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Surface receptor Toso controls B cell–mediated regulation of T cell immunity

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Introduction

A key feature of the immune system is the maintenance of a delicate balance between protecting against infectious agents and minimizing immune-mediated tissue damage. To achieve this critical equilibrium, tight regulatory mechanisms have evolved to control protective immune responses and to avoid misguided or excessive inflammation. It is now well established that a subset of CD4+ T cells, termed regulatory T cells, exhibit immunosuppressive function and are crucial for the maintenance of normal immune homeostasis by controlling inflammation and preventing autoimmunity (I). More recently, it has been recognized that B cells can also negatively regulate T cell responses in an antibody-independent manner. A suppressive role of B cells in pathogenic T cell responses was already suggested in 1996 by Janeway and colleagues, who showed that B cell–deficient mice exhibited exacerbated disease in experimental autoimmune encephalomyelitis (EAE) (2). An increasing number of reports has since indicated immunoregulatory function of B cells in various disease models, including T cell–dependent autoimmune models (3–8), transplantation (9), inflammation (10), and cancer (11), which has led to the concept of regulatory B cells (Bregs). Bregs exhibit their regulatory function primarily via the release of IL-10, although other mechanisms may also be involved (12). In vivo identification of Bregs is complicated by the description of multiple different Breg subsets with partially overlapping phenotypes and surface marker characteristics in different mouse and human model systems (13). Until now, no Breg-defining transcription factor or unique lineage marker has been identified, and Bregs are mainly defined by their ability to secrete IL-10. The nature and origin of Bregs are still controversial, and it is unclear whether they represent a distinct B cell lineage or a dynamic cellular state.

Toso, also known as Faim3 (Fas apoptosis inhibitory molecule 3) or FcyR (Fc receptor for IgM), is a type I transmembrane protein belonging to the immunoglobulin gene superfamily. Expression of Toso is restricted to lymphoid organs, where it is particularly highly expressed in B cells. In humans, a tight association of Toso overexpression with B cell malignancy has been observed in patients with chronic lymphocytic leukemia (14–16). Toso was originally identified as a surface molecule with negative regulatory function on lymphocyte apoptosis (17, 18). Subsequently, additional studies have identified Toso as an Fc receptor for soluble IgM (Fcy receptor) (19, 20). More recently, it has been demonstrated that Toso physically interacts with membrane IgM-containing B cell antigen receptor (BCR) complexes on the surface of mature B cells and/or within the trans-Golgi network of developing B cells (21, 22). Functionally, increasing evidence suggests that Toso serves as a physiologically important immunoregulatory molecule for B and T cells. Toso-deficient mice have been reported to show enhanced serum levels of IgM and IgG autoantibodies (21, 23–25), which, however, are not associated with autoimmune pathology (26). Furthermore, studies on Toso-deficient mice have revealed strong immunoprotective function of Toso in a model of Listeria infection (27) and during lymphocytic choriomeningitis.
virus infection (28). Toso-deficient mice are also largely resistant to the development of EAE and exhibit reduced pathogenic T cell responses (29). The mechanism underlying the phenotypic defects of Toso-deficient mice remains a controversial issue, and models involving different effector mechanisms and different immune cell types have been proposed (21, 22, 27, 29). Particularly, it is unclear whether the effects of Toso on tolerance in the B cell compartment are interrelated with impaired immune protection in Toso-deficient mice.

We demonstrate here that the specific deletion of Toso on B cells results in impaired antiviral T cell responses. We provide evidence that links this immunoregulatory function of B cells on T cell immunity to a specific set of IL-10–competent B cells. Our data show that these Bregs are negatively regulated by Toso and exhibit high prevalence for self-reactivity. Thus, via control of the pool of Bregs, Toso exhibits a dual role in immune homeostasis: it maintains normal self-tolerance within the B cell compartment and, at the same time, ensures protective T cell immunity against infection.

Results
Toso deficiency results in increased mortality and reduced production of proinflammatory cytokines by T cells upon influenza infection. To assess the impact of Toso on immune responses during acute viral infection, we intranasally infected WT and Toso–/– mice with 1,000 PFU of influenza virus strain A/PR8 (H1N1). Whereas 84% of WT animals survived infection, Toso–/– mice exhibited significantly increased mortality; most died between days 10 and 15 postinfection (p.i.), and only 23% survived (Figure 1A). Pulmonary viral titers in the bronchoalveolar lavage fluid were comparable between WT and Toso–/– mice at day 4 p.i., indicating normal viral replication and infectivity, but were relatively increased in Toso–/– mice during the clearance phase (day 7 p.i.) (Supplemental Figure 1A; supplemental material available online with this article; https://doi.org/10.1172/JCI97280DS1). Thus, increased influenza-induced mortality of Toso–/– mice was associated with delayed viral clearance.

Antiviral immunity and recovery from influenza infection are largely dependent on effectors T cell responses (30, 31), which usually peak around days 9–10 p.i., just when Toso–/– mice start to become moribund. We thus next examined virus-specific T cell responses in Toso–/– mice. Viral antigen-specific CD4+ and CD8+ T cell populations were enumerated in the lungs of infected animals at day 9 p.i. by tetramer staining for the immunodominant CD4 T cell epitope NP366-374/I-Ak (NP311) or the CD8 T cell epitope NP366-354/Dk (NP366). Both frequency and absolute numbers of virus-specific NP311-tetramer-positive CD4+ T cells and NP366-dextramer-positive CD8+ T cells were comparable between WT and Toso–/– mice (Figure 1, B and C), indicating normal antigen-specific priming and clonal expansion of virus-specific T cells in Toso–/– mice.

Effector T cells contribute to viral control and elimination by the production of potent proinflammatory cytokines such as TNF-α and IFN-γ. The percentage as well as absolute numbers of IFN-γ– and TNF-α–producing T cells declined in lungs of influenza A–infected mice was significantly reduced in Toso–/– mice with both CD4+ and CD8+ T cells being affected (Figure 1, D–G).

Reduced production of these important antiviral cytokines (IFN-γ and TNF-α) by Toso–/– T cells was also observed in the spleen of infected animals, irrespective of whether mice were infected with a high dose (1,000 PFU) or a low dose (50 PFU) of influenza virus (Supplemental Figure 1, B–G, and data not shown), the latter not inducing any mortality in WT or Toso–/– mice.

Thus, although T cells in Toso–/– mice were capable of being activated and expanding in response to viral infection, these T cells were compromised in mounting an efficient antiviral cytokine response.

Conditional deletion of Toso in B cells results in impaired protective T cell immunity and limits immunopathological tissue damage. To assess whether reduced production of proinflammatory cytokines by T cells in Toso–/– mice is a T cell–intrinsic defect that depends on the specific deletion of Toso in T cells, or whether this is an indirect effect mediated by other cell types, such as antigen-presenting dendritic cells or B cells, we used a conditional gene targeting approach. To this end, we crossed TosoCKO mice onto different Cre-recombinase–expressing transgenic mouse lines — CD4-Cre mice, CD11c-Cre mice, and CD19-Cre mice — to specifically delete Toso in T cells, dendritic cells, and B cells, respectively (Supplemental Figure 2).

Upon influenza A infection, virus-induced mortality, as well as IFN-γ and TNF-α production by T cells, was not affected by ablation of Toso in T cells (TosoCKO/CD4-CreCKO mice) (Figure 2A and Supplemental Figure 3, A–D). Overall survival and T cell responses were also normal in TosoCKO/CD11c-CreCKO mice (Figure 2B and Supplemental Figure 3, E–H), indicating that conditional deletion of Toso in dendritic cells does not compromise their capacity to efficiently prime T cell activation. To our surprise, increased virus-induced lethality and impaired production of proinflammatory cytokines by T cells were only observed upon specific deletion of Toso in B cells (TosoCKO/CD19-CreCKO mice) (Figure 2, C–G). Similarly to straight Toso–/– mice (Figure 1A), most TosoCKO/CD19-CreCKO mice died between days 10 and 15 p.i. (only 11% survival), whereas 88% of CD19-CreCKO control mice survived infection (Figure 2C).

Also, the functional ability of CD4+ and CD8+ T cells to produce IFN-γ and TNF-α was significantly impaired in TosoCKO/CD19-CreCKO mice compared with CD19-CreCKO control mice (Figure 2, D–G). These data strongly indicate that the functional defect of T cells in Toso-deficient mice is not a T cell–intrinsic phenotype, but rather is indirectly induced by Toso-deficient B cells. Upon influenza infection, TosoCKO/CD19-CreCKO mice also had significantly more CD4+ T cells expressing PD-1 (Figure 2H and Supplemental Figure 4A), an inhibitory surface receptor that has been associated with T cell exhaustion (32). Interestingly, increased PD-1 expression on CD4+ T cells upon Toso deletion in B cells correlated with increased expression of its cognate ligand PD-L2 on CD19+ B cells (Figure 2I and Supplemental Figure 4B).

We further extended our studies on the B cell–specific role of Toso to a model of infection-induced intestinal pathology, using infection with attenuated Salmonella Typhimurium. In this model, chronic Salmonella infection of the murine gastrointestinal tract is associated with severe immunopathology that manifests in tissue fibrosis and extensive damage to the gut tissue along with the expression of a characteristic Th1-dominated inflammatory cytokine profile (33). Using this model, conditional deletion of Toso on
B cells in Toso<sup>−/−</sup>/CD19<sup>Cre</sup> mice led to impaired production of TNF-α by CD4<sup>+</sup> and CD8<sup>+</sup> T cells, which was associated with significantly attenuated overall cecal pathology (mainly attributable to reduced tissue damage in the epithelium and the mucosa) and relative protection from weight loss compared with CD19<sup>Cre</sup> control mice (Supplemental Figure 5).

Together, our data suggest that while expression of Toso on B cells is associated with enhanced protective T cell immunity during acute infection, it may also contribute to T cell-mediated immunopathological tissue damage under chronic inflammatory conditions.

**Toso deficiency results in increased numbers of IL-10–producing B cells.** The unexpected finding that T cell effector function is indirectly regulated by Toso expression on B cells prompted us to analyze functional characteristics of Toso-deficient B cells. Here, we first assessed the capacity of Toso<sup>−/−</sup> B cells to produce the antiinflammatory cytokine IL-10. To this end, purified B cells from WT and Toso<sup>−/−</sup> mice were treated for 24 hours with BAFF, LPS, anti-CD40, or anti-IgM or were control treated. Most interestingly, Toso<sup>−/−</sup> B cells exhibited a strongly increased capacity to produce IL-10 when compared with WT B cells (Figure 3A).

Depending on the stimulus, splenic B cells from Toso<sup>−/−</sup> mice induced up to 4-fold higher frequency and numbers of IL-10–producing cells (Figure 3, B and C). Increased production of IL-10 by B cells from Toso-deficient mice is a B cell–intrinsic phenotype, as it was specifically observed upon conditional deletion of Toso in B cells (Toso<sup>f/f/CD19-Cre</sup>/− mice), whereas B cells from Toso<sup>f/f/CD4-Cre</sup> and Toso<sup>f/f/CD11c-Cre</sup> mice exhibited normal IL-10 production (Figure 3, D and E). B cell cytokine production was, however, not generally affected by Toso deficiency, as activation-induced TNF-α production was normal in Toso<sup>−/−</sup> B cells (Supplemental Figure 6).
While only small numbers of IL-10−competent B cells are found in naive mice, number and frequency of IL-10−producing cells expand considerably upon influenza A infection (Figure 3, F and G). Importantly, also under these conditions of an in vivo viral infection, splenic B cells from Toso−/− mice had a significantly higher capacity to produce IL-10, as compared with B cells from WT mice (Figure 3, F and G). Total B cell counts in the spleen and numbers of GL7+CD95+ germinal center B cells were similar between WT and Toso−/− mice upon influenza A infection (Supplemental Figure 7). Finally, upon acute infection with influenza A virus, significantly higher frequency and numbers of IL-10−competent B cells were detected in the lungs of Toso−/−/CD19−Cre−/− mice compared with CD19−Cre−/+ control mice (Figure 3H). Thus, taken together, our data suggest that Toso expression on B cells exhibits a B cell–intrinsic negative regulatory function on the capacity of these cells to produce the antiinflammatory cytokine IL-10.
IL-10 both under steady-state conditions and in an inflammatory setting during viral infection.

**Phenotypic characteristics of IL-10–competent B cell subsets.** We next extended our analysis of IL-10–producing B cells onto different B cell subsets. Flow cytometric analysis of B cells from Vert-X IL-10 reporter mice — an IL-10-IRES-GFP knock-in mouse strain — showed that, upon LPS induction, essentially all splenic B1 cells (CD19+B220hi), as well as a small but significant fraction of B2 cells (CD19+B220lo), produced IL-10, as indicated by GFP expression (Supplemental Figure 8A). Thus, IL-10–competent B cell subsets reside within both B1 and B2 B cell compartments. IL-10–producing B1 cells exhibited a CD5+ phenotype and showed low expression for CD1d, while, in line with previous reports (34), IL-10–producing B2 B cells were mainly characterized as CD1d-CD5+ cells. Importantly, intracellular IL-10 staining of activated WT and Toso−/− B cells revealed that both of these IL-10–producing B cell populations — IL-10–producing B1a cells (B220hi) and IL-10–producing B2 cells (B220lo) — were markedly increased in B cell cultures from Toso−/− mice, irrespective of whether IL-10 production was induced by treatment with BAFF/L-21, LPS, or CpG oligonucleotides (Figure 4A–C).

Based on B220 surface staining and the characteristic expression of CD1d on IL-10–producing B2 B cells, we were able to efficiently identify IL-10–competent B1 and B2 B cell subsets within untreated naive CD19+ B cells. High-purity cell sorting experiments showed that more than 90% of naive sorted B220lo B1 cells could be induced to produce IL-10 (Figure 4D, E and D). IL-10–competent B cells were also highly enriched within naive B220loCD1d+ B2 B cells, where approximately 50% of sorted cells could be induced to express IL-10 (Figure 4E). In contrast, the large population of B220loCD1d− B2 B cells was largely unable to produce IL-10. We also examined whether IL-10–competent B220loCD1d+ B2 cells are interrelated with B220loAA4.1+ transitional B2 cells; however, these two B2 B cell subsets were clearly distinct by CD1d versus AA4.1 surface expression (Figure 4F), and, importantly, IL-10 production could not be induced in B220loAA4.1+ transitional B cells (Figure 4G), thus also functionally confirming the different nature of these two different B cell subsets. Marginal zone (M2) precursor B cells, which have been implicated in IL-10 production in a model of experimental arthritis (4), were also largely unable to produce IL-10 in our system (Supplemental Figure 8, B–E).

Further phenotypical analysis showed that the B220hi B1 B cell population expresses high levels of CD5 and CD43, typical for B1a B cells, while IL-10–competent B220loCD1d+ B2 cells exhibit a CD5loIgMloIgDhiCD21hiCD23hi phenotype reminiscent of MZ B cells (Figure 4H and Supplemental Figure 8F). IL-10–incompetent B220loCD1d− cells were mainly IgMhiIgDloCD21loCD23hi follicular B cells (Figure 4H).

Detection of IL-10–producing B cells from influenza-infected mice required short-term ex vivo restimulation. Here, we observed that IL-10–producing B1 and B2 B cell subsets are substantially expanded in both lung and spleen from influenza A–infected mice compared with uninfected control mice (Supplemental Figure 9, A–C). Moreover, IL-10–producing B2 cells (B2/IL-10 cells) from infected mice were also characterized by high expression of CD1d, and, under such an inflammatory context, IL-10–producing B1 and B2 B cells showed strong expression of Tim-1, CD73, and FasL (Supplemental Figure 9, D and E).

Most importantly, in Toso−/− mice both of the IL-10–competent B cell subsets (B220hiCD1d+ B2 cells and B220loB1a cells) were significantly increased in frequency and numbers (Figure 4D). Interestingly, however, on a per-cell basis, the cell-intrinsic capacity to produce IL-10 was comparable between the respective B cell subsets from WT and Toso−/− mice (Figure 4E). Thus, Toso−/− IL-10–competent B cells appeared to be functionally normal, but were present in significantly higher quantities upon genetic ablation of Toso.

**Suppressive function of IL-10–producing Bregs.** IL-10–producing B cells have been described as Bregs that exhibit immune regulatory functions and can downmodulate T cell responses and inflammatory reactions (35, 36). Thus, to demonstrate immunosuppressive activity of IL-10–producing B1 and B2 B cell subsets, we performed in vitro T cell–B cell coculture experiments. To this end, B cells from Vert-X IL-10/GFP reporter mice were purified by FACS into IL-10–producing B1 cells (B220loGFP+; “B1/IL-10 cells”) and B2 cells (B220hiGFP+; “B2/IL-10 cells”), as well as GFP-negative control B cells (B220loGFP−; B2/effecter cells). Sorted B cell populations were added to cultures containing purified naive CD4+ or CD8+ T cells. Cultures were stimulated with anti-CD3 mAbs, and proinflammatory cytokine production by CD4+ or CD8+ T cells was assayed.

The frequency of IFN-γ+ and TNF-α+ CD8+ T cells was significantly reduced in cocultures with B1/IL-10 cells and B2/IL-10 cells, compared with cultures with B2/effecter control cells (Figure 5, A and B). A similar suppressive effect of IL-10–producing B1 and B2 B cell subsets was observed in cocultures with naive CD4+ T cells (Figure 5C).

Next, we assessed whether freshly isolated naive B220lo B1a cells and B220loCD1d+ B2 cells can also exhibit immunosuppressive function on T cell immunity. For these experiments on nonactivated B cell subsets, we performed adoptive transfer of purified naive B cell subsets followed by in vivo viral challenge. We adoptively transferred 1 × 10⁶ FACs-sorted CD19+ B220hi B1a cells, CD19+B220hiCD1d+ B2 B cells, and CD19+B220loCD1d− B2
Figure 4. Phenotypic characteristics of IL-10–competent B cell subsets. (A–C) Purified B cells from WT and Toso−/− (KO) mice were cultured for 16 hours with BAAF plus IL-21 (A), LPS (B), or CpG oligonucleotides (C). For the last 5 hours, cells were treated with PMA/ionomycin in the presence of BFA/monesin and CD19+ B cells analyzed for IL-10 production. (D) B220 versus CD1d staining on naive CD19− B cells from WT and Toso−/− (KO) mice. (E) B220+ B2-CD1d+ B cells (black), B220− B2-CD1d− B cells (blue), and B220+ B1 B cells (red) from WT and Toso−/− (KO) mice were purified by FACs. Cells were stimulated for 16 hours with LPS plus PMA/monesin/BFA/monesin during the last 5 hours and subsequently analyzed for IL-10 production. (F) CD19+B220+ B2-Bregs were analyzed for CD1d versus AA4.1 (CD93) staining to identify AA4.1+ CD1d− B2-effector cells, AA4.1+ transitional B2 cells (B2-trans), and CD1d+ B2-Bregs. (G) AA4.1+ CD1d+ B2-effector cells, AA4+ B2-transitional cells, and CD1d− B2-Bregs were purified from IL-10/GFP reporter (Vert-X) mice by flow cytometric cell sorting. Cells were treated for 16 hours with LPS plus PMA/monesin during the last 5 hours and analyzed for GFP (IL-10) expression. (H) Flow cytometric analysis of naive B cells from C57BL/6/J mice. Left panel is gated on CD19+ B cells and shows gating for total B2 cells (B220hi), B220hiCD1d− B2 cells, B220hiCD1d+ B2 cells, and B220lo B1 cells. FACs profiles on the right show expression of IgM versus IgD (top panel) and CD23 versus CD21 (bottom panel) on the indicated B cell subsets. (I) Number and frequency of splenic B220+ B1 B cells and B220+CD1d− B2 B cells in WT and Toso−/− (KO) mice. Each symbol represents an individual mouse; horizontal lines indicate the mean ± SEM. n = 5; **P < 0.01; Student’s t test. Data are representative of at least 3 independent experiments.

B cells from untreated Toso−/− mice into C57BL/6J recipient mice. One day after adoptive transfer, mice were intranasally infected with influenza A, and cytokine responses in lung T cells were analyzed on day 9 p.i. (Figure 5D). In animals that had received CD19+B220hiCD1d+ cells, virus-induced TNF-α or IFN-γ production by CD4+ and CD8+ T cells was comparable to that in normal control animals that had not received any transferred cells (“no transfer”; Figure 5, E–H). Adoptive transfer of naive CD19+B220+ B1a cells, however, had clear suppressive effects on virus-induced cytokine production by CD4+ and CD8+ T cells (Figure 5, E–H). Reduced TNF-α and IFN-γ production by T cells was also observed upon transfer of naive CD19+B220hiCD1d− B2 B cells, although its effects were less pronounced than those of CD19+B220+ B1a cells, consistent with the greater enrichment of IL-10 competency of CD19+B220+ B1a cells (Figure 4E). Based on their immunoregulatory function, CD19+B220hiCD1d− B2 cells are here termed as “B1-Bregs” and CD19+B220hiCD1d+ B2 cells as “B2-Bregs.” Adoptively transferred naive B1- and B2-Bregs isolated from IL-10−/− mice did not exhibit measurable suppressive activity on proinflammatory cytokine production by CD4+ and CD8+ T cells during influenza A infection (Supplemental Figure 10), suggesting that IL-10 is a critical effector molecule of Bregs in vivo, although the involvement of alternative effector mechanisms, such as CD73-mediated adenosine generation, which has been reported to be affected in IL-10−/− B cells (37), cannot be fully ruled out.

Our findings on adoptive transfer of as little as 1 × 106 cells demonstrate that naive B1-Bregs and B2-Bregs can both act as physiological regulators of T cell function by suppressing virus-induced T cell cytokine production during acute influenza A infection. Moreover, these data also provide a mechanistic explanation for the impaired T cell responses in Toso−/− mice during influenza A infection, which are likely caused by the higher numbers of immunosuppressive B1 and B2 Breg subsets in these mice.

B cell development in Toso-deficient mice. Toso surface expression is not restricted to B1 and B2 immunoregulatory B cells, but Toso is rather expressed on all peripheral B cells, with relatively highest expression on B220hiCD1d+ effector (follicular) B cells (Supplemental Figure 11A). Thus, how Toso specifically affects the generation of regulatory B cell subsets is still puzzling. Consistent with previous reports (24, 25) and the absence of Toso surface expression in early developmental B cell stages, we observed normal development of B cells in the bone marrow of Toso−/− mice (Supplemental Figure 11, B–G). Analysis of splenic B cells from Toso−/− mice revealed reduced frequency of mature IgMhiIgDlo B cells, while the population of IgMloIgDhi transitional B cells was significantly increased (Supplemental Figure 12, A and B). This was further accompanied by an increase in CD21hi/CD23hi MZ B cells (Supplemental Figure 12, C and D), suggesting enhanced differentiation toward this particular B cell subset, which is also consistent with increased numbers of MZ-like B2-Bregs in Toso−/− mice.

In addition to slightly increased surface expression of IgM, we also noted that overall CD21 and CD23 expression levels, as well as CD62L surface levels, were slightly downregulated in Toso−/− B cells, further indicating alterations in peripheral B cell maturation/differentiation (Supplemental Figure 12, E–H). Expression of CD19, B220, IgD, MHC-II, and CD44 was not affected by Toso deficiency (Supplemental Figure 12H).

Furthermore, higher numbers of B220hi B1a cells were found not only in the spleen of Toso−/− mice (Figure 4I and Supplemental Figure 13, A and B), but also in the peritoneal cavity, where an increase in B1a cells was accompanied by a corresponding decrease in B1b cells (Supplemental Figure 13, C and D). Analysis of peritoneal B cells revealed that B1a cells have an extremely high capacity to produce IL-10, whereas B1b cells are substantially less potent in IL-10 production (Supplemental Figure 13, E and F). Altogether, our analysis of B cell development in Toso−/− mice suggests that Toso is dispensable for B cell development in the BM, but fine-tunes the maturation/differentiation of specific B cell subsets in the periphery.

Self-reactivity of regulatory B cell subsets. Development of peripheral B cell compartments is tightly regulated, and even small changes in B cell maturation/differentiation may alter the balance of peripheral B cell tolerance. We thus next examined serum antibody levels and autoantibody production in Toso−/− mice. Basal levels of serum IgM and IgG were comparable between 8- to 10-month-old WT and Toso−/− mice (Figure 6A). However, consistent with published reports (21, 23–25), we detected elevated serum titers of autoreactive antibodies in Toso−/− mice. IgM and IgG antibodies directed against dsDNA or ssDNA were significantly increased in sera from aged Toso−/− mice compared with WT controls (Figure 6, B and C), supporting a role of Toso in the maintenance of self-tolerance.

To elucidate the major source of self-reactive antibodies in Toso−/− mice, we analyzed the capacity of different B cell subsets to produce autoantibodies. High-purity sorted B1- and B2-Bregs from Toso−/− mice exhibited strong production of anti-dsDNA and anti-ssDNA antibodies upon in vitro cultivation with LPS, while, in marked contrast, B2-effector cells and AA4.1+ transitional B2 B cells were largely unable to produce self-reactive antibodies (Figure 6D). Efficient production of autoantibodies was not a specific feature of Toso−/− Bregs, but was similarly observed in WT B1- and

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A

B

C

D

E

F

G

H
Figure 5. Suppressive function of IL-10–producing Bregs. (A–C) Bregs suppress inflammatory cytokine production in T cells in vitro. B cells from IL-10/GFP reporter (Vert-X) mice were treated for 16 hours with LPS, and PMA/ionomycin was added during the last 5 hours. B2/effector cells (B220+CD4−), B2/IL-10 cells (B220+GFP+), and B1/IL-10 cells (B220+GFP−) were then purified by FACS and were subsequently cocultured with naive CD8+ T cells (A and B) or naive CD4+ T cells (C). Cultures were stimulated with anti-CD3 for 48 hours and restimulated with PMA/ionomycin in the presence of BFA/menom for 5 hours. Percentage of IFN-γ–producing (A) and TNF-α–producing (B and C) T cells was determined by intracellular cytokine staining. (D–H) Bregs suppress inflammatory cytokine production in T cells during antiviral immune response in vivo. (D) Experimental model. Briefly, naive CD19−B220+CD1d− B2 B cells (gray), CD19−B220+CD1d− B2 B cells (blue), and CD19+ B220+ B1a cells (red) were purified from Toso−/− (KO) mice by FACS and were adaptively transferred into C57BL/6J mice. Mice were infected i.n. with 1,000 PFU influenza virus strain A/PR8 (H1N1). On day 9 p.i., lung cells were isolated and analyzed for cytokine staining. (E–H) Number and frequency of TNF-α–producing (E and F) and IFN-γ–producing (G and H) CD4+ T cells (E and G) and CD8+ T cells (F and H). Mice that had not received adaptively transferred cells (no transfer; open squares) but were also infected with influenza were used as positive control; uninfected mice served as a negative control (open circles). Data are expressed as mean ± SEM; symbols represent individual mice. (A–C) n = 3; (E–H) n = 4–9. *P < 0.05; **P < 0.01; ***P < 0.001; 1-way ANOVA and Dunnett’s post hoc test. Data are representative of at least 3 independent experiments.

B2-Bregs (Supplemental Figure 14A). B2-effector cells and AA4.1+ transitional B2 B cells exhibited only minimal autoantibody production under these conditions, even though they showed efficient blast formation and expression of the plasma cell marker CD138 (Supplemental Figure 14B). Together, these data indicate that self-reactive B cells are highly prevalent among B1-Bregs and B2-Bregs. Interestingly, IL-10–competent immunoregulatory B cell numbers rise as mice age, and increased numbers of Bregs in Toso−/− versus WT mice become even more pronounced in older mice (Figure 6, E and F). It is thus likely that the higher numbers of Bregs in Toso−/− mice are responsible for increased autoantibody levels in Toso−/− mice.

Toso fine-tunes B cell antigen receptor responsiveness. Regulation of B cell antigen receptor (BCR) signaling is a critical determinant of peripheral B cell differentiation/maintenance and the establishment of tolerance (38–41). We thus next evaluated the effects of Toso deficiency on BCR responsiveness and B cell activation/survival. Reflecting their different maturation/differentiation state, overall responsiveness to IgM triggering was also observed in Toso−/− B2-Bregs and B2-transitional cells, while, owing to their cell type–intrinsic low BCR responsiveness, B1-Bregs largely failed to respond to anti-IgM stimulation (Figure 6K). Together, these data indicate that Toso acts as a signal amplifier to fine-tune BCR responsiveness.

Anti-Toso treatment modulates IL-10–competent B cell numbers at sites of inflammation and results in impaired T cell responses upon influenza infection. Given the negative regulatory effect of Toso on IL-10–competent B cells and their immunosuppressive function on T cell immunity, we sought to investigate the effects of anti-Toso antibody treatment on B and T cell responses in the influenza-induced lung inflammation model and to evaluate its potential for immunomodulatory therapeutic applications. To induce virus-mediated lung inflammation, mice were infected intranasally with influenza A. One day before infection and on days 2 and 5 p.i., mice were treated with either anti-Toso mAb or control IgG, and B cells in influenza lungs were analyzed on day 9 p.i. Notably, Toso is highly expressed on essentially all peripheral B cells; however, in vivo anti-Toso mAb application did not result in B cell depletion, as comparable numbers of B cells were detected in lungs of anti-Toso and control IgG–treated mice (Figure 7A). Treatment with anti-Toso mAb did, however, induce a striking increase in both frequency and absolute numbers of IL-10–competent B cells in lungs from influenza-infected animals (Figure 7B). Importantly, consistent with our findings on Toso-deficient mice, increased numbers of IL-10–competent B cells correlated with impaired T cell responses at sites of inflammation, as virus-induced TNF-α and IFN-γ production by CD4+ and CD8+ T cells was significantly reduced in lungs of mice that had received Toso mAb compared with control IgG–treated mice (Figure 7, C–F). Together, these data re-emphasize the immunoregulatory role of Toso during inflammatory disease. Furthermore, as Toso is conserved between mice and humans and is also expressed on human B cells (ref. 19 and Supplemental Figure 16), the data also suggest that Toso may provide a promising therapeutic target to modulate IL-10–competent B cell compartments and to dampen excessive T cell responses at local sites of inflammation.

Discussion

Using conditional gene deletion, we here demonstrate that impaired antiviral T cells responses upon influenza A infection in...
Toso−/− mice were not due to T cell–inherent defects, but rather were induced by a previously unrecognized role of Toso in B cells. Our findings thus reveal an unexpected regulatory activity of B cells on T cell function during viral infection. Deletion of Toso on B cells results in a strong increase of IL-10–competent B cells, and, as we further demonstrate, this specific subtype of B cells mediates immunosuppressive activity on T cell responses during viral infection, most likely via an IL-10–dependent mechanism. Immunosuppressive function of IL-10 during influenza infection has been demonstrated in studies on IL-10−/− mice (42, 43). Moreover, B cells have been shown to be a relevant source of IL-10 during viral infection (44). B cells upregulate IL-10 expression during infection with murine cytomegalovirus, and this B cell–derived IL-10 decreases virus-specific IFN-γ responses in CD8+ T cells (44). Considering our data, we thus propose that Toso promotes efficient antiviral T cell cytokine responses by restricting the size of the IL-10–competent B cell pool. Specifically, B cell– intrinsic expression of Toso restricted differentiation/maintenance of IL-10–competent B cells in vivo. At the cellular level, Toso−/− Bregs exhibited normal functions. Hence, the impaired T cell responses observed in Toso−/− mice following influenza infection likely reflect the increased numbers of Bregs in Toso-deficient mice.

The present study demonstrates that IL-10–competent B cells are highly enriched within 2 distinct subpopulations of B cells, the small fraction of splenic B220+CD1d−CD5− B2 cells and the fraction of B220+CD5+B1a cells. These findings are consistent with earlier descriptions of B1a B cells as highly potent producers of IL-10 (45) and recent reports of an IL-10–competent population of CD19+CD1d+CD5+ B cells, termed B10 cells, that exhibits immunoregulatory function in models of autoimmunity (6, 8, 34, 46). Interestingly, IL-10–competent B220+CD1d+CD5+B cells share many surface marker characteristics with MZ B cells.

Adoptive transfer of naive B cell subsets showed that both major IL-10–competent B cell compartments — B220+CD1d+C-
deficiency on B cells have increased numbers of Bregs and, thus, a “dysregulated” system, which results in impaired (“suppressed”) proinflammatory T cell responses (see model in Supplemental Figure 17). Our data indicate that during acute influenza infection, in which antiviral immune protection is largely dependent on effector T cells, such suppressed T cell responses are detrimental and are damage) by autoreactive Bregs induces their immunosuppressive activity to dampen the inflammatory response and limit immunopathology. We thus propose that the population of Bregs is normally tightly controlled, to ensure a well-balanced immune response that allows for efficient immune protection against pathogens while minimizing immunopathological tissue damage. Mice with Toso deficiency on B cells have increased numbers of Bregs and, thus, a “dysregulated” system, which results in impaired (“suppressed”) proinflammatory T cell responses (see model in Supplemental Figure 17). Our data indicate that during acute influenza infection, in which antiviral immune protection is largely dependent on effector T cells, such suppressed T cell responses are detrimental and are

Figure 7. Immunomodulatory effect of Toso-blocking antibody treatment on influenza-induced lung inflammation. Mice were infected i.n. with influenza virus strain A/PR8 (H1N1) to induce pulmonary inflammation. On day −1, day 2, and day 5 p.i., mice were treated with anti-Toso mAb or control IgG (200 μg/mouse; i.v.). Lung cells isolated on day 9 p.i. were restimulated ex vivo and analyzed for cytokine production in T and B cells by intracellular cytokine staining. (A–C) Quantification of total CD19+ B cells (A) and frequency (B) and number (C) of IL-10–positive B cells in lungs of infected animals. (C–F) Quantification of frequency and numbers of TNF-α–producing (C and D) and IFN-γ–producing (E and F) CD4+ T cells (C and E) and CD8+ T cells (D and F). Each symbol represents an individual mouse; horizontal lines indicate the mean ± SEM. PBS control, n = 2; influenza A–infected mice, n = 5. *P < 0.05; **P < 0.01; ***P < 0.001; Student’s t test. Data are representative of 2 independent experiments.
associated with impaired immune protection and an increased risk of mortality. In contrast, under chronic inflammatory conditions, such as the chronic bacterial-induced model of colitis, in which effector T cells are more associated with immunopathological tissue damage, higher numbers of Bregs and, thus, impaired T cell effector function are beneficial, as this limits T cell-mediated tissue destruction (Supplemental Figure 17).

Differentiation and homeostasis of peripheral B cell subsets is critically influenced by signaling through the BCR (38–41). In line with altered peripheral B cell compartments in Toso-deficient mice, we here demonstrate that Toso shifts the threshold for BCR-mediated cellular activation/survival pathways. The exact molecular mechanism of how Toso affects BCR responsiveness is currently unknown. As an IgM-binding molecule, Toso may interact directly with membrane-bound IgM-containing BCR complexes (22) or, alternatively, may indirectly affect B cell signaling via recognition of soluble IgM immune complexes. Increased tonic signaling in Toso-deficient B cells (21) could not be confirmed in our study and may be related to the unusual occurrence of a lymphoproliferative disorder in this particular strain of mice, which has not been observed in any other strain of Toso-deficient mice.

Infection with influenza virus is frequently associated with severe pulmonary immune pathology in human patients. The anatomical structures in the lung are highly sensitive to tissue destruction, necessitating a fine balance between pro- and anti-inflammatory responses during pulmonary infection. In particular, after viral clearance, excessive release of proinflammatory cytokines by continually recruited CD8+ T cells can cause severe lung tissue injury. In the present study, we show that during influenza A–induced pulmonary inflammation the application of Toso blocking antibody selectively induces IL-10–competent B cells at the site of inflammation, an effect that was associated with reduced production of proinflammatory cytokines by lung T cells. These data suggest that clinical targeting of Toso may provide a novel therapeutic approach to control pathogenic T cell responses via the modulation of IL-10–competent B cell compartments at local sites of inflammation.

Methods

Mice and viral infection. Mice with constitutive Toso knockout (Toso−/−) and the generation of mice with a conditional floxed Toso allele (Tosofl mice) have been described before (18). In brief, the targeting vector was designed to have exons 4–7 flanked by loxP sites. After transfection into C57BL/6 embryonic stem cells, targeted embryonic stem cell clones were identified by Southern blotting and were injected into blastocysts. Upon germline transmission the flt-flanked neomycin-selection cassette was removed by breeding with C57BL/6 flp deleter mice. The resulting floxed-targeted mouse lines were crossed with transgenic mouse lines expressing Cre-recombinase under the control of the cdl9 promoter [Cdl9-Cre mice: Tg(Cd19-cre)1Cwi] (http://www.informatics.jax.org/reference/J:73127), the cdl11c [lgatax] promoter [Cdl11c-Cre mice: Tg(lgatax-cre,-EGFP)4097Ach] (http://www.informatics.jax.org/reference/J:123556), or the cdl19 promoter [CD19-Cre mice: Cd19cre] (http://www.informatics.jax.org/reference/J:67676). Vert-X IL-10 reporter mice [B6(Cg)-Il10tm1End ] (http://www.informatics.jax.org/reference/J:151551) to carry an internal ribosome entry site (IRES)–enhanced green fluorescent protein (eGFP) fusion protein downstream of exon 5 of the interleukin-10 (Il10) gene were used for some experiments. Cre-expressing transgenic mouse lines, IL-10 reporter mice, and IL-10–deficient mice (B6.129P2-Ili0tm1End ) were all on C57BL/6J background and were originally obtained from The Jackson Laboratory. Mice were housed in individually ventilated cages under specific pathogen–free conditions in the barrier animal facility at Hannover Medical School. Mice were infected at 10–13 weeks of age. Controls were sex- and age-matched. For in vivo influenza infection, mice were anesthetized with ketamine-xylazine. Virus was diluted in sterile PBS, and mice were infected by intranasal (i.n.) administration of a total volume of 40 μl of PR8 influenza virus. Mice were monitored daily for weight loss, signs of illness, and survival. Anti-Toso mAb or control IgG (catalog 012-000-003, Jackson ImmunoResearch) was administered i.v. on days –1, 2, and 5 p.i. (200 μg/mouse/time point). To examine the primary immune response, cells were harvested from lungs and spleen on the indicated days postinfection. Bronchoalveolar lavage fluid was collected for viral titer measurements.

Salmonella infection studies. Streptomycin (20 mg/mouse) was given by oral gavage to mice aged 16 weeks. Twenty-four hours after antibiotic administration, mice were infected with Salmonella Typhimurium ΔaroA at a dose of 3 × 108 bacteria in 100 μl HEPES buffer (100 mM, pH 8.0). Control mice (mock infection) were given 100 μl HEPES buffer. For histopathological analysis, tissues were fixed in 10% neutral buffered formalin overnight and embedded in paraffin. Cecum sections (5 μm) were deparaffinized and stained with H&E. Histological scores in the ceca of infected mice were determined as previously described (63). Briefly, pathological changes were assessed by evaluation of various parameters such as presence of luminal cells, infiltrating immune cells, crypt abscesses, and the formation of edema in the respective layer of the intestinal bowel wall including the surface epithelium, mucosa, and submucosa.

Influenza virus. Influenza virus strain A/PR8 (A/Puerto Rico/8/34 [H1N1]) was obtained from ATCC. Virus was grown in Madin–Darby canine kidney (MDCK) cells (obtained from ATCC). Viral titers were determined by standard MDCK plaque titration assay. Briefly, serial 10-fold dilutions of virus stock or bronchoalveolar lavage fluid from infected mice were allowed to adsorb onto 90% confluent MDCK cells on a 24-well plate. After 2.5 hours of incubation, cells were overlaid with 1.2% Avicel RC-581 (IMDC Deutschland GmbH) in DMEM (Gibco) supplemented with 0.1% BSA, 1% L-glutamine, penicillin, streptomycin, and 1 μg/ml TPCK Trypsin (Thermo Fisher Scientific) and cultured for 24 hours at 37°C in 5% CO2. Cells were washed, fixed, and permeabilized, and virus plaques were visualized and enumerated by staining with an mAb against influenza A nucleoprotein (A5AH, AbD Serotec). Viral titers were calculated as PFU per milliliter.

Flow cytometry (FACS). Single-cell suspensions of spleen, bone marrow, and peritoneum were prepared from fresh tissue using standard procedures. To isolate pulmonary lymphocytes, lung lobes were minced and strained through nylon mesh. Following red blood cell lysis, cells were blocked with anti-CD16/32 (clone 93, Biolegend) and
subsequently stained with fluorescent-labeled mAbs as summarized in Supplemental Table 1. Anti-Toxo mAb (rat IgG2a) directed against the extracellular domain of murine Toxo was generated by DNA vaccination (18) and was directly conjugated with DyLight 649 (Thermo Fisher Scientific). APC-labeled MHCI I F-α^/NP_366-374 (NP311) tetramer (containing QYSLRPENPAHK of influenza virus nucleoprotein) was obtained from the NIH Tetramer Core Facility (Emory University Vaccine Center, Atlanta, Georgia, USA), and APC-labeled D^b/NP_366-374 (NP366) dextramer (containing ASNENMETM of influenza virus nucleoprotein) was purchased from Immunodex. For discrimination of live versus dead cells, we used the fixable viability dye eFlour450 (eBioscience). For the detection of intracellular cytokines, cells were restimulated ex vivo in the presence of brefeldin A and monensin (both eBioscience). After cell surface staining, cells were fixed with paraformaldehyde and permeabilized using Perm/Wash buffer (eBioscience) and stained with mAbs against TNF-α (MP6-XT22), IFN-γ (XMG1.2), or II-10 (JES5-16E3; all eBioscience). Flow cytometric measurements were performed on a FACS Canto II cell analyzer (BD Biosciences). Data were analyzed with FlowJo software (version 9.9.3, Mac; version 10.0.7, PC; Tree Star).

**Cell sorting and adoptive transfer experiments.** For high-purity cell sorting experiments, splenic B cells were first enriched by magnetic isolation using negative selection (pan-B cell isolation kit; Miltenyi Biotec and Stemcell Technologies). B cell subpopulations were then further purified following immunofluorescence surface staining using high-speed flow cytometry cell sorters (FACSARia Fusion and FACSARia IIu, both BD Biosciences). To obtain highly pure B cell subsets with minimal contamination by other cell types, we pre-gated on viable cells that were CD19^+ but negative for CD4, CD8, F4/80, and NK1.1. Further gating of B cell subsets was primarily based on B220, CD1d, and AA4.1 surface expression or B220 versus GFP (II-10) expression. Sorted B cell subsets had greater than 98% purity.

For adoptive transfer experiments, FACS-sorted B cell subsets were immediately transferred i.v. into syngeneic C57BL/6j recipient mice (1 x 10^6 cells per recipient mouse). One day after adoptive transfer, mice were infected i.n. with influenza virus strain A/PR8. On day 9 p.i., lung cells were isolated, restimulated ex vivo, and subjected to flow cytometric analysis.

**Ex vivo cell restimulation, cell activation, and B cell cultures.** For the detection of T cell cell cytokine production, cells were restimulated ex vivo for 5 hours in the presence of brefeldin A and monensin (both eBioscience) on tissue culture plates that had been precoated with anti-CD3 (10 μg/ml; clone 145-2C11, eBioscience) and anti-CD28 (2 μg/ml; clone 37.51, Biologend). Where indicated, splenocytes were restimulated for 6 hours with influenza A virus peptide of amino acids 366–374 of the viral nucleoprotein (NP366 peptide), an H-2K^b-restricted epitope that is specific for CD8^+ T cells, in the presence of brefeldin A and monensin. To assess IL-10 production by B cells during influenza A virus infection, cells were restimulated ex vivo for 5 hours with PMA (50 ng/ml; Sigma-Aldrich) and ionomycin (500 ng/ml; Sigma-Aldrich) in the presence of brefeldin A and monensin. Following ex vivo restimulation, cells were subjected to cell surface and intracellular FACS staining.

For the in vitro analysis of IL-10 production by B cells, isolated leukocytes or purified B cell populations were cultured in the indicated times with ultrapure LPS (500 ng/ml; InvivoGen), CpG oligonucleotides (10 μg/ml; InvivoGen), BAF (200 ng/ml; Biolegend), anti-CD40 (10 μg/ml; clone 1C10, Biolegend), anti-IgM [10 μg/ml; goat anti-mouse F(ab')2, fragment, Jackson ImmunoResearch], or a combination of BAFF (200 ng/ml) plus IL-21 (50 ng/ml; Biolegend). PMA (50 ng/ml; Sigma-Aldrich) and ionomycin (500 ng/ml; Sigma-Aldrich) plus brefeldin A and monensin (both eBioscience) were added during the last 5 hours of culture. Cells were subsequently analyzed by intracellular flow cytometry.

For some experiments, FACS-sorted B cell populations were stimulated for the indicated times with titrated amounts of anti-IgM (goat anti-mouse F(ab')2, fragment, Jackson ImmunoResearch), and cultures were subsequently analyzed by flow cytometry for cell survival and upregulation of activation markers. To analyze in vitro autoantibody production, FACS-sorted B cell populations were stimulated for 3 days as indicated. Culture supernatants were then collected for the detection of autoantibodies, and cells were analyzed by flow cytometry.

For the analysis of B cell signaling, FACS-sorted B effector cells were stimulated with anti-IgM [10 μg/ml; goat anti-mouse F(ab')2, fragment, Jackson ImmunoResearch]. Stimulation was stopped by fixation in paraformaldehyde. Cells were then permeabilized using Perm/Wash buffer (eBioscience), stained with anti-phospho-Btk/Itk (Tyr551, Tyr511; eBioscience), and analyzed by flow cytometry.

**ELISA and detection of autoantibodies.** Serum titers of IgM and IgG were determined by specific ELISA kits (eBioscience) according to the manufacturer’s protocol. To detect autoantibodies in serum and cell culture supernatants, high-binding ELISA plates (Greiner) were coated overnight with 2 μg/ml ssDNA or dsDNA from calf thymus (Sigma-Aldrich). ssDNA was obtained by heat denaturation of dsDNA (95°C, 10 minutes) followed by rapid cooling on ice. Coated plates were blocked with 1% BSA, 0.5% gelatin in TBS for 2 hours at room temperature, and diluted samples were incubated overnight at 4°C in TBS 1% BSA. Bound anti-ssDNA or anti-dsDNA antibodies were detected with HRP-conjugated anti-mouse IgG (eBioscience) or with anti-mouse IgM-biotin (Jackson ImmunoResearch) and streptavidin-HRP (R&D Systems) followed by TMB substrate solution (eBioscience). Absorbance was measured at 450 nm.

**In vitro B cell suppression assay.** Splenic B cells from Vert-X IL-10 reporter mice were isolated by positive selection using CD19-coupled MicroBeads (Miltenyi Biotec) and were cultured for 16 hours with ultrapure LPS (500 ng/ml) plus addition of PMA (50 ng/ml) and ionomycin (500 ng/ml) during the last 5 hours. B cell cultures were surface-stained, and indicated CD19^+ B subpopulations were purified with greater than 98% purities by FACS based on B220 versus GFP (II-10) expression. Naïve CD4^+ and naïve CD8^+ T cells were isolated from C57BL/6j WT mice using respective naïve T cell isolation kits (Miltenyi Biotec). For suppression assays, FACS-sorted B cell subpopulations were cocultured with purified naïve T cells (5 x 10^5 T cells to 6 x 10^5 T cells) in 48-well plates that had been precoated with anti-CD3 (10 μg/ml; clone 145-2C11, eBioscience) for 48 hours. During the last 5 hours, PMA/ionomycin plus brefeldin A and monensin was added to the cultures. Cells were subjected to surface and intracellular FACS staining and analyzed by flow cytometry.

**Statistics.** Data are presented as mean values ± SEM. Statistical analysis was performed using GraphPad Prism (version 6.0h, Mac OS X). Unless stated otherwise, differences between means were assessed using 2-tailed Student’s t test. Statistical analysis of Kaplan-Meier survival curves was performed by the log-rank test. A P value of less than 0.05 was considered statistically significant.
Study approval. Animal experiments were performed in accordance with institutional guidelines and were approved by the local authorities (Lower Saxony State Office for Consumer Protection and Food Safety, Germany).

Author contributions
KHL designed the study. JY and VHHD performed the majority of the experiments, with specific contributions by AW, AS, and GAG. Technical assistance was provided by KW. KB provided valuable resources. ACC provided key reagents and feedback on the manuscript. NF and KHL supervised the study, analyzed data, and wrote the manuscript. Funding acquisition was handled by KHL.

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