T-box transcription factor T-bet, a key player in a unique type of B-cell activation essential for effective viral clearance

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IgG2a is known to be the most efficient antibody isotype for viral clearance. Here, we demonstrate a unique pathway of B-cell activation, leading to IgG2a production, and involving synergistic stimulation via B-cell antigen receptors, toll-like receptor 7 (TLR7), and IFNγ receptors on B cells. This synergistic stimulation leads to induction of T-box transcription factor T-bet expression in B cells, which, in turn, drives expression of CD11b and CD11c on B cells. T-bet/CD11b/CD11c positive B cells appear during antiviral responses and produce high titers of antiviral IgG2a antibodies that are critical for efficient viral clearance. The results thus demonstrate a previously unknown role for T-bet expression in B cells during viral infections. Moreover, the appearance of T-bet$^+$ B cells during antiviral responses and during autoimmunity suggests a possible link between these two processes.

interferon gamma | virus

During viral infections, antigen-specific B cells are critical for the generation of protective antibodies and provide an important component of immune memory to the virus in question. B cells secrete a wide spectrum of viral-specific antibodies of different isotypes that define their function. Intensive studies have been performed to establish which isotype is the most potent for viral clearance and the protection of the host against the pathogen (1). Several reports indicate that mouse IgG2a/c (hereinafter referred to as IgG2a) is the major isotype produced during antiviral immune responses and, compared with other isotypes, is the most efficient in creating antibody-mediated protection against viruses in vivo (1–3). However, the factors leading to the isotype switch to IgG2a during viral infection have not been exhaustively studied. IFN gamma (IFNγ) is known to induce switching to IgG2a (4), and T-bet expression has been shown to be critical for this process (4–6). However, whether the same stimuli are required for antiviral IgG2a production during pathogen clearance and which B-cell subset is the target of this process has never been studied.

In this report, we investigate a previously unknown, synergistic effect of B-cell antigen receptor (BCR), toll-like receptor 7 (TLR7), and IFN gamma receptor (IFNγR) signaling that results in high levels of T-bet expression and IgG2a production in B cells both in vitro and in vivo.

T-bet, in addition to being a Th1 lineage defining transcription factor, is expressed in other cell types such as dendritic cells (7), natural killer (NK) cells (8, 9), CD8 T cells (10, 11), and B cells (12–14). Several reports have described the role of T-bet in B cells, mainly focusing on its requirement for class switching to IgG2a and the appearance of IgG2a-expressing memory B cells (5, 6, 14). Expression of T-bet in B cells has been shown to be driven by engagement of their BCRs, IFNγR, and TLR9 or TLR7 or CD40 (12, 15–18). However, the significance of T-bet expression in B cells during pathogen clearance has never been established.

In this report, we show that T-bet–expressing B cells also appear at the peak of antiviral responses and are the major producers of viral-specific antibodies. The appearance of these cells depends on signaling via BCR and IFNγRs and TLRs on the B cells. The IFNγ involved is produced by cells other than B cells and, in the absence of other stimuli, is most effectively induced by TLR7 signaling. Clearance of virus is abrogated in the absence of the T-bet–expressing B cells. The virus-specific T-bet–expressing B cells are similar in their induction requirements and properties to a type of B cell that we and others have recently described, termed age-associated B cells (ABCs). This subset of B cells accumulates in aged females and autoimmune prone mice and humans and produces auto-antibody upon stimulation and is absolutely dependent on T-bet expression for its appearance (19–21). Thus, the results suggest a unique and unexpected link between antiviral responses and autoimmunity, indicating that the same type of B-cell activation may be involved in both processes.

Results

TLR7, BCR, and IFNγR Signaling Synergize in the Up-Regulation of T-bet Expression in B Cells in Vitro. Others have reported that T-bet expression in B cells is crucial for IgG2a class switching (6) and IgG2a memory formation (14) and that, moreover, a combination of BCR and IFNγR signaling induces T-bet production in B cells (17).

To explore this phenomenon in more detail, we decided to test the contributions to the induction of T-bet in B cells of different types of stimuli, using spleen cells from C57BL/6 (WT) mice

Significance

Here, we show that signals delivered by antigen engagement, IFNγ, and toll-like receptor 7 [TLR7] induce T-box transcription factor T-bet and IgG2a switching in B cells. The IgG2a product of these signals is important for viral immunity. For example, the titers of mouse gammaherpesvirus 68, an Epstein–Barr-related virus, are not well reduced if the B cells cannot express T-bet and consequently cannot switch to production of IgG2a. The T-bet expressing B cells resemble a subset of B cells that appears in autoimmune prone mice and women. Thus, a B-cell differentiation pathway that has evolved to promote immunity to viruses may also contribute to autoimmunity.

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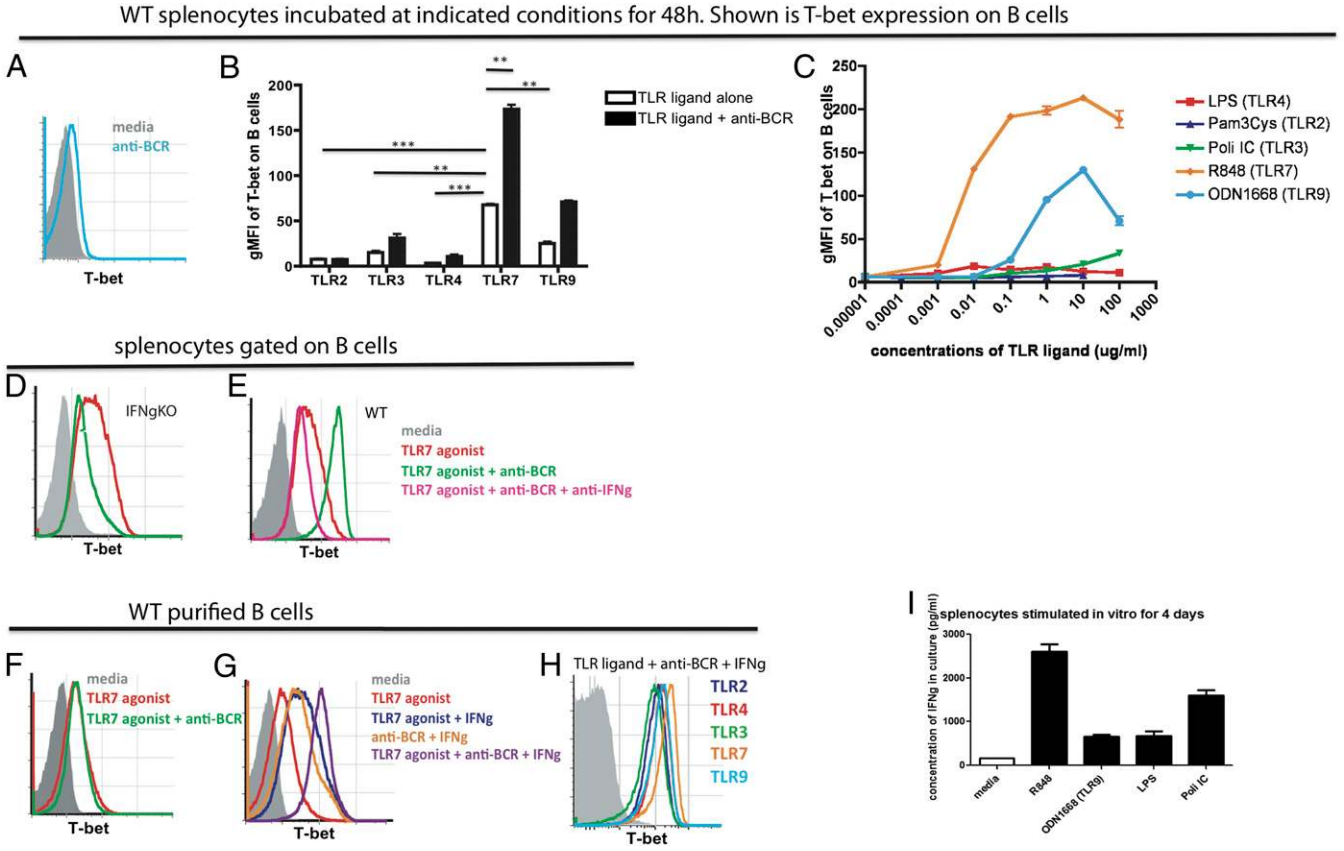

Fig. 1. TLR7, BCR, and IFNγR signaling synergize in the up-regulation of T-bet expression in B cells in vitro. (*A*–*C*) Unseparated splenocytes from WT mice were cultured for 48 h under the indicated conditions. After incubation, T-bet expression in B cells was analyzed. (*D* and *E*) IFNγR1$^{-/-}$ or WT splenocytes were cultured for 48 h under the indicated conditions, and T-bet expression in B cells was then analyzed. (*F*–*H*) Purified WT B cells were incubated under the indicated conditions for 48 h, and T-bet expression in B cells was then analyzed. (*A*–*H*) Histograms and titrations represent the levels of T-bet expressed in B cells, gated as live/B220^{+}/CD4^{-}/CD8^{-}/CD19^{+}. (*I*) Unseparated WT splenocytes were incubated under the indicated conditions for 4 d. The supernatants were then analyzed by ELISA for the presence of IFNγ. In all relevant sections of the figure, bars represent the means ± SEM of $n = 3$ mice per group. $^{*}P < 0.05$, $^{**}P < 0.001$, $^{***}P < 0.0001$ (*t* test). All data are representative of at least three independent experiments.

cultured for 48 h. Mere cross-linking of the BCRs on B cells induced almost no T-bet (Fig. 1*A*) irrespective of the concentration of BCR cross-linking antibody (Fig. S1). T-bet was induced to some extent in the B cells in WT spleen cell cultures incubated with TLR agonists (Fig. 1*B*). Of the agonists, TLR7 was the most effective, regardless of concentration. Culture of WT spleen cells with both anti-BCR and TLR agonists induced yet more T-bet, indicating a synergistic effect of these two stimuli. Again, the TLR7 agonist was the most effective, regardless of agonist concentration (Fig. 1 *B* and *C*). Because of this feature, we decided to focus our study on the effects of TLR7 agonists.

IFNγ has been shown to play a role in T-bet induction in B cells (17); therefore, we decided to test the role of this cytokine in the synergistic induction of T-bet by BCR and TLR7. Splenocytes from IFNγR1$^{-/-}$ mice were incubated with TLR7 agonist and/or anti-BCR, and B-cell expression of T-bet was analyzed. The absence of IFNγR did not affect the levels of T-bet induced in response to TLR7 agonist alone but considerably reduced the amount of T-bet induced in B cells in response to the combination of TLR7 agonist and anti-BCR (compare Fig. 1 *D* and *E*, green histograms). Moreover, addition of blocking anti-IFNγ antibodies to cultures of WT splenocytes incubated with anti-BCR and TLR7 agonist inhibited T-bet induction (Fig. 1*E*). Thus, IFNγR is a third component of the signaling proteins needed for high T-bet induction in B cells.

To confirm that IFNγ was really involved and to demonstrate that the cytokine acted directly on the target B cells, B cells were purified from the spleens of WT mice and cultured with or without anti-BCR, TLR7 agonist, and IFNγ. IFNγ alone had no effect on T-bet expression by the purified B cells (Fig. S2). T-bet was induced to low and similar levels in B cells cultured with TLR agonist alone, but addition of anti-BCR, unlike in whole splenocytes, did not induce higher level of T-bet expression in purified B cells (Fig. 1*F*), indicating that the synergistic effect of TLR7 and BCR require this cytokine. However, T-bet was induced to maximal levels by culture of B cells with all three stimuli (Fig. 1*G*), and, moreover, in the presence of exogenously added IFNγ, the TLR7 agonist was not noticeably superior to agonists for other TLRs (Fig. 1*H*). This effect was also manifest in similar experiments with purified follicular B cells (FO B cells), the major B-cell population in spleens (Fig. S3). Also, the effect was not due to differential cell proliferation or death under the different culture conditions (Fig. S4).

These results suggested that T-bet is best induced on the target, purified B cells themselves, by a combination of BCR, IFNγR, and TLR signaling, regardless of the TLR engaged. Why then, did a TLR7 agonist have superior activity when unseparated spleen cells were cultured in the absence of exogenously added IFNγ? The most straightforward explanation is that, of all of the TLR agonists, the TLR7 agonist induced the greatest amount of IFNγ production by splenic non-B cells. To check this hypothesis, WT spleen cells were cultured for 4 d with optimal concentrations of different TLR agonists, and their supernatants were analyzed for concentrations of IFNγ by

ELISA. As shown in Fig. 1*I*, the TLR7 agonist induced the highest concentration of IFNγ in culture. Our preliminary data indicate that T cells produce IFNγ in response to TLR7 stimulation in these cultures (data not shown).

STAT1 is required for T-bet induction in different cell types; however, induction of T-bet in B cells by TLR9 has been reported to be STAT1-independent (12, 22, 23). We investigated whether STAT1 was required for induction of T-bet in B cells in the system studied here. As shown in Fig. S5, STAT1$^{-/-}$ B cells could up-regulate low levels of T-bet in response to TLR7 ligation alone, indicating that this is a STAT1-independent process. However, the synergistic effect of TLR7, BCR, and IFNγR ligation was almost completely abolished in STAT1$^{-/-}$ B cells. Thus, it seems that the T-bet–inducing activity of TLR7 ligand is STAT1-independent but that the additional inducing effects of IFNγ and BCR require STAT1 activity.

Together, these results suggest that T-bet is best induced in B cells by a synergistic combination of signaling on the B cell, by BCR, IFNγR, and any TLR. However, in the absence of other stimuli, TLR7 agonists are the best inducers of IFNγ. Therefore, as far as spleen cell cultures are concerned, overall, B-cell expression of T-bet is best induced by anti-BCR and TLR7 agonists.

BCR and TLR7 Synergize to Drive T-bet Expression in B Cells in Vivo. Our in vitro data indicate that innate and adaptive immune receptors can synergize in B cells, leading to high levels of T-bet expression, a phenomenon that has not been previously described. However, does this phenomenon also happen in vivo? To answer this question, MD4 transgenic B cells, bearing BCRs specific for hen egg lysozyme (HEL) were transferred into B6.SJL congenic hosts, followed by immunization with HEL, TLR7 agonist, or a mixture of both, 24 h after B-cell transfer. The level of T-bet expression in donor and host B cells was assessed 24 h after immunization. As shown in Fig. 2 *A* and *B*, MD4, but not the endogenous host B cells showed a slight increase of T-bet expression in response to immunization with HEL protein alone, probably because only the MD4 B cells detected the antigen. The MD4 B cells also expressed a slightly higher level of T-bet after exposure to TLR7 agonist than WT B cells did, a result that is statistically significant although of unknown causes (Fig. 2 *A* and *B*). However, when mice received a mixture of HEL and TLR7 ligand, MD4, but not endogenous WT B cells, expressed three times more T-bet than B cells exposed to TLR7 agonist alone (Fig. 2 *A* and *B*). All of the MD4 cells increased their levels of T-bet, in line with our observation that the stimuli act on most of the B cells in spleen, that is, the FO B cells (Fig. S5). The average amounts of T-bet expression achieved in vivo did not, however, reach those detected in vitro after stimulation with anti-BCR, TLR7 agonist, and IFNγ (Fig. 1). Perhaps the levels of IFNγ reached in vivo did not match those apparent in our in vitro cultures. Overall, however, these experiments indicate that BCR, TLR7, and IFNγR synergize, not only in vitro, but also in vivo to induce high levels of T-bet expression in B cells.

To find out what kind of B cells is induced by the procedures described here, we assessed the phenotype of the MD4 B cells in vivo after immunization with HEL and a TLR7 agonist. Twenty-four hours after immunization, there was a significant increase of MD4, but not WT B cells expressing CD11c and CD11b (Fig. 2*C* and Fig. S6). This phenomenon occurred only in animals immunized with a mixture of HEL and TLR7 ligand (Fig. 2*D* and Fig. S6). The joint expression by these cells of T-bet, CD11b, and CD11c is reminiscent of another population of B cells, so-called

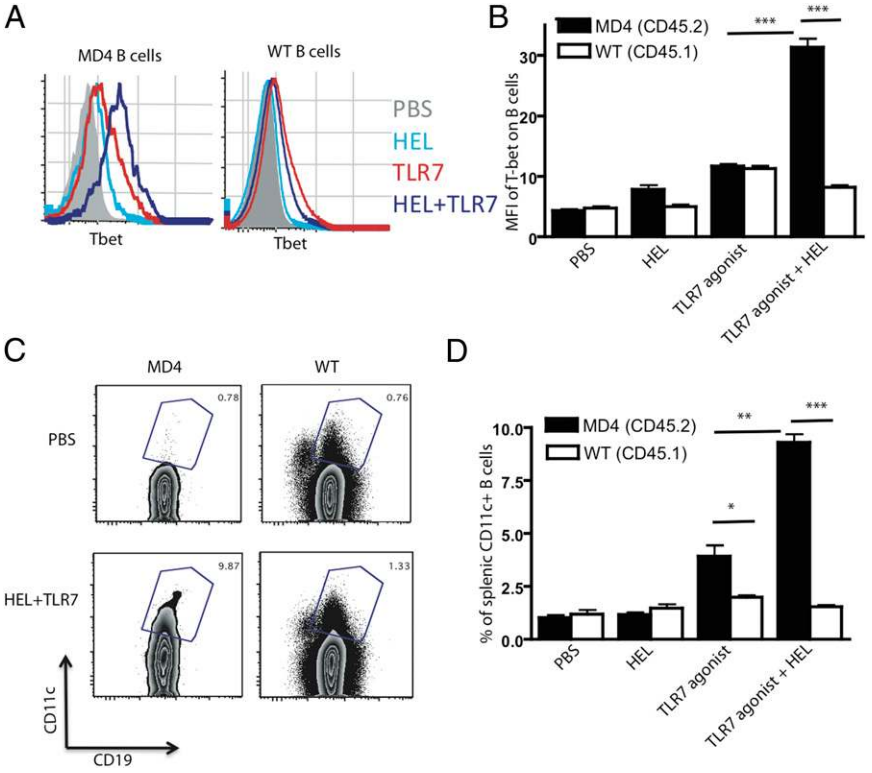

Fig. 2. BCR and TLR7 synergize in T-bet induction in vivo driving CD11c and CD11b expression on B cells. MD4 transgenic B cells were transferred into B6.SJL recipients followed by immunization of recipient mice with HEL, a TLR7 agonist (R848), or a mixture of both i.p. 24 h after cell transfer. (*A* and *B*) Splenocytes were analyzed for T-bet expression in B cells (gated as B220$^+$, CD19$^+$, CD4$^-$, CD8$^-$) or (*C* and *D*) the presence of CD11c$^+$ B cells in donor MD4 B cells (black bars) and host WT B cells (white bars). Bars represent the means ± SEM of $n = 3$ mice per group. *$P < 0.05$, **$P < 0.001$, ***$P < 0.0001$ (*t* test). Data are representative of three independent experiments.

ABCs, which we and others have recently reported (19, 21), a result we consider in *Discussion*.

About 10% of the immunized MD4 cells acquired CD11b and CD11c in this experiment (Fig. 2 *C* and *D* and Fig. S6) even though T-bet levels were increased in all of the MD4 B cells (Fig. 2*A*). A plot of T-bet levels against CD11c showed that the expression of CD11c did not correlate with the level of T-bet in these cells (data not shown); therefore, we cannot explain in the moment why only a proportion of the T-bet$^+$ B cells expressed CD11c on their surface. The result may be due to the very short time course (24 h) of this experiment or to the different locations in which the transferred MD4 cells reside.

These results demonstrate that in vivo BCR and TLR7 signaling, in the presence of IFNγ, synergize to induce high levels of T-bet expression in B cells leading to their differentiation into CD11b/c$^+$ B cells.

T-bet Overexpression in B Cells Is Sufficient for Their Differentiation into CD11b$^+$/CD11c$^+$ B Cells. To determine whether T-bet expression in B cells is sufficient to drive them to express CD11b and CD11c, we generated immature B cells in vitro from T-bet$^{-/-}$ bone marrow cells cultured, as previously described (24), in the presence of IL-7 for 4–5 d. The immature B cells were transduced with either a control GFP-expressing retrovirus [pMIG (pMCSV-IRES-GFP)] or a retrovirus encoding GFP [pMIG+T-bet (pMCSV-IRES-T-bet-GFP)] and T-bet with the efficiency of transduction (monitored by GFP expression) at 15–30% (data not shown). The cells were transferred into sublethally irradiated congenic hosts. Transduced cells were not purified by sorting before transfer, thus allowing the internal control of GFP-negative (nontransduced) cells in the same host for comparison. Splenic B cells were analyzed for the presence of CD11b and CD11c on day 10 by FACS. In mice given GFP control-transduced B cells, the levels of expression of CD11b and CD11c were similar whether or not the donor B cells expressed GFP (Fig. 3 *A* and *B*), indicating that retroviral transduction itself did not promote the expression of these markers on B cells. In contrast, in mice receiving T-bet–transduced B cells, the levels of CD11b and CD11c expressed by GFP$^+$ B cells were significantly higher than those of the GFPneg B cells (Fig. 3 *A* and *B*). The level of CD11c expression correlated directly with the level of GFP in GFP$^+$ T-bet–transduced, but not GFP control-transduced, cells (Fig. 3*C*), indicating that the level of expression of these markers depended on the level of T-bet expression. The levels of CD11b and, to some extent, CD11c achieved in these in vivo experiments were not as high as those observed in cultured cells (Fig. 1). Perhaps this phenomenon is due to the relative immaturity of the B cells studied in the in vivo experiments. Alternatively, in these in vivo experiments, the B cells are not receiving any signals (for example via their BCRs, TRL7, or IFNγRs) other than those delivered via T-bet itself. It is possible that signals from

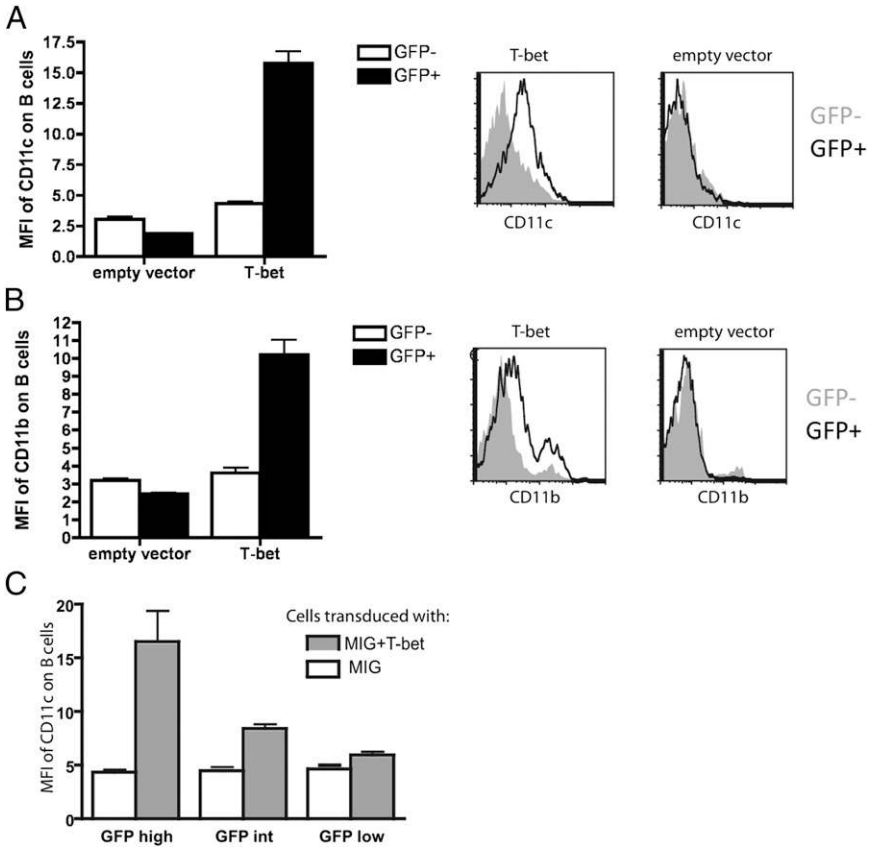

Fig. 3. T-bet expression in B cells is sufficient for their differentiation into CD11c$^+$/CD11b$^+$ B cells. Immature B cells from T-bet$^{-/-}$ mice were generated by culture of bone marrow-derived cells for 5 d in IL-7 and transduced with retroviruses expressing GFP alone or GFP plus T-bet. The cells were transferred into sublethally irradiated congenic hosts, and B cells in the spleens of the mice were analyzed for expression of GFP and CD11b and CD11c 10 d later. (*A* and *B*) Sample histograms and bar graphs show the expression of CD11c (*A*) and CD11b (*B*) on splenic donor B cells. The B cells were gated as CD4$^-$/CD8$^-$/CD19$^+$/CD45.1$^+$. Bars represent means ± SEM of B cells from $n = 5$ mice per group. (*C*) Donor GFP$^+$ B cells were subsequently gated as GFP high, intermediate, and low. The mean fluorescent intensity (MFI) of CD11c was analyzed for each population. Bars represent the means ± SEM of cells from $n = 5$ mice per group. Data are representative of two independent experiments.

these receptors contribute to the phenotype of the B cells via means in addition to their induction of T-bet.

Overall, these data demonstrate that elevated levels of T-bet expression in B cells are sufficient to drive CD11b and CD11c expression on B cells. This fact leads to the hypothesis that synergistic stimulation via TLR7, BCR, and IFNγ leads to the induction of T-bet in B cells, which, as a transcription factor, directly or indirectly induces CD11b and CD11c.

T-bet/CD11b/CD11c Positive B Cells Appear at the Peak of Antiviral Response and Produce Viral Specific IgG2a Antibodies. So far, we have shown that TLR7, BCR, and IFNγ synergistically drive T-bet and consequent CD11b and CD11c expression in B cells. The next question we asked was why this pathway of B-cell activation has evolved. Does it play any role in protective immune responses to pathogens?

Because it has been previously reported that T-bet drives IgG2a isotype switching (5, 25), we decided to test whether synergistic activation of B cells via TLR7, BCR, and IFNγ also leads to IgG2a production. To address this question, purified B cells were incubated for 7 d in the presence of different stimuli, and the levels of different IgG isotypes in supernatants were then evaluated. Synergistic activation of B cells with TLR7, BCR, and IFNγ drove the highest levels of IgG2a and IgG2b production, compared with other stimulation conditions (Fig. 4). Thus, one outcome of synergistic induction of T-bet in B cells is an isotype switch to IgG2a production.

IgG2a is well known to be the most potent isotype for antibody-dependent cellular cytotoxicity (ADCC) and is the major isotype produced during antiviral responses (1, 2, 25, 26). To address whether T-bet expression in B cells plays a role in antiviral responses, we examined the phenotype of spleen B cells at the peak of the antibody response (10 or 15 dpi) (26) to murine gammaherpesvirus 68 (gHV68), lymphocytic choriomeningitis virus (LCMV), and vaccinia infection. As shown in Fig. 5 *A* and *B*, a significant percentage of B cells converted into T-bet–expressing $CD11b^+/CD11c^+$ B cells at the peak of the response to any of these viruses. The percentage of T-bet positive B cells was significantly reduced in $TLR7^{-/-}$ and $IFN\gamma^{-/-}$ mice infected with gHV68 (data not shown). Because T-bet has been recently reported to be necessary for formation and maintenance of IgG2a memory B cells (14), we tested whether $T\text{-}bet^+$ B cells appearing at the peak of antiviral response also represent a subset of memory B cells. The results demonstrated the lack of CD73 [a memory B-cell marker (27, 28)] on $T\text{-}bet^+$ B cells at 15 d after gHV68 infection (data not shown), indicating that, at this time, this population of B cells is not a subset of memory B cells but might, however, be a precursor for such cells.

To assess the function of this B subset in responses to viruses, we isolated $CD11c^+$ B cells and FO B cells from gHV68-infected mice at 15 d postinfection (dpi) and measured the amounts of antiviral antibody made by the two types of cells in vitro. As shown in Fig. 5*C*, $CD11c^+$ B cells produced the vast majority of antiviral IgG2a antibodies.

To address the function of T-bet–expressing B cells during an antiviral response more directly, mixed bone marrow chimeras were generated in which all cells, except B cells, could express T-bet. Thus, lethally irradiated C57BL/6 mice received a mixture of bone marrow cells from B-cell–deficient μMT mice and either $T\text{-}bet^{-/-}$ or WT mice. The chimeric mice were later infected with

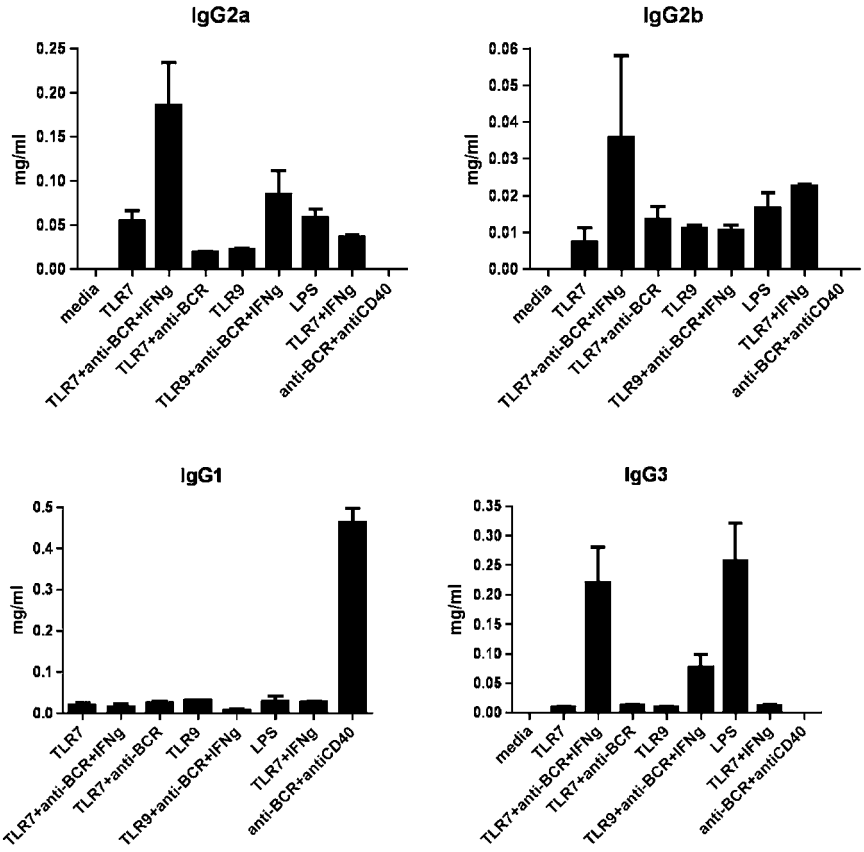

Fig. 4. T-bet induction in B cells by TLR7, BCR, and IFNγR leads to IgG2a production. Purified B cells from young C57BL/6 mice were cultured under the indicated conditions, and supernatants were analyzed for the presence of different IgG isotypes by ELISA at day 7. Bars represent the means ± SEM of $n = 3$ samples per group. Data are representative of three independent experiments.

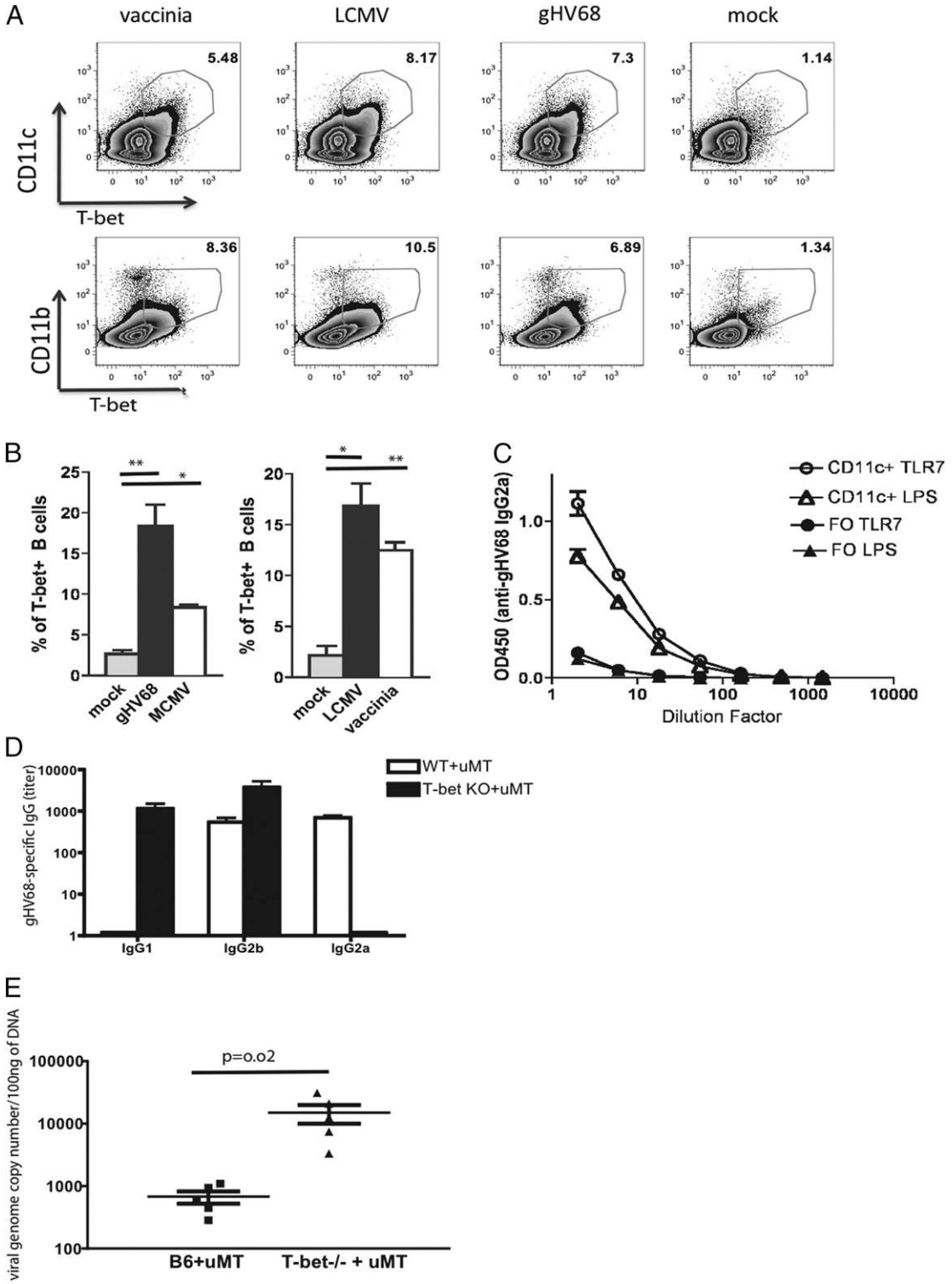

Fig. 5. T-bet$^+$/ CD11c$^+$/CD11b$^+$ B cells appearing at the peak of antiviral response secrete antiviral IgG2a and essential for effective viral clearance. C57BL/6 mice were infected with gHV68 or mock infected for 14 d or alternatively infected with LCMV or vaccinia or mock infected for 10 d. (*A*) The appearance of T-bet$^+$/CD11c$^+$/CD11b$^+$ B cells in spleen was analyzed by FACS. FACS plots present the data for B cells gated as CD19$^+$/B220$^+$/CD4$^-$/CD8$^-$. (*B*) The percentages of splenic T-bet positive B cells in infected mice is shown. Bars represent the means ± SEM for $n = 5$ mice per group. (*C*) CD11c$^+$ and follicular B cells from gHV68-infected mice were sorted at day 14 postinfection with gHV68 and stimulated in vitro for 7 d with LPS or TLR7 agonist. Supernatants were analyzed at day 7 for the presence of antiviral antibodies by ELISA ($n = 3$ samples per group). (*D* and *E*) WT mice were lethally irradiated and reconstituted with mixtures of bone marrow cells from WT and μMT or T-bet$^{-/-}$ and μMT mice, as indicated. Seven weeks later, the chimeras were infected with gHV68 or mock infected. (*D*) Serum levels of antiviral IgG of different isotypes were analyzed by ELISA 18 dpi. Bars represent the means ± SEM of $n = 5$ mice per group. (*E*) Spleens from the bone marrow chimeric mice were analyzed for the viral loads by real-time PCR at 18 dpi. The viral genome copy number per 100 ng of DNA is shown. $P = 0.02$ (*t* test). All data are representative of three independent experiments.

gHV68, and their viral load and antiviral antibody response were assessed 17 d after infection. Mice containing WT B cells produced high titers of antivirus IgG2a and IgG2b. Mice containing T-bet–deficient B cells, on the other hand, contained similar levels of virus-specific IgG2b but failed to produce antiviral IgG2a and, instead, produced high titers of antiviral IgG1 (Fig. 5*D*). This result directly demonstrates that T-bet expression in B cells is required for antiviral IgG2a antibody production.

To find out whether the absence of antiviral IgG2a had any impact on viral load, we measured the amount of gHV68 in the spleens of infected mice using real-time PCR. Indeed, mice with T-bet–deficient B cells had increased viral titers and therefore impaired viral clearance compared with WT animals (Fig. 5*E*).

gHV68 is a DNA virus; therefore, it is possible that infection with this virus generates T-bet positive B cells via TLR9 rather than TLR7 engagement. However, many cells die during the acute phase of infection with gHV68 so it is also possible that self or viral RNA released from these dead cells provides a ligand for TLR7. Therefore, gHV68 infection might induce T-bet in B cells via this most efficient pathway.

Overall our data indicate that, during viral infection, B cells receive simultaneous stimulation through BCR, TLR7, and IFNγR. This stimulation leads to up-regulation of T-bet expression and conversion of the B cells into $CD11c^+$ B cells, the major precursors for antiviral IgG2a antibody production (Fig. S7), the isotype that best contributes to effective viral clearance and ADCC (1, 25, 29).

Discussion

In this report, we have characterized a unique B-cell activation pathway that leads to antiviral IgG2a secretion during viral infection. We demonstrate that the pathway requires BCR, TLR7, and IFNγR signaling that synergize in the B cells to induce T-bet expression. In turn, T-bet drives the differentiation of these cells toward IgG2a production.

The results reported here raise the question why this type of B-cell activation has evolved in the first place. Our data indicate that the process appears to be crucial for the generation of the precursors for the cells that secrete antiviral IgG2a in response to virus infections. How do virus infections lead to activation of the BCR/IFNγ/TLR7 pathway? Our model suggests that, in the animal during viral infection, viral proteins bind to the BCRs of follicular B cells specific for these proteins. Virus infection itself will lead to production and release of viral RNA, which may be taken up nonspecifically by nearby B cells and also, if the viral RNA is associated with viral proteins, by virus-specific B cells. At the same time, viral infection causes host cell death, which leads to release of self RNA, which can engage TLR7. Viral infections also induce plentiful production of IFNγ by different cell types (30). Thus, all three required stimuli can be delivered to viral-specific B cells during infection, and the cells will consequently up-regulate T-bet and differentiate into $CD11^+/CD11b^+$ B cells. Simultaneously, T-bet expression will induce preferential class switching of the B cell toward IgG2a production (Fig. S7). It may be that antigen-specific T cells are not the only source of IFNγ because we found, as described here, that stimulation of T cells by TLR7 induces IFNγ production even in the absence of engagement of T-cell receptors on the T cells.

This idea leads to the next question: why are $CD11b^+/CD11c^+$ $IgG2a^+$ B cells needed during antiviral responses? Are they particularly important for defense against viruses? It has been shown by Markine-Goriaynoff and Coutelier that, among IgG1, IgG2a, IgG2b, and IgG3 switch variants of the same monoclonal antibody specific for VP3 envelope glycoprotein of lactate dehydrogenase-elevating virus (LDV), the IgG2a variant showed the best protection of host (1). Kipps et al. demonstrated that antibodies of different isotypes but the same binding affinities have different abilities to direct antibody-dependent cellular cytotoxicity (ADCC), with IgG2a being the most efficient (25). Nimmerjahn and Ravetch (29) and Nimmerjahn et al. (31) provided the mechanism for the superior efficiency of IgG2a in ADCC, elegantly demonstrating that this antibody isotype has the highest affinity for activating Fc receptors (29, 31). Thus, we can conclude that, evolutionarily, this type of B-cell activation has been selected for the induction of rapid and efficient antiviral humoral immune responses.

The significance of CD11b and CD11c expression by the virally induced B cells is less clear. Perhaps expression of this combination of integrins locates the B cells in particular areas because of their relative adherence to, for example, fibrinogen (32–35). Alternatively, these integrins may affect the function of the B cells bearing them by signaling or binding to complement components.

The phenotype of the T-bet–expressing B cells that are induced by TLR7 engagement and virus infections is very similar to that of a B-cell population, composed of age-associated B cells (ABCs), that we and others have previously described (19–21, 36). Both the virus-specific B cells and ABCs are $T\text{-}bet^+$ $CD11b^+$ $CD11c^+$, and both are most efficiently induced by TLR7 engagement (Fig. S8). However, many of the $T\text{-}bet^+$ B cells induced by virus infection produce antiviral antibodies whereas ABCs express autoantibodies, particularly if they are derived from autoimmune mice. It is likely that the two populations are induced by similar pathways. For virus-specific cells, the pathway probably occurs via BCR engagement, internalization of virus antigens and associated RNA, consequent TLR7 activation, and concurrent IFNγ secretion by T cells. For ABCs, induction might involve BCR binding of self ribonuclear proteins or self RNA and internalization of the BCRs in complex with the RNA-containing self antigen, which would then be delivered to endosomal compartments and there be sensed by intracellular TLR7. The potentially autoreactive B cells would thus, like the virus-specific B cells, receive signals through their BCRs and TLR7. If IFNγ were also present, these B cells would then induce T-bet, express CD11b and CD11c, and switch to production of damaging IgG2a autoantibody. Thus, a pathway that has been evolutionarily selected to provide protection against virus infections might inadvertently also lead, sometimes, to autoreactive responses. In the present report we also suggest that other splenic cells produce IFNγ in response to TLR7 activation. The involvement of IFNγ in this pathway is very intriguing because several reports indicated reduction in autoimmunity upon deletion of IFNγ or IFNγR (37–40).

A critical role for this type of dual B-cell stimulation through BCR and TLRs for the development of autoimmunity and breaking tolerance has been previously reported (15, 41–43). A report by Leadbetter et al. proposed for the first time that, upon ligation, BCR not only provides strong BCR signaling but also internalizes the antigen, bringing it to intracellular compartments where it can be recognized by TLR9 (41). Subsequent work extended this concept to mice expressing a BCR, 564Igi, specific for ssDNA, ssRNA, and nucleosomes (15). In these mice, autoantibody production was largely dependent on TLR7 signaling, and the autoreactive B cells, although anergic, were very similar in phenotype to ABCs, with low levels of CD21, intermediate CD5 expression, and elevated levels of T-bet expression (15). However, in such publications, the downstream events following this dual stimulation were not extensively studied.

As far as T-bet and autoimmunity is concerned, various reports have described various effects of T-bet, depending on the autoimmune syndrome in question (44, 45). Mice lacking T-bet suffer less in some models of autoimmunity, including collagen antibody-induced arthritis (CAIA) (46), myelin oligodendrocyte glycoprotein (MOG)-induced experimental autoimmune encephalomyelitis (EAE) (47) and lupus-like disease in MRL/lpr animals (6). In contrast, worsening of proteoglycal or *Staphylococcus aureus*-induced arthritis has been reported in $T\text{-}bet^{-/-}$

mice (48). All these studies are complicated by the fact that the diseases have been studied in mice in which all cell types lack T-bet expression. Because T-bet is involved in the functions of different cell types [T cells, NK cells, dendritic cells (DCs), and B cells], it is difficult to know which cell types are responsible for the improvement or worsening of the disease in the absence of T-bet. For this reason, we believe that cell type-specific deletion of T-bet will shed more light on its role in autoimmunity.

In conclusion, our present report demonstrates a unique B-cell activation pathway that is crucial for the effective antiviral humoral immune response and may also contribute to autoimmunity.

Materials and Methods

Mice. B6. SJL, C57BL/6, and μMT mice were purchased from The Jackson Laboratory. T-bet$^{-/-}$, IFNγR1$^{-/-}$ and MD4-tg mice were originally purchased from The Jackson Laboratory and bred at the National Jewish animal facility. Female mice were used for all experiments. All animals were handled in strict accordance with good animal practice as defined by the relevant national and/or local animal welfare bodies, and all animal work was approved by the National Jewish Health Animal Care and Use Committee.

Infections. C57BL/6 mice were inoculated intraperitoneally with 10^6 pfu of gHV68, 10^6 pfu of mouse cytomegalovirus (MCMV), 2×10^5 pfu of LCMV Armstrong strain, or 5×10^6 pfu of vaccinia virus. gHV68 virus was generated as was previously described (49). MCMV was obtained from Dr. C. Kulesza (Princeton University, Princeton) and was generated as previously described (50). LCMV was obtained from Dr. D. Homann (University of Colorado, Denver) and was generated as previously described (51). Vaccinia virus was obtained from Dr. R. Kedl (National Jewish Health, Denver) and was generated as previously described (52). All manipulations were performed in accordance with the National Jewish Institutional Animal Care and Use Committee.

Generation of Bone Marrow Chimeras. Bone marrow cells were isolated from C57BL/6, T-bet$^{-/-}$, and μMT mice. C57BL/6 or T-bet$^{-/-}$ bone marrow cells were mixed with μMT cells at a 1:4 ratio, and 5×10^6 cells were injected i.v. into lethally irradiated (900 rad) C57BL/6 mice. Mice were rested for 5–6 wk before other manipulations were performed.

Production of Retroviral Particles. The pMSCV-T-bet-IRES-GFP and pMSCV-IRES-GFP plasmids were the kind gift of Dr. L. Glimcher (Harvard University, Cambridge, MA) and were provided by Dr. L. Gapin (National Jewish Health). Retroviral plasmids were cotransfected into Phoenix cells with the pCL-Eco accessory plasmid using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Viral supernatants were harvested 24 and 48 h after transfection.

Retroviral Transduction of Immature B Cells. Immature B cells were generated and transduced as previously described (24, 53). In brief, bone marrow cells were cultured in complete media in the presence of IL-7 for 3 d. On day 3, cells were resuspended in complete media mixed with retroviral supernatant, polybrene (1.6 μg/mL) and IL-7 and spinfected at $1{,}124 \times g$ for 2 h at 37 °C. Spinfection was repeated 24 h later. Spinfected immature B cells were analyzed for transduction efficiency and injected i.v. into sublethally (500 rad) irradiated congenic mice (5-6 $\times$ 10^6 cells per mouse) 24 h after last spinfection.

In Vitro Cultures. Whole splenocytes were cultured at 5×10^6 cells per mL in 96-well plates for 48 h at various conditions as indicated. B cells were purified as a CD43 negative fraction using anti-CD43 beads (Miltenyi) and cultured at 5×10^6 cells per mL for 48 h or 7 d as indicated. TLR7 agonist R848 (InvivoGen) was used at 1 μg/mL, TLR2 agonist Pam3Cys at 250 ng/mL, TLR3 agonist Poly I:C at 50 μg/mL, TLR4 agonist LPS at 20 μg/mL, and TLR9 agonist ODN1668 (InvivoGen) at 1 μg/mL; "anti-BCR" (Fab′)2 anti-IgM (The Jackson Laboratory) was used at 5 μg/mL, anti-IFNγ (clone R4-6A2, eBioscience) at 40 μg/mL, and IFNγ (Amgen) at 100 U/mL.

MD4 B-Cell Transfer. Splenic B cells from MD4 transgenic (Tg) mice were obtained and purified as CD43 negative fractions using CD43 microbeads (Miltenyi). The 5×10^6 MD4 B cells were injected i.v. into B6.SJL mice. Mice were immunized i.p. 24 h after cell transfer with 50 μg of HEL (Sigma), 50 μg of R848 (Invivogen), or a combination of both. Splenic cells were analyzed 24 h after immunization.

Flow Cytometry. Cells were stained with antibodies to mouse CD4 (clone GK1.5), CD8 (clone 53–6.7), B220 (clone RA3-6B2), CD11b (clone M1/70), CD11c (clone N418), CD19 (clone 1D3), CD21 (clone 7G6), CD45.1 (A20), and CD45.2 (clone 104) purchased from eBioscience, BD Pharmingen, or Biolegend. For intracellular T-bet staining, cells were surface stained, washed in PBS, and stained using Fixable Viability Dye (eBioscience), fixed and permeabilized with FoxP3 staining buffer set (eBioscience), and stained with anti-human/mouse T-bet antibodies (clone 4B10) (eBioscience). Cells were analyzed by flow cytometry on a CyAn (Beckman-Coulter) instrument, and data were analyzed using FlowJo software (Treestar).

ELISA. Plates were coated with goat anti-mouse total IgG antibodies (The Jackson Laboratory) or with viral antigen [generated as previously described (54)] to detect total or gHV68-specific antibodies, respectively. Supernatant or serum IgG was detected with AP-conjugated goat anti-mouse IgG1, IgG2b, IgG2c, IgG3, or total IgG (The Jackson Laboratory) as indicated. For IFNγ detection, BD OptEIA mouse IFN-γ ELISA Set (BD) was used.

Real-Time PCR. DNA was isolated from whole splenocytes form γHV68-infected mice at indicated time point using QIAGEN DNeasy Blood and Tissue Kit. Detection of 70-bp region of the γHV68 gB gene was used to measure viral load by real-time PCR using forward 5′-GGCCCAAATTCAATTTGCCT-3′ and reverse 5′-CCCTGGACAACTCCTCAAGC-3′ primers and the SYBR Green PCR Master Mix. The analysis was performed on a 7300 Fast Real-Time PCR System (Applied Biosystems). Standard curves were generated using plasmid containing the γHV68 gB gene (55). Cycle threshold values were converted to copy numbers of the gB gene. Copy numbers were standardized to the amount of input DNA and were expressed as copies of viral genome per 100 ng of genomic DNA. Each sample was measured in triplicate.

Statistics. Data were analyzed with Prism 5 (GraphPad Software) using using Student *t* test. Graphs show the mean ± SEM (*$P < 0.05$, **$P < 0.001$, ***$P < 0.0001$).

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