

# 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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Contributed by Philippa Marrack, July 5, 2013 (sent for review May 22, 2013)

**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 IFN $\gamma$  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<sup>+</sup> B cells during antiviral responses and during autoimmunity suggests a possible link between these two processes.**

interferon gamma | virus

**D**uring 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 (IFN $\gamma$ ) 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 (IFN $\gamma$ R) 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, IFN $\gamma$ R, 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 IFN $\gamma$ Rs and TLRs on the B cells. The IFN $\gamma$  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 IFN $\gamma$ R 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 IFN $\gamma$ R 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, IFN $\gamma$ , 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.**

Author contributions: K.R., A.V.R., L.F.v.D., J.W.K., and P.M. designed research; K.R. and A.V.R. performed research; K.R. and L.F.v.D. contributed new reagents/analytic tools; K.R., A.V.R., and P.M. analyzed data; and K.R., A.V.R., L.F.v.D., and P.M. wrote the paper.

The authors declare no conflict of interest.

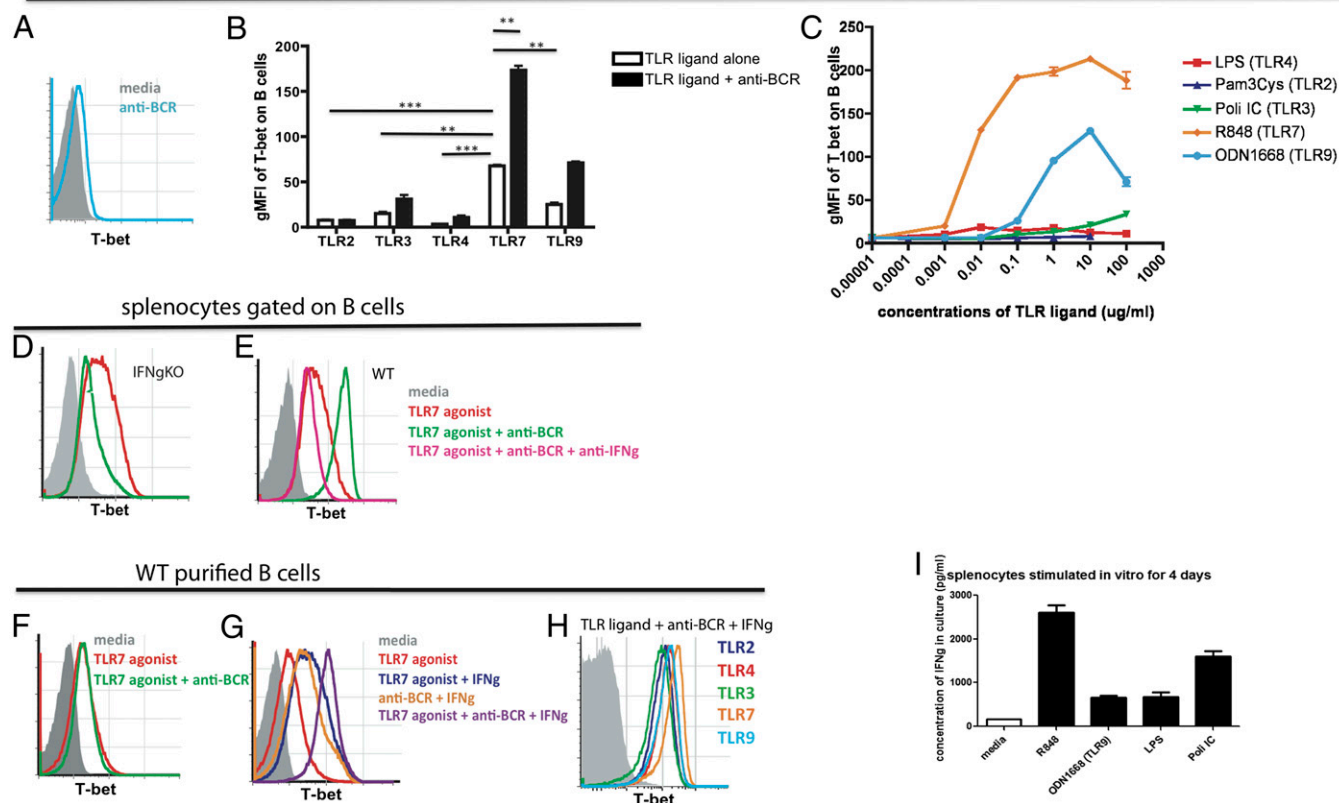
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This article contains supporting information online at [www.pnas.org/lookup/suppl/doi:10.1073/pnas.1312348110/-DCSupplemental](http://www.pnas.org/lookup/suppl/doi:10.1073/pnas.1312348110/-DCSupplemental).

WT splenocytes incubated at indicated conditions for 48h. Shown is T-bet expression on B cells



**Fig. 1.** TLR7, BCR, and IFN $\gamma$  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) IFN $\gamma$ R1 $^{-/-}$  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 IFN $\gamma$ . In all relevant sections of the figure, bars represent the means  $\pm$  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. 1A) 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. 1B). 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. 1B and C). Because of this feature, we decided to focus our study on the effects of TLR7 agonists.

IFN $\gamma$  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 IFN $\gamma$ R1 $^{-/-}$  mice were incubated with TLR7 agonist and/or anti-BCR, and B-cell expression of T-bet was analyzed. The absence of IFN $\gamma$ R 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. 1D and E, green histograms). Moreover, addition of blocking anti-IFN $\gamma$  antibodies to cultures of WT splenocytes incubated with anti-BCR and TLR7 agonist inhibited T-bet induction (Fig. 1E). Thus, IFN $\gamma$ R is a third component of the signaling proteins needed for high T-bet induction in B cells.

To confirm that IFN $\gamma$  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 IFN $\gamma$ . IFN $\gamma$  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. 1F), 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. 1G), and, moreover, in the presence of exogenously added IFN $\gamma$ , the TLR7 agonist was not noticeably superior to agonists for other TLRs (Fig. 1H). 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, IFN $\gamma$ R, 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 IFN $\gamma$ ? The most straightforward explanation is that, of all of the TLR agonists, the TLR7 agonist induced the greatest amount of IFN $\gamma$  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 IFN $\gamma$  by



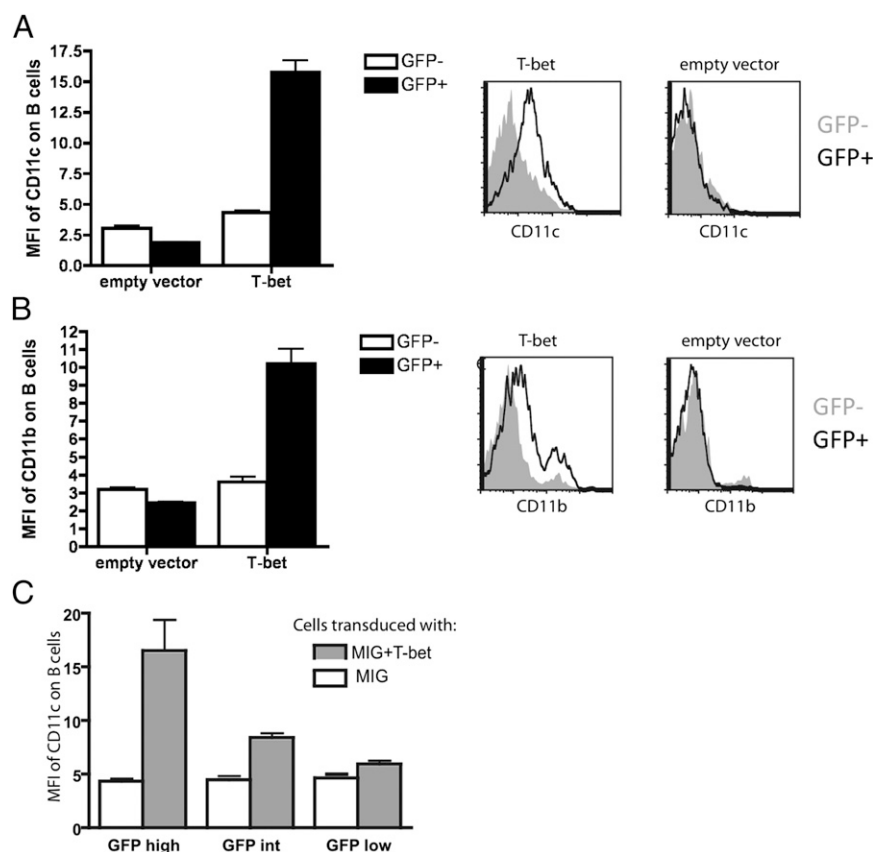
ABCs, which we and others have recently reported (19, 21), a result we consider in *Discussion*.

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<sup>+</sup> 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 IFN $\gamma$ , synergize to induce high levels of T-bet expression in B cells leading to their differentiation into CD11b/c<sup>+</sup> B cells.

**T-bet Overexpression in B Cells Is Sufficient for Their Differentiation into CD11b<sup>+</sup>/CD11c<sup>+</sup> 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<sup>-/-</sup> 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

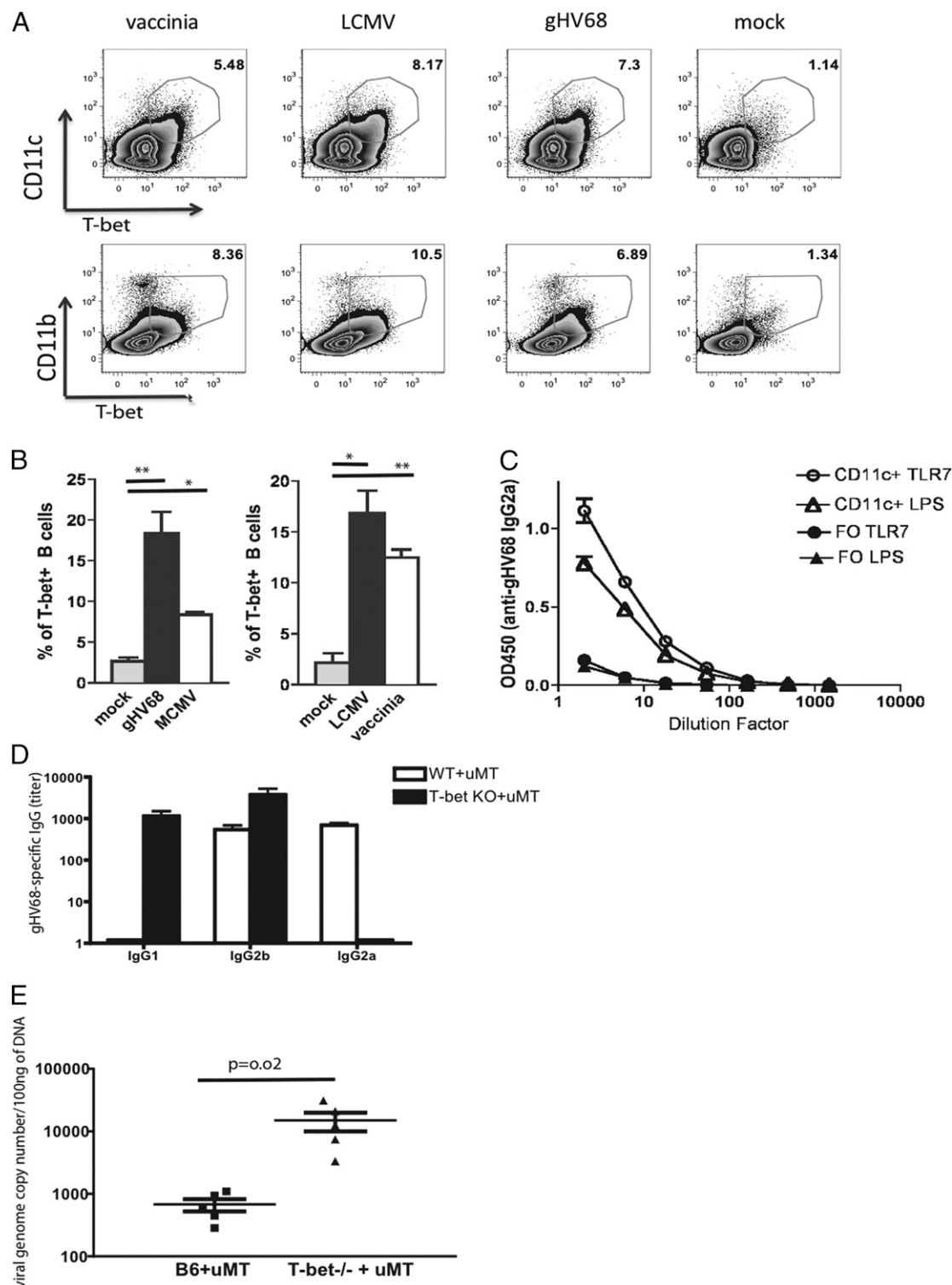
of transduction (monitored by GFP expression) at 15–30% (data not shown). The cells were transferred into sublethally irradiated congenic hosts. Transduced cells were not purified by sorting before transfer, thus allowing the internal control of GFP-negative (nontransduced) cells in the same host for 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<sup>+</sup> B cells were significantly higher than those of the GFP<sup>neg</sup> B cells (Fig. 3*A* and *B*). The level of CD11c expression correlated directly with the level of GFP in GFP<sup>+</sup> 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, TLR7, or IFN $\gamma$ Rs) 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<sup>+</sup>/CD11b<sup>+</sup> B cells. Immature B cells from T-bet<sup>-/-</sup> 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<sup>+</sup>/CD8<sup>+</sup>/CD19<sup>+</sup>/CD45.1<sup>+</sup>. Bars represent means  $\pm$  SEM of B cells from  $n = 5$  mice per group. (*C*) Donor GFP<sup>+</sup> 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  $\pm$  SEM of cells from  $n = 5$  mice per group. Data are representative of two independent experiments.







**Fig. 5.** T-bet<sup>+</sup>CD11c<sup>+</sup>/CD11b<sup>+</sup> 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<sup>+</sup>CD11c<sup>+</sup>/CD11b<sup>+</sup> B cells in spleen was analyzed by FACS. FACS plots present the data for B cells gated as CD19<sup>+</sup>/B220<sup>+</sup>/CD4<sup>+</sup>/CD8<sup>-</sup>. (B) The percentages of splenic T-bet positive B cells in infected mice is shown. Bars represent the means  $\pm$  SEM for  $n = 5$  mice per group. (C) CD11c<sup>+</sup> 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  $\mu$ MT or T-bet<sup>-/-</sup> and  $\mu$ MT 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  $\pm$  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 antiviral 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. 5D). 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. 5E).

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 IFN $\gamma$ R. This stimulation leads to up-regulation of T-bet expression and conversion of the B cells into CD11c<sup>+</sup> 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 IFN $\gamma$ R 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/IFN $\gamma$ /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 IFN $\gamma$  by different cell types (30). Thus, all three required stimuli can be delivered to virus-specific B cells during infection, and the cells will consequently up-regulate T-bet and differentiate into CD11<sup>+</sup>/CD11b<sup>+</sup> 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 IFN $\gamma$  because we found, as described here, that stimulation of T cells by TLR7 induces IFN $\gamma$  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<sup>+</sup>/CD11c<sup>+</sup> IgG2a<sup>+</sup> 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-bet<sup>+</sup> CD11b<sup>+</sup> CD11c<sup>+</sup>, and both are most efficiently induced by TLR7 engagement (Fig. S8). However, many of the T-bet<sup>+</sup> 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 IFN $\gamma$  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 IFN $\gamma$  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 IFN $\gamma$  in response to TLR7 activation. The involvement of IFN $\gamma$  in this pathway is very intriguing because several reports indicated reduction in autoimmunity upon deletion of IFN $\gamma$  or IFN $\gamma$ R (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 proteoglycan or *Staphylococcus aureus*-induced arthritis has been reported in T-bet<sup>−/−</sup>

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  $\mu$ MT mice were purchased from The Jackson Laboratory. T-bet<sup>-/-</sup>, IFN $\gamma$ R1<sup>-/-</sup> 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<sup>6</sup> pfu of gHV68, 10<sup>6</sup> pfu of mouse cytomegalovirus (MCMV), 2  $\times$  10<sup>5</sup> pfu of LCMV Armstrong strain, or 5  $\times$  10<sup>6</sup> 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<sup>-/-</sup>, and  $\mu$ MT mice. C57BL/6 or T-bet<sup>-/-</sup> bone marrow cells were mixed with  $\mu$ MT cells at a 1:4 ratio, and 5  $\times$  10<sup>6</sup> 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  $\mu$ g/mL) and IL-7 and spininfected at 1,124  $\times$  g for 2 h at 37 °C. Spinfection was repeated 24 h later. Spininfected immature B cells were analyzed for transduction efficiency and injected i.v. into sublethally (500 rad) irradiated congenic mice (5–6  $\times$  10<sup>6</sup> cells per mouse) 24 h after last spinfection.

**In Vitro Cultures.** Whole splenocytes were cultured at 5  $\times$  10<sup>6</sup> 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  $\times$  10<sup>6</sup> cells per mL for 48 h or 7 d as indicated. TLR7 agonist R848 (InvivoGen) was used at 1  $\mu$ g/mL, TLR2 agonist Pam3Cys at 250 ng/mL, TLR3 agonist Poly I:C at 50  $\mu$ g/mL, TLR4 agonist LPS at 20  $\mu$ g/mL, and TLR9 agonist ODN1668 (InvivoGen) at 1  $\mu$ g/mL; "anti-BCR" (Fab')<sub>2</sub> anti-IgM (The Jackson Laboratory) was used at 5  $\mu$ g/mL, anti-IFN $\gamma$  (clone R4-6A2, eBioscience) at 40  $\mu$ g/mL, and IFN $\gamma$  (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  $\times$  10<sup>6</sup> MD4 B cells were injected i.v. into B6.SJL mice. Mice were immunized i.p. 24 h after cell transfer with 50  $\mu$ g of HEL (Sigma), 50  $\mu$ g 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 Biologend. 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 IFN $\gamma$  detection, BD OptEIA mouse IFN- $\gamma$  ELISA Set (BD) was used.

**Real-Time PCR.** DNA was isolated from whole splenocytes from  $\gamma$ HV68-infected mice at indicated time point using QIAGEN DNeasy Blood and Tissue Kit. Detection of 70-bp region of the  $\gamma$ HV68 gB gene was used to measure viral load by real-time PCR using forward 5'-GGCCCAATT-CAATTTGCCT-3' and reverse 5'-CCCTGGACAACCTCTCAAGC-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  $\gamma$ HV68 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 Student t test. Graphs show the mean  $\pm$  SEM (\*P < 0.05, \*\*P < 0.001, \*\*\*P < 0.0001).

**ACKNOWLEDGMENTS.** We thank Drs. L. Glimcher and L. Gapin for providing the retroviral control and T-bet expressing constructs, Drs. J. Cambier and A. Getahun for MD4-Tg mice, Dr. R. Kedl for STAT1<sup>-/-</sup> mice and vaccinia virus, Dr. L. Lenz for IFN $\gamma$ R1<sup>-/-</sup> mice, Dr. D. Homann for LCMV, and Dr. C. Kulesza for MCMV. We also thank Drs. M. Munks and M. Phillips for critical comments on the manuscript. The work was supported in part by US Public Health Service Grants AI-18785 and AI-22295. This work was supported in part by a T32 (AI074491) training grant award (to K.R.).

- Markine-Goriaynoff D, Coutelier JP (2002) Increased efficacy of the immunoglobulin G2a subclass in antibody-mediated protection against lactate dehydrogenase-elevating virus-induced polioencephalomyelitis revealed with switch mutants. *J Virol* 76(1):432–435.
- Coutelier JP, van der Logt JT, Heessen FW, Vink A, van Snick J (1988) Virally induced modulation of murine IgG antibody subclasses. *J Exp Med* 168(6):2373–2378.
- Schmitz N, et al. (2012) Universal vaccine against influenza virus: Linking TLR signaling to anti-viral protection. *Eur J Immunol* 42(4):863–869.
- Snapper CM, Paul WE (1987) Interferon-gamma and B cell stimulatory factor-1 reciprocally regulate Ig isotype production. *Science* 236(4804):944–947.
- Gerth AJ, Lin L, Peng SL (2003) T-bet regulates T-independent IgG2a class switching. *Int Immunol* 15(8):937–944.
- Peng SL, Szabo SJ, Glimcher LH (2002) T-bet regulates IgG class switching and pathogenic autoantibody production. *Proc Natl Acad Sci USA* 99(8):5545–5550.

- Lugo-Villarino G, Ito S, Klinman DM, Glimcher LH (2005) The adjuvant activity of CpG DNA requires T-bet expression in dendritic cells. *Proc Natl Acad Sci USA* 102(37):13248–13253.
- Robbins SH, Tessmer MS, Van Kaer L, Brossay L (2005) Direct effects of T-bet and MHC class I expression, but not STAT1, on peripheral NK cell maturation. *Eur J Immunol* 35(3):757–765.
- Townsend MJ, et al. (2004) T-bet regulates the terminal maturation and homeostasis of NK and Valpha14i NKT cells. *Immunity* 20(4):477–494.
- Cruz-Guilloty F, et al. (2009) Runx3 and T-box proteins cooperate to establish the transcriptional program of effector CTLs. *J Exp Med* 206(1):51–59.
- Pearce EL, et al. (2003) Control of effector CD8+ T cell function by the transcription factor Eomesodermin. *Science* 302(5647):1041–1043.
- Liu N, Ohnishi N, Ni L, Akira S, Bacon KB (2003) CpG directly induces T-bet expression and inhibits IgG1 and IgE switching in B cells. *Nat Immunol* 4(7):687–693.



13. Peng SL, Li J, Lin L, Gerth A (2003) The role of T-bet in B cells. *Nat Immunol* 4(11), 1041, author reply 1041 (lett).
14. Wang NS, et al. (2012) Divergent transcriptional programming of class-specific B cell memory by T-bet and ROR $\alpha$ . *Nat Immunol* 13(6):604–611.
15. Berland R, et al. (2006) Toll-like receptor 7-dependent loss of B cell tolerance in pathogenic autoantibody knockin mice. *Immunity* 25(3):429–440.
16. Durali D, et al. (2003) In human B cells, IL-12 triggers a cascade of molecular events similar to Th1 commitment. *Blood* 102(12):4084–4089.
17. Xu W, Zhang JJ (2005) Stat1-dependent synergistic activation of T-bet for IgG2a production during early stage of B cell activation. *J Immunol* 175(11):7419–7424.
18. Yoshimoto T, et al. (2004) Induction of IgG2a class switching in B cells by IL-27. *J Immunol* 173(4):2479–2485.
19. Rubtsov AV, et al. (2011) Toll-like receptor 7 (TLR7)-driven accumulation of a novel CD11c<sup>+</sup> B-cell population is important for the development of autoimmunity. *Blood* 118(5):1305–1315.
20. Rubtsova K, Marrack P, Rubtsov AV (2012) Age-associated B cells: Are they the key to understanding why autoimmune diseases are more prevalent in women? *Expert Rev Clin Immunol* 8(1):5–7.
21. Hao Y, O'Neill P, Naradikian MS, Scholz JL, Cancro MP (2011) A B-cell subset uniquely responsive to innate stimuli accumulates in aged mice. *Blood* 118(5):1294–1304.
22. Lighvani AA, et al. (2001) T-bet is rapidly induced by interferon-gamma in lymphoid and myeloid cells. *Proc Natl Acad Sci USA* 98(26):15137–15142.
23. Afkarian M, et al. (2002) T-bet is a STAT1-induced regulator of IL-12R expression in naive CD4<sup>+</sup> T cells. *Nat Immunol* 3(6):549–557.
24. Melamed D, Kench JA, Grabstein K, Rolink A, Nemazee D (1997) A functional B cell receptor transgene allows efficient IL-7-independent maturation of B cell precursors. *J Immunol* 159(3):1233–1239.
25. Kipps TJ, Parham P, Punt J, Herzenberg LA (1985) Importance of immunoglobulin isotype in human antibody-dependent, cell-mediated cytotoxicity directed by murine monoclonal antibodies. *J Exp Med* 161(1):1–17.
26. Sangster MY, et al. (2000) Analysis of the virus-specific and nonspecific B cell response to a persistent B-lymphotropic gammaherpesvirus. *J Immunol* 164(4):1820–1828.
27. Tomayko MM, Steinle NC, Anderson SM, Shlomchik MJ (2010) Cutting edge: Hierarchy of maturity of murine memory B cell subsets. *J Immunol* 185(12):7146–7150.
28. Anderson SM, Hannum LG, Shlomchik MJ (2006) Memory B cell survival and function in the absence of secreted antibody and immune complexes on follicular dendritic cells. *J Immunol* 176(8):4515–4519.
29. Nimmerjahn F, Ravetch JV (2005) Divergent immunoglobulin g subclass activity through selective Fc receptor binding. *Science* 310(5753):1510–1512.
30. Sarawar SR, et al. (1996) Cytokine production in the immune response to murine gammaherpesvirus 68. *J Virol* 70(5):3264–3268.
31. Nimmerjahn F, Bruhns P, Horiuchi K, Ravetch JV (2005) FcgammaRIV: A novel FcR with distinct IgG subclass specificity. *Immunity* 23(1):41–51.
32. Georgakopoulos T, Moss ST, Kanagasundaram V (2008) Integrin CD11c contributes to monocyte adhesion with CD11b in a differential manner and requires Src family kinase activity. *Mol Immunol* 45(13):3671–3681.
33. Wolfenson H, Lavelin I, Geiger B (2013) Dynamic regulation of the structure and functions of integrin adhesions. *Dev Cell* 24(5):447–458.
34. Han C, et al. (2010) Integrin CD11b negatively regulates TLR-triggered inflammatory responses by activating Syk and promoting degradation of MyD88 and TRIF via Cbl-b. *Nat Immunol* 11(8):734–742.
35. Myones BL, Dalzell JG, Hogg N, Ross GD (1988) Neutrophil and monocyte cell surface p150,95 has iC3b-receptor (CR4) activity resembling CR3. *J Clin Invest* 82(2):640–651.
36. Rubtsov AV, Rubtsova K, Kappler JW, Marrack P (2013) TLR7 drives accumulation of ABCs and autoantibody production in autoimmune-prone mice. *Immunol Res* 55(1–3): 210–216.
37. Balomenos D, Rumold R, Theofilopoulos AN (1998) Interferon-gamma is required for lupus-like disease and lymphoaccumulation in MRL-lpr mice. *J Clin Invest* 101(2): 364–371.
38. Haas C, Ryffel B, Le Hir M (1997) IFN-gamma is essential for the development of autoimmune glomerulonephritis in MRL/lpr mice. *J Immunol* 158(11):5484–5491.
39. Haas C, Ryffel B, Le Hir M (1998) IFN-gamma receptor deletion prevents autoantibody production and glomerulonephritis in lupus-prone (NZB x NZW)F1 mice. *J Immunol* 160(8):3713–3718.
40. Schwarting A, Wada T, Kinoshita K, Tesch G, Kelley VR (1998) IFN-gamma receptor signaling is essential for the initiation, acceleration, and destruction of autoimmune kidney disease in MRL-Fas(lpr) mice. *J Immunol* 161(1):494–503.
41. Leadbetter EA, et al. (2002) Chromatin-IgG complexes activate B cells by dual engagement of IgM and Toll-like receptors. *Nature* 416(6881):603–607.
42. Green NM, Marshak-Rothstein A (2011) Toll-like receptor driven B cell activation in the induction of systemic autoimmunity. *Semin Immunol* 23(2):106–112.
43. Viglianti GA, et al. (2003) Activation of autoreactive B cells by CpG dsDNA. *Immunity* 19(6):837–847.
44. Peng SL (2006) The T-box transcription factor T-bet in immunity and autoimmunity. *Cell Mol Immunol* 3(2):87–95.
45. Lazarevic V, Glimcher LH (2011) T-bet in disease. *Nat Immunol* 12(7):597–606.
46. Wang J, et al. (2006) Transcription factor T-bet regulates inflammatory arthritis through its function in dendritic cells. *J Clin Invest* 116(2):414–421.
47. Bettelli E, et al. (2004) Loss of T-bet, but not STAT1, prevents the development of experimental autoimmune encephalomyelitis. *J Exp Med* 200(1):79–87.
48. Hultgren OH, Verdrengh M, Tarkowski A (2004) T-box transcription-factor-deficient mice display increased joint pathology and failure of infection control during staphylococcal arthritis. *Microbes Infect* 6(6):529–535.
49. Suárez AL, van Dyk LF (2008) Endothelial cells support persistent gammaherpesvirus 68 infection. *PLoS Pathog* 4(9):e1000152.
50. Kulesza CA, Shenk T (2006) Murine cytomegalovirus encodes a stable intron that facilitates persistent replication in the mouse. *Proc Natl Acad Sci USA* 103(48): 18302–18307.
51. Korn Johnson D, Homann D (2012) Accelerated and improved quantification of lymphocytic choriomeningitis virus (LCMV) titers by flow cytometry. *PLoS ONE* 7(5): e37337.
52. Kedl RM, et al. (2000) T cells compete for access to antigen-bearing antigen-presenting cells. *J Exp Med* 192(8):1105–1113.
53. Rowland SL, DePersis CL, Torres RM, Pelanda R (2010) Ras activation of Erk restores impaired tonic BCR signaling and rescues immature B cell differentiation. *J Exp Med* 207(3):607–621.
54. Larson JD, et al. (2012) Murine gammaherpesvirus 68 infection protects lupus-prone mice from the development of autoimmunity. *Proc Natl Acad Sci USA* 109(18): E1092–E1100.
55. Nguyen Y, McGuffie BA, Anderson VE, Weinberg JB (2008) Gammaherpesvirus modulation of mouse adenovirus type 1 pathogenesis. *Virology* 380(2):182–190.