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Induction of IL-10-producing regulatory B cells following *Toxoplasma gondii* infection is important to the cyst formation

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ABSTRACT

Toxoplasma gondii is a protozoan parasite that infects humans and animals via congenital or postnatal routes. During parasite infection, IL-10-producing Bregs are stimulated as part of the parasite-induced host immune responses that favor infection. In this study, we investigated whether *T. gondii* infection induces immune regulatory cells including IL-10-producing $CD1d^{high}CD5^{+}$ regulatory B cells (Bregs) and whether Breg induction is critical for the development of chronic infection of *T. gondii*. Furthermore, B cell-deficient (μMT) mice revealed that the IL-10-producing B cells might be associated with the development of chronic *T. gondii* infection. To better understand the mechanism underlying the accumulation of IL-10-producing B cells upon *T. gondii* infection, we determined the effect of products released by *T. gondii* on the induction and differentiation of IL-10-producing B cells during the acute stage of infection using transgenic green fluorescent protein (GFP)-expressing *T. gondii* strain. We demonstrated that products secreted at the stage of cell lysis by fully replicated tachyzoites induced the differentiation of naive B cells to IL-10-producing Bregs. Our results indicated that the downregulation of the immune response via Bregs during *T. gondii* infection is related to cyst formation in the host brain and to the establishment of chronic infection.

1. Introduction

Toxoplasma gondii is an intracellular protozoan parasite with a worldwide distribution that infects a variety of warm-blooded mammals and causes toxoplasmosis, one of the most common chronic parasitic infections in humans: approximately one-third of the world population carries *T. gondii* [1]. Toxoplasmosis is usually clinically asymptomatic in healthy individuals but it can lead to severe complications in pregnant women and immunocompromised patients. The life cycle of *T. gondii* includes two asexual stages: rapidly dividing tachyzoites are found in the spleen and non-lymphoid tissues in the first week of infection and define the acute phase, whereas slowly dividing bradyzoites, residing mainly inside tissue cysts in the brain, appear around the second week and mark the beginning of the chronic phase [2]. Acute infection is characterized by tachyzoite proliferation and is known to cause lymphadenitis and congenital infection of fetuses [3]. During chronic infection, the parasite forms cysts, preferentially in the brain, and this phase is characterized by the balance between host immune responses and the parasite immune evasion.

Parasites employ various strategies to evade host immune responses that normally thwart infection. The establishment and maintenance of chronic infection involves a balance between host immunity and parasite evasion of the immune response [4]. Although immune evasion supposedly evolved to favor parasite establishment within the host, some particular immune-escaping strategies might, quite paradoxically, be beneficial for the host [5]. Regulatory B cells (Bregs), regulatory T cells (Tregs), and alternatively activated macrophages have been identified as key components of the immune regulatory network functioning during helminth infections [6–8]. These immunoregulatory cells expand during parasite infection and may promote the development of chronic infection and parasite survival, as well as influence unrelated immune-driven pathology such as allergy and autoimmune diseases [7,9].

B cells typically function as antibody-producing cells but they are also involved in other immune functions including cytokine and chemokine secretion and antigen presentation. In addition, IL-10-producing B cells have been shown to participate in the induction of immune tolerance and the suppression of inflammation [9–11]. The IL-10-producing subset of Bregs was first demonstrated to play a critical role in limiting disease severity in autoimmune conditions [6]. In recent studies, it has been found that these Bregs are also significantly induced during parasite infection [6,12–14].

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Several studies have demonstrated that during parasite infection, IL-10-producing Bregs are stimulated as part of the parasite-induced host immune responses that favor infection [13,14]. Indeed, blockade of these Bregs increased host resistance and reduced parasite burden, while also increasing immune-driven pathology [13,14]. Interestingly, IL-10-producing B cells induced by parasite infection have been shown to suppress allergic inflammatory and autoimmune diseases. In animal models, various helminths alleviated the symptoms of experimental allergic and autoimmune diseases via the induction of IL-10-producing B cells [12,15–17]. These results have prompted clinical trials in which the introduction of live helminths to treat patients with autoimmune diseases was tested [18]. However, the clear understanding of the complex cellular mechanism that regulates the host immune response to parasitic infections through the activation of the immune regulatory network remains a key issue in the prevention and control of immune-mediated diseases.

T. gondii causes the induction of strong cell-mediated immunity characterized by a highly polarized Th1 response in the early stages of infection. IFN-γ-dependent, cell-mediated immunity is the major host resistance mechanism against chronic *T. gondii* infection [19]. Recent studies have reported that IL-10-producing $CD1d^{high}CD5^{+}$ B cells suppress the IFN-γ production and type 1 immune responses during intracellular bacterial infection [20]. In addition, chronic graft-versus-host disease-derived T cells cultured with IL-10-producing Bregs showed significantly reduced IFN-γ release, suggesting that these Bregs might be deeply involved in IFN-γ-dependent immunity [21]. However, the role of IL-10-producing B cells in the course of *T. gondii* infection is poorly understood.

The aim of this study was to investigate the influence of *T. gondii* infection on the accumulation of IL-10-producing $CD1d^{high}CD5^{+}$ B cell as immune regulatory cells.

2. Materials and methods

2.1. Ethics statement

The study were reviewed and approved by the Korean Centers for Disease Control and Prevention and the Institutional Animal Care and Use Committee (KCDC-IACUC; approval number KCDC-12-039-2A). All experimental and animal care protocols were performed in accordance with the guideline for the Care and Use of Laboratory Animals of the Korean Centers for Disease Control and Prevention. Pathogen-free6- to 7-week-oldfemale C57BL/6 mice were purchased from Orient Bio (Korea). B cell-deficient μMT (B6.129S2-*Igh-6*^{tm1Cgn}/J) mice were obtained from Jackson Laboratory (Bar Harbor, ME, USA), and housed in a specific pathogen-free facility in individually ventilated and filtered cages. For in vivo studies, mice were anesthetized by 2% isoflurane in 0.3 L/min oxygen inhalation during the procedure. The adequacy of anesthesia was monitored by disappearance of pedal withdrawal reflex. For ex vivo studies, mice were euthanized by cervical dislocation. The adequacy of sacrifice was monitored from the disappearance of heart beat and breath.

2.2. Parasites and infection

Cysts of the *T. gondii* ME49 strain were obtained from the brains of chronically infected mice: μMT and control mice were infected with 15 cysts intraperitoneally and the brains were removed at indicated days after infection and homogenized with 1 mL of Dulbecco's phosphate-buffered saline (Gibco/Life Technologies, Grand Island, NY, USA) by using a 23-gauge needle. The brain cysts were examined in 20-μL brain homogenates mounted on glass slides by using a light microscope at a magnification of 20 × and the total number of cysts was determined in five 20-μL per sample. For the identification of the exact stage of the parasite lytic cycle, we a used transgenic green fluorescent protein (GFP)-expressing *T. gondii* strain generated by Prof. Yoshifumi Nishikawa (National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Japan). GFP-positive *T. gondii* tachyzoites were kindly provided by Dr. Young-Ha Lee (Chungnam National University School of Medicine, Korea). HeLa cells (ATCC, Manassas, VA, USA) were inoculated with GFP-positive *T. gondii* tachyzoites at a multiplicity of infection of 1:6 for 3 days and supernatants containing excretory/secretory products (ESPs) released during *T. gondii* infection were collected at 4, 24, 48, and 72 h post-infection. After centrifugation for 5 min at 6000 rpm, the protein concentration in the ESP-containing supernatants was determined using the Bradford assay and the samples were stored at −70 °C until use.

2.3. Antibodies and flow cytometry

Splenocytes were isolated using a 40-μm nylon cell strainer (BD Biosciences, San Jose, CA, USA); RBCs were lysed with buffer containing 0.14 NH_4Cl and 0.017 M Tris-base (pH 7.5). IL-10-producing $CD1d^{high}CD5^{+}CD19^{+}$ Bregs were analyzed by flow cytometry after immunostaining of surface markers with the following monoclonal antibodies: fluorescein isothiocyanate (FITC)-conjugated anti-CD19 (clone 1D3) (BD Biosciences, San Jose, CA, USA), phycoerythrin (PE)-conjugated anti-CD1d (clone 1B1; isotype, Rat IgG2b, κ) (BD Biosciences), allophycocyanin (APC)-conjugated anti-CD5 (clone 53–7.3) (eBioscience, San Diego, CA, USA), and peridinin chlorophyll-protein complex (PerCP)- or PE-conjugated anti-IL-10 (clone JES5–16E3) (eBioscience). For subsequent intracellular IL-10 staining, the Cytofix/Cytoperm kit (BD Biosciences) was used according to the manufacturer's protocol (BD Biosciences). Intracellular transport processes were inhibited with BD GolgiStop containing the transport inhibitor monesin and Fc receptors were blocked by treatment with rat anti-mouse CD16/32 antibody (BD Bioscience) for 15 min. The FlowJo software (Tree Star Inc., Ashland, OR, USA)was used to analyze the flow cytometry data. To determine background staining, non-reactive isotype-matched control monoclonal antibodies (eBioscience) were used and cells were gated to exclude ≥98% of non-reactive cells.

2.4. B cell isolation and stimulation

The regulatory B cell isolation kit (Miltenyi Biotech) was used to purify naive $CD19^{+}$ B cells according to the manufacturer's instructions. For analysis of the cytokine production, 4×10^5 purified $CD19^{+}$ B cells were cultured with ESPs, total lysate of *T. gondii* tachyzoites, or LPS (*Escherichia coli* serotype 0111:B4, Sigma-Aldrich, St. Louis, MO, USA) for 24 h. Culture supernatant was collected to assess the cytokine production, and the cells were analyzed by flow cytometry.

2.5. Statistical analysis

Statistical analyses were performed with the GraphPad Prism v.3 software. All data are shown as the mean ± SD. The significance of the differences between sample means was determined using Student's *t*-test or with one-way ANOVA followed by Bonferroni's post-test for multiple comparisons. A *P*-value < 0.05 was considered significant.

3. Results

3.1. IL-10-producing B cells are increased during T. gondii infection

Our previous study and another recent study demonstrated that IL-10 production by B cells is required for host susceptibility to protozoan parasite infection [13,14]. To determine whether these B cells are induced by *T. gondii* infection, the IL-10 production by B cells in *T. gondii*-infected and uninfected control mice was verified by direct intracellular cytokine staining. At day 15 post-infection, the ratio to total B cells and the total number of spleen IL-10-producing B cells was higher in infected mice than in uninfected mice ($P < 0.01$) (Fig. 1A). It has been shown that IL-10-producing B cells function as Bregs with the $CD1d^{high}CD5^{+}CD19^{+}$ phenotype [12,22]. Therefore, we examined the cell phenotype to formally identify the IL-10-producing B cell subpopulation. As expected, the majority of $IL\text{-}10^{+}CD19^{+}$ spleen cells expanded in response to *T. gondii* infection exhibited the $CD1d^{high}$ $CD5^{+}$ phenotype (Fig. 1B). To confirm the increase in these regulatory immune cells during *T. gondii* infection, we monitored the dynamics of the IL-10-producing B cell subpopulation during the stage of cyst formation in the brain, which is characteristic of chronic infection. A large number of cysts with increased size in the brain and the increased population of IL-10-producing B cells in the spleen were observed in chronically infected mice, respectively. (Fig. 2). These data demonstrated that *T. gondii* infection induces the accumulation of IL-10-producing Bregs.

3.2. Cyst formation during T. gondii infection is markedly suppressed in B cell-deficient mice

To investigate whether the induction of IL-10-producing B cells could facilitate cyst formation, we used μMT mice that lack mature B cells. By using flow cytometry, we confirmed that B cells with the $CD5^{+}CD1d^{+}CD19^{+}$ phenotype were not present in these mice (Fig. 3A). C57BL/6 wild-type control and μMT mice were infected with *T. gondii* and monitored for brain cyst burden. Comparison of the course of *T. gondii* infection in age- and sex-matched μMT and C57BL/6 wild-type mice revealed that the number of cysts was 4-fold lower in the μMT mice than in the control mice ($P < 0.001$) (Fig. 3B). Spleen enlargement is a typical symptom of protozoal infection such as toxoplasmosis. However, infected μMT mice demonstrated smaller spleens than the wild-type mice (Fig. 3C).

T. gondii is known to induce an antigen-specific type 1 T cell immune response in the infected host [23]. In addition, analysis of the Th1/Th2-associated serum cytokines showed that the levels of IFN-γ and TNF-α increased in the *T. gondii*-infected μMT mice. In contrast, IL-10 production was considerably lower in these mice

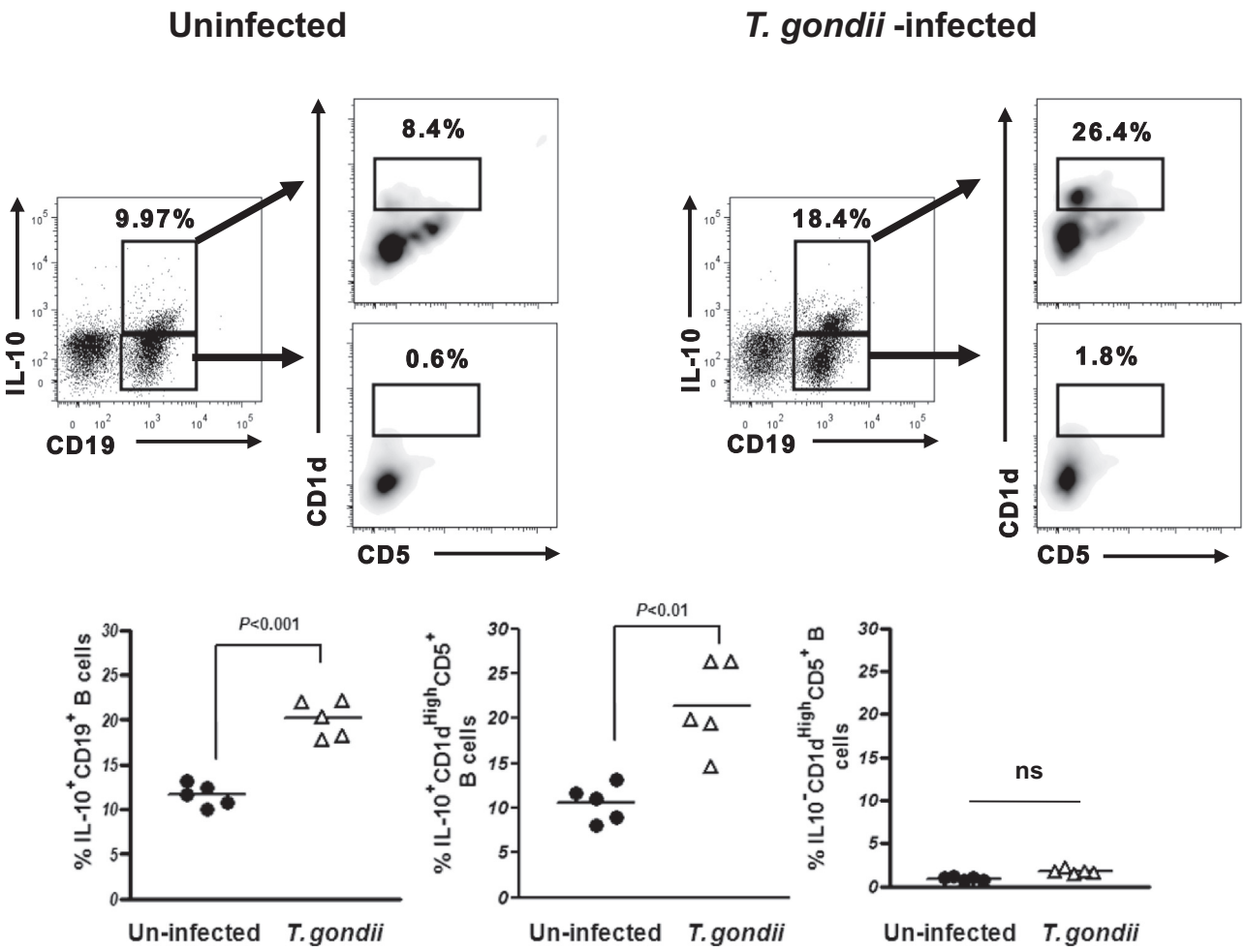

Fig. 1. Identification of IL-10-producing $CD1d^{high}CD5^{+}CD19^{+}$ Bregs induced by *T. gondii* infection. Splenocytes were isolated from uninfected and *T. gondii*-infected mice at day 15 post-infection. To detect IL-10-producing B cells, splenocytes were stained with antibodies against B220, CD19, CD1d, CD5, and IL-10. B220 staining was used as the initial gate for identifying B cells. (A) Analysis of IL-10-producing B cell population (left panels). The bar graphs indicate the ratio (%) and total numbers of B cells that produced IL-10 in the spleen of uninfected and infected mice. (B) The gating strategy used to identify IL-10-producing $CD1d^{high}CD5^{+}$ B cell is shown. The proportion of $IL\text{-}10^{+}CD1d^{high}CD5^{+}$ B cells is expressed as the mean ± SD. Cells from 5 mice per group were analyzed and data are representative of 3 independent experiments. $^{**}P < 0.01$ vs. uninfected control mice (Student's *t*-test).

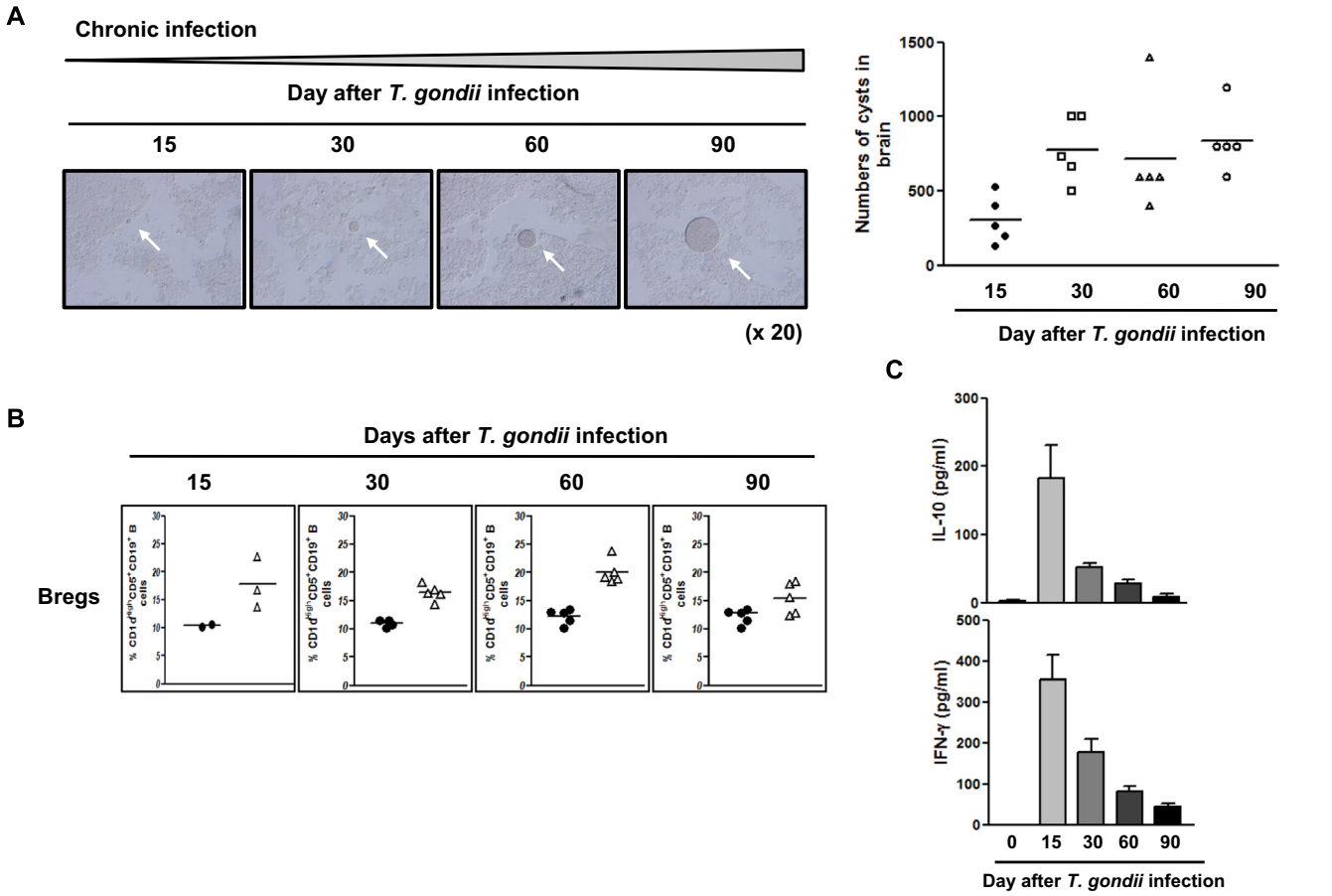

Fig. 2. Cyst formation and Breg accumulation during *T. gondii* infection. Mice were infected with 15 *T. gondii* cysts by intraperitoneal injection and sacrificed at the indicated time points. (A) The brain was analyzed for cyst burden. Cysts in the brain were observed under a light microscope (magnification 20 ×; upper panels). The graph presents the dynamics of cyst formation in the brain during the course of infection. (B) The spleen were analyzed populations of immunoregulatory cells. The IL-10^{+} Breg population was monitored during the acute and chronic stages of infection. Bars graphs represent the mean ± SD (n=5) and data are representative of 2 independent experiments with comparable results.

than in the wild-type group (Fig. 3D). There was no difference between the levels of IL-5 and IL-13 in wild-type and μMT mice (Fig. 3D). Taken together, although the complete absence of B cells is not the same as the absence of IL-10-secreting B cells, these results indirectly suggested that IL-10-producing B cells maybe involved in *T. gondii* pathogenesis and in Treg accumulation following parasite infection.

3.3. *Effect of parasite-derived ESPs released during different stages of T. gondii infection on the induction of IL-10-producing B cells*

To better understand the mechanism underlying the accumulation of IL-10-producing B cells upon *T. gondii* infection, we examined the effect of ESPs released by *T. gondii* on the induction and differentiation of IL-10-producing B cells during the acute stage of infection. *Toxoplasma* can infect and replicate within virtually any nucleated mammalian cell; its lytic cycle includes host cell attachment and invasion, intracellular vacuole formation, and parasite replication and egress [24]. To identify distinct stages of the parasite's lytic cycle, we monitored the infection of HeLa cells with GFP-expressing *T. gondii* (Fig. 4A). ESP-containing cell supernatants were harvested at different infection stages and used to treat naive CD19^{+}B cells isolated from the spleens of uninfected mice and the effect of the ESPs on the differentiation of naive B cells into IL-10-producing cells was determined after 24 h by flow cytometry. Culture supernatants of uninfected cells, LPS, or medium alone were used as controls. Among the ESPs obtained at different infection stages, the fraction collected at the stage of cell lysis and release of *T. gondii* tachyzoites, at 72 h after inoculation, was the most potent in enhancing the differentiation of naive B cells into IL-10-producing CD5^{+}CD1^{high}CD19^{+} Bregs (Fig. 4B and C). It should be noted that B cell differentiation was not induced by stimulation with the *T. gondii* total lysate and was only slightly induced by live tachyzoites (Supplementary Fig. S1).

4. Discussion

The development of chronic parasite infection is believed to result from the suppression of host immune responses. As *T. gondii* infection is usually chronic, it is likely that the parasite modulates the host immune system to prevent its own destruction. The findings in this study indicated that the induction of IL-10-producing Bregs in response to *T. gondii* infection may play an important role in the development of chronic infection as a part of the parasite evasion strategy against host immune mechanisms (Fig. 5).

We observed that a significant increase of the IL-10-producing CD1^{high}CD5^{+}CD19^{+} Breg subset during *T. gondii* infection was accompanied by increased cyst formation in the brain, which is a characteristic feature of chronic *T. gondii* infection (Figs. 2A and B). To investigate indirectly the relationship between the induction of IL-10-producing B cells and the facilitation of cyst formation, we used μMT mice that cannot generate mature IL-10-producing CD1^{high}CD5^{+}CD19^{+} B cells (Fig. 3A). In this experiment, we observed that *T. gondii* cyst formation in the brain of B cell-deficient

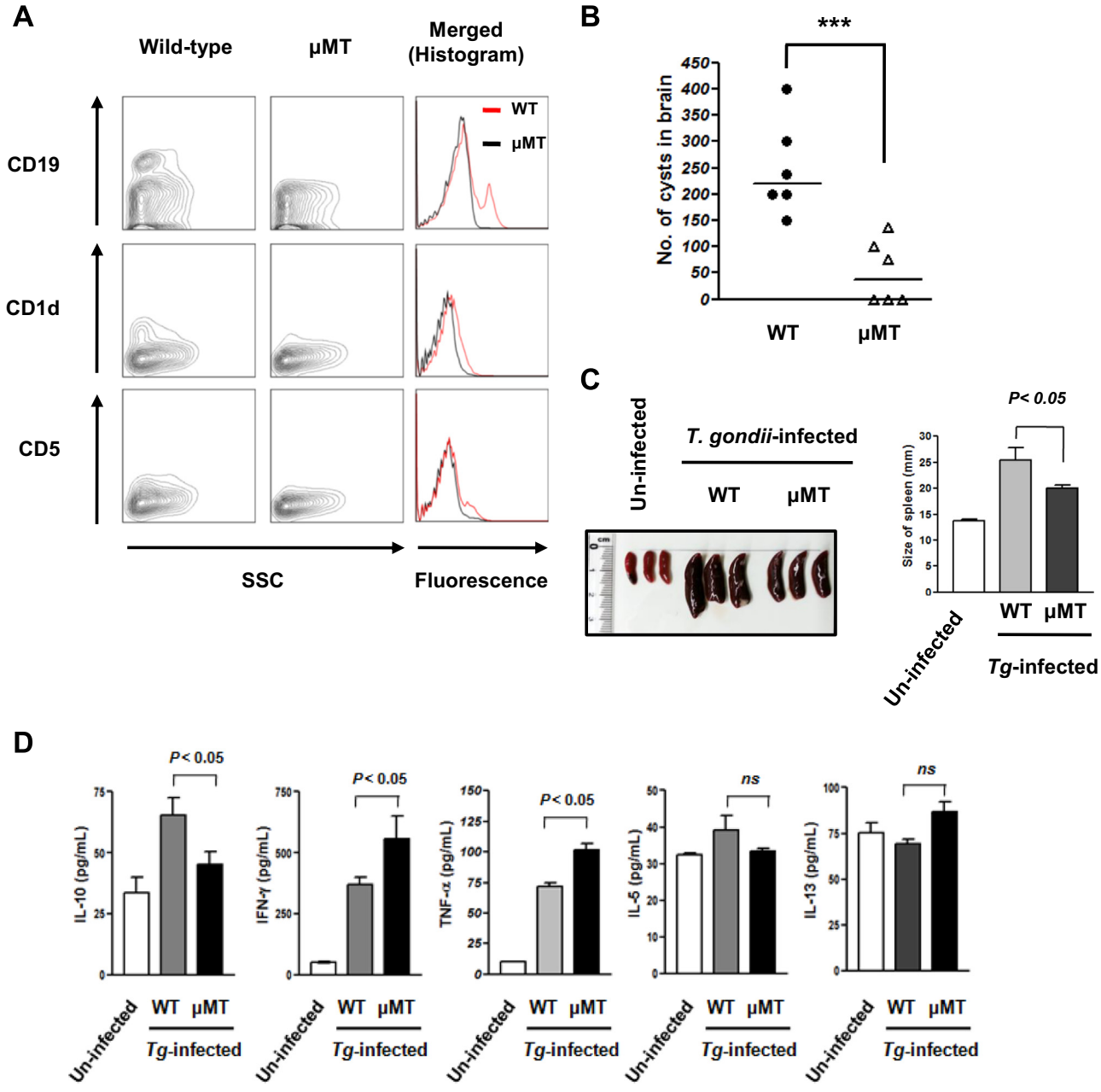

Fig. 3. Cyst formation in B cell deficient mice was significantly suppressed during *T. gondii* infection. (A) Identification of the absence of Bregs in μMT mice. Splenocytes from μMT and wild-type mice were stained with anti-mouse CD19, CD5, and CD1d antibodies and analyzed by flow cytometry. Black and red histograms represent μMT and wild-type mice, respectively. μMT and wild-type (WT) mice were infected with *T. gondii*. (A) The number of brain cysts 15 days post-infection. (B) Spleen enlargement in the infected mice. (C) Analysis of serum cytokine levels in the infected mice. (D) Flow cytometric analysis after staining for CD25 and FoxP3 in gated spleen $CD4^+$ T cells and the ratio (%) of $CD4^+CD25^+FoxP3^+$ T cells to total $CD4^+$ T cells in the splenocytes. The results are expressed as the mean ± SD (n=5). $^*P<0.05$ and $^{***}P<0.001$ vs. wild-type mice.

mice was significantly suppressed (Fig. 3B). Furthermore, splenomegaly, one of the classic features of toxoplasmosis, was moderate in infected B cell-deficient compared to wild-type mice (Fig. 3C). Thus, these results suggest that the induction of IL-10-producing B cells during *T. gondii* infection is critical for cyst formation and the establishment of chronic parasite infection. These results provide the first evidence for the importance of IL-10-producing B cells in the development of chronic *T. gondii* infection.

Furthermore, we shed light on the mechanism induced by these Bregs to support chronic infection. The proliferation of tachyzoites during the acute stage is important for the establishment of chronic infection but is limited by the IFN-γ-dependent host immune response [19]. We found that IL-10-producing B cells contributed to the suppression of these cell-mediated immune responses as evidenced by the higher IFN-γ serum levels in the μMT mice compared to wild mice (Fig. 3D).

Although several studies have reported Breg accumulation in response to helminth infection, the data on protozoal infections are limited and it is unclear whether parasite-derived molecules affect the induction of IL-10-producing B cells. Thus, we studied the effect of parasite-derived products secreted during different stages of *T. gondii* infection on the induction of Bregs in vitro. *T. gondii*, as an obligate intracellular pathogen, passes through four distinct life cycle stages: attachment and invasion, formation of the parasitophorous vacuole and intracellular growth, replication, and lysis of the host cell and release of the parasites (Fig. 4A). We demonstrated that the ESPs released at the stage of cell lysis by fully replicated tachyzoites induced the differentiation of naive B

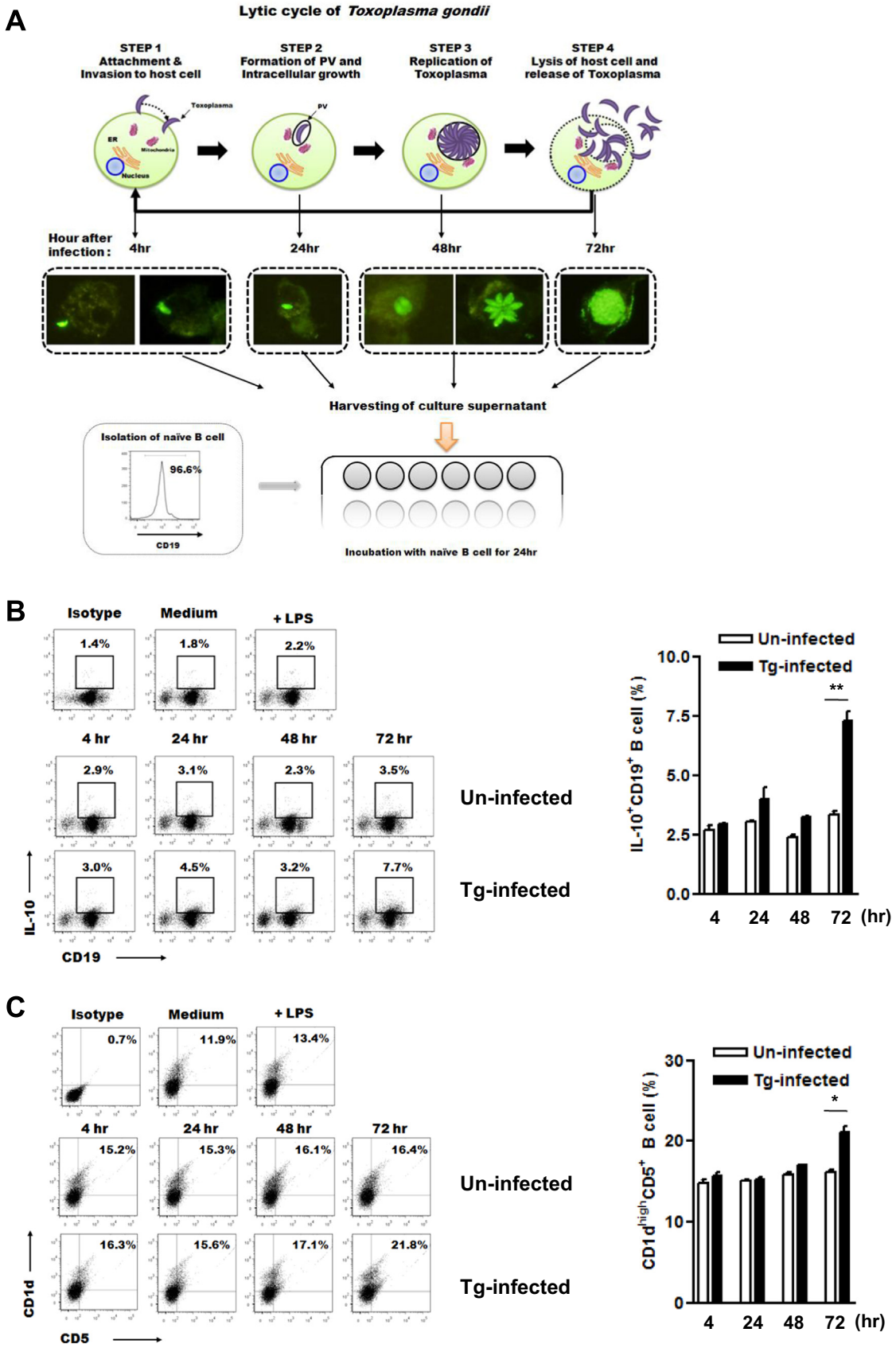

Fig. 4. IL-10-producing CD1d^{high}CD5^{+} Bregs are stimulated by *T. gondii*-infected cell supernatants. (A) Life cycle of *T. gondii*. For the identification of the lytic cycle stage, HeLa cells were inoculated at a multiplicity of infection of 1:6 with GFP-expressing *T. gondii* tachyzoites and incubated for 3 days, and tachyzoite transformation was monitored by fluorescent microscopy. Supernatants containing ESPs released during *T. gondii* infection were collected at 4, 24, 48, and 72 h post-infection and used to treat naive CD19^{+} B cells isolated from wild-type mice for 24 h. LPS (1 μg/mL) and medium were used as positive and negative control, respectively. PV, parasitophorous vacuole. (B–C) To quantify the IL-10^{+}CD19^{+}and CD1d^{high}CD5^{+}CD19^{+} B cells after the treatment, the cells were stained with CD19, CD5, and CD1d antibodies or with the Rat IgG2b, κ isotype control antibody. To detect intracellular IL-10, the cells were permeabilized and stained with IL-10 antibody or JES5-16E3 isotype control antibody. The ratio (%) of IL-10-producing CD19^{+} or CD1d^{high}CD5^{+}CD19^{+} B cells to total CD19^{+} B cells is presented. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with control group.

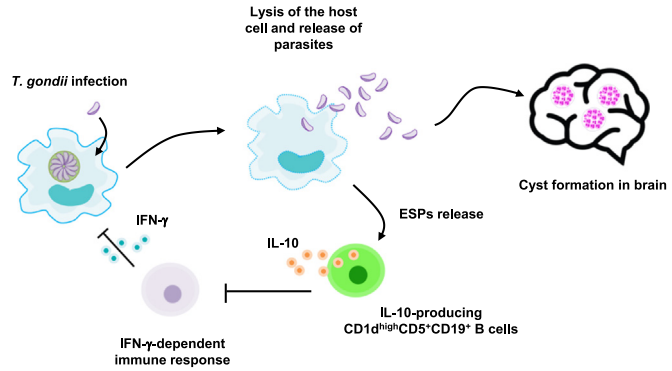

Fig. 5. The mechanism pertaining to the role of IL-10 producing regulatory B cells in *T. gondii* infection. The induction of IL-10-producing Bregs in response to *T. gondii* infection modulates the host immune system to establish the chronic infection of *T. gondii*.

cells into IL-10-producing Bregs (Fig. 4B). However, it is not clear whether the ESP was from host or parasitic origin. This finding indicated that the induction of IL-10-producing Bregs is enhanced by host cell lysis and parasite release, and offers new insights into the mechanism related to the differentiation and induction of IL-10-producing B cells by parasite infection.

In this study, we demonstrated that a significant accumulation of the IL-10-producing $CD1^{high}CD5^{+}CD19^{+}$ B cells subset was induced during *T. gondii* infection. Although considerable works have been conducted on the immune response to *Toxoplasma*, there are no studies investigating the role of B cells subset with a unique IL-10-producing $CD1d^{high}CD5^{+}$ phenotype in this process. Our results provided evidence that modulation of the immune response via IL-10-producing B cells induced in the course of *T. gondii* infection is related to the establishment of chronic infection (Fig. 5).

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Appendix A. Transparency document

Transparency document associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrep.2016.05.008.

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