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The Regulation of CD95 (Fas) Ligand Expression in Primary T Cells: Induction of Promoter Activation in CD95LP-Luc Transgenic Mice

Lyse A. Norian,* Kevin M. Latinis,* Steve L. Eliason,† Krzysztof Lyson,† Chunmei Yang,‡ Timothy Ratliff,*§ and Gary A. Koretzky**

The interaction between CD95 (Fas) and CD95L (Fas ligand) initiates apoptosis in a variety of cell types. Although the regulation of CD95L expression on activated T cells is an area of intense study, knowledge related to the induction of CD95L promoter activity in primary T cells is lacking. In this report we describe the generation of a novel transgenic mouse strain, CD95LP-Luc, in which murine CD95L promoter sequence controls the expression of a luciferase reporter gene. We use these mice to illustrate several important findings related to transcriptional regulation of CD95L in primary T cells. We demonstrate that maximal CD95L promoter activity occurs only after prolonged T cell stimulation and requires costimulation through CD28. We provide evidence that thymocytes express CD95L/luciferase after strong TCR ligation and that inducible CD95L promoter activation is present, but unequal, in both Th1 and Th2 effector cells. We also illustrate that while agonist peptide presentation by APCs generates robust proliferation during a primary T cell response, the same stimulus induces only modest CD95L promoter activity. These results suggest alternate explanations for the well-characterized delay in CD95-mediated activation-induced cell death following initial ligation of the TCR. The Journal of Immunology, 2000, 164: 4471–4480.

A apoptotic cell death is a tightly regulated process. Within the T lymphocyte compartment of the immune system, apoptosis contributes to homeostasis and peripheral tolerance as well as to cytolytic effector functions (reviewed in Refs. 1–3). Control of these events is crucial for normal progression of immune responses. Two key mediators of T cell apoptosis are the receptor-ligand pair of CD95 (Fas) and CD95L (Fas ligand). Both molecules are present at low or undetectable levels on the surface of resting T cells and are inducibly expressed at much higher levels following T cell activation (1–3). Therefore, activated T cells possess the means to limit their own expansion through the interaction of these two molecules, leading to the apoptotic death of the CD95-bearing cell (4–7). This process, referred to as activation-induced cell death (AICD),3 is believed to be essential for maintaining T cell homeostasis in the periphery. Defects in the expression or function of either CD95 or CD95L result in unchecked lymphoproliferation and autoimmunity in both mice and humans (8–10). However, the processes that regulate expression of these molecules, and therefore T cell apoptosis, are currently incompletely understood.

Early studies examining the regulation of inducible CD95L expression demonstrated that CD95L mRNA is not detectable in resting T cells, but is present following TCR cross-linking with Ab (4–7, 11, 12). These results led to the conclusion that inducible CD95L surface expression is mediated primarily at the level of transcription. Secondary stimulation of murine CD4+ cells augments their CD95L-mediated cytolytic capabilities compared with those seen during a primary response (13), and recent evidence suggests this effect may be due in part to vesicular release of CD95L (14), rather than solely to enhanced CD95L promoter transcriptional activation.

We have focused on understanding the transcriptional regulation of inducible CD95L expression on activated T cells. Using the Jurkat T cell line as a model system, we and others have demonstrated a role for NF-AT in mediating transcriptional activation of the CD95L promoter (15–18). Other transcription factors, such as NF-kB, Sp1, and Egr-2 and -3, also contribute to inducible CD95L promoter activity (19–22). New evidence suggests that NF-AT and Egr proteins bind to a series of composite response elements within the promoter, thereby cooperatively regulating transcription (23).

Although much has been learned about how CD95L expression is regulated, an important limitation of the majority of work in this area is that most studies have been conducted with T cell lines or hybridomas. Differences are frequently observed between responses of primary vs immortalized cell lines or clones (19, 21, 24), emphasizing the need to verify observations derived from the latter cell types with freshly isolated cells. Another limitation of studies of CD95L is that detection of protein levels has proven difficult and often unreliable. Thus, most studies have measured either CD95L mRNA levels, generally by RT-PCR, or protein expression, indirectly through bioassays. Studies of the CD95L promoter have relied almost exclusively on transient or stable
transfections of promoter reporter constructs into T cell lines (15–23, 25).

To address some of these limitations, we have generated a transgenic strain of mice in which 2.2 kb of murine CD95L promoter sequence is used to drive expression of a luciferase reporter gene. This segment of the promoter extends in a 5′ direction from the translational start site and encompasses all previously identified response elements (15–23). In the present study we have used this strain, designated CD95LP-Luc, to examine the regulation of CD95L promoter induction of luciferase activity (CD95L/luciferase) in a variety of primary murine T cell populations.

Materials and Methods

Generation of the CD95LP-Luc construct

pBSA-Luc was generated by cloning a 2.0-kb human growth hormone polyadenylation signal into pBluescript downstream of the 1.9-kb luciferase gene from the Clontech luciferase plasmid (Palo Alto, CA). CD95L promoter (2.2 kb) immediately 5′ of the translational start site was amplified by PCR using murine genomic DNA from a A/II library. The sequence of the PCR product was confirmed by fluorescent automated sequencing (University of Iowa DNA Facility, Iowa City, IA). This PCR product was cloned into the HindIII site of pBSA-Luc. The orientation of the insert was confirmed by PCR using a sense primer from the promoter sequence (5′-TCAGCTTCACAGACAGCCCAATTGGAACTTCCAG AGAC-3′) and a luciferase-specific antisense primer (5′-CACG CCATATCGTTTCAT-3′), followed by BamHI digestion of the PCR product.

Mice

CD95LP-Luc reporter transgenic mice were generated by microinjection of the 6.1-kb reporter construct into fertilized C57BL6/SJLJF1 eggs as previously described (26). Three luciferase transgene-positive founder lines were identified by Southern blot analysis and were established by backcrossing onto C57BL6/6 mice (National Cancer Institute, Frederick, MD). All transgenic lines demonstrated similar luciferase activity within the lymphocyte compartment. Progeny were assessed for the presence of the luciferase transgene by PCR using the sense and antisense primers listed above. Do11.10 TCR transgenic mice and CD95LP-Luc mice were bred to generate CD95LP-Luc × Do11.10 double-transgenic mice. Screening for the presence of the luciferase transgene was performed by PCR using the primers described above and for the presence of the transgenic TCR via flow cytometric analysis of PBL with anti-CD4-PE, anti-CD8-PerCP, and a clonotype-specific mAb, KJ-126-PE (CalTag, Burlingame, CA).

Preparation and activation of cells

For experiments using unfractionated cell populations, single-cell suspensions of splenocytes and lymph node cells, or thymocytes, were obtained from mice following RBC lysis. Splenocytes were cultured for the indicated times in vitro RPMI 1640 supplemented with 10% FCS, penicillin (1000 U/ml), streptomycin (1000 U/ml), and gentamicin (200 U/ml) at 8.3 × 10^6 cells/ml in six-well tissue culture plates at 37°C and 5% CO_2_. For stimulations, wells were precoated with bound anti-CD3ε (145-2C11, PharMingen) at 8 μg/ml. When used, cyclosporin A (Sigma, St. Louis, MO) was added at 200 ng/ml for the first 24 h of culture, then removed by three rounds of washing and resuspension in complete medium. Viable cells, determined by trypan blue exclusion, treated in this manner were then recounted and the presence of anti-CD3ε Ab was confirmed above as described above.

When used, CD4+ or CD3ε-enriched populations were obtained by negative selection of Ficoll-purified mononuclear cells using a magnetic concentration system (Dynal, Oslo, Norway). Briefly, for CD4+ enrichment, cells were incubated for 30 min with anti-CD8, anti-I-Aδ, anti-I-Aα, and anti-NK1.1 (PharMingen) before removal with IgG-coated magnetic beads (Cortex Biochem, San Leandro, CA). CD3ε enrichment was performed similarly, except that the following primary Abs were used: anti-I-Aα, anti-B220, and anti-NK1.1. CD3ε depletions were also performed via negative selection through binding to anti-CD4 and anti-CD8. CD4+ -enriched cells (>90% CD3ε, 81% CD4ε) were stimulated in vitro with bound anti-CD3ε as described above plus 10 μg/ml anti-CD28 (PharMingen) or peptide-pulsed APCs. APCs were prepared from RBC-depleted BALB/c splenocytes and lymph node cells and were either left unstimulated or were pre-treated for 24 h with 5 μg/ml anti-CD40 (1C10, R&D Systems, Minneapolis, MN) before irradiation (3000 rad). OVA peptides 323–339, 323–338, 323–336, or 324–334 (100 μM; HPLC purified to >90%); Re-
sarch Genetics, Huntsville, AL) were incubated with APCs for 2 h, then unbound peptide was removed by a series of three washes.

 Luciferase assays

Luciferase activity was determined as described previously (15) or, where noted, with the Promega Luciferase Assay System (Promega, Madison, WI) according to the manufacturer’s protocol. In all experiments 5 × 10^6 splenocytes plus lymph node cells or 50 × 10^6 thymocytes, cultured as described above, were used per treatment. Each treatment was performed in triplicate. For assessment of constitutive luciferase activity in testes, organs were excised and placed in RPMI medium. Tissues were then weighed and manually homogenized in 2000 μl of lysis buffer/g of tissue. For luciferase assays, 100 μl of each tissue lysate was used.

Proliferation assays

Responder cells (1 × 10^6/well) were cultured with either bound anti-CD3ε as described above or 5 × 10^3 peptide-pulsed irradiated APCs in flat-bottom 96-well plates at 37°C in 5% CO_2 in a total volume of 200 μl medium/well. After 2 days, the cultures were pulsed for 20 h with 1 μCi/ml of [3H]thymidine, harvested, and counted on a beta counter. Each treatment was performed in triplicate.

Generation of Th1 and Th2 cultures

Differentiation of Th0 CD4+ cells into Th1 and Th2 phenotypes was performed as described by others (27). Briefly, CD4+ -enriched cells were cultured at ~1 × 10^6 cells/ml in complete RPMI 1640 for 4 days with bound anti-CD3ε and anti-CD28 in the presence of the following: for Th1, recombinant murine (rm) IL-2 at 10 ng/ml (PeproTech, Rocky Hill, NJ), rmIL-12 at 350 ng/ml, and neutralizing anti-IL-4 at 10 μg/ml (both from R & D Systems); and for Th2, rmIL-2 at 10 ng/ml and rmIL-4 (PeproTech) at 10 ng/ml. Cells were then harvested, washed three times, counted, and either left unstimulated or restimulated with bound anti-CD3ε plus anti-CD28. Supernatant was collected at 24 h for both stimulated and unstimulated effectors and was used for the assessment of cytokine production as evidence for differentiation into a Th1 vs Th2 phenotype.

Cytokine ELISA

IL-4 and IFN-γ cytokine secretion was determined by sandwich ELISA using purified capture (2 μg/ml) and HRP-conjugated (1 μg/ml) anti-IL-4, or purified capture (2 μg/ml) anti-IFN-γ with polyclonal rabbit anti-murine IFN-γ and HRP-conjugated donkey anti-rabbit IgG (all gifts from Dr. Elizabeth Field, Iowa City, IA). The TMB Liquid Substrate System (Sigma) was used as a chromogen. Recombinant murine IL-4 (PeproTech) and IFN-γ (R&D Systems) were used as standards.

Detection of CD95L mRNA by RT-PCR

Cells were stimulated as indicated and cultured for the times given. Total RNA was isolated from 5 × 10^6 using RNA STAT-60 (Tel-Test, Friendswood, TX) or Trizol reagent (Life Technologies, Grand Island, NY) according to the manufacturer’s protocol. cDNA synthesis and PCR were performed using the Perkin-Elmer GeneAmp RNA PCR kit (Roche Molecular Systems, Branchburg, NJ) according to the manufacturer’s protocol. RT reactions were conducted using extension from oligo(dT)s. The following CD95L-specific primers were used: forward, 5′-CTTGGGCGGTC TCCAGGGTCTAGT-3′; and reverse, 5′-TCTCCATTAGCACA GA TTC-3′. The following Grb2-specific primers were used: forward, 5′-GGGGATCCCGAGGATCCGCCCATCCATATGACTTC-3′; and reverse, 5′-GGGAATTCCAGACGTTCCCGGTTACGACGAC-3′.

Results

Generation and initial characterization of CD95LP-Luc reporter transgenic mice

To study the transcriptional regulation of CD95L in primary murine T cells, we generated transgenic mice (CD95LP-Luc) in which 2.2 kb of murine CD95L promoter sequence is used to drive expression of a luciferase reporter gene. This system allows quantitative detection of luciferase, as a surrogate for endogenous CD95L expression, in tissues in which the CD95L promoter is transcriptionally active. Before the development of transgenic mice, the activity of the reporter construct was assessed by transient transfection into the Jurkat and Sertoli TM4 cell lines. We and others have shown that transcriptional activation of the CD95L
promoter is induced in Jurkat T cells following ligation of the TCR (15–22) and is constitutive in the testicular Sertoli TM4 cell line (15, 28). As predicted, transient transfection of the 6.1-kb CD95LP-pBSA-Luc construct produced minimal luciferase activity in resting Jurkat T cells and enhanced activity (10- to 15-fold increases over unstimulated controls) in response to plate-bound anti-TCR stimulation (data not shown). Transiently transfected Sertoli TM4 cells demonstrated constitutive CD95L promoter activity (data not shown), illustrating that the new construct functioned as expected.

After transgenic mice were generated, founders were identified by Southern blot analysis of genomic DNA for the integrated luciferase gene. Three founder lines were established, and functional luciferase expression was examined in a variety of tissues from each. All lines demonstrate constitutive luciferase activity in the testes of male transgene-positive (Luc+) mice, with minor variability in luciferase expression detected over progeny within lines (Fig. 1A). As a control, transgene negative littermates (Luc−) were shown to possess no luciferase activity. In all subsequent experiments constitutive luciferase activity in testicular tissue was used as a control for the presence of functional protein in male mice, and animals were used interchangeably from two of the three founder lines.

We first asked whether splenocytes from the CD95LP-Luc mice would respond to anti-TCR stimulation with inducible luciferase activity. Unfractionated splenocytes were harvested from Luc+ mice and cultured in vitro with plate-bound anti-TCR Ab over a period of 120 h. As shown in Fig. 1B, freshly isolated Luc+ splenocytes possessed no luciferase activity, but a gradual increase was detected in response to TCR ligation. Inducible CD95L/luciferase activity was maximal at ~96 h and waned thereafter. During this time, viable cell numbers remained fairly constant; although increases in total cell numbers were observed, large numbers of dead or dying cells were present in cultures by 72 h of stimulation (data not shown). As an additional control, TCR-dependent CD95L/luciferase activity was inhibited in cultures that were treated with the pharmacologic agent cyclosporin A during the initial 24 h of stimulation as described in Materials and Methods (Fig. 1B, inset). The concentration of cyclosporin A used was shown previously to inhibit NF-AT nuclear translocation in T lymphocytes (15, 16). This suggests that, as previously demonstrated in T cell lines and hybridomas, transcriptional induction of CD95L in primary T cells is dependent upon the activity of the cyclosporin A-sensitive phosphatase calcineurin and its substrate NF-AT (29–31).

To determine whether our luciferase detection system correlates with transcription of endogenous CD95L mRNA, semiquantitative RT-PCR analysis was performed on the same splenocyte preparations. Levels of endogenous CD95L mRNA reflect a similar trend, with no endogenous mRNA detected in freshly isolated cells and high levels present at 72–96 h of culture with TCR stimulation (Fig. 1C). This is observed in splenocytes from both Luc− and Luc+ mice, indicating that introduction of additional copies of the CD95L promoter in Luc+ mice has not artificially delayed expression of the endogenous CD95L transcript due to competition for limiting amounts of transcription factors.

The observed peak in CD95L promoter activity occurred at a later time than that described previously for CD95L promoter activity and CD95L functional expression in T cell lines and hybridomas (4–7, 19). This disparity could result from differences in the cell cycle status of peripheral T cells vs T cell lines or hybridomas; a majority of the former were isolated in a truly quiescent state, and the latter types displayed a partially activated phenotype. Our data are consistent, however, with early studies that characterized the phenomenon of AICD, in which peripheral T cells stimulated in vitro showed inhibition of proliferation and increased apoptosis only after at least 3 days of primary stimulation and subsequent restimulation (32, 33).

We next assessed the ability of other cell types present in unfractionated splenocyte and lymph node cell preparations to contribute to observed CD95L/luciferase activity. We did this by culturing unfractionated splenocytes and lymph node cells in vitro for 72 h with plate-bound anti-CD3 as before and then compared luciferase activity in 5 × 10⁶ unfractionated cells, T-enriched cells, and T-depleted cells. As shown in Fig. 1D, CD95L/luciferase activity was induced in the unFractionated population. Flow cytometric analysis of this population with a combination of anti-CD4-FITC and anti-CD8 FITC illustrated that ~74% of the cells in this culture were T lymphocytes (Fig. 1E). Following T cell enrichment via negative selection, luciferase activity increased from a mean of 3761 in the unfractionated population to a mean of 4737 (Fig. 1D). The T-enriched luciferase value therefore represents an ~26% increase over that of the unFractionated value. Flow cytometric data show that CD4+ and CD8+ cells comprise 96% of the T-enriched population, a value that represents a 29% increase over unfractionated cultures (Fig. 1E).

Following T cell depletion, the percentage of T cells present no longer correlated with the amount of luciferase activity detected. As shown in Fig. 1E, CD4+ and CD8+ cells comprised only ~5% of cells present in the T-depleted population. In contrast, luciferase activity remained at ~52% of that seen in the unFractionated population (mean, 1964; Fig. 1D). These findings imply that non-T lymphocytes present in the 72-h unFractionated cultures can also inducibly generate luciferase. Notably, the amount of luciferase produced by equivalent numbers of T-depleted cells is less than half that produced by enriched T cells (compare 4737 light units/5 × 10⁶ T-enriched cells vs 1964 light units/5 × 10⁶ T-depleted cells). Thus, although non-T lymphocytes can produce luciferase under these culture conditions, they contribute marginally to luciferase activity in unFractionated splenocyte populations, where they make up just over 20% of the cells present by 3 days in culture.

The cellular source responsible for this luciferase activity and the stimulus that induces their response require further examination. Likely candidates include B lymphocytes and macrophages, as both cell types have been reported to express CD95L (34–37). Interestingly, B lymphocytes from CD95LP-Luc mice were not induced to produce luciferase in response to in vitro LPS stimulation, and bone marrow-derived macrophages did not produce measurable amounts of luciferase during phagocytosis of Ig-coated SRBCs (data not shown).

Naïve T cells require costimulation through CD28 as well as engagement of the TCR to undergo proliferation and become fully activated. Because many TCR-mediated signal transduction events regulate both IL-2 production and CD95L transcription, we hypothesized that induction of maximal CD95L promoter activity would also require costimulatory signals through CD28. We addressed this issue by culturing CD3−-enriched cells (~90% CD3−) with plate-bound anti-CD3 Ab alone or in combination with anti-CD28. Fig. 2A illustrates that, as with unfractionated splenocyte cultures, CD3− enriched cultures also exhibited a gradual increase in detectable CD95L/luciferase activity over the course of 96 h. As expected, CD3−-enriched populations that simultaneously received strong costimulation through CD28 ligation and anti-CD3 stimulation displayed greatly increased CD95L/luciferase activity compared with cells that received stimulation through the TCR alone (Fig. 2B). These results indicate that co-stimulatory signals initiated downstream of CD28 are required for
FIGURE 1. Initial characterization of CD95L/luciferase activity in cells from CD95LP-Luc mice. A, Mice were screened for the presence of the luciferase transgene by PCR (inset), and testes from three Luc+ mice and a Luc− littermate control were assessed for constitutive luciferase activity as described in Materials and Methods. B, Unfractionated splenocytes from Luc+ mice were stimulated in vitro with plate-bound anti-TCR Ab at $5 \times 10^6$ cells/well, harvested at the indicated times, and assessed for luciferase activity. Results are expressed as the mean ± SD of triplicate samples and are representative of five independent experiments. The inset shows inhibition of CD95L/luciferase activity at 72 h in response to treatment with cyclosporin A during the initial 24 h of culture. C, RT-PCR analysis for endogenous CD95L or Grb2 mRNA from the Luc+ splenocytes used in B. As a control, analysis of endogenous transcripts from Luc− splenocytes stimulated under the same conditions is also shown. D, Unfractionated splenocytes and lymph node cells from Luc+ mice were cultured in vitro for 72 h as in A. Cells were then harvested, and luciferase activity was assessed by use of the Promega system in $5 \times 10^6$ unfractionated cells, T-enriched cells, or T-depleted cells. Results are expressed as the mean ± SD of triplicate samples and represent three independent experiments. E, Flow cytometric analysis of propidium iodide-excluding cells from the three 72-h populations examined in D. Cells were stained with a combination of anti-CD4-FITC and anti-CD8-FITC.
optimal TCR-dependent CD95L promoter activity in primary T lymphocytes.

**Inducible CD95L/luciferase expression in thymocytes**

We next examined thymocytes to determine whether they inducibly express CD95L/luciferase in a manner similar to that seen in mature T cells. Two recent reports have demonstrated that HSA^{high}CD4^{+}CD8^{−} thymocytes (designated partially immature by the investigators) undergo CD95-dependent apoptosis in response to strong TCR stimuli (38, 39). This is in contrast to previous work, based on studies of lpr and gld mice, indicating that thymic maturation progresses normally in the absence of functional CD95 or CD95L (40). We therefore wanted to determine whether thymocytes inducibly express CD95L in response to strong TCR stimulation. Unfractionated CD95LP-Luc thymocytes were cultured in vitro with 8 μg/ml of plate-bound anti-TCR Ab for a period of 72 h. As in mature peripheral T cells, minimal luciferase expression was detected at 0 and 24 h, and high levels of expression were present at 72 h of culture (Fig. 3A). Unstimulated thymocytes cultured over the same 72-h period demonstrated no luciferase activity. We again used RT-PCR analysis of endogenous CD95L mRNA to confirm that these luciferase results accurately reflect endogenous transcript production. As shown in Fig. 3B, CD95L mRNA was also observed following 72 h of stimulation, but no transcript was detected at that time in unstimulated cultures. To determine the population of thymocytes responsible
for the production of luciferase, we stained cells at 0 and 72 h for the expression of CD4 and CD8 (Fig. 3C). Initial thymocyte cultures contained the predicted ratios of CD4+CD8+, CD4+CD8−, and CD4−CD8+ cells. However, following 72 h of culture with anti-TCR Ab, single-positive CD4+ and CD8+ thymocytes comprised the majority of remaining viable cells. Unstimulated thymocytes that were cultured over the same 72-h period retained a staining profile similar to that observed in freshly isolated thymocytes (data not shown). Therefore, luciferase activity and CD95L mRNA detected at this time were likely produced by these single-positive thymocytes. This finding is consistent with results indicating that CD95L expression in double-positive thymocytes is inhibited by the activity of RORγt, a protein whose expression is lost as thymocytes mature to the single-positive stage (41).

*Th1 effector cells induce CD95L/luciferase more efficiently than Th2 cells*

Once in the periphery, T cells can differentiate into discrete effector populations, such as Th1 and Th2, which possess unique functional characteristics. CD95L expression has been examined previously in the context of Th1 vs Th2 cells, but conflicting results have been obtained. Early work using cell lines indicated the presence of CD95L mRNA in a variety of Th1 cell lines, but an absence of CD95L mRNA in all but one Th2 line examined (12). More recently, however, CD95L protein has been reported in both Th1 and Th2 differentiated primary cultures and clones (27, 42, 43), although it is still unclear whether expression is equivalent in the two populations. We therefore wanted to compare CD95L promoter activity in Th1 vs Th2 effector populations.

For our studies CD4+ cells were obtained from CD45LP-Luc mice via negative selection and were then cultured for 4 days in the presence of anti-TCR Ab plus anti-CD28 with IL-2, IL-12, and neutralizing anti-IL-4 for differentiation to Th1 effectors or with IL-2 and IL-4 for differentiation to Th2 effectors. Effectors were then harvested, washed to remove exogenous cytokines, plated at equal cell numbers, and restimulated as before or left unstimulated. Under these conditions, we found that at the end of the 96-h differentiation period, both Th1 and Th2 cells possessed transcripts for endogenous CD95L, as determined by RT-PCR (Fig. 4A, lanes 2 and 3). As shown previously, freshly isolated Th0 CD4+ cells did not express detectable amounts of CD95L mRNA (Fig. 4A, lane 1).

To quantify the relative amounts of CD95L promoter activity in the two differentiated cell populations, we again employed cells from CD45LP-Luc transgenic mice. As shown in Fig. 4B (second group of bars), while neither Th1 nor Th2 cells displayed CD95L/luciferase at the end of the differentiation period, promoter activity was severalfold greater in Th1 cells relative to Th2 cells. This difference becomes even more pronounced following a 24-h restimulation (Fig. 4B, third group of bars). Interestingly, CD95L/luciferase activity is rapidly lost if differentiated effector cells are cultured in medium alone during this same 24-h period (Fig. 4B, last group of bars). Similar trends reflecting increased levels of endogenous CD95L mRNA upon restimulation or decreased levels upon culture without stimulation, are observed in both Th1 and Th2 populations (data not shown). Cytokine profiles from TCR-stimulated culture supernatants illustrate that complete differentiation of cultures was obtained (Fig. 4C). Thus, both the RT-PCR and luciferase data indicate that Th2 as well as Th1 cells can be induced to transcribe the CD95L gene. Clearly, however, although there is a statistically significant increase in promoter activity (p < 0.05, by rank sum Mann-Whitney U test) in the Th2 population, the magnitude is substantially less than that seen in the Th1 cells. The enhanced CD95L promoter activity we observed in Th1 cells may be mediated by subset-specific transcription factors, but it may also be due to quantitative differences in the transcriptional activity of factors expressed in both Th1 and Th2 effector populations.

**Differential requirements for induction of proliferation vs CD95L promoter activity**

Physiological stimulation of T cells with peptide/MHC triggers qualitatively different responses compared with Ab engagement of the TCR. Furthermore, similar peptides containing specific amino acids induce CD95L promoter activity in Th1 cells. Interestingly, however, CD95L promoter activity in Th2 cells is not significantly enhanced by TCR engagement of T cells with anti-CD3 and anti-CD28 Abs (Fig. 4D). This finding is consistent with results indicating that CD95L expression in double-positive thymocytes is inhibited by the activity of RORγt, a protein whose expression is lost as thymocytes mature to the single-positive stage (41).

The enhanced CD95L promoter activity we observed in Th1 cells is not due to concomitant induction of proliferation. Induction of proliferation was measured by 3H-thymidine incorporation (data not shown). As shown previously, freshly isolated Th0 CD4+ cells were enriched for CD4+ cells through negative selection and differentiated into Th1 or Th2 effectors as described in Materials and Methods. Endogenous CD95L or Grb2 transcripts from 5 × 10^6 cells were visualized by RT-PCR. Lane 1, Th0 cells; lane 2, Th1 cells after 96-h differentiation; lane 3, Th2 cells after 96-h differentiation. B, CD4+ cells were differentiated into Th1 or Th2 effectors as in A. At the end of the 96-h differentiation period, cells were washed and replated at 5 × 10^6 cells/well in the presence or the absence of plate-bound anti-TCR plus anti-CD28. Luciferase activity was assessed in CD4-enriched Th0 cells, at the end of the differentiation period and following an additional 24 h of either rest or restimulation. Data are expressed as the mean ± SD of triplicate samples and are representative of three independent experiments. C, Supernatants were collected from the cultures used in B at the 120 h point from either unstimulated or TCR-restimulated Th1 and Th2 cells. Sandwich cytokine ELISA was performed as described to detect IFN-γ and IL-4 and was quantified against a standard curve of recombinant murine cytokine.
Differential induction of proliferation and CD95L promoter activity in response to peptide-pulsed APCs. A. Inhibition of agonist peptide-induced proliferation by altered peptides was determined by culturing unfractionated D011.10 TCR\(^+\) cells for the initial 24-h culture period with 10 \(\mu\)M of the indicated OVA peptide. After this time, cells were washed and replated in the presence of the agonist 323–339 peptide only. Cells were pulsed with \(^{3}H\)thymidine as described above, and incorporated label was assessed after a total of 72 h of culture. Data are expressed as the mean ± SD of triplicate cultures and are representative of six independent experiments.

B. Proliferative responses of D011.10 cells stimulated with Ab controls or either the 323–339 or 323–338 peptide as in A. We then stimulated enriched CD4\(^+\) cells from double-transgenic models with either irradiated H-2\(^{a}\) or H-2\(^{b}\) APCs pulsed with each of the four OVA peptides or a combination of plate-bound anti-TCR plus anti-CD28 and again measured their proliferative responses. CD4\(^+\) cells stimulated with Ab controls or either the 323–339 or 323–338 peptide respond with robust proliferation over the course of a 72-h stimulation. As expected, proliferation in response to the 324–334 peptide (Fig. 5A) is greatly diminished, and little or no proliferation is detected in response to the 324–334 peptide stimulation. This observation is consistent with results generated in systems using other models of TCR antagonism (49, 50).

Because the responses of D011.10 TCR transgenic T cells to these different OVA peptides have not been well described, we first assessed the ability of truncated, partially agonistic peptides (323–338, 323–336, and 324–334) to inhibit proliferative responses to subsequent agonistic 323–339 peptide stimulation. As shown in Fig. 5A, when splenocytes from CD95LP-Luc mice were exposed initially to either the 323–336 or 324–334 peptide, then washed and restimulated with only the agonistic 323–339 peptide, a marked inhibition of T cell proliferation results. This observation is consistent with results generated in systems using other models of TCR antagonism (49, 50).

We also assessed the ability of the peptide-pulsed APCs to induce luciferase activity in CD4\(^+\) cells from these cultures. We were surprised to find that even the agonist 323–339 and 323–338 peptides induced only a fraction (10–25%) of the CD95L promoter activity in primary T cells, while full agonists would induce strong promoter activation. To address this, we bred CD95LP-Luc mice (H-2\(^{b}\)) with mice expressing the D011.10 transgenic TCR (H-2\(^{a}\)). The D011.10 receptor is specific for the agonistic OVA\(_{323-339}\) peptide presented in the context of I-A\(^d\) (47). Truncations of this amino acid sequence produce peptides that function as partial agonists or antagonists for T cells bearing this clonotypic TCR (47).

For the following experiments we obtained enriched populations of naive CD4\(^+\) cells from D011.10 \times CD95LP-Luc mice by negatively selecting CD8\(^+\) cells, MHC class II\(^+\) cells, and NK cells. Although the D011.10 TCR is also weakly allogeneic against I-A\(^b\), and some F\(_{1}\) progeny possessed detectable numbers of CD4\(^+\) CD8\(^-\) cells in the periphery, the enriched CD4\(^+\) cells used in these studies exhibited normal responses to agonistic stimuli, as had been reported previously in a similar double-transgenic model system (48).

Because the responses of D011.10 TCR transgenic T cells to these different OVA peptides have not been well described, we first assessed the ability of truncated, partially agonistic peptides (323–338, 323–336, and 324–334) to inhibit proliferative responses to subsequent agonistic 323–339 peptide stimulation. As shown in Fig. 5A, when splenocytes from CD95LP-Luc \times D011.10 mice are exposed initially to either the 323–336 or 324–334 peptide, then washed and restimulated with only the agonistic 323–339 peptide, a marked inhibition of T cell proliferation results. This observation is consistent with results generated in systems using other models of TCR antagonism (49, 50).

We then stimulated enriched CD4\(^+\) cells from double-transgenic mice with either irradiated H-2\(^{a}\) APCs pulsed with each of the four OVA peptides or a combination of plate-bound anti-TCR plus anti-CD28 and again measured their proliferative responses. CD4\(^+\) cells stimulated with Ab controls or either the 323–339 or 323–338 peptide respond with robust proliferation over the course of a 72-h stimulation. As expected, proliferation in response to the 323–336 pulsed APCs is greatly diminished, and little or no proliferation is detected in response to the 324–334 peptide stimulation (Fig. 5B).

We also assessed the ability of the peptide-pulsed APCs to induce luciferase activity in CD4\(^+\) cells from these cultures. We were surprised to find that even the agonist 323–339 and 323–338 peptides induced only a fraction (10–25%) of the CD95L promoter activity in response to the 324–334 peptide (Fig. 5A). We then stimulated enriched CD4\(^+\) cells from double-transgenic models with either irradiated H-2\(^{a}\) APCs pulsed with each of the four OVA peptides or a combination of plate-bound anti-TCR plus anti-CD28 and again measured their proliferative responses. CD4\(^+\) cells stimulated with Ab controls or either the 323–339 or 323–338 peptide respond with robust proliferation over the course of a 72-h stimulation. As expected, proliferation in response to the 323–336 pulsed APCs is greatly diminished, and little or no proliferation is detected in response to the 324–334 peptide stimulation (Fig. 5B).
activation seen with anti-TCR treatment (Fig. 5C). Longer incubations in these experiments and modifications of culture conditions, such as increasing the ratio of APCs to CD4+ cells or including exogenous IL-2, yielded no increase in the level of agonist peptide-induced CD95L/luciferase activity (data not shown).

This raised the possibility that our culture conditions, which used freshly isolated splenocytes as APCs, were not providing sufficient costimulation to generate high levels of CD95L promoter activity. We then attempted to increase surface expression of costimulatory molecules such as B7.2 on the APCs by preincubation for 24 h with soluble anti-CD40. This did augment CD95L promoter activity in 323–339-stimulated CD4+ cells (generating between 45–65% of anti-TCR controls), but had no effect on the levels of T cell proliferation detected (data not shown). In all cases the pattern of responses to the four peptides was similar, in that APCs pulsed with 323–339 and 323–338 induced modest increases in CD95L/luciferase activity over unstimulated controls, while 323–336 and 324–334 did not (Fig. 5C).

These data suggest that primary T cells demonstrate modest CD95L promoter activity in response to initial exposure to agonistic antigenic stimuli, and undetectable levels of activity in response to partial agonist or antagonistic stimuli. Therefore, while the tested agonistic peptide/MHC complexes can act as potent inducers of T cell activation in terms of proliferation, their induction of CD95L appears to be modest. These observations are consistent with findings from Bonfoco et al., who demonstrated that AICD of primary T cells subsequent to superantigen exposure is dependent upon inducible CD95L expression on nonlymphoid cells, rather than on the activated T cells (51). Our data indicate that one possible explanation for their observation is that during an initial response to physiologic stimuli, CD95L promoter activity in primary T cells is insufficient to produce the density of surface CD95L required to mediate apoptotic suicide or fratricide.

Discussion

The receptor-ligand pair of CD95 and CD95L mediates apoptotic cell death in a wide variety of different cell populations. The importance of these interactions has been convincingly demonstrated in mice and humans possessing loss-of-function mutations in either molecule. Activated T cells are unique in that biochemical signals triggered by ligation of the TCR culminate in the surface expression of both CD95 and CD95L. Based on studies conducted with T cell lines or hybridomas, several laboratories demonstrated that this dual expression could mediate apoptosis within populations of activated T cells (referred to as AICD), and a paradigm for T cell homeostasis in the periphery was established (4–7). Understanding the regulation of induced CD95L expression has been the focus of intense study, and while significant advances have been made in recent years, most studies have used only T cell lines, clones, or hybridomas. The reasons for this have ranged from difficulties in detecting CD95L protein with available Abs to poor transfection efficiency of primary cells for use in examination of CD95L promoter activation. However, to fully elucidate the mechanisms that control inducible CD95L expression, studies must use primary T cells as well.

In this report we present several findings regarding TCR-mediated activation of the CD95L promoter in murine lymphocytes. These findings tie together many previous observations on various aspects of TCR-mediated signal transduction events and AICD. First, we demonstrate that freshly isolated peripheral T cells from CD95LP-Luc mice possess no luciferase activity or endogenous mRNA. This is in contrast to work by Mountz and colleagues (25), performed with stably transfected CD95L promoter-green fluorescence protein (GFP) Jurkat cells, that clearly shows a subset of unstimulated cells with constitutive GFP expression. These differences may be due to the partially activated phenotype of the transformed Jurkat T cell line.

In response to anti-TCR stimulation, peripheral T cells from CD95LP-Luc mice illustrate a gradual increase in CD95L promoter activity that peaks after ~96 h, reflecting the pattern of endogenous CD95L mRNA transcript levels. Optimal induction of CD95L promoter function appears to be dependent upon costimulatory signals generated through the ligation of CD28. Interestingly, we do not find comparable induction of CD95L promoter activity in response to agonist peptide/MHC stimulation. Although agonist peptide-pulsed APCs stimulate T cells sufficiently during a primary response to proliferate strongly, we observe a marked disparity in terms of their ability to induce CD95L/luciferase. Freshly isolated B cells, which comprise the majority of cells in splenocyte preparations used as APCs, are poor stimulators of naive T cells. To establish whether, during the course of a primary T cell response to Ag, induction of CD95L promoter activity requires a greater level of costimulation than that needed to effect proliferation, we pretreated APC splenocytes with anti-CD40 for 24 h. Although this produces a 2-fold enhancement in the level of CD95L/luciferase activity, it remains consistently less than that seen in response to treatment with Ab directed against the TCR.

These data therefore suggest that upon primary encounter of a naive T cell with an APC, the T cell is stimulated to proliferate, but produces only low levels of CD95L. This would allow for clonal expansion of the T cell, and significant CD95L promoter activation may come only as a response to secondary encounter with Ag. This model is supported by previous work showing that secondary stimulation of peripheral T cells with superantigen enhances CD95-dependent cytolytic activity relative to primary stimulation (13). To address this hypothesis more directly, we are currently investigating CD95L promoter activity in primary vs secondary immune responses.

This model of delayed CD95L promoter activity would provide another potential explanation for the failure of recently activated T cells to undergo AICD in vivo. The pattern of delayed CD95L/luciferase activity we observe in response to anti-TCR stimulation correlates with the well-described decline in responsive CD4+ Vβ8+ peripheral T cell numbers that occurs at ~72 h post-staphylococcal enterotoxin B administration (51, 52). It has been shown previously that expression of FLICE inhibitory protein, a known inhibitor of CD95-mediated signal events, is high in resting T cells and declines following T cell activation (53). The presence of FLIP is believed to protect recently activated T cells from undergoing AICD, but an absence of sufficient CD95L on the surface of T cells would also prohibit AICD in this population.

During the course of our examination of CD95L promoter function in unfractionated splenocyte and lymph node cultures, we also detected luciferase activity in a non-T cell population. This was detected luciferase activity in a non-T cell population. This was
contributions to the promoter activity detected under these conditions remain to be determined. Although strong TCR ligation in the periphery leads to activation and proliferation, the same stimulation causes deletion of cells in the thymus (54). We observe comparable induction of CD95L/luciferase activity in mature T cells and thymocytes. However, this ability of thymocytes to inducibly express CD95L probably has minimal impact on negative selection during thymic maturation. One previous examination of thymic tissue showed CD95L expression only on nonlymphoid cells (55), indicating that, at most, few CD95L thymocytes are present within the thymus at a given time. Interestingly, purified populations of HSA<sup>+</sup>CD<sup>+</sup>CD<sup>−</sup> single-positive thymocytes have been demonstrated to undergo CD95-dependent apoptosis in vitro in response to a strong TCR stimulus provided by bound anti-TCR Ab (38, 39). The majority of thymocytes are CD<sup>−</sup>CD<sup>−</sup> double-positive cells, and these express a thymus-specific isoform of an orphan nuclear receptor, RORγt, that prohibits CD95L expression (41). Therefore, the number of thymocytes capable of producing CD95L is likely very small, and previous methods used for detection may not have had sufficient sensitivity to do so. It is possible that during the course of maturation, CD95/LCD95 interactions mediate apoptosis for only a small percentage of developing thymocytes that possess the highest affinity for self Ags.

We also provide data that illustrate CD95L promoter activity in both Th1 and Th2 effectors. Although this is consistent with reports by other groups (27, 42, 43), use of the CD95LP-Luc strain allowed us to demonstrate that CD95L promoter activity differs between these cell populations. Several transcription factors have been described that show a divergence in expression patterns or activity between differentiated Th1 vs Th2 populations. We and others previously reported that the transcription factor NF-AT contributes to CD95L promoter activation in Jurkat T cells, and other transcription factors, such as Egr-2 and -3 and NF-κB, participate in transcriptional regulation of the promoter as well (15–23). Therefore, these proteins constitute likely candidates for regulating CD95L promoter activity in Th1 and Th2 populations. Using a transgenic mouse model, Rincon and Flavell (56) determined that NF-AT activity is much greater in differentiated Th2 cells relative to Th1 cells. Their results imply that while NF-AT may regulate CD95L promoter activity in Th2 cells, factors other than NF-AT are activated in Th1 cells during the differentiation process. The contributions of NF-AT and the identification of other transcription factors that mediate differential CD95L promoter activity in Th1 and Th2 cells remain to be determined.

Our CD95LP-Luc model system provides a novel method for investigating CD95L promoter activity in primary cells. Still, several caveats must be considered, such as potential differences in the stability of luciferase protein relative to endogenous CD95L, and potential differences in the response of murine vs human lymphocytes. Another factor that cannot be addressed in our system is the contribution of vesicular cytoplasmic CD95L compared with surface CD95L. However, this system does enable us to investigate CD95L promoter activity in primary T cells, something that was only possible previously through the use of RT-PCR analysis of mRNA levels. The use of luciferase as a surrogate for CD95L has been widely used in transient transfection assays in cell lines, and its use here allows us to easily monitor CD95L promoter activity in primary cells in a quantitative fashion. This model system therefore provides an advantageous alternative to methods currently available for examination of CD95L promoter activity in a variety of cell types.

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