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Reciprocal expression of TRAIL and CD95L in Th1 and Th2 cells: role of apoptosis in T helper subset differentiation

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Abstract

Upon activation, naïve T helper cells can differentiate into two major distinct subsets, T helper 1 (Th1) and T helper 2 (Th2), as defined by their effector functions and cytokine secretion patterns. Cytokine milieu and costimulatory molecules have been shown to play an essential role in determining T helper differentiation. However, it is still unclear how the effects of signals of costimulatory molecules and cytokines are exerted during T helper differentiation. We show evidence suggesting that while cytokine signals initiate the differentiation program, the selective action of death effectors determines the end point balance of differentiating T helper subsets. We examined the expression of TNF-related apoptosis-inducing ligand (TRAIL) and CD95L in cloned and *in vitro* differentiated Th1 and Th2 cells. We found that activation-induced expression of TRAIL is exclusively observed in Th2 clones and primary T helper cells differentiated under the Th2 condition, while the expression of CD95L is mainly in Th1 cells. Furthermore, these two subsets exhibit distinct susceptibilities to TRAIL- and CD95L-mediated apoptosis. Th2 cells are more resistant to either TRAIL- or CD95L-induced apoptosis than Th1 cells. More importantly, both Th1 and Th2 cells could induce apoptosis in labeled Th1 but not Th2 cells. Blocking TRAIL and CD95L significantly enhance IFN-γ production *in vitro*. Likewise, young MRL/MpJ-*lpr/lpr* mice also showed more Th1 response to ovalbumin immunization as compared to MRL/MpJ+/+. Therefore, apoptosis mediated by CD95L and TRAIL is critical in determining the fate of differentiating T helper cells.
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Keywords: apoptosis; T helper cell type 1; T helper cell type 2; TRAIL; CD95 ligand

Abbreviations: TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; CD95L, CD95 ligand; Th1, T helper cell type1; Th2, T helper cell type 2; AICD, Activation-induced cell death

Introduction

In response to antigenic stimulation, T helper cells differentiate into at least two distinct subsets, T helper 1 (Th1) and T helper 2 (Th2), as defined by immune functions they mediate[1] and cytokines they secrete.[2] This classification paradigm has provided valuable information for understanding the balance of immune responses, and for designing therapeutic strategies to stimulate humoral and cellular immunities.[3,4] Th1 cells secrete IL-2, IFN-γ, and lymphotoxin and are important in promoting cellular immunity. On the other hand, Th2 cells secrete IL-4, IL-5, IL-10, and IL-13 and are crucial in supporting humoral immune responses.[4,5] T helper precursors could commit to either Th1 or Th2 phenotype within a few days upon activation.[6] Although several factors, such as dose and route of antigen administration and engagement of costimulatory molecules, have been shown to influence the differentiation of T helper subsets, the most potent regulators of T helper differentiation are undoubtedly cytokines.[7] Indeed, T helper subsets reciprocally regulate each other, as it has been shown that IFN-γ inhibits the proliferation and functions of Th2 cells, whereas IL-4 and IL-10 suppress Th1 cells.[3,8–10]

Even though strong effects of cytokines on inducing Th1 and Th2 development from naïve T cells were clearly demonstrated in bulk T cell culture,[4] it is not known whether a naïve precursor T helper cell is preprogrammed to become Th1 or Th2 lineage upon activation.[5] The stochastic model predicts that the differentiation orientation is genetically determined. The selection signals will determine which subset in a T helper population will grow out.[9,11,12] The instructive model proposes that a naïve T cell is malleable and fully capable of becoming either Th1 or Th2 cells. The differentiation process is completely dependent on the instructive signals such as the cytokine milieu and costimulation molecules.[13] Although more evidence supports the stochastic model,[14–17] the resolution of this paradox still awaits for single-cell-based experiments. Regardless, after the initiation of differentiation, the establishment of a proper type of immune response is likely maintained by regulating cell survivability under specific conditions. In this paper, we show that TRAIL and CD95L, which are critical for the regulation of lymphocyte apoptosis, are expressed in distinct patterns in both cloned and *in vitro* differentiated Th1 and Th2 cells upon activation through the T-cell antigen receptor (TCR). TRAIL is

exclusively observed in Th2 cells, while CD95L is mainly detected in Th1 cells. Furthermore, Th2 cells are more resistant to either TRAIL- or CD95L-induced apoptosis than Th1 cells. Blocking TRAIL and CD95L significantly enhanced IFN-γ secretion, and slightly decreased IL-4 in activated T helper cells. Additionally, we found that young MRL/MpJ-lpr/lpr (MRL/lpr) mice demonstrate a higher Th1 response than MRL/MpJ+/+ (MRL/+) in response to ovalbumin immunization. Therefore, CD95L- and TRAIL-mediated apoptosis plays a critical role in the development of Th1 and Th2 cells from the activated T-cell population.

Results

TRAIL is differentially expressed in C57BL/10 and Balb/c mice

Activation of TCR can induce the expression of CD95L and TRAIL in T-cell hybridomas[18–23] and primary T cells.[18,24–26] However, the role of genetic background in the expression of CD95L and TRAIL is not known. We examined TRAIL expression in splenocytes of BALB/C and C57BL/10 mice at 6 h after anti-CD3 stimulation. We found that anti-CD3-induced surface expression of TRAIL is much higher in BALB/c than in C57BL/10 mice (Figure 1a). Furthermore, when T-cell blasts (generated according to Lenardo,[27] >95% are T cells) were further activated for different times and examined for TRAIL expression by Northern blotting analysis, we found significant expression of TRAIL only in cells from BALB/c, but not C57BL/10 mice (Figure 1b). We also found that DBA mice express TRAIL at low levels upon activation (data not shown).

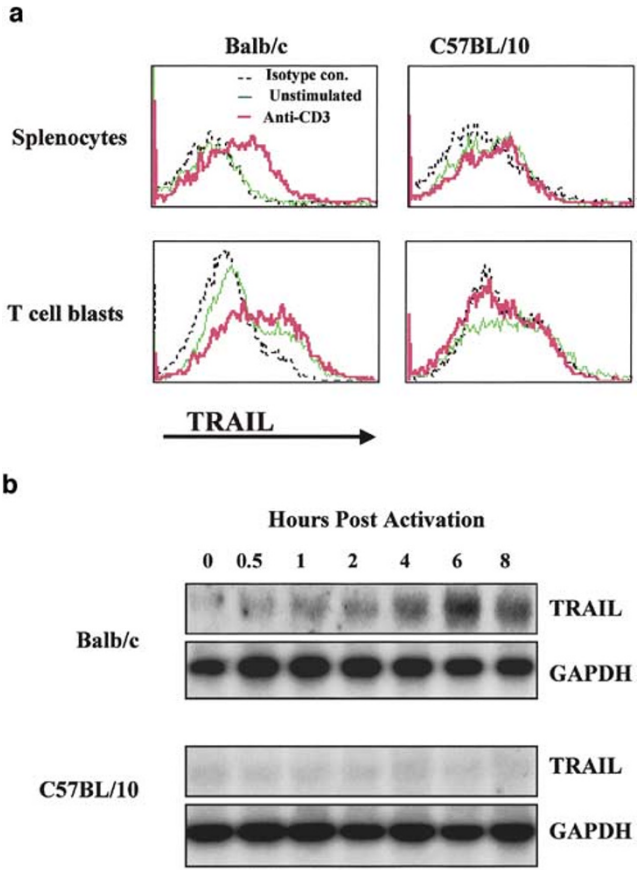

Figure 1 TRAIL expression in BALB/c and C57BL/10 mice. (**a**) Freshly isolated splenocytes and T-cell blasts (generated by stimulation with anti-CD3 for 48 h followed by a 48-h IL-2 activation) were stimulated with anti-CD3 for 6 h. TRAIL was detected by flow cytometry upon surface staining with anti-TRAIL. (**b**) T-cell blasts were activated again with anti-CD3 for 0.5, 1, 2, 4, 6, and 8 h and TRAIL mRNA was detected by Northern blotting analysis. GAPDH was used to control RNA loading

Differential expression of TRAIL by Th1 and Th2 cells

Since BALB/c mice are more likely to develop Th2-type immune responses than C57BL/10 and DBA, it is conceivable that TRAIL is differentially expressed in Th1 and Th2 cells.

We next examined the level of TRAIL in T-cell clones and in *in vitro* polarized T helper cells. Both T-cell clones and *in vitro* established Th1 and Th2 cells (verified by their production of signature cytokines) were stimulated with plastic bound anti-CD3 for 4 h and total RNA was isolated. As shown in Figure 2a, the expression of TRAIL was observed in both cloned and *in vitro* differentiated Th2 cells, while TRAIL was barely detectable in either cloned or *in vitro* polarized Th1 cells. This is the first demonstration of a skewed expression pattern of TRAIL in different T-cell subsets. Interestingly, when the same RNA blot membranes were analyzed for CD95L expression, we found an opposite pattern: activation-induced increase in CD95L expression was more significant in Th1 than that in Th2 cells (Figure 2a).

To verify the expression of TRAIL in Th2 cells, we activated both cloned and *in vitro* differentiated T helper cells with anti-CD3 for 6 h and stained with a murine TRAIL-specific monoclonal antibody. As shown in Figure 2b, TRAIL protein was only detectable in Th2, but not in Th1 cells. In cloned Th2 cells, TRAIL was already expressed on the surface substantially and was further increased upon activation. The expression of TRAIL on *in vitro* derived Th2 cells was low before activation (Figure 2c). We believe that the higher basal expression of TRAIL on cloned Th2 cells may be the result of repeated activation. Nonetheless, repeated activation of Th1 cells did not increase the expression of TRAIL, further indicating the specificity of TRAIL expression in Th2 cells. Therefore, TRAIL protein is only expressed on the surface of Th2 cells, correlating with its RNA expression pattern.

Th1 cells are sensitive to TRAIL and CD95L-mediated apoptosis

Stimulation of activated T-cell blasts often leads to activation-induced cell death (AICD).[27] AICD has been shown to occur in Th1 and to some degree in Th2 cells.[28] To investigate the role of TRAIL and CD95L in AICD, we activated both Th1 and Th2 cells with plastic-bound anti-CD3 for 5 h and analyzed apoptotic cells by AnnexinV and propidium iodide (PI) double staining. The AnnexinV single positive cells represent early apoptotic cells. As shown in Figure 3, AICD in differentiated

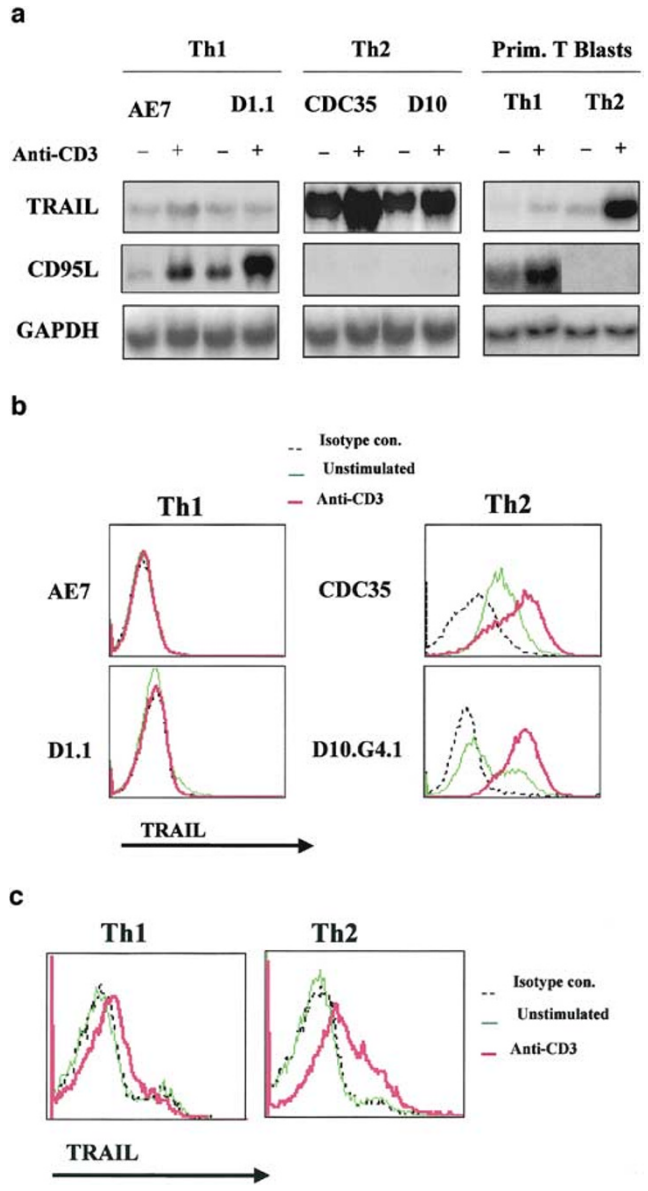

Figure 2 TRAIL and CD95L expression in cloned and primary Th1 and Th2 cells. (**a**) Th1 clones (AE7 and D1.1), Th2 clones (CDC35 and D10) and freshly generated T-cell blasts were activated with plastic-bound anti-CD3 for 4 h. The expression of TRAIL and CD95L was determined by northern blotting analysis. GAPDH was used as a control for RNA loading. (**b**) Th1 clones (AE7 and D1.1) and Th2 clones (CDC35 and D10) were activated with plastic coated anti-CD3 for 6 h. Surface TRAIL was detected by flow cytometry upon staining with FITC-anti-TRAIL antibody. (**a**) Primary Th1 and Th2 cells were generated by adjusting cytokine availability. TRAIL expression on cell surface was determined 6 h after activation with plastic-bound anti-CD3

Th1 cells can be inhibited by anti-CD95L, but not by soluble DR5. On the other hand, AICD in Th2 cells, although less prominent, is not significantly affected by either soluble DR5 or anti-CD95L, indicating that AICD in differentiated Th2 cells is independent of both CD95L and TRAIL. In addition, we found that the combination of both DR5 and anti-FasL could not inhibit AICD in Th2 cells. Surprisingly, we have recently shown that caspase inhibitor Z-VAD could only block activation-induced apoptosis in Th1 cells, but not in Th2 cells (data not shown).

To test directly the role of CD95L and TRAIL in AICD, we treated *in vitro* differentiated Th1 and Th2 cells with either recombinant murine CD95L or recombinant TRAIL. Murine CD95L (R&D systems, Minneapolis, MN, USA) was added directly to cell culture, while TRAIL (R&D systems, Minneapolis, MN, USA) was used as anti-6-his oligomerized form. Apoptosis was analyzed by staining with propidium iodide combined with AnnexinV or by DNA content analysis. We found that both cloned and *in vitro* polarized Th1 cells are sensitive to CD95L- or TRAIL-mediated apoptosis, while Th2 cells are resistant (Figure 4). Oligomerization of TRAIL was found to be necessary for the induction of apoptosis in Th1 cells. We also tested differentiated T helper cells for their sensitivity to agonist antibody to CD95, JO2, and found that only Th1 cells are sensitive to JO2-induced apoptosis (data not shown). Therefore, CD95L expressed on Th1 cells can lead to the suicide of Th1 cells, while TRAIL expressed on Th2 cells may only mediate fratricide of Th1 cells.

Since Th1 cells are sensitive to TRAIL- and CD95L-induced apoptosis, these cells could be the targets of TRAIL- or CD95L-expressing cells. To test directly the fratricidal capability of Th1 and Th2 cells, we performed a cytotoxicity assay. Th1 and Th2 cells were used as both effectors and targets. Targets were labeled with ^{51}Cr and mixed with effectors. As shown in Figure 5, when Th1 cells were used as effectors, about 30% of Th1 cells were killed at the effector : target ratio of 30 : 1; Th2 cells were resistant. On the other hand, when Th2 cells were used as effector cells, about 15% of Th1 cells were killed while Th2 cells were completely resistant. In addition, Th1-mediated killing was inhibited by anti-CD95L, while Th2-mediated killing was inhibited by soluble TRAIL receptor DR5. These results suggest that Th1 cells can kill themselves by CD95/CD95L and can be killed by Th2 cells via TRAIL.

Blocking of CD95L- and TRAIL-mediated apoptosis promotes Th1 differentiation

Although several models regarding the differentiation process have been proposed, the exact mechanisms by which cytokine signals promote Th1 and Th2 differentiation are still being debated. Our results of the difference between Th1 and Th2 cells in susceptibility to TRAIL- and CD95L-mediated apoptosis strongly suggest a potential role for apoptosis in the differentiation of T-cell populations. To investigate whether apoptosis participates in the regulation of Th1 and Th2 development, we tested the effect of anti-CD95L and DR5. We found that during *in vitro* differentiation of Th1 cells, the presence of anti-CD95L significantly increased the production of IFN-γ (Figure 6a). Although, soluble DR5 alone did not have much effect on IFN-γ production, the combination of DR5 and anti-CD95L *additively* increased the production of IFN-γ. On the other hand, during the differentiation of Th2 cells the presence of anti-CD95L and DR5 decreased the production of IL-4. When isolated $CD4^+$ cells were activated under neutral unbiased conditions, the presence of anti-CD95L dramatically increased the IFN-γ production, while at the same time,

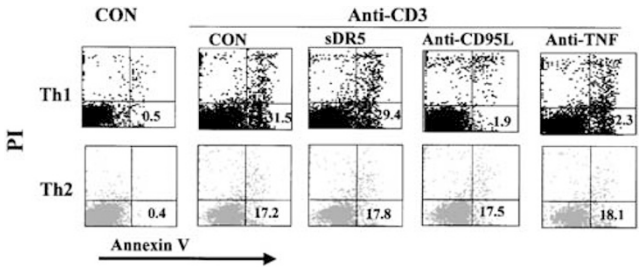

Figure 3 AICD in Th1 cells. Primarily differentiated Th1 and Th2 cells were stimulated with plastic-bound anti-CD3 in the presence or absence of soluble DR5, a blocker of TRAIL, anti-FasL, or anti-TNF-α for 5 h. Apoptosis was determined by propidium iodide permeability and AnnexinV binding. Cells recently committed to apoptosis are AnnexinV positive and PI negative. Cells dead for a prolonged time are positive for both AnnexinV and PI. The numbers indicates the percentage of cells that died recently and are only positive for AnnexinV

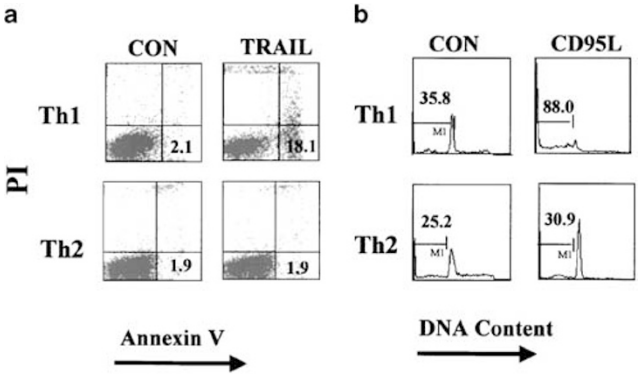

Figure 4 Sensitivity of Th1 and Th2 cells to TRAIL and CD95L. (**a**) *In vitro* generated Th1 and Th2 cells were treated with anti-6-his oligomerized TRAIL (R&D systems) for 20 h. Cell viability was assessed by staining with PI and AnnexinV. The number in the figure represents cells that died recently and were stained positive for only AnnexinV. (**b**) Th1 and Th2 cells were treated with CD95L for 12 h and apoptosis was analyzed by DNA content analysis. The percentage cells with subdiploid DNA content are indicated

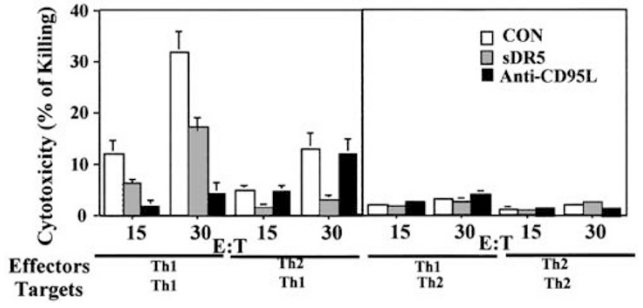

Figure 5 Th1 cells, but not Th2 cells, are killed by Th1 and Th2 cells via CD95L and TRAIL, respectively. Th1 or Th2 cells were labeled with ^{51}Cr as targets and mixed with Th1 or Th2 cells as effectors at different ratios. Cytotoxicity was determined at 4 h by measuring ^{51}Cr release in the culture supernatant

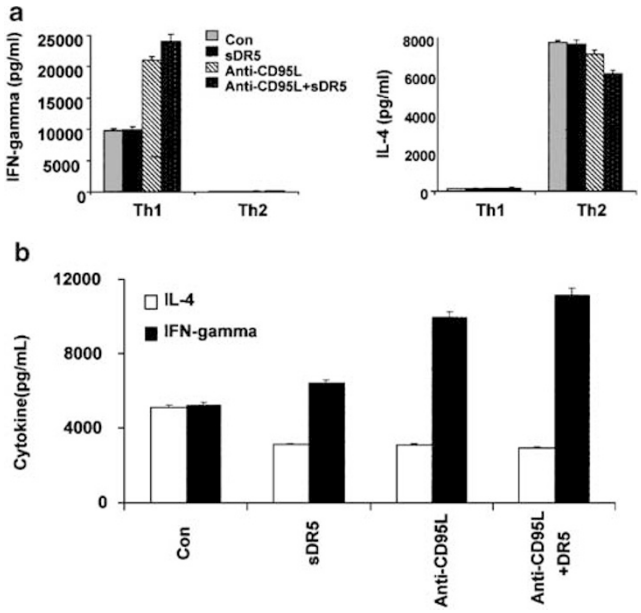

Figure 6 Blockade of TRAIL and CD95L enhances IFN-γ production in *in vitro* differentiated Th1 cells. (**a**) Sorted resting CD4$^+$ T cells were activated with anti-CD3 and anti-CD28 under Th1 or Th2 cytokine conditions in the presence or absence of soluble DR5 or anti-CD95L. IFN-γ and IL-4 were detected at 24 h after second anti-CD3 activation. (**b**) Sorted CD4$^+$ T cells were activated with anti-CD3 and anti-CD28 without addition of skewing cytokines

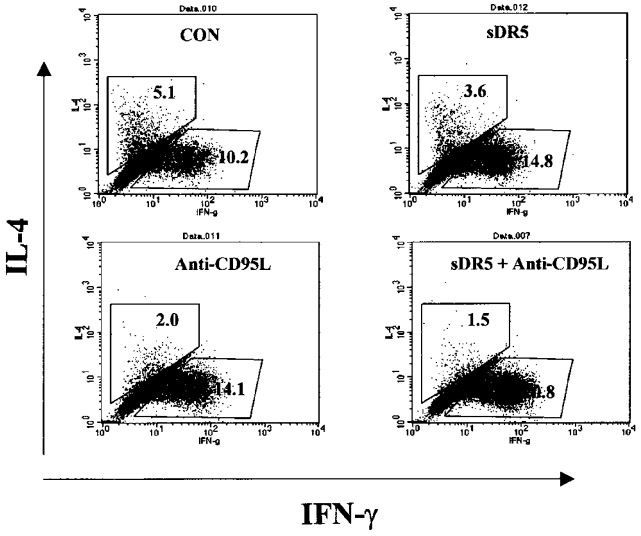

Figure 7 Blockade of TRAIL- and CD95L-mediated apoptosis enhances IFN-γ and reduces IL-4 producing cells. Sorted resting CD4$^+$ T cells were activated for 7 days with anti-CD3 and anti-CD28 in the presence of soluble DR5 (blocker of TRAIL) or MFL3 (blocker of FasL). IFN-γ and IL-4 were detected by intracellular staining at 6 h after activation with PMA and ionomycin

decreased IL-4 production. Similarly, although soluble DR5 only had minimal effect on cytokine production, the combination of DR5 with anti-CD95L demonstrated an additive effect (Figure 6b). To further investigate the effect of blocking CD95L and DR5 on cytokine production at single-cell levels, we activated purified CD4$^+$ T cells for 7 days with anti-CD3 and anti-CD28 in the presence or absence of DR5 or anti-CD95L. The number of cells producing IFN-γ and IL-4 was determined by intracellular staining. We found that the number of cells producing IL-4 was reduced, while the number of cells producing IFN-γ increased (Figure 7). Therefore,

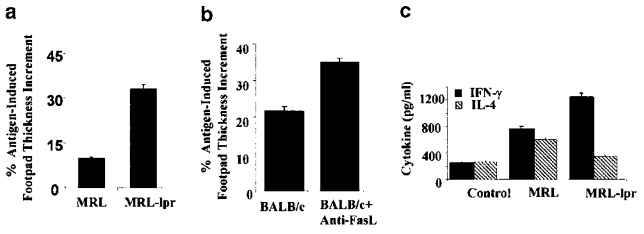

Figure 8 CD95 mutation increases Th1 response. MRL/MpJ-lpr/lpr and MRL/MpJ-+/+ mice (5-week old) were immunized with 10 μg OVA in 50 μl saline mixed with 50 μl incomplete Freund's adjuvant at tail base. Mice received saline mixed with incomplete Freund's adjuvant in absence of OVA were as control. (**a**) Mice were tested for DTH reaction with aggregated OVA on day 5 after immunization and footpad thickness increment as a measure of DTH was determined 24 h later. The percent of antigen-specific footpad thickness increment was determined by comparing aggregated OVA injected left footpad with PBS injected right footpad. (**b**) Five-weeks-old BALB/c mice were also immunized as above, except one group received 100 μg anti-FasL every day for the first 4 days after immunization. DTH was assessed. (**c**) Serum cytokine level was detected on day 6 after immunization by ELISA

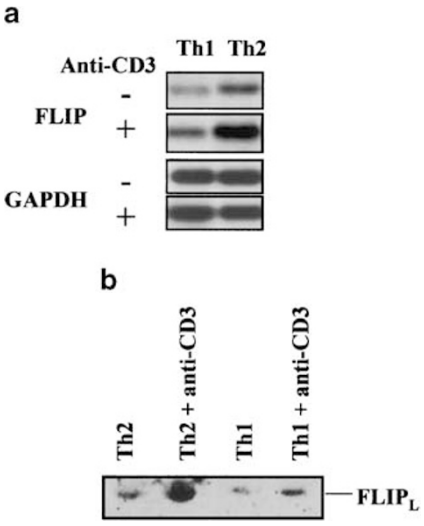

Figure 9 FLIP expression. *In vitro* differentiated Th1 and Th2 cells were activated with plastic-bound anti-CD3 for 4 h and total RNA were isolated. The expression of FLIP RNA was determined by Northern blotting analysis (**a**). The Th1 and Th2 cells were also activated for 5 h and the level of FLIP protein was also detected by Western blotting analysis (**b**)

blocking CD95L and TRAIL function during T-cell activation favors the development of Th1 cells.

To further investigate the role of apoptosis in Th1 and Th2 response, we immunized 5-week-old MRL*LPR/lpr* mice, which bear autosomal recessive mutation in CD95, and their appropriate background control, MRL$^{+/+}$ mice, with OVA in incomplete Freund's adjuvant. DTH response to OVA challenge, a characteristic Th1 response, was assessed 5 days after immunization. We found that MRL*LPR/lpr* mice showed higher DTH response as compared to MRL$^{+/+}$ mice (Figure 8a). Unimmunized mice did not show response to aggregated OVA. It is important to point out that our immunization protocol did not include *Mycobacterium tuberculosis* and therefore did not induce a high-level DTH response in MRL$^{+/+}$ mice. Nevertheless, a significant increase in DTH response was observed in MRL*LPR/lpr* mice. In addition, we also tested the effect of *in vivo* administration of anti-FasL on DTH response in Balb/c mice and also found that anti-FasL significantly increased DTH response (Figure 8b). We examined the levels of IL-4 and IFN-γ in their serum. We found that MRL*LPR/lpr* produced more IFN-γ and less IL-4 than MRL$^{+/+}$ mice **(**Figure 8c). Therefore, young mice with a loss of function mutation of CD95 showed higher Th1 response, further supporting a role for apoptosis in Th1 and Th2 development.

Th2 cells express high levels of FLIP

Our studies have clearly shown that Th2 cells are more resistant to AICD, and to CD95L- and TRAIL-induced apoptosis. To investigate the mechanisms controlling this resistance, we examined the expression of FLIP, a molecule known to block TNF receptor family member trimerization-induced apoptosis signals. Total RNA was isolated from *in vitro* differentiated and cloned Th1 and Th2 cells. The level of FLIP was determined by Northern blotting analysis. As shown in Figure 9a, FLIP mRNA expression is higher in Th2 cells than in Th1 cells. We also examined FLIP protein in Th1 and Th2 cells by Western blot analysis. As shown in Figure 9b, the expression of FLIP is higher in Th2 cells than in Th1 cells. Therefore, the higher level of FLIP in Th2 cells may confer the resistance to apoptosis.

Discussion

The discovery of two major T helper cell subsets (Th1 and Th2) is a landmark for the understanding of the mechanisms regulating the immune responses during infections, autoimmunity, and cancer pathogenesis.[5,8,10] Great efforts have been devoted to the understanding of the molecular mechanisms controlling the differentiation of Th1 and Th2 cells. However, the exact mechanisms by which these signals modulate the differentiation process are not well defined. In this paper, we show that two TNF superfamily members, TRAIL and CD95L, are expressed in distinct patterns in Th1 and Th2 cells upon activation through TCR: TRAIL is only observed in Th2 cells, while CD95L is mainly detected in Th1 cells. Th2 cells are more resistant to either TRAIL- or CD95L-induced apoptosis than Th1 cells. Blocking TRAIL and CD95L significantly enhanced IFN-γ secretion while moderately decreased IL-4 production. Therefore, our findings provide novel information for the understanding of the mechanisms controlling Th1 and Th2 responses.

Recent studies have revealed that the initial expression of cytokines is cell cycle dependent, with IFN-γ appearing during the initial cell division and IL-4 emerging only after at least three cell divisions.[29] Therefore, it seems that the T helper cell fate decision has intrinsic heterogeneity and both the instructive and the stochastic models may operate simultaneously and contribute to T helper cell differentiation. Recently, Coffman and Reiner[30] proposed to devise "the equivalent of an embryologist's fate map" to account for the birth, differentiation, and death of all descendants of an

individual T cell stimulated under particular cytokine conditions. We have examined the role of cell death in T helper cell differentiation. The data presented here revealed that during T helper cell differentiation, CD95L expressed on Th1 cells and TRAIL on Th2 cells will preferentially kill Th1. It should be noted that although Th2 cells are resistant to both CD95L- and TRAIL-mediated apoptosis, they still undergo AICD. AICD in Th2 cells is independent of TRAIL and CD95L (Figure 3). In addition, AICD in Th2 is not inhibited by caspase inhibitor Z-VAD (data not shown), suggesting that AICD in Th1 and Th2 is mediated by different pathways. Nevertheless, except when there is a sustained Th1 cytokine environment to support continuous Th1 differentiation, the immune response will be pushed towards the Th2 type by apoptosis mediated by CD95L and TRAIL. The best evidence is that in the absence of exogenous cytokines, inhibition of apoptosis by anti-CD95L and soluble DR5 leads to an increase in Th1 cells. Therefore, the process of Th1 and Th2 differentiation is a result of continuous cell proliferation and apoptosis. Figure 10 depicts our model illustrating the critical role of apoptosis in the differentiation of T helper cells. We propose that cytokine signals initiate the differentiation program, while apoptosis determines the destiny of T helper subsets.

The loss-of-function by mutations in the CD95 *(lpr)* and CD95L *(gld)* genes cause lymphocyte accumulation and autoimmune diseases.[31] It is interesting to note that in lpr or *gld* mice, there is a significant increase in the production of IFN-γ at their early stage of autoimmune diseases.[32,33] Although various studies with different model systems have demonstrated that Th2 plays a critical role in the development of SLE both in humans and in mice, the cytokines generated by Th1 cells have also been shown to accelerate the onset of SLE. This may reflect the dynamics of SLE pathogenesis. It should be noted that with the progression of autoimmune disease, there is gradually more IL-4 and less IFN-γ. To test directly the effect of CD95 mutation on immune responses, we selected young MPJ/lpr mice (predisease). Upon immunization with OVA, we found that mice bearing lpr mutation showed higher DTH response and higher IFN-γ production, providing direct *in vivo* evidence for the role of CD95 in the development of T helper cells. In a rat kidney transplantation model, adenovirus-mediated intragraft CD95L gene transfer could prolong kidney survival by suppressing Th1 cytokines, while at the same time progressively increasing Th2 cytokines,[34] again arguing that overexpression of CD95L preferentially eliminates Th1 cells while sparing Th2 cells.

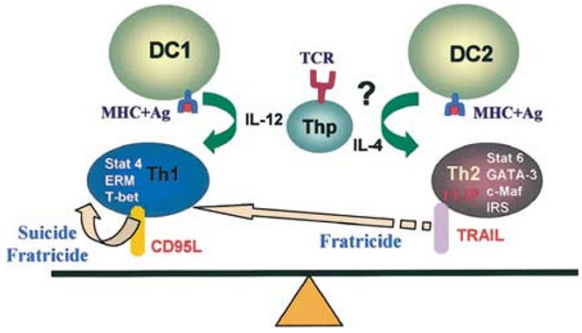

Figure 10 Diagrammatic representation of the role of CD95L and TRAIL in Th1 and Th2 differentiation. Resting T helper cells (ThP) are activated by antigens (Ag) presented by different types of dendritic cells (DC). Due to the availability of specific cytokines in the environment and the transcription factors inside the cells, T helper cells may differentiate into either Th1 or Th2 cells. CD95L expressed on Th1 cells and TRAIL expressed on Th2 cells could downregulate Th1 and skew the immune response towards Th2 type if Th1-inducing cytokines are not continuously available

It is well established that cytokine milieu plays a critical role during the differentiation of Th1 and Th2 cells. The modulation of the availability of key cytokines such as IL-4, IFN-γ, and IL-12 leads to the distinct patterns of T helper cell differentiation. The role of these cytokines in T helper differentiation is believed to affect the activities of transcription factors. Upon binding to their respective receptors, IL-12 and IL-4 activate STAT4- and STAT6-dependent pathways, respectively, and ultimately regulate the development of IFNγ and IL-4 production. In addition to STAT4 and STAT6, significant progress has been made in identifying the transcription factors that control the differentiation process of T helper precursors (Thp) into a Th1 or Th2 cells. Many transcription factors including c-Maf, NF-AT, NIP45, and GATA-3 have also been shown to regulate T helper subset differentiation.[5] In fact, it has been shown that ectopic expression of a particular factor such as GATA-3 is sufficient to induce Th2 cytokine expression in developing T cells. Egr3, a transcription factor know to regulate CD95L expression, has been found to be enriched in Th1 cells. However, the exact mechanisms by which these factors affect the transcriptional activity of CD95 L and TRAIL during T helper cell differentiation are yet to be investigated . Nevertheless, we have found that mice deficient in STAT6ao express less TRAIL (data not shown). Since Th2 cells have elevated IRS2 expression,[35] we transfected T-cell hybridoma A1.1 cells with IRS-2 and found that IRS2 promoted TRAIL expression (data not shown). We believe that further investigation in the role of these factors in the modulation of the expression of CD95L and TRAIL will undoubtedly provide information for better understanding of the mechanisms by which Th1 and Th2 cells are regulated.

Traditionally, Th1 and Th2 cells are classified by their cytokine expression patterns either via ELISA or by intracellular staining. Recent studies have revealed that several molecules are unique to, or preferentially expressed on, Th1 or Th2 cells. For example, Th1 cells preferentially expressed IFNγ receptor β chain, IL-12 receptor β chain, IL-18 receptor, and CCR5. On the other hand, Th2 cells express T1/ST2, CCR3, CCR4 and CCR8.[10] A recent study has shown that among 6000 genes compared, 215 genes are found to be differentially expressed in the two types of T cells.[36] Our finding of the preferential expression of TRAIL in Th2 and CD95L in Th1 cells provides another potential marker for the distinction of Th1 and Th2 cells.

The Th1 and Th2 dichotomy has provided an important model for understanding the regulation of the immune system. Although it is clear that the cytokine milieu is critical in Th1 and Th2 differentiation, it is not known which factors are involved in the establishment and stability of each type of immune response. Our findings of the differential expression of CD95L and TRAIL and the role of apoptosis mediated by these molecules in the balance between Th1 and Th2

development, demonstrate that cytokine signals initiate the direction, while apoptosis determines their destiny.

Materials and Methods

Reagents and mice

Recombinant DR5 was a gift of Dr. Youhai Chen (University of Pennsylvania, Philadelphia, PA, USA). Anti-murine CD95L (MFL3), anti-murine CD95 (JO2), and AnnexinV were from Pharmingen (La Jolla, CA, USA). Antibodies to IFNγ, IL-4, and IL-12 were purchased from eBioscience (La Jolla, CA, USA). Anti-TNFα, TRAIL, anti-6-his, and IFNγ were from R & D Systems (Minneapolis, MN, USA). Chicken ovalbumin (OVA, fraction V) was from Sigma Chemical Co. (St. Louis, MO, USA). Female BALB/c and C57BL/10 mice at 4–6 weeks old were obtained from NCI (Frederick, MD, USA). MRL$^{+/+}$ and MRL/lpr mice were obtained from the Jackson Laboratories (Bar Harbor, ME, USA).

Cell culture

Murine splenocytes were isolated from 8 to 10-week-old Balb/c and C57BL/10 mice. Murine Th1 clones, D1.1 and AE7, and Th2 clones, D10 and CDC35, were described previously.[37–39] All cells were maintained in RPMI 1640 medium supplemented with 2 mM L-glutamine, 50 mM 2-mercaptoethanol, 10% heat-inactivated fetal bovine serum, and 10 mM gentamycin. Th1 clones were also provided with exogenous IL-2 and Th2 clones with 10% supernatant of concanavalin A-activated rat splenocytes.

Immunization and DTH testing

MRL$^{+/+}$ and MRL*LPR/lpr* mice (5-week-old) were immunized with OVA. Each received 10 μg in 50 μl saline mixed with 50 μl incomplete Freund's adjuvant at tail base. Delayed-type hypersensitivity (DTH) was tested by injecting 200 μg aggregated OVA in 30 μl saline to the right hind footpad 5 days after immunization. The left footpad was injected with 30 μl of saline as control. Footpad thickness was measured with a caliper 24 h after challenge. The caliper plunger was allowed to rest freely on the footpad. Antigen-induced footpad thickness increments were calculated according to the following formula: $(R_{\text{immunized}}-L_{\text{immunized}})-(R_{\text{unimmunized}}-L_{\text{unimmunized}})$. Serum was also collected from each mouse on day 6 after immunization.

Differentiation of T Helpers

Pooled splenocytes and lymph node cells were labeled with phycoerythrin (PE)-conjugated anti-CD4 (GK1.5). CD4$^+$ T cells were sorted to >98% purity using a FACStar Sorting Flow Cytometer (BD, San Jose, CA, USA). Th1 and Th2 subsets were differentiated as described by Ho *et al.*, 1996. Briefly, purified CD4$^+$ T cells at 10^6/ml were activated with plastic bound anti-CD3 and anti-CD28. In addition, anti-IL-4 at 10 μg/ml was included for Th1 cells. Anti-IL-12 and anti-IFNγ at 10 μg/ml were supplied for Th2 cells. IL-2 at 50 U/ml was added to all cultures, and 10 ng/ml IL-12 and 5 ng/ml IL-4 were added to Th1 and Th2 cultures, respectively, 24 h later. Cells were divided at a 1 : 4 ratio 3 days later and maintained under the above cytokine conditions for respective T helper subsets in the absence of anti-CD3 and anti-CD28 for another 3 days.

Microcytotoxicity assay

The ^{51}Cr release microcytotoxicity assay was performed as described.[40] Briefly, target cells were labeled with ^{51}Cr for 1 h at 37°C and mixed with effector cells at various ratios. Supernatant was harvested 4 h after culture and radioactivity was measured by a gamma-counter. Spontaneous release from ^{51}Cr-labeled target cells was usually $<15\%$. Percent of specific release was calculated using the following formula: (experimental release−spontaneous release)/(total release−spontaneous release).

Cytokine measurement

IFN-γ and IL-4 were measured by ELISA using kits obtained from Biosource (Camarillo, CA, USA) according to the manufacturer's instruction. Recombinant murine IL-4 and IFN-γ were used as standard. Briefly, tissue culture supernatant or serum was diluted in standard buffer in kits and incubated in microtiter plates coated with the first antibodies specific to respective cytokines for 1.5 h at 37°C. After washing, the bound cytokines were detected with biotinylated second cytokine specific antibodies and streptavidin peroxidase. Cytokine amount was determined by the addition of peroxidase substrate, tetramethyl benzidine.

IL-4 and IFN-γ were also detected by intracellular staining. During the last 3.5 h of specific treatments, Brefeldin A was added at 10 μg/ml . Upon fixing with 4% paraformaldehyde, cells were resuspended in 50 μl of permeabilization buffer (PBS, 2% FCS, 0.02% sodium azide, 0.5% saponin) and stained with FITC-labeled rat anti-murine IFN-γ or PE-labeled anti-IL-4 and analyzed by flow cytometry. PE- or FITC-conjugated rat IgG1 was used as isotype controls.

Northern blotting

Total RNA was isolated with QIAGEN columns. RNA samples were fractionated on denaturing agarose gels, and transferred onto a Nytran membrane. The DNA probes (mouse TRAIL, CD95L, GAPDH, FLIP) were labeled with ^{32}P-dCTP by random priming (Boehringer Mannheim, Indianapolis, IN, USA) according to the manufacturer's protocols. Prehybridization and hybridization were carried out at 42°C in a solution containing 5 × SSC (10 × SSC is 1.5 M NaCl, 0.15 M sodium citrate), 2.5 mM EDTA, 0.1% SDS, 5 × Denhardt's solution, 2 mM sodium pyrophosphate, 50 mM sodium phosphate, and 50% formamide. Membrane was washed with 0.2 × SSC, 0.1% SDS at 56°C for 1 h and hybridization signals were detected by autoradiography.

Western blotting

Equal numbers of Th1 and Th2 cells were lysed in RIPA lysis buffer, which was composed of 1% NP-40, 50 mM HEPES (pH 7.4), 150 mM NaCl, 500 μM orthovanadate (Fisher Scientific, Fairlawn, NJ, USA), 50 mM $ZnCl_2$, 2 mM EDTA, 2 mM phenylmethylsufonyl fluoride, 0.1% SDS, and 0.1% deoxycholate. Samples were incubated at 4°C for 10 min and then centrifuged at 10,000 × g for 15 min at 4°C. The supernatants were transferred, mixed, and boiled in SDS sample buffer. The lysates were separated by polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Biorad, Hercules, CA, USA). The membrane was then incubated at room temperature in a blocking solution composed of 5% skim milk powder dissolved in 1 × TBS (10 mM Tris, pH 8.0, and 140 mM NaCl) for 1 h followed by incubation with the blocking solution containing anti-FLIP (Sigma) at 1 μg/ml for 4 h at room temperature. The membrane was washed three times in TBS (5 min each), and then incubated with horseradish peroxidase conjugated protein A in the

blocking solution. The blot was then exposed by ECL (Amersham, Arlington Heights, IL, USA) after another three washes in TBS.

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