Expanded B cell population blocks regulatory T cells and exacerbates ileitis in a murine model of Crohn disease

Timothy S. Olson,† Giorgos Bamias,‡ Makoto Naganuma,‡ Jesús Rivera-Nieves,‡ Tracy L. Burcin,§ William Ross,§ Margaret A. Morris,∥ Theresa T. Pizarro,∥ Peter B. Ernst,‡ Fabio Cominelli,§ and Klaus Ley‡

†Department of Molecular Physiology and Cardiovascular Research Center, and ‡Digestive Health Center of Excellence, University of Virginia Health Science Center, Charlottesville, Virginia, USA. §Department of Biomedical Engineering and Cardiovascular Research Center, University of Virginia, Charlottesville, Virginia, USA.

SAMP1/YitFc mice develop discontinuous, transmural inflammatory lesions in the terminal ileum, similar to what is found in human Crohn disease. Compared with the mesenteric lymph nodes (MLNs) of AKR control mice, SAMP1/YitFc MLNs contain a 4.3-fold expansion in total B cell number and a 2.5-fold increased percentage of CD4+ T cells expressing the αEβ7 integrin. Although αEβ7+CD4+ T cells possess a regulatory phenotype (CD25+·L-selectinlo·and CD45RBhi), express IL-10, and suppress effector T cell proliferation in vitro, they cannot prevent ileitis development in SCID mice adoptively transferred with effector CD4+ T cells, although the CD4αEβ7+ subset, which overlaps with the αEβ7+CD4+ subset, prevents colitis. The αEβ7+CD4+ T cells express high levels of ICOS, a costimulatory molecule that augments B cell function, suggesting their involvement in the increase in B cells, IgA+ cells, and soluble IgA found within the MLNs and ileum of SAMP1/YitFc mice. MLN B cell numbers correlate with ileitis severity in SAMP1/YitFc mice, and cotransfer of SAMP1/YitFc MLN B cells along with CD4+ T cells increases ileitis severity in SCID mice compared with transfer of CD4+ T cells alone. SAMP1/YitFc B cells prevent αEβ7+CD4+ T cells from suppressing effector T cell proliferation. We conclude that SAMP1/YitFc MLN B cells contribute to the development of SAMP1/YitFc ileitis.

Introduction
Inflammatory bowel disease (IBD) requires increased host genetic susceptibility, immune system dysregulation, and altered interactions of host cells with pathogens and normal flora within the intestinal mucosa (1). Although CD4+ T cell subset functions have been studied in great detail (2), the contributions of other immune cells to the development of IBD are just beginning to be understood (3, 4). In particular, irregularities in B cell development and antigen-specific immunoglobulin production may be critical for understanding the pathogenesis of IBD (5–7).

Abnormal immunoreactivity of serum or mucosal antibodies toward enteric bacterial flora has been reported in both animal models and IBD patients (7, 8). IgA production plays a critical role in preventing the intestinal invasion of both pathogenic and commensal bacteria (9). Although classic follicular B2 lineage cells produce considerable IgA, roughly half of the intestinal IgA-producing plasma cells are derived from B1 cells that develop and differentiate within the peritoneal cavity (10). B1 cells produce IgM or IgA in a T cell–independent manner, with specificities for common enteric bacterial antigens (11). Within lymphoid organs, B2 B cell proliferation and isotype class switching are promoted by follicular helper T cells (Tfh cells). Although Tfh cells lack Th1 or Th2 cytokine production, they greatly enhance B cell IgG and IgA production (12). Tfh cells express high levels of inducible T cell costimulator (ICOS), a molecule that, through binding of ICOS ligand on B cells, is required for T cell–mediated B cell help and antibody class switching (13).

CD4+CD45RBhiCD25+ regulatory T cells (Treg cells) prevent colitis induced by transferring effector CD4+CD45RBhiCD25+ T cells into SCID mice through mechanisms involving TGF-β and IL-10 (14). Recently, expression of the αEβ7 integrin was used to define novel subsets of CD4+ Treg cells also capable of preventing colitis (15). The αEβ7 integrin binds E-cadherin on epithelial cells and an unknown ligand on endothelial cells (16, 17) and is required for the maintenance of normal lymphocyte numbers within the epithelium and lamina propria (18). In contrast to CD4+CD25+·cells, CD4+CD25– Treg cells preferentially express αEβ7 (19). These Treg cells also express high levels of the glucocorticoid-induced TNF receptor (GITR), a TNF receptor family member that regulates T cell proliferation and activation-induced apoptosis (20). Stimulation of Treg cells with anti-GITR or through binding of GITR ligand (GITRL), expressed by subsets of APCs, reverses the suppression of effector T cell proliferation by Treg cells in vitro, suggesting a role for GITR in the promotion of proinflammatory responses (19, 21, 22). Whether this pathway is important in the context of IBD has not been examined.

The SAMP1/YitFc spontaneous ileitis model provides an excellent system for the study of interactions between leukocyte subsets participating in the development of intestinal inflammation. SAMP1/YitFc mice develop Crohn-like discontinuous, transmural ileitis without chemical, genetic, or immunological manipulation (23). The lesions contain many histopathological features seen in Crohn disease, including villous atrophy, crypt hyperplasia, and infiltration of both acute and chronic inflammatory cells (23).
The lesions are associated with a Th1-type inflammatory response that can be downregulated by antibiotic therapy (24). Importantly, mesenteric lymph node (MLN) CD4+ T cells from SAMP1/YitFc mice adoptively transfer ileitis to SCID recipients (25).

In this study, we have investigated abnormalities in B cell homeostasis and functionality in SAMP1/YitFc mice, beginning with the observation that B cell numbers are greatly increased in MLNs of SAMP1/YitFc versus wild-type mice. To link B cell population expansion to abnormalities of Treg cell function in this model, we investigated both T cell activation of B cells and the effects of B cells on T cell function. To address a causal relationship between B cell population expansion and disease severity, we adoptively transferred B cells along with T cells and measured ileitis. In contrast to models of colitis in which B cells have been shown to decrease disease severity (5, 26), our data demonstrate that B cells play an important proinflammatory role in the development of SAMP1/YitFc ileitis through mechanisms that may involve inhibition of Treg cell function.

Results

Expanded B cell and non-naive CD4+ T cell populations in SAMP1/YitFc MLNs. One of the hallmarks of the SAMP1/YitFc ileitis phenotype is a macroscopically enlarged MLN (27). SAMP1/YitFc MLNs from mice older than 30 weeks of age had a threefold increase in total lymphocyte number (52 ± 5 million cells, mean ± SEM) compared with MLNs from age-matched AKR mice (17 ± 1 million cells) (Figure 1A), the strain from which SAMP1/YitFc mice were derived (23). Using flow cytometry subset analysis, we found the largest expansion occurred in the B cell subset, which was increased 4.3-fold in SAMP1/YitFc compared with AKR MLNs. The CD4+ T cell number was increased 2.5-fold and, compared with AKR CD4+ T cells, an increased percentage of SAMP1/YitFc CD4+ T cells expressed CD25 (30% ± 2% versus 15% ± 1%), and a decreased percentage expressed high levels of L-selectin (55% ± 4% versus 84% ± 2%) and CD45RB (58% ± 5%, and CD45RB (53% ± 6%), most αβ+CD4+ T cells were CD69 (59% ± 3%), CD25 (75% ± 3%), L-selectin (66% ± 4%), and CD45RB (74% ± 6%) (Figure 2A), consistent with a regulatory phenotype (15). To test whether this expanded αβ+CD4+ T cell subset possessed regulatory or ileitis-producing capability, we analyzed the cytokine expression of isolated MLN αβ+CD4+ T versus αβ+CD8+ T cells in 2-day cultures by cytometric bead

![Figure 1](image-url)

Figure 1

Expanded B cell and αββ+CD4+ T cell populations in SAMP1/YitFc versus AKR MLN. (A) Total lymphocyte numbers (mean ± SEM) presented as the percentage of total cells in AKR and SAMP1/YitFc MLNs — as determined by lymphocyte-gated flow cytometry — that are CD4+ T cells (n = 16 and 32, respectively), CD8+ T cells (n = 10 and 19, respectively), and B cells (n = 13 and 24, respectively). Fold increases in the overall size of each of these populations in SAMP1/YitFc versus AKR are indicated. (B) Top, CD4+ T cell—gated histograms of β7 integrin chain expression on MLN cells from SAMP1/YitFc and AKR mice, showing that SAMP1/YitFc mice have an increased percentage of CD4+ T cells expressing high levels of β7. Bottom, the αββ+ cells express β7 as a dimer with the αEβ7 integrin chain, as αEβ7 cells display a 1:1 correlation of αE to β7 expression in β7 versus αE dot plots that is not seen in isotype controls. (C) Comparison of the percentage (mean ± SEM) of MLN CD4+ T cells that are αE+ in SAMP1/YitFc mice (n = 31) versus AKR mice (n = 21). *Significantly greater (P < 0.05) than AKR cell percentage.
array. The αββ7-CD4+ T cells produced eight- to tenfold lower levels of TNF-α and IL-2 and almost threefold greater amounts of IL-10 than did αββCD4+ T cells (Figure 2B).

To test the function of SAMP1/YitFc MLN αββ7-CD4+ T cells, we adoptively transferred 5 × 10^6 unfractionated, αββ7-CD4+, or αββ7-CD4+ T cells intraperitoneally into C3H/HeJ SCID mice. After 6 weeks, ilea were removed from the recipients and analyzed for ileitis severity with a standardized histopathological scoring system (33) (Figure 3A). Compared with mice receiving SAMP1/YitFc αββ7-CD4+ T cells, SCID mice receiving SAMP1/YitFc αββ7-CD4+ T cells had significantly lower chronic inflammatory indices (1.3 ± 0.3 versus 5.6 ± 0.4, mean ± SEM) and total inflammatory scores (8 ± 1 versus 21 ± 1), suggesting that αββ7-CD4+ T cells contain the major ileitis-producing subset.

Since the percentage of CD4+ T cells expressing αββ increases with age in SAMP1/YitFc MLNs (data not shown) and the severity of SAMP1/YitFc ileitis stabilizes with age (27), αββ7-CD4+ T cell populations might expand in an attempt to curb inflammation in older SAMP1/YitFc mice. To test whether αββ7-CD4+ T cells can downregulate ileitis, we injected 5 × 10^6 αββCD4+ T cells into SCID mice 3 weeks before, at the same time as, or 3 weeks after injection of 5 × 10^6 αββCD4+ T cells (Figure 3A). No changes were seen in the inflammatory scores in any of the three groups compared with scores obtained from mice receiving αββ7-CD4+ T cells alone, indicating that SAMP1/YitFc αββ7-CD4+ T cells cannot downregulate ileitis produced by αββCD4+ T cells. To test whether SAMP1/YitFc αββCD4+ T cells might have defective regulatory function compared with wild-type regulatory cells, we transferred 5 × 10^6 SAMP1/YitFc CD4+ T cells alone or a combination of SAMP1/YitFc plus AKR (5 × 10^5 each) CD4+ T cells into a separate cohort of SCID mice (Figure 3B). AKR MLN CD4+ T cells were unable to prevent the ileitis produced by SAMP1/YitFc CD4+ T cells.

To test regulatory cell function in the well-characterized CD45RB-CD4+ and CD25-CD4+ T cell adoptive transfer models (14) using Treg populations that overlap with the αββCD4+ population (15), we transferred 5 × 10^6 unfractionated, CD45RB-, CD45RB+, CD25-, or CD25- SAMP1/YitFc MLN CD4+ T cells into a third cohort of SCID mice and determined both ileitis and colitis severity (Figure 3C). CD45RB-CD4+ or CD25-CD4+ T cells produced levels of ileitis similar to those produced by unfractionated CD4+ T cells. CD25-CD4+ T cells produced significantly less ileitis than unfractionated CD4+ T cells. CD45RB-CD4+ T cells produced levels of ileitis similar to those produced by CD45RB-CD25+ T cells, suggesting that CD45RB-CD4+ T cells contain subsets of cells that are proinflammatory in the context of ileitis.

In contrast, SCID mice receiving SAMP1/YitFc MLN CD45RB-CD4+ or CD25-CD4+ T cells exhibited substantially more colitis than mice receiving unfractionated CD4+ T cells containing Treg cells (Figure 3C). This finding strongly suggests that SAMP1/YitFc CD4+ Treg cell populations (CD45RB-CD25+, CD25-), or αββ) are not defective, as they can suppress the development of colitis in a fashion similar to Treg cells in other adoptive transfer models.

αββ-CD4+ T cells may be involved in B cell help. We next tested whether αββCD4+ T cells might be involved in the B cell population expansion seen in SAMP1/YitFc MLNs. In contrast to the majority of αββCD4+ T cells, most SAMP1/YitFc αββCD4+ T cells (73% ± 2%, mean ± SEM) expressed ICOS, a costimulatory molecule that plays a critical role in germinal center formation and immunoglobulin class switching (34) (Figure 4B). We found a positive correlation between the percentage of MLN CD4+ T cells that expressed αββ and MLN B cell number, as a percentage of total cells, in individual mice (Figure 4A). In mice in which more than 15% of CD4+ T cells expressed αββ, B cells comprised 55% ± 4% (mean ± SEM) of total MLN lymphocytes, whereas in mice in which αββ cells constituted less than 15% of the CD4+ population, only 34% ± 3% of total MLN lymphocytes were B cells. This correlation, along with the high level of ICOS expression by αββCD4+ T cells, suggests that this subset may directly interact with B cells in SAMP1/YitFc MLNs.

SAMP1/YitFc B cells produce increased IgA. Compared with AKR supernatants, SAMP1/YitFc MLN supernatants contained more IgG1, κ (3.4-fold), IgAκ (4.5-fold), IgMκ (2.3-fold), IgAγ (7.9-fold), and IgMγ (3.7-fold), as determined by cytometric bead array (Figure 5A). Cocultures of SAMP1/YitFc CD4+ T cells and B cells contained more IgA and IgM, but not IgG2a, than did cocultures of AKR CD4+ T cells and B cells (Figure 5C), recapitulating the in vivo expression pattern. Mixed cocultures of SAMP1/YitFc CD4+...
T cells plus AKR B cells, as well as those of AKR CD4+ T cells plus SAMP1/YitFc B cells, produced levels of IgA similar to that of cocultures of AKR T cells and AKR B cells, suggesting that both SAMP1/YitFc B cells and T cells are required for the SAMP1/YitFc expression pattern (data not shown). SAMP1/YitFc B cells cultured with εαεεCD4+ T cells produced mostly IgA; whereas B cells cultured with εεεεCD4+ T cells produced increased IgM and IgG2a (data not shown). Serum IgA was elevated 2.7-fold in SAMP1/YitFc mice (23 ± 5 μg/ml, mean ± SEM) compared with AKR mice (8.7 ± 0.8 μg/ml) (Figure 5B), suggesting that the increase in MLN IgA production may lead to elevated systemic levels.

Immunostaining and flow cytometry showed that SAMP1/YitFc MLNs contained many more IgA+ cells than did AKR MLNs (Figure 6A and B). Most of the IgA expression was found on mature B cells (B220lo), while some was also found on B220hi cells thought to be differentiating plasmablasts (35). IgA+ cells were IgM− and Syndecan-1− (data not shown), suggesting that these cells had yet to differentiate into plasma cells. Less than 20% of SAMP1/YitFc MLN B cells possessed a B1 cell phenotype (CD23− IgD−), indicating that most MLN B cells belonged to the B2 class. The CD23− IgD− population may also include memory or early antigen-secreting cells derived from B2 cells, suggesting that the B1 population may be even smaller than the CD23− IgD− populations seen in Figure 6B. Most IgA+ cells were found in the CD23+ B2 cell population (data not shown). In contrast to the B cell and IgA+ cell population expansion in SAMP1/YitFc versus AKR MLNs, spleens and Peyer’s patches of SAMP1/YitFc versus AKR mice contained similar percentages of B cells as well as IgA+ cells (data not shown).

In AKR ileal sections, IgA+ cells were found as single cells or in small clusters, evenly distributed throughout the intestine (Figure 6C and D). Most of the free IgA staining was located within crypts or near the base of villi. In SAMP1/YitFc ilea, IgA+ cells were found in large clusters and were distributed in a focally concentrated pat-

Figure 3
SAMP1/YitFc Treg populations block colitis, but not ileitis, in the CD4+ T cell adoptive transfer model. (A) Comparison of adoptively transferred ileitis severity (6 weeks after transfer, mean ± SEM) in SCID recipients (n = 4 in each group) induced by 5 × 106 SAMP1/YitFc total MLN CD4+ T cells, εεεεCD4+ T cells, εεεεεεCD4+ T cells, or combination treatment using εεεεεεCD4+ T cells injected 3 weeks before, at the same time as, or 3 weeks after εεεεεεCD4+ T cells. (B) In a separate cohort of SCID mice, severity of ileitis induced by 5 × 106 SAMP1/YitFc CD4+ T cells (n = 4) was not decreased by coinjection of 5 × 106 AKR MLN CD4+ T cells (n = 5). Total inflammatory scores represent the sum of three individual histological indices, including active inflammation, chronic inflammation, and villus architectural distortion. (C) In a third cohort, adoptively transferred ileitis and colitis severities were compared among SCID mice 6 weeks after mice received 5 × 106 SAMP1/YitFc MLN unfractionated CD4+, CD45RB−/CD4+, CD45RB−/CD4−, CD25−/CD4+ or CD25−/CD4− T cells. Because villus distortion is not measured in colitis, the sum of active and chronic inflammatory scores was used for this comparison. Data are expressed as mean ± SEM. *Significantly decreased (P < 0.05) compared with ileitis severity in mice receiving εεεεεεCD4+. #Significantly increased compared with colitis severity in mice receiving unfractionated CD4+ T cells.

Figure 4
Correlation between B cell expansion and αεεεεεεCD4+ T cells. (A) Left, correlation (r = 0.6) between B cell number as a percentage of all MLN cells and the percentage of MLN CD4+ cells expressing αεεεεεε in individual SAMP1/YitFc mice (n = 21). Right, comparison of the percentage of B cells in MLNs of mice with αεεεεεεCD4+ cells comprising greater than versus less than 15% of the MLN CD4+ population (line represents mean). (B) Representative CD4+ gated dot plot and average quadrant percentages (n = 10) showing expression of β7 integrin versus ICOS on SAMP1/YitFc MLN CD4+ T cells.
Figure 5
Soluble IgA production increased in SAMP1/YitFc versus AKR mice. (A) Representative dot plots of κ (1:100 dilution, FL2) and λ (1:1, FL1) light chain antibody isotype levels within MLN supernatants from AKR mice (n = 2) and SAMP1/YitFc mice (n = 2), measured with a cytometric bead array containing isotype-specific beads with preset FL3 intensities. MLNs were resuspended in 5 ml, and centrifuged to remove the cell pellet from the tested supernatants. An increase of at least twofold in IgG1 κ, IgA κ, IgM κ, IgA λ, and IgM λ was seen in SAMP1/YitFc versus AKR MLNs, as measured by mean fluorescence intensity of the isotype-specific beads. (B) ELISA detecting IgA antibody concentrations (mean ± SEM) in serum samples collected via cardiac puncture from SAMP1/YitFc mice (n = 12) and wild-type AKR mice (n = 13). (C) Concentrations of IgA (n = 4), IgM (n = 2), and IgG2a (n = 2), measured in triplicate by ELISA, from 3-, 7-, and 11-day anti-CD3–stimulated cocultures of SAMP1/YitFc CD4+ T cells and B cells versus AKR CD4+ T cells and B cells (10^6 T cells/well and 10^6 B cells/well). *Significantly greater than AKR concentrations (P < 0.05).

Discussion
In SAMP1/YitFc mice, an expanded αβ⁺CD25⁺CD45RB⁺CD4° MLN T cell subset is associated with increased B cells that contribute to ileitis. These T cells have a phenotype similar to that of αβ⁺CD4° T cells.
of Treg cells that prevent inflammation in adoptively transferred colitis models (15, 36, 37), but they cannot prevent ileitis in our adoptive transfer model. The lack of anti-inflammatory capacity of SAMP1/YitFc Treg cells in the adoptive transfer model suggests a failure of regulatory pathways in the donor SAMP1/YitFc ileitis model as well.

Wild-type AKR MLN CD4+ T cells, presumably containing functional Treg populations, did not prevent ileitis when cotransferred into SCID mice with SAMP1/YitFc CD4+ T cells. SAMP1/YitFc Treg cells (CD45RBloCD25+) prevented colitis development. Furthermore, SAMP1/YitFc αE+CD4+ T cells suppressed αE−CD4+ T cell proliferation in vitro. The αE−CD4+ and CD25−CD4+ T cells represent overlapping subsets of Treg cells, and the ability of SAMP1/YitFc αE−CD4+ T cells to block colitis was not directly tested. Given that SAMP1/YitFc αE−CD4+ T cells block effector T cell proliferation, and that previous studies have shown that αE−CD25− Treg cells are more effective at preventing colitis than is the αE−CD25+ subset (15), it is likely that SAMP1/YitFc αE− cells block colitis at least as well as SAMP1/YitFc CD25+ cells. Taken together, these findings strongly suggest that aberrant proinflammatory signals that override anti-inflammatory pathways, and not inherently defective regulatory cells, are likely the cause of SAMP1/YitFc ileitis.

The increased B cells present within the MLNs of SAMP1/YitFc mice expressed GITRL, increased the severity of adoptively transferred ileitis, and abrogated Treg cell function in vitro. Therefore, B cells may be the primary cell population responsible for overriding anti-inflammatory or regulatory signals in vivo and promoting the development of SAMP1/YitFc ileitis. Alternatively, or in addition, as homing of T cells to the ileum versus the colon requires differential chemokine receptor expression (38), colitis-preventing Treg...
A cell population expansion precedes and induces the expansion of thymus versus normal mice could be explained by a model in which action of T reg cell function that is further enhanced by transfer of SAMP1/YitFc MLN B cells.

The positive correlation between αE+CD4+ T cell populations through GITR/GITRL–mediated interactions, which have been shown previously to induce IL-2 responses through opsonization or immune complex formation, would drive IgA production as part of their anti-inflammatory repertoire (46). However, most IgA that binds to enteric bacteria is derived from B1 cells, whereas B2-derived IgA like neutrophil cytoplasmic antibody (pANCA) (43); I2, a bacterial transcription factor (44); and Saccharomyces cerevisiae subspecies (45).

The spontaneous colitis seen in C3H/HeJ/Bir mice is associated with a mainly IgG2a response to enteric bacteria (8). However, the inflammation in these mice is limited to the cecum and the colon (41), and thus immunoglobulin specificity may be different from that of Crohn-like ileitis. While some studies indicate that mucosal IgG, not IgA, is elevated in Crohn patients (7), other studies suggest that serum IgA, but not IgG, immunoreactivity is stronger in Crohn patients than in controls (42). Relative to normal individuals, many Crohn patients have elevated serum IgA specifically recognizing HupB, a mycobacterial homolog of the antigen recognized by anti-neutrophil cytoplasmic antibody (pANCA) (43); I2, a bacterial transcription factor (44); and Saccharomyces cerevisiae subspecies (45). Under homeostatic conditions, IgA is translocated across intestinal epithelial cells to the luminal surface, where it prevents bacteria from exiting the lumen, thereby functioning to prevent initiation of immune responses (9). From this perspective, it makes sense that Treg cells, which produce IgA-promoting TGF-β in other models, would drive IgA production as part of their anti-inflammatory repertoire (46). However, most IgA that binds to enteric bacteria flora is derived from B1 cells, whereas B2-derived IgA like that present in SAMP1/YitFc MLNs recognizes potentially pathogenic, invasive bacteria and aids in their elimination (47, 48). In SAMP1/YitFc ileitis, where epithelial barrier integrity is likely diminished as part of the ongoing inflammatory process, the coating of invasive bacteria by IgA may initiate proinflammatory responses through opsonization or immune complex formation, as discussed above. This role, if relevant, may also be played by antibodies of other isotypes, such as IgM or IgG1, which are also present in SAMP1/YitFc MLNs recognizes potentially pathogenic bacteria flora is derived from B1 cells, whereas B2-derived IgA like that present in SAMP1/YitFc MLNs recognizes potentially pathogenic, invasive bacteria and aids in their elimination (47, 48). In SAMP1/YitFc ileitis, where epithelial barrier integrity is likely diminished as part of the ongoing inflammatory process, the coating of invasive bacteria by IgA may initiate proinflammatory responses through opsonization or immune complex formation, as discussed above. This role, if relevant, may also be played by antibodies of other isotypes, such as IgM or IgG1, which are also present in SAMP1/YitFc MLNs.

B cells themselves have recently been shown to directly modulate intestinal inflammation. Backcrossing TCR−/− mice with Igμ−/− mice results in mice with more severe chronic colitis than in mice receiving SAMP1/YitFc T cells alone, while mice receiving AKR B cells and SAMP1/YitFc T cells had an intermediate phenotype.

Figure 8 Cotransfer of MLN B cells increases ileitis severity in the CD4+ T cell adoptive transfer model. (A and B) A total of $5 \times 10^5$ CD4+ T cells from pooled SAMP1/YitFc MLNs (n = 6) were injected intraperitoneally into SCID mice either alone (n = 4) or in combination with $2 \times 10^6$ SAMP1/YitFc (n = 8) or AKR (n = 4) MLN B cells intravenously. (A) After 6 weeks, SCID mice receiving both SAMP1/YitFc T cells and SAMP1/YitFc B cells had higher chronic and total inflammatory scores than those of mice receiving T cells alone, while mice receiving AKR B cells and SAMP1/YitFc T cells had an intermediate phenotype. (B) Immunostaining of paraffin-embedded ileal sections, revealing an increase in T cell (CD3+ cells) infiltrates in SCID mice receiving cotransfer of B cells from either strain compared with that of mice receiving T cells alone, and an increased neutrophil (GR-1+ cells) infiltrate in mice receiving specifically SAMP1/YitFc B cells. PMN, polymorphonuclear granulocytes.
that seen in mice that are TCR–/– alone, and reconstitution of TCR–/–Igα–/– mice with mature B cells ameliorates this increased inflammation severity (26). The anti-inflammatory B cell subset in this model expresses IL-10 and high levels of CD1D, a marker of B1 cell–like spleen marginal zone cells (6, 49). B cells involved in colitis are likely quite different than B cells that participate in ileitis, as trafficking of B cell lineage subsets to colon versus ileum also relies on distinct pathways (50, 51). CD45RBhi T cells from Fvβ–/– mice can induce ileitis when transferred to SCID mice only when cotransferred with B cells (52), consistent with our data suggesting that the proinflammatory activity of intestinal B cells may be particularly relevant in the ileum. Furthermore, in the Gα2–/– model of IBD, a selective deficit in B1 cells and an increase in B2 cells are linked to the development of intestinal inflammation (5). The present findings are consistent with a model in which mucosal B2 cells are proinflammatory and B1 cells are anti-inflammatory in the context of IBD.

In conclusion, we have established a link between an increased regulatory CD4+ T cell subset expressing the αEβ7 integrin, an expanded MLN B cell population, and elevated serum IgA in SAMP1/YitFc mice. The increased number of IgA-expressing MLN B cells correlates with severity of inflammation in SAMP1/YitFc host ileitis. SAMP1/YitFc Treg cells are incapable of modulating the severity of adoptively transferred ileitis, but can prevent colitis. SAMP1/YitFc MLN B cells cotransferred with CD4+ T cells increase the severity of overall ileitis, and specifically T cell infiltration, in SCID mice. These B cells express GITR and abrogate αE–CD4+ T cell regulatory function in vitro. Taken together, our results demonstrate the importance of T cell/B cell interactions and B cell function in the pathogenesis of a Crohn-like murine ileitis model.

Methods

Mice. SAMP1/YitFc mice, a substrain of the SAMP1/Yit line (27), were obtained from established colonies at the University of Virginia Health Science Center vivarium (Charlottesville, Virginia, USA). Age-matched wild-type AKR/J mice and 6- to 8-week-old SCID mice on the C3H/HeJ background were obtained from Jackson Laboratories (Bar Harbor, Maine, USA). All animals were housed in a specific pathogen–free facility, and all experiments were approved by the institutional committee for animal use.

Flow cytometry. MLNs were crushed through 70-μm filters into staining buffer consisting of PBS with 2% FCS. Cells were counted using Trypan Blue (Sigma-Aldrich, St. Louis, Missouri, USA). Cells were stained with combinations of biotinylated anti–mouse GITR (BAF524) (R&D Systems, Minneapolis, Minnesota, USA); streptavidin–allophycocyanin; FITC–, phycoerythrin– (PE–), peridinin chlorophyll protein–, allophycocyanin–, or biotin-labeled rat anti–mouse CD4 (RM4-5), CD8 (53-6.7), CD19 (1D3), eFluor299 (M290), β7 (M293), ICOS (7E.17G9), CD25 (PC61), L-selectin (ME-14), CD45RB (16a), B220 (RA3-6B2), IgM (R6-60.2), IgA (C10-3), IgD (11-26c.2a), and CD23 (B3B4); and hamster anti–mouse CD69 (H1-2F3) (BD Biosciences – Pharmaningen, San Diego, California, USA). Cells were stained for 20–30 minutes at 4°C, washed twice in staining buffer and fixed in 1% paraformaldehyde. Flow cytometry data were acquired on a FACSCalibur flow cytometer with CellQuest software (BD Biosciences, San Diego, California, USA) and were analyzed with WinMIDII 2.8 (J. Trotter, Scripps Research Institute, La Jolla, California, USA). All animals were housed in a specific pathogen–free facility, and all experiments were approved by the institutional committee for animal use.

Cell isolations and adoptive transfer. SAMP1/YitFc or AKR MLN CD4+ T cells were isolated magnetically by positive selection with anti–mouse CD4 microbeads or by negative selection with the mouse CD4 negative isolation kit (Miltenyi Biotec, Auburn, California, USA). All selections were performed according to the manufacturer’s instructions. B cells were isolated by positive selection with anti–mouse CD19 microbeads or by negative selection with anti–mouse CD43 microbeads (Miltenyi Biotec). By flow cytometry, B cell and CD4+ T cell fractions were more than 97% and 95% pure, respectively. The αE– and αE+ subsets were isolated either before or after CD4+ cell selection with FITC– or PE-labeled rat anti–mouse IgG1, IgM, or biotin-labeled rat anti–mouse CD4 (RM4-5), CD8 (53-6.7), CD19 (1D3), and CD23 (B3B4); and hamster anti–mouse CD69 (H1-2F3) (BD Biosciences – Pharmaningen, San Diego, California, USA) and were analyzed with WinMIDII 2.8 (J. Trotter, Scripps Research Institute, La Jolla, California, USA). All animals were housed in a specific pathogen–free facility, and all experiments were approved by the institutional committee for animal use.

For adoptive transfer, cells were counted, washed, and resuspended in PBS for injection into SCID recipients. T cells and B cells were
injected in 500 μl of PBS at doses of 5 × 10^6 cells intraperitoneally and 2 × 10^6 cells i.v. (tail vein), respectively. The ilea and/or colons of SCID recipients were harvested 6 weeks after transfer.

Cell culture, cytokine analysis, and T cell proliferation assays. Cells were cultured in 96-well plates at 37°C with 5% CO2 in RPMI media containing 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10 μg/ml plate-bound anti-mouse CD3 (145-2C11) (BD Biosciences — Pharmingen) with or without 2 μg/ml anti-mouse CD28 (37.51) (BD Biosciences — Pharmingen). For cytokine analysis, T cell subsets were stimulated with plate-bound anti-CD3 and soluble anti-CD28 and were cultured for 48 hours at a density of 5 × 10^5 cells/well in 100 μl of media. Supernatants were assayed for TNF-α, IL-2, and IL-10 levels by cytometric bead array using the mouse inflammation and mouse Th1/Th2 cytokine kits (BD Biosciences — Pharmingen) according to the manufacturer’s instructions. In T cell/B cell coculture experiments, 10^5 MLN CD4^+ T cells and 10^6 MLN B cells were cultured in 200 μl/well for 3, 7, or 11 days in wells containing plate-bound anti-CD3 with or without soluble anti-CD28.

For proliferation assays, 10^5 irradiated (3,000 rad) splenic APCs were cultured for 3 days with plate-bound anti-CD3 (10 μg/ml) and combinations of α/CD4^+ T cells, α/CD4^+ T cells, and B cells, each at a density of 5 × 10^5 cells/well. Each condition was assayed in triplicate. Incorporation of [3H]thymidine (1 μCi/well) (MP Biomedicals, Irvine, California, USA) was determined with the mouse immunoglobulin isotyping detection antibody was used at a dilution of 1:1,000. Plates were developed according to the manufacturer’s instructions, using 1:100 and 1:1 dilutions of supernatant for detection of α/CD4^+ T cells and soluble anti-CD28.

For cytokine analysis, T cell subsets were stimulated with plate-bound anti-CD3, soluble anti-CD28, and were cultured for 48 hours at a density of 5 × 10^5 cells/well in 100 μl of media. Supernatants were assayed for TNF-α, IL-2, and IL-10 levels by cytometric bead array using the mouse inflammation and mouse Th1/Th2 cytokine kits (BD Biosciences — Pharmingen) according to the manufacturer’s instructions. In T cell/B cell coculture experiments, 10^5 MLN CD4^+ T cells and 10^6 MLN B cells were cultured in 200 μl/well for 3, 7, or 11 days in wells containing plate-bound anti-CD3 with or without soluble anti-CD28.

For proliferation assays, 10^5 irradiated (3,000 rad) splenic APCs were cultured for 3 days with plate-bound anti-CD3 (10 μg/ml) and combinations of α/CD4^+ T cells, α/CD4^+ T cells, and B cells, each at a density of 5 × 10^5 cells/well. Each condition was assayed in triplicate. Incorporation of [3H]thymidine (1 μCi/well) (MP Biomedicals, Irvine, California, USA) was determined with the mouse immunoglobulin isotyping detection antibody was used at a dilution of 1:1,000. Plates were developed according to the manufacturer’s instructions, using 1:100 and 1:1 dilutions of supernatant for detection of α/CD4^+ T cells and soluble anti-CD28.

For cytokine analysis, T cell subsets were stimulated with plate-bound anti-CD3, soluble anti-CD28, and were cultured for 48 hours at a density of 5 × 10^5 cells/well in 100 μl of media. Supernatants were assayed for TNF-α, IL-2, and IL-10 levels by cytometric bead array using the mouse inflammation and mouse Th1/Th2 cytokine kits (BD Biosciences — Pharmingen) according to the manufacturer’s instructions. In T cell/B cell coculture experiments, 10^5 MLN CD4^+ T cells and 10^6 MLN B cells were cultured in 200 μl/well for 3, 7, or 11 days in wells containing plate-bound anti-CD3 with or without soluble anti-CD28.

For proliferation assays, 10^5 irradiated (3,000 rad) splenic APCs were cultured for 3 days with plate-bound anti-CD3 (10 μg/ml) and combinations of α/CD4^+ T cells, α/CD4^+ T cells, and B cells, each at a density of 5 × 10^5 cells/well. Each condition was assayed in triplicate. Incorporation of [3H]thymidine (1 μCi/well) (MP Biomedicals, Irvine, California, USA) was determined with the mouse immunoglobulin isotyping detection antibody was used at a dilution of 1:1,000. Plates were developed according to the manufacturer’s instructions, using 1:100 and 1:1 dilutions of supernatant for detection of α/CD4^+ T cells and soluble anti-CD28.

For cytokine analysis, T cell subsets were stimulated with plate-bound anti-CD3, soluble anti-CD28, and were cultured for 48 hours at a density of 5 × 10^5 cells/well in 100 μl of media. Supernatants were assayed for TNF-α, IL-2, and IL-10 levels by cytometric bead array using the mouse inflammation and mouse Th1/Th2 cytokine kits (BD Biosciences — Pharmingen) according to the manufacturer’s instructions. In T cell/B cell coculture experiments, 10^5 MLN CD4^+ T cells and 10^6 MLN B cells were cultured in 200 μl/well for 3, 7, or 11 days in wells containing plate-bound anti-CD3 with or without soluble anti-CD28.

For proliferation assays, 10^5 irradiated (3,000 rad) splenic APCs were cultured for 3 days with plate-bound anti-CD3 (10 μg/ml) and combinations of α/CD4^+ T cells, α/CD4^+ T cells, and B cells, each at a density of 5 × 10^5 cells/well. Each condition was assayed in triplicate. Incorporation of [3H]thymidine (1 μCi/well) (MP Biomedicals, Irvine, California, USA) was determined with the mouse immunoglobulin isotyping detection antibody was used at a dilution of 1:1,000. Plates were developed according to the manufacturer’s instructions, using 1:100 and 1:1 dilutions of supernatant for detection of α/CD4^+ T cells and soluble anti-CD28.

For cytokine analysis, T cell subsets were stimulated with plate-bound anti-CD3, soluble anti-CD28, and were cultured for 48 hours at a density of 5 × 10^5 cells/well in 100 μl of media. Supernatants were assayed for TNF-α, IL-2, and IL-10 levels by cytometric bead array using the mouse inflammation and mouse Th1/Th2 cytokine kits (BD Biosciences — Pharmingen) according to the manufacturer’s instructions. In T cell/B cell coculture experiments, 10^5 MLN CD4^+ T cells and 10^6 MLN B cells were cultured in 200 μl/well for 3, 7, or 11 days in wells containing plate-bound anti-CD3 with or without soluble anti-CD28.

For proliferation assays, 10^5 irradiated (3,000 rad) splenic APCs were cultured for 3 days with plate-bound anti-CD3 (10 μg/ml) and combinations of α/CD4^+ T cells, α/CD4^+ T cells, and B cells, each at a density of 5 × 10^5 cells/well. Each condition was assayed in triplicate. Incorporation of [3H]thymidine (1 μCi/well) (MP Biomedicals, Irvine, California, USA) was determined with the mouse immunoglobulin isotyping detection antibody was used at a dilution of 1:1,000. Plates were developed according to the manufacturer’s instructions, using 1:100 and 1:1 dilutions of supernatant for detection of α/CD4^+ T cells and soluble anti-CD28.
CMH/HeJBlir mice demonstrate selective antibody reactivity to antigens of the enteric bacterial flora. J. Immunol. 159:44–52.


