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Identification of IFN-γ-producing innate B cells

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Although B cells play important roles in the humoral immune response and the regulation of adaptive immunity, B cell subpopulations with unique phenotypes, particularly those with non-classical immune functions, should be further investigated. By challenging mice with Listeria monocytogenes, *Escherichia coli*, vesicular stomatitis virus and Toll-like receptor ligands, we identified an inducible CD11a^{hi}FcγRIII^{hi} B cell subpopulation that is significantly expanded and produces high levels of IFN-γ during the early stage of the immune response. This subpopulation of B cells can promote macrophage activation via generating IFN-γ, thereby facilitating the innate immune response against intracellular bacterial infection. As this new subpopulation is of B cell origin and exhibits the phenotypic characteristics of B cells, we designated these cells as IFN-γ-producing innate B cells. Dendritic cells were essential for the inducible generation of these innate B cells from the follicular B cells via CD40L-CD40 ligation. Increased Bruton's tyrosine kinase activation was found to be responsible for the increased activation of non-canonical NF-κB pathway in these innate B cells after CD40 ligation, with the consequent induction of additional IFN-γ production. The identification of this new population of innate B cells may contribute to a better understanding of B cell functions in anti-infection immune responses and immune regulation.

***Keywords*:** B cells; IFN-γ; innate response; dendritic cells; CD40

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Introduction

B lymphocytes are known to dominate the humoral immunity by producing antibodies, and are also involved in opsonization and complement fixation. B cells have also been shown to play important roles in the induction and regulation of T cell immune responses through antigen presentation and optimal CD4$^+$ T cell activation, thus contributing to the differentiation of naïve CD4$^+$ T cells and the polarization of T helper 1 (Th1) and T helper 2 (Th2) subsets [1, 2]. B cell subsets with different characteristics, such as B1 B cells, follicular B (FO B) cells and marginal zone B (MZ B) cells, have been identified and extensively investigated in past decades [3-6]. Recently, B cells have been reported to be able to mediate antibody-independent functions, mainly by secreting different types of cytokines [2, 7, 8]. Furthermore, additional B cell subsets with distinctive cytokine-secreting profiles have been characterized [8-11]. For example, regulatory IL-10-producing CD1d^{hi}CD5$^+$ B cells (named B10 cells) suppress the CD4$^+$ T cell-mediated contact hypersensitivity reaction and prevent the induction of autoimmune diseases in several mouse models [2, 10-13]. It has also recently been demonstrated that B cells are the relevant source of IL-17 induced by *Trypanosoma. cruzi* trans-sialidase via a unique pathway that is independent of the transcription factor RORγt [14]. Additionally, B10 and even CD40-activated B cells can induce the generation of both CD4$^+$ and CD8$^+$ regulatory T cells, which subsequently control the immune response [13, 15, 16]. Increasing evidence from clinical observations and basic research reveals the great heterogeneity of B cells, indicating that, in addition to B10 cells, there are likely more cytokine-producing subsets of B cells that exert multiple antibody-independent, non-classical functions

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during pathological processes than previously thought. For example, the innate function of B cells has recently attracted considerable attention, and further investigation is necessary to examine the existence of unidentified B cell subsets, particularly in the innate immune response against infection.

Dendritic cells (DCs) are the most potent professional antigen (Ag)-presenting cells in the initiation and control of the T cell adaptive immune response against pathogen infection, and are able to regulate the functions of different types of lymphocytes. With regard to DC-B cell interactions, it is reported that different DC populations can influence the development, proliferation and activation of B cells through various mechanisms. For example, activated mature DCs enhance B cell activation and differentiation by providing a series of cytokines, such as B cell-activating factors and proliferation-inducing ligands [17, 18]. Mouse immature bone marrow (BM)-derived DCs can suppress anti-IgM-induced B cell activation and enhance the Ag-induced apoptotic response of the BM-derived B cells [17]. In addition, $CD11c^{lo}$ immature DCs provide critical survival signals to Ag-specific MZ B cells and promote their differentiation into the IgM-secreting plasmablasts [19]. Our recent study also showed that regulatory DCs can program B cells to differentiate into $CD19^{hi}Fc\gamma RIIb^{hi}$ regulatory B cells through IFN-β and CD40L [20]. Although many studies have been performed to investigate the relationship between DC and B cells, there is still no direct evidence as to whether DCs are capable of regulating the differentiation and functions of B cells during the innate defense against pathogens.

Interferons (IFNs), both type I (IFN-α/β) and type II (IFN-γ), have multiple functions in innate and adaptive immune responses, and the efficient induction of IFN-α/β production to eliminate an invading virus is an active topic in infection and immunity research. Indeed, many efforts have been made to elucidate the molecular mechanisms for IFN-α/β production against viral infection via the Toll-like receptor (TLR) or RIG-I pathway in the last decade [21-24]; however, the mechanisms for IFN-γ production during the innate immune response remain unclear to date. IFN-γ, which is considered to be mainly produced by NK cells and $CD4^+$ T cells, can strengthen innate immunity via induction of antimicrobial factors or degradative pathways in other immune cells, such as macrophages. IFN-γ directly inhibits viral replication and activates immune responses for the elimination of viruses, thus protecting the host against virus-induced pathogenesis and lethality [25]. IFN-γ is essential for controlling intracellular bacterial infection; for example, mice deficient in IFN-γ or its cognate receptors are more susceptible to *Listeria monocytogenes* (LM) infection [26, 27]. Our previous studies also showed that the Th1 cytokines IFN-γ and IL-18 can protect the host against chronic parasite infection [28, 29]. Considering the important role of IFN-γ in the innate immune response against intracellular infection and in the regulation of adaptive immune responses, it is of great significance to identify new types of immune cells that can produce high levels of IFN-γ during infection, and to comprehensively investigate the function and underlying mechanisms of IFN-γ-producing cells in innate immunity.

In this study, we challenged mice with pathogens including LM, *Escherichia coli* (*E. coli*) and vesicular stomatitis virus (VSV), or TLR ligands, and then analyzed the phenotypic changes of B cells expanded *in vivo*. Using this approach, we identified a pathogen-inducible $CD11a^{hi}Fc\gamma RIII^{hi}CD19^+$ cell subpopulation during the early stage of the immune response, which has a B cell origin with an FO B cell-like phenotype and a unique cytokine profile with high production of IFN-γ. The pathogen-expanded new subpopulation of B cells can promote innate responses against intracellular bacterial infection via generating IFN-γ through a feedback mechanism. Our results contribute to a better understanding of B cell immunobiology and provide mechanistic insight into the role of IFN-γ in the innate immune response.

Results

Mouse $CD11a^{hi}Fc\gamma RIII^{hi}CD19^+$ cells expand in response to pathogen infection and TLR ligand challenge

To investigate the function of B cells in the innate immune response against infection, we analyzed the phenotypic changes of B cells from LM-infected mice. Interestingly, a subset of $CD11a^{hi}CD16/CD32^{hi}CD19^+$ splenic cells was found to be significantly increased in the LM-infected mice (Figure 1A). As the available anti-CD16/CD32 mAb could recognize activating receptor FcγRIII (CD16) and inhibitory receptor FcγRIIb (CD32b), we purified the $CD11a^{hi}CD16/CD32^{hi}CD19^+$ B cell subset and $CD11a^{lo}CD16/CD32^{lo}CD19^+$ conventional B cells and analyzed the mRNA levels of FcγRIIb and FcγRIII in each population. The expression of FcγRIII, but not FcγRIIb, in the pathogen-induced $CD11a^{hi}CD16/CD32^{hi}CD19^+$ cells was upregulated more significantly than in the $CD11a^{lo}CD16/CD32^{lo}$ conventional B cells (Figure 1B), suggesting that the FcγRIII is overexpressed on $CD11a^{hi}CD16/CD32^{hi}CD19^+$ cells. Therefore, LM infection induced a distinct new population of $CD19^+$ B cells, $CD11a^{hi}Fc\gamma RIII^{hi}CD19^+$ cells, in the spleens of C57BL/6 mice.

The number of $CD11a^{hi}Fc\gamma RIII^{hi}CD19^+$ cells in the spleen started to increase on day 2, reached the

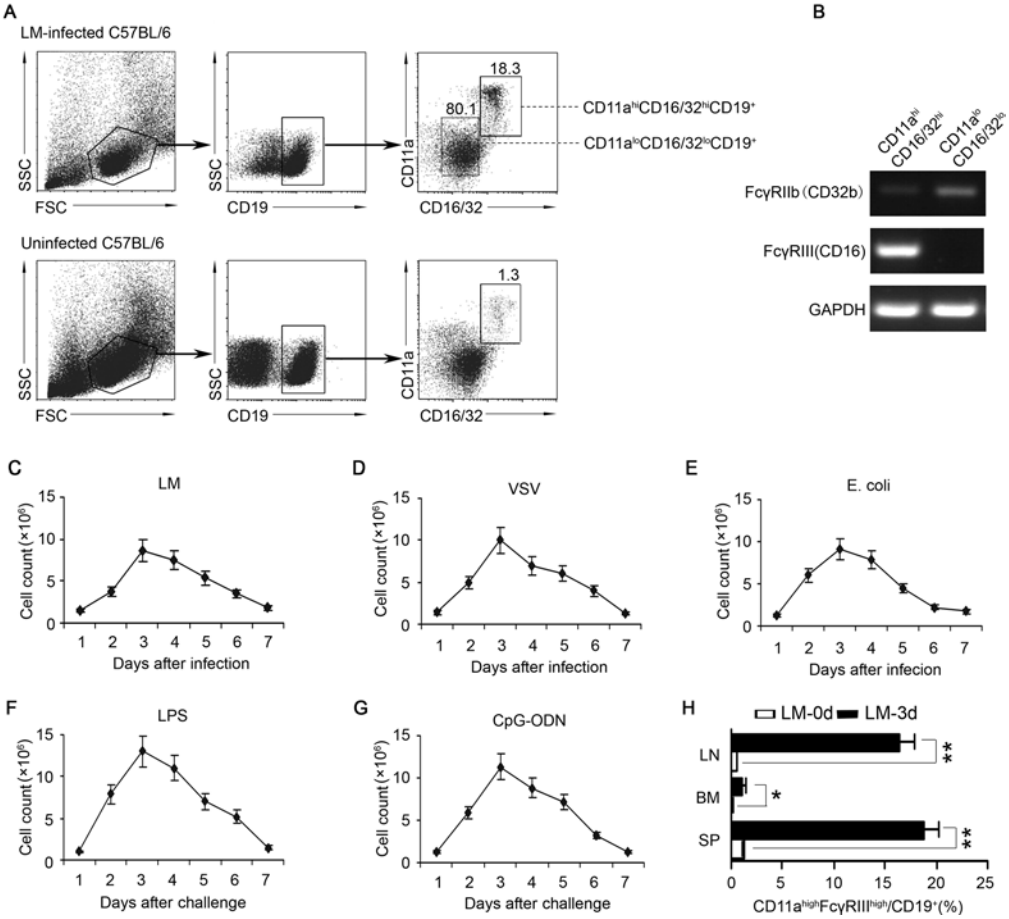

Figure 1 Generation of CD11a^{hi}FcγRIII^{hi}CD19^{+} cells in mice infected with pathogens or challenged with TLR ligands. Naïve C57BL/6 mice were i.p. infected with 2 × 10^{6} LM **(A-C**, **H)**, 5 × 10^{6} PFU VSV **(D)**, or 1 × 10^{6} *E. coli* **(E)**. **(A)** Splenocytes were isolated on day 3 post-infection, and the percentage of CD11a^{hi}FcγRIII^{hi}CD19^{+} cells in the CD19^{+} B cells was analyzed. **(B)** The FcγRIIb and FcγRIII mRNA expression of splenic CD11a^{hi}FcγRIII^{hi} CD19^{+} or CD11a^{lo}FcγRIII^{lo} CD19^{+} cells was assessed by RT-PCR. The transcript of the mouse *GAPDH* gene was used as an amplification control. **(C**-**E)** The number of CD11a^{hi}FcγRIII^{hi} B cells in 10^{8} splenocytes was examined within 7 days after infection with LM **(C)**, VSV **(D)**, and *E. coli* **(E)**. **(F**, **G)** Naïve C57BL/6 mice were i.p. injected with LPS (0.5 mg/kg weight) **(F)** or CpG-ODN (2.5 mg/kg weight) **(G)**. The numbers of CD11a^{hi}FcγRIII^{hi} B cells in 10^{8} splenocytes were dynamically examined within 7 days after the challenge. **(H)** The percentages of CD11a^{hi}FcγRIII^{hi} B cells in the CD19^{+} B cells in the lymph nodes (LN), spleens (SP), and BM were analyzed on day 0 or day 3 post-LM infection. Data shown represent the mean ± SD. $^{**}P < 0.01$, $^{*}P < 0.05$.

peak on day 3, and then declined gradually until day 7 after LM infection (Figure 1C). Similarly, splenic CD11a^{hi}FcγRIII^{hi}CD19^{+} cells were also expanded in mice infected with VSV and *E. coli* (Figure 1D and 1E). After being challenged with TLR ligands, such as Lipopolysaccharide (LPS) and CpG-ODN, the number of splenic CD11a^{hi}FcγRIII^{hi}CD19^{+} cells increased rapidly, peaking on day 3 after the challenge and decreasing during the ensuing 4 days (Figure 1F and 1G).

To further investigate whether the CD11a^{hi} FcγRIII^{hi}CD19^{+} cells were widely distributed in other lymph organs in the innate response, we collected mononuclear cells from the lymph nodes and BM of C57BL/6 mice 3 days after LM infection. The data showed that CD11a^{hi}FcγRIII^{hi}CD19^{+} cells were also significantly expanded in the mesenteric lymph nodes and slightly increased in the BM (Figure 1H). Therefore, microbial infection could induce the systemic expansion of a new population of CD11a^{hi}FcγRIII^{hi}CD19^{+} cells in both central and peripheral lymph organs during the early period of the immune response.

CD11a^{hi}FcγRIII^{hi}CD19^{+} cells originate from FO B cells

We then further characterized the origin of these

CD11a^{hi}FcγRIII^{hi}CD19^{+} cells. Electron microscopy of splenic CD11a^{hi}FcγRIII^{hi}CD19^{+} cells revealed a typical lymphocytic morphology. The cells had a diameter of 6-8 μm and a smooth and round shape, with a compact nucleus, limited amounts of cytoplasm, abundant mitochondria and an extensive Golgi apparatus (Figure 2A).

We performed a microarray analysis of the CD11a^{hi}FcγRIII^{hi} B cells and conventional CD11a^{lo} FcγRIII^{-} B cells derived from LM-infected mice to identify the specific transcriptome and gene signature of each type of cells. Differentially expressed genes in the CD11a^{hi}FcγRIII^{hi} B cells relative to conventional B cells were then selected for an unsupervised hierarchical cluster analysis. Transcriptional similarity was observed between the CD11a^{hi}FcγRIII^{hi} and conventional B cells, indicating a close relationship between these cells (Figure 2B). Because cellular antigens and transcription factors may reflect and determine the identity of specific cell subsets, we clustered these types of genes that were highly expressed in the CD11a^{hi}FcγRIII^{hi} B cells, and similarity was also observed between the CD11a^{hi}FcγRIII^{hi} and conventional B cells. However, the CD11a^{hi}FcγRIII^{hi} B cells did express their own cellular antigens including fcer1a, cxcr3 and ifitm1, and transcription factors including gata2, mef2b and csrp2 (Figure 2C and 2D). These data suggested that CD11a^{hi}FcγRIII^{hi} B cells have a transcriptional pattern that is similar to that of the conventional B cells, while these cells have their own transcriptional signature as a specific cell subset.

To further elucidate the origin and specific function of these new pathogen-expanded B cells, we selected three sets of genes that are highly expressed in both CD11a^{hi}FcγRIII^{hi} and conventional B cells, and performed an immune-related pathway enrichment analysis. The CD11a^{hi}FcγRIII^{hi} B cell-specific transcripts were mostly enriched in the group of cytokines and cytokine receptors, suggesting that CD11a^{hi}FcγRIII^{hi} B cells may exert their specific function by secreting cytokines (Supplementary information, Table S1).

To uncover the functional relationship between the CD11a^{hi}FcγRIII^{hi} and conventional B cells, genes with high expression in CD11a^{hi}FcγRIII^{hi} B cells were chosen for a cluster analysis with regard to four B cell-related pathways in the KEGG database. These results also showed a close relationship between the CD11a^{hi}FcγRIII^{hi} and conventional B cells (Figure 2E-2H). Furthermore, the CD11a^{hi}FcγRIII^{hi} and conventional B cells had their own unique highly transcribed genes in these pathways.

In addition to high expression of both CD11a and FcγRIII on the cell surface, CD11a^{hi}FcγRIII^{hi} B cells displayed a unique phenotype of mIgM^{int}mIgD^{hi}B220^{hi}CD40^{hi}CD40L^{hi}CD5^{-}CD11b^{-}CD43^{lo}CD80^{-}CD86^{lo}I-A/I-E^{lo}CD21^{lo}CD23^{hi} (Figure 2I), which is similar to that of FO B cells (mIgM^{int}mIgD^{hi}CD21^{lo}CD23^{hi}). However, the CD11a^{hi}FcγRIII^{hi} B cells displayed reduced expression of MHC-II and co-stimulation molecules compared to FO B cells, indicating a weak role in T cell-related adaptive immunity. Moreover, after LPS stimulation, the CD11a^{hi}FcγRIII^{hi} B cells secreted IgG2a, IgG2b, IgG3 and IgM, though at relatively lower levels compared to the conventional B cells (Figure 2J). Together with the observation that CD11a^{hi}FcγRIII^{hi} B cells can be induced from FO B cells *in vitro* when co-cultured with DCs in the presence of pathogen components (see results shown later), these results indicate that CD11a^{hi}FcγRIII^{hi} B cells with unique phenotypic and functional characteristics originate from FO B cells in the innate response.

DCs induce generation of CD11a^{hi}FcγRIII^{hi} B cells from FO B cells through CD40L-CD40 ligation

Upon recognition of invading pathogens, professional Ag-presenting cells, including macrophages and DCs, will become activated or undergo maturation, and then provide activating signals to T, B and NK cells, which jointly contribute to a full activation of immune response against infection. In order to investigate what kinds of immune cells and molecule(s) might be responsible for the peripheral expansion of CD11a^{hi}FcγRIII^{hi} B cells in response to pathogens, we isolated splenic CD19^{+} B cells from wild-type (WT) mice and examined their conversion into CD11a^{hi}FcγRIII^{hi} B cells in the *in vitro* co-culture systems with different kinds of immune cells in the presence of heat-killed LM (HKLM). We found that CD19^{+} B cells alone could not be converted into CD11a^{hi}FcγRIII^{hi} B cells in response to HKLM stimulation (Figure 3A). When co-cultured with DCs, but not with NK cells, macrophages, CD4^{+} or CD8^{+} T cells, CD19^{+} B cells could be converted into CD11a^{hi}FcγRIII^{hi} B cells in response to HKLM stimulation (Figure 3A). To identify which B cell subset can differentiate into CD11a^{hi}FcγRIII^{hi} B cells, purified FO B, MZ B or B1 B cells were co-cultured with DCs in the presence of HKLM. We found that CD11a^{hi}FcγRIII^{hi} B cells could be induced from FO B cells *in vitro* (Figure 3B).

Once separated by a transwell system, DCs could not induce the generation of CD11a^{hi}FcγRIII^{hi} B cells from the FO B cells (Figure 3C), indicating that DCs induce the generation of CD11a^{hi}FcγRIII^{hi} B cells via cell-cell contact. The cross-talk between DCs and NK cells or B cells has been extensively investigated, and the CD40/CD40L interaction has been shown to be critical for cross-activation. We found that a neutralizing anti-CD40 or anti-CD40L antibody significantly inhibited the HKLM-induced generation of CD11a^{hi}FcγRIII^{hi} B

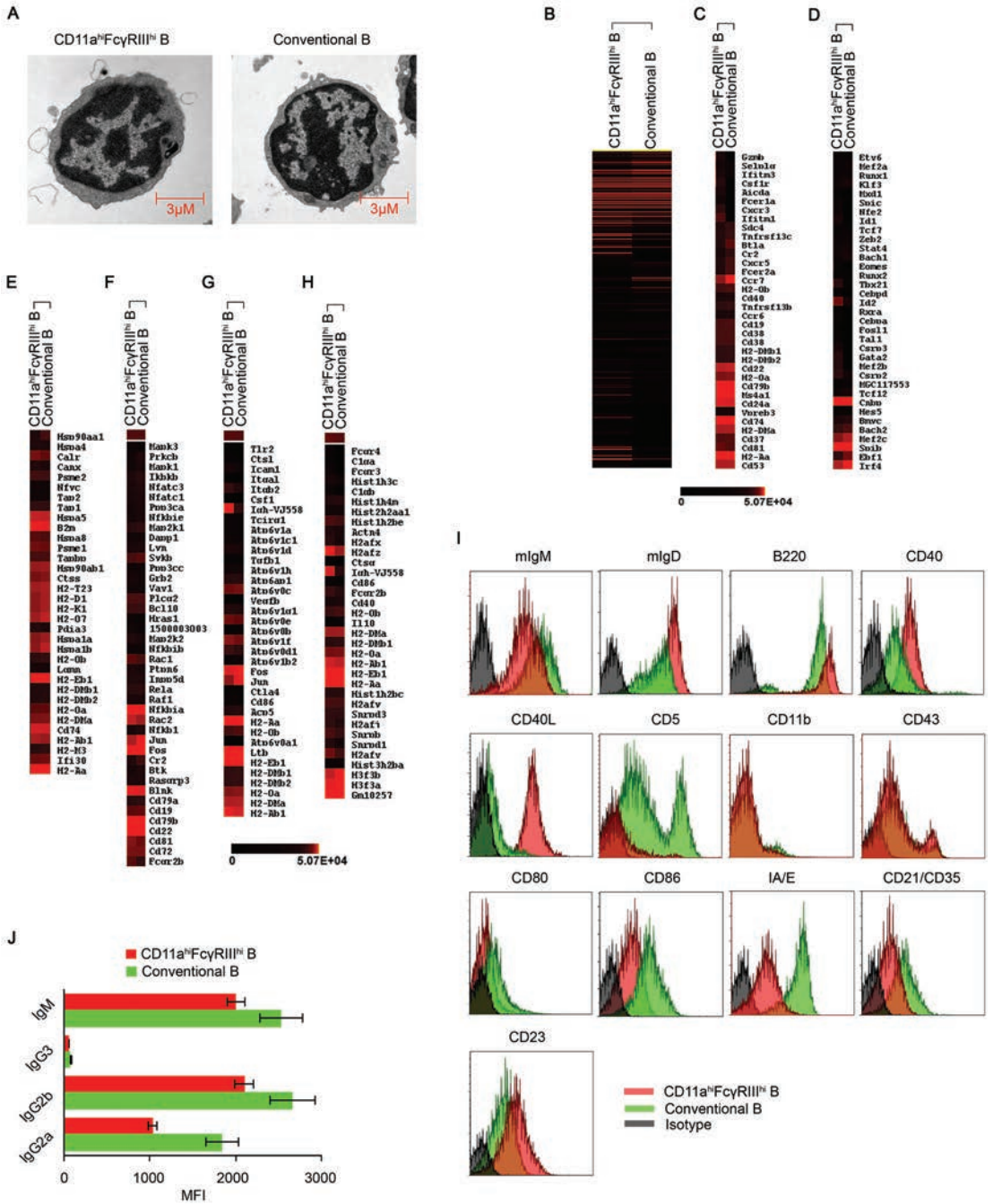

Figure 2 Morphological and gene signature characteristics of the CD11a^{hi}FcγRIII^{hi} B cells. C57BL/6 mice were infected with 2 × 10^6 LM. CD11a^{hi}FcγRIII^{hi} B cells and CD11a^{lo}FcγRIII$^-$ conventional B cells were sorted from the splenocytes of the infected mice 3 days later. **(A)** Electron microscopic observation of CD11a^{hi}FcγRIII^{hi} and conventional B cells. **(B)** Unsupervised clustering analysis of differentially expressed genes of the CD11a^{hi}FcγRIII^{hi} and conventional B cells based on microarray data. Red and black correspond to high and low expression levels, respectively. **(C**, **D)** Heat-map of clustering analysis of differentially expressed cellular antigens and transcription factors in the CD11a^{hi}FcγRIII^{hi} and conventional B cells. The gene symbols are listed. **(E**-**H)** Highly expressed genes in the CD11a^{hi}FcγRIII^{hi} B cells were subjected to a cluster analysis with regard to the antigen processing and presentation pathway, mmu04612 **(E)**, B cell receptor signaling pathway, mmu04662 **(F)**, rheumatoid arthritis, mmu05323 **(G)**, and systemic lupus erythematosus, mmu05322 **(H)**, based on the KEGG database. The gene symbols are listed. **(I)** The surface markers of CD11a^{hi}FcγRIII^{hi} and conventional B cells. **(J)** Secretion of immunoglobulins by CD11a^{hi}FcγRIII^{hi} and conventional B cells after stimulation with 1 μg/ml LPS for 24 h. Data shown represent the mean ± SD. **P* < 0.05.

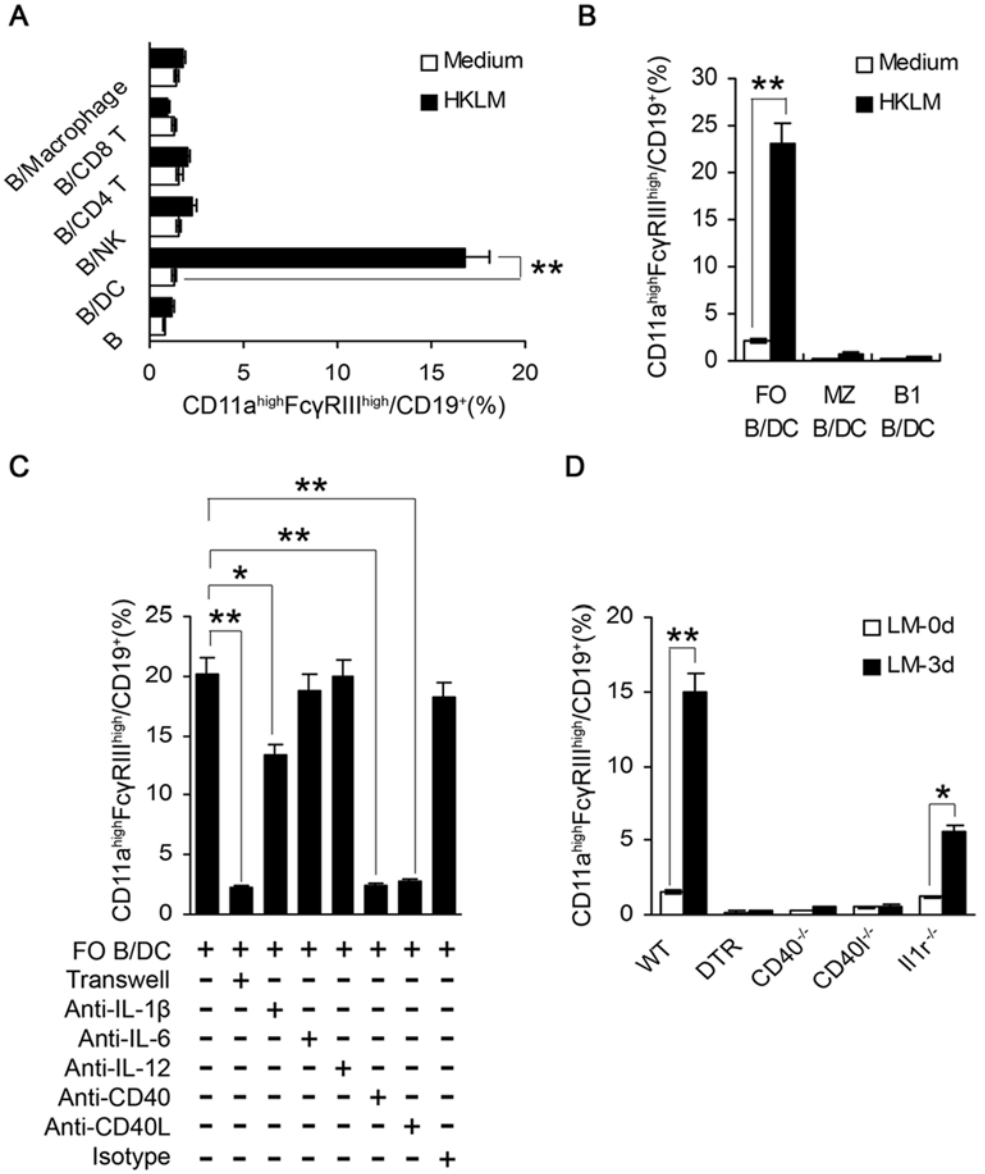

Figure 3 Cellular and molecular mechanisms for the inducible generation of CD11a^{hi}FcγRIII^{hi} B cells. **(A)** NK cells, DC, macrophages, CD4$^+$ T cells, and CD8$^+$ T cells were sorted from C57BL/6 mice and then co-cultured with CD19$^+$ B cells from naïve C57BL/6 mice (1:1) in the presence of HKLM (10^8/ml). The proportions of CD11a^{hi}FcγRIII^{hi} cells in the CD19$^+$ B cells were analyzed 48 h later. **(B)** Splenic FO (CD93$^-$CD21^{lo}CD23^{hi}), MZ (CD93$^-$CD21^{hi}CD23^{lo}), and B1 (B220$^+$CD5$^+$) B cells were purified and co-cultured with DCs. HKLM was added in the co-culture system. The percentages of CD11a^{hi}FcγRIII^{hi} cells in the CD19$^+$ B cells were assessed 48 h later. **(C)** DCs and FO B cells from WT mice were co-cultured in the presence of HKLM. Anti-IL-1β (5 μg/ml), anti-IL-6 (5 μg/ml), anti-IL-12 (5 μg/ml), anti-CD40 (5 μg/ml), or anti-CD40L (5 μg/ml) was added or a 0.4-μm transwell system was used, as indicated. The expression of CD11a and FcγRIII on B cells was examined after 48 h. **(D)** CD11c-DTR mice were injected with diphtheria toxin (DT) (100 ng) for the depletion of conventional DCs. The CD11a^{hi}FcγRIII^{hi} cell population in splenic B cells was determined in WT, DC-depleted (DTR), *Cd40*$^{-/-}$, *Cd40l*$^{-/-}$ and *Il1r*$^{-/-}$ mice 3 days after LM infection. Data shown represent the mean ± SD of triplicate experiments. *P < 0.05, **P < 0.01.

cells in the DC/FO B co-culture system (Figure 3C). Furthermore, LM infection failed to induce the generation of CD11a^{hi}FcγRIII^{hi} B cells in DC-depleted DTR, *CD40*$^{-/-}$ or *CD40l*$^{-/-}$ mice (Figure 3D). Thus, the CD40/CD40L pathway was required for the generation of CD11a^{hi}FcγRIII^{hi} B cells induced by pathogen-activated DCs. We also found that blocking IL-1β suppressed the HKLM-induced generation of CD11a^{hi}FcγRIII^{hi} B

cells in the DC/FO B co-culture system (Figure 3C). Consistently, the numbers of LM infection-expanded CD11a^{hi}FcγRIII^{hi} B cells were limited in *Il1r*$^{-/-}$ mice and much less than that in LM-infected WT mice (Figure 3D), suggesting that IL-1β is also partially involved in the process. However, when cultured in the presence of agonistic anti-CD40 mAbs and recombinant IL-1β *in vitro*, the FO B cells alone failed to convert into CD11a^{hi}FcγRIII^{hi} B cells (Supplementary information, Figure S1), indicating that other signal(s) may be required to cooperate with CD40 ligation to effectively induce the generation of CD11a^{hi}FcγRIII^{hi} B cells *in vivo*. Together, pathogen-activated DCs can induce the conversion of FO B cells into CD11a^{hi}FcγRIII^{hi} B cells via the CD40-CD40L pathway, leading to the generation of this new B cell subset.

CD11a^{hi}FcγRIII^{hi} B cells preferentially produce IFN-γ

A microarray assay showed that the CD11a^{hi}FcγRIII^{hi} B cell-specific transcripts were mostly enriched for cytokines and cytokine receptors, suggesting that these new B cells may exert their specific function by secreting cytokines. Thus, to uncover the function of CD11a^{hi}FcγRIII^{hi} B cells, we assessed their cytokine production using intracellular staining and ELISA. As CD40 ligation is essential for the DC-mediated CD11a^{hi}FcγRIII^{hi} B cell generation *in vitro,* and these B cells express higher levels of CD40 (Figure 2I), we chose an agonistic CD40 mAb as the stimulator. We found that the CD11a^{hi}FcγRIII^{hi} B cells expressed intracellular IL-1, IL-6 and IFN-γ in response to CD40 ligation/activation (Figure 4A). As detected by ELISA, both the CD11a^{hi}FcγRIII^{hi} and conventional B cells have the ability to produce IL-1, IL-2 and IL-6, and a small amount of IL-12p70 and TNF-α (Figure 4B-4E and 4G). Interestingly, much higher levels of IFN-γ were detected in the supernatants of the CD11a^{hi}FcγRIII^{hi} B cell culture systems than in that of the conventional B cells in response to CD40 activation (Figure 4F). Considering that NK cells have long been considered as the major source of IFN-γ, we compared the IFN-γ production ability between CD11a^{hi}FcγRIII^{hi} B cells and NK cells. Interestingly, both intracellular staining and ELISA showed that the CD11a^{hi}FcγRIII^{hi} B cells could produce a comparabe amount of IFN-γ to NK cells (Supplementary

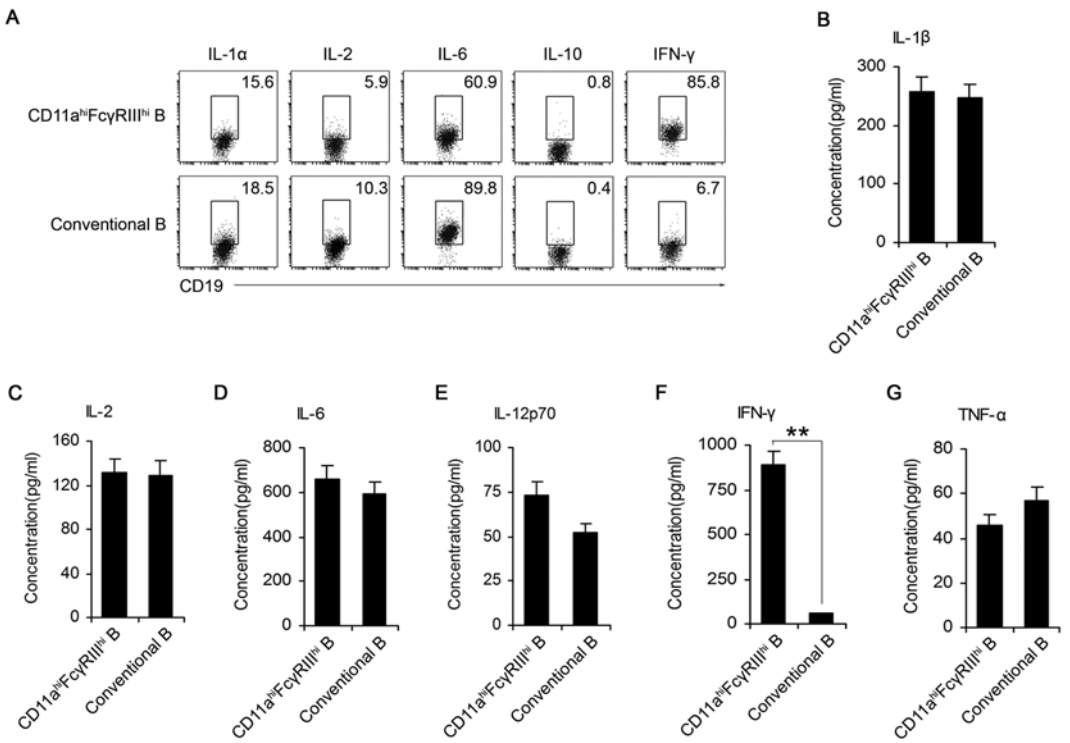

Figure 4 CD11a^{hi}FcγRIII^{hi} B cells produce a high level of IFN-γ in response to CD40 ligation. C57BL/6 mice were infected with 2 × 10^6 LM. **(A)** The intracellular expression of IL-1α, IL-2, IL-6, IL-10 and IFN-γ in CD11a^{hi}FcγRIII^{hi} and conventional B cells was assayed on day 3 post-infection. **(B-G)** Splenic CD11a^{hi}FcγRIII^{hi} and conventional B cells were sorted on day 3 post-infection. IL-1β, IL-2, IL-6, IL-12p70, IFN-γ, or TNF-α secretion by CD11a^{hi}FcγRIII^{hi} and conventional B cells was detected by ELISA. Data shown represent the mean ± SD of triplicate experiments. *P < 0.05, **P < 0.01.

information, Figure S2). As IFN-γ is proven important for the function of several types of immune cells in an autocrine manner [30], we speculated whether IFN-γ signaling is essential for the generation of CD11a^{hi}FcγRIII^{hi} B cells. We found that CD11a^{hi}FcγRIII^{hi} B cells could be induced by LM infection in both *Ifnγ*$^{-/-}$ and *Ifngr1*$^{-/-}$ mice (Supplementary information, Figure S3), indicating that the inducible generation of CD11a^{hi}FcγRIII^{hi} B cells is not through an IFN-γ autocrine manner. Taken together, in addition to NK cells, CD11a^{hi}FcγRIII^{hi} B cells also account for a large part of IFN-γ production during the early stage of LM infection.

CD40 ligation induces preferential IFN-γ production by enhancing Btk and non-canonical NF-κB activation in CD11a^{hi}FcγRIII^{hi} B cells

CD11a^{hi}FcγRIII^{hi} B cells were found to express a higher level of CD40 than conventional B cells (Figure 2I). As observed above, the CD40 signal was responsible for the inducible generation of CD11a^{hi}FcγRIII^{hi} B cells. As a member of the TNF receptor (TNFR) superfamily, CD40 signaling ultimately activates a variety of transcription factors, including canonical NF-κB p65, non-canonical p52, and AP-1, initiated by the interaction of at least four TNFR-associated factors (TRAFs). Thus, we analyzed CD40 ligation-triggered signaling pathways and found that the activation of the canonical NF-κB and MAPK signaling pathways was comparable in the CD11a^{hi}FcγRIII^{hi} B cells and conventional B cells (Figure 5A). However, the CD40 ligation-induced activation of Bruton's tyrosine kinase (Btk) in the CD11a^{hi}FcγRIII^{hi} B cells was much more significant than in the conventional B cells (Figure 5A); the increased nuclear translocation of p52 further confirmed the increased activation of the non-canonical NF-κB pathway (Figure 5B). Furthermore, the increased activation of the non-canonical NF-κB pathway was suppressed in the CD11a^{hi}FcγRIII^{hi} B cells after pretreatment with the Btk inhibitor PCI-32765 (Figure 5C), subsequently reducing IFN-γ expression in the CD11a^{hi}FcγRIII^{hi} B cells (Figure 5D). These results indicated that Btk activation is upstream of the non-canonical NF-κB activation in the induction of IFN-γ expression in CD11a^{hi}FcγRIII^{hi} B cells activated by CD40 ligation. Therefore, the CD40 signal, perhaps from pathogen-activated DCs, induces IFN-γ expression in CD11a^{hi}FcγRIII^{hi} B cells by promoting Btk phosphorylation and consequently enhancing the activation of the non-canonical NF-κB pathway.

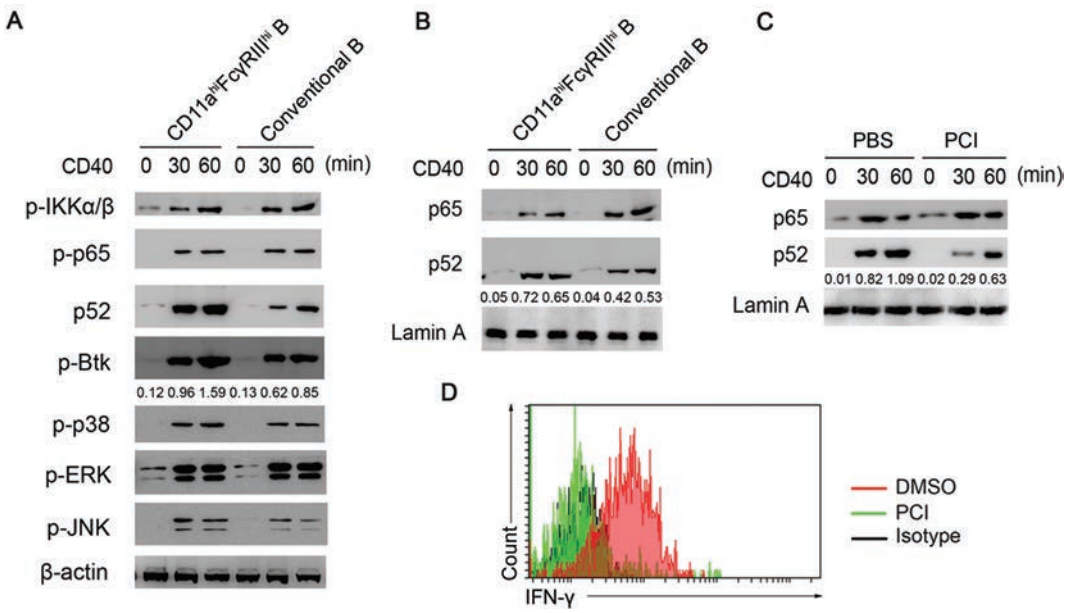

Figure 5 Increased activation of the Btk and non-canonical NF-κB pathways is responsible for the increased IFN-γ production in CD40-triggered CD11a^{hi}FcγRIII^{hi} B cells. **(A**, **B)** Signaling pathways in the CD11a^{hi}FcγRIII^{hi} and conventional B cells stimulated with activating anti-CD40, with actin **(A)** and lamin A **(B)** as loading controls. The data are representative of three independent experiments with similar results. The numbers below the lanes (top) indicate Btk **(A)** and p52 **(B**, **C)** band densities, presented relative to the β-actin **(A)** and lamin A **(B**, **C)** expression in the same lane (below). **(C)** Nuclear translocation of p65 and p52 in CD11a^{hi}FcγRIII^{hi} B cells pretreated with the Btk inhibitor PCI-32765 (5 nM) for 60 min. **(D)** Intracellular IFN-γ expression in CD11a^{hi}FcγRIII^{hi} B cells pretreated with neutralizing CD11a mAbs or the Btk inhibitor PCI-32765.

The tyrosine kinase Btk is required for the innate response against LM infection, at least partially, via the generation of CD11a^{hi}FcγRIII^{hi} B cells

Btk is a cytoplasmic tyrosine kinase belonging to the Tec family of kinases and is by far the most studied member of this family. Btk is expressed in almost all hematopoietic lineages except T cells, and has been given much attention because of its predominant expression during different developmental stages of B lymphocytes, from hematopoietic stem cells, the common lymphoid progenitor, to pre-B, pro-B, immature, and mature B cells, but not plasma cells [31]. Accordingly, Btk has been shown to be crucial for B cell development, differentiation, signaling, and function [31]. To investigate whether Btk is important in anti-LM responses, we infected *Btk*$^{-/-}$ mice with LM intraperitoneally and estimated the severity of infection by measuring the bacterial load in the spleen and liver, and the serum IFN-γ level within 7 days post-infection. We found that the bacterial load in the spleen (Figure 6A) and liver (Figure 6B) was higher in the *Btk*$^{-/-}$ mice than in the WT controls after LM infection. LM infection also significantly increased the serum levels of IFN-γ in WT but not in the *Btk*$^{-/-}$ mice (Figure 6C). Thus, the *Btk*$^{-/-}$ mice exhibited impaired protection against LM infection. We also examined the proportion of CD11a^{hi}FcγRIII^{hi} cells in B cells and found that LM infection failed to induce the generation of CD11a^{hi}FcγRIII^{hi} B cells in the *Btk*$^{-/-}$ mice (Figure 6D).

CD11a^{hi}FcγRIII^{hi} B cells increase the resistance of macrophages to LM infection through IFN-γ in vitro

Considering that macrophages, which can be activated by IFN-γ, are the major target of LM infection, we addressed whether CD11a^{hi}FcγRIII^{hi} B cells generated in the early stage of LM infection may increase the resistance of macrophages to LM infection. We used an agonistic anti-CD40 mAb to activate the CD11a^{hi}FcγRIII^{hi} B cells and co-cultured them with LM-infected BM-derived macrophages (BMDMs). After co-culturing for several hours, we found that the CD11a^{hi}FcγRIII^{hi} B cells, which were isolated from LM-infected mice and activated by CD40 ligation, significantly inhibited intracellular bacterial growth/survival in macrophages in comparison to CD40-activated conventional B cells (Figure 7A). In contrast, the CD40-activated CD11a^{hi}FcγRIII^{hi} B cells derived from LM-infected *Ifnγ*$^{-/-}$ mice did not show this inhibitory effect (Figure 7B). These *in vitro* results indicate that CD11a^{hi}FcγRIII^{hi} B cells may control the growth of LM in macrophages through the production of IFN-γ.

To further clarify the effects of CD11a^{hi}FcγRIII^{hi} B cells on the activation of macrophages, we co-cultured

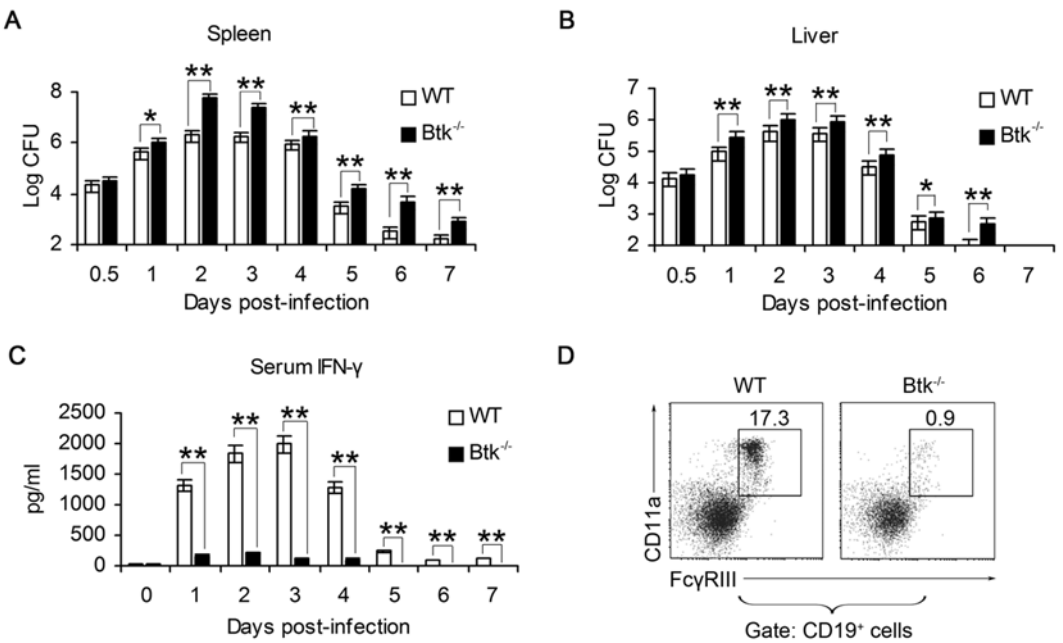

Figure 6 *Btk*$^{-/-}$ mice with impaired CD11a^{hi}FcγRIII^{hi} B cell generation are more susceptible to LM infection. WT and *Btk*$^{-/-}$ mice were infected with LM. CFUs in the spleen **(A)** and liver **(B)** and serum IFN-γ **(C)** were analyzed at the indicated time. **(D)** The CD11a^{hi}FcγRIII^{hi} cell proportion in splenic B cells was determined in WT and *Btk*$^{-/-}$ mice on day 3 post-LM infection. Data shown represent the mean ± SD of triplicate experiments. **P* < 0.05, ***P* < 0.01.

CD11a^{hi}FcγRIII^{hi} B cells with BMDMs in the presence or absence of HKLM stimulation and then assessed the production of TNF-α and nitrite (as a marker of iNOS-mediated NO production), both of which are pro-inflammatory factors known to be required for the resistance to *Listeria*. The CD40-activated CD11a^{hi}FcγRIII^{hi} B cells alone were capable of enhancing the production of TNF-α and NO by macrophages; the presence of HKLM promoted this effect and elicited much higher levels of production of both TNF-α and NO by macrophages (Figure 7C and 7D). Together, these data suggest that DC-induced CD11a^{hi}FcγRIII^{hi} B cells can promote the activation of macrophages via generating IFN-γ and subsequently inhibit bacterial growth in LM-infected macrophages.

CD11a^{hi}FcγRIII^{hi} B cells promote the innate immune response against LM infection in vivo

Considering that *Btk*$^{-/-}$ mice could not generate CD11a^{hi}FcγRIII^{hi} B cells, we used *Btk*$^{-/-}$ mice as a model to investigate whether CD11a^{hi}FcγRIII^{hi} B cells contribute to the innate defense against LM infection *in vivo*. We purified the CD11a^{hi}FcγRIII^{hi} B cells and conventional B cells from LM-infected WT mice and adoptively transferred these cells into *Btk*$^{-/-}$ mice and found that the CD11a^{hi}FcγRIII^{hi} B cells provided potent protection against LM infection (Figure 8A and 8B). Accordingly, the serum IFN-γ level was significantly higher in the *Btk*$^{-/-}$ mice after CD11a^{hi}FcγRIII^{hi} B cell adoptive transfer (Figure 8C). In contrast, the adoptive transfer of CD11a^{hi}FcγRIII^{hi} B cells derived from LM-infected *Ifnγ*$^{-/-}$ mice into *Btk*$^{-/-}$ mice did not have such a protective effect (Figure 8A-8C). Therefore, CD11a^{hi}FcγRIII^{hi} B cells promote the innate resistance to LM infection at an early stage of the immune response via IFN-γ production.

Discussion

B cells are critical for the humoral immunity and have

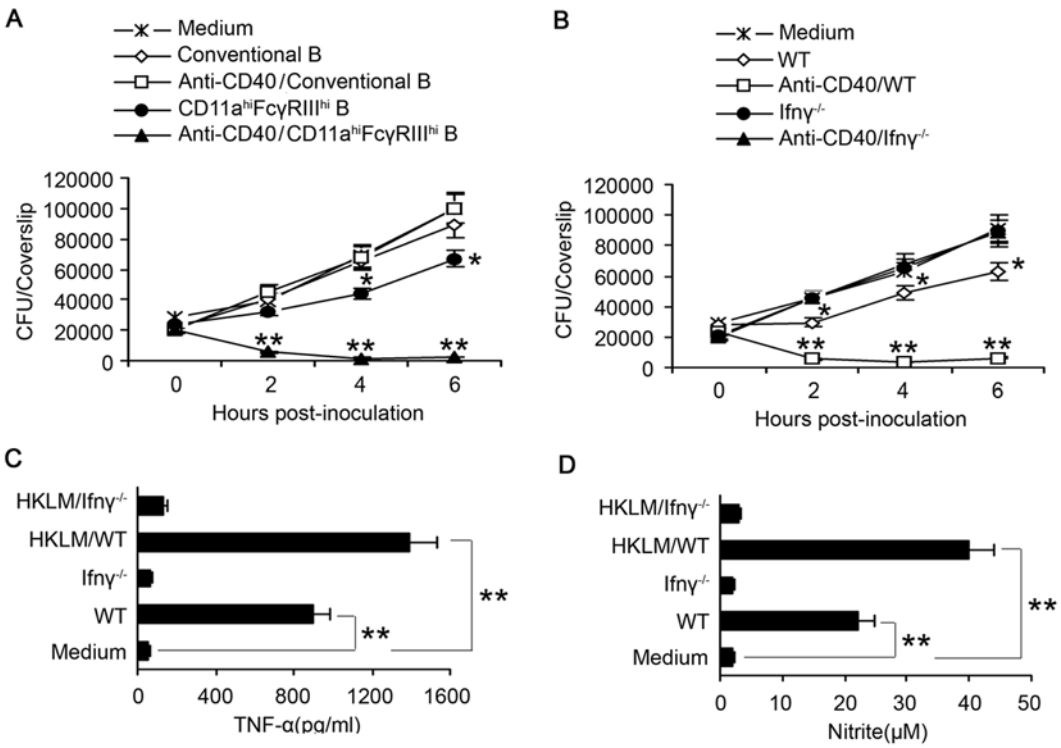

Figure 7 CD11a^{hi}FcγRIII^{hi} B cells enhance the resistance of macrophages to LM infection through IFN-γ. **(A**, **B)** BMDMs from WT mice were infected with LM. **(A)** LM-infected BMDMs were co-cultured with CD11a^{hi}FcγRIII^{hi} or conventional B cells from WT mice with or without anti-CD40 pretreatment. **(B)** LM-infected BMDMs were co-cultured with CD11a^{hi}FcγRIII^{hi} B cells from WT or *Ifnγ*$^{-/-}$ mice with or without anti-CD40 pretreatment. **(A**, **B)** CFUs/coverslip (mean ± SD) were determined at 0, 2, 4, and 6 h post-infection. **(C**, **D)** BMDMs were co-cultured with anti-CD40-pretreated CD11a^{hi}FcγRIII^{hi} B cells from WT or *Ifnγ*$^{-/-}$ mice in the presence or absence of HKLM. After 48 h, the TNF-α **(C)** and nitrite **(D)** levels in the supernatants were examined. *P < 0.05, **P < 0.01.

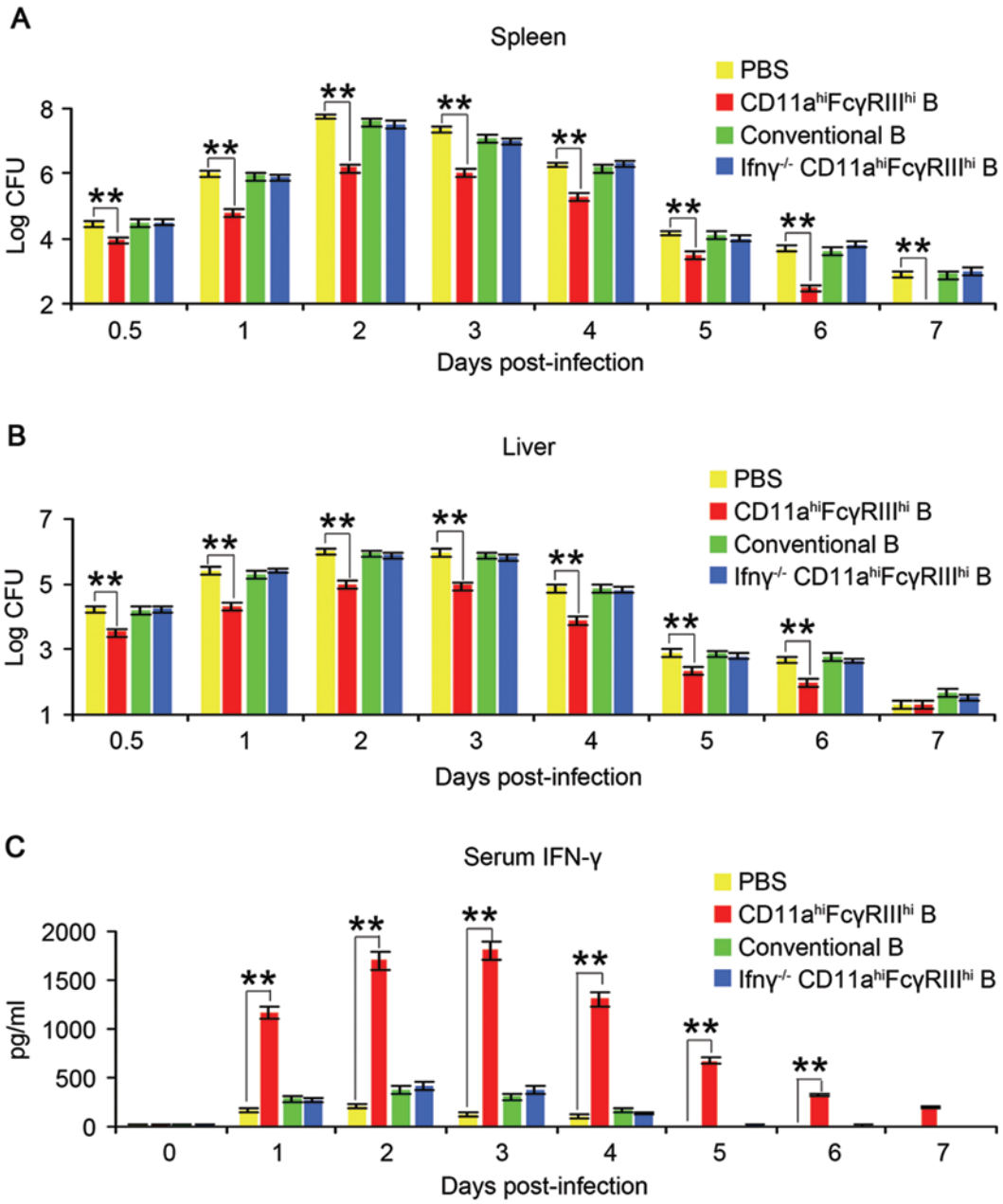

Figure 8 Adoptive transfer of CD11a^{hi}FcγRIII^{hi} B cells promotes the innate defense of *Btk*$^{-/-}$ mice against LM infection. CD11a^{hi}FcγRIII^{hi} and conventional B cells were purified from the spleen of WT or *Ifnγ*$^{-/-}$ mice on day 3 after infection with LM using Dako MoFlo^{TM} XDP; the cells were then transferred intravenously into *Btk*$^{-/-}$ mice infected with LM. CFUs in the spleen **(A)** and liver **(B)**, and serum IFN-γ **(C)** were examined at the indicated time. Data shown represent the mean ± SD of triplicate experiments. **P* < 0.05, ***P* < 0.01.

antibody-independent, non-classical functions. These cells can differentiate into various functional subsets in response to pathogen infections, and then participate in or regulate the innate and adaptive immune responses. Some B cell subsets contribute to the clearance of pathogenic agents, thereby providing effective protection against microbial infection [4-6]. Accordingly, the innate function of B cells has attracted much attention in recent years. Indeed, B cells express many types of innate receptors that can initiate the innate function of B cells in response to invading pathogens [32, 33]. Natural antibody-producing CD11b^{+}CD5$^{+/-}$ B1 B cells, Ag-present-

ing $CD24^+CD21^+B220^+$ FO B cells, and $CD1d^{hi}CD21^{hi}$ MZ B cells with the potential to differentiate into short-lived plasma cells have been identified as directly or indirectly to mediate active immunoprotection [34]. Our previous work demonstrated that an IFN-α-producing $PDCA\text{-}1^+Siglec\text{-}H^-CD19^+$ B cell subset mediates the innate defense against LM infection by activating NK cells [35]. More recently, the atypical chemokine receptor D6, but not CD11b and CD5, was found to be another key marker of innate-like B cells and therefore, was used to identify a novel scavenging B1 B cell subset [36]. B cells are divided into Be1 and Be2 according to their unique cytokine production profiles and the type of Th responses that they mediate *in vitro* [37]. Stimulation of combined TLR ligands, IL-12/IL-18 or PMA/ionomycin can trigger B cells to make many kinds of cytokines including IFN-γ *in vitro* [37-41]. Observations from some infectious diseases indicated the potential role of B cell-derived IFN-γ in the adaptive immune response [37, 41]. Given that the IFN-γ-dependent innate immune response is of great importance in host defense against invading pathogens, whether IFN-γ-producing B cells have a role in this process continues to spur vigorous research efforts. In this study, we identified a new IFN-γ-producing innate B cell subset ($CD11a^{hi}Fc\gamma RIII^{hi}$) and showed the phenotypic and morphological characteristics and microarray results of these pathogen-induced B cells. Together with their generation in the early phase of intracellular bacterial infection, the observation that the innate response was regulated by these IFN-γ-producing $CD11a^{hi}Fc\gamma RIII^{hi}$ B cells supports this B cell subset as an important innate-like cell population.

These $CD11a^{hi}Fc\gamma RIII^{hi}$ B cells have the unique phenotype of $mIgM^{int}mIgD^{hi}CD40^{hi}CD40L^{hi}$ $CD5^-CD11b^-$ $CD43^{lo}CD80^-CD86^{lo}$I-A/I-$E^{lo}CD21^{lo}$ $CD23^{hi}$, distinguishing them from B1 B cells ($CD5^+$ $CD43^+CD11b^+$) and MZ B cells ($CD21^{hi}CD23^{int}$), yet linking them to FO B cells ($mIgM^{int}$ $mIgD^{hi}$ $CD21^{int}$ $CD23^{hi}$). However, their low expression of MHC II and co-stimulation molecules may indicate that these $CD11a^{hi}Fc\gamma RIII^{hi}$ B cells do not participate in the T cell-mediated adaptive immune response. As expected, acting as a new innate B cell subset, $CD11a^{hi}Fc\gamma RIII^{hi}$ B cells activate macrophages via generating IFN-γ in the innate response, and thus promote the resistance to intracellular bacteria in the early period of infection. Therefore, our results provide insights into the innate function of B cells against intracellular bacterial infection.

It should be noted that the innate IFN-γ response in the spleen appears approximately 14 h after LM infection, with a maximum IFN-γ production induced at 20 h post-infection, which is convincingly mediated by NK cells [42]. Therefore, rapidly activated NK cells are the main source of IFN-γ in the initial phase of innate responses, and the activated macrophages may be important players for eliciting the downstream effects to control bacterial growth by maintaining the level of serum IFN-γ [43, 44]. Our study showed that IFN-γ-producing innate B cells are not induced so rapidly, as their number peaked on day 3 post-infection, suggesting that these B cells may participate in the innate defense against LM after NK cells first impact the activation of macrophages. This could be another important source of IFN-γ to complement the functions of NK cells.

DCs have the potential to activate B cells, and in turn DC functions can be regulated by these major immune cells. DCs are thought to activate B cells and trigger their class switching via the production of various cytokines [45]. Moreover, B cells are reported to modulate DC maturation and steady-state migration by producing natural IgG antibodies [46]. However, whether and how DCs influence the B cell function during innate immune response remains poorly defined. Our results demonstrate that DCs, and not NK or T cells, activated by invading pathogens or pathogenic components, can induce the generation of $CD11a^{hi}Fc\gamma RIII^{hi}$ B cells from FO B cells through the CD40-CD40L pathway, thus providing new insights into the DC-B cell interaction. CD40 has long been demonstrated to be one of the most important signal molecules during B cell development, maturation, activation and immunoglobulin secretion [47]. Our results, together with others, demonstrate the crucial role of CD40 in the peripheral B cell differentiation. Interestingly, we also found that $CD11a^{hi}Fc\gamma RIII^{hi}$ innate B cells were present, albeit in relatively small numbers, in the BM; however, we cannot yet explain their generation in a central immune organ.

The molecular mechanisms for innate IFN-γ production in NK cells and even B cells remain unclear to date. Several transcriptional factors, such as T-bet and Eomes, are verified to be critical for IFN-γ production in T cells. Although IFN-γ has been shown to be mainly produced by NK, NKT and $CD4^+$ T cells, macrophages and DCs have been recently reported to produce IFN-γ in some cases [48]. IFN-γ plays an important role in the upregulation of membrane molecule expression and the activation and differentiation of many cell types, particularly the activation of macrophages and neutrophils [49]. Our data presented here demonstrate that $CD11a^{hi}Fc\gamma RIII^{hi}$ innate B cells are one of the main sources of IFN-γ after LM infection and indicate that the higher IFN-γ production in these B cells occurs in a CD40L/CD40-dependent manner initiated by a cell-cell contact between DCs and B cells. This is the first report of CD40 eliciting IFN-γ

production. It has been reported that IFN-γ induction mainly depends on NF-κB, NFAT, STAT, T-bet, AP-1, CREB-ATF, GATA-3 and yin-yang-1, all of which co-operate to regulate the induction of IFN-γ expression. Additionally, IFN-γR signal was found to be essential for Th1 cell- or IL-12/IL-18-mediated IFN-γ production by B cells [50], whereas our experiments showed that generation of CD11a^{hi}FcγRIII^{hi} B cells by interaction with DCs after LM infection depended on CD40 ligation rather than IFN-γR signal, implying that IFN-γ release by B cells may be influenced by the type of stimuli and accessory cells that they interact with. Therefore, as stated above, the exact mechanism for IFN-γ induction needs to be further demonstrated. By screening changes in the signaling pathways, we showed that an increased Btk activation and subsequent non-canonical NF-κB activation in CD11a^{hi}FcγRIII^{hi} innate B cells resulted in higher IFN-γ secretion, outlining a new mechanistic explanation for IFN-γ production in B cells.

In conclusion, we identified a new population of IFN-γ-producing CD11a^{hi}FcγRIII^{hi} innate B cells that can promote the innate response against intracellular infection by activating macrophages via the release of IFN-γ.

Materials and Methods

Mice

C57BL/6 mice (Joint Venture Sipper BK Experimental Animals Co., Shanghai, China), *Btk*$^{-/-}$, *Ifnγ*$^{-/-}$, *Il1r*$^{-/-}$, *Cd40*$^{-/-}$, *Cd40l*$^{-/-}$, *Ifngr1*$^{-/-}$, and CD11c-DTR mice (Jackson Laboratory, Bar Harbor, Maine, USA) were maintained in a specific pathogen-free facility and used at 6-10 weeks of age. All the animal experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, with the approval of the Scientific Investigation Board of the Second Military Medical University, Shanghai, China.

Reagents

The neutralizing anti-mouse IL-6 (MP5-20F3), anti-CD40L (208109), anti-IL-1β (30311), anti-IL-12 (C17.8), anti-IL-6 (MP5-20F3), and isotype control mAbs were purchased from R&D Systems (Minneapolis, Minnesota, USA). Fluorescence-conjugated mAbs against CD4 (RM4-5), CD5 (53-7.3), CD8 (53-6.7), CD11a (2D7), CD11b (M1/70), CD11c (HL3), CD16/32 (2.4G2), CD19 (1D3), CD21/CD35 (7G6), CD23 (B3B4), CD40 (3/23), CD40L (TRAP1), CD43 (S7), CD45R/B220 (RA3-6B2), CD80 (16-10A1), CD86 (GL1), CD93 (C1qRp) (R139), F4/80 (6F12), NK1.1 (PK136), I-A/I-E (2G9), IgD (11-26c.2a), IgM (G155-228), IL-1α (364-3B3-14), IL-2 (MQ1-17H12), IL-6 (MP5-20F3), IL-10 (JES3-19F1), IFN-γ (B27), IgD (11-26c.2a) and IgM (G155-228) and agonistic anti-CD40 (HM40-3) and neutralizing CD11a mAbs (M17/4) were purchased from BD Biosciences Pharmingen (San Diego, CA, USA). LPS was obtained from Sigma-Aldrich.

Infection with bacteria or viruses

Mice were infected intraperitoneally with 2×10^6 LM (strain 10403S), a kind gift from Dr Hao Shen (University of Pennsylvania School of Medicine, USA), 1×10^6 *E. coli*, a kind gift from Dr Hangping Yao (Zhejiang University, China), or 5×10^6 PFU VSV. The spleens and livers from LM-infected mice were harvested at various time points after infection, and the bacterial CFUs were determined as previously described [51].

Purification and culture of splenic cell subsets

Splenocytes from C57BL/6 mice, with or without LM infection, were incubated with monoclonal anti-CD19, anti-CD3, anti-CD4, anti-CD8, anti-NK1.1, anti-CD11c, anti-F4/80, anti-CD11a, anti-CD16/CD32, anti-CD21, anti-CD23, and anti-CD93 antibodies (BD Biosciences, San Jose, CA, USA). The cells were then sorted using fluorescence-activated Dako MoFlo^{TM} XDP to a purity of > 98% [35]. For the co-culture experiments, different subsets of B cells were seeded at a density of 1×10^6 cells/well and incubated for 24 h. NK cells (CD3$^-$NK1.1$^+$), DCs (CD3$^-$CD11b$^+$CD11c$^+$), macrophages (CD11b$^+$F4/80$^+$CD11c$^-$), CD4$^+$ T cells (CD3$^+$CD4$^+$NK1.1$^-$) and CD8$^+$ T cells (CD3$^+$CD8$^+$NK1.1$^-$) cells were then added at a density of 1×10^6 cells/well in the co-culture system. For the other co-culture experiments, splenic FO (CD93$^-$CD21^{lo}CD23^{hi}), MZ (CD93$^-$CD21^{hi}CD23^{lo}), and B1 (B220$^+$CD5$^+$) B cells were purified and co-cultured with DCs. HKLM (10^8/ml), prepared as described previously [52], was used as a pathogenic stimulator in the co-culture system.

RT-PCR analysis of CD16/CD32 expression

RT-PCR was performed to analyze CD16 and CD32 mRNA expression, as described previously [20]. In brief, total RNA was isolated with the TRIzol reagent from 2×10^6 cells following the manufacturer's instructions. For retrotranscription, 1 μg of total RNA was used to synthesize cDNA with an oligo(dT)$_{18}$ primer and 200 units of SuperScriptII (Gibco BRL, Rockville, MD, USA). The sequences of the specific primers used in this study were as follows: CD16, forward primer (5′-ATGAAAATGATGTGGGCCTG-3′), reverse primer (5′-CACTCTGCCTGTCTGCAAAAG-3′); CD32, forward primer (5′- ATGGGAATCCTGCCGTTCCTA-3′), reverse primer (5′- CCGTGAGAACACATGGACAGT-3′). The cDNA was amplified in a final volume of 20 μl containing 2.5 mM magnesium dichloride, 1.25 units Ex Taq polymerase (Takara, Dalian, China), and 1 μl specific primers. All the PCR products were analyzed by 1.5% agarose gel electrophoresis and visualized by staining the gel with ethidium bromide.

Flow cytometry

Flow cytometry analyses were conducted using an LSR II (BD Biosciences, Mountain View, CA, USA). The data were analyzed with CELLQuest or FACSDiva software (BD Biosciences, San Jose, CA, USA), as described previously [53].

Cytokine assay

Splenic CD11a^{hi}FcγRIII^{hi} B cells (CD19$^+$NK1.1$^-$CD11a^{hi}FcγRIII^{hi}) and conventional B cells (CD19$^+$NK1.1$^-$CD11a^{lo}FcγRIII^{lo}) were purified from mice infected with LM for 3 days. Each subset of B cells was cultured with stimulation of agonistic CD40 mAbs. Supernatants were collected 48 h later and IFN-γ, IL-1β, IL-2, IL-6, IL-12p70, and TNF-α were then quantified using ELISA kits (R&D system).

Splenocytes derived from LM-infected mice (day 3) were incu-

bated with agonistic CD40 mAbs and brefeldin A. After 6 h, intracellular staining for the detection of IL-1α, IL-2, IL-6, IL-10, and IFN-γ in splenocytes was performed as described [31].

BMDM experiments

BM was collected from C57BL/6 mice and cultured as described to generate BMDMs [54]. CD11a^{hi}FcγRIII^{hi} B cells and conventional CD11a^{-}FcγRIII^{-} B cells from WT or *Ifnγ*$^{-/-}$ mice were sorted on day 3 post-LM infection and cultured with or without CD40 stimulation for 1 day.

To assess the effects of CD11a^{hi}FcγRIII^{hi} B cells on intracellular LM growth in BMDMs, BMDMs were plated at 2.5 × 10^5 cells/well on 12-mm glass coverslips in 24-well plates. After 2 h incubation at 37 °C, the coverslips were then placed in the CD11a^{hi}FcγRIII^{hi} B cell or conventional B cell culture system. The number of CFUs/coverslip at various time points post-infection was examined to assess LM growth in the macrophages.

To assess the effects of CD11a^{hi}FcγRIII^{hi} B cells on the BMDM responses to HKLM, macrophages were co-cultured with CD11a^{hi}FcγRIII^{hi} B cells or conventional B cells; the supernatants were collected after 48 h and assessed for nitrite and TNF-α.

Adoptive transfer of B cells into Btk$^{-/-}$ mice infected with LM

Splenic mononuclear cells from C57BL/6 mice on day 3 post-LM infection were stained with fluorescence-conjugated mAbs against NK1.1, CD11a, CD19 and FcγRIII, and were purified using Dako MoFlo™ XDP for the preperation of CD11a^{hi}FcγRIII^{hi} B cells (CD19^{+}NK1.1^{-}CD11a^{hi}FcγRIII^{hi}) and conventional B cells (CD19^{+}NK1.1^{-}CD11a^{lo}FcγRIII^{lo}). Each B cell subset (8 × 10^6) was intravenously injected into *Btk*$^{-/-}$ mice 3 h after infection with LM. The spleens and livers from the LM-infected *Btk*$^{-/-}$ mice were harvested at various times after infection, and the bacterial CFUs were determined as described [35]. Sera were collected at different time points, and IFN-γ was assayed by ELISA.

Immunoblotting

Cells were lysed with RIPA buffer (Cell Signaling Technology, Beverly, MA, USA) supplemented with a protease inhibitor cocktail. The protein concentrations of the extracts were measured with the BCA assay (Pierce). Immunoblotting was performed as previously described [55].

Microarray data analysis

Microarray raw data were processed to expression signals. Genes were classified into different functional categories according to KEGG orthology database [56]. Genes that belong to cellular antigen and transcription factor classes were respectively picked out and hierarchical clustered. Higher expressed genes (> 2 folds) of splenic CD11a^{hi}FcγRIII^{hi} B compared to conventional B cells were picked out and subjected to functional pathway enrichment analysis according to KEGG pathway database. *P* values were calculated based on hypergeometric distribution analysis.

Statistical analysis

The data are shown as the mean ± SD of three or more independent experiments. The statistical analysis for the comparison of the different groups was performed using Student's *t*-test. A value of $P < 0.05$ was considered statistically significant.

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(**Supplementary information** is linked to the online version of the paper on the *Cell Research* website.)