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Nonredundant Roles for B Cell-Derived IL-10 in Immune Counter-Regulation

Rajat Madan,* Filiz Demircik,§ Sangeetha Surianarayanan,¶ Jessica L. Allen,* Senad Divanovic,* Aurelien Trompette,* Nir Yogev,¶ Yuanyuan Gu,¶ Marat Khodoun,† David Hildeman,† Nicholas Boespflug,* Mariela B. Fogolin,¶ Lothar Gröbe,¶ Marina Greweling,¶ Fred D. Finkelman,†# Rhonda Cardin,‡ Markus Mohrs,** Werner Müller,** Ari Waisman,§ Axel Roers,¶ and Christopher L. Karp*†

IL-10 plays a central role in restraining the vigor of inflammatory responses, but the critical cellular sources of this counter-regulatory cytokine remain speculative in many disease models. Using a novel IL-10 transcriptional reporter mouse, we found an unexpected predominance of B cells (including plasma cells) among IL-10-expressing cells in peripheral lymphoid tissues at baseline and during diverse models of in vivo immunological challenge. Use of a novel B cell-specific IL-10 knockout mouse revealed that B cell-derived IL-10 nonredundantly decreases virus-specific CD8+ T cell responses and plasma cell expansion during murine cytomegalovirus infection and modestly restrains immune activation after challenge with foreign Abs to IgD. In contrast, no role for B cell-derived IL-10 was evident during endotoxia; however, although B cells dominated lymphoid tissue IL-10 production in this model, myeloid cells were dominant in blood and liver. These data suggest that B cells are an underappreciated source of counter-regulatory IL-10 production in lymphoid tissues, provide a clear rationale for testing the biological role of B cell-derived IL-10 in infectious and inflammatory disease, and underscore the utility of cell type-specific knockouts for mechanistic liming of immune counter-regulation. The Journal of Immunology, 2009, 183: 2312–2320.

Although the immune system is essential for host protection against pathogens, all immune responses themselves have potential for harming the responding host. It is thus not surprising that immune responses are tightly regulated in time, space, and character. Despite this, dysregulated inflammation is central to the pathogenesis and expression of a broad range of diseases, both infectious and noninfectious. Although immunologists have traditionally focused on the mechanisms underlying immune response activation and class specification, control of immune response amplitude and resolution is just as important. Numerous, often overlapping and redundant mechanisms of immune counter-regulation have been identified in recent years, including counter-regulatory proteins and lipids, specialized regulatory cell types, inhibitory receptor/ligand pairs, and modulators of intracellular signaling pathways. Among these diverse mechanisms, IL-10 appears to play a uniquely important role.

Originally identified as a product of Th2-polarized CD4+ T cells that inhibited Th1 cell cytokine production (“cytokine synthesis inhibitory factor”), IL-10 has subsequently been shown to be produced by a plethora of adaptive and innate immune cell types and to have pleiotropic, largely counter-regulatory activities across the immune system (1). Murine studies have underscored the central role played by IL-10 in restraining inflammatory responses, both local and systemic, innate and adaptive, and polarized along diverse axes of response such as Th1, Th2, or Th17 (1–3). During some experimental infections (e.g., toxoplasmosis), IL-10-mediated down-modulation of inflammatory responses is essential to prevent the development of lethal immunopathology (4). In other experimental infections (e.g., cutaneous leishmaniasis and infection with lymphoplastic choriomeningitis virus), the counter-regulatory activities of IL-10 are required to induce latency or chronicity (5–7). IL-10 also restrains the expression of autoimmune and allergic disease (1) and is required to prevent mice from developing pathological gut inflammation in response to endogenous gut flora (8, 9).

Cells of the innate immune system (including monocytes, macrophages, dendritic cells, neutrophils, eosinophils, and innate lymphocyte populations), cells of the adaptive immune system (including CD4+ T cells, CD8+ T cells, gut intraepithelial T cells, and B cells), and even nonimmune cells (e.g., keratinocytes and hepatocytes) can all express IL-10 (10, 11–14). This profusion of IL-10-producing cell types, along with difficulties in detecting IL-10 via intracellular staining without relatively nonphysiological stimulation, has made it difficult to disentangle the roles played by specific IL-10-expressing cell types in regulating immune responses. The focus has largely been on CD4+ T cells in infectious models, where experimental attention has

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*Division of Molecular Immunology, †Division of Immunobiology, ‡Division of Infectious Diseases, Cincinnati Children’s Hospital Research Foundation and the University of Cincinnati College of Medicine, Cincinnati, Ohio 45229; †First Medical Department, University of Mainz, Mainz, Germany; ‡Division of Dermatology, University of Cologne, Cologne, Germany; ††Division of Experimental Immunology, Helmholtz Centre for Infection Research, Braunschweig, Germany; ‡‡Cincinnati Veteran’s Affairs Medical Center, Cincinnati, OH 45220; **Trudeau Institute, Saranac Lake, New York 12983; and ¶Faculty of Life Sciences, University of Manchester, Manchester, United Kingdom

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2 Current address: Cell Biology Unit, Institute Pasteur de Montevideo, Uruguay.
3 Address correspondence and reprint requests to Dr. Christopher Karp, Division of Molecular Immunology, Cincinnati Children’s Hospital Medical Center, 3333 Burnet Avenue, Cincinnati OH 45229. E-mail address: chris.karp@chmcc.org

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been paid to the regulatory role of IL-10 production by CD4+ T cell subpopulations (2).

To better define IL-10 production in vivo, we generated an IL-10 transcriptional reporter mouse. This reporter revealed an unexpected dominance of B cells among IL-10-expressing cells in lymphoid tissues of naive mice and during in vivo immunological challenge. Of particular interest, a significant fraction of plasma cells and plasmablasts expressed IL-10 in both naive and challenged mice. Use of a novel B cell-specific IL-10 knockout mouse revealed biologically important, nonredundant roles for B cell-derived IL-10 in immune counter-regulation during infection with murine cytomegalovirus (MCMV), including restraint of the amplitude of virus-specific CD8+ T cell responses and down-modulation of plasma cell expansion. B cell-derived IL-10 also modestly restrained immune activation after challenge with foreign Abs to IgD. B cell-derived IL-10 production was not required to restrain LPS-induced immune activation. However, despite B cell dominance in lymphoid organs, myeloid cells were the dominant IL-10-expressing cells in blood and liver after LPS challenge. These data suggest that B cells are an underappreciated source of counter-regulatory IL-10 production in lymphoid tissues and underscore the utility of cell type-specific knockouts for mechanistic analysis of immune counter-regulation.

Materials and Methods

IL-10 transcriptional reporter (Vert-X) mice

A 20-kb genomic fragment containing Ili0 along with flanking sequences was isolated via recombining from BAC clone RP23-1222PS (BACPAC Resource Center, Children’s Hospital, Oakland, CA). A 6.8 kb EcoRI fragment containing the fifth exon, the endogenous poly(A), and the 3′ untranslated region of Ili0 was subsequently subcloned into pBluescript II KS (Stratagene). A floxed neomycin-internal ribosomal entry site (IRES)-enhanced GFP (eGFP) (15) was cloned into the HindIII site between the endogenous stop and poly(A) sites of Ili0, followed by subcloning of an HSV-TK cassette (where TK is thymidine kinase) into the Sall site of pBluescript II KS. After linearizing with NorI, the targeting vector was electroporated into a C57BL/6 embryonic stem cell line, and embryonic stem cell clones were selected with G418 and ganciclovir. Correctly targeted clones, screened initially by PCR followed by Southern blot confirmation (17), were injected into C57BL/6 albino blastocysts implanted into C57BL/6 females. Male chimeric mice were bred to C57BL/6 mice (The Jackson Laboratory), and correctly targeted heterozygous mice were interbred to generate homozygous Vert-X (where Vert is the French word for green and X is the roman numeral for 10) mice. Genotyping of Vert-X mice was performed by PCR using the following oligonucleotides: Ili0 5′-AGGCTGTTCTAACAAAGCCATGAATGAATT-3′; GFP 5′-GGAGAGGAATTTGCAATCGATTGCTGAGATT-3′; and Ili0 3′-5′-CAGAGGCAGACAAACATACACCATTCCCA-3′.

B cell-specific, IL-10-deficient mice

Ili0floxed mice, with loxp sites that flank exon 1 of Ili0 (18, 19), were bred with CD19-Cre mice (20) to generate mice with B cell-specific inactivation of Ili0. Southern analysis of cell-type specificity and efficiency of Cre-mediated deletion was performed as described (18). T and B cells were immunostained with anti-F4/80 (Caltag Laboratories). Genotyping was performed by Southern blot analysis as described earlier (8, 18) or by PCR using the following oligonucleotides: IL10-5′-CCACCATAGAGGACTGCTGCTGCTCAG-3′ and 5′-GCCATGCGCCCTTATTGCCATG-3′. All mice were on the C57BL/6 background. Mice were housed under standard specific pathogen-free conditions. All animal experimentation was done in accordance with institutional guidelines.

In vivo challenge reagents

TLR4-specific Escherichia coli (K12) LPS was from InvivoGen; E. coli (055:B5) and Salmonella typhimurium LPS was from Sigma-Aldrich. CpG DNA (oligodeoxynucleotide 1826) was from Coley Pharmaceuticals or Metabion. Goat polyclonal anti-eGFP was generated as described (21). Parent virus stock of MCMV strain K181 was provided by E. Mo-carski, Stanford University School of Medicine, Stanford, CA) and MCMV strain SMV-1939 (provided by M. J. Reddehase (Johannes Gutenberg University, Mainz, Germany)) were prepared in NIH 3T3 cells (22). All virus stocks were stored at −70°C.

Flow cytometry

Single cell leukocyte suspensions were generated from mouse lymphoid organs as described (23). Peritoneal cells were harvested by lavage as described (24). Liver leukocytes were isolated as described (25), with minor modifications. Briefly, mouse livers were perfused with PBS via the hepatic portal vein, followed by removal of the gall bladder. Livers were mechanically dissociated with scissors, incubated with collagenase IV (Roche), and passed through a 100-μm nylon cell strainer (BD Falcon). After the nonleukocyte fraction was pelleted by low-speed centrifugation, the leukocyte fraction was pelleted and subjected to Percoll gradient purification. Erythrocyte lysis was performed with ACK (ammonium chloride/potassium) lysis buffer (BioWhittaker). Salivary glands devoid of adjacent lymph nodes were excised, homogenized, and incubated with collagenase IV (Roche). Digested samples were passed through a 40-μm nylon cell strainer (BD Falcon) and washed with PBS. Blood leukocytes were isolated from the inferior vena cava and transferred into EDTA-containing capillary blood collection Microtubes (Sarstedt). Pelleted cells were stained, followed by red cell lysis as described above.

To blockade of Fc receptors with CD16/32 blocking Ab (eBio-science), leukocytes were stained using mAbs to the following cell surface markers: CD19-PE, CD19-PE-Cy7, CD1d-PE, B220-allophycocyanin-Alexa Fluor 750, IgM-PE-Cy7, CD11b-Pacific Blue, CD11c-allophycocyanin, CD1c-Alexa Fluor 700, TCR-β-allophycocyanin, CD4-PE-Cy7, CD14-allophycocyanin-Alexa Fluor 750, CD5-PerCP-Cy5.5, CD8-Pacific Blue, CD25-PE, and CD16/32 blocking Ab (all from eBioScience); CD8-PE-Cy7, CD138-allophycocyanin, CD11b-PerCP-Cy5.5, IgM-PE, and CD19- FITC, (all from BD Pharmingen); as well as isotype controls (eBioScience and BD Pharmingen). For intracellular staining, cells were stained for cell surface markers, treated with BD Cytofix/Cytoperm and BD Perm/Wash buffer, and then stained with IFN-γ-PE or IFN-γ-FITC (BD Pharmingen and eBioScience, respectively). Data were acquired using LSR II, FACS Canto, and FACSScan (Becton Dickinson) flow cytometers and analyzed using CellQuest and FloJo software. Splenic leukocyte subpopulations were purified using a FACS Vantage SE FACS sorter (Becton Dickinson).

Peptide restimulation

Splenic leukocytes were restimulated with MCMV-specific peptides (M45, HIGIRNASFI, M141, VIDAFAAF, M38, SSPPMRV; M78, VDYSSYP; M57, SCEIFQWQR; M139, TYYGFCLL; IE3, RALEYKNI; M36, GTGVLNTSV, 1 μM; IPT Peptide Technologies) (26) for 6 h in the presence of brefeldin A (Applied Biosystems).

ELISPOT and ELISA

FACS-sorted splenic leukocytes were plated in 96-well polystyrene di-fluoride membrane ELISPOT plates (Millipore) and analyzed for IL-10 protein expression using a mouse IL-10 ELISPOT kit (eBioScience). Spots were quantified on an ELISPOT 3B analyzer (Cellular Technology). Goat IgG-specific Abs and systemic IL-10 were quantified from the harvested serum by ELISA as described previously (27, 28).

Quantitative RT-PCR

mRNA expression was quantified by quantitative RT-PCR, using a Light-Cycler system (Roche) as described (23) with the following primer sets: IL-10-5′-GAAGCATGGCCCAGAAATCA-3′ and 5′-TGCTCCACTGC-3′; CTTGCTCTTT-3′ and 5′-GCCATGCGCCCTTATTGCCATG-3′. All mRNA expression was normalized to β-actin (5′-GGGCCAGAAGCCAGAGGTATG-3′ and 5′-CATTGAGGAGTTGAAGGAGGTAG-3′).

Results

Generation of an IL-10 transcriptional reporter mouse

To track IL-10-producing cells in vivo, a floxed neomycin-IRES-eGFP cassette (15) was inserted between the endogenous stop site and the poly(A) site of Ili0 (Fig. 1a). The neomycin resistance
Fidelity of reporter expression in Vert-X mice

To address the fidelity of reporter expression, splenic leukocytes from Vert-X mice were stimulated in vitro with CpG DNA. Eighteen hours after stimulation, CD19\(^+\) GFP\(^+\) and CD19\(^+\) GFP\(^-\) cells were FACS sorted and mRNA expression (GFP, IL-10) and protein secretion (IL-10) were quantified by RT-PCR and ELISPOT, respectively. IL-10 mRNA expression also segregated with GFP mRNA and protein secretion (IL-10) were quantified by RT-PCR and ELISPOT, respectively. As shown in Fig. 2, a and b, IL-10 mRNA expression and protein secretion segregated with GFP mRNA and protein expression, respectively; IL-10 mRNA expression also segregated with GFP protein expression. Similar fidelity of reporter expression was seen in CD11c\(^+\) cells (data not shown).

To address whether IL-10 is transcribed from both alleles, splenic leukocytes from Vert-X heterozygous and homozygous mice (along with wild-type controls to control for baseline autofluorescence) were stimulated in vitro with CpG DNA. Twenty-four, 48, and 72 h after stimulation, GFP expression was quantified by flow cytometry in CD19\(^+\) B220\(^+\) B cells. Although the percentage of GFP\(^+\) B cells was similar in heterozygotes and homozygotes (Fig. 2c), the mean fluorescence intensity (MFI) of GFP expression in B cells from heterozygous mice was about half that of homozygous mice (Fig. 2d). Similar results were seen following challenge with LPS and Pam3Cys (data not shown). These data suggest that IL-10/reporter expression is biallelic, as previously reported (29).

**Splen ic IL-10 reporter expression in naive mice**

Approximately 1.3% of splenic leukocytes were found to express GFP in naive or PBS-stimulated mice. Flow cytometric analysis revealed that the majority of splenic leukocytes exhibiting baseline GFP expression were B cells (CD19\(^+\) B220\(^+\) and CD19\(^+\) B220\(^{low/–}\) cells (presumptive plasmablasts; see Ref. 30) and CD138\(^+\) plasma cells) and CD4\(^+\) T cells (both CD25\(^+\) and CD25\(^–\)) (Fig. 3). When analyzed differently, by defining the percentage of cells in a given splenic leukocyte subpopulation that were GFP\(^+\) (as opposed to the percentage of GFP\(^-\) cells accounted for by a given splenic leukocyte subpopulation), the highest proportion of baseline GFP expression was exhibited by CD19\(^+\) B220\(^{low/–}\) cells, at least one-third of which exhibited baseline GFP expression (Table I). Indeed, a right shift in the fluorocytogram of CD19\(^+\) B220\(^{low/–}\) cells from Vert-X mice, compared with that from wild-type mice (data not shown), suggests that all such cells may express IFN, at least to some extent. Splenic marginal zone (CD19\(^+\) B220\(^+\) CD21\(^{high}\) CD23\(^{low}\)) and follicular (CD19\(^+\) B220\(^+\) CD21\(^{int}\) CD23\(^{+}\)) B cells also exhibited baseline GFP expression, albeit at lower percentages (6.2 ± 0.3 and 0.5 ± 0.06%, respectively).

![FIGURE 1. Generation of Vert-X mice: targeting of an IRES-eGFP reporter into the il10 locus. a, Maps of the il10 locus (top), the targeting construct (middle), and the mutated il10 locus (bottom). A genomic 6.8-kb EcoRI fragment of the wild-type C57BL/6 locus (top), isolated by recombineering techniques from a BAC clone, was mutat ed by the following: 1) the introduction of a loxP-flanked (filled triangles) neomycin cassette (Neo), an IRES element, eGFP, and a polyadenylation signal (pA), all cloned into a HindIII site between the fifth exon (5) and the endogenous pA of il10; and 2) the 5' addition of an HSV-derived thymidine kinase (HSV-TK) cassette. After verification (by PCR and Southern blotting) of successful targeting into C57BL/6 embryonic stem cells, positive clones were injected into blastocysts of C57BL/6 albino females. The floxed Neo was deleted by Cre-mediated recombination using C57BL/6 Zp3 Cre deleter mice (The Jackson Laboratory), generating the targeted alleleil10. Southern blot analysis of DNA from tail biopsies of the progeny of heterozygous breeding. The novel IRES site introduced by the targeting construct results in a 3-kb fragment in the correctly targeted allele in place of the 10-kb fragment of the wild-type allele. +, Wild type; Vert-X, targeted allele.](http://www.jimmunol.org/content/172/6/2314/F1)

marker was excised by breeding with Zp3-Cre mice. Successful Cre-deletion was confirmed by Southern blot analysis (Fig. 1b). All steps, from the BAC clone source of genomic DNA through Cre-mediated deletion were done with C57BL/6 mice to obviate the need for backcrossing. Homozygous targeted mice, designated Vert-X, developed normally and were viable, fertile, and without obvious phenotype; in particular, such mice lacked evidence of inflammatory bowel disease.
Vert-X mice were stimulated as described above. At the indicated time points cells were harvested, stained with Abs to CD19 and B220, and analyzed by flow cytometry. Splenic leukocytes from wild-type C57BL/6 mice, treated identically, were used to correct for baseline autofluorescence in CD19+ cells. Thus, B cells are the dominant splenic population of IL-10 expressing leukocytes after systemic LPS challenge. Among B cell subpopulations, the fraction of cells expressing GFP was highest in marginal zone and follicular B cells. Although marginal zone and follicular B cells are not distinguishable by surface marker expression after stimulation, cells were immunostained for CD19 and sorted by flow cytometry into CD19+ populations. IL-10 and eGFP mRNA expression was quantified by real-time RT-PCR in purified subsets and normalized for levels of β-actin mRNA expression. Data represent means ± SE from a single mouse (representative of an experiment where n = 2). b, Splenic cells from Vert-X mice were stimulated and sorted as described above, followed by ELISPOT analysis of IL-10-secreting cell numbers in purified subsets. Data represent means (± SEM) of triplicate samples from a single mouse, representative of an experiment where n = 5. Similar data were obtained with CD11c+ cells (data not shown). c and d, Splenic leukocytes from heterozygous and homozygous Vert-X mice were stimulated as described above. At the indicated time points cells were harvested, stained with Abs to CD19 and B220, and analyzed by flow cytometry. Splenic leukocytes from wild-type C57BL/6 mice, treated identically, were used to correct for baseline autofluorescence. c, Percentage of CD19+ B220− cells that were GFP+. d, MFI of GFP+ CD19+ B220− cells. Data represent means (± SEM) of three different mice and are representative of data from two independent experiments.

**Figure 2.** Fidelity and biallelic expression of the targeted locus. a, Splenic leukocytes from age- and sex-matched Vert-X mice were stimulated in vitro with 2 μM CpG (oligodeoxynucleotide 1826). Eighteen hours after stimulation, cells were immunostained for CD19 and sorted by flow cytometry into CD19+ GFP+ and CD19+ GFP− populations. IL-10 and eGFP mRNA expression was quantified by real-time RT-PCR in purified subsets and normalized for levels of β-actin mRNA expression. Data represent means ± SE from a single mouse (representative of an experiment where n = 2). b, Splenic cells from Vert-X mice were stimulated and sorted as described above, followed by ELISPOT analysis of IL-10-secreting cell numbers in purified subsets. Data represent means (± SEM) of triplicate samples from a single mouse, representative of an experiment where n = 5. Similar data were obtained with CD11c+ cells (data not shown). c and d, Splenic leukocytes from heterozygous and homozygous Vert-X mice were stimulated as described above. At the indicated time points cells were harvested, stained with Abs to CD19 and B220, and analyzed by flow cytometry. Splenic leukocytes from wild-type C57BL/6 mice, treated identically, were used to correct for baseline autofluorescence. c, Percentage of CD19+ B220− cells that were GFP+. d, MFI of GFP+ CD19+ B220− cells. Data represent means (± SEM) of three different mice and are representative of data from two independent experiments.

**Table 1.** Gfp expression by splenic leukocytes at baseline and after in vivo stimulation with LPS.

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Vert-X (naive)</th>
<th>Vert-X LPS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total leukocytes</td>
<td>1.3 ± 0.1</td>
<td>10.4 ± 0.6***</td>
</tr>
<tr>
<td>CD19+ B220−</td>
<td>1.1 ± 0.1</td>
<td>14.1 ± 0.8***</td>
</tr>
<tr>
<td>CD19+ B220low−</td>
<td>35.4 ± 3.4</td>
<td>56.7 ± 5.0*</td>
</tr>
<tr>
<td>CD19+ CD138+</td>
<td>7.1 ± 1.2</td>
<td>64.6 ± 3.5***</td>
</tr>
<tr>
<td>TCR− CD4+ CD25+</td>
<td>4.7 ± 0.2</td>
<td>5.6 ± 0.3*</td>
</tr>
<tr>
<td>TCR+ CD4+ CD25−</td>
<td>1.2 ± 0.1</td>
<td>1.9 ± 0.2*</td>
</tr>
<tr>
<td>TCR− CD8+</td>
<td>0.4 ± 0.06</td>
<td>3.5 ± 0.3***</td>
</tr>
<tr>
<td>CD11b+</td>
<td>0.5 ± 0.1</td>
<td>7.5 ± 1.2***</td>
</tr>
<tr>
<td>CD11b+ GR1+</td>
<td>0.6 ± 0.2</td>
<td>1.2 ± 0.4 (NS)</td>
</tr>
<tr>
<td>CD11chigh</td>
<td>0.7 ± 0.2</td>
<td>2.2 ± 0.4**</td>
</tr>
<tr>
<td>NK1.1+ TCR+</td>
<td>0.4 ± 0.1</td>
<td>1.2 ± 0.5 (NS)</td>
</tr>
<tr>
<td>NK1.1+ TCR−</td>
<td>0.7 ± 0.1</td>
<td>3.9 ± 0.2***</td>
</tr>
</tbody>
</table>

*Age- and sex-matched wild-type and Vert-X mice were challenged i.v. with TLR4-specific LPS (E. coli K12; 20 μg) or left unchallenged. Forty-eight hours after stimulation, splenic leukocytes were analyzed by flow cytometry. Data represent the mean percentage (± SE) of each cell type that were GFP+ (8 mice/group). *p < 0.05; **p < 0.001; ***p < 0.0001 (Student’s t test). Results similar to those in naive mice were seen after in vivo challenge with PBS (data not shown).
challenge; whereas 5.7 (± 0.8) × 10^6 cells were CD19^+ GFP^+ 48 h after i.v. challenge with LPS, only 3.1 (± 0.67) × 10^6 of such cells were CD1d^{hi}CD5^-.

B cells were similarly dominant IL-10 producers in the peritoneal cavity, where 5.1 ± 0.3% of all leukocytes expressed GFP after i.v. LPS challenge. Flow cytometric analysis revealed that the majority of these cells (77.8 ± 4.3%) were CD19^+CD5^- B1a cells, with CD19^+CD5^- B1b and B2 cells accounting for another 6.2 ± 0.9%. Notably, however, similar percentages of total peritoneal leukocytes and peritoneal B1a cells expressed GFP in naive mice, suggesting that i.v. LPS challenge fails to induce IL-10 expression over baseline levels in peritoneal cells (data not shown).

Such B lymphocyte predominance of IL-10 competence after LPS stimulation was not the case in blood or liver leukocytes, however; 3.5 ± 0.6% of blood and 4.3 ± 0.3% of liver leukocytes were GFP^+ 24 h after systemic LPS challenge (compared with ~0% at baseline). Under such conditions, B cells accounted for <4% of the GFP^+ cells in blood and liver. Myeloid (CD11b^-) cells were the dominant IL-10-expressing population in blood after LPS challenge (accounting for 87.3 ± 0.6% of GFP^+ cells); myeloid (CD11b^-) cells (49.1 ± 2.7%) and NK (NK1.1^-TCR^-) cells (28.5 ± 3.5%) dominated in the liver.

In diverse other models of immunological challenge in which IL-10 has been shown to play an important counter-regulatory role, including systemic challenge with another TLR ligand (CpG DNA) and goat anti-mouse IgD Ab (36) and s.c. infection with MCMV, B cells also represented the dominant population of IL-10-expressing leukocytes in the spleen (Table II). A similar dominance of B cell-derived reporter expression was seen in draining lymph node following s.c. infection with MCMV Smith strain (s.c.) or mock-challenge with endotoxin-free PBS. Data depicted represent the mean percentage (± SEM) of total GFP^+ cells represented by CD138^+ splenic plasmacytes (PBS (left side), LPS, and anti-IgD) or lymph node (PBS (right side) and MCMV) leukocytes in 5–10 mice. In the case of challenge with MCMV strain, CD138^+ cells represented 57.3 ± 2.6% of total GFP^+ cells in draining lymph nodes (not shown), *p < 0.005 (Student’s t test).

We have previously shown that intestinal inflammation is a sensitive indicator of inflammatory bowel disease in IL-10 mutant mice (18). As previously reported, IL-10^-/- and IL10flox/floxCD4^+Cre mice exhibited rectal prolapse of variable penetrance by the age of 6 mo (18). In contrast, 0 of 68 IL10flox/floxCD19-Cre mice aged 6 mo or older developed prolapse. The absence of intestinal inflammation was confirmed by histological analysis of H&E-stained

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Table II. Cell dominance of IL-10/GFP^+ expression by splenic leukocytes after diverse in vivo stimuli

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>LPS</th>
<th>CpG</th>
<th>Goat Anti-IgD</th>
<th>MCMV</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD19^-</td>
<td>89.7 ± 0.5</td>
<td>84.1 ± 1.1</td>
<td>83.9 ± 0.9</td>
<td>59.4 ± 2.7</td>
</tr>
<tr>
<td>TCR^-</td>
<td>5.0 ± 0.4</td>
<td>7.3 ± 0.7</td>
<td>10.8 ± 1.3</td>
<td>22.2 ± 2.2</td>
</tr>
<tr>
<td>CD11c^{hi}</td>
<td>0.2 ± 0.02</td>
<td>0.3 ± 0.03</td>
<td>0.8 ± 0.2</td>
<td>2.8 ± 0.2</td>
</tr>
<tr>
<td>CD11b^-</td>
<td>3.8 ± 0.5</td>
<td>3.6 ± 0.3</td>
<td>1.1 ± 0.1</td>
<td>5.4 ± 0.2</td>
</tr>
<tr>
<td>NK1.1^-</td>
<td>1.7 ± 0.1</td>
<td>1.8 ± 0.2</td>
<td>0.8 ± 0.1</td>
<td>0.6 ± 0.1</td>
</tr>
</tbody>
</table>

Vert-X mice were challenged with LPS (E. coli K12; 20 μg i.v.; harvested at 48 h), CpG (oligodeoxynucleotide 1826; 50 μg i.v.; 24 h), goat anti-mouse IgD (400 μl i.p.; 6 days), or MCMV Smith strain (1 × 10^5 PFU s.c. in the foot pad; 7 days). Splenic leukocytes were analyzed by flow cytometry. The data shown depict the percentage (± SEM) of the total number of GFP^+ cells accounted for by cells expressing the indicated surface markers. Please note: because cells can express more than one of these markers, the total does not always add up to 100%. Data depicted represent the mean percentages of GFP^+ cell types in eight (LPS), three (CpG), seven (anti-IgD), and five (MCMV) mice, respectively.
paraffin sections of small intestine and colon from Il10flox/Il10flox CD19-Cre mice (data not shown). Thus, B cell-specific IL-10 production does not appear to have a critical, nonredundant function in protection from pathological inflammation of the gut.

**B cell-derived IL-10 restrains Ag-specific CD8<sup>+</sup> T cell responses and plasma cell numbers during acute MCMV infection**

IL-10 protects against host morbidity during experimental MCMV infection; IL-10-deficient mice exhibit increased disease in the face of more efficient control of virus replication (37). IL-10 also facilitates the establishment of chronic infection in salivary glands, restraining CD4<sup>+</sup> T cell-mediated viral clearance and facilitating horizontal transmission (38, 39). Quantification of Ag-specific CD8<sup>+</sup> T cell responses by flow cytometric analysis of MCMV peptide-driven IFN-γ production revealed a significant increase in the percentage of MCMV-specific CD8<sup>+</sup> cells in Il10flox/flox CD19-Cre mice, compared with wild-type mice (Fig. 6a) 7 days after infection. Thus, B cell-derived IL-10 plays a nonredundant role in restraining the acute MCMV-specific CD8<sup>+</sup> T cell response. B cell-derived IL-10 also decreased plasma cell numbers during acute MCMV infection (Fig. 6b).

**B cell-derived IL-10 plays a modest counter-regulatory role after challenge with foreign Abs to IgD**

Injection of mice with a foreign Ab to mouse IgD activates a B cell-dependent, Th2-polarized immune response against antigenic determinants derived from the foreign Ab (36). B cells activated by membrane IgD cross-linking facilitate subsequent CD4<sup>+</sup> T cell activation by processing and presentation of the anti-IgD Ab. In turn, activated CD4<sup>+</sup> T cells secrete cytokines and provide contact-dependent help that leads to further B cell proliferation and differentiation (36). Endogenously produced IL-10 regulates this model, as shown by the dramatic increase in nearly all splenic leukocyte populations in anti-IgD-inoculated IL-10 knockout mice, compared with wild-type mice (Fig. 7, a–d). Il10flox/flox CD19-Cre mice produced significantly less IL-10 in this model (Fig. 7e). Despite the dramatic increase in plasma cell numbers and...
numbers of IL-10-expressing plasma cells observed after challenge of wild-type mice with foreign Abs to IgD and the heightened increase in plasmablast and plasma cell expansion observed in IL-10-deficient mice (23). That said, the increase in plasmablast and plasma cell expansion observed in wild-type mice with foreign Abs to IgD and the heightened sensitivity to LPS (but not CpG) intermediate between that of wild-type mice and IL-10-deficient mice (18), whereas mice with selective deletion of IL-10 in T cells have unaltered sensitivity to endotoxic shock (18), whereas mice with selective deletion of IL-10 in macrophages and neutrophils exhibit sensitivity to LPS (but not CpG) intermediate between that of wild-type and IL-10-deficient mice (19). To define whether B cell-derived IL-10 has a major, nonredundant role in immune counter-regulation during MCMV infection, a more modest role in restraining immune activation after challenge with foreign Abs to IgD, and no evident nonredundant role during endotoxemia. B cell-specific IL-10 knockout mice also fail to develop inflammatory bowel disease, indicating that B cell-derived IL-10 is not required for protection against pathological inflammation of the gut.

The reporter fidelity and robustness exhibited by Vert-X mice suggest that they will provide a useful tool for further mechanistic studies of IL-10-mediated counter-regulation. In particular, the Vert-X mouse appears to report IL-10 expression with greater sensitivity than the previously described tiger mice, whose T cells appear to exhibit a gene dose-dependent decrease in IL-10 protein production (29). Vert-X mice provide information complementary to that provided by a recently published translational IL-10 reporter mouse (40), an experimental system in which cells that have expressed IL-10 protein remain marked for sustained periods. An advantage of transcriptional reporter mice is their ability to unveil the in vivo kinetics and localization of the initiation of gene expression. Subsequent events in the translation and release of cytokine protein are not addressed. There may be qualitative differences in GFP and IL-10 translation; the IRES sequence allows for cap-independent translation of GFP, whereas that of IL-10 is likely to be cap dependent. Furthermore, IL-10 is thought to be secreted rapidly, whereas the GFP reporter remains intracellular. Finally, IL-10 has been reported to undergo posttranscriptional regulation due to mRNA destabilizing sequences in the 3′-untranslated region (41, 42); it is possible that this process is modified by the inserted construct. Indeed, use of the In Vivo Cytokine Capture Assay (28) revealed somewhat increased systemic IL-10 production in Vert-X compared with wild-type mice, both in the basal state and during cutaneous infection with Leishmania major (data not shown). Despite this and the exquisite sensitivity of L. major infection to IL-10, the course of infection proceeded identically in Vert-X and wild-type mice (data not shown). Therefore, although the data provided by all such reporters must be interpreted with caution, these mice enable unique access to in vivo information about IL-10 gene expression.

One example of the novel and important information provided by our study of these mice is the relative importance of IL-10 produced by B cells vs other cell types for regulation of immune activation induced by different stimuli. Our results reveal a nonredundant role for B cell-derived IL-10 during MCMV infection, a more modest role after anti-IgD challenge, and no evident role after TLR challenge, even though B cells are a major source of IL-10 in all three models. Possible reasons for this variation include differences in the location, timing, and overall vigor of the
resulting immune responses in these models. Although B cells are the dominant IL-10-expressing cell type in peripheral lymph organs after LPS challenge, myeloid cells dominate in blood and liver, and \textit{Il10}\textsuperscript{flox/flox}\textsuperscript{LysM-Cre} mice exhibit increased sensitivity to LPS challenge (19). Additionally, LPS-induced toxicity appears to be largely dependent on myeloid cell secretion of inflammatory cytokines; such secretion may only be suppressed efficiently by autocrine production of IL-10. Early MCMV infection is associated with local viral replication and local immune responses in lymphoid tissue, whereas the dramatic systemic immune activation of multiple IL-10-producing cell types following TLR challenge may lead to functional redundancy among cellular sources of this cytokine. Finally, as noted above, IL-10 expression may not always directly reflect IL-10 protein secretion. However, these data do suggest the likelihood that B cells play an underappreciated role in immune counter-regulation and provide a clear rationale for testing the biological role of B cell-derived IL-10 in these and other models. Previous attempts to address such issues have been beset by technical problems. B cell reconstitution of \mu\textit{MT} or RAG-deficient mice leads to a reversed ratio of B cells to T cells and a reversed ratio of marginal zone to follicular B cells in spleen (data not shown). Bone marrow chimera studies involving \mu\textit{MT} or IL-10\textsuperscript{-/-} and B cell-deficient (IL-10 wild type) mice, although often compelling (43), can lead to variable proportions of non-B cells expressing IL-10. Adoptive transfer of in vitro activated IL-10 wild-type vs knockout B cells (44) may provide clear evidence that B cells can provide IL-10-mediated counter-regulation without necessarily demonstrating that they normally do this in vivo. Future such studies should be facilitated by \textit{Il10}\textsuperscript{flox/flox} CD19-Cre and other cell type-specific IL-10 knockout mice. Increasing percentages of mature B cells, plasmablasts, and plasma cells express IL-10, both in naive and immunologically challenged mice. This may represent an instance of what may be a general phenomenon: the more differentiated or polarized a cell type is, the more likely it is to express IL-10, which is arguably why IL-10 was first discovered in Th2 clones, given the increased cellular divisions needed for Th2 vs Th1 differentiation (45, 46). However, as IL-10 can act as an in vitro differentiation factor for human plasma cells (47–49), such IL-10 production by B cells might act in \textit{cis}, facilitating plasma cell development. Naive IL-10 knockout mice exhibit no deficit in plasma cell numbers in spleen or bone marrow, however (data not shown), and such mice exhibit increased plasma cell numbers after anti-IgG challenge. Furthermore, a lack of B cell-derived IL-10 led to increased plasma cell numbers during MCMV infection. Thus, B cell-derived IL-10 either inhibits plasma cell expansion in \textit{cis} in mice in vivo or, more likely, acts in \textit{trans} to provide counter-regulatory activity for the overall immune response at the level of Ag presentation.

In addition to well-recognized immunopathogenic roles, particularly in autoimmune disease, B cells have also long been recognized to be able to play immunoregulatory roles. The paradigmatic B cell-derived effector molecule, Ig, can dampen immune responses through engagement of ITIM-bearing Fc receptors (30), something that has been exploited therapeutically (51). Obviously, Igs can also regulate the amplitude of immune responses through neutralization of pathogens and pathogen-derived molecules. The current studies add to previous evidence that B cell-derived IL-10 can play an immunoregulatory role in autoimmune disease. Both experimental allergic encephalomyelitis induced by active immunization and inflammatory bowel disease developing in TCR\textalpha-deficient mice are exacerbated by the absence of mature B cells (52, 53). In experimental allergic encephalomyelitis, B cell deficiency was associated with a lack of recovery from what is normally a monophasic disease (52); bone marrow chimera systems provided strong evidence that the critical missing counter-regulatory factor in \mu\textit{MT} mice was B cell-derived IL-10 (43). In the case of inflammatory bowel disease, TCR\textalpha/-IgG\textsuperscript{-/-} double-deficient mice developed more severe disease than TCR\textalpha-deficient mice, which was ameliorated by the transfer of wild-type but not IL-10-deficient B cells (53, 54). B cells have also been shown to be able to provide protection against arthritis in the collagen-induced arthritis model through an IL-10-dependent mechanism (44). These models, as well as models implicating IL-10 from neonatal B cells in the impaired activation of neonatal dendritic cells by TLR ligands (55, 56), will likely benefit from reexamination with currently available experimental approaches. More broadly, careful use of reporter and cell type-specific knockout mice should facilitate mechanistic analysis of the role played by IL-10 and other central counter-regulatory mediators in immune homeostasis and disease.

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References
