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Innate Response Activator B Cells Protect Against Microbial Sepsis

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Abstract

Recognition and clearance of bacterial infection is a fundamental property of innate immunity. Here we describe an effector B cell population that protects against microbial sepsis. Innate response activator (IRA)-B cells are phenotypically and functionally distinct, develop and diverge from B1a B cells, depend on pattern recognition receptors, and produce GM-CSF. Specific deletion of IRA-B cell activity impairs bacterial clearance, elicits a cytokine storm, and precipitates septic shock. These observations enrich our understanding of innate immunity, position IRA-B cells as gatekeepers of bacterial infection, and identify new treatment avenues for infectious diseases.

Sepsis is characterized by whole-body inflammation to overwhelming infection (1). Over the last thirty years, sepsis' incidence has risen, indicating a need for a better understanding of its complex pathophysiology (2, 3). The growth factor granulocyte macrophage colony stimulating factor (GM-CSF) elicits multiple changes in cells expressing its cognate receptor. Yet, despite GM-CSF's multiple functions and known relationship with innate leukocytes, its in vivo cellular source and role in sepsis remain uncertain (4).

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Supporting Online Material

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Materials and Methods

Figs. S1 to S12

Table S1

References (29–36)

Profiling of GM-CSF expression by flow cytometry led to a surprising observation. Among the organs, the bone marrow and spleen contained the majority of GM-CSF⁺ cells in the steady state ($1.0 \pm 0.1 \times 10^6$ and $2.9 \pm 0.8 \times 10^5$ cells, respectively) (Fig. 1A) (5). In response to lipopolysaccharide (LPS), a component of gram negative bacteria, GM-CSF⁺ cells increased in number preferentially in the spleen ($3.2 \pm 0.2 \times 10^6$ cells), and were predominantly B220⁺ MHCII⁺ CD19⁺ IgM⁺ B cells (Fig. 1B and fig. S1, A and B). This is surprising because GM-CSF is believed to be produced in vivo by non-hematopoietic cells, macrophages, and, in some cases, T cells (4, 6). Nevertheless, B cells constituted the largest GM-CSF⁺ population under these conditions (fig. S1C), a finding that we confirmed by Western blot analysis (Fig. 1C). We named these B cells innate response activator (IRA) B cells because of GM-CSF's known role in activating innate leukocytes. Numerous IRA-B cells accumulated in the spleen in a mouse model of sepsis (fig. S2, A and B) (7) and in response to *Escherichia coli* infection (fig. S2C), indicating that IRA-B cell expansion is a general feature of the body's response to bacteria. In humans, we detected CD19⁺ CD20⁺ IRA-B cells expressing varying levels of CD43, CD27 (fig. S2, D and E), and CD284 (TLR4) (fig. S2F) (8). We therefore elected to characterize murine IRA-B cells in more detail.

Immunofluorescence of spleen sections from LPS recipients co-localized the GM-CSF signal with round mononuclear cells expressing IgM, B220, PAX5, and CD19 (Fig. 1D and fig. S1D) in the red pulp (Fig. 1, E and F). RT-PCR experiments conducted on sorted cells and unprocessed tissue from wild type or B cell-deficient μ MT mice indicated that B cells produce GM-CSF (Fig. 1G). Serum GM-CSF levels were negligible (i.e., below the 7.8 pg/ml detection limit of the assay), a finding that is consistent with the observation that GM-CSF is rapidly removed through receptor-mediated clearance (9). Collectively, these data indicate that inflammation expands the IRA-B cell population in vivo.

B cells are linked developmentally, reside in different regions, and mediate distinct functions (10–14). We profiled IRA-B cells according to several well-established methods (13, 15, 16). Our experiments revealed that (CD19⁺ B220⁺ MHCII⁺ GM-CSF⁺) IRA-B cells are phenotypically unique. They are: IgM^{high} CD23^{low} CD43^{high} CD93⁺ (Fig. 2, A and B, and fig. S3A); IgD^{low} CD21^{low} (fig. S3B); CD138⁺ VLA4^{high} LFA1^{high} CD284⁺ (Fig. 2C and fig. S3, C and D); and CD5^{int} (fig. S3, E and F). IRA-B cells contained large stores of intracellular IgM (fig. S4A) and spontaneously secreted IgM, but not IgA or IgG₁ (fig. S4, B and C). In addition to GM-CSF, IRA-B cells produced IL-3 but not pro-IL-1 β , IL-6, and TNF α (fig. S4D). We failed to detect IL-10 expression by IRA-B cells in any of the conditions. Thus, IRA-B cells have a unique B cell phenotype and are functionally distinct from other B cells, including the recently described IL-10-producing B10 B cells (17).

The ability to sort IRA-B cells according to their surface phenotype (fig. S5A) allowed us to profile their transcriptome. Unsupervised hierarchical clustering (Fig. 2D) and principal component analysis (PCA) (Fig. 2E) grouped IRA-B cells in a separate population from T1, FO, MZ, B1a and PC. IRA-B cells also gave rise to a unique transcriptome signature (fig. S5, B to D, and table S1), and expressed genes relevant to B cell biology (fig. S5D).

To decipher where IRA-B cells fit in the B cell lineage we performed several parabiosis and fate-mapping studies. First, we reasoned that if IRA-B cells derive from a circulating precursor they should have high chimerism in a parabiosis setting. Joining CD45.1⁺ with CD45.2⁺ mice revealed high chimerism among IRA-B cells (Fig. 3A), T1 and FO B cells (fig. S6A), but markedly lower chimerism for the spleen-resident MZ B cells and their precursors (fig. S6A). Thus, IRA-B cells derive from a circulating cell.

Second, to identify the IRA-B cell precursor, we adoptively transferred B cell subsets to mice receiving LPS for 3 days (fig. S6, B to E). Among the subsets (splenic T1, FO, MZ, B1 and peritoneal B1a, B1b, B2) only peritoneal B1 B cells (Fig. 3B) gave rise to IRA-B cells. Of these, B1a B cells were the dominant precursor. B1a-derived IRA-B cells readily proliferated (fig. S6E), and developed in the spleen after relocating from the peritoneum (fig. S7). These findings confirm that B1a B cells travel to the spleen in response to peritoneal TLR stimuli (18, 19), and indicate that, upon splenic accumulation, B1a B cells can differentiate to IRA-B cells.

The ontogenic relationship between B1a and IRA-B cells raised the question whether IRA-B cells constitute a distinct subset. To elucidate this, we first placed peritoneal B1a B cells in culture. In response to LPS, B1a B cells separated into three discrete populations: CD138⁻ cells resembling “unchanged” B1a B cells, and two populations of CD138⁺ cells, IRA-B cells among them (fig. S8A). In vitro, IRA-B cells spontaneously secreted GM-CSF (fig. S8B). Second, we sorted peritoneal B1a B cells, IRA-B cells, and splenic CD43⁺ CD138⁺ cells, and followed their fate in vivo. B1a B cells gave rise to multiple cell types (fig. S9A), including IRA-B and CD43⁺CD138⁺ cells, whereas (CD43^{high} CD138⁺) IRA-B and CD43⁺ CD138⁺ cells remained phenotypically segregated (fig. S9, B and C). The data suggest that B1a B cells give rise to distinct cells. IRA-B cells are a subset of this group.

Surface phenotype and fate-mapping studies, though important, reveal little about function. How IRA-B cells arise was our next question. Expectedly, B cell-deficient μ MT (20) and *Cd19*^{-/-} (21) mice did not develop IRA-B cells (Fig. 3, C and D). Surprisingly, *Tnfrsf13c*^{-/-} mice lacking the B-cell activating factor receptor (BAFFR) failed to generate IRA-B cells; BAFFR is believed to be dispensable to B1 B cells (22). At the level of microbial recognition, mice lacking the LPS receptor TLR4 or its adaptor MyD88, but not TRIF, did not generate IRA-B cells (Fig. 3, C and D), indicating a specific MyD88-dependent pathway. The process could depend on direct B1a binding to LPS via TLR4, or on indirect, extrinsic factors such as TLR4-expressing macrophages. To discriminate between these two possibilities, we adoptively transferred B1a B cells from wt mice into *Tlr4*^{-/-} mice (Fig. 3E). B1a wt B cells, but not endogenous *Tlr4*^{-/-} B cells, differentiated to IRA-B cells, indicating that direct TLR4 signaling on B1a B cells is sufficient to generate IRA-B cells.

To test whether IRA-B cells are restricted to TLR4-mediated recognition, we injected TLR ligands Pam3CSK4 (ligand for TLR1/2), Poly(I:C) (TLR3), FLA-ST (TLR5), FSL-1 (TLR2/6), R848 (TLR7/8), and CpG ODN1668 (TLR9). The ligands Pam3CSK4, FSL-1 and R848 yielded IRA-B cells (fig. S10A), a finding that we confirmed in vitro (fig. S10B). We also wondered whether GM-CSF can play an autocrine role for B1a-IRA-B cell conversion (23). B1a cells expressed *Csf2rβ* (CD131) (fig. S11A) and, when placed in culture with antibodies against CD131, failed to give rise to IRA-B cells (fig. S11, B and C), but remained alive and gave rise to CD43⁺ CD138⁺ cells. Thus, IRA-B cells develop via MyD88-dependent pathways and use GM-CSF as an autocrine factor.

The spleen's open circulation (24) allows blood leukocytes to enter and exit easily. To reside in the spleen, leukocytes resort to adhesive ligands; MZ B cells, for example, rely on VLA-4 and LFA-1 (25). We wondered whether splenic IRA-B cells, which express VLA-4 and LFA-1 at high levels, might behave similarly. Injection of neutralizing antibodies to VLA-4 and LFA-1 diminished IRA-B cell numbers, revealing that, indeed, the two integrins are responsible for retention (Fig. 3F).

Are IRA-B cells functionally important? To answer this, we focused on the cecal ligation and puncture (CLP) sepsis model (26). We generated mixed chimeras by reconstituting lethally irradiated mice with μ MT and GM-CSF-deficient (*Csf2*^{-/-}) bone marrow cells. In

these mice (called GM/ μ MT chimeras), the μ MT marrow contributed all leukocytes except B cells whereas the *Csf2*^{-/-} marrow contributed only *Csf2*^{-/-} cells. Consequently, the only population completely lacking the capacity to produce GM-CSF in the reconstituted mice were B cells. We tested the quality of the chimeras and their controls by PCR (fig. S11, A and B) and by flow cytometry (fig. S11, C and D).

In response to severe CLP, 40% of control mice survived and recovered, but every GM/ μ MT chimera died within 2 days (Fig. 4, A and B). To characterize this phenotype further, we profiled GM/ μ MT chimeras and controls for several sepsis-relevant indices 20 hours after CLP, prior to any mortalities. Compared to IRA-B cell-containing controls (fig. S11E), the peritoneal cavity of GM/ μ MT chimeras had more leukocytes, mostly neutrophils (Fig. 4C), and experienced a severe IL-1 β , IL-6 and TNF α cytokine storm in the serum (Fig. 4D) and peritoneum (Fig. 4E). This inflammatory signature typically associates with a defect in bacterial clearance. Indeed, neutrophils from the GM/ μ MT chimeras phagocytosed bacteria poorly (Fig. 4F). The GM/ μ MT chimeras, moreover, had a modest reduction of serum IgM but not IgG (Fig. 4G), and developed severe liver and lung pathologies (Fig. 4H). Finally, bacterial titre measurements revealed that GM/ μ MT chimeras were more infected than controls (Fig. 4, I and J). Although it is possible that other bone marrow cells contribute GM-CSF for the protection against sepsis in this setting, the most likely explanation is that IRA-B cells protect against septic shock by controlling the organism's ability to clear bacteria.

GM-CSF is a pleiotropic cytokine that influences the production, maturation, function, and survival of its target cells. GM-CSF's role in sepsis has remained elusive because its indiscriminate ablation is protective (27) but its supplementation can be beneficial (28). The *in vivo* identification of GM-CSF-producing B cells illustrates a previously unrecognized locational specificity that dictates the cytokine's function. IRA-B cells differ from other subsets because their pathogen recognition pathways and tissue distribution license GM-CSF expression. The function is important in sepsis and gives rise to questions as to how IRA-B cells participate in other infectious and inflammatory diseases.

Supplementary Material

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Acknowledgments

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Fig. 1.

Innate response activator (IRA) B cells are GM-CSF-producing B cells that increase in number during inflammation. **(A)** Quantification of GM-CSF-producing cells retrieved from tissues in the steady state and in response to 4 daily i.p. injections of LPS (means $\pm$ SEM, $n = 3-5$). $*P < 0.05$. **(B)** Identification of GM-CSF-producing cells in the spleen. Representative plots show percentage of B cells and their production of GM-CSF retrieved from spleens during inflammation. Data represent at least ten independent experiments. **(C)** Western blot for GM-CSF conducted on sorted cells. One of three independent experiments is shown. **(D)** Co-localization of representative GM-CSF-producing cells with IgM. **(E)** Red pulp sections with markers against CD11b (green) and GM-CSF (red) (left panel) and B220 (green) and GM-CSF (red) (right panel). Co-localization of green and red cells is yellow and the scale bar is shown in white. **(F)** Quantification of GM-CSF⁺ B cells and other cells on histological sections of the spleen in the red pulp and white pulp in the steady state and after LPS (means $\pm$ SEM, $n = 3-4$). $*P < 0.05$. **(G)** Splenic GM-CSF expression detected by RT-PCR and conducted on sorted cells and on unprocessed spleen tissue taken from wild type and B cell knockout (μ MT) mice (means $\pm$ SEM, $n = 3-4$). $*P < 0.05$.

Fig. 2.

IRA-B cells are a distinct subset with a unique phenotypic signature. **(A)** Flow cytometric analysis of the phenotype of IRA-B cells. Plots show B cell phenotypes retrieved from spleens during steady state and inflammation. Representative from $n > 10$ is shown. **(B)** Plots show the phenotype of GM-CSF-producing cells in the spleens during inflammation. IRA-B cells are IgM^{high}, CD23^{low} CD43⁺ CD93⁺. **(C)** Plots show the phenotype of IRA-B cells with respect to VLA4 and CD138 expression as determined by flow cytometry. Representative from $n > 5$ is shown. **(D)** Hierarchical clustering dendrogram based on whole-genome microarray data of sorted samples of B cell subsets retrieved from LPS-treated animals and steady-state B1a. **(E)** Principal Component Analysis (PCA) of the different cell subsets shown in (D).

Fig. 3.

IRA-B cells develop from B1a B cells via TLR4/MyD88 and reside in tissue through LFA-1/VLA-4. (A) Flow cytometric analysis of the percent chimerism is shown in spleens of CD45.1⁺ mice that had been in parabiosis with CD45.2⁺ mice for 3 weeks prior to LPS injection. Mice were sacrificed 2 days after LPS injection. Representative plots from two independent experiments are shown. (B) Adoptive transfer of peritoneal B1a B cells yields IRA-B cells. Cells from steady state CD45.2⁺ mice were transferred to CD45.1⁺ mice that then received LPS for 3 days. Animals were analyzed 72 hours after transfer. Representative plots from flow cytometric analysis of $n = 4-5$ mice are shown. (C) Flow cytometric analysis of the development of IRA-B cells in *Tlr4*^{-/-}, *Myd88*^{-/-}, *Ticam1*^{-/-} (the gene that encodes TRIF), μ MT, *Tnfrsf13c*^{-/-} (the gene that encodes BAFRR), and *Cd19*^{-/-} mice. Representative plots from $n = 4$ mice are shown. (D) Enumeration of IRA-B cells in steady state and inflammation in wt (C57BL/6) mice and in the mice shown in (D) (means $\pm$ SEM, $n = 4-10$). * $P < 0.05$. (E) Flow cytometric analysis of the adoptive transfer of CD45.1⁺ B1a cells into congenic *Tlr4*^{-/-} CD45.2⁺ mice injected with LPS. Representative from $n = 3$ mice is shown. (F) Flow cytometric analysis of the effect of blocking VLA-4/LFA-1 on IRA-B cell retention in the spleen. Representative from $n = 3$ mice is shown.

Fig. 4.

IRA-B cells protect against polymicrobial sepsis. **(A)** Generation of mixed chimeras (GM/μMT). **(B)** Kaplan-Meier curve showing survival of GM/μMT and control animals after cecal ligation and puncture (CLP). $n = 10$ – 20 /group. **(C)** Enumeration of total leukocytes and neutrophils in the peritoneum of GM/μMT (dark red) and control (black bars) mice 20 h after CLP. **(D)** Serum levels and **(E)** peritoneal levels of inflammatory cytokines in GM/μMT (dark red) and control (black bars) mice 20 h after CLP. **(F)** Ex vivo phagocytosis assay showing capacity of neutrophils to phagocytose *E. coli* from GM/μMT (dark red) and control (black bars) mice 20 h after CLP. **(G)** serum levels of IgM and IgG 20 h after CLP in same groups as above. **(H)** Representative H&E stain of liver and lung sections 20 h after CLP in same groups as above. **(I)** Blood from GM/μMT and control mice 20 h after CLP was plated for 1 day. Representative plate shows bacterial colonies. **(J)** Enumeration of bacteremia in the peritoneum and blood of GM/μMT (dark red) and control (black bars) mice 20 h after CLP. $*P < 0.05$ [means $\pm$ SEM, $n = 10$ – 20 /group for (C)–(G), (J). Four independent experiments were performed and data were grouped].