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The Binding Site for TRAF2 and TRAF3 but Not for TRAF6 Is Essential for CD40-Mediated Immunoglobulin Class Switching

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Summary

To define the role of TRAF proteins in CD40-dependent isotype switching in B cells, we introduced wild-type (WT) and mutant CD40 transgenes that lacked the binding motifs for TRAF6 (CD40ΔTRAF6), TRAF2 and TRAF3 (CD40ΔTRAF2/3), or both (CD40ΔTRAFs) into B cells of CD40$^{-/-}$ mice. The in vivo isotype switch defect in CD40$^{-/-}$ mice was fully corrected by WT and CD40ΔTRAF6, partially by CD40ΔTRAF2/3, and not at all by CD40ΔTRAFs transgenes. CD40-mediated isotype switching, proliferation, and activation of p38, JNK, and NFκB in B cells were normal in WT and CD40ΔTRAF6 mice, severely impaired in CD40ΔTRAF2/3, and absent in CD40ΔTRAFs mice. These results suggest that binding to TRAF2 and/or TRAF3 but not TRAF6 is essential for CD40 isotype switching and activation in B cells.

Introduction

Immunoglobulin class switching in the course of the response to T-dependent antigens involves deletional switch recombination and requires two signals (Bacharier et al., 1998). One signal is delivered by cytokines which target specific C heavy chain (CH) locus for switch recombination by causing their transcription. The other signal is delivered by ligation of the B cell surface antigen CD40.

CD40 is a member of the TNFR family of surface molecules and is expressed on all B cells (Banchereau et al., 1994). Its ligand (CD40L) is expressed transiently on activated T cells. The critical role of CD40-CD40L interactions in isotype switching is illustrated by several observations: anti-CD40 mAb bypasses the requirement for T cells in IL-4-driven IgE isotype switching in vitro (Jabara et al., 1990); mutations in the CD40L underlie the isotype switch defect in patients with X-linked HyperIgM syndrome (Fuleihan et al., 1993); and mice deficient in CD40 or CD40L fail to undergo isotype switching in response to T-dependent (TD) antigens (Castigli et al., 1994; Kawabe et al., 1994; Xu et al., 1994).

The intracellular domain of human CD40 has a binding site for Jak3 in its proline-rich Box 1 membrane proximal region (aa 222–229) (Hanissian and Geha, 1997), a TRAF6 binding KxxPxE motif (aa 231–236) (Pullen et al., 1998), and a PxQxT motif (aa 250–254), that binds TRAF2 and TRAF3 (Cheng et al., 1995; Lee et al., 1999; Pullen et al., 1998; Sutherland et al., 1999). TRAF2 and TRAF3, respectively, bind TRAF1 and TRAF5. The intracellular domain of murine CD40, like that of human CD40, has a proline-rich membrane proximal region (aa 222–230), a conserved TRAF6 binding motif, RQDPQE (aa 234–239), and a 32 aa stretch (aa 247–278) that is 100% homologous to aa 246–277 of human CD40 and that contains the TRAF2 and TRAF3 binding motif, PxQxT (aa 251–255).

CD40 ligation in B cells causes activation of the MAP kinases JNK and p38 (Li et al., 1996) and of the transcription factor NFκB (Berberich et al., 1994; Iciek et al., 1997). Studies in B cell lines suggest that TRAF proteins play an important role in CD40 signaling. TRAF2 lacking an amino-terminal RING finger domain is a dominant-negative inhibitor of CD40 activation of NFκB (Rothe et al., 1995). TRAF2 and TRAF6 synergize in NFκB activation (Lee et al., 1999; Tsukamoto et al., 1999). TRAF6, TRAF2, and TRAF3 all have been reported to be involved in JNK and p38 activation by CD40 (Grammer et al., 1998; Leo et al., 1999a; Reinhard et al., 1997; Song et al., 1997; Sutherland et al., 1999).

The role of TRAF proteins in CD40-mediated isotype switching is not well defined. Both TRAF6 and TRAF2/3 binding sites are important for NFκB-dependent CD40-mediated activation of the Cγ1 and Cε promoters (Leo et al., 1999b). Although isotype switching in B cells of TRAF3$^{-/-}$ and TRAF6$^{-/-}$ mice is defective, these mice die perinatally and have generalized defects in T and B cell activation, making the findings hard to interpret (Lomaga et al., 1999; Naito et al., 1999; Xu et al., 1996). TRAF2$^{-/-}$ mice also die perinatally, but TRAF2$^{-/-}$ TNFR1$^{-/-}$ mice are viable and have impaired T helper-dependent antibody responses (Nguyen et al., 1999; Yeh et al., 1997). B cells from TRAF5$^{-/-}$ mice switch to IgG in response to TD antigens, although affinity maturation is impaired and their B cells show defective production of IgG in response to stimulation with anti-CD40+IL-4 (Nakano et al., 1999). Isotype switching is intact in TRAF1$^{-/-}$ mice (Tsitsikov et al., 2001).

To examine the role of TRAF proteins in CD40 isotype switching and to circumvent the potential deleterious effects of the disruption of TRAF genes on immune cells, we examined the ability of transgenes coding for CD40 mutants that lack one or both TRAF binding sites to reconstitute isotype switching in CD40$^{-/-}$ B cells.

Results

Reconstitution of CD40$^{-/-}$ Mice with CD40 Transgenes

WT and mutant murine CD40 constructs were generated as illustrated in Figure 1A. CD40 Δ231–246, designated CD40ΔTRAF6, lacks the sequence ^{234}RQDPQE^{239}, which shows homology to the TRAF6 binding motif KxxPxE in human CD40 (Pullen et al., 1998). CD40 T255A, designated CD40ΔTRAF2/3, carries a T255A point mutation in the

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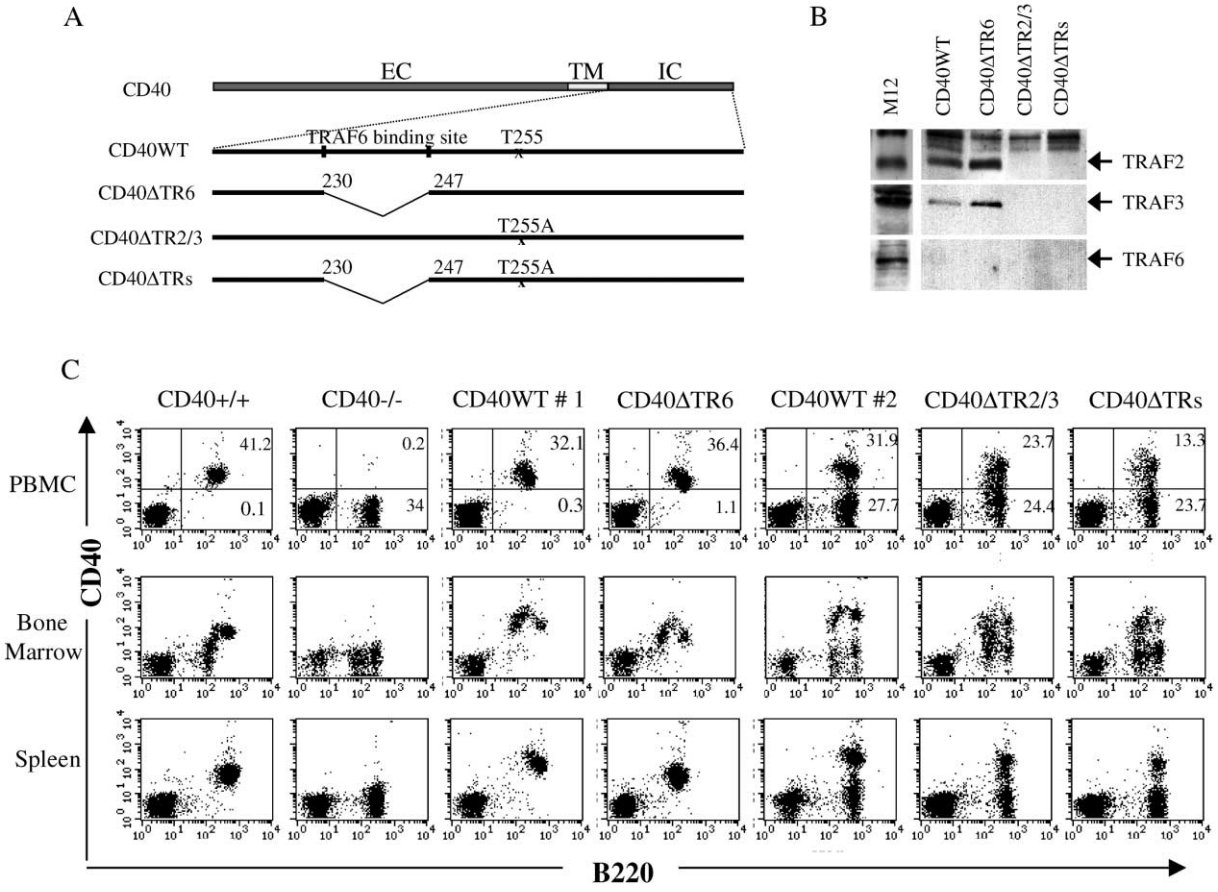

Figure 1. Characterization of the CD40 Mutants

(A) Schematic representation of WT CD40 and mutant transgenes used: WT (CD40WT), CD40ΔTRAF6 (CD40ΔTR6), CD40ΔTRAF2/3 (CD40ΔTR2/3), and CD40ΔTRAFs (CD40ΔTRs).

(B) Association of TRAF proteins with CD40 mutants. Left panel: expression of TRAF proteins in M12 B cells; right panel: GST-mCD40 pull-down assay with M12 B cell lysates. Bound proteins were probed for the presence of TRAF proteins.

(C) Representative FACS analysis of CD40 surface expression in PBMCs, bone marrow, and spleen of 8- to 12-week-old transgenic mice after staining with anti-B220-FITC and anti-CD40-PE.

^{251}PxQxT^{255} sequence, which corresponds to the T254A mutation in human CD40 that abolishes TRAF2 and TRAF3 binding (Pullen et al., 1998). CD40Δ231–246/T255A, designated CD40ΔTRAFs, carries both mutations present in ΔTRAF6 and ΔTRAF2/3.

To determine if the CD40 mutants lacked the expected associations with TRAF molecules, GST-WT CD40 and mutant fusion proteins were examined for their capacity to bind TRAF proteins in lysates of the murine B cell line M12, that expresses TRAF2, TRAF3, and TRAF6 (Figure 1B, left panel). GST-CD40WT and GST-CD40ΔTRAF6 associated with TRAF2 and TRAF3. In contrast, GST-CD40ΔTRAF2/3 and GST-CD40ΔTRAFs did not. GST-CD40WT and all three mutant fusion proteins showed no detectable association with TRAF6 (Figure 1B, right panel). Furthermore, GST-CD40WT did not bind TRAF6 from lysates of HEK 293 cells that overexpressed mTRAF6 (data not shown).

mCD40 and mTRAF6 interact in the yeast two-hybrid assay (Ishida et al., 1996). Our failure to detect their interaction in a pull-down assay may be due to the fact that it is weak or unstable, and does not rule out a role for TRAF6 binding in CD40 signaling. Therefore, constructs containing WT and all three mutant CD40 cDNAs were used to create transgenic mice which were bred on the CD40$^{-/-}$ background. Lines that expressed CD40 only on B220^{+} cells and in amounts comparable to those expressed in CD40$^{+/+}$ mice were selected for study. For each of the constructs, at least two transgenic lines derived from separate founders were studied and similar results were obtained, with the exception of CD40ΔTRAF6 where one out of four lines studied gave aberrant results and was excluded from analysis.

FACS analysis of CD40 expression on peripheral blood lymphocytes from representative lines of reconstituted CD40$^{-/-}$ mice is shown in Figure 1C. Of the two lines reconstituted with WT CD40 studied, one line (CD40WT#1) expressed CD40 on all B cells and was used as a control for CD40ΔTRAF6 mice which expressed CD40 on all B cells. The second line (CD40WT#2) expressed CD40 on ~50% of the B cells. It was used as a control for CD40ΔTRAF2/3 and CD40ΔTRAFs mice which expressed CD40 on 45%–50% and ~30%–35% of their B cells, respectively. The mean fluorescence intensity of

CD40 expression on B cells from all mouse lines studied was comparable to that of B cells from $CD40^{+/+}$ mice. CD40 expression has remained stable for more than three generations in each of the lines analyzed.

Phenotypic Analysis of Lymphoid Organs

Like $CD40^{-/-}$ mice (Castigli et al., 1994; Kawabe et al., 1994), bone marrow (BM) from all transgenic mouse lines had normal cellularity and normal percentage and expression profile of IgM^+ and $B220^+$ cells (data not shown). This suggests that introduction of the transgenes did not interfere with B cell development. In all transgenic lines, the fraction of $B220^+$ BM cells that expressed CD40 matched that in peripheral blood (Figure 1C). In normal BM, CD40 is highly expressed on $B220^{high}$ cells but is absent or poorly expressed on $B220^{low}$ cells. In contrast, in all transgenic lines, both $B220^{low}$ and $B220^{high}$ BM cells expressed high levels of CD40. This likely reflects the fact that the $E\mu V_H$ promoter, used to drive transgene expression, is active at earlier stages of B cell development than the endogenous CD40 gene promoter, which is poorly active in pre-B cells and immature B cells (Castigli et al., 1996).

Spleens were normal in size and cellularity in all transgenic mice. We previously reported that the number and phenotype of B cells in the spleen of $CD40^{-/-}$ mice are normal as assessed by staining for B220 and IgM (Castigli et al., 1994). The numbers of $CD3^+$ and $B220^+$ cells and the expression of IgM on $B220^+$ cells in the spleens of all transgenic mice were similar to those of $CD40^{+/+}$ mice (data not shown). In all lines, the expression of CD40 on $B220^+$ cells in spleen and peripheral blood was comparable (Figure 1C). The thymus was normal in size, architecture, and numbers of CD4, CD8, and CD3 positive cells in all transgenic lines (data not shown).

Serum Immunoglobulin Levels

$CD40^{-/-}$ mice have decreased serum IgG1 and IgG2a, undetectable IgE, and normal or modestly decreased IgM, IgG2b, IgG3, and IgA (Castigli et al., 1994; Kawabe et al., 1994). Introduction of the CD40WT transgene in the $CD40^{-/-}$ background resulted in normalization of serum IgG1, IgG2a, and IgE levels, both in the line where CD40 was expressed on all B cells, CD40WT#1, and in the line where CD40 was expressed on ~50% of the B cells, CD40WT#2 (Figure 2). Reconstitution with CD40Δ TRAF6 resulted in normalization of serum IgG1, IgG2a, and IgE to levels not significantly different from those of CD40WT#1 controls. Reconstitution with CD40Δ TRAF2/3 partially restored serum IgG1 and IgE levels, but IgG2a levels were poorly restored. Serum IgG1, IgG2a, and IgA were significantly lower in CD40ΔTRAF2/3 mice than in CD40WT#2 controls. Reconstitution with CD40ΔTRAFs completely failed to restore serum IgG1, IgG2a, and IgE levels. Serum IgG1, IgG2a, IgA, and IgE were significantly lower in CD40ΔTRAFs mice than in CD40WT#2 controls. Serum levels of IgG2b and IgG3 were in the normal range in all reconstituted mice.

Antibody Response and Germinal Center Formation to the TD Antigen KLH

As previously reported (Kawabe et al., 1994), $CD40^{-/-}$ mice made normal IgM and IgG3 antibody responses to KLH, poor IgG2a and IgG2b responses, and no detectable IgG1 and IgE responses (Figure 3A). Mice reconstituted with CD40WT mounted a normal antibody response comparable to that of $CD40^{+/+}$ mice in all Ig classes examined, regardless of whether CD40 was expressed on all B cells (CD40WT#1) or on ~50% of the B cells (CD40WT#2). CD40ΔTRAF6 normalized the antibody response to KLH. CD40ΔTRAF2/3 reconstituted IgG2b and IgE responses to normal, but IgG1 and IgG2a responses were poorly restored (Figure 3A). CD40Δ TRAFs reconstituted the IgG2b response but not the IgG1, IgG2a, and IgE responses. All three CD40 mutant transgenic mice made normal IgM and IgG3 antibody responses.

Spleens from KLH-immunized $CD40^{+/+}$ mice revealed the presence of multiple germinal centers (Figure 3B). These were absent from spleens of nonimmunized mice (data not shown) and from spleens of immunized $CD40^{-/-}$ mice, as previously reported (Castigli et al., 1994; Kawabe et al., 1994). Mice reconstituted with CD40WT had prominent germinal centers in their spleens with numbers similar to those found in $CD40^{+/+}$ mice, regardless of whether CD40 was expressed on all B cells (CD40WT#1) or on only ~50% of the B cells (CD40WT#2). Reconstitution with CD40ΔTRAF6 fully restored germinal center formation to normal. Germinal center formation was partially restored in CD40ΔTRAF2/3 mice. These mice had smaller and less developed germinal centers than control CD40WT#2 mice. Germinal centers were absent from spleens of CD40ΔTRAFs mice.

CD40-Mediated Isotype Switching In Vitro

CD40 ligation in the presence of IL-4 causes isotype switching to IgE and IgG1 (Hasbold et al., 1998; Jabara et al., 1990). Since CD40ΔTRAF2/3 and CD40ΔTRAFs mice expressed CD40 on only a fraction of their B cells, $CD40^+$ B cells were purified from spleens and were stimulated with hamster IgM anti-mouse CD40 and IL-4. The purification procedure by itself did not activate the B cells because unstimulated purified $CD40^+$ B cells did not proliferate nor secrete IgE or IgG1 (see legend of Figure 4). B cells from mice reconstituted with CD40WT or with CD40ΔTRAF6 secreted comparable amounts of IgE and IgG1 in response to anti-CD40+IL-4 as B cells from $CD40^{+/+}$ mice (Figure 4A). In contrast, B cells from CD40ΔTRAF2/3 and CD40ΔTRAFs mice secreted very little or no detectable amounts of IgE and IgG1 following stimulation with anti-CD40+IL-4 (Figure 4A). This was not due to a general impairment in the ability of these B cells to undergo isotype switching or to respond to IL-4, because they secreted normal amounts of IgE and IgG1 in response to LPS+IL-4 (Figure 4A).

CD40 ligation synergizes with IL-4 in activating a number of molecular events that result in class switch recombination (CSR) to IgE and IgG1. These include expression of $C\epsilon$ and $C\gamma1$ germline transcripts (GLTs), expression of the gene for activation-induced deaminase (AID), followed by $S\mu \rightarrow S\epsilon$ and $S\mu \rightarrow S\gamma1$ deletional switch recombination and expression of mature $I\mu$-$C\epsilon$ and $I\mu$-$C\gamma1$ transcripts (Manis et al., 2002). The ability of CD40 mutants to activate these events was examined in B cells from transgenic mice. CD40 ligation and IL-4 each induced expression of $C\gamma1$ GLT and synergized in

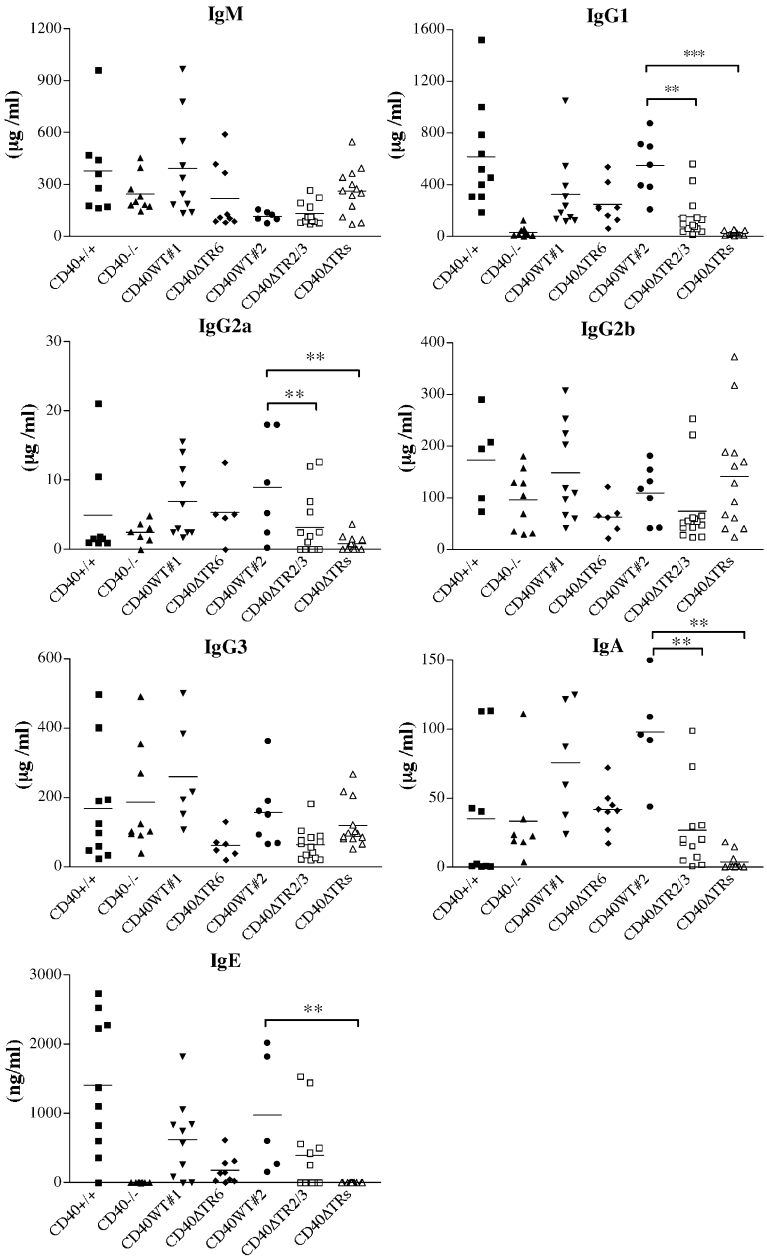

Figure 2. Serum Immunoglobulins in CD40$^{-/-}$ Mice Reconstituted with CD40 Transgenes

Serum immunoglobulin levels from nonimmunized 12-week-old CD40$^{-/-}$ mice reconstituted with CD40 transgenes, CD40$^{+/+}$ littermates (+/+), and CD40$^{-/-}$ mice (−/−). Mann Whitney test was used to compare CD40ΔTR6 to CD40WT#1 and CD40ΔTR2/3 and CD40ΔTRs to CD40WT#2 mice. Significantly different values are indicated: * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

inducing Cγ1 GLT in normal CD40$^{+/+}$ B cells (Figure 5A). CD40 ligation synergized with IL-4 in inducing Cε GLT in normal B cells, but neither stimulus induced Cε GLT by itself at the time point examined (4 days poststimulation). B cells from CD40WT and CD40ΔTRAF6 mice expressed Cγ1 and Cε GLT comparably to normal B cells. In contrast, CD40 ligation failed to induce Cγ1 GLT and to upregulate IL-4-induced Cγ1 GLT in B cells from CD40ΔTRAF2/3 and CD40ΔTRAFs mice. CD40 ligation and IL-4 induced weak expression of Cε GLT in B cells from CD40ΔTRAF2/3 mice and no detectable Cε GLT expression in B cells from CD40ΔTRAFs mice. B cells from all mice expressed comparable amounts of Cγ1 GLT in response to IL-4 and of Cγ1 and Cε GLT in response to LPS+IL-4.

CD40 ligation and IL-4 each induced weak expression of AID, but synergized to induce strong AID gene expression in B cells from CD40$^{+/+}$ mice. B cells from CD40WT and CD40ΔTRAF6 mice expressed AID comparably to normal CD40$^{+/+}$ B cells (Figure 5A). CD40 ligation induced weak expression of AID in B cells from CD40ΔTRAF2/3 and CD40ΔTRAFs mice and poorly upregulated AID expression induced by IL-4 in these B cells. All B cells expressed comparable amounts of AID following stimulation with IL-4 and LPS+IL-4.

CD40 ligation synergized with IL-4 in causing Sμ→Sγ1 and Sμ→Sε deletional switch recombination (Figure 5B) and in inducing mature Iμ-Cγ1 and Iμ-Cε transcripts in B cells from CD40$^{+/+}$ mice (Figure 5C), but neither stimulus induced these events by itself (data not shown).

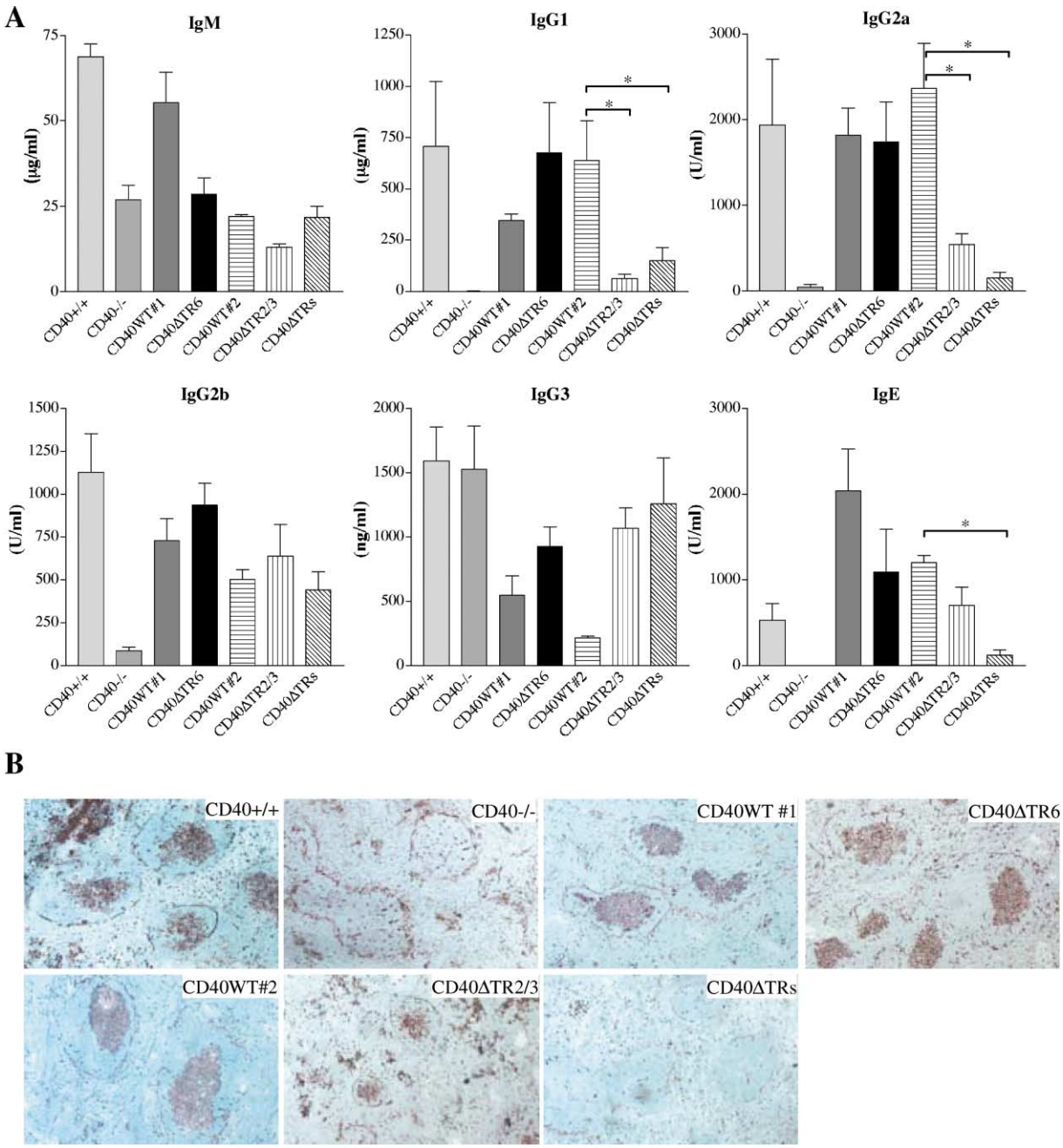

Figure 3. Antibody Response and Germinal Center Formation in Response to KLH

(A) IgM, IgG subclasses, and IgE antigen-specific antibody responses to KLH following immunization with the TD antigen in CD40$^{-/-}$ mice reconstituted with CD40 transgenes, CD40$^{+/+}$ littermates (+/+), and CD40$^{-/-}$ mice (−/−). Statistical analysis was performed as in Figure 3.
(B) Spleen sections (×40) from mice immunized with KLH examined for PNA binding by immunohistochemistry.

Deletional switch recombination and expression of mature transcripts in response to anti-CD40+IL-4 were normal in B cells from CD40WT and CD40ΔTRAF6 mice. In contrast, they were markedly diminished in B cells from CD40ΔTRAF2/3 mice and undetectable in B cells from CD40ΔTRAFs mice. All B cells underwent Sμ→Sγ1 and Sμ→Sε deletional switch recombination and expressed mature Iμ-Cγ1 and Iμ-Cε in response to LPS+IL-4.

CD40-Mediated B Cell Proliferation

CD40 ligation causes B cell proliferation (Banchereau et al., 1994). B cells from mice reconstituted with CD40WT or with CD40ΔTRAF6 proliferated in response to CD40 crosslinking to an extent comparable to that of B cells from CD40$^{+/+}$ mice. In contrast, CD40^{+} B cells from CD40ΔTRAF2/3 and CD40ΔTRAFs mice proliferated very poorly to stimulation with anti-CD40-IgM (Fig-

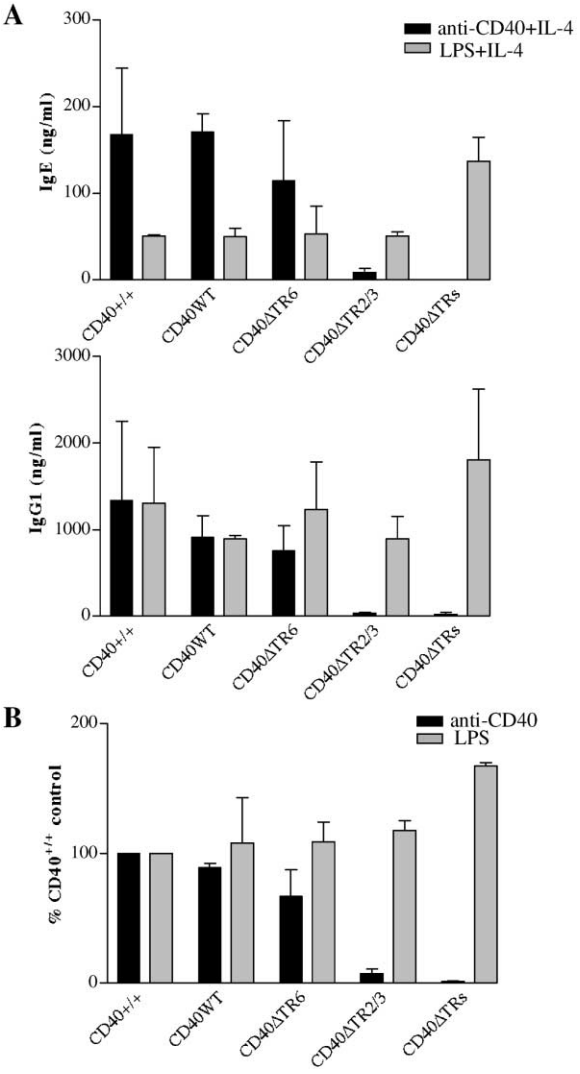

Figure 4. Immunoglobulin Synthesis and B Cell Proliferation In Vitro

(A) Net IgG1 and IgE synthesis by $CD40^+$ B cells. IgG1 and IgE in supernatants of unstimulated cells were <4 and <7 ng/ml, respectively, and were subtracted from values of stimulated cultures. Results shown are the mean of three experiments.

(B) Proliferation of $CD40^+$ B cells. Results are derived from three experiments and expressed as percentage of the proliferation of B cells from $CD40^{+/+}$ mice (mean $\pm$ SD cpm for anti-CD40 = 27,279 $\pm$ 15,034 and for LPS = 74,257 $\pm$ 16,652). B cells cultured in media alone incorporated <1100 cpm.

ure 4B). This was not due to a general impairment in B cell proliferation, because all B cells proliferated normally in response to LPS.

CD40-Mediated Upregulation of CD23, CD54, and CD86 Expression on B Cells

CD40 ligation upregulates the expression of a number of surface markers on B cells, including CD23, CD54, and CD86 (Banchereau et al., 1994). $CD40^+$ B cells from mice reconstituted with CD40WT or CD40ΔTRAF6 upregulated CD23, CD54, and CD86 expression following sCD40L stimulation to an extent comparable to that of B cells from $CD40^{+/+}$ mice (Figure 6). CD40-mediated upregulation of these markers was preserved in $CD40^+$ B cells from CD40ΔTRAF2/3 mice. In contrast, it was severely diminished in $CD40^+$ B cells from CD40ΔTRAFs mice. $CD40^+$ B cells from all transgenic lines upregulated CD23, CD54, and CD86 expression normally in response to LPS (Figure 6).

CD40 Activation of NFκB in B Cells

CD40 ligation in B cells causes the nuclear translocation and activation of NFκB (Banchereau et al., 1994). Electromobility shift assay (EMSA) was used to assess the translocation of NFκB following CD40 ligation in purified $CD40^+$ B cells. Figure 7A shows that addition of nuclear extracts from unstimulated $CD40^{+/+}$ B cells to a radiolabeled oligonucleotide encoding an NFκB consensus sequence derived from the HIV1 gene gives rise to a retarded complex. This complex was inhibited by an excess of cold self-competitor but not of irrelevant AP-1 binding consensus sequence oligonucleotide (data not shown). In three experiments, CD40 ligation on B cells from $CD40^{+/+}$ mice resulted in upregulation of the intensity of the specific nuclear complex (1.9 $\pm$ 0.4-fold). CD40-mediated nuclear translocation of NFκB in B cells from CD40WT mice (2.1 $\pm$ 0.1-fold) and CD40ΔTRAF6 mice (2.1 $\pm$ 0.9-fold) was comparable to that in B cells of $CD40^{+/+}$ mice. In contrast, it was impaired in B cells from CD40ΔTRAF2/3 (0.9 $\pm$ 0.05-fold, $p < 0.05$ compared to CD40WT mice) and CD40ΔTRAFs mice (0.9 $\pm$ 0.1-fold, $p < 0.05$) (Figure 7A). The latter had consistently increased baseline nuclear NFκB content.

CD40 ligation in B cells causes NFκB-dependent upregulation of bcl-xL and A20 gene expression (Dadgostar et al., 2002). Expression of both genes was normally upregulated in B cells of CD40WT and CD40ΔTRAF6 mice. CD40 ligation upregulated bcl-xL and A20 expression in B cells from CD40ΔTRAF2/3 mice but not in B cells from CD40ΔTRAFs mice (Figure 7C). The latter had elevated baseline levels of bcl-xL and A20 mRNA, which correlated with their increased baseline nuclear NFκB content but were lower than the levels observed in CD40-stimulated control B cells from CD40WT#2 mice.

CD40 Activation of MAP Kinases in B Cells

CD40 ligation in resting mouse B cells results in the phosphorylation and activation of the MAP kinases p38 and JNK but not Erk (Aicher et al., 1999 and our unpublished data). CD40-mediated phosphorylation of p38 and JNK in $CD40^+$ B cells from CD40WT and CD40ΔTRAF6 mice was comparable to that in B cells of $CD40^{+/+}$ mice. In contrast, it was not detectable in B cells from CD40ΔTRAF2/3 and CD40ΔTRAFs mice (Figure 7B).

CD40 ligation causes p38-dependent downregulation of expression of a number of genes that include diacylglycerol kinase α (DAG kinase α) in normal B cells (Dadgostar et al., 2002). Expression of DAG kinase α was normally downregulated in B cells of CD40WT and CD40ΔTRAF6 mice. In contrast, CD40 ligation caused no detectable downregulation of DAG kinase α expression in B cells from CD40ΔTRAF2/3 and CD40ΔTRAFs mice (Figure 7C).

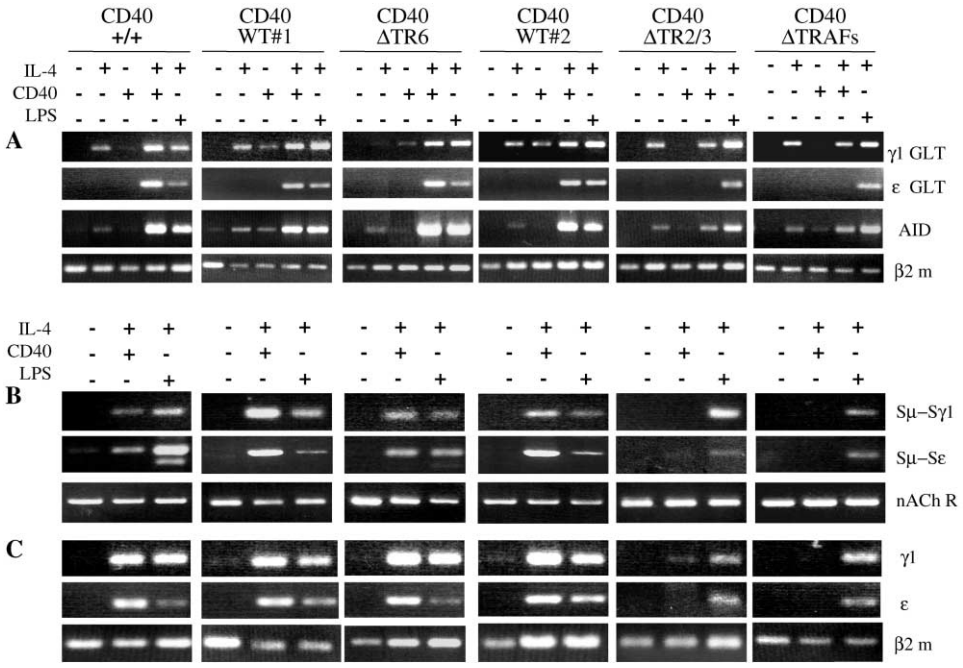

Figure 5. Molecular Events in Isotype Switching

(A) Expression of Cε and Cγ1 germline and AID transcripts measured by RT-PCR in B cells stimulated for 4 days with IL-4, CD40, CD40+IL-4, and LPS+IL-4 as control.

(B) Sμ→Sε and Sμ→Sγ1 deletional switch recombination measured by DC-PCR at day 6.

(C) Expression Iμ-Cε and Iμ-Cγ1 mature transcripts by RT-PCR at day 4. Similar results were obtained in three independent experiments.

Discussion

Our results suggest that binding to TRAF2 and/or TRAF3 but not TRAF6 is essential for CD40-mediated isotype switching and activation of murine primary B cells.

The transgene reconstitution strategy we have used to dissect the role of TRAF proteins in CD40-dependent isotype switching was validated by the observation that expression of CD40WT transgene in B cells of $CD40^{-/-}$ mice completely restored CD40-dependent immunoglobulin class switching and B cell activation (Figures 2–7). In vivo immunoglobulin class switching was normalized even when CD40WT was expressed on only ~50% of the B cells (Figures 2 and 3), suggesting that CD40 expression on a fraction of B cells is sufficient to support normal in vivo Ig class switching.

GST-mCD40 bound TRAF2 and TRAF3 in mouse B cell lysates (Figure 1B). However, despite the presence of a conserved TRAF6 binding sequence in mCD40 and the fact that mCD40 and mTRAF6 interact in the yeast two-hybrid assay (Ishida et al., 1996), we were unable to demonstrate an interaction between GST-mCD40 and TRAF6. Binding of TRAF6 to GST-hCD40 has not been consistently demonstrated, suggesting that their interaction is weak and may require a conformational change in CD40 that could be mimicked by the overexpression of trimerized CD40 intracellular domains (Lee et al., 1999; Leo et al., 1999a; Werneburg et al., 2001).

Introduction into B cells of $CD40^{-/-}$ mice of CD40ΔTRAF6, in which the TRAF6 binding motif is deleted but which retains the ability to bind TRAF2 and TRAF3, restored CD40-dependent isotype switching in vivo and in vitro, as well as CD40 induction of proliferation, upregulation of CD23, CD54, and CD86 expression, and activation of NFκB, JNK, and p38 (Figures 2–7). These results suggest that binding of TRAF6 to CD40 may not be necessary for isotype switching and activation of primary mouse B cells. Moreover, our finding that mice reconstituted with CD40ΔTRAF2/3, in which the TRAF6 binding site is intact, have severe defects in CD40-mediated isotype switching, proliferation, and activation of NFκB, JNK, and p38 suggests that binding of TRAF6 to CD40 is not sufficient for CD40 triggering of these events.

Our results are consistent with previous findings that TRAF6 plays a minimal role in CD40-mediated surface marker expression and NFκB activation in a B cell line (Jalukar et al., 2000). In contrast, studies in B cell lines that overexpress dominant-negative (DN) TRAF6 mutants (Jalukar et al., 2000), or trimerized mutant CD40 intracellular domains (Werneburg et al., 2001), have implicated TRAF6 in CD40 activation of NFκB. However, overexpression of DN TRAF6 may affect the function of other TRAFs, and overexpression of trimerized CD40 intracellular domains may not mimic physiologic signaling by transmembrane CD40. TRAF6 has also been implicated in CD40 signaling based on studies with B cells from $TRAF6^{-/-}$ mice (Lomaga et al., 1999; Naito et al., 1999). However, TRAF6 deficiency may interfere generally with B cell development and function. It remains possible that TRAF6 plays a role in CD40-mediated B cell activation independently of its binding to CD40.

Reconstitution of B cells from $CD40^{-/-}$ mice with the CD40ΔTRAF2/3 transgene partially restored CD40-depen-

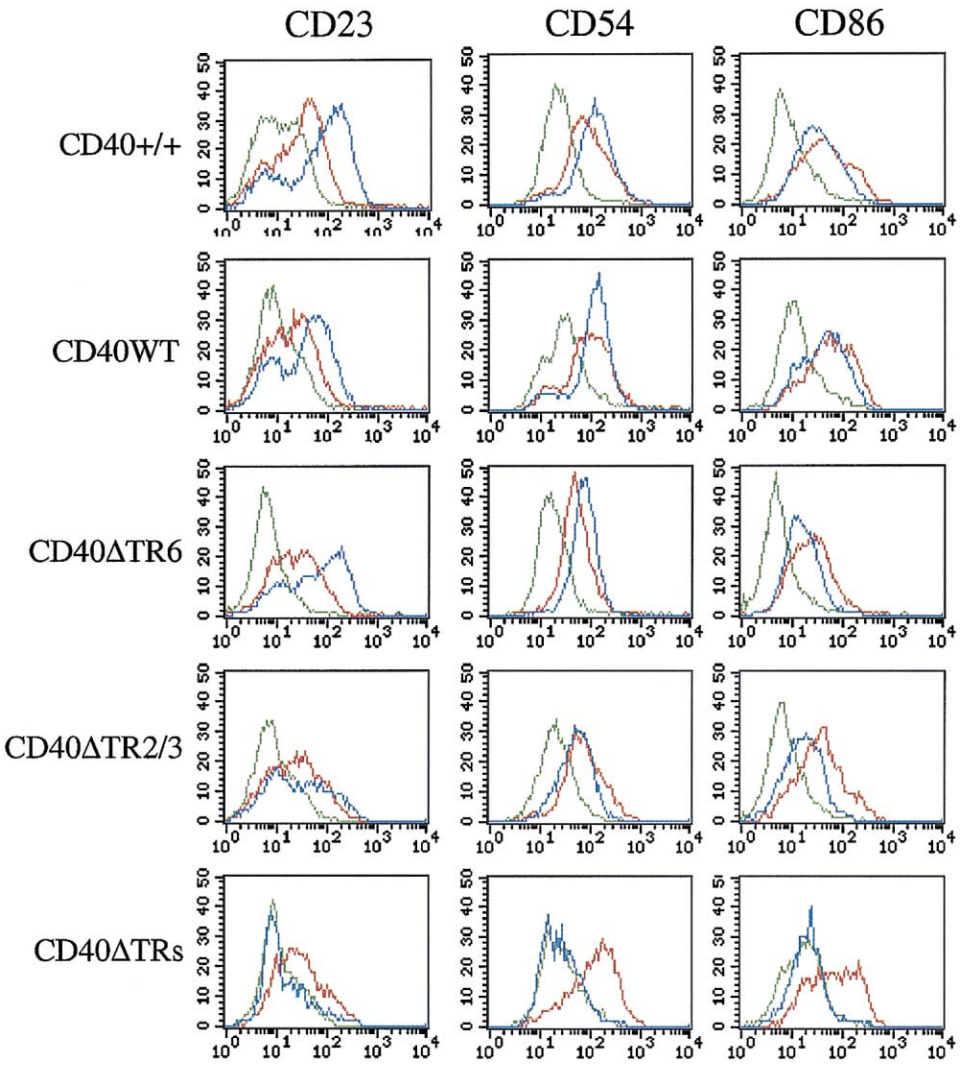

Figure 6. Upregulation of CD23, CD54, and CD86 Expression on B Cells

B cell-enriched splenocytes were left unstimulated (green) or were stimulated overnight with sCD40L from supernatants of CD40L transfected J558L cells (blue) or LPS (red), then stained with anti-CD40-PE- and FITC-conjugated mAbs to either CD23, CD54, or CD86 and with the relevant isotype controls. Stimulation with control supernatants of J558L cells transfected with empty plasmid caused no upregulation of CD23, CD54, or CD86 (data not shown).

dent serum isotype levels, TD antibody responses, and germinal center formation in vivo (Figures 2 and 3). This partial restoration is unlikely to be due to the fact that only half of the B cells from these mice expressed CD40, because in vivo isotype class switching and germinal center formation were fully restored in a line of CD40$^{-/-}$ mice reconstituted with CD40WT (CD40WT#2), in which a similar percentage of the B cells expressed CD40. In vitro isotype switching to IgE and IgG1 in response to CD40/IL-4 stimulation, as well as proliferation to anti-CD40, were severely impaired in B cells from CD40ΔTRAF2/3 mice (Figure 4). These results suggest an important role for TRAF2/3 binding in CD40-driven isotype switching. TRAF1 and TRAF5 bind to CD40 via TRAF2 and TRAF3, respectively (Leo et al., 1999a). However, CD40-dependent isotype switching to IgG in response to TD antigen and CD40 activation of NFκB are intact in TRAF1$^{-/-}$ mice and minimally affected in TRAF5$^{-/-}$ mice (Nakano et al., 1999; Tsitsikov et al., 2001). Thus, it is unlikely that loss of binding of TRAF1 or TRAF5 accounts for the isotype switch defect in CD40ΔTRAF2/3 mice.

Examination of germline transcription, AID expression, deletional switch recombination, and mature transcripts expression demonstrated severely defective CD40-directed isotype switching to Cε and Cγ1 in B cells from CD40ΔTRAF2/3 mice (Figure 5). The residual in vitro switching observed in these cells is consistent with the residual in vivo isotype switching observed in CD40ΔTRAF2/3 mice. Additional signals delivered to the B cells in vivo that synergize with residual signaling by CD40ΔTRAF2/3 to activate class switching may explain the observation that in vivo isotype switching is less affected than in vitro CD40 isotype switching in CD40ΔTRAF2/3 mice. CD40 upregulation of CD23, CD54, and CD86 expression in B cells of ΔTRAF2/3 mice

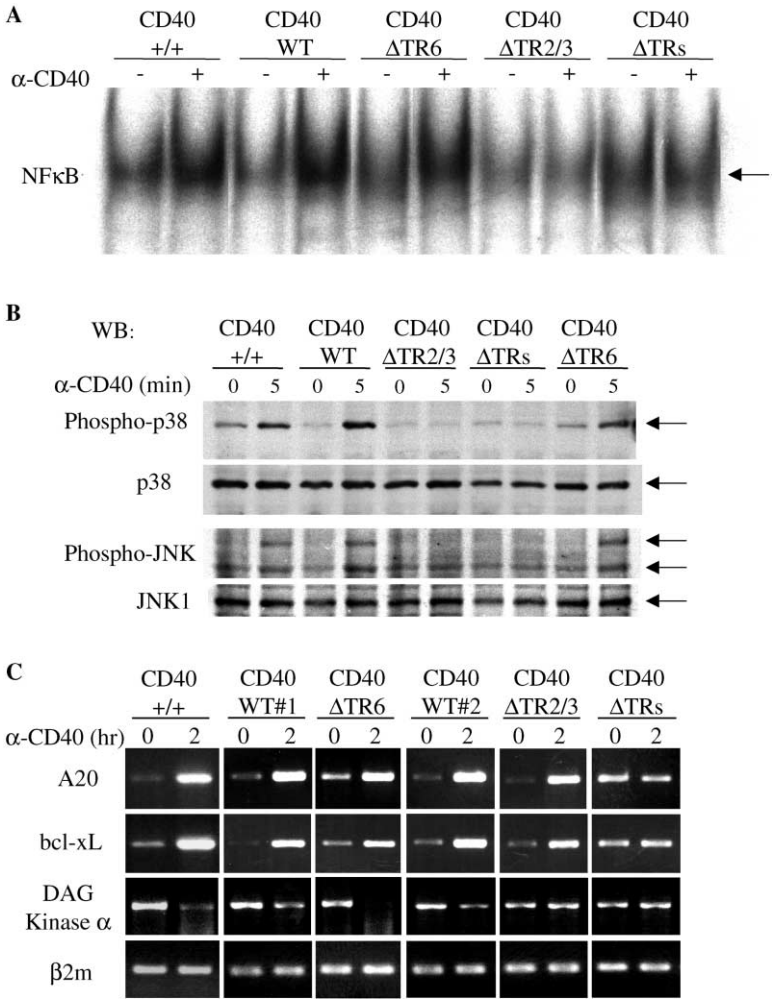

Figure 7. CD40 Activation of NFκB and MAP Kinases in Spleen B Cells

(A) EMSA using nuclear extracts from CD40$^+$ spleen B cells stimulated with anti-CD40 for 30 min at 37°C, and a consensus ^{32}P-labeled NFκB oligonucleotide probe.

(B) Phosphorylation of p38 and JNK. B cells were stimulated for 5 min with anti-CD40, and lysates were analyzed for phosphorylation of p38 and JNK by Western blotting with phospho-specific antibodies. Membranes were reprobed with kinase-specific antibodies for loading controls.

(C) A20, bcl-xL, and DAG kinase α mRNA measured by RT-PCR following 2 hr stimulation with anti-CD40.

was largely preserved (Figure 6). This is consistent with findings in B cell lines (Lee et al., 1999) and suggests that CD40-mediated gene upregulation is not strictly dependent on TRAF2 and/or TRAF3 binding to CD40.

CD40 activation of NFκB, p38, and JNK was impaired in B cells from CD40ΔTRAF2/3 mice, although residual activation of NFκB was suggested by preserved CD40 upregulation of bcl-xL and A20 gene expression (Figure 7). Several effectors that lie in the NFκB activation pathway have been shown to play an important role in CD40-mediated isotype switching and proliferation. They include IKKα, IKKγ/NEMO, p50-, RelA, c-Rel, and p52 (Sha, 1998; Doffinger et al., 2001; Jain et al., 2001; Lee et al., 1999; Richards and Katz, 1997). Furthermore, we have found that the p38 inhibitor SB203580 and the JNK inhibitor SP600125 inhibit IgE and IgG1 secretion in response to anti-CD40+IL-4 but not to LPS+IL-4 (H.H.J. et al., unpublished data). Thus, it is likely that defective CD40 activation of NFκB, p38, and JNK in B cells from CD40ΔTRAF2/3 mice contributes to their defective CD40-dependent isotype switching and proliferation.

The CD40ΔTRAFs transgene completely failed to correct CD40-dependent isotype switching in vivo and in vitro (Figures 2–5). Examination of Cε and Cγ1 germline transcription, AID expression, deletional switch recombination, and mature transcripts expression revealed no detectable CD40-directed isotype switching in B cells from CD40ΔTRAFs mice (Figure 5). Furthermore, these B cells completely failed to proliferate, upregulate surface markers, and activate NFκB and MAP kinases in response to CD40 ligation (Figures 4–7). The fact that in vivo and in vitro CD40-directed isotype switching and activation were partially restored in CD40ΔTRAF2/3 mice but not at all in ΔTRAFs mice suggests that TRAF6 binding to CD40, although undetectable in our pull-down assay, may occur in B cells and synergize with residual signals delivered via CD40ΔTRAF2/3. However, we cannot rule out a role for molecules, other than TRAF6, such as TTRAP, whose association with CD40 may be disrupted by the ΔTRAF6 deletion, although TTRAP itself inhibits CD40 activation of NFκB (Pype et al., 2000).

Our results suggest that CD40 binding to TRAF2 and/or TRAF3 but not to TRAF6 plays an essential role in CD40-mediated isotype switching in murine B cells. Since the 32 aa long CD40 intracellular region that surrounds the TRAF2/3 binding site is fully conserved between mouse and man, it is likely that our results are applicable to human B cells. However, since the affinity of hCD40 for TRAF6 may be higher than that of mCD40,

TRAF6 binding may play a more important role in CD40-mediated isotype switching in human B cells. In addition to binding TRAF proteins, the intracellular domain of human CD40 binds Jak3 (Hanissian and Geha, 1997; Revy et al., 1999). However, studies with B cells from Jak3-deficient patients have failed to reveal a role for Jak3 in CD40-mediated isotype switching, proliferation, and gene upregulation (Jabara et al., 1998). Furthermore, reconstitution of CD40$^{-/-}$ mice with a mutant mCD40 transgene that lacks the proline-rich aa 222–230 sequence that binds to Jak3 resulted in normal CD40-mediated isotype switching and B cell activation (data not shown). This result, together with our finding that deletion of aa 231–246 in ΔTRAF6 does not affect CD40 function and with the fact that the terminal 11 aa of mCD40 are absent from hCD40, narrows the region of CD40 important for isotype switching, with the exclusion of the 6 juxtamembrane aa, to aa 247–278. This 32 aa region contains the PxQxT motif essential for TRAF2 and TRAF3 binding. However, this motif is also present in TNFR family members which bind TRAF2 and TRAF3, yet do not cause isotype switching in B cells, e.g., CD27 (Nagumo et al., 1998; Yamamoto et al., 1998). This suggests that other elements of the aa 247–278 sequence or, alternatively, the transmembrane and/or extracellular domain of CD40 may play a critical role in isotype switching. Current studies are aimed to examine these possibilities.

Experimental Procedures

Generation of CD40$^{-/-}$ Mice with CD40 Transgenes

PCR-generated CD40 WT and mutated gene products (Figure 1A) were cloned in the pBSVE6BK vector containing an Igμ enhancer and Ig heavy chain (IgV$_H$) promoter (Shaw et al., 1999). Not1-Pvu1 fragments of the CD40 cloned products were injected into pronuclei of fertilized oocytes from (C57BL/6/SJL) mice. Founders identified by PCR and Southern blot analysis of tail DNA were crossed with CD40$^{-/-}$ mice on a C57BL/6/129Sv background. F2 animals that were CD40$^{-/-}$ transgene positive were further screened for CD40 surface expression by FACS analysis. CD40$^{+/+}$ control mice were on a mixed C57BL/6/SJL/129Sv background.

Flow Cytometry Analysis

Single–cell suspensions were stained with fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated antibodies in PBS containing 5% rat serum (Sigma), 0.05% sodium azide, and Fc-block (Pharmingen), washed, fixed in 2% paraformaldehyde, and analyzed on a FACSCalibur cytometer (Becton Dickinson). FITC- or PE-conjugated mAbs used in these studies were: CD3 (145-2C11), CD4 (L3T4), CD8α (53-6.7), B220 (RA3-6B2), anti-CD40 (3/23), anti-IgM (R6-60.2), rat IgG2a (R35-95), and hamster IgG (A19-3) (Pharmingen).

Coprecipitation of TRAF Proteins with CD40-GST Fusion Proteins

Intracellular regions corresponding to WT and mutant CD40 were amplified from transgenic constructs by PCR using sense (AAG GAATTCTGGTCAAGAAACCAAAGGATAATGA) and antisense (AAA CTCGAGTCAGACCAGGGGCCTCAA) oligonucleotides and subcloned into the EcoRI/XhoI sites of GST expression vector pGEX-5x-2 (Pharmacia). The fusion proteins were prepared on Glutathione Sepharose beads (Pharmacia) according to the manufacturer's recommendations. M12 B cell (1 × 10^6 cells) lysates in 0.5% Nonidet P-40/150 mM NaCl/10 mM KCl/0.5 mM DTT/0.2 mM/1× Complete Protease Inhibitor (CPI) cocktail (Roche) were centrifuged, and the supernatant was incubated with WT or mutant GST-CD40 Sepharose beads. Beads were washed in lysis buffer; bound proteins were resolved by SDS PAGE gels and transferred onto nitrocellulose membrane. Membranes were blocked with 2% BSA/0.2% gelatin in PBS for 2 hr at 37°C and incubated with mAbs to the mouse TRAF3 (1:500), TRAF6 (1:500) (Santa Cruz Biotechnology), and rabbit anti-mouse TRAF2 (1:250, MBL International) followed by protein G-conjugated to horseradish peroxidase (Bio-Rad). Positive bands were detected by using ECL reagents (NEN).

Immunizations

Mice received an intraperitoneal and a subcutaneous injection each of 200 μg of an alum precipitate of KLH (Calbiochem) containing pertussis toxin (300 ng/mouse, List Biologicals). A booster dose of 30 μg of KLH in aqueous solution was injected intraperitoneally on day 14. Day 21 mice were sacrificed, their spleens were frozen in OCT compound (Ames), and blood was collected.

Germinal Center Formation

Frozen spleen tissue sections, 4 μm thick, were stained with biotin-labeled peanut agglutinin (Vector Laboratories) followed by avidin-biotinylated peroxidase complex (Dako), and developed with 3-amino-9-ethylcarbazole (Aldrich).

ELISA Assays

Immunoglobulins were assayed by ELISA as described (Spergel et al., 1998). To measure anti-KLH-specific antibody, plates were coated with KLH (10 μg/ml) in sodium carbonate buffer (pH 9.0). The wells were blocked with 2% BSA for 2 hr and incubated with diluted sera overnight at 4°C. Alkaline phosphatase-conjugated isotype-specific antibodies (Pharmigen) were used as revealing antibodies. Anti-KLH-specific standards (Pharmingen) were used to determine IgM, IgG1, and IgG3 concentrations. For all other isotypes, a serum pool of ten normal mice was run in parallel, and the readings at 1:25, 1:300, and 1:300 for IgE, IgG2a, and IgG2b, respectively, was taken as an arbitrary unit of 1000, to which the serum dilutions were compared.

RT-PCR

RNA was extracted from cultured splenic B cells using TRIzol (Invitrogen) and was reverse transcribed by Superscript II RT (Invitrogen) according to manufacturer's instructions. PCR primers used were as described previously for Cε and Cγ1 germline and mature transcripts (Muramatsu et al., 2000) and for β2 microglobulin (β2 m) (Spergel et al., 1998). For AID, A20, Bcl-xl, and DAG kinase α transcripts, the following primers were used: AIDF, 5′-GGAGACC GATATGGACAGCCTTCTG-3′; AIDR, 5′-TCAAAATCCCAACATAC GAAATGC-3′; A20F, 5′-ATGGCTGAACAACTTCTTCCTCAGGC-3′; A20R, 5′-GGTCTTCTGAGGATGTTGCTGAGG-3′; bcl-xlF, 5′-ATG TCTCAGAGCAACCGGGAGC-3′; bcl-xlR, 5′-CCAGCCGCCGTTCT CCTGGATCC-3′; DAG kinase αF, 5′-CCAAAGAGAAGGGCCTCAT CAGC-3′; and DAG kinase αR, 5′-GTGCTGACTTCACAAGGCT GGG-3′. All PCR reactions were performed on various dilutions of cDNA to ensure that the products measured were in the linear range. Depending on the PCR product being measured, 5 to 30 ng of cDNA fell within this range.

DC-PCR

Genomic DNA was isolated from cultured splenic B cells on day 6. DNA was digested with EcoR1, circularized, and used as template for PCR using primers as reported previously for Sμ-Sγ1 and nicotinic acetylcholine receptor β unit (nAChR) (Chu et al., 1992) and for Sμ-Sε (Xu and Rothman, 1994). 5 ng of circular DNA was amplified for Sμ-Sγ1 and nAChR (35 cycles), and 20 ng was amplified for Sμ-Sε (one round of 34 cycles) .

Proliferation and IgE Synthesis of B Cells

CD40$^+$ B cells were purified from spleen cell suspensions using Dynabeads M-450 sheep anti-rat IgG (Dynal) coated with rat IgG anti-mouse CD40 mAb (IC10, R&D Systems). Purified cells were >95% B220$^+$ CD40$^+$ by FACS analysis and were suspended in RPMI containing 10% FCS, L-Glutamine, and 50 μM β-mercaptoethanol (complete medium) and rested 24 hr before stimulation. For Ig synthesis, CD40$^+$ B cells (0.5 × 10^6/ml) were cultured in complete medium alone, hamster IgM anti-mouse CD40 (anti-CD40-IgM, 1 μg/ml, HM40-3, Pharmingen)+IL-4 (50 ng/ml, R&D systems), or LPS (20 μg/ml)+IL-4 (50 ng/ml). Supernatants were collected after 6 days

and assayed for IgE and IgG1 by ELISA. For proliferation, $CD40^+$ spleen cells (0.5×10^6/ml) were cultured in complete medium alone or in the presence of LPS (20 μg/ml), anti-CD40-IgM (1 μg/ml) for 72 hr, pulsed with 1 μCi [^{3}H]-thymidine for an additional 16 hr, then harvested and scintillation counted.

Surface Expression of CD23, CD54, and CD86

Purified splenic B cells (>90% $B220^+$) obtained using Dynabeads M-280 Streptavidin (Dynal) after incubation with biotinylated antibody cocktail Thy1.2 (53-2.1), Mac1 (M1/70), and Gr-1 (RB6-8C5) (Pharmingen) were cultured overnight with medium, sCD40L (1:20 dilution of supernatants from muCD40L:muCD8 transfected J558L cells), control supernatants (1:20 dilution of J558L cells transfected with empty plasmid), or LPS (20 μg/ml). Cells were then washed and double stained as described above with anti-CD40-PE and FITC-conjugated mAbs to either CD23 (B3B4), CD54 (3E2), or CD86 (GL1) or with the appropriate isotype controls, all from Pharmingen. Analysis was performed by gating on $CD40^+$ B cells.

EMSA for NFκB

Spleen cells were stained with PE-conjugated rat anti-mouse CD40, and $CD40^+$B cells were sorted using a FACSVantage SE cell sorter (Becton Dickinson). Purified $CD40^+$B cells (1×10^6/ml) were cultured alone or with anti-CD40-IgM (1 μg/ml) for 30 min. EMSA was performed on nuclear extracts (0.8 μg/lane) as described (Tsitsikov et al., 2001). Oligonucleotides used were: NFκB sequence of human immunodeficiency virus (HIV) 1 Long terminal repeat TCGCTGGGG ACTTTCCAGGGA; consensus AP-1 sequence, CGCTTGATGAG TCAGCCG (Promega). The densitometric analysis of the scanned bands was evaluated using the NIH Image program 1.62. Statistical analysis was performed using unpaired t test with Welch's correction.

MAP Kinase Phosphorylation

FACS sorted $CD40^+$ splenic B cells were suspended in RPMI-1640 medium for 2 hr at 37°C before stimulation with anti-CD40-IgM (1 μg/ml). Activated MAP-kinases were detected by immunoblotting lysates from 1.5×10^6 cells with phospho-p38 and phospho-SAPK/JNK (New England Biolabs). Membranes were reprobed with kinase-specific antibodies to p38 (New England Biolabs) and JNK1 (Santa Cruz Biotech.) as loading controls. Immunoreactive bands were detected by enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech).

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