Two NFAT Transcription Factor Binding Sites Participate in the Regulation of CD95 (Fas) Ligand Expression in Activated Human T Cells*

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Antigen receptor engagement on T lymphocytes activates transcription factors important for stimulating cytokine gene expression. This is critical for clonal expansion of antigen-specific T cells and propagation of immune responses. Additionally, under some conditions antigen receptor stimulation initiates apoptosis of T lymphocytes through the induced expression of CD95 ligand and its receptor. Here we demonstrate that the transcription factor, NFAT, which is critical for the inducible expression of many cytokine genes, also plays a critical role in the regulation of T cell receptor-mediated CD95 ligand expression. Two sites within the CD95 ligand promoter, identified through DNase I footprinting, bind NFAT proteins from nuclear extracts of activated T cells. Although both sites appear important for optimal expression of CD95 ligand in activated T cells, mutational analysis suggests that the distal NFAT site plays a more significant role. Furthermore, these sites do not appear to be required for constitutive CD95 ligand expression in Sertoli cells.

Adaptive immune responses require the activation of T lymphocytes through antigen-specific T cell receptor (TCR) stimulation. Signaling events initiated by TCR ligation lead to the activation of transcription factors that regulate expression of cytokine genes, such as IL-2. This is important for the clonal expansion of antigen-specific T cells and propagation of immune responses (1, 2). However, once the antigenic stimulus has been cleared, the expanded population of cells must be eliminated to prevent accumulation of excessive lymphocytes (2, 3). Recently, it has been proposed that one mechanism by which this occurs is through the induced expression of CD95 ligand (4–7). Once expressed, CD95 ligand engages its receptor, CD95, also expressed on the population of activated lym-

phocytes (8). In the absence of costimulatory signals that can delay apoptosis (9–12), CD95 ligation rapidly initiates the programmed cell death machinery thus efficiently eliminating excessive activated lymphocytes (8). Additionally, autoreactive T cells that are inappropriately activated in the periphery are believed to undergo apoptosis through a similar process (13).

The significance of CD95 ligand expression in the process of activation-induced cell death has been highlighted by recent studies that demonstrate that blocking CD95/CD95 ligand interactions prevents apoptosis of TCR-stimulated lymphocytes (5, 7). Interestingly, in addition to its inducible expression on activated lymphocytes, CD95 ligand is constitutively expressed on epithelial cells within the eye and Sertoli cells within the testes (14–16). Constitutive CD95 ligand expression participates in maintenance of the “immune privileged” status of these tissues by inducing apoptosis in infiltrating, CD95-bearing, activated lymphocytes (17). Despite improved understanding of the important physiological roles for CD95 ligand in immune privileged sites and in controlling T cell homeostasis, little is yet known about the regulation of CD95 ligand expression in these various cell types.

In contrast, much is known about the signaling pathways that couple TCR ligation to expression of cytokine genes. Engagement of the TCR leads to rapid activation of protein tyrosine kinases of the Src and Syk families (18). These protein tyrosine kinases then couple to the activation of the Ras and phospholipase Cγ1 signaling intermediates (19). Ras activation drives signals that ultimately lead to induction of members of the AP-1 family of transcription factors, important for regulation of the IL-2 gene promoter (20, 21). Stimulation of phospholipase Cγ1 leads to the calcium-dependent activation of the serine phosphatase, calcineurin (22). Activated calcineurin then functions to dephosphorylate nuclear factor of activated T cell (NFAT) family members. Dephosphorylated NFAT proteins then enter the nucleus where they also serve an essential role in regulating the expression of many cytokine genes, including IL-2 (20, 23).

The immunosuppressant cyclosporin A (CsA) inhibits NFAT-dependent transcriptional events by binding calcineurin and blocking its enzymatic activity, thus preventing the redistribution of NFAT to the nucleus (24). Previous studies demonstrated that treatment of lymphocytes with CsA also inhibits TCR-mediated CD95 ligand expression (6, 25, 26). Additionally, lymphocytes from mice with targeted gene disruption of CsA, cyclosporin A; EMSA, electrophoretic mobility shift assays; bp, base pair(s).

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regulates CD95 ligand expression in activated lymphocytes. Using DNase I footprint analysis, we define two potential NFAT binding sites within the CD95 ligand promoter region. Both sites bind NFAT proteins independently, in an inducible and specific fashion. Yet, mutational analysis demonstrates that the distal NFAT site is more important than the proximal site for regulation of the CD95 ligand promoter in activated T cells. In contrast to the findings in T cells, experiments examining constitutively expressed CD95 ligand in Sertoli cells demonstrate that neither NFAT site is required for constitutive promoter activity in these cells.

**EXPERIMENTAL PROCEDURES**

**Antibodies, Reagents, and cDNA Constructs**—The following reagents were used in this study: sodium 1-luciferin (Sigma), CsA (Sigma), phorbol myristate acetate (PMA; Sigma; used for stimulation at 50 ng/ml), ionomycin (Sigma; used for stimulations at 1 μM). The following antibodies were used in this study: anti-NF-kappaB clone TCR γ chain mAb, C305 (29); MOPC IgG2a (Organon Teknika Corp., West Chester, PA); anti-NFATp (mAb G1-D10) and anti-NFATc (mAb 7A6) were gifts of G. Crabtree. The binding reactions were incubated for 30 min at room temperature and then stimulated with 0.005 M isopropyl-1-thio-

**Preparation of Recombinant Glutathione S-Transferase-NFATc Protein**—Double-stranded oligonucleotide probes were used in these experiments: double-stranded oligonucleotides end-labeled with [γ-32P]ATP (Amersham Corp.) using T4 polynucleotide kinase. A 1000-fold excess of unlabeled specific and nonspecific oligonucleotides were used as competitors where indicated. Supershifts were performed by addition of 1 μl of both α-NFATp and α-NFATc antibodies to the binding reactions or 1 μl MOPC as a negative control. In addition to the CD95 ligand promoter probes described in Fig. 1B, the following double-stranded oligonucleotide probes were used in these experiments: nonspecific, 5′-TGTCGAATGCAAATCACTAGAA-3′; IL-2 dist WT, 5′-GGTCAGAGGAGGAGGAGGAAATGTCGAACTACCG-3′; luciferase assay—Transformed cells were treated as indicated, followed by lysis in 100 μl of harvest buffer (100 mM KPO4, pH 7.8), 1.0 mM DTT, 1% Triton X-100. Lysates were then mixed with 100 μl of assay buffer (200 mM KPO4, (pH 7.8), 10 mM ATP, 20 μM MgCl2) followed by addition of 100 μl of 1.0 mM luciferin. Luciferase activity, expressed in arbitrary light units, was determined in triplicate for each experimental condition using a luminometer (Monolight 100; Analytical Luminescence Laboratory, San Diego, CA).

**RESULTS**

**Two Regions of the CD95 Ligand Promoter Bind NFAT Protein**—We have shown previously that a CD95 ligand reporter comprised of 486 base pairs of DNA immediately 5′ of the translational start site of the human CD95 ligand gene contains critical promoter elements for TCR-induced transcriptional activation in the Jurkat T cell line (28). Additionally, this...
reporter reflects constitutive expression of CD95 ligand in the TM4 Sertoli cell line. This previous study also suggests that NFAT may play a role in the induced but not constitutive expression of CD95 ligand.

To explore the role of NFAT in CD95 ligand expression further, we first attempted to map NFAT binding sites within the CD95 ligand promoter. As shown in Fig. 1A (lanes 3 and 4), DNase I footprint analysis reveals two regions within the 486-base pair probe that are protected from enzymatic digestion through interactions with recombinant NFAT protein. We designated these regions as putative NFAT distal and NFAT proximal based on their location relative to the predicted TATA box. Lane 1 demonstrates that in the absence of recombinant protein both NFAT sites are DNase I-sensitive. The two sites appear to bind recombinant protein with grossly equivalent affinities as each site is partially protected from DNase I cleavage with 50 ng of protein and protected more with 500 ng. Additionally, NFAT appears to bind both DNA strands, as labeling either strand individually provides NFAT-mediated DNase I-protected sites at similar regions of the promoter (data not shown).

Each protected site corresponds to approximately 20 base pairs within the CD95 ligand promoter. Sequence analysis reveals that both regions contain an identical GGAAA sequence that differs sufficiently from previously defined NFAT binding sites to fail recognition by a GCG computer search using the data bases described in Refs. 30 and 31. Many of the previously described NFAT sites within cytokine promoters bind NFAT family members cooperatively with other transcription factors (23). Interestingly, there are no consensus AP-1, ATF-2, Oct-1, or other recognizable transcription factor binding sites surrounding the CD95 ligand promoter NFAT elements.
This suggests that the putative NFAT sites we have identified in the CD95 ligand promoter might bind NFAT proteins in the absence of these other transcription factors. In support of this, DNase footprint analysis using recombinant AP-1 protein failed to demonstrate AP-1 binding either independently or in cooperation with recombinant NFAT protein (data not shown). It is possible, of course, that other unidentified transcription factors may interact with NFAT proteins at these sites.

To address further the specificity of NFAT binding to the two sites, each was mutated independently in the context of the 486-base pair promoter (Fig. 1A), all proximal and distal NFAT site DNA probes are illustrated in Fig. 1B, A and B. A radiolabeled probe containing the distal NFAT binding site was incubated with the indicated nuclear extracts (Stimulus). Bands corresponding to NFAT protein-DNA complexes, NFAT protein-DNA-mAb supershifted complexes, and nonspecific complexes are indicated with arrows. A, binding reactions were incubated in the absence of DNA or antibody competitors (NONE) or in the presence of 1000-fold excess of unlabeled nonspecific probe (Non-sp), a probe specific for the distal NFAT site (Sp-dist), a probe specific for the proximal NFAT site (Sp-prox), or mAbs specific for NFATp and NFATc (NFAT Ab) or isotype control mAb (Cont Ab). B, binding reactions were incubated in the presence of 1000-fold excess of unlabeled nonspecific probe (Non-sp), a probe specific for the distal NFAT site (Sp-dist), or a probe specific for the IL-2 distal NFAT site (IL-2 NF). C and D, a radiolabeled probe containing the proximal NFAT binding site was incubated with the indicated nuclear extracts (Stimulus). Bands corresponding to NFAT protein-DNA complexes, NFAT protein-DNA-mAb supershifted complexes, and nonspecific complexes are indicated with arrows. C, binding reactions were incubated in the absence of DNA or antibody competitors (NONE) or in the presence of 1000-fold excess of unlabeled nonspecific probe (Non-sp), a probe specific for the proximal NFAT site (Sp-prox), a probe specific for the proximal NFAT site (Sp-prox), a probe specific for the distal NFAT site (Sp-dist), or a probe specific for the IL-2 distal NFAT site (IL-2 NF).
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TCR or with PMA plus ionomycin induces complex formation with both the distal and proximal probes (lanes 2 and 3, Fig. 2, A and C). Treatment with CsA inhibits complex formation with both distal and proximal probes (lane 4, Fig. 2, A and C).

To address the specificity of binding in these experiments both probes were mixed with nuclear extracts from PMA and ionomycin-activated Jurkat cells in the presence of various DNA competitors. An excess of unlabeled nonspecific DNA probe does not inhibit complex formation (lane 5, Fig. 2, A and C and lane 11, Fig. 2, B and D) nor do probes that incorporate mutations in the NFAT binding sites (lane 7, Fig. 2, A and C). Yet excess unlabeled probes specific for either the distal or the proximal NFAT sites compete complex formation with the labeled distal or proximal NFAT probes, respectively (lane 6, Fig. 2, A and C and lane 12, Fig. 2, B and D).

To begin to address whether the composition of the complexes associated with the distal and proximal NFAT sites are similar, a cross competition experiment was performed. Excess unlabeled proximal probe competes complex formation with the labeled distal probe (lane 8, Fig. 2A) and unlabeled distal probe competes complex formation with the labeled proximal probe (lane 8, Fig. 2C). This suggests that the proteins from activated nuclear extracts that form complexes with either probe are likely to have similar binding characteristics.

To assess further whether the putative NFAT sites bind to NFAT proteins from activated Jurkat cell nuclear extracts, supershift experiments were performed with NFAT-specific antibodies. Antibodies against NFATp and NFATc (which constitute the T cell-specific isoforms of NFAT (32, 33)) were incubated with nuclear extracts from PMA and ionomycin-activated Jurkat cells in the presence of labeled distal and proximal NFAT probes. In both instances the specific complexes are supershifted (lane 9, Fig. 2, A and C), indicating that each probe binds NFAT proteins from activated nuclear extracts. A nonspecific isotype control antibody does not induce a supershift (lane 10, Fig. 2, A and C). Interestingly, in contrast to what has been shown for the IL-2 NFAT sites, antibodies specific for AP-1 transcription factor components fail to supershift complexes formed with distal and proximal CD95 ligand NFAT probes, whereas supershift formation is detected with a control probe containing an AP-1 site (data not shown).

Finally, we examined whether the specificities of NFAT complex formation with the CD95 ligand NFAT sites are similar to that of an NFAT site from the IL-2 promoter. Complex formation with either labeled distal or proximal CD95 ligand NFAT probe is competed efficiently with a probe containing a canonical NFAT binding site derived from the IL-2 gene promoter (lane 13, Fig. 2, B and D). This suggests that NFAT protein binding specificities for each site are similar. Collectively, these results indicate that both the distal and proximal CD95 ligand promoter NFAT sites are capable of binding nuclear NFAT proteins from T cells in an inducible and specific fashion.

Both Proximal and Distal CD95Ligand Promoter NFAT Sites Can Function Independently to Support TranscriptionalActivation in T Cells—Having determined that both the distal and proximal CD95 ligand promoter NFAT sites are capable of binding NFAT proteins from activated lymphocytes, experiments were designed to test whether these sites are capable of activating transcription in cells. We created reporter constructs containing triplicate copies of the distal NFAT or the proximal NFAT site placed upstream of the previously defined IL-2 minimal promoter. The reporters were transfected transiently into Jurkat cells and stimulated with immobilized α-TCR antibody, alone or in the presence of CsA. As shown in Fig. 3, the minimal IL-2 promoter exhibits little reporter activity in TCR-stimulated cells. In contrast, reporter constructs driven by either the distal or proximal NFAT sites are activated substantially by TCR engagement relative to unstimulated controls. As predicted, the inducible response for both triplicated reporters is blocked completely by the addition of CsA during TCR stimulation, suggesting that the observed reporter activity depends on NFAT nuclear translocation. These results indicate that each NFAT binding site within the CD95 ligand promoter region can activate transcription independently. Surprisingly, in contrast to studies that demonstrate the capacity of calcium ionophore stimulation to partially activate the full-length, 486-bp CD95 ligand reporter (28), the triplicated reporters are not activated by calcium signals alone (data not shown). Despite our observations that these NFAT sites do not cooperate with AP-1 factors, this result suggests that their regulation may require other, as yet unidentified, factors. Furthermore, the calcium-responsive nature of the full-length reporter may involve other calcium-dependent transcriptional regulators in addition to NFAT.

Mutation of the Distal NFAT Site Has a More Pronounced Effect on CD95Ligand Promoter Activity in T Cells Than Does Mutation of the Proximal Site—We next addressed the functional importance of the two NFAT sites for TCR-mediated CD95 ligand expression. Each site was mutated independently or together in the context of the full-length, 486-bp CD95 ligand reporter. The mutant constructs incorporate the sequence alterations shown in Fig. 1B which prevent NFAT protein binding. Jurkat cells were transfected with the wild type reporter or one of the mutant reporters and then left unstimulated or stimulated with immobilized anti-TCR mAb or anti-TCR mAb plus CsA. As shown in Fig. 4A, engagement of the TCR on cells transfected with the wild type reporter results in a 24-fold...
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The role of the distal and proximal NFAT sites for constitutive CD95 ligand expression, we performed experiments using a Sertoli cell line which, as previously reported, constitutively expresses CD95 ligand transcripts (28). By utilizing cytotoxicity assays with CD95-sensitive target cells, we confirmed that this cell line expresses functional CD95 ligand (data not shown). This Sertoli line was then transfected with either the wild type CD95 ligand reporter, a reporter containing the proximal NFAT site mutation, or a control luciferase reporter lacking a promoter. Cells were cultured for 72 h post-transfection and then assayed for luciferase activity. As shown in Fig. 5, the wild type reporter as well as both reporters containing NFAT site mutations also exhibit high basal levels of reporter activity relative to controls (data not shown). However, in three independent experiments there is somewhat

increase in luciferase activity relative to unstimulated cells or cells stimulated in the presence of CsA. Shown also, in agreement with prior studies (28), the distal NFAT site mutant reporter exhibits markedly diminished background luciferase expression, and TCR stimulation is much less effective at inducing luciferase activity in cells transfected with this reporter. In contrast, mutation of the proximal NFAT site appears to have a less dramatic effect on activation of the CD95 ligand promoter. TCR stimulation of cells transfected with this reporter still results in a 19-fold increase in luciferase activity over unstimulated cells. However, in each of four independent experiments, the proximal NFAT binding site mutant reporter is significantly less inducible than the wild type reporter. Similar to the wild type reporter, this activity is inhibited completely by CsA. As shown in Fig. 4B, a reporter containing both NFAT site mutations also exhibits minimal luciferase production in TCR-activated Jurkat cells, similar to the effect of the distal site mutation. Collectively, these results indicate that both NFAT sites participate in the maximal activation of the CD95 ligand reporter; however, the distal site contribution appears greater.

Interestingly, truncation mutants of the CD95 ligand promoter behave differently from the NFAT site-specific mutant constructs. When the distal NFAT site is eliminated by truncating the promoter 3' to this site, the resulting reporter is still activated to approximately 50% of the level seen with the full-length reporter (data not shown). In contrast, as shown in Fig. 4A, the distal site mutant consistently provides a more pronounced diminution in reporter activity. One explanation for this discrepancy is that a transcriptional repressor site may exist in the region deleted by the truncations. This model predicts that in the absence of the repressor, other factor binding sites within the remaining promoter region may be capable of activating transcription. In contrast, the repressor would still function in the distal NFAT site mutant reporter, thus limiting the degree of reporter activation. Efforts are currently underway to elucidate the mechanism for this difference between the truncation and point mutant reporters.

**Individual NFAT Sites Are Not Essential for Constitutive CD95 Ligand Expression in Sertoli Cells**—Next, to address the role of the distal and proximal NFAT sites for constitutive CD95 ligand expression, we performed experiments using a Sertoli cell line which, as previously reported, constitutively expresses CD95 ligand transcripts (28). By utilizing cytotoxicity assays with CD95-sensitive target cells, we confirmed that this cell line expresses functional CD95 ligand (data not shown). This Sertoli line was then transfected with either the wild type CD95 ligand reporter, a reporter containing the distal NFAT site mutation, a reporter containing the proximal NFAT site mutation, or a control luciferase reporter lacking a promoter. Cells were cultured for 72 h post-transfection and then assayed for luciferase activity. As shown in Fig. 5, the wild type reporter as well as both reporters containing NFAT site mutations exhibit high levels of luciferase activity relative to the control reporter, indicating high basal activity for each. The double NFAT site mutant reporter also exhibits high basal levels of reporter activity relative to controls (data not shown).
in the regulation of activation-induced CD95 ligand expression in T cells. Through DNase I footprinting and gel shift analysis, we identified two NFAT binding sites within the first 486 base pairs of the CD95 ligand promoter. When analyzed independently each site can facilitate transcription through a calcineurin-dependent mechanism. However, the distal site plays a more important role in driving TCR-mediated expression of CD95 ligand. These data support the notion that NFAT participates in the regulation of CD95 ligand expression in activated T cells. It is important to note, however, that relative to reporter activity in unstimulated cells, neither NFAT single mutant nor the double mutant completely blocks TCR inducibility, suggesting that other regions of the promoter likely contribute to the inducible expression of CD95 ligand in T cells.

Much of what is known about the function of NFAT proteins has been derived from analysis of cytokine gene promoters in lymphocytes (23). To date, four isoforms of NFAT proteins have been cloned (32, 33, 38–40). Interestingly, each isoform binds to similar DNA sequences via an internal region of the protein which is homologous to the Rel family of transcription factors (41). NFATp and NFATc are the predominant isoforms expressed in mature lymphocytes (32, 33). They function as key regulators of IL-2, IL-3, granulocyte monocyte colony-stimulating factor, IL-4, interferon-γ, and tumor necrosis factor-α expression (23). Furthermore, TCR-mediated nuclear translocation of each isoform is inhibitable with CsA.

Studies of the IL-2 promoter indicate that as many as five NFAT binding sites participate in the regulation of this gene (20, 42). Recent mutational analysis indicates that all five NFAT sites are essential for optimal TCR-mediated activation of this promoter (42). Thus NFAT acts as a key transcription factor regulating cytokine gene expression critical for the clonal expansion of lymphocytes. Our results indicate that NFAT proteins are also critical for the expression of CD95 ligand, a molecule whose induction is important for triggering apoptosis in activated T cells. Similarly, other TCR-mediated signaling events, such as Ras activation, have been shown to participate in both IL-2 induction (43, 44) as well as CD95 ligand expression (28). Thus, it appears that many of the same signaling events are important for induction of cellular activation and termination of immune responses. Whether TCR engagement leads to proliferation or cell death may in fact depend on signals delivered via receptors other than the TCR. Recent work from numerous laboratories has focused on signaling events initiated by CD28 ligation which lead to protection from apoptosis (9–12). Although inspection of the CD95 ligand promoter does not suggest the presence of consensual CD28 response elements, further studies are needed to determine whether co-receptors play a role in the regulation of CD95 ligand expression.

In this study we explored the role played by NFAT in the regulation of activation-induced CD95 ligand expression in T cells. Through DNase I footprinting and gel shift analysis, we identified two NFAT binding sites within the first 486 base pairs of the CD95 ligand promoter. When analyzed independently each site can facilitate transcription through a calcineurin-dependent mechanism. However, the distal site plays a more important role in driving TCR-mediated expression of CD95 ligand. These data support the notion that NFAT participates in the regulation of CD95 ligand expression in activated T cells. It is important to note, however, that relative to reporter activity in unstimulated cells, neither NFAT single mutant nor the double mutant completely blocks TCR inducibility, suggesting that other regions of the promoter likely contribute to the inducible expression of CD95 ligand in T cells.

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This study additionally supports the existence of NFAT binding sites that appear to function independently of AP-1 cofactors. Previous studies of numerous cytokine gene promoters have described NFAT binding sites that couple with additional transcription factor binding sites immediately 3’ to the NFAT sequence (23). For instance, several NFAT sites within the IL-2 promoter are activated by both NFAT and AP-1 factors binding cooperatively (23). This likely explains the requirement for both calcium signals and Ras-dependent signals for activation of these sites, as increases in calcium lead to calcineurin-dependent nuclear translocation of NFAT proteins, and Ras activation leads to stimulation of AP-1 factors. In contrast, the CD95 ligand promoter NFAT sites we have found, as well as recently described NFAT binding sites within the TNF-α promoter (45) and an enhancer region of the IL-3 gene (46), seem
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to function without cooperative assistance from AP-1 factors. These NFAT sites do not possess surrounding identifiable transcription factor binding sites. Furthermore, the purine-rich regions that dictate NFAT binding in the CD95 ligand promoter as well as the NFAT sites of the tumor necrosis factor-α promoter and IL-3 enhancer consist of a GGAAA core DNA sequence. In contrast, the previously defined human IL-2 NFAT/AP-1 consensus sequence is GGAAAACTGTITCA (23).

It is important to note that previous work has shown that Ras-dependent signals are also important for regulating CD95 ligand expression in activated T cells (28). Since these signals do not seem to influence AP-1 cooperative binding of the CD95 ligand promoter NFAT sites, it is likely that other regions of the CD95 ligand promoter contain transcription factor binding sites that are dependent on Ras-mediated signals. Further promoter analysis directed at identifying these sites should provide more insight into the inducible regulation of CD95 ligand expression in activated T cells.

Finally, this study begins to address the regulation of CD95 ligand expression in cells that constitutively express this molecule. Our data suggest that NFAT sites in the CD95 ligand promoter that are critical for activation-induced expression in T cells are not required for constitutive expression of CD95 ligand in Sertoli cells. In agreement with this, NFAT factors have not been described to play a major role in the regulation of constitutively expressed genes in cells outside of the immune system. It is interesting to speculate, however, that certain cells contained in immune privileged tissues of the body possess transcriptional regulatory factors that are responsible for the constitutive expression of CD95 ligand. Identification of these factors could provide insight into the regulation of immune privilege status within these tissues.

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