

# Innate secretory antibodies protect against natural *Salmonella typhimurium* infection

Odilia L.C. Wijburg,<sup>1,2</sup> Tania K. Uren,<sup>1</sup> Kim Simpfendorfer,<sup>1,2</sup> Finn-Eirik Johansen,<sup>3</sup> Per Brandtzaeg,<sup>3</sup> and Richard A. Strugnell<sup>1,2</sup>

<sup>1</sup>Cooperative Research Center for Vaccine Technology and <sup>2</sup>Australian Bacterial Pathogenesis Program at the Department of Microbiology and Immunology, The University of Melbourne, VIC 3010, Australia

<sup>3</sup>Laboratory for Immunohistochemistry and Immunopathology, Institute of Pathology, Rikshospitalet, N-0027 Oslo, Norway

The production of IgA is induced in an antigen-unspecific manner by commensal flora. These secretory antibodies (SABs) may bind multiple antigens and are thought to eliminate commensal bacteria and self-antigens to avoid systemic recognition. In this study, we addressed the role of "innate" SABs, i.e., those that are continuously produced in normal individuals, in protection against infection of the gastrointestinal tract. We used polymeric immunoglobulin receptor (pIgR<sup>-/-</sup>) knock-out mice, which are unable to bind and actively transport dimeric IgA and pentameric IgM to the mucosae, and examined the role of innate SABs in protection against the invasive pathogen *Salmonella typhimurium*. In vitro experiments suggested that innate IgA in pIgR<sup>-/-</sup> serum bound *S. typhimurium* in a cross-reactive manner which inhibited epithelial cell invasion. Using a "natural" infection model, we demonstrated that pIgR<sup>-/-</sup> mice are profoundly sensitive to infection with *S. typhimurium* via the fecal-oral route and, moreover, shed more bacteria that readily infected other animals. These results imply an important evolutionary role for innate SABs in protecting both the individual and the herd against infections, and suggest that the major role of SABs may be to prevent the spread of microbial pathogens throughout the population, rather than protection of local mucosal surfaces.

One of the most characteristic features of the mucosal immune system of most mammals is the dominant presence of SABs, particularly secretory IgA (SIgA), an antibody class unique to the mucosae. At least 80% of all Ig-producing plasma cells in the body are found in the intestinal lamina propria, and most of these cells produce polymeric IgA (pIgA; mainly as dimers) (1, 2). Both pIgA and pentameric IgM are actively transcytosed across the secretory epithelium that lines the mucosal surfaces to external secretions after binding to the polymeric Ig receptor (pIgR), a glycoprotein expressed on the basolateral surface of secretory columnar and crypt epithelial cells, also called membrane secretory component (3–6). Some 40 mg SIgA kg<sup>-1</sup> body weight is transported to the gut lumen by the pIgR every day in a healthy adult human (7). The development of the gastric-associated lymphoid tissue (GALT)

and the production of IgA is initiated by colonization of the gut with commensal organisms (8). Neonates, in which SIgA antibodies are barely detectable, depend on maternal IgG transferred through the placenta, and a supply of SABs (mainly SIgA) from breast milk providing passive immunization of the gut. Similarly, colonization of germ-free mice with commensal bacteria stimulates the development of an otherwise undeveloped immune system and results in the production of IgA (9). Monoassociation of germ-free mice with commensal microbes demonstrated that ≥85% of IgA produced by these animals was not reactive with the colonizing bacterium, and this IgA is referred to as natural IgA or innate IgA (9–11). The origin, requirements for production, and the specificity of innate IgA are the subject of ongoing debate (11–13). It has been shown that innate IgA originates from both conventional B2 B cells and CD5<sup>+</sup> B1 B cells (11, 14, 15). B1 cells, unlike B2 cells, do not require cognate interaction with CD4<sup>+</sup> T cells and germinal center reactions in Peyer's patches (PP), but are thought to depend on "bystander"

## CORRESPONDENCE

Richard A. Strugnell:  
rastru@unimelb.edu.au

Abbreviations used: GALT, gastric-associated lymphoid tissue; MDCK, Madine-Darby canine kidney; pIgA, polymeric IgA; pIgR, polymeric immunoglobulin receptor; PP, Peyer's patches; SAB, secretory antibody; SIgA, secretory IgA.

OL.C. Wijburg and T.K. Uren contributed equally to this work. T.K. Uren's present address is Lab. of Hepatitis Viruses, Div. of Viral Products, Center for Business and Economic Research/Food and Drug Administration, Bethesda, MD.

CD4<sup>+</sup> T cell help in the form of cytokines (IL-5, IL-6, IL-10) only to produce IgA (12, 14). However, a report by MacPherson et al. demonstrated that IgA responses to commensal bacteria occur in specific pathogen-free TCR- $\beta^{-/-}\delta^{-/-}$  mice, suggesting that a CD4<sup>+</sup> T cell-independent pathway for production of IgA may exist (13). B1 cells may be stimulated in an antigen-specific manner through the BCR (13), but considering the large proportion of total IgA that does not bind the commensal bacterium that was used to colonize monoassociated germ-free mice, it is more likely that polyclonal stimulation of B1 cells, through for instance Toll-like receptors, induces production of innate IgA (10, 11, 16).

The specificity of innate IgA that is secreted into the mucosal lumen is largely unknown, although SIgA present in intestinal washings and saliva has been shown to react with commensal bacteria and autoantigens (13, 17, 18). Because of its ability to bind multiple antigens, innate IgA has been referred to as polyreactive (19, 20). A recent study, however, has shown a restricted use of V<sub>H</sub> genes that harbor somatic mutations by IgA-producing plasma cells in the gut, suggesting that although the IgA repertoire may be restricted and not driven by affinity maturation the use of such V<sub>H</sub> genes would generate antibodies with a distinct specificity (21).

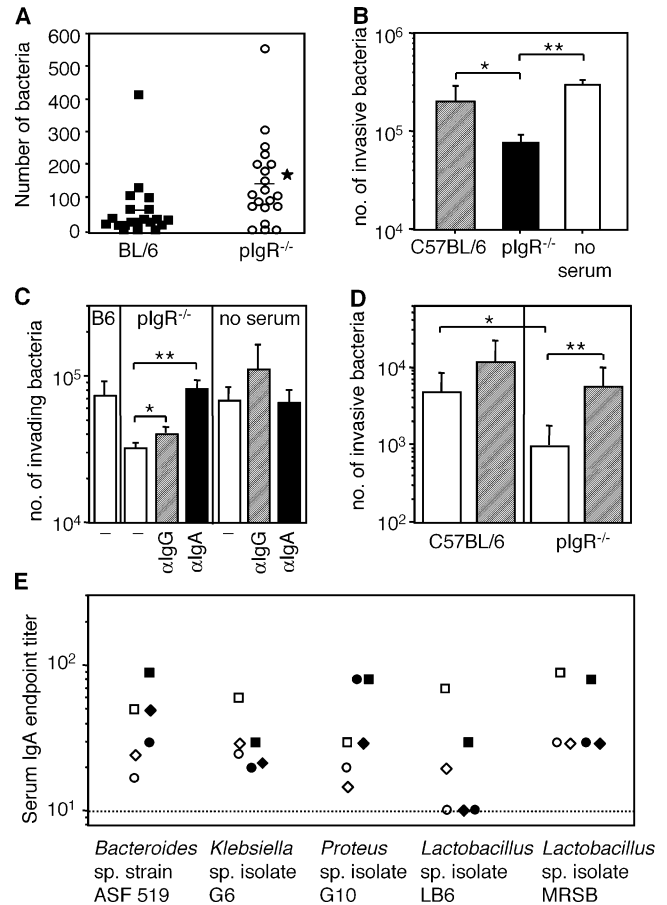
It has been suggested that SAb specific for commensal organisms function by immune exclusion, preventing the translocation of luminal flora and induction of systemic immune reactions (1, 2, 22). However, a role for innate SAb in protection of the gut against pathogenic organisms is less clear. In this study, pIgR<sup>-/-</sup> mice, which are unable to bind and actively transport dimeric IgA and pentameric IgM to the mucosae (5, 6), were used to examine the role of innate SAb in protection against the invasive gastrointestinal pathogen *Salmonella typhimurium*.

## RESULTS AND DISCUSSION

### Innate SAb inhibit epithelial cell invasion by *S. typhimurium*

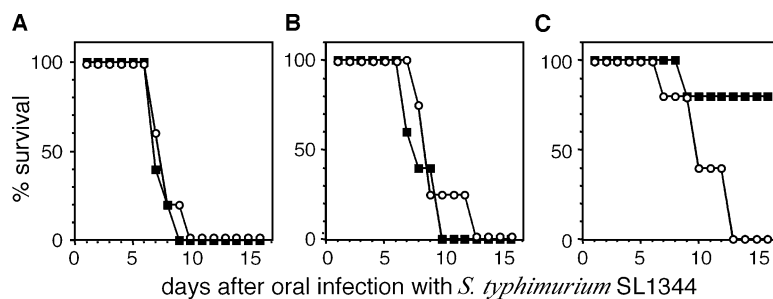
*S. typhimurium* is an invasive enteric pathogen that preferentially uses the M cells of the PP as portals of entry, after which the bacteria spread to the mesenteric LN and in mice, disseminate to cause a systemic disease (23). We investigated whether innate SAb block invasion of the gut epithelium by comparing the number of bacteria detected in the PP and small intestinal mucosa of B6 and pIgR<sup>-/-</sup> mice 6 or 12 h after oral infection with 10<sup>7</sup> CFUs *S. typhimurium*. The combined results of three independent experiments showed that *S. typhimurium* invasion is significantly increased (P = 0.011) in pIgR<sup>-/-</sup> mice (137 ± 129) compared with B6 mice (59 ± 88) (Fig. 1 A).

In pIgR<sup>-/-</sup> mice, pIgA destined for secretion into the mucosal lumen accumulates in the blood, resulting in 100-fold greater serum levels than in B6 mice, whereas IgG and IgM concentrations are similar (5, 6). The use of pIgR<sup>-/-</sup> serum allowed us to examine the interaction between “accumulated” innate SIgA in its dimeric form (6) and *S. typhimurium*. We used an in vitro invasion assay to demonstrate that serum



**Figure 1. Innate IgA inhibits *S. typhimurium* invasion.** (A) Naive mice were orally infected with 10<sup>7</sup> CFUs *S. typhimurium*. Shown are the numbers of *S. typhimurium* detected in PP 6 or 12 h after oral infection, or in ileal mucosa 12 h after infection. Presented are the combined results of three independently performed experiments. \*P = 0.011. (B) An in vitro invasion assay was used to determine the ability of serum from naive B6 (hatched columns) or pIgR<sup>-/-</sup> mice (black columns) to inhibit *S. typhimurium* invasion. Presented is the mean invasion ± 1 SD of triplicate cultures of one representative out of three performed experiments. \*P = 0.04; \*\*, P < 0.001. (C) Goat anti-mouse IgG (hatched columns), goat anti-mouse IgA (black columns), or culture medium (white columns) was added to serum from pIgR<sup>-/-</sup> mice before use in an in vitro invasion assay. As controls, B6 serum or goat antibodies added to the assay in the absence of serum was used. Presented is the mean invasion ± 1 SD of triplicate cultures. \*, P = 0.4; \*\*, P = 0.01. (D) Normal (white bars) and preabsorbed serum (hatched bars) from B6 or pIgR<sup>-/-</sup> mice (pools of three) was assayed for its ability to block invasion of MDCK cells by *S. typhimurium*. Shown is the mean invasion ± 1 SD of quadruplicate cultures of one representative out of six independently performed experiments. \*, P = 0.04; \*\*, P = 0.01. (E) ELISA was used to determine the IgA titers specific for gut microflora in normal (white bars) and preabsorbed (hatched bars) serum from pIgR<sup>-/-</sup> mice. Symbols represent serum pools formed from three or four mice (n = 10 in total). The dotted line indicates the detection limit of the assay.

from pIgR<sup>-/-</sup> mice blocked Madine-Darby canine kidney (MDCK) epithelial cell invasion by *S. typhimurium* (Fig. 1 B). After preincubation of the MDCK cells with serum obtained



**Figure 2.** The *S. typhimurium* LD<sub>50</sub> is reduced in pIgR<sup>-/-</sup> mice. Groups of five naive B6 (■) or pIgR<sup>-/-</sup> mice (○) were orally infected with either

10<sup>6</sup> (A), 10<sup>5</sup> (B), or 10<sup>4</sup> (C) CFUs *S. typhimurium*. For ethical reasons, mice were killed when they became moribund; these mice were deemed "nonsurvivors."

from naive pIgR<sup>-/-</sup> mice, a significantly reduced number of invaded *S. typhimurium* was detectable ( $7.6 \times 10^4 \pm 1.5 \times 10^4$ ) compared with the effect of serum from naive B6 mice ( $2 \times 10^5 \pm 9 \times 10^4$ ;  $P = 0.04$ ) or with no serum control ( $3 \times 10^5 \pm 3 \times 10^4$ ;  $P < 0.001$ ). Similar results were obtained with heat-inactivated serum (unpublished data), suggesting that IgG antibodies inducing complement activation did not contribute to the obtained results. The inhibitory effect of pIgR<sup>-/-</sup> serum was abolished by the addition of goat anti-mouse IgA antibodies but not by goat anti-mouse IgG antibodies, suggesting that IgA in pIgR<sup>-/-</sup> serum was inhibiting invasion of MDCK cells by the bacteria (Fig. 1 C).

The production of IgA is induced in an antigen-unspecific manner by commensal flora (9–11). Although some reports have suggested that SIgA is polyreactive in nature, other findings point to a restricted specificity that may be cross-reactive (13, 17–19, 21, 24). In further experiments, we examined the reactivity of dIgA in pIgR<sup>-/-</sup> serum with *S. typhimurium*. Serum samples were preabsorbed against *S. typhimurium* by overnight incubation at 4°C, which resulted in a nonsignificant reduction in the amount of total IgA in the samples ( $6.13 \pm 1.6$  mg/ml versus  $4.31 \pm 0.85$  mg/ml in preabsorbed serum). Fig. 1 D shows that compared with untreated pIgR<sup>-/-</sup> serum, preabsorbed pIgR<sup>-/-</sup> serum was no longer able to effectively inhibit invasion of MDCK cells, suggesting that a small proportion of total pIgA was able to bind *S. typhimurium* and block host cell invasion.

In previous studies, we characterized the composition of the ileal flora of pIgR<sup>-/-</sup> mice, which was similar to that of B6 mice (25). Subsequent studies demonstrated that pIgR<sup>-/-</sup> have increased levels of serum IgA specific for members of the gut flora (unpublished data). To further investigate the polyreactive nature of pIgA in serum from pIgR<sup>-/-</sup> mice, isolated species of normal gut flora were cultured (25; unpublished data) and used as antigens in an ELISA to measure binding of normal pIgR<sup>-/-</sup> serum and pIgR<sup>-/-</sup> serum preabsorbed against *S. typhimurium*. Fig. 1 E shows that after preabsorption binding of pIgR<sup>-/-</sup> serum to flora isolates was either increased or not altered.

In contrast to findings by Bouvet et al. (19, 24), preabsorption of pIgR<sup>-/-</sup> serum with *S. typhimurium* only removed a small fraction of the total IgA and did not reduce the bind-

ing to other commensals. SIgA forms large multivalent complexes with mucins and/or pFv after secretion (26), which may affect the quantitative analysis of binding of SIgA to antigens. Whereas we used serum samples containing dIgA, Bouvet et al. used samples of saliva and gut washings containing large aggregates of SIgA, and preabsorbing to a cross-reactive antigen may therefore have resulted in removal of a large proportion of IgA. Collectively, our results suggest that pIgA in serum of pIgR<sup>-/-</sup> mice is not polyreactive but restricted in nature, and some of the innate pIgA antibodies are able to bind *S. typhimurium* in a cross-reactive manner. Further investigations which are beyond the scope of this paper, e.g., analysis of IgV<sub>H</sub> gene usage and the use of Ig allotype chimeric mice (15), may provide more insight into the exact nature and origin of the dIgA that binds *S. typhimurium*.

#### pIgR<sup>-/-</sup> mice are more susceptible to infection with *S. typhimurium*

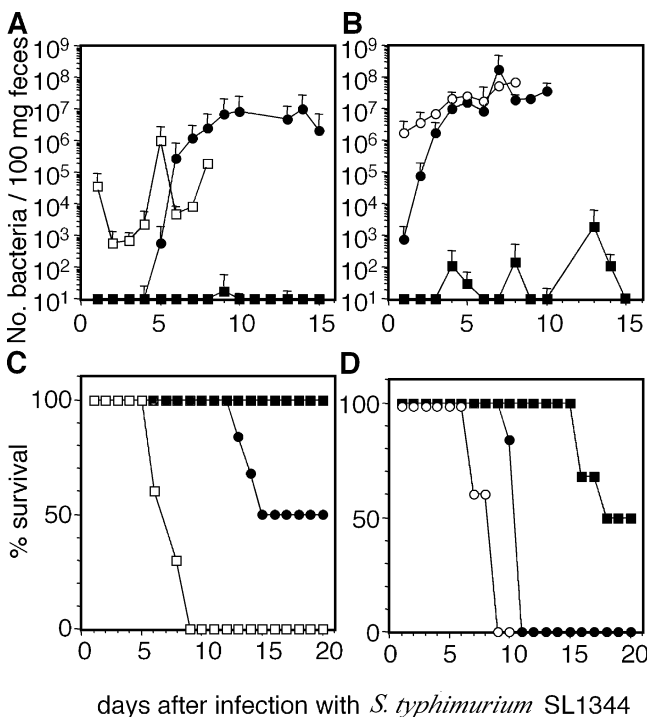
We further investigated whether the increased microbial invasion in vivo as a result of lack of innate SAb augmented susceptibility of pIgR<sup>-/-</sup> mice to infection with virulent *S. typhimurium*. By orally infecting naive mice with decreasing doses of *S. typhimurium* and monitoring their survival, we first determined whether the lack of SAb in pIgR<sup>-/-</sup> mice renders these animals more sensitive to infection with virulent bacteria than wild-type B6 mice (Fig. 2). At an oral dose of 10<sup>6</sup> or 10<sup>5</sup> CFUs, pIgR<sup>-/-</sup> and B6 mice were equally susceptible to infection. However, infection of pIgR<sup>-/-</sup> mice with 10<sup>4</sup> CFUs *S. typhimurium* resulted in 100% mortality, whereas this dose was lethal in only 20% of B6 mice. Although this difference in mortality of pIgR<sup>-/-</sup> mice compared with B6 mice was demonstrated using a low inoculation dose (Fig. 2), the in vivo invasion experiments (Fig. 1 A) were performed with a higher infectious dose to enable recovery of intracellular bacteria within 6 h after infection from the gut tissues (27). Collectively though, these results strongly suggest that innate SAb protect mice against pathogenic bacterial invasion.

#### Innate SAb reduce spread of *S. typhimurium* during fecal-oral transmission

We subsequently wished to mimic the natural fecal-oral route of transmission of *S. typhimurium* infection by cohousing

orally infected mice with naive animals. These experiments were performed with three cages in which one orally infected B6 mouse was cohoused with two naive B6 and two naive pIgR<sup>-/-</sup> mice, and with three cages in which one orally infected pIgR<sup>-/-</sup> mouse was cohoused with two naive B6 and two naive pIgR<sup>-/-</sup> mice. B6 mice infected with *S. typhimurium* shed  $4 \times 10^4$  ( $\pm 5 \times 10^4$ ) bacteria 1 d after infection; this increased to  $2 \times 10^5$  ( $\pm 700$ ) bacteria at day 8 (Fig. 3 A). One out of three orally infected B6 mice was shedding significantly higher numbers of bacteria on day 5 after infection before it became moribund and was killed, resulting in an increased average bacterial count ( $10^6 \pm 1.8 \times 10^6$ ) in the feces on this day. All infected B6 mice had succumbed to the infection by day 9 after oral inoculation (Fig. 3 C).

Only one out of the six naive B6 mice cohoused with the orally inoculated B6 mice acquired the infection and had detectable bacteria in its feces on day 9 but none of them died (Fig. 3 C). Conversely, three out of six naive pIgR<sup>-/-</sup> mice cohoused with the orally infected B6 mice started shedding the *S. typhimurium* at day 5 after infection, and the number of bacteria shed in the feces rapidly increased to  $\sim 8 \times 10^6$  bacteria around day 9 after infection (Fig. 3 A); these mice all became moribund by day 15 (Fig. 3 C).



**Figure 3. Naive pIgR<sup>-/-</sup> mice are susceptible to "natural" infection with virulent *S. typhimurium*.** One B6 mouse (□, A) or one pIgR<sup>-/-</sup> mouse (○, B) was orally inoculated with *S. typhimurium* and cohoused with naive B6 mice (■) and naive pIgR<sup>-/-</sup> mice (●). The course of the infection in all mice ( $n = 3$ , orally inoculated mice;  $n = 6$ , naive mice) was followed by measuring the number of bacteria shed in feces. Mice were killed when they became moribund, and the survival was recorded (C and D). The experiment was performed with three cages for each strain of mice, and the data represent the mean  $\pm$  1 SD.

The number of bacteria detected in feces of orally inoculated pIgR<sup>-/-</sup> mice (Fig. 3 B) was at any time point significantly ( $P < 0.01$ ) higher than that in feces of B6 mice (Fig. 3 A), starting at  $2 \times 10^6$  ( $\pm 2 \times 10^5$ ) bacteria one day after infection, and increasing to  $\sim 6.5 \times 10^7$  ( $\pm 2 \times 10^6$ ) bacteria on day 7 and 8 after infection (Fig. 3 B), at which point the animals had all been killed (Fig. 3 D). This suggested that in the absence of SAbs, the number of replicating *S. typhimurium* was dramatically increased.

Naive pIgR<sup>-/-</sup> mice cohoused with the *S. typhimurium*-infected pIgR<sup>-/-</sup> mice rapidly acquired the infection and were shedding bacteria in their feces as early as 1 d after being cohoused with the orally inoculated pIgR<sup>-/-</sup> mice. After 5 d of cohousing, the naive pIgR<sup>-/-</sup> mice were shedding  $>10^7$  bacteria (Fig. 3 B), and by day 11 all had succumbed to the infection (Fig. 3 D). Interestingly, naive B6 mice cohoused with orally infected pIgR<sup>-/-</sup> mice were also more sensitive to the infection compared with cohousing with orally infected B6 mice, suggesting that the lower number of bacteria shed from B6 mice less readily infected other animals compared with bacteria shed from pIgR<sup>-/-</sup> mice. *S. typhimurium* was detectable in feces of three out of six B6 mice cohoused with infected pIgR<sup>-/-</sup> mice (Fig. 3 B), and the infection was lethal for these mice (Fig. 3 D).

It has been demonstrated that circulating natural IgM antibodies can protect against pathogenic microorganisms (28). Natural (or innate) SIgA production is induced by colonization with commensal flora (9–12), and SAbs are thought to act by "immune exclusion" or "immune elimination" (1, 2, 19, 22). Because of its ability to bind multiple antigens, whether in a restricted or polyreactive manner, binding of innate dIgA to commensal flora or autoantigens and subsequent removal from the lamina propria via transport by the pIgR may prevent systemic recognition and undesirable pathological immune responses. In support of this hypothesis is the finding that SIgA in breast milk is reactive with members of the mother's gut microbiota that were present in the third trimester and at birth, providing the neonate protection against commensal organisms while the GALT is undeveloped (8).

In line with this concept, our studies show that the large amounts of innate SAbs normally actively exported into the gut lumen by the pIgR provide a primitive type of front line defense against invading bacterial pathogens in the naive host. Our data demonstrate for the first time that, in the absence of SAbs, increased numbers of *S. typhimurium* invade the gastrointestinal epithelium, suggesting that innate SAbs normally present in the gut block bacterial adhesion and penetration. Indeed, our in vitro experiments verified that serum obtained from naive pIgR<sup>-/-</sup> mice, which contains pIgA that is normally destined for secretion to the mucosae (5, 6), binds to *S. typhimurium* in a cross-reactive manner and inhibits the invasion of MDCK cells by *S. typhimurium*.

More importantly, however, our study shows that the innate SAbs may also reduce the shedding of pathogens and the spread of infections to other individuals which may not only

be because of increased numbers of bacteria, but could also be the result of the absence of a SAB “coat” on the excreted bacteria, which may normally prevent the bacteria from invading a new host (9, 29, 30). Therefore, we propose that although innate SABs may protect locally at mucosal surfaces their major role may be to prevent the spread of pathogens throughout the population. This may explain why the immune system has evolved to produce much larger amounts of IgA than any other Ig class, and why such large amounts of SABs are continuously transported across the mucosae into external secretions and breast milk.

In conclusion, our data demonstrates that the innate SAB system, which in evolutionary terms is considered a primitive front line defense against induction of autoimmunity and invasion by microbial pathogens (19), is crucial for both the individual host and its herd. This finding has important implications for the development of novel immunotherapeutics designed to protect against pathogens that invade via the mucosae, which are often devised to activate the production of antigen-specific SIgA while ignoring the protective roles of innate SABs.

## MATERIALS AND METHODS

**Animals.** pIgR<sup>-/-</sup> mice were generated on a pure C57BL/6 (B6) genetic background as previously described (6). All mice were bred and reared under conventional conditions at the animal facility of The University of Melbourne, Department of Microbiology and Immunology. Mice were age and sex matched for each experiment, and used at 6–8 wk of age. All animal experiments were approved by The University of Melbourne Animal Ethics and Experimentation Committee, and complied with the Prevention of Cruelty to Animals Act (1986) and the National Health and Medical Research Council (NHMRC) Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (1997).

**Oral infection of mice with *S. typhimurium*.** In this study, mice were orally infected with the virulent *S. typhimurium* strain SL1344 as described before (27).

**Natural *S. typhimurium* infection model.** To mimic the natural fecal-oral route of *S. typhimurium* transmission, a single mouse was infected with 10<sup>7</sup> CFU *S. typhimurium* SL1344 by oral gavage and placed in a cage with naive animals. The acquisition and development of *S. typhimurium* infection in the naive animals was monitored by detection of *S. typhimurium* in feces. Absence of fecal *S. typhimurium* was confirmed before the start of each experiment in all animals.

**Bacterial colonization of organs.** The number of live *S. typhimurium* in PP or entire small intestines was determined by plating serial dilutions of the homogenates on LB agar plates containing streptomycin, as described previously (27).

Shedding of *S. typhimurium* was determined by counting the number of bacteria in fresh fecal extracts. Individual mice were placed in clean boxes, and the collected fecal pellets were weighed, dissolved in sterile PBS (100 mg/ml), and vortexed vigorously. Serial dilutions were plated on selective indicator media (DCA and MacConkey agar plates containing streptomycin) to enumerate the number of *S. typhimurium* in the samples.

**Invasion assays.** Invasion of MDCK cells by *S. typhimurium* SL1344 was determined as described before (27). In brief, monolayers of MDCK cells were infected with mid-log growth phase *S. typhimurium* at multiplicity of infection of 10. Serial dilutions of serum, pooled from three naive B6 or

pIgR<sup>-/-</sup> mice, were added to the MDCK cells 5 min before addition of the bacteria. After 1 h, MDCK cells were washed and incubated for 1.5 h with culture media containing 100 µg/ml gentamicin to kill extracellular bacteria. After this period, cells were lysed with 0.1% (wt/vol) Triton X-100 in PBS, and the number of bacteria present in the lysates was determined by viability count. Preabsorbed serum was prepared by incubating the serum with *S. typhimurium* SL1344 for 12 h at 4°C while rotating, after which the bacteria were removed by centrifugation. In some experiments, 1 mg of goat anti-mouse IgG or goat anti-mouse IgA (Sigma-Aldrich), diluted in culture media, was mixed with 200 µl serum before use in the assay.

**Statistical analysis.** The nonparametric two-tailed Mann-Whitney U test was used for statistical analysis of the results. Differences were considered significant when  $P < 0.05$ .

This work was carried out with the financial support of the Australian Government's Cooperative Research Center Program and the NHMRC. O.L.C. Wijburg is a NHMRC P. Doherty Research Fellow.

The authors have no conflicting financial interests.

Submitted: 17 October 2005

Accepted: 2 December 2005

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