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)[1] 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). Breading 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-$2^{b/b}$) express a Tg TCR reactive against the L^d 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-$2^{b/b}$)

[1]*Abbreviations used in this paper:* AIA, activation-induced apoptosis; ASTC, antigen-specific T cells; dm2, BALB/c H-2-dm2.

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background and then bred with dm2 mice (a BALB/c L^d loss mutant) to obtain $2C_{F1}$ mice (H-$2^{b/d}$, L^{d-}, $1B2^+$) and used as lymphocyte donors. C.B-17 *scid* (effectively BALB/c congenic to B6 at the IgH locus, H-$2^{d/d}$) and B6 *scid* (H-$2^{b/b}$) mice were bred in the animal colony at the Ontario Cancer Institute. $scid_{F1}$ mice (H-$2^{b/d}$, L^{d+}) were obtained by breeding C.B-17 *scid* mice with B6 *scid* mice and used as recipients. Viable lymphocyte suspensions were prepared from spleen and pooled axillary, inguinal, and mesenteric LN of $2C_{F1}$ mice and injected i.v. into $scid_{F1}$ 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 FACScan® 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 $scid_{F1}$ 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 10–10^5/well) were cocultured with irradiated (20 Gy) F1 (BALB/cxB6) splenocytes (H-$2^{b/d}$, L^{d+}) in α-MEM supplemented with 10% FCS in the presence or absence of 25 U/ml rIL-2. After 5 d of culture, percentage lysis of specific target cells was measured by ^{51}Cr 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 supernatant, containing less than 2% B cells, was harvested after placing the cell suspension in a magnetic field. Since almost all $CD8^+$ cells are $1B2^+$, to isolate $1B2^+CD8^+$ cells, the B-depleted cells were incubated with an anti-CD8 biotinylated antibody followed by incubation with streptavidin coated magnetic beads (Dynal A.S.; Oslo, Norway). Almost all the $CD8^+$ cells (>99%) can bind to the beads 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 prepared 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′-ATGTACAGCATGCAGCTC-GCATCCTGTGTC-3′ and 5′-CTGCTTGGGCAAGTAAAA-TTTGAAGGTGAGC-3′; IL-4, 5′-ATGGGTCTCAACCCC-CAGCTAGTTGTC-3′ and 5′-CTTCGTTGCTGTGAGGAC-GTTTGGCAC-3′; IL-10, 5′-CCCAGAAATCAAGGAGCA-TTTG-3′; and 5′-CATATGCTTCTATGCAGTTG-3′; IFN-γ, 5′-CTTCTTGGATATCTGGAGGAACTGGCAAAA-3′ and 5′-CTCAAACTTGGCAATACTCATGAATGCATC-3′; FAS, 5′-ATCCGAGCTCTGAGGAGGCGGGTTCATGAAAC-3′ and 5′-GGAGGTTCTAGATTCAGGGTCATCCTG-3′; FAS-L, 5′-CAGCTCTTCCACCTGCAGAAGG-3′ and 5′-AGATTC-CTCAAAATTGATCAGAGAGAG-3′. β-actin, 5′-ATGGAT-GACGATATCGCT-3′ and 5′-ATGAGGTAGTCTGTCAGGT-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

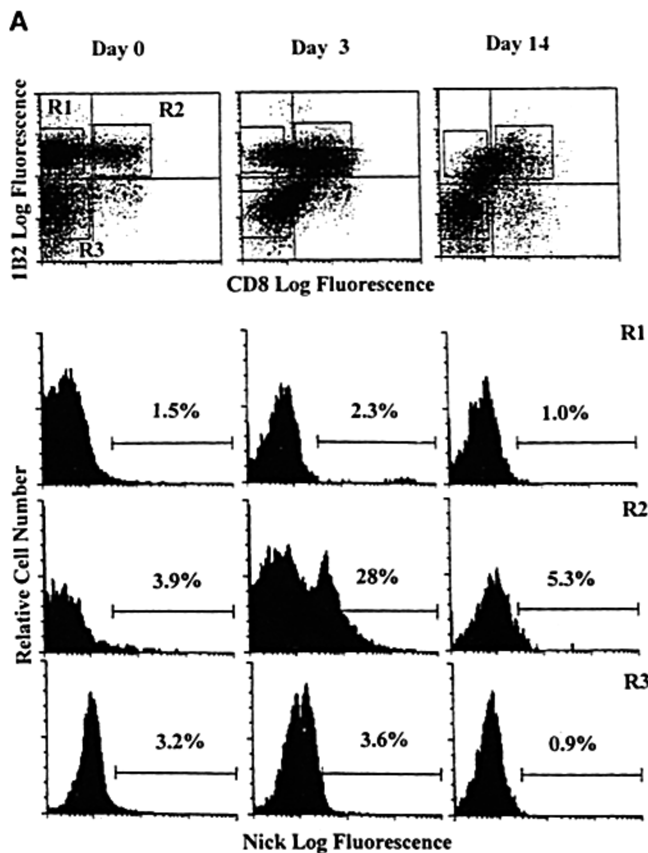
Establishment of An Adoptive Transfer System. Since in most previous studies antigens were introduced into adult mice that possessed a large number of ASTC (1–3, 6–10), it is possible that some ASTC persisted simply because they did not have a chance to contact the antigen due to a limited quantity and/or half-life of the antigen. To exclude this possibility, we have established an adoptive transfer system. B62C mice were bred with dm2 mice (a BALB/c L^d loss mutant). The subsequent $2C_{F1}$ mice, which were H-$2^{b/d}$, L^{d-}, $1B2^+$, were used as lymphocyte donors. A limited number, 3–4 millions, of viable $1B2^+CD8^+$ cells from $2C_{F1}$ mice were intravenously injected into a $scid_{F1}$ mouse (H-$2^{b/d}$, L^{d+}, obtained by breeding C.B-17 *scid* with B6 *scid* mice). A group of B6-*scid* mice (H-$2^{b/b}$, L^{d-}) were injected with the same number of $1B2^+CD8^+$ cells from B62C (H-$2^{b/b}$) transgenic mice as negative controls. In this system the only immunological response was that of $1B2^+CD8^+$ cells of donor origin to the L^d 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 L^d Ag on all the nucleated cells in $scid_{F1}$ guaranteed the persistence of a high dose of Ag and greatly increased the chance for $1B2^+CD8^+$ cells to encounter the L^d 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 $scid_{F1}$ 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 $scid_{F1}$ 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 *A*, *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 $scid_{F1}$ 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 $scid_{F1}$ mice are of BALB/c background, they constitutively express the L^d 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



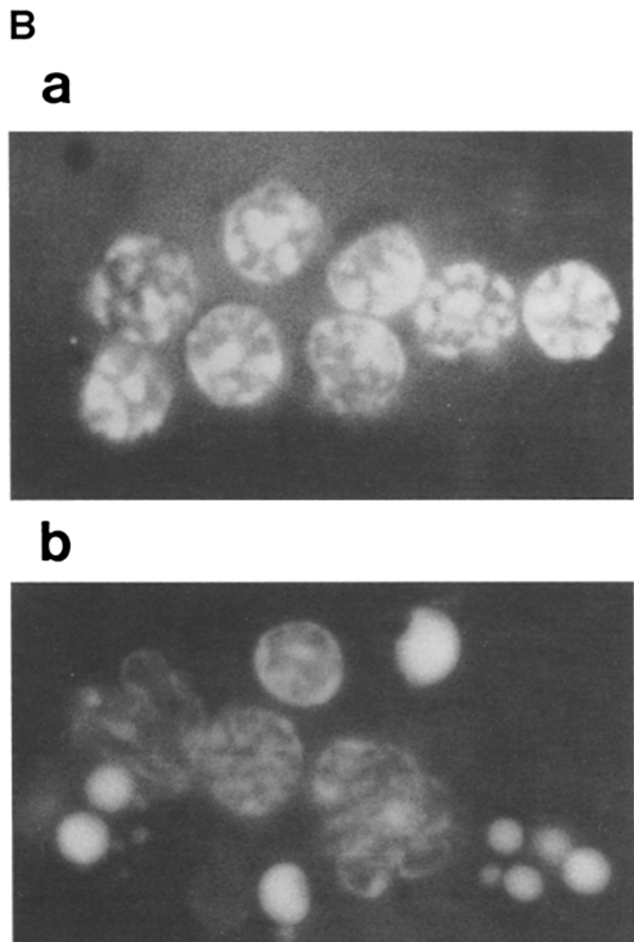


Figure 1. Activation induced apoptosis in the majority, and persistence of a small population of ASTC after encountering antigen in vivo. (*A*) $scid_{F1}$ mice were injected with 3×10^7 viable lymphocytes containing 12–15% $1B2^+CD8^+$ cells from $2C_{F1}$ mice. LN and spleen from $scid_{F1}$ mice were collected on days 0, 3, and 14 after injection and lymphocytes were stained for the transgenic TCR, CD8, and DNA nicks as described in Materials and Methods. Data were acquired and analyzed on a FACScan® using Lysis II software. The dot plots of anti-CD8 vs 1B2 staining can be divided into three regions as indicated in the top panel: region 1 (R1), $1B2^+CD8^-$ cells, region 2 (R2), $1B2^+CD8^+$ cells and region 3 (R3), $1B2^-CD8^-$ cells. Each of these regions was separately analyzed for the presence of DNA nicks (*bottom*) where percentages of apoptotic cells in each region are indicated. (*B*) Acridine Orange staining of lymphocytes from $scid_{F1}$ mice before (*a*) and 5 d after injection (*b*).

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 $scid_{F1}$ mice that received transgenic cells previously would not act as good stimulators in an MLR. To test this possibility, LNC from $2C_{F1}$ mice were used as responders and stimulated by irradiated splenocytes from $scid_{F1}$ 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 $scid_{F1}$ 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 $2C_{F1}$ cells, lymphocytes were collected from $scid_{F1}$ 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 L^d 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 $scid_{F1}$ 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

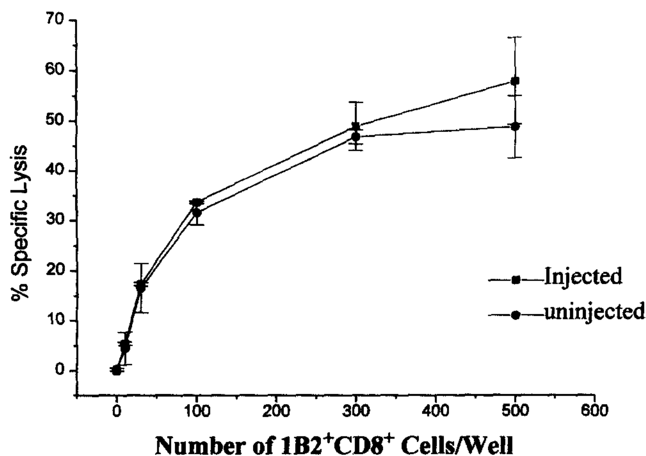


Figure 2. Persistence of some ASTC is not due to lack of antigen-presenting cells. Splenocytes from $2C_{F1}$ mice were stimulated by irradiated (20Gy) splenocytes from $scid_{F1}$ mice either uninjected or injected with $2C_{F1}$ cells 2 wk previously. Percentage lysis of specific target cells was measured 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.

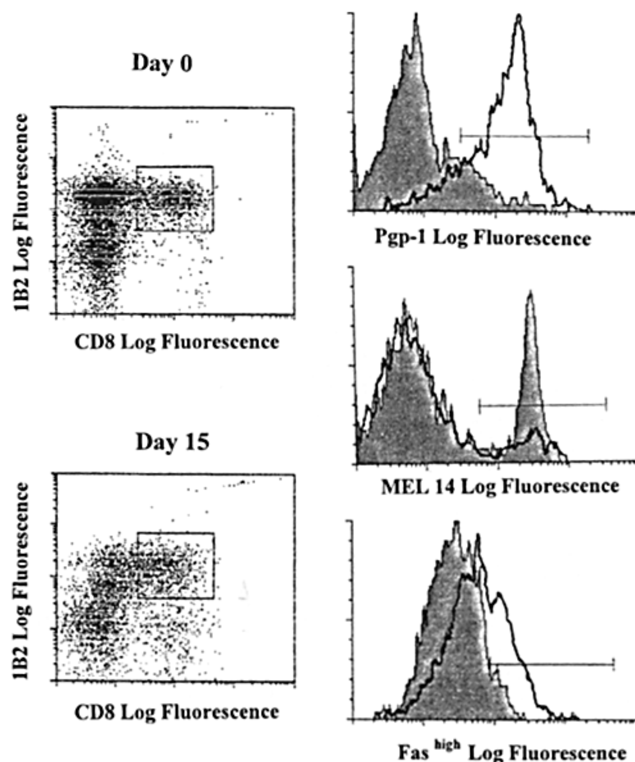


Figure 3. Expression of activation markers on $1B2^+CD8^+$ cells. Lymphocytes from $scid_{F1}$ were stained with 1B2 and CD8 followed by staining one of the activation markers. Pgp-1, MEL-14, and Fas^{high} histograms are of gated $1B2^+CD8^+$ cells. Lymphocytes from day 0 (*grey areas*) are compared to that 15 d after injection of $2C_{F1}$ cells (*areas within black lines*).

Table 1. *Percentage Expression of Activation Markers on 1B2⁺ CD8⁺ Cells*

Activation markers	LN		Spleen	
	Day 0	Day 15	Day 0	Day 15
$Pgp1^{+}$	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 $scid_{F1}$ mice on day 0, 5, and 21 after injection of $2C_{F1}$ 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 semiquantatative 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 $scid_{F1}$ mice 1 wk after injection were cultured with irradiated $(B6xBALB/c)_{F1}$ splenocytes in the presence of exogenous rIL-2 and compared to the response of naive $1B2^{+}CD8^{+}$ cells. The latter could kill L^{d+} target cells whereas the former did not show any cytotoxicity to L^{d+} cells even in the presence of exogenous IL-2 (Fig. 6). When the unsorted remaining $1B2^{+}CD8^{+}$ cells from $scid_{F1}$ 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 $scid_{F1}$ mice 4 d after receiving lymphocytes from

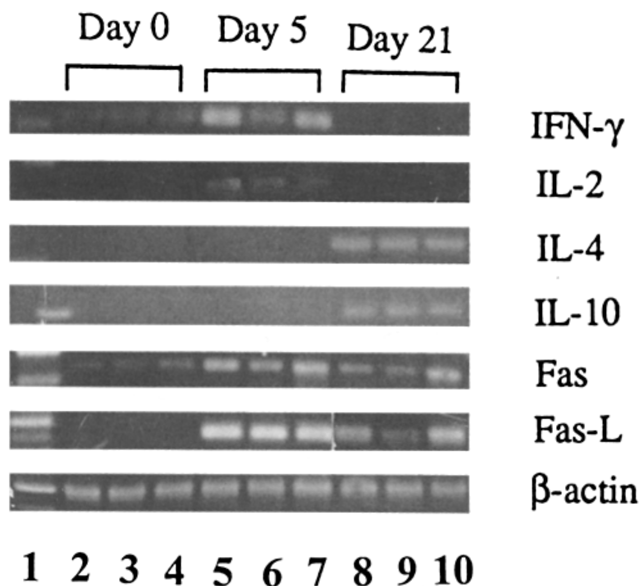


Figure 4. Residual ASTC express Fas/Fas-L as well as IL-4 and IL-10. Total RNA was extracted from purified $1B2^{+}CD8^{+}$ cells (5×10^{6}) on days 0, 5, and 21. Semiquantitative analysis of the mRNA levels of IFN-γ, IL-2, IL-4, IL-10, Fas, Fas-L, and β-actin was performed by RT-PCR at varying time 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.

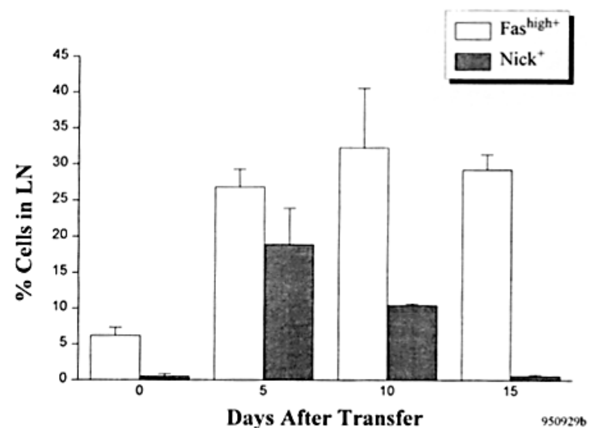


Figure 5. No correlation between % Fas^{high+} and % $nick^{+}$ cells after encounter with Ag in vivo. Lymphocytes were collected from $scid_{F1}$ mice on days 0, 5, 10, and 15 after transfer and stained for Fas with PE-conjugated anti-Fas mAb or for DNA nicks as described in Fig. 1 *B*. Percentages 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).

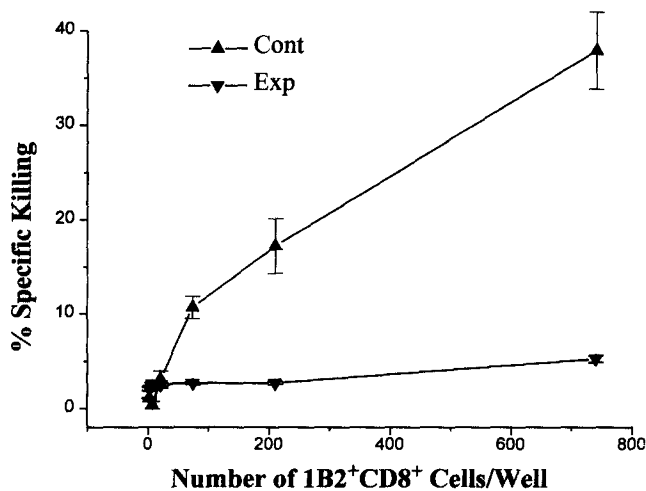


Figure 6. Residual ASTC did not kill target cells bearing L^d Ag in the presence of exogenous IL-2. 1 wk after transfer, residual $1B2^+CD8^+$ cells (*Exp*) were purified by cell sorting from $scid_{F1}$ mice, and naive $1B2^+CD8^+$ cells were also purified as controls. Varying numbers of purified $1B2^+CD8^+$ cells were cocultured with irradiated (20 Gy) $(BALB/cxB6)_{F1}$ splenocytes ($H\text{-}2^{b/d}$, L^{d+}) in α-MEM supplemented with 10% FCS in the present of 25 U rIL-2. 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.

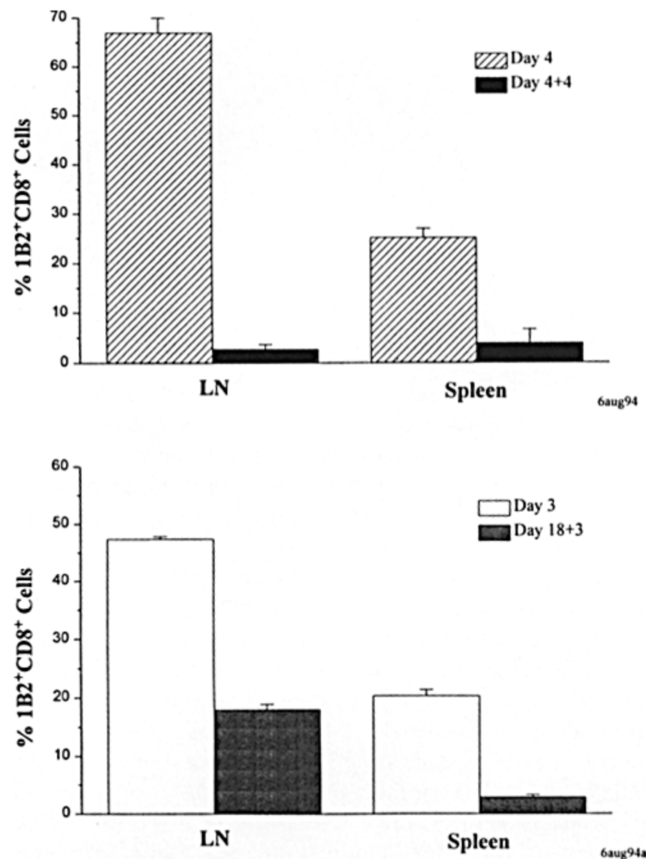


Figure 7. Non-responsiveness of residual ASTC in vivo upon antigen restimulation. (*A*) $Scid_{F1}$ mice were injected with $2C_{F1}$ lymphocytes. 4 d after injection recipient mice were killed and viable lymphocytes were isolated and transferred into new $scid_{F1}$ mice (3×10^7/mouse) (*grey bar, experimental group*). On the same day of the secondary transfer, another four naive $scid_{F1}$ mice were each injected with 3×10^7 freshly collected $2C_{F1}$ cells as day four controls (*white bar*). After another 4 d, LNC and splenocytes were collected from both groups of mice and stained with anti-1B2 and anti-CD8. Data were analyzed on two-color flow cytometry. Mean percentage of $1B2^+CD8^+$ cells in LN and spleen are shown. (*B*) The same experiment was performed 18 d after the first injection and 3 d after the secondary transfer.

$2C_{F1}$ mice. As controls, age- and sex-matched $scid_{F1}$ mice were injected with the same number of naive lymphocytes from $2C_{F1}$ mice. 4 d after transfer, LNC and splenocytes from $scid_{F1}$ mice were stained with mAbs against 1B2 and CD8. Unlike in the control $scid_{F1}$ mice that received naive $1B2^+CD8^+$ cells, LNC and splenocytes in the $scid_{F1}$ 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 $2C_{F1}$ 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 $scid_{F1}$ 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, $scid_{F1}$ mice were injected with 3×10^7 viable lymphocytes from $2C_{F1}$ mice. 15 d later, these $scid_{F1}$ mice were given a second injection of the same number of viable naive lymphocytes from $2C_{F1}$ mice. 5 d after the second injection, the total numbers of $1B2^+CD8^+$ cells in LN and spleen were compared to those of $scid_{F1}$ which received only one injection 5 or 20 d before. It was found that the total number of $1B2^+CD8^+$ cells in the $scid_{F1}$ mice which received two injections was comparable to the $scid_{F1}$ mice which received one injection 20 d before, but significantly lower than observed in the $scid_{F1}$ mice that 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 $scid_{F1}$ 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-

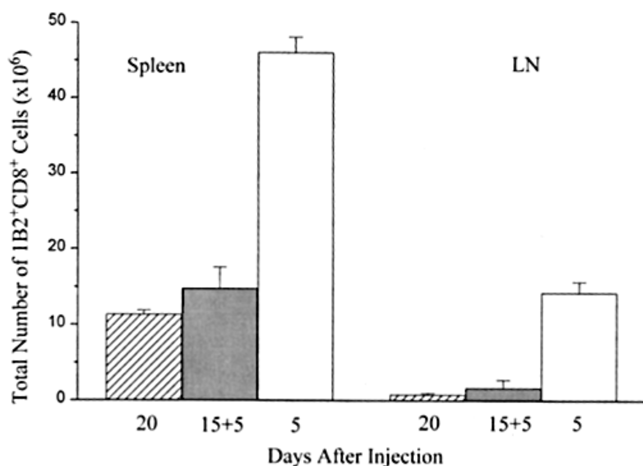


Figure 8. Inhibition of ASTC response to L^d antigen in vivo by residual cells. *$Scid_{F1}$* mice were injected with 3×10^7 $2C_{F1}$ cells. 15 d after injection, some of them were left for another 5 d without the second injection (*grey bar*); some received a second injection of 3×10^7 2C cells (*hatched bar*). At the time, another group of naive *$scid_{F1}$* 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 ovalbumin 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 hyporesponsive 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) L^{d+} 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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