Characterization of Apoptosis-resistant Antigen-specific T Cells In Vivo

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Summary

Clonal deletion via activation-induced apoptosis (AIA) of antigen-specific T cells (ASTC) plays a very important role in the induction of peripheral tolerance. However, none of the studies performed so far has shown a complete deletion of ASTC, a small population always persisting in the periphery. The mechanism by which this small population of ASTC escapes AIA has not been determined. Since the existence of these ASTC may influence the outcome of autoimmune diseases and long-term graft survival, we have characterized the properties of these residual ASTC in vivo with the objective of determining mechanisms that may contribute to their persistence. It was found that the resistance of the residual ASTC to AIA is not due to lack of activation or Fas/Fas-L expression. Compared to those susceptible to AIA, the residual ASTC express a high level of Th2-type cytokines that may help them to escape from AIA. Furthermore, they are able to suppress proliferation of other ASTC, suggesting they may, in fact, prolong tolerance in vivo.

Recent studies have demonstrated that the in vivo encounter with antigen following injection of bacterial superantigens (1–3) or allogenic cells (4) into normal mice or injection (5–9) or feeding (10) of transgenic mice with specific antigens leads to a deletion of the majority of antigen-specific T cells (ASTC) in the periphery by a process termed activation-induced apoptosis (AIA). However, the deletion has never been complete. There is always a small percentage (∼10–30% of total) of ASTC that are able to escape from AIA and persist in the periphery of the host. The persistence of this small population of ASTC does not seem to depend on the type of antigen used (SEB, Mls, MHC, or HY), route of antigen administration (oral, i.p., or i.v.) or the dosage of antigen used (1–9). A similar phenomenon has also been observed after in vitro induction of apoptosis in mature peripheral T cells and T cell hybridomas (11–18).

Why do T cells that possess the same Ag specificity and phenotype (CD4 or CD8) undergo a different fate after encountering antigen? What are the important factors in determining whether or not a T cell dies? To date, studies on peripheral tolerance have focused only on the majority of dying cells. No systematic studies have been done on the residual cells to reveal the mechanism why these cells are capable of escaping AIA, and their function, if any, in maintaining tolerance in vivo is not clear. In this paper we study the molecular, cellular, and functional characteristics of the apoptosis-resistant ASTC in vivo to delineate possible mechanisms by which some ASTC can escape from clonal deletion whereas others undergo apoptosis. We found that there was no significant difference between apoptosis-resistant and -sensitive ASTC in terms of activation or Fas/Fas-L expression. However, there was a difference in their cytokine expression: apoptosis-resistant ASTC expressed significantly higher levels of IL-4 and IL-10 than apoptosis-sensitive ASTC. Functional studies revealed that the apoptosis-resistant ASTC have become unresponsive to further Ag stimulation, in vivo and in vitro, and that they can suppress the activation of other ASTC.

Materials and Methods

Mice and Adoptive Transfer. C57BL/6 (B6), (B6xBALB/c)F1 (BYJ F1) and BALB/c H-2-dm2 (dm2) mice were purchased from Jackson Laboratories (Bar Harbor, ME). Breeding stock of 2C transgenic mice were kindly provided by Dr. Dennis Y. Loh (Howard Hughes Medical Institute, St. Louis). A large fraction of T cells in the periphery of the 2C mouse (H-2b/b) express a Tg TCR, reactive against the L3 class I MHC antigen. These T cells can be detected by a clonotypic mAb 1B2 and are predominantly CD8+ (19, 20). 2C transgenic mice were first backcrossed onto B6 mice for 6–8 generations to obtain the transgene on B6 (H-2b/b).
background and then bred with dm2 mice (a BALB/c Ld loss mutant) to obtain 2Cfl mice (H-2k/d, Ld, 1B2+) and used as lymphocyte donors. CB-17 std (effectively BALB/c congeneric to B6 at the IgH locus, H-2k/d) and B6 std (H-2k/d) were bred into the animal colony at the National Cancer Institute. $sdf_1$ mice (H-2k/d, Ld+) were obtained by breeding CB-17 std mice with B6 std mice and used as recipients. Viable lymphocyte suspensions were prepared from spleen and pooled axillary, inguinal, and mesenteric LN of 2Cfl mice and injected i.v. into $sdf_1$ mice (3–4 × 10^7 cells/mouse).

**Cell Surface Marker Staining.** To follow the fate of adoptively transferred ASTC in vivo, lymphocytes from LN and spleen of recipient mice were collected and analyzed at different times after injection. Lymphocytes were stained with biotinylated 1B2 mAb (which recognize the transgenic TCR; hybridoma kindly provided by Dr. Herman Eisen, MIT) and FITC-conjugated anti-CD8 (Pharmingen, San Diego, CA) followed by staining with streptavidin red 670 (GIBCO BRL, Gaithersburg). Data were acquired and analyzed using FACSscan Lysis II software (Becton Dickinson, Mountain View, CA).

**Staining of Antigen-specific Apoptotic Cells.** To detect apoptotic antigen specific T cells in vivo, we have developed a technique which combines cell surface marker staining with an in situ nick translation assay, as described elsewhere (21). Briefly, following surface marker staining, cells were fixed, permeabilized and then incubated with Pol I and biotin-conjugated-nucleotide. Cells containing DNA breaks were then visualized by the fluorescent detection of the newly synthesized biotin-containing DNA. Fluorescence is limited to the nucleus of the cell. Cell surface staining using FITC and Red670-labeled monoclonal antibodies before fixation for nick translation makes it possible to define which cell subsets are undergoing apoptosis in the whole population.

**Cell Sorting and Cytotoxicity Assay.** 4 wk after transfer, lymphocytes from the spleen of $sdf_1$ mice were stained with mAbs 1B2 and CD8 as described above. 1B2+CD8+ cells were sorted by using a cell sorter (Coulter Epics, Hialeah, FL), and the sorted cells were used as responder cells in a cytotoxicity assay. Varying numbers of responder cells (from 10^5 to 10^7/well) were cocultured with 1B2+CD8+ cells from B6-scid mice in the presence or absence of 25 U/ml IL-2. After 5 d of culture, percentage lysis of specific ASTC was determined by ¹¹¹In release assay.

**Purification of 1B2+CD8+ Cells by Magnetic Beads.** After lysing red blood cells, lymphocytes were incubated with anti-mouse IgG(H+L)-coated magnetic beads (Advanced Magnetic, Inc., Cambridge, MA). The supematant, containing less than 2% B cells, was harvested after placing the cell suspension in a magnetic field. The bead-binding cells were more than 95% pure 1B2+CD8+ cells and will be held by the magnetic field. The bead-binding cells were more than 95% pure 1B2+CD8+ cells and more than 98% viable.

**Determination of mRNA Levels of Cytokines and Fas/Fas-L by RT-PCR.** Total RNA was extracted from purified 1B2+CD8+ cells (5 × 10^6) with TriZol reagent (GIBCO BRL). cDNA was synthesized from RNA with 50 pmol oligo(dT)18 and 200 units of murine MLV reverse transcriptase (GIBCO BRL); 2 μl of the cDNA mixture was used in a PCR reaction with 10 pmol of forward and reverse primers and 2.5 U of Taq DNA polymerase (Perkin-Elmer Cetus, Norwalk, CT). The sequences of the specific sense and anti-sense oligonucleotide primer pairs, 5' and 3', were as follows (22–27): IL-2, 5'-ATGACACGTGACGCTGCACTGCTGAC-3' and 5'-CCGTTGGGCAATGCTCAG-3'; IFN-γ, 5'-ATCGTGGCGCTCCAGAATTCCACTGGACAATGCACTG-3' and 5'-CATGGTTGTTGAGATGATGCTGAGAC-3'; IL-4, 5'-ATGTTGCTTCACTGCCCAAGGAGGCAATCCTGGAGAC-3'; IL-10, 5'-ATGCGTCCCTGCTGGCAATCCTGAC-3'; and 5'-CTCAAACTGCTAATGCTAGGTTTGTGAATGAC-3'. Samples were amplified through 30 cycles at an annealing temperature of 58°C in a PCR Thermal Cycler (Perkin-Elmer Cetus). The products were separated on agarose gel by electrophoresis and stained with ethidium bromide. The specificity of the reactions was confirmed by direct sequencing of the PCR products with a cyclic sequencing kit (Stratagene Inc., LaJolla, CA). Semiquantitative analysis of the mRNA levels of IFN-γ, IL-2, IL-4, IL-10, Fas, Fas-L, and β-actin was performed by RT-PCR at varying time points.

**Results**

**Establishment of An Adoptive Transfer System.** Since in most previous studies antigens were introduced into adult mice that possessed a large number of ASTC (1–3, 6–10), it is possible that some ASTC persisted simply because they did not have a chance to contact the antigen due to a limited quantity and/or half-life of the antigen. To exclude this possibility, we have established an adoptive transfer system. B6C2 mice were bred with dm2 mice (a BALB/c Ld loss mutant). The subsequent 2Cfl mice, which were H-2k/d, Ld, 1B2+, were used as lymphocyte donors. A limited number, 3–4 millions, of viable 1B2+CD8+ cells from 2Cfl mice were intravenously injected into a $sdf_1$ mouse (H-2k/d, Ld, obtained by breeding C.B-17 std with B6 std mice). A group of B6-scid mice (H-2k/d, Ld) were injected with the same number of 1B2+CD8+ cells from B6C2 mice (H-2k/d) transgenic mice as negative controls. In this system the only immunological response was that of 1B2+CD8+ cells of donor origin to the Ld antigen expressed on the recipients, since scid mice exhibit defective recombination of antigen receptor genes leading to an early arrest in T and B lymphocyte development, allowing for survival and growth of transplanted foreign cells. In addition, constant expression of the Ld Ag on all the nucleated cells in scid mice guaranteed the persistence of a high dose of Ag and greatly increased the chance for 1B2+CD8+ cells to encounter the Ld antigen.

**Activation, Proliferation and Apoptosis of the Majority and Persistence of the Minority of ASTC after Encountering Ag In Vivo.** The fate of 1B2+CD8+ cells was followed in recipient scid mice in vivo after adoptive transfer. The total number of 1B2+CD8+ cells started to increase in both LN and spleen 3 d after transfer into scid mice and reached a peak by 5 d. 1 wk after transfer, the total number of 1B2+CD8+ cells started to decline in the periphery. By 2 wk, the majority (less than 10% of peak number) of 1B2+CD8+...
cells had disappeared from the periphery. However, the disappearance of ASTC was not complete. There were ~20–30% of ASTC persistent in the periphery for at least 2 wk after injection (Fig. 1, top). No significant change in either the total numbers of 1B2⁺CD8⁻ (Fig. 1) or 1B2⁻CD8⁻ cells in the same animal or of 1B2⁺CD8⁺ cells in the B6 scid control mice could be observed (not shown).

To see whether the loss of 1B2⁺CD8⁺ cells in scidF1 mice was due to death by AIA in vivo after encountering antigen, we developed a technique in which staining of cell surface markers is combined with the detection of nuclear DNA strand breaks. This allows one to detect apoptosis in a defined lymphocyte population in vivo at the single cell level (21). Using this technique, we found that before encountering antigen, there was a low number of apoptotic cells in each subset, which may represent either a normal rate of cell death in the 2C mice or background staining. 3 d after encountering antigen in vivo, marked apoptosis was detected in 1B2⁺CD8⁺ cells but not in 1B2⁺CD8⁻ or 1B2⁻CD8⁻ cells. 2 wk after injection, very few apoptotic cells could be detected. (Fig. 1 A, bottom). This finding was confirmed by staining cells before and 5 d after injection with Acridine Orange. As shown in Fig. 1 B, before encountering antigen, no apoptotic cells could be seen (Fig. 1 a). However, 5 d after injection, a high proportion of cells displayed nuclear chromatin condensation and fragmentation (Fig. 1 b). These results provide direct evidence that after encountering antigen in vivo, there is a transient expansion followed by AIA of the majority, and persistence of the minority of ASTC in the periphery. As the scidF1 mice are of BALB/c background, they constitutively express the Ld antigen on all nucleated cells, therefore make it unlikely that some ASTC survived due to failure to encounter antigen.

Persistence of Some ASTC Is Not Due to Lack of Antigen-presenting Cells. Another possibility could be that some ASTC survive AIA because the activated 1B2⁺CD8⁺ cells

![Figure 1](image-url)
killed all the antigen presenting cells in the recipient mice so that the rest of the 1B2+CD8+ cells could not be activated and deleted. If this were the case, splenocytes from scidF1 mice that received transgenic cells previously would not act as good stimulators in an MLR. To test this possibility, LNC from 2CF1 mice were used as responders and stimulated by irradiated splenocytes from scidF1 mice that had been injected with 1B2+CD8+ cells 2 wk earlier, or from naive scid mice as control. As shown in Fig. 2, cells from both injected and naive scidF1 mice were equally effective in stimulating the generation of cytotoxic T cells. These experiments demonstrate that the fact that some ASTC survive AIA is not due to lack of antigen presenting cells in the recipient and suggest that residual cells themselves may possess different cellular or molecular characters from those susceptible to AIA.

Persistence of Some ASTC Is Not Due to Lack of Activation. Since it has been reported that not all ASTC can be activated after encountering antigen in vivo (28), it is possible that some ASTC escape AIA not because these cells are more resistant to apoptosis but because they are unable to be activated due to lack of receptors for co-stimulators. Accordingly, we examined residual ASTC for a set of T cell activation markers. 15 d after receiving 2CF1 cells, lymphocytes were collected from scidF1 mice and triple stained with mAbs specific for 1B2 and CD8 in combination with one of the following T cell activation markers: pgp-1, MEL 14, or Fas. The expression levels of those activation markers on the residual 1B2+CD8+ cells were compared to naive 1B2+CD8+ cells (Fig. 3). The results are summarized in Table 1. There is a clearly increased expression of pgp-1 and decreased expression of MEL 14 on the residual 1B2+CD8+ cells compared to that on the naive 1B2+CD8+ cells. After activation, the percentage of Fas+ cells was also increased (not shown). This increase was more marked for the percentage of Fas^{high}+ cells. These data strongly suggest that the residual 1B2+CD8+ cells have in fact been activated after encountering the Ld antigen in vivo.

Persistence of Some ASTC Is Not Due to Lack of Fas/Fas-L Expression. Recently, many studies have shown that interaction between Fas and Fas-ligand (Fas-L) plays an important role in AIA in mature T lymphocyte both in vitro (16–18) and in vivo (25, 26, 29–31). We therefore asked whether some ASTC survived AIA because of a lack of or downregulation of Fas and/or Fas-L expression. To test this, 1B2+CD8+ cells were first purified from scidF1 mice on day 0, 5, and 21 after injection and their expression of Fas and Fas-L mRNA was compared using RT-PCR. As shown in Fig. 4, lanes 2–4, naive 1B2+CD8+ cells express a low level of Fas mRNA, but no detectable Fas-L mRNA. 5 d after encountering antigen in vivo, both Fas and Fas-L expression were significantly increased (lanes 5–7), which may account for the marked apoptosis detected in ASTC at this time. Interestingly, at 3 wk after injection, the levels of expression of both Fas and Fas-L mRNA were still high in the remaining resting 1B2+CD8+ cells, although a little less than at 5 d after injection (lane 8–10).

Expression of Fas on the 1B2+CD8+ cells at different times after injection was also analyzed by flow cytometry.

![Figure 2](image2.png)

**Figure 2.** Persistence of some ASTC is not due to lack of antigen-presenting cells. Splenocytes from 2CF1 mice were stimulated by irradiated (20Gy) splenocytes from scidF1 mice either un.injected or injected with 2CF1 cells 2 wk previously. Percentage lysis of specific target cells was measured by ^51^Cr release assay after 5 d culture. Data are plotted as percentage specific killing vs number of 1B2+CD8+ cells/well. Each data point represents five replicates.

![Figure 3](image3.png)

**Figure 3.** Expression of activation markers on 1B2+CD8+ cells. Lymphocytes from scidF1 were stained with 1B2 and CD8 followed by staining one of the activation markers. Pgp-1, MEL-14, and Fas^{high}+ histograms are of gated 1B2+CD8+ cells. Lymphocytes from day 0 (grey areas) are compared to that 15 d after injection of 2CF1 cells (areas within black lines).
Table 1. Percentage Expression of Activation Markers on 1B2+ CD8+ Cells

| Activation markers | LN Day 0 | LN Day 15 | Spleen Day 0 | Spleen Day 15 |
|--------------------|---------|----------|-------------|--------------|
| Pgpl+              | 30.5 ± 3.3 | 88.7 ± 0.7 | 20.7 ± 3.9 | 85.6 ± 2.1   |
| MEL 14+            | 28.6 ± 1.0 | 12.0 ± 0.7 | 26.2 ± 4.2 | 16.5 ± 0.2   |
| Fas high+          | 7.3 ± 1.0 | 28.8 ± 4.5 | 7.7 ± 0.5  | 15.4 ± 3.6   |

and the correlation between the percentage of Fas high+ cells and the percentage of apoptotic cells was studied. As shown in Fig. 5, the percentage of Fas high+ cells increased about fourfold on day 5, fivefold on day 10 and fivefold on day 15 in LNC compared to what was found on day 0. There was no correlation between the percentages of Fas high+ cells and the percentage of apoptotic cells (Fig. 5). These data strongly argue against the possibility that some ASTC survived AIA due to a lack of Fas (Fig. 5) or Fas-L (Fig. 4) expression and indicate that to die or not to die is not solely dependent on Fas/Fas-L expression, suggesting that additional regulatory factors are involved in the survival of ASTC after tolerance induction.

Apoptosis-resistant ASTC Express High Levels of Th2-like Cytokines. Next, we examined the pattern of cytokine expression in apoptosis-resistant ASTC. Purified 1B2+CD8+ cells from scidF1 mice on day 0, 5, and 21 after injection of 2CF1 cells were obtained and total RNA was extracted. The expression levels of mRNA for IFN-γ, IL-2, IL-4, and IL-10 were determined by a semiquantitative RT-PCR (Fig. 4). It was found that, except for IFN-γ, naive ASTC did not express detectable cytokine mRNA. 5 d after encountering Ag in vivo, significant levels of mRNA for IFN-γ and IL-2 were detected but there was no mRNA for IL-4 or IL-10, i.e., the cells exhibited a Th1-like pattern of cytokine expression (32,33). In contrast, the surviving 1B2+CD8+ cells expressed high levels of both IL-4 and IL-10 mRNA, i.e., the cells exhibited a Th2-like pattern of cytokine expression (32,33). These results indicate that CD8+ cells can produce Th1- or Th2-like cytokines, and suggest that ASTC which express Th2-like cytokines are more resistant to AIA than those that express Th1-like cytokines.

Unresponsiveness of the Apoptosis-resistant ASTC to Ag Restimulation In Vitro and In Vivo. To characterize the biological function of apoptosis-resistant ASTC, we first examined their ability to generate cytotoxic T cells in vitro. Purified residual 1B2+CD8+ cells from scidF1 mice 1 wk after injection were cultured with irradiated (B6xBALB/c)F1 spleenocytes in the presence of exogenous rIL-2 and compared to the response of naive 1B2+CD8+ cells. The latter could kill Lα+ target cells whereas the former did not show any cytotoxicity to Lα+ cells even in the presence of exogenous IL-2 (Fig. 6). When the unsorted remaining 1B2+CD8+ cells from scidF1 mice 4 wk after injection were tested in the same way, similar results were found (not shown). These data indicate that after AIA, the surviving cells can not kill antigen specific targets in vitro even in the presence of exogenous IL-2.

The functional status of the residual cells was also examined in vivo by secondary transfer of the residual ASTC into naive scidF1 mice 4 d after receiving lymphocytes from

![Figure 4](https://example.com/figure4.png)

*Figure 4.* Residual ASTC express Fas/Fas-L as well as IL-4 and IL-10. Total RNA was extracted from purified 1B2+CD8+ cells (5 × 10⁶) on days 0, 5, and 21. Semiquantitative analysis of the mRNA levels of IFN-γ, IL-2, IL-4, IL-10, Fas, Fas-L, and β-actin was performed by RT-PCR at varying time points: lanes 2–4, day 0; lanes 5–7, day 5; lanes 8–10, day 21; lane 1, molecular markers. Each time point contained samples obtained from three different mice.

![Figure 5](https://example.com/figure5.png)

*Figure 5.* No correlation between % Fas high+ and % nick cells after encounter with Ag in vivo. Lymphocytes were collected from scidF1 mice on days 0, 5, 10, and 15 after transfer and stained for Fas with PE-conjugated anti-Fas mAb or for DNA nicks as described in Fig. 1 B. Percentages of Fas high+ and nick+ cells in LN were plotted. Each time point contains data from 2–5 mice. Similar results were obtained in spleen (not shown).
Figure 6. Residual ASTC did not kill target cells bearing Ld Ag in the presence of exogenous IL-2. 1 wk after transfer, residual 1B2+CD8+ cells (Exp) were purified by cell sorting from scidF1 mice, and naive 1B2+CD8+ cells were also purified as controls. Varying numbers of purified 1B2+CD8+ cells were cocultured with irradiated (20 Gy) (BALB/cxB6)F1 splenocytes (H-2b/a, Ld*) in α-MEM supplemented with 10% FCS in the presence of 25 U rIL-2. Percentage lysis of specific target cells was measured by 51Cr release assay after 5 d culture. Data are plotted as percentage specific killing vs number of 1B2+CD8+ cells/well. Each data point represents five replicates.

2CF1 mice. As controls, age- and sex-matched scidF1 mice were injected with the same number of naive lymphocytes from 2CF1 mice. 4 d after transfer, LNC and splenocytes from scidF1 mice were stained with mAbs against 1B2 and CD8. Unlike in the control scidF1 mice that received naive 1B2+CD8+ cells, LNC and splenocytes in the scidF1 mice that received residual 1B2+CD8+ cells did not show blast transformation, and the percentage of 1B2+CD8+ cells was significantly lower than that in the control mice (Fig. 7 A). Similar experiments were performed 18 d after receiving lymphocytes from 2CF1 mice and 3 d after secondary transfer, and the same results were obtained (Fig. 7 B). Both experiments showed that the residual 1B2+CD8+ cells proliferated little, if at all, after being transferred into naive scidF1 mice that provided new source of Ag and APC. These results not only support the notion that persistence of some of ASTC after AIA is not due to lack of antigen/antigen presenting cells, but also argues against the residual 1B2+CD8+ cells being traditional memory cells (34).

Apoptosis-resistant ASTC Possess Suppressive Function In Vivo. To see whether the residual ASTC have any immunoregulatory role in vivo, scidF1 mice were injected with 3 × 107 viable lymphocytes from 2CF1 mice. 15 d later, these scidF1 mice were given a second injection of the same number of viable naive lymphocytes from 2CF1 mice. 5 d after the second injection, the total numbers of 1B2+CD8+ cells in LN and spleen were compared to those of scidF1 which received only one injection 5 d earlier (Fig. 8). When the remaining ASTC were mixed with naive 1B2+CD8+ cells (at 1:1 ratio) and injected into naive scidF1 mice, the total number of 1B2+CD8+ cells in spleen and LN 5 d after injection was found to be significantly lower than in those mice that received the same number of naive 1B2+CD8+ cells alone (not shown). These data suggest that proliferation of newly injected 1B2+CD8+ cells in vivo has been suppressed by the residual Th2-like ASTC.

Discussion

There are two published studies using adoptive transfer systems to study the fate of ASTC in vivo (5, 7). Rocha and von Boehmer (5) transferred anti-male HY transgenic T cells into male nude mice that carry male HY Ag. Kear-
Figure 8. Inhibition of ASTC response to L α antigen in vivo by residual cells. ScidF1 mice were injected with 3 × 10⁷ 2C1 cells. 15 d after injection, some of them were left for another 5 d without the second injection (grey bar); some received a second injection of 3 × 10⁷ 2C cells (hatched bar). At the time, another group of naive scidF1 mice were injected with the same number of cells from the same donors as controls (clear bar). 4 d later, all mice were killed and the total number of 1B2+CD8 + and 1B2+CD8 - cells in LN were analyzed by flow cytometry.

ney et al. (7) transferred limiting number of anti-chicken ovalalbumin peptide TCR transgenic cells into normal syngenic BALB/c mice and then exposed the recipients to the peptide Ag in vivo. In both systems, it was observed that ASTC initially proliferated extensively after encounter with Ag. Most of the ASTC then rapidly disappeared from the periphery, leaving behind a population that was hypo-responsive to antigenic restimulation. We have confirmed these findings in a different adoptive transfer system and have extended their studies by further characterization of the remaining ASTC in terms of their activation markers, Fas/Fas-L expression, cytokine profiles, and their effect on naive ASTC in vivo.

We have demonstrated that the small population of ASTC that was able to escape from AIA did not do so due to a lack of activation or lack of Fas/Fas-L expression on these cells. Our finding that high levels of Fas and Fas-L expression could be observed both at an early stage after activation and on the residual ASTC at later times suggests that expression of Fas/Fas-L may be important in the induction of some but not all ASTC death in our system. This result is consistent with findings that peripheral T cell deletion takes place in lpr mice, and autoimmune syndromes occur in mouse strains without Fas or Fas-L defects (35, 36). Recently, Zheng et al. (35) found that Fas/Fas-L interaction alone accounted for almost all CD4+ T cell apoptosis, whereas tumor necrosis factor caused most CD8+ T cell death in vitro. Since in our system the ASTC are CD8+ cells, it is possible that the remaining 1B2+CD8+ cells may use a different pathway (i.e., tumor necrosis factor) for autoregulatory apoptosis.

It has been reported that cytokines are involved in regulation of apoptosis in both T and B lymphocytes in vitro (37–41). For instance, interferon γ (IFN-γ) has been shown to promote cell death induced by anti-TCR mAbs in the absence of costimulatory cells (37). IL-10 could prevent apoptosis in EBV infected human T cells in vitro (38, 39). So far, however, no in vivo study has shown a correlation between cytokine production pattern and apoptosis. The fact that high levels of IL-4 and IL-10 could only be observed on the residual AIA-resistant ASTC, but not on the naive or early activated ASTC suggests that Th2-like CD8+ cells are more resistant to AIA than Th1-like CD8+ cells. Currently, it is not clear what determines that a T cell to expresses high levels of IL-4/IL-10. It is possible that high levels of expression of mRNA of IL-4 and IL-10 is the consequence of selective deletion of Th1-like cells or of conversion of Th0 or Th1-like cells into Th2-like cells. As regards to what role IL-4/IL-10 could play in AIA in vivo, it is possible that Th2-like cytokines might serve as "salvation signals." After activation and expression of Fas/Fas-L, a death signal may be transduced if no further salvation signal is provided. However, if a salvation signal (IL-10/IL-4?) is supplied, it may block the death signal and help T cells to escape from AIA. Further studies such as blocking IL-4/IL-10 by anti-IL-4/IL-10 mAbs would help in delineating the role of Th2-like cytokines in preventing apoptosis.

Another interesting finding in this study is that the residual 1B2+CD8+ cells could inhibit proliferation of naive 1B2+CD8+ cells in vivo, which suggests that Th2-like CD8+ cells may function as suppressor cells. Our preliminary studies have indicated an enhanced (over 100 d) Lα+ skin graft survival in the presence of the residual 1B2+ CD8+ cells. Further studies should delineate the underlying mechanism of this suppression. Taken together, these studies not only open a new window for our understanding of the mechanisms involved in the induction of peripheral tolerance, but may have potential therapeutic applications in both autoimmune diseases and long-term graft rejection.

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