Polymeric IgR knockout mice are more susceptible to mycobacterial infections in the respiratory tract than wild-type mice

Anna Tjärlund¹, Ariane Rodríguez¹, Pere-Joan Cardona², Evelyn Guirado², Juraj Ivanyi³, Mahavir Singh⁴, Marita Troye-Blomberg¹ and Carmen Fernández¹

¹Department of Immunology, Wenner-Gren Institute, Stockholm University, Svante Arrhenius väg 16, 10691 Stockholm, Sweden
²Unitat de Tuberculosi Experimental, Fundació Institut Germans Trias i Pujol, Badalona, Catalonia, Spain
³Department of Oral Medicine and Pathology, Guy’s Hospital, London SE1 9RT, UK
⁴Gesellschaft für Biotechnologische Forschung mbH, 38124 Braunschweig, Germany

Keywords: mucosal immunity, mycobacteria, plgR, secretory IgA

Abstract

It is generally accepted that cellular, and not humoral immunity, plays the crucial role in defense against intracellular bacteria. However, accumulating data indicate the importance of humoral immunity for the defense against a number of intracellular bacteria, including mycobacteria. We have investigated the role of secretory IgA, the main isotype found in mucosal tissues, in protection against mycobacterial infection, using polymeric IgR (pIgR)-deficient mice. Characterization of the humoral response induced after intra-nasal immunizations with the mycobacterial antigen PstS-1 revealed a loss of antigen-specific IgA response in saliva from the knockout mice. IgA level in the bronchoalveolar lavage of knockout mice was similar to wild-type level, although the IgA antibodies must have reached the lumen by other means than plgR-mediated transport. Infection with Mycobacterium bovis bacillus Calmette–Guerin (BCG) demonstrated that the immunized plgR⁻/⁻ mice were more susceptible to BCG infection than immunized wild-type mice, based on higher bacterial loads in the lungs. This was accompanied by a reduced production of both IFN-γ and tumor necrosis factor-alpha (TNF-α) in the lungs. Additionally, the plgR⁻/⁻ mice displayed reduced natural resistance to mycobacterial infection proved by significantly higher bacterial growth in their lungs compared with wild-type mice after infection with virulent Mycobacterium tuberculosis. The knockout mice appeared to have a delayed mycobacteria-induced immune response with reduced expression of protective mediators, such as IFN-γ, TNF-α, inducible nitric oxide synthase and regulated upon activation normal T cell sequence, during early infection. Collectively, our results show that actively secreted IgA plays a role in protection against mycobacterial infections in the respiratory tract, by blocking entrance of bacilli into the lungs, in addition to modulation of the mycobacteria-induced pro-inflammatory response.

Introduction

The mucosal immune system provides the first line of defense against entrance of a multitude of ingested and inhaled microorganisms. The most characteristic component of the mucosal immunity is secretory IgA (SlgA), which is the predominant Ig isotype in mucosal tissue [reviewed in (1)]. Polymeric IgR (pIgR) expressed at the basolateral surface of epithelial cells mediates active transport of dimeric IgA, and also pentameric IgM, to exocrine secretions (2), leaving the secretory component (SC) bound to dimeric IgA. SlgA protects the epithelial barrier by a mechanism termed immune exclusion (3). It also protects against intracellular pathogens by interacting with and neutralizing viruses intracellularly as it is transcytosed via the plgR (4, 5). Furthermore, through cross-linking of SlgA bound to the FcγR, protective functions such as phagocytosis, degranulation (6), respiratory burst activity (7) and antibody-dependent cytotoxicity (8) can be initiated.

Mycobacterium tuberculosis is one of the most ubiquitous pathogens in the world, with an estimated one-third of the
The studies were performed using C57BL/6 and pIgR knockout mice. Both BCG and virulent M. tuberculosis were used as challenge organisms for infection of the mice. The aim of our study was to investigate the importance of SIgA antibodies versus systemic immunity in protection against mycobacteria, as has been demonstrated previously in virus infection studies (25). The protection against challenge, after i.n. vaccination with the mycobacterial antigen PstS-1, has been shown to be associated with a strong local and systemic IgA response (21), suggesting a protective role for IgA in the respiratory tract. It was recently demonstrated that i.n. inoculations with monoclonal IgA antibodies against the x-crystalline antigen of M. tuberculosis in mice significantly reduced lung bacterial counts after either aerosol or i.n. challenge with M. tuberculosis (22).

We have recently shown that IgA knockout mice are more susceptible to i.n. infection with Mycobacterium bovis bacillus Calmette–Guérin (BCG), characterized by significantly higher bacterial load in the lungs compared with wild-type mice (23). All these data led us to think that vaccination targeting the respiratory mucosa, achieved through i.n. immunization, could provide an advantage in order to improve the currently used subcutaneous immunization with BCG (21, 24).

pIgR knockout mice present a unique opportunity to explore the relative contribution of secretory antibodies versus systemic immunity in protection against mycobacteria, as has been demonstrated previously in virus infection studies (25). The aim of our study was to investigate the importance of SIgA in protection against mycobacteria infection using pIgR−/− mice. Both BCG and virulent M. tuberculosis were used as challenge organisms for infection of the mice.

Our results clearly show that actively secreted IgA plays a role against mycobacterial infection. This was indicated by significantly higher bacterial loads, and diminished expression of factors important for an efficient immunity against tuberculosis, in the lungs of infected pIgR−/− mice with respect to wild-type mice.

**Methods**

**Mice**

The studies were performed using C57BL/6 and pIgR−/− mice with a C57BL/6 background (provided by M. Nanno, Yakult Central Institute for Microbiological Research, Tokyo, Japan), 8–12 weeks of age. These mice lack exon 2 of the pIgR gene and were generated by a gene-targeting strategy as previously described (26), and backcrossed 10 times to C57BL/6 mice, resulting in knockout mice with 99.9% C57BL/6 background. Animals were kept at the Animal Department at the Arrhenius Laboratories, Stockholm University, Sweden. All experiments performed were in accordance with the relevant ethical committee in Stockholm. Mice were supervised every day and sentinel animals were used to check specific pathogen-free conditions in the facility.

The M. tuberculosis aerosol infection experiments were performed using specific pathogen-free, 6–8 weeks old pIgR−/− and C57BL/6 mice, obtained from Harlan Iberica, S.L. (Sant Feliu de Codines, Catalonia, Spain). All the animals were kept under controlled conditions in a BL3 High Security Facility with sterile food and water ad libitum and weighed once a week. They were supervised every day under a protocol paying attention to weight loss, apparent good health (bristled hair and wounded skin) and behavior (signs of aggressiveness or isolation). Animals were euthanized with halothane (Fluothane, Zeneca Farma, Madrid, Spain) overdose so as to avoid any suffering. All experimental proceedings were approved and supervised by the Animal Care Committee of ‘Germans Trias i Pujol’ University Hospital in agreement with the European Union Laws for protection of experimental animals.

**Immunizations**

The endotoxin-free recombinant PstS-1 protein (GBF, Braunschweig, Germany) was used as antigen for immunizations. As adjuvant, for targeting the mucosal immune system, cholera toxin (CT) (List Biological Laboratories Inc., Campbell, CA, USA) was used.

Groups of four to six mice for characterization of immune responses and four to seven mice for protection studies were immunized i.n. using the PstS-1 protein (10 μg per dose) formulated with CT (1 μg per dose) in PBS. Each group of mice received three immunizations separated by 3-week intervals. i.n. immunizations were performed on mice anesthetized with isofluorane (Baxter Medical AB, Kista, Sweden) and the antigen (30 μl) was applied to the external nares, 15 μl in each nostril, using a micropipette.

**Sample collection**

Serum, saliva and bronchoalveolar lavage (BAL) were collected 14 days after the last immunization. Mice were bled by retro-orbital puncture and serum was collected after centrifugation of coagulated blood. The BAL was obtained by flushing 1.5 ml of sterile PBS into the lungs of sacrificed mice. Saliva was collected after intra-peritoneal injection of 100 μl of pilocarpine 1 mg ml−1 (Tika Läkemedel AB, Lund, Sweden). The mononuclear cells were collected from lungs 14 days after the final immunization. Briefly, the lungs were homogenized in balanced salt solution using a glass homogenizer. The cell suspension was thereafter subjected to gradient separation using Lympholyte M (Cederlane laboratories, Ontario, Canada). After centrifugation at 1500 g for 20 min at 24°C, the interface was collected and the cells were washed twice in balanced salt solution.

**Antibody detection**

Specific antibodies and total IgA in serum, saliva and BAL were analyzed by ELISA. ELISA plates (Costar, NY, USA) were coated with either the PstS-1 protein (2 μg ml−1) or unlabeled...
anti-mouse Ig (1 μg ml⁻¹) (Southern Biotechnology Associates, Inc., Birmingham, AL, USA) in coating buffer, pH 9.4, overnight at room temperature. Plates were washed three times with PBS–TWEEN 0.05% (vol/vol). After washing, the samples were applied to the wells in serial dilutions starting from 1:200 for serum, 1:4 for saliva and 1:50 for BAL and the plates were incubated overnight at room temperature. Following incubation, the plates were washed and incubated for 2 h with alkaline phosphatase-labeled goat anti-mouse Ig isotypes (Southern Biotechnology Associates), and finally the enzyme substrate reaction was developed using p-nitrophenyl phosphate (SIGMA Chemical Co., St Louis, MO, USA) as substrate. Optical density (OD) was read in a multispan plate reader (Anthos Labtec Instruments, Salzburg, Austria) at 405 nm. Background level was defined as OD obtained using PBS–TWEEN 0.05% (vol/vol) instead of samples. OD dilution curves were plotted (after background value correction) and titers were defined as the inverse log₁₀ of the lowest dilution giving OD of 0.1 for BAL and serum and an OD of 0.2 for saliva samples.

Cytokine ELISA

Cell suspensions obtained from lungs of individual mice, 4 weeks after i.n. infection with BCG, were cultured (2 × 10⁵ cells per well) with BCG (1:2 ratio) as stimulation. Supernatants were collected after 48 h and production of IFN-γ (Mabtech, Stockholm, Sweden) and tumor necrosis factor-alpha (TNF-α) (R&D system, Minneapolis, MN, USA) was assayed by ELISA, according to the manufacturer's recommendations with some modification. Streptavidin conjugated to alkaline phosphatase, instead of HRP, was used at a 1:1000 dilution. The enzyme–substrate reaction was developed using p-nitrophenyl phosphate (SIGMA Chemical Co.). OD was read in a multispan plate reader at 405 nm and concentrations were determined after background value correction.

mRNA quantification

The procedures are described elsewhere (27). In short, total RNA from the middle right lobe of the lungs of aerosol-infected mice was extracted with a commercial phenol–chloroform method, RNAzol (Cinna/Biotecx, Friendswood, TX, USA). After a deoxynucleoside treatment with DNA-free kit (Ambion, Woodward Austin, TX, USA), a denaturing agarose gel was used to assess the stability of RNA. Five micrograms of RNA was reverse transcribed using a Superscript Reverse Transcription kit (Gibco BRL, Grand Island, NY, USA) following the manufacturer's recommendations to obtain cDNA. The quantitative analysis for IFN-γ, regulated upon activation normal T cell sequence (RANTES), inducible nitric oxide synthase (iNOS) and TNF-α was performed using a LightCycler™ System (Roche Biochemicals, Idaho Falls, ID, USA). A real-time PCR was carried out in glass capillaries to a final volume of 10 μl in the presence of 1 μl of 10 × reaction buffer (Taq polymerase, deoxynucleoside triphosphates, MgCl₂, SYBRGreen, Roche Biochemicals), 1 μl of cDNA (or water as negative control, which was always included), MgCl₂ to a final concentration of 2–5 mM, and primers to a final concentration of 0.5 μM were also added. A single peak was obtained for each PCR product by melting curve analysis and only one band of the estimated size was observed on the agarose gel. Hypoxanthine guanine phosphoribosyl transferase (HPRT) mRNA expression was analyzed for every target sample to normalize for efficiency in cDNA synthesis and RNA loading. A ratio based on the HPRT mRNA expression was obtained for each sample.

Intra-nasal infection and quantification of bacterial load

BCG (Pasteur strain) was cultured in a chemically defined liquid culture medium (28) over a period of 5 days. Seed lots of bacteria were stored at −70°C. Mice were i.n. inoculated with 10⁶ colony-forming units (CFU) of live BCG, in 30 μl PBS, under anesthesia with isoﬂurane 2 weeks after the last immunization with PstS-1. At weeks 1 and 4 after infection, mice were sacrificed and the numbers of viable bacteria in the lungs were determined. The right lung from individual mice was homogenized in 2 ml PBS supplemented with 0.15 M NaCl and 0.05% Tween 80 (vol/vol), using glass homogenizers. Serial dilutions of the lung homogenates were plated on Middlebrook 7H11 agar plates. The numbers of CFU were determined after 2–4 weeks of incubation at 37°C.

M. tuberculosis aerosol infection

M. tuberculosis H37Rv was grown in Proskauer Beck medium containing 0.01% Tween 80 to mid-log phase and stored at −70°C. Mice were placed in the exposure chamber of an airborne infection apparatus (Glas-col Inc., Terre Haute, IN, USA). The nebulizer compartment was filled with 7 ml of the bacillar suspension at a previously calculated concentration to provide an uptake of ~20 viable bacilli within the lungs. Ten mice were used for each time point in every experimental group. The numbers of viable bacteria in the left lung homogenates on weeks 3 and 8 were followed by plating serial dilutions on nutrient Middlebrook 7H11 agar and counting bacterial colonies after 21 days incubation at 37°C.

Histology and morphometry

Procedures have been described in previous works (29). Briefly, two right lung lobes from each mouse were fixed in buffered formalin and subsequently embedded in paraffin. Every sample was stained with hematoxillin–eosin. For histometry, 5-μm thick sections from each specimen were stained with hematoxillin–eosin and photographed at ×6 using a Stereoscopic Zoom SMZ800 microscope (Nikon, Tokyo, Japan) and a Coolpix 990 digital camera (Nikon). Sections of eight lung lobes were studied in each case. A sequence of appropriate software programs were used: Scion Image (Scion Corporation, Frederick, MD, USA) and Photoshop 5.0 (Adobe Systems Incorporated, San José, CA, USA) to determine the area of each single lesion and the total tissue area on photomicrographs at each time point. Sections were blindly evaluated in order to get a more objective measurement.

Statistical analysis

Experimental groups were compared by analysis of variance followed by Tukey post-test. Differences in bacterial loads between wild-type and plgR⁻/⁻ mice were compared by Mann–Whitney U-test. The level of significance was set at P < 0.05.
Results

Ig levels in the respiratory tract and serum of plgR<sup>-/-</sup> mice

To study the significance of the plgR gene with respect to antibody responses, total Ig titers in saliva, BAL and serum from both wild-type and gene-depleted mice were analyzed. The only Ig isotype detected in saliva from either mouse strain was IgA (Table 1). Total IgA titers were 20% lower in saliva from plgR<sup>-/-</sup> knockout mice compared with wild-type mice. No major differences in Ig titers in BAL were seen between the two experimental groups (Table 1). The plgR<sup>-/-</sup> mice showed significantly ($P < 0.01$) elevated levels of IgA in serum with respect to wild-type mice (Table 1). IgG and IgM titers remained the same between the two mouse strains.

Antibody responses induced by immunization with the PstS-1 antigen

We next wanted to study the effect of disrupting the plgR gene with regard to local induction of specific immune responses. Since the route of entry of the tubercle bacillus into the body is via the respiratory tract, we wanted to investigate the antibody response induced in the respiratory tract in immunized mice. plgR<sup>-/-</sup> mice and wild-type mice were immunized with the PstS-1 antigen given i.n., and the immune response induced in the respiratory tract was assessed by measuring antigen-specific antibody titers in saliva and BAL from the immunized mice. One important finding was that no specific IgA antibody responses could be detected in saliva from plgR knockout mice, although wild-type mice showed a strong IgA response (Table 2). However, when the antigen-specific antibody responses in BAL were compared, no differences for any of the isotypes between knockout and wild-type mice were found (Table 2).

The systemic immune response induced in immunized mice was also evaluated, and the results showed a significant increase ($P < 0.01$) of PstS-1-specific IgA titers in sera from plgR knockout mice compared with the wild-type mice (Table 2). IgG was the major isotype found in sera from both mouse strains, with similar levels between the two groups, which was also the case for antigen-specific IgM levels.

plgR<sup>-/-</sup> mice are more susceptible to mycobacterial infection

The susceptibility of plgR<sup>-/-</sup> mice to mycobacterial infection was evaluated by comparing bacterial loads in the lungs to wild-type mice after infection. Control mice were administered CT i.n. according to the immunization protocol and subsequently infected with BCG. The results revealed that the bacterial growth in lungs was higher in the gene-depleted control mice than in wild-type control mice, and increasing from week 1 to week 4 for both mouse strains (Fig. 1A). However, the higher bacterial load seen in the plgR<sup>-/-</sup> mice compared to wild-type mice was not statistically significant. When PstS-1-immunized mice where i.n. infected with BCG, a pronounced difference between the groups was demonstrated. The results showed a significantly higher ($P < 0.01$) bacterial load in lungs from plgR<sup>-/-</sup> mice at both week 1 and week 4 compared with wild-type mice (Fig. 1B).

### Table 1. Total Ig levels in saliva, BAL and serum from plgR<sup>-/-</sup> and wild-type C57BL/6 mice<sup>a</sup>

<table>
<thead>
<tr>
<th></th>
<th>Saliva</th>
<th>BAL</th>
<th>Serum</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6</td>
<td>IgA</td>
<td>3.26</td>
<td>3.44 ± 0.18</td>
</tr>
<tr>
<td></td>
<td>IgG</td>
<td>ND</td>
<td>3.60 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>IgM</td>
<td>ND</td>
<td>3.11 ± 0.11</td>
</tr>
<tr>
<td>plgR&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>IgA</td>
<td>2.62</td>
<td>3.70 ± 0.20</td>
</tr>
<tr>
<td></td>
<td>IgG</td>
<td>ND</td>
<td>2.93 ± 0.45</td>
</tr>
<tr>
<td></td>
<td>IgM</td>
<td>ND</td>
<td>3.28 ± 0.22</td>
</tr>
</tbody>
</table>

ND = Not detected.

<sup>a</sup>Results are presented as mean ± SEM from BAL and serum or values from testing pooled saliva samples. A representative of two different experiments is shown ($n = 4–6$).

<sup>b</sup>$P < 0.01$ versus wild-type mice as calculated by analysis of variance followed by Tukey post-test.

### Table 2. PstS-1-specific antibody titers in saliva, BAL and serum from immunized plgR<sup>-/-</sup> and wild-type C57BL/6 mice<sup>a</sup>

<table>
<thead>
<tr>
<th></th>
<th>Saliva</th>
<th>BAL</th>
<th>Serum</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6</td>
<td>IgA</td>
<td>2.50 ± 0.40</td>
<td>3.25 ± 0.12</td>
</tr>
<tr>
<td></td>
<td>IgG</td>
<td>ND</td>
<td>3.52 ± 0.15</td>
</tr>
<tr>
<td></td>
<td>IgM</td>
<td>ND</td>
<td>2.79 ± 0.30</td>
</tr>
<tr>
<td>plgR&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>IgA</td>
<td>ND&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.14 ± 0.35</td>
</tr>
<tr>
<td></td>
<td>IgG</td>
<td>ND</td>
<td>3.32 ± 0.18</td>
</tr>
<tr>
<td></td>
<td>IgM</td>
<td>ND</td>
<td>2.52 ± 0.17</td>
</tr>
</tbody>
</table>

ND = Not detected.

<sup>a</sup>Results are presented as mean ± SEM from BAL, serum and saliva. A representative of two different experiments is shown ($n = 4–6$).

<sup>b</sup>$P < 0.01$ versus wild-type mice as calculated by analysis of variance followed by Tukey post-test.

### BCG-infected plgR<sup>-/-</sup> mice exhibit reduced IFN-γ and TNF-α production in the lungs

It is well recognized that protective immunity against <i>M. tuberculosis</i> requires antigen-specific CD4<sup>+</sup> T cell responses with accompanying IFN-γ production (13), a central factor in activation of anti-mycobacterial activities in macrophages (30). In addition, other cytokines, in particular TNF-α, also participate by regulating the formation and maintaining the structural integrity of lung granulomas (31–33). In order to investigate a possible mechanism responsible for the higher bacterial burden found in the lungs of immunized plgR<sup>-/-</sup> mice, the production of IFN-γ and TNF-α was assayed in the lungs of the mice at week 4 after infection. Isolated mononuclear cells from the lungs of infected mice were stimulated in vitro with BCG for 48 h and the IFN-γ and TNF-α production in the supernatants were analyzed by ELISA. The results revealed that the plgR<sup>-/-</sup> mice had a significant reduction in the production of both IFN-γ ($P < 0.01$) and TNF-α ($P < 0.01$) compared with wild-type mice (Fig. 2).

### plgR<sup>-/-</sup> mice display delayed natural protection against <i>M. tuberculosis</i> infection compared with wild-type mice

To further investigate the role of secreted IgA in defense against mycobacterial infection <i>in vivo</i>, non-immunized plgR<sup>-/-</sup> mice
were subjected to aerosol infection with virulent *M. tuberculosis*, and bacterial growth in the lungs were determined. The difference in bacterial load between the plgR<sup>−/−</sup> mice and the wild-type mice was most pronounced at 3 weeks post-infection, at which time point the knockout mice displayed a significant increase (*P* < 0.01) in the bacterial load in the lungs compared with wild-type mice (Fig. 3). No difference between the two groups could be detected at week 8 after infection.

**Histological analysis**

The granulomatous response plays a critical role in controlling mycobacterial dissemination. We therefore examined the granulomatous infiltration in the lungs of *M. tuberculosis*-infected mice. There was a slightly reduced infiltration in the lungs of knockout mice at week 3 when compared with wild-type mice (Fig. 4A). The opposite was seen at week 8 where the granulomatous infiltration was significantly higher (*P* < 0.01) in the knockout mice compared with wild-type mice. For both mouse strains, the percentage of infiltration increased from week 3 to week 8.

Histological analyses were performed to investigate the nature of the granulomatous infiltration. Qualitative differences in lung infiltration were seen at week 3 post-infection. Histological appearance of granulomas in wild-type mice was then characterized by clustering of neutrophils, macrophages and lymphocytes that gave rise to pregranulomas (Fig. 4B). Minimal necrosis and karyorrhexis could be detected. In the knockout mice, the infiltration of neutrophils was more intense quantitatively, whereas numbers of macrophages and lymphocytes were reduced (Fig. 4C). Additionally, more necrosis and karyorrhexis among the neutrophils were detected. At week 8 post-infection, there were no major qualitative differences.
between the two mouse strains regarding the nature of the granulomas and cellular composition (data not shown).

**Decreased mRNA levels of IFN-γ, TNF-α, iNOS and RANTES in the lungs of *M. tuberculosis*-infected plgR−/− mice**

To investigate whether the enhanced susceptibility of plgR−/− mice is associated with an impaired production of these molecules, mRNA from lungs of aerosol-infected mice was prepared and quantified using real-time PCR. IFN-γ mRNA levels in the lungs of plgR−/− mice were significantly reduced compared with control mice at both week 3 (P < 0.01) and week 8 (P < 0.05) after infection (Fig. 5). A significant decrease (P < 0.01) in TNF-α mRNA levels was also detected in the knockout mice 3 weeks after infection, whereas no difference at the later time point could be seen (Fig. 5). The reduction in TNF-α mRNA levels in wild-type mice from week 3 to 8 was pronounced, whereas the levels in plgR−/− mice decreased only slightly. Production of reactive nitrogen intermediates is considered a defense mechanism against microbial pathogens, exerted by innate immune cells. The analysis of mRNA levels of iNOS in the lungs of infected mice revealed a significant reduction (P < 0.01) in the plgR−/− mice at both time points investigated (Fig. 5). The C–C chemokine RANTES, produced by a variety of cells, is involved in attracting monocytes and lymphocytes to sites of infection, as well as promoting Tn1 type of responses (34–36). The mRNA levels of this chemokine were quantified, and as for TNF-α, plgR−/− mice displayed significant reduced (P < 0.01) levels of RANTES mRNA at week 3 after infection, whereas no difference was seen at week 8 after infection (Fig. 5). The levels decreased from week 3 to week 8 in wild-type mice, while in the knockout mice the levels remained the same.

**Discussion**

In the present study, we evaluated the role of mucosal IgA antibodies in providing protection against mycobacterial infections, using plgR−/− mice. IgA represents the most prominent antibody class at mucosal surfaces. The significance of SlgA in the first-line mucosal defense is well recognized, although its precise role remains unclear (37). We have previously found that IgA−/− mice are more susceptible to BCG infection compared with wild-type littermates (23). Furthermore, we demonstrated that these IgA-deficient mice are more susceptible to i.n. infection with another intracellular bacteria, *Chlamydia pneumoniae*, than their wild-type littermates (38), indicating a protective role for IgA against intracellular bacteria. Although infection studies with *M. tuberculosis* have previously been performed in B cell-deficient mice (15, 16), mice deficient in actively secreted antibodies, plgR−/− mice, have never been investigated in this context.

Several studies have shown a decrease in total IgA levels in both small and large intestine in plgR−/− mice (26, 39), but less is known about the antibody responses in the respiratory tract. Analyzed total Ig levels in saliva, BAL and serum revealed no differences between the plgR−/− mice and wild-type mice except a significant increase in serum IgA in plgR−/− mice (26, 39, 40). Evaluation of the induced antibody responses in immunized mice displayed high levels of antigen-specific IgA antibodies in both saliva and BAL from wild-type mice. Interestingly, although IgA levels in BAL from the plgR−/− mice were comparable to those seen in wild-type mice, no antigen-specific IgA antibodies could be detected in the saliva from immunized knockout mice. The finding of IgA antibodies in some of the mucosal secretions from plgR−/− mice may indicate an alternative way for IgA to reach pulmonary secretions, for instance through leakage. Alternatively, active transport via plgR-mediated transcytosis of IgA, passive transport or plgR expression may be different in the upper and lower respiratory tracts. Indeed, previous studies have revealed higher plgR expression, and subsequently greater IgA transport, in the upper airways compared with the lower respiratory tract (reviewed in (41)). Additionally, in the alveolar spaces of the lower respiratory tract, IgG is the predominant Ig and not IgA (42). This further supports the idea of increased passive transport in the lower respiratory tract since IgG found in external secretions originates predominantly from the circulation.

Infection of wild-type and knockout mice with BCG, after i.n. immunization with either the mycobacterial antigen PstS-1 combined with CT or with CT alone (control mice), clearly demonstrated that the plgR−/− mice, both immunized and control, are more susceptible to BCG infection. However, the results indicate that SlgA might not be crucial for natural immunity to BCG infection, but important for the immune response elicited by mucosal immunization, using CT as adjuvant, upon a subsequent BCG infection. The function of the plgR extends beyond ensuring efficient secretion of dimeric IgA at mucosal surfaces. SC, the part of the plgR remaining
after transcytosis, has been implicated in several functions (43–49). SC has been shown to increase the stability of SIgA and to enhance resistance to proteolysis, and SC-associated glycans may interact with bacteria and interfere with their attachment to mucosal epithelium (48, 50, 51). Moreover, mucin, which is abundant in most external secretions, binds SIgA and thereby enhances entrapment of SIgA-coated bacteria in the mucus layer (52, 53). In agreement with this are previous studies demonstrating that actively secreted IgA plays an important role in cross-protection against variant virus infections in the upper respiratory tract (25, 54).

Lung mononuclear cells from immunized pIgR knockout mice, after BCG challenge, displayed a significant reduction in production of both IFN-\(\gamma\) and TNF-\(\alpha\). This defect in the mycobacterium-induced pro-inflammatory immune response in the pIgR\(^{-/-}\) mice is in agreement with the high bacterial load found in the same mice. We have previously found that IgA\(^{-/-}\) mice have an impaired T\(_\text{h}1\) immune response after BCG infection, with reduced production of IFN-\(\gamma\) and TNF-\(\alpha\) (23). It could possible be due to lack of IgA binding to Fc\(\gamma\)Rs and subsequent activation of the receptor-bearing cell. We are currently conducting experiments to assess the mechanistic details behind the impaired pro-inflammatory response found in both IgA-, and plgR-deficient mice. Noteworthy is a recent study reporting an immunomodulatory role for SIgA in the gastrointestinal tract (55). Luminal to subepithelial transported SIgA was able to stimulate a mucosal immune response via its activating action on dendritic cells.

Evaluation of natural resistance to virulent \(M.\) tuberculosis infection demonstrated that plgR knockout mice were significantly more susceptible to infection during the early stage compared with wild-type mice. No difference between the two mouse strains was seen at the later stage of infection, indicating that SIgA plays its major role during early infection. In agreement with our findings is the study by Quan et al. (56) showing the presence of natural polyreactive antibodies of the SIgA isotype in human colostrums and saliva. The reactivity of these antibodies with a large number of bacterial antigens suggests involvement in protection against pathogens.

The impaired control of \(M.\) tuberculosis growth in the plgR-deficient mice correlated with markedly reduced mRNA levels of several protective factors in the lungs. Expression of the chemokine RANTES was significantly lower in plgR\(^{-/-}\) mice during early infection when compared with wild-type mice.

---

**Fig. 4.** Histological analysis of \(M.\) tuberculosis-infected mice. (A) Granulomatous infiltration in the lungs of \(M.\) tuberculosis-infected mice was assessed by analyzing two lung lobes from each mouse \((n = 4)\). Data are expressed as percentage, calculated by dividing the granuloma-involved area by the total tissue area. A representative of two different experiments is shown. **\(^{**}\) \(P < 0.01\) versus wild-type mice. (B) and (C) Histopathological analysis of lung sections from \(M.\) tuberculosis-infected mice 3 weeks post-infection. The representative sections shown are from infected wild-type (B) and infected plgR\(^{-/-}\) (C) mice. Photomicrographs from granulomas were taken at a magnification of \(\times 400\), with scale bar 200 \(\mu\)m. Labels indicate macrophages (arrow), lymphocytes (diamond), neutrophils (u) and karyorrhexis (asterisk).
Since RANTES is important for attraction of monocytes and lymphocytes to sites of infection (34–36), lower numbers of those cell populations could be expected in the lungs of the plgR-deficient mice. Support for this comes from histological analysis that revealed differences between the wild-type and knockout mice, with lower numbers of macrophages and lymphocytes in the pregranulomas of the knockout mice at week 3 post-infection. Impaired attraction of these immune cells could then affect the protective immunity against infection. In accordance with this, our results revealed reduced expression of the pro-inflammatory cytokines IFN-γ and TNF-α in the lungs of infected plgR−/− mice early during infection. IFN-γ activates macrophages, leading to iNOS expression and consequently production of nitric oxide and other reactive nitrogen intermediates, important for bactericidal capacity of macrophages (57). Indeed, the higher susceptibility to infection seen in plgR−/− mice was associated with a significant reduction of iNOS mRNA levels in the lungs. Therefore, plgR−/− mice initially display lower expression of factors important for a proper granuloma formation and protective immunity against tuberculosis, which most likely leads to an impaired control of M. tuberculosis growth in the lungs. However, the mechanisms behind the impaired expression of these protective molecules in the lungs of plgR knockout mice remain to be elucidated. The higher infiltration and number of granulomas found in the knockout mice at the later stage of infection is most likely a consequence of the higher bacillary bulk detected in the same mice at the early time point, and subsequently higher dissemination rate through the parenchyma. Thus, a higher cellular attraction is triggered and a greater inflammatory response is required to control this initial dissemination in the knockout mice. Interestingly, human SC has been demonstrated to bind and inactivate the chemokine IL-8 (47), which is important for chemotaxis of neutrophils. No rodent homologue for human IL-8 has been identified, although a murine IL-8R homologue has been reported (58), and mice deficient in this receptor display impaired neutrophil responses and reduced leukocyte migration (59–61). If the chemokines binding to the murine IL-8R homologue display similar binding properties to the SC, the possibility of higher activity of these chemokines in mucosal secretions from plgR−/− mice arises. This in turn could result in increased chemotaxis of neutrophils in the knockout mice. In our study, we could observe an increased infiltration of neutrophils in the knockout mice compared with wild-type mice during early infection.

Collectively, our results demonstrate that the plgR plays a role for protection against mycobacterial infection in the respiratory tract. SIgA could act by antigenic specificity, in addition to polyreactive binding to the bacteria, and blocking the entrance of the bacilli into the lungs. In addition, our data point to a role for the SC, alternatively SIgA, in modulation of mycobacteria-induced pro-inflammatory immune responses and subsequently for protection against mycobacterial infections.

![Graph of mRNA levels of protective substances in M. tuberculosis-infected plgR−/− mice.](https://academic.oup.com/intimm/article-abstract/18/5/807/2950967)

Fig. 5. Reduced mRNA levels of protective substances in M. tuberculosis-infected plgR−/− mice. mRNA from the lungs of mice infected with M. tuberculosis was prepared and quantified by real-time PCR. Each expression level is normalized to HPRT mRNA expression. The mean ± SEM of four to five individual mice is shown. *P < 0.05, **P < 0.01 versus wild-type mice. A representative of two different experiments is shown.
Acknowledgements

A. Tjärnlund and A. Rodríguez shared first authorship. We are grateful to P. Marsh and A. Williams for providing the BCG and for insightful comments and suggestions about this work. We thank A. Salerno, F. Dieli, R. Reljic, J. Tree and M. Comini for helpful suggestions and discussions and E. Julián for critical reading of the manuscript. We are also grateful to M. Nanno for providing the pIgR−/− mice. This work was financially supported by the European Commission specific RTD program QLK2-CT-1999-00367 and Hjärt-Lungfonden.

Abbreviations

BAL bronchoalveolar lavage
BCG Mycobacterium bovis bacillus Calmette-Guérin
CFU colony-forming unit
CT cholera toxin
HPRT hypoxanthine guanine phosphoribosyl transferase
i.n. intra-nasal
iNOS inducible nitric oxide synthase
OD optical density
pIGR polymeric IgR
RANTES regulated upon activation normal T cell sequence
SIgA secretory IgA
SC secretory component
TNF-α tumor necrosis factor-alpha

References

Mycobacterial infection in pIgR−/− mice


53 Magnusson, K. E. and Stjernström, I. 1982. Mucosal barrier mechanisms. Interplay between secretory IgA (SIgA), IgA and mucins on the surface properties and association of salmonellae with intestine and granulocytes. Immunology 45:239.


