CD43⁻, but not CD43⁺, IL-10-producing CD1d^{hi}CD5⁺ B cells suppress type 1 immune responses during *Chlamydia muridarum* genital tract infection

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Regulatory B (Breg) cells are known to modulate immune responses through predominantly interleukin-10 (IL-10)-dependent mechanisms and can be hypothetically divided into innate and adaptive subsets based on the nature of their activating signals. However, the specific role of different Breg subsets in modulating immune responses remains ambiguous. Here we have shown that *Chlamydia* induces IL-10-producing splenic B-cell populations consisting of CD43⁺ and CD43⁻ subsets of IgM^{hi}IgD^{lo} innate-like B (ILB) cells *in vitro*. While CD43⁺ IL-10-producing B cells displayed innate type features and were readily induced by *Chlamydia* via Toll-like-receptor (TLR) signaling, CD43⁻ IL-10-producing B cells required additional B-cell activating factor (BAFF)-mediated signals from dendritic cells (DCs) for their differentiation and activation, thereby classifying them as adaptive type Bregs. Importantly, CD43⁻, but not CD43⁺, IL-10-producing ILB cells displayed *bona fide* Breg activity by potently suppressing interferon- γ (IFN- γ) production *in vitro* in an IL-10-dependent manner. Furthermore, a novel CD43⁻ CD1d^{hi}CD5⁺ IL-10-producing Breg population was predominantly induced by *Chlamydia* genital infection *in vivo*. Correspondingly, mixed bone marrow chimeric mice with B-cell-specific IL-10 deficiency exhibited significantly increased type 1 immune responses, decreased bacterial burden, and reduced oviduct pathology upon infection. Our data demonstrate for the first time a distinct role for CD43⁻ CD1d^{hi}CD5⁺-adaptive Bregs over CD43⁺ innate counterparts in controlling mucosal responses against intracellular bacterial infection.

INTRODUCTION

Chlamydia species are obligate intracellular bacterial pathogens that infect mucosal epithelium lining the ocular, respiratory, and genital tract (GT). *Chlamydia trachomatis* is the most common sexually transmitted bacterial pathogen worldwide.¹ While a large portion of *Chlamydia* GT infections are initially subclinical, such infections often become persistent, leading to chronic inflammation with serious long-term complications including pelvic inflammatory disease and infertility.¹⁻³ In mice, intravaginal inoculation of *Chlamydia muridarum* (Cm) results in tubal pathology similar to that observed in women.³

Due to the intracellular lifestyle of *Chlamydia*, cell-mediated immune responses are important in controlling the infection. In particular, CD4⁺ T helper 1 (Th1) cells are critical for resolving primary infection and preventing bacterial ascension into the upper GT via IFN- γ production.^{1,2} Mice with genetic deletions in CD4, IFN- γ , or IFN- γ receptor have profoundly impaired abilities to resolve *Chlamydia* infection and display massive inflammatory responses in the GT.⁴⁻⁶ In both mice and humans, CD4⁺ Th1 responses are highly regulated and weakened Th1 responses commonly lead to reduced host resistance and prolonged *Chlamydia* infection.^{1,2,7}

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B lymphocytes are essential to humoral immunity and are also important modulators of cellular responses via their interactions with DCs and T lymphocytes within the lymphoid organs. In mice, three mature B subsets with different functional activities, phenotypes, and/or topographic locations are identified: B1 cells (including CD5⁺ B1a and CD5[−] B1b subsets), follicular B (FOB) cells, and marginal zone B (MZB) cells.^{8,9} Although FOB and MZB cells belong to the “B2” lineage that develops after B1 cells during ontogeny, these subsets have distinct functional roles. FOB cells, also called adaptive B cells, are responsible for classic T-cell-dependent B-cell responses and the production of high-affinity antigen-specific antibodies. In contrast, MZB cells, as well as B1 cells, produce low-affinity IgM antibody upon microbial stimulation independently of T-cell help and are therefore termed innate-like B (ILB) cells.^{8–12} Whereas FOB cells comprise the vast majority of B cells in the peripheral lymphoid organs, B1 cells are relatively rare in peripheral lymphoid organs but highly enriched in the coelomic cavities.¹³ Initial rapid T-cell-independent IgM responses from ILB cells are the first line of host defense against blood-borne and mucosal pathogens.^{11,12} However, B cells also possess important antibody-independent functions including antigen presentation and cytokine production, particularly production of immune suppressive cytokine interleukin-10 (IL-10).^{8,9,14} Hence, B cells can positively or negatively regulate immune responses depending on the engagement of specific functions of different B-cell subsets. Previous attempts to characterize the role of B cells in anti-*Chlamydia* immunity using B-cell deficient mice in models of respiratory, GT, and peritoneal infection have yielded conflicting results.^{15–18} Although *Chlamydia* infection elicits substantial antibody responses, *Chlamydia*-specific antibodies are generally considered to provide minimal protection during primary genital infection.^{1,2,16,19} However, passive immunization with immune serum containing anti-*Chlamydia* antibodies is able to enhance Th1 responses and confer protection against secondary *Chlamydia* genital challenge.^{19,20} Despite numerous investigations examining the contribution of antibody in *Chlamydia* infection, however, the role of other functional activities of B cells in host responses to *Chlamydia* remains elusive.

Recently, regulatory B (Breg) cells have become recognized as discrete B-cell entities that modulate immune responses in numerous disease models, including systemic bacterial infection, mainly via production of IL-10.^{9,13,14,21–24} Two Breg populations—CD1d^{hi}CD21⁺CD23⁺ T2-MZ precursor²⁵ and CD1d^{hi}CD5⁺ B10 cells²⁶—have been described in mice, but the relationship of these populations to conventional innate-like and adaptive B subsets is unclear. Alternatively, IL-10-producing Bregs can be classified as “innate type” or “adaptive type” based on signal(s) required for their activation/induction.^{9,14} Major signaling pathways involved in Breg generation include TLR ligation and signals involved in adaptive immunity such as B-cell receptor stimulation and multiple co-stimulatory signals, particularly the CD40–CD40L pathway.^{9,14,21} Bregs induced by TLR stimulation alone are proposed to be innate type whereas

Bregs requiring B-cell receptor and/or CD40–CD40L signals in the presence or absence of TLR stimulation are proposed to be adaptive type.^{9,14} However, no definitive marker currently exists to differentiate innate from adaptive type and the specific role of innate vs. adaptive Bregs in modulating immune responses is ambiguous.

In this study, we examined the phenotype and function of *Chlamydia*-induced IL-10-producing B cells *in vitro* and *in vivo*. We demonstrated that CD43 is a novel indicator of innate and adaptive type IL-10-producing B-cell populations activated by TLR signals alone or TLR plus DC-derived signals, respectively. Unlike CD43⁺ IL-10-producing B cells, CD43[−] IL-10-producing B cells displayed *bona fide* Breg activity and potently modulated host responses to *Chlamydia* infection *in vivo*. Our data for the first time demonstrate a distinct role for CD43[−] adaptive Bregs over CD43⁺ innate counterparts in controlling mucosal responses against intracellular bacterial infection.

RESULTS

Chlamydia induces robust IL-10 production from multiple ILB cell subsets *in vitro*

To identify immune cells that produce IL-10 in response to *Chlamydia* (*Cm*), *Cm*-stimulated splenocytes from IL-10 green fluorescent protein (IL-10GFP) mice were analyzed by flow cytometry. Remarkably, *Cm* induced a distinct IL-10GFP⁺ population that comprises >94% of CD19⁺B220⁺ B cells and ~5% of CD11c⁺ DCs (**Figure 1a**). IL-10 production from purified B (B220⁺) cells was significantly greater than non-B (B220[−]) fraction at 48 h post infection, confirming that B cells are a major source of IL-10 induced by *Cm in vitro* (**Figure 1b**). Notably, *Cm* elicited large quantities of IL-6 in addition to IL-10 but appeared to suppress low levels of endogenous IFN- γ production from purified B cells. No detectable amounts of IL-4, IL-17A, or IL-12p70 were measured (**Supplementary Figure 1** online).

Phenotypical analyses were conducted by comparing IL-10GFP⁺ with IL-10GFP[−] B-cell populations. While IL-10GFP[−] B cells displayed a classic IgM^{lo}IgD^{hi} FOB phenotype,^{8–10} IL-10GFP⁺ B cells exhibited a striking IgM^{hi}IgD^{lo} phenotype—a characteristic feature of ILB cells, including B1 and MZB cells.^{9,10} In addition, IL-10GFP⁺ B cells displayed higher fluorescence intensities of CD1d, CD5, and CD43 relative to IL-10GFP[−] B cells (**Figure 1c**). While CD1d was only expressed by a small fraction (~6%) of IL-10GFP[−] B cells, it was generally upregulated by all IL-10GFP⁺ B cells (**Figure 1d**). Approximately 28% of IL-10GFP⁺ B cells concurrently carried CD1d^{hi}CD5⁺ B10 (**Figure 1d**) and CD43⁺CD5⁺ B1a phenotypes (**Figure 1e**). Thus, ~50% of *Cm*-induced IL-10GFP⁺ B cells expressed CD43 and half of these CD43⁺IL-10GFP⁺ cells were CD43⁺CD5⁺CD1d^{hi} (**Figure 1d,e**). Other *Cm*-induced IL-10GFP⁺ B populations were CD43[−]CD1d^{hi/low}CD5^{+/−} (~50%) and CD43⁺CD1d^{hi/low}CD5[−] (~25%) (**Figure 1f**), indicating that *Chlamydia* induces IL-10 production from a heterogeneous population of ILB cells *in vitro*. Heat-killed *Cm*, lipopolysaccharide (LPS), and CpG also stimulated IL-10

Figure 1 *Chlamydia muridarum* (*Cm*) induces robust IL-10 production from multiple innate-like B subsets *in vitro*. (a) Splenocytes derived from IL-10GFP or C57BL/6 mice were stimulated with *Cm* (MOI 0.1) for 48 h *in vitro*. IL-10GFP⁺ cells were analyzed by flow cytometry. (b) IL-10 levels were measured by ELISA in culture supernatants of B220⁺ and B220⁻ fractions of C57BL/6 splenocytes 48 h PI. Data are presented as mean $\pm$ s.e.m. of triplicate wells and are representative of two independent experiments. $^{**}P \leq 0.01$ using two-way ANOVA test. (c–f) Representative FACS plots of surface marker expression of IL-10GFP⁺ and IL-10GFP⁻ CD19-gated B cells. Filled histogram represents isotype control. Data are representative of three independent experiments. (g–h) B1, MZB, and FOB cells were purified by sorting and stimulated with *Cm* (MOI 0.1) for days 1, 2, and 3 *in vitro*. IL-10, IL-6, and GM-CSF production was determined by ELISA. Data are presented as mean $\pm$ s.e.m. of triplicate wells. $^{**}P \leq 0.01$, $^{***}P \leq 0.001$ using two-way ANOVA test compared with FOB group. ANOVA, analysis of variance; ELISA, enzyme-linked immunosorbent assay; FACS, fluorescence-activated cell sorting; FOB; follicular B cells; GFP, green fluorescence protein; GM-CSF, granulocyte macrophage colony-stimulating factor; IL, interleukin; MOI, multiplicity of infection; MZB, marginal zone B; PI, post infection.

production from B cells, although a reduced potency was evident (**Supplementary Figure 2**).

Consistent with our phenotypic analysis, purified B1 and MZB cells, but not FOB cells, produced large amounts of IL-10 upon *Cm* stimulation. B1, but not MZB cells, also released granulocyte macrophage colony-stimulating factor whereas all B-cell subsets were able to produce IL-6 (**Figure 1g-i**), indicating that *Cm*-induced IL-10-producing B cells likely have diverse lineages/origins.

The development of CD43⁺, but not CD43⁻, IL-10-producing ILB cells upon *Cm* infection requires DC-mediated signals

The concept of innate and adaptive Bregs has been proposed,^{9,14} so we wondered whether *Cm* may elicit such Breg populations. We noticed that the proportions of CD43⁺ IL-10GFP⁺ ILB cells and total IL-10GFP⁺ cells were markedly reduced in purified B-cell cultures compared with the whole splenocyte cultures (**Figure 2a**). This indicates a requirement

for other immune cells to induce or amplify CD43⁺ IL-10GFP⁺ cells. Furthermore, IL-10GFP⁺ B cells appeared to proliferate more within whole splenocyte cultures than in purified B-cell cultures (**Figure 2a**), suggesting that CD43⁺ IL-10GFP⁺ ILB cells may undergo greater expansion than CD43⁺ counterparts. DCs are known to provide important signals to B cells including CD40/CD40L interaction and BAFF production, which promote B-cell proliferation and survival.^{27–29} Therefore, a simple DC-depletion method was used to examine whether DCs are required for the generation of CD43⁺ IL-10GFP⁺ ILB cells. DC depletion markedly reduced the frequencies of IL-10GFP⁺ B cells by ~45% (8.4% vs. 4.7%) in *Cm*-treated cultures. Notably, reduced frequencies of IL-10GFP⁺ ILB cells were due almost entirely to elimination of the CD43⁺ IL-10GFP⁺ B population (**Figure 2b**). Similar phenotype was also observed in LPS-stimulated cultures (**Figure 2c**). Together, these data suggest that CD43⁺ and CD43⁻ IL-10⁺ ILB cells have different signaling requirements—*Cm*-triggered TLR signals alone vs. TLR- plus DC-mediated

Figure 2 DC-derived signals are critically required for IL-10 production from CD43⁺, but not CD43⁻, ILB cells upon *Cm* or LPS stimulation (**a**) Frequency and phenotype of IL-10GFP⁺ B cells in cultures of purified B cells alone or whole splenocytes 72 h PI. Values in bar graph represent the mean ± s.e.m. of triplicate samples. Histogram shows eFluor670 dilution-based proliferation rate of IL-10GFP⁺CD19⁺ B cells from purified B cell or whole splenocyte cultures 72 h PI (**b,c**) Frequency and phenotype of IL-10GFP⁺ B cells in whole splenocyte or CD11c⁺ DC-depleted splenocyte cultures 48 h following *Chlamydia* or LPS (100 ng ml⁻¹) stimulation. (**d**) Phenotype of sorted B1, MZB and FOB cells in whole splenocyte cultures 48 h post-*Cm* stimulation. *Cm*, *Chlamydia muridarum*; DC, dendritic cells; FOB, follicular B cells; IL, interleukin; ILB, innate-like B cells; LPS, lipopolysaccharide; MZB, marginal zone B; PI, post infection.

signals—that potentially classify these B subsets as innate and adaptive Breg populations,^{9,14} respectively. B10 cells are reported to contain both CD43⁺ and CD43[−] fractions,³⁰ so we analyzed CD43 levels on IL-10GFP⁺CD1d^{hi}CD5⁺ cells in *Cm*-stimulated and LPS-stimulated cultures. Consistently, DC-depletion markedly attenuated generation of CD43[−] B10 cells in *Cm*-stimulated and LPS-stimulated whole splenocyte cultures (Figure 2b,c). Collectively, our data demonstrate that CD43 is a novel indicator distinguishing innate and adaptive IL-10-producing ILB populations.

Although CD43 is generally expressed by innate B1 cells, it can be transiently upregulated by B2 cells under certain conditions.^{13,31} We therefore tracked the origin of CD43⁺ and CD43[−] IL-10-producing ILB cells following *Cm* stimulation in co-cultures of whole splenocytes from CD45.1 congenic mice mixed with B1, MZB, or FOB cells from IL-10GFP mice (CD45.2). Following *Cm* stimulation, almost all B1-derived IL-10GFP⁺ cells remained CD43⁺, whereas MZB-derived IL-10GFP⁺ cells displayed both CD43⁺ and CD43[−] phenotypes (Figure 2d). A large portion of MZB-derived GFP⁺ cells lost their surface CD23 expression (data not shown). Furthermore, MZB-derived IL-10GFP⁺ cells expressed higher levels of CD1d but comparable levels of CD5 relative to their B1-derived counterparts (Figure 2d). CD43 expression was also upregulated in a fraction of FOB cells upon *Cm* stimulation; however, IL-10GFP⁺ cells originating from FOB cells were only detected at background levels. Thus, *Cm*-induced CD43[−]CD1d^{hi}CD5⁺ adaptive Bregs derive solely from MZB cells.

DC-derived BAFF production is essential for the development of adaptive Bregs upon *Cm* infection

Next, we examined the potential role of TLR signaling in the development of *Cm*-induced innate and adaptive Bregs using MyD88 or TRIF knockout (KO) mice. Given the dominant role of TLR2-dependent signals in mediating *Cm*-induced pathology,³² TLR2KO mice were also included in these investigations. Remarkably, purified B cells from MyD88KO mice produced little to no IL-10 following *Cm* stimulation. Similar results were observed using whole splenocytes (Figure 3a), highlighting an essential role of MyD88-dependent signals for IL-10 production from all subsets of B cells. Conversely, TRIF-dependent signals inhibited IL-10 production by innate Bregs in purified B-cell cultures. Interestingly, TLR2-dependent signals were only required for IL-10 production from splenocyte cultures, indicating a role for TLR2 in the development of adaptive Bregs, specifically (Figure 3a). Notably, IL-10 levels correlated directly with the degree of B-cell expansion within each *Cm*-stimulated culture, with CD19⁺ B cells comprising over 60% of cells in wild type (WT) and TRIFKO cultures but only 21 and 39% in MyD88KO and TLR2KO cultures, respectively (Figure 3b). Furthermore, B cells recovered from MyD88KO and TLR2KO splenocyte cultures displayed reduced CD40 and CD40L expression compared with WT and TRIFKO counterparts. A similar pattern of reduced CD40 expression was also observed on CD11b⁺ myeloid DCs, but

CD40L levels on CD4⁺ T cells were comparable among groups (Figure 3b).

DC-derived BAFF has been shown to induce the development of IL-10-producing Bregs,³³ so we next examined BAFF production by bone marrow-derived DCs in response to *Cm* stimulation. As shown in Figure 3c, BAFF levels were significantly elevated in WT and TRIFKO BMDC-derived supernatants of *Cm*- or LPS-stimulated cultures compared with unstimulated cultures. However, *Cm*-induced BAFF was attenuated in MyD88KO and TLR2KO (Figure 3c). Blocking BAFF using the soluble decoy receptor TACI-Ig significantly reduced *Cm*-induced IL-10 production in WT, but not TLR2KO, splenocytes (Figure 3d). Of interest, unlike the CD40 expression pattern observed in DCs from splenocyte cultures (Figure 3b), *Cm* stimulation only marginally induced CD40 in WT BMDC and did not appreciably induce CD40 in KO bone marrow-derived DCs (Figure 3e). These data indicate that the release of BAFF is an essential mechanism utilized by DC to promote adaptive Bregs from MZB cells during *Cm* infection and that DC-derived BAFF induces B-cell activation and survival in a MyD88-dependent, TLR2-dependent but TRIF-independent manner. This mechanism may be directly or indirectly coupled with CD40/CD40L upregulation on B cells to further enhance adaptive Breg development via an autocrine pathway.

Cm-induced CD43[−], but not CD43⁺, IL-10-producing B cells suppress IFN- γ responses *in vitro*

Next, we used an *in vitro* suppression assay to determine whether *Cm*-induced IL-10-producing B cells are able to suppress T-cell responses. As demonstrated in Figure 4a, *Cm*-stimulated B cells, but not unstimulated B cells, significantly reduced CD4⁺ T-cell proliferation in a dose-dependent manner with ~50% suppression at 1:1 ratio of B cells to responders compared with responder cells alone. Furthermore, *Cm*-stimulated B cells, but not unstimulated B cells, markedly suppressed IFN- γ production in a dose-dependent manner (Figure 4b). In contrast, IL-4 levels were significantly promoted by *Cm*-stimulated B cells, but suppressed by unstimulated B cells, although the overall level is low (Figure 4c). Relative to Th1/Th2 cytokine profiles, B cells had a minor effect on IL-17A/Th17 response (Figure 4d).

Notably, *Cm*-stimulated B cells, but not unstimulated B cells, generally increased IFN- γ levels in co-cultures compared with responder cells alone while also showing dose-dependent suppression (Figure 4b). This observation suggests that *Cm* stimulation induces both immune stimulatory and inhibitory B-cell subsets. Given the distinct innate vs. adaptive nature of CD43⁺ and CD43[−] IL-10-producing ILB cells, we next sought to determine whether these subpopulations differed in function. To this end, CD43⁺ and CD43[−] IL-10GFP⁺ subsets were sorted from *Cm*-stimulated splenocytes and used in the suppression assay with μ MT splenocytes as responders (Figure 4e). Remarkably, CD43[−] IL-10GFP⁺ B cells significantly suppressed IFN- γ production in a dose-dependent

Figure 3 Role of MyD88, TRIF, and TLR2 in *Cm*-induced IL-10 production, DC-derived BAFF production and CD40/CD40L expression on B cells. Purified B cells or whole splenocytes were isolated from WT (C57BL/6), MyD88KO, TRIFKO, or TLR2KO mice and stimulated with *Cm* for 40 h. **(a)** IL-10 levels were measured by ELISA. Data are presented as the mean $\pm$ s.e.m. of triplicate wells and are representative of three independent experiments. $^{*}P \leq 0.05$, $^{***}P \leq 0.001$ vs. WT using one-way ANOVA test. **(b)** Frequency of CD19⁺ B cells and CD4⁺ T cells in whole splenocyte cultures 40 h following *Cm* stimulation (top panels). Relative CD40 and CD40L expression on gated immune cells from all examined mouse strains are indicated by histogram overlays (bottom panels). **(c)** BMDCs were treated with medium, *Cm*, or LPS for 24 h. BAFF levels in the culture supernatants were determined by ELISA. Data are presented as the mean $\pm$ s.e.m. of triplicate wells from one representative experiment. $^{***}P \leq 0.001$ vs. medium alone in each group using two-way ANOVA test. **(d)** Whole splenocyte cultures from WT and TLR2KO mice were stimulated with *Cm* alone or *Cm* plus TACI-Ig for 48 h. IL-10 levels were determined by ELISA. Data are presented as the mean $\pm$ s.e.m. of 3–6 wells that were pooled from two representative experiments. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$ vs. *Cm* stimulation group using one-way ANOVA test. **(e)** CD40 expression on BMDCs of WT, MyD88KO, TRIFKO, and TLR2KO treated with medium, *Cm*, or LPS for 24 h. ANOVA, analysis of variance; BAFF, B-cell activating factor; BMDC, bone marrow-derived dendritic cell; *Cm*, *Chlamydia muridarum*; DC, dendritic cells; ELISA, enzyme-linked immunosorbent assay; IL, interleukin; KO, knockout; ND, no difference; WT, wild type.

manner, whereas CD43⁺ counterparts markedly promoted IFN- γ production (**Figure 4e**). While CD43⁺ and CD43⁻ IL-10GFP⁺ cells exhibited comparable GFP intensities during sorting, post-culture examination revealed only low levels of IL-10 in CD43⁺-containing cultures. Rather, high levels of IL-6 and detectable levels of granulocyte macrophage colony-stimulating factor were found in conditions containing CD43⁺, but not CD43⁻, ILB cells (**Figure 4e**). Our data suggest that CD43⁺ and CD43⁻ IL-10-producing ILB cells have distinct molecular profiles, and only CD43⁻, but not CD43⁺, IL-10-producing ILB cells are the *bona fide* Breg population induced by *Cm*.

To investigate the role of IL-10 in CD43⁻ Breg-mediated suppressive function, we utilized CD19⁺CD43⁻ B cells derived from *Cm*-stimulated wild-type vs. IL-10KO splenocytes. While wild-type CD43⁻ B cells appeared to have

only a minor inhibitory effect over IFN- γ responses, IL-10-deficient CD43⁻ B cells markedly promoted IFN- γ production in a dose-dependent manner, indicating a reversal of Breg suppressive function to an activating phenotype (**Figure 4f**). Similar results were obtained in wild-type CD43⁻ B-cell cultures treated with anti-IL-10R antibody (**Figure 4f**), demonstrating that IL-10 from Bregs is essential in suppressing or restraining IFN- γ responses. Notably, IL-10 from CD43⁻ B cells had no qualitative effect over the kinetics of IL-4, IL-17A, as well as IL-2 production (**Figure 4g**).

Cm* infection induces expansion of CD43⁻CD1d^{hi}CD5⁺IL-10⁺ B cells *in vivo

Having observed potent IL-10 production by functionally distinct CD43⁺ and CD43⁻ B cells in response to *Cm* stimulation *in vitro*, we next examined B-cell responses to *Cm*

in vivo. *Cm* infection significantly increased the total number of B cells, as well as the frequency and absolute number of IL-10-producing B cells in the iliac lymph node (ILN), which all peaked at day 5 post infection and returned to basal levels by day 10 (Figure 5a–c). Notably, IL-10 production was concurrently induced in both B and non-B cells at day 5 post infection, with the frequency of IL-10⁺ non-B cells being significantly greater than IL-10⁺ B cells (Figure 5b,d).

Importantly, a large majority of IL-10⁺ B cells induced in the ILN by day 3 post infection were CD43[−] and this remained the dominant IL-10⁺ B-cell phenotype in the ILN at days 5 and 10 post infection. The number of IL-10-producing CD43[−] B cells was significantly greater than CD43⁺ counterparts at day 5 post infection (Figure 5e). Approximately 70% of these IL-10-producing B cells carried a B10 phenotype of CD43[−]CD1d^{hi}CD5⁺ (Figure 5c,e). In contrast to

Figure 5 *Cm* genital tract infection induces expansion of CD43⁻ CD1d^{hi} CD5⁺ IL-10⁺ B cells in the ILN but not in the genital tract *in vivo*. C57BL/6 mice were intravaginally infected with *Cm* and killed at various time points ($n = 6$ mice per time point). (a, b, d, e) The absolute number of total B cells, the frequency of IL-10⁺ of total B cells, and the absolute number of IL-10⁺ B cells and IL-10⁺ non-B cells, as well as the frequency of CD43⁺ vs. CD43⁻ IL-10⁺ B cells in the ILN were determined on days 0, 3, 5, and 10 PI ($n = 2-3$ mice per timepoint for CD43 staining) (c) Representative dot plots of IL-10 expression in CD19⁺ B cells and CD19⁻ non-B cells in the ILN on days 0, 3, 5, and 10 PI by ICCS. Dot plots in bottom panel show CD19 and CD43 expression by IL-10⁺ CD19⁺-gated cells. Density plots represent CD1d and CD5 expression by IL-10⁺ CD19⁺ or IL-10⁺ CD19⁻-gated cells at day 5 post infection. Values in quadrants represent percent of gated cells. (f, g) The absolute number of total B cells and IL-10⁺ B cells, as well as IL-10⁺ non-B cells in the spleen on days 0, 3, 5, and 10 PI (h) Representative dot plots of IL-10 expression in CD19⁺ B cells and CD19⁻ non-B cells in the spleen on days 0, 3, 5, and 10 PI by ICCS. (i) Isotype control for IL-10 staining in spleen and ILN samples. (j) IL-10 expression by CD19⁺ B cells on days 5 and 10 PI in the GT cells by ICCS. * $P \leq 0.05$ vs. day 0 using one-way ANOVA test in a/b; *** $P \leq 0.001$ vs. day 0 using one-way ANOVA test in d; * $P \leq 0.05$, ** $P \leq 0.01$ using two-way ANOVA test in d/g. *Cm*, *Chlamydia muridarum*; GT, genital tract; ICCS, intracellular cytokine staining; IL, interleukin; ILN, iliac lymph node; PI, post infection.

Figure 4 *Cm*-induced CD43⁻, but not CD43⁺, IL-10-producing ILB cells suppress type 1 response via IL-10-dependent mechanism *in vitro*. B cells were prestimulated with *Cm* (MOI 1.0) for 24 h, irradiated, and then co-cultured at 1:1, 1:2, 1:5, or 1:10 ratio with eFluor670-labeled OT-II splenocytes with or without anti-CD3 for 72–90 h. (a) Percentage of CD4⁺ T-cell proliferation in the presence of different densities of unstimulated or *Cm*-stimulated B cells. Data are pooled from two individual experiments and presented as the mean $\pm$ s.e.m. of *Cm*-stimulated B cells from five mice. Unstimulated B cells were derived from one mouse. * $P \leq 0.05$, *** $P \leq 0.01$ vs. responder cells alone using one-way ANOVA test. (b) IFN- γ , (c) IL-4, and (d) IL-17A levels measured by ELISA from co-culture supernatants. Data are presented as the mean $\pm$ s.e.m. of triplicate wells from one representative experiment. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$ vs. responder cells alone using one-way ANOVA test and, for IFN- γ ELISA data, # $P \leq 0.05$ vs. 1:10 using one-way ANOVA test. (e) IL-10GFP splenocytes were stimulated with *Cm* (MOI 0.1) for 40 h. CD43⁺ IL-10GFP⁺ and CD43⁻ IL-10GFP⁺ cells were sorted, irradiated, and co-cultured at different ratios with μ MT splenocytes with or without anti-CD3/anti-CD28 for 72 h. IFN- γ , IL-10, IL-6, and GM-CSF levels were measured by ELISA from co-culture supernatants. Data are presented as the mean $\pm$ s.e.m. of triplicate wells and are representative of two independent experiments. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$ vs. responder cells alone “0” using one-way ANOVA test. (f, g) WT and IL-10KO splenocytes were stimulated with *Cm* for 40 h and CD19⁺ CD43⁻ cells were sorted, irradiated, and co-cultured at different ratios with μ MT splenocytes with or without anti-CD3/anti-CD28 for 72 h. In one set of wells, anti-IL-10R (10 μ g ml⁻¹) was applied. IFN- γ , IL-4, IL-17, and IL-2 levels were measured by ELISA. Data are presented as the mean $\pm$ s.e.m. of triplicate wells of one representative experiment. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$ vs. responder cells alone “0” using one-way ANOVA test. ANOVA, analysis of variance; *Cm*, *Chlamydia muridarum*; ELISA, enzyme-linked immunosorbent assay; GFP, green fluorescence protein; GM-CSF, granulocyte macrophage colony-stimulating factor; IFN- γ , interferon gamma; IL, interleukin; ILB, innate-like B cells; KO, knockout; MOI, multiplicity of infection; WT, wild type.

observations in the ILN, total B-cell numbers in the spleen remained relatively stable throughout the course of infection (Figure 5f). However, the number of splenic IL-10⁺ B cells significantly decreased at day 5 post infection, suggesting effluxion of IL-10⁺ B cells from the spleen to ILN (Figure 5g,h). Although B cells were detected in the GT at both day 5 and day 10 post infection, these B cells were not IL-10 producers (Figure 5j). Together, these data show that *Cm*-induced CD43⁺CD1d^{hi}CD5⁺ Bregs are preferentially expanded and/or recruited to draining ILN in response to *Cm* infection.

To verify these IL-10-producing CD43⁺ B cells are inhibitory *in vivo*, we sorted IL-10GFP⁺CD43⁺ and IL-10GFP⁺CD43⁺ B cells from *Cm*-stimulated splenocytes and adoptively transferred each population into μ MT recipient 4 h post-*Cm* infection. Impressively, mice receiving IL-10GFP⁺CD43⁺ B cells exhibited markedly reduced frequencies and total numbers of IFN- γ -producing CD4⁺ and CD8⁺ T cells in the ILN at day 5 post infection compared with mice receiving IL-10GFP⁺CD43⁺ or PBS alone (Figure 6a,b). Total numbers

of ILN CD4⁺ and CD8⁺ T cells were also slightly decreased in IL-10GFP⁺CD43⁺ B-cell recipients (Figure 6c). An alternative adoptive transfer approach using IL-10KO or wild-type B cells further verified the critical role of B-cell-derived IL-10 on type 1 response *in vivo* (Figure 6d). Collectively, our results indicate that *Cm* genital tract infection naturally induces the development of CD43⁺CD1d^{hi}CD5⁺ Bregs in the ILN, which functionally restrain the induction of type 1 response in an IL-10-dependent manner.

IL-10 deficiency in B cells markedly enhances cellular responses and host resistance against *Cm* infection

As CD43⁺CD1d^{hi}CD5⁺ Bregs were accumulated in the ILN along with other IL-10-producing cells following *Cm* infection, we next generated mixed bone marrow (BM) chimeric mice with B-cell-specific IL-10 deficiency to examine the unique impact of B-cell-derived IL-10 on host responses to *Cm* infection. Following 6 weeks of BM reconstitution, the frequencies of B and CD4⁺ T cells in the peripheral blood of IL-10KO B and wild-type B chimeras were comparable. IL-10

Figure 6 CD43⁺IL-10GFP⁺ B cells inhibit IFN- γ responses upon *Cm* genital tract infection *in vivo*. IL-10GFP splenocytes were stimulated with *Cm* (MOI 0.1) for 40 h. IL-10GFP⁺CD43⁺ and IL-10GFP⁺CD43⁺ cells were sorted and adoptively transferred into μ MT recipient. (a) Frequency of IFN- γ ⁺CD4⁺ T cells in ILN of mice receiving PBS, IL-10GFP⁺CD43⁺ or IL-10GFP⁺CD43⁺ at day 5 post infection. (b) Total cell number of IFN- γ ⁺CD4⁺ and IFN- γ ⁺CD8⁺ T cells and (c) total cell number of CD4⁺ and CD8⁺ T cells in ILN of mice receiving PBS, IL-10GFP⁺CD43⁺, or IL-10GFP⁺CD43⁺. (d) The absolute numbers of total cells, frequency of IFN- γ ⁺ among CD4⁺ T cells, and absolute number of total IFN- γ ⁺CD4⁺ cells recovered from the ILN (top panels) and spleen (bottom panels) of μ MT recipients of wild-type or IL-10^{-/-} B cells at day 10 post infection ($n = 4$ mice per group). * $P \leq 0.05$, ** $P < 0.01$ vs. PBS group using two-way ANOVA test in panel b. * $P \leq 0.05$ using one-tailed Student's *t*-test in d. ANOVA, analysis of variance; *Cm*, *Chlamydia muridarum*; GFP, green fluorescence protein; IFN- γ , interferon gamma; IL, interleukin; ILN, iliac lymph node; MOI, multiplicity of infection; PBS, phosphate-buffered saline.

Figure 7 IL-10 deficiency in B cells markedly enhances cellular responses and host resistance against *Cm* genital tract infection. **(a)** Characterization of frequency of B220⁺ and CD4⁺ cells in peripheral WBCs and IL-10-producing B cells in the spleen of wild-type B and IL-10^{-/-} B chimeras at day 1 ($n = 4$ –6 per group). **(b,c)** The absolute numbers of total cells, and the frequency and absolute number of B220⁺ and CD4⁺ cells recovered from ILN **(b)** and spleen **(c)** of wild-type B and IL-10KO B chimeras at day 10 post infection. Data are pooled from two independent experiments and presented as mean $\pm$ s.e.m. of 6–9 mice per group. * $P \leq 0.05$, using Student's *t*-test. ** $P \leq 0.01$ using two-way ANOVA test. **(d)** The total number and frequencies of IFN- γ ⁺CD4⁺ and IFN- γ ⁺CD4⁻ cells in the ILN of wild-type B vs. IL-10KO B chimeras at day 10 post infection ($n = 6$ –9 mice per group). **(e)** *Cm* level in the genital swab samples from wild-type B and IL-10KO B chimeras were quantified. ($n = 4$ –8 mice per group per time point). * $P \leq 0.05$ using two-way ANOVA test. **(f)** Oviducts from wild-type B and IL-10KO B chimeras at day 30 post infection were stained with H&E and oviduct dilation was measured ($n = 10$ –11 mice per group). Data are presented as box whiskers plot: line within box represents median, box limits 25 and 75 percentiles, whiskers extend to the minimum and maximum values. * $P \leq 0.05$ using Mann–Whitney *U*-test. ANOVA, analysis of variance; *Cm*, *Chlamydia muridarum*; H&E, hematoxylin and eosin; IL, interleukin; ILN, iliac lymph node; KO, knockout; NS, no significance; WBCs, white blood cells.

production by B cells from IL-10KO B chimeras was confirmed to be markedly reduced compared with wild-type B counterparts (**Figure 7a**). At day 10 post infection, IL-10KO B chimeras had markedly enhanced cellular responses in the ILN and spleen relative to wild-type B chimeras (**Figure 7b,c**). The increased cellular response in the ILN of IL-10KO B chimeras was attributed to a marked expansion of B cells and a trend of

increase in CD4⁺ T cell numbers (**Figure 7b,c**). Consistent with a role of Bregs in modulating type 1 responses, IL-10KO B chimeras had significantly increased Th1 cell numbers and IFN- γ -producing non-CD4 populations in the ILN compared with wild-type B chimeras (**Figure 7d**). In comparison, Th17 responses were not significantly affected by B-cell-derived IL-10 (**Supplementary Figure 3**). Most strikingly, IL-10KO

B-cell chimeric mice exhibited significantly reduced bacterial burden by day 20 post infection compared with wild-type counterparts (**Figure 7e**). Furthermore, accelerated bacterial clearance in IL-10KO B-cell chimeras corresponded with reduced severity of hydrosalpinx by day 30 post infection (**Figure 7f**).

DISCUSSION

The objective of this study was to examine the role of IL-10-producing B cells in host responses to *Chlamydia* infection *in vitro* and *in vivo*. We have identified CD43 as a novel cell surface marker distinguishing innate type from adaptive type IL-10-producing B cells. Remarkably, *Chlamydia*-induced CD43[−], but not CD43⁺, B cells possessed potent IL-10-dependent suppressive activity over IFN- γ responses *in vitro*. Furthermore, the majority of IL-10-producing B cells induced by *Chlamydia in vivo* carried a CD43[−]CD1d^{hi}CD5⁺ phenotype. Finally, mixed BM chimeric mice with IL-10 deficiency restricted to the B-cell compartment showed significantly increased Th1 responses, enhanced bacterial clearance, and reduced pathology during *Chlamydia* infection relative to wild-type controls.

IL-10 is a potent immunosuppressive cytokine strongly implicated in the negative regulation of Th1 responses in *Chlamydia*-infected hosts.³⁴ Genetic analyses on gene polymorphisms in the IL-10 promoter region in *Chlamydia*-infected individuals have established a link between allelic variants in levels of IL-10 production and the risk of developing pathological sequelae.^{35–37} Similarly, IL-10KO mice are highly resistant to *Chlamydia*, displaying augmented Th1 responses, rapid clearance, and less severe GT pathology compared with wild-type counterparts.³⁸ However, cellular sources of IL-10 during *Chlamydia* infection are somewhat ambiguous, as IL-10 is produced by a broad range of immune and non-immune cells.^{34,39} In a DC-based vaccination model, IL-10-deficient DCs stimulated robust protective Th1 immunity against genital *Chlamydia* infection that was superior to that induced by wild-type DCs, suggesting that endogenous IL-10 produced by DCs at the immune induction site potentially affects the strength/quality of antichlamydial Th1 responses.^{38,40} In addition, IL-10-producing DCs were reported to be enriched in the relatively less-inflamed lower GT but not in the heavily inflamed upper GT during *Chlamydia* infection, supporting an anti-inflammatory role for IL-10-producing DCs in controlling cellular responses at the immune effector site.⁴¹ In this study, we found that CD43[−]CD1d^{hi}CD5⁺ Bregs were localized to the ILN, indicating that Bregs specifically modulate immune responses in the ILN but not at the immune effector site.

The concept of innate and adaptive Breg subsets was initially proposed by Mizoguchi¹⁴ and supported by others subsequently,^{9,30} however, the origin and the role of different subsets of Bregs in modulating host responses are largely under-studied. To date, the identification of Breg populations—particularly B10 cells—in many published studies requires an *in vitro* stimulation/amplification procedure using LPS and phorbol 12-myristate 13-acetate plus ionomycin following agonistic

anti-CD40 antibody prestimulation.^{23,30} This protocol is likely to amplify both innate and adaptive Bregs and, indeed, the B10 population is reported to contain both CD43⁺ and CD43[−] fractions.³⁰ Herein, we have established that innate and adaptive Bregs can be identified based on their CD43 expression. We demonstrated a dramatic attenuation of CD43[−]IL-10-producing B cells in purified B-cell cultures and DC-depleted splenocyte cultures in comparison with whole splenocyte cultures upon *Chlamydia* or LPS stimulation. While MyD88-dependent signals are required for IL-10 production by all B-cell subsets, the requirement for DC-derived signals in IL-10 production by CD43[−], but not CD43⁺, B cells would classify these cells as adaptive and innate Bregs, respectively. Based on this understanding, a novel *in vitro* co-culture system allowed us to track the origin of innate vs. adaptive Bregs. We have provided clear evidence demonstrating that CD43⁺ innate Bregs may originate from either B1 or MZB cells whereas CD43[−] adaptive Bregs are solely derived from MZB cells. A distinct CD43⁺CD1d^{hi}CD5⁺ population accounted for 25–35% of IL-10⁺ B cells in whole splenocyte cultures and ~60% of DC-depleted splenocyte cultures. This population phenotypically overlaps a subset of B10 and B1a cells and closely resembles a splenic IgM^{hi}IgD^{lo}B220⁺CD1d^{hi}CD43⁺ population previously described to produce IL-10 in response to a human *Chlamydia trachomatis* serovar independently of T cells *in vitro*.⁴² However, despite being potent IL-10 producers, our *in vitro* suppression assay results did not support a claim that *Chlamydia*-induced CD43⁺IL-10⁺ B cells are true immunosuppressive Bregs. Rather, they appear to have immune stimulatory function.

In contrast, CD43[−]CD1d^{hi}CD5⁺ B cells demonstrated potent IL-10-dependent suppressive activity in our *in vitro* suppression assay and *in vivo* adoptive transfer experiment, supporting that these cells are a *bona fide* Breg population. We demonstrated here that the generation of CD43[−]IL-10⁺ Bregs was fundamentally different from that of CD43⁺IL-10⁺ B cells in that it required the presence of DCs. We further demonstrated that the specific DC-derived signals controlling the development of CD43[−]IL-10⁺ Bregs were MyD88 dependent, TLR2 dependent, but TRIF independent. Direct B–DC interaction has been shown to be necessary for B-cell proliferation and survival.²⁷ Both membrane bound molecules (e.g. CD40) and soluble molecules such as IFN- β and BAFF produced by DCs are reported to promote Breg differentiation.^{33,43} In our system, both DC-derived BAFF production and CD40/CD40L expression by B cells were controlled in a MyD88-dependent, TLR2-dependent but TRIF-independent manner. While both molecular mechanisms are clearly required in the development of Bregs, we believe the process starts with *Chlamydia*-induced BAFF production by DCs, which, in turn, promotes B-cell survival/activation and upregulates CD40/CD40L on B cells directly or indirectly.

Collectively, our data demonstrate for the first time a distinct role for MZB-derived CD43[−]CD1d^{hi}CD5⁺ adaptive Bregs over CD43⁺ innate-type counterparts in controlling mucosal immune responses. It is also the first report that B cells are a key

cellular source of counterregulatory IL-10 produced during *Chlamydia* infection and potentially suppress protective host Th1 responses to *Chlamydia* *in vivo*.

METHODS

Mice. C57BL/6 mice (6–8 weeks) were purchased from Charles River Laboratory. Breeding pairs of congenic CD45.1-B6 mice, B-cell-deficient (μ MT) mice and IL-10KO mice were purchased from Jackson Laboratory. Breeding pairs of IL-10GFP reporter mice⁴⁴ were obtained from Dr Richard Flavell (Yale University). MyD88KO, TLR2KO, TRIFKO, and OT-II mice were obtained from Dr Jean Marshall (Dalhousie University). Mice were maintained under specific-pathogen-free conditions at the InVivo Laboratory of the IWK Health Centre. All animal procedures were approved in accordance with the guidelines of the Canadian Council on Animal Care.

To generate BM chimeric mice, recipient CD45.1-B6 mice received consecutive doses of 750 rads and 450 rads of gamma irradiation 18 h apart. Recipient mice were then reconstituted with $\sim 10^7$ CD45.2⁺ T-depleted donor BM cells consisting of 80% μ MT and 20% IL-10KO or wild-type cells to generate IL-10KO B and wild-type B chimeras, respectively. BM cells were prepared by flushing the femurs and tibia of wild-type C57BL/6, μ MT and IL-10KO mice (all CD45.2⁺) with RPMI 1640 containing 5% bovine serum. Red blood cells were lysed by ACK (0.1 mM EDTA, 0.15M NH₄Cl, 1 mM KHCO₃) buffer. To deplete mature T cells, BM cell preparations were labeled with CD4, CD8 α , and Thy1.2-directed antibodies and negative selection was performed using MACS beads. T-depleted BM cells were resuspended in 100 μ l of PBS and IV transferred to recipient mice and left to reconstitute for 6 weeks. Peripheral blood analysis of CD45.1/CD45.2 expression showed that recipient mice were generally 96% reconstituted with donor cells. Antibiotic-laced drinking water (0.2% w/v neomycin) was administered *ad libitum* for 72 h before irradiation and for 2 weeks after BM reconstitution.

Cm stimulation and B-cell suppression assay *in vitro*. *Chlamydia muridarum* (Cm) was propagated in McCoy cells according to procedures described previously.^{45–47} Purified Cm was stored in small aliquots at -80°C until use. Aliquots of purified Cm were inactivated at 65°C for 30 min and used as heat-killed Cm.

To assess IL-10 production, splenocytes isolated from IL-10GFP mice were stimulated with Cm (multiplicity of infection = 0.1). At various timepoints, the culture supernatants were collected for determining IL-10 level by enzyme-linked immunosorbent assay and cell pellets were stained with fluorescent antibodies recognizing MHCII, CD19, B220, CD11c, and CD3. The IL-10-producing immune subsets were assessed by GFP expression by flow cytometry. In some experiments, splenocytes were depleted with DCs using CD11c-conjugated MACS beads (Miltenyi Biotech, Toronto, ON, Canada) before Cm stimulation. In other experiments, various B subsets were sorted and then stimulated with live Cm.

To setup suppression assays, whole splenocytes were prestimulated *in vitro* with Cm for 24 or 40 h. B cells or B subsets were purified by sorting, gamma irradiated (1,500 rad) to halt proliferation and then co-cultured in titrated ratios with 2×10^5 responder cells, from OT-II or μ MT mice, in the presence of anti-CD3 ($2 \mu\text{g ml}^{-1}$) or anti-CD3 plus anti-CD28 ($4 \mu\text{g ml}^{-1}$) or media alone. In some experiments, anti-IL-10R antibody (clone1B1.3a) was used at $10 \mu\text{g ml}^{-1}$. To assess T-cell proliferation, responder cells were labeled with $5 \mu\text{M}$ eFluor670 (eBioscience, San Diego, CA). Following 72–90 h of co-culture, the culture supernatants were collected for cytokine measurement and the cell pellets were used to determine T-cell proliferation based on eFluor670 dilution.

Cm infection *in vivo*. Three and 10 days before infection, mice were injected subcutaneously with 2.5 mg of Depo-Provera (Pfizer, Kirkland, Canada). Mice were anesthetized and inoculated

intravaginally with 5×10^5 inclusion-forming units of Cm. To monitor bacterial shedding, mice were swabbed vaginally twice per week and the samples were stored at -80°C until bacterial load quantification by inclusion-forming units assay.^{45–47}

In adoptive transfer experiments, B cells were purified from wild-type or IL-10KO mice using CD19-MACS beads and $\sim 15\text{--}20 \times 10^6$ cells were transferred IV into μ MT-recipient mice 24 h before intravaginal infection. Alternatively, CD43⁺IL-10GFP⁺ or CD43⁺IL-10GFP⁺ B cells were sorted from Cm-stimulated splenocytes and $\sim 2 \times 10^6$ cells were transferred IV into μ MT-recipient mice 4 h post intravaginal infection.

Fluorescence-Activated Cell Sorting analysis and intracellular cytokine staining. To phenotype immune cells in the spleen, ILN, and GT, single-cell suspensions were isolated, blocked with anti-mouse CD16/CD32 antibody, and then labeled with fluorochrome-conjugated antibodies recognizing the following: CD3, CD4, CD19, CD21, CD23, B220, IgM, IgD, CD1d, CD5, and CD43. Anti-CD43 antibody clones S7 and R2/60 were purchased from BD Pharmingen (Mississauga, ON, Canada) and eBioscience, respectively. To determine the cell type and the frequency of IFN- γ , IL-17A γ , and IL-10-producing cells, single-cell suspensions were re-stimulated in culture with heat-killed Cm (multiplicity of infection ~ 0.01) for 72 h followed by amplification with phorbol 12-myristate 13-acetate (50 ng ml^{-1}) plus ionomycin ($2 \mu\text{g ml}^{-1}$) in the presence of Brefeldin A ($10 \mu\text{g ml}^{-1}$) and monensin ($2 \mu\text{M}$) for the last 4–6 h of culture. The cells were surface labeled then fixed, permeabilized, and intracellularly labeled with IFN- γ , IL-17A, IL-10, or isotype-matched control antibody (rat IgG1 and rat IgG2a) as described previously.^{45–47} The data were analyzed using FCS Express 4 Flow Cytometry software (De Nova Software).

Histology. BM chimeric mice were killed at day 30 post infection; the GT were fixed and embedded. Longitudinal 5- μm sections were cut and stained with hematoxylin and eosin. The oviduct dilation was assessed blindly by measuring the cross-sectional diameter of the oviduct in left and right uterine horns using ImageJ software (National Institute Health).⁴⁷

Enzyme-linked immunosorbent assay. The level of cytokines in the supernatants was measured by enzyme-linked immunosorbent assay using antibody pairs specific for mouse IFN- γ , IL-17A, IL-4, IL-10, IL-2, IL-6, granulocyte macrophage colony-stimulating factor, and IL-12p70 (eBioscience) and BAFF (R&D System, Minneapolis, MN).

Statistical analyses. Tests for statistical significance were performed using GraphPad Prism 5 software. The specific analysis is indicated in each figure. *P* values ≤ 0.05 were considered statistically significant.

SUPPLEMENTARY MATERIAL is linked to the online version of the paper at <http://www.nature.com/mi>

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DISCLOSURE

The authors declared no conflict of interest.

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