event or a more general phenomenon, we examined the serum sensitivity of *P. aeruginosa* isolates taken from 11 different patients with bronchiectasis and chronic *Pseudomonas* infection (Table 1). Serum was collected from each patient and 20 healthy individuals. Each patient (P) and their isolated bacterium (B) and serum (S) were assigned the same number; patient P1, with serum S1, is colonized by *P. aeruginosa* B1. We found that eight patients had serum (S4–11) that could kill their cognate colonizing strain (B4-11), but three patients had serum (S1–3)

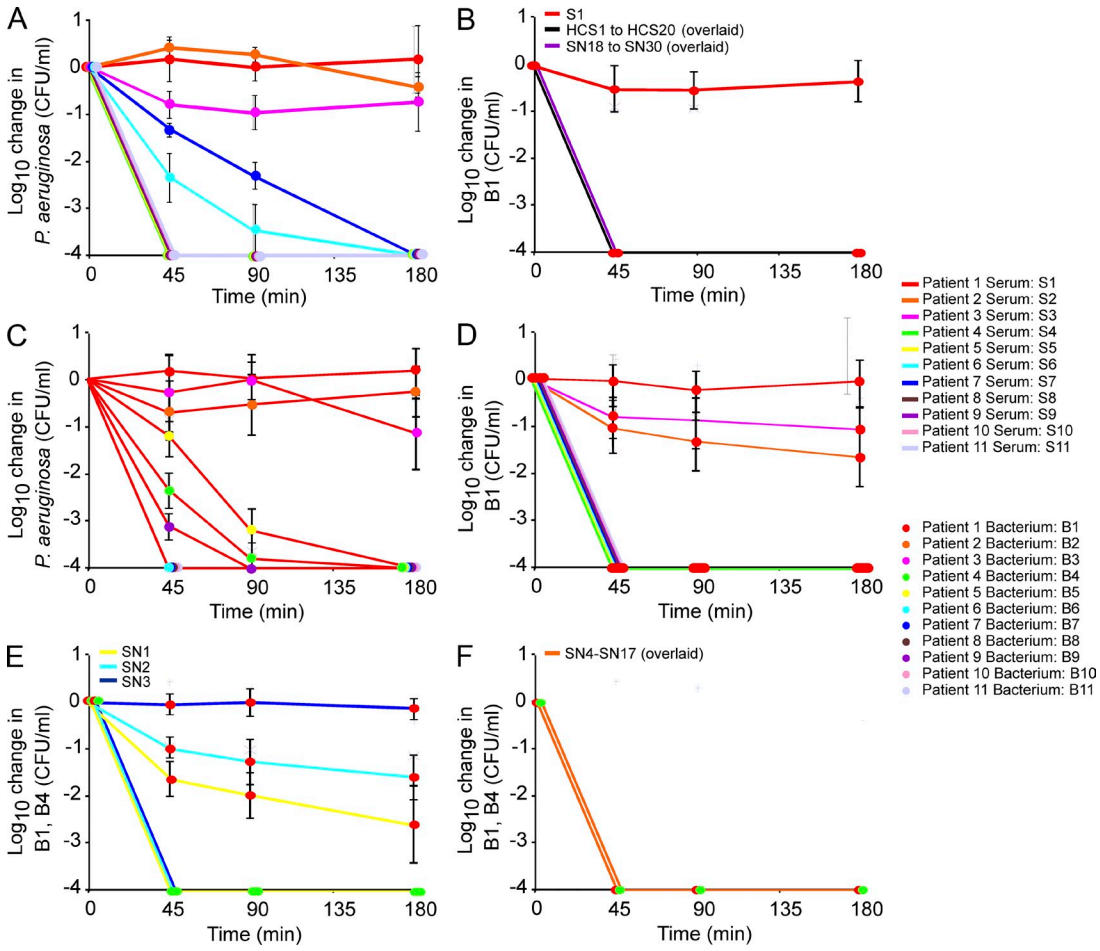

Figure 1. Identification of patients with impaired serum killing. (A) Killing curves of *P. aeruginosa* strains isolated from bronchiectasis patients with their autologous serum at 45, 90, and 180 min. Negative values correspond with a decrease in viable *P. aeruginosa* compared with initial concentration. (B) Killing of B1 by sera taken from 20 healthy people at 45, 90, and 180 min. Killing of B1 by sera from patients with bronchiectasis but without *P. aeruginosa* colonization (SN18–SN30) is also shown. The curves depicting killing by HCS1-HCS20 and SN18-SN30 are overlaid to simplify. (C) Killing curves of all strains (B1–B11) by serum (S1). (D) Killing curves of *P. aeruginosa* strain B1 by patient serum (S1–S11). (E) Killing curves of B1 and B4 by sera SN1, 2, and 3 (SN1–3). (F) Killing curves of B1 and B4 by sera SN4–SN17. The curves depicting killing by SN4–SN17 are overlaid to simplify graphs. For all serum bactericidal assays, error bars represent the mean ± SD for a minimum of three independent experiments.

that failed to kill their infecting strains (B1–3; Fig. 1 A). The bactericidal activity of the eight sera (S4–11) was inactivated by heat treatment, implying that serum killing was caused by the action of complement (unpublished data). The strains from patients with impaired bacterial killing were not innately resistant to killing, as sera from 20 healthy human controls (HCS) and sera from patients with bronchiectasis but without *P. aeruginosa* colonization (SN18–30) killed these three strains within 45 min (Fig. 1 B). Similar results were found for B2 and B3 (unpublished data).

Next, we tested each patient's serum against all 11 of the *P. aeruginosa* isolates. We found that S1–3 could not kill B1–3 but could kill the *P. aeruginosa* strains from the other 8 patients (Fig. 1 C). In contrast, S4–11 could kill B1–3 (Fig. 1 D). This suggests the factors mediating resistance to serum killing are common to B1–3 and S1–3 but absent from the other strains and sera. We extended this analysis by testing sera (SN1–17) isolated from patients with bronchiectasis from a geographically distinct cohort who were colonized with *P. aeruginosa* (Table 1). Three sera (SN1–3) failed to kill strain B1 but could kill strain B4, whereas the remainder (SN4–17) could affect serum-mediated killing of both B1 and B4 (Table 1 and Fig. 1, E and F). A similar phenomenon was observed for a small sample of patients with cystic fibrosis (Table 1). Thus, ∼20% of the patients with bronchiectasis and *P. aeruginosa* infection had impaired serum killing of their strains, and the factor involved appeared to be specific both to the patient sera and the infecting *P. aeruginosa* strain.

Impaired serum contains a blocking factor

We next explored whether the impaired serum killing results from an inhibitory factor present within the serum or from the lack of a serum component required for bactericidal activity. Specific anti–*P. aeruginosa* IgG, IgA, and IgM were present

in the sera with impaired capacity to kill, at levels comparable to or greater than those in HCS that killed all the bacterial isolates (Fig. 2 A). Furthermore, IgG and complement components C1q, C3, and the C5b-9 membrane attack complex (MAC) were deposited on all strains (Fig. 2, A and B). Antibody binding and complement deposition were confirmed by immunofluorescence microscopy (unpublished data). Thus, the impaired serum killing is not due to a lack of complement or antibody binding.

To determine if the lack of bacterial killing was due to a blocking factor in the serum, we mixed serum with impaired killing with HCS. Addition of HCS to S1–3 (50:50) did not restore serum killing, whereas HCS similarly diluted with PBS readily killed *P. aeruginosa* (Fig. 2 C). These data suggest that impaired serum-killing by S1–3 is caused by the presence of a factor inhibiting serum-mediated killing. In fact, complete killing by S1–3 was only restored when HCS represented 94, 70, and 80%, respectively, of the mixed sera (Fig. 2 D), indicating the patients serum had a potent capacity to inhibit killing.

IgG blocks the ability for serum to kill specific *Pseudomonas* strains

We established that the impaired serum killing of patients' cognate *Pseudomonas* strains is due to a blocking factor in their serum. To identify the inhibitor, S1 was fractionated, based on molecular weight, and fractions were added to HCS. Inhibition was observed when the 100–300-kD fraction was added to HCS (Fig. 3 A). As this fraction contains IgG antibody, we investigated whether depleting antibody could restore bactericidal activity. Antibody was depleted by passing S1 over either a Protein A or a Protein G column, reducing total IgG titers ∼100-fold (Fig. 3 B). We found that the inhibitory serum S1, when depleted of antibody using either a Protein A or G column, could kill B1 within 180 min. Moreover, when mixed with HCS (50:50), B1 was rapidly killed indicating that the antibody-depleted serum no longer had the capacity to inhibit the bactericidal activity of HCS (Fig. 3 C). Importantly, antibodies eluted from the Protein A and G columns, in a volume equal to that of S1 originally applied to the column, inhibited the bactericidal activity of HCS, even when added at low concentrations (4–6%; Fig. 3 D). Similar observations were made for S2 and S3 (unpublished data). Due to the different antibody binding affinities of Protein A and G (Table S1), these findings suggest IgG is the blocking factor present in S1–3.

IgG2 is the inhibitory factor in impaired serum

All of the initial cohort of 11 patients had normal proportions of the four IgG subclasses overall (Table 2); however, to determine

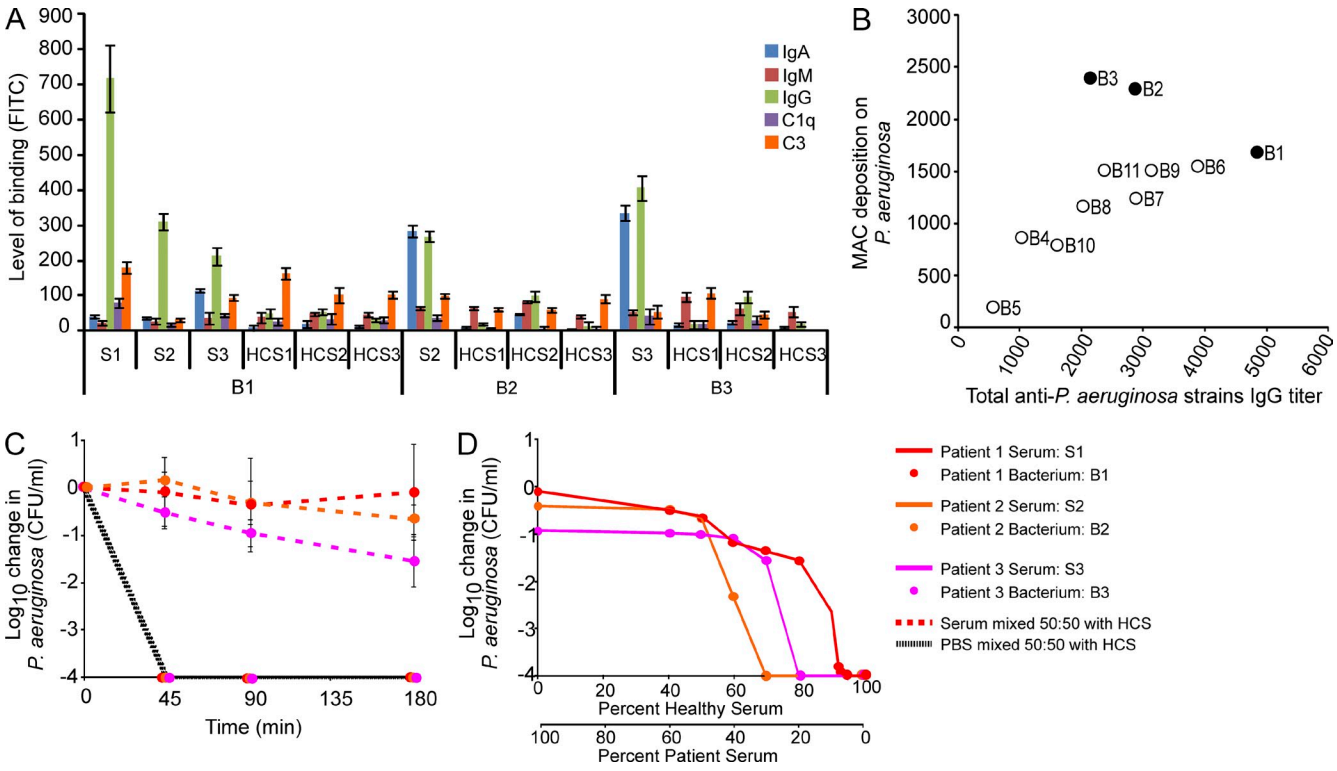

Figure 2. Impaired serum is caused by a blocking factor, not a lack of antibody or complement. (A) Binding of specific IgG, IgM, IgA antibodies and C1q and C3 complement factors from indicated patient serum to B1, B2, and B3. Each strain was tested with autologous serum and at least three separate healthy controls. Data are representative of three independent experiments. (B) Serum titers of *P. aeruginosa*–specific IgG compared with C5b-9 MAC deposition on autologous strains. (C) Inhibition of HCS-mediated killing of strains B1–3 with a 50:50 mix of HCS and autologous patient sera (S1–3). Dashed lines represent HCS mixed 50:50 with buffer. (D) Killing of *P. aeruginosa* strains B1–3 at 180 min by mixed sera consisting of different percentages of HCS mixed with autologous serum. For all data, error bars represent the mean ± SD for a minimum of three independent experiments.

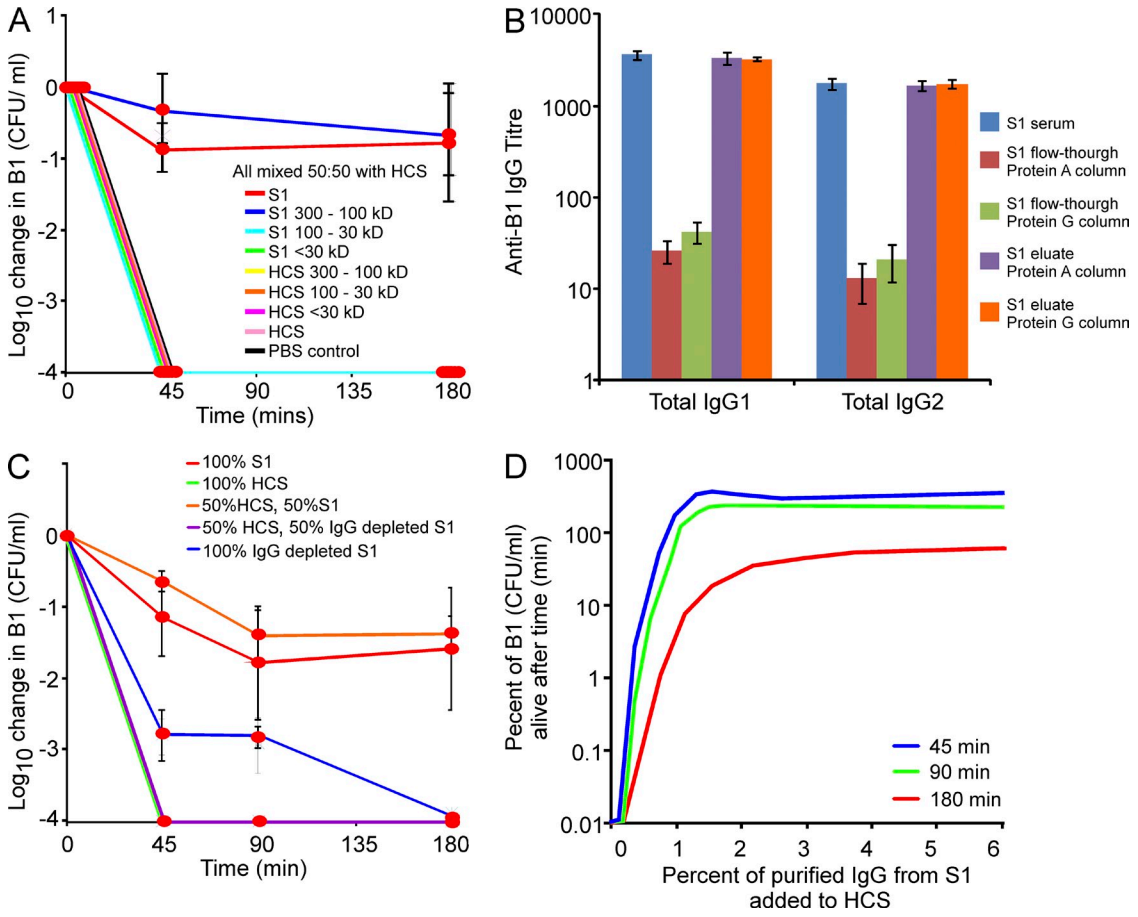

Figure 3. IgG inhibits serum-mediated killing. (A) Killing of B1 by HCS mixed 50:50 with S1 and HCS fractionated into indicated size ranges. The killing curves for several fractions overlap and have been separated to allow for visualization. Data are representative of three independent experiments. (B) Titer of total IgG1 and IgG2 of S1 serum before and after passing through Protein A and G columns. Data are representative of two independent experiments. (C) Killing curves of *P. aeruginosa* strain B1 by serum S1. Killing was measured for S1 depleted of antibody using a Protein G column, before and after addition of HCS (50:50). Data are representative of three independent experiments. (D) IgG purified from S1 using a Protein G column was resuspended in PBS to the same volume of serum loaded on the column. Purified IgG was added to HCS in indicated concentrations and measured for its ability to kill B1 at 45, 90, and 180 min. Data are representative of three independent experiments. Error bars represent the mean ± SD.

if a specific IgG isotype could be responsible for the impaired killing of bacteria by serum seen in 3 patients, the titer of each IgG sub-class specific for *P. aeruginosa* was determined. Anti–*P. aeruginosa* IgG1 titers were not statistically different between impaired and normal killing sera groups (Fig. 4 A), and neither were levels of IgG3 and IgG4 (not depicted). In contrast, S1–3 had significantly ($P < 0.001$) increased titers of anti–*P. aeruginosa* IgG2 compared with sera displaying normal bactericidal activity (Fig. 4 A). To test if IgG2 is the inhibitory factor, we purified IgG2 from S1 by passing the serum over an affinity column coated with a monoclonal antibody against human IgG2 (Jefferis et al., 1992). The IgG2-depleted flow-through lost its inhibitory capacity. In contradistinction, IgG2 eluted from the column, in the same volume of serum loaded on the column, blocked the serum bactericidal activity of HCS (Fig. 4 B).

LPS is the target of the inhibitory IgG2

To determine if the inhibitory IgG2 antibody targeted a specific bacterial factor, we performed Western immunoblotting of outer membrane protein and polysaccharide fractions with patient serum and anti–human IgG. S1 contained antibodies that recognized proteins from all strains (Fig. 5 A). In contrast, the serum only recognized the O-antigen side chains of LPS from B1–3 and did not recognize O-antigen of strains from patients without impaired serum killing (Fig. 5 B). Similar results were obtained for S2 and S3, whereas HCS had no detectable anti–O-antigen antibody to B1–3 (Fig. 5 C). SDS-PAGE and silver staining of LPS fractions revealed that the strains B1, B2, and B3 produced significant amounts of long-chain O-antigen but the other strains did not (Fig. 5 D).

By binding the LPS isolated from B1 to an ELISA plate we determined that the patients with impaired serum-mediated

Table 2. Total antibody titers of bronchiectasis patients

Patient	IgG	IgA	IgM
P1	10.73	2.2	1.07
P2	12.66	2.17	0.82
P3	10.69	4.49	1.05
P4	11.81	4.06	1.29
P5	11.18	1.51	4.19
P6	11.91	3.4	1.64
P7	16.64	10.37[a]	0.73
P8	15.6	3	3.08
P9	14.5	3.59	1.15
P10	13.89	2.78	0.99
P11	12.5	1.87	0.77
Normal range	6.0–16.00	0.8–4.0	0.50–2.00

[a]No paraprotein detected by IMFIX.

bacterial killing had high levels of anti-LPS IgG by ELISA (Fig. 5 E). To test if the level of anti-LPS antibody is responsible for this effect, rather than simply the presence of anti-LPS antibodies per se, we purified anti-LPS antibodies from S4, a serum that has normal bactericidal activity. The eluted antibodies, concentrated 10-fold on the column (Fig. 5 F), inhibited the bactericidal activity of HCS in a dose-dependent manner (Fig. 5 G), indicating the titer of anti-LPS antibody in serum is critical for inhibition.

Antibodies against O-antigen, but not lipid A or core, inhibit serum killing

The three strains that could not be killed by serum containing blocking IgG2 possessed high amounts of O-antigen. These observations suggest the long-chain O-antigen of LPS is the target of inhibitory antibody. To test this, LPS purified from B1 was immobilized on a polymyxin-B agarose column and S1 was passed through the column to remove antibody specific to the LPS (Fig. 6 A). The recovered flow-through fraction was now able to kill B1 and no longer inhibited the killing activity of HCS (Fig. 6 B). Conversely, anti-LPS antibody eluted from the column inhibited the bactericidal activity of HCS in a dose-dependent manner (Fig. 6, A and C). Immunofluorescence microscopy revealed that the flow-through fraction lacked detectable anti–*P. aeruginosa* IgG2 (unpublished data). In contrast, anti–*P. aeruginosa* IgG1 remained detectable in the serum depleted of anti-LPS antibody (Fig. 6 A).

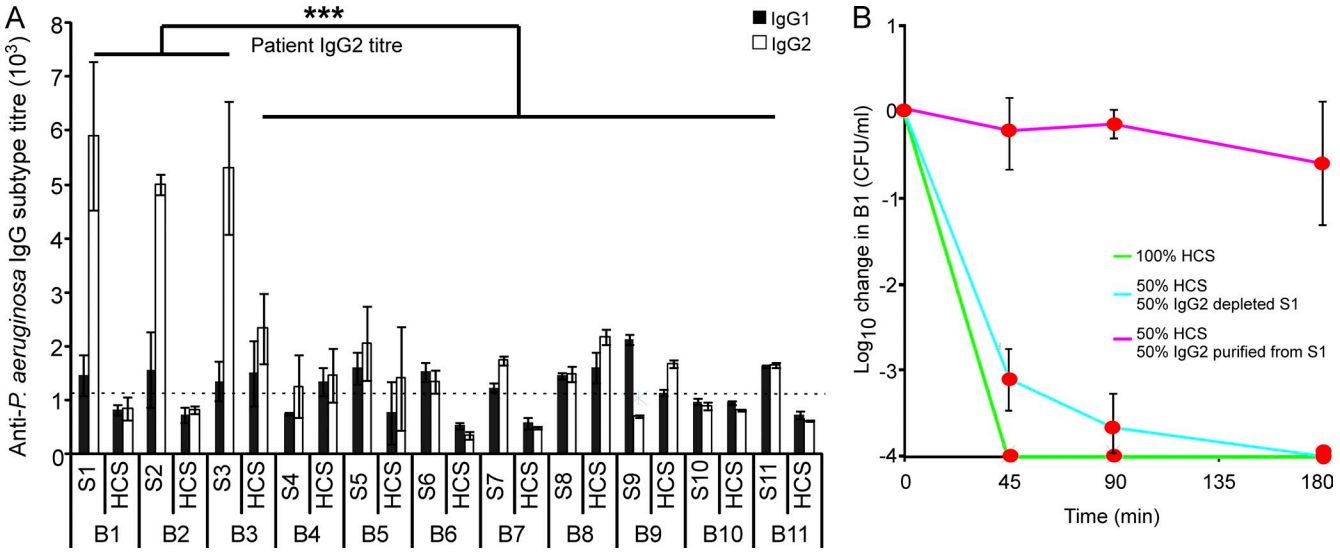

Figure 4. IgG2 inhibits serum-mediated killing. (A) Titers of IgG1 and IgG2 isotypes in patient serum and HCS that is specific to infecting strains. Dashed line indicates the median IgG2 titer from sera S4–S11. Data are representative of three independent experiments. ***, $P < 0.001$. (B) Killing of B1 was measured with S1 depleted of IgG2 mixed with HCS or with a mixture HCS supplemented with IgG2 purified from S1 or HCS alone. For all data, error bars represent the mean ± SD for a minimum of three independent experiments.

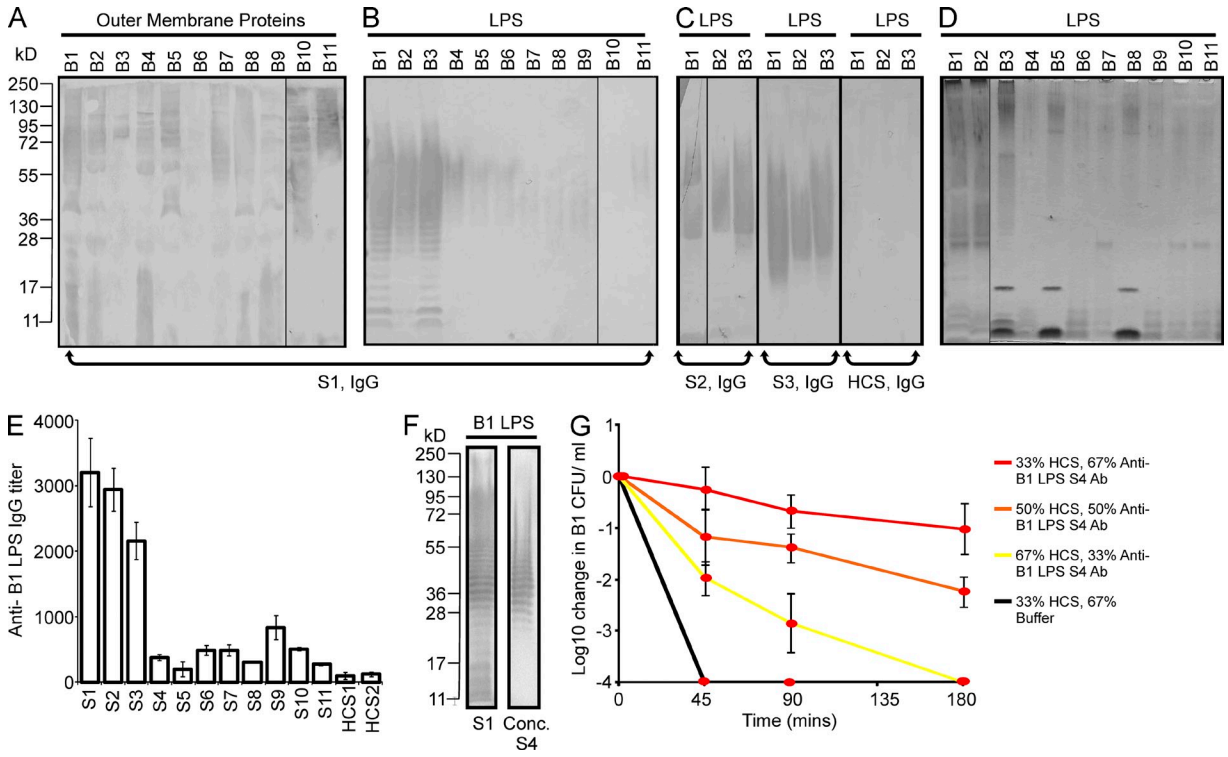

Figure 5. Inhibitory antibody recognizes *P. aeruginosa* LPS and is dose dependent. (A) Western blot of outer membrane protein fractions obtained from B1–B11 probed with S1 as a primary antibody and anti-human IgG as the secondary antibody. (B) Polysaccharide-only preparations from *P. aeruginosa* isolates analyzed by Western blot probed with S1 serum as a primary antibody and anti-IgG as a secondary antibody. (C) Western blot of LPS purified from B1, B2, and B3 probed with S2, S3, or HCS as the primary antibody and anti-human IgG as the secondary antibody. S2 and S3 have high LPS-specific IgG response. In contrast, no anti-LPS IgG is detected in HCS. All Western blots (A–C) are representative of three independent experiments. (D) Polysaccharide-only preparations from *P. aeruginosa* isolates are analyzed by silver stain. Data are representative of six independent experiments. (E) Patient serum IgG titer specific for LPS isolated from B1 determined by ELISA. LPS was isolated from *P. aeruginosa* strain B1 and attached to a 96-well plate. ELISA was performed with dilutions of patient or healthy sera and anti-human IgG conjugated to alkaline phosphatase. Error bars represent mean ± SD for three independent experiments. (F) Antibodies specific for B1 LPS were purified and concentrated from S4. S1 and anti-LPS antibodies concentrated from S4 were used as primary antibodies in a Western blot against B1 LPS. Data represents three independent experiments. (G) Killing curve of B1 with anti-LPS antibodies concentrated from S4 mixed with HCS. HCS serum similarly diluted with buffer is used as a control. Error bars represent mean ± SD for three independent experiments.

All *P. aeruginosa* strains contain lipid A and core oligosaccharide of LPS. Consequently, we depleted S1 of antibody to lipid A and the core elements by passing S1 over a polymyxin B column on which LPS isolated from B4, which lacks O-antigen, was immobilized (Fig. 6 A). The flow-through antibody had a 30-fold lower level of binding to lipid A and core oligosaccharide compared with both the native serum and the antibody eluted from the column (Fig. 6 A). However, the flow through from this column still recognized O-antigen–containing LPS purified from B1 at a level similar to native S1 (Fig. 6 A). This flow-through inhibited the killing of B1 by HCS, but the antibody recognizing lipid A and core oligosaccharide eluted from the column did not (Fig. 6 D). These data are summarized in Fig. S1.

The role of inhibitory antibodies in the lung

The role of serum-mediated killing in controlling bacterial growth during lung infection is not widely recognized. However, previously high levels of antibody were shown to be present in the lungs of patients suffering from bronchiectasis (Hill et al., 1998). We hypothesized that patients with impaired immunity and *P. aeruginosa* colonization would have high titers of IgG2 present in the lung. To confirm this, the sol phase of sputum from P2 (impaired killing) and P4 (normal killing) was harvested and the levels of IgG1 and IgG2 were measured. P2 sol phase sputum had 512 and 220 mg/liter IgG1 and IgG2, respectively, whereas P4 sol phase contained 315 mg/liter IgG1 and 158 mg/liter IgG2.

Having demonstrated the presence of antibody in the lung, we next sought to determine whether this antibody played a role in protecting bacteria from serum-mediated killing in vivo. To establish this, we first investigated whether *P. aeruginosa* was opsonized by antibody in vivo. Immunofluorescence microscopy of sputum smears revealed bacteria present within the sputum were labeled with anti–human IgG2-FITC (Fig. 7 A). Additional investigations revealed cultured bacteria could also be labeled with IgG2 when opsonized with 1:200 sol phase sputum (Fig. 7 A). To confirm that this opsonization protects

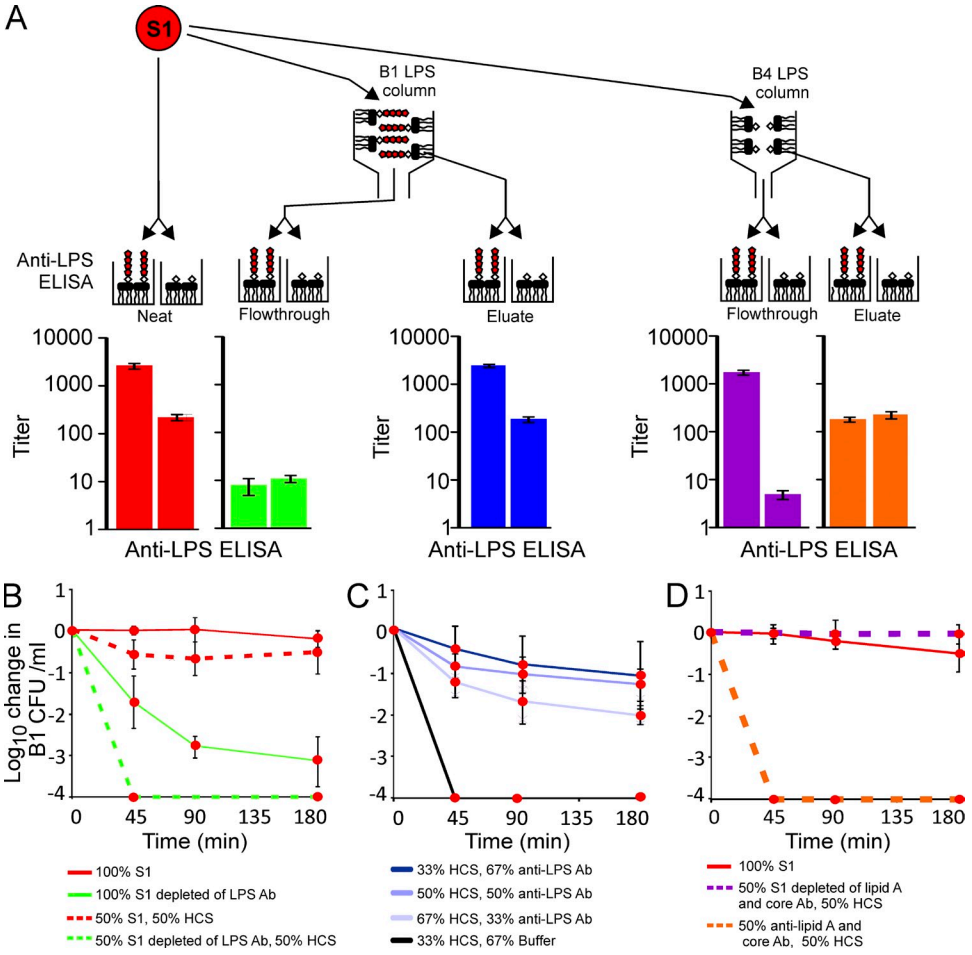

Figure 6. Inhibitory antibodies are specific for the O-antigen. (A) Schematic of treatment of S1 serum and resultant IgG titers specific to LPS purified from either B1 (with O-antigen) or B4 (without O-antigen). S1 titers against the two LPS extracts are measured after no treatment, passage through, or elution from a column containing purified B1 LPS or passage through or elution from a column containing B4 LPS. In each case, all column fractions were resuspended in PBS to the same volume of serum added to the column. (B) Killing curve of *P. aeruginosa* B1 treated with S1 or S1:HCS (50:50) depleted of anti-LPS antibodies. (C) Killing curve of B1 after incubation with anti-LPS antibodies purified from S1 and mixed with HCS at different concentrations. (D) Killing curve of *P. aeruginosa* B1 treated with HCS mixed with either S1 depleted of antibodies to the lipid A and core oligosaccharides of LPS or antibodies that recognize lipid A and core oligosaccharide. For all data, error bars represent the mean ± SD of three independent experiments.

bacteria from complement-dependent killing, we explored whether HCS could kill bacteria from sputum. The unfiltered sol phase of sputum from P2, containing opsonized bacteria, was mixed 50:50 with HCS; however, there was no reduction in bacterial numbers over 180 min of incubation (Fig. 7 B). To confirm this phenomenon, B1 was incubated with a mixture of HCS and sterile sol-phase from P2. No complement-dependent killing was observed over 180 min. In contrast, B1 incubated with a mixture of HCS and sterile sol-phase from P4 was rapidly killed within 45 min (Fig. 7 B).

Opsonization is important for cell-mediated killing, which is known to play a vital protective role within the lung (Whitters and Stockley, 2012). We hypothesized that inhibitory antibodies may also play a role in cell-mediated killing. Thus, we investigated killing of B1 and B4 by washed peripheral blood cells. B1 opsonized with HCS was rapidly killed on incubation with peripheral blood cells. Similarly, opsonization of B4 with either HCS or S1 led to rapid killing of the bacteria. In contrast, B1 opsonized with S1 was not killed (Fig. 7 C).

These data suggest an important role for inhibitory antibody in protecting bacteria within the lung from immune-mediated clearance. However, it is accepted that *P. aeruginosa* resides in a biofilm within the lung. Therefore, we investigated the effect of serum on an established biofilm. B1 forms a thick biofilm in a 96-well plate over 24 h. Incubation of the B1 biofilm with HCS and S4 sera for 2 h drastically reduced the amount of biofilm. In contrast, S1 had no effect on the amount of biofilm over a similar period (Fig. 7 D).

Patients with inhibitory antibodies have worse lung function

The results of the aforementioned in vivo and in vitro studies suggest that the presence of inhibitory antibody may have clinical relevance. Thus, we sought to determine whether patients with bronchiectasis and inhibitory levels of anti-LPS IgG2

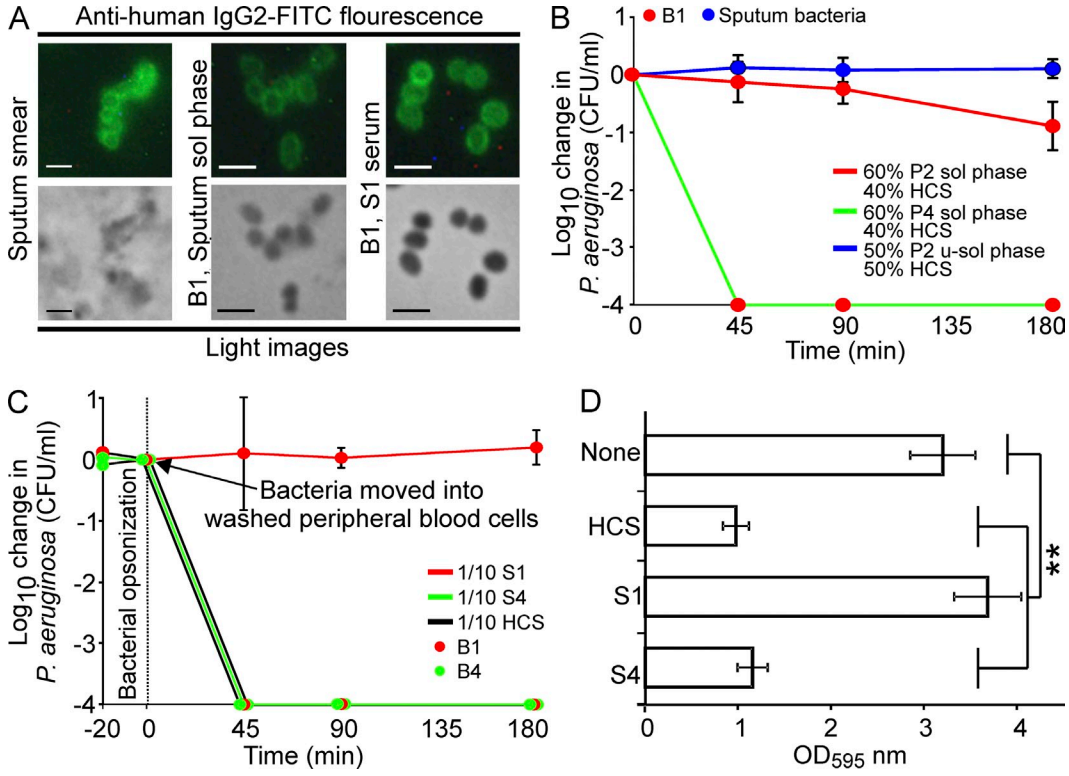

Figure 7. Significance of inhibitory antibodies in vivo. (A) Immunofluorescence labeling of bacteria present in sputum with anti-human IgG2-FITC (top left). Immunofluorescence labeling of cultured B1 bacteria with sol-phase sputum (top middle) or patient serum (top right) used as the source of primary antibody and anti-human IgG2-FITC. Bottom images are corresponding light images. Bar, 2 µm. (B) Killing curves for bacteria already present in P2 sputum after mixing the unfiltered sol phase with HCS 50:50. Killing curves for B1 with a 40:60 mix of HCS and sterile sol phase sputum isolated from either P2 or P4 are also shown. Error bars represent the mean ± SD of three independent experiments. (C) Killing curves for B1 or B4 by washed peripheral blood cells after 20-min opsonization with a 1/10 dilution of HCS, S1, or S4. Negative values correspond with a decrease in viable *P. aeruginosa* compared with initial concentration. Error bars represent the mean ± SD of three independent experiments. (D) Biofilm formation of B1 after overnight growth followed by no treatment or exposure to HCS, S1, or S4 serum for 2 h. Biofilm formation was examined in polystyrene microtiter plates. Error bars represent the mean ± SD of 16 independent experiments. **, $P < 0.01$.

antibody had more marked disease severity than those patients whose serum could mediate killing. We used forced expiratory volume in 1 s (FEV1) as a measure of lung function. Individuals colonized with *P. aeruginosa* who also possessed inhibitory antibody had poorer lung function when compared with individuals colonized with *P. aeruginosa* whose serum displayed normal killing ($P < 0.002$) and patients with bronchiectasis who were not colonized with *P. aeruginosa* ($P < 0.05$; Fig. 8 A and Table 1). This indicates the impaired capacity to kill bacteria has clinical consequences. Interestingly, a similar proportion of patients from two different cohorts displayed IgG2-mediated inhibition of serum killing, suggesting there may be an underlying genetic, rather than acquired, basis for an elevated response (Table 1).

DISCUSSION

Antibody is usually associated with protection against infectious disease. In contrast, antibody-dependent enhancement of infection is seen for some microbial organisms, most notably viruses such as dengue fever (Halstead and O'Rourke, 1977), but to a lesser extent parasitic organisms such as leishmaniasis (Halstead et al., 2010). In the case of dengue fever, circulating antibodies bind to the newly infecting virus but do not neutralize infection. Instead, these antibodies enhance viral entry via efficient interaction of the virus–antibody complex with Fc receptors (Halstead et al., 2010; Flipse et al., 2013). However, the action of antibody in exacerbating bacterial infectious disease is less well understood. Our results indicate that in patients with bronchiectasis, who are chronically colonized with *P. aeruginosa*, the presence of high titers of IgG2 antibodies specific for the O-antigen of LPS impairs serum-mediated killing of the infecting strain and is associated with a poorer lung function. Here, we describe antibody-dependent enhancement of bacterial infection and demonstrate the mechanism is different to that for dengue.

Lack of serum bactericidal activity against *P. aeruginosa* has previously been noted for patients with CF (Waisbren and Brown, 1966; Guttman and Waisbren, 1975). Moreover, increased anti-LPS antibody titers have been noted in CF patients chronically infected with *P. aeruginosa* (Fick et al., 1986). Separately, high levels of IgG3 and IgG2 specific for lipid A and O-antigen were shown to correlate with deteriorating pulmonary function

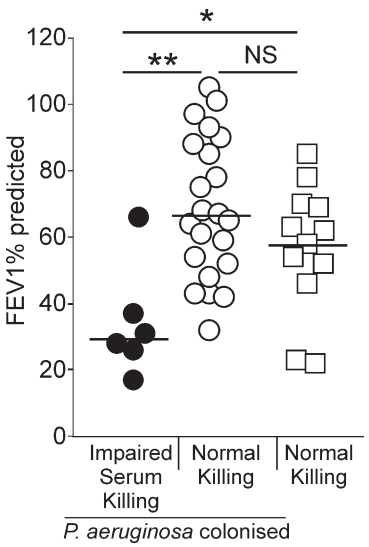

Figure 8. Inhibitory IgG2 antibody is associated with poor lung function. (A) Comparison of FEV1% predicted values for patients from two non-CF bronchiectasis cohorts that are colonized with *P. aeruginosa* and display inhibition of serum mediated killing (●), patients who are colonized with *P. aeruginosa* and display normal serum-mediated killing (○), or patients who are not colonized with *P. aeruginosa* (□). The horizontal bars represent the median for each group. FEV1% scores represent mean of three independent measurements. *, $P < 0.05$; **, $P < 0.01$.

(Kronborg et al., 1993). In contrast, our data demonstrate that in bronchiectasis patients, high titers of IgG2 specific for the O-antigen of LPS are sufficient to impair serum-mediated killing of *P. aeruginosa*. Importantly, high titers of IgG2 in the sputum are associated with phenotypes within the lung, including opsonization of infecting bacteria, inhibition of cell-mediated killing, and lack of biofilm clearance.

The biological properties of IgG2 may be a factor in its role as an inhibitor of serum- and/or cell-mediated killing. Switching to IgG2 is particularly associated with responses to bacterial polysaccharides (Siber et al., 1980) but, in contrast to IgG1 and IgG3, the C1q-binding sites on IgG2 are frequently not exposed on antigen binding (Brüggemann et al., 1987; Schroeder and Cavacini, 2010). IgG2 also binds to only one class of FcγR (FcγRII), whereas other IgG classes bind multiple classes (Normansell, 1987; Schroeder and Cavacini, 2010). Indeed, IgG2 antibodies have been seen to exert antiphagocytic effects on *P. aeruginosa* (Hornick and Fick, 1990). However, we hypothesize that anti–O-antigen IgG2 inhibits killing of the *P. aeruginosa* strains by a mechanism similar to that recently described for nontyphoidal *Salmonella enterica* infection in some HIV-infected Malawian adults (MacLennan et al., 2010). Thus, inhibitory IgG2 antibodies bind O-antigen, a target distal on the LPS molecule, and exert their inhibitory effect either by activating and depositing complement away from the bacterial membrane and preventing MAC insertion or by blocking access of protective antibody (Brown et al., 1983; Moffitt and Frank, 1994; MacLennan et al., 2010). However, we have yet to establish whether low titers of anti–O-antigen IgG2 can promote bacterial killing without the addition of other protective antibodies (Taborda et al., 2003). Notably, in the *Salmonella* study, although IgG was found to be inhibitory in the serum, the specific isotype conferring inhibition was not identified. Furthermore, in the current study, the impaired serum killing is not associated with HIV infection or an immunocompromised state.

Our findings have significant implications for vaccine design. Currently, LPS is thought to be an optimal target for protective antibodies. Three O-antigen–based vaccines against *P. aeruginosa*, Pseudogen, PEV-01, and Aerugen, have reached phase II or III trials (Pennington et al., 1975; Langford and Hiller, 1984; Cryz et al., 1997). However, two vaccines resulted in worse clinical status in the vaccinated group and the third trial was suspended (Cryz et al., 1989; Döring and Pier, 2008). These studies have not detailed the IgG subclasses induced in response to the vaccine. The current study provides a potential mechanistic basis for the failure of these vaccines strategies. It indicates that candidate O-antigen polysaccharide-based vaccines may elicit imbalanced anti–O-antigen (IgG2 dominant) antibody induction, rendering the vaccine ineffective while increasing the susceptibility to life-threatening *P. aeruginosa* infections. Furthermore, historical reports of the association of impaired serum killing with other bacterial infections suggest this mechanism may be common for a wide variety of Gram-negative bacterial infections (Waisbren and Brown, 1966). Importantly, understanding the impact elevated levels of IgG2 have on infections could provide opportunities to attenuate disease in several clinical settings.

MATERIALS AND METHODS

Patient details, strains, and samples. Bronchiectasis patients with and without chronic *P. aeruginosa* colonization were identified and confirmed by CT scan. Eleven bronchiectasis patients with chronic *P. aeruginosa* colonization were identified. *P. aeruginosa* was isolated by sputum culture on chocolate blood agar and *Pseudomonas* isolation agar and subsequently cultured in Luria broth. Serum was collected from each patient and 20 healthy individuals. Each patient (P), their isolated bacterium (B), and serum (S) were assigned the same number; patient P1, with serum S1, is colonized by *P. aeruginosa* B1 (Table 1). In the absence of a widely recognized disease severity index in bronchiectasis, the degree of lung function impairment was evaluated using forced expiratory volume in 1 s (FEV1) as a percent predicted of a normal FEV1. This work was performed in compliance with the human ethical approval guidelines granted by the Birmingham Ethics Committee (code RRK3404) and Newcastle and North Tyneside Research Ethics committee (code 12/NE/0248). Additional serum samples were obtained from patients with bronchiectasis regardless of whether they had *P. aeruginosa* colonization or not. These samples were from a distinct geographical location (Newcastle) and each patient (PN), their isolated *P. aeruginosa* if present (BN), and serum (SN) were assigned the same number. Serum samples from eight patients with cystic fibrosis (SCF) and *Pseudomonas* colonization were from Birmingham. Colonization was defined by positive *P. aeruginosa* culture from sputum on at least two separate occasions.

Analysis and manipulation of serum. Serum bactericidal assays were performed in triplicate using a modification of the method described MacLennan et al. (2010). In brief, bacteria were grown overnight in 5 ml of LB at 37°C and resuspended in PBS to a final concentration of 10^7 CFU/ml; 10 µl was then mixed with 90 µl of undiluted human serum at 37°C with shaking (180 rpm), and viable counts were determined. Serum mixing experiments were performed by first mixing the serum with either PBS, concentrated antibodies, other sera, unfiltered sol phase of sputum or sterile sol phase of

sputum at the ratios described in text in a final volume of 90 µl before addition of bacteria. Killing was confirmed as caused by the activity of complement by 56°C heat inactivating the serum as a control. Killing of *Pseudomonas* by washed peripheral blood cells was performed as previously described (Gondwe et al., 2010). In brief, bacteria were grown and resuspended in PBS as above before 10 µl was added to 90 µl of 1/10 dilution of sera (or PBS) for 20-min opsonization. At this point 10 µl this suspension was added to 90 µl of blood cells washed twice in RPMI. Samples were incubated on a rocker plate at 20 rpm at 37°C and numbers of viable *Pseudomonas* were determined after 45, 90, and 180 min by serial dilution on Luria Bertani agar.

Complement deposition and antibody binding were quantified essentially as previously described (MacLennan et al., 2010). In brief, 5 µl *Pseudomonas* at an OD_{600} = 0.6 was mixed with 45 µl 10% serum (antibody determination) or undiluted serum (complement deposition) for 1 h at room temperature. After 3 washes with PBS a final incubation with FITC-conjugated anti–human immunoglobulin (Total IgG, IgG1, IgG2, IgG3, IgG4, IgA, IgM; Sigma-Aldrich) and anti-C1, C3, and C5b-9 (Dako). The C5b-9 antibody recognizes a neo-epitope on the MAC that only forms when the MAC assembles. After this final incubation, the cells were washed as before and analyzed on a FACSAria II (BD). Total IgG subtype concentrations in sol phase sputum and serum samples were determined using the Human IgG Subclass Single Dilution Bindarid kit (Binding Site).

Fixation and preparation of *Pseudomonas* and sputum for cell imaging was performed as described previously (Leyton et al., 2011). In brief, poly L-lysine–coated coverslips loaded with fixed cells or a sputum streak were washed three times with PBS, and nonspecific binding sites were blocked for 1 h in PBS containing 1% BSA (Europa Bioproducts). Coverslips were incubated with 1:500 diluted serum or sol-phase sputum for 1 h, washed three times with PBS, and incubated for an additional 1 h with FITC-conjugated anti–human immunoglobulin (total IgG, IgG1, IgG2, IgG3, IgG4, IgA, IgM; Sigma-Aldrich). The coverslips were then washed three times with PBS, mounted onto glass slides, and visualized using either phase contrast or fluorescence using Leica DMRE fluorescence microscope (100× objective)-DC200 digital camera system.

Serum was fractionated with ultrafiltration columns (Vivascience) with 300, 100, and 30-kD size exclusion filters. In brief, 1 ml of serum was passed first through the 300-kD column as per manufacturer's instructions. Both the flow-through fraction and the retained fraction were diluted to a final concentration of 1 ml with PBS. The 1 ml flow-through fraction was then passed through the 100-kD column in the same way before the final passage through the 30-kD column. All four fractions (>300, 300–100, 100–30, and <30 kD) were brought to 1 ml final volume with PBS.

Antibodies were removed from serum using Protein A–Sepharose 4B, Protein G-Sepharose (GE Healthcare) or anti–human IgG2 monoclonal HP6200-Sepharose according to the manufacturer's instructions. All fractions retained were buffer exchanged into PBS to the desired volume before use in assays. Anti-LPS antibodies were removed from serum in the following manner. First, the LPS fraction was purified and quantified from the *Pseudomonas* using the method described below. The LPS preparation was diluted to 1 mg/ml and 1 ml mixed in microcentrifuge tube with 1 ml polymyxin-B agarose (Sigma-Aldrich) overnight at 4°C. The polymyxin B agarose has a binding capacity of 500 µg/ml so should be saturated with *Pseudomonas* LPS. The resin mix was then loaded onto the column and washed with 10 ml of 0.1 M ammonium bicarbonate buffer (pH 8.0). The serum was then passed over the column and washed with an additional 10 ml of buffer. Finally, bound antibody was eluted with a pH gradient of citric acid before buffer exchange into PBS.

P. aeruginosa biofilm formation was grown as described previously (Wells et al., 2008). In brief, 150 µl low-density *P. aeruginosa* culture was incubated in a 96-well plate overnight at 37°C shaking. Nonadherent culture was then removed and replaced with 150 µl of serum or LB and incubated at 37°C for 2 h. Supernatant was then removed and the biofilm stained with crystal violet. Biofilm intensity was measured at 595 nm.

Analysis of bacterial fractions. Bacterial cell fractions were isolated and analyzed as previously described (Browning et al., 2003; Parham et al., 2004). In brief, outer membrane proteins were isolated by first separating the cell envelopes from the cytoplasm, after French pressure lysis of bacterial cells, by centrifugation (48,000 *g* for 60 min at 4°C). The envelopes were retained and were resuspended in 3 ml of buffer (2% [vol/vol] Triton X-100, 10 mM Tris-HCl, pH 7.5) and incubated at 25°C for 15 min to solubilize inner membrane components. Triton X-100–extracted envelopes were harvested by centrifugation at 48,000 *g* for 60 min at 4°C and washed four times in 30 ml of 10 mM Tris-HCl, pH 7.5. Insoluble fractions were resuspended in 1 ml 10 mM Tris-HCl pH 7.5 and stored at −20°C.

LPS was isolated as previously described (Browning et al., 2003). In brief, *Pseudomonas* was grown overnight at 37°C. The equivalent of 1 ml of OD_{600} = 1 culture was spun and the pellet resuspended in 100 µl of lysing buffer (1 M Tris, pH 6.8, 2% SDS, and 4% 2-mercaptoethanol). The suspension was then boiled for 10 min, spun down, and supernatant was moved to a fresh Eppendorf. 5 µl of 5 mg/ml Proteinase K was added to each sample before incubation at 60°C for 1 h. Finally, the LPS preparation was heated at 98°C for 10 min and stored at −20°C. LPS isolations were quantified by running the sample on an SDS-PAGE gel and comparing to five standards (10, 5, 1, 0.5, and 0.1 mg/ml) of commercially available *Pseudomonas aeruginosa* serotype 10 LPS (Sigma-Aldrich).

Bacterial cell fractions were visualized using SilverQuest kit (Invitrogen) or Western blotting (Raghunathan et al., 2011) using patient serum (1:200) and secondary antibody (1:5,000 alkaline phosphatase conjugated anti–human IgG, IgM or IgA; Sigma-Aldrich) before detection with nitro-blue tetrazolium and 5-bromo-4-chloro-3′-indolyphosphate as the substrate.

Statistical methods. All experiments were performed at least three times unless otherwise stated. Correlation was determined using Spearman's rank and Pearson product-moment correlation coefficients. Statistical significance between patient groups was determined by Student's *t* test. Error bars represent ± 1 standard error.

Online supplemental material. Fig. S1 gives an overview of all experiments performed with S1 serum and a summary of their results. Table S1 lists affinity of Protein A and Protein G for human immunoglobulins. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20132444/DC1.

We thank Catherine Wardius and Pratiba Bharadwa for laboratory and clinical microbiology support. We offer our sincere thanks to Prof. Ian MacLennan FRS for critical appraisal of the manuscript.

The research leading to these results was supported by the European Union Seventh Framework Programme under grant agreement no. PIIF-GA-2009-254733 to TJW and IRH and funds from the University of Birmingham to I.R. Henderson. D. Whitters received support from an unrestricted educational grant from Grifols Inc. A. De Soyza received support from HEFCE, NIHR Biomedical Research Centre, and the NIHR Northumbria Tyne and Wear Comprehensive local research network. C.A. MacLennan is the recipient of a clinical research fellowship from GlaxoSmithKline.

C.A. MacLennan is currently employed by by Novartis Vaccines for Global Health. There are no additional competing financial interests.

Author contributions: I.R. Henderson, R.A. Stockley, C.A. MacLennan, A.F. Cunningham, T.J. Wells, D. Whitters, and A. De Soyza designed research; T.J. Wells, D. Whitters, Y.R. Sevastsyanovich, J.N. Heath, J. Pravin, A. Cranston, and D.F. Browning performed research; T.J. Wells, D. Whitters, I.R. Henderson, A.F. Cunningham, C.A. MacLennan and R.A. Stockley analyzed data; and I.R. Henderson, A.F. Cunningham, T.J. Wells and C.A. MacLennan wrote the paper.

Submitted: 25 November 2013
Accepted: 10 July 2014

REFERENCES

Bilton, D. 2008. Update on non-cystic fibrosis bronchiectasis. *Curr. Opin. Pulm. Med.* 14:595–599. http://dx.doi.org/10.1097/MCP.0b013e328312ed8c

Brown, E.J., K.A. Joiner, and M.M. Frank. 1983. The role of complement in host resistance to bacteria. *Springer Semin. Immunopathol.* 6:349–360. http://dx.doi.org/10.1007/BF02116279

Browning, D.F., D.E. Whitworth, and D.A. Hodgson. 2003. Light-induced carotenogenesis in *Myxococcus xanthus*: functional characterization of the ECF sigma factor CarQ and antisigma factor CarR. *Mol. Microbiol.* 48:237–251. http://dx.doi.org/10.1046/j.1365-2958.2003.03431.x

Brüggemann, M., G.T. Williams, C.I. Bindon, M.R. Clark, M.R. Walker, R. Jefferis, H. Waldmann, and M.S. Neuberger. 1987. Comparison of the effector functions of human immunoglobulins using a matched set of chimeric antibodies. *J. Exp. Med.* 166:1351–1361. http://dx.doi.org/10.1084/jem.166.5.1351

Cryz, S.J. Jr., J.C. Sadoff, A.S. Cross, and E. Fürer. 1989. Safety and immunogenicity of a polyvalent *Pseudomonas aeruginosa* O-polysaccharide-toxin A vaccine in humans. *Antibiot Chemother (1971).* 42:177–183.

Cryz, S.J. Jr., A. Lang, A. Rüdeberg, J. Wedgwood, J.U. Que, E. Fürer, and U. Schaad. 1997. Immunization of cystic fibrosis patients with a *Pseudomonas aeruginosa* O-polysaccharide-toxin A conjugate vaccine. *Behring Inst. Mitt.* 98:345–349.

Döring, G., and G.B. Pier. 2008. Vaccines and immunotherapy against *Pseudomonas aeruginosa*. *Vaccine*. 26:1011–1024. http://dx.doi.org/10.1016/j.vaccine.2007.12.007

Fick, R.B. Jr., J. Olchowski, S.U. Squier, W.W. Merrill, and H.Y. Reynolds. 1986. Immunoglobulin-G subclasses in cystic fibrosis. IgG2 response to *Pseudomonas aeruginosa* lipopolysaccharide. *Am. Rev. Respir. Dis.* 133:418–422.

Flipse, J., J. Wilschut, and J.M. Smit. 2013. Molecular mechanisms involved in antibody-dependent enhancement of dengue virus infection in humans. *Traffic*. 14:25–35.

Gondwe, E.N., M.E. Molyneux, M. Goodall, S.M. Graham, P. Mastroeni, M.T. Drayson, and C.A. MacLennan. 2010. Importance of antibody and complement for oxidative burst and killing of invasive nontyphoidal Salmonella by blood cells in Africans. *Proc. Natl. Acad. Sci. USA*. 107:3070–3075. http://dx.doi.org/10.1073/pnas.0910497107

Guttman, R.M., and B.A. Waisbren. 1975. Bacterial blocking activity of specific IgG in chronic *Pseudomonas aeruginosa* infection. *Clin. Exp. Immunol.* 19:121–130.

Halstead, S.B., and E.J. O'Rourke. 1977. Dengue viruses and mononuclear phagocytes. I. Infection enhancement by non-neutralizing antibody. *J. Exp. Med.* 146:201–217. http://dx.doi.org/10.1084/jem.146.1.201

Halstead, S.B., S. Mahalingam, M.A. Marovich, S. Ubol, and D.M. Mosser. 2010. Intrinsic antibody-dependent enhancement of microbial infection in macrophages: disease regulation by immune complexes. *Lancet Infect. Dis.* 10:712–722. http://dx.doi.org/10.1016/S1473-3099(10)70166-3

Hann, S., and D.S. Holsclaw. 1976. Interactions of *Pseudomonas aeruginosa* with immunoglobulins and complement in sputum. *Infect. Immun.* 14:114–117.

Hill, S.L., J.L. Mitchell, D. Burnett, and R.A. Stockley. 1998. IgG subclasses in the serum and sputum from patients with bronchiectasis. *Thorax*. 53:463–468. http://dx.doi.org/10.1136/thx.53.6.463

Hornick, D.B., and R.B. Fick Jr. 1990. The immunoglobulin G subclass composition of immune complexes in cystic fibrosis. Implications for the pathogenesis of the Pseudomonas lung lesion. *J. Clin. Invest.* 86:1285–1292. http://dx.doi.org/10.1172/JCI114836

Jefferis, R., C.B. Reimer, F. Skvaril, G.G. de Lange, D.M. Goodall, T.L. Bentley, D.J. Phillips, A. Vlug, S. Harada, J. Radl, et al. 1992. Evaluation of monoclonal antibodies having specificity for human IgG subclasses: results of the 2nd IUIS/WHO collaborative study. *Immunol. Lett.* 31:143–168. http://dx.doi.org/10.1016/0165-2478(92)90141-A

Kronborg, G., T. Pressler, A. Fomsgaard, C. Koch, and N. Høiby. 1993. Specific IgG2 antibodies to *Pseudomonas aeruginosa* lipid A and lipopolysaccharide are early markers of chronic infection in patients with cystic fibrosis. *Infection*. 21:297–302. http://dx.doi.org/10.1007/BF01712448

Langford, D.T., and J. Hiller. 1984. Prospective, controlled study of a polyvalent pseudomonas vaccine in cystic fibrosis—three year results. *Arch. Dis. Child.* 59:1131–1134. http://dx.doi.org/10.1136/adc.59.12.1131

Leyton, D.L., Y.R. Sevastsyanovich, D.F. Browning, A.E. Rossiter, T.J. Wells, R.E. Fitzpatrick, M. Overduin, A.F. Cunningham, and I.R. Henderson. 2011. Size and conformation limits to secretion of disulfide-bonded loops in autotransporter proteins. *J. Biol. Chem.* 286:42283–42291. http://dx.doi.org/10.1074/jbc.M111.306118

MacLennan, C.A., J.J. Gilchrist, M.A. Gordon, A.F. Cunningham, M. Cobbold, M. Goodall, R.A. Kingsley, J.J. van Oosterhout, C.L. Msefula, W.L. Mandala, et al. 2010. Dysregulated humoral immunity to nontyphoidal Salmonella in HIV-infected African adults. *Science*. 328:508–512. http://dx.doi.org/10.1126/science.1180346

Martínez-García, M.A., M. Perpiñá-Tordera, P. Román-Sánchez, and J.J. Soler-Cataluña. 2005. Quality-of-life determinants in patients with clinically stable bronchiectasis. *Chest*. 128:739–745. http://dx.doi.org/10.1378/chest.128.2.739

Martínez-García, M.A., J.J. Soler-Cataluña, M. Perpiñá-Tordera, P. Román-Sánchez, and J. Soriano. 2007. Factors associated with lung function decline in adult patients with stable non-cystic fibrosis bronchiectasis. *Chest*. 132:1565–1572. http://dx.doi.org/10.1378/chest.07-0490

Moffitt, M.C., and M.M. Frank. 1994. Complement resistance in microbes. *Springer Semin. Immunopathol.* 15:327–344. http://dx.doi.org/10.1007/BF01837364

Normansell, D.E. 1987. Human immunoglobulin subclasses. *Diagn. Clin. Immunol.* 5:115–128.

Parham, N.J., U. Srinivasan, M. Desvaux, B. Foxman, C.F. Marrs, and I.R. Henderson. 2004. PicU, a second serine protease autotransporter of uropathogenic *Escherichia coli*. *FEMS Microbiol. Lett.* 230:73–83. http://dx.doi.org/10.1016/S0378-1097(03)00862-0

Pasteur, M.C., S.M. Helliwell, S.J. Houghton, S.C. Webb, J.E. Foweraker, R.A. Coulden, C.D. Flower, D. Bilton, and M.T. Keogan. 2000. An investigation into causative factors in patients with bronchiectasis. *Am. J. Respir. Crit. Care Med.* 162:1277–1284. http://dx.doi.org/10.1164/ajrccm.162.4.9906120

Pasteur, M.C., D. Bilton, and A.T. Hill. British Thoracic Society Non-CF Bronchiectasis Guideline Group. 2010. British Thoracic Society guideline for non-CF bronchiectasis. *Thorax*. 65:577. http://dx.doi.org/10.1136/thx.2010.142778

Pennington, J.E., H.Y. Reynolds, R.E. Wood, R.A. Robinson, and A.S. Levine. 1975. Use of a *Pseudomonas Aeruginosa* vaccine in pateints with acute leukemia and cystic fibrosis. *Am. J. Med.* 58:629–636. http://dx.doi.org/10.1016/0002-9343(75)90498-2

Raghunathan, D., T.J. Wells, F.C. Morris, R.K. Shaw, S. Bobat, S.E. Peters, G.K. Paterson, K.T. Jensen, D.L. Leyton, J.M. Blair, et al. 2011. SadA, a trimeric autotransporter from *Salmonella enterica* serovar Typhimurium, can promote biofilm formation and provides limited protection against infection. *Infect. Immun.* 79:4342–4352. http://dx.doi.org/10.1128/IAI.05592-11

Schroeder, H.W. Jr., and L. Cavacini. 2010. Structure and function of immunoglobulins. *J. Allergy Clin. Immunol.* **125**(2, Suppl 2):S41–S52. http://dx.doi.org/10.1016/j.jaci.2009.09.046

Siber, G.R., P.H. Schur, A.C. Aisenberg, S.A. Weitzman, and G. Schiffman. 1980. Correlation between serum IgG-2 concentrations and the antibody response to bacterial polysaccharide antigens. *N. Engl. J. Med.* 303:178–182. http://dx.doi.org/10.1056/NEJM198007243030402

Taborda, C.P., J. Rivera, O. Zaragoza, and A. Casadevall. 2003. More is not necessarily better: prozone-like effects in passive immunization with IgG. *J. Immunol.* 170:3621–3630. http://dx.doi.org/10.4049/jimmunol.170.7.3621

Waisbren, B.A., and I. Brown. 1966. A factor in the serum of patients with persisting infection that inhibits the bactericidal activity of normal serum against the organism that is causing the infection. *J. Immunol.* 97:431–437.

Wells, T.J., O. Sherlock, L. Rivas, A. Mahajan, S.A. Beatson, M. Torpdahl, R.I. Webb, L.P. Allsopp, K.S. Gobius, D.L. Gally, and M.A. Schembri. 2008. EhaA is a novel autotransporter protein of enterohemorrhagic *Escherichia coli* O157:H7 that contributes to adhesion and biofilm formation. *Environ. Microbiol.* 10:589–604. http://dx.doi.org/10.1111/j.1462-2920.2007.01479.x

Whitters, D., and R. Stockley. 2012. Immunity and bacterial colonisation in bronchiectasis. *Thorax*. 67:1006–1013. http://dx.doi.org/10.1136/thoraxjnl-2011-200206

Wilson, C.B., P.W. Jones, C.J. O'Leary, D.M. Hansell, P.J. Cole, and R. Wilson. 1997. Effect of sputum bacteriology on the quality of life of patients with bronchiectasis. *Eur. Respir. J.* 10:1754–1760. http://dx.doi.org/10.1183/09031936.97.10081754