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TLR Signaling Fine-Tunes Anti-Influenza B Cell Responses without Regulating Effector T Cell Responses

Alex K. Heer,* Abdijapar Shamshiev,† Alena Donda,‡ Satoshi Uematsu,‡ Shizuo Akira,‡ Manfred Kopf,2,3* and Benjamin J. Marsland2,3*

Influenza is a ssRNA virus that has been responsible for widespread morbidity and mortality. To date, vaccines that generate influenza strain-specific Abs have proved effective in neutralizing this infectious agent; however, antigenic drift and shift preclude the long-term effectiveness of individual vaccines. Protective immunity against a primary infection with lethal type A influenza virus is to a large degree mediated by the humoral response, as shown by studies in which mice lacking T cells directly and TLR-induced production of IFN-α, which acted to reduce IgG1 and increase IgG2a/c class switching. Notably, direct TLR signaling on B cells or T cell help through the CD40-CD40L interaction was sufficient to support B cell proliferation and IgG1 production, whereas IFN-α was critical for fine-tuning the nature of the isotype switch. Taken together, these data reveal that TLR signaling is not required for anti-influenza T cell responses, but through both direct and indirect means orchestrates appropriate anti-influenza B cell responses. The Journal of Immunology, 2007, 178: 2182–2191.

Influenza is a highly contagious respiratory virus that has been responsible for widespread morbidity and mortality. To date, vaccines that generate influenza strain-specific Abs have proved effective in neutralizing this infectious agent; however, antigenic drift and shift preclude the long-term effectiveness of individual vaccines. Protective immunity against a primary infection with lethal type A influenza virus is to a large degree mediated by the humoral response, as shown by studies in which mice lacking B cells rapidly succumb to influenza infection despite the ability to mount a vigorous CD8 T cell response (1, 2). Class switch recombination to Ab isotypes other than IgM is largely dependent upon T cell help, although Abs of the IgG isotypes are detectable at reduced titers in the absence of T-B interaction (3–5). Detailed analysis of the efficacy of Ab isotypes showed that IgG isotypes are primarily responsible for neutralizing influenza virus during primary and challenge infections (6, 7), and earlier studies identified IgG2a as a key isotype for Ab-mediated effector functions in C57BL/6 mice (8–10). Of note, later studies revealed that in fact the C57BL/6 mouse strain with the Igh-1b allele does not have the gene for IgG2a, but rather the IgG2c isotype (11–13). Thus, whereas many studies report IgG2a levels in this mouse strain, they may in fact be detecting IgG2c using IgG2a cross-reacting Abs. Therefore, below we refer to these isotypes as IgG2a/c.

Although many studies have examined the relative importance of the different effector arms of the adaptive immune response against influenza infection, surprisingly little is known about the early innate immunological mechanisms that drive adaptive immune responses in vivo. Key instigators of innate immunity are pattern recognition receptors, including the TLR family, which bind conserved microbial products. The influenza virus genome, which consists of ssRNA, is recognized by TLR7, whereas dsRNA, an intermediate product during viral replication, is recognized by TLR3 (14, 15). Signaling through TLR7 and TLR3 requires the adaptor molecules MyD88 and Toll/IL-R domain-containing adaptor-inducing IFN-β (TRIF),4 respectively (16, 17). After binding of ssRNA or dsRNA to the relevant TLRs, signaling cascades activate inflammatory responses that lead to the up-regulation of costimulatory molecule surface expression and production of cytokines and chemokines (18). This process leads to full maturation of dendritic cells (DCs), and consequently, the activation of Ag-specific T cells (19). Besides the importance of TLRs for DC maturation and the subsequent activation of T cells, recent reports suggest that TLR ligands also act directly upon B cells to support B cell proliferation and promote class switching to IgG isotypes (20, 21). However, although prior studies have shown that viral pathogen-associated molecular patterns can promote DC and

4 Abbreviations used in this paper: TRIF, Toll/IL-R domain-containing adaptor-inducing IFN-β; BAL, bronchoalveolar lavage; DC, dendritic cell; MDA5, melanoma differentiation-associated gene 5; RIG-I, retinoic acid-inducible protein 1; RT, room temperature.

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B cell activation, the in vivo implications of these data on host defense against influenza virus remain to be elucidated.

We sought to determine the role of such innate signals on the quality of the anti-influenza immune responses in vivo. Surprisingly, we found that the known TLR pathways (TLR3/TRIF; TLR7/MyD88) were irrelevant for CD4 and CD8 T cell proliferation, activation, and effector functions following influenza infection. In addition, TLR3 deficiency did not influence the outcome of the humoral response following influenza infection. However, TLR7 and MyD88 deficiency resulted in a striking dysregulation of influenza-specific Ab isotype switching. Specifically, these mice exhibited exaggerated influenza-specific IgG1 titers compared with wild-type or littermate controls, and MyD88-deficient mice had significantly reduced levels of influenza-specific IgG2a/c. Further in vivo and in vitro experiments suggested that B cell switching to IgG isotypes required the simultaneous presence of at least two signals: either TLR or CD40 stimulation directly on B cells to promote proliferation and IgG1 isotype switching, or either one of these signals together with IFN-α to drive class switching to IgG2a/c. Taken together, these data show that whereas TLR signaling influences B cell responses both directly and indirectly through IFN-α induction, it is not required for effective anti-influenza T cell responses.

**Materials and Methods**

**Animals, virus, and infection**

TLR7/−/− (16), TLR3/−/− (22), MyD88/−/− (23), CD40/−/− (24), CD40L/−/− (25), and IFN-αR/−/− (26) mice were backcrossed at least six generations onto the C57BL/6 background and maintained in specific pathogen-free conditions (Biosupport). TRIFRS2-mutant mice generated by random germline mutagenesis in C57BL/6 mice using the alkylating agent N-ethyl-N-nitrosourea (27) were provided by M. Freudenberg (Max Planck Institute for Immunology, Freiburg, Germany) with permission of B. Beutler. BAL cells were added to a 96-well plate and then serially diluted in 3-fold steps. A total of 1 × 10⁵ EL-4 cells was added to the BAL cells. After 5-h incubation at 37°C, 25 μl of supernatant was mixed with 150 μl of scintillation fluid, and release of 51Cr due to EL-4 cell lysis was measured on a scintillation reader.

**Bronchoalveolar lavage (BAL)**

Mice were sacrificed at day 10 after infection and restrained on the back. A small incision on the trachea was performed through which a catheter was introduced. The lungs were flushed with 1 ml of PBS. BAL cells were harvested by centrifugation. Total cell numbers per BAL were determined by a Coulter Counter (Instrumenten Gesellschaft), and cells were processed for further analysis. BAL fluid was used to measure virus-specific Ig levels.

**Determination of virus-specific CD8⁺ T cells**

BAL cells (1 × 10⁵) were incubated with 5 μg/ml NP34⁴ PE-conjugated tetramers (provided by A. Donda, University of Lausanne, Epalinges, Switzerland) at 4°C for 45 min. CD4-FITC and CD8-allophycocyanin (eBioscience) were subsequently added for 20 min at 4°C. Cells were washed and analyzed by flow cytometry (FACSCalibur; BD Biosciences).

**Ex vivo cytotoxicity assay**

EL-4 cells were incubated with 2 μCi of ⁵¹Cr and 30 ng of NP34 peptide for 90 min and then washed three times. Between 1.5 × 10⁵ and 1.8 × 10⁶ BAL cells were added to a 96-well plate and then serially diluted in 3-fold steps. A total of 1 × 10⁴ EL-4 cells was added to the BAL cells. After 5-h incubation at 37°C, 25 μl of supernatant was mixed with 150 μl of scintillation fluid, and release of ⁵¹Cr due to EL-4 cell lysis was measured on a scintillation reader.

**Specific restimulation of BAL cells**

At day 9 after infection, spleens from naive mice were taken and cells were positively selected for CD11c by MACS, according to the manufacturer’s instructions (Miltenyi Biotec). A total of 1.5 × 10⁶ CD11c⁺ DCs was then incubated overnight with 1.6 × 10⁶ PFU of UV-inactivated virus (PR8) in 96-well plates. At day 10 after infection, these CD11c⁺ DCs were pulsed with 1 μg/ml NP34 peptide for 2 h before BAL cells from individual mice were added. After 3 h of incubation at 37°C, brefeldin A (Sigma-Aldrich), and cells were again incubated at 37°C for another 2 h. Cells were harvested, stained with FITC-labeled anti-TNF-α (eBioscience), PE-labeled anti-CD4 (eBioscience), biotin-labeled anti-CD8 (eBioscience), and allophycocyanin-labeled anti-IFN-γ (eBioscience), as previously described (28), and analyzed by flow cytometry (FACSCalibur; BD Biosciences).

**Proliferation of splenic CD4⁺ and CD8⁺ T cells**

Mediastinal lymph nodes were taken at day 10 after infection. Cells from individual mice were incubated with UV-inactivated virus (PR8) in IMDM-10% FCS at 37°C for 4 days. Cells were then harvested, stained as described above, and analyzed by flow cytometry (FACSCalibur; BD Biosciences).

**Detection of virus-specific Abs**

At the indicated time points, serum or BAL fluid was measured for virus-specific IgA and IgG Ab isotype levels. Ninety-six-well plates (Maxisorp; Nunc) were coated with UV-inactivated influenza virus (PR8) in PBS overnight at 4°C. Plates were washed and incubated with PBS-1% BSA for 2 h at room temperature (RT) for blocking. Serum and BAL fluids from individual mice were serially diluted in PBS-0.1% BSA starting with a 1/1 dilution for BAL fluids and a 1/50 dilution for serum, followed by incubation at RT for 2 h. Plates were washed five times and incubated with alkaline-phosphate-labeled goat anti-mouse Abs to IgG1, IgG2a, IgG2c, IgG2b, IgG3, or IgA (Southern Biotech) at 1/1000 dilution in PBS-0.1% BSA at RT for 2 h. Thereafter, plates were washed five times and substrate p-nitrophenyl phosphate (Sigma-Aldrich) was added. ODs were measured on an ELISA reader (Bucher Biotec) at 405 nm.

**In vitro B cell assay**

Splenic cells from naive mice were positively sorted for CD19 by MACS (Miltenyi Biotec). A total of 2 × 10⁵ CD19⁺ cells was incubated in 96-well plates with R837 (Imiquimod; 1 μg/ml; InvivoGen), anti-CD40 (5 μg/ml; provided by Cytos Biotechnology), rIFN-α (10 ng/ml; PBL Biomedical Laboratories), and combinations thereof in a final volume of 250 μl at 37°C for 6 days. Supernatant (150 μl) was harvested, and plates were washed once with IMDM-10% FCS before being incubated again with the substances indicated above at the same concentrations at 37°C for 4 days. A total of 150 μl of supernatant was harvested, and IgG1 and IgG2a/c Ab isotype levels were measured by ELISA.

**Results**

TLR7 and MyD88 are dispensable for T cell activation and effector function upon influenza infection

The recognition of self vs nonself through the simultaneous uptake of Ag and stimulation of TLR ligands has become a central paradigm in immunology. Prior studies have demonstrated that the receptor responsible for the recognition of viral ssRNA is TLR7, which signals through the adaptor protein MyD88, and indeed, cells deficient in either TLR7 or MyD88 fail to respond to this ligand alone, or in the context of a ssRNA virus (14). To assess whether these findings were also relevant to in vivo immune responses against influenza infection, we infected MyD88⁻/⁻ and TLR7-deficient mice with influenza virus and analyzed the ensuing CD4⁺ and CD8⁺ T cell responses at day 10 after infection. Surprisingly, no difference was observed in the number of virus-specific CD8⁺ T cells between TLR7⁻/⁻ (Fig. 1A) or MyD88⁻/⁻ (Fig. 1B) mice and their respective littermate controls. Furthermore, the proportion of CD4⁺ T cells in the airways was similarly unimpaired (data not shown). We next examined influenza-specific
CD4$^+$ and CD8$^+$ T cell effector functions by means of intracellular staining for IFN-γ after restimulation with influenza virus-bearing DCs. Again, we observed no difference between TLR7$^{-/-}$ or MyD88$^{-/-}$ mice and their respective controls (Fig. 1C). To check for a potential skew in the type of cytokines produced, we measured IL-4 protein levels by ELISA in BAL fluid, by intracellular staining of CD4$^+$ and CD8$^+$ cells, and performed quantitative real-time PCR analysis of whole lung tissue. In each case, minimal IL-4 was detectable, and levels were comparable in all groups (data not shown). To ensure that the similarities were not limited to expansion and cytokine production, we assessed Ag-specific cytotoxicity using a conventional $^{51}$Cr release assay directly ex vivo and found that specific cell lysis was also comparable (Fig. 1D). Taken together, these data suggest that TLR7 and its adaptor molecule MyD88 are not required for in vivo T cell responses against influenza virus.

Absence of TLR3 or its adapter molecule TRIF has no influence on the outcome of T cell responses against influenza virus infection

Many RNA viruses replicate via a dsRNA intermediate product, which is recognized by TLR3. Considering the TLR7/MyD88 pathway was not required for T cell responses against influenza, the recognition of this virus might instead have been achieved through the TLR3/TRIF pathway. We thus infected TLR3$^{-/-}$, TRIF-deficient (TRIF$^{-/-}$LPS2/LPS2), and C57BL/6 mice with influenza virus and examined the inflammatory infiltrate in the airways at day 10 postinfection. Total BAL CD4$^+$, CD8$^+$ cell counts (data not shown) and the proportion of CD8$^+$ NP34-tetramer-staining cells (Fig. 2, A and B) were comparable in all three groups. We then examined T cell effector functions and found no apparent difference in IFN-γ production by CD4$^+$ and
CD8\(^+\) T cells (Fig. 2C). In addition, we expanded cells from the draining lymph nodes of individual mice for 5 days and tested their cytotoxic potential. Consistent with the other T cell effector function readouts, we did not see any significant difference in cytolytic activity between wild-type, TLR3\(^{−/−}\) / LPS2/LPS2, and TRIF\(^{−/−}\) mice (Fig. 2D). Overall, these two previously described TLR pathways of RNA recognition do not appear to play major roles in the induction of T cell responses upon influenza infection.

**TLR7 and MyD88 control class switching of B cells to IgG isotypes**

For many years, LPS has been added to B cell cultures and found to increase proliferation, and more recent data have revealed that the mechanism underlying this phenomenon is signaling through TLRs (20, 21). However, the relevance of this activation pathway during an in vivo influenza infection remains to be established. Although the TLR3 and TLR7 pathways do not appear to be important for anti-influenza T cell responses, we sought to determine whether they were important for B cell responses. We thus infected TLR7\(^{−/−}\) and MyD88-deficient mice with influenza virus and obtained serum and BAL fluid for examination of influenza-specific Ab isotype switching. As mentioned previously, whereas IgG2a is often reported in studies investigating Ab isotype switching in C57BL/6 mice, the Igh1-b allele in this inbred strain in fact encodes IgG2c (11–13). In the current investigation, we have assessed both anti-IgG2a and anti-IgG2c Abs, both of which are likely to detect Igh1-b-encoded IgG2c in C57BL/6 mice. Analysis of the serum and BAL fluid of TLR7-deficient mice revealed significantly elevated virus-specific IgG1 titers, whereas virus-specific IgG2a/c titers were similar at day 10 compared with littermate controls (Fig. 3A and data not shown). At the same time point, virus-specific IgG2b and IgG3 also did not differ when compared with littermate controls (data not shown). We next analyzed serum and BAL Ab levels from influenza-infected MyD88-deficient mice. Previous reports showed that MyD88\(^{−/−}\) mice have reduced IgG1 titers and a complete lack of IgG2a Ab isotype when mice were immunized with OVA (29). However, in line with the
TLR7−/− data, we found higher virus-specific IgG1 titers in MyD88−/− mice as compared with the wild-type group (Fig. 3B and data not shown), but reduced levels of virus-specific IgG2a/c (Fig. 3B and data not shown). We additionally measured virus-specific IgA in the BAL fluid and found no difference between the knockout and littermate control groups (data not shown). MyD88 also acts as the adaptor molecule for TLR4 and TLR9, which have been reported to play a role in some viral infections (30, 31). To assess an involvement of TLR4 and TLR9 in the recognition of influenza virus and the outcome of class switching, we measured Ab isotypes in TLR4−/− and TLR9−/− mice at day 10 postinfection and found no difference as compared with wild-type controls (data not shown). We next investigated the IgG1 and IgG2a/c Ab titers in serum and BAL of TLR3−/− (Fig. 3C) and TRIFLPS2/LPS2 mice (Fig. 3D). Whereas all IgG isotypes were similar in TLR3−/− mice as compared with wild-type mice, TRIFLPS2/LPS2 mice had slightly elevated levels of IgG1. However, taken together with data from Fig. 2, TLR3 and its associated signaling molecule, TRIF, appear to play little or no role in the initiation of T and B cell responses against influenza virus. Overall, these data show that stimulation of TLR7 and its respective signaling molecule MyD88 has a profound influence on the induction of B cell class switching to IgG isotypes. However, it appears that the importance of TLR7 signaling is limited to inhibition of IgG1 class switching, and another MyD88-dependent pathway additionally promotes IgG2a/c switching in this system.

FIGURE 3. TLR7 and MyD88 regulate virus-specific IgG1 Ab responses. Groups of TLR7+/+, MyD88+/+, TLR3+/+, TRIFLPS2/LPS2, and appropriate control mice were infected with 50 PFU of influenza virus. Mice were bled at day 10 after infection, and levels of Ab isotypes were measured using the indicated commercial Ig by ELISA. Serum IgG1 and IgG2a/c Ab isotypes in TLR7−/− (A), MyD88−/− (B), TLR3−/− (C), and TRIFLPS2/LPS2 (D) mice. Graphs are representative of at least three independent experiments with four to seven mice per group.
Redundant signals induce B cell class switching to IgG2a/c in vitro and in vivo

Clearly, TLR signaling plays an important part in regulating the isotype of anti-influenza Abs. To ascertain the factors involved in driving B cell class switching directly, we used an in vitro system in which we could stimulate sorted B cells with each of the most plausible candidates: specifically, the TLR7 ligand R837, anti-CD40, rIFN-α, and combinations thereof. After 10 days of culture with the indicated stimuli, the supernatants were harvested and IgG1 and IgG2a/c Ab isotype levels were measured. We found that when given alone, R837 and anti-CD40 induced some IgG1 secretion, but not switching to IgG2a (Fig. 4). However, when both R837 and anti-CD40 were present in the culture, class switching to IgG2a/c also occurred (Fig. 4). rIFN-α alone could not induce IgG1 or IgG2a Ab isotype secretion (data not shown), but together with R837 and anti-CD40 it induced robust levels IgG2a (Fig. 4). These data suggest that B cell TLR signaling and T cell help share redundant signaling pathways for the initiation of IgG1 class switch recombination, as one signal alone was sufficient for class switching to IgG1. Comparatively, these signals can act in concert in the presence of IFN-α, to induce IgG2a/c switching. The strength of this in vitro system is that it allows the impact of individual signals to be addressed; however, by nature such an assay is not physiologically relevant. We thus sought to test these data in vivo. We infected CD40L−/− and CD40−/− mice with influenza and then assessed influenza-specific IgG1 and IgG2a/c levels. In line with prior investigations, we found that both of these IgG isotypes were reduced in the absence of either CD40 or CD40L (Fig. 5A and data not shown). In these mice, while the CD40-CD40L interaction was missing, the B cells still received signals to undergo IgG1 and IgG2a/c isotype switching. Notably, whereas the total levels of the isotypes were reduced, there was no skewing toward either IgG1 or IgG2a/c as seen in the absence of TLR7 or MyD88. We then addressed the role of T cell help and MyD88 signaling together. Accordingly, MyD88−/− and C57BL/6 were administrated with neutralizing anti-CD40L Ab before and during the influenza infection, and on day 12 postinfection, we assessed IgG1 and IgG2a/c levels. Administration of the

![FIGURE 5.](http://www.jimmunol.org/)

CD40 signaling promotes IgG1 and IgG2a/c production, whereas MyD88 signaling regulates IgG1 class switching in vivo. A, CD40L−/− mice were infected with 50 PFU of influenza virus. Mice were bled 10 days after infection, and serum IgG1 and IgG2a/c Ab isotype levels were determined by ELISA. B, MyD88−/− mice (knockout (KO)) were injected with 200 μg of anti-CD40L mAb i.p. 1 day before infection with 50 PFU of influenza virus. At day 4 after infection, mice were again injected with 200 μg of anti-CD40L i.p. Mice were bled 11 days after infection, and serum IgG1 and IgG2a/c Ab isotype levels were determined by ELISA. Data are representative of two independent experiments.
anti-CD40L Ab led to a negligible reduction in IgG1 and a more striking decrease in influenza-specific IgG2a/c (Fig. 5B). These data support the conclusion that the primary role of the CD40-CD40L interaction is in supporting B cell switching overall, whereas MyD88 signaling plays a dual role in supporting the magnitude of the Ab response in addition to fine-tuning the nature of the IgG isotype.

*IFN-α* regulates the appropriate switching of IgG isotypes during influenza infection

Our in vitro data implicated *IFN-α* as playing a supporting role in regulating Ab isotype switching. Notably, plasmacytoid DC and airway epithelium have been described to produce *IFN-α* upon exposure to influenza virus. We thus examined the anti-influenza response of *IFN-αR*−/− mice to assess the contribution of type I IFNs to Ab class switching. Similar to MyD88−/− mice, mice deficient in type I IFN receptor had higher IgG1 and lower IgG2a/c levels as compared with control mice, consistent with recent results from Coro et al. (32) (Fig. 6A). However, upon infection with low-dose influenza, the role of type I IFN was less pronounced because IFN-αR−/− mice exhibited comparable IgG2a/c levels to wild-type mice, whereas IgG1 titers were increased (Fig. 6B). Taken together, these data point to a central role for *IFN-α* in suppressing IgG1 switching and a secondary role in enhancing IgG2a/c switching when *IFN-α* levels are not limiting.

**Discussion**

Numerous studies describe the importance of TLR-dependent maturation of DCs in triggering adaptive immune responses. Upon TLR activation, DCs become fully mature costimulator-bearing, cytokine-producing cells that can trigger full T cell differentiation (33–35). Surprisingly, the data from our current study diverge from this paradigm by showing that CD4+ and CD8+ T cell responses are not impaired upon influenza infection in the absence of known TLR signals. Barchet et al. (36) reported that DCs matured and produced inflammatory cytokines and *IFN-α* in response to influenza virus through a TLR7/MyD88- and protein kinase R-independent pathway in vitro. Together with our results, one can conclude that activation of T cells during anti-influenza immune responses relies on mechanisms other than the TLR7/MyD88, TLR3/TRIF, and protein kinase R pathways. It is becoming increasingly clear that the immune system has evolved redundant mechanisms in innate viral recognition. Retinoic acid-inducible protein 1 (RIG-I) was reported to be involved in the detection of dsRNA and the subsequent activation of the transcription factors NF-κB and IFN regulatory factor-3, two major components leading to type I IFN and inflammatory cytokine production (37, 38). Another candidate for innate viral recognition is melanoma differentiation-associated gene 5 (MDA5), which can induce the production of IFN-β upon binding of dsRNA (39, 40). Recently, Kato et al. (41) analyzed the differential roles of RIG-I and MDA5 in influenza virus detection, and they found no induction of type I IFNs in mouse embryonic fibroblasts when using wild-type influenza virus due to IFN suppression by the viral protein NS1. Only when mouse embryonic fibroblasts were infected with mutant ΔNS1 influenza virus did RIG-I become essential for *IFN-α* and *IFN-β* production. It is plausible that pathways...
such as RIG-I and MDA5, or as yet undescribed TLRs, may contribute to innate viral recognition, but their exact role remains to be elucidated. Another plausible pathway for the compensation of TLR signaling may lie in the strength of TCR signaling and costimulation, which could be sufficient to drive adaptive immune responses against influenza infection in the absence of TLR stimulation. Comparatively, in systems in which an innocuous, nonreplicating Ag such as OVA is used, TLR ligands (e.g., LPS) or other adjuvants (e.g., alum) are required to provide sufficient stimulation for full T cell activation. There is accumulating, albeit controversial (42), evidence that TLR signaling directly on B cells is critical for their full activation. For over 30 years, LPS has been known to induce Ig production in vitro (43). More recently, other TLR ligands, including CpG, have been shown to efficiently induce proliferation and secretion of Abs by B cells in vivo and in vitro (44). Pasare and Medzhitov (20) found that TLR4 signaling is mandatory for IgM and IgG1 production when mice were immunized with OVA-LPS or human serum albumin LPS, and that T help is required for isotype class switching. They concluded that optimal B cell responses require both T cell help and TLR signaling. In light of these data, it was surprising that we found no reduction of class switching to virus-specific IgG1 in TLR7−/− or MyD88−/− mice after influenza infection; in fact, the IgG1 response was enhanced. These data are in accordance with reports that TLR ligands that signal through MyD88 may negatively regulate IgG1 and promote IgG2a/c Ab isotype class switching (45). Our data indicate that TLR signaling plays a robust, yet redundant role for B cell Ab production in vivo. TLR stimulation directly on B cells led to proliferation and IgG1 production, but alone failed to fully regulate isotype switching. It appears that during influenza infection, TLR stimulation of other cell types, most likely plasmacytoid DC and airway epithelial cells, leads to IFN-α production, which fine-tunes the antiviral Ab response. During influenza infection, this fine-tuning appeared to occur between days 5 and 8 postinfection because elevated IgG1 switch transcripts from TLR7−/− and MyD88−/− mice could be detected (data not shown). Our in vitro data indicated that the TLR7 signal on B cells was not sufficient to induce the correct Ab isotypes, because it increased proliferation and production of IgG1, but not IgG2a/c. However, combined with anti-CD40 stimulation or IFN-α, IgG2a/c isotype switching was detectable. Notably, CD40 stimulation of B cells in vitro resulted in the same IgG isotype profile as seen with TLR7 stimulation alone. It is thus plausible that TLR7 and CD40 stimulation directly on B cells both provide signals for proliferation, and that the class switch to IgG1 occurs by default upon stimulation with either one of these signals. The addition of rIFN-α alone to the in vitro B cell cultures resulted in neither IgG1 nor IgG2a/c class switching, but in combination with agonistic anti-CD40 and a TLR7 ligand, it led to IgG2a/c production.

To assess the role of type I IFNs in vivo, we infected IFN-αR−/− mice with either a normal dose or a low dose of influenza virus in an attempt to clarify the importance of high levels vs limiting FIGURE 7. TLR7 regulates appropriate Ab isotype switching directly through signaling in B cells, and indirectly through the induction of IFN-α. A, T cell help through the CD40-CD40L interaction drives B cell proliferation and low level production of IgG1, but not IgG2a/c. B, TLR stimuli directly upon B cells induce B cell proliferation and production of IgG1. C, In the combined presence of the CD40-CD40L interaction and TLR-ligand stimulation, B cells proliferate and produce both IgG1 and IgG2a/c. D, Type I IFNs in combination with CD40-CD40L interaction and TLR7 stimulus drive the production of IgG2a/c at the expense of IgG1.
levels of IFN-α. We found that mice deficient in type I IFN signaling showed decreased IgG2a/c class switching when infected with a normal dose of influenza virus; however, under limiting IFN-α conditions, no difference in IgG2a/c was detectable. These data show that IFN-α is not requisite for IgG2a class switching; however, it can enhance IgG2a/c levels when present in sufficient amounts. Comparatively, during both high and low-dose infections, the absence of IFN-α signaling led to dysregulated IgG1 production, highlighting the central role this cytokine plays in fine-tuning the response (46, 47).

Whereas influenza-specific IgG2a/c levels were normal in the absence of TLR7 signaling, the absence of MyD88 led to reduced IgG2a/c levels. This disparity indicates that an additional MyD88 pathway may be important for activating B cells during infection. During stress responses and lung tissue damage, heat shock proteins are released into the tissue. It was shown that heat shock protein 70 can induce proinflammatory cytokines via TLR2/TLR4, which signal through MyD88 (48). Thus, local tissue damage may play a role in priming B cells for a full IgG2a/c response. MyD88 is also a key adaptor molecule for IL-1 signaling; however, we have shown previously that IL-1 is not important for IgG2a/c class switching during influenza infection (49), making it an unlikely candidate to reconcile this disparity.

In conclusion, we show that TLR signaling is not critical for the initiation of adaptive T cell immune responses against influenza infection. We further demonstrate that TLR signaling has both direct and indirect effects upon B cells. Specifically, CD40-CD40L interactions and TLR signaling on B cells result in proliferation and initiate IgG1 and IgG2a/c class switching, whereas TLR-induced type I IFN production fine-tunes the antiviral response, decreasing IgG1 and increasing IgG2a/c (Fig. 7). Thus, TLR signaling plays a central role in regulating the quality of the humoral response while being redundant for the development of anti-influenza T cell immunity.

Disclosures

The authors have no financial conflict of interest.

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