The Journal of Immunology

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J Immunol 2006; 176:4343-4351; ;
doi: 10.4049/jimmunol.176.7.4343
http://www.jimmunol.org/content/176/7/4343

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Type I IFN Receptor Signals Directly Stimulate Local B Cells Early following Influenza Virus Infection[1]

Elizabeth S. Coro, W. L. William Chang, and Nicole Baumgarth[2]

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.**

Following 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 $CD8^+$ T 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-ζ (limitin) use one widely expressed surface IFNR (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 (DC[3]; 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) ($IFNR^{-/-}$) 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 1×10^6 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 $IFNR^{-/-}$ C57BL/6 (cells provided by J. Cyster, University of California, San Francisco, CA, with permission from M.-K. Kaja, University of Washington, Seattle,

Center for Comparative Medicine, University of California, Davis, CA 95616

Received for publication November 3, 2005. Accepted for publication January 25, 2006.

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[1] This work was supported by a grant from the National Institutes of Health (NIH) National Institute of Allergy and Infectious Diseases (AI51354) and was conducted in part in a facility constructed with support from Research Facilities Improvement Grant (C06 RR-12088) from the National Center for Research Resources/NIH.

[2] Address correspondence and reprint requests to Dr. Nicole Baumgarth, Center for Comparative Medicine, University of California, County Road 98 and Hutchison Drive, Davis, CA 95616. E-mail address: nbaumgarth@ucdavis.edu

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

0022-1767/06/$02.00

WA), or BALB/c mice (provided by J. Durbin, Ohio State University, Columbus, OH) at different ratios. Chimeras were kept in filter top cages and provided with acidified drinking water. Mice were infected with 1.6×10^6 PFU of the influenza A virus reassortant Mem71 (H3N1) as described (20). All experiments were performed in accordance with protocols approved by the University of California (Davis, CA) Animal Use and Care Committee.

Virus plaque assay

For determination of virus titers, lung tissue and spleens of mice at various times after infection were homogenized in 1 ml of PBS and aliquots were stored at −80°C until use. Plaque assay using Marbin-Darby canine kidney cells were performed as described (21).

Cell preparation and flow cytometry

Lymph node and bone marrow tissue cell preparations were generated as previously described (9). Inguinal lymph nodes were used as source for resting lymph node B cells, as mediastinal lymph nodes (MLN) are undetectable without infection. Lung tissue cell suspension was generated after flushing blood from the vascular bed with mouse-tonicity balanced saline solution in situ, collagenase I/DNase (Worthington) digestion, and Percoll (Pharmacia) gradient centrifugation as described (9). Live cell counts were obtained by trypan-blue exclusion using a hemocytometer. Cell suspensions were stained as described (21) with the following directly conjugated Abs at previously determined optimal concentrations: CD69-FITC, CD86-PE, CD19-PE, CD19-allophyocyanin (eBioscience), CD138-allophycocyanin (BD Biosciences) CD38-FITC, and B220-Cy5.5PE. Noncommercial reagents were generated as described (⟨www.drmr.com⟩). Propidium iodide was used at 1 μg/ml to discriminate dead cells. Data acquisition was done using a FACSCalibur and a FACSAria (BD Biosciences), the latter equipped with three lasers and appropriate dichroics and bandpass filters for 12-color, 14-parameter analysis. FACS sorting was done using a MoFlo high-speed cell sorter (DakoCytomation), equipped with three lasers and appropriate dichroics and bandpass filters for 11-color, 13-parameter analysis and sorting. Purities were >94%. Data analysis was conducted with FlowJo software (Tree Star).

Subtraction library

A cDNA subtraction library was generated using the PCR-Select cDNA Subtraction kit, and the SMART PCR cDNA Synthesis kit (BD Clontech) according to manufacturer's instructions with total RNA isolated from FACS purified resting lymph node $B220^+CD69^-$ B cells and activated $B220^+CD69^+$ MLN B cells 30 h following influenza virus A/Mem71 infection. Twenty-seven randomly chosen clones were submitted for sequencing (Davis Sequencing). Identification of genes occurred by comparison of sequences to GenBank.

Real-time RT-PCR

Total RNA was extracted using the RNAeasy kit (Qiagen) and cDNA synthesized from purified B cells or total lung, spleen, and mediastinal tissues using random hexamers (Promega) and Superscript II (Invitrogen Life Technologies) according to manufacturer's instructions. Real-time PCR was set-up using the cDNA to measure expression levels of the following genes: *IFIT2* ("Ifi1" Assays-by-Design; Applied Biosystems), *IFIT3* (forward: 5′-CGCCATGTTCCGCCTAGA-3′; reverse: 5′-CCAGGAGAACTTTCAGGTACTGGTT-3′; probe: FAM-CTGAGGATTCAACTCCATGGCCTGCT-TAMRA), *CD69* (forward: 5′-AGAATTTAACAGCTGGTTCAACTTGA-3′; reverse: 5′-CCAGTGGAAGTTTGCCTCACA-3′; probe: 5′-TTCACGGACACGCACCTCCCAG-3′), *IFN-α* (forward: 5′-ATGGCTAGGCTCTGTGCTTT-3′; reverse: 5′-CTCTTGTTCCTGAGGTTAT-3′; probe 1: 5′-TGAGCTACTGGTCAACCTG-3′; probe 2: 5′-TGAGCTACTGGCCAACCTG-3′), *IFN-β* ("Ifn β" Assays-by-Design; Applied Biosystems) and *IFN-γ* (forward: 5′-TCAAGTGGCATAGATGTGGAAGAA-3′; reverse: 5′-TGGCTCTGCAGGATTTTCATG-3′; probe: 5′-TCACCATCCTTTTGCCAGTTCCTCCAG-3′). An ABI Prism 7700 (Applied Biosystems) was used for amplification, data acquisition, and data analysis. GAPDH (forward: 5′-TGTGTCCGTCGTGGATCTGA-3′; reverse: 5′-CCTGCTTCACCACCTTCTTGAT-3′; probe: 5′-CCGCCTGGAGAAACCTGCCAAGTATG-3′) was used as a housekeeping gene to control for RNA input. Fold changes in gene expression were calculated following data normalization to GAPDH.

Magnetic cell separation and tissue culture

For B cell purification by MACS, spleen single-cell suspensions were stained as for FACS analysis with biotinylated Abs to CD3, CD4, CD8, DX-5 (eBioscience), GR-1, and F4/80 followed by streptavidin coupled to MACS beads (Miltenyi Biotec). B cells were enriched using an autoMACS (Miltenyi Biotec) and collection of the negative fraction. B cell purities were >93% as determined by FACS following staining with anti-B220. FACS- or MACS-purified B cells were cultured for 6 h at 10^7cells/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% CO_2 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 ELISPOTs 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 10^7/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 ELISA 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 3 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 ($<1 \times 10^6$ cells/mouse) to being usually detectable ($3–5 \times 10^6$/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 and data not shown). This was evidenced by increased mean fluorescent staining intensities for these two surface markers and higher frequencies of B cells expressing these receptors at detectable levels (Fig. 1*B*). In contrast, changes in expression of MHC II or CD40 were not measurably affected at this time (data not shown). MLN B cell surface expression of CD69 was maximally induced by 24–48 h, decreased after day 2, and was gone by day 3 after infection (data not shown). CD69 expression was induced significantly also in a small subset of lung tissue B cells, but not in splenic B cells (Fig. 1). ELISPOT analysis showed the presence of Ab-secreting cells as early as day 2 after infection (Fig. 1*C*). Comparing the number of total Ig-secreting cells with that of virus-specific cells showed that the virus-specific component represented only ~25% of the overall Ab-secreting cells. Whether the virus-binding cells might constitute cross-reactive natural Ab producing B-1 cells (9, 24) remains to be determined. Similar polyclonal B cell activation was reported in mesenteric lymph nodes following rotavirus infection (25). Because most regional lymph node B cells displayed these phenotypic changes,

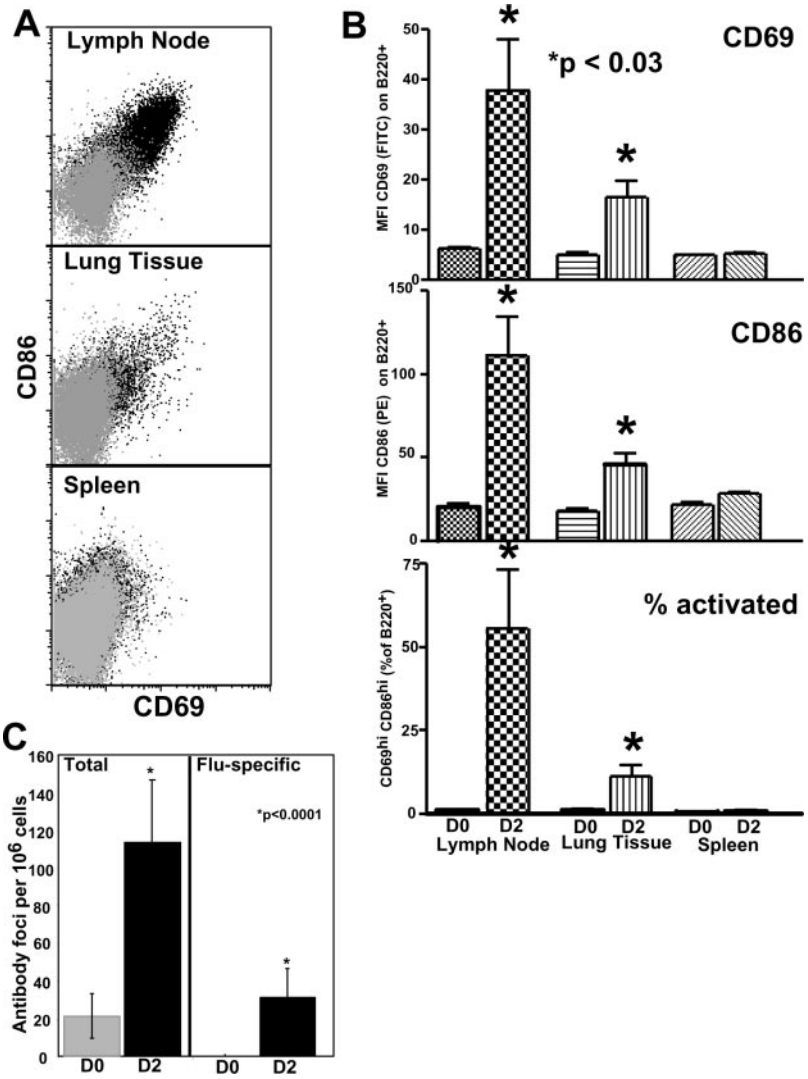

FIGURE 1. Early activation of MLN B cells during influenza virus infection. *A*, Infection-induced increase in CD69 and CD86 expression on local B cells. Shown are overlay dot plots of CD69 and CD86 expression on live $B220^+$ B cells from MLN (*top panel*), lung tissues (*middle panel*), and spleens (*bottom panel*) of a representative BALB/c mouse, noninfected (gray dots) and infected for 2 days with influenza A/Mem71 (black dots). *B*, Mean fluorescence intensities (MFI) of CD69 (*top panel*) and CD86 (*middle panel*) staining on B cells were calculated from three individual mice per group before (D0) and 2 days (D2) following infection with influenza A/Mem71. B cells were identified as described for *A*. CD69 and CD86 expression on B cells from MLN and lung tissue were significantly increased 2 days following influenza virus infection ($p < 0.03$). *Bottom panel*, Frequencies of CD69- and CD86-expressing B cells. Frequencies of live cells and $B220^+$ cells in lymph nodes were not significantly different between lymph nodes from noninfected (84.3 ± 0.47% live cells, 23.1 ± 3.3% $B220^+$) and infected (81.7 ± 3.7 live cells, 29.6 ± 5.9% $B220^+$) mice at this time after infection. Results are a representative from >20 experiments performed with C57BL/6, 129SV/EV, and BALB/c mouse strains. Although CD69 expression on B cells disappeared by day 3 after infection, increased CD86 continued to be expressed at higher levels until after day 7 (data not shown). *C*, Results of ELISPOT analysis on pooled lymph nodes from noninfected and day 2-infected mice when tested for presence of Ig-secreting cells (*left panel*) and virus-specific Ab-secreting cells (*right panel*). Results are representative of three performed.

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 $CD69^+B220^+$ B cells of BALB/c mice infected for 30 h with influenza virus and from $CD69^-B220^+$ B cells isolated from peripheral inguinal and axillares lymph nodes of noninfected 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 represented sequences from the same gene (*IFIT3*, GenBank accession number AK077459) and one represented a distinct gene (*IFIT2*, GenBank accession number NM_008332). Real-time RT-PCR analysis using aliquots of RNA originally used for the subtraction library confirmed that IFIT2 and IFIT3 expression in the $CD69^+$ B cells of MLN from influenza-infected mice was 40- and 28-fold higher, respectively, compared with expression in $CD69^-$ lymph node B cells from noninfected mice. *CD69* gene expression differed only 2.3-fold between these B cells, possibly due to mRNA degradation known to regulate short-term induction of CD69 expression (26).

Induction of "IFN-induced protein" expression in resting B cells is mediated in vitro by type I but not type II IFN

Expression, regulation, and function of the *IFIT* gene family have not been studied in B cells. We determined next whether and which type of IFN could induce *IFIT2* and *IFIT3* gene expression in resting lymph node B cells. In vitro exposure of MACS-purified B cells from peripheral lymph nodes of noninfected BALB/c mice showed a dose-dependent induction of *IFIT2* and *IFIT3* with recombinant IFN-α and IFN-β (type I IFN) but not with IFN-γ (Fig. 2*A*). 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. 2*B*), 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,

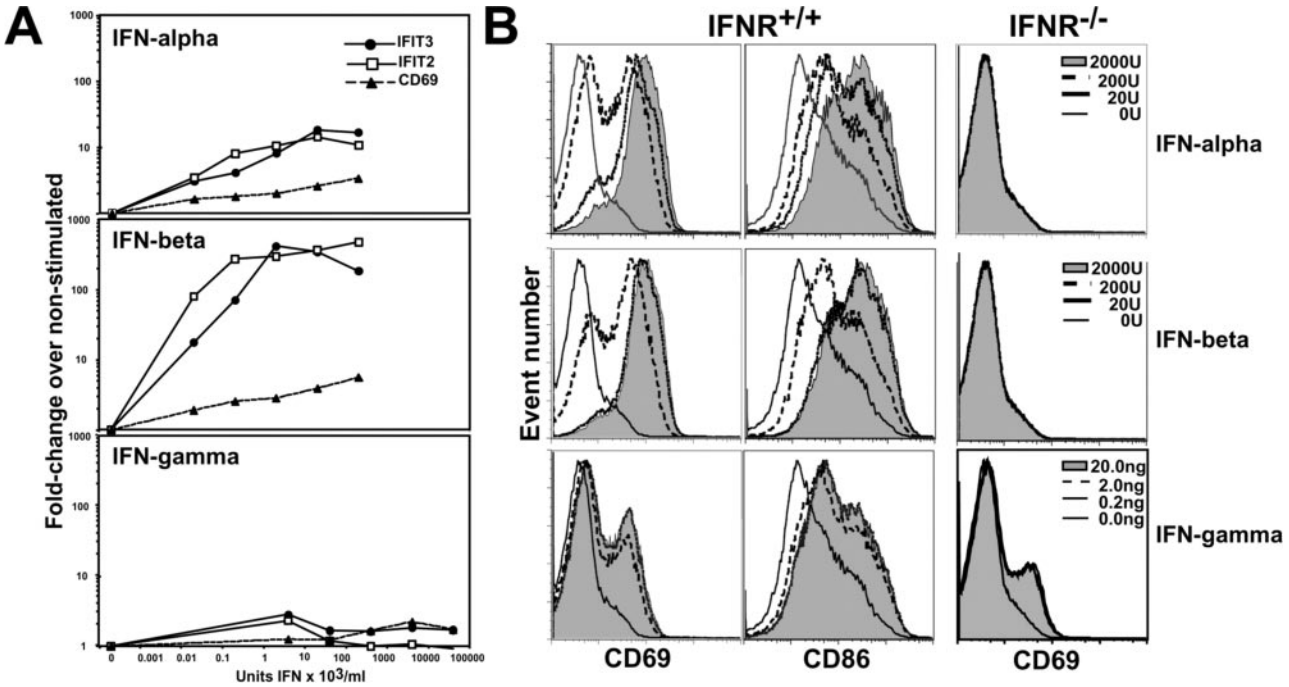

FIGURE 2. Type I but not type II IFN induces genetic and phenotypic changes similar to those observed in B cells early during influenza infection. *A*, IFN-dose-dependent induction of *IFIT2*, *IFIT3*, and *CD69* gene expression by IFN-α, IFN-β, but not IFN-γ. Purified B cells from lymph nodes of noninfected 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.

but not in lymph node B cells from mice that lack expression of the IFNR (IFNR$^{-/-}$) (Fig. 2*B*).

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

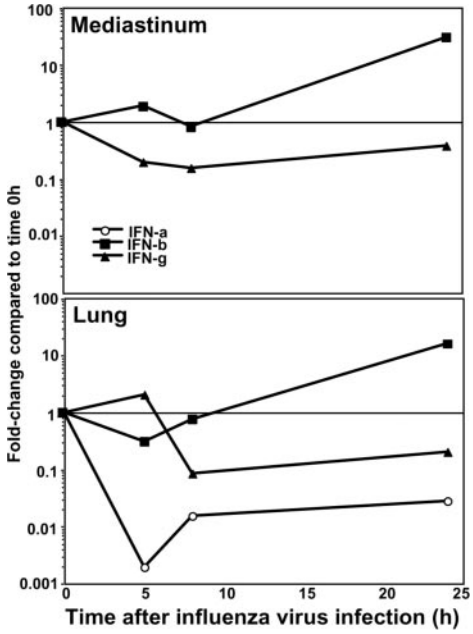

FIGURE 3. Rapid induction of IFN-β but not IFN-α and IFN-γ in the respiratory tract following influenza virus infection. Total RNA was isolated from lung tissue and mediastinum of two BALB/c mice before and 5, 7, and 24 h after influenza virus infection. IFN-α (○), IFN-β (■), and IFN-γ (▲) mRNA expression 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).

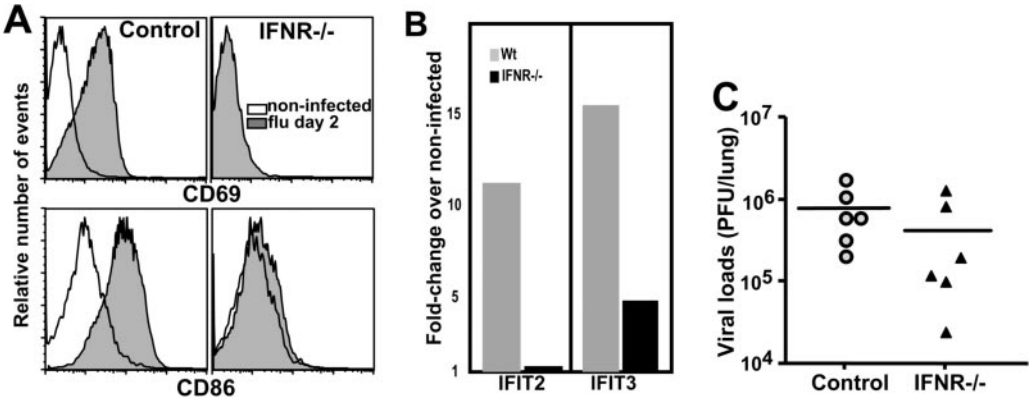

FIGURE 4. IFNR signaling is required for early B cell activation and accumulation following influenza virus infection. *A*, Influenza virus infection-induced up-regulation of CD69 and CD86 does not occur in IFNR$^{-/-}$ mice. Shown are histogram profiles for CD69 and CD86 expression on mediastinal lymph 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- and132-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.

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. 4*A*). 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 compared with cells from noninfected wild-type mice (data not shown). Two days following infection, IFIT2 induction in the mediastinal lymph node B cells could only be observed in the wild-type but not the IFNR$^{-/-}$ mice (Fig. 4*B*), thus demonstrating the dependence of IFIT2 induction on IFNR signals. 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. 4*B*). 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

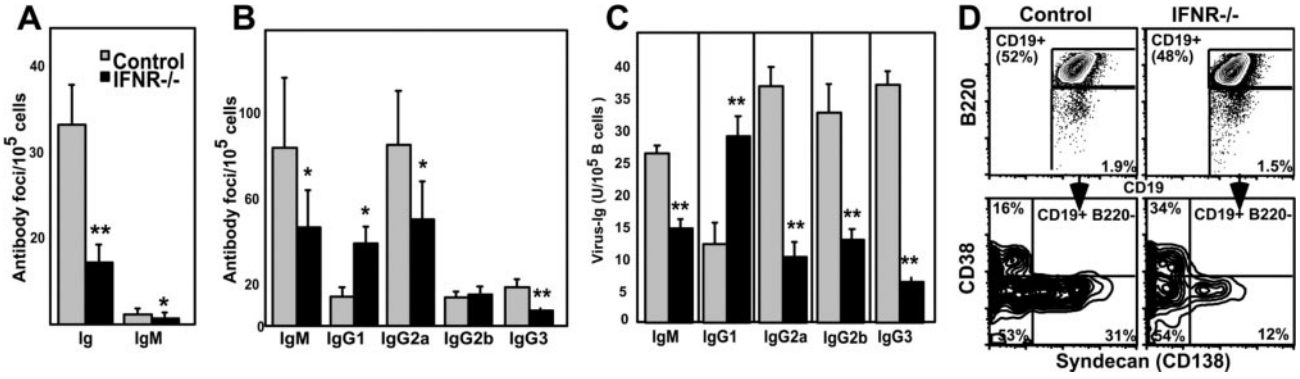

FIGURE 5. Lack of IFNR signaling strongly affects early local Ab responses following influenza virus infection. *A*, Enumeration of virus-specific Ig-secreting cells on day 2 after influenza virus infection. Shown are mean counts IgM and total Ig-secreting cells ± SD from triplicate cultures of MLN from wild-type control (▨) and IFNR$^{-/-}$ (■) mice. Low frequency of IgG2a production was seen only in two of the four experiments performed (data not shown). No virus-specific ELISPOTs were detected in lymph nodes from noninfected mice (data not shown). *B*, Enumeration of virus-specific Ig-secreting cells on day 5 after influenza virus infection. Data are mean counts ± SD from triplicate cultures after normalization for B cell input numbers calculated from the frequency of CD19$^+$ cells determined by FACS. Representative results from one of three experiments performed. *C*, 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 a total of three 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$^+$B220^{low} (activated) B cells. Numbers indicate frequencies among gated cell population. Similar analysis was performed on day 7 resulting in even more marked reduction in B220^{low}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.

after infection with a sublethal dose of influenza A/Mem71 (H3N1) between IFNR$^{-/-}$ and IFNR$^{+/+}$ mice (Fig. 4*C*). 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 demonstrate that signaling through the IFNR is a necessary and sufficient stimulatory signal for local lymph node B cells shortly after influenza virus infection.

IFNR 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 ($7.6 \pm 1.9 \times 10^6$ cells/IFNR$^{-/-}$ mice and $7.2 \pm 1.8 \times 10^6$ 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 ($43.5 \pm 7.7\%$ vs $36.9 \pm 4.5\%$ 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. 5*A*). 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 was 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. 5*B*). MLN B cells from IFNR$^{-/-}$ mice showed a much greater reduction in the amounts of Ab secreted into the supernatant (Fig. 5*C*) than expected from the more moderate reductions in the frequencies of B cells secreting Abs compared with controls (Fig. 5*B*). 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 plasmablast 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) plasmablasts in IFNR$^{-/-}$ compared with controls (Fig. 5*D* 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 I 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*

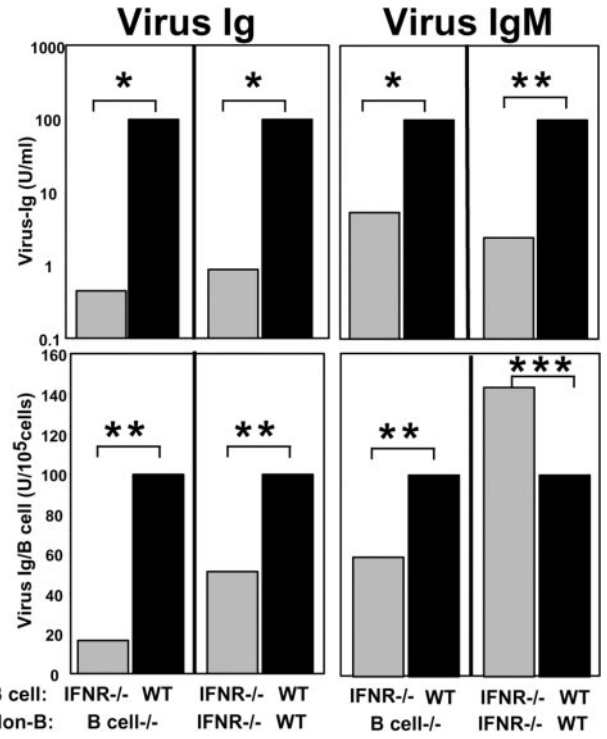

FIGURE 6. Lack of IFNR expression on B cells only reduced virus-specific Ab secretion at day 5 after influenza virus infection. Mixed bone marrow irradiation chimeras were created with lethally irradiating wild-type (WT) C57BL/6 recipients reconstituted with 75% bone marrow from B cell-deficient mice (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. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$

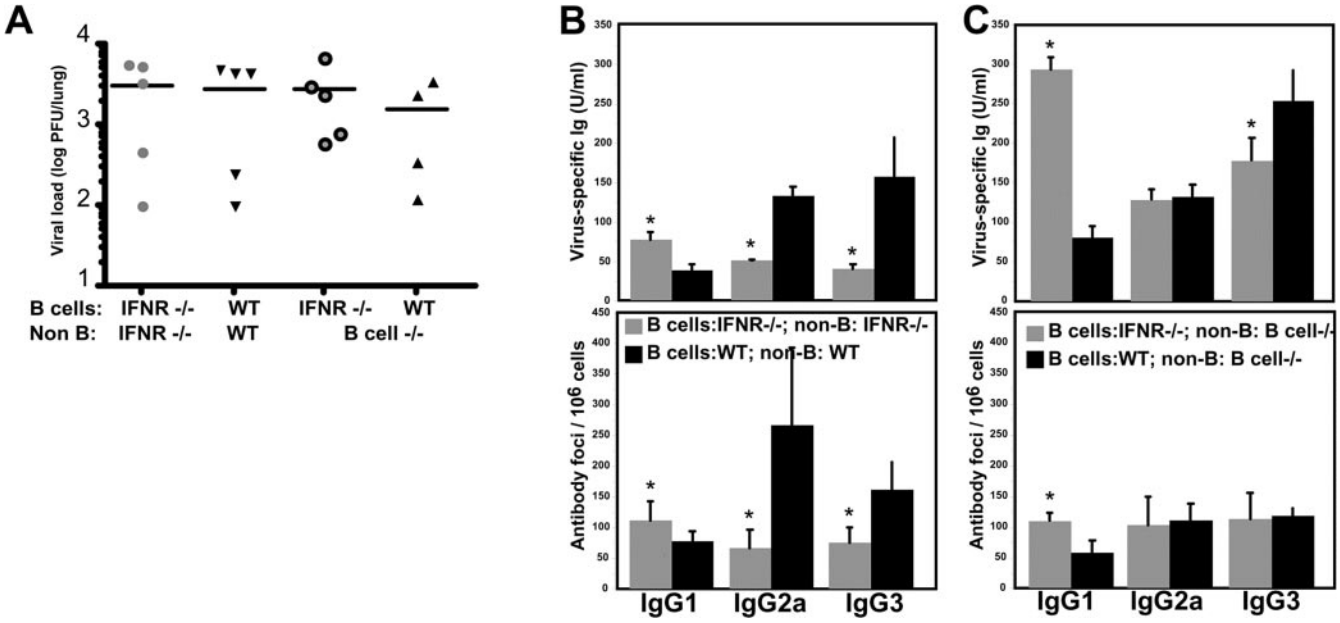

FIGURE 7. Lack of IFNR on B cells causes alterations in isotype profile of the local virus-specific Ab response. Mixed bone marrow irradiation chimeras were created by reconstituting wild-type (WT) mice with bone marrow from either WT or IFNR$^{-/-}$ mice or with a mix of bone marrow from B cell-deficient 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. *, $p < 0.05$.

panel). 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. 7*A*). Those levels were one to two orders of magnitude lower than the peak viral loads of wild-type and IFNR$^{-/-}$ mice (Fig. 4*B*). Thus, all groups of chimeras seemed to be clearing the infection, consistent with studies in mice in which all cells lack the IFNR (Fig. 4*B* and Refs. 27 and 29). In three independent experiments, using pooled tissues from three to five mice per group, we used ELISPOT and ELISA analysis on MLN cultures to assess the role of direct IFNR signaling on B cells for the regulation of local virus-specific B cell responses. Consistent with results in IFNR$^{-/-}$ mice (Fig. 5), we found that the levels of virus-specific IgG1 secretion and the frequency of IgG1-secreting B cells were significantly enhanced and the levels of IgG2a and IgG3 were reduced in chimeras in which all hemopoietic cells lack expression of the IFNR compared with control chimeras with bone marrow from wild-type mice (Fig. 7*B*). A similar change was seen also for virus-specific IgG1 and IgG3 in the chimeras lacking the IFNR only on B cells (Fig. 7*C*). In contrast to those data, the lack of IFNR on B cells only did not affect the levels of virus-specific IgG2a (Fig. 7*C*), suggesting additional indirect effects of IFN on the regulation of the B cell response. Taken together, the chimera data demonstrate that direct IFNR-mediated B cell stimulation affects both the magnitude and quality of the local influenza virus-specific Ab response.

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 IFNR 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

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. 5*C*).

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 I 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. 7*C*). 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 IFNR-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. 4*C*, (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 IFNR 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 motifs 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, IFIT2 and IFIT3 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 IFIT family of genes might be part of the overall stress response to microbial stimuli. It is interesting to note that enhanced expression of IFIT2 but not IFIT3 was dependent entirely on IFNR-mediated signals (Fig. 4*B*). Multiple pathways might exist that induce different members of this family of genes depending on the type of microbial stimulus. Another member of the IFIT family (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 IFNR-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 IFNR 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 IFNR 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 IFIT2 and IFIT3 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 IFNR-mediated 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 Ab-mediated autoimmune diseases.

Acknowledgments

We thank Drs. Charles Bevins, Yueh-hsiu Chien, David Tarlinton, and Andrew Fell for critically reading the manuscript and for helpful discussions and suggestions as well as Virginia Doucett for excellent technical help. We also thank Drs. Joan Durbin, Jason Cyster, and Murali-Krishna Kaja for valuable reagents.

Disclosures

The authors have no financial conflict of interest.

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