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.
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 \( \text{Pseudomonas aeruginosa} \) who died despite treatment (Waisbren and Brown, 1966). We hypothesized that similar impaired serum-killing also exists for other bronchiectasis patients with chronic \( \text{Pseudomonas} \) infections and that this contributes to disease severity. Indeed, IgG antibody to \( \text{Pseudomonas aeruginosa} \) 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 \( \text{Pseudomonas} \) 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 \( \text{Pseudomonas aeruginosa} \) 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 \( \text{Pseudomonas aeruginosa} \) isolates taken from 11 different patients with bronchiectasis and chronic \( \text{Pseudomonas} \) 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 \( \text{Pseudomonas aeruginosa} \) 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) 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.

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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 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–SN30) 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.

**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.
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.
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
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).

Table 2. Total antibody titers of bronchiectasis patients

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

*aNo paraprotein detected by IMFIX.

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.
in the lungs of patients suffering from bronchiectasis (Hill et al., 1998). We hypothesized that patients with impaired immunity and \textit{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 \textit{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

All \textit{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 \textit{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.

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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...
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.

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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
In contrast, our data demonstrate that mean of three independent measurements. *, P < 0.05; **, P < 0.01.

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 Malavian 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 Aegeren, 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 (Waishbren 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 at 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 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 10⁶ 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 36°C heat inactivating the serum as a control. Killing of P. aeruginosa 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 OD600 = 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 Bindaris 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 (Vivaspin) 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 IgG 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 strain by methods 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 is described previously (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 OD600 = 1 culture was spun and the pellet resuspended in 100 µl of lysis 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 quantitated 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 phosphate 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.

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

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REFERENCES


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