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# A Novel Mechanism of B Cell–Mediated Immune Suppression through CD73 Expression and Adenosine Production

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**Immune suppression by regulatory T cells and regulatory B cells is a critical mechanism to limit excess inflammation and autoimmunity. IL-10 is considered the major mediator of B cell–induced immune suppression. We report a novel mechanism for immune suppression through adenosine generation by B cells. We identified a novel population of B cells that expresses CD73 as well as CD39, two ectoenzymes that together catalyze the extracellular dephosphorylation of adenine nucleotides to adenosine. Whereas CD39 expression is common among B cells, CD73 expression is not. Approximately 30–50% of B-1 cells ($B220^+CD23^-$) and IL-10–producing B (B10) cells ($B220^+CD5^+CD1d^{hi}$) are $CD73^{hi}$, depending on mouse strain, whereas few conventional B-2 cells ($B220^+CD23^+AA4.1^-$) express CD73. In keeping with expression of both CD73 and CD39, we found that $CD73^+$ B cells produce adenosine in the presence of substrate, whereas B-2 cells do not. $CD73^{-/-}$ mice were more susceptible to dextran sulfate sodium salt (DSS)-induced colitis than wild type (WT) mice were, and transfer of $CD73^+$ B cells ameliorated the severity of colitis, suggesting that B cell CD73/CD39/adenosine can modulate DSS-induced colitis. IL-10 production by B cells is not affected by CD73 deficiency. Interestingly, adenosine generation by $IL\text{-}10^{-/-}$ B cells is impaired because of reduced expression of CD73, indicating an unexpected connection between IL-10 and adenosine and suggesting caution in interpreting the results of studies with $IL\text{-}10^{-/-}$ cells. Our findings demonstrate a novel regulatory role of B cells on colitis through adenosine generation in an IL-10–independent manner.** *The Journal of Immunology*, **2014, 193: 5904–5913.**

The balance of proinflammatory and anti-inflammatory function is tightly regulated in the immune system. Inflammatory reactions must be transient, lasting only until infectious agents are cleared from the body, because excess inflammation can cause tissue damage or even autoimmunity (1, 2). Immune cells contain inhibitory populations (3) such as regulatory T cells (Tregs) (4, 5), regulatory myeloid cells (Mregs) (6, 7) and regulatory B cells (Breg) (8–16). A major anti-inflammatory factor is IL-10, which suppresses production of inflammatory cytokines such as IFN-γ, IL-2, IL-3, TNF-α, and GM-SCF from macrophages and activated T cells; inhibits IL-6R signaling in a SOCS3-dependent manner; diminishes Ag presentation by APCs; and induces IL-10 production (producing a feed-forward mechanism of IL-10 production) from macrophages and dendritic cells (17).

During the past decade, accumulating evidence has demonstrated that B cells play a nonredundant role in the suppression of inflammation as well as the production of protective Abs (8–11). Bregs were first defined by Kazs et al. (18) in a hypersensitivity model. Since then, additional studies have revealed that Bregs can suppress various experimental mouse disease entities, such as experimental autoimmune encephalomyelitis (EAE) (19) (12, 20), collagen-induced arthritis (21–23), OVA-induced asthma (24), contact hypersensitivity (14, 25), TCR-α knockout (KO), IL-2 KO, and IL-10 KO spontaneous colitis (26–28), type 1 diabetes (29) and systemic lupus erythematosus (SLE) (30) in an IL-10-dependent manner. IL-10 is produced by peritoneal B-1 cells, marginal zone (MZ) B cells, and transitional 2–MZ precursor cells upon activation (11). A landmark study performed by Yanaba et al. (14) showed that IL-10–secreting B cells (termed B10 cells) are found in the $B220^+CD1^{hi}CD5^+$ B cell fraction. Importantly, this phenotypic profile covers all IL-10–producing B cell populations such as B-1 cells, MZ B cells, and transitional 2–MZ precursor cells (11). However, some studies suggest that other suppressive factors might be responsible for B cell–mediated immune suppression (10). Teichmann et al. (31) recently reported using B cell–specific IL-10–deficient mice, that IL-10 is not required for amelioration of SLE symptoms, although depletion of B cells accelerated SLE disease (30). Furthermore, Ray et al. (32) suggested an IL-10–independent mechanism of B cells in EAE recovery. Thus, unknown suppressive factors other than IL-10 might be involved in B cell–mediated amelioration of SLE and EAE.

Tregs dampen inflammation by secreting IL-10 and TGF-β (33). Recent results indicate that, in addition, Tregs express CD39 and CD73 (34, 35) along with traditional markers like Foxp3 and CD25, and they suppress inflammation in a CD39- and CD73-dependent manner (35). More recently, it was reported that Th17 cells differentiated from naive T cells with IL-6 and TGF-β in vitro also express CD39 and CD73, and this Th17 population plays a suppressive role in cancer immunity (36). CD39 and CD73 are ectoenzymes (37). CD39 catalyzes the breakdown of extracellular ATP to ADP and AMP, while CD73 catalyzes the conversion of AMP to adenosine (37). Extracellular ATP plays a proinflammatory role, whereas adenosine plays an anti-inflammatory role (38). Therefore, regulating the balance of extracellular ATP and adenosine concentration is important to maintain homeostasis.

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Abbreviations used in this article: B10, IL-10–producing B; Bmem, memory B; Breg, regulatory B cell; DSS, dextran sulfate sodium salt; KO, knockout; Mreg, regulatory myeloid cell; MZ, marginal zone; SLE, systemic lupus erythematosus; Treg, regulatory T cell.



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CD39-deficient (39) and CD73-deficient mice (40, 41) show exaggerated features of chemically induced colitis. Furthermore, SNPs in the human *CD39* gene are associated with the spontaneous colitis, known as Crohn disease (CD) (39). These data suggest CD73 and CD39 play important roles in suppressing colitis in both mouse and human, presumably through generation of adenosine.

Mouse B cells can be divided into two subsets, acquired-type conventional B-2 cells and innate-type B-1 cells, which can be further divided into B-1a cells and B-1b cells according to CD5 expression (42). B-1a cells are the primary source of natural Ab, which can also be contributed by MZ B cells, whereas B-1b cells contribute long-lasting memory to some kinds of bacteria or virus infections (43) (44). In addition to CD5, recent studies have revealed that B-1 cell populations can be subdivided based on the expression of PD-L2 (CD273) (45, 46), CD25 (47), and PC1 (also termed ENPP1) (48). It was originally reported that CD73 is expressed on a few mouse splenic B cells (49), and more recent data show that CD73 is expressed by memory B (Bmem) cells (50, 51). However, whether CD73 is expressed by B-1 cells is still unknown, although B-1 cells are known to function in a regulatory, anti-inflammatory manner (52–56).

In this study, we examined whether B-1 cells express CD73 and whether adenosine generation by CD73 is involved in B-1 cell–mediated immunosuppression. We identified a novel means of dividing B-1 cells based on CD73 expression. We showed that CD73$^{hi}$ B-1 cells generate adenosine and inhibit experimental colitis. This finding represents a novel Breg mechanism for the anti-inflammatory effect mediated by B cells.

## Materials and Methods

### *Abs and reagents*

Anti-CD3ε (145-2C11), anti-CD16/CD32 (2.4G2), PE-anti-CD73 (TY23), APC-anti-CD39 (TÜ66), FITC-anti-CD21/35 (7G6), PE– and APC–anti-PD-L2 (TY25), and FITC–anti-IgM$^a$ (DS-1) were obtained from BD Biosciences (San Diego, CA). Alexa Flour 647-anti-CD73 (TY11.8),

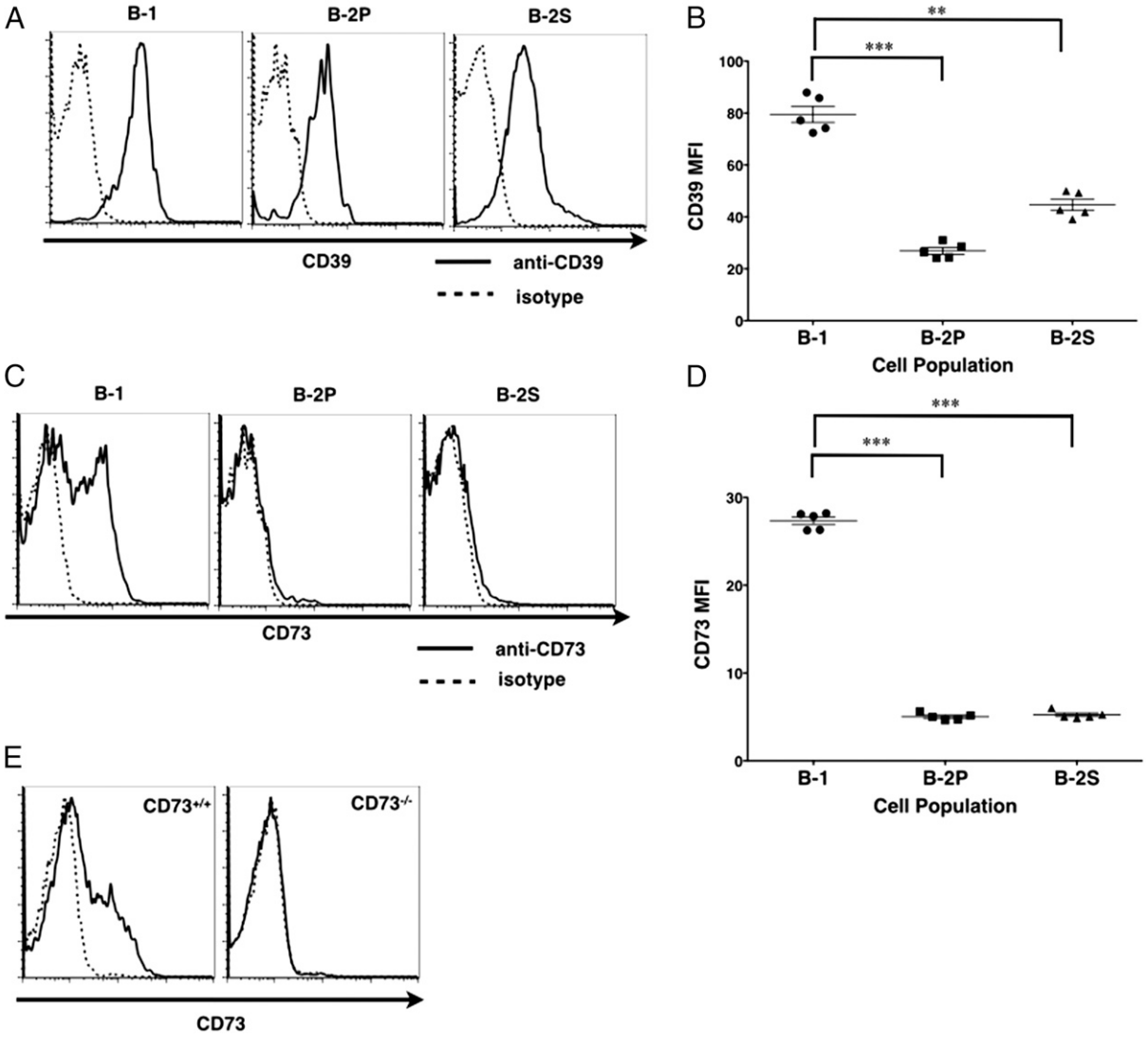


**FIGURE 1.** CD73 and CD39 are expressed by B-1 cells. Peritoneal cavity cells and spleen cells obtained from C57BL/6 mice were immunofluorescently stained with anti-CD39 (**A** and **B**), anti-CD73 (**C**–**E**), or isotype control (A–E), as indicated, and analyzed by flow cytometry. B-1 cells from the peritoneal cavity were identified as B220$^+$CD23$^{lo}$. B-2 cells from the peritoneal cavity (B-2P) were identified as B220$^+$CD23$^{hi}$. B-2 cells from the spleen (B-2S) were identified as B220$^+$CD93$^-$CD21$^{lo}$CD23$^{hi}$. Results are representative (A and C) or inclusive (B and D) of five mice. Mean fluorescence intensity (MFI) of stained CD39 (B) or CD73 (D) was calculated using FlowJo. (E) Peritoneal cavity cells from CD73$^{+/+}$ (*upper panel*) or CD73$^{-/-}$ (*lower panel*) mice were immunofluorescently stained with anti-CD73 (line) or isotype control (dashed line), and CD73 expression on B-1 cells is shown. Results are representative of three independent experiments. $^{**}p < 0.01$, $^{***}p < 0.001$.

FITC- and perCP-Cy5.5-anti-B220 (RA3-6B2), perCP-Cy5.5-F4/80, Alexa Fluor 647-anti-CD5 (53-7.3), APC-anti-CD93 (AA4.1), and APC-anti-Gr-1 (RB6-8C5) were obtained from BioLegend. PE-Cy7-anti-CD23 (2G8) was obtained from Abcam. PE-anti-IL-10 (JES5-16E3) was obtained from eBioscience (San Diego, CA). Anti-CD40 (1C10) was obtained from R&D Systems. Affinity-purified $F(ab')_2$ fragments of goat anti-mouse IgM (anti-Ig) were obtained from Jackson Immunoresearch Laboratories. LPS from *Salmonella typhimurium* and 5′-AMP were obtained from Sigma-Aldrich. Adenosine deaminase (ADA) was obtained from Roche Diagnostics (Indianapolis, IN).

### *Mice*

BALB/cByJ, C57BL/6, and CB17 SCID mice, along with IL-$10^{-/-}$ mice on BALB/c, were obtained from The Jackson Laboratory (Bar Harbor, ME). CD73-deficient mice (CD73$^{-/-}$) on BALB/c were provided by Dr. Linda Thompson (Oklahoma Medical Research Foundation, Oklahoma City, OK). Mice at 7–10 wk of age were housed at least 1 wk before experimentation. Mice were cared for and handled in accordance with National Institutes of Health and institutional guidelines.

### *Flow cytometry*

Cell surface phenotype was analyzed as described previously (57). Cells pretreated with 2.4G2 (5 μg/ml) were incubated with fluorescence-conjugated monoclonal Abs in staining buffer (PBS containing 2% FCS and 0.05% $NaN_3$) on ice for 30 min and then washed with staining buffer. Stained cells were analyzed with a FACSCalibur or LSRII (BD Biosciences) or Gallios (Beckman Coulter) instrument. In some experiments, 7-amino-actinomycin D (1 μg/ml; BD Biosciences) was used to exclude dead cells from analysis. For each sample, at least $1 \times 10^4$ cells were collected and were analyzed with FlowJo software (Tree Star). Compensation was adjusted with FlowJo software using none-staining and single-color staining controls for each flow experiment.

### *Purification of CD73$^{hi}$ and CD73$^{lo}$ B-1 cells and B-2 cells*

CD73$^{hi}$ and CD73$^{lo}$ B-1 cell populations were obtained by sort-purifying B220$^{+}$CD23$^{-/lo}$CD73$^{hi}$ and B220$^{lo}$CD23$^{-/lo}$CD73$^{lo}$ cells from peritoneal washouts with an Influx instrument (Becton Dickinson), respectively. Splenic B-2 cells (B-2S) were obtained by sort-purifying B220$^{+}$AA4.1$^{-}$CD21$^{lo}$CD23$^{hi}$ cells from spleen cell suspensions. Cells were cultured in RPMI 1640 medium (HyClone) containing 10% FCS, 10 mM HEPES, 2 mM L-glutamine, 0.1 mg/ml penicillin and streptomycin, and 50 μM 2-ME (complete medium) or in X-VIVO 10 medium (Lonza) containing 10 mM HEPES, 2 mM L-glutamine, 0.1 mg/ml penicillin and streptomycin, and 50 μM 2-ME (serum-free medium).

### *Measurement of adenosine by CREB reporter assay using CHO-ADORA2B cells*

Sort-purified cells ($1 \times 10^5$) were incubated in 96-well microtiter plates in 200 μl X-VIVO 10 serum free medium (Lonza) for the indicated times with or without AMP or ATP. Supernatants were then harvested and kept at −20°C until analysis. Adenosine receptor $A_{2B}$ (ADORA2B) stimulation of CREB, reflected in CREB-driven luciferase activity was used as the



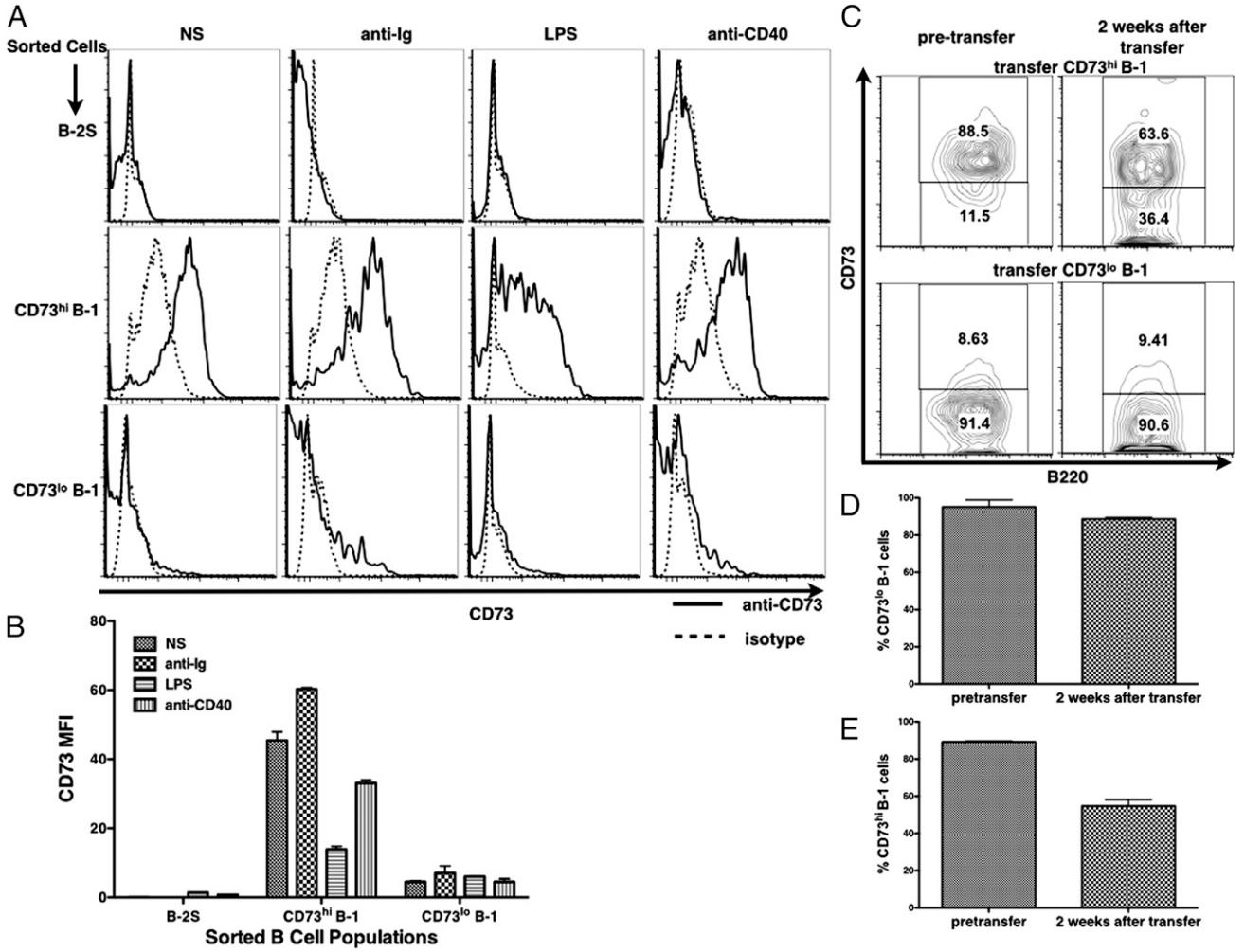


**FIGURE 2.** CD73 expression on B-1 cells is stable in vitro and in vivo. (**A**) Sort-purified CD73$^{hi}$ or CD73$^{lo}$ B-1 cells, or B-2S cells, were cultured for 72 h with or without anti-Ig (10 μg/ml), LPS (10 μg/ml), or anti-CD40 (2 μg/ml) in complete RPMI 1640 medium. Cells were harvested and restained with anti-CD73 or isotype control, as indicated, and analyzed by flow cytometry. Results are representative or inclusive (**B**) of two independent experiments. (**C** and **D**) Sort-purified CD73$^{hi}$ or CD73$^{lo}$ B-1 cells ($1 \times 10^6$) were adoptively transferred i.p. into CB17 SCID mice. After 2 wk, peritoneal cavity cells were immunofluorescently stained with anti-CD73 and anti-B220 and analyzed by flow cytometry. Results are representative (C) or inclusive (D and **E**) of four recipient mice. The percentage of CD73$^{lo}$ (D) or CD73$^{hi}$ (E) B-1 cells before or after transfer are shown, respectively.

readout for adenosine in supernatants. PathDetect pCRE-luc Cis-Reporter Plasmid (Agilent Technologies) and pRL-TK vector (Promega) were cotransfected into Chinese Hamster Ovary (CHO) cells that overexpress adenosine receptor $A_{2B}$ (CHO-ADORA2B) (DiscoveRx), using Amaxa Nucleofector (Lonza) according to the manufacturer's instructions. Transfected cells were cultured in F-12K medium (American Type Culture Collection) containing 10% FCS, 10 mM HEPES, 2 mM L-glutamine, 0.1 mg/ml penicillin and streptomycin for 24 h at a density of $1 \times 10^4$ cells per 100 μl per well in 96-well microtiter plates. After washing transfected cells with X-VIVO 10 twice, supernatants from primary cell cultures were incubated with pCRE-luc transfected CHO-ADORA2B cells for 4 h. Luciferase activity in cell lysates was analyzed by the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions, using a 20/20n Single Tube Luminometer (Turner Biosystems), as reported previously (46). Relative luciferase activity was calculated as firefly-luciferase activity/renilla-luciferase activity. Concentrations of adenosine were calculated using a standard regression curve generated using adenosine (Sigma-Aldrich) diluted in culture medium.

### *Measurement of adenosine by HPLC*

Sort-purified cells ($1 \times 10^6$) were incubated in 24-well plates in 1 ml X-VIVO 10 serum free medium for 2 h with or without AMP or ATP. Supernatants were then harvested and kept at −20°C until analysis. Phenylboronic acid was added to the supernatants, which were then incubated at room temperature for 20 h. Each sample was extracted with ethyl acetate three times, and the combined organic portion was concentrated and dried under a nitrogen stream and then dissolved in pyridine. Acetic anhydride was added, and the solution was incubated at 60°C for 2 h. The solvent was removed under a nitrogen stream, and the residue was dried with a vacuum pump for 20 h. The dried residue was dissolved in methanol and injected directly for HPLC analysis (Waters 600 Controller and Waters 996 Photodiode Array). Column type: Phenomenex Luna 5u C18 100A 250 × 4.6 mm. Flow rate: 1 ml/min with a linear gradient from water (0.05% TFA): MeOH (0.05% TFA) [90:10] to water (0.05% TFA): MeOH (0.05% TFA) [10:90] in 20 min. Wavelength: 256 nm.

### *Adoptive transfer into SCID*

Sort-purified $CD73^{hi}$ and $CD73^{lo}$ B-1 cells, or total B-1 cells, were washed with PBS twice and then suspended in PBS. B-1 cells ($1 \times 10^6$) were i.p. injected into CB17 SCID mice. Mice were sacrificed 2 wk after the transfer, and CD73 expression on B-1 cells was analyzed with flow cytometry after restaining with fluorescently labeled anti-CD73.

### *Dextran sulfate sodium salt mouse colitis model*

Mice were administered 5% dextran sodium sulfate (DSS; molecular size range, 36–50 kDa; ) in drinking water for 7 d, followed by normal drinking water. The parameters for colitis evaluation recorded in the experiments were body weight, colon length, and colon density. In some experiments, total colon proteins were extracted using lysis buffer (200 mM NaCl, 5 mM EDTA, 10 mM Tris, 10% glycine) containing Complete Protease Inhibitor Cocktail (Roche Diagnostics) and ground with Sample Grinding Kit (GE Healthcare) according to the manufacturer's instructions. In some experiments, sort-purified $1 \times 10^6$ cells were washed with PBS twice and then suspended in PBS and i.p. injected into $CD73^{-/-}$ mice 1 wk before DSS administration.

### *IL-1β*

Colon protein lysates were evaluated for content of IL-1β by ELISA (eBioscience) according to the manufacturer's instructions.

### *Statistics*

All quantitative data are expressed as mean ± SEM. Student *t* test was used for statistical determinations. The *p* values $< 0.05$ are considered statistically significant ($*p < 0.05$, $**p < 0.01$, or $***p < 0.001$, as indicated in the figure legends).

## Results

### *Peritoneal B-1 cells express CD39 and CD73*

We investigated whether B-1 cells express CD39 and CD73—cell surface ectoenzymes that together can generate immunosuppressive adenosine. Splenocytes and peritoneal cells from male C57BL/6 mice were tested for surface expression of these molecules by immunofluorescent staining and flow cytometry. Although all B cells express CD39 (Fig. 1A), we found expression of CD39 on B-1 cells to be 3-fold higher than that of peritoneal B-2 (B-2P) cells and splenic B-2 (B-2S) cells (Fig. 1B). In contrast, expression of CD73 is much more restricted. We found that CD73 is expressed on approximately half of B-1 cells, but on few conventional B-2P or B-2S cells (Fig. 1C). Overall, B-1 cell expression of CD73 is 5-fold higher than expression of CD73 by peritoneal and splenic B-2 cells (Fig. 1D). The intensity of immunofluorescently stained CD73 in B-1 cells bearing low amounts of CD73 is still a little higher than isotype control staining (Fig. 1C), and there was no staining for CD73 above isotype control when B-1 cells from $CD73^{-/-}$ mice were examined (Fig. 1E), indicating that the minor shift of CD73 intensity in the CD73 low fraction is due to a very small degree of CD73 expression and is not due to nonspecific binding. On this basis, we subdivided B-1 cells into two populations that express high ($CD73^{hi}$ B-1 cells) and low ($CD73^{lo}$ B-1 cells) amounts of CD73. B-1 cells from male BALB/c mice show a pattern of CD73 and CD39 expression that is similar to B-1 cells from male C57BL/6 mice (Supplemental Fig. 1). We analyzed CD73 and CD39 expression on B cells from female BALB/c and C57BL/6 mice, and we did not find any difference between male and female cells (data not shown). These results indicate that higher expression of CD39, and bimodal expression of CD73 and CD39 by B-1 cells are not strain or gender specific.

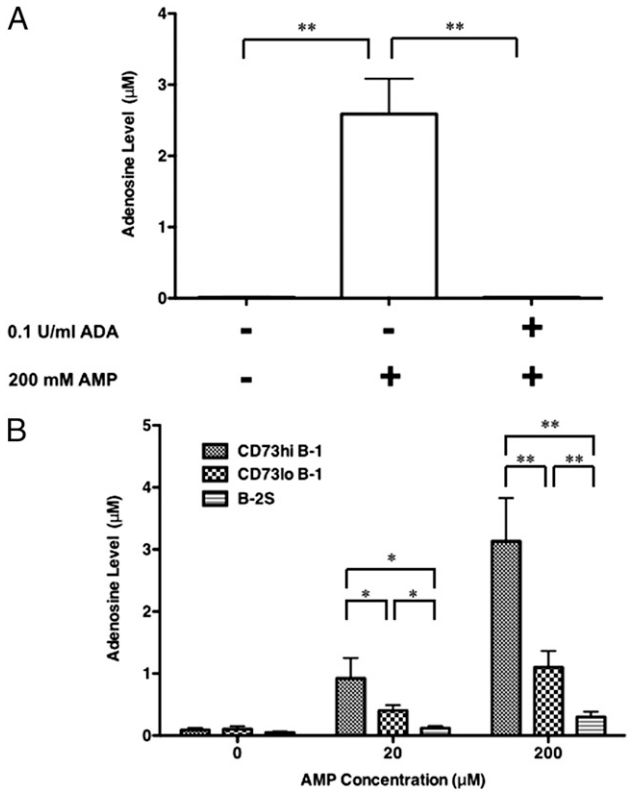


**FIGURE 3.** Adenosine is produced by $CD73^{hi}$ B-1 cells in the presence of 5′AMP in vitro. (**A**) Sort-purified total B-1 cells were cultured in serum-free X-VIVO medium with or without the indicated concentrations of 5′AMP for 2 h. Some cells were pretreated with 0.1 U/ml ADA. (**B**) Sort-purified $CD73^{hi}$ or $CD73^{lo}$ B-1 cells, or B-2S cells, were cultured in serum-free X-VIVO medium with or without the indicated concentrations of 5′AMP for 2 h. Supernatants were harvested, and adenosine levels were measured by CREB luciferase reporter assay using CHO-ADORA2B cells. Data shown are mean values ± SEM from (A) four and (B) eight independent experiments, respectively. $*p < 0.05$, $**p < 0.01$.

*CD73 expression is not an inducible marker*

We tested the possibility that CD73 expression on CD73$^{hi}$ B-1 cells may be transient, or that CD73 expression can be upregulated on CD73$^{lo}$ B-1 cells or CD73$^{-}$ B-2S cells. CD73$^{hi}$ and CD73$^{lo}$ B-1 cells and B-2S cells were sort-purified and then cultured with or without B cell mitogens for 72 h, after which B cells were restained with anti-CD73. We found that CD73 expression did not change in any of the B cell populations during culture without stimulation (Fig. 2A, 2B). Furthermore, there was almost no enhancement of CD73 expression from CD73$^{lo}$ B-1 cells or CD73$^{-}$ B-2S cells after mitogen stimulation with anti-Ig or anti-CD40 (Fig. 2A, 2B). Although CD73 expression was a little affected by anti-Ig or anti-CD40 stimulation, LPS stimulation downregulated CD73 expression (Fig. 2A, 2B). Next, sort-purified CD73$^{hi}$ and CD73$^{lo}$ B-1 cells were adoptively transferred into CB17 SCID. After 2 wk, peritoneal washout cells were collected, and expression of CD73 by B-1 cells was examined by immunofluorescent staining and flow cytometry. We found that CD73 expression stayed low when CD73$^{lo}$ B-1 cells were transferred (Fig. 2C, 2D), and most CD73 expression stayed high when CD73$^{hi}$ B-1 cells were transferred (Fig. 2C, 2E). However, we observed that CD73 expression on some CD73$^{hi}$ B-1 cells decreased, presumably because of environmental factors such as LPS derived from commensal bacteria, which downregulated CD73 expression in vitro (Fig. 2A, 2B). These results suggest that CD73 expression on CD73$^{hi}$ B-1 cells can be downregulated after activation and that CD73 expression on CD73$^{lo}$ B-1 cells or CD73$^{-}$ B-2 cells are not inducible.

*CD73$^{hi}$ and CD73$^{lo}$ B-1 cells are similar in many functional characteristics*

We characterized CD73$^{hi}$ and CD73$^{lo}$ B-1 cell populations further by examining several phenotypic and functional features. We found that CD73$^{hi}$ and CD73$^{lo}$ B-1 cells are similar in terms of cell size (FSC) and cell density (SSC), and of surface expression of IgM, IgD, Mac1, B220, PD-L2, and CD23 as detected by immunofluorescent staining and flow cytometry (Supplemental Fig. 2A). There was also no difference in phosphatidylcholine binding between CD73$^{hi}$ and CD73$^{lo}$ B-1 cells (Supplemental Fig. 2B), with the variance between PD-L2$^{+}$ and PD-L2$^{-}$ B-1 cells (45) providing a positive control (Supplemental Fig. 2C). There was no significant difference in CD73 expression levels between B-1a cells and B-1b cells (data not shown). We evaluated induction of IL-10 secretion by intracellular staining after stimulation by PMA or LPS and again found no difference between CD73$^{hi}$ and CD73$^{lo}$ B-1 cells (Supplemental Fig. 2D). We further examined proliferative responses by thymidine incorporation with a variety of mitogenic stimuli and found similar positive responses to PMA,

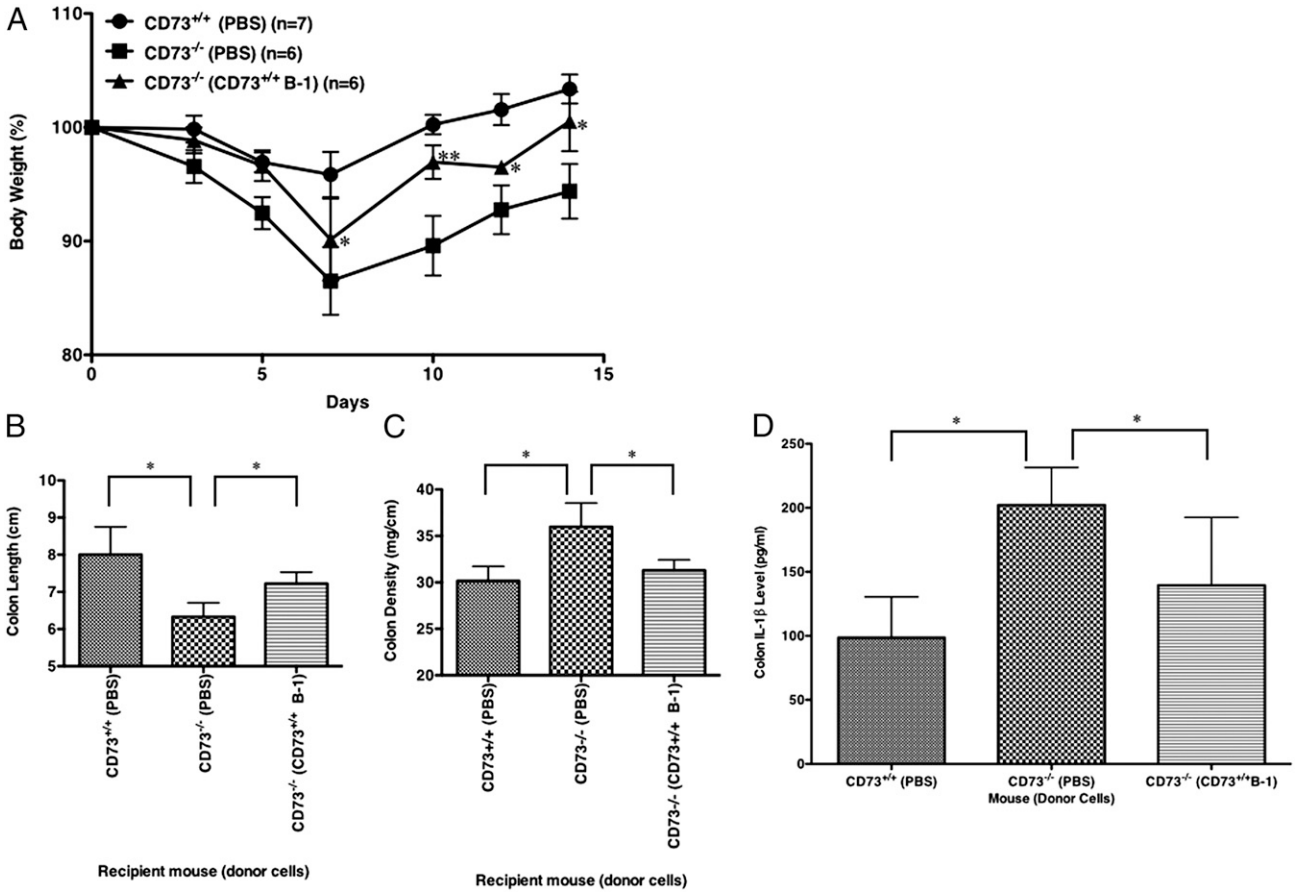


**FIGURE 4.** WT B-1 cells confer resistance to DSS-induced colitis in CD73$^{-/-}$ mice. B-1 cells ($1 \times 10^6$) from WT (CD73$^{+/+}$) mice were i.p. injected into CD73$^{-/-}$ mice or CD73$^{-/-}$ mice were given PBS i.p., 1 wk before DSS treatment began. Mice were administered 5% DSS in drinking water for 7 d, followed by normal drinking water. (**A**) Body weight was monitored and is shown as the percent of initial weight prior to DSS (mean ± SEM). Student *t* test was used to analyze the significance of the weight loss of CD73$^{-/-}$ mice that had received B-1 cells (▲) compared with CD73$^{-/-}$ mice that instead had received only PBS (■). (**B**) Colon length and weight were recorded 1 wk after the end of DSS administration. Colon length is shown. (**C**) Colon density was calculated as weight/length and is shown. (**D**) Proteins were extracted from colonic tissue, and IL-1β levels were quantified by ELISA. Data shown are mean values ± SEM from at least five mice for each group pooled from two independent experiments. *$p < 0.05$, **$p < 0.01$.

LPS, and anti-CD40, which are known B-1 cell mitogens (58) with little or no response to BCR crosslinking, as expected (Ref. 59 and data not shown).

We determined the capacity for B-1 cell Ig secretion by ELISA quantification of IgM in serum collected from CB17 SCID mice 2 wk after transfer of $CD73^{hi}$ or $CD73^{lo}$ B-1 cells (Fig. 2B). We found no difference in IgM levels between the two recipient groups (data not shown), indicating that $CD73^{hi}$ and $CD73^{lo}$ B-1 cells both spontaneously secrete natural IgM Ab and do so similarly. Collectively, these results indicate that B-1 cell expression of CD73 does not correlate with differences in numerous known phenotypic or functional B-1 cell characteristics.

### *$CD73^{hi}$ B-1 cells produce substantially more adenosine than $CD73^{lo}$ B-1 cells*

Next, we focused on CD73 enzyme activity. CD73 converts AMP to adenosine outside cells (37). $CD73^{hi}$ and $CD73^{lo}$ B-1 cells and B-2S cells were sort-purified and cultured with or without the indicated concentrations of AMP in serum-free medium for 2 h. Supernatants were harvested and tested for adenosine. To measure adenosine in supernatant fluids, we established a CREB luciferase reporter assay using CHO-ADORA2B cells (see *Materials and Methods*). This new method relies on induction of CREB activation by adenosine. We demonstrated that activation of the CREB luciferase reporter is highly sensitive because the assay reliably measures adenosine concentrations $>0.01$ μM (Supplemental Fig. 3A). We calculated the concentration of adenosine using a standard regression curve generated by diluting adenosine in culture medium. The firefly luciferase activity was completely blocked by pretreatment of ADA, indicating that this new adenosine detection method using CHO-ADORA2B cells and CREB luciferase reporter is highly specific to adenosine generation (Fig. 3A). We found that $CD73^{hi}$ B-1 cells produced substantially more adenosine in response to increasing doses of AMP than did $CD73^{lo}$ B-1 cells (Fig. 3B). In direct contrast, B-2S cells did not produce any adenosine at any dose of AMP (Fig. 3B). We confirmed these results with HPLC analysis (Supplemental Fig. 3B). Collectively, these findings indicate that CD73 on B-1 cells is a functional ectoenzyme and that higher levels of CD73 on $CD73^{hi}$ B-1 cells produce much more adenosine in response to extracellular AMP as compared with $CD73^{lo}$ B-1 cells and B-2S cells.

### *CD73 expressed by B-1 cells ameliorates DSS-induced acute colitis*

We evaluated the role of B-1 cells and B-1 cell CD73 in vivo. We examined DSS-induced colitis in mice because suppression of this condition has been shown in separate studies to be dependent on B cells (26, 27, 52) and to require adenosine produced through CD39 and CD73 enzyme activity (39–41). Consistent with the latter, $CD73^{-/-}$ mice are more susceptible to DSS-induced colitis than WT mice are (41), whereas B-1 cell numbers are normal. Bynoe et al. (41) reported that after DSS administration, $CD73^{-/-}$ mice lose more weight and exhibit colons characterized by shorter length, higher density, and more IL-1β, resulting from increased colonic inflammation, as compared with control mice, which we confirmed in preliminary experiments (Fig. 4A). We hypothesized that DSS-induced colitis would be suppressed by B-1 cells bearing CD73, which are capable of generating adenosine. To test this hypothesis, we adoptively transferred BALB/c B-1 cells to $CD73^{-/-}$ mice 7 d before administration of DSS. We found that DSS administration to $CD73^{-/-}$ mice that received B-1 cells resulted in less severe colitis in comparison with $CD73^{-/-}$ mice that instead received only PBS, as evidenced by reduced weight loss (Fig. 4A), increased colon length (Fig. 4B), and diminished colon density (Fig. 4C). Furthermore, B-1 cell transfer reduced IL-1β levels in colonic extracts of DSS-treated $CD73^{-/-}$ mice as compared with PBS-treated $CD73^{-/-}$ mice (Fig. 4D), although we did not find any significant differences in IL-10 and TNF-α levels in colonic extracts (data not shown). However, these parameters still reflected more inflammation than was present in DSS-treated WT ($CD73^{+/+}$) mice (Fig. 4). Regardless, these results strongly suggest that CD73 on B-1 cells is responsible for reducing the severity of experimental inflammatory colitis.

### *$IL\text{-}10^{-/-}$ B-1 cells produce less adenosine because of reduced expression of CD73*

Like B-1 cells, Tregs also express CD73 (35). Ring et al. (60) recently reported that Tregs from $IL\text{-}10^{-/-}$ mice fail to generate adenosine in the presence of ATP, suggesting an unexpected connection between IL-10 and adenosine production. We investigated the possibility that adenosine production by B-1 cells may be affected by IL-10 just like Tregs. We sort-purified B-1 cells from WT or $IL\text{-}10^{-/-}$ mice and cultured B-1 cells with or without the indicated concentrations of AMP for the indicated times, after which supernatants were harvested and analyzed for adenosine by

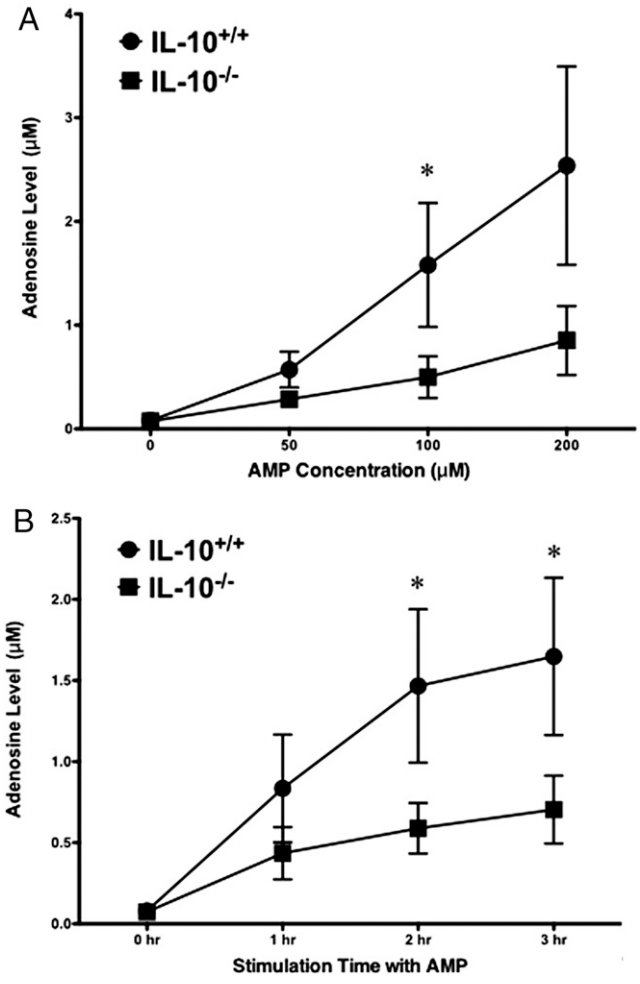


**FIGURE 5.** $IL\text{-}10^{-/-}$ B-1 cells produce less adenosine in the presence of AMP in vitro than WT B-1 cells did. Sort-purified B-1 cells from WT ($IL\text{-}10^{+/+}$) (●) or $IL\text{-}10^{-/-}$ mice (■) were (**A**) cultured in serum-free X-VIVO medium for 2 h with or without the indicated concentrations of AMP or (**B**) cultured in serum-free X-VIVO medium with 100 μM AMP for the indicated times. Adenosine levels in supernatants were measured by CREB luciferase reporter assay using CHO-ADORA2B cells. Data shown are mean values ± SEM from eight independent experiments. $*p < 0.05$.

CREB reporter assay. Intriguingly, we found adenosine generated by B-1 cells from IL-$10^{-/-}$ mice was reduced 50–70% compared with that from WT B-1 cells at any concentration of AMP or ATP substrate at any time point (Fig. 5 and data not shown). This difference was not due to altered cell viability, because B-1 cells from WT and IL-$10^{-/-}$ mice were 80–95% viable during the period of cell culture (data not shown). We next analyzed CD39 and CD73 surface expression on IL-$10^{-/-}$ B-1 cells. Surprisingly, we found that CD73 expression was much lower on IL-$10^{-/-}$ B-1 cells as compared with WT B-1 cells ($p < 0.01$; Fig. 6A–C), whereas CD39 expression trended slightly higher, although this difference was not statistically significant ($p = 0.06$; Fig. 6D, 6E). Taken together, these results indicate that IL-$10^{-/-}$ B-1 cells generate less adenosine because of reduced expression of CD73, as compared with WT B-1 cells. B10 cells are characterized by $CD5^{+}CD1d^{hi}$ B cells and IL-10 production after activation. We hypothesized that B10 cells express CD73 because $CD5^{+}CD1d^{hi}$ B cells include splenic B-1a cells. To test this idea, we analyzed CD73 expression on $CD5^{+}CD1d^{hi}$ B cells. As we expected, ~30% of splenic B10 cells expressed CD73 (Fig. 6F). Interestingly, CD73 expression was much lower on IL-$10^{-/-}$ B10 cells as compared with WT B10 cells (Fig. 6F). In contrast, as reported by Ring et al. (60), CD73 expression by IL-$10^{-/-}$ Tregs was comparable to that of IL-$10^{+/+}$ Tregs (Fig. 6G), suggesting that the reduction of CD73 expression on IL-$10^{-/-}$ mice is a defect specific to B cell populations. These data indicate that IL-10 plays a role in CD73 expression on B cell lineages (Fig. 7).

## Discussion

We have identified, to our knowledge for the first time, expression of the 5′-nucleotidase CD73 by B-1 cells and B10 cells, thereby distinguishing $CD73^{hi}$ and $CD73^{lo}$ subsets and marking a new way to divide this population. In contrast to B-1 cells and B10 cells, CD73 is not expressed by conventional B-2 cells. CD73 is an ectoenzyme that is capable of generating adenosine from AMP. We also showed that $CD73^{hi}$ but not $CD73^{lo}$ B-1 cells produce adenosine in vitro when substrate is provided, and that $CD73^{+}$ but not CD73− B-1 cells suppress inflammation associated with DSS-induced colitis in vivo. Together, these results describe a new mechanism for immunosuppression by a select subset of the small B-1 cell population, separate and apart from IL-10 secretion. $CD73^{hi}$ B-1 cells may thus be considered a novel type of Breg.

CD73 expression by $CD73^{hi}$ B-1 cells is stable and CD73 is not induced on $CD73^{-}$ B-1 cells by a variety of B cell mitogens, suggesting that CD73 is not an activation marker. Rather,

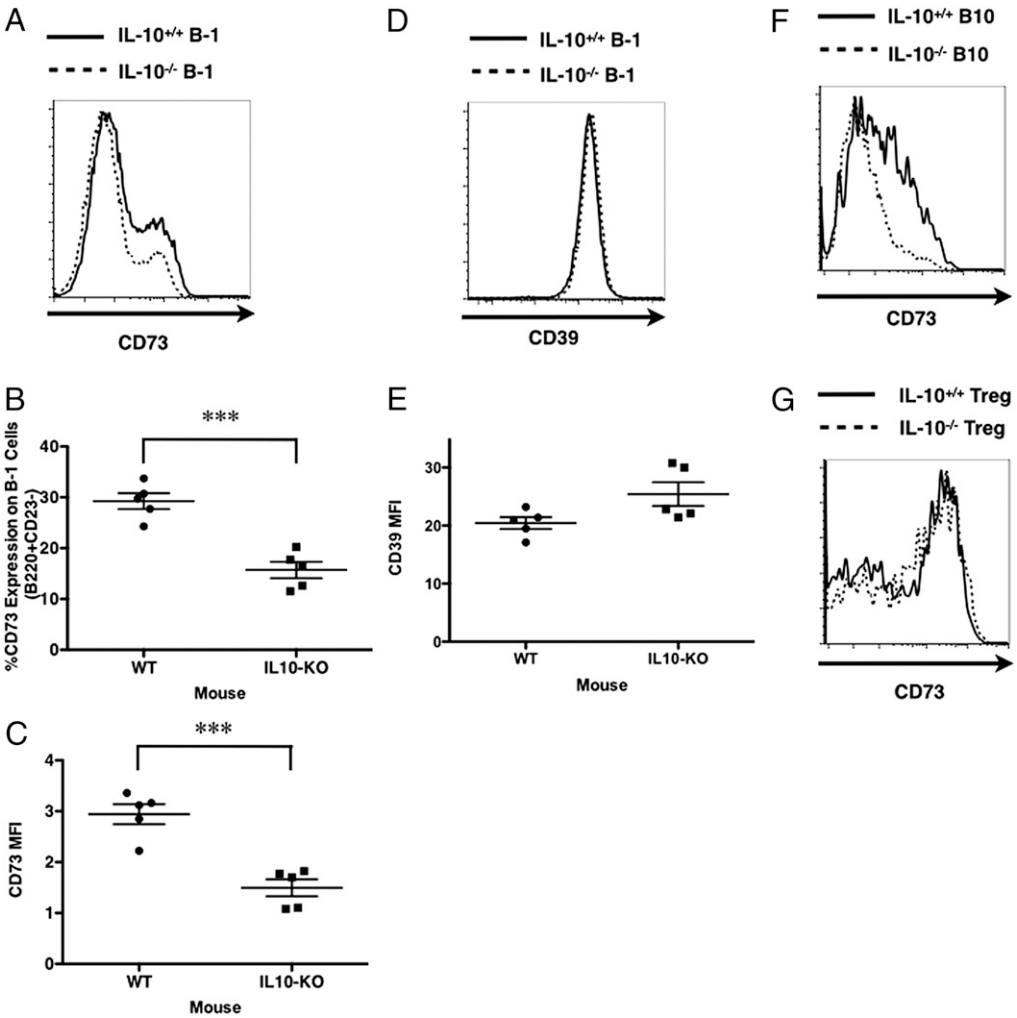


**FIGURE 6.** CD73 expression on B-1 cells and B10 cells is impaired in IL-$10^{-/-}$ mice. (**A**–**E**) Peritoneal cavity cells from WT (IL-$10^{+/+}$) or IL-$10^{-/-}$ mice were immunofluorescently stained to identify B-1 cells ($B220^{+}CD23^{lo}$) and to gauge expression of CD73 (A) and CD39 (D) by flow cytometry. The fraction of CD73-expressing B-1 cells (B) and mean fluorescence intensity (MFI) of CD73 (C) or CD39 (E) expression on B-1 cells were calculated with FlowJo. (**F** and **G**) Wild type (WT) splenocytes were immunofluorescently stained to identify B10 ($B220^{+}CD5^{+}CD1d^{hi}$) (F) and Tregs ($CD4^{+}CD25^{+}$) (G) and to gauge expression of CD73. Results are representative of three mice. ***$p < 0.001$.

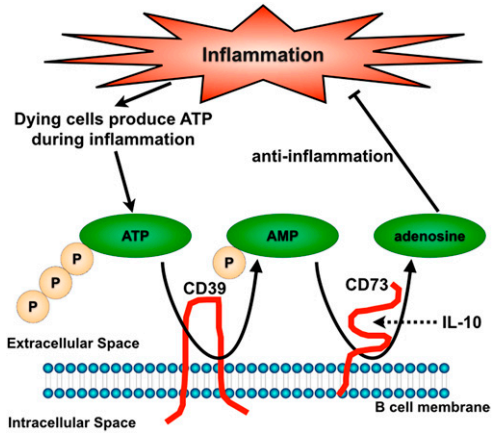


**FIGURE 7.** Model of B cell–mediated anti-inflammation thorough CD73/adenosine. In areas of ongoing inflammation, ATP is released from dead or dying cells. At such sites, ATP is converted to adenosine through CD73-expressing B cells. Extracellular adenosine can suppress ongoing inflammation to maintain homeostasis. Our results also indicate that IL-10 is a positive regulator of CD73 expression on B cells.

CD73 identifies a specific CD73$^{hi}$ subset that differs little from CD73$^{-}$ B-1 cells in many functional parameters with the notable exception of ectoenzyme activity. However, CD73 expression is not the only surface Ag that distinguishes B-1 cell subpopulations, as B-1 cell phenotypic heterogeneity extends to expression of PD-L2 (45), CD80 (61), CD25 (47), PC1 (48), and CD5 (42). Interestingly, Shlomchik's group recently reported that CD73, PD-L2, and CD80 are expressed by some Bmem cells, suggesting that Bmem cells are phenotypically heterogeneous (50, 51), much like B-1 cells. The similarity between B-1 cells and Bmem cells, with respect to expression of CD73, PD-L2, and CD80, may relate to developmental selection by Ag in both populations and suggests that some B-1 cells are Bmem cells or that some Bmem cells derive from B-1 cells. The existence of memory function within the B-1 cell pool has been delineated by recent work from Herzenberg et al. (62, 63) supporting the existence of memory B-1 (B-1mem) cells. Further study will be required to elucidate connections that might be present between these two populations.

CD73$^{hi}$ B-1 cells concurrently express the ATP-diphosphohydrolase, CD39, a ubiquitous surface Ag and ectoenzyme that converts ATP to ADP and AMP. Coordinated expression of CD39 and CD73 on B-1 cells is capable of generating adenosine from extracellular ATP as well as AMP, as we have shown (Figs. 3, 5, and data not shown). These latter molecules leak from dead and dying cells during an inflammatory response, and so with respect to CD73$^{hi}$ B-1 cells, inflammation may seed its own feedback inhibition (Fig. 7). ATP may also be actively released (64) or secreted by commensal bacteria (65), or both. Considering the peritoneal location of murine B-1 cells, adenosine generation could have a role under normal circumstances in circumventing potential immune responses against gut bacteria, as suggested by the ability of B-1 cells to ameliorate DSS-induced colitis in CD73-deficient animals (Fig. 4). The ectoenzyme–adenosine mechanism of immunosuppression is clearly distinct from IL-10–mediated immune inhibition, although the B-1 cell population participates in both. Although IL-10 has been considered a major inhibitory factor of B cell–mediated immune suppression, we suggest that CD73-mediated adenosine production by B cells could have a more important role than IL-10 in opposing inflammation in vivo for several reasons. First, the absolute number of IL-10–secreting cells is extremely limited in the steady state (8), whereas 30–50% of resting B-1 cells (Fig. 1) and B10 cells (Fig. 6) express CD73. Second, potent IL-10 secretion requires strong stimulation such as that provided by anti-CD40 + LPS + PMA + Ionomycin (8), whereas CD73-mediated adenosine production is constitutive so long as substrate is present (Fig. 5). In keeping with this, Teichmann et al. (31) recently reported that lineage-specific deletion of IL-10 from B cells does not alter disease progression in a mouse model of lupus, suggesting that another activity of B cells, such as B-1 expression of CD73, mediates suppression. Furthermore, CD73$^{hi}$ B-1 cells might be more potent in suppressing inflammation than Tregs, considering that transfer of Tregs did not ameliorate the severity of colitis in these mice (41).

Despite the distinction between CD73- and IL-10–mediated immune suppression, these two mechanisms appear to be linked. IL-10 deficiency affects both Tregs (60) and CD73$^{hi}$ B-1reg cells in ways that reduce adenosine generation and thus adenosine-mediated immunosuppression. In B-1 cells, this results from a straightforward reduction in CD73 expression (Fig. 6), whereas in Tregs, CD73 expression is not affected and the mechanism is less well defined. Regardless of mechanism, this interaction affects the interpretation of studies conducted with IL-10–deficient animals. Loss of immunosuppression in the absence of IL-10 has been invoked often to support an IL-10–dependent regulatory pathway in inflammatory diseases and disease models including colitis (26, 28), EAE (12, 20), collagen-induced arthritis (21–23), asthma (24), contact hypersensitivity (14, 25), type 1 diabetes (29), and SLE (30). However, our results and the results of others raise the possibility that the true mediator of immunosuppression is adenosine, the generation of which is impaired in by IL-10 deficiency, which now must be considered in studies that rely on IL-10$^{-/-}$ mice, tissues, or both.

The molecular mechanism by which CD73 expression is regulated in B-1 cells remains unknown. Recent results on Th17 cells differentiated in vitro indicate that expression of CD73 and CD39 is upregulated by IL-6–induced STAT3 activation and TGF-β–induced Gfi-1 downregulation, with both transcription factors binding to the ectoenzyme promoters; these result in production of an immunosuppressive Th17 effector cell (36). B-1 cells constitutively express activated, phosphorylated STAT3 (66), and this may predispose to constitutive CD73 expression, although it is not known whether B-1 cells that express CD73 are the same that express pSTAT3.

A role for adenosine in immune regulation by B cells is emphasized by the recent report that up to 90% of human circulating B cells coexpress CD73 and CD39 and generate adenosine (67). Although the particular B cell populations that express CD73 and CD39 have not been delineated, the situation in human B cells stands in contrast to mouse B cells, of which only a portion of the small B-1 cell population and some memory B cells coexpress CD73 and CD39. It is unclear at the present time whether a larger fraction of human B cells can actively participate in adenosine-mediated immunosuppression as compared with mouse B cells, or whether only a fraction of CD73-bearing human B cells is immunosuppressive, such as the recently identified human B-1 cell population that may express higher levels of CD73 than human conventional B cells do (68). Inasmuch as CD39-expession is not accompanied by coexpression of CD73 among most human circulating T cells (67), it may be that adenosine-mediated immunosuppression is a dominant mode of effector cell immune inhibition for B cells but not other cell types.

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## Disclosures

The authors have no financial conflicts of interest.

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