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Marginal Zone B Cell Is a Major Source of IL-10 in *Listeria monocytogenes* Susceptibility

Chen-Cheng Lee* and John T. Kung*†

*Rag-1*–knockout (KO) mice are highly resistant to *Listeria monocytogenes* infection. The role played by the many *Rag-1*–dependent lymphocyte lineages was studied using a genetic approach in which each *Rag-1*–dependent lymphocyte lineage was eliminated one at a time. Only B cell-deficient *Igh*-KO mice displayed reduced bacterial load and improved survival upon *Listeria* infection. *Listeria* infection of *Rag-1*–KO and *Il-10*–KO hosts that had been adaptively transferred with wild-type marginal zone B (MZB) cells, but not follicular B cells, resulted in heightened bacterial load and increased IL-10 production in the spleen, but not the liver. This MZB cell-dependent increase in bacterial load was eliminated by anti–IL-10 mAb. In addition, *Listeria* infection of MZB cell-deficient *Rbpj*-KO mice showed decreased bacterial load and increased survival. Whereas multiple cell types have been shown to be capable of IL-10 production, our results indicate that the MZB cell is the most dominant and relevant IL-10 source in the context of *Listeria* susceptibility. In marked contrast to the generally protective nature of MZB cells in defending against pathogenic infection, our results demonstrate that MZB cells play a detrimental role in *Listeria* infection and possibly other infections as well. *The Journal of Immunology*, 2012, 189: 3319–3327.

Innate and adaptive immune responses play distinct, complementary, and integrated protective roles in the defense against pathogenic infections. An exception to this generality is seen in the case of *Listeria monocytogenes* infection, in which adaptive lymphocytes have been shown to play a detrimental role (1). Systemic *L. monocytogenes* infection of mice is a well-established model for studying innate and adaptive immune responses (2). TNF-α and IFN-γ produced by the host have been shown to play protective roles in early *L. monocytogenes* infection (3, 4). In marked contrast, IL-10 plays a detrimental role in *L. monocytogenes* infection and is thus a susceptibility factor (5, 6). Multiple cell types, including macrophages, DCs, NK cells, B cells, and T cells, have been demonstrated to produce IL-10 upon infection by intracellular pathogens (reviewed in Ref. 7), although it is unclear whether one cell type or multiple cell types actually provide the relevant IL-10 that mediates increased susceptibility. The fact that spleen marginal zone is the entry site for bloodstream pathogens indicates that cells located within the immediate vicinity of the marginal zone are the most likely candidates for IL-10 production. Other cells located anatomically away from the marginal zone, despite possessing the ability to produce IL-10, do not do so because they do not come into direct contact with the invading pathogen. Based on the anatomical location of marginal zone B (MZB) cells, they are one of the very first cells that come into contact with bloodstream pathogens and are therefore poised to mount the first-line host defense response (8). Indeed, pathogens trapped in the marginal zone activate MZB cells to thereby become either IgM-secreting plasma cells or APCs (9, 10). A similarly protective role played by MZB cells is evidenced by the increased susceptibility to *Staphylococcus aureus* infection of mice genetically modified to be highly deficient in MZB cells (11). In addition, Ab-mediated depletion of MZB cells results in increased bacterial burden in mice infected with *Borrelia burgdorferi* (12). Further, adoptive transfer of MZB cells is protective against lethal polyoma virus infection of SCID mice through the production of viral-specific Abs (13). Thus, the weight of the evidence so far supports a generally protective role for MZB cells in pathogenic infection. Whether an exception to this protective first-defense MZB cell role exists is unknown, although it is clear that MZB cells do mount rapid first-line responses against bloodstream pathogens. As purified MZB cells produce IL-10 in vitro (C.C. Lee and J.T. Kung, unpublished observations and data shown in Fig. 2D, 2E) in response to heat-killed (HK) *L. monocytogenes*, we hypothesized that IL-10 production is one among a battery of in vivo responses to *L. monocytogenes* infection. In this article, through the use of genetic and adoptive cell transfer approaches, we show that the MZB cell is a dominant and relevant II-10–producing cell in the context of host susceptibility/resistance; we also uncover a detrimental role played by MZB cells in *L. monocytogenes* infection through IL-10 production.

Materials and Methods

**Mice and *L. monocytogenes* infection**

The wild-type (WT) C57BL/6 mice and genetically modified *Igh*-knockout (KO), *Rag-1*–KO, *Tlr2*–KO, *Tlr4*–KO, *H2-Ab*–KO, *β2m*–KO, *Cd1d*–KO, *Tcrd*–KO, *Ifn-γ*–KO, *Ifnar*–KO, and *Cd19*<sup>−/−</sup> mice on C57BL/6 background and II-10–KO mice on C57BL/10 background were originally obtained from The Jackson Laboratory. The C57BL/6 mice with floxed *Rbpsuh* gene (*Rbpj*<sup>fl/+</sup>) were kindly provided by Dr. Tasuku Honjo (Department of Medical Chemistry, Graduate School of Medicine, Kyoto University, Kyoto, Japan) (11). MZB-deficient mice were generated first by mating *Rag-1*–KO with *Igh*-KO mice to obtain *Tlr2*–KO, *Tlr4*–KO, *H2-Ab*–KO, *β2m*–KO, *Cd1d*–KO, *Tcrd*–KO, *Ifn-γ*–KO, *Ifnar*–KO, and *Cd19*<sup>−/−</sup> mice on C57BL/6 background and II-10–KO mice on C57BL/10 background. The C57BL/6 mice with floxed *Rbpsuh* gene (*Rbpj*<sup>fl/+</sup>) were kindly provided by Dr. Tasuku Honjo (Department of Medical Chemistry, Graduate School of Medicine, Kyoto University, Kyoto, Japan) (11). MZB-deficient mice were generated first by mating *Cd19*<sup>−/−</sup> mice with *Rbpj*<sup>fl/+</sup> mice to obtain *Cd19*<sup>−/−</sup>*Rbpj*<sup>fl/+</sup> F1, which were backcrossed to *Rbpj*<sup>fl/+</sup> mice. Offspring from this cross were geno-

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The online version of this article contains supplemental material.

Abbreviations used in this article: BHI, brain–heart infusion; cKO, conditional knockout; DC, dendritic cell; FOB, follicular B; HK, heat-killed; KO, knockout; MZB, marginal zone B; PALS, periarteriolar lymphoid sheath; PI, propidium iodide; Tip-DC, Tip-DC and inducible NO synthase-producing dendritic cell; WT, wild-type.

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typed by PCR, as previously reported (14). The MZB cell-deficient Cd1d−/−Rag2−/− mice were used for experiments or further breeding. Mice from 8 to 12 wk old were used for experiments. All mouse strains were bred and housed under specific pathogen-free conditions at the Institute of Molecular Biology Animal Facility (Academia Sinica, Taipei, Taiwan).

Freshly grown L. monocytogenes was washed, resuspended in PBS, and i.v. injected at a dose of 10^7 CFU per mouse, except where specifically indicated. The dose of L. monocytogenes inoculated was always confirmed by performing colony counts on brain–heart infusion (BHI) agar plates. All L. monocytogenes-infected mice were housed in isolators (Bell Isolation Systems, Livingston, Scotland) that were kept under constant negative-pressure operation.

All procedures involving live L. monocytogenes and mice were performed in accordance with institutional guidelines and had been approved by Academia Sinica BioSafity Committee and Institutional Animal Care and Use Committee. All procedures involving live L. monocytogenes were performed in accordance with Biosafety Level 2 guidelines.

Abs
Neutralizing anti–IL-10 mAb (clone JES5-2A5) was purchased from Bio X Cell. FITC–anti-CD21 and PE–anti-CD23 mAbs were purchased from BDIS. All other mAbs were prepared from hybridoma culture supernatant by affinity purification and conjugated with indicated fluorochrome, as previously described (15, 16).

Preparation of live and HK L. monocytogenes
The streptomycin-resistant L. monocytogenes strain, 10403S, was kindly provided by Dr. Jeffrey Miller (Department of Microbiology and Immunology, School of Medicine and Molecular Biology Institute, University of California, Los Angeles). Aliquots of L. monocytogenes in log growth were frozen in 20% glycerol at −70°C. Prior to L. monocytogenes inoculation, an aliquot of the frozen L. monocytogenes was thawed and inoculated 1:100 into BHI broth (Difco Becton Dickinson) and cultured at 37°C in a shaker. Exponentially growing L. monocytogenes was harvested by centrifugation at 4°C and washed twice with ice-cold PBS, then resuspended in ice-cold PBS at appropriate concentrations. For heat inactivation, exponentially grown L. monocytogenes was washed with ice-cold PBS, heat-inactivated at 70°C for 1 h, resuspended in PBS, and stored frozen (−70°C) in aliquots until use.

FACS
Spleen cells were depleted of RBCs, as previously described (17). T cells were depleted by complement-mediated lysis of spleen cells that had been incubated with anti-CD4, anti-CD8, and anti–Thy-1 mAbs, as described earlier (18). To isolate MZB and follicular B (FOB) cells, T cell-depleted spleen cells were incubated with FITC–anti-CD21 and PE–anti-CD23. Cy5–anti-CD3 (500AA2), Alexa Fluor 647–anti-CD11c (N418), Alexa Fluor 680–anti-CD11b (M170), in the presence of anti-FcR (2.4G2) mAb (19). After applying propidium iodide (PI) to cells, CD11c−, CD11b−, and CD19− cells were sorted simultaneously, FOB and MZB cells were sorted on the criteria of CD21^lowCD23^low and CD21^highCD23^marker expression, respectively (FACSaria cell sorter; BD Biosciences). To obtain spleen cells depleted of B cells and T cells (non-B/T cells), spleen cells were first depleted of T cells, then incubated with Cy5–anti-CD3, PE–anti-CD19, and PI to positively identify the few residual T cells. B cells, and dead cells, respectively. Cells that were PI−CD19− and CD11c− as non-B/T cells. Sorted non-B/T, MZB, and FOB cells were always reanalyzed, and purities >99% were always obtained. A summary of the staining and sorting procedure for B cell subsets, including purity analysis of sorted cells, is shown in Supplemental Fig. 1.

Cytokine induction, neutralization, and measurements
II-10 produced by HK L. monocytogenes-stimulated MZB cells, FOB cells, and non-B/T cells that were set up in tissue culture was determined by ELISA (DuoSet ELISA Development System; R&D Systems). For tissue cytokine measurements, spleens and livers of L. monocytogenes-infected mice were harvested and weighed, then individually homogenized (100 mg wet tissue weight per milliliter of PBS containing 0.5% Triton X-100, 0.05% sodium azide, and protease inhibitor mixture; Sigma-Aldrich). Tissue homogenates were next subjected to one round of freeze-thaw, followed by sonication (Cole-Palmer Ultrasonic, 10 min), as previously published (20); then they were incubated at 4°C for 1 h, centrifuged (12,000 × g, 10 min), and the supernatants collected for II-10, Ifn-γ, and Tnf-α determinations (DuoSet ELISA Development System; R&D Systems). Tissue Ifn-α was determined by VetriKine Mouse Ifn-α ELISA (PBL InterferonSource).

Determination of spleen and liver CFU
Harvested spleens and livers were individually weighed, placed in PBS containing 1% saponin, and homogenized with the aid of tissue grinders (Tenbroeck 7 ml; Wheaton). The homogenates were serially diluted and plated on BHI agar plates (30–300 CFU per plate), and colonies counted after overnight incubation at 37°C. Bacterial load was always expressed as CFU per entire spleen or liver.

Statistical analysis
Survival of L. monocytogenes-infected mice on different genetic backgrounds was analyzed using the log-rank test. Correlation between CFU and cytokine production was examined by two-tailed Spearman’s correlation coefficient test. Except when results of independently performed experiments were pooled, statistical differences were analyzed by unpaired two-tailed Student’s t test. When results of multiple experiments were combined, the Wilcoxon matched-pairs signed-rank test was used for statistical analysis. Comparisons between groups with p < 0.05 were considered significantly different from each other.

Results
B cell-deficient mice display increased resistance to L. monocytogenes infection
Rag-1−/−KO mice are highly resistant to L. monocytogenes infection (1). To ascertain the roles played by B cells and other lymphocyte subsets in mediating the strikingly increased resistance of Rag-1−/−KO mice to L. monocytogenes infection, we examined day 3 bacterial load in the spleen and liver of L. monocytogenes-infected Igh−KO, H2-2B−/−, B2m−/−, Cd1d−/−, and Tcrd-KO mice. We found decreased resistance in β2m−/− and Cd1d-deficient mice, consistent with a protective role played by MHC-I–restricted CD8 T cells and Cd1d-restricted NK T cells (Supplemental Fig. 2B, 2C). Little change in resistance was observed for H2-2B−/− and Tcrd-KO mice, indicating MHC-II–restricted CD4 T cells and TCR-γδ T cells most likely did not play significant roles during the early phase of L. monocytogenes infection (Supplemental Fig. 2A, 2D). For B cell-deficient Igh−KO mice, however, significant improvement in survival and decreased bacterial load were seen. At a dose of 10^4 inoculated L. monocytogenes, 67% of WT mice died by day 6 compared with no death for Igh−KO mice (Fig. 1A). A 5-fold higher L. monocytogenes inoculum was required for Igh−KO mice to reach a similar level of mortality observed for WT mice. In addition, day 3 L. monocytogenes bacterial loads in spleens and livers of L. monocytogenes-infected WT mice were 61- and 29-fold greater than those seen in Igh−KO mice, respectively (Fig. 1B, 1C).

L. monocytogenes-infected Igh−KO mice show decreased II-10 production
To gain understanding of possible cytokine contribution to the decreased bacterial load in L. monocytogenes-infected Igh−KO mice, we examined the expression of Tnf-α and Ifn-γ, two key cytokines known to mediate L. monocytogenes elimination (3, 4). II-10, a cytokine known to exacerbate L. monocytogenes infection, was also examined. As the cytokines that play dominant decisive roles in the regulation of bacterial load are expected to display either positive or negative correlation with bacterial load, we first determined the relationship between these three cytokines and bacterial load in the spleen and the liver of L. monocytogenes-infected WT mice. At an early time point of day 1.5 after L. monocytogenes infection, only II-10 and spleen bacterial load showed a strong positive correlation (r = 0.806, p = 0.005; Fig. 2A). At day 1.5 post L. monocytogenes infection, no significant correlation was observed for II-10 and liver bacterial load, for Ifn-γ and spleen/liver bacterial load, and for Tnf-α and spleen/liver bacterial load. At day 3 post L. monocytogenes infection, the positive correlation seen at day 1.5 was maintained between II-10 and spleen bacterial load (Fig. 2B). A relatively weak but statistically nonsignificant correlation between II-10 and bac-
either tissue Il-10 or Ifn-γ mice than for WT mice (Fig. 2C). No significant difference in ocytogenes was significantly higher for Igh

mediating L. monocytogenes infection. If so, the highly L. monocytogenes-resistant nature of Rag-1–KO and II-10–KO mice would be compromised upon adoptive transfer of MZB, but not FOB, cells. We first adoptively transferred 1.5 × 10⁶ MZB cells or 2 × 10⁷ FOB cells per mouse into a cohort of Rag-1–KO hosts. The number of transferred MZB and FOB cells was similar to and based on the total number of MZB and FOB cells in a WT spleen. Our finding of highly reduced bacterial load in L. monocytogenes-infected Rag-1–KO mice is consistent with heightened resistance of Rag-1–KO mice against L. monocytogenes infection (1). Adoptive transfer of MZB cells, but not FOB cells, into Rag-1–KO mice caused an increase in L. monocytogenes load in the spleen that was 9.7-fold that of the Rag-1–KO control group (Fig. 3A). This increase in bacterial load in the spleen was accompanied by increased II-10 and decreased Ifn-γ production. In marked contrast, adoptive transfer of MZB cells into Rag-1–KO hosts had no effect on bacterial load and II-10/Ifn-γ production in the liver. Although these results support a detrimental role for MZB cells in L. monocytogenes infection in the spleen environment, the possible contribution of the altered anatomical structure of Rag-1–KO mice in mediating L. monocytogenes resistance, such as the complete lack of B cell follicles and periarteriolar lymphoid sheath (PALS), is difficult to rule out. To rule out the possible contribution of altered anatomical microenvironment associated with Rag-1–KO mice in L. monocytogenes susceptibility, II-10–KO mice were chosen as adoptive hosts because of their normal spleen architecture. Although less striking in magnitude than in Rag-1–KO mice, heightened L. monocytogenes resistance in II-10–KO mice was highly significant and consistent with previously published findings (5, 6). Adoptive transfer of MZB cells into II-10–KO hosts caused an increase in L. monocytogenes load in the spleen that was 13.2-fold that of the II-10–KO control group (Fig. 3B). This increase in bacterial load in the spleen was accompanied by increased II-10 and decreased Ifn-γ production. On the contrary, adoptive transfer of MZB cells into II-10–KO hosts had no effect on bacterial load and II-10/Ifn-γ production in the liver. Even though significant increases in bacterial load and II-10 production in the spleen were observed following L. monocytogenes infection of Rag-1–KO and II-10–KO hosts that had been adoptively transferred with MZB cells, the increased levels in bacterial load and II-10 production did not reach those seen in L. monocytogenes-infected WT

MZB cells inhibit innate immunity against L. monocytogenes through II-10 production

On the basis of our finding that MZB cells are the most potent II-10-producing cells in vitro, and that they are poised to make the first contact with bloodborne Ags, we hypothesized that MZB cells are the dominant II-10–producing cells in response to L. monocytogenes infection. In marked contrast, the role of MZB cells in mediating resistance to L. monocytogenes infection is minimal compared to the role of FOB cells. Since the FOB cells are the dominant Ifn-γ-producing cells in response to L. monocytogenes infection, the relatively poor II-10 production by MZB cells in response to L. monocytogenes infection is consistent with our previous observation that MZB cells are not major contributors to the systemic II-10 response to L. monocytogenes infection (1). Despite this, the MZB cells are the most potent Il-10–producing cells in response to L. monocytogenes infection. We next determined spleen and liver tissue Ifn-γ and spleen/liver bacterial load was observed for day 3-infected, but not earlier day 1.5-infected, WT mice (Fig. 2B). No significant correlation was seen between Tnf-α and bacterial load in the spleen and the liver at day 3 post L. monocytogenes infection (Fig. 2B). On the basis of these results, we concentrated on tissue II-10 and Ifn-γ measurements in subsequent studies. We next determined spleen and liver tissue Ifn-γ and II-10 of L. monocytogenes-infected WT and Igh-KO mice (Fig. 2C). Spleen tissue II-10 was significantly higher for L. monocytogenes-infected WT mice than for Igh-KO mice. On the contrary, spleen tissue Ifn-γ was significantly higher for L. monocytogenes-infected Igh-KO mice than for WT mice (Fig. 2C). No significant difference in either tissue II-10 or Ifn-γ was observed for the liver of L. monocytogenes-infected WT and Igh-KO mice. Because B cells are missing in Igh-KO mice, we tested the ability of B cells to produce II-10 in response to L. monocytogenes stimulation. In the absence of added accessory cells, MZB cells produced readily detectable II-10 upon stimulation HK L monocytogenes in a dose-dependent manner (Fig. 2D). The amount of II-10 produced by MZB cells was significantly augmented when they were stimulated in the presence of II-10–KO accessory cells. B cell- and T cell-depleted spleen cells, a cell population enriched in macrophages and dendritic cells (DCs), produced lower amounts of II-10 than did MZB cells; addition of II-10–KO accessory cells did not increase their level of II-10 production. On their own, FOB cells showed extremely poor II-10 production response to HK L monocytogenes, although addition of II-10–KO accessory cells did promote weak II-10 production. In the presence of II-10–KO accessory cells, the amount of II-10 produced by MZB cells in response to the highest dose of HK L monocytogenes was 4.8-fold and 9.4-fold more than those produced by B/T cell-depleted spleen cells and FOB cells, respectively. II-10 production was triggered through Tlr-2 and Tlr-4, as highly striking and significant reductions in II-10 production were observed for HK L monocytogenes-stimulated MZB cells obtained from Tlr4-KO and Tlr2-KO genetic backgrounds, respectively (Fig. 2E).

MZB cells inhibit innate immunity against L. monocytogenes through II-10 production

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FIGURE 1. B cell-deficient mice are more resistant to systemic L. monocytogenes infection. (A) C57BL/6 (WT, n = 12) and B cell-deficient (Igh-KO, n = 8) mice were given L. monocytogenes (i.v., 10⁶ CFU per mouse), and their survival was monitored over an observation period of 30 d. Another cohort of Igh-KO mice (n = 8) was infected with a higher dose of L. monocytogenes (5 × 10⁶ CFU per mouse), and their survival was also observed over a 30-d period. For WT and Igh-KO mice given 10⁴ L. monocytogenes CFU, their survival rate showed highly significant difference (**p < 0.01, log-rank test). Data shown are typical of one of three independently performed experiments. WT (i.v., 10³ CFU/mouse), and bacterial loads were determined at the level of the individual mouse for each of the indicated five postinfection time points (3 WT and Igh-KO mice for each of the time points) for the spleen (B) and the liver (C). As ~50% WT mice will die when infected with L. monocytogenes at a dose of 10⁶ CFU, a lower dose of 10⁵ CFU was given per mouse to avoid their death, to enable determination of bacterial load as a function of time without the complication associated with deaths of WT mice, but not Igh-KO mice. On day 3 of infection, the L. monocytogenes loads (mean ± SD) in the spleen and the liver for WT mice were 61- and 29-fold those of Igh-KO mice, respectively. Data shown are typical of one of two independently performed experiments.

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FIGURE 2. Il-10 production by B cells in response to L. monocytogenes infection positively correlates with L. monocytogenes load in the spleen. A cohort of WT mice (n = 22) was infected by L. monocytogenes (i.v., 10^4 CFU per mouse). On day 1.5 and day 3 postinfection, 10 and 12 mice were sacrificed, respectively, and their spleens and livers removed and homogenized, and the presence of tissue Il-10, Ifn-γ, Tnf-α, as well as CFU, was determined [day 1.5, (A); day 3, (B)]. Correlation (r) and statistical significance (p) calculated by Spearman’s correlation between each of the three cytokines versus CFU are shown. (C) WT mice (n = 4, open bars) and Igh-KO mice (n = 4, filled bars) were infected with L. monocytogenes (i.v., 10^4 CFU per mouse). On day 3 postinfection, their spleens and livers were removed, homogenized, and assayed for tissue Il-10 and Ifn-γ. Groups showing significant difference calculated by unpaired Student t test (two-tailed) are indicated: *p<0.05, **p<0.01. Data (mean ± SD) shown are typical from one of two independently performed experiments. (D) MZB, FOB, and non-B/T cells were purified by FACS sorting from WT spleens. T cell-depleted spleen cells from Il-10-KO mice prepared according to the Materials and Methods section were used as accessory cells (AC). WT MZB, FOB, and non-B/T spleen cells were set up in culture in triplicate (10^5 cells per well per 0.2 ml), with or without addition of Il-10-KO AC (10^5 cells per well per 0.2 ml), then stimulated with HK L. monocytogenes as indicated. At 48 h after HK L. monocytogenes stimulation, the amount of Il-10 released into culture medium was determined. Il-10 production by MZB cells versus other cell types was analyzed by the unpaired Student t test. For cultures without AC, MZB versus FOB, p = 0.005, 0.009, and 0.031 for high-, intermediate-, and low-dose L. monocytogenes stimulation, respectively; MZB versus non-B/T, p = 0.126, 0.615, and 0.139 at high-, intermediate-, and low-dose L. monocytogenes stimulation, respectively. For cultures with added AC, MZB versus FOB, p = 0.006, 0.002, and 0.0003 at high-, intermediate-, and low-dose HK L. monocytogenes stimulation, respectively; MZB versus non-B/T, p = 0.010, 0.002, and 0.0004 at high-, intermediate-, and low-dose HK L. monocytogenes stimulation, respectively. Il-10 production by MZB cells from WT, Tlr2-KO, and Tlr4-KO mice were isolated by FACS, placed into culture (10^5 cells per well per 0.2 ml) in the presence of added Il-10-KO accessory cells, and stimulated by HK L. monocytogenes, as in (D). Il-10 production by WT versus Tlr-KO MZB cells was analyzed by the Student t test: WT versus Tlr2-KO, p = 0.028, 0.010, and 0.008 at high-, middle-, and low-dose HK L. monocytogenes stimulation, respectively; WT versus Tlr4-KO, p = 0.006, 0.001, and 0.0002 at high-, middle-, and low-dose HK L. monocytogenes stimulation, respectively. Data from Il-10 production (mean ± SD) shown in (D) and (E) are typical of one of two independently performed experiments.
mice. A likely explanation lies in the $1.5 \times 10^6$ MZB cells we adoptively transferred into each host mouse. Because only a fraction of adoptively transferred MZB cells will find their way back to the spleen, the degree of increase in bacterial load and IL-10 production caused by adoptively transferred MZB cells is likely to be less potent than that of resident MZB cells in the spleen of WT mice, owing to the relatively smaller number of adoptively transferred MZB cells reaching the spleen, compared with resident MZB cells in a WT host.

To confirm that the MZB cell-mediated increase in bacterial load in the spleen was indeed caused by IL-10 production, we adoptively transferred WT MZB cells into II-10−/− KO hosts, followed by L. monocytogenes infection. In this instance, we again observed an inhibitory effect exerted by MZB cells on innate immunity against L. monocytogenes infection (Fig. 3C). As the II-10−/− KO hosts were unable to produce any IL-10, the only source of IL-10 was from the adoptively transferred MZB cells. The adverse effect MZB cells exerted on L. monocytogenes load was blocked by anti–IL-10 mAb, providing support for IL-10 as the actual mediator through which MZB cells exerted inhibitory action on innate immune response against L. monocytogenes infection (Fig. 3C).

**MZB cell-deficient Rbpg-cKO mice display increased resistance to L. monocytogenes infection**

*Rbpg-cKO* mice, conditional knockout (cKO) mice with B cell-specific *Rbpg* deletion, had been shown to possess normal numbers of FOB cells, but highly deficient MZB cells (11). We subjected *Rbpg-cKO* mice to L. monocytogenes infection and examined their survival along with bacterial load and tissue cytokine production (Fig. 4). Under an identical dose of L. monocytogenes challenge ($10^6$ CFU per mouse), 5 of 8 (62.5%) WT mice died within 5 d, with the remainder (37.5%) surviving for the entire 30-d observation period. In marked contrast, a highly significant reduction in mortality was observed for *Rbpg-cKO* mice such that 8 of 10 (80%) survived and of the 20% that died, significant delay in their death was seen. Significantly reduced bacterial load in the spleen was seen as early as 1.5 d after L. monocytogenes infection of *Rbpg-cKO* mice, as the bacterial load for WT mice was 4.1-fold that for *Rbpg-cKO* mice (Fig. 4B; $p = 0.0001$). By 3 d post L.
monocytogenes infection, spleen bacterial load for WT mice had increased to 70.7-fold that for Rbpj-cKO mice (Fig. 4C; \( p = 0.009 \)). Similar bacterial load levels were seen in the liver of day 1.5 and day 3 L. monocytogenes-infected WT and Rbpj-cKO mice. The increased resistance of Rbpj-cKO mice to L. monocytogenes infection was due to the missing MZB cells because adoptive transfer of sorted MZB cells into Rbpj-cKO mice, followed by L. monocytogenes infection, resulted in significantly increased bacterial load in the spleen (Fig. 4D). Consistent with results already shown in Fig. 3, adoptive transfer of MZB cells had no effect on bacterial load in the liver of Rbpj-cKO mice (Fig. 4D).

Increased L. monocytogenes resistance in Rbpj-cKO mice is correlated with reduced II-10, but not Ifn-α, production

An average of 3.58 pg of II-10 per milligram of tissue was detected for day 1.5 L. monocytogenes-infected WT spleens (Fig. 5A). In striking contrast, an ~10-fold reduced II-10 level (0.37 pg/mg) was found for the spleens of L. monocytogenes-infected Rbpj-cKO mice. The difference in II-10 production between WT and Rbpj-cKO mice was highly significant (\( p = 0.004 \)). By day 3, the difference in II-10 production between L. monocytogenes-infected WT and Rbpj-cKO mice had become smaller, but significant difference was still observed (\( p = 0.0495 \)). For day 3 L. monocytogenes-infected WT mice, spleen tissue Ifn-γ was 10.27 pg/mg. The 13.85 pg/mg of spleen tissue Ifn-γ observed for L. monocytogenes-infected Rbpj-cKO mice was a modest 35% more than the 10.27 pg/ml observed for L. monocytogenes-infected WT mice, although this difference was statistically significant (\( p = 0.027 \)). To address possible relatedness between II-10 and type I Ifn, tissue Ifn-α was determined. As no significant difference was observed for the amount of Ifn-α produced by WT and Rbpj-cKO mice (Fig. 5), no apparent relatedness existed between II-10 and Ifn-α.

II-10 neutralization further reduces bacterial load in L. monocytogenes-infected Ifnar-KO mice

To more directly address the relatedness between Ifn-α/β and the II-10 detrimental pathway in L. monocytogenes infection, we...
infected Ifnar-KO mice with or without II-10 neutralization. Consistent with increased resistance of Ifnar-KO mice against L. monocytogenes infection, significantly lower bacterial load (∼10^6 per spleen) was observed for L. monocytogenes-infected Ifnar-KO mice (Fig. 6), in comparison with the ∼10^7 CFU per spleen of L. monocytogenes-infected Ifnar-KO spleen. This result indicates that in the total absence of Ifnar signaling, II-10 still plays a detrimental role. Thus, Ifnar signaling is not required for II-10 production, and little, if any, relatedness exists between detrimental Ifn-α/β and II-10 pathways. The relatively weak effect of II-10 neutralization on liver bacterial load in L. monocytogenes-infected Ifnar-KO mice is consistent with the idea that the II-10 detrimental pathway is more dominant in the spleen than the liver, most likely a consequence of II-10–producing MZB cells in the spleen, but not the liver.

Discussion

MZB cells line the outer border of the anatomically distinct white pulp structure of the spleen and are commonly regarded as the cells that mount first-line protective response against bloodborne pathogens (9–13). Even though many cell types are capable of producing II-10 (reviewed in Ref. 7), a cytokine that plays a detrimental role in L. monocytogenes infection (5, 6), we report in this article that the MZB cell is the most relevant source of II-10 in the context of L. monocytogenes susceptibility. Our finding of a detrimental MZB cell role in L. monocytogenes infection stands in stark contrast to the well-established protective role MZB cells play in pathogenic infection and raise the possibility that innate response by MZB cells to bloodborne pathogens may also play detrimental rather than protective roles in other infections.

As adaptive lymphocytes in general play a protective role in the defense against pathogenic infections, we were intrigued by the highly resistant nature of the lymphocyte-deficient Rag-1–KO mouse against L. monocytogenes infection (1). We report in this article that the production of II-10 by MZB cells causes an increase in bacterial load in the spleen of L. monocytogenes-infected mice. II-10 and Ifn-α/β are the two best-known detrimental cytokines in Lm infection (5, 6, 21, 22). It is unclear whether the II-10 pathway is distinct from the Ifn-α/β pathway or whether they converge onto a common inhibitory mechanism. Tnf-α and inducible NO synthase-producing dendritic cells (Tip-DCs) have been identified as the major Ifn-β producer (23), although the cellular source of II-10 in response to L. monocytogenes infection has until now not been clearly defined. Increased resistance to L. monocytogenes infection in Ifnar-KO mice has been explained by the effect of Ifn-α/β in sensitizing lymphocytes to lysteriolysin O–mediated killing, followed by phagocytosis of apoptotic cells by macrophages, which in turn results in the production of the anti-inflammatory cytokine II-10 (24). In this scenario, II-10 is downstream of Ifn-α/β production and Ifnar signaling. As direct supporting evidence for this attractive proposal is lacking, the possibility that the II-10 pathway is distinct and does not overlap with the Ifn-α/β pathway deserves serious consideration. Our results clearly show that II-10 production by MZB cells alone was sufficient to exacerbate L. monocytogenes infection. In addition, MZB cell-deficient Rhps-cKO mice displayed increased resistance against L. monocytogenes infection. As the MZB cell-deficient mice were generated by conditional deletion of Rbps in B cells only (11), the development of and ability to produce Ifn-α/β by the non-B lineage Tip-DC is expected to be unaltered. L. monocytogenes-infected WT and Rhps-cKO mice are therefore expected to produce similar amounts of Ifn-α/β, which is confirmed by our experimental results (Fig. 5). Also, there are similar numbers of T cells in WT and Rhps-cKO mice (11), and if Ifn-α/β–mediated apoptosis of T cells is the cause of elevated II-10 production (24), both WT and Rhps-cKO mice are expected to be equally susceptible to L. monocytogenes infection, which is contrary to our finding of increased resistance against L. monocytogenes infection in MZB cell-deficient Rhps-cKO mice (Fig. 4). In addition, the beneficial effect of II-10 neutralization we observed in L. monocytogenes-infected Ifnar-KO mice is incompatible with II-10 being downstream of Ifn-α/β action (Fig. 6). Taken together, our results indicate that the detrimental effect exerted by MZB cells is mediated through II-10 production, and represents a unique detrimental pathway distinct from the Ifn-α/β pathway.

The results we present in this article clearly show the MZB cell as the most relevant II-10–producing cell in the context of L. monocytogenes susceptibility/resistance. However, how II-10 mediates the detrimental effect is less than clear. Some possible mechanisms through which II-10 promotes L. monocytogenes growth have been considered. II-10 production by MZB cells is accompanied by a decreased level of Ifn-γ production, which is consistent with the reported inhibitory action of II-10 on Ifn-γ production by NK cells (25) and increased Ifn-γ production by innate CD8+ T cells in the absence of II-10 (26, 27). We found ∼10-fold higher bacterial load in the spleen of L. monocytogenes-infected Ifn-γ−/− mice than in that of Ifn-γ+ mice (Supplemental Fig. 3). If we make the assumption that mice with two functional Ifn-γ alleles produce twice as much Ifn-γ as mice with one functional Ifn-γ allele, then the ∼2-fold increase in Ifn-γ in the spleen of L. monocytogenes-infected Igk-KO mice (Fig. 2) can be expected to mediate a drop of L. monocytogenes load by ∼10-fold. The experimentally determined drop was, however, a much higher 61-fold (Fig. 1), a result consistent with the existence of Ifn-γ-dependent and –independent inhibitory pathways of innate response against L. monocytogenes. This brings up the possibility that the production of a single cytokine, II-10, can inhibit innate immune response against L. monocytogenes through multiple pathways. II-10, by exerting modest inhibitory effects on different arms of the innate defense pathways, may end up with a much more potent overall inhibition through additive or synergistic effects. The reported inhibitory effect of II-10 on bacterial killing may represent yet another of the multiple II-10 targets (28). Another possible target of II-10 action may be the generation of TipDC, a cell known to play an indispensable role in clearing L. monocytogenes infection (29). If so, we would expect to find in-

**FIGURE 6.** II-10 neutralization further reduces bacterial load in L. monocytogenes-infected Ifnar-KO mice. Two groups of Ifnar-KO mice were set up. Control group: Ifnar-KO mice were treated with PBS 6 h prior to infection. II-10-neutralizing group: Ifnar-KO mice were treated with anti–II-10 mAb (100 μg per mouse) 6 h prior to infection. All mice were infected with L. monocytogenes (i.v., 10^7 CFU per mouse). On day 3 postinfection, spleens and livers were removed and homogenized, and bacterial loads were determined. Data (mean ± SD) shown are typical of one of two independently performed experiments; they were also analyzed by unpaired Student t test. *p < 0.05.
creased numbers of Tip-DCs in *L. monocytogenes*-infected II–10–KO and Rhpj-cKO mice. On the contrary, we observed, in preliminary experiments, reduced numbers of Tip-DCs in *L. monocytogenes*-infected II–10–KO and Rhpj-cKO mice. Migration of DCs from the marginal zone into PALS (T cell zone) is correlated with increased bacterial load (30). DC migration into PALS is presumably dependent on chemokine(s) produced by cells residing within PALS. Alterations of the anatomical organization of PALS may therefore be expected to cause reduced chemokine production and decreased DC migration into PALS, a condition that has previously been shown to limit *L. monocytogenes* growth (30). By the same token, an altered marginal zone structure that prevents the ingestion of bloodborne *L. monocytogenes* by DCs there will also result in reduced bacterial load. The marginal zone comprises four types of cells (8). At the outer rim, marginal zone macrophages are found. Marginal zone metallophilic macrophages form the inner ring and are immediately adjacent to the white pulp. Between these two rings of macrophages are the MZB cells and DCs. Other than missing MZB cells, the marginal zone organization of Rhpj-cKO mice is normal in that it contains DCs, MARCO+ macrophages (Supplemental Fig. 4A), and MOMA+ macrophages (11). We have observed similar levels of DC migration into PALS in *L. monocytogenes*-infected WT, Rhpj-cKO, and II–10–KO mice (Supplemental Fig. 4B), indicating little, if any, II–10 role in promoting DC migration into PALS. It is equally possible that the detrimental effect mediated by II–10 works by an as yet unknown but dominant mechanism. Further studies are required to distinguish between the single- or multiple-target models by which II–10 exerts harmful effects on *L. monocytogenes* infection.

Biological effects mediated by MZB cells are more likely to be felt in the spleen microenvironment because MZB cells are found only in the spleen and not elsewhere. This is indeed what we have observed. Adoptive transfer of MZB cells into Rag–1–KO mice resulted in heightened bacterial load in the spleen but not the liver. However, using Rag–1–KO mice as adoptive hosts may confound the apparent clear-cut detrimental MZB cell effect in that their spleens do not possess the many specialized anatomical structures such as B cell follicles and PALS. This concern led us to study genetically modified Rhpj-cKO mice that are highly deficient in MZB cells, but not FOB cells. Indeed, *L. monocytogenes* infection of Rhpj-cKO mice resulted in a highly significant ~70-fold reduced bacterial load in the spleen, but with only a 6-fold reduction for the liver (Fig. 4). In addition to missing MZB cells, however, Rhpj-cKO mice may have other deficiencies, such as reduced SIGN-R1 expression by marginal zone macrophages (31). Although it is possible that the deficient SIGN-R1 expression in Rhpj-cKO mice may contribute to increased *L. monocytogenes* resistance, this possibility appears unlikely for the following reasons. First, the detrimental effects we report in this article for MZB cells that had been adoptively transferred into Rag–1–KO, II–10–KO, and Rhpj-cKO hosts were completed within a short period of 3 d, with clearly measurable effects within 1.5 d. In contrast, no SIGN-R1 recovery was observed within the first 3 d of adoptive transfer of MZB cells back into MZB cell-deficient hosts, as the recovery of SIGN-R1 expression takes much longer than 3 d (31). Second, SIGN-R1 expression has been found to be normal in II–10–deficient mice (31); the normal SIGN-R1 expression in II–10–KO mice is incompatible with a SIGN-R1 role in the resistance-compromising effect we report in this article for MZB cells that had been adoptively transferred into II–10–KO hosts (Fig. 5).

Even though many similarities are shared in the immune defense mechanisms deployed by the spleen and the liver against *L. monocytogenes* infection, organ-specific unique resistance and susceptibility factors are also evident. Our finding of Ifn-α production in *L. monocytogenes*-infected spleen, but not liver, further underscores the difference of the spleen and liver in their immune response to *L. monocytogenes* infection (Fig. 5). This finding may on first glance be difficult to reconcile with the highly reduced bacterial load in the spleen as well as the liver of *L. monocytogenes*-infected Ifnar-KO mice (21). It is possible that the Ifn-α/β produced by cells in the spleen can travel to distant sites such as the liver to exert its detrimental effect. Our observation that Ifn-γ production in the spleen, but not the liver, was dampened by the adoptive transfer of MZB cells (Fig. 3A, 3B) further supports the uniqueness of the spleen and the liver in the defense against *L. monocytogenes* infection.

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**Disclosures**

The authors have no financial conflicts of interest.

**References**


Figure S1. Isolation of MZB and FOB cells by fluorescence-activated cell sorting (FACS). (A) Spleen cells removed of red blood cells and depleted of T cells were incubated with FITC-anti-CD21, and PE-anti-CD23, Cy5-anti-CD3 (500AA2), AF647-anti-CD11c (N418), AF680-anti-CD11b (M1/70) in the presence of unlabeled anti-FcR (2.4G2) mAb. (A) Correlated CD3+CD11c versus CD11b expression is shown as a contour plot. Fluorescence emission of Cy5-CD3 and AF647-CD11c was detected by the same detector as Cy5 and AF647 possess similar spectral properties. (B) To achieve highly enriched viable B cells, PI<sup>-</sup>, CD3<sup>-</sup>/CD11c<sup>-</sup>, and CD11b<sup>-</sup> sorting gates were simultaneously engaged (blue rectangle in A) to respectively remove dead (PI<sup>+</sup>), T cells/dendritic cells (CD3<sup>+</sup>/CD11c<sup>+</sup>), and macrophages (CD11b<sup>+</sup>). The cells within this sort gate is highly enriched for B cells as they were >98% CD19<sup>+</sup>. (B) CD21 versus CD23 correlated expression of this enriched B cell population was acquired and the frequencies of MZB cells (CD21<sup>hi</sup>/CD23<sup>low</sup>) and FOB cells (CD21<sup>low</sup>/CD23<sup>hi</sup>) shown. MZB and FOB cells defined by correlated CD21/CD23 expression were sorted (FACS Aria cell sorter, BD Bioscience). (C, D) Greater than 99% pure MZB cells and FOB cells were obtained as seen by re-analysis of sorted MZB and FOB cells.
Figure S2. T cells are not susceptibility factors in *Lm* infection. Indicated numbers of WT mice, *H2-Aβ-KO* mice devoid of MHC-II-restricted CD4+ T cells, *β2m-KO* mice devoid of MHC-I-restricted CD8+ T cells, *Cd1d-KO* mice devoid of NKT cells, and *Tcrd-KO* mice devoid of TCR-γδ T cells, were infected with *Lm* (i.v., 10^4 CFU/mouse). On d3 post-*Lm* infection, spleens and livers were removed, homogenized, and bacterial loads determined. Data (mean ± SD) shown are typical of one of two independently performed experiments; they were also analyzed by unpaired Student’s *t* test, *, *p* < 0.05.
Figure S3. Allelic effects of Ifn-γ gene on determination of Lm loads. WT mice (Ifn-γ+/+, n = 3), Ifn-γ+/mice (n = 5) and Ifn-γ-KO mice (n = 3) were infected with Lm (10^4 CFU/mouse). On d3 post-Lm infection, spleens and livers were removed, homogenized, and bacterial loads determined. Data (mean ± SD) shown are typical of one of two independently performed experiments.
Figure S4. (A) Normal marginal zone architecture of Rbpj-cKO mice. WT and Rbpj-cKO spleens were frozen-sectioned, stained simultaneously by AF488-anti-CD11c, PE-anti-MARCO, and AF647-anti-B220, to detect DCs, MZ macrophages, and B cells, respectively, followed by confocal immunofluorescence analysis. For indicated WT and Rbpj-cKO spleens, fluorescence images depicting DC, MZ macrophage and B cell presence are as follows: DC: a, e; MZ macrophage: b, f; B cell: c, g; merged (DC, MZ macrophage and B cell): d, h. Data shown are typical of one of three experiments independently performed (white bar, 50 μm). (B) Similar levels of DC migration into PALS upon Lm infection of WT, Rbpj-cKO, and Il-10-KO mice. WT, Rbpj-cKO, and Il-10-KO mice were infected with Lm (i.v., 10^4 CFU/mouse). At 24h post-infection, the mice were perfused by 2% paraformaldehyde, spleen frozen sections made, then incubated simultaneously with AF488-anti-CD11c, AF546-anti-B220 to detect DCs and B cells, respectively. Subsequently, spleen sections were incubated with Hoechst 33342 for nuclear staining. The degree of compactness of revealed by nuclear staining was used to define white pulp and red pulp areas. For indicated WT, Rbpj-cKO, and Il-10-KO spleens, fluorescence images depicting nuclei, DCs, and B cells are as follows: nuclei: a, e, i; DCs: b, f, j; B cells: c, g, k; merged (DCs, B cells): d, h, l. Data shown are typical of one of three independently performed experiments (white bar, 100 μm).