

Egr Family Members Regulate Nonlymphoid Expression of Fas Ligand, TRAIL, and Tumor Necrosis Factor during Immune Responses†

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The Fas ligand (FasL)/Fas pathway is crucial for homeostasis of the immune system and peripheral tolerance. Peripheral lymphocyte deletion involves FasL/Fas in at least two ways: coexpression of both Fas and its ligand on T cells, leading to activation-induced cell death, and expression of FasL by nonlymphoid cells, such as intestinal epithelial cells (IEC), that kill Fas-positive T cells. We demonstrate here that superantigen *Staphylococcus enterotoxin B* (SEB) induced a dramatic upregulation of *FasL*, *TRAIL*, and *TNF* mRNA expression and function in IEC from BALB/c and C57BL/6 mice. Using adoptive transfer in which CD4⁺ T cells from OT-2 T-cell receptor transgenic mice were transferred into recipients, we observed an induction in IEC of *FasL*, *TRAIL*, and *TNF* mRNA after administration of antigen. Specific Egr-binding sites have been identified in the 5' promoter region of the *FasL* gene, and *Egr-1*, *Egr-2*, and *Egr-3* mRNA in IEC from mice treated with SEB and from transgenic OT-2 mice after administration of antigen was upregulated. Overexpression of *Egr-2* and *Egr-3* induced endogenous ligand upregulation that was inhibited by overexpression of Egr-specific inhibitor Nab1. These results support a role for Egr family members in nonlymphoid expression of *FasL*, *TRAIL*, and *TNF*.

Apoptosis-inducing members of the tumor necrosis factor (TNF) family, the so-called death ligands, include TNF, FasL, and TNF-related apoptosis-inducing ligand (TRAIL). These ligands and their receptors play pivotal roles in the regulation of the immune system, especially via induction of apoptosis in activated lymphocytes. In particular, they have been implicated in the process of activation-induced cell death (AICD) in T lymphocytes, in which repeated antigen stimulation of T cells induces apoptosis through the induction of death ligand expression and sensitization to death receptor-induced apoptosis (3, 6, 13, 44). Death ligand-mediated AICD is at least one mechanism for peripheral deletion, the process whereby expanding clones of activated T cells subsequently decrease in number (31).

The roles for death ligands in immune regulation include expression of death ligands in nonlymphoid tissues. Immune privilege in the anterior chamber of the eye requires functional FasL expression in this organ, which induces apoptosis in infiltrating lymphocytes (9). Similarly, functional FasL expression on the cornea contributes to graft acceptance in an animal model of cornea transplant (42). More recent studies have shown that epithelial cells of the eye express, in addition to FasL, TRAIL (21) and TNF (8), which may also contribute to immune privilege.

Other nonlymphoid tissues express FasL only during immune responses involving extensive T-cell activation, and this is also likely to contribute to peripheral deletion (36). Such

tissues include the lung, liver, and small intestine. More recently, we demonstrated that expression of FasL on intestinal epithelial cells (IEC) is induced by TNF in response to antigen (35). TNF-induced FasL expression in IEC was found to depend on activation of NF- κ B and its effect on the *FasL* promoter.

Cyclosporine has been shown to interfere with AICD (41), suggesting that cyclosporine-sensitive transcription factors may be involved. Indeed, several studies have demonstrated roles for the cyclosporine-sensitive nuclear factor of activated T cells (NFAT) transcription factor and for early-growth-response transcription factors, mainly *Egr-2* and *Egr-3*, in T-cell expression of the *FasL* gene following T-cell receptor (TCR) ligation (7, 38). NFAT is a family of transcription factors that are present in the cytoplasm in a phosphorylated form. After dephosphorylation mediated by the phosphatase calcineurin, these factors are rapidly translocated into the nucleus, where they activate different genes, including those encoding cell surface receptors, signaling molecules, and cytokines. Double knockout mice lacking both NFATp and NFAT4 exhibit impaired induction of FasL and a lymphoproliferative disorder, suggesting a key role for these transcription factors in *FasL* transcription (37).

While several studies postulate that NFAT may be a direct regulator of *FasL* (19, 20, 23), it has also been demonstrated that the cyclosporine-inhibitable *Egr-2* and *Egr-3* transcription factors are potent regulators of *FasL* expression (29, 30). This family is composed of four members: *Egr-1* (NGFI-A, *krox24*, *zif268*), *Egr-2* (*krox20*), *Egr-3* (PILOT), and *Egr-4* (NGFI-C). All of these members share a highly conserved DNA-binding domain composed of three zinc finger motifs that bind and transactivate transcription from the consensus sequence GCG GGGCG (33). Similar to some other transcription factors, Egr members associate with corepressor proteins Nab1 and

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Nab2, which can modulate transcription of Egr-dependent genes. These factors bind to Egr-1, Egr-2, and Egr-3 by direct protein-protein interactions with a conserved R1 domain, inhibiting the transactivating potential of these transcription factors (32).

Here, we extend our observations on FasL expression in IEC to other death ligands and transcription factors. We report that *Egr-1*, *Egr-2*, and *Egr-3* mRNA is upregulated in IEC from BALB/c and C57BL/6 mice challenged with superantigen *Staphylococcus enterotoxin B* (SEB) and from transgenic OT-2 mice after administration of antigen. Overexpression of Egr-2, Egr-3, or TNF in IEC induces endogenous *FasL*, *TRAIL*, and *TNF* upregulation, whereas Nab1 coexpression completely abolishes these effects. These results support a role for Egr family members in nonlymphoid expression of *FasL*, *TRAIL*, and *TNF*.

MATERIALS AND METHODS

Cell lines and animals. All animals were bred in house or purchased from Jackson Laboratories (Bar Harbor, Maine) and housed under pathogen-free conditions at the La Jolla Institute for Allergy and Immunology. All cell lines were obtained from the American Type Culture Collection (Manassas, Va.), including L1210 and HT29 cells, and grown in suspension culture medium RPMI 1640. Primary IEC were isolated from small intestines according to the procedure previously described (22) and cultured in Dulbecco's modified Eagle's medium. Each medium was supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 U of penicillin/ml, and 100 µg of streptomycin/ml, and the cells were grown in 5% CO₂ at 37°C.

Adoptive transfer and antigenic stimulation. CD4⁺ lymphocyte subsets were enriched as previously described (36), and lymphocytes (5 × 10⁶ to 10 × 10⁶) were introduced into the tail veins of recipient animals. Superantigen SEB (75 to 100 µg/mouse; Sigma) was injected intraperitoneally (i.p.) into BALB/c mice. OT-2 TCR transgenic animals and recipients of OT-2 lymphocytes were treated subcutaneously (s.c.) with the ovalbumin (OVA) peptide consisting of amino acids 323 to 339 (OVA 323-339 peptide) at 300 µg per mouse. The OVA 323-329 peptide introduced s.c. was delivered in complete Freund's adjuvant (15).

Functional assay for FasL and TRAIL expression. FasL and TRAIL activities were detected by the ability of IEC to induce DNA fragmentation in Fas-positive and TRAIL receptor 2 (TRAIL-R2)-positive target cells (3, 21). Briefly, parental L1210 and L1210-Fas, both containing TRAIL-R2, were labeled with 5 µCi of [³H]thymidine/ml for 4 h (39). After incubation, cells were rinsed twice with Hanks balanced salt solution (HBSS) to remove unincorporated thymidine. Target cells were then incubated in complete medium with dissociated epithelial cells from the small intestines of control or treated mice in 96-well plates in the presence or absence of Fas-Fc and/or TRAIL-R2-Fc (R&D Systems, Minneapolis, Minn.). After 18 h, cells were harvested with a multiautomated system harvester, and DNA was counted in a beta counter. DNA fragmentation was determined as previously described (2).

Real-time reverse transcription-PCR (RT-PCR). Total RNA was isolated from primary IEC with Trizol (Gibco BRL, Rockville, Md.) according to the manufacturer's instructions. RNA was reverse transcribed by Moloney murine leukemia virus reverse transcriptase (Gibco BRL) with random hexamers (Gibco BRL). For FasL gene expression, primers were located in separate exons in genomic DNA in order to eliminate the possibility of genomic DNA contamination. Sequence-specific primers for murine FasL, TNF, β-actin, 18S, and luciferase were previously described (35). Specific forward and reverse primers were, respectively, as follows: murine TRAIL, 5'-CTCTCGGAAAGGGCAT TC-3' and 5'-TCCTGCTCGATGACCAGCT-3'; Egr-1, 5'-GGCGATGGTGG AGACGAGT-3' and 5'-CGGCCAGTATAGGTGATGGG-3'; Egr-2, 5'-TCTC CGTGCCAGAGAGATCC-3' and 5'-GAGGACAGGGAAACGGCTTT-3'; Egr-3, 5'-CACTGGAGACCATCAAGGCC-3' and 5'-GGCAGGCTGCCGA A-3'; TRAIL-R2, 5'-TGCTGCTCAAGTGGCGC-3' and 5'-GGCATCCAGCA GATGGTTG-3'; human FasL, 5'-CAGTCCACCCCTGAAAAAAA-3' and 5'-CCAGAGGATGGACCTTGGAG-3'; 18S, 5'-ACATCAAAGGAAGGCAG-3' and 5'-TCGTCACCTACTCCCGG-3'; LacZ, 5'-AATGGCTTTCGC TACCTGGA-3' and 5'-CCATCGCTGGGCGTA-3'; green fluorescent protein (GFP), 5'-TGGCCCTGTCTTTTACCAG-3' and 5'-TTTTCGTTGGGA TCTTTCGAA-3'; *Renilla*, 5'-AATTTGCAGCATATCTTGAACCATT-3' and

5'-GGATTTCACGAGGCCATGAT-3'. Real-time PCR was performed with AmpliTaq Gold polymerase in a PE Biosystems 5700 thermocycler using the SyBr Green detection protocol as outlined by the manufacturer. Briefly, 12 ng of total cDNA, 50 nM (each) primer, and 1× SyBr Green mixture were used in a total volume of 25 µl.

Plasmid constructs and retrovirus gene transfer. Murine Egr-2, Egr-3, and Nab1 cDNAs were amplified by RT-PCR from total RNA using the Egr-2 forward primer (5'-ATGAACGGAGTGGCGGGAGATGGC-3'), Egr-2 reverse primer (5'-TCACGGTGTCTGGTTCGAGAGGT-3'), Egr-3 forward primer (5'-ATGACCGCAAACCTCGCCGAG-3'), Egr-3 reverse primer (5'-T CAGGCGCAGTGGTGACCAC-3'), Nab1 forward primer (5'-ATGCCAC AGCCTTACCTAGGACCCTG-3'), and Nab1 reverse primer (5'-CTATCTTG AGTCTTCAGGCTCTG-3') at the respective ATG start and stop codons. All amplified fragments were cloned directly into the pcDNA3.1V5-TOPO vector (Invitrogen, Carlsbad, Calif.) and sequenced according to the manufacturer's instructions.

The hemagglutinin-tagged Egr-3 product was generated by PCR using the Egr-3 forward primer containing an *EcoRI* restriction site (5'-TATAGAATTC ATGACCGCAAACCTCGCCG-3') and the Egr-3 reverse primer containing a *NotI* restriction site (5'-TATAGCGGCCGCTTATGCATAATCTGGTACATC GTATGGATAGGCGCAGGTGGTGACCACAGGGGC-3'). The PCR product was digested with *EcoRI* and *NotI* and cloned into the retroviral pMX-IRES-GFP vector, digested with *EcoRI* and *NotI*. Retrovirus production, HT29 cell infection, and sorting were conducted as previously described (5).

Human *FasL* promoter constructs were generated by PCR. The bp -300 and -240 regions were cloned with the 5'-CCCAAGCTTCGGCTGTTATCAGAA AATTGTGGG-3' and the 5'-CCCAAGCTTCTCTCAGCTTCAGCTGCAA AGCG-3' sense primers together with the 5'-ACGCGTCGACCATGGCAGC TGGTGGTTCAGGCCAGC-3' antisense primer. Egr and NFAT with mutations in the bp -300 region were generated by PCR using the 5'-CCCAAGCTTCG GCTGTTATCAGAAAATTTTAAACGGAAACTTCC-3' and the 5'-CCCAA GCTTCGGCTGTTATCAGAAAATTTGTGGGCGTCCCGATCCAGG-3' sense primers, respectively, and Egr with mutations in the bp -240 region was generated by PCR using the 5'-CCCAAGCTTCTCTCAGCTTCAGCTGCAAAGCTCTA AATGTTTCT-3' sense primer. All amplified fragments were cloned directly into the pGLOW vector (Invitrogen) and sequenced according to the manufacturer's instructions.

Transient-transfection and reporter assays. In transient-expression experiments, all transfections were performed with Superfect (Qiagen, Valencia, Calif.) according to the manufacturer's instructions and 0.5 µg of each vector. Transfected IEC, HT29, and Egr3-HT29 cells were incubated for 18 h at 37°C before analysis by real-time PCR. Due to the low efficiency of transient transfection, TNF, Egr2, Egr3, and Nab1 overexpression was checked by real-time PCR. The antagonist anti-TNF receptor type 1 (TNFR1) antibody (clone 55R-170) was used at a concentration of 2 µg/ml. GFP mRNA was analyzed by real-time RT-PCR, and transfection efficiencies were measured by analysis of *Renilla* or luciferase reporter mRNA expression.

Protein extraction and Western blot analysis. The antibodies used in this study included anti-TRAIL, anti-FasL (Santa Cruz Biotechnology, Santa Cruz, Calif.), anti-TNF (a gift from Carl F. Ware), and antiactin (ICN, Aurora, Ohio). For protein extraction, cells were washed twice in phosphate-buffered saline (PBS), lysed in buffer containing 150 mM NaCl, 50 mM pH 8.0 Tris-HCl, 0.1% Sodium dodecyl sulfate (SDS), and 0.5% Na deoxycholate in the presence of protease inhibitors for 30 min at 4°C and then centrifuged (20 min; 15,000 × g) and supernatants were collected. For Western blots, after SDS-polyacrylamide gel electrophoresis and electrotransfer to nitrocellulose membranes (Amersham Biosciences), the membranes were incubated for at least 1 h with 5% nonfat milk in TPBS (0.1% Tween 20, 1× PBS) and for 2 h at room temperature with primary antibodies. Horseradish peroxidase-conjugated goat anti-rabbit, anti-hamster, or anti-mouse secondary antibodies were then added for 30 to 60 min, and proteins were revealed by enhanced chemiluminescence reagent (Pierce, Rockford, Ill.) and autoradiography.

RESULTS

***FasL*, *TRAIL*, and *TNF* mRNA are upregulated in IEC after SEB injection.** We have previously established that the superantigen SEB induces a dramatic upregulation of *FasL* expression and function in nonlymphoid cells of the liver and small intestine (2). Using real-time RT-PCR, we studied the expression of other death ligands of the TNF family in IEC from

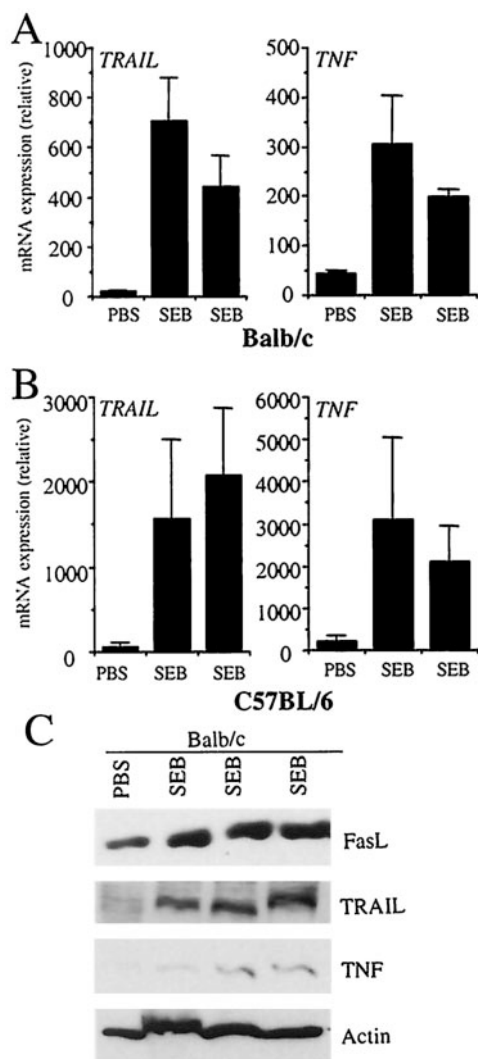


FIG. 1. IEC FasL, TRAIL, and TNF are induced in response to superantigen. BALB/c (A) and C57BL/6 (B) mice were injected i.p. with superantigen SEB (75 to 100 μ g), and after 3 days IEC total mRNA was isolated and assessed by real-time RT-PCR for *TRAIL* and *TNF* mRNA expression. Independent data from two SEB-treated mice are shown for each strain. Absolute mRNA values were determined, normalized to β -actin, and reported as arbitrary units. (C) FasL, TRAIL, and TNF protein levels were also assessed by Western blotting. Independent results from three SEB-treated mice are shown.

BALB/c and C57BL/6 mice injected with PBS or SEB. Interestingly, we observed a dramatic upregulation of *TRAIL* and *TNF* mRNA in these cells 3 days after SEB injection (Fig. 1A and B). In keeping with our previous observations, an increase in FasL protein levels in response to superantigen-induced activation of peripheral lymphocytes and an increase in TRAIL and TNF protein levels were also observed (Fig. 1C).

OT-2 transfer induces *FasL*, *TRAIL*, and *TNF* mRNA up-regulation. hFlp-lacZ transgenic mice express a *LacZ* construct under the control of the proximal promoter of the human *FasL* gene (36), and antigenic challenge of these mice resulted in increased expression of nonlymphoid *FasL* as well as *LacZ* in the epithelium of the small intestine (36). To study

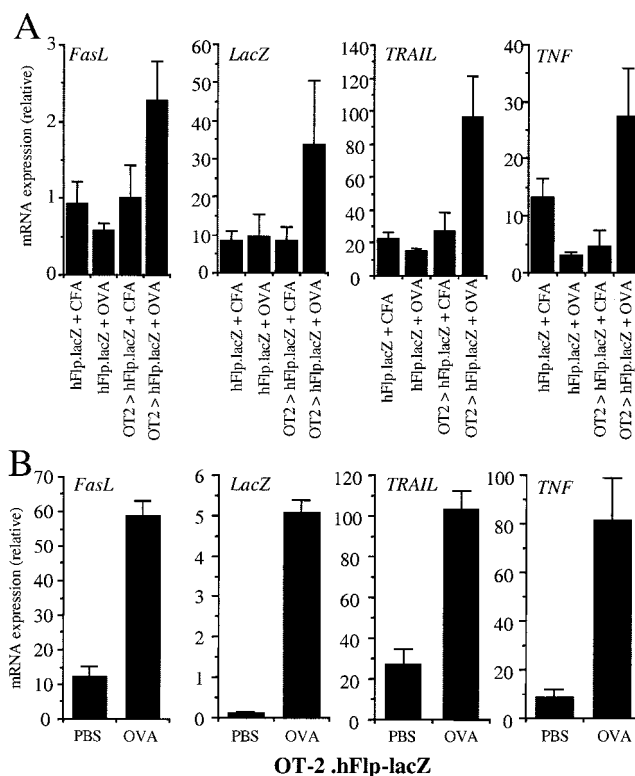


FIG. 2. OT-2 transfer into hFlp-lacZ transgenic mice and antigen stimulation in OT-2 \times hFlp-lacZ double-transgenic mice simultaneously induce *FasL*, *TRAIL*, and *TNF* upregulation. CD4⁺ T cells from OT-2 TCR transgenic mice were transferred into C57BL/6 recipients. OT-2 \rightarrow hFlp-lacZ recipients (A) and OT-2 \times hFlp-lacZ double-transgenic (B) mice were challenged with OVA 323-339, and IEC were isolated. The OVA peptide introduced s.c. was delivered in complete Freund's adjuvant. Total mRNA was isolated and assessed by real-time RT-PCR for *FasL*, *LacZ*, *TRAIL*, and *TNF* mRNA expression. Absolute mRNA values were determined, normalized to β -actin, and reported as arbitrary units.

the expression of the different death ligands in IEC, we employed an adoptive-transfer approach in which CD4⁺ T cells from OT-2 TCR (OVA-plus-I-A^b-responsive) transgenic mice were transferred into transgenic hFlp-lacZ recipient mice (OT-2 \rightarrow hFlp-lacZ mice). OT-2 \rightarrow hFlp-lacZ recipients were challenged with the OVA peptide; 3 days later IEC were isolated and RNA was assessed by real-time RT-PCR for endogenous *FasL*, *LacZ*, *TRAIL*, and *TNF* expression. In keeping with our previous findings, we observed that induction of expression of *LacZ* mRNA correlated with expression of *FasL* mRNA after administration of antigen (Fig. 2A). We also observed a significant induction of *TRAIL* and *TNF* mRNAs, demonstrating that activated lymphocytes induced accumulation of these different ligands at the mRNA level. A similar effect was observed when hFlp-lacZ \times OT-2 double-transgenic animals were immunized directly with OVA (Fig. 2B).

***FasL*, *TRAIL*, and *TNF* induction in IEC is TNF dependent.** We recently reported that FasL expression in IEC is induced in activated T cells through a TNF-dependent mechanism (35). To determine if *TRAIL* and *TNF* mRNA expression in the IEC is also induced by TNF production, we injected TNF into mice and examined ligand expression in the epithelium of the small

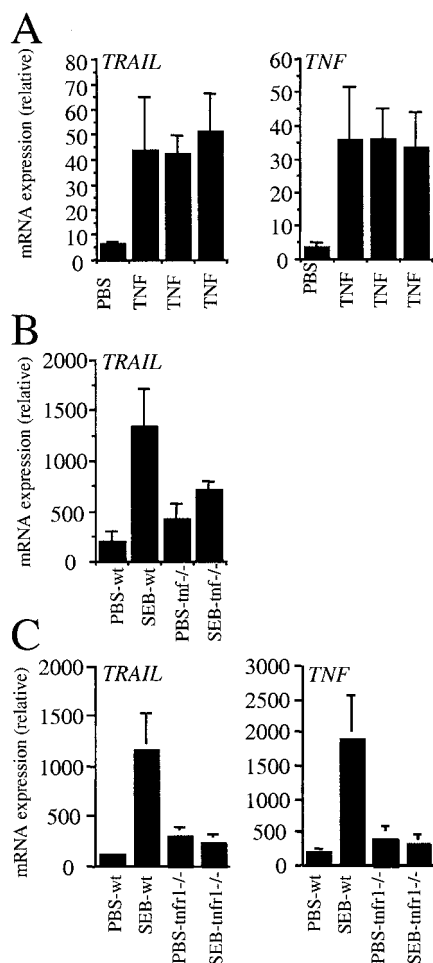


FIG. 3. Induction of nonlymphoid *TRAIL* and *TNF* mRNA is dependent on TNF. (A) BALB/c mice were injected with TNF (2 μ g), and IEC were harvested 12 h later and analyzed for *TRAIL* and *TNF* mRNA expression. Absolute mRNA values were determined, normalized to 18S RNA, and reported as arbitrary units. Data from three TNF-treated mice are shown. (B) B6129SF2 and *TNF*^{-/-} mice were injected with SEB. IEC were isolated 3 days after SEB administration and analyzed for *TRAIL* mRNA expression. Absolute mRNA values were determined, normalized to β -actin, and reported as arbitrary units. (C) C57BL/6 and *TNFR1*^{-/-} mice were injected with SEB. IEC were isolated 3 days after SEB administration and analyzed for *TRAIL* and *TNF* mRNA expression. Absolute mRNA values were determined, normalized to β -actin, and reported as arbitrary units.

intestine. Injection of TNF (2 μ g/mouse) resulted in a significant increase in *TRAIL* and *TNF* expression in IEC in vivo (Fig. 3A). We then asked if TNF is necessary for upregulation of *TRAIL* in IEC as it is for upregulation of *FasL*. To address this question, wild-type and *TNF*^{-/-} mice were injected with SEB and 3 days later IEC mRNA was isolated and assayed by real-time RT-PCR for *TRAIL* mRNA expression. We observed a significant increase of *TRAIL* mRNA expression in wild-type mice after SEB injection, whereas little *TRAIL* mRNA was seen in *TNF*^{-/-} mice (Fig. 3B). We have previously shown that IEC constitutively express *TNFR1* (p55) (35) and not *TNFR2* (data not shown). To confirm that the observed effect is dependent on *TNFR1*, wild-type and *TNFR1*^{-/-} mice were injected with SEB. Three days later total IEC

mRNA was isolated, and *TRAIL* and *TNF* mRNA expression was assessed by real-time RT-PCR (Fig. 3C). Levels of *TRAIL* and *TNF* mRNA expression in wild-type IEC were significantly increased after SEB injection, whereas no upregulation was seen for *TRAIL* and *TNF* in IEC from *TNFR1*^{-/-} mice (Fig. 3C). Taken together, these results suggest that TNF is involved in *TRAIL* expression following exposure to antigen.

IEC express functional FasL and TRAIL. FasL present in nonlymphoid tissue has been shown to delete reactive lymphocytes during viral infection and after exposure to SEB or an OVA peptide (2, 9, 36). To evaluate the function of nonlymphoid FasL and TRAIL, we isolated IEC from SEB-treated and untreated animals and tested their ability to kill Fas⁺ target cells. As shown in Fig. 4, IEC from SEB-treated mice killed targets bearing the Fas receptor (L1210-Fas), confirming our previous observation (Fig. 4A). Because L1210-Fas also expresses the TRAIL-R2 (Fig. 4B, inset), we used these cells to examine the function of TRAIL by culturing the IEC and target cells in the presence of Fas-Fc or TRAIL-R2-Fc. The apoptosis induced by IEC in L1210-Fas cells was partially inhibited when Fas-Fc or TRAIL-R2-Fc was included in the culture (Fig. 4B). We also observed a greater protection when both Fas-Fc and TRAIL-R2-Fc were added together to the culture in which IEC and targets were at a higher ratio (10:1), suggesting a possible cooperation between these two ligands in killing. However, the addition of both Fas-Fc and TRAIL-R2-Fc did not completely inhibit cell death, and therefore it is possible that other factors are also involved.

To further evaluate the function of nonlymphoid TRAIL, we isolated IEC from SEB-treated C57BL/6-*gld* animals and tested their ability to kill TRAIL-R2⁺ target cells (L1210). IEC from SEB-treated C57BL/6 animals induced apoptosis in L1210 cells, and this apoptosis was partially inhibited when TRAIL-R2-Fc was included in the culture (Fig. 4C). IEC from SEB-treated C57BL/6-*gld* animals induced less apoptosis in L1210 cells, presumably due to nonfunctional FasL, but the observed death was almost completely inhibited when TRAIL-R2-Fc was included in the culture (Fig. 4C). Again, the addition of TRAIL-R2-Fc did not completely inhibit cell death in the higher-ratio (10:1) culture, suggesting that other factors could be involved. Thus, the FasL and the TRAIL induced on IEC by in vivo administration of SEB are biologically active.

Egr transcription factors are upregulated in IEC after SEB and OVA injection. The *Egr-1* transcription factor has been shown to be involved in *TNF* expression, while *Egr-2* or *Egr-3* expression can induce *FasL* transcription in nonlymphoid cells (17, 30). These observations suggest a possible role for *Egr* transcription factors in *FasL* and *TNF* expression and perhaps *TRAIL* expression. *Egr* family members are zinc finger proteins that are rapidly induced in diverse cell types following cell activation by different stimuli. *Egr-1* mRNA is known to be constitutively expressed in many tissues, whereas *Egr-2* and *Egr-3* mRNA is not found in unactivated tissues (34), arguing in favor of de novo synthesis mechanisms. Using real-time RT-PCR, we studied endogenous *Egr-1*, *Egr-2*, and *Egr-3* gene expression in IEC from BALB/c, C57BL/6, B6129SF2 wild-type, *TNF*^{-/-}, and *TNFR1*^{-/-} mice injected with PBS or SEB. We observed that *Egr-1* mRNA is the major *Egr* mRNA expressed in these cells, while *Egr-2* and *Egr-3* mRNA is expressed at lower levels (Fig. 5). Constitutive *Egr-2* and *Egr-3*

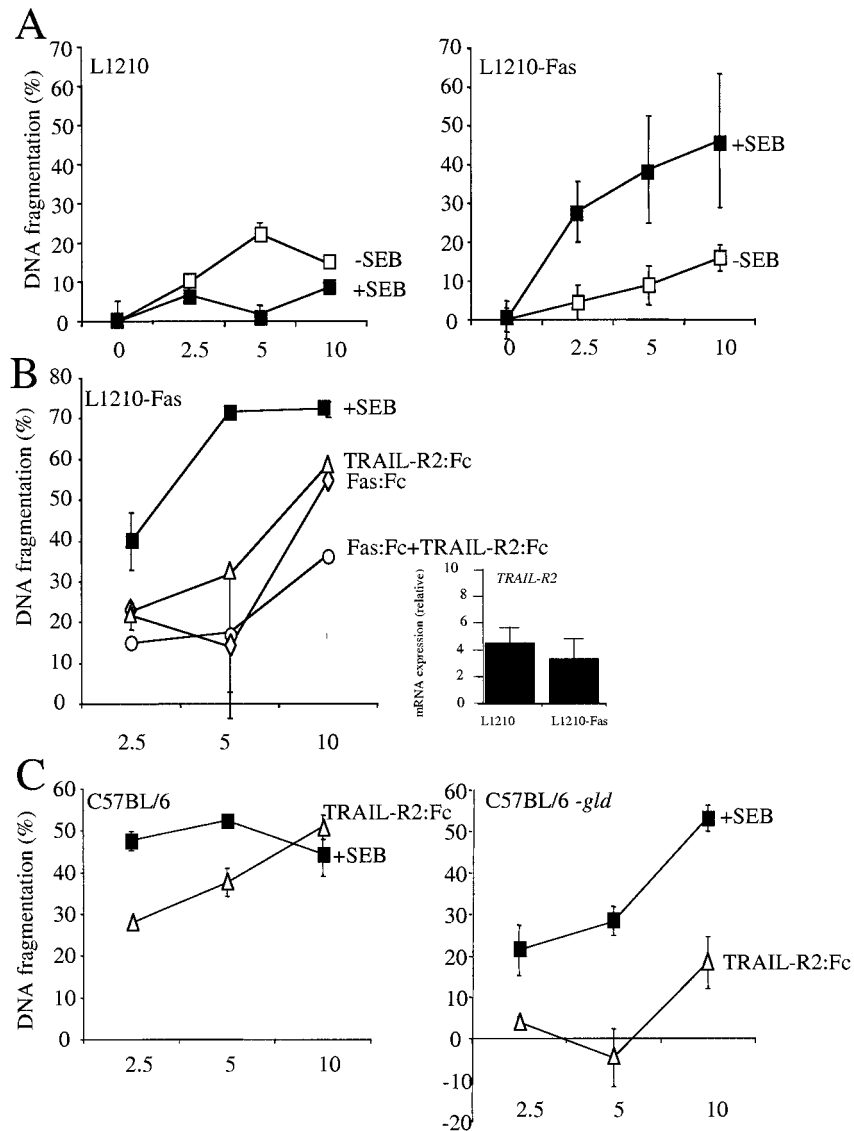


FIG. 4. Epithelial cells from the small intestines of SEB-treated mice have functional FasL and TRAIL. BALB/c mice were challenged in vivo with PBS or SEB, and IEC were prepared. (A) IEC were cocultured with [3 H]thymidine (TdR)-labeled L1210 or L1210-Fas target cells for 18 h at the ratios indicated on the x axes. Induction of apoptosis in the targets was assessed as the percentage of specific DNA fragmentation. (B) The contribution of FasL and TRAIL to the killing of L1210-Fas targets expressing TRAIL-R2, as assessed by real-time RT-PCR (inset), was studied by inclusion of 5 mg of Fas:Fc and/or TRAIL-R2-Fc/ml. Induction of apoptosis in the L1210-Fas targets at the indicated ratios was assessed as the percentage of specific DNA fragmentation. (C) C57BL/6 and C57BL/6-*gld* mice were challenged in vivo with SEB, and IEC were prepared. The IEC were cocultured with [3 H]TdR-labeled L1210 target cells for 18 h at the indicated ratios. The contribution of TRAIL to the killing of L1210 targets was studied by inclusion of 5 mg of TRAIL-R2-Fc/ml. Induction of apoptosis in the L1210 targets at the indicated ratios was assessed as the percentage of specific DNA fragmentation.

mRNA expression in some tissues is not consistent with previous observations (34) but can be explained by the fact that real-time PCR is more sensitive than Northern analysis or conventional RT-PCR.

In addition, we observed a dramatic upregulation of *Egr-1*, *Egr-2*, and *Egr-3* mRNA in wild-type IEC 3 days after SEB injection (Fig. 5A). The kinetics of appearance of *FasL*, *TRAIL*, and *TNF* mRNA in these cells after SEB injection correlated with *Egr* expression (Fig. 1A and B). *Egr-1*, *Egr-2*, and *Egr-3* mRNA was also induced in IEC from hFlp-lacZ \times OT-2 double-transgenic mice after direct immunization with

OVA (Fig. 5B). To determine if *Egr* mRNA expression in IEC was dependent on TNF, wild-type and *TNF^{-/-}* mice were injected with SEB and 3 days later IEC mRNA was isolated and *Egr* expression was assessed by real-time RT-PCR (Fig. 6A). *Egr-1* and *Egr-3* mRNA expression in wild-type IEC was significantly increased after SEB injection, whereas no upregulation was seen for *Egr-1*, *Egr-2*, or *Egr-3* in IEC from *TNF^{-/-}* mice (Fig. 6A).

We have shown that IEC express TNFR1 (35). To confirm that the observed effect is dependent on TNFR1, wild-type and *TNFR1^{-/-}* mice were injected with SEB and 3 days later IEC

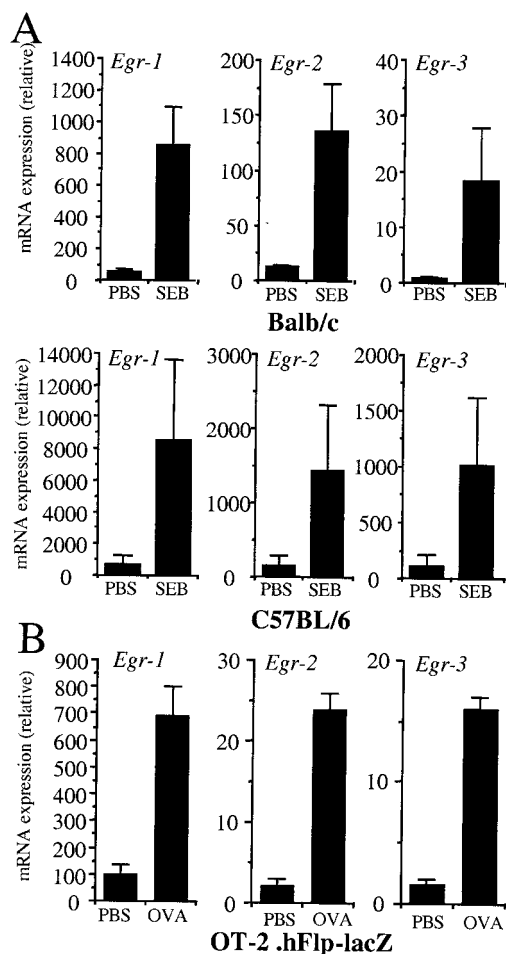


FIG. 5. IEC *Egr-1*, -2, and -3 mRNA is induced in response to superantigen and after antigen stimulation in OT-2 × hFlp-lacZ double-transgenic mice. BALB/c and C57BL/6 (A) and OT-2 × hFlp-lacZ double-transgenic (B) mice were injected i.p. with superantigen SEB (75 to 100 μg) (A) or challenged with OVA (B), and after 3 days IEC total mRNA was isolated and assessed by real-time RT-PCR for *Egr-1*, *Egr-2*, and *Egr-3* mRNA expression. Absolute mRNA values were determined, normalized to β-actin, and reported as arbitrary units.

mRNA was isolated and Egr expression was assessed by real-time RT-PCR (Fig. 6B). *Egr-2* and *Egr-3* mRNA expression in wild-type IEC was significantly increased after SEB injection, whereas no upregulation was seen for *Egr-1*, *Egr-2*, or *Egr-3* in IEC from TNFR1^{-/-} mice (Fig. 6B).

Egr-2 and Egr-3 transcription factors induce endogenous *FasL*, *TRAIL*, and *TNF* upregulation in IEC. Egr family transcription factors contain no known dimerization domains and are known to bind to DNA and activate transcription as monomers (32). Since *Egr-1* did not appear to have a major role in activation-induced upregulation of *FasL* transcription (30), we studied endogenous ligand expression after *Egr-2* or *Egr-3* overexpression in primary IEC. *Egr-2* or *Egr-3* overexpression was sufficient to induce endogenous *FasL* mRNA accumulation in both cell types (Fig. 7). Interestingly, we also observed an increase in endogenous *TRAIL* and *TNF* mRNA after *Egr-2* or *Egr-3* overexpression in primary IEC (Fig. 7).

Recently, a novel family of corepressors, named Nab, that

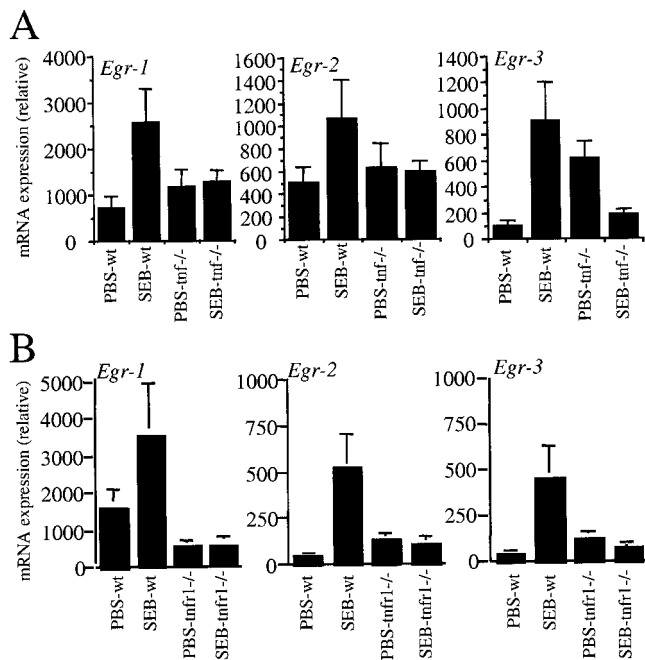


FIG. 6. IEC *Egr-1*, -2, and -3 mRNA expression is dependent on TNF. B6129SF2 and TNF^{-/-} (A) and C57BL/6 and TNFR1^{-/-} (B) mice were injected i.p. with superantigen SEB (75 to 100 μg), and after 3 days IEC total mRNA was isolated and assessed by real-time RT-PCR for *Egr-1*, *Egr-2*, and *Egr-3* mRNA expression. Absolute mRNA values were determined, normalized to β-actin, and reported as arbitrary units.

inhibits the transcriptional activity of the Egr family of zinc finger transcription factors was identified (43). To determine if endogenous *FasL*, *TRAIL*, and *TNF* mRNA expression is Egr dependent, murine Nab1 was cloned and coexpressed with *Egr-2* or *Egr-3*. As expected, Nab1 expression inhibited Egr-induced *FasL*, *TRAIL*, and *TNF* upregulation in primary IEC (Fig. 7). To further analyze the effects of TNF and Egr mem-

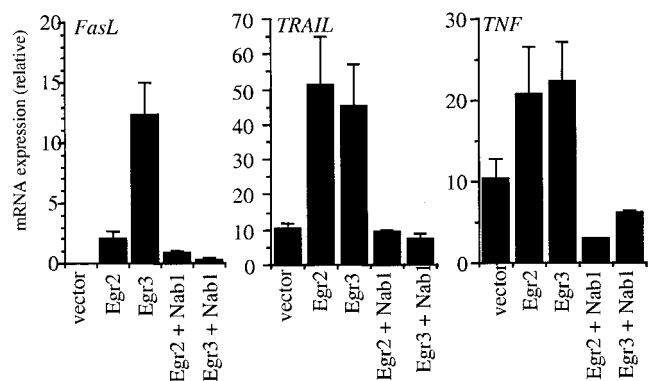


FIG. 7. Transient overexpression of *Egr-2* and *Egr-3* induces endogenous *FasL*, *TRAIL*, and *TNF* mRNA upregulation in primary IEC. Primary IEC prepared from BALB/c mice were transfected with different constructs expressing murine *Egr-2*, *Egr-3*, and/or Nab1. Twenty hours later total mRNA isolated from transfected IEC was analyzed for endogenous *FasL*, *TRAIL*, and *TNF* mRNA expression. Absolute mRNA values were determined, normalized to 18S RNA, and reported as arbitrary units.

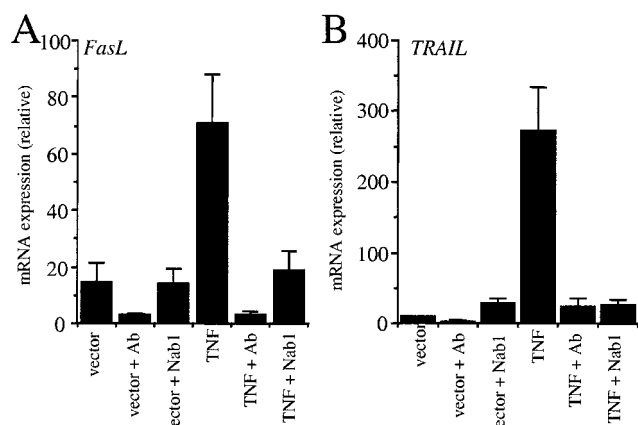


FIG. 8. Endogenous *FasL* and *TRAIL* mRNA upregulation induced by TNF is inhibited by Nab1 overexpression in IEC. Primary IEC prepared from BALB/c mice were transfected with different constructs expressing murine Egr-2 or Egr-3 either alone, in the presence of a neutralizing TNFR1 antibody (Ab), or with Nab1. Twenty hours later total mRNA was isolated from transfected IEC and analyzed for *FasL* (A) and *TRAIL* (B) mRNA by real-time RT-PCR. Absolute mRNA values were determined, normalized to 18S RNA, and reported as arbitrary units.

bers on IEC, we expressed TNF in primary IEC by transient transfection and assayed the effects on *FasL* and *TRAIL* mRNA levels (Fig. 8). Primary IEC were transfected with TNF-pcDNA in the presence or absence of an anti-TNFR1 antagonistic antibody, and 20 h later RNA was analyzed by real-time RT-PCR. As we have previously shown (35), there was a significant increase in *FasL* mRNA levels in TNF-transfected IEC, and we also observed an increase in *TRAIL* mRNA (Fig. 8). The TNF-dependent effects on *FasL* and *TRAIL* expression were reduced to background levels when an anti-TNFR1 antagonistic antibody was added to the medium (Fig. 8). We then examined the effect of Nab1 expression in the cells expressing TNF (note that the expression of TNF allowed us to examine the effects of Nab1 coexpression on endogenous ligand expression in the same cells). As shown in Fig. 8, the expression of the Egr-specific inhibitor Nab1 inhibited TNF-induced *FasL* and *TRAIL* upregulation. These results suggest that TNF production induces Egr-dependent expression of *FasL* and *TRAIL* in IEC.

To identify DNA elements that control the regulation of *FasL* transcription, known regions of the human *FasL* gene 5' of the translation start site containing Egr-binding sites were used to drive the expression of a GFP reporter in primary IEC (Fig. 9). Due to the difficulty in transfecting primary cells with high efficiency to study reporter gene expression by conventional assays, we developed an assay to study reporter expression at the mRNA level by real-time RT-PCR. In transient transfections with the indicated GFP reporter constructs, expression of Egr-2 led to a weak induction of the bp -300 human *FasL* promoter reporter while Egr-3 induced strong transcription (Fig. 9A and B). Surprisingly, truncation of the *FasL* promoter to nucleotide -240, deleting putative overlapping NFAT and Egr sites, did not inhibit the transcriptional activity induced by Egr-2 but eliminated the response to Egr-3, indicating that Egr-3 binds only the putative overlapping

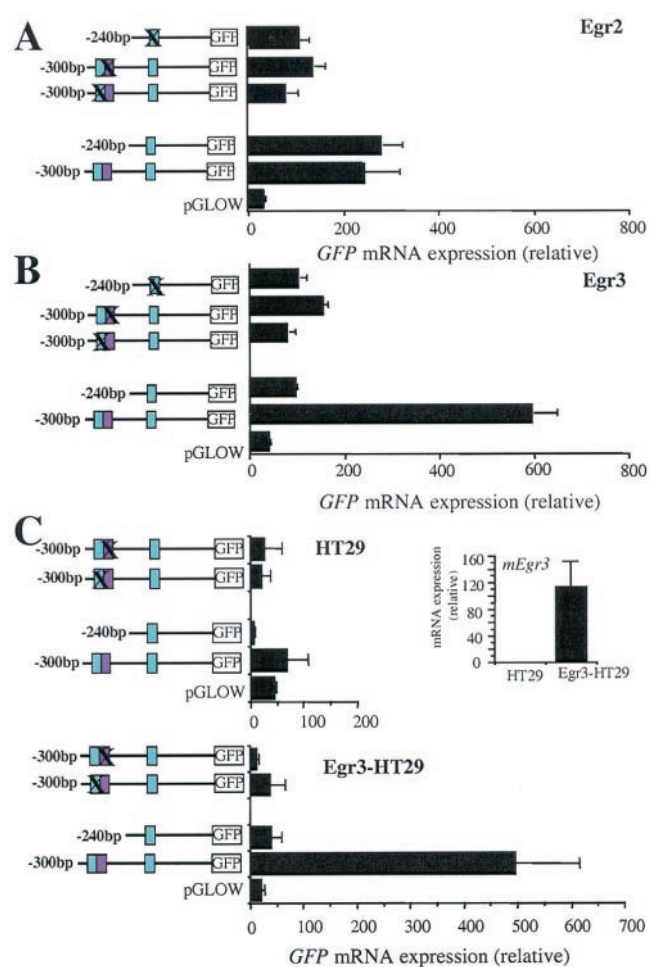


FIG. 9. Expression of exogenous Egr-3 and Egr-2 protein is sufficient to induce *FasL*-responsive-element-dependent transcription in nonlymphoid cells. Primary IEC prepared from BALB/c mouse cells were cotransfected with different constructs expressing murine Egr-2 (A) or Egr-3 (B) with the indicated GFP reporter plasmids with schematic Egr (blue boxes) and NFAT (purple boxes) sites. X, mutated Egr and NFAT sites. GFP mRNA was analyzed by real-time RT-PCR and normalized to *luciferase* mRNA expression as an efficiency control. (C) HT29 cells were infected with either an internal ribosomal entry site (IRES)-GFP control retrovirus or an IRES-GFP retrovirus expressing wild-type murine Egr-3. Stably infected cells were sorted by fluorescence-activated cell sorter to >95% GFP-expressing cells. *Egr-3* mRNA expression was assessed by real-time RT-PCR. (Inset) HT29 and Egr3-HT29 cells were cotransfected with the indicated GFP reporter plasmids and a *Renilla* vector as a control. GFP mRNA was analyzed by real-time RT-PCR and normalized to *Renilla* mRNA.

NFAT and Egr sites. To determine if these sites are required for *FasL* induction in IEC, we tested *FasL* promoter activity using promoter-GFP constructs with specific mutations in the binding sites for these transcription factors. Mutations within the Egr or NFAT sites completely abolished the activity of the bp -300 construct upon Egr-3 overexpression, suggesting that mutation in the NFAT site affects Egr-3 binding to its own site (Fig. 9). The role of Egr-3 at the putative overlapping NFAT and Egr sites in the *FasL* promoter was confirmed by using HT29 cells overexpressing Egr-3 (Fig. 9C). In this experiment,

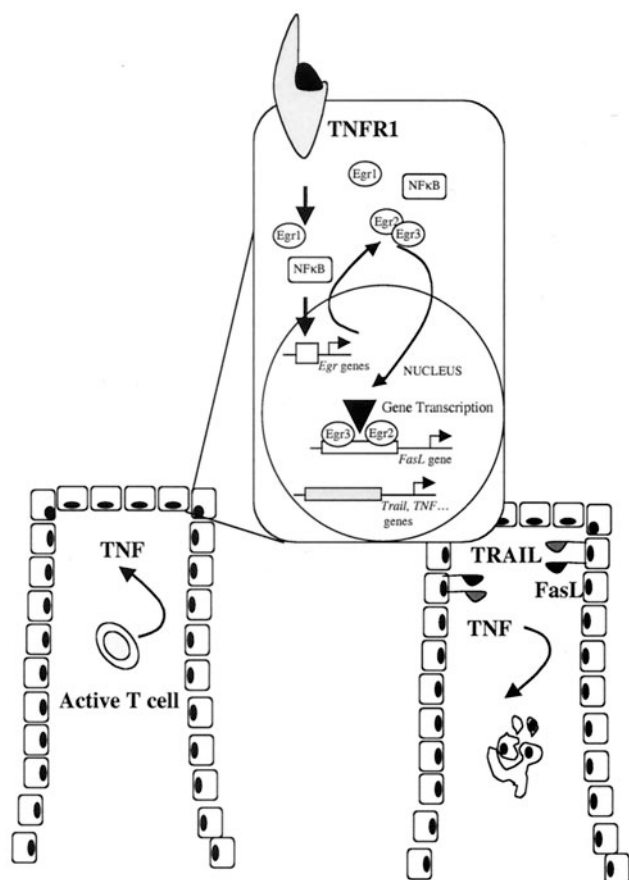


FIG. 10. Model of nonlymphoid ligand induction in response to the presence of activated lymphocytes. Activated lymphocytes produce TNF that binds its receptor, TNFR1, constitutively expressed on the surfaces of IEC (35). TNFR1 activation induces several transcription factors, such as Egr and NF- κ B, that in turn induce *TNF*, *FasL*, and *TRAIL* expression in IEC. IEC expressing functional FasL and TRAIL then induce apoptosis of infiltrating Fas- and TRAIL-R-bearing lymphocytes.

this site was again required for Egr-3-induced *FasL* promoter activity.

DISCUSSION

In this paper, we have shown that IEC express not only FasL but also TRAIL and TNF in response to superantigen- or peptide-induced activation of peripheral lymphocytes. In keeping with our previous observations, we found that the expression of these different ligands in IEC depends on TNF (35). Our results suggest a model in which TNF, produced by lymphoid cells after activation by superantigen or peptide (16), induces Egr-dependent expression of FasL, TRAIL, and TNF in IEC through TNFR1. Biologically active FasL and TRAIL expressed in the small intestine may then kill infiltrating Fas⁺ and TRAIL-R2⁺ T cells (Fig. 10).

One function of the TNF ligand family is the induction of apoptosis. Constitutive expression of *TRAIL* mRNA has been reported in most normal tissues and cell types (10). TRAIL is not expressed on freshly isolated T cells; however, both TRAIL

and the TRAIL receptor are upregulated upon T-cell activation (12, 25, 40). For example, CD4⁺ V α 24NKT cells express TRAIL only when the cells are activated by α -galactosylceramide-pulsed dendritic cells and can kill malignant target cells (32). However, the function of this cytokine in normal tissues is still not clear. Recently, a study using mice deficient in TRAIL found an essential role for TRAIL in thymocyte apoptosis and inhibition of autoimmunity (18). Our results show that functional FasL and TRAIL are expressed on normal IEC during immune responses, suggesting a possible cooperation of these ligands in peripheral lymphocyte deletion. Our data are in line with recent publications demonstrating cross-activation of different death systems, enabling autoamplification of apoptosis following ligation of FasL, TRAIL, and TNF (11). Following the same concept, Lee et al. recently reported the expression of a functional TRAIL protein in an immune-privileged site, the anterior chamber of the eye (21). In addition, the pattern of expression of TRAIL in the eye is remarkably similar to that of FasL, suggesting a possible cooperation between these different ligands in the elimination of activated T cells, thereby limiting the damage to the tissues into which the T cells infiltrate.

While the idea that T-cell-induced nonlymphoid expression of molecules such as FasL, TRAIL, and TNF contributes to peripheral deletion is attractive, a possible problem concerns the effect of cyclosporine in these systems. Egr is inhibited by cyclosporine (29), and this accounts in part for the inhibition of FasL expression in activated T cells treated with cyclosporine (4). In contrast, peripheral deletion is not inhibited by cyclosporine (2). At present, we envision at least two possibilities. First, it is possible that Egr in IEC and other nonlymphoid cells is not inhibitable by cyclosporine. Alternatively, some nonlymphoid cells may express FasL (and other related molecules) that is not dependent on Egr. For example, constitutive expression of FasL in the testes is not inhibited by cyclosporine (19).

Studies with the *FasL* promoter have established the presence of several sites important in its transcription, such as binding sites for NF- κ B (14, 26, 27), NFAT (19, 20), activating protein 1 (AP-1) (28), and Egr-2 and -3 (29, 30), although the relative functional importance of these sites might differ among species. We found that injection of the superantigen SEB into wild-type mice or of OVA into OT-2 mice induced *Egr-1*, *Egr-2*, and *Egr-3* expression in IEC and that this was not observed with mice deficient in TNF expression. Egr-2 and Egr-3 have recently emerged as important transcription factors for FasL expression in lymphoid and nonlymphoid cells (29, 30). Because induction of both Egr-2 and Egr-3 was shown to be cyclosporine sensitive in T cells (29), the expression of the *Egr-2* and -3 genes might be dependent on NFAT. In fact, a transcriptionally active NFAT site was identified in the *Egr-3* promoter, confirming this hypothesis (24). In addition, it was recently shown that NFAT-mediated induction of Egr-2 and/or Egr-3 is essential for optimal FasL synthesis in vivo (38). We have provided evidence that mutation within distal Egr- or NFAT-overlapping sites completely abolished the transcriptional activity of the bp -300 construct in primary IEC and Egr3-HT29 cells, demonstrating that mutation in the NFAT site affects Egr-3-induced FasL expression. However, this ob-

ervation does not exclude a possible interaction between Egr and endogenous NFAT. We also demonstrated that Egr-3 has no effect on a second putative Egr-binding site using the bp -240 construct in primary IEC and Egr3-HT29 cells, suggesting that Egr-2 may act exclusively at this site. In primary IEC, Egr-2 drove reporter expression of constructs containing either the distal Egr- or NFAT-overlapping site or the proximal Egr-binding site of the human *FasL* promoter.

We also found that overexpression of Egr-2 and Egr-3 in IEC was sufficient to induce expression of *FasL*, *TRAIL*, and to a lesser extent *TNF* mRNA. This role for Egr members in death ligand expression is relevant, because expression of the Egr corepressor Nab1 inhibited TNF-induced expression of these ligands in IEC. Therefore, the Egr transcription factor family is likely to be involved in the transcriptional regulation of several members of the TNF ligand family in IEC.

We have also recently reported that expression of *FasL* by TNF is dependent on NF- κ B, one component of the TNFR signaling pathway (35). These results are not contradictory since mutations within both the Egr- and the κ B1-binding sites silence the murine *FasL* promoter (28). Taken together, these results confirm that NF- κ B and Egr-2 and -3 are important transcription factors for the expression of murine *FasL*.

Sequences proximal to bp -182 on the *TNF* promoter contain motifs for several transcription factors, including AP-1, Egr-1, NF- κ B, and SP1. For example, lipopolysaccharide activation of macrophages induces the binding of c-Jun, Egr-1, and NF- κ B to binding sites within the proximal bp -182 region of the *TNF* promoter (45). We also observed an Egr-2- and -3-dependent induction of endogenous *TNF* in A1.1 T cells (N. M. Droin and D. R. Green, data not shown), suggesting a redundancy between the different members of this family.

Studies with the *TRAIL* promoter have recently established the importance of several NF- κ B binding sites for *TRAIL* transcription in T cells (1). Sequence analysis of the *TRAIL* promoter also revealed several potential NFAT and AP-1 sites and one potential Egr site (N. M. Droin and D. R. Green, data not shown), suggesting that the involvement of the Egr family members in *TRAIL* regulation may be direct. Thus, regulation of *TRAIL* may parallel in many respects that of *FasL* and *TNF*. Further functional studies of cooperative interactions between transcription factors (Egr members and NF- κ B) bound to these different regulatory elements will be essential to our understanding of the transcriptional regulation of the *FasL*, *TRAIL*, and *TNF* genes in epithelia after activation of peripheral lymphocytes.

Taken together, these data suggest a model of ligand transcriptional regulation converging on the recruitment of complexes composed by shared components such as the Egr members and NF- κ B. This hypothesis suggests a very subtle regulation of these common complexes by different combinations of transcription factors in the context of different enhancer sequences. We must now determine which transcription factors are required physiologically for expression of each ligand in nonlymphoid tissues and activated T cells. Such studies will shed light on the specificity of these complexes and help us to understand the expression of *FasL*, *TRAIL*, and *TNF* in different cell types during immune responses.

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