Differential Susceptibility of Naïve and Activated Human γδ T Cells to Activation-Induced Cell Death by T-Cell Receptor Cross-Linking

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Abstract

Background: T cells undergo activation-induced cell death (AICD) through repeated stimulation of their T cell receptors (TCRs). Activated human γδ T cells were found to die by apoptosis when their TCRs were cross-linked by antibodies, whereas naïve γδ T cells freshly isolated from blood did not. Therefore, we investigated the factors that could contribute to this differential susceptibility.

Materials and Methods: γδ T cells were isolated from the peripheral blood of healthy human volunteers and their TCRs were cross-linked either directly (naïve) or after an *in vitro* incubation of 11 days (activated). Their cell cycle profiles, cytokine, Fas and FasL mRNA messages, and surface expression of Fas and FasL were determined.

Results: The naïve cells were cycling while the activated T cells exited from the G1 to subG1 phase upon TCR cross-linking. IL-2 and IL-4 mRNAs and surface expression of FasL were detected only in activated T cells in the time period examined. In addition, cFLIP mRNA expression was found only in naïve γδ T cells and activated T cells treated with cyclosporin A (CsA), which inhibited AICD in the activated T cells. CsA also downregulated the surface expression of FasL in activated T cells.

Conclusions: The differential expression of cytokines, apoptotic inducers and inhibitors provide the basis for the differential susceptibility of naïve and activated γδ T cells to AICD upon TCR cross-linking. This contributes to our understanding of the regulation and maintenance of γδ T-cell homeostasis, which would be important in many infectious as well as autoimmune diseases, where γδ T cells have been implicated.

Introduction

Activation-induced cell death (AICD) of lymphocytes is an important mechanism for homeostatic regulation of lymphocyte activation and proliferation (1) and for maintaining peripheral tolerance to self-antigens (2). AICD of T cells describes a process whereby repeated stimulation through the T cell receptor (TCR) results in apoptotic death mediated through Fas (CD95) and FasL (CD95L) interactions (3–5). It is a tightly regulated process and involves both apoptosis-inducing and inhibiting moieties, accounting for the AICD-susceptible or resistant phenotypes seen in different T cells (6,7). For example, naïve T cells stimulated through their TCR do not undergo AICD due to high levels of cellular FLICE-like inhibitor protein (cFLIP), which competitively inhibits the binding of FLICE (FADD-like IL-1β-converting enzyme), a zymogen of caspase 8, and shuts off Fas signaling (8). It is also known that IL-2 regulates and potentiates AICD (9), and naïve T cells need to progress into S phase of the cell cycle before becoming susceptible (10).

However, most studies examining AICD were done using CD4⁺ αβ T cells or T cell hybridomas. Little is known about the regulation of AICD in γδ T cells, although AICD has been reported in murine intestinal intraepithelial γδ T cells and was shown to be augmented by IL-2 (11). γδ T cells isolated from *Mycobacteria tuberculosis*-infected patients were also more susceptible to AICD than normal individuals and death was mediated by Fas and FasL interactions (12).

γδ T cells are involved in natural anti-infectious immunity (13,14). They rapidly respond in an MHC-unrestricted manner to phosphate-containing non-peptide molecules (15–17) and alkylamines (18) present in many microbes. Therefore, in addition to the control through T-cell co-receptors (19), the regulation of AICD in these cells may be an important element in guiding innate T-cell immunity responses. We have compared the cell cycle and cytokine profiles, as well as the Fas, FasL and cFLIP expression of naïve versus activated γδ T cells stimulated by TCR cross-linking. This allows us to identify factors that could account for the differential susceptibility of naïve and activated γδ T cells to AICD.

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Materials and Methods
Cell Culture

Human peripheral blood lymphocytes (PBL) were isolated from the blood of healthy volunteers drawn

by venipuncture on a Ficoll density gradient (Sigma, St. Louis, MO). The PBLs were cultured in RPM1 1640 medium supplemented with 10% FBS (Biological Industries, Israel), 100 μg/ml penicillin, 100 U/ml streptomycin, 2 mM L-glutamine (Sigma) and 50 U/ml of IL-2 (GibcoBRL, Gaithersburg, MD). To activate the $\gamma\delta$ T cells, mitomycin C-treated Daudi cells were added to the culture at a ratio of 1 Daudi cell to 5 PBLs. On the sixth day of culture, half of the media was removed and replaced with an equal amount of new media.

Stimulation and Magnetic Cell Sorting (MACS) of $\gamma\delta$ T Cells

Stimulation of both naïve and activated $\gamma\delta$ T cells by TCR cross-linking was accomplished through the MACS procedure. For activated cells that had been in culture for 11–12 days, the $\gamma\delta$ T cells were positively selected by MACS as described (20). Briefly, the cells were first stained with anti-TCRδ1 antibody from the supernatant of 5A6.E9 hybridoma (ATCC, Rockville, MD) followed by goat-anti-mouse κ antibody conjugated to biotin (PharMingen, San Diego, CA) and streptavidin microbeads (Miltenyi biotec, Sunnyvale, CA). To stimulate and purify naïve $\gamma\delta$ T cells, PBLs isolated from blood were subjected to MACS as above without prior *in vitro* culture. The eluted positive fraction typically contained $\gamma\delta$ T cells with a purity greater than 90%. The cells were plated in media and aliquots were taken out for subsequent experiments at various time-points after the separation. For cyclosporin A (CsA) (Sigma) treatment, cells were preincubated in CsA for 1 hour before stimulation and CsA was added to the purified cells plated in wells after MACS.

Apoptosis of $\gamma\delta$ T Cells

The percentage of apoptotic cells at each time point was determined by double staining of cells with annexin V-FITC and PI as described in the ApoAlert Annexin V FITC detection kit (Clontech, Palo Alto, CA). The bright staining PI cells were excluded as necrotic cells. Cells were analyzed by flow cytometry (FACScan, Becton Dickinson, San Jose, CA). Apoptosis was confirmed by microscopy and the viability of cells was determined by trypan blue dye exclusion method.

Cell Cycle and Flow Cytometric Analysis

The cells (0.8 x 10^6) were fixed in 1% formaldehyde and permeabilised with a solution of 12.5% RPMI, 12.5% FBS and 52.5% ethanol overnight. To detect the DNA content of the cells, 10 μg/ml propidium iodide (PI) (Sigma) and 100 U/ml RNaseA (Sigma) were added for 1 hour at room temperature. To stain the surface of cells for Fas and FasL expression, cells were stained with anti-Fas ZB4 antibody (I munotech, Marseille, France) followed by rat-antimouse IgG1 conjugated to PE (Becton Dickinson), and biotinylated anti-FasL NOK-1 antibody (PharMingen) followed by streptavidin-PE (Becton Dickinson). Cells were preincubated in 5 mM EDTA for at least 4 hours before staining for FasL. The cell cycle positions and surface expression were assessed by flow cytometry.

RT-PCR Analysis

RNA was extracted from 1.2 x 10^6 cells at each time-point using TRIzol reagent (GibcoBRL) according to manufacturer's instructions. The RNA was reverse transcribed into cDNA by incubating 5 μg of RNA, 0.2 μM oligo-dT, 1 μM dNTPs, 12 U RNAsin (Promega, Madison, WI) and 20 U of Moloney murine leukemia virus reverse transcriptase (New England Biolabs, Beverly, MA) at 37°C for 1 hour. PCR was performed using an MJ-Research thermal cycler in a reaction mix consisting of cDNA, 0.4 μM sense and anti-sense primers, 0.2 μM dNTPs, 2 μM MgCl$_2$, 10x reaction buffer and 1 U Taq DNA polymerase (Biotools, Madrid, Spain). Each PCR amplification was carried out for 35 cycles. The primer sequences were as follows:

β-actin, 5'-AAATCGTGCGTGACATTAACG-3' and 5'-AGCACTGTGTTGGCGTACAG-3'; IL-2, 5'-CATTGCACTAAGTCTTGCACTTGTCA-3' and 5'-CGTTGATATTGCTGATTAAGTCCCTG-3'; IL-4, 5'-AACACAACTGAGAAGGAAACCTTC-3' and 5'-GC-TCGAACACTTTGAATATTTCTC-3'; IL-7, 5'-ATGTT-CCATGTTTCTTTTAGGTAT-3' and 5'-TGCATTTCT-CAAATGCCCTAATCC-3'; IL-15, 5'-TGGAGCACA-GAAATCAATG-3' and 5'-GAAACAGCCCAAAATG-AAG-3'; TNF-α, 5'-CAAGCCTGTAGCCCATGTTG-TAGCA-3' and 5'-ATCCCAAAGTAGACCTGCCCA-GACT-3'; IFN-γ, 5'-TTTGGGTTCTCTTGGCTGTTA-CTGC-3' and 5'-TTCGCTTCCCTGTTTTAGCTGCT-GG-3'; Fas, 5'-ATCTGGACCCTCCTACCTCT-3' and 5'-AGATCTGGATCCTTCCTCTT-3'; FasL, 5'-GGA-TTGGGCCTGGGGATGTTTCA-3' and 5'-TTGTGGC-TCAGGGGCAGGTTGTTG-3'; and cFLIP, 5'-GGGA-GAAGTAAAGAACAAAG-3' and 5'-CGTAGGCAC-AATCACAGCAT-3'.

The primers were all designed to give a different product length or none with genomic DNA. PCR products were size fractionated on 1% agarose gel and normalized according to the amount of β-actin detected in the same mRNA sample. The amount of cDNA template used for the reactions was predetermined by dilution to fall within the linear portion of amplification curve. Relative mRNA levels were determined using the BioProfile software of a photoimager (Vilber Lourmat, Marne La Vallee, France).

Results

Differential Susceptibility of Naïve and Activated $\gamma\delta$ T Cells to AICD

$\gamma\delta$ T cells that had been activated *in vitro* with IL-2 and Daudi cells (a Burkitt's lymphoma EBV-transformed cell-line known to be stimulatory for human $\gamma\delta$ T cells) underwent AICD when they were restimulated by TCR cross-linking during the positive MACS selection procedure (21). However, it was noticed that
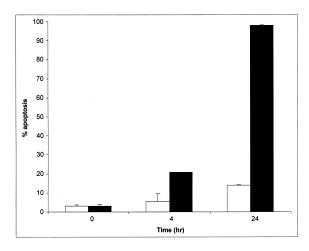
Fig. 1. **Percentage of naïve and activated γδ T cells undergoing apoptosis at various time-points after TCR cross-linking.** Results represent the average of duplicates at each time-point and are representative of at least 3 different experiments. Percentage of apoptotic naïve γδ T cells (white bars ± SE) typically does not exceed 20% while apoptotic activated cells (black bars ± SE) range from 50–98% in these experiments.

naïve γδ T cells isolated from blood and subjected to the same separation procedure without prior *in vitro* culture survived. The percentage of viability for naïve cells was typically around 70–90% while it ranged from 10–40% for activated cells at 24 hours after stimulation. The cells were found to die by apoptosis as determined by annexin V staining (Fig. 1). The percentage of apoptotic cells was below 20% for naïve cells while it ranged from 50–90% for activated γδ T cells 24 hours after stimulation in different experiments. The same phenomenon was observed when naïve or activated cells were cross-linked with the same TCR antibodies, but without passing through the separation column. However, any TCR cross-linking mentioned in this study refers to the γδ TCRs being cross-linked during the MACS procedure.

Cell Cycle Profiles of γδ T Cells after TCR Cross-Linking

The cell cycle profiles of both naïve and activated γδ T cells show that most cells remained in G1 phase of the cell cycle. In the naïve cell population, a portion of the cells entered into G1 4 hours after stimulation and by 24 hours, some cells began to enter the S phase (Fig. 2a), reflecting the asynchronous nature of the culture. In contrast, the activated cells showed a decline of cells in the G1 phase and a corresponding increase in cells exhibiting sub G1 DNA content (Fig. 2b). Very few cells were in the S and G2/M phase throughout the time-points examined. The activated cells were exiting from G1 to the sub G1 phase. The percentage of apoptotic cells as assessed by cell cycle was always lower than that by annexin staining, this could be that the latter method detects cells early in apoptosis, while PI staining of DNA detects cells in late apoptosis and is less sensitive, as incomplete DNA loss resulted in a broader G1 peak (data not shown).
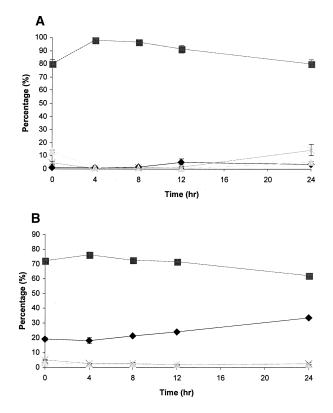

Fig. 2. **Percentages of naïve and activated γδ T cells during various stages of cell cycle at different time-points after TCR cross-linking (A–naïve, B–activated).** Each ■ × △ time-point represents the average of duplicates of cells at G1( ), S ( ), G2/M( ) and sub G1( ) phases of the cell cycle. The graphs are representative of at least 3 separate experiments.

Differential mRNA Expression in Naïve and Activated γδ T Cells

Cytokines, most notably IL-2, have been implicated in driving cells into the cell cycle and therefore inducing AICD (9). We found that there were significant differences in the expression of cytokine messages between naïve and activated cells after TCR cross-linking (Table 1). The presence of IL-2 and IL-4 mRNAs 4 hours after cross-linking in activated γδ T cells was detected whereas they were absent in naïve T cells. IFN-γ was detected only in activated T cells while TNF-α was detected in both activated and naïve T cells. Interestingly, TNF-α mRNA was well expressed in naïve T cells and showed a 4-fold increase during the 24 hour observation period. In contrast, TNF-α mRNA was only detected after a 4-h stimulation in activated T cells (data not shown). In addition to IL-2 and IL-4, IL-7 and IL-15 can also support the growth and development of γδ T cells (22). However, they could not be detected in any of the samples. This was not surprising, as they were not known to be produced by T cells, although IL-15 had been detected in intestinal intraepithelial lymphocytes (11). We have also examined the expression of Fas and FasL. Fas was not detected in naïve T cells

Table 1. mRNA expression of cytokines, Fas, FasL and FLIP of naïve and activated γδ T cells after TCR cross-linking

Time (h)	Fas			FasL			FLIP			IL-2			IL-4			TNFα		IFNγ	
	N	A	CsA	N	A	CsA	N	A	CsA	N	A	CsA	N	A	CsA	N	A	N	A
0	−−	++	++	++	++	++	+−	−−	−−	−−	−−	−−	−−	+−	++	++	−−	−−	++
4	−−	++	++	++	++	++	++	−−	++	−−	++	−−	−−	++	++	++	++	−−	++
8	−−	++	++	+−	++	++	−−	−−	−−	−−	−−	−−	−−	+−	−−	+−	−−	−−	++
12	−−	++	++	+−	++	++	+−	−−	−−	−−	−−	−−	−−	−−	−−	+	−−	−−	−−
24	ND	−	ND	−	ND	+	+	−	+	−	−	−	−	−	−	−	−	ND	−

mRNA expression was examined by RT-PCR for naïve (N), activated (A) and CsA treated γδ T cells at various time-points after stimulation through MACS. The data set for each time-point for both naïve and activated cells represents two sets of experiments from different cultures, where + denotes the presence and − the absence of the mRNA in question. The "+/−" indicates the situation, where one experimental data set showed the presence of the given mRNA, whereas the other set showed its absence. If both experiments were positive or negative, it is denoted as ++ or −−, respectively. ND denotes not determined as several samples at t24 showed degradation of RNA and did not show a consistent signal for β-actin amplification. The rest of the data for t24 is from one experiment.

while it was present at all time-points in activated T cells. In contrast, FasL was detected in both naïve and activated cells, with a stronger expression in activated T cells (Fig. 3). cFLIP was detected in naïve but not activated T cells, suggesting an anti-apoptotic protective role of cFLIP in naïve γδ T cells. In all our RT-PCR analyses, the negative fractions, which had no contaminating γδ T cells as assessed by flow cytometry, were used for comparisons. Contaminating cells in the γδ positive fraction did not contribute to the message detected, as the profile of the negative fractions did not match that of the positive fractions. Furthermore, attempts to amplify the relevant message using the amount of template from the negative fraction corresponding to the number of contaminating cells in each positive fraction sample did not give a detectable signal as that in the positive fraction.

Surface Expression of Fas and FasL

Since Fas and FasL have to be expressed on the surface of cells to exert AICD, we examined their expression on naïve and activated γδ T cells after stimulation. It was found that naïve γδ T cells did not express Fas until 24 hours after stimulation while activated γδ T cells expressed Fas before the

FasL   β-actin

Fig. 3. FasL and β-actin mRNA expression in naïve and activated γδ T cells at various time-points after TCR cross-linking. mRNA was transcribed into cDNA before PCR amplification as described in Materials and Methods.

stimulation and at all time-points examined after stimulation (Fig. 4A). Naïve cells also did not express FasL on the surface while FasL could be seen on activated γδ T cells most clearly at 8 hours after stimulation, although it was also present at low levels at other time-points examined with the possible exception of t0 (Fig. 4B).
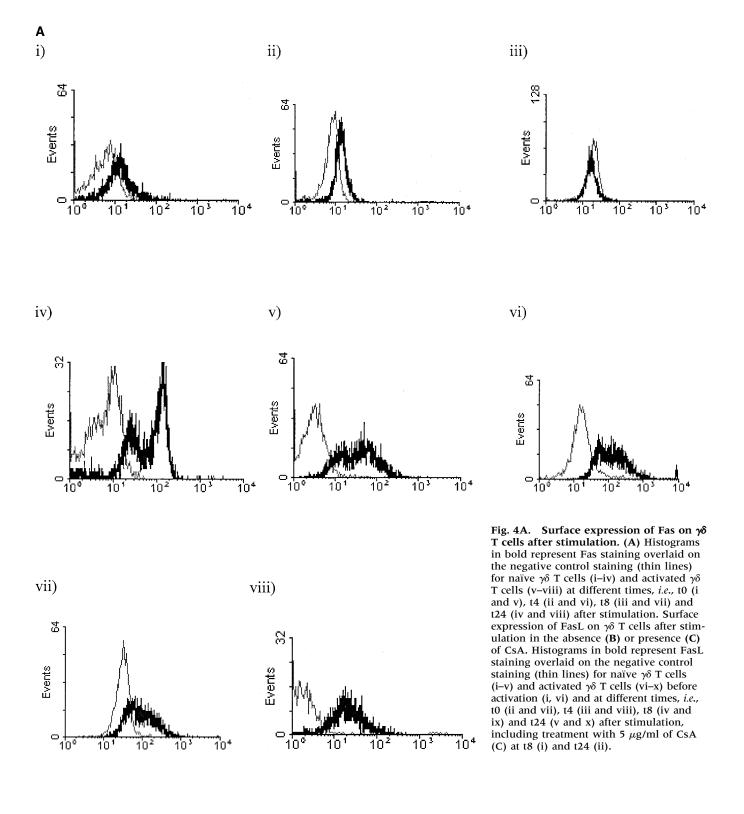
Cyclosporin A Inhibition of AICD

Cyclospo1rin A has been shown to inhibit AICD in other systems through the inhibition of FasL expression (23,24). We found that CsA could significantly inhibit AICD of activated γδ T cells (Table 2). The treatment with CsA blocked IL-2 but not IL-4 expression. Although CsA had no effect on the Fas and FasL mRNA expression in activated γδ T cells, the surface expression of FasL was abrogated with CsA treatment (Fig. 4C). The expression of FLIP was detected at 4 hours for one sample, and 24 hours after stimulation for another (Table 1).

Discussion

It is known that freshly isolated peripheral T cells are resistant to AICD, in contrast to mature activated T cells or hybridomas, but acquire the susceptibility to AICD upon primary activation (9,25). Memory T cells could also be inhibited from undergoing AICD when correctly stimulated by a foreign antigen (26), and human T helper 1 (Th1) lymphocytes are more susceptible to AICD than Th2 lymphocytes (27). These findings showed that regulation of AICD could depend on the cell cycle status of the cells, their cytokine environment, presence of costimulatory molecules and differential expression of apoptosis-inducers or inhibitors (28). In agreement with previous studies with αβ T cells, we found that naïve human γδ T cells isolated from peripheral blood were resistant to AICD

upon TCR cross-linking, in contrast to their activated counterparts. When we compared the cell cycle of naïve and activated γδ T cells upon TCR cross-linking, we found that activated cells mostly remained in G1 phase throughout the 24-h examination period and exited to the sub G1 phase from G1. The experiments were stopped at t24 because the majority of cells had undergone AICD by then. In contrast, naïve cells

began to enter the S phase by 24 hours after stimulation. This is consistent with the finding that naïve T cells had to progress through S phase before acquiring sensitivity to AICD (10). The activated cells, having been *in vitro* culture for 11–12 days, had already undergone several divisions before the restimulation.

Activated cells also expressed IL-2 and IL-4 after stimulation whereas naïve T cells did not in the time
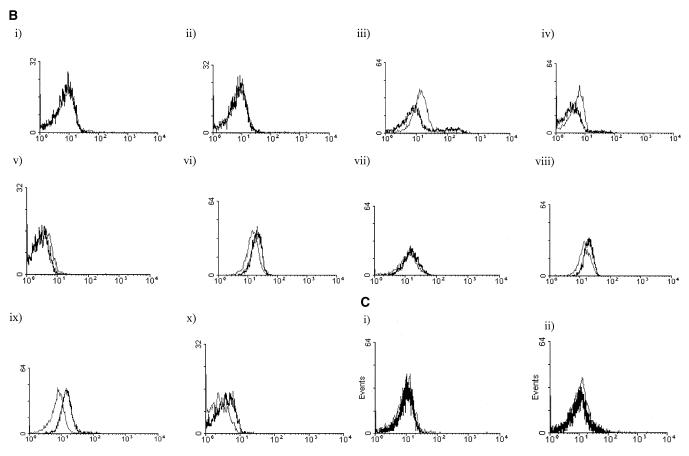
A

Fig. 4A.   Surface expression of Fas on γδ T cells after stimulation. (A) Histograms in bold represent Fas staining overlaid on the negative control staining (thin lines) for naïve γδ T cells (i–iv) and activated γδ T cells (v–viii) at different times, *i.e.*, t0 (i and v), t4 (ii and vi), t8 (iii and vii) and t24 (iv and viii) after stimulation. Surface expression of FasL on γδ T cells after stimulation in the absence (**B**) or presence (**C**) of CsA. Histograms in bold represent FasL staining overlaid on the negative control staining (thin lines) for naïve γδ T cells (i–v) and activated γδ T cells (vi–x) before activation (i, vi) and at different times, *i.e.*, t0 (ii and vii), t4 (iii and viii), t8 (iv and ix) and t24 (v and x) after stimulation, including treatment with 5 μg/ml of CsA (C) at t8 (i) and t24 (ii).

B

Fig. 4B and C   (*Continued*).

period examined. As IL-2 promotes S phase entry and potentiates AICD, its early expression could drive activated cells to death. This is further supported by the fact that CsA inhibited AICD and IL-2 expression in the activated cells. IL-4 expression was not inhibited by CsA, which suggests that IL-4 is not

Table 2.   Effect of CsA on AICD of activated $\gamma\delta$ T cells

	Viability (%)		Apoptosis (%)	
	T0	T24	T0	T24
Experiment 1				
Untreated	96.3	34.2	3.0	69.6
1μg/ml CsA	94.2	60.0	2.5	17.9
Experiment 2				
Untreated	74.8	9.3	ND	96.1
5μg/ml CsA	87.7	57.5	ND	20.7

The percent viability was determined by trypan blue dye exclusion and percentage of apoptotic cells was determined by the annexin V assay as described in Materials and Methods. ND denotes not determined. The table is a composite of two separate experiments, where CsA was used at 1 μg/ml for one and 5 μg/ml for the other.

involved in AICD. There was no difference in the rate and extent of cell death in naïve and activated T cells whether or not IL-2 was present in the culture after the TCR stimulation. This shows that it predisposes cells to AICD before the actual death signal through the TCR and not after the death signal. It is noteworthy that naïve $\gamma\delta$ T cells expressed more TNF-α than activated T cells. Therefore, the possibility of positive contribution of TNF-α to this form of death appears unlikely. In addition, IFN-γ was not detected in naïve cells, indicating that the cytokine is produced later during activation of $\gamma\delta$ T cells.

Naïve T cells also did not express Fas on their surface until 24 hours after stimulation. Although they expressed FasL mRNA, the surface expression of FasL could not be detected. Thus, FasL is most likely preformed and activation leads to surface expression (29). This could account for the lack of AICD in these cells. Furthermore, cFLIP could be detected in naïve cells only, which would further prevent AICD even during later time-points when naïve cells began to express Fas. Since CsA treatment seemed to reverse the expression of cFLIP in activated cells to that of the naïve phenotype, it is possible that this could be at least partially responsible for the protection from AICD afforded by CsA. CsA has been shown to inhibit AICD in other systems by

suppressing FasL induction (23,24) through the presence of a NF-AT binding site in the promoter of FasL (30), and by preventing the down-regulation of cFLIP (29). However, another study showed that CsA inhibited cFLIP expression in memory T cells (26). In our system, we found that the expression of cFLIP in naïve $\gamma\delta$ T cells correlated with survival and CsA treatment in activated cells prevented the down-regulation of cFLIP. Although FasL mRNA was still expressed in the presence of CsA, the surface expression was downregulated, suggesting that calcineurin is involved in the regulation of FasL surface expression (29). Therefore, our results support the model where cell activation leads to the upregulation of Fas, FasL and cFLIP, as in the naïve cells. At this stage, the cells are resistant to AICD. When the cells have progressed through the S phase of the cell cycle and IL-2 production begins, restimulation results in AICD as cFLIP is downregulated. The regulation of cFLIP is linked to the S phase progression (31), and consequently to the production of IL-2. As $\gamma\delta$ T cells have been found to be activated and expanded in numbers in various microbial infections, this mechanism of AICD in $\gamma\delta$ T cells may be crucial for maintaining $\gamma\delta$ T cell homeostasis under these circumstances. The ligands recognized by $\gamma\delta$ T cells are common in both microbes and man and are likely to be released when cells die during inflammation (15, 16). Consequently, it may be important to downregulate the $\gamma\delta$ T-cell response when the offending pathogen has been removed since chronic activation of the cells may result in autoimmunity. Understanding the mechanism of AICD also creates opportunities for possible therapeutic interventions in some retroviral and mycobacterial infections (12,14,32–35), where preventing the premature deletion or increased susceptibility of $\gamma\delta$ T cells to apoptosis may be desirable.

Acknowledgments

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