Published in final edited form as:
J Immunol. 2009 August 1; 183(3): 1667–1674. doi:10.4049/jimmunol.0900056.

Fas Apoptosis Inhibitory Molecule Enhances CD40 Signaling in B Cells and Augments the Plasma Cell Compartment[1]

Hiroaki Kaku and **Thomas L. Rothstein**[2]
Center for Oncology and Cell Biology, The Feinstein Institute for Medical Research, Manhasset, NY 11030

Abstract

Fas apoptosis inhibitory molecule (FAIM) was cloned as a mediator of Fas resistance that is highly evolutionarily conserved but contains no known effector motifs. In this study, we report entirely new functions of FAIM that regulate B cell signaling and differentiation. FAIM acts to specifically enhance CD40 signaling for NF-κB activation, IRF-4 expression, and BCL-6 down-regulation in vitro, but has no effect on its own or in conjunction with LPS or anti-Ig stimulation. In keeping with its effects on IRF-4 and BCL-6, FAIM overexpression augments the plasma cell compartment in vivo. These results indicate that FAIM is a new player on the field of B cell differentiation and acts as a force multiplier for a series of events that begins with CD40 engagement and ends with plasma cell differentiation.

Fas apoptosis inhibitory molecule (FAIM)[3] was cloned (1) via differential display from primary B cells, in which sensitivity to Fas-mediated apoptosis had been inducibly down-modulated and, consistent with its origin, expression of exogenous FAIM rendered BAL-17 B cells relatively Fas-resistant (1–3). In subsequent studies, FAIM has been shown to inhibit death receptor-induced apoptosis in other cell types and other species (4–6). The *Faim* gene is located at 9f1 in mouse (and at the syntenic region 3q22 in human) and encodes an ~1.2 kb transcript that produces a 179 amino acid protein of ~20 kDa (1,7). FAIM is highly evolutionarily conserved (from worm to fly to mouse to human) but contains no known effector motifs and the means by which FAIM opposes apoptosis remains uncertain. In keeping with the way in which it was identified, FAIM expression is up-regulated in primary B cells by the combined action of CD40L and anti-Ig (1).

The full-length FAIM gene was originally cloned by screening a murine thymus cDNA library. Subsequent screening of a murine brain cDNA library yielded a novel clone that possessed an insert upstream of the putative FAIM start methionine but that was otherwise identical with the *Faim* gene first described, resulting in a sequence longer by 66 N-terminal nucleotides (7). This longer FAIM isoform was termed FAIM-long (FAIM-L) and the previously identified shorter FAIM form was renamed FAIM-short (FAIM-S). Characterization of the murine

[1]This work was supported by United States Public Health Service grant AI40181 awarded by the National Institute of Health in part by USPHS Grant AI40181.

2 Address correspondence and reprint requests to Dr. Thomas L. Rothstein, Center for Oncology and Cell Biology (Immunobiology Laboratory), The Feinstein Institute for Medical Research, 350 Community Drive, Manhasset, NY 11030. tr@nshs.edu.

Disclosures
The authors have no financial conflict of interest.

[3]Abbreviations used in this paper: FAIM, fas apoptosis inhibitory molecule; BCL-6, B cell lymphoma-6; Blimp-1, B lymphocyte-induced maturation protein 1; EMSA, electrophoretic mobility shift assay; FAIM-L, FAIM-Long; FAIM-S, FAIM-Short; GC, germinal center; IRF, IFN regulatory factor; NGF, nerve growth factor; PAX-5, paired box gene 5; XBP-1, X-box binding protein 1.

Faim genomic locus revealed that the two FAIM isoforms result from alternative splicing, with FAIM-S lacking exon 2 of six exons, and FAIM-L incorporating all six exons (7). FAIM-L differs dramatically from FAIM-S in terms of its highly restricted expression pattern; whereas FAIM-S is characterized by wide tissue distribution, FAIM-L is expressed almost exclusively in the brain (7).

B cells must be stimulated to up-regulate Fas expression and acquire sensitivity to Fas-mediated apoptosis (2). Because many anti-apoptotic genes are NF-κB responsive (8) and inducible Fas resistance in B cells requires NF-κB (9), we considered the possibility that FAIM might oppose Fas killing by up-regulating NF-κB induction in activated B cells. The studies reported in this paper asked whether FAIM would enhance CD40 induction of NF-κB in B cells (10–12). It did, and this led to subsequent studies showing that FAIM enhances CD40-mediated IFN regulatory factor (IRF)-4 expression in vitro, that FAIM enhances CD40-mediated BCL-6 down-regulation in vitro, and that, in keeping with enhanced expression of IRF-4 and diminished expression of BCL-6, FAIM augments plasma cell differentiation in vivo.

Materials and Methods

FAIM-S-expressing A20 transductants

A20 B lymphoma cells were obtained from the American Type Culture Collection. FAIM-S and FAIM-L were amplified by PCR using LA-*Taq* (Takara Shuzo). The products were cloned into the MIGW.IRES.GFP vector (13), which was amplified in BOSC packaging cells cultured in DMEM medium containing 10% FCS, 10 mM HEPES, 2 mM L-glutamine, and 0.1 mg/ml penicillin and streptomycin, as previously described (14). A20 cells were transduced with MIGW.FAIMS.IRES.GFP, MIGW.FAIML.IRES. GFP, or empty vector MIGW.IRES.GFP, as described (14). At 10 days following transduction, GFP^+ A20 cells were sort-purified. At the time of experimentation, transduced A20 cells were >95% GFP-expressing.

Reporter plasmids

The NF-κB-dependent firefly-luciferase vector was obtained from BD Clontech and was cotransfected with pRL-TK (renilla-luciferase) vector using the nucleofector kit V (Amaxa Biosystems) according to the manufacturer's instructions. At 24 h after transfection, luciferase activity in cell lysates was analyzed by the dual-luciferase reporter assay (System-Promega) according to the manufacturer's instructions, using a 20/20n single tube luminometer (Turner BioSystems). Relative luciferase activity was calculated as firefly-luciferase activity/renilla-luciferase activity and the data shown represent the mean fold induction of two to three experiments.

Electrophoretic mobility shift assay (EMSA) for NF-κB activation

Nuclear extracts were prepared and assayed for NF-κB by EMSA, as previously described (15). NF-Y binding was used to verify that equal amounts of nuclear extract protein were present in each sample. The probe sequences are listed in Table I.

Western blotting

Proteins were extracted from B cell pellets with Nonidet P-40 lysis buffer and equal amounts of protein for each condition were subjected to SDS-PAGE followed by immunoblotting, as previously described (16).

Gene expression

Total RNA was extracted using Ultraspec reagent (Biotecx Laboratories) according to the manufacturer's instructions, reverse transcribed, amplified by real-time PCR, and normalized

to expression of β_2-microglobulin, as previously described (17). The sequences of the primer sets are shown in Table I.

Mice

Male BALB/cByJ mice at 8–14 wk of age were obtained from The Jackson Laboratory. Mice were housed at least 1 wk before experimentation. Mice were cared for and handled in accordance with National Institutes of Health and The Feinstein Institute for Medical Research's guidelines.

Chimera mice expressing FAIM-S

Bone marrow chimera mice were generated by MSCV transduction and adoptive transfer, as previously described (14). Briefly, bone marrow cells harvested from the hind legs of 5-fluorouracil-treated BALB/c mice were transduced with FAIM-S-expressing MSCV.FAIMS.IRES.GFP vector. Cells were treated with 100 ng/ml stem cell factor, 100 ng/ml flt3 ligand, and 50 ng/ml thrombopoietin (R&D Systems) during three rounds of transduction over a period of 24 h. MSCV.FAIMS.IRES.GFP-transduced bone marrow cells (1×10^6) were adoptively transferred to lethally irradiated (900 rad) BALB/c recipient mice via the tail vein.

Flow cytometry

For FACS analysis, cells pretreated with 5 μg/ml 2.4G2 were stained with FITC-, PE-, or PE-Cy5-conjugated mAb in staining buffer (PBS containing 2% FCS and 0.05% NaN_3) on ice for 30 min and washed with staining buffer. Stained cells were analyzed on a FACSCalibur instrument (BD Biosciences) equipped with CellQuest software. To exclude dead cells from the analysis, 7-amino-actinomycin D (2 μg/ml) (BD Pharmingen) was used. For each sample, at least 1×10^4 cells were collected and analyzed.

Splenic B2 cells

Splenic B2 cells were obtained by negative selection with anti-Thy1.2 Ab and rabbit complement, as previously described (2). Isolated B2 cells were >95% B220 positive. B cells were cultured in RPMI 1640 medium containing 10% FCS, 10 mM HEPES, 2 mM L-glutamine, 0.1 mg/ml penicillin and streptomycin, and 50 μM 2-ME.

Caspase activation

Splenic B2 cells were stimulated with CD40L for 48 h, washed, and then stimulated with anti-Fas Ab (Jo-2; BD Pharmingen) for 4 h. Cells were exposed to Red-DEVD-FMK (MBL International) for 1 h according to the manufacturer's instructions. Cells were analyzed by flow cytometry on a FACSCalibur instrument.

Antibodies and reagents

CD40L was prepared and used as previously described (11,15,18). Affinity-purified $F(ab')_2$ goat anti-mouse IgM (anti-Ig) was obtained from Jackson ImmunoResearch Laboratories. LPS from *Salmonella typhimurium* was obtained from Sigma-Aldrich. Monoclonal anti-FcγR Ab 2.4G2, monoclonal anti-Fas Ab Jo-2, FITC-conjugated RA3-6B2 (anti-B220), and PE-conjugated anti-CD80, anti-CD86, and anti-CD138 were obtained from BD Pharmingen. Anti-CD40-PE was obtained from Southern Biotech. Affinity-purified anti-FAIM Ab was obtained from rabbits immunized with CYIKAVSSRKRKEGIIHTLI peptide (located near the C-terminal region of FAIM). Abs against RelB and IκBα were obtained from Cell Signaling Technology. Anti-Lamin B1 and anti-c-*Rel* Abs were from Santa Cruz Biotechnology. Anti-

tubulin Ab was obtained from Calbiochem and anti-actin Ab was obtained from Sigma-Aldrich. Red-DEVD-FMK was obtained from MBL International.

Results

FAIM-S enhances CD40L-induced NF-κB activation

To investigate the influence of FAIM on NF-κB activation, we cloned FAIM-S and, separately, FAIM-L into the MSCV retroviral vector MIGW.IRES.GFP (19,20) and then transduced A20 cells with FAIM-S-expressing MIGW.FAIMS.IRES.GFP, FAIM-L-expressing MIGW.FAIML.IRES.GFP, or empty vector. We then selected transduced A20 cells by fluorescence-activated cell sorting to generate stable transductants. We verified FAIM expression by Western blotting, and thereby demonstrated that FAIM-S-transduced A20 B cells contained 10-fold more FAIM-S than the low endogenous FAIM-S level present in empty vector-transduced cells, and that FAIM-L-transduced A20 cells contained an incalculably greater amount of FAIM-L as compared with vector-transduced A20 cells because the latter did not express detectable levels of FAIM-L (Fig. 1*A*). We then obtained nuclear extracts from each population of transduced A20 cells before and after stimulation with CD40L, anti-Ig, or LPS and tested these extracts for κB-binding activity by EMSA with a probe containing a consensus NF-κB binding motif (Fig. 1*B*). We found that FAIM-S overexpression produced marked enhancement of CD40L-induced κB-binding activity in A20 cells as compared with vector-transduced A20 cells. There was no difference in CD40 expression between FAIM-S-transduced and empty vector-transduced A20 cells (data not shown). In contrast, FAIM-L was completely inactive. Interestingly, neither FAIM-S nor FAIM-L overexpression enhanced anti-Ig- or LPS-induced κB-binding activity. Thus, FAIM-S specifically enhances CD40L-induced κB-binding activity in murine B cells.

To examine FAIM-enhanced, CD40L-induced NF-κB activity further, we transfected FAIM-S-, FAIM-L-, and vector-transduced A20 B cells with a κB-dependent firefly luciferase reporter plasmid and evaluated luciferase activity before and after stimulation with CD40L. We found that FAIM-S overexpression produced heightened activation of the NF-κB reporter plasmid after stimulation by CD40L as compared with transduction of empty vector ($p < 0.05$, $n = 4$) (Fig. 1*C*). In contrast, FAIM-L was completely inactive ($p > 0.3$, $n = 4$). Similar results were obtained when CD40 was triggered by 1C10 monoclonal rat anti-mouse CD40 Ab. FAIM-S, but not FAIM-L, enhanced NF-κB-dependent reporter activity (data not shown). As with EMSA, these results were specific for CD40 signaling; neither FAIM-S nor FAIM-L transduction enhanced either anti-Ig- or LPS-induced κB-binding activity (data not shown). These results indicate that FAIM-S specifically enhances NF-κB activity in B cells, as detected by reporter gene function and that FAIM-L lacks the NF-κB-enhancing activity of FAIM-S.

There are two NF-κB activation pathways induced by CD40L; the canonical pathway, in which nuclear translocation of p50, p65, and c-Rel is induced though IKKβ activity and IκBα degradation, and the noncanonical pathway, in which translocation of p52 and RelB is induced though NIK and IKKα activity (21,22). To examine which pathway is enhanced by FAIM-S, we first evaluated IκB degradation by Western blotting. We found that CD40L-induced IκBα degradation was accentuated in FAIM-S A20 transductants as compared with empty vector transductants (Fig. 1*D*). FAIM-S enhancement of IκBα degradation was similarly evident when CD40 was triggered by anti-CD40 Ab (data not shown). Thus, enhancement of CD40L-induced NF-κB activity produced by FAIM-S occurs in conjunction with enhanced IκBα degradation, suggesting that FAIM-S acts on the canonical pathway. We then directly tested the role of the noncanonical pathway by examining nuclear accumulation of RelB. We found that CD40L produced an enhanced increase in nuclear RelB, as well as c-Rel, in FAIM-S A20 transductants as compared with empty vector transductants (Fig. 1*E*). Thus, FAIM-S

enhances CD40-induced NF-κB activation through both canonical and noncanonical mechanisms.

To further address the physiological activity of FAIM-S-enhanced NF-κB, we analyzed target gene expression of A20, IκBα, CD80, and IL-12 p35 induced by CD40L (23–26) in A20 cells transduced with FAIM-S or with empty vector. We found that FAIM-S enhanced the CD40L-induced expression of all four NF-κB target genes studied as compared with expression produced by CD40L in vector-transduced A20 cells (Fig. 1*F*). Thus, enhancement of CD40L-induced NF-κB activity produced by FAIM-S results in enhanced activation of physiological NF-κB targets. Of note, however, is that enhancement of some genes, such as *Cd80* and *IL-12 p35*, was more substantial and more prolonged than others. These latter genes are regulated by IRF transcription factors as well as by NF-κB (27–29). All together these results indicate that, by multiple criteria, FAIM-S enhances CD40L-stimulated NF-κB activation.

FAIM-S enhances CD40L-induced IRF4 activation

Our results above showing disproportionate enhancement of some NF-κB target gene responses by FAIM-S raised the possibility that FAIM affects IRF family members as well as NF-κB. The IRF family contains nine members. The activity of IRF1 and IRF4 is regulated by transcription, whereas the activity of other family members, particularly IRF3 and IRF7, is regulated by serine and threonine phosphorylation. To determine whether FAIM enhances IRF activation induced by CD40L, we first evaluated the levels of IRF3 and IRF7 phosphorylation, because IRF3 and IRF7 family members have been implicated in regulating CD80 expression in macrophages (27,28). We prepared lysates from FAIM-S- and vector-transduced A20 cells before, and at various times after, CD40L stimulation, and then conducted Western blotting with anti-pIRF3 and anti-pIRF7 Abs. We found that phosphorylation of IRF3 and IRF7 at baseline was similar in FAIM-S-transduced A20 cells as compared with vector-transduced A20 cells, and that CD40L failed to stimulate pIRF3 or pIRF7 in either population (data not shown). Next, we analyzed the levels of *Irf1* and *Irf4* gene expression in separate groups of FAIM-S- and vector-transduced A20 cells. We prepared RNA before, and at various times after, CD40L stimulation of these cells, and then conducted real-time PCR. We found no CD40L-stimulated induction of *Irf1* expression above the background observed in empty vector-transduced A20 cells (data not shown). In contrast, CD40L produced up-regulation of *Irf4* expression (30,31) in empty vector-transduced A20 cells and, importantly, we found that this CD40-mediated induction of *Irf4* was markedly enhanced by FAIM-S (Fig. 2*A*). We further examined IRF4 protein. We prepared lysates from FAIM-S-transduced and vector-transduced A20 cells before, and at two times after, CD40L stimulation, and then conducted Western blotting with anti-IRF4 Ab. We found that FAIM-S greatly enhanced the induction of IRF4 protein produced by CD40L-stimulation of A20 cells (Fig. 2*B*). Thus, FAIM-S enhances CD40L-stimulated IRF4 expression, recapitulating the activity of FAIM-S in enhancing CD40L-stimulated NF-κB activation.

FAIM-S enhances CD40L-induced down-regulation of BCL-6 expression

CD40 engagement in B cells produces down-regulation of BCL-6 expression through IRF4-mediated transcriptional inhibition (30–33). In view of our finding that FAIM enhances CD40L-stimulated IRF4, we reasoned that FAIM might augment CD40L-mediated down-regulation of BCL-6. To address this, we stimulated stably transduced A20 cells with CD40L, prepared RNA from stimulated cells at various times, and examined *Bcl-6* gene expression by real-time PCR. As predicted by previous work, we found that the level of CD40L-induced down-regulation of *Bcl-6* expression was accentuated (i.e., experienced a further decline) in the presence of FAIM-S (Fig. 3). Thus, FAIM-mediated enhancement of CD40L-stimulated NF-κB activation and IRF4 expression in B cells is accompanied by reinforcement of *Bcl-6* down-regulation as well.

FAIM-S augments plasma cell differentiation in vivo

IRF4 is a key determinant of plasma cell production (34,35). The capacity of FAIM-S to enhance IRF4 expression, and to further diminish *Bcl-6* expression, suggested a possible role for FAIM in influencing plasma cell development. Moreover, FAIM is up-regulated in germinal center B cells (Ref. 36 and our unpublished observations), which puts FAIM in the right location to influence B cell differentiation. Furthermore, our previous finding that FAIM expression is stimulated in response to BCR and CD40 engagement also supports the notion that FAIM responds to signals involved in late B cell differentiative processes. To address the effect of FAIM-S on B cells in vivo, we transduced bone marrow cells from normal mice with MIGW.FAIMS.IRES.GFP, as described in *Materials and Methods*. We then adoptively transferred these mixed (retrovirally transduced and nontransduced) bone marrow cells into lethally irradiated recipients, and evaluated and compared GFP+ (retrovirally transduced) lymphocytes with GFP– (nontransduced) lymphocytes obtained from adoptive hosts 8–12 wk later. We first evaluated FAIM-S expression in splenic B cells by Western blotting. We found that FAIM-S expression in GFP+ B cells was much higher (greater than 5-fold higher) than the low endogenous level present in GFP– B cells, as might be expected (Fig. 4*A*). As expected, this increased level of FAIM-S eliminated the susceptibility to Fas-mediated apoptosis displayed by primary B cells, as judged by the relative level of activated caspase 3 produced by anti-Fas Ab Jo-2 in CD40L-treated GFP+ and GFP– B cells (Fig. 4*B*).

We then analyzed splenic, peritoneal cavity, and bone marrow cell populations. We found no difference between GFP+ and GFP– cells in terms of the fraction of B220+ cells in spleen or bone marrow populations, nor in the fraction of B220+ cells or of B220loCD5+ cells in the peritoneal cavities, of these chimeric mice (data not shown). Neither did we find differences in T cell development or CD4:CD8 ratios (data not shown). Importantly, however, we found a dramatic difference in CD138+ (syndecan+) cells, in that the number of CD138+B220+ cells among GFP+ B220+ bone marrow cells was 3-fold higher ($n = 5$, $p < 0.01$) than the number of CD138+B220+ cells among GFP–B220+ bone marrow cells (Fig. 4, *C* and *D*). In contrast, there was no increase in CD138+B220+ cells among GFP+B220+ bone marrow cells as compared with the number of CD138+B220+ cells among GFP–B220+ bone marrow cells in adoptive recipients of empty vector-transduced bone marrow stem cells (data not shown). These data indicate that FAIM-S enhances plasma cell differentiation during normal physiological processes in vivo.

The chimeric mice presented the opportunity to test whether FAIM-S affects NF-κB target gene expression in primary B cells by evaluating CD80. We stimulated splenic B cells from bone marrow chimera mice with CD40L, anti-Ig, or LPS. After 24 h, we examined stimulated and nonstimulated B cells for CD80 expression, comparing GFP+ and GFP– populations. We found that the increase in CD80 expression produced by CD40L was enhanced in the presence of exogenous FAIM-S (Fig. 4*E*). In contrast, CD80 expression induced by anti-Ig and by LPS was little affected by FAIM-S expression (data not shown). As a control, we monitored expression of CD86, induction of which was little changed by FAIM-S under any of the stimulatory conditions (data not shown). Thus, FAIM-S specifically enhances CD40L-induced expression of an NF-κB/IRF4-responsive cell surface marker in primary B cells.

Discussion

The present results introduce a new player in B cell differentiation. As shown here, FAIM in B cells enhances CD40 signaling for NF-κB activation, IRF4 expression, and BCL-6 down-regulation in vitro, and FAIM overexpression leads to expansion of the plasma cell compartment in vivo. All of these features are connected to, and fully consistent with, current paradigms for the regulation of B cell differentiation and plasma cell generation. What is remarkable is the role of FAIM in acting to promote CD40-driven outcomes.

Induction of plasma cell generation revolves around a cascade of four sequentially inhibiting transcription factors (reviewed in Ref. 37): B cell leukemia/lymphoma-6 (BCL-6), B lymphocyte-induced maturation protein 1 (BLIMP-1), paired box gene 5 (PAX-5) and X-box binding protein 1 (XBP-1). In brief, BCL-6 represses BLIMP-1 (which can, conversely, repress BCL-6, resulting in relatively stable states of BCL-6-dominant or BLIMP-1-dominant expression); BLIMP-1 represses PAX-5; and PAX-5 represses XBP-1. Whereas BCL-6 is required for germinal center development (38,39), BLIMP-1 is considered the master regulator of plasma cell differentiation (40,41). Among many other activities, up-regulation of BLIMP-1 represses PAX-5, a gene product that maintains B cell identity (42,43). Repression of PAX-5, in turn, leads to, among other effects, up-regulation of XBP-1, which expands elements of the secretory pathway, enhances the unfolded protein response, and promotes plasma cell differentiation (44–46). B cells express abundant levels of BCL-6 and PAX-5, but little BLIMP-1 or XBP-1, whereas plasma cells express abundant amounts of BLIMP-1 and XBP-1, but little BCL-6 or PAX-5. At the top of this cascade lies BCL-6, and loss of BCL-6 is the key event that initiates transformation of the BCL-6^{high}/PAX-5^{high} phenotype to the BLIMP-1^{high}/XBP-1^{high} phenotype, leading to plasma cell differentiation. In keeping with this, BCL-6 expression is tightly regulated by several distinct mechanisms (30,32,33,47,48). Most prominently, and directly related to our results with FAIM, BCL-6 is transcriptionally down-regulated as a result of CD40 engagement (30,32,33).

The transcription factor cascade leading to plasma cell differentiation takes place within germinal center (GC) B cells (reviewed in Ref. 49), which experience CD40 signaling, as evidenced by the CD40 transcriptional signature in a subset of GC centrocytes in situ (30). This result is supported by additional studies indicating that CD40 signaling plays a key role in centrocyte survival and differentiation (30,50–52). It has been shown that CD40 engagement induces NF-κB activation in B cells (10–12). In several landmark studies, Dalla-Favera and colleagues elucidated the functional relationships among CD40, NF-κB, and BCL-6, by identifying IRF4, known to be induced by CD40 (30) and known to be required for plasma cell generation (34), as the key intermediary (31). They demonstrated that NF-κB binds to the *IRF4* promoter and induces *IRF4* transcription and that IRF4 binds to the *BCL-6* promoter and suppresses *BCL-6* expression. To confirm the physiological relevance of these results, Dalla-Favera and colleagues examined primary GC B cells and found that, following CD40L stimulation, induction of nuclear NF-κB is accompanied by up-regulation of IRF4 and that up-regulation of *IRF4* transcripts and IRF4 protein is accompanied by down-regulation of *BCL-6* transcripts and BCL-6 protein. Finally, Saito et al. showed by in situ staining that GC centrocytes that express nuclear NF-κB lack BCL-6 expression, and vice versa (31). All together, these results indicate that CD40 signaling produces activation of NF-κB, which then stimulates IRF4 expression, which in turn represses *BCL-6* transcription, and thereby promotes terminal differentiation of GC B cells.

FAIM expression is relatively enriched in GC B cells (Ref. 36 and our unpublished observations) and FAIM enhances each of the CD40-triggered events noted above that are connected to each other and to plasma cell differentiation: NF-κB activation, IRF4 expression, and BCL-6 down-regulation. This strongly suggests that the positive influence of FAIM on CD40 signaling produces, perforce, a positive increase in the plasma cell compartment. Recent work by Sciammas et al. demonstrating that the level of IRF4 determines the level of plasma cell differentiation (35) supports the notion that more CD40 signaling in B cells equals more plasma cell generation, fully in keeping with the effects of FAIM noted here. Thus, work reported by Dalla-Favera and colleagues (30,31) provides a foundation for our results with FAIM. FAIM-S does not stimulate on its own but rather acts as a force multiplier for a series of events that begins with CD40 engagement and ends with plasma cell differentiation. However, it cannot be said with certainty that the signaling enhancement produced by FAIM-S in A20 cells occurs in primary B cells, nor can it be said at this time whether the level of

FAIM-S produced by transduction of A20 cells or the level present in primary B cells derived from FAIM-S-transduced bone marrow stem cells approximates the level that occurs physiologically in GC B cells. In addition, we cannot rule out the possibility that apoptosis protection contributes to increased plasma cell generation in the presence of FAIM-S, although the normal development of B cells up to the plasma cell stage weighs against this, nor that constitutive (and thus potentially early) FAIM-S expression plays a role.

We found that the specificity of FAIM-S in enhancing CD40 signaling in B cells, but not signaling produced by other stimuli, is matched by the specificity embodied in the failure of FAIM-L to recapitulate the effects of FAIM-S. This inversion of FAIM-S/FAIM-L activities, which has been noted in other systems (4,53), is unexpected, inasmuch as FAIM-L includes the entire sequence of FAIM-S, differing only in an additional 22 N-terminal amino acids. This suggests that the N-terminal domain in general, or the nonspliced additional N-terminal 22 amino acids of FAIM-L in particular, are regulatory in nature and influence the activity of a more C-terminal effector motif. Because the evolutionarily conserved FAIM-S sequence contains no known effector motifs, and because the FAIM-S structure expresses a unique fold that has no known homologues (54), it is highly likely that, when defined, the FAIM-S effector domain will be found to be a currently unrecognized and novel sequence. The mechanism by which FAIM-S effects enhancement of CD40 signaling in B cells remains unknown; however, inasmuch as NF-κB stimulates IRF4 expression and IRF4 inhibits BCL-6 expression, it appears that the various facets of FAIM activity are determined by its amplification of NF-κB activation. Because the levels of NF-κB produced by other NF-κB-inducing stimuli are not affected by FAIM-S, elucidation of the molecular details by which FAIM acts might properly focus on upstream events surrounding CD40 triggering.

As expected from our early work (1), FAIM-S opposed Fas-mediated apoptosis in primary B cells. Our new finding that FAIM-S enhances CD40-mediated NF-κB activation provides information that may be relevant to Fas-resistance. Many anti-apoptotic genes are NF-κB-dependent (8), and we previously showed that inducible Fas-resistance in B cells requires NF-κB (9). Further, we found that CD40-induced expression of BCL-xL and FLIP were enhanced by FAIM-S (data not shown). Note that although FAIM-S affects CD40 signaling, FAIM-S itself is induced not by CD40L alone, but by anti-Ig stimulation of CD40L-treated B cells, and in our previous experiments, CD40L was present when anti-Ig was added to induce Fas-resistance (1). Thus, it seems likely that the mechanism responsible for the anti-apoptotic effect of FAIM relates to its ability to enhance NF-κB activation, shown here, and that the FAIM-S features of signaling enhancement and anti-apoptosis would seem to be of one piece, with the latter dependent on the former.

Sole et al. evaluated the function of FAIM in neuronal tissue by studying PC12 cells and superior cervical ganglion neurons and found that FAIM-S (but not FAIM-L) enhanced nerve growth factor (NGF)-induced neurite outgrowth (53). They further showed that NF-κB activation induced by NGF, which is critical for neurite outgrowth, was enhanced by FAIM-S (53). Together with the present results, it is clear that FAIM-S is capable of enhancing NF-κB activation in two very distinct tissues (B cells and neural cells) in conjunction with triggering of two independent receptors (CD40 and NGFR) that, however, are members of the same overall family (55,56). TNFR is a member of the same family of cysteine-rich pseudorepeat receptors and in preliminary experiments we found that FAIM-S (but not FAIM-L) enhanced TNF-induced NF-κB activation in 3T3 cells (our unpublished observations). In view of these results, and considering the evolutionary conservation of FAIM and of NGFR family members (57), it may be speculated that these molecules have been functionally interrelated for some time, and that FAIM can effect enhancement of signaling following engagement of multiple NGFR/TNFR family members even beyond CD40, NGFR, and TNFR.

Acknowledgments

We are grateful to our colleagues for helpful discussions and technical assistance throughout the course of this study.

References

1. Schneider TJ, Fischer GM, Donohoe TJ, Colarusso TP, Rothstein TL. A novel gene coding for a Fas apoptosis inhibitory molecule (FAIM) isolated from inducibly Fas-resistant B lymphocytes. J. Exp. Med 1999;189:949–956. [PubMed: 10075978]
2. Rothstein TL, Wang JK, Panka DJ, Foote LC, Wang Z, Stanger B, Cui H, Ju ST, Marshak-Rothstein A. Protection against Fas-dependent Th1-mediated apoptosis by antigen receptor engagement in B cells. Nature 1995;374:163–165. [PubMed: 7533263]
3. Foote LC, Schneider TJ, Fischer GM, Wang JK, Rasmussen B, Campbell KA, Lynch DH, Ju ST, Marshak-Rothstein A, Rothstein TL. Intracellular signaling for inducible antigen receptor-mediated Fas resistance in B cells. J. Immunol 1996;157:1878–1885. [PubMed: 8757305]
4. Segura MF, Sole C, Pascual M, Moubarak RS, Perez-Garcia MJ, Gozzelino R, Iglesias V, Badiola N, Bayascas JR, Llecha N, et al. The long form of Fas apoptotic inhibitory molecule is expressed specifically in neurons and protects them against death receptor-triggered apoptosis. J. Neurosci 2007;27:11228–11241. [PubMed: 17942717]
5. Choi CY, Reimers K, Allmeling C, Kall S, Choi YH, Vogt PM. Inhibition of apoptosis by expression of antiapoptotic proteins in recombinant human keratinocytes. Cell Transplant 2007;16:663–674. [PubMed: 17912957]
6. Yu LY, Saarma M, Arumae U. Death receptors and caspases but not mitochondria are activated in the GDNF- or BDNF-deprived dopaminergic neurons. J. Neurosci 2008;28:7467–7475. [PubMed: 18650325]
7. Zhong X, Schneider TJ, Cabral DS, Donohoe TJ, Rothstein TL. An alternatively spliced long form of Fas apoptosis inhibitory molecule (FAIM) with tissue-specific expression in the brain. Mol. Immunol 2001;38:65–72. [PubMed: 11483211]
8. Sen R. Control of B lymphocyte apoptosis by the transcription factor NF-κB. Immunity 2006;25:871–883. [PubMed: 17174931]
9. Schram BR, Rothstein TL. NF-κB is required for surface Ig-induced Fas resistance in B cells. J. Immunol 2003;170:3118–3124. [PubMed: 12626568]
10. Lalmanach-Girard AC, Chiles TC, Parker DC, Rothstein TL. T cell-dependent induction of NF-κB in B cells. J. Exp. Med 1993;177:1215–1219. [PubMed: 8459216]
11. Francis DA, Karras JG, Ke XY, Sen R, Rothstein TL. Induction of the transcription factors NF-κB, AP-1 and NF-AT during B cell stimulation through the CD40 receptor. Int. Immunol 1995;7:151–161. [PubMed: 7537532]
12. Berberich I, Shu GL, Clark EA. Cross-linking CD40 on B cells rapidly activates nuclear factor-κB. J. Immunol 1994;153:4357–4366. [PubMed: 7525701]
13. Cherry SR, Biniszkiewicz D, van Parijs L, Baltimore D, Jaenisch R. Retroviral expression in embryonic stem cells and hematopoietic stem cells. Mol. Cell Biol 2000;20:7419–7426. [PubMed: 11003639]
14. Holodick NE, Repetny KJ, Zhong X, Rothstein TL. Adult bone marrow generates CD5+ B1 cells containing abundant N-region additions. Eur. J. Immunol. 2009in press
15. Mizuno T, Rothstein TL. Cutting edge: CD40 engagement eliminates the need for Bruton's tyrosine kinase in B cell receptor signaling for NF-κB. J. Immunol 2003;170:2806–2810. [PubMed: 12626529]
16. Mizuno T, Rothstein TL. B cell receptor (BCR) cross-talk: CD40 engagement creates an alternate pathway for BCR signaling that activates IκB kinase/IκBα/NF-κB without the need for PI3K and phospholipase Cγ. J. Immunol 2005;174:6062–6070. [PubMed: 15879100]
17. Frances R, Tumang JR, Rothstein TL. B-1 cells are deficient in Lck: defective B cell receptor signal transduction in B-1 cells occurs in the absence of elevated Lck expression. J. Immunol 2005;175:27–31. [PubMed: 15972627]

18. Lane P, Brocker T, Hubele S, Padovan E, Lanzavecchia A, McConnell F. Soluble CD40 ligand can replace the normal T cell-derived CD40 ligand signal to B cells in T cell-dependent activation. J. Exp. Med 1993;177:1209–1213. [PubMed: 7681469]
19. Van Parijs L, Refaeli Y, Lord JD, Nelson BH, Abbas AK, Baltimore D. Uncoupling IL-2 signals that regulate T cell proliferation, survival, and Fas-mediated activation-induced cell death. Immunity 1999;11:281–288. [PubMed: 10514006]
20. Repetny KJ, Zhong X, Holodick NE, Rothstein TL, Hansen U. LBP-1a binds Sμ and Sα, but not Sγ1, immunoglobulin switch regions in vivo, and represses heavy chain class switching only to IgA, but not to IgG1. Eur. J. Immunol 2009;39:1387–1394. [PubMed: 19384868]
21. Gilmore TD. Introduction to NF-κB: players, pathways, perspectives. Oncogene 2006;25:6680–6684. [PubMed: 17072321]
22. Hoffmann A, Baltimore D. Circuitry of nuclear factor κB signaling. Immunol. Rev 2006;210:171–186. [PubMed: 16623771]
23. Sarma V, Lin Z, Clark L, Rust BM, Tewari M, Noelle RJ, Dixit VM. Activation of the B-cell surface receptor CD40 induces A20, a novel zinc finger protein that inhibits apoptosis. J. Biol. Chem 1995;270:12343–12346. [PubMed: 7539000]
24. Goldstein MD, Debenedette MA, Hollenbaugh D, Watts TH. Induction of costimulatory molecules B7-1 and B7-2 in murine B cells: the CBA/N mouse reveals a role for Bruton's tyrosine kinase in CD40-mediated B7 induction. Mol. Immunol 1996;33:541–552. [PubMed: 8700170]
25. Vaidyanathan H, Gentry JD, Weatherman A, Schwartzbach SD, Petro TM. Differential response of the murine IL-12 p35 gene to lipopolysaccharide compared with interferon-γ and CD40 ligation. Cytokine 2001;16:1–9. [PubMed: 11669581]
26. Li Q, Verma IM. NF-κB regulation in the immune system. Nat. Rev. Immunol 2002;2:725–734. [PubMed: 12360211]
27. Romieu-Mourez R, Solis M, Nardin A, Goubau D, Baron-Bodo V, Lin R, Massie B, Salcedo M, Hiscott J. Distinct roles for IFN regulatory factor (IRF)-3 and IRF-7 in the activation of antitumor properties of human macrophages. Cancer Res 2006;66:10576–10585. [PubMed: 17079482]
28. Lim W, Gee K, Mishra S, Kumar A. Regulation of B7.1 costimulatory molecule is mediated by the IFN regulatory factor-7 through the activation of JNK in lipopolysaccharide-stimulated human monocytic cells. J. Immunol 2005;175:5690–5700. [PubMed: 16237059]
29. Goriely S, Molle C, Nguyen M, Albarani V, Haddou NO, Lin R, De Wit D, Flamand V, Willems F, Goldman M. Interferon regulatory factor 3 is involved in Toll-like receptor 4 (TLR4)- and TLR3-induced IL-12p35 gene activation. Blood 2006;107:1078–1084. [PubMed: 16219795]
30. Basso K, Klein U, Niu H, Stolovitzky GA, Tu Y, Califano A, Cattoretti G, Dalla-Favera R. Tracking CD40 signaling during germinal center development. Blood 2004;104:4088–4096. [PubMed: 15331443]
31. Saito M, Gao J, Basso K, Kitagawa Y, Smith PM, Bhagat G, Pernis A, Pasqualucci L, Dalla-Favera R. A signaling pathway mediating down-regulation of BCL6 in germinal center B cells is blocked by BCL6 gene alterations in B cell lymphoma. Cancer Cell 2007;12:280–292. [PubMed: 17785208]
32. Allman D, Jain A, Dent A, Maile RR, Selvaggi T, Kehry MR, Staudt LM. BCL-6 expression during B-cell activation. Blood 1996;87:5257–5268. [PubMed: 8652841]
33. Niu H, Cattoretti G, Dalla-Favera R. BCL6 controls the expression of the B7-1/CD80 costimulatory receptor in germinal center B cells. J. Exp. Med 2003;198:211–221. [PubMed: 12860928]
34. Klein U, Casola S, Cattoretti G, Shen Q, Lia M, Mo T, Ludwig T, Rajewsky K, Dalla-Favera R. Transcription factor IRF4 controls plasma cell differentiation and class-switch recombination. Nat. Immunol 2006;7:773–782. [PubMed: 16767092]
35. Sciammas R, Shaffer AL, Schatz JH, Zhao H, Staudt LM, Singh H. Graded expression of interferon regulatory factor-4 coordinates isotype switching with plasma cell differentiation. Immunity 2006;25:225–236. [PubMed: 16919487]
36. Shen Y, Iqbal J, Xiao L, Lynch RC, Rosenwald A, Staudt LM, Sherman S, Dybkaer K, Zhou G, Eudy JD, Delabie J, McKeithan TW, Chan WC. Distinct gene expression profiles in different B-cell compartments in human peripheral lymphoid organs. BMC Immunol 2004;5:20. [PubMed: 15369600]

37. Lin KI, Tunyaplin C, Calame K. Transcriptional regulatory cascades controlling plasma cell differentiation. Immunol. Rev 2003;194:19–28. [PubMed: 12846804]
38. Fukuda T, Yoshida T, Okada S, Hatano M, Miki T, Ishibashi K, Okabe S, Koseki H, Hirosawa S, Taniguchi M, Miyasaka N, Tokuhisa T. Disruption of the Bcl6 gene results in an impaired germinal center formation. J. Exp. Med 1997;186:439–448. [PubMed: 9236196]
39. Tunyaplin C, Shaffer AL, Angelin-Duclos CD, Yu X, Staudt LM, Calame KL. Direct repression of prdm1 by Bcl-6 inhibits plasmacytic differentiation. J. Immunol 2004;173:1158–1165. [PubMed: 15240705]
40. Shaffer AL, Lin KI, Kuo TC, Yu X, Hurt EM, Rosenwald A, Giltnane JM, Yang L, Zhao H, Calame K, Staudt LM. Blimp-1 orchestrates plasma cell differentiation by extinguishing the mature B cell gene expression program. Immunity 2002;17:51–62. [PubMed: 12150891]
41. Shapiro-Shelef M, Lin KI, McHeyzer-Williams LJ, Liao J, McHeyzer-Williams MG, Calame K. Blimp-1 is required for the formation of immunoglobulin secreting plasma cells and pre-plasma memory B cells. Immunity 2003;19:607–620. [PubMed: 14563324]
42. Horcher M, Souabni A, Busslinger M. Pax5/BSAP maintains the identity of B cells in late B lymphopoiesis. Immunity 2001;14:779–790. [PubMed: 11420047]
43. Lin KI, Angelin-Duclos C, Kuo TC, Calame K. Blimp-1-dependent repression of Pax-5 is required for differentiation of B cells to immunoglobulin M-secreting plasma cells. Mol. Cell Biol 2002;22:4771–4780. [PubMed: 12052884]
44. Reimold AM, Iwakoshi NN, Manis J, Vallabhajosyula P, Szomolanyi-Tsuda E, Gravallese EM, Friend D, Grusby MJ, Alt F, Glimcher LH. Plasma cell differentiation requires the transcription factor XBP-1. Nature 2001;412:300–307. [PubMed: 11460154]
45. Shaffer AL, Shapiro-Shelef M, Iwakoshi NN, Lee AH, Qian SB, Zhao H, Yu X, Yang L, Tan BK, Rosenwald A, et al. XBP1, downstream of Blimp-1, expands the secretory apparatus and other organelles, and increases protein synthesis in plasma cell differentiation. Immunity 2004;21:81–93. [PubMed: 15345222]
46. Yoshida H, Matsui T, Yamamoto A, Okada T, Mori K. XBP1 mRNA is induced by ATF6 and spliced by IRE1 in response to ER stress to produce a highly active transcription factor. Cell 2001;107:881–891. [PubMed: 11779464]
47. Niu H, Ye BH, Dalla-Favera R. Antigen receptor signaling induces MAP kinase-mediated phosphorylation and degradation of the BCL-6 transcription factor. Genes Dev 1998;12:1953–1961. [PubMed: 9649500]
48. Bereshchenko OR, Gu W, Dalla-Favera R. Acetylation inactivates the transcriptional repressor BCL6. Nat. Genet 2002;32:606–613. [PubMed: 12402037]
49. Calame KL, Lin KI, Tunyaplin C. Regulatory mechanisms that determine the development and function of plasma cells. Annu. Rev. Immunol 2003;21:205–230. [PubMed: 12524387]
50. Allen CD, Okada T, Cyster JG. Germinal-center organization and cellular dynamics. Immunity 2007;27:190–202. [PubMed: 17723214]
51. Huntington ND, Xu Y, Puthalakath H, Light A, Willis SN, Strasser A, Tarlinton DM. CD45 links the B cell receptor with cell survival and is required for the persistence of germinal centers. Nat. Immunol 2006;7:190–198. [PubMed: 16378097]
52. Meyer-Hermann ME, Maini PK, Iber D. An analysis of B cell selection mechanisms in germinal centers. Math. Med. Biol 2006;23:255–277. [PubMed: 16707510]
53. Sole C, Dolcet X, Segura MF, Gutierrez H, Diaz-Meco MT, Gozzelino R, Sanchis D, Bayascas JR, Gallego C, Moscat J, Davies AM, Comella JX. The death receptor antagonist FAIM promotes neurite outgrowth by a mechanism that depends on ERK and NF-κB signaling. J. Cell Biol 2004;167:479–492. [PubMed: 15520226]
54. Hemond M, Rothstein TL, Wagner G. Fas apoptosis inhibitory molecule contains a novel β-sandwich in contact with a partially ordered domain. J. Mol. Biol 2009;386:1024–1037. [PubMed: 19168072]
55. Naismith JH, Sprang SR. Modularity in the TNF-receptor family. Trends Biochem. Sci 1998;23:74–79. [PubMed: 9538693]
56. Vogel LA, Noelle RJ. CD40 and its crucial role as a member of the TNFR family. Semin. Immunol 1998;10:435–442. [PubMed: 9826576]

57. Lanave C, Colangelo AM, Saccone C, Alberghina L. Molecular evolution of the neurotrophin family members and their Trk receptors. Gene 2007;394:1–12. [PubMed: 17379456]

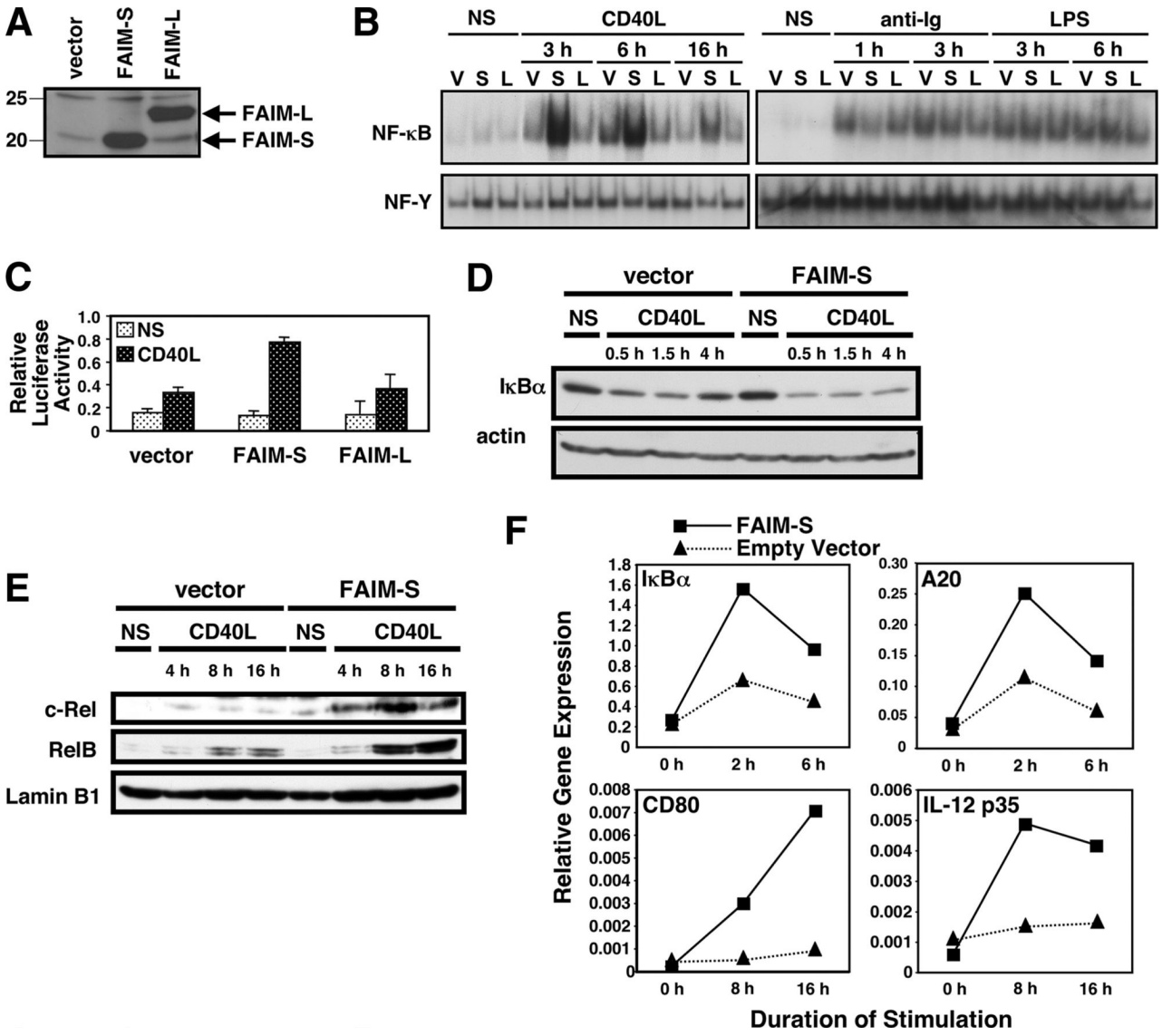

FIGURE 1.

FAIM-S enhances CD40L-induced NF-*κ*B activation. *A* and *B*, A20 cells were transduced with MSCV.IRES.GFP vectors expressing either FAIM-S or FAIM-L or with empty vector alone. Transduced A20 cells were selected on the basis of GFP expression by cell sorting, after which isolated A20 cells were evaluated for expression of FAIM-S and FAIM-L by Western blotting with rabbit anti-mouse FAIM Ab, as indicated (*A*). These GFP-expressing, transduced A20 cells were stimulated with CD40L, anti-Ig, or LPS for various times as indicated, after which nuclear extracts were obtained and tested for binding to a consensus NF-*κ*B binding site by EMSA. Nuclear extracts were also tested for NF-Y binding as a positive control. S, FAIM-S transductants; L, FAIM-L transductants; V, empty vector transductants; NS, not stimulated. One of three comparable experiments is shown. *C*, FAIM-S- and empty vector-transduced A20 cells were transiently transfected with a *κ*B-dependent firefly luciferase reporter plasmid and a thymidine kinase promoter-dependent renilla luciferase expression vector, and were either not stimulated or were stimulated by CD40L for 24 h, after which lysates were prepared and firefly luciferase activity was measured and reported in relation to the renilla luciferase control. Mean values for three independent experiments are shown. *D*, FAIM-S- and empty vector-

transduced A20 cells were either not stimulated or were stimulated with CD40L for the times indicated, after which cell lysates were prepared and evaluated for expression of IκBα by Western blotting. Blots were stripped and reprobed for tubulin content as a loading control. One of three comparable experiments is shown. *E*, FAIM-S- and empty vector-transduced A20 cells were either not stimulated or were stimulated with CD40L for the times indicated, after which nuclei were collected and evaluated for expression of RelB and c-Rel by Western blotting. Blots were stripped and reprobed for lamin B1 content as a loading control. One of three comparable experiments is shown. *F*, FAIM-S- and empty vector-transduced A20 cells were either not stimulated (0 h) or were stimulated with CD40L for the times indicated, after which RNA was prepared, reverse transcribed, and evaluated for expression of the genes indicated by real-time PCR. Expression levels were normalized to β_2-microglobulin and one of many (>3) comparable experiments is shown.

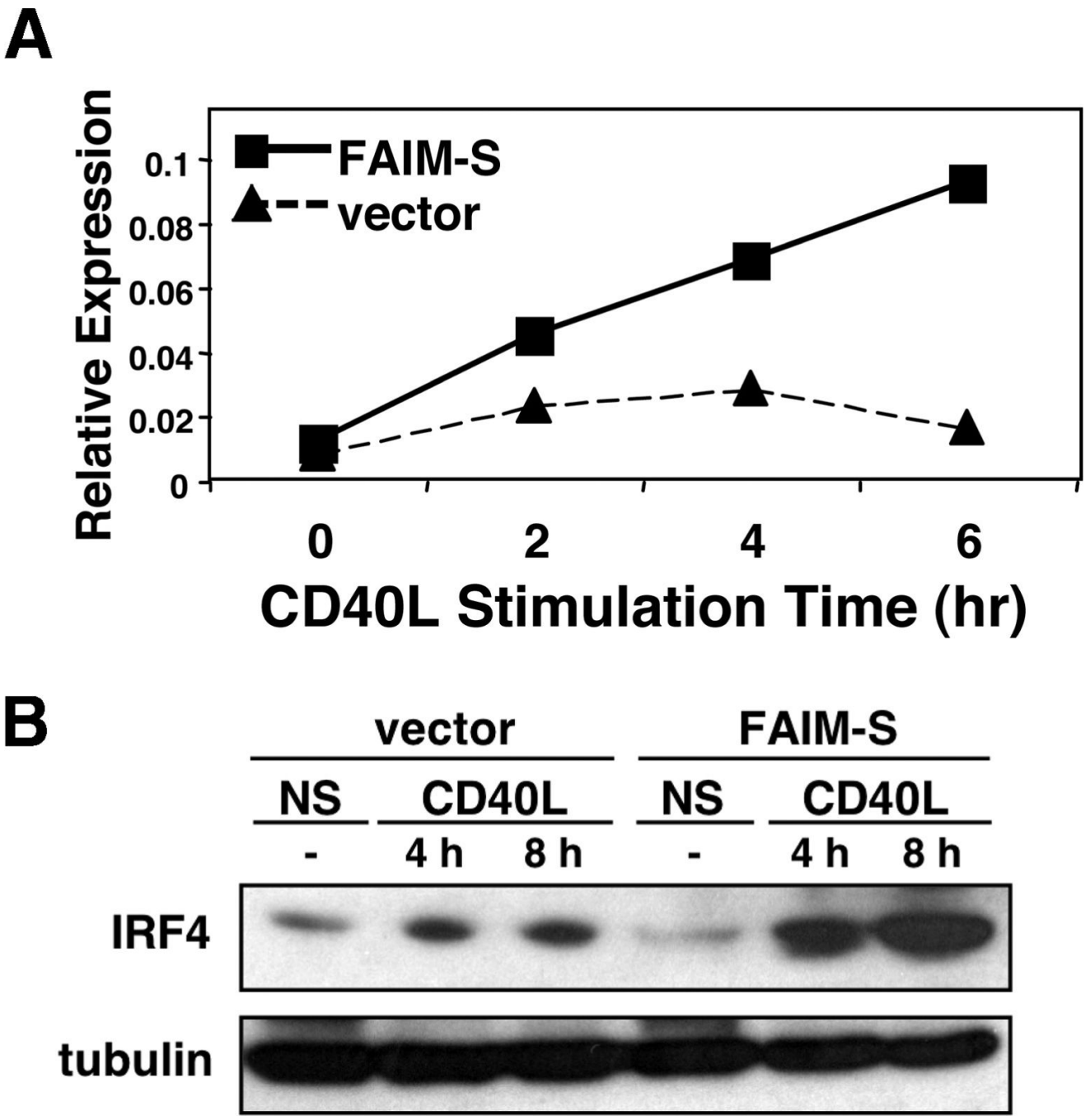

FIGURE 2.

FAIM-S enhances CD40L-induced IRF4 activation. *A*, FAIM-S- and empty vector-transduced (vector) A20 cells were either not stimulated (0 h) or were stimulated by CD40L for the times indicated, after which RNA was prepared, reversed transcribed, and evaluated for *Irf-4* gene expression by real-time PCR. Results were normalized to expression of β_2-microglobulin. One of three comparable experiments is shown. *B*, FAIM-S- and empty vector-transduced (vector) A20 cells were either not stimulated (NS) or were stimulated by CD40L for the times indicated, after which cell lysates were prepared and evaluated for expression of IRF4 by Western blotting. Blots were stripped and reprobed for tubulin content as a loading control. One of three comparable experiments is shown.

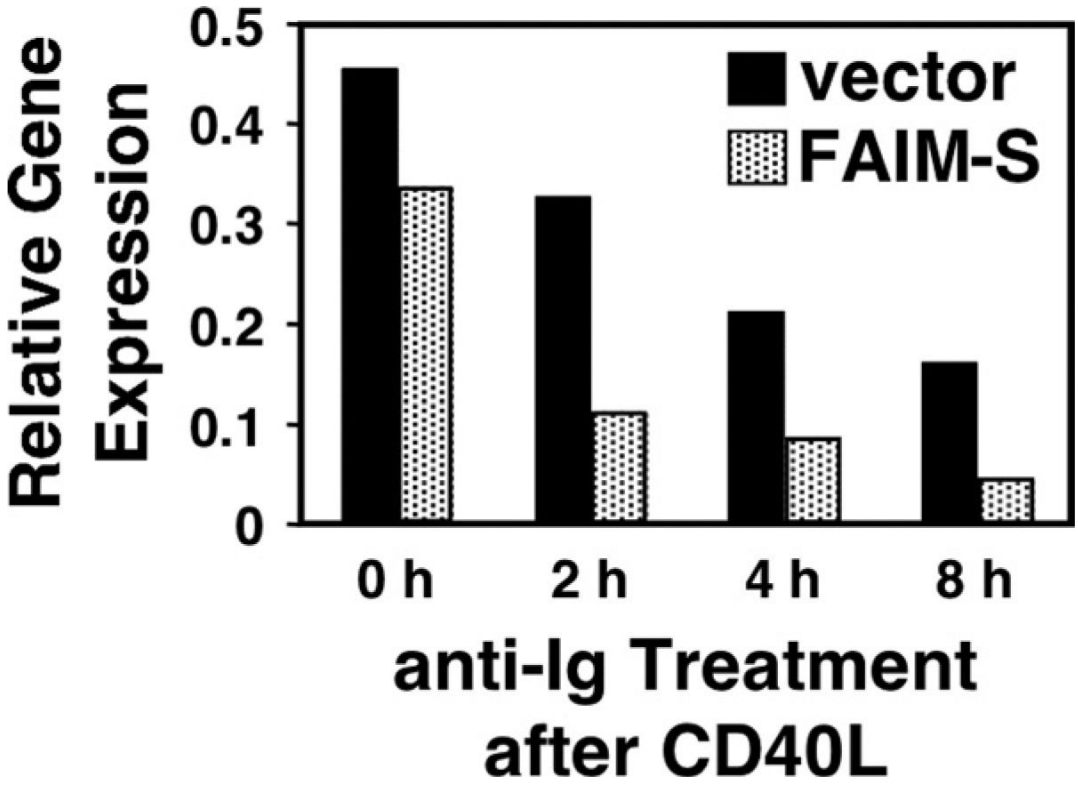

FIGURE 3.

FAIM-S enhances CD40L-induced down-regulation of BCL-6 expression. FAIM-S- and empty vector-transduced (vector) A20 cells were treated with CD40L for 48 h, during which anti-Ig was added for the final number of hours indicated. From these cells, RNA was prepared, reverse transcribed, and evaluated for *Bcl-6* gene expression by real-time PCR. Expression levels were normalized to β_2-microglobulin, and one of three comparable experiments is shown.

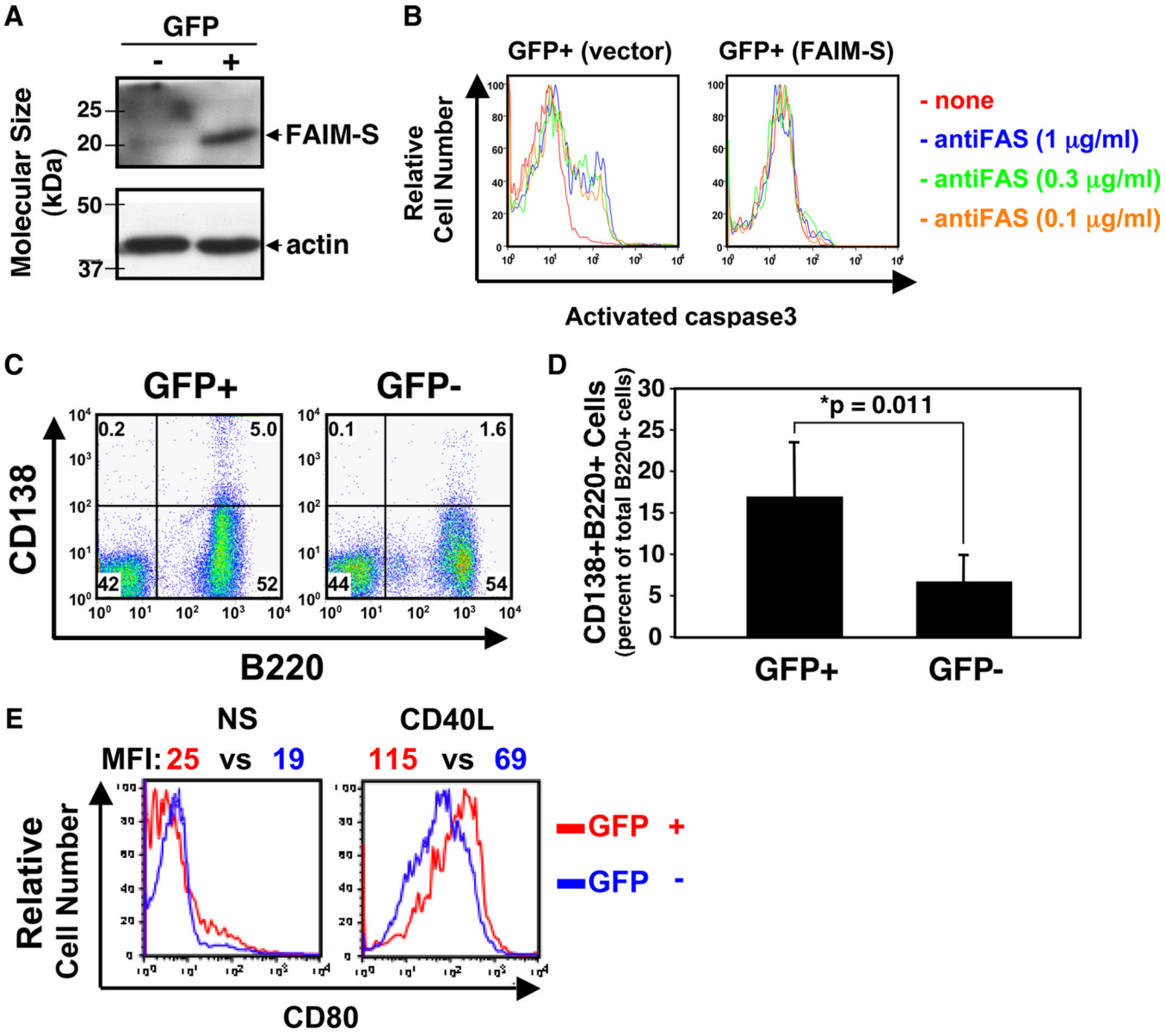

FIGURE 4.

FAIM-S augments plasma cell differentiation and CD40-mediated signaling in vivo. Bone marrow cells obtained from normal mice were transduced with FAIM-S-expressing MSCV.FAIMS.IRES.GFP vector and then adoptively transferred to lethally irradiated recipients. Following hematopoietic reconstitution of chimeric mice 8–12 wk later, GFP+ (transduced) lymphocytes were evaluated and compared with GFP– (nontransduced) lymphocytes in *A*, *C*, *D*, and *E*. *A*, Lysates were prepared from sort-purified splenic B cells and evaluated for expression of FAIM-S by Western blotting. Blots were stripped and reprobed for expression of actin as a loading control. *B*, GFP+ splenic B cells were obtained from adoptive hosts of MSCV.FAIMS. IRES.GFP-transduced and from empty vector-transduced bone marrow cells and were stimulated with CD40L for 24 h and then treated with Jo-2 anti-Fas Ab for 4 h, after which B cells were treated with cell permeable red-DEVD-FMK and activated caspase 3 was detected by flow cytometry. *C* and *D*, Bone marrow cells were stained for B220 and CD138 and analyzed by flow cytometry. A typical experiment is shown in *C*, and results

compiled from five such experiments are displayed in *D*, wherein $B220^+CD138^+$ cells are represented as a percentage of $B220^+$ cells (mean ± SD). *E*, Sort-purified B cells were either not stimulated (NS) or were stimulated by CD40L for 48 h, after which CD80 expression was evaluated by immunofluorescent staining and flow cytometric analysis. The mean fluorescence intensity (MFI) of GFP+ vs GFP– B cells is provided above each of the panels. One of two comparable experiments is shown.

Table I

Oligonucleotide sequences used for quantitative PCR and EMSA[a]

	Forward	Reverse
Quantitative PCR		
*β*2-microglobulin	CTGACCGGCCTGTATGCTAT	TTTTCCCGTTCTTCAGCATT
FAIM	TGCAATGGTCAGAAAATGGA	CGCTGCTCACAGCTTTTATG
CD80	GATGCTCACGTGTCAGAGGA	CAACGATGACGACGACTGTT
IL-12 p35	CATCGATGAGCTGATGCAGT	GAAGCAGGATGCAGAGCTTC
IRF4	CTACCCCATGACAGCACCTT	CCAAACGTCACAGGACATTG
BCL-6	GACGTTGTCATCGTGGTGAG	GGTTGCATTTCAACTGGTCA
A20	GGCGCCAAAAGAATCAGAGC	AGAAAAAGGCTGGGTGCTGTG
I*κ*B*α*	TTGGTGACTTTGGGTGCTGAT	GAGCGAAACCAGGTCAGGATT
EMSA		
NF-*κ*B	AGCTTCAGAGGGGACTTTCCGAGAGG	TCGACCTCTCGGAAAGTCCCCTCTGA
NF-Y	AGCTGATCTGAGAATTTTCTGATTGGTTCTGGCGAGTTTGGTCGACCAAACTCGCCAGAACCAATCAGAAAATTCTCAGATC	

[a] All sequences are presented in the 5′ to 3′ direction.