B1b lymphocyte-derived antibodies control *Borrelia hermsii* independently of opsonophagocytosis

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Running title: Bactericidal IgM produced by B1b cells

Key words: B1b cells, IgM, Complement, *Borrelia hermsii*, Fcα/μR

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Abstract

The critical role of IgM in controlling pathogen burden has been demonstrated in a variety of infection models. In the murine model of *Borrelia hermsii* infection, IgM is necessary and sufficient for the rapid clearance of bacteremia. Convalescent, but not naïve, B1b cells generate an IgM-mediated protective response against *B. hermsii*, but the mechanisms of control are unknown. Here we show that neither Fcα/μR, a high-affinity receptor for IgM that mediates opsonophagocytosis, nor IgM-dependent complement-activation were required for controlling *B. hermsii*. Bacteria in diffusion chambers were killed when chambers were implanted either into convalescent or passively immunized mice. Furthermore, adoptively transferred convalescent B1b cells produced IgM that killed bacteria in diffusion chambers. These results demonstrate that clearance of *B. hermsii* does not require opsonophagocytosis and suggest that a mechanism for *in vivo* B1b cell-mediated protection is through the secretion of bactericidal IgM.

Introduction

IgM is the first antibody produced during immune responses, and thus it is important for the early control of infection. Natural IgM is generated spontaneously in the absence of antigenic stimulation and can contribute to early defense by limiting the initial pathogen burden [1, 2]. IgM is also generated in an antigen-specific manner during the early phases of the immune response to specific pathogens. Natural IgM as well as antigen-induced IgM has been demonstrated in protective immunity to a number of pathogens, such as *Streptococcus pneumoniae* and influenza virus, underscoring the non-redundant roles of natural and antigen-induced IgM [3, 4].

The vital role of IgM in the rapid control of bacteremia due to infection with relapsing fever bacteria is well defined [5, 6]. To study the role of IgM in controlling bacteremia we have been utilizing the murine model of infection by *Borrelia hermsii*, a relapsing fever spirochete that causes recurrent episodes of high-level (~10^8 bacteria/ml blood) bacteremia associated with the outgrowth of individual bacterial serotypes [7, 8]. These serotypes are defined by the transient expression of antigenically distinct major surface proteins of *B. hermsii*, the variable major proteins (Vmps) [8]. Control of each wave of bacteremia is concurrent with the rapid production of specific IgM [9-14]. Mice that are deficient in the secreted form of IgM – but not other isotypes – exhibit persistent bacteremia that is identical to that found in mice that lack B cell development [14]. In contrast, mice deficient in activation-induced cytidine deaminase – which produce only IgM but no other antibody isotypes – control infection indistinguishably from wildtype mice [15]. These results demonstrate that IgM is required and sufficient for the resolution of *B. hermsii* bacteremia.

Concurrent with the resolution *B. hermsii* infection there is an expansion of B1b lymphocytes (IgM^{hi}, IgD^{lo}, $CD23^{-}$, $CD5^{-}$, $Mac1^{+}$), a minor subset of mature B cells that are abundant in the peritoneal cavity [14, 15]. Reconstitution of immunodeficient *Rag1*$^{-/-}$ mice (lacking T and B cells) with expanded B1b cells from convalescent mice confers complete immunity, whereas reconstitution with naive B1b cells provides only partial protection, demonstrating that convalescent B1b cells have acquired immunological memory [15]. Like conventional memory B cells, the reconstituted population persists but does not spontaneously secrete *B. hermsii*-specific antibodies; rather they maintain quiescence. Upon a subsequent exposure to antigen, however, memory-like B1b cells rapidly differentiate into antigen-specific IgM secreting plasma cells, resulting in a specific antibody response that is faster and to a greater magnitude than that of naïve B1b cells [15]. Although the lymphocyte subsets that respond to *B. hermsii* and FhBA, a surface protein of *B. hermsii* that is recognized by B1b cell-derived IgM has been defined [15, 16], it is not clear how such IgM controls this infection *in vivo*.

The pentameric structure of IgM is known to efficiently induce the classical complement pathway (11). Despite this, $C3^{-/-}$ and $C5^{-/-}$ mice control relapsing fever Borrelia infection as efficiently as wildtype mice demonstrating that antigen-specific IgM eliminates its targets independently of complement [5, 13, 17, 18]. Although IgM is not typically known as an opsonin, the discovery of an Fc receptor (FcR) for IgM has suggested a role for IgM in opsonophagocytosis. The high-affinity Fcα/μR, which also binds the Fc region of IgA molecules, is expressed on macrophages and B cells as well as on non-hematopoietic tissues such as kidney and intestine [19-21]. Furthermore, Fcα/μR has been demonstrated to facilitate the phagocytosis

of IgM-coated *Staphylococcus aureus in vitro*, further suggesting its role in clearance of IgM-coated pathogens [19]. In support of this, specific antibody-coated *B. hermsii* bacteria are engulfed by neutrophils in a complement-independent manner, indicating that antibody-mediated opsonophagocytosis is a potential mechanism of clearance of *B. hermsii* [22]. Nevertheless, it is unknown whether Fcα/μR-mediated endocytosis plays a role in facilitating IgM-mediated control of pathogens such as *B. hermsii in vivo*.

In addition to promoting opsonophagocytosis and complement activation, antibodies of various isotypes have been shown to exert direct anti-bacterial activities. These antibodies appear to exert these effects possibly by catalyzing the production of oxidative molecules [23-25], interference with pathogen iron uptake [26, 27], or by directly damaging bacterial membrane [5, 18]. Among the IgM antibodies that have been described to be bactericidal, CB515 recognizes a Vmp expressed by a related relapsing fever Borrelia, lyses bacteria *in vitro*, and confers a partial protection in complement-deficient mice *in vivo* [18]. The scFv fragments of CB515 retain the lytic activity of the parent monoclonal, demonstrating that the bactericidal activity resides in the variable region of the antibody [28].

In this study, we examined the mechanism of B1b cell-derived antibody in control of *B. hermsii* infection *in vivo*. Here we show that IgM-mediated phagocytosis of *B. hermsii* is not required for control of infection. Using a diffusion chamber model to examine host-pathogen interactions, we demonstrate that B1b cells contribute to protective immunity by producing *B. hermsii*-specific antibodies that are directly bactericidal.

Materials and Methods

Mice. The Institutional Animal Care and Use Committee have approved these studies. Mice were housed in micro-isolator cages with free access to food and water, and were maintained in a specific pathogen-free facility at Thomas Jefferson University. C57BL/6J (B6), Balb/cJ, and C57BL/6J-Rag1^{tm1Mom}/J (Rag1$^{-/-}$) mice were purchased from The Jackson Laboratories (Bar Harbor, ME). Fcα/μR$^{-/-}$ mice were described previously [20]. C3-deficient mice on C57BL6 background (C3$^{-/-}$) were provided by Robert Eisenberg, University of Pennsylvania.

Infections and cobra venom factor treatment. Mice were infected intravenously (i.v.) via the tail vein with 5x10^4 bacteria of a fully virulent *B. hermsii* strain DAH-p1 (from the blood of an infected mouse), and the bacteremia was monitored by dark-field microscopy. To deplete C3, mice were treated i.p. with 30 μg (14.0 units) cobra venom factor (CVF) (Quidel, San Diego, CA) one day prior to infection and again on three days post infection as described [29]. This dose of CVF is sufficient to maintain C3 levels below 5% of normal levels for four days [30].

Passive immunizations. To obtain convalescent immune serum, blood was obtained via cardiac puncture from wildtype mice that had been infected (>60 days post infection) with *B. hermsii*. To initiate clotting, blood was pooled and incubated at 37°C for 1 hr. After clotting, blood was centrifuged at 1000 x *g*, and serum was collected and filtered through 0.22 μm PES membranes (Millipore, Billerica, MA). Passive immunizations were performed by injecting mice with 250 μl serum via the tail vein.

Enzyme-linked immunosorbent assay (ELISA). IgM levels were measured with ELISA kits according to the manufacturer's instructions (Bethyl Laboratories, Montgomery, TX). *B. hermsii*-specific IgM was determined by coating 96-well plates (ICN Biomedicals Inc., Aurora, OH) with *in vivo* grown *B. hermsii* DAH (10^5 wet bacteria/well). Plates were washed and blocked with 2% BSA in PBS pH 7.2 for 2 hr at room temperature. Blood samples of immunized mice were diluted 1:250 and samples were centrifuged (16000 x *g* for 10 min.) and supernatant was used. Bound IgM was measured using HRP-conjugated goat anti-mouse IgM. Specific antibody levels were interpreted as ng/μl equivalents using IgM standards.

Diffusion chambers. Mixed cellulose ester (MCE) membranes (0.05 μm pore size; Millipore) were adhered to lucite diffusion chamber rings (weight - 0.4 g; diameter - 14 mm; Width - 5 mm; Millipore) using cement as described previously [31]. Chambers were loaded with 200 μl of *in vivo*-adapted *B. hermsii* strain DAH in BSK-H (Sigma-Aldrich, St. Louis, MO) supplemented with 6% rabbit serum (Sigma-Aldrich) at 6.67 x 10^6 bacteria/ml (1.33 x 10^6 bacteria per chamber), and chambers were sealed. Mice were anesthetized with 3% isoflurane, and using aseptic technique, chambers were implanted in a subcutaneous pocket formed by making an incision of approximately 1.5 cm in the dorsal-anterior skin of each mouse. Chambers were removed after 24 hours, and contents were collected. Bacterial density in the chamber was measured by dark-field microscopy. Chamber contents were stored at -20°C until analyzed. In some experiments, mice were passively immunized with a) 250 μl convalescent immune serum or b) treated with 14.0 units CVF one day prior to implantation as described above.

B1b cell reconstitution. Rag1$^{-/-}$ mice were reconstituted with 10^5 MACS-purified B1b cells from convalescent (i.e., resolved *B. hermsii* DAH infection) mice as described [15]. Because *Rag1*$^{-/-}$ mice reconstituted with convalescent B1b cells do not generate a specific IgM response without bacterial stimulation, they were infected i.v. with 5 x 10^4 bacteria of the *B. hermsii* DAH ten days after adoptive transfer of B1b cells.

Statistical analysis. Statistics were performed using the Prism 5 software program (GraphPad Software, Inc., La Jolla, CA). To analyze statistical significance, two-tailed unpaired Student's *t*-test or two-way ANOVA were used as necessary.

Results

Clearance of *B. hermsii* does not require Fcα/μR-mediated opsonophagocytosis. $C3^{-/-}$ and $C5^{-/-}$ mice control relapsing fever infection, suggesting that other mechanisms such as opsonophagocytosis play a role in IgM-mediated control of *B. hermsii* [13]. Indeed, antibody has been shown to enhance opsonophagocytosis of *B. hermsii* by neutrophils *in vitro* [22]. Recently Fcα/μR, a specific receptor for IgM, has been shown to mediate opsonophagocytosis of IgM-coated *S. aureus* [19]. Since antibodies in particular IgM is critical for the protection against *B. hermsii*, to examine whether Fcα/μR plays a role in IgM-mediated opsonophagocytosis, we infected mice deficient in Fcα/μR. The magnitude and duration of *B. hermsii* bacteremia during the first episode in these mice were comparable to that of wildtype (Figure 1A). Moreover, passive immunization confers protection to $Fc\alpha/\mu R^{-/-}$ mice indicating that immune serum control *B. hermsii* in an Fcα/μR-independent manner (Figure 1B). Consistent with the critical role for IgM in control of this bacterium, $Fc\alpha/\mu R^{-/-}$ mice rapidly generated a *B. hermsii*-specific IgM response to a similar extent as in wildtype mice (Figure 1C). Although infection with *B. hermsii* does eventually elicit an IgG response, this does not occur until after the first wave of bacteremia is cleared. Thus, the IgM-mediated clearance of the first wave does not require Fcα/μR-mediated opsonophagocytosis.

Control of *B. hermsii* in the absence of Fcα/μR-mediated opsonophagocytosis is independent of C3. It is known that control of relapsing fever bacteremia occurs independently of the complement system, as bacterial clearance occurs in the absence of C3 and C5 [13]. These results suggest that complement either does not contribute to clearance or that other mechanisms such as opsonophagocytosis operate in a redundant fashion. To examine whether complement-

mediated lysis or complement-mediated opsonophagocytosis was plays a critical function in the absence of Fcα/μR-mediated opsonophagocytosis, we depleted C3 in wildtype and Fcα/μR$^{-/-}$ mice with cobra venom factor (CVF). One day prior to infection, mice were treated with 30 μg of CVF (14.0 units) i.p., which is sufficient to deplete C3 to less than 5% of normal levels for four days [29, 30]. At three days post-infection, mice were treated with the same dose to maintain C3 depletion. Despite a deficiency in both Fcα/μR and C3, the magnitude of the bacteremia was similar in CVF-treated and -untreated wildtype and Fcα/μR$^{-/-}$ mice (Figure 2). These results indicate that the effector mechanism of *B. hermsii*-specific IgM is neither C3- nor Fcα/μR-dependent.

Antibody-mediated control of *B. hermsii* does not require phagocytic cell contact. Since phagocytosis can be promoted by a variety of other mechanisms, we tested whether IgM-mediated *in vivo* control of *B. hermsii in vivo* requires cell contact. To test this, we adapted a diffusion chamber system originally devised to model the host-pathogen interaction during parasite infections [31]. The permeability of these chambers (0.05 μm) was large enough to allow free diffusion of IgM (300 Å) while restricting both the escape of *B. hermsii* (~0.25 x ~5.0 μm) and the entrance of host cells. Each chamber contained 1.33 x 10^6 *in vivo*-adapted *B. hermsii* spirochetes in bacterial growth medium. A schematic for the diffusion chamber model is represented in Figure 3A. After 24 hours in naïve mice, the number of bacteria in each chamber was approximately threefold higher than the initial number in each chamber. In contrast, the number of bacteria in chambers implanted in convalescent mice was nearly undetectable (Figure 3B).

The numbers of *B. hermsii* were also reduced significantly in the chambers implanted in passively immunized mice (Figure 3B). Interestingly, the degree of bacterial killing in these mice was to a lesser extent than what was observed in the chambers implanted in convalescent mice, suggesting that the amount of specific IgM present in the chamber had a direct effect on bacterial killing inside the chambers. Therefore, we examined specific IgM levels in the chambers taken from naïve mice, convalescent mice, and naïve mice that were given convalescent immune serum. As expected, we found that specific IgM had entered the chambers implanted in convalescent mice and passively immunized naïve mice. Furthermore, increasing amounts of specific IgM in each chamber inversely correlated with bacterial viability (Figure 3C). These results demonstrate that lysis of *B. hermsii* in diffusion chambers is IgM-dependent and does not require cell contact.

Killing of *B. hermsii* in diffusion chambers is C3-independent. As described above, IgM controls *B. hermsii* independent of C3 or Fcα/μR-mediated opsonophagocytosis. To test whether cell contact-independent killing of *B. hermsii* in diffusion chambers is also complement independent, we depleted C3 in naïve and convalescent wildtype mice with CVF one day prior to implantation of diffusion chambers. As expected, bacterial growth was unhindered in naïve mice regardless of whether complement was depleted (Figure 4). In contrast, there were no bacteria remaining in chambers removed from convalescent mice irrespective of the presence of C3 (Figure 4A).

To independently confirm that IgM is bactericidal *in vivo* independent of the complement system, we used mice deficient in both the alternative and classical complement pathways due to

a targeted deletion in C3. Diffusion chambers containing 1.33 x 10^6 *B. hermsii* bacteria were implanted into convalescent wildtype and $C3^{-/-}$ mice. In agreement with the above results, bacteria in diffusion chambers were killed in both wildtype and $C3^{-/-}$ mice (Figure 4B). These results demonstrate that C3 is not required for antibody-mediated lysis of *B. hermsii* in the diffusion chambers.

B1b cell-derived antibodies are sufficient to kill *B. hermsii* in diffusion chambers. Clearance of *B. hermsii* is T cell-independent, and expansion of the peritoneal B1b cell pool is observed concurrent with the resolution of infection [15]. B1b cells exhibit TI memory, and reinfection with *B. hermsii* results in the rapid generation of specific IgM that is capable of controlling disease in the absence of any other adaptive immune cells [15]. To determine whether B1b-derived IgM is capable of clearing *B. hermsii* independently of opsonophagocytosis, we tested whether IgM generated by adoptively transferred convalescent B1b cells was able to kill *B. hermsii* in diffusion chambers.

$Rag1^{-/-}$ mice reconstituted with purified convalescent B1b cells and primed with *B. hermsii* generated a *B. hermsii*-specific IgM response (Figure 5A). To measure cell-independent killing by B1b-derived IgM, we implanted diffusion chambers containing 1.33 x 10^6 spirochetes in reconstituted $Rag1^{-/-}$ mice at ten days post-infection. As expected, *B. hermsii* bacteria in diffusion chambers proliferated vigorously when implanted in unreconstituted $Rag1^{-/-}$ recipients. On the other hand, we observed a significant decrease in the number of live bacteria in chambers implanted in mice reconstituted with convalescent B1b cells (Figure 5B). These results demonstrate that IgM generated by B1b cells is directly bactericidal *in vivo*.

Discussion

IgM is the dominant isotype secreted upon antigen encounter and plays a critical role in immunity to a number of infections [2]. The production of specific IgM is required and sufficient to control bacteremia during relapsing fever infection [14, 15]. We have previously shown that B1b cells generate *B. hermsii*-specific IgM that is capable of controlling *B. hermsii* bacteremia. Here we show that the IgM-mediated control of *B. hermsii* bacteremia is independent of Fcα/μR and C3 and does not require cell contact. Our data indicate that the mechanism of B1b-mediated control of *B. hermsii* is through the secretion of bactericidal antibodies.

Although antibodies tend to exert their protective function through the activation of the complement cascade or via Fc receptor engagement, some exert direct damage to pathogens in a variety of ways. For example, a *Pseudomonas aeruginosa* LPS-specific IgG alters cell wall permeability, formation of cell wall vesicular structures, elongation, and inhibition of cell division [32]. The bactericidal IgG CB2, specific for OspB of *Borrelia burgdorferi*, induces the formation of 2.8 - 4.4 nm pores in the outer membrane of the bacterium [33]. Paradoxically, this antibody does not induce lysis of OspB-expressing *E. coli*, indicating that IgG-induced lysis is dependent on the cell membrane structure of *B. burgdorferi* or the expression of another *B. burgdorferi* protein. In fact, the lytic activity of CB2 depends on cholesterol domains in the outer membrane of *B. burgdorferi*, suggesting that CB2 alters the fluid structure of the bacterial outer membrane [34]. In addition, *B. burgdorferi* has been shown to harbor a prophage holin or holin-like system containing the genes *blyA* and *blyB* on the conserved circular plasmid cp32 [35]. Transformation of *E. coli* with *blyA* induces cell membrane damage in the host cell that is dependent on a host cytolysin ClyA acting in *trans*, demonstrating that the gene product of *blyA*

is functional [36]. Remarkably, treatment of *B. burdgorferi* spirochetes with sublethal quantities of CB2 induced the expression of cp32 gene products BlyA and BlyB, suggesting that the bactericidal activity of CB2 may be dependent on the induced expression of bacteriophage gene products [37]. Finally, as described above, bactericidal activity of the V region of an IgM specific for a Vmp expressed by a related relapsing fever Borrelia results in direct lysis of the bacterial outer membrane [18]. In spite of this observation, the precise biochemical mechanism of this lysis is unknown. Due to the homology between *B. burdgorferi,* Spanish relapsing fever and *B. hermsii*, it is tempting to speculate that bactericidal B1b-derived IgM lyses *B. hermsii* in the same mechanism as described by Benach and colleagues. More extensive studies will be required to determine the exact mechanism by which bactericidal IgM kills its targets.

B1b cells have been shown to play a role in controlling a number of bacterial pathogens including *B. hermsii*, *Streptococcus pneumoniae* and non-typhoidal Salmonella [4, 15, 38]}. In the absence of all other B cell types, convalescent B1b cells are sufficient to provide protection to infection of immunodeficient mice with *B. hermsii*. We have shown that FhBA, an outer surface protein of *B. hermsii* is specifically recognized by the IgM secreted by convalescent B1b cells [16]. The previously described anti-*Borrelia* antibodies recognize major surface bacterial proteins such as OspB and Vmps rather than FhBA. Despite having shown that B1b cells secrete *B. hermsii*-specific IgM that is capable of leading to direct bacterial lysis, the biochemical basis for this lysis of *B. hermsii* is unknown. The generation of B1b-derived monoclonal antibodies to FhBA of B. hermsii the characterization of such antibodies would provide a better insight on how B1b cell-derived antibodies exert their antibacterial activity in the complete absence of a cell-contact.

Acknowledgements

We thank Jessica Hess and Sandra Bonne-Année for assistance with diffusion chamber experiments and Dr. Robert Eisenberg for providing $C3^{-/-}$ mice. This work was supported by NIH grant R01 AI065750 to KRA.

Figure Legends

Figure 1. Fcα/μR-mediated opsonophagocytosis is not required for clearance of *B. hermsii*. (A). Balb/cJ or Fcα/μR$^{-/-}$ mice were infected i.p. with *B. hermsii* strain DAH, and bacteremia was monitored by dark-field microscopy. Each plot represents an individual mouse. Dotted lines represent limit of detection. (B).. One day prior to infection with strain DAH, serum from convalescent (80 days post-infection) wildtype mice was passively transferred either into Balb/cJ or Fcα/μR$^{-/-}$ mice. Bacteremia was monitored by dark-field microscopy. Dotted lines represent the limit of detection. (C). *B. hermsii*-specific IgM was measured by ELISA. Data points represent mean ± SD. Kinetics of anti-*B. hermsii* IgM responses were not statistically significant between wildtype and Fcα/μR$^{-/-}$ mice.

Figure 2. Fcα/μR- and C3-mediated opsonophagocytosis are not required for clearance of *B. hermsii*. (A) Balb/cJ or (B) Fcα/μR$^{-/-}$ mice were treated without or with 14.0 units of cobra venom factor (CVF) to deplete complement component C3 on the days indicated (arrows). Mice were infected with *B. hermsii*, and bacteremia was monitored by dark-field microscopy. Each plot represents an individual mouse. Dotted lines represent limit of detection.

Figure 3. Antibody-mediated lysis in diffusion chambers. (A) Diffusion chamber model. *B. hermsii* (size: ~0.25 μm x ~5.0 μm) and eukaryotic cells are unable to pass through the 0.05 μm pores size membrane of the diffusion chambers. IgM (300 Å) passively diffuses through the chamber membrane. (B) Chambers containing 1.33 x 10^6 *B. hermsii* were implanted into various groups of B6 mice (n= 4-7 per group) as shown in *A*. Chambers were removed after 24 hours and live bacteria were enumerated by dark-field microscopy. Data represent mean ± SD. Dotted line

represents starting number of bacteria in each chamber. Data were analyzed by two-tailed unpaired Student's *t* test, with significance reached at $p < 0.05$. (C) *B. hermsii*-specific IgM in diffusion chambers was measured by ELISA, and specific IgM values were matched with chamber bacterial density. Non-linear regression analysis measuring the effect of specific IgM concentration on bacterial death was performed.

Figure 4. Lysis of *B. hermsii* in diffusion chambers is C3-independent. (A) Cobra venom factor **(**CVF, 14.0 units) was given to naïve (n=4) or convalescent (n=5) B6 mice one day prior to implantation of diffusion chambers containing *B. hermsii*. Control groups of naïve (n=3) or convalescent (n=3) mice were given DPBS. (B) Chambers containing 1.33×10^6 *B. hermsii* were implanted into naïve B6 mice (n=4), convalescent B6 mice (n=4), or convalescent $C3^{-/-}$ mice (n=3). For *A* and *B*, chambers were removed from mice after 24 hours, and bacteria were counted by dark-field microscopy. Mean numbers of bacteria ± SD are shown. (N.D. – not detectable). Dotted line indicates starting number of bacteria in each chamber. Data were analyzed by two-tailed unpaired Student's *t* test, with significance reached at $p < 0.05$.

Figure 5. B1b cell-derived IgM is sufficient to kill *B. hermsii* in diffusion chambers. $Rag1^{-/-}$ mice were reconstituted without or with B1b cells (10^5 cells per mouse) from convalescent B6 mice. After 10 days, B1b cell reconstituted mice were infected with *B. hermsii* in order to stimulate IgM production. (A) *B. hermsii*-specific IgM in non-reconstituted (n=4) or B1b-reconstituted mice (n=4) was determined by ELISA. Means ± SD are shown. Data were analyzed by two-way ANOVA, with significance reached at $p < 0.05$. (B) On day 10 post-infection, chambers containing *B. hermsii* were implanted into mice from each group. Twenty-four hours

later chambers were removed, and live bacteria were counted by dark-field microscopy. Dotted line represents the starting number of *B. hermsii* in each chamber. Data were analyzed by two-tailed unpaired Student's *t* test, with significance reached at $p < 0.05$.

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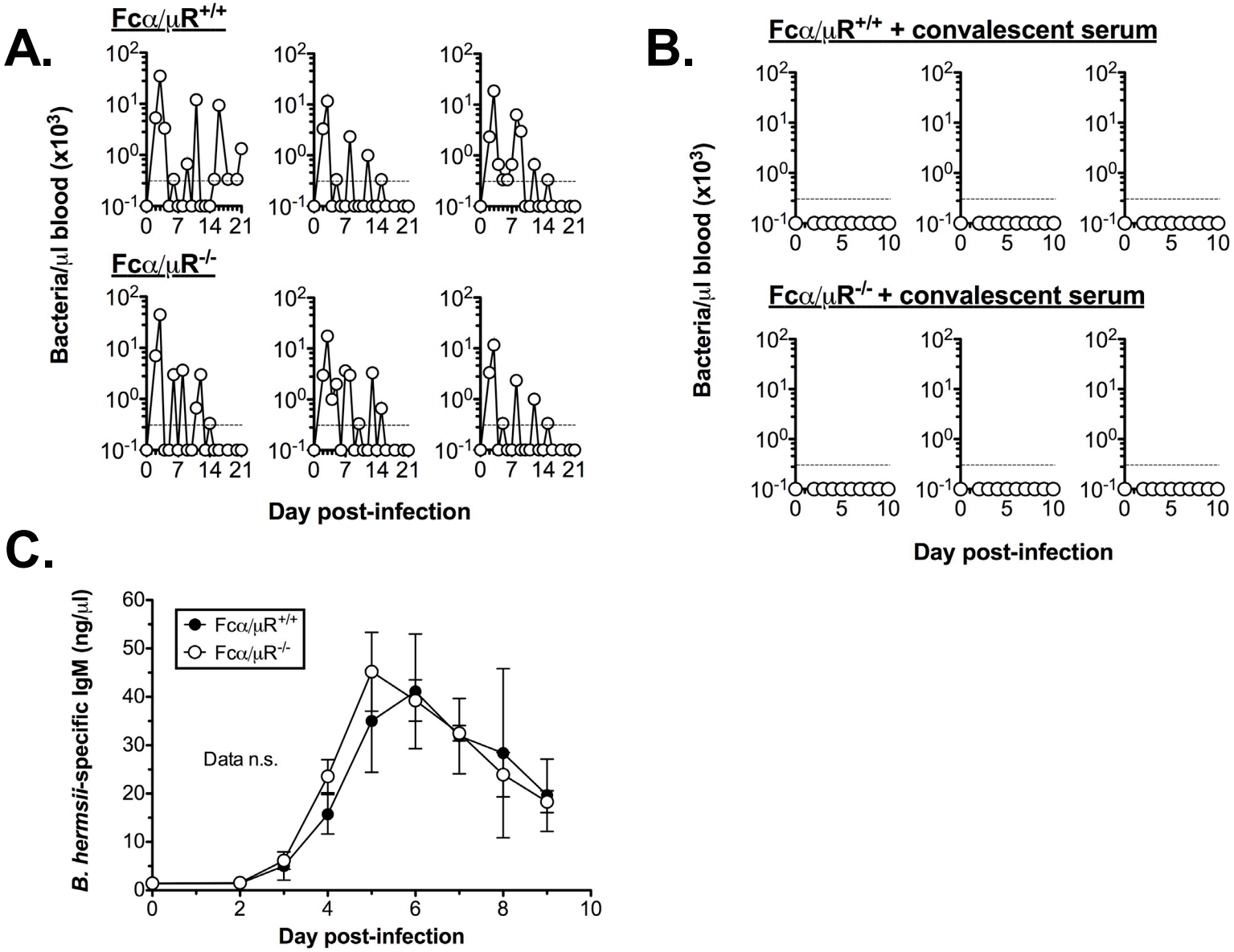
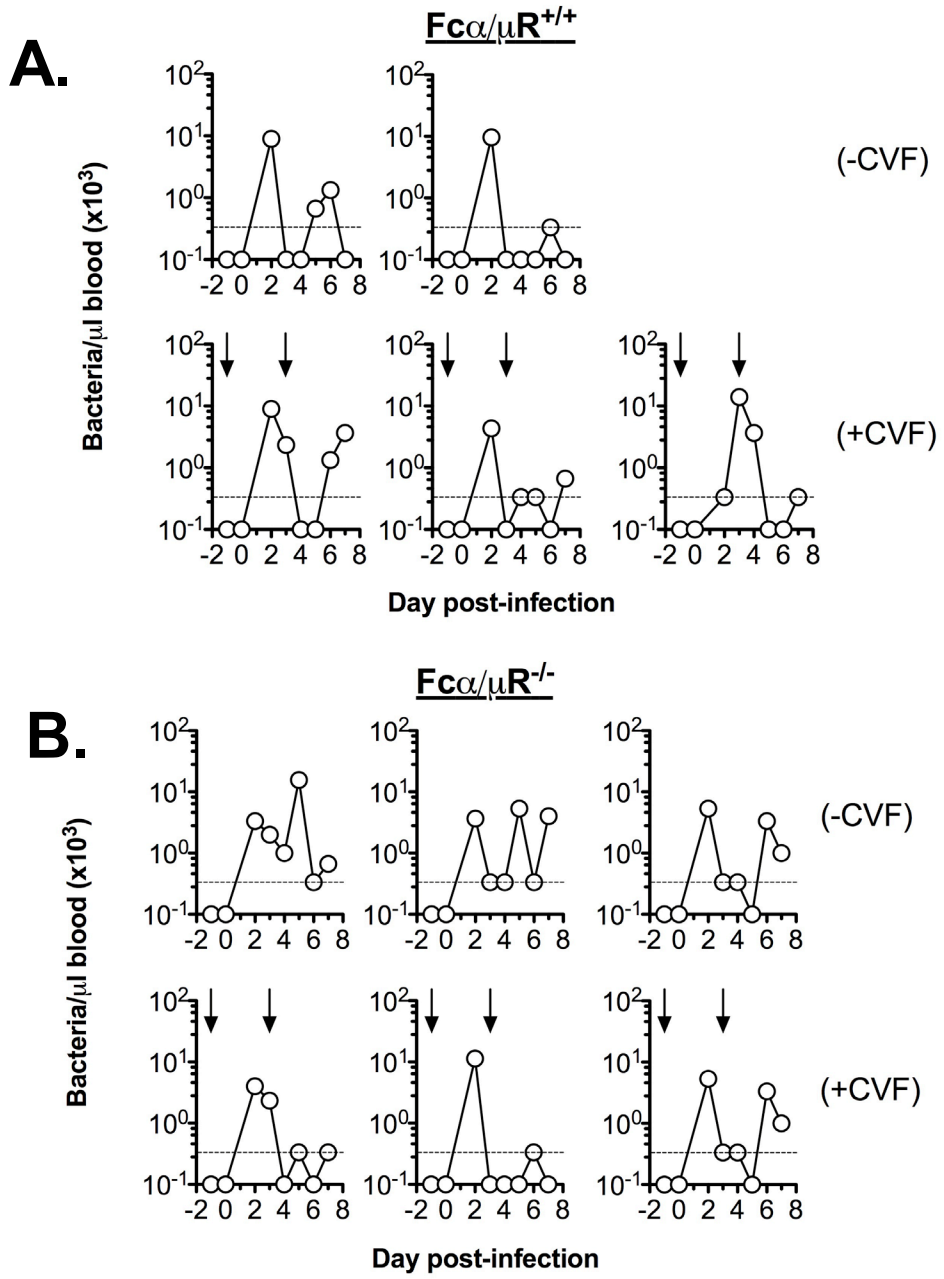
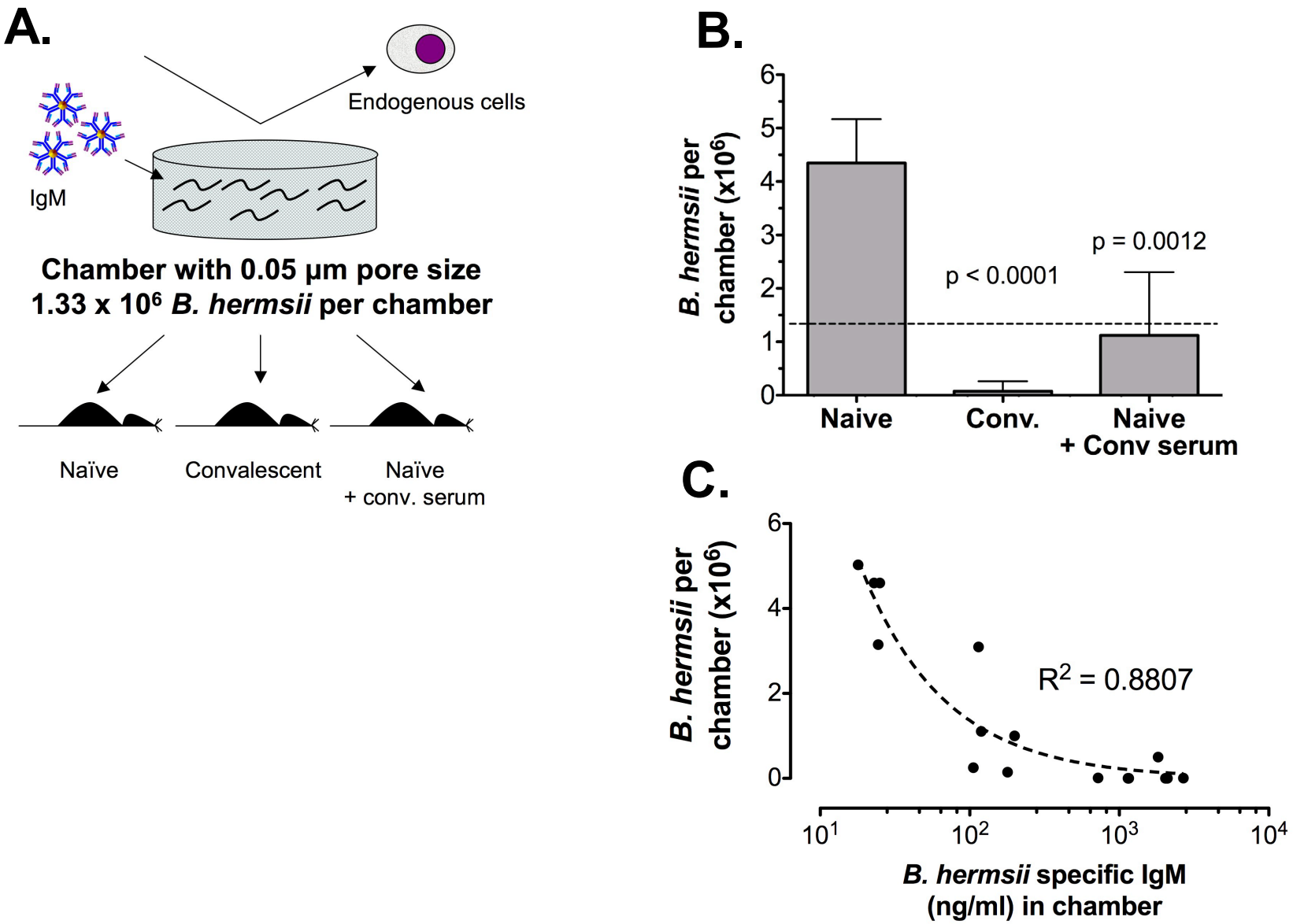


Colombo et al., Figure 1



Colombo et al., Fig 2



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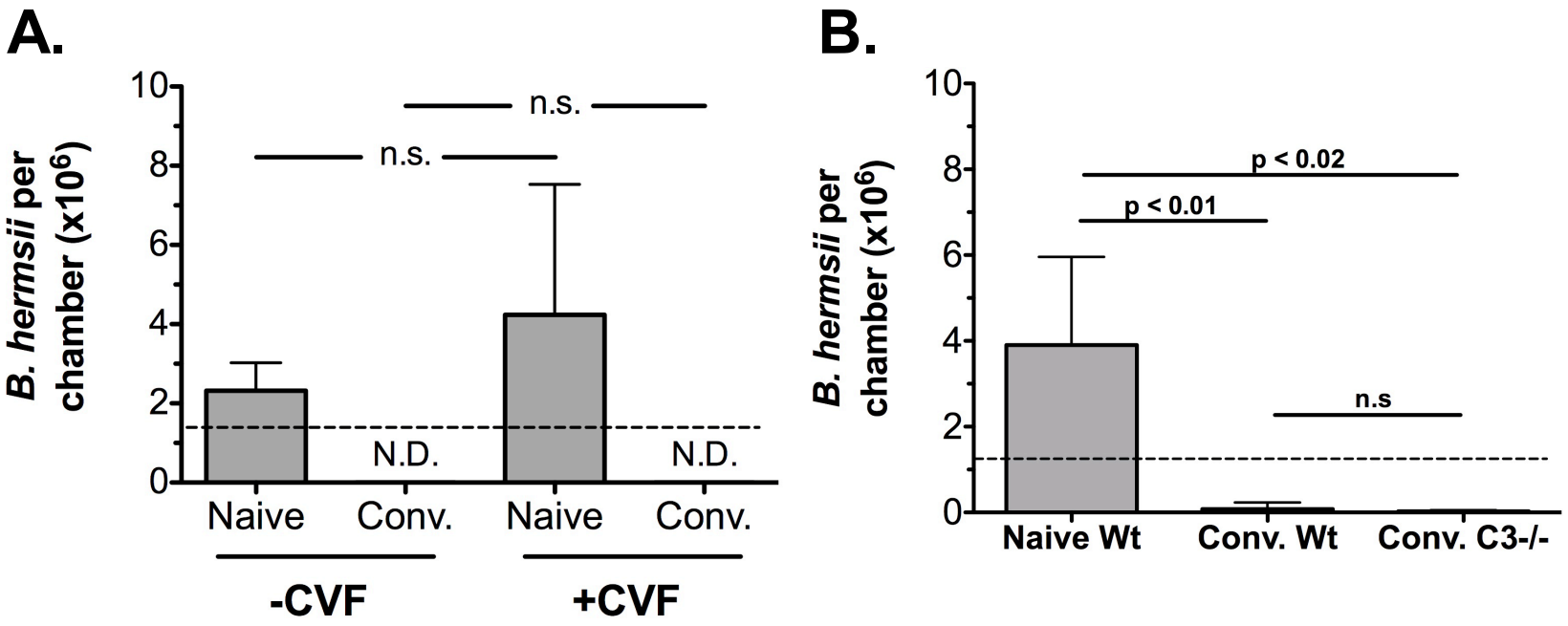
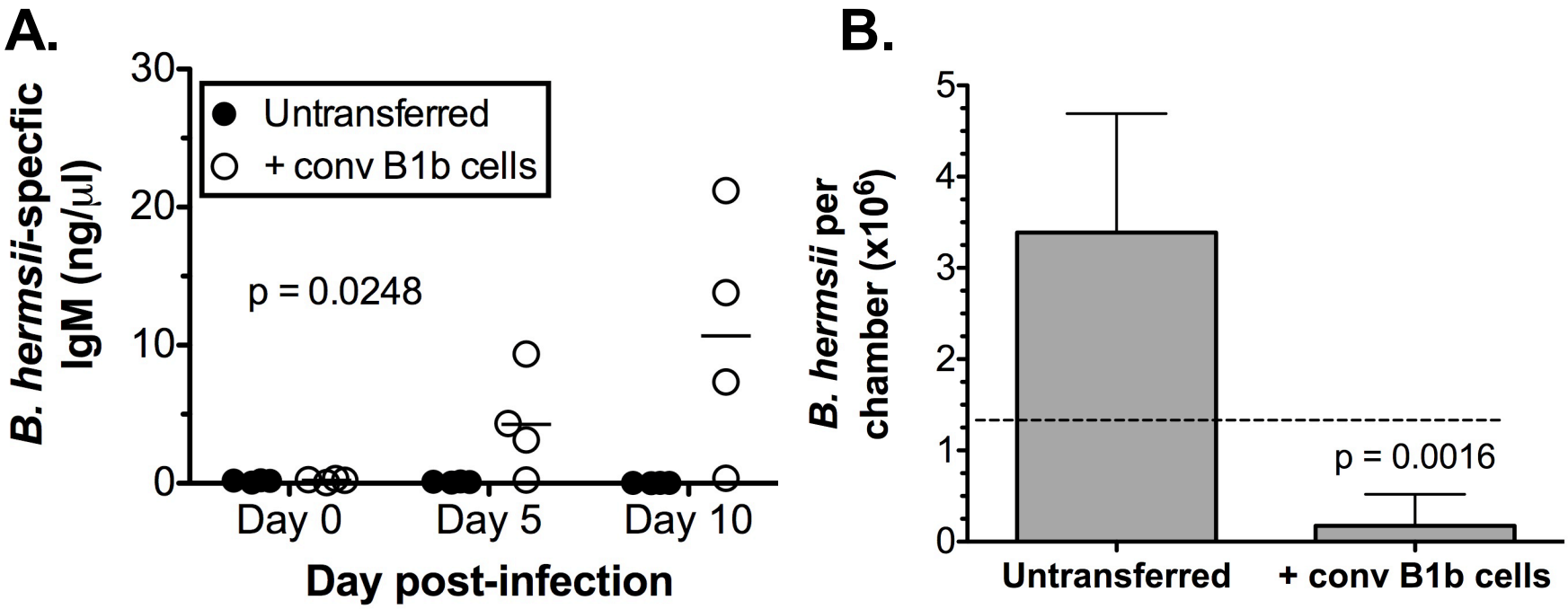


Colombo et al., Fig 4



Colombo et al., Fig 5