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The Absence of the Transcription Activator TFE3 Impairs Activation of B Cells In Vivo

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TFE3 is a ubiquitously expressed member of the TFE3/mi family of basic helix loop helix zipper transcription factors. TFE3 binds to μE3 sites located in the immunoglobulin heavy-chain (IgH) intronic enhancer, heavy-chain variable region promoters, the Ig kappa intronic enhancer, and regulatory sites in other genes. To understand the role of TFE3 in Ig expression and lymphoid development, we used embryonic stem (ES) cell-mediated gene targeting and RAG2$^{-/-}$ blastocyst complementation to generate mice which lack TFE3 in their B and T lymphocytes. TFE3^{-} ES cells fully reconstitute the B- and T-cell compartments, giving rise to normal patterns of IgM^{+} B220^{+} B cells and CD4^{+} and CD8^{+} T cells. However, TFE3^{-} B cells show several defects consistent with poor B-cell activation. Serum IgM levels are reduced twofold and IgG and IgA isotypes are reduced three- to sixfold in the TFE3^{-} chimeras even though in vitro, the TFE3^{-} splenocytes secrete normal levels of all isotypes in response to lipopolysaccharide activation. Peripheral TFE3^{-} B cells also show reduced surface expression of CD23 and CD24 (heat-stable antigen).

The immunoglobulin heavy-chain (IgH) gene has served as a highly informative paradigm for understanding tissue-specific and developmentally regulated gene transcription (8, 19). However, understanding the precise mechanisms by which the IgH promoters and enhancers act is complicated by the finding that many functionally important sites within these DNA elements are recognized by more than one protein, and it is difficult to determine which protein(s) actually participates in transcriptional regulation. One way to address this problem is by mutation of the genes encoding specific DNA-binding proteins by using homologous recombination (38).

We have used this approach to study the role of TFE3, a transcriptional activator which binds to μE3 sites (6, 40). μE3 sites are functionally important in the IgH intronic enhancer (49) and in some IgH variable-region promoters; they are also found in the Ig kappa intronic enhancer (35) and in other genes. Although several proteins recognize μE3 sites, we do not know which of these actually activates IgH gene transcription (8). Identification of functional μE3-binding protein(s) is necessary to understand IgH enhancer and promoter activity and also to fully understand the B-cell specificity of the IgH intronic enhancer. The enhancer μE3 site is occupied in a B-cell-specific manner (18) even though none of the known proteins which recognize the site are B-cell specific, suggesting that occupation of the site may require a generally expressed protein to associate with or be modified by a B-cell-specific protein or for accessibility of the site to be modified in a B-cell-specific manner.

μE3-binding proteins all share basic, helix-loop-helix zipper (bHLHzip) motifs which are responsible for DNA binding and dimerization. They are divided into three subgroups based on their ability to heterodimerize with one another: (i) the Myc family containing the proteins N-Myc, L-Myc, and c-Myc, and their Max or Max-like heterodimerization partners (7); (ii) the USF family containing the genes USF1 and USF2 (22, 46); and (iii) the TFE3 family containing TFE3 (6, 41), TFEB (9, 20), TFEC (52), and mi (26). Within the TFE3 family, TFE3 and TFEB are activators which are highly homologous and are both expressed in B cells, TFEC is a dominant negative when heterodimerized with other family members, and mi is an activator which is not expressed in B cells. TFE3 contains an N-terminal acidic transcriptional activation domain and, C-terminal to the bHLHzip region, a proline-rich transcriptional activation domain (4, 6). The two activation domains of TFE3 act synergistically, and an alternatively spliced form of TFE3 lacking the acidic activation domain has dominant negative activity (40).

Several facts suggest that TFE3, and/or TFEB, may be important for μE3 site-dependent activity of IgH gene transcription. In cotransfection assays using μE3 site-dependent promoters, TFE3 is a much stronger activator than USF or c-Myc. Furthermore, we have shown that TFE3-mediated activation is synergistic in reporters which, like the IgH gene, have both proximal and distal μE3 sites (3). Thus, TFE3 could be important for mediating interactions between the IgH intronic enhancer and V_H promoters. Transdominant TFE3 but not USF constructs have been shown to block μE3 site activity, thus implicating the TFE3 family in μE3 activity in vivo (10). However, it is not known if TFE3 and TFEB play separate, overlapping, or redundant roles in IgH transcription, and it is not known if USF or Myc family members also play a role.

In this report, we describe generation of TFE3^{-} embryonic stem (ES) cells which are viable and which are able to contribute to a variety of tissues in chimeric animals. Chimeric mice made with TFE3^{-} ES cells and RAG2$^{-/-}$ (recombinase-activating gene 2 homozygous null) blastocysts (11, 45) have normal numbers of B cells and T cells. However, the TFE3^{-} B cells have decreased surface expression of some markers associated with mature or activated B cells. The TFE3^{-} chimeras also have decreased serum concentrations of all Igs. Nevertheless, when TFE3^{-} splenocytes are activated in vitro with lipo-

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polysaccharide (LPS), they secrete normal levels of all Ig isotypes. Thus, TFE3$^-$ B cells appear to have a defect in their ability to respond in vivo to one or more normal B-cell activation signals.

MATERIALS AND METHODS

Targeting construct. We used a BALB/c genomic clone of TFE3 which had been cloned previously in our lab (40). The 7.0-kb 3′ *Eco*RI fragment was subcloned into pUC19. A 4.0-kb fragment from *Bgl*II to *Sma*I was removed by partial digestion. This was replaced by a promoterless neomycin resistance (*neo*) cassette to make the TFE3/NEO construct. The cassette was a PCR product generated with oligonucleotides which flanked the entire *neo* gene, including the start and stop codons. The PGK-NEO construct was used as a template. The oligonucleotides were designed so that the 5′ end of the *neo* fragment would have a *Bgl*II site which would result in an in-frame fusion of the *neo* gene and the *tfe3* gene. The 5′ homology segment was generated by PCR using oligonucleotides which hybridized in exons 3 and 4. The 5′ oligonucleotide also included an *Eco*RI site. The genomic clone was used as a template. The PCR product was digested with *Eco*RI and ligated with an *Eco*RI partial digestion of the TFE3/NEO construct to generate the targeting construct.

Gene targeting and generation of chimeric mice. CCE ES cells were maintained on neomycin-resistant (Neo^R) STO cells, which produced lymphocyte inhibitory factor (LIF). Cells were electroporated with 20 μg of *Xmn*I-linearized targeting construct and selected with 0.2 mg of G418 per ml. Cell culture, transfection, and selection were all based on previously published protocols (39). Transfections generally yielded between 5 and 50 Neo^R clones. Targeted clones were identified by Southern blotting of genomic DNA digested with either *Xba*I or *Nco*I and probed with the probe indicated in Fig. 1. Appropriate clones showed complete loss of the wild-type band, appearance of the appropriate mutant band, and no extra bands indicating nonhomologous insertions.

We had a targeting frequency of 1:2 to 1:20, varying with each transfection. Targeted clones were resubcloned to remove any contaminating wild-type cells and karyotyped for a normal diploid complement of 40 chromosomes.

Two clones, 66-3 and 66-8, were used for the generation of chimeric mice with RAG2$^{-/-}$ blastocysts according to previously published protocols. Chimeric mice were identified by staining peripheral blood leukocytes for B220 and CD5. Only chimeras with normal numbers of peripheral B and T cells were used for further studies. Chimeras derived from both clones were used in all experiments, with no differences seen between the two clones.

Fluorescence-activated cell sorting (FACS) analysis. Preparation, staining, and analysis of cells were performed as described previously (28). Prepared cells were stained with fluorescein isothiocyanate (FITC)-phycoerythrin-, allophycocyanin (APC)-, or biotin-conjugated antibodies. Biotin staining was revealed with streptavidin-Texas red (TR). Antibodies used included anti-CD43 (S7), anti-CD40 (3/23), anti-CD23 (B3B4), and anti-B7-1 (1G10), obtained from Pharmingen Inc. Anti-CD4 (GK1.5), anti-CD8 (53-6.7), anti-B220 (RA3-6B2), anti-IgD (11-26), anti-IgM (331), anti-heat-stable antigen (HsAg) (30-F1), and anti-HsAg (53-10) were prepared in our lab.

LPS cultures. Total splenocytes harvested and prepared as above for FACS analysis were cultured in RPMI–10% fetal calf serum (FCS) 20 μg of LPS per ml at a density of 10^6 cells/ml. Cells were fed daily to maintain a density of 10^6 cells/ml and harvested after 96 h. Harvested cells were then used for FACS analysis and for Western and Southern blotting. Culture supernatants were saved for measurement of Ig isotype concentrations.

Southern blots. Genomic DNA was prepared by resuspending 5×10^6 cells in TES buffer (10 mM Tris [pH 8.0], 400 mM NaCl, 2 mM EDTA, 1% sodium dodecyl sulfate [SDS]) with 0.5 mg of proteinase K per ml and incubating them at 55°C overnight. The solution was then phenol-chloroform extracted and ethanol precipitated. Ten micrograms of DNA was used per digest. DNA was separated on a 0.7% gel and blotted to a Hybond N (Amersham) membrane. The membrane was UV cross-linked and hybridized to the random-primed probe in 1.5× SSPE (1× SSPE is 0.18 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA [pH 7.7])–10% polyethylene glycol–7% SDS overnight at 68°C. The blot was washed three times in 0.2× SSPE–0.1% SDS at 68°C for 30 min.

Western blots. Cells were washed twice in phosphate-buffered saline (PBS) and resuspended in lysis buffer (50 mM Tris [pH 8.0], 0.5% Nonidet P-40, 10% glycerol, 0.1 mM EDTA, 400 mM NaCl). The mixture was incubated on ice for 30 min and centrifuged for 15 min in a microcentrifuge to remove debris. An equivalent of 5×10^6 cells was loaded on an SDS–10% polyacrylamide gel. The gel was electroblotted to nitrocellulose overnight in 25 mM Tris–190 mM glycine–20% methanol at 30 V. The membrane was treated for 1 min with isopropanol and blocked for 2 h in 5% nonfat milk–0.2% Tween 20 in PBS. The primary antibody was a rabbit polyclonal serum generated against recombinant TFE3. The secondary antibody was Boehringer Mannheim catalog no. 606 220. Primary and secondary incubations were done at room temperature in 2% milk PBS for 2 h and were followed by three washes in PBS–0.2% Tween 20. The blot was visualized by using an Amersham ECL kit.

Enzyme-linked immunosorbent assay (ELISA) Ig quantitation. Serum and LPS culture supernatants were analyzed by using the SBA Clonotyping system from Southern Biotechnology Associates. All assays were done in duplicate with serial threefold dilutions of serum and culture supernatants starting at 1:1,000 and continuing to 1:2,180,000. The only change from Southern Biotechnology's protocol was the substitution of 1 mg of 4-methylumbelliferyl phosphate per ml for *p*-nitrophenyl phosphate in the substrate solution. Results were read on a Fluoroscan plate reader, and absolute values were determined by comparison to a standard curve from isotypes of known concentration obtained from Southern Biotechnology. All processing of samples was done in parallel with age-matched controls. Mice were all 6 to 8 weeks old. The presented results are the products of two separate experiments. The first used half of the chimeras plus wild-type C57B6/CBA F_1 mice as controls; the second used the remaining TFE3$^-$ chimeras plus wild-type chimeras as controls.

Tritiated thymidine incorporation. Splenocytes were prepared as for FACS analysis. They were then put in 100 μl of Dulbecco modified Eagle medium–10% FCS at a concentration of 10^6 cells/ml for LPS cultures or 5×10^5 cells/ml for CD40L cultures. LPS was added at concentrations of 0, 1, 10, and 100 μg/ml. CD40L membranes were prepared as previously described (30) and were serially diluted such that 0, 0.1, 1, or 10 μl of the original stock was added in 100 μl of Dulbecco modified Eagle medium–10% FCS. Cultures were incubated at 37°C in 5% CO_2 for 48 h, and then 0.1 ml of tritiated thymidine (0.1 mCi/ml) was added for 12 h. Cells were lysed by multiple freeze-thaw cycles and harvested onto glass fiber filters for counting in a scintillation counter. All cultures were done in triplicate.

RESULTS

Generation of TFE3$^-$ ES cells. In the murine *tfe3* (*mtfe3*) gene, (Fig. 1A), the basic region and helix 1 are encoded by exons 5 and 6. Since point mutations in the basic and helix-loop-helix regions of bHLH proteins ablate the DNA-binding ability of TFE3 (40), we reasoned that removal of these domains should yield a null *mtfe3* allele. Furthermore, since we observed that *mtfe3* is transcribed in ES cells (not shown), we chose to use a promoterless targeting strategy to ablate the *mtfe3* gene in ES cells (27). The targeting construct contained the *neo* gene fused in frame to the fourth exon of *mtfe3* (Fig. 1B). The construct lacks a 3.0-kb fragment of the endogenous gene which encodes the basic region and helix 1 of the HLH domain. Homologous recombination will result in the indicated structure (Fig. 1C). This will code for a Neo^R fusion protein which contains TFE3's amino terminus, but the protein should not be able to bind μE3 sites or dimerize with other TFE3 family members.

The targeting construct was transfected into 2×10^7 CCE ES cells (39). An average of 11 Neo^R clones were obtained for each electroporation. This low number of resistant clones is consistent with the promoterless nature of the targeting construct. Neo^R clones were analyzed by PCR and Southern blotting. Figure 1D shows a typical Southern blot of *Nco*I-digested DNA from three ES cell clones which was probed with a gene fragment indicated in Fig. 1C. The first lane is from a wild-type clone and shows the expected 3.5-kb band. The next two are from clones with mutated *mtfe3* genes and show a 2.5-kb band which is predicted for homologous recombinants. Since the *mtfe3* gene is X linked (25, 41), the Southern blot shows both the appearance of the mutated band and the loss of the wild-type band. With this analysis, we are able to identify and eliminate from further study clones which had extra copies of the targeting construct randomly integrated in the genome. We reconfirmed the identity of all TFE3$^-$ clones by Southern blotting using a second restriction enzyme and by PCR analyses (data not shown). Of 110 initial Neo^R clones, 10 were confirmed by Southern blotting and PCR to be homologous recombinants.

Generation of RAG2$^{-/-}$ TFE3$^-$ chimeric mice. To examine the effect of the *mtfe3* null allele on lymphocyte development, we made chimeric animals by injecting TFE3$^-$ ES cells into RAG2$^{-/-}$ blastocysts. Wild-type CCE ES cells were also injected into RAG2$^{-/-}$ blastocysts to make chimeric controls. Since RAG2$^{-/-}$ animals cannot rearrange their Ig and T-cell receptor genes, they lack B and T cells (45); therefore all of the

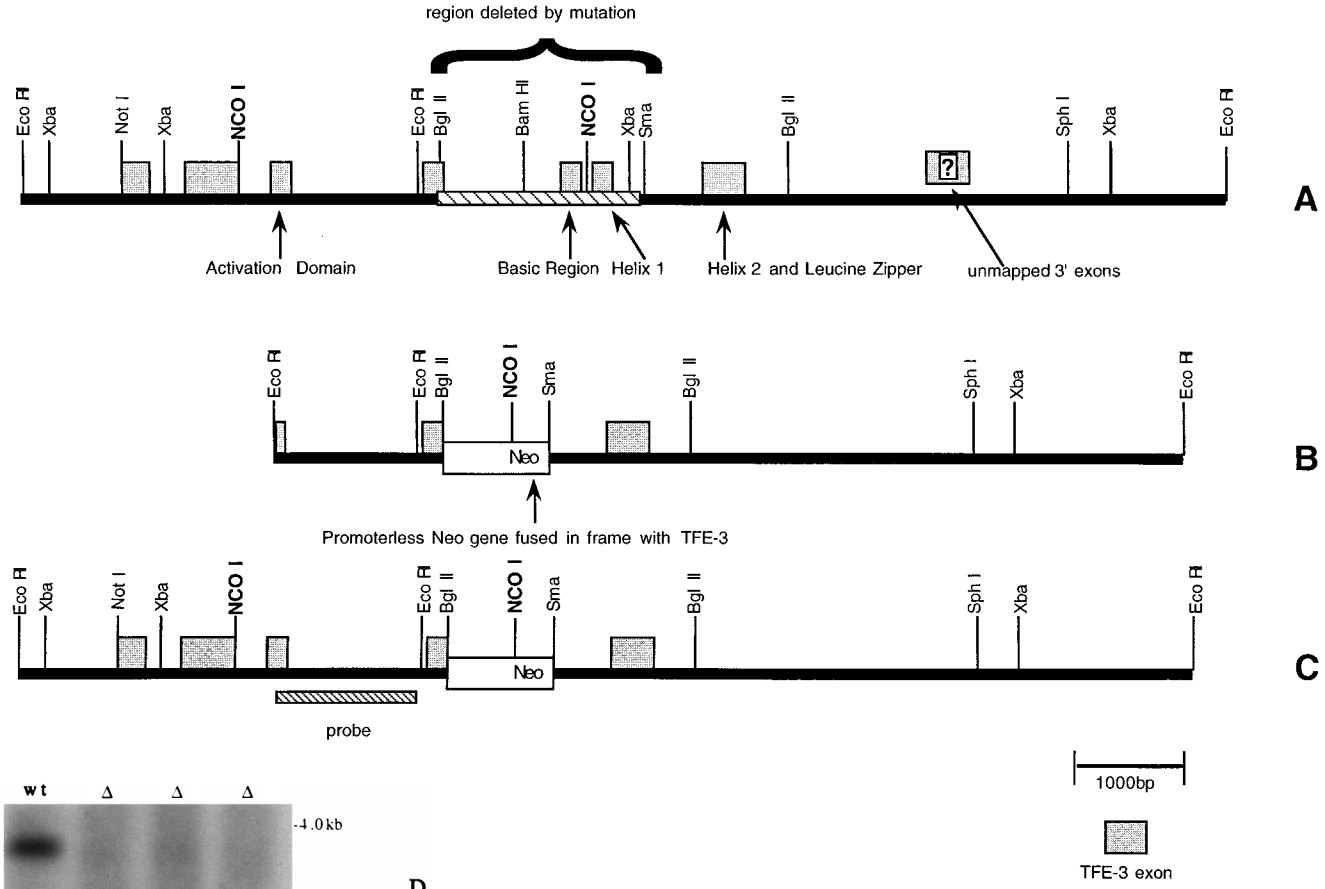

FIG. 1. (A) Map of the *mtfe3* genomic locus. Exons are indicated by stippled boxes. The region deleted by the targeting construct is indicated by a hashed box and brace. (B) TFE3 targeting construct. The *neo* gene is indicated by the open box. (C) Map of the mutated *mtfe3* allele. The probe used for Southern blots is indicated by a hashed box. (D) Southern blot of ES cell DNA from wild-type (wt) or TFE3$^-$ (Δ) cell lines digested with *Nco*I and probed with the probe indicated in panel C. Size markers are indicated. (E) Southern blot of DNA from day 4 LPS-cultured splenocytes from TFE3$^-$ RAG2$^{-/-}$ chimeras (D), C57B6/CBA F_1 wild-type (wt) mice, and TFE3$^-$ ES cells cut with *Nco*I and probed as described above. (F) Western blot of whole-cell extracts from TFE3$^-$ chimeras (D) and wild-type (wt) mice run on an SDS–10% polyacrylamide gel and probed with a polyclonal serum against mouse TFE3. Size standards are indicated.

lymphocytes in the RAG2$^{-/-}$ chimeras must be derived from the TFE3$^-$ ES cells which contain a wild-type *RAG2* gene (11). Preliminary analyses to detect T and B cells in peripheral blood were used to identify putative chimeras (not shown). To confirm that these chimeras had been reconstituted with TFE3$^-$ ES cells, splenocytes were isolated and cultured in vitro with LPS. After 4 days in culture, >90% of the cells were B220 positive (data not shown). DNA from these cells was analyzed by Southern blot to confirm the presence of the *mtfe3* null allele (Fig. 1E). The DNA in this experiment was digested and probed as described for Fig. 1D. The mutant band is visible in DNA from both chimeras (lanes 3 and 4) but not in the control (lane 2), demonstrating reconstitution of B cells in the RAG2$^{-/-}$ mice from TFE3$^-$ ES cells.

In addition, proteins in whole-cell extracts from the LPS-cultured splenocytes were analyzed by immunoblotting with antiserum against TFE3 protein (Fig. 1F). The wild-type TFE3, migrating as an 80-kDa band (lane 1), is clearly missing from the TFE3$^-$ splenocytes (lanes 2 and 3). We did not see an abnormal-size protein in the mutant splenocytes. This may be due to decreased stability of the abnormal fusion protein or the loss of most of the TFE3 sequences which were used to generate the antibody. We conclude that lymphocytes in the chimeric mice were derived from TFE3$^-$ ES cells and that the *mtfe3* mutation resulted in loss of detectable TFE3 protein.

TFE3$^-$ RAG2$^{-/-}$ chimeras have normal populations of T cells in the thymus. The chimeric mice were analyzed by flow cytometry to determine how completely their lymphoid compartments had been reconstituted. The total numbers of cells in TFE3$^-$ and wild-type thymuses (Table 1) were not signifi-

TABLE 1. Distribution of T cells[a]

Group	Spleen			Lymph node			Peripheral blood leukocytes		Thymus				
	Total no. of cells (10^7)	% $B220^+$	% $CD4^+$	% $CD8^+$	% $B220^+$	% $CD4^+$	% $CD8^+$	% $B220^+$	% $CD5^+$	Total no. of cells (10^7)	% $CD4^+$	% $CD8^+$	% $CD4^+$ $CD8^+$	% $CD4^-$ $CD8^-$
$TFE3^-$ chimeras	7.0 ± 4.1	36 ± 8.1	33 ± 2.7	12 ± 1.0	28 ± 3.0	51 ± 1.5	19 ± 1.8	11 ± 3.2	30 ± 9.0	8.4 ± 4.1	6.1 ± 0.2	4.7 ± 0.2	82 ± 2.9	6.7 ± 2.9
Wild type	6.5 ± 3.0	39 ± 1.0	20 ± 1.8	14 ± 3.8				11 ± 0.3	44 ± 2.5					
C57B6/CBA F_1 controls	6.5 ± 1.0	52 ± 2.0	23 ± 2.8	13 ± 0.3	29 ± 4.0	43 ± 3.6	22 ± 2.2			6.7 ± 1.3	7.1 ± 0.8	3.6 ± 0.7	86 ± 0.7	3.1 ± 0.8

[a] Values are means ± standard deviations for two to five individual animals.

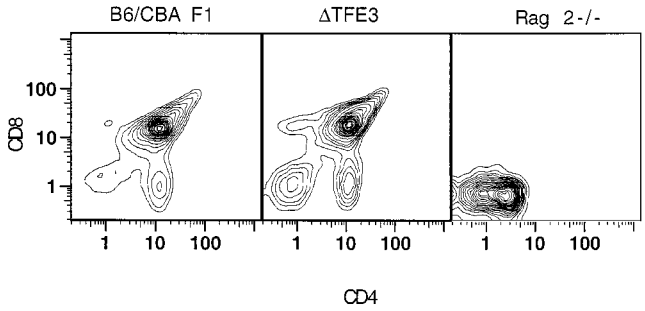

FIG. 2. FACS plots using 5% probability contours of wild-type C57B6/CBA F_1 (B6/CBA F_1), $TFE3^-$ chimera (ΔTFE3), and $RAG2^{-/-}$ mice thymocytes stained with anti-CD8-FITC and anti-CD4-APC.

cantly different. Furthermore, the frequency of thymic subpopulations in wild-type C57B6/CBA F_1 control mice and $RAG2^{-/-}$ $TFE3^-$ chimeras showed no significant differences (Fig. 2). The $TFE3^-$ thymocytes showed a slight increase in double-negative cells, but this likely includes the double-negative population normally found in $RAG2^{-/-}$ mice. Therefore, we conclude that lack of TFE3 does not affect the generation of single- and double-positive T cells in the thymus and that T-cell development, judged by expression of CD4 and CD8, is normal in $TFE3^-$ animals.

$TFE3^-$ $RAG2^{-/-}$ chimeras have normal bone marrow populations but a defect in HsAg expression. $TFE3^-$ bone marrow was also analyzed for the presence of B-cell precursors. The patterns of CD43 and B220 expression (Fig. 3) are similar in wild-type and $TFE3^-$ chimeras, indicating similar development of pre-B cells and immature B cells. Interestingly, the $CD43^{dull}$ $B220^+$ cells, which should exclude the $RAG2^{-/-}$ cells (45), show a difference in the expression of HsAg on $TFE3^-$ bone marrow lymphocytes. Surface HsAg expression is usually low early in B-cell development and increases in late pro-B cells, pre-B cells, and immature B cells (24), consistent with the HsAg pattern in Fig. 3 for wild-type cells. However, the $HsAg^{bright}$ population is missing from the $TFE3^-$ animals.

$TFE3^-$ $RAG2^{-/-}$ chimeras have normal reconstitution of peripheral B- and T-cell compartments. Splenocytes from $TFE3^-$ and wild-type chimeric animals were analyzed with stains for T-cell markers CD4 and CD8 (Fig. 4A, upper panel) and B-cell markers B220, IgM, IgD, and CD40 (Fig. 4A, lower panels). The total number of lymphocytes and the percentages of $CD4^+$, $CD8^+$, and $B220^+$ cells are shown in Table 1. Mutant and wild-type chimeras have comparable percentages of peripheral B and T cells. Furthermore, the patterns of IgM, IgD, and CD40 expression in wild-type and $TFE3^-$ B cells are indistinguishable. Thus, $TFE3^-$ chimeras have full reconstitution of peripheral B and T cells. These peripheral cells appear phenotypically normal with respect to several important surface markers, including IgD and IgM.

To confirm these results, cells from peritoneal lymph nodes were also analyzed for expression of B220, IgM, CD4, and CD8. Similar to splenic lymphocytes, these cells showed similar distributions of IgM^+ $B220^+$ B cells and $CD4^+$ and $CD8^+$ T cells in wild-type and $TFE3^-$ chimeras. Examination of peritoneal and peripheral blood lymphocytes also showed normal B- and T-cell populations, including B-1 cells (data not shown). The percentages of B and T cells in lymph nodes and peripheral blood are also shown in Table 1. These results demonstrate that TFE3 is not necessary for the reconstitution of

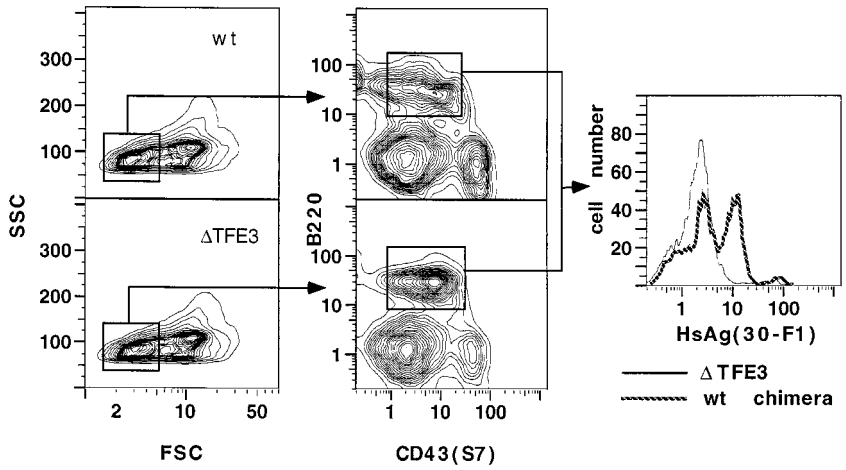

FIG. 3. FACS plots using 5% probability contours of bone marrow from TFE3$^-$ chimeras (ΔTFE3) and wild-type (wt) chimeras. The first column represents forward scatter (FSC) versus orthogonal scatter (SSC). The population of small, smooth cells typical of lymphocytes was selected and plotted in the second column with the stains anti-B220-APC and anti-CD43 (S7)-TR. Cells were then further gated for pre-B and immature B cells by selecting CD43$^+$ and B220$^+$ cells. These cells were then plotted in the third column for level of staining with anti-HsAg (30-F1)-FITC.

lymphoid compartments in the bone marrow, thymus, or periphery.

Expression of CD23 and HsAg is defective in TFE3$^-$ splenic B cells. To better characterize the TFE3$^-$ B cells, control and TFE3$^-$ splenocytes were stained for several surface markers characteristic of peripheral B-cell differentiation or activation. Patterns of IgM compared with CD5, CD43, IgD, and interleukin-2 (IL-2) receptor alpha chain were not significantly different between wild-type and TFE3$^-$ chimeras (not shown). However, CD23 expression was significantly decreased on

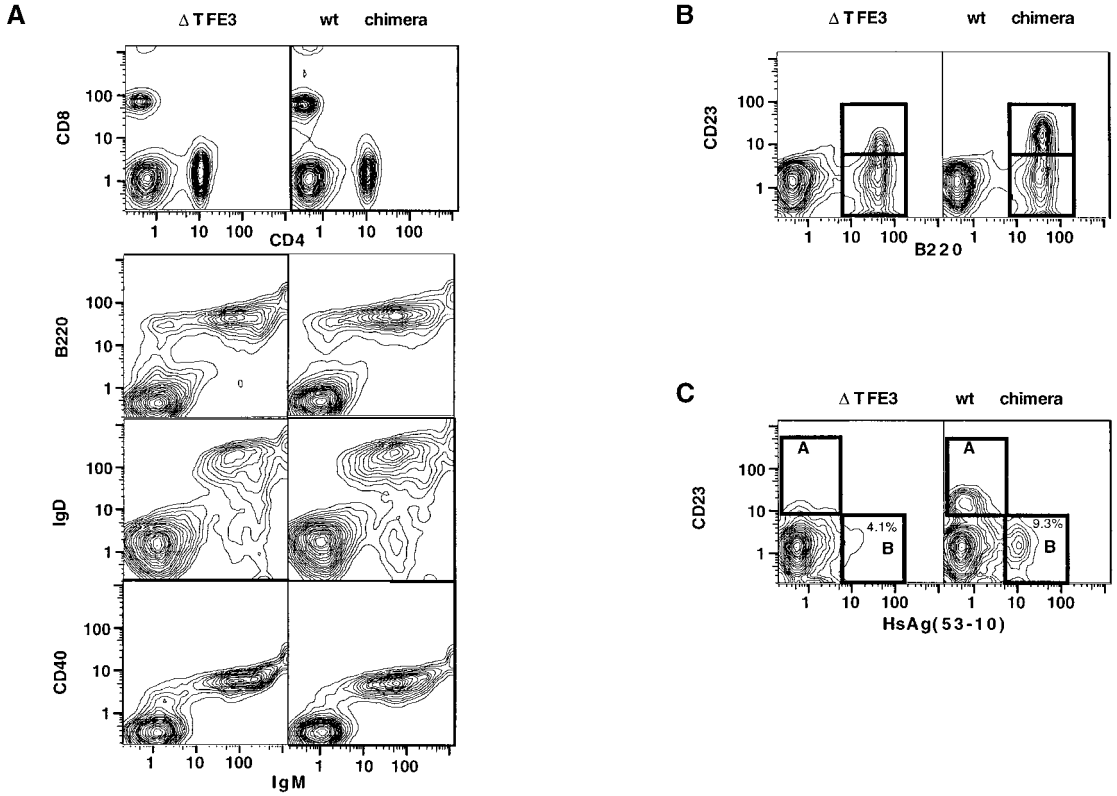

FIG. 4. FACS plots using 5% probability contours of TFE3$^-$ chimeras (ΔTFE3) and wild-type (wt) chimeras. (A) Splenocytes stained with anti-CD8-APC and anti-CD4-FITC (top row), anti-B220-FITC and anti-IgM-APC (second row), anti-IgD-TR and anti-IgM-APC (third row), or anti-CD40-FITC and anti-IgM-APC (bottom row). (B) Splenocytes stained with anti-CD23-TR and anti-B220-FITC. Gates used to determine the percentage of CD23$^+$ B cells are indicated by thick lines. (C) Splenocytes stained with anti-CD23-TR and anti-HsAg (53-10)-FITC. CD23$^+$ (A) and HsAg$^+$ (B) populations are indicated. The percentage of HsAg$^+$ cells is shown in the upper right corner of each plot.

TFE3$^-$ splenic B cells compared to control cells. As seen in Fig. 4B, 11% of TFE3$^-$ B cells are CD23$^+$, compared to 30% of wild-type B cells. In addition, the CD23$^+$ B cells in TFE3$^-$ mice expressed lower levels of CD23 than wild-type B cells. A similar defect in CD23 expression was observed on TFE3$^-$ B cells from peritoneal lymph nodes (data not shown).

As seen in the bone marrow, HsAg expression was also decreased on TFE3$^-$ B cells from spleen (Fig. 4C). Wild-type animals have a population of CD23$^+$ cells (sector A) which is mostly mature, follicular B cells and an HsAg$^+$ population (sector B) which contains B1 cells, immature B cells, and activated B cells (48). Both markers are significantly decreased on TFE3$^-$ B cells. HsAg is heavily glycosylated, and lower levels of detection could be due to changes in glycosylation in TFE3$^-$ mice. However, we have shown decreased levels of HsAg in assays using two other antibodies, 30-F1 and M1/69 (data not shown). M1/69 has been shown to recognize the unglycosylated HsAg protein (29). Interestingly, we also stained TFE3$^-$ and wild-type thymocytes with an anti-HsAg antibody, J11-D, and saw no difference in HsAg expression between wild-type and TFE3$^-$ thymocytes (data not shown). Thus, the abnormality in HsAg levels reflects expression and not faulty glycosylation and is evident in B cells but not in T cells.

HsAg expression is abnormally low in bone marrow B cells (Fig. 3) as well as splenic B cells of the TFE3$^-$ chimeras. However, TFE3$^-$ chimeras have normal numbers of mature resting B cells, germinal center B cells, and B1 cells, as judged by surface expression of IgM, IgD, CD43, CD5, and B220 in spleen and peritoneal cells. Therefore, CD23 and HsAg expression in TFE3$^-$ B cells appears to represent a specific decrease in expression of these proteins rather than a generalized defect in B-cell development.

TFE3$^-$ RAG2$^{-/-}$ chimeras have decreased serum Ig levels. The lack of CD23 and HsAg on the surface of TFE3$^-$ B cells is consistent with a defect in B-cell activation or maturation. Furthermore, μE3 sites are found in the IgH intronic enhancer, V_H gene promoters, and the kappa-chain intronic enhancer. Therefore, although surface IgM levels are normal, levels of secreted Ig might be affected by lack of TFE3 if IgH or kappa gene transcription were compromised. Based on this reasoning, we examined the effect of the TFE3 null allele on serum Ig levels.

The concentrations of Ig isotypes in sera from wild-type control mice and from wild-type chimeric controls were compared to those in sera from TFE3$^-$ chimeras which had full reconstitution of peripheral B and T cells as determined by FACS analysis of spleen, thymus, and peripheral blood (Fig. 5). Although the wild-type mice and wild-type chimeras had similar levels of Igs, the TFE3$^-$ chimeras had significant reductions in their serum Ig concentrations. The levels of serum IgM were approximately twofold lower in the TFE3$^-$ chimeras than in control mice and wild-type chimeras. Levels of the other isotypes were more significantly affected. Serum IgG1 was reduced approximately sixfold, serum IgG3 and IgA were reduced approximately fourfold, and serum IgG2a and IgG2b were reduced approximately threefold.

To ensure that the decrease in serum Ig concentration was not caused by a decrease in the number of secreting cells in TFE3$^-$ animals, we measured the frequency of cells secreting each isotype from unstimulated splenocytes, using an ELISA spot assay (15). This assay detects individual secreting cells but is not quantitative for the amount of Ig being secreted by each cell. The only significant difference observed was a twofold increase in the number of IgM-secreting cells in TFE3$^-$ B cells. Numbers of IgG1- and IgE-secreting cells were too low to be measured, and all other isotypes showed no significant differences (data not shown). We conclude there is not a significant decrease in the number of Ig-secreting cells in the spleens of TFE3$^-$ chimeras but that TFE3$^-$ B cells have a defect in their ability to secrete normal amounts of Ig.

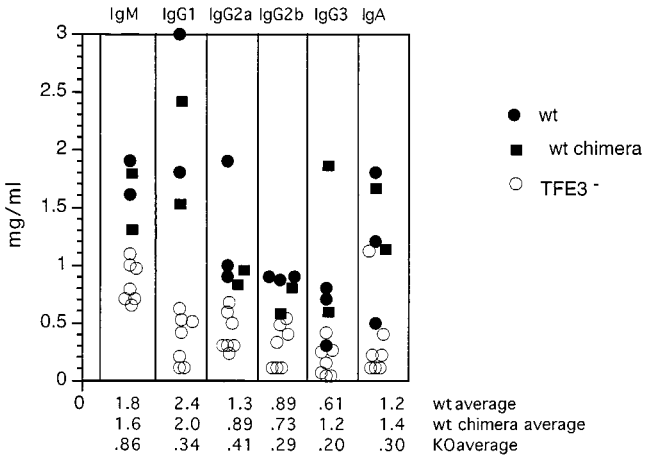

FIG. 5. Quantification of serum Ig concentration by ELISA. Isotypes are indicated on top of each column. Each point represents the average of two independent assays. Closed circles, C57B6/CBA F_1 wild-type (wt) sera; closed boxes; wild-type chimeras; open circles, TFE3$^-$ chimeric sera. Averages for all animals are shown at the bottom of each column. KO, knockout. Results were obtained from two separate experiments. In the first experiment, three mutants and three age-matched wild-type C57B6/CBA mice were processed entirely in parallel. The second experiment was run identically to the first with four mutants and two age-matched wild type chimeras. All mice were 6 to 8 weeks old.

Germinal center morphology is normal in TFE3$^-$ chimeras. Signals which activate B cells to undergo isotype switching and terminal differentiation to Ig-secreting plasma cells are received in germinal centers, where B cells encounter T cells and antigen (32). The defect in Ig secretion suggested that TFE3$^-$ chimeras might be defective in germinal center formation. In addition, HsAg is an activation marker which is expressed on germinal center B cells. Finally, targeted mutation of CD40 affects B-cell activation and CD23 expression and blocks germinal center formation. Therefore, we analyzed germinal center formation in spleens from control and TFE3$^-$ mice. Frozen sections of spleens were stained for B220 and with peanut agglutinin to visualize germinal centers. Comparison of TFE3$^-$ chimeras to wild-type mice revealed no difference in the size, number, or morphology of germinal centers (data not shown).

TFE3$^-$ splenocytes activated in vitro secrete Ig normally. Since TFE3$^-$ lymphocytes were defective in their ability to secrete Ig and in expression of surface markers associated with activation, we wanted to examine their ability to be activated in vitro. We first activated splenocytes with two mitogens, LPS and CD40L-containing membranes (30). Incorporation of tritiated thymidine was used as a measure of cell division. Figure 6A shows tritium incorporation versus mitogen concentration for CD40L and LPS. Splenocytes derived from TFE3$^-$ and wild-type chimeras showed similar responses to both mitogens.

We also wanted to measure the ability of TFE3$^-$ B cells to secrete Igs following in vitro stimulation. Figure 6B shows levels of Igs present in the media after 4 days of LPS culture. TFE3$^-$ splenocytes expressed all isotypes at levels comparable to those for wild-type- or wild-type chimera-derived splenocytes. Thus, TFE3$^-$ splenocytes are capable of normal Ig secretion when they are activated in vitro with LPS.

The ability of in vitro-activated lymphocytes to express

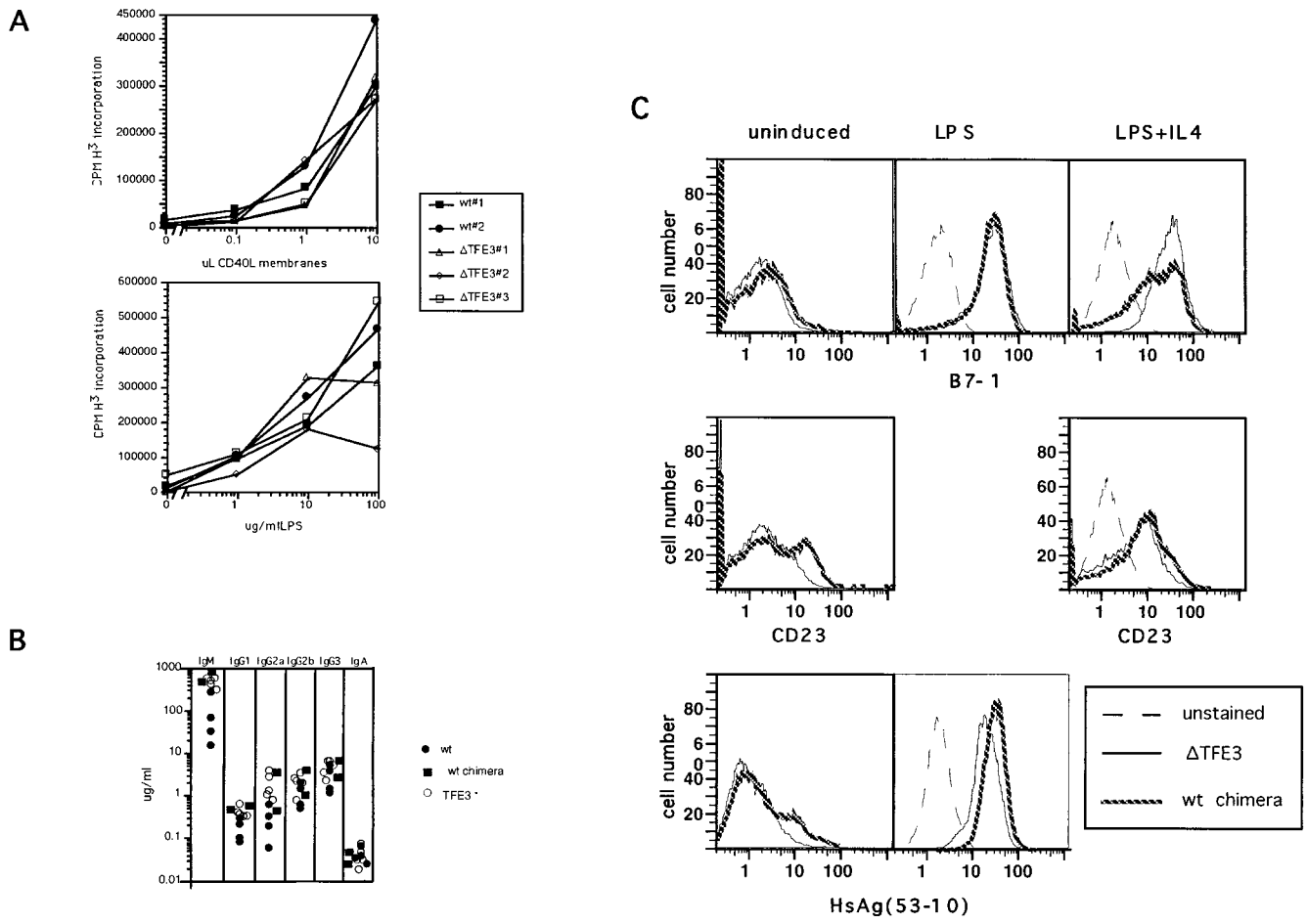

FIG. 6. (A) Tritiated thymidine incorporation in splenocytes cultured with CD40L (top)-containing membranes or LPS (bottom). Wild-type (wt) or TFE3$^-$ splenocytes were cultured in the presence of 0, 0.1, 1, or 10 μl of CD40L membrane stock per 100 μl of culture or 0, 1, 10, and 100 μg of LPS per ml. Total tritium incorporation for each culture is plotted on the *y* axis. Each point represents the average of three separate cultures. All splenocytes were from chimeric animals. (B) Ig concentrations in media from day 4 LPS-cultured splenocytes. (C) Expression of activation markers on B cells following culture with LPS or LPS plus IL-4 for 4 days. Levels of anti-B7-1–TR (first row), anti-CD23-TR (second row), and anti-HsAg (53-10)-FITC (third row) staining are shown. The first column shows uninduced B220$^+$ splenocytes, the second shows LPS-cultured splenocytes, and the third shows LPS–IL-4-cultured splenocytes.

CD23 and HsAg, markers which were decreased in vivo, was also measured. Splenocytes were cultured in vitro for 4 days in the presence of LPS or LPS and IL-4 and then analyzed for the expression of activation markers including HsAg, CD23, and B7-1 (Fig. 6C). B7-1 is clearly induced in both TFE3$^-$ and wild-type cells under both culture conditions. LPS plus IL-4 induced CD23 equally on TFE3$^-$ and control cells. HsAg is induced when both TFE3$^-$ and normal cells are activated by LPS, although the levels are slightly lower on the TFE3$^-$ cells.

TFE3$^-$ T cells can be activated normally in vitro. Although the defects observed in TFE3$^-$ splenocytes occurred in activated B cells, they could be the result of a defect in T-cell activation which affected subsequent activation of B cells by T cells. Therefore, we also examined the ability of TFE3$^-$- and wild-type chimera-derived splenic T cells to be activated. CD28 and CD40L are two markers of T-cell activation (2, 23). Although they were not detectable on significant numbers of T cells in vivo, we were able to induce their expression in vitro with phorbol myristate acetate and ionomycin. We saw significant and indistinguishable increases in the levels of both markers on TFE3$^-$- and wild-type chimera-derived splenic T cells following 12 h of activation in vitro (data not shown). Therefore, TFE3$^-$ T cells can be activated normally in vitro.

DISCUSSION

We have mutated the *mtfe3* gene in ES cells by homologous recombination. TFE3$^-$ ES cells are viable, contribute to somatic tissues in chimeric animals, and are able to reconstitute fully the B- and T-cell compartments in RAG2$^{-/-}$ chimeric animals. The number and distribution of B and T cells in lymphoid compartments of the TFE3$^-$ chimeras are similar to those in wild-type mice and wild-type chimeras. However, TFE3$^-$ chimeras have decreased levels of serum Ig, with particularly low levels of IgG1, IgG3, and IgA. TFE3$^-$ B cells also show abnormally low surface expression of CD23 and HsAg. In contrast, TFE3$^-$ B cells respond normally in vitro to stimulation with LPS and display normal Ig secretion, isotype switching, and surface expression of CD23 and HsAg. Although we have not characterized the full range of T-cell functions in TFE3$^-$ chimeras, we do observe complete reconstitution of all T-cell compartments and normal T-cell activation in vitro. If the TFE3$^-$ cells were important contributors to other somatic tissues in the chimeras, we could postulate that a TFE3-dependent process in a nonlymphoid tissue was responsible for the B-cell phenotype. However, in chimeras made with TFE3$^-$ ES cells in other mouse strains, we always saw less than 30%

ES cell contribution to somatic tissues. The defects observed in the TFE3$^-$ chimeras are most consistent with a failure of B cells to respond normally to in vivo activation signals.

TFE3 is not required for development of many cell lineages, including lymphocytes. Given that TFE3 is expressed ubiquitously in adult tissues (40) and is expressed in ES cells, the transcription factor might perform an essential function. However, we were able to obtain multiple TFE3$^-$ ES clones. Therefore, TFE3 is not required for cell division or viability. Furthermore, we have been able to generate chimeric animals by injection of TFE3$^-$ ES cells into mouse blastocysts. These chimeras showed ES cell contribution to erythrocytes, heart, brain, skeletal muscle, and skin (data not shown). In addition, TFE3$^-$ B and T cells appear to fully reconstitute lymphoid compartments in RAG2$^{-/-}$ chimeric mice. Thus, while TFE3 may play a role in regulating the expression of genes in many tissues, it is not strictly required. It is likely that in many situations the related protein TFEB, or members of the Myc or USF subfamilies, can substitute for TFE3.

A role for TFE3 in B-cell activation. The phenotypic and functional characteristics altered in TFE3$^-$ B cells, Ig secretion and surface expression of CD23 and HsAg, have been used as indicators of both B-cell development and B-cell activation. Surface Ig expression is the hallmark of naive and activated B cells, whereas secretion is characteristic of activated B cells and terminally differentiated plasma cells (34). CD23 is the low-affinity receptor for IgE, and it has been suggested that soluble forms of CD23 function as B-cell growth factors. Expression of CD23 is induced by treatment of isolated splenocytes with IL-4, and addition of LPS induces it even further (16, 42). HsAg has been used as a stage-specific marker during early B-cell development in that it is expressed at high levels in immature and pre-B cells but decreases as cells mature (1). However, HsAg is also induced when B cells are activated and HsAg expression is increased on germinal center B cells (48).

Defective expression of CD23 and HsAg and lack of Ig secretion could be caused by a block in early development of TFE3$^-$ B cells prior to the stage when HsAg is expressed in the bone marrow. This could result in immature B cells which were unable to secrete Ig and would be consistent with the observed lack of HsAg in bone marrow precursors as well as peripheral B cells. However, the phenotype of the peripheral B cells is not consistent with this hypothesis. The peripheral B cells in TFE3$^-$ chimeras have normal expression of many markers which are associated with mature follicular B cells, including high levels of IgD and B220 with lower levels of IgM. In addition, TFE3$^-$ chimeras have germinal centers with normal morphology. Finally, although it is decreased, there is secreted Ig in TFE3$^-$ chimeras, demonstrating the presence of mature B cells. Therefore, although a partial block in B-cell development cannot be entirely ruled out, the data do not support models in which TFE3 is required for an early step in B-cell development.

Another model, more consistent with the data, is that TFE3 is required for B cells to respond normally to activation signals which they receive in vivo. Signals which activate B cells might induce or activate TFE3, which in turn would directly activate transcription of Ig, CD23, and HsAg genes. Alternatively or in addition, TFE3 might be directly required for transcription of genes encoding proteins which participate in signal transduction during B-cell activation. The TFE3-dependent path would ultimately result in expression of CD23 and HsAg and Ig secretion.

There are data to support a direct role for TFE3 in Ig transcription. TFE3 binds μE3 sites which are found in the IgH intronic enhancer, some heavy-chain promoters, and the kappa intronic enhancer (35). Mutation of the μE3 site in the IgH intronic enhancer results in a 50% reduction in enhancer activity (49). Furthermore, TFE3, but not other μE3-binding proteins like USF, is able to mediate a synergistic interaction between enhancer and promoter μE3 sites (3). Finally, TFE3 transdominant negative constructs can repress the activity of isolated μE3 sites in vivo (10). Thus, the loss of TFE3 could easily result in a decrease in expression from the Ig genes. There is currently no known requirement for TFE3 or μE3 sites in transcription of the CD23 and HsAg genes. Promoters for both genes have been cloned and partially characterized, but no functionally important μE3 sites have been identified (17, 37, 51). However, such sites may be identified in the future. A direct role for TFE3 in the transcription of Ig, CD23, and HsAg genes is consistent with the loss of HsAg expression at two distinct stages of B-cell development, mature activated B cells and pre-B/immature B cells, in TFE3$^-$ mice. It also explains why some markers of B-cell maturation or activation but not others are affected. A direct role for TFE3 in the transcription of CD23, HsAg, and Ig would explain why TFE3$^-$ B cells are unable to upregulate these genes in vivo.

An important prediction of this model is that TFE3 would be induced or activated by B-cell activation. Consistent with this prediction, we have observed increases in TFE3 mRNA levels following LPS activation of pre-B cells (39a). If part of the signaling machinery which upregulates HsAg in mature B cells also regulates the gene in the bone marrow, this would also explain why TFE3$^-$ pre-B cells have decreased HsAg levels.

However, TFE3 could also be required for the transcription of a gene encoding a protein which is a component of the signal transduction machinery leading to increased Ig, CD23, and HsAg expression following B-cell activation. The fact that apparently normal germinal centers are present in TFE3$^-$ chimeras suggests that if this is so, the protein must not be required for many other aspects of B-cell activation. However, it is interesting that CD20 is a good candidate for such a signal transduction protein. The CD20 promoter has a functionally important μE3 site, and CD20 has been implicated in B-cell activation (30a). Currently no monoclonal antibodies against murine CD20 are available; therefore, we were not able to test CD20 expression on the TFE3$^-$ B cells.

An intriguing feature of the TFE3$^-$ B cells is the contrast between their failure to respond normally to activation in vivo and their ability to respond normally to LPS activation in vitro. These data emphasize that there are important differences between activation in vitro with LPS and normal signaling in vivo. It may be that LPS signaling in vitro delivers a stronger signal than in vivo signaling so that other members of the TFE3 family or other transcription factors acting on the same genes may be sufficiently induced to circumvent the lack of TFE3. Alternatively, LPS activation may use signaling pathways different from those used in vivo, and these paths might be TFE3 independent. It is interesting that in recent studies of OCA-B-deficient mice, one group found that OCA-B-deficient B cells also fail to secrete normal levels of Ig in vivo but secrete normally in response to LPS activation in vitro (31, 43). In contrast, mutation of the IgH 3′ enhancer leads to a more severe effect on class switching in vitro than in vivo (14). These data, in conjunction with our results, favor a model of different signaling pathways between in vivo activation and LPS activation in vitro.

Phenotypes of mice lacking genes for Ig transcription factors. In the last several years, gene targeting experiments have been used to help understand the role of several transcription factors and transcriptional control elements in the immune system (36). A few, like the IgH enhancer-, the OCA-B-, and

the NF-κB-targeted mutations have affected Ig expression in ways which might have been predicted from the known function of the gene (13, 31, 43, 47). However, two phenotypes which complicate our ability to understand the roles of other genes in the immune system have appeared. A number of mutations have resulted in a complete block to B-cell development or even a block to lymphocyte development. Examples include *ikaros* and genes for BSAP, Pu.1, and E2A (5, 21, 44, 50). Conversely, other transcription factor mutations like c-*jun* and N-*myc* mutations have had no obvious effect on B-cell development (12, 33).

Mutation of the *mtfe3* gene results in a unique B-cell phenotype which is most consistent with a defective response to in vivo activation signals. The expression of serum Ig is decreased, which might be expected given the requirement for μE3 sites in the IgH and Ig kappa-chain genes. However, other surface markers, CD23 and HsAg, which are not known to be regulated by TFE3, are also decreased in vivo. Whether this represents a problem in the signaling cascade which upregulates these genes upon B-cell activation and/or a problem with their transcription will require further evaluation. The TFE3$^-$ mice should allow identification of other TFE3-dependent genes, and further study of TFE3$^-$ B cells should lead to further understanding of mechanisms responsible for activation of mature B cells and stimulation of Ig secretion.

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