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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 II-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 II-10 production in the spleen, but not the liver. This MZB cell-dependent increase in bacterial load was eliminated by anti-II-10 mAb. In addition, Listeria infection of MZB cell-deficient Rbpj-cKO mice showed decreased bacterial load and increased survival. Whereas multiple cell types have been shown to be capable of II-10 production, our results indicate that the MZB cell is the most dominant and relevant II-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.

I nnate 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, II-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 II-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 II-10 that mediates increased susceptibility. The fact that spleen marginal zone is the entry site for bloodborne pathogens indicates that cells located within the immediate vicinity of the marginal zone are the most likely candidates for II-10 production. Other cells located anatomically away from the marginal zone, despite possessing the ability to produce II-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 bloodborne 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 rapidly 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 bloodborne pathogens. As purified MZB cells produce II-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 II-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 II-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–α-β–KO, β2m–KO, Cd1d–KO, Tcrd–KO, Ikn-γ–KO, Ifnar–KO, and Cd19–KO mice on C57BL/6 background and Il-10–KO mice on C57BL/10 background were originally obtained from The Jackson Laboratory. The C57BL/6 mice with floxed Rbpj gene (Rbpjfl/−) 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 C57BL/6 mice and genetically modified Igh-knockout (KO), Rag-1–KO, Tlr2–KO, Tlr4–KO, H2–α-β–KO, β2m–KO, Cd1d–KO, Tcrd–KO, Ikn-γ–KO, Ifnar–KO, and Cd19–KO mice on C57BL/6 background and Il-10–KO mice on C57BL/10 background. The wild-type (WT) C57BL/6 mice and genetically modified Igh-knockout (KO), Rag-1–KO, Tlr2–KO, Tlr4–KO, H2–α-β–KO, β2m–KO, Cd1d–KO, Tcrd–KO, Ikn-γ–KO, Ifnar–KO, and Cd19–KO mice on C57BL/6 background and Il-10–KO mice on C57BL/10 background were originally obtained from The Jackson Laboratory. The C57BL/6 mice with floxed Rbpj (Rbpjfl/−) gene 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–cre KO mice with Rbpjfl/− mice to obtain Cd19cre/+:Rbpjfl/− F1, which were backcrossed to Rbpjfl/− mice. Offspring from this cross were geno-
typed by PCR, as previously reported (14). The MZB cell-deficient Cd19\textsuperscript{cre/+}Rbpj\textsuperscript{f/f} mice were used for experimentation 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\textsuperscript{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 protocols with active protocols that had been approved by Academia Sinica Biosafety 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 JESS-2A5) was purchased from Bio X Cell. FITC–anti-CD21 and PE–anti-CD23 mAbs were purchased from BD Biosciences. 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 removed 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 mAb, 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), CD11b\textsuperscript{−}, CD11c\textsuperscript{−}, and CD3\textsuperscript{−} gates simultaneously, FOB and MZB cells were sorted on the criteria of CD22\textsuperscript{−}CD24\textsuperscript{−} and CD21\textsuperscript{−}CD23\textsuperscript{−} marker expression, respectively (FACS aria 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 CD3 CD19\textsuperscript{−} cells were sorted 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; 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 described (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 Tinfl-α determinations (Duoset ELISA Development System; R&D Systems). Tissue Ifn-α was determined by VeriKine 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 (Tenbroneck 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\textsuperscript{KO}, H2-2β\textsuperscript{A}, B2m\textsuperscript{−}, Cdl\textsuperscript{+}, and Tcrd-KO mice. We found decreased resistance in β2m\textsuperscript{−} and Cdl\textsuperscript{−}KO mice, consistent with a protective role played by MHC-I–restricted CD8\textsuperscript{+} T cells and Cdl\textsuperscript{−}restricted NKT cells (Supplemental Fig. 2B, 2C). Little change in resistance was observed for H2-2β\textsuperscript{A} and Tcrd-KO mice, indicating MHC-II–restricted CD4\textsuperscript{+} T cells and TCR-γ6 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\textsuperscript{KO} mice, however, significant improvement in survival and decreased bacterial load were seen. At a dose of 10\textsuperscript{5} inoculated L. monocytogenes, 67% of WT mice died by day 6 compared with no death for Igh\textsuperscript{KO} mice (Fig. 1A). A 5-fold higher L. monocytogenes inoculum was required for Igh\textsuperscript{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\textsuperscript{KO} mice, respectively (Fig. 1B, 1C).

L. monocytogenes-infected Igh\textsuperscript{KO} mice show decreased II-10 production

To gain understanding of possible cytokine contribution to the decreased bacterial load in L. monocytogenes-infected Igh\textsuperscript{KO} mice, we examined the expression of Ifn-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 Tinfl-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-
tential load in the liver was seen on day 3 post- 
*L. monocytogenes* infection. A negative correlation between 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 Il-10 and Ifn-γ measurements in subsequent studies.

We next determined spleen and liver tissue Ifn-γ and Il-10 of 
*L. monocytogenes* -infected WT and Ifg-KO mice (Fig. 2C). Spleen tissue Il-10 was significantly higher for 
*L. monocytogenes*-infected WT mice than for Ifg-KO mice. On the contrary, spleen tissue Ifn-γ was significantly higher for 
*L. monocytogenes*-infected Ifg-KO mice than for WT mice (Fig. 2C). No significant difference in either tissue Il-10 or Ifn-γ was observed for the liver of 
*L. monocytogenes*-infected WT and Ifg-KO mice. Because B cells are missing in Ifg-KO mice, we tested the ability of B cells to produce Il-10 in response to 
*L. monocytogenes* stimulation. In the absence of added accessory cells, MZB cells produced readily detectable Il-10 upon stimulation HK 
*L. monocytogenes* in a dose-dependent manner (Fig. 2D). The amount of Il-10 produced by MZB cells was significantly augmented when they were stimulated in the presence of Il-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 Il-10 than did MZB cells; addition of Il-10–KO accessory cells did not increase their level of Il-10 production. On their own, FOB cells showed extremely poor Il-10 production response to HK 
*L. monocytogenes*; although addition of Il-10–KO accessory cells did promote weak Il-10 production. In the presence of Il-10–KO accessory cells, the amount of Il-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. Il-10 production was triggered through Tlr-2 and Tlr-4, as highly striking and significant reductions in Il-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 Il-10 production**

On the basis of our finding that MZB cells are the most potent Il-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 Il-10–producing cells in response to 
*L. monocytogenes* infection. If so, the highly 
*L. monocytogenes*-resistant nature of Rag-1–KO and Il-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 Il-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 Il-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 periartherial 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, Il-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 Il-10–KO mice was highly significant and consistent with previously published findings (5, 6). Adoptive transfer of MZB cells into Il-10–KO hosts caused an increase in 
*L. monocytogenes* load in the spleen that was 13.2-fold that of the Il-10–KO control group (Fig. 3B). This increase in bacterial load in the spleen was accompanied by increased Il-10 and decreased Ifn-γ production. On the contrary, adoptive transfer of MZB cells into Il-10–KO hosts had no effect on bacterial load and Il-10/Ifn-γ production in the liver. Even though significant increases in bacterial load and Il-10 production in the spleen were observed following 
*L. monocytogenes* infection of Rag-1–KO and Il-10–KO hosts that had been adoptively transferred with MZB cells, the increased levels in bacterial load and Il-10 production did not reach those seen in 
*L. monocytogenes*-infected WT mice.
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., 10⁴ 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., 10⁴ 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 (10⁵ cells per well per 0.2 ml), with or without addition of IL-10–KO AC (10⁵ cells per well per 0.2 ml), then stimulated with high dose *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 HK *L. monocytogenes* stimulation, respectively; MZB versus non-B/T, *p* = 0.126, 0.615, and 0.139 at high-, intermediate-, and low-dose HK *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. (E) MZB cells from WT, Tlr2–KO, and Tlr4–KO mice were isolated by FACS, placed into culture (10⁵ cells per well per 0.2 ml) in the presence of added IL-10–KO accessory cells, and stimulated by high dose *L. monocytogenes* as in (D). IL-10 produced 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 IL-10−/− hosts that received no donor cells, and then observed an inhibitory effect exerted by MZB cells on innate immunity against 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 IL-10−/− 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 Rbpj-cKO mice display increased resistance to L. monocytogenes infection

Rbpj-cKO mice, conditional knockout (cKO) mice with B cell-specific Rbpj deletion, had been shown to possess normal numbers of FOB cells, but highly deficient MZB cells (11). We subjected Rbpj-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 Rbpj-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 Rbpj-cKO mice, as the bacterial load for WT mice was 4.1-fold that for Rbpj-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-\( \alpha \), 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 \( \sim \)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 (\( p = 0.0495 \)).

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

To more directly address the relatedness between Ifn-\( \alpha/\beta \) and the II-10 detrimental pathway in L. monocytogenes infection, we

**FIGURE 4.** MZB cell-deficient Rbpj-cKO mice are more resistant to L. monocytogenes infection. (A) WT mice \( (n = 8) \) and Rbpj-cKO mice \( (n = 10) \) mice were infected with L. monocytogenes \( (10^4 \text{ CFU per mouse}) \), and their survival was monitored over a 30-day observation period. Significant statistical difference in survival \( (p < 0.05, \text{ log-rank test}) \) was observed. (B and C) WT \( (n = 8) \) and MZB cell-deficient Rbpj-cKO mice \( (n = 12) \) were infected by L. monocytogenes \( (10^4 \text{ CFU per mouse}) \). On day 1.5 post L. monocytogenes infection, spleens and livers from 4 WT and 6 Rbpj-cKO mice were removed and homogenized, and bacterial loads were determined (B). On day 3 post L. monocytogenes infection, spleens and livers from the remaining 4 WT and 6 Rbpj-cKO mice were removed and homogenized, and bacterial loads were determined (C). (D) Three groups of host mice were set up. The first group \( (n = 3) \) consisted of control WT mice that did not receive any donor cells. The second group \( (n = 3) \) consisted of Rbpj-cKO mice that did not receive any donor cells. The third group \( (n = 3) \) consisted of Rbpj-cKO hosts, each of which received an adoptive transfer of WT MZB cells \( (2 \times 10^6 \text{ cells per host}) \). At 1 d following cell transfer, all mice were subjected to L. monocytogenes infection \( (10^6 \text{ CFU per mouse}) \). On day 3 post L. monocytogenes infection, spleens and livers were removed, and bacterial loads were individually determined. Data (mean \( \pm \) SD) shown in (B), (C), and (D) are typical of one of two independently performed experiments; they were also analyzed by unpaired Student \( t \) test. *\( p < 0.05 \), **\( p < 0.01 \).

**FIGURE 5.** Similar Ifn-\( \alpha \) production for L. monocytogenes-infected WT and Rbpj-cKO mice. WT and Rbpj-cKO mice were subjected to L. monocytogenes infection \( (10^6 \text{ CFU per mouse}) \). On day 1 and day 3 post L. monocytogenes infection, spleens and livers from WT \( (n = 3) \), and Rbpj-cKO mice \( (n = 3) \), and Rbpj-cKO mice \( (n = 3) \) were removed, and tissue Ifn-\( \alpha \) was individually determined. On day 1.5 and day 3 post L. monocytogenes infection, spleens and livers from WT \( (1.5, n = 5; \text{ day 3, } n = 5) \), and Rbpj-cKO mice \( (1.5, n = 5; \text{ day 3, } n = 5) \), respectively, were removed, and tissue II-10 and Ifn-\( \gamma \) were individually determined. Data for tissue cytokines for the relatively early (day 1 and day 1.5) and late (day 3) time points are shown in (A) and (B), respectively. Data (mean \( \pm \) SD) shown are typical of one of two independently performed experiments; they were also analyzed by unpaired Student \( t \) test. *\( p < 0.05 \), **\( p < 0.01 \). nd, None detected.
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 WT mice (Fig. 1). Administration of anti-II-10 mAb further and significantly reduced bacterial load in *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 bloodstream 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 on sensitizing lymphocytes to listeriolysin 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 Rbpj-cKO mice displayed increased resistance against *L. monocytogenes* infection. As the MZB cell-deficient mice were generated by conditional deletion of Rbpj 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 Rbpj-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 Rbpj-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 Rbpj-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 Rbpj-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 Ifgk-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 Tip-DC, 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., 10^6 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.](image-url)
creased numbers of Tip-DCs in *L. monocytogenes*-infected II-10–KO and Rbpj-cKO mice. On the contrary, we observed, in preliminary experiments, reduced numbers of Tip-DCs in *L. monocytogenes*-infected II-10–KO and Rbpj-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 bloodstream *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 metallorphic 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 Rbpj-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, Rbpj-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 Rbpj-cKO mice that are highly deficient in MZB cells, but not FOB cells. Indeed, *L. monocytogenes* infection of Rbpj-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, Rbpj-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 Rbpj-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 adaptively transferred into Rag-1–KO, II-10–KO, and Rbpj-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 adaptively 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
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References


