HHS Public Access

Author manuscript

J Immunol. Author manuscript; available in PMC 2020 September 15.

Published in final edited form as:

J Immunol. 2020 March 15; 204(6): 1508–1520. doi:10.4049/jimmunol.1901120.

B cell activating factor (BAFF) produced by neutrophils and dendritic cells is regulated differently and has distinct roles in Ab responses and protective immunity against West Nile virus

Daniela Giordano[1], **Runa Kuley**[1,*], **Kevin E. Draves**[1,*], **Kelsey Roe**[1], **Ursula Holder**[1], **Natalia V. Giltiay**[2], **Edward A. Clark**[1,2]

[1]Department of Immunology, University of Washington, Seattle, WA, USA

[2]Department of Medicine, Division of Rheumatology, University of Washington, Seattle, WA, USA

Abstract

B cell activating factor (BAFF) is essential for B cells to develop and respond to Ags. Dysregulation of BAFF contributes to the development of some autoimmune diseases and malignancies. Little is known about when, where and how BAFF is produced *in vivo* and about which BAFF-producing cells contribute to B cell responses. To better understand BAFF functions, we created BAFF reporter mice (BAFF-RFP) and *Baff* floxed ($Baff^{fl/fl}$) mice. Splenic and bone marrow (BM) neutrophils (Nphs) from BAFF-RFP mice expressed the highest constitutive levels of BAFF; other myeloid subsets including conventional dendritic cells (cDCs) and monocyte (MO) subsets expressed lower levels. Treatment of BAFF-RFP mice with Poly(I:C) increased BAFF expression in splenic $Ly6C^{hi}$ inflammatory MOs, $CD11b^{hi}$ activated NK subset, and in BM myeloid precursors. After infection with West Nile virus (WNV), BAFF increased in $CD8^{-}$ cDCs and Nphs, and $BAFF^{+}$ $CD11b^{hi}$ NK cells expanded in draining lymph nodes. The cell- and tissue-specific increases in BAFF expression were dependent on type I IFN signaling. MAVS signaling also was required or contributed to BAFF expression in DC and MO subsets, respectively. Mice with deletion of *Baff* in either cDCs or Nphs had reduced Ab responses after NP-Ficoll immunization; thus, BAFF produced by both cDCs and Nphs contributes to T cell-independent Ab responses. Conversely, mice with a cDC *Baff*-deficiency had increased mortality after WNV infection and decreased WNV-specific IgG and neutralizing Ab responses. BAFF produced by Nphs and cDCs is regulated differently and has key roles in Ab responses and protective immunity.

Correspondence: Daniela Giordano: giordano@uw.edu.

*Contributed equally to this study

Author Contributions

Conceptualization, Daniela Giordano, Edward A. Clark; Data curation, Daniela Giordano, Runa Kuley, Kevin E. Draves, Kelsey Roe; Formal analysis, Daniela Giordano, Runa Kuley, Kelsey Roe; Funding acquisition, Edward A. Clark, Daniela Giordano, Natalia V. Giltiay; Investigation, Daniela Giordano, Runa Kuley, Kevin E. Draves, Kelsey Roe, Ursula Holder; Methodology, Daniela Giordano, Runa Kuley, Kelsey Roe; Project Administration, Daniela Giordano, Edward A Clark; Resources, Edward A. Clark; Supervision, Daniela Giordano, Edward A. Clark; Visualization, Daniela Giordano; Writing – original draft, Daniela Giordano; Writing – review and editing, Daniela Giordano, Edward A Clark, Runa Kuley, Kevin E. Draves, Kelsey Roe, Natalia V. Giltiay.

Introduction

B-cell-activating factor (BAFF) (also known as BLyS or Tnfsf13b) is a member of the TNF family of ligands that binds to three different TNF receptor family members: BAFFR, TACI and BCMA (1–3). *Baff*$^{-/-}$ and *Baffr*$^{-/-}$ mice develop few or no mature B cells displaying a block in B cell development from the transitional 1 (T1) to T2 stage onward (4–6). BAFF signaling, like signaling through the BCR, functions as a key survival factor for maintaining mature B cell homeostasis (5, 6). BAFF is overexpressed in patients with autoimmune diseases such as Sjögren's Syndrome, systemic lupus erythematosus (SLE), type I diabetes, B cell lymphoma and chronic lymphocytic leukemia (3, 7–9). In addition to BAFF's contributing to the development of autoimmune diseases (10), we and others have shown that BAFF plays an important role in infectious diseases, including HIV and West Nile virus (WNV) (11–15). BAFF may also contribute to the pathology of HIV (16). Moreover, BAFF has been associated to inflammatory conditions, such as inflammatory bowel disease (17) and allergic airway inflammatory diseases (18).

Although BAFF dysregulation has been implicated in a number of human diseases, little is known about the regulation of BAFF expression and function *in vivo*. Most studies have focused on the effects of blocking BAFF receptors and the development of BAFF-blocking drugs (17, 19, 20). However, which BAFF-producing cells contribute to specific B cell responses and B cell-associated diseases is not entirely clear. Studies primarily done *in vitro* have established that BAFF can be expressed by different immune cell types including monocytes (MOs), macrophages (MΦs), dendritic cells (DCs), neutrophils (Nphs), and follicular dendritic cells (FDCs) as well as epithelial cells and stromal cells (1, 11, 21–23). In particular, BAFF produced by DCs, Nphs, epithelial cells, FDCs and stromal cells helps to regulate humoral immune responses (24). Several cytokines including IFN-γ, IL-10, G-CSF and GM-CSF upregulate BAFF expression in different cell types (1, 21, 25–28).

Type I IFN is a key regulator of BAFF. IFNα induces DCs to produce BAFF, which can play a role in class switch recombination (CSR) and drive B cells to become Ab producing cells (29–33). In particular, Type I IFN-dependent BAFF produced by DCs is likely to play a key role in T-independent type 2 (TI-2) Ab responses (29, 32–35). Both ssRNA and dsRNA viruses induce Type I IFN via RNA-sensing pathways, including TLR3, TLR7 and the RIG-I/MAVS pathway (36). Poly(I:C) promotes BAFF production in tonsillar mononuclear cells, which in turn triggers IgA CSR and aggravates IgA nephropathy (37). However which specific myeloid cell subset is responsible for this effect is not known. Infection with either ssRNA or dsRNA viruses also induces BAFF production *in vitro* in human salivary gland epithelial cells, DCs and MOs (11).

To define the BAFF-producing cells implicated in specific immune responses, we developed two novel transgenic (Tg) mouse lines, BAFF reporter (BAFF-RFP) mice and *Baff* floxed (*Baff*$^{fl/fl}$) mice. Using BAFF-RFP reporter mice to monitor BAFF levels, we detected changes in BAFF expression in specific myeloid populations after *in vivo* activation of RNA-sensing pathways. Changes in BAFF production by MOs, cDCs and Nphs were mostly dependent on IFNAR but differentially affected by the absence of MAVS. Furthermore, we identified a new BAFF-expressing CD11b^{hi} NK subset induced by either Poly(I:C) or WNV

infection. Selective removal of *Baff* from either cDCs or Nphs revealed that BAFF produced from both DCs and Nphs is required for optimal TI-2 Ag-specific Ab responses. Mice lacking BAFF expression on cDCs were more susceptible to infection with WNV and had reduced WNV-specific IgG and neutralizing Abs as compared to controls. Thus, BAFF produced by cDCs is required to protect against a lethal viral infection. These findings underscore that BAFF produced by Nphs and cDCs is regulated differently and has distinct roles in Ab responses and protective immunity.

Materials and Methods

Mice

C57BL/6J, (B6) mice were purchased from Jackson Labs (Bar Harbor, ME). BAFF-RFP and $Baff^{fl/fl}$ mice (on the B6 background) were developed at the University of California at Davis Mouse Biology Program (UC Davis, CA) (see supplemental Fig. S1A). $Mavs^{-/-}$ (B6×129Sv/Ev) mice were kindly provided by Dr. S. Akira (Osaka University, Osaka, Japan) and backcrossed onto the B6 background (38). $Ifnar^{-/-}$ B6 mice were a kind gift from Dr. Michael Gale (University of Washington, Seattle, WA). $Mrp8^{Cre}$ and zDC^{Cre} B6 mice were purchased from Jackson Labs (Bar Harbor, ME). All mice were age- and sex-matched for experiments and used at 8–11 wks of age. Mice were housed in a specific pathogen free environment; all procedures were approved by the University of Washington Institutional Animal Care and Use Committee.

Generation of BAFF-RFP reporter and $Baff^{fl/fl}$ mice

A two-construct approach was used to generate BAFF reporter mice expressing a Tag-Red Fluorescence Protein-T (RFP) (39) under the *Tnfsf13b/Baff* promoter (BAFF-RFP) and $Baff^{fl/fl}$ mice to conditionally knockout the *Tnfsf13b/Baff* gene. First, the endogenous reporter BAFF-RFP mice were generated by replacing a *Tnfsf13b* allele with a targeting construct expressing IRES-RFP between exon 2 and exon 3 of *Baff* gene and containing loxP sites between exon 3 and exon 7. In the BAFF-RFP allele *Baff* is functionally knocked-out where the endogenous reporter expresses BAFF-IRES-RFP (BAFF-RFP), the RFP protein under the control of the BAFF promoter but with an internal ribosome entry site (IRES). In a second step, $Baff^{fl/fl}$ mice were generated by excising the RFP sequence and leaving wild type *Baff* gene with floxed exons 3–7 (for details about the construct and the generation of the transgenic mice see supplemental Fig. S1A, B). The $Baff^{fl/fl}$ mice express wild type *Baff* until Cre-mediated deletion of exons 3–7 render the *Baff* gene inactive. $Baff^{fl/fl}$ mice were crossed with $Mrp8^{Cre}$ mice (40, 41) and zDC^{Cre} mice (42, 43), to generate a conditional knockout (cKO) where *Baff* is selectively deleted in Nphs ($Baff^{fl/fl}$ $Mrp8^{Cre}$) or cDCs ($Baff^{fl/fl}$ zDC^{Cre}).

For *in vivo* experiments we used heterozygous BAFF-IRES-$RFP^{+/-}$ (BAFF-$RFP^{+/-}$) mice, which still have mature B cells and, therefore, are suitable for functional studies. Unless otherwise specified, “BAFF-RFP”, indicates heterozygous BAFF-$RFP^{+/-}$ mice. BAFF-$RFP^{+/-}$ mice were crossed with $Ifnar^{-/-}$ mice or $Mavs^{-/-}$ mice to generate BAFF-RFP $Ifnar^{-/-}$ mice and BAFF-RFP $Mavs^{-/-}$ mice, respectively.

Injection of TLR agonists and harvesting of PBMC, spleen and bone marrow (BM) cells

Mice were injected i.p. with 200μg/mouse Poly(I:C), 200μg/mouse R848 or 20nmol/mouse CpG; 6 h after injection of the TLR ligand, Brefeldin A (eBioscience, San Diego, CA, USA) was administered i.v. (44, 45). Brefeldin A was injected *in vivo* to optimize the retention of the BAFF-RFP signal inside the cells after TLR agonist administration; 24 h after TLR agonist injection, spleens and BMs were harvested for cell subsets analysis. Spleens from naïve or TLR-injected mice were harvested and dissociated into single cell suspensions as previously described with some modification (33). Briefly spleens were removed and dissociated by enzymatic digestion at 37 °C with liberase TL and Dnase I (Roche, Indianapolis, IN, USA), followed by mincing the tissue between the ends of two frosted microscope glass slides to obtain a single cell suspension. BM cells were isolated by cutting one end of the femur and flushing BM cells out of the bone by centrifugation. For PBMC harvesting, blood was collected by retroorbital eye bleeds using heparinized capillary tubes. After erythrocytes were lysed, PBMC, splenocytes and BM cell suspensions were processed for staining for flow cytometry. In initial experiments, BAFF-RFP mice were injected with Brefeldin A only for 18 h as controls, no significant differences in the BAFF-RFP signal/subset by flow cytometry, between naïve BAFF-RFP mice and Brefeldin A only injected BAFF-RFP mice were observed.

West Nile virus infections and inguinal LN (iLN) harvesting

The pathogenic lineage 1 WNV-TX infectious clone, derived from the Texas 2002-HC strain, was generously provided by Dr. Michael Gale (University of Washington, Seattle, WA) and prepared as described (13). For infections for iLN analysis, mice were inoculated under anesthesia with 1000 PFU WNV-TX subcutaneously into both footpads (f.p.) in a total volume of 40 μL; 20 μL (500 PFU) were injected in each f.p. (13). 24 h or 48 h after WNV infection iLNs were removed, minced into small fragments and digested for 40 min at 37 °C with liberase TL and Dnase I (Roche, Indianapolis, IN, USA), as described (46). Single cell suspensions were processed for staining for flow cytometry. For survival studies infected mice were inoculated under anesthesia with 100 PFU WNV-TX subcutaneously into one f.p. in a total volume of 20 μL. Mice were monitored at least once daily for survival. Serum was isolated from blood, collected via the retro-orbital route at d 4 and d 7, and stored at −80°C until use.

WNV RNA quantitation, WNV-specific Ab ELISA and FRNT50

Viral RNA was extracted from sera by using QiAMP viral RNA extraction kit (Qiagen, Valencia, CA, USA). Sequences for primers and TaqMan probes used, were as described (13). WNV envelope protein (WNVE)-specific IgG was quantitated by ELISA assay using a recombinant WNV-E protein (MyBioSource, San Diego, CA, USA) as described (13). Titers of WNV neutralizing Abs were quantitated by a focus forming reduction neutralization test (FRNT) using Vero cells as described (47). The $FRNT_{50}$ titers were calculated as the lowest dilution of serum with less than 50% of the average number of spots in the virus only wells.

NP-Ficoll immunization and NP-Ab ELISA

Mice were administered i.p. with 20 ug/mouse of NP (4-Hydroxy-3-nitrophenylacetic) hapten conjugated to AminoEthylCarboxyMethyl-FICOLL (AECM-FICOLL), NP-Ficoll (Biosearch Technologies, Petaluma, CA, USA). At the indicated time points mice were bled and serum was isolated. Serum NP Ab titers were measured as described (33). Plates were developed with tetramethylbenzidine substrate and read at 450 nm absorbance. Values were compared with known dilutions of IgM or IgG to calculate Ab concentrations.

Flow cytometry

Splenocytes, PBMCs, BM cells and iLNs were processed into single cell suspensions as above and stained for flow cytometry analysis as described (13). Cells were incubated with an Aqua Live-Dead fixable viability dye (Molecular Probes, Life Technologies, Waltham, MA, USA) in the absence of FBS, to discriminate dead cells. Cells were then blocked using an anti-F_C receptor Ab (anti-CD16/CD32) (2.4G2) (BioLegend, (San Diego, CA, USA) and stained for surface markers and then fixed in 1–2% paraformaldehyde. Cells were stained with mAbs conjugated to FITC, allophycocyanin, eFluor450, allophycocyanin-eFluor780, PerCPCy5.5, PE-Cy7, AlexaFluor647, BUV395, BV605, BV421, BV711, BV650 and BUV395. For analysis of splenic or BM cell subsets eleven- to twelve-colors flow cytometry was performed using combinations of mAbs against: CD19 (1D3), CD11b (M1/70) and CD11c (N418) from eBioscience (San Diego, CA, USA); B220 (RA3–6B2), CD93 (AA4.1) and Ly6C (AL-21) from BD Horizon/Biosciences (San Jose, CA, USA); CD19 (1D3), B220 (RA3–6B2), CD3 (17A2), NK1.1 (PK136), CD8α (53–6.7), Ly6G (1A8), SiglecH (440c), Ly49c (14B11), CD49b (DX5), NKp46/CD335 (29A1.4), CD127 (SB/199), CD21/35 (7E9), CD23 (B3B4) and CD24 (M1/69) from BioLegend (San Diego, CA, USA). The BAFF-RFP signal was detected in the PE channel. Cells were processed with an LSRII flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) using a FACSDiva software and data were analyzed using FlowJo (v.10, Tree Star). See supplemental Fig S2A and Fig. S2B for gating strategies for spleens (and iLNs) and BM, respectively. Splenic myeloid cell subsets were defined as described previously (13), as in supplemental Fig S2A. For BM we identified 5 populations of myeloid cells and precursors in the **CD11b$^+$**B220$^-$CD19$^-$Ly6G$^-$SSC$^-$ gate and defined them based on the literature (48–50) (See supplemental Fig. S2B and supplemental Table S1). A similar gating strategy as for spleen cells was used for PBMC. PBMC subsets were defined as follows: **Nphs**, CD11b^{hi}Ly6G^{hi}Ly6C^{int}SSC^{int-}NK1.1$^-$; **Ly6C^{hi} MOs**, CD11b^{hi}Ly6C^{hi}CD11c$^-$SSC$^-$Ly6G$^-$NK1.1$^-$SiglecH$^-$; **DCs**, CD11b$^+$Ly6C$^-$CD11c$^+$SSC$^-$Ly6G$^-$NK1.1$^-$SiglecH$^-$; **NK cells**, NK1.1^{hi}CD11b^{int}; **T cells**, CD3$^+$CD19$^-$; **B cells**, CD3$^-$CD19$^+$.

Splenic B cells subsets were defined as described previously (13, 51). After gating out debris, doublets and dead cells, B cells subsets in the CD19$^+$ B220$^+$ gate were defined as follows: FO B cells, CD24^{mid}CD21/35^{mid}CD93$^-$CD23$^-$; Marginal zone (MZ) B cells, CD24^{hi}CD21/35^{hi}CD93$^-$CD23$^-$; MZ B cell precursors CD24^{hi}CD21/35^{hi}CD93^{lo}CD23$^+$; T2 B cells, CD24^{hi}CD21/35$^{int/hi}$CD93$^+$CD23$^+$; T1 B cells CD24^{hi}CD21/35^{lo}CD93$^+$CD23$^-$. For BM B cell precursors and long lived plasma cells (**PCs**) (52) we gated out NK cells, pDCs and CD11b$^+$ cells. In the NK1.1$^-$SiglecH$^-$CD11b$^-$ gate we defined **PreProB** cells as B220$^-$CD43$^+$CD19$^-$; **ProB** cells as B220^{lo}CD43$^+$CD19$^+$; **PreB** cells as

B220^{+}CD43^{-}CD19^{+}IgM^{-}IgD^{-}; newly formed B cells (**NFB**) as B220^{+}CD43^{-}CD19^{+}IgM^{+}IgD^{-}; mature B cells (**MatB**) as B220^{+}CD43^{-}CD19^{+}IgM^{+}IgD^{+}; **PreBNFMatB** (B220^{+}CD43^{-}). Splenic **PCs** and long lived **PCs** in the BM were defined as B220^{lo}CD138^{+}. Splenic T cell subsets are defined as follows: **CD8 T cells** as CD3^{+} CD8^{+}CD19^{-}, **CD4 T cells** as CD3^{+} CD8^{-}CD19^{-}.

Sorting Strategy for BAFF-RFP mice and BAFF cKO mice verification and BAFF qPCR analysis

BM cells from WT and BAFF-RFP (+/−) mice were harvested as described above and cell subsets sorted with FACS Aria cell sorter (Becton Dickinson, Franklin Lakes, NJ, USA). Nphs were sorted from WT and BAFF-RFP mice as CD11b^{+}Ly6G^{hi}Ly6C^{int}SSC^{int-}. B cells were sorted from WT mice as Ly6G^{-}B220^{+} cells, and as Ly6G^{-}B220^{+}RFP^{+} B cells and Ly6G^{-}B220^{+}RFP^{-} B cells from BAFF-RFP mice.

Splenocytes were harvested from *Baff*$^{fl/fl}$, *Baff*$^{fl/fl}$ *MRP8*^{Cre} or *Baff*$^{fl/fl}$ *zDC*^{Cre} mice as described above and enriched with CD11b^{+} CD11c^{+} magnetic bead positive selection (Milteny, CA, USA). Cell populations were sorted using a FACS Aria cell sorter (Becton Dickinson, Franklin Lakes, NJ, USA). For gating strategies see Supplemental Fig. S2A. Sorted cells were lysed and RNA extracted for qRT-PCR analysis as described (33). The primer sequences used were as follows: *mBaff*-F 5'-AGGCTGGAAGAAGGAGATGAG-3' and *mBaff*-R 3'- CAGAGAAGACGAGGGAAGGG −5'. See Supplemental (Fig. S1C, S1D) for verification of BAFF Nph cKO (*Baff*$^{fl/fl}$ MRP8^{Cre}) mice or BAFF cDCs cKO (*Baff* $^{fl/fl}$ zDC^{Cre}) mice. As additional controls, sorted cells from WT mice, MRP8^{Cre} mice and zDC^{Cre} mice all showed similar levels of *Baff* mRNA as *Baff*$^{fl/fl}$ mice (not shown).

Statistical analyses

Survival data were analyzed by Mantel-Cox log-rank test. Timeline data were analyzed using a 2-way ANOVA with Tukey's multiple comparison test. Analysis between more than two groups were performed using 1-way ANOVA with Holm-Sidak method for multiple comparisons. Analyses between two groups were performed using unpaired Student t test. GraphPad Prism 7 was used for statistical analyses. Differences of $p<0.05$ were considered significant.

Results

BAFF-RFP expression in BM and splenic cell populations

We developed mouse models to define how BAFF expression by cell subsets is regulated and what BAFF-producing cells are required for B cell responses. We used a two-construct approach to generate BAFF IRES-TagRFP-T (BAFF-RFP) reporter mice concurrently with *Baff*$^{fl/fl}$ mice (see supplemental Fig. S1A for details). In BM from naïve BAFF-RFP (+/−) mice most Nphs were BAFF-RFP^{+} and a small population of BAFF-RFP^{+} B220^{+} B cells was detectable (Fig. 1A). To determine whether RFP expression in BAFF-RFP mice correlated with *Baff* mRNA expression, we sorted BM Nphs, BAFF-RFP^{+} B220^{+} cells and BAFF-RFP^{-} B220^{+} B cells and quantified *Baff* mRNA levels (Fig. 1B, for sorting strategy see Methods). As expected *Baff* mRNA levels in Nphs from heterozygous BAFF-RFP mice

were about half the levels detected in WT mice. *Baff* mRNA expression was lower in B cells than in Nphs in both WT and BAFF-RFP$^{+/-}$ mice. Importantly, *Baff* mRNA levels were higher in BAFF-RFP^{+} B cells compared to BAFF-RFP^{-} B cells or unsorted WT B cells (Fig. 1B). Therefore, BAFF-RFP detected by flow cytometry correlates with *Baff* mRNA expression.

Analysis of cell populations from spleens or PBMCs (see Methods and supplemental Fig. S2A for gating strategy) from naïve BAFF-RFP$^{+/-}$ mice revealed that BAFF is constitutively expressed at high levels in Nphs, expressed at lower levels in DCs and MOs and had very low expression or was undetectable in other leukocyte subsets (Fig. 1C and 1D, Fig. S3A and S3B). These data are consistent with previous reports (1, 11, 21). Although BAFF-RFP expression overall was very low in B cells, BAFF was detectable particularly in MZ B cells and MZ B cell precursors and T2 B cells and in small numbers of FO and T1 B cells (Fig. S3A), in agreement with *Baff* mRNA expression in B cells subsets (53).

Interestingly, in the BM of naïve BAFF-RFP$^{+/-}$ mice, in addition to Nphs, other myeloid cell subsets and myeloid precursors constitutively expressed significant levels of BAFF-RFP (Fig. 2). Using mice with either one *Rfp* allele (RFP$^{+/-}$) or two *Rfp* alleles (RFP$^{+/+}$), we examined the CD11b^{+} BM population in detail and designated subsets based on their cell surface phenotypes (see Methods and supplemental Fig. S2B and Table S1). In particular, a high percentage of Nph precursors, CD11b^{+} myeloid precursors and preDCs expressed BAFF-RFP compared to MOs (Fig. 2A). BAFF-RFP expression levels were also greater in Nphs and other myeloid precursors compared to MOs, as shown by their higher levels of expression (Fig. 2B). The BAFF-RFP signals in both BAFF-RFP$^{+/-}$ and BAFF-RFP$^{+/+}$ were significantly different from RFP background detection in WT mice (Fig. 2A–D). The higher levels of BAFF-RFP in BAFF-RFP$^{+/+}$ mice vs BAFF-RFP$^{+/-}$ mice in all BM populations, confirmed that BAFF-RFP protein detection by flow cytometry correlates with the amount of *Baff* gene expression (Fig. 2A–D).

We also analyzed BM B cell subsets and precursors. BAFF-RFP was expressed at low levels in B220^{lo} CD138^{+} long-lived plasma cells (LLPCs) and in a subset of CD11b^{+} B220^{+} CD19^{+} B cells but not in ProB cells or more mature B cells precursors (Fig. 2C and 2D). As expected, BAFF-RFP$^{+/-}$ mice had fewer mature B cells precursors in the BM than WT mice, while BAFF-RFP$^{+/+}$ mice had almost no mature B cells and normal numbers of earlier B cell precursors (Fig. S2C and S2D). Although the BAFF-RFP$^{+/-}$ mice had lower expression of BAFF, they were still capable of producing mature B cells and thus could be used to measure B cell functions and Ab responses *in vivo*. For subsequent studies we used BAFF-RFP$^{+/-}$ mice and refer to them simply as BAFF-RFP mice.

Cell and tissue specific upregulation of BAFF-RFP in myeloid subsets after *in vivo* administration of TLR agonists or WNV infection

To address how the activation of RNA-sensing pathways regulate BAFF expression in specific cell subsets *in vivo*, we injected BAFF-RFP mice with TLR3 agonist Poly(I:C) or the TLR7/8 agonist R848 and analyzed BAFF-RFP expression in splenic cell populations 24 h later (Fig 3A–D). Poly(I:C) administration led to significant increases in BAFF-RFP MFI (Fig. 3A and 3B) and the percentage of BAFF-RFP^{+} cells (not shown) in inflammatory

Ly6C^{hi} MOs but not in cDCs, Nphs or B cells. In contrast, R848 administration had no effect on BAFF-RFP expression in splenic Ly6C^{hi} MOs but slightly upregulated BAFF-RFP in Nphs (Fig. 3C and 3D). Furthermore, the TLR9 agonist, CpG did not affect BAFF-RFP expression in either Ly6C^{hi} MOs or Nphs (Fig. 3D). Thus, changes in BAFF-RFP expression in myeloid subsets varied depending on the TLR-ligand used.

Next we challenged BAFF-RFP mice with WNV-TX (WNV), a pathogenic ssRNA virus, and examined BAFF-RFP expression in myeloid cells within the draining iLNs 24 h and 48 h after infection. Similarly to the effect of the TLR3 agonist in the spleen, WNV infection slightly increased BAFF-RFP levels in Nphs from iLNs (Fig. 3E), and in addition, significantly up-regulated BAFF-RFP expression in CD8^{-} cDCs 24 h post-infection (Fig. 3E). BAFF-RFP levels did not change in iLN CD8^{+} cDC, MO subsets, B cells or T cells after WNV infection (Fig. 3E and not shown).

Interestingly, after either Poly(I:C) administration or WNV infection, we detected increased numbers of an activated NK1.1 subset, which expressed high levels of both BAFF-RFP and CD11b (CD11b^{hi} NK). In contrast, CD11b^{int-} NK cells were BAFF-RFP^{-} (Fig. 3F, 3G, 3H ***left panel*** and 3I). This BAFF-RFP^{+} CD11b^{hi} NK subset was not upregulated after *in vivo* administration of R848 or CpG (Fig. 3H *right panel*). A phenotypic analysis revealed that the CD11b^{hi} NK cells, like other NK cell subsets, express CD49b, NKp46 and Ly-49C, and in addition express Ly6G, a marker found on Nphs (Supplemental Fig. S3C). Furthermore, CD11b^{hi} NK cells did not express CD127 suggesting they were not innate lymphoid cell 1 (ILC1) (54). In summary, by using BAFF-RFP reporter mice we detected cell- and tissue-specific up-regulation of BAFF after *in vivo* challenge with different RNA-sensing TLR agonists or WNV infection.

Requirement for Type I IFN and MAVS signaling pathways for BAFF-RFP up-regulation in myeloid subsets

Both dsRNA and ssRNA viruses trigger three RNA sensing pathways: TLR3 (dsRNA), TLR7/TLR8 (ssRNA) and RIG-I/MDA5/MAVS (ssRNA, dsRNA), which induce type I IFN production in plasmacytoid DCs or other cells (36, 55). Type I IFN is a major inducer of BAFF. However, direct regulation of BAFF by TLR engagement also has been described (22). Thus, to test whether Type-1 IFN was required for BAFF upregulation, we crossed BAFF-RFP mice with *Ifnar*$^{-/-}$ mice and examined BAFF-RFP expression after either Poly(I:C) administration or WNV infection (Fig. 4). Unlike in BAFF-RFP WT mice, BAFF-RFP *Ifnar*$^{-/-}$ mice did not upregulate BAFF-RFP in Ly6C^{hi} MOs and Ly6C^{hi} DCs in response to Poly(I:C), while there was no change in the constitutive BAFF-RFP expression in Nphs (Fig. 4A and 4B, and not shown). Furthermore, BAFF-RFP *Ifnar*$^{-/-}$ mice didn't upregulate BAFF in either iLN Nphs or CD8^{-} cDC after infection with WNV (Fig. 4C and not shown). Thus, the increases in BAFF expression in splenic inflammatory monocyte subsets in response to Poly(I:C) immunization and in iLN Nphs and CD8^{-} cDCs after WNV infection all require type I IFN signaling.

Since both dsRNA and ssRNA can induce type I IFN via the MAVS pathway, we next tested whether Poly(I:C)- or WNV-induced changes in BAFF-RFP expression required MAVS. Using BAFF-RFP *Mavs*$^{-/-}$ mice, we found that myeloid subsets differed in their

requirement for MAVS to regulate BAFF expression. Poly(I:C)-induced increases in BAFF-RFP MFI in Ly6C^{hi} DCs required MAVS, while the BAFF-RFP MFI increase in Ly6C^{hi} MOs and Ly6C^{lo} MOs was only partially inhibited in BAFF-RFP *Mavs*$^{-/-}$ mice (Fig. 5A and 5B). As expected, there was no change in the BAFF-RFP expression in Nphs. These data suggest that for MO subsets, type I IFN production that up-regulates BAFF, occurs through either the TLR3 pathway or the MAVS pathway. In contrast, the BAFF increase in inflammatory Ly6C^{hi} DCs is dependent solely on type I IFN produced via the MAVS pathway.

We also analyzed BM cells from BAFF-RFP *Ifnar*$^{-/-}$ and BAFF-RFP *Mavs*$^{-/-}$ mice after Poly(I:C) immunization. Both the IFNAR and MAVS signaling pathways were required to induce increases in the percentage of inflammatory BAFF-RFP^{+} Ly6C^{hi} MOs and BAFF-RFP^{+} Ly6C^{hi} CD11c^{+} cells as well as in other myeloid precursors and BAFF^{+} CD11b^{+} B cells (Supplemental Fig. S3D and S3E). Thus, BAFF is differentially regulated in both a cell–specific manner and in a tissue-specific fashion. For example, BAFF regulation in Ly6C^{hi} MOs is only partially dependent on MAVS in the spleen, but requires MAVS in the BM.

BAFF MFI was not significantly upregulated in CD8^{-} cDCs in BAFF-RFP *Mavs*$^{-/-}$ mice after WNV infection; however, BAFF upregulation in Nphs after infection was independent of MAVS (Fig. 5C). Therefore, after WNV infection the BAFF increase in CD8^{-} cDCs requires the activation of the MAVS pathway, while TLR7/8 pathway may be required for increasing BAFF expression in Nphs. In summary, the changes in BAFF expression by myeloid subsets induced after Poly(I:C) immunization or WNV infection require type I IFN signaling but only in some cases are dependent on MAVS.

The expansion of BAFF^{+} CD11b^{hi} NK cells and BAFF^{+} Ly6C^{hi} MOs and DCs is regulated by IFNAR and MAVS signaling

BAFF-RFP reporter mice allowed us not only to detect BAFF expression per cell but also to analyze changes in populations of BAFF-RFP producing cells. Several BAFF-RFP^{+} myeloid cell subsets including Nphs, inflammatory MOs and cDCs were expanded in the iLNs 24 h after WNV infection (Fig. 6A). A number of factors, including chemokines and chemokine receptors, could regulate migration and the expansion of myeloid cells upon infection. Therefore, we tested whether MAVS/IFNAR signaling pathways were required for the changes in cell numbers. Interestingly, MAVS signaling contributed to BAFF-RFP^{+} inflammatory Ly6C^{hi} MO (Fig. 6A) and Ly6C^{hi} DC (not shown) expansion but not to the increased numbers of BAFF^{+} CD8^{-} cDCs and BAFF^{+} Nphs (Fig. 6A). Thus, even though BAFF-RFP expression per cell in CD8^{-} cDC required MAVS signaling, the expansion of CD8^{-} cDCs was MAVS independent. The requirements for IFNAR in the expansion of myeloid subsets were similar to the MAVS requirements (not shown). Taken together these results suggests that RIG-I/MAVS-Type I IFN signaling axis plays a role in the expansion of inflammatory Ly6C^{hi} MOs and Ly6C^{hi} DCs, while other factors are responsible for the increased numbers of Nphs and cDCs upon WNV infection.

The expansion of the BAFF-RFP^{+} NK CD11b^{hi} cells in the iLN after WNV infection required the IFNAR signaling pathway, but was independent of MAVS signaling (Fig. 6B).

In contrast, administration of Poly(I:C) to either BAFF-RFP *Ifnar*$^{-/-}$ or BAFF-RFP *Mavs*$^{-/-}$ mice failed to expand BAFF^{+} CD11b^{hi} NK cells (Fig. 6C). Taken together these data suggest that type I IFN-dependent expansion of activated BAFF^{+} CD11b^{hi} NK cells, upon WNV infection is mostly dependent on TLR7/8 signaling. In contrast, after dsRNA challenge, the type I IFN-dependent expansion of this BAFF^{+} NK subset, occurs via engagement of the MAVS pathway rather than TLR3 activation.

BAFF produced by DCs is required for protective immune responses to WNV infection

BAFF expression increases in cDCs and Nphs, and BAFF^{+} cDCs and BAFF^{+} Nphs expand after WNV infection (Fig. 3E, 6A). To examine the role of these sources of BAFF during infection, we generated *Baff* conditional knockout (BAFF cKO) mice, where we specifically deleted *Baff* in cDCs or Nphs, *Baff*$^{fl/fl}$ mice (See Supplemental Fig. S1A and Methods) were crossed with either *zDC*^{Cre} mice or *Mrp8*^{Cre} mice to generate mice deficient in BAFF expression on cDCs (*Baff*$^{fl/fl}$ *zDC*^{Cre}), and mice deficient in BAFF expression on Nphs (*Baff*$^{fl/fl}$ *Mrp8*^{Cre}). Analysis of the expression of *Baff* mRNA in purified cDCs or Nphs confirmed that BAFF expression was knocked out appropriately in the respective BAFF cKO mice (Fig. S1C, S1D). *Baff* mRNA reduction was restricted to cDCs in *Baff*$^{fl/fl}$ *zDC*^{Cre} mice. BAFF mRNA in *Baff*$^{fl/fl}$ *Mrp8*^{Cre} mice was dramatically reduced in Nphs, but some reduction was evident in MOs. In agreement with previous findings suggesting that radiation-resistant but not hematopoietic cells were responsible for B cell homeostasis (56), we found that depleting BAFF from either Nphs or DCs did not alter the splenic B cell compartment in naïve mice (data not shown).

We infected *Baff*$^{fl/fl}$ *zDC*^{Cre} mice and *Baff*$^{fl/fl}$ *Mrp8*^{Cre} mice and monitored their survival and Ab responses. *Baff*$^{fl/fl}$ *zDC*^{Cre} mice had significantly increased mortality compared to *Baff*$^{fl/fl}$ and *zDC*^{Cre} controls (Fig. 7A). In contrast, *Baff*$^{fl/fl}$ *Mrp8*^{Cre} mice had similar survival rate as *Baff*$^{fl/fl}$ mice (Fig. 7A). Furthermore, *Baff*$^{fl/fl}$ *zDC*^{Cre} mice had increased virus titers (Fig. 7B) and decreased WNV-specific IgG and WNV neutralizing Abs (Fig. 7C and 7D) compared to controls. Thus, BAFF produced by DCs is required for optimal protective immune responses to WNV infection. Apparently, BAFF from cDCs helps to sustain or promote B cell humoral responses to WNV, since WNV-specific Ab responses are decreased in mice lacking BAFF expression on cDC.

BAFF from Nphs and cDCs contributes to TI-2 IgG Ab responses

BAFF produced by DCs, Nphs or MOs has been reported to contribute to TI-2 Ab responses (25, 32, 33, 57). To examine further whether BAFF from Nphs or cDCs play a role in TI-2 Ab responses, we immunized *Baff*$^{fl/fl}$ *Mrp8*^{Cre} mice and *Baff*$^{fl/fl}$ *zDC*^{Cre} mice with the TI-2 Ag, NP-Ficoll (Fig. 8). Both *Baff*$^{fl/fl}$ *Mrp8*^{Cre} mice and *Baff*$^{fl/fl}$ *zDC*^{Cre} mice had reduced NP-specific IgM and IgG3 production (Fig. 8A, and 8B, respectively). Thus, both Nph and cDC BAFF sources are required for normal Ag-specific Ab responses to TI-2 Ags. Since BAFF depletion in Nph in *Baff*$^{fl/fl}$ *Mrp8*^{Cre} mice was selective but not restricted to Nphs, we cannot formally exclude that BAFF from MOs may also contribute to the reduced Ab response to NP-Ficoll. However, *Baff*$^{fl/fl}$ *Cx3cr1*^{Cre} mice, which have reduced BAFF expression in MOs, did not have reduced NP-specific Ab responses compared to control mice (Fig S1E).

Discussion

BAFF is essential for the host response to pathogens and provides a critical link between innate and adaptive immune responses (1, 58). Although BAFF dysregulation has been implicated in several diseases, studies investigating BAFF producing cells have been limited either to *in vitro* experiments or used models where BAFF or its receptors were completely blocked or ablated (13, 29, 32, 33, 57, 59). Here we developed and used BAFF-RFP reporter mice and *Baff*$^{fl/fl}$ mice to better understand BAFF sources and their functions in B cell responses. We found that BAFF is expressed in several blood cell subsets as well as lymphoid tissues, including the spleen and LNs. The major cells expressing BAFF are Nphs, DCs and MO subsets. Furthermore, BAFF expression can undergo cell- and tissue-specific up-regulation upon *in vivo* activation of different RNA-sensing pathways. In addition, selective deletion of BAFF expression in cDCs or Nphs revealed that BAFF from both cDCs and Nphs contribute to TI-2 Ab responses, while cDC BAFF, unlike Nph BAFF, is required for optimal protective immunity against WNV.

Nphs express the highest BAFF levels in blood and in every tissue we tested, including the spleen, iLNs and BM. These data are consistent with previous reports describing Nphs as a major source of BAFF (21, 60, 61). BAFF production by Nphs has been reported in human autoimmune diseases and mouse models (62–64). However, BAFF-production by Nphs during viral infections has not been previously noted. BAFF expression by Nphs was slightly up-regulated after *in vivo* challenge with two ssRNA stimuli: R848 and WNV. WNV-induced BAFF increase in Nphs was IFNAR-dependent but MAVS-independent, suggesting that Type-1 IFN production that regulates BAFF in Nphs might be induced through the TLR7/8 pathway. Although, Nph BAFF production increased after viral infection, it is not required for protective immunity to WNV. In contrast, BAFF produced by Nphs is required for protective immunity against *Salmonella typhimurium* infection (Kuley et al. manuscript in preparation). Further investigation is required to elucidate the possible roles of BAFF produced by Nphs during viral and bacterial infections. Puga et al. (56) previously reported that BAFF produced by human Nphs may contribute to TI Ab responses (57). In support of this study, we demonstrated that selective depletion of BAFF from Nphs reduces Ag-specific Ab responses to a TI-2 Ag. Nphs strategically located in the peri-marginal zone of the spleen may help B cells to rapidly respond to blood-borne TI Ags (57, 65, 66).

Earlier studies also implicated DCs in TI Ab responses, and more recently, several cDC subsets were reported to be localized in the marginal zone (32, 67–69). In agreement with these findings, we found that BAFF from cDCs is required for optimal humoral responses to a TI-2 Ag. The reduction but not full ablation of Ab responses to NP-Ficoll in either BAFF Nph cKO or BAFF cDC cKO mice, suggests that both Nphs and cDCs contribute to TI-2 Ab responses. It is also possible that some BAFF-producing cells compensate for the absence of BAFF-production by another source. Further studies are needed to identify specific niches in lymphoid tissues where BAFF-producing cDCs and Nphs provide help to B cell responses (70, 71).

BAFF plays an important protective role during viral infections (13, 72). In particular, BAFF signaling is an important regulator of humoral responses and required for survival against

lethal WNV infection (73). Here we found that cDC BAFF is essential for protection from WNV infection. In contrast to BAFF Nph cKO mice, BAFF cDC cKO mice after WNV infection, had increased mortality and increased virus titers. The reduced WNV-specific IgG and WNV neutralizing Abs in BAFF cDC cKO mice suggest that BAFF from cDCs is essential for the development of protective humoral responses to WNV. How BAFF-producing cells contribute to control WNV replication, whether it is through the regulation of B cells responses or other mechanism needs further investigation. Consistent with the protective role of cDC-derived BAFF, WNV infection up-regulated BAFF expression in $CD8^-$ cDCs. WNV induced up-regulation of BAFF in $CD8^-$ cDCs was dependent on MAVS, suggesting that this occurred through WNV activation of the RIG-I pathway (36, 55). Since both pDCs and cDCs express the RIG-I/MAVS pathway and can produce Type I IFN upon viral challenge (47, 55, 74), it remains to be determined if BAFF upregulation in cDCs after WNV infection is dependent on Type I IFN produced by cDCs or by pDCs.

The IFNAR-dependent and MAVS-dependent BAFF regulation in inflammatory MO subsets by Poly(I:C) most likely occurs via MDA5 triggered by long dsRNA (36, 55, 75). Our finding that Poly(I:C) increased BAFF levels in splenic inflammatory $Ly6C^{hi}$ MO and $Ly6C^{hi}$ DC subsets is consistent with previous reports. Poly(I:C) and virus-induced Type I IFN production by pDCs upregulates BAFF levels in MOs and myeloid DCs (12, 31, 76). Inflammatory MO/DCs also express TLR3 and produce IFNα in response to Poly(I:C) and dsRNA (74, 77–80). Furthermore, the MAVS signaling pathway has been implicated in type I IFN production by inflammatory MOs and MO-derived DCs (74, 79). Further studies are needed to establish whether the MAVS/IFNAR requirement for Poly(I:C)-induced BAFF up-regulation in inflammatory MO subsets occurs via type I production by pDCs, or in an autocrine fashion.

Using BAFF-RFP mice we identified a murine NK cell subset expressing BAFF induced by the activation of RNA-sensing pathways. The increased expression of CD11b, NKp46 and Ly49C in $BAFF^+$ $CD11b^{hi}$ NK cells, compared to $CD11b^{int/-}$ NK cells, suggests that the $BAFF^+$ $CD11b^{hi}$ NK cells are activated (54, 81). The fact that the expansion of $BAFF^+$ $CD11b^{hi}$ NK cells is dependent on IFNAR suggests type I IFN plays a major role not only in the up-regulation of BAFF expression by NK cells but also in the induction of this activated NK subset. These findings are in agreement with previous reports implicating type I IFN in the activation of NK cells during viral infections (82–85). NK cells play an important role in antiviral immunity not only through the NK-mediated killing of virus infected cells but also by regulating adaptive immune responses (84, 86, 87). NK cells can enhance Ag presentation by B cells and can promote TI Ab responses against bacteria or in autoimmune settings (88–90). Also a human NK subset has been shown to produce BAFF (91). BAFF expression by human NK cells inhibits sensitivity to treatment of chronic lymphoid leukemia, and has been correlated to a SNP associated with susceptibility to autoimmune disease (92, 93). It would be interesting to investigate whether the activated BAFF expressing NK cells we have characterized play a role in regulating B cell responses during infections or during the development of autoimmune diseases.

Remarkably, in the BM not only Nphs, but also some $CD11b^+$ myeloid precursors express high levels of BAFF. Interestingly, Poly(I:C)-dependent BAFF regulation in BM MO subsets

was similar to their counterparts in the spleen, and required the MAVS/IFNAR pathway. These BAFF^{+} myeloid precursors are most likely late precursors since they express CD11b. Among the myeloid precursors expressing higher BAFF levels, the population we define as CD11b^{+} myeloid precursor lacking Ly6C may be an earlier progenitor of a precursor not committed to a specific granulocyte/monocyte lineage, as described by Yanez et al. (48, 50). Consistent with reports of BM-derived DCs being a source of BAFF (33), we found that BM preDCs expressed substantial BAFF levels. Despite the high BAFF expression in Nph and DC precursors in the BM and in spleens, these cell sources were not required to maintain the general mature B cell compartment. Our results are in agreement with earlier findings suggesting that BAFF produced by radiation-resistant cells, possibly stromal cells, are responsible for normal B cell homeostasis (56),

Interestingly, a subset of CD19^{+}B220^{+}CD11b^{+} B cells in the BM expressed BAFF; these cells were CD43^{+lo}CD5^{-}CD11c^{-}, and some were IgM^{+} and IgD^{+}. Further analysis is required to define this subset better. They may belong to the B1 B cell lineage because since they express CD11b and BAFF (53, 94) or be a BM B cell subset providing BAFF to BM LLPCs as a survival factor (1, 5, 95). We also found that BAFF is expressed in some peripheral B cell subsets; further studies are required to define the functions of this source of BAFF.

Our new BAFF-RFP reporter and *Baff*$^{fl/fl}$ mouse lines may be useful for identifying BAFF sources and defining their *in vivo* functions. Localization of BAFF-RFP positive cells in the spleen and lymph nodes, would also be very helpful to identify specific niches of BAFF producing cells during immune responses. Given the major role of BAFF in the host response to pathogens (58) and in autoimmune diseases, more insights into the localization, timing and function of BAFF-producing cells regulating B cell responses may help in the development of more targeted vaccines and therapies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We thank Dr. Keith Elkon for helpful insights and discussion. We thank Michel Black and the Cell and Analysis Flow Cytometry and Imaging Core in the Department of Immunology at the University of Washington for their support. The authors declare they have no competing financial interests.

This work was supported by a grant from the National Institutes of Health (R01AI44257, EA Clark).

Abbreviations

B6	C57BL/6
BAFF	B cell activating factor
BM	bone marrow
cKO	conditional knockout

cDC	conventional DC
DC	dendritic cell
FO	follicular
f.p.	footpad
IFNAR	Type I IFN receptor
KO	knockout
cKO	conditional knockout
LLPC	long-lived plasma cell
MAVS	mitochondrial activator of virus signaling
MΦ	macrophage
MO	monocyte
MZ	marginal zone
Nph	neutrophil
NP	4-hyrody-3-nitrophenacetyl
Poly(I:C)	polyinosinic:polycytidylic acid
RIG-I	retinoic acid-inducible gene I
Tg	transgenic
WNV	West Nile Virus
WNVE	West Nile Virus E protein

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Key points

1. Cell- and tissue-specific type I IFN-dependent BAFF increases in myeloid subsets
2. Neutrophils and DCs BAFF is required for optimal T-independent 2 humoral responses
3. BAFF produced by DCs is required for protective immunity to West Nile virus

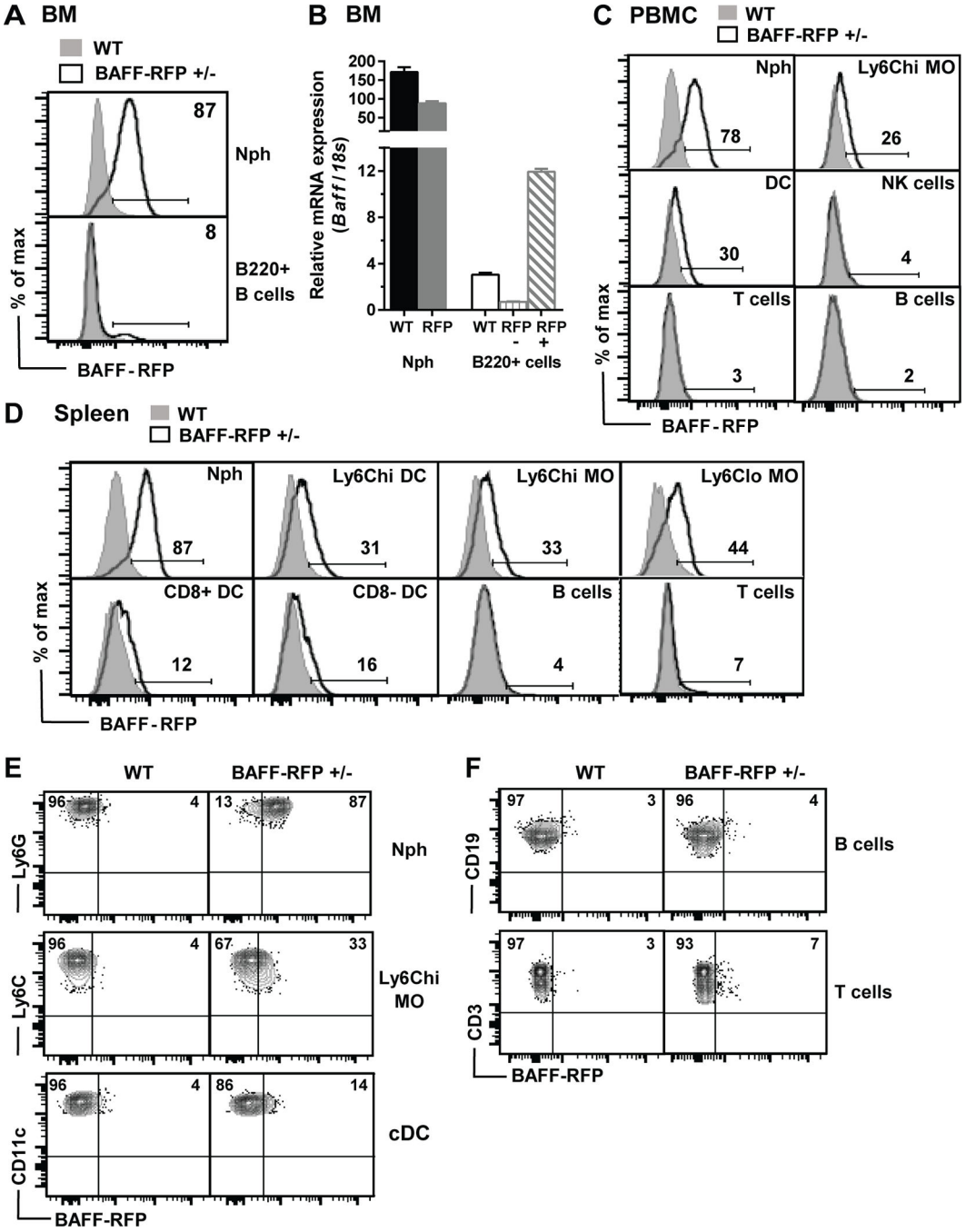

Figure 1. BAFF-RFP detection in blood and splenic cell subsets.

A. BAFF-RFP by flow cytometry in BM Nph and a subset of B220^{+} B cells in BAFF-RFP $^{+/-}$ mice (See Methods for gating strategy). **B.** BAFF mRNA by qPCR in sorted Nphs, B220^{+} B cells from WT mice, B220^{+}RFP^{+} B cells and B220^{+}RFP^{-} B cells from BAFF-RFP $^{+/-}$ mice. *Baff* mRNA expression in arbitrary units relative to 18S. **A** and **B**, data show one representative of two experiments. **C** and **D**, expression of BAFF-RFP in PBMC (**C**) and splenic (**D, E and F**) cell populations from naïve BAFF-RFP$^{+/-}$ mice. **C** and **D**, data show one representative of two (**C**) or more than five (**D, E and F**) experiments. In **A**, **C** and **D**,

WT (C57BL/6): *grey filled histograms*; BAFF-RFP$^{+/-}$ mice: *black empty histograms*. **E** and **F**, dot plots of BAFF-RFP expression in myeloid (**E**), B cells and T cells (**F**). In **A**-**E**, Neutrophils, Nph; Ly6C^{hi} monocytes, Ly6C^{hi} MO; dendritic cells, DC, conventional DC, cDC. For gating strategy of cell populations in Spleen (**D, E and F**) see Fig. S2B; and for cell populations in BM (**A**) and PBMCs (**C**) see Methods PBMCs (**C**) see Methods.

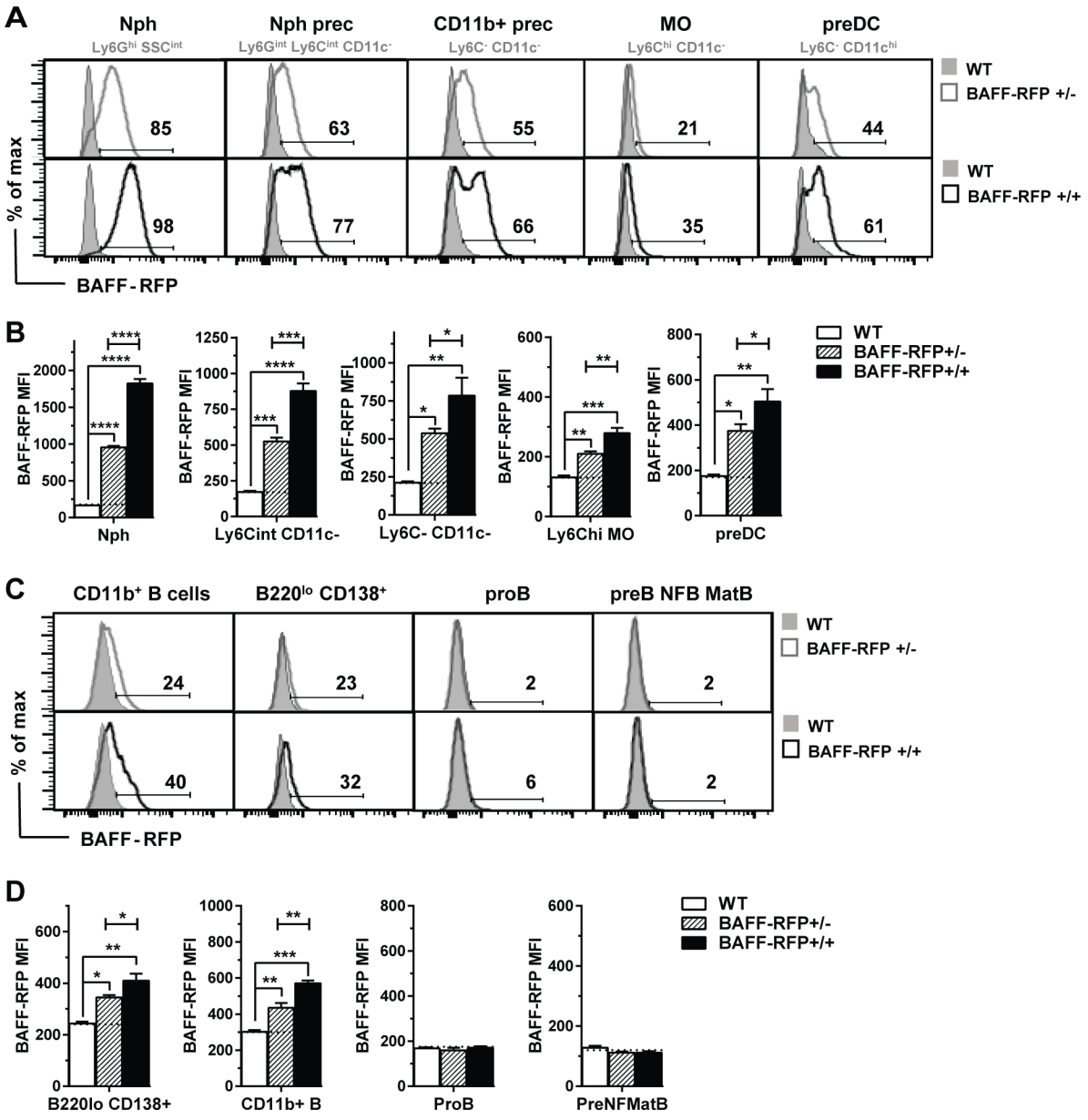

Figure 2. BAFF-RFP detection in BM cell populations.

A-D, BAFF-RFP detected by flow cytometry in BM myeloid cell populations (**A-B**) or B cell populations (**C-D**) from naïve WT mice, BAFF-RFP$^{+/-}$ mice and BAFF-RFP$^{+/+}$ mice. For gating strategy of myeloid cell populations (**A** and **B**) in BM see Fig. S2B. For gating strategy of B cell populations (**C** and **D**) see Methods. Data shown are representative histograms (**A** and **C)** or mean ± SEM of RFP MFI (**B** and **D**) from one of two independent experiments performed with three mice per group each. **B** and **D**, dotted lines show RFP MFI background in WT control. Statistics were performed by one-way ANOVA with Holm-Sidak method for multiple comparisons; * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.

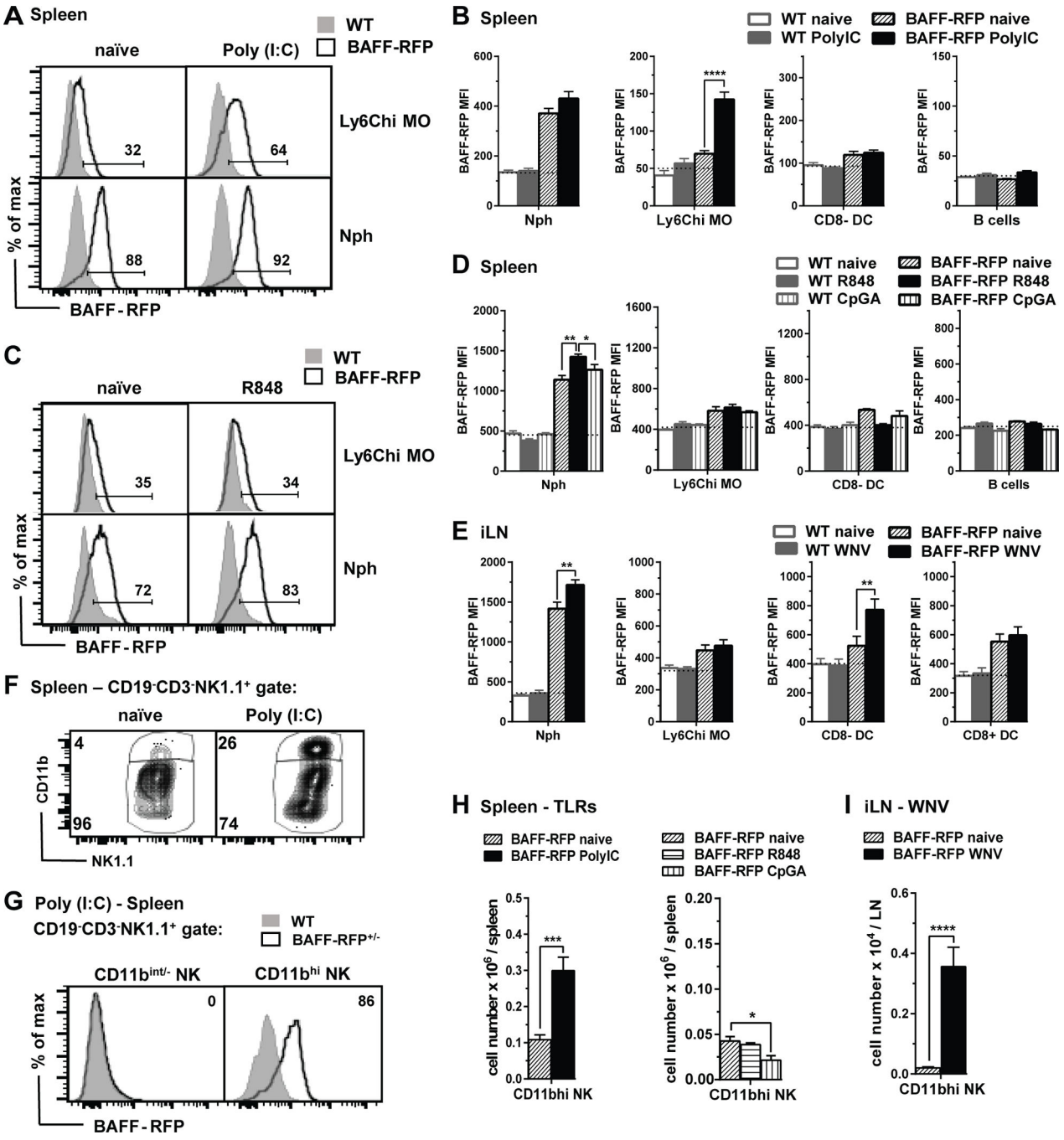

Figure 3. Cell and tissue specific up-regulation of BAFF-RFP in myeloid subsets after *in vivo* administration of TLR agonists or WNV infection.

A-D and **F-G**, WT and BAFF-RFP mice were injected i.p. or not for 24 h with 200μg/mouse Poly(I:C) (**A,B,F** and **G**), 200μg/mouse R848 (**C,D** and **G**), or 20nmol/mouse CpG (**D** and **G**). **A-D** and **F-G,** 6 h after *i.p.* injection of the TLR ligand, Brefeldin A was administered *i.v.* (see Methods). In **E** and **H**, WT and BAFF-RFP$^{+/-}$ mice were infected via f.p. with 1000pfu WNV. **A-H**, BAFF-RFP^{+} cell populations from spleens (**A-D** and **F-G**) or iLNs (**E** and **H**) were analyzed by flow cytometry and RFP MFI was compared to WT mice as

controls. In **F-H,** $CD11b^{hi}$ NK cell subset upregulated in spleen after Poly(I:C) injection (**F** and **G**) or in iLN after WNV infection (**H**). **A, F** and **C**, data are shown as representative histograms from more than three independent experiments (**A** and **F**, Poly(I:C)), or two independent experiments (**C**, R848). **B, D** and **E** bar graphs show means ± SEM of RFP MFI. **H** and **I**, bar graphs show total cell numbers of BAFF-RFP^{+} $CD11b^{hi}$ NK cells in spleens from Poly(I:C) (**H, *left panel***), R848 or CpG (**H, *right panel***), or in iLN from WNV infected mice **(I)**. Graphs summarize data from three independent experiments (**B, E, H, *left panel*** and **I (**N=9)); or are from one representative of two independent experiments (**D** and **H *right panel*** (N=3–4). In **B, D** and **E,** dotted lines show RFP MFI background in WT control mice. Statistics were performed by one-way ANOVA with Holm-Sidak method for multiple comparisons; except unpaired Student t test was used in **G *left panel*** and **H**; * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

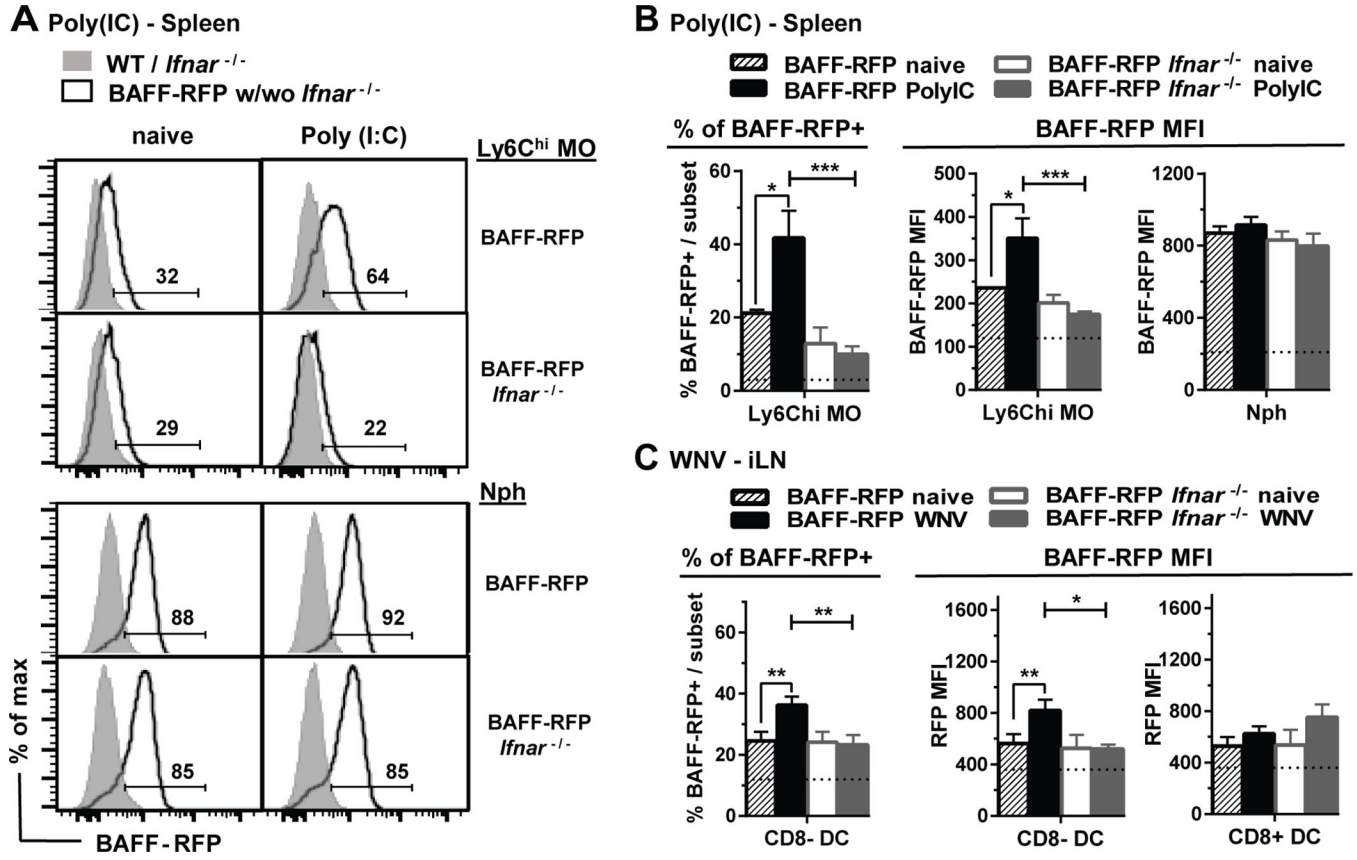

Figure 4. The type I IFN receptor (IFNAR) is required for BAFF up-regulation in MOs and cDCs induced by Poly(I:C) and WNV.

BAFF-RFP mice and BAFF-RFP *Ifnar*$^{-/-}$ mice were treated or not for 24 h with 200μg/mouse Poly(I:C) (**A** and **B**) by i.p. injection or f.p. infection with WNV (1000pfu) (**C**). Data show BAFF-RFP levels by flow cytometry in myeloid populations from spleens (**A** and **B**) and iLNs (**C**). **A,** data shown are representative histograms from two independent experiments, the numbers indicate % of BAFF-RFP^{+} cells. **B** and **C** bar graphs show means ± SEM of % of BAFF-RFP^{+} cells (***left panels***) or RFP MFI (***right panels***) and summarize data from two independent experiments (N=6–7). **B** and **C**, dotted lines show % of RFP^{+} cells or RFP MFI background in WT and *Ifnar*$^{-/-}$ mice. Statistics were determined with one-way ANOVA with Holm-Sidak method for multiple comparisons test; *** p<0.001, **** p<0.0001.

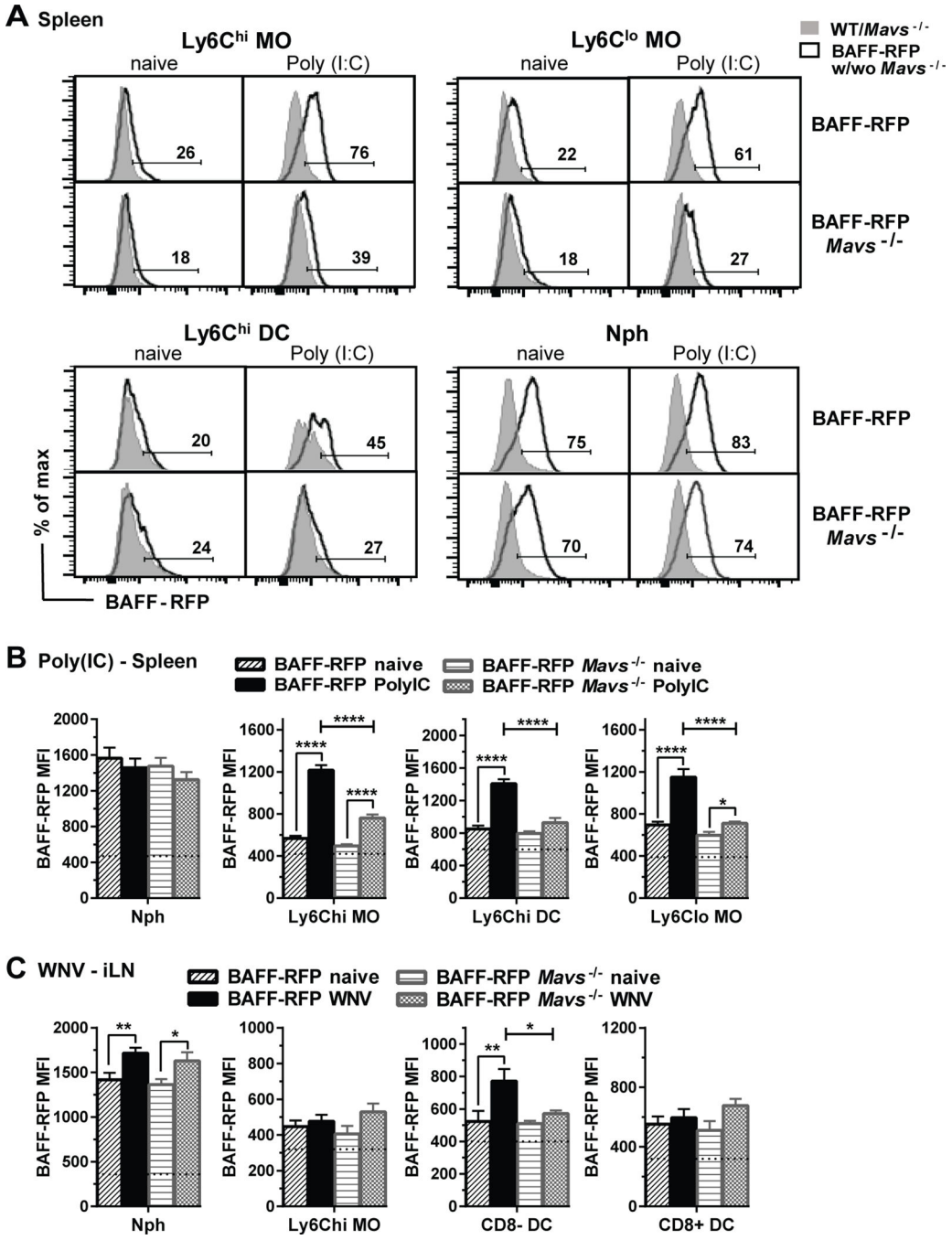

Figure 5. Differential requirement of MAVS for BAFF changes induced by Poly(I:C) and WNV in different myeloid subsets.

A-C. BAFF-RFP mice and BAFF-RFP *Mavs*$^{-/-}$ mice were left untreated or treated i.p. with 200μg/mouse Poly(I:C) or infected with 1000pfu WNV via the f.p. for 24 h. Data show BAFF-RFP levels by flow cytometry in myeloid populations from spleens (**A** and **B**) and iLNs (**C**). In **A,** data shown are representative histograms from two independent experiments, the numbers indicate % of BAFF-RFP^{+} cells. In **B** and **C** bar graphs show means ± SEM of BAFF-RFP MFI and summarize data from two independent experiments

(N=6–7). In **B** and **C**, dotted lines show RFP MFI background in WT and *Mavs*$^{-/-}$ mice that were included as controls in each experiment. Statistics were determined with one-way ANOVA with Holm-Sidak method for multiple comparisons test; ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

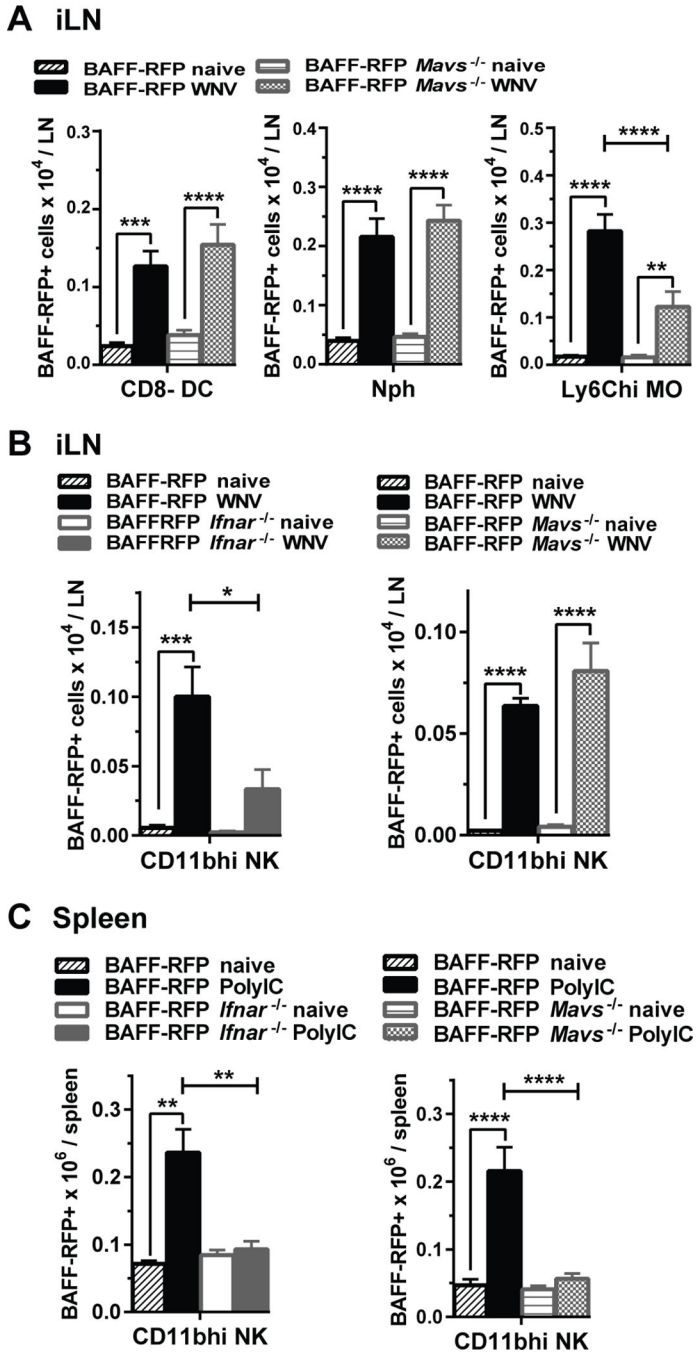

Figure 6. Regulation by MAVS and IFNAR signaling of BAFF$^+$ CD11b^{hi} NK cells and BAFF$^+$ Ly6C^{hi} MO/DC expansion in response to WNV and Poly(I:C).

BAFF-RFP mice, BAFF-RFP *Mavs*$^{-/-}$ mice (**A**) BAFF-RFP *Ifnar*$^{-/-}$ mice (**B** and **C**) or WT mice were infected or not with 1000pfu WNV (f.p.) (**A and B**) or injected i.p with 200μg/mouse Poly(I:C) (**C**). Data show numbers of BAFF-RFP$^+$ myeloid cell subsets from iLNs harvested 24 h post-infection (**A and B**), or from spleens 24 hrs post- Poly(I:C) immunization (**C**). Bar graphs show means ± SEM of BAFF-RFP$^+$ cell numbers per iLN and summarize data in **A** and **B** ***right panel*** from three independent experiments (N=9); in **B** ***left***

panel from two independent experiments (N=6–9), in **C *left panel*** and **C *right panel*** from two independent experiments each (N=6–7). Statistics were performed using one-way ANOVA with Holm-Sidak method for multiple comparisons test; * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

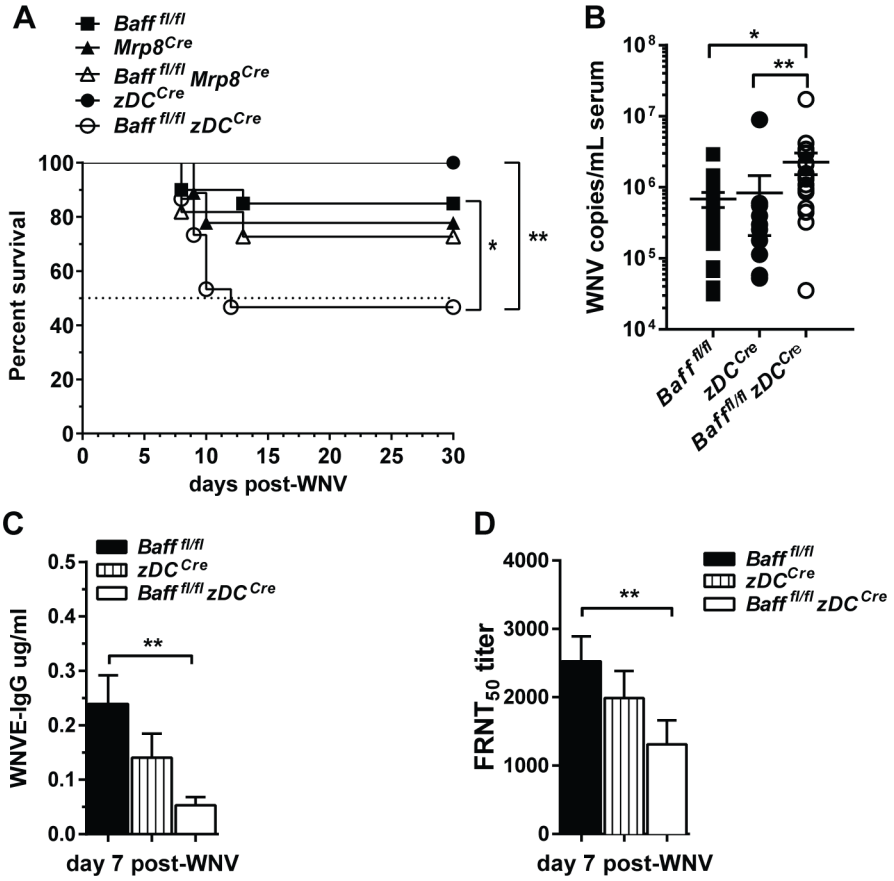

Figure 7. BAFF produced by DCs is required for protective immune responses to WNV infection.

A-D, *Baff*$^{fl/fl}$, *Mrp8*^{Cre}, *Baff*$^{fl/fl}$ *Mrp8*^{Cre} mice, *zDC*^{Cre}, *Baff*$^{fl/fl}$ *zDC*^{Cre} mice were infected via f.p. with WNV (100pfu). Mice were monitored daily for survival (**A**). Viral titers in sera were quantified using real time qPCR (**B**). WNVE-specific IgG were analyzed by ELISA (**C**). Neutralizing Abs were analyzed by $FRNT_{50}$ in Vero cells (**D**). **A-D**, Data are combined from three independent experiments: *Baff*$^{fl/fl}$, N=20; *Mrp8*^{Cre}, N=9; *Baff*$^{fl/fl}$ *Mrp8*^{Cre}, N=11; *zDC*^{Cre}, N=14; *Baff*$^{fl/fl}$ *zDC*^{Cre}, N=15. Statistics were performed using a log-rank test for significance (**A**), or one-way ANOVA corrected with Holm-Sidak method for multiple comparisons test (**B-D**); * $p<0.05$, ** $p<0.01$.

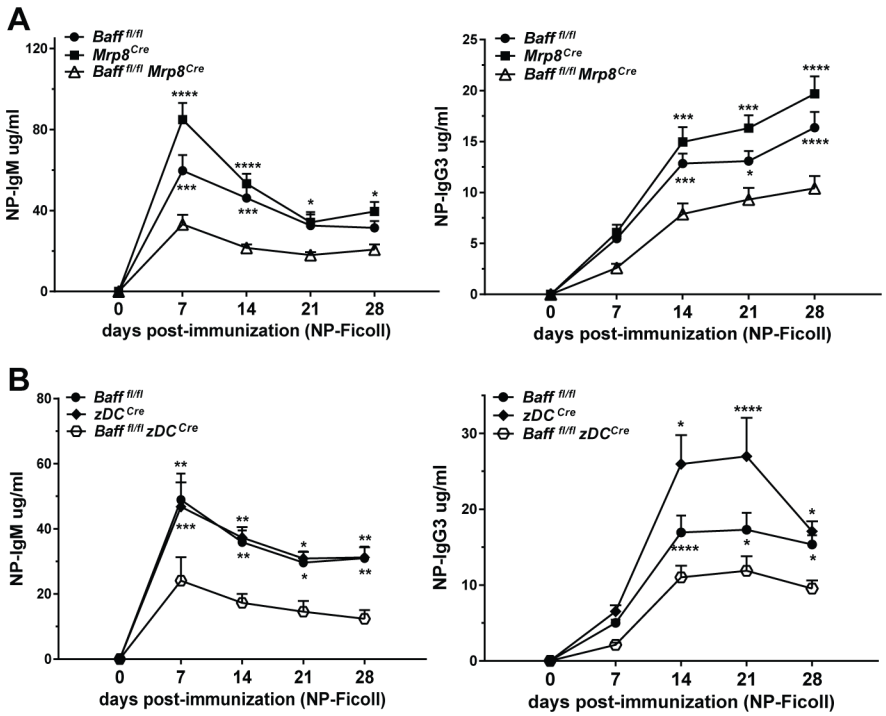

Figure 8. BAFF from Nphs and cDCs contributes to TI-2 IgG Ab responses.

Baff$^{fl/fl}$ *Mrp8*^{Cre} mice (**A**) or *Baff*$^{fl/fl}$ *zDC*^{Cre} mice (**B**) were immunized i.p. with 20 μg of NP-Ficoll, bled at the indicated time points, and NP-specific IgM and IgG3 were analyzed by ELISA. **A** and **B**, The data presented show means ± SEM of NP-IgM and NP-IgG3 and are combined from three independent experiments for each cKO genotype; **A**, *Baff*$^{fl/fl}$ mice, N=17; *Mrp8*^{Cre} mice, N=7; *Baff*$^{fl/fl}$ *Mrp8*^{Cre} mice, N=17. **B**, *Baff*$^{fl/fl}$ mice, N=17; *zDC*^{Cre} mice, N=8; *Baff*$^{fl/fl}$ *zDC*^{Cre} mice, N=15. Statistics were performed using 2-way ANOVA with Tukey's multiple comparison test. Statistics shown are comparison between *Baff*$^{fl/fl}$ mice or Cre mice controls vs. BAFF cKO mice; * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.