Type I IFN Receptor Signals Directly Stimulate Local B Cells Early following Influenza Virus Infection

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Rapidly developing Ab responses to influenza virus provide immune protection even during a primary infection. How these early B cell responses are regulated is incompletely understood. In this study, we show that the first direct stimulatory signal for local respiratory tract B cells during influenza virus infection is provided through the type I IFNR. IFNR-mediated signals were responsible for the influenza infection-induced local but not systemic up-regulation of CD69 and CD86 on virtually all lymph node B cells and for induction of a family of IFN-regulated genes within 48 h of infection. These direct IFNR-mediated signals were shown to affect both the magnitude and quality of the local virus-specific Ab response. Thus, ligand(s) of the type I IFNR are direct nonredundant early innate signals that regulate local antiviral B cell responses. The Journal of Immunology, 2006, 176: 4343–4351.

Follow ing infection of a susceptible host, influenza virus rapidly replicates within the epithelial cells of the respiratory tract. Lung viral loads peak about day 3 after infection in mice (1). Subsequent clearance of the virus seems mediated by the activation of various innate and adaptive immune mechanisms, including type I IFN (2–4), NK cells (5), cytotoxic CD8T cells (6, 7), and B cells (8, 9). The extremely rapid kinetics of the antiviral B cell responses, with measurable Ab secretion occurring before peak lung viral loads, are consistent with an involvement of B cells in viral clearance during primary infection (8). Local Ab production in the respiratory tract is measurable as early as 2 days after infection and includes isotype-switched Ig (this study). In the serum, virus-induced Abs can be measured within 4–5 days following infection (8, 10). The mechanisms that regulate early humoral responses to viruses and the exact nature of the signals that B cells receive during an infection are currently unknown.

Influenza infection causes rapid induction of type I IFN secretion in the respiratory tract (2, 3). IFN is a well-known family of antiviral cytokines that can be secreted by virtually all host cells (11). All currently known 13 subtypes of murine IFN-α as well as IFN-β and a novel member of the cytokine family, IFN-γ (limitin) use one widely expressed surface IFNAR (12). The powerful role of IFN in antiviral immunity is highlighted by the fact that many viruses, including influenza virus, have evolved immune evasion strategies that counteract the antiviral effects of IFN (4). One major role of IFN is its ability to induce a so-called “antiviral state,” rendering cells protected from viral infection and viral replication by inhibiting RNA and protein synthesis (13). More recently, it has been appreciated that IFN also plays pleiotropic roles in immunomodulation, both at the innate and adaptive immune level (11, 14, 15). Although early studies reported both inhibitory as well as enhancing effects of IFN on T and B cell responses, more recent studies provided evidence that IFN enhances adaptive immune responses, mainly via its potent immunomodulatory effects on dendritic cells (DC3; Refs. 11, 14, 15).

Two recent studies concluded that while IFN enhances B cell responses, it does so only indirectly via activation of DC (16, 17). Studies by Tough and colleagues (16) showed that bacillus Calmette-Guérin-induced IFN production enhanced the ability of DC to induce B cell responses in vivo following their adoptive cell transfer. An in vitro study by Jego et al. (17) using human DC and B cells showed that IFN induces activation and IL-6 secretion by DC, which was shown to be required for the differentiation of B cells to Ab-secreting cells in 15-day cultures. These studies have led to the development of a model in which plasmacytoid DC will activate myeloid DC via IFN to produce IL-6 thereby driving B cell activation and differentiation (15); a model that has been used to explain the role of IFN in development of the autoimmune disease systemic lupus erythematosus (SLE). However, others reported that IFN can also directly enhance BCR signal-mediated B cell activation in vitro (18).

The studies outlined here demonstrate that the rapid influenza virus infection-induced stimulation of local B cell populations in the respiratory tract is mediated directly through the type I IFNR.

Materials and Methods

Mice and virus

Female 8- to 12-wk-old BALB/c mice (Charles River Laboratories), C57BL/6 mice (The Jackson Laboratory), 129 SV/EV wild-type mice, and mice lacking the type I IFNR via targeted mutation of the IFNAR1 gene (19) (IFNAR−/−) on 129 SV/EV background (both originally obtained from B&K Universal) were kept in filter top cages under conventional housing conditions for the duration of the experiment. To generate irradiation chimeras, C57BL/6 or BALB/c recipient mice received a lethal dose (850 rad) of gamma irradiation. Twelve hours later, they were reconstituted with 1 × 107 mixed bone marrow cells. Bone marrow cell mixes contained cells from B cell-deficient C57BL/6 or BALB/c mice (Igh6−/−; The Jackson Laboratory) and wild-type C57BL/6 and BALB/c or IFNAR−/− C57BL/6 (cells provided by J. Cyster, University of California, San Francisco, CA, with permission from M.-K. Kaj, University of Washington, Seattle, WA) for 1 week in a total volume of 1 ml of CS-179 media

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3 Abbreviations used in this paper: DC, dendritic cell; SLE, systemic lupus erythematosus; MLN, mediastinal lymph node.

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were calculated following data normalization to GAPDH. Keeping gene to control for RNA input. Fold changes in gene expression were >93% as determined by FACS following staining with anti-B220. FACS- or MACS-purified B cells were cultured for 6 h at 10^6 cells/ml at variable concentrations of IFN-α, IFN-β, and IFN-γ in medium (RPMI 1640, 2 mM l-glutamine, 100 U of penicillin/ml, 100 μg of streptomycin/ml, 10% heat inactivated FCS, 50 μM 2-ME) at 37°C with 95% air/5% CO₂ before analysis by FACS or RNA extraction.

**ELISPOT and ELISA**

Lymph node cell suspensions were directly placed at 10^6 cells/well and 2-fold serial dilutions in medium in duplicates or triplicates into ELISPOT plates (MultiScreen HA Filtration; Millipore). Virus- and isotype-specific ELISPsots were done as described previously (21). Mean spot counts ± SD/10^5 input cells were calculated from all wells containing countable spot numbers. For detection of virus-specific Abs by ELISA, cell suspensions were placed in duplicates or triplicates at 10^6/ml in RPMI 1640/10% FCS into 96-well microtiter plates. Supernatants collected 24 h after culture onset were analyzed by virus- and isotype-specific ELISAs as previously described (21). Relative units per milliliter were calculated from comparison to a standard hyperimmune serum.

Commercial ELISA kits (PBL Biomedical Laboratories) were used to determine serum levels of IFN-α and IFN-β in wild-type and IFNR−/− mice (n = 6) at day 5 following influenza infection. Serum was used at 1/10.

**Statistical analysis**

Statistical analyses for FACS, ELISA, and ELISPOT data were done using a two-tailed Student’s t test or a two-way repeat measure ANOVA. Data were regarded as statistically significant at p < 0.05.

**Results**

*Influenza virus infection causes rapid stimulation of lymph node B cells*

MLN draining the lower respiratory tract rapidly increased in size and cellularity from being only detectable by histology before infection (<1 × 10^6 cells/mouse) to being usually detectable (3–5 × 10^6/mouse) 2 days following intranasal infection of anesthetized mice. Early B cell responses to the virus are induced primarily at this site. Known direct activation signals for specific B cells include what is believed to be the first activation signal via the B cell Ag receptor (“signal 1”) as well as costimulatory signals provided by activated Th cells (“signal 2”) (22, 23). In apparent contrast to this so-called “two-signal model,” we noted that during experimental respiratory tract infection of mice with influenza virus most B cells in the MLN showed increased surface expression of the early activation marker CD69 and of CD86 within 24–48 h after infection (Fig. 1). B cell responses to the virus are induced primarily at this site. Known direct activation signals for specific B cells include what is believed to be the first activation signal via the B cell Ag receptor (“signal 1”) as well as costimulatory signals provided by activated Th cells (“signal 2”) (22, 23). In apparent contrast to this so-called “two-signal model,” we noted that during experimental respiratory tract infection of mice with influenza virus most B cells in the MLN showed increased surface expression of the early activation marker CD69 and of CD86 within 24–48 h after infection (Fig. 1).
Ag-specific activation signals were unlikely responsible. Therefore, we sought to identify the mediator(s) of this rapid and localized global B cell activation.

Increased expression of a family of IFN-induced genes in lymph node B cells following influenza virus infection

To determine the gene expression changes that accompany this rapid activation of B cells, a cDNA subtraction library was generated from RNA isolated from highly FACS-purified MLN CD69<sup>+</sup>B220<sup>+</sup> B cells of BALB/c mice infected for 30 h with influenza virus and from CD69<sup>+</sup>B220<sup>+</sup> B cells isolated from peripheral inguinal and axillares lymph nodes of noninfected BALB/c mice. Of 27 clones isolated and sequenced, 3 were identified as belonging to a family of “IFN-induced proteins with tetratricopeptide repeats (IFIT)” of unknown function. Two of those clones representing to a family of “IFN-induced proteins with tetratricopeptide (IFIT)” were seen only in lymph node B cells from wild-type mice, not affected (data not shown). The effects of recombinant type I IFN showed a dose-dependent induction of IFIT2 and IFIT3 with recombiant IFN-α and IFN-β (type I IFN) but not with IFN-γ (Fig. 2A). Strong induction of IFIT2 and IFIT3 gene expression was seen also in WEHI231 and WEHI279 B cell lines following IFN-β treatment (data not shown).

Furthermore, in vitro exposure of B cells to type I IFN induced similar phenotypic changes to those observed in lymph node B cells following influenza virus infection. Within 6 h after exposure to IFN-α and IFN-β but only to a limited extent after exposure to IFN-γ, B cells strongly increased surface expression of CD69 and CD86 in an IFN-dose-dependent manner (Fig. 2B), consistent with a report by others (18). MHC II and CD40 surface expression was not affected (data not shown). The effects of recombinant type I IFN were seen only in lymph node B cells from wild-type mice,
but not in lymph node B cells from mice that lack expression of the IFNR (IFNR−/−) (Fig. 2B). Consistent with a local role for type I IFN in the induction of CD69 and CD86 on respiratory tract but not on splenic B cells (Fig. 1), serum levels of IFN-α and IFN-β on day 3 after infection were below the threshold of detection (625 and 150 pg/ml, respectively) both in wild-type (n = 6) and in IFNR−/− mice (n = 6). In contrast, we found mRNA induction of IFN-β but not IFN-γ (or IFN-α) in the lung and mediastinum of mice infected for 24 h with influenza virus (Fig. 3). Purified leukocyte suspensions of lung tissue and MLN, including FACS-purified T cells, B cells, and APCs contained no detectable mRNA for IFN (in two of two experiments performed, data not shown), suggesting that virus-infected respiratory tract epithelial cells were the main source for IFN-β at this time.

Together, these data show that type I IFN is sufficient to induce the in vivo-observed induction of IFIT2 and IFIT3 and phenotypic changes in MLN B cells. They furthermore suggested that this family of cytokines induced in response to infection with influenza virus is responsible for early polyclonal B cell stimulation in the respiratory tract.

**IFNR signaling is required for influenza virus-mediated induction of early B cell activation**

To determine whether type I IFN was responsible also for the observed early MLN B cell stimulation following influenza virus infection in vivo, we studied infection in IFNR−/− 129SV/EV mice (19). The results showed that expression of CD69 and up-regulation of CD86 on MLN B cells in vivo following influenza virus infection was dependent almost entirely on IFNR-mediated

**FIGURE 2.** Type I but not type II IFN induces genetic and phenotypic changes similar to those observed in B cells early during influenza infection. A. IFN-dose-dependent induction of IFIT2, IFIT3, and CD69 gene expression by IFN-α, IFN-β, but not IFN-γ. Purified B cells from lymph nodes of noninfected mice were stimulated for 17 h with indicated concentrations of IFN-α (top panel), IFN-β (middle panel), and IFN-γ (bottom panel). Real-time RT-PCR analysis was performed on cDNA generated from duplicate cultures. Data shown are mean fold differences in gene expression compared with untreated control cultures. Results are representative from two experiments performed. B. Dose-dependent induction of CD69 and CD86 surface expression by type I IFN. Shown are histogram profiles for CD69 and CD86 surface expression by MACS-purified resting lymph node B cells from wild-type IFNR+/+ (left panels) and IFNR−/− mice (right panels), identified by lack of propidium iodide uptake and staining for B220, following a 6-h culture in the presence and absence of indicated concentrations of IFN-α (top panel), IFN-β (middle panel), and IFN-γ (bottom panel). Results are from a representative experiment of two performed.

**FIGURE 3.** Rapid induction of IFN-β but not IFN-α and IFN-γ in the respiratory tract following influenza virus infection. Total RNA was isolated from lung tissue and mediastinum of two BALB/c mice before and 5, 7, and 24 h after influenza virus infection. IFN-α (○), IFN-β (■), and IFN-γ (▲) mRNA expression was measured for individual mice using real-time RT-PCR. Data are shown as mean fold changes of RNA expression levels in mediastinum (top panel) compared with mean levels measured in tissues from noninfected mice. IFN-α was not expressed in the mediastinum at any time point tested. Results are from one of two experiments that yielded similar results. No IFN signals were detected after preparation of leukocyte cell suspensions from these tissues (data not shown).
signals. Although there was strong induction of CD69 and CD86 in IFNR−/− controls, up-regulation of these receptors on MLN B cells was virtually absent from IFNR−/− mice. (Fig. 4A). Confirming these results, quantitative RT-PCR showed greatly lower IFIT2 and IFIT3 steady-state expression (26- and 132-fold, respectively) by purified lymph node B cells from noninfected IFNR−/− mice (right panels). Live B cells were identified by lack of propidium iodide uptake and staining for B220. Data are representative of 10 experiments that yielded similar results.

B cells from controls but not from IFNR−/− mice strongly induced IFIT2 and IFIT3 gene expression in response to influenza virus infection. Real-time RT-PCR analysis for expression of IFIT2 (left panel) and IFIT3 (right panel) by MACS-purified mediastinal lymph node B cells from control mice (●) and IFNR−/− mice (■) isolated 2 days after influenza virus infection compared with expression in purified lymph node B cells before infection. Results are from one of six experiments that yielded similar results. For this experiment, steady-state levels of IFIT2 and IFIT3 (±1-fold change) were, respectively, 26- and 132-fold lower in IFNR−/− mice compared with controls.

IFIT3 was induced >15-fold in the infected wild-type mice far exceeding the low levels of induction (from a >130-fold lower starting point) in IFNR−/− mice (Fig. 4B). The differences in the virus-induced phenotypic and genetic changes of B cells in IFNR−/− and wild-type controls were unlikely due to differences in the course or magnitude of influenza virus replication in these mice. Others had previously shown that infection of IFNR−/− mice with influenza A/X31 (H3N2) and A/PR8/34 (H1N1) showed similar infection kinetics and clearance in lung tissue compared with wild-type mice (1, 27). Our data are in agreement with those studies showing that there were no significant differences in day 3 peak lung viral loads.

control (Fig. 4C). Supernatants from lymph node cultures set up with aliquots from the same cells as shown in B were analyzed by ELISA for secretion of virus-specific Ig isotypes. Shown are mean relative units ± SD calculated from triplicate cultures of cells from controls (●) and IFNR−/− cells after normalization for B cell input numbers. Data are a representative from one of three experiments performed. D, Shown is a 5% contour plot with outliers from FACS analysis of MLN suspensions from control (left panels) and IFNR−/− (right panels) mice 5 days after influenza A/Mem71 infection after gating on live CD3, CD4, CD8-negative CD19+ cells (upper panels). Lower panels, MLN cells after additional gating on CD19+/CD8−/CD138+ (plasmablasts) in IFNR−/− mice (data not shown). Shown is a representative sample from four mice analyzed. Analysis was one of three independent experiments performed.

**FIGURE 4.** IFNR signaling is required for early B cell activation and accumulation following influenza virus infection. A, Influenza virus infection-induced up-regulation of CD69 and CD86 does not occur in IFNR−/− mice. Shown are histogram profiles for CD69 and CD86 expression on mediastinal lymph node B cells before (unfilled) and 2 days following infection with influenza virus A/Mem71 (gray) of controls (left panels) and IFNR−/− mice (right panels). Live B cells were identified by lack of propidium iodide uptake and staining for B220. Data are representative of 10 experiments that yielded similar results. B, B cells from controls but not from IFNR−/− mice strongly induced IFIT2 and IFIT3 gene expression in response to influenza virus infection. Real-time RT-PCR analysis for expression of IFIT2 (left panel) and IFIT3 (right panel) by MACS-purified mediastinal lymph node B cells from control mice (●) and IFNR−/− mice (■) isolated 2 days after influenza virus infection compared with expression in purified lymph node B cells before infection. Results are from one of six experiments that yielded similar results. For this experiment, steady-state levels of IFIT2 and IFIT3 (±1-fold change) were, respectively, 26- and 132-fold lower in IFNR−/− mice compared with controls. C, Groups of six wild-type (control, □) and congenic IFNR−/− (▲) mice were infected with influenza A/Mem71. On day 3 of infection, peak viral loads were determined in the lungs of individual mice. Data are expressed as PFU per lung. No significant differences in viral loads were observed.
after infection with a sublethal dose of influenza A/Mem71 (H3N1) between IFNR−/− and IFNR+/+ mice (Fig. 4C). IFNR−/− mice were reported to harbor virus outside the respiratory tract following infection with highly virulent strains of influenza (27). Viral load determination on spleens of wild-type and IFNR−/− BALB/c mice failed to detect any replicating virus at days 3, 5, 7, or 10 postinfection with our low pathogenic Mem71 strain (data not shown). Overall, these data demonstrated that signaling through the IFNR is a necessary and sufficient stimulatory signal for local lymph node B cells shortly after influenza virus infection.

**IFN signaling is required for maximal early antiviral Ab responses**

We next determined the effects of IFNR signaling on the local B cell responses to influenza virus infection. We initially compared B cell responses in the mediastinal lymph nodes in IFNR+/+ control and IFNR−/− mice. These mice showed no significant differences in the overall lymph node size (7.6 ± 1.9 × 10⁶ cells/IFNR−/− mice and 7.2 ± 1.8 × 10⁶ cells/control mice by day 5 after infection, n = 6). There was consistently a slightly reduced frequency of B cells in the lymph nodes of IFNR−/− mice (43.5 ± 7.7% vs 36.9 ± 4.5% CD19+ lymphocytes) at this time. However, these differences did not reach statistical significance (p = 0.067).

Analysis of virus-specific MLN B cell responses showed significant reduction in total and IgM virus-specific Ab-secreting B cells in IFNR−/− mice compared with wild-type controls as early as 2 days after infection (Fig. 5A). Very few IgG2a Ab-secreting cells were detected at that time (data not shown). The reduction in virus-specific Ab-secreting cells was not simply due to the reduced frequencies of B cells in the lymph nodes of IFNR−/− mice, as the reduction in B cell frequency was at most 15%, whereas the virus-specific responses were decreased by 50% or more. By day 5 after influenza virus infection, IFNR−/− mice showed significant reductions for virus-specific IgM, IgG2a, and IgG3 Ab-secreting MLN B cells and significant increases for IgG1-secreting cells compared with controls (Fig. 5B). MLN B cells from IFNR−/− mice showed a much greater reduction in the amounts of Ab secreted into the supernatant (Fig. 5C) than expected from the more moderate reductions in the frequencies of B cells secreting Abs compared with controls (Fig. 5B). This reduction in secreted Abs was consistent with a noticeable reduction in diameter of the ELISPOTs (data not shown) and suggested that IFN affects the differentiation of isotype-switched B cells to Ab-secreting plasma cells. In contrast, similar levels of reductions were observed for IgM and IgG1 secretion and the numbers of cells secreting these isotypes in IFNR−/− mice compared with controls (Fig. 5, B and C).

IFNR signals were indeed required for maximal early plasma-blast differentiation in vivo following influenza virus infection. FACS analysis of MLN at days 5 and 7 following influenza virus infection revealed a strong reduction in CD19+B220+CD138+ (Syndecan) plasma blasts in IFNR−/− compared with controls (Fig. 5D and data not shown). Immunohistochemistry demonstrated the absence of germinal centers in MLN of either control or IFNR−/− mice at day 5 after influenza virus infection (data not shown), indicating that plasma blast differentiation at that time following infection occurs mainly in extrafollicular foci (28) rather than in germinal centers. Thus, our data show that type 1 IFN is a crucial innate signal that drives maximal induction of those extrafollicular B cell responses that provide immediate immune protection through rapid Ab production.

**B cells are direct targets of IFN-mediated activation**

Previous reports had suggested that IFN affects humoral immune responses indirectly through their effects on DCs (16, 17). To determine whether and which effects of IFN on the anti-influenza virus-specific B cell responses are due to IFN signals directly provided to the B cells or indirectly via altering the function of other hemopoietic cells, we created two sets of mixed bone-marrow irradiation chimeras. The first set of mice was reconstituted to create mice in which only B cells lacked the IFNR. To generate these mice and their controls, lethally irradiated wild-type mice received 75% bone marrow from IFNR-carrying B cell-deficient mice (Igh6−/−; The Jackson Laboratory) plus as a source for B cells, an additional 25% of bone marrow from either congenic IFNR−/− mice (gift of Dr. J. Cyster, University of California, San Francisco, CA with permission from Dr. M.-K. Kaja, University of Washington, Seattle, WA, and Dr. J. Durbin, Ohio State University, Columbus, OH), or wild-type mice. A second set of chimeras was created in which most bone marrow-derived cells lacked IFNR−/−. In the present study, irradiated wild-type mice were reconstituted with 75% of IFNR−/− or as control wild-type bone marrow plus 25% of B cell-deficient bone marrow.

In two independent experiments on both C57BL/6 and BALB/c backgrounds, virus-specific Ab production from MLN cultures at day 5 after infection showed reduced total and IgM-antiviral Ab responses in mice that lacked IFNR only on B cells (Fig. 6, upper panel). Lack of IFNR expression on B cells only reduced virus-specific Ab secretion at day 5 after influenza virus infection. Mixed bone marrow irradiation chimeras were created with lethally irradiating wild-type (WT) C57BL/6 recipients reconstituted with 75% bone marrow from B cell-deficient mice (B cell−/−) plus 25% bone marrow from IFNR−/−, or C57BL/6 controls as source for B cells. Thus, creating mice in which only B cells were IFNR deficient or in which all cells expressed the IFNR. A second set of chimeras received 75% bone marrow from either IFNR−/− or wild-type mice plus 25% bone marrow from B cell-deficient mice. In these mice, most of the bone marrow-derived cells were either IFNR deficient or IFNR−/−. Upper panels. Mean levels of virus-specific Ab secretion in supernatants of duplicate MLN cultures (all isotypes left panel, IgM right panel). Data were normalized to B cell input numbers calculated from frequencies of CD19+ cells as determined by FACS and compared with the relevant control chimeras. Lower panels. Ab secretion per secreting B cell as determined by ELISPOT. *, p < 0.05; **, p < 0.01; ***, p < 0.001.
IFNR cell-deficient mice plus IFNR cultured at various titrations. units of virus-specific Ig loads were not significantly different in any of the groups tested. A/Mem71. Mice were analyzed at day 7 following infection. The IgM responses were less affected compared with the overall humoral response. The additional lack of IFNR on most other bone marrow-derived cells appeared to make little difference to the overall reduction (Fig. 6, upper panel). Because frequencies of CD19+ MLN B cells were reduced by ~30% in chimeras with IFNR+/−-derived B cells as assessed by flow cytometry (data not shown), we further normalized virus-specific total Ab production to B cell input numbers. This showed that the reduction in Ab production was due not simply to reduced numbers of B cells in the MLNs (Fig. 6, lower panels). IFNR+/− B cells produced less total virus Ig/cell compared with their controls, consistent with the lack of differentiation to plasma blasts seen in vivo in the IFNR−/− mice. The IgM responses were less affected compared with the overall humoral response. The additional lack of IFNR on most other bone marrow-derived cells appeared to make little difference to the overall reduction (Fig. 6, upper panel). Because frequencies of CD19+ MLN B cells were reduced by ~30% in chimeras with IFNR+/−-derived B cells as assessed by flow cytometry (data not shown), we further normalized virus-specific total Ab production to B cell input numbers. This showed that the reduction in Ab production was due not simply to reduced numbers of B cells in the MLNs (Fig. 6, lower panels). IFNR+/− B cells produced less total virus Ig/cell compared with their controls, consistent with the lack of differentiation to plasma blasts seen in vivo in the IFNR−/− mice. These studies, like any reconstitution experiments must be interpreted with the caveat that adoptive transfer of bone marrow cells into lethally irradiated mice might not fully reconstitute all aspects of the host immune system. Indeed, in contrast to nonirradiated mice (Fig. 5), all chimeras, including those with B cells from wild-type bone marrow, lacked detectable levels of virus-specific IgG2a 5 days after influenza virus infection and only mice reconstituted with bone marrow from C57BL/6 mice showed a small number of IgG2a-secreting cells by ELISPOT (data not shown), suggesting overall delayed kinetics in the B cell responses of the reconstituted mice. Further analyses of virus-specific B cell responses were therefore conducted with similarly generated irradiation chimeras at day 7 following influenza virus infection.

Lung virus titers in these chimeras showed comparably low levels of replicating virus in all groups by day 7 after infection (Fig. 7A). Those levels were one to two orders of magnitude lower than the peak viral loads of wild-type and IFNR−/− mice (Fig. 4B). Thus, all groups of chimeras seemed to be clearing the infection, consistent with studies in mice in which all cells lack the IFNR

Discussion

This study provides both genetic and functional evidence that the first direct stimulatory signal for local lymph node B cells during influenza virus infection is mediated through type I IFN and that this signal affects both the quality and magnitude of the earliest antiviral humoral responses. Taken together with studies by others (16, 17) that provided evidence for an indirect role of type I IFN, our data demonstrate the importance of innate immune signals such as type I IFN in the regulation of B cell responses to pathogens.

Our data show that IFNR-signaling affects early antiviral B cell responses at multiple levels: first during early B cell stimulation, as

FIGURE 7. Lack of IFNR on B cells causes alterations in isotype profile of the local virus-specific Ab response. Mixed bone marrow irradiation chimeras were created by reconstituting wild-type (WT) mice with bone marrow from either WT or IFNR−/− mice or with a mix of bone marrow from B cell-deficient mice plus IFNR−/− or WT bone marrow at a ratio of 75:25. Two months following reconstitution, mice were infected with influenza A/Mem71. Mice were analyzed at day 7 following infection. A, Comparison of lung viral loads in each of the four groups of chimera (n = 5/group). Viral loads were not significantly different in any of the groups tested. B, Virus-specific and isotype-specific ELISPOT (upper panel) and ELISA (lower panel) were performed on MLN from mice reconstituted with IFNR−/− bone marrow (∗) compared with controls reconstituted with wild-type bone marrow (■). Cellularity of the lymph nodes and the frequencies of CD19+ B cells were similar in all of the groups studied (data not shown). Shown are mean relative units of virus-specific Ig ± SD calculated from triplicate cultures (top panel) and mean frequencies of Ab-secreting foci ± SD calculated from triplicate cultured at various titrations. C, Similar analysis as in B on chimeras that lacked IFNR only on B cells (∗) compared with controls (■). Results are representative of at least three independent experiments performed. *, p < 0.05.
shown by the induction of IFN-induced genes in local lymph node B cells, including the induction of CD69 and CD86 and two members of a family of IFN-induced proteins; second, during the induction of Ab generation shown by reduced frequencies and altered isotypes of the early virus-specific B cell responses both in IFNR−/− mice (Fig. 5, A and B) as well as in chimeras lacking IFNR only on B cells (Figs. 6 and 7); and third, at the level of B cell differentiation to Ab-secreting cells, shown by the reduction in virus-specific Ab production per specific B cell (Fig. 6) and the lack of CD138+ plasmablasts in IFNR−/− mice (Fig. 5C).

The data from the IFNR−/− mice and chimeras lacking IFNR on all hemopoietic cells are consistent with earlier reports (27, 29, 30) that indicated a role for type I IFN in the reciprocal regulation of IgG2a and IgG1. The effects of IFN on the isotype profile of the virus-specific B cell response might be regulated at least in part indirectly through IFN-γ, because type I IFN is known to strongly enhance IFN-γ signaling (11), the cytokine directing Ig isotype switching toward IgG2a and IgG3 (30, 31). This cytokine, like type IFN, also induces STAT1, which has recently been shown to induce T-bet activation for IgG2a production (32). Therefore, it was surprising that the lack of IFNR on B cells only while strongly enhancing the IgG1 and reducing the IgG3 responses, did not alter virus-specific IgG2a responses on day 7 after infection (Fig. 7C). This was confirmed in four independent experiments. A more complex relationship might thus exist between virus-specific IgG1 and IgG2a production than a simple switch from one to the other. Specifically, IgG2a seems to be more strongly affected by IFN-mediated signals to cells other than B cells compared with the other measured IgG subtypes. The exact mechanisms underlying this difference are unclear at this point.

Type I IFN is one of the earliest induced innate immune mechanisms to influenza virus that directly limit viral spread by inducing an antiviral state in the vicinity of virus-infected IFN-producing host cells (3, 11). We show here that this early innate signal can also directly affect the earliest B cell responses at a time when the acute virus infection is not yet cleared. It is required for a strong early wave of Ab production immediately following influenza virus infection. We have shown previously that the presence of virus-binding natural Abs at the time of infection is crucial for survival from influenza virus infection (9). Rapid induction and secretion of Abs in the respiratory tract following infection likely represents an additional B cell-mediated mechanism that reduces influenza virus spread. Our findings have important practical implications for vaccine design, as they suggest that IFN could enhance vaccine-induced immediate early immune protection, of particular importance for vaccinations during an ongoing (natural or man-made) pandemic to slow down and eventually halt the spread of infections among exposed populations.

Given the pluripotent role of type I IFN in antiviral defense and immune regulation, it is surprising that following infection influenza lung viral loads are similar in wild-type and IFNR−/− mice (Fig. 4C, 1, 27). Others have shown previously that the lack of IFNR will expand the tissue tropism of highly pathogenic strains of this virus (27). We did not detect any virus outside the respiratory tract in our studies, likely due to the fact that we are using a low pathogenic influenza virus strain (Mem71). This is an advantage for our study as it allowed us to compare the B cell responses in mice carrying similar antigenic loads. One of the major downstream events of IFN signaling is the induction of STAT1. The lack of STAT1 confers strong susceptibility to a number of viruses, including influenza virus (29, 33). During infection with a low pathogenic strain of influenza virus, it is possible that induction of STAT1 by cytokines other than type I IFN is sufficient to mediate major antiviral activities that contribute to virus clearance.

It is also possible that the observed increased production of IgG1 could have compensated for the reduction in IgG2a and IgG3 expression in the IFNR−/− providing virus neutralization from around day 5 during the infection. It remains to be studied whether such changes in the isotype profile of the response might cause increased pathology in the respiratory tract.

The molecular mechanisms that regulate the effects of IFN on B cell activation and differentiation remain to be identified. Interestingly, two transcription factors induced by IFN-β, IFN regulatory factors 1 and 2, show recognition of similar binding motives than Blimp-1, a transcriptional repressor known to drive plasma cell differentiation (34). In addition, Blimp-1 itself represses IFN-β transcription (35), suggesting possible negative feedback regulation of Blimp-1 on IFN-β production. Our data open up the possibility that IFN-induced plasma cell differentiation constitutes a Blimp-1-independent B cell differentiation process that involves the IFN-induced transcription factors IFN regulatory factors 1 and 2; two transcription factors with known affects on B cell differentiation (36, 37).

To our knowledge, IFT1 and IFT3 expression by B cells has not previously been reported and their function(s) remain unknown to date. Previously, related genes were shown to be induced by LPS treatment of murine 3T3 fibroblasts and peritoneal macrophages (38). The IFTT family of genes might be part of the overall stress response to microbial stimuli. It is interesting to note that enhanced expression of IFTT2 but not IFTT3 was dependent entirely on IFN-mediated signals (Fig. 4B). Multiple pathways might exist that induce different members of this family of genes depending on the type of microbial stimulus. Another member of the IFTT family (IFT1/IFIT1 or P58 inhibitor of the dsRNA-activated protein kinase) is recruited by influenza virus to inhibit autophosphorylation and dimerization of type I IFN-activated dsRNA-activated protein kinase and thus its ability to down-modulate protein synthesis, as part of the antiviral responses (39, 40). Enhanced expression of this gene was recently shown to occur in patients suffering from SLE (41, 42).

Type I IFN has been implicated in the pathogenesis of Ab-mediated autoimmune disorders, particularly SLE (11, 41–44). Ab-mediated autoimmunity is also a known complication from IFN-α treatment in hepatitis C virus-infected patients (45–47). Most efforts in delineating the mechanisms of autoantibody induction via IFN have focused on the DCs as a potential source as well as recipient of IFN signals (15). By demonstrating that IFN-mediated signals strongly and directly activate B cells during a viral infection, our data suggest that the IFN-mediated induction or worsening of autoimmune disorders might be due to deregulated B cell development or differentiation, supporting studies in lpr mice (11, 44).

It will be of importance to identify the IFNγR ligand(s) that induce the changes reported in this study in B cell stimulation during early infection. Our data indicate that IFN-β exerts stronger biological effects on B cells (per unit activity) compared with IFN-α (Fig. 2 and our unpublished observations). This correlated with the up-regulation of IFN-β but not IFN-α (Fig. 3) in the respiratory tract early following influenza virus infection. However, it is unclear whether altering concentrations of various IFN ligands, including different ratios of IFN-α subtypes, might differentially affect B cell stimulation. Discrete activities for various IFN-α subtypes on B cells have been reported (48). Gene expression levels for α were actually slightly reduced following infection (Fig. 3). However, we do not know whether this is due to reduction in all or just a small number of IFN subtypes. Most recently, we also tested IFN-ζ, a cytokine that seems to act particularly strongly on B cells (49) and found that it also induces IFTT2 and IFTT3 expression (E. S. Coro and N. Baumgarth, unpublished observations). Its expression during influenza virus infection remains to be
studied. Understanding how a balance is achieved between IFN-related signals for the induction of maximal responses and B cell-mediated pathology seems key for the development of better vaccines and the identification of targets for therapies against Autoimmune diseases.

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Disclosures

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