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Stat1-Dependent Synergistic Activation of T-bet for IgG2a Production during Early Stage of B Cell Activation¹

Weifeng Xu and J. Jillian Zhang²

During the adaptive phase of an immune response, naïve B cells receive multiple signals to become activated. Among them are the engagement of the B cell Ag receptor and stimulation by cytokines. Specifically for an anti-microbial response, the recognition of viral or bacterial Ags by the BCR and the stimulation of IFN-γ result in the predominant production of IgG2a. The T-bet protein has been shown to be required for class switching to IgG2a. In this report we further investigated the regulation of T-bet gene expression during the early stage of B cell activation. We show that there is a striking synergistic activation of T-bet in primary B cells when both the BCR and IFN-γ signaling pathways are activated. The synergistic activation of T-bet correlates with a 100% increase in the number of B cells that produce IgG2a. This transcription synergy on T-bet is transient in the first 24 h of B cell activation. Furthermore, we demonstrate that the synergistic activation of T-bet is dependent on Stat1 and that Stat1 is required for the IgG2a germline transcription and the production of IgG2a in response to the simultaneous signaling of BCR and IFN-γ. Finally, we show that Stat1 directly regulates the expression of T-bet by binding to the T-bet promoter. These results reveal the mechanism of regulation of T-bet expression and uncover a novel physiological function of Stat1 for B cell activation. The Journal of Immunology, 2005, 175: 7419–7424.

To establish humoral immunity during an anti-microbial response, naïve B cells in the peripheral organs are activated by multiple signals to become Ag-specific effector cells (1, 2). Among the many signals naïve B cells receive, the engagement of the BCR by foreign Ags and cytokines secreted by T cells is critical for the activation of naïve B cells and their differentiation into Ab-secreting plasma cells (3–5). To achieve a diverse array of effector functions (6), activated B cells undergo class switching to produce various isotypes of Abs (7). The class switching to specific isotypes of Abs is regulated by the induction of germline transcription in the IgH locus (8). For example, during an anti-viral response, IFN-γ induces the Ig2a germline transcription (9, 10) and the class switching to IgG2a for elimination of viral pathogens (11, 12).

The transcription factors that specifically mediate the cytokine-induced germline transcription play critical roles in the regulation of class switch recombination. For the production of IgG2a during an anti-viral response, analyses of T-bet-deficient mice showed that the T-bet protein was required for the Ig2a germline transcription and production of IgG2a induced by IFN-γ (13). The T-bet protein was initially identified as a transcription factor critical for the development of Th1 cells (14, 15). The expression of T-bet is restricted to the Th1 cells during Th cell differentiation, and its expression in Th cells requires Stat1 (14, 16). For the role of T-bet in B cell activation, it was found that constitutive expression of exogenous T-bet from a plasmid resulted in the activation of Ig2a germline transcription and class switching to IgG2a (13). In addition, only T cell-independent switching to IgG2a was found to require T-bet (17). Furthermore, it was demonstrated that treatment of B cells with the bacterial product CpG resulted in a Stat1-independent induction of T-bet and inhibition of IL-4/CD40-induced class switching to IgE and IgG1 (18). It has also been suggested that aberrant expression of T-bet in B cells may lead to lymphoproliferative disorders (19). However it is not clear, under physiological conditions, how endogenous T-bet expression is induced and regulated during B cell activation.

In this study, we investigated the regulation of T-bet in primary B cells in response to IFN-γ and BCR cross-linking during B cell activation. We show that there is a striking synergistic activation of T-bet expression when naïve splenic B cells are stimulated by IFN-γ and BCR cross-linking. This activation of T-bet is transient and decreases after 24 h of stimulation. Furthermore, using Stat1−/− primary B cells, we demonstrate that the synergistic activation of T-bet is dependent on Stat1. In addition, we demonstrate that Stat1 is required for the Ig2a germline transcription and the production of IgG2a in response to the simultaneous signaling of BCR and IFN-γ. Finally, we show that T-bet is a direct Stat1 target gene in B cells. These results reveal the mechanism of regulation of T-bet expression and uncover a novel physiological function of Stat1 for B cell activation.

Materials and Methods

Cell culture, Abs, and reagents

A20, 2PK-3 (American Type Culture Collection), M12.4.1 (provided by F. Alt, Harvard Medical School, Boston, MA), CH27 cells (provided by L. K. Densin, Memorial Sloan-Kettering Cancer Center, New York, NY) and purified primary B cells were cultured in RPMI 1640 with 10% FBS (Gemi- ini Bioproducts). For long-term primary B cell culture, 20 μg/ml LPS was included and fresh medium and ligands were added on the third day. Re- combinant mouse IFN-γ was from Roche. Anti-Stat1C for the chromatin immunoprecipitation (ChIP)¹ assays was from Santa Cruz Biotechnology. Abs for FACS analysis: biotin anti-mouse IgG2a (R19-15), FITC anti-mouse CD45R/B220 (RA3-B2B), and streptavidin-PE were from BD

¹Abbreviations used in this paper: ChIP, chromatin immunoprecipitation; IP, immunoprecipitated.

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Pharmingen. Anti-IgG F(ab')2, and anti-IgM F(ab')2, were from Zymed Laboratories. Cells were treated with mIFN-α at 50 U/ml for A20 cells, 10 U/ml for CH27 and primary B cells, goat anti-mouse IgG F(ab')2 at 5 μg/ml, goat anti-mouse IgM F(ab')2 at 5 μg/ml.

**FACS**

One million cells were collected on day 4 of culture, washed twice with PBS/5% FBS, stained with 1 μl of 1/5 diluted FITC-anti-mouse B220, or 1 μl of biotin-purified anti-mouse IgG2a in 100 μl of PBS/5% FBS for 20 min at 4°C. Cells were washed twice with PBS/5% FBS and stained with 1 μl of streptavidin-PE for another 20 min at 4°C. Cells were then washed and resuspended in PBS/5% FBS followed by FACS analyses (BD Biosciences, FACSCalibur).

**Cell extracts and Western blot analysis**

Whole cell extracts were prepared as previously described (20) and separated by 6% SDS-PAGE. Western blot analyses were done using chemiluminescence (DuPont/NEN).

**ChIP assay**

ChIP experiments were performed as previously described (21). Briefly, 107 cells were resuspended in SDS lysis buffer, cross-linked with formaldehyde, sonicated, and immunoprecipitated (IP) with anti-Stat1. Genomic DNAs were isolated from the IP complex and used as templates for PCR with primers specific for the T-bet promoter. ChIP PCR primer pair sequences for the murine T-bet promoter: 5'-CTGCTCCTGGCCCTTC TCTG and 5'-TGCCAGGCCGTGAGAATG. 32P-labeled PCR products were separated on acrylamide gels, visualized by autoradiography, and further quantitated by a PhosphorImager. One representative result from two to six independent experiments is shown. Quantitation of one experiment was further confirmed by real-time PCR.

**Nuclear extract**

Nuclear extracts were prepared from 5 × 107 CH27 cells untreated or treated with 40 U/ml mIFN-γ for 20 min. After washing with cold PBS, cells were spun down, resuspended in 3 vol of buffer A (20 mM HEPEs (pH 7.9), 10 mM KCl, 0.1 mM Na3VO4, 1 mM EDTA, 0.2% NP40, 10% glycerol, 1 mM PMSF, 1 mM DTT, and protease inhibitors), and placed on ice for 5 min, and the nuclei were collected by centrifugation. The nuclei pellets were then resuspended in 3 vol of buffer B (420 mM NaCl, 20% glycerol, 20 mM HEPEs (pH 7.9), 10 mM KCl, 0.1 mM Na3VO4, 1 mM EDTA, 1 mM PMSF, 1 mM DTT, and protease inhibitors) followed by centrifugation. The supernatant nuclear extracts were immediately stored at –80°C. The amount of protein was determined using the Bio-Rad protein assay (Bio-Rad).

**EMSA**

Double-stranded oligonucleotides corresponding to putative STAT-1 binding sites in the murine T-bet gene mTbet ES1–4: 5'-AGCTTAGT TAGTGGATAAGAA (located at –901) and mutant ES1–4: 5'-AGCT TAGTGGATCGAAAAGA (located at –901) and mutant ES1–4: 5'-AGCT TAGTGGATCGAAAAGA were synthesized and labeled with 32P by Klenow filling. Total of 1 ng of labeled probe was incubated with 5 μg of nuclear extracts at room temperature for 10 min. For the supershift, anti-STAT1 C-terminal Ab or control mouse IgG was preincubated with nuclear extracts for 5 min on ice. Competitive EMSA was done by adding 10-fold molar excess of unlabeled double-stranded oligonucleotides to the mixture. Samples were loaded on a 5% PAGE gel and run at constant voltage of 200 V for ~3 h in cold room.

**Purification of primary B cells**

Primary B cells were purified from 8- to 12-wk-old BALB/c mice, C57BL/6 and Stat1−/− mice, or congenic 129Sv wild-type mice (Taconic) essentially as described previously (22). Briefly, splenocytes were depleted of RBC with ammonium chloride potassium lysis buffer and then incubated with rabbit complement (Cedarlane Laboratories) together with anti-Thyl.2 (eBioscience) and anti-CD4 (Accurate Biochemicals) to deplete T cells and macrophages. Discontinuous Percoll (1.129 g/ml; American Pharmacia Biotech) density centrifugation was performed to recover the primary B cells. The purity of B cells (>95%) was determined by FACS with anti-B220 and anti-CD3. The studies have been reviewed and approved by the Institutional Animal Care and Use Committee at Weill Medical College of Cornell University.

**Real-time PCR**

Real-time PCR experiments were performed as previously described (22). Primer sequences are: GAPDH, 5'-ACGACCCCTTCTAGGACC and 5'-AGACACCCATGACTCCACG; T-bet, 5'-GCCAGGACCGCTTA TAGT and 5'-GACGATCATTGGTCATACATTGT.

**Results**

**Synergistic activation of T-bet in primary B cells in response to simultaneous IFN-γ and BCR signaling**

Analyses of T-bet-deficient mice show that T-bet protein is essential for Th1 cell development and required for production of IgG2a Abs by B cells in MRL mice (13, 15). Because the production of IgG2a requires IFN-γ (11, 12), we first investigated whether IFN-γ can induce T-bet expression in primary B cells from normal mice. Splenic primary B cells were purified from 8- to 10-wk-old BALB/c mice and treated with IFN-γ, anti-IgM, or IFN-γ plus anti-IgM for lengths of time indicated. RNA samples were analyzed by real-time RT-PCR for the expression of T-bet and an internal control GAPDH. Results shown are T-bet expression normalized with that of GAPDH. One representative result from three independent experiments is shown. B, Splenic primary B cells were purified from 8- to 10-wk-old C57BL/6 mice and treated with IFN-γ, anti-IgM, or IFN-γ plus anti-IgM for lengths of time indicated. RNA samples were analyzed by real-time RT-PCR for the expression of T-bet and an internal control GAPDH. Results shown are T-bet expression normalized with that of GAPDH. C, Splenic primary B cells were purified from 8- to 10-wk-old C57BL/6 mice and treated with IFN-γ, anti-IgM, or IFN-γ plus anti-IgM for lengths of time indicated. Whole cell lysates were analyzed by Western blotting.
by real-time PCR for the expression of T-bet and an internal control GAPDH. T-bet expression was very low in resting naive B cells (Fig. 1). After stimulation with IFN-γ for 2 h, T-bet expression was induced up to 12-fold (Fig. 1). Because engagement of the BCR by viral Ags is one of the critical signals during B cell activation, we also treated the splenic B cells with anti-IgM (in the F(ab')2 form to avoid FcR activation). Treatment with anti-IgM induced ~5-fold induction in T-bet expression (Fig. 1). Because naive B cells receive multiple signals simultaneously to achieve activation, we also treated the splenic primary B cells with IFN-γ plus anti-IgM. Strikingly, there was a much more rapid induction of T-bet and the level of T-bet mRNA reached to more than 60-fold after 2 h of treatment with IFN-γ plus anti-IgM (Fig. 1A). To rule out the possibility that this synergy is specific to the BALB/c strain, we purified splenic primary B cells from C57BL/6 mice and treated the cells similarly as above. Real-time RT-PCR analyses showed that T-bet was also synergistically activated in primary B cells from C57BL/6 (Fig. 1B). Furthermore, Western blotting analyses of T-bet protein expression showed that when primary B cells were stimulated with both IFN-γ and anti-IgM, T-bet protein level was significantly enhanced (Fig. 1C, lanes 8–10). These results demonstrate that T-bet expression is synergistically activated in primary B cells stimulated with the cytokine IFN-γ and cross-linking of the BCR.

**BCR signaling enhances IFN-γ-induced IgG2a production in primary B cells**

To test whether the synergistic activation of T-bet in primary B cells correlates with enhanced IgG2a production, we purified splenic B cells from BALB/c or C57BL/6 mice and cultured the cells in the presence of LPS and further stimulated them with IFN-γ alone, anti-IgM alone, or IFN-γ plus anti-IgM for 4 days followed by FACS analyses of cell surface IgG2a expression. The addition of LPS in the culture medium was necessary for long-term cell culture of primary B cells but had little effect in IgG2a expression (Fig. 2). Primary B cells cultured with LPS plus anti-IgM did not express significant levels of IgG2a on their surface either (Fig. 2). IFN-γ could induce ~5% of the cells from BALB/c or C57BL/6 to express surface IgG2a, respectively (Fig. 2). When the B cells were stimulated with IFN-γ plus anti-IgM, the number of cells that produced IgG2a more than doubled compared with the cells treated with IFN-γ in both strains (Fig. 2). These results demonstrate that there is a physiological correlation between the synergistic activation of T-bet and the optimal production of IgG2a during B cell activation.

**The synergistic activation of T-bet is transient in naive B cells**

To further understand the regulation of T-bet expression in primary B cells during B cell activation, we isolated RNA from the
B cells that were cultured as in Fig. 2 and analyzed T-bet expression in a time course of 2, 12, 24, 48, 72, and 96 h. As shown in Fig. 3A, after 12 h of treatment with IFN-γ alone or IFN-γ plus anti-IgM, the level of T-bet expression decreased ~70% compared with the level of T-bet after 2-h treatment. The level of T-bet continued to decrease to almost background levels after 48 h of culture even with addition of new medium and ligands at the 48-h time point (Fig. 3A). This result indicates that the induction of T-bet expression is transient, only at the early stage of B cell activation.

We further analyzed the status of T-bet expression in a panel of B cell lines: IgM-positive (IgM⁺) CH27 cell line (23) and the IgG-positive (IgG⁺) cell lines A20 (24), 2PK-3 (25), and M12.4.1 (26). These cells were treated with IFN-γ, anti-IgM, or IFN-γ plus anti-IgM in a time course up to 150 min. In the IgM⁺ CH27 cells, IFN-γ could induce an ~7-fold increase in T-bet expression after 90 min, although anti-IgM could induce ~2-fold increase (Fig. 3B). When CH27 cells were treated with IFN-γ plus anti-IgM, T-bet expression increased ~20-fold (Fig. 3B). These expression profiles are very similar to that of the naive primary B cells shown in Fig. 1. However, the IgG⁺ cells showed a very different pattern. IFN-γ did not induce a significant increase in T-bet expression, whereas anti-IgG alone or IFN-γ plus anti-IgG induced about a 5-fold increase in T-bet expression in A20 or 2PK-3 cells (Fig. 3B). In M12.4.1 IgG⁺ cells, very little T-bet expression was induced by any ligand (Fig. 3B). The failure of IFN-γ to induce T-bet expression was not due to any defect in the IFN-γ signaling pathway in all three IgG⁺ cells because the expression of another IFN-γ-responsive gene, IRF-1, was induced ~20-fold (data not shown). Therefore, the synergistic activation of T-bet seems only to occur in IgM⁺ B cell line, but not in IgG⁺ B cell lines.

The synergistic activation of T-bet is dependent on Stat1

Previous reports have shown that T-bet expression in T cells is Stat1-dependent (16), although in B cells it can be induced by CpG in a Stat1-independent manner (18). To investigate whether the synergistic activation of T-bet in primary B cells also requires Stat1, we analyzed the expression of T-bet in Stat1⁻/⁻ splenic primary B cells. Congenic sv129 Stat⁺/⁺ primary B cells were also purified and analyzed in parallel. Treatment of IFN-γ plus anti-IgM induced a synergistic activation of T-bet in Stat1⁺/⁺ primary B cells, similar to that of primary B cells from BALB/c mice (Fig. 4A). However, in Stat1⁻/⁻ primary B cells, there was no synergistic activation of T-bet by IFN-γ plus anti-IgM and no induction by IFN-γ alone (Fig. 4B). The small increase in T-bet expression in response to anti-IgM was still present in Stat1⁻/⁻ B cells. These results demonstrate that Stat1 is critical for the synergistic induction of T-bet during B cell activation.

Stat1 is required for class switching to IgG2a for humoral immune response

To further demonstrate the physiological importance of regulation of T-bet expression by Stat1, we analyzed the production of IgG2a in Stat1⁻/⁻ splenic B cells. A significant percentage of congenic Stat⁺/⁺ primary B cells could produce IgG2a in response to IFN-γ treatment and the number of IgG2a-positive B cells was increased with IFN-γ plus anti-IgM treatment (Fig. 5A, top panels). However, very few of the Stat1⁻/⁻ splenic B cells could produce IgG2a in a wide range of anti-IgM concentration (Fig. 5A, bottom panels). We further analyzed the expression of the IgG2a germline transcripts by real-time RT-PCR. In Stat1⁺/⁺ primary B cells, IFN-γ and IFN-γ plus anti-IgM-induced Iγ2a germline transcription by ~4-fold (Fig. 5B). However, no induction of IgG2a germline transcripts was detected in Stat1⁻/⁻ primary B cells. Together these results demonstrate that Stat1 is required for the activation of IgG2a germline transcription and class switching to Iγ2a during the humoral immune response.
IFN-γ cells were purified from 8- to 10-wk-old BALB/c mice and treated with an anti-Stat1 Ab. Genomic DNA were purified from the pre-nuclear extracts from CH27 cells treated with IFN-γ was further confirmed by real-time PCR. Four independent experiments is shown. Quantitation of two experiments further quantitated by a PhosphorImager. One representative result from

To further analyze the role of Stat1 in regulating T-bet expression, we used ChIP assay to see whether Stat1 is present on the T-bet promoter. Splenic primary B cells from BALB/c mice were stimulated with IFN-γ for 30 or 60 min, cross-linked with formaldehyde, sonicated, and IP with anti-Stat1 Ab. Genomic DNAs were isolated from the IP complex and used as templates for PCR with primers specific for the T-bet promoter. The 32P-labeled PCR products were separated by electrophoresis and visualized by autoradiography. B, Splenic primary B cells were purified from 8- to 10-wk-old BALB/c mice and treated with the indicated ligands for ChIP analyses. 32P-labeled PCR products of the T-bet promoter region were separated on acrylamide gels, visualized by autoradiography, and further quantitated by a PhosphorImager. One representative result from four independent experiments is shown. Quantification of two experiments was further confirmed by real-time PCR. C, EMSAs were performed with nuclear extracts from CH27 cells treated with IFN-γ for 20 min and a 32P-labeled probe containing a putative Stat1 binding site from the T-bet promoter. Supershift was performed with an anti-Stat1 Ab and normal mouse IgG. Ten-fold excess of cold wild-type or mutant probes were used for competition binding assays. S1, Stat1; wt, wild type; mut, mutant.

**FIGURE 6.** T-bet is a direct Stat1 target gene. A, Splenic primary B cells were purified from 8- to 10-wk-old BALB/c mice and treated with IFN-γ for 30 or 60 min, cross-linked with formaldehyde, sonicated, and IP with anti-Stat1 Ab. Genomic DNAs were isolated from the IP complex and used as templates for PCR with primers specific for the T-bet promoter. The 32P-labeled PCR products were separated by electrophoresis and visualized by autoradiography. B, Splenic primary B cells were purified from 8- to 10-wk-old BALB/c mice and treated with the indicated ligands for ChIP analyses. 32P-labeled PCR products of the T-bet promoter region were separated on acrylamide gels, visualized by autoradiography, and further quantitated by a PhosphorImager. One representative result from four independent experiments is shown. Quantification of two experiments was further confirmed by real-time PCR. C, EMSAs were performed with nuclear extracts from CH27 cells treated with IFN-γ for 20 min and a 32P-labeled probe containing a putative Stat1 binding site from the T-bet promoter. Supershift was performed with an anti-Stat1 Ab and normal mouse IgG. Ten-fold excess of cold wild-type or mutant probes were used for competition binding assays. S1, Stat1; wt, wild type; mut, mutant.

T-bet is a direct Stat1-target gene

To further analyze the role of Stat1 in regulating T-bet expression, we used ChIP assay to see whether Stat1 is present on the T-bet promoter. Splenic primary B cells from BALB/c mice were stimulated with IFN-γ for 30 and 60 min, cross-linked with formaldehyde, and sonicated to generate lysate for immunoprecipitation with an anti-Stat1 Ab. Genomic DNA were purified from the precipitated Stat1-DNA complexes and subjected to PCR analyses with a pair of primers flanking several putative Stat1 sites in the T-bet promoter. As shown in Fig. 6A, after treatment with IFN-γ for 30 min, there was a dramatic increase in the amount of Stat1 on the T-bet promoter (Fig. 6A, lane 2). The amount of Stat1 on the T-bet promoter decreased after 60 min, correlating with the status of Stat1 Tyr phosphorylation (data not shown). This result demonstrates that T-bet is a direct Stat1 target gene and Stat1 regulates the expression of T-bet by transcription activation.

To see whether the simultaneous signaling of IFN-γ and anti-IgM affect the binding of Stat1 to the T-bet promoter, ChIP analyses were performed on primary B cells either untreated or treated with IFN-γ alone, anti-IgM alone, or IFN-γ plus anti-IgM. Anti-IgM did not induce any detectable binding of Stat1 to the T-bet promoter (Fig. 6B, lane 2). The binding of Stat1 to the T-bet promoter were similar in cells treated with IFN-γ alone or IFN-γ plus anti-IgM (Fig. 6B, lanes 3 and 4), suggesting that the activation of the BCR signaling pathway does not influence the IFN-γ-induced binding of Stat1 to the T-bet promoter.

To further identify the specific Stat1 binding site(s) in the T-bet promoter, we synthesized four sets of oligonucleotides that contained sequences similar to the consensus Stat1 binding site TTTN5AA and performed gel shift assays with nuclear extracts from IFN-γ-treated CH27 cells. One of the oligonucleotide sequence detected a Stat1 containing complex when cells were treated with IFN-γ (Fig. 6C, lane 2), and this complex could be supershifted by an anti-Stat1 Ab, but not by a control mouse IgG (Fig. 6C, lanes 3 and 4). Furthermore, this Stat1-containing complex can be competed away with cold wild-type probe but not with a mutant probe (Fig. 6C, lanes 5 and 6). These results indicate that there is a Stat1-specific binding site in the T-bet promoter through which Stat1 regulates the expression of T-bet in the early stage of B cell activation.

**Discussion**

Regulation of Ab class switching by cytokines is critical for the generation of diverse effector function during the humoral response (6). The various cytokines induce germline transcription in the specific isotype locus, a necessary step preceding class switching. One of the families of transcription factors that play essential roles in cytokine signaling is the STAT family of transcription factors. The STAT proteins are directly activated by various cytokines through tyrosine phosphorylation and translocate into the nucleus to activate gene expression. The induction of germline transcription by cytokines could be mediated directly by the STATs as in the case for Ie induction by Stat6 (27–29). It is also possible that the expression of other transcription factors can be induced by STAT-mediated transcription activation, and these factors, in turn, directly bind to the germline promoter to activate its expression. In this report, we demonstrate that, for IFN-γ-induced Iγ2a expression and IgG2a production, Stat1 plays a critical role by regulating the expression of T-bet, which has been shown to be required for IgG2a switching through analyses of T-bet knockout mice (13).

In normal resting naive B cells, T-bet expression is very low (Fig. 1). However, T-bet expression is quickly induced when the naive B cells are stimulated by IFN-γ. Most strikingly, when the B cells are stimulated by both IFN-γ and cross-linking of BCR, T-bet expression increased more than 60-fold and was easily detectable after only 30 min of treatment (Fig. 1). This synergistic activation of T-bet by two signaling pathways enables the B cells to undergo class switching more efficiently, resulting in a doubling of the number of B cells that can produce IgG2a (Fig. 2). For the long-term cell cultures in this study, we included LPS to maintain cell
growth and survival. Although LPS by itself does not induce production of IgG2a (Fig. 2), similar bacteria-released products may have a role in promoting class switching during an anti-bacterial immune response in vivo. It is conceivable that such synergy between different signaling pathways is critical for the host to mount a maximal humoral response.

The mechanism to achieve the synergy remains to be further investigated. Scanning of the T-bet promoter revealed the presence of other transcription factors binding sites in addition to multiple Stat1 sites. Our results show that the T-bet promoter is a direct Stat1 target and the synergy in activating T-bet by IFN-γ and anti-IgM is dependent on Stat1 (Fig. 1). It has been reported that NF-κB activity is required for the Stat1-independent induction of T-bet by CpG in primary B cells (18). Using the c-Rel-deficient mice (30, 31), we show that the synergistic activation of T-bet by IFN-γ and anti-IgM does not involve c-Rel, the predominant member of the NF-κB family in mature B cells (Ref. 32 and our unpublished observations). Although LPS-TLR4 has been shown to be able to activate IRF-3/7 and NF-κB, it alone cannot induce any T-bet expression or IgG2a production, nor can it affect IFN-γ or anti-IgM induced T-bet expression or IgG2a production (Fig. 2 and data not shown), further which suggests that NF-κB may not be involved in the synergistic T-bet expression in our system. It is likely that several transcription factors can induce T-bet transcription independently in different signaling pathways. When these transcription factors are simultaneously activated by multiple signaling pathways as in the case of B cell activation in vivo, perhaps together they can form a more stable enhancer on the T-bet promoter to synergistically activate T-bet.

Another interesting finding in this study is that the dramatic synergistic induction of T-bet is only in the first few hours of ligand stimulation and T-bet level is quickly down-regulated (Fig. 3A). Furthermore, it seems that the synergistic activation of T-bet only occurs in IgM B cells, but not in IgG + B cells (Fig. 3B), with the implication that once the B cells undergo class switching, T-bet is no longer needed. All together, these results would suggest that the role of T-bet for B cell activation is highly specific. It has been reported that in a significant subset of B cell lymphoproliferative disorders such as lymphoblastic leukemia and lymphoma, T-bet is constitutively expressed (19). Together with our results, it is likely that several transcription factors can induce T-bet transcription independently in different signaling pathways. When these transcription factors are simultaneously activated by multiple signaling pathways as in the case of B cell activation in vivo, perhaps together they can form a more stable enhancer on the T-bet promoter to synergistically activate T-bet.