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Novel Suppressive Function of Transitional 2 B Cells in Experimental Arthritis

Jamie G. Evans,* Karina A. Chavez-Rueda,‡ Ayad Eddaoudi,† Almut Meyer-Bahlburg,‡ David J. Rawlings,‡ Michael R. Ehrenstein,3,4* and Claudia Mauri3,4*

The immune system contains natural regulatory cells important in the maintenance of tolerance. Although this suppressive function is usually attributed to CD4 regulatory T cells, recent reports have revealed an immunoregulatory role for IL-10-producing B cells in the context of several autoimmune diseases including collagen-induced arthritis. In the present study, we attribute this suppressive function to a B cell subset expressing high levels of CD21, CD23, and IgM, previously identified as transitional 2-marginal zone precursor (T2-MZP) B cells. T2-MZP B cells are present in the spleens of naive mice and increase during the remission phase of arthritis. Following adoptive transfer to immunized DBA/1 mice, T2-MZP B cells significantly prevented new disease and ameliorated established disease. The suppressive effect on arthritis was paralleled by an inhibition of Ag-specific T cell activation and a reduction in cells exhibiting Th1-type functional responses. We also provide evidence that this regulatory subset mediates its suppression through the secretion of suppressive cytokines and not by cell-to-cell contact. The ability to regulate an established immune response by T2-MZP B cells endows this subset of B cells with a striking and previously unrecognized immunoregulatory potential. The Journal of Immunology, 2007, 178: 7868–7878.

Over the last few years new evidence has shown that IL-10-producing B cells can play a “regulatory” role in immune responses against several autoantigens (1). In particular, IL-10-producing B cells control the progression of inflammatory diseases including inflammatory bowel disease in TCRα−/− mice (2), experimental autoimmune encephalomyelitis (3), and the collagen-induced arthritis (CIA) model (4). Our previous results demonstrating that the adoptive transfer of anti-CD40-treated splenic B cells into syngeneic mice prevented arthritis suggested that one of the B cell subsets populating the spleens could be responsible for controlling arthritis development.

In the periphery, distinct subsets of both immature (transitional) and mature B cells can be distinguished based on their variation in antigenic specificity, relative responsiveness to BCR, TLR, and coreceptor signaling, and patterns of cytokine production, suggesting a range of functional diversity (5–9). In addition, splenic B cells are distinguished according to a unique combination of markers delineating their stages of development (6, 8, 10, 11). The most immature transitional B cells, transitional 1 (T1) B cells, are predominantly localized within the periarterial lymphatic sheath (7, 12, 13). This population is distinguished by its relative expression of specific surface markers and includes cells that are predominantly AA4highCD23−CD24highCD21−IgMhighIgDlowCD1d+. As T1 B cells mature they exhibit progressively higher expression levels of CD21, CD23, and IgD and newly express CD1d.

In this report, we have assessed the relative role that B cells at different stages of development have in the immunoregulation of CIA. Our results show a previously unrecognized protective effect of transitional 2 (T2)-marginal zone (MZ) precursor (MZP) B cells in the pathogenesis of CIA. The transfer of T2-MZP cells, but not mature B cell subsets, during the induction phase of CIA inhibited the pathogenic Th1 response in vivo and the delayed-type hypersensitivity (DTH) response and prevented the development of arthritis through the production of IL-10.

Materials and Methods

Antibodies

Anti-CD3 or B220 PE or FITC, anti-CD21 FITC (clone 7G6), anti-CD23 PE (clone B3B4), anti-IgM allophycocyanin (clone II/41), anti-CD4-FITC (clone H129.19), anti-CD1d-PE (clone 1B1), anti-A4-1 (PE), anti-IL-10-allophycocyanin (clone JES5-16F3), anti-TNF-α-allophycocyanin (clone MP6-XT22), and anti-IFN-γ-allophycocyanin (clone XMG1.2) were from BD PharMingen. For in vitro neutralization, the IL-10 mAb (clone 1B1.2) was used.

Mice, induction, assessment of arthritis, and histological examination

IL-10 knockout (KO) in the H2b background was generated by backcrossing the original IL-10 KO/H2b with DBA/1 H2b mice. The mice were typed by PCR and IL-10 KO−/− H2b were further backcrossed into DBA/1. Mice from the 10th generation (DBA/II11–10 KO−/−) were used for the transfer experiments. Male DBA/1 (8- to 12-wk old) were immunized with 100 μg of type II bovine collagen (CII) emulsified in CFA (Difco Laboratories) (14). Of note, the medium, CII, and purified Abs used in all described

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experiments were endotoxin free. The development of arthritis was assessed daily for the duration of the experiment. The clinical severity of arthritis was graded as follows: 0, normal; 1, slight swelling and/or erythema; 2, pronounced edematous swelling; 3, pronounced edematous swelling plus joint rigidity; and 4, laxity. Each limb was graded, allowing a maximal clinical score of 16 for each animal. All clinical evaluations were performed in a blinded manner. All the mice are kept in accordance to the local guidelines.

Hind paws were removed post mortem and fixed in 10% (w/v) buffered formalin and decalified in 5% EDTA. The paws were subsequently embedded in paraffin, sectioned, and stained with H&E.

All animals were bred and maintained at the University College of London (London, U.K.). These studies have been reviewed and approved by the Home Office U.K.

In vivo depletion of CD25+ Cells

Endogenous CD25+ cells were depleted by i.p. injection with 1 mg of the anti-CD25 Ab clone PC61 (rat IgG1) 2 days before the induction of CIA. Control mice received an i.p. injection of 1 mg of MAC49 (rat IgG1; anti-phytochrome). Confirmation of CD25+ cell depletion by PC61 was determined by staining peripheral blood 7 and 15 days after treatment with an mAb that recognizes a different epitope of CD25 (clone 7D4), resulting in >90% CD25+ cell depletion (data not shown).

Adoptive transfer to DBA/1 mice

Splenocytes were incubated with anti-CD43 magnetic microbeads followed by capture with MACS LD separation columns (Miltenyi Biotech). The resulting B cell populations were routinely >97% pure as assessed by B220 or CD19 expression. B cells were stained either with anti-mouse anti-CD21-FITC, anti-CD23-PE, and anti-IgM-allophycocyanin or with anti-CD21-FITC and anti-CD23-PE and sorted by a MoFlo cytometer (DakoCytomation). B cell subsets (4 × 10^5) unless otherwise stated, were injected into the tail veins of 9- to 10-wk-old DBA/1 at the time of CIA/CFA immunization or 2 days after disease onset.

Isolation of CD4+ T cells

CD4+ T cells were isolated from the lymph nodes of naive or arthritic DBA/1 mice by negative selection using a Pan T cell isolation kit and passage through a MACS LS column (Miltenyi Biotech) (>98% purity).

Flow cytometric analysis of intracellular cytokines synthesis

Intracellular cytokine analysis was performed as previously described (4). Briefly, splenocytes or purified lymphocytes were resuspended at 5 × 10^6 cells/ml in complete medium or CII (50 μg/ml) for 24 h. PMA (50 ng/ml; Sigma-Aldrich), ionomycin (500 ng/ml; Sigma-Aldrich), and GolgiStop (BD Biosciences) were added for an additional 6 h. Cells were stained with anti-B220-PE-Cy7, anti-CD21-FITC, anti-CD23-PE, and anti-IgM-allophycocyanin conjugated mAbs. Permeabilized cells were incubated with anti-mouse IFN-γ, IL-10-, and TNF-α-allophycocyanin conjugated mAbs (BD Pharmingen). To demonstrate the specificity of staining, fixed/permeabilized cells were incubated with an excess of unlabeled anti-CD25 mAb (clone 7D4), resulting in >90% CD25+ cell depletion (data not shown).

Statistical analysis

For the statistical analysis of the data, the Mann-Whitney U test and the Fisher exact test were applied to analyze clinical results. Unpaired t tests were applied on cytokine quantification experiments. p < 0.05 was considered significantly different.

Results

Phenotypical characterization of IL-10-producing splenic B cells

To phenotypically identify the protective B cell subset(s) responsible for IL-10 production in the CIA model, B220−IL-10+ B cells were analyzed for the expression of CD21 and CD23 (these markers are commonly used to distinguish mature vs immature B cells) (6, 8, 10, 11). B cells were isolated by negative selection from mice during the acute and remission phase of disease as well as from naive mice and the expression of IL-10 measured by intracellular staining. Considerably more IL-10 is produced by B cells derived from mice in the remission phase than in the acute phase of disease or from naive mice (Fig. 1A). The results in Fig. 1B show that the majority of B220−IL-10+ B cells expressed high levels of CD21 and were CD23+. Further phenotypic analysis revealed that these IL-10−B220+CD21intCD23+ B cells were also IgMbright (Fig. 1C; for clarity of presentation only data from remission mice are shown) and expressed high levels of the CD1d CD1d cell surface marker (not shown). This surface phenotype was previously attributed to T2-MZP B cells (17, 18). IL-10−B220−CD21intCD23+ B cells also uniformly expressed an intermediate level of the complement receptor ClqRp, recognized by the mAb AA4.1 (Fig. 1D). This marker is highly expressed on recently formed bone marrow B cells as well as transitional B cells in the periphery (8, 19). The level of AA4 expression on IL-10−B220−CD21intCD23+ was lower than that present on T1 B cells (data not shown) but significantly higher than that expressed by either CD23lowCD21highMZ (data not shown) or CD23midCD21int follicular (FO) B cells where ‘int’ in CD23intCD21int is intermediate). These results suggested that the majority of IL-10− B cells might be derived from relatively recent emigrants from the bone marrow
and were consistent with cells of a T2-MZP phenotype. To ascertain whether the absolute number of a B cell subset changes as a consequence of treatment with collagen with or without PMA and ionomycin, we compared the number of T2-MZP, FO, and MZ B cells to the number before stimulation. There were no significant numerical changes in any B cell compartment in response to these treatments (data not shown).

The results in Fig. 1A show that the percentage of B cells producing IL-10 decreases during the acute stage of disease and dramatically increases in the remission phase, where nearly half of the B cells produced IL-10. We measured whether the increase in IL-10-producing B cells in mice in the remission phase of arthritis was mirrored by a variation in the absolute numbers of T2-MZP B cells throughout the course of the disease. Splenocytes were

![Figure 1](http://www.jimmunol.org/Downloadedfrom/)

**FIGURE 1.** Characterization of IL-10-producing cells at different phases of arthritis. A, Splenocytes isolated from DBA/1 mice during the acute or remission phase of disease and from naive mice were cultured with CII (50 µg/ml) for 24 h followed by 8 h of stimulation with PMA and ionomycin. The histograms display the percentage of B220+ B cells expressing IL-10. The profiles are representative of results obtained from nine mice. Identical results were obtained when CD19 was used as an alternative for B220 (data not shown). B, Differential expression of CD21 and CD23 on B220+ IL-10+ gated B cells derived from naive, acute, and remission mice. Data are representative of four independent experiments with four mice each. C and D, Representative histograms showing the expression of IgM (C) and AA4 (D) on T2-MZP and FO B cells from remission mice. The faint line represents the isotype control. E, Absolute number of T2-MZP B cells in naive, acute, and remission mice based on the gates shown in B and Fig. 2A. The data are representative of four independent experiments. F, T2-MZP B cells were isolated by FACS. The postsorted B220+CD23+CD21high IgM+ T2-MZP B cells profile is shown on the left (0 h). Purified T2-MZP B cells (5 x 10^5) were cultured in medium alone, anti-IgM F(ab’)2 (10 µg/ml), or LPS (1 µg/ml) for 96 h. Cells were stained again for CD21 and IgM markers. The percentages of T2-MZP with an unchanged phenotype and newly differentiated mature B cells are as indicated. G, Proliferative response of remission sorted T2-MZP and MZ B cells in response to anti-IgM F(ab’)2 (10 µg/ml) or LPS (1 µg/ml) stimulation. Cells were pulsed with [3H]thymidine for 12 h before harvesting. Data are shown as mean ± SEM of triplicate wells and are representative of two experiments. Similar results were obtained using B cells from mice in the naive phase of disease (data not shown). H, Deltex1 and Hes1 expressions are upregulated in remission T2-MZP and in MZ B cells. Data are representative of two independent experiments.
isolated from naive as well as from mice in the acute or remission phase of disease and B cells were stained with B220, CD21, CD23, and IgM (gated depicted in Figs. 1B and 2A). The decrease in IL-10-producing B cells observed during the acute phase of disease was paralleled by a significant decrease in the percentage of T2-MZP B cells. Similarly, mice in remission exhibited a significant increase in T2-MZP B cells and an increased absolute number of T2-MZP IL-10⁺ B cells was observed during the remission phase of arthritis compared with the T2-MZP IL-10⁺ number present in the spleens of naive mice (Fig. 1E and data not shown).

In vitro, T2-MZP B cells proliferate in response to BCR engagement and down-modulate CD23, leading to a phenotype that is consistent with mature FO B cells (6, 20). Furthermore, this subset also proliferates in response to LPS. These combined functional activities distinguish T2-MZP from both MZ B cells, which proliferate in response to LPS but not BCR engagement, and from FO B cells, which proliferate in response to BCR but not LPS (6,
The major producers of IL-10 (Fig. 2, MZP B cells (also displaying high levels of CD21 and IgM) were immunized. The transfer of MZ B cells did not alter the course of arthritis, as this group developed disease at rates similar to those of the untreated controls (Fig. 2, D and E). A slight delay in arthritis development was observed following transfer with FO B cells; however, all mice in this group developed arthritis by day 46 after immunization. In contrast, only 40% of mice treated with T2-MZP B cells developed arthritis. Furthermore, the clinical disease score was significantly less severe in these animals compared with that in the untreated group (Fig. 2, D and E). A similar protective effect was also obtained when B cells were first purified by negative selection using the MACS system. T2-MZP B cells were then sorted using CD21 and CD23 alone without IgM (Fig. 2, F and G).

Adoptive transfer of T2-MZP B cells isolated from the remission phase of disease prevented the induction of arthritis

We next addressed the question of whether T2-MZP B cells have a "regulatory" capacity and might prevent arthritis development. T2-MZP, FO, and MZ B cell splenic subsets were purified from DBA/1 mice during the remission phase of arthritis by FACS sorting according to relative expression levels of B220, CD23, IgM, and CD21 as depicted in Fig. 2A. After FACS isolation, reanalysis of the sorted samples consistently revealed purity of ~98% (data not shown). The production of IL-10 by purified B cell subsets was measured by intracellular staining and the supernatant was tested for IL-10 by ELISA before the addition of PMA and ionomycin (Fig. 2, B and C). These data confirmed that B220⁺CD23⁻ T2-MZP B cells (also displaying high levels of CD21 and IgM) were the major producers of IL-10 (Fig. 2, B and C). Purified T2-MZP, FO, and MZ B cells (4 x 10⁵) from remission animals were transferred i.v. into syngeneic DBA/1 mice on the day of CIA/CFA immunization. The transfer of MZ B cells did not alter the course of the arthritis, as this group developed disease at rates similar to those of the untreated controls (Fig. 2, D and E). A slight delay in arthritis development was observed following transfer with FO B cells; however, all mice in this group developed arthritis by day 46 after immunization. In contrast, only 40% of mice treated with T2-MZP B cells developed arthritis. Furthermore, the clinical disease score was significantly less severe in these animals compared with that in the untreated group (Fig. 2, D and E). A similar protective effect was also obtained when B cells were first purified by negative selection using the MACS system. T2-MZP B cells were then sorted using CD21 and CD23 alone without IgM (Fig. 2, F and G).

At the end point of the experiment (48 days after immunization), joints were analyzed to determine whether the protection conferred by the transfer of T2-MZP B cells correlated with reduced joint damage as assessed by histology (Fig. 3A). The majority of the joints from the control group were severely damaged, exhibiting proliferation of the synovial membrane and the accumulation of inflammatory cells. A similar pattern of inflammation was observed in mice treated with FO and MZ B cells. No histological evidence of tissue damage was observed in the joints of protected mice that received T2-MZP B cells, and minimal cartilage damage and cellular infiltration were detected in the joints of T2-MZP-treated mice that had developed arthritis (data not shown).

To examine the impact of B cell subset transfer on the levels of the autoreactive Abs required for disease pathogenesis in this model, peripheral blood was drawn 30 days after CIA/CFA immunization. T2-MZP B cell transfer significantly suppressed the CIA-specific IgG response and induced a switch away from a "pathogenic" Th1 IgG2a toward a more "protective Th2 like" IgG1 response (Fig. 3B).

**FIGURE 3.** Histological and serological evaluation of B cell subsets transfer. A, Histological assessment of arthritic joints in T2-MZP, FO, and MZ B cell treated mice vs control group (CTRL). Note the severe synovitis with erosion of articular cartilage in all sections except the joint from a mouse treated with T2-MZP B cells. B, T2-MZP B cell transfers inhibited CIA-specific IgG Ab response. On day 30 after CIA/CFA immunization, control and mice treated with T2-MZP, MZ, or FO B cells were bled and the serum levels of total, IgG1, and IgG2a CIA-specific IgG Abs were measured by ELISA.

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<th>B</th>
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CII/CFA immunization. The results in Fig. 4A demonstrate that splenocytes derived from the control, MZ, and FO B cell group strongly proliferated in vitro in response to CII restimulation. In contrast, splenocytes derived from the recipients of T2-MZP B cell transfers exhibited a dramatic reduction in proliferation following CII stimulation. This observation suggested that the transfer of T2-MZP B cells might have altered the initial T cell priming in response to CII in vivo. We also assessed the cytokines produced by splenocytes isolated from these different treatment groups. Fig. 4B shows that splenocytes isolated from mice treated with FO or MZ B cells 15 days after the transfer produced similar amounts of IFN-\(\gamma\) and IL-10 compared with the control group. In contrast, a significantly reduced amount of IFN-\(\gamma\) and a trend toward a reduction in IL-12 production was observed in mice that received T2-MZP B cells. Furthermore, an increase in IL-10 production was detected exclusively in the T2-MZP-treated group. To determine the cellular population(s) responsible for these alterations in cytokine production, CII specific IFN-\(\gamma\) and IL-10 expression levels were also measured by intracellular staining in B vs T lymphocytes. In keeping with the results shown in Fig. 4B, a significant reduction of IFN-\(\gamma\) produced by T cells, paralleled by an increase in the IL-10 produced by B cells, was detected in T2-MZP B cell-treated mice (Fig. 4C). Overall, there was a marked increase in the relative ratios of B cell IL-10/T cell IFN-\(\gamma\) in mice that had received T2-MZP B cells. Together, these results suggested that the adoptive transfer of T2-MZP B cells at an early stage of disease renders autoreactive T cells unresponsive to CII and unable to produce cytokines essential for the pathogenesis of arthritis.

**T2-MZP B cells transfer results in an inhibition of CII specific Th1 response**

We studied the DTH response induced in mice injected with either T2-MZP or mature B cells subset after immunization with CII/CFA. As shown in Fig. 4D, when mice treated with MZ or FO B cells or PBS were challenged with bovine CII, the severity of footpad swelling was significantly greater than that elicited in mice treated with T2-MZP B cells. Interestingly, T2-MZP B cells isolated from mice immunized with CII in CFA failed to inhibit an
OVA-dependent DTH response in mice immunized with OVA in CFA (data not shown). The data indicate that the suppression induced by T2-MZP B cells is specific for the Ag to which the T2-MZP B cells have been exposed.

**T2-MZP B cells isolated from naive mice can also partially suppress CIA induction**

We next addressed whether naive T2-MZP B cells can also limit arthritis development or whether this suppressive capacity is an “acquired feature” of remission T2-MZP cells. The results in Fig. 5A show that the transfer of 4 × 10^5 naive T2-MZP B cells delayed the disease onset of arthritis in recipient mice. All mice in the control group developed severe arthritis within 43 days after CII/CFA immunization. In contrast, only 60% of the mice treated with remission T2-MZP B cells developed arthritis. Similar to the results reported in Fig. 2, mice treated with remission T2-MZP B cells developed milder disease compared with the control group.

These data suggested that naive T2-MZP B cells are not equally "potent" as the remission T2-MZP B cells in interfering with the arthritogenic immune response. This could be due to an enrichment of IL-10-producing cells within the T2-MZP B cell pool during the remission phase of the disease. To begin to test this idea, we transferred increased numbers of naive or remission T2-MZP B cells (8 × 10^5 T2-MZP B cells) into syngeneic mice coincident with CII/CFA immunization. The results in Fig. 5B show that both groups of mice displayed a significant decrease in the incidence and clinical score of disease. This suggested that a threshold of naive T2-MZP B cells was likely required for efficient disease inhibition.

**T2-MZP B cells inhibit IFN-γ production via the release of IL-10**

To further confirm the potential immunoregulatory capacity of T2-MZP and to unravel the mechanism of action, we set up an in vitro experiment in which remission T2-MZP, MZ, or FO B cells were cocultured with naive CD4+ T cells for 24 h with medium or CII followed by 6 h of stimulation with PMA and ionomycin. Although T2-MZP B cells significantly inhibited the generation of IFN-γ-producing T cells, cytokine levels remained unaffected when T cells were cocultured with either MZ or FO B cells (Fig. 6A). As previously shown, the effector suppressive function of B cells was further enhanced in the presence of CII (4). Of note, no differences in the relative numbers of IL-10-producing CD4+ T cells were observed in any of the conditions tested (Fig. 6B).

We also addressed the question of whether remission T2-MZP B cells are able to down-modulate the response of CD4+ T cells isolated from spleens of arthritogenic mice. CD4+ T cells (5 × 10^5) were cultured at different ratios with purified T2-MZP B cells from remission donors for 24 h with medium, CII, OVA (as an irrelevant Ag), or anti-IgM F(ab’2) (as a nonspecific polyclonal BCR activator) followed by 6 h with ionomycin and PMA. T2-MZP B cells significantly inhibited the differentiation of IFN-γ-producing T cells upon CII stimulation. In contrast, the Th1 response remained unaffected when T cells were cocultured with T2-MZP B cells stimulated with an irrelevant Ag (OVA) or in the presence of cells stimulated with anti-IgM (Fig. 6, C and D). T cell proliferation induced by CII was also profoundly suppressed when cultured with T2-MZP B cells (Fig. 6E). These results confirm the immunoregulatory capacity of T2-MZP B cells and the requirement for CII for their suppression.

We next assessed whether IL-10 is critical for the immunosuppressive effect of T2-MZP B cells on IFN-γ-producing T cell differentiation. Fig. 6C shows the relative percentage of inhibition of IFN-γ production in CD4+ T cells. Suppression was dependent on IL-10 production because the addition of neutralizing anti-IL-10R/anti-IL-10 mAbs prevented IFN-γ inhibition (Fig. 6F). Transwell experiments were used to determine whether suppression was mediated by cell contact (Fig. 6F). Suppression was contact independent because the separation of target T cells from T2-MZP B cells by a permeable membrane did not abrogate suppression. Thus, taken together these results demonstrated that the immunoregulatory capacity of T2-MZP is achieved via the provision of IL-10 and does not require cell contact.

**Prevention of arthritis is abrogated if T2-MZP B cells lack the capacity to secrete IL-10**

To establish whether the T2-MZP B cells mediate their protective effects via the release of IL-10, T2-MZP B cells were isolated from wild-type or CII/CFA-immunized IL-10−/− DBA/1 mice. Mice were immunized with CII/CFA and sacrificed 4 wk after disease onset. Of note, only wild-type mice will be in the remission phase
of disease, as the IL-10−/− mice display an exacerbated and prolonged inflammation period (24). Mice treated with wild-type T2-MZP B cells were effectively protected, with only two of eight mice developing mild disease compared with eight of eight control mice developing severe arthritis. In contrast, 100% of mice that received IL-10-deficient T2-MZP B cells developed a severe arthritis (Fig. 7, A and B). A similar outcome was obtained when T2-MPZ were isolated from naive IL-10−/− mice (data not shown). This confirms that T2-MZP B cells protect against arthritis development via the provision of IL-10.

The immunoregulatory effect of T2-MZP B cells is independent of the presence of endogenous CD4+CD25+ regulatory T cells (Treg).

To assess whether immunoregulation by T2-MZP requires the presence of endogenous Treg, CD4+CD25+ T cells were depleted as previously shown (25). Administration of the anti-CD25 mAb (clone PC61) resulted in the depletion of >90% of CD4+CD25+ T cells (tested on days 15 and 30 after depletion; data not shown). Depleted and nondepleted mice were immunized and treated on the day of CII/CFA immunization with T2-MZP B cells or with PBS. As previously shown, in vivo depletion of CD25+ Treg resulted in an exacerbation of the clinical symptoms paralleled by a higher incidence of arthritis compared with undepleted mice (isotype control treated). In contrast, 50% of CD25-depleted mice treated with T2-MZP B cells developed disease and the severity of arthritis was significantly milder compared with that in the group of mice lacking CD25+ Treg or in the control group (Fig. 7, C and D). The incidence and the severity of arthritis in undepleted mice treated with remission T2-MZP B cells was very similar to the disease in the T2-MZP-depleted group and was omitted for clarity of presentation. These results demonstrated that T2-MZP B cells suppress disease independently of the presence of endogenous CD4+CD25+ Treg.

T2-MZP B cells ameliorate established arthritis

In the following experiments we have tested the therapeutic potential of the T2-MZP B cells transfer.

**FIGURE 6.** The inhibition of Th1 differentiation by T2-MZP B cells is IL-10 dependent and contact independent. A and B, Intracellular production of IFN-γ (A) and IL-10 (B) by naive CD4+ T cells (5 × 10^5) cocultured with 5 × 10^5 purified T2-MZP, FO, and MZ B cells (1:1) from remission mice. Cells were stimulated with medium or CII (50 μg/ml) for 24 h followed by 8 h of stimulation with ionomycin and PMA. Data represent mean ± SEM. Data are representative of three independent experiments. C, T2-MZP B cells were purified from the spleens of remission mice and cultured at different ratios with CD4+ T cells isolated from the lymph nodes of arthritic mice. Cell cultures were stimulated with either medium alone or CII (50 μg/ml), OVA (50 μg/ml), or Fab′(a)2 (10 μg/ml) in the presence of 5 × 10^5 irradiated APCs. Samples were stimulated with ionomycin and PMA for 24 h followed by 6 h of stimulation with GolgiStop. The results indicate the percentage of IFN-γ-producing CD4+ T cells. The results are representative of two independent experiments each with four mice. D, Samples were cultured for 72 h and IFN-γ production was measured by ELISA. E, Samples were cultured for 48 h and pulsed with [3H]thymidine (3H-TdR) for 12 h before harvesting. ***, p < 0.001; ****, p < 0.0001 vs respective T cells cultured alone. F, Remission T2-MZP B cells were cocultured (1:1) with naive CD4+ T cells and stimulated with CII in the presence of isotype control (10 μg/ml) (data not shown) or anti-IL-10R/anti-IL-10 mAb (5 μg/ml). A Transwell membrane was also added to prevent cell-to-cell contact. PMA and ionomycin were added for the last 6 h of culture. The results indicate the percentage of inhibition of the cytokine production relative to the naive T cells stimulated with CII and are representative of three independent experiments.
were randomly assigned to a treatment group on the day of CII/CFA immunization. Two days after disease onset mice were treated with \(7 \times 10^5\) T2-MZP B cells (i.v.) or with PBS. The treatment was repeated on day 5 of disease onset. The results in Fig. 7 show that the transfer of T2-MZP B cells after disease onset significantly improved the severity of arthritis. The transfer of T2-MZP B cells also significantly reduced the levels of anti-CII IgG autoreactive Abs (Fig. 7F).

**Discussion**

Each of the key physiological functions of B cells, including production of Ab, Ag presentation, and activation of naïve T cells, has been suggested as being performed almost exclusively by mature B cells (1). In comparison to the wealth of literature dissecting the pleiotropic actions of mature B cells, less attention has been paid to the possibility that distinct transitional B cell subsets might play independent physiological roles not shared by their fully “mature” counterparts.

In this study we have demonstrated that B cells at an immature stage of development subset protect mice from CIA. The protection from disease was paralleled by an inhibition of Th1 response as shown by the reduced amount of IFN-γ and an inhibition of the DTH response to CII in mice treated with T2-MZP compared with control group. These “regulatory” B cells express AA4^+^, CD21^high^, CD23^+^, CD24^high^, IgM^high^, IgD^+^, and CD1d^+^, characteristic markers of a splenic subpopulation that has been alternatively designated as either “T2” (12, 19) or “MZP” B cells (26). Although initial work suggested that this cell population represented an immature bipotent precursor of both FO and MZ B cells (12), additional studies suggest that this subset is predominantly enriched for progenitors of MZ B cells (13, 19, 20, 21). The evidence that we have supplied in this work strongly supports the notion that T2-MZP B cells have a regulatory capability. These cells, for example, expressed intermediate levels of the immature B cell surface marker AA4, consistent with a late transitional B cell stage (20). In addition, T2-MZP B cells exhibit a unique constellation of functional activities. The “regulatory” T2-MZP B cells isolated from the spleens of either naïve and remission mice in this study also exhibited each one of these functional properties. Specifically, “regulatory” T2-MZP B cells proliferated and down-modulated CD21 in response to BCR cross-linking, proliferated in response to LPS stimulation, and exhibited high levels of Notch2...
target genes consistent with active Notch2-dependent signaling in vivo. Moreover, the transfer of unmanipulated naive T2-MZP B cells protected mice from developing arthritis. Although it may be possible that in response to immunization a mature B cell subset could acquire new surface markers (i.e., up-regulation of CD23 and CD21), the naive T2-MZP B cells described here should not differ from those previously reported. These combined data strongly support the conclusion that the "regulatory" B cell subset identified in this study is phenotypically and functionally identical with the T2-MZP B cell subset.

Our results demonstrate that T2-MZP B cells exert their protective effect through the production of IL-10, in agreement with previous studies on "regulatory" B cells (2–4, 27, 28). Both the transfer of T2-MZP B cells isolated from IL-10 deficient mice and our in vitro experiments (Figs. 7 and 6, respectively) confirm that T2-MZP B cells suppress disease and proinflammatory cytokines production by T cells via the release of IL-10. Our in vitro experiments demonstrating that contact is not required for the suppression of IFN-γ producing T cells further suggest that cognate interaction is not essential, at least in regard to the inhibition of Th1 differentiation. IL-10 is a key regulator of inflammation and it has been shown to inhibit both Th1- and Th2-type immune responses (29). IL-10 can also facilitate down-modulation of co-stimulatory molecules on APCs, including B cells (28, 30). It is conceivable that the IL-10 released by T2-MZP B cells might reduce the expression of MHCII, CD80, and CD86 and affect the maturation of other APCs (i.e., mature B cells and dendritic cells), leading to induction of T cell anergy or even the generation of Treg cells in vivo (31–35). However, despite the lack of T cell response to CII, our study shows neither numerical change in CD4+CD25+ Treg cells following T2-MZP B cell transfer (data not shown) nor the differentiation of Tr1 as indicated by the lack of differentiation of IL-10-producing CD4+ T cells (36). Moreover, the results in Fig. 7, C and D, demonstrated that the suppressive effect of T2-MZP B cells is independent of the presence of endogenous Treg cells, because the depletion of CD4+CD25+ Treg cells did not diminish their inhibitory capacity. Nevertheless, we cannot exclude the possibility that regulatory or suppressor T cells other than those expressing CD25 or producing IL-10 could be induced upon transfer (i.e., CD4+CD45RB or CD4+ T cells producing TGFβ) (37). One experiment that might address this issue would be the purification of T cells after coculture with T2-MZP B cells and an assay of the suppressive capacity of the former on syngeneic effector T cells.

It is unclear why the endogenous T2-MZP B cells fail to protect mice from developing arthritis. Similar issues have arisen, but only recently have they begun to be unraveled in the field of Treg cells in autoimmune disease. Recent studies have revealed that either a reduction in the number of Treg cells or a defective function is present in autoimmune diseases (38–40). We could envisage a similar situation for the endogenous T2-MZP B cells. Thus, the frequency of B cells producing IL-10 could be reduced as the inflammatory arthritis ensues and/or the functional ability of T2-MZP B cells could be impaired by the proinflammatory milieu. Our results showed that after the intense inflammatory phase, characteristic of CIA, the number of T2-MZP B cells increased. Whether this increase in T2-MZP B cells during the remission phase of the disease is responsible for the resolution of the inflammatory process or is merely consequence of this process remains to be established. Additional experiments are also required to determine the immunoregulatory role of endogenous as opposed to transferred T2-MZP B cells.

Another possible explanation is that endogenous T2-MZP B cells, resident in the spleens, are unable to reach the site of inflammation. These cell populations have been previously defined as non-recirculating cells (10). Therefore, it might be possible that transferred T2-MZP B cells, as opposed to endogenous cells, can home to the periphery where they are mostly needed. We have addressed this specific point by taking advantage of CSFE technique; although we found that T2-MZP B cells repopulate and survive in the spleens (data not shown), the low number of T2-MZP B cells recovered by the joints did not yield sufficiently conclusive results. Future experiments using transgenic BCR mice as donors should help to unravel this issue.

At present, we cannot entirely rule out the possibility that T2-MZP B cells, consistent with their "transitional" nature, might differentiate after transfer into a mature B cell effector subset and thereby exert their protective effect. The lack of protection observed after the transfer of mature MZ B cells in the CIA model could be due, for example, to a failure of homing capacity, a lack of survival signals, or competitive "unfitness". However, the percentage of T2-MZP, FO and MZ DAPI CFSE+ B cell subsets recovered after B cells transfer equals the percentage of B cell subsets normally present in the spleens, demonstrating that both mature and T2-MZB cells have the same capacity to survive and traffic to the spleens (data not shown). These findings are also consistent with observations that T2-MZP cells cycle in vivo (12) and are maintained for long periods following the transplantation of purified splenic B cells into B cell-deficient, μMT recipient mice (A. Meyer-Bahlburg and D. J. Rawlings, unpublished observations) in the absence of immature B cell production in the bone marrow. Therefore the survival results, together with our in vitro results showing that MZ or FO B cells fail to suppress proinflammatory cytokine production while T2-MZP B cells inhibit the differentiation of Th1-like responses, favor the hypothesis that the immunoregulatory effect observed in vivo is directly mediated by T2-MZP B cells or at least a proportion of these IL-10 producing cells.

The suppressive role of T2-MZP B cells has also been demonstrated in the MRL/lpr mouse model for lupus. In this model we have shown that the stimulation of splenocytes with an agonistic anti-CD40 mAb leads to the differentiation of IL-10-producing T2-MZP B cells (K. A. Chavez and C. Mauri, unpublished observations). The expansion of this population is also consistent with the capacity of T2-MZP cells to proliferate in response to CD40 signals (D. J. Rawlings, unpublished observations). Dalwadi et al. demonstrated that Gnt2 KO mice are more prone to inflammatory colitis and that this correlates with both a decrease in the absolute numbers of T2-MZP B cells and a reduction in the number of IL-10-producing B cells in this strain (41). Although not yet formally tested, this observation is also consistent with an immunoregulatory role for T2-MZP B cells in this colitis disease model.

A particular B cell subset expressing CD1d has been previously associated with IL-10 production and suppression of disease in a model of inflammatory colitis (2). However, CD1d alone is not sufficient to identify the regulatory B cell subset because our results clearly demonstrate that whereas MZP B cells are protective, their mature counterparts, which also express CD1d, lack a regulatory function. Indeed, the data presented in Fig. 2 suggest a trend toward worsening disease upon MZ B cell transfer.

Collectively, our results open the intriguing possibility that T2-MZP B cells sentinel the microenvironment where autoantigens are presented to T cells and are involved in the maintenance or restoration of tolerance. The findings demonstrating that the transfer of T2-MZP B cells not only prevented but also ameliorated established arthritis, together with the identification of T2-MZP B

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cells as a potent regulatory population, provide potential new opportunities for the manipulation of these cells in the treatment of autoimmune disease.

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Disclosures
The authors have no financial conflict of interest.

References