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Apoptotic Cells Induce Tolerance by Generating Helpless CD8⁺ T Cells That Produce TRAIL¹

Thomas S. Griffith,* Hirotaka Kazama,† Rebecca L. VanOosten,* James K. Earle, Jr.,* John M. Herndon,† Douglas R. Green,‡ and Thomas A. Ferguson2†

The decision to generate a productive immune response or immune tolerance following pathogenic insult often depends on the context in which T cells first encounter Ag. The presence of apoptotic cells favors the induction of tolerance, whereas immune responses generated with necrotic cells promote immunity. We have examined the tolerance induced by injection of apoptotic cells, a system in which cross-presentation of Ag associated with the dead cells induces CD8⁺ regulatory (or suppressor) T cells. We observed that haptenated apoptotic cells induced CD8⁺ suppressor T cells without priming CD4⁺ T cells for immunity. These CD8⁺ T cells transferred unresponsiveness to naive recipients. In contrast, haptenated necrotic cells stimulated immunity, but induced CD8⁺ suppressor T cells when CD4⁺ T cells were absent. We further found that CD8⁺ T cells induced by these treatments displayed a “helpless CTL” phenotype and suppress the immune response by producing TRAIL. Animals deficient in TRAIL were resistant to tolerance induction by apoptotic cells. Thus, the outcome of an immune response taking place in the presence of cell death can be determined by the presence of CD4⁺-mediated Th cell function. *The Journal of Immunology, 2007, 178: 2679–2687.

Dead cells arise during normal tissue turnover or from pathogenic conditions, and the immune system must deal with these cells without the induction of autoimmunity or suppression of productive immune responses. Although cells can be induced to die by various methods (e.g., death receptor, toxicity, etc.), there are generally two types of cell death—apoptotic and necrotic (1–3). Apoptosis is a highly regulated process induced by specific stimuli; apoptotic cells tend to induce a tolerogenic or immunosuppressive response. Necrosis, conversely, results from the failure to control cellular homeostasis after injury and can result in stimulation of immunity. A number of recent studies have compared the effects of apoptotic and necrotic cells and the consensus is that dendritic cells (DC)⁴ are central players in determining the immunological outcome. Apoptotic cells can stimulate T cells without the benefit of DC maturation (and co-stimulation), leading to immune tolerance (4, 5). Necrotic cells release proinflammatory molecules, such as high mobility group box protein 1 (6), uric acid (7), or heat shock protein 70 (8), which can stimulate the DC to mature, leading to immunity. Mature DC, for example, stimulate CD4⁺ T cells to provide help for CD8⁺ T cells, encouraging their expansion into effector cells (5, 9, 10).

Although apoptotic cells can induce tolerance by deleterious mechanisms (11), recent studies suggest that tolerance following presentation of apoptotic cells is “infectious” and mediated by regulatory T cells (Treg) (12). The phenomenon of infectious immunological tolerance was described over 30 years ago in Ag-modified self and heterologous erythrocyte models (13–15). The idea that T cells can regulate (or suppress) immunity unfortunately never progressed beyond the phenomenological stage, as the cells and factors proposed to mediate tolerance escaped molecular characterization. Interestingly, many of the earliest descriptions of infectious tolerance described the function of CD8⁺ regulatory T cells (henceforth called T suppressor or Ts cells); however, with the exception of a few studies with T cell vaccination (16, 17) and autoimmunity (18), little is known about the function of the CD8⁺ Ts cells. Mechanisms proposed to account for CD8⁺ T cell suppression have been regulatory cytokines (IL-10) (19), as well as CD8 immune deviation (20). With the availability of new technology, there has been a renewed interest in T cell suppression, evidenced by the many studies focused on the ability of CD8⁺ Treg to suppress immunity in multiple systems (21).

Our investigation into the molecular mechanisms of infectious tolerance uses a classical tolerance system of presenting hapten-modified spleen cells to the immune system. Injection of these cells i.v. leads to their apoptosis (via Fas/FasL), entry into the cross-priming pathway of the CD8ar⁺ DC, and presentation to CD8⁺ T cells (12). The induced CD8⁺ T cells are capable of transferring tolerance to nontolerant individuals (i.e., infectious tolerance). In the current study, we explored the stimulation of CD8⁺ T cells with apoptotic cells and necrotic cells. Our results demonstrate that haptenated apoptotic cells stimulate CD8⁺ T cells, which upon restimulation secrete TRAIL (22, 23) as an effector molecule in the tolerance pathway. Apoptotic cells do not stimulate Th cell function. Interestingly, necrotic cells, which can stimulate CD4⁺ T cell-mediated immunity, do not induce TRAIL production by CD8⁺ T cells unless the Ag is presented in the absence of CD4⁺.
T cells. Thus, the response of the immune system after encountering dead cells depends on the stimulation of CD4+ T cell help.

Materials and Methods

Animals

C57BL/6 mice were purchased from The Jackson Laboratory. C57BL/6 TRAIL−/− mice were obtained from Amgen (24) and bred in our own facility at the University of Iowa (UI), according to UI Institutional Animal Care and Use Committee (IACUC) guidelines. They are >10 generations backcrossed to C57BL/6. All animal procedures were performed according to National Institutes of Health guidelines and approved by the UI or Washington University IACUC.

Abs and reagents

Anti-CD4 (GK1.5) and anti-CD8 (clone 2.43) Ab for in vivo depletion were purified from hybridoma supernatants. Mouse anti-TRAIL Ab (N2B2) and the control mouse IgG2a were purchased from eBioscience. 2,4,6 trinitro-benzene sulfonic acid (TNBS) was purchased from Sigma-Aldrich. Recombinant mouse TRAIL (rTRAIL) and zVAD-fmk were purchased from BIOMOL. Anti-CD40 (FGK45) has been described (25) and mice received a single i.v. injection of 300 μg of FGK45.

Trinitrophenyl (TNP) coupling of spleen cells

Splenic cells were isolated and coupled with TNP as previously described (12). Briefly, 107 cells were incubated in 0.5 ml of HBSS and 0.5 ml of 10 mM TNBS for 7–10 min at room temperature. After incubation, cells were washed three times with HBSS before use. Cells were irradiated (3000 R) to induce apoptosis. Necrotic cells were prepared by alternating five freeze/thaw cycles using a liquid nitrogen/37°C water bath. TNP-coupled cells were injected at 107 cells (apoptotic) or equivolance (necrotic) per mouse. All cells were delivered i.v. via the retro-orbital plexus.

Immune response

Mice were immunized with 0.1 ml of 10 mM TNBS s.c. Four days later, mice were challenged with 0.033 ml of 10 mM TNBS in PBS in the right footpad and 0.033 ml of PBS in the left footpad. Measurements were taken 24 h postinjection by a masked observer. Values are expressed in micrometers 43°C. Our initial studies examined the difference between the challenged and unchallenged footpad in unimmunized mice.

T cell isolation

T cell populations were obtained using EasySep Mouse CD4/CD8+ T Cell enrichment kit(s) (Stem Cell Technologies) as per the manufacturer’s instructions. Cell populations were used only when purity was >95% as determined by flow cytometry using anti-CD4 and anti-CD8 Abs.

Preparation of immune and tolerant supernatants

Mice were injected with TNP-apoptotic cells i.v. and immunized with 0.1 ml of 10 mM TNBS s.c. 7 days later. Spleen cells were then isolated 1 day later, cultured in vitro for 48 h in RPMI 1640 medium with 10% FBS. Supernatants from tolerant mice or immune mice were then mixed with 4-day immune CD4+ cells for 1 h. Following washing, the CD4+ T cells were transferred to naive mice that were challenged immediately. In some groups, 1 μg of anti-TRAIL Ab (N2B2) or 1 μg of isotype control IgG2a was added to the tolerant supernatant. The percent killing in each group of mice was determined by the formula 100 × [(% TNP-spl unimmunized/% spl unimmunized) × 100].

Adoptive transfer of immunity

Mice were immunized with 0.1 ml of 10 mM TNBS. Four days later, spleens were harvested, erythrocyte-free suspensions were prepared (12), and the CD4+ T cells were negatively selected and purified using the Stem Cell Technologies kit. Ten million CD4+ cells per mouse were infused into naive recipients. In some experiments, CD4+ cells were incubated with immune supernatant, tolerant supernatant, ZVAD-fmk, or rTRAIL before transfer. Mice were immediately challenged and measurements were taken as described above.

Quantitative RT-PCR

Total RNA was harvested from cell populations with TRIzol reagent (Invitrogen Life Technologies). Total RNA (2 μg) was reverse-transcribed using Superscript II. The real-time quantitative RT-PCR primer/probe sets for mouse TRAIL, FasL, TNF, FLIP, Bcl-xL, DR5, and rRNA were purchased from Applied Biosystems. A total of 250 ng of cDNA was used as a template for TaqMan assays for all transcripts and the internal rRNA control. The TaqMan PCR was conducted as described previously (26).

In vivo cytotoxicity

TNP-coupled spleen (TNP-spl) or normal spleen cells (spl) were suspended at 1 × 107 cells/ml in warmed PBS/0.1% BSA. CFSE (1.25 μl/ml of 5 mM stock) was added to the TNP-spl for the CFSEhigh population (reference population). Cells were incubated 10 min at 37°C (water bath). The reaction was stopped by the addition of ice-cold PBS containing 10% FBS. Cells were washed three times with PBS/FBS, counted, and resuspended to the appropriate volume. Ten million target cells (CFSElow) and 10 million reference cells (CFSEhigh) were injected into naive or tolerant C57BL/6 or TRAIL−/− recipients. Spleens were harvested 18 h later and analyzed by flow cytometry. A total of 3–5000 events in the reference population were collected and the number of target cells recovered was enumerated. Unimmunized C57BL/6 mice were used as controls. The percent reduction in the number of recovered CFSElow cells (TNP-spl) in the unimmunized vs tolerant mice was considered the percent cytotoxicity. Individual mice were analyzed and the percent killing in each group of mice was determined by the formula 100 − (((% TNP-spl tolerant/% spl tolerant)/(% TNP-spl unimmunized/% spl unimmunized)) × 100).

Statistical analysis

Significant differences between groups were evaluated using a two-tailed Student’s t test (p < 0.01).

Results

Necrotic, but not apoptotic, haptenated cells prime for immunity

We sought to understand the molecular basis of infectious immunological tolerance induced by the administration of apoptotic cells to the immune system (12). Our initial studies examined the immunological response to either apoptotic (induced by irradiation) or necrotic (induced by freeze/thaw) syngeneic TNP-coupled cells injected i.v. into naive C57BL/6 mice. Mice injected with haptenated necrotic cells demonstrated immunity after footpad challenge with TNBS, whereas mice injected with haptenated apoptotic cells did not (Fig. 1). Depletion of CD8+ T cells in vivo

FIGURE 1. Apoptotic cells do not prime for immunity. C57BL/6 mice were injected i.v. with apoptotic (107) or necrotic (108 equivalence) TNP-coupled spleen cells. Some groups of mice were depleted of CD8+ cells by three daily 100-μg doses of anti-CD8 (αCD8, mAb 2.43). Four days later, mice were challenged with 0.033 ml of 10 mM TNBS in the right footpad and 0.033 ml of PBS in the left footpad. Measurements (micrometer ± SE) were taken 24 h later and represent the difference between right footpad (Ag challenge) and left footpad (PBS challenge). Immune control groups were injected with 0.1 ml of 10 mM TNBS s.c. The percent killing in each group of mice was determined by the formula 100 × (((% TNP-spl tolerant/% spl tolerant)/(% TNP-spl unimmunized/% spl unimmunized)) × 100).

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before immunization did not affect these results. These data show
that apoptotic cells do not prime for CD4-mediated immunity
while necrotic cells are able to induce this type of immune
response.

CD8⁺ T cells transfer tolerance induced by haptenated
apoptotic cells

We next determined whether injection of apoptotic cells led to the
generation of the CD8⁺ T cells that could regulate the immune
response. Haptenated apoptotic or necrotic cells were injected into
untreated or CD4⁺ T cell-depleted naive C57BL/6 mice. After 7
days, CD8⁺ T cells were isolated from the spleens of these mice
and injected into naive C57BL/6 mice. These animals were im-
mediately immunized with TNBS, challenged after 4 days, and
immunity was measured 1 day later. The transfer of CD8⁺ T cells
from mice that had received haptenated apoptotic cells made re-
cipients unresponsive to Ag, while those receiving CD8⁺ T cells
from mice that had received haptenated necrotic cells were readily
immunized (Fig. 2A). Therefore, the generation of regulatory CD8⁺ T cells in this system appears to correlate
with the failure to activate CD4⁺ T cells.
Apoptotic cells induce TRAIL production by CD8+ T cells

Activation of CD8+ T cells with Ag typically generates CTL that kill target cells via perforin/granzyme or FasL pathways (27). Recent studies have indicated that CD8+ CTL primed in the absence of CD4+ T cell help (“helpless CTL”) fail to undergo secondary expansion because they produce TRAIL and undergo activation-induced cell death (AICD) (28). CD8+ cells primed in the presence of CD4+ T cell help, in contrast, do not produce TRAIL and expand upon secondary exposure to Ag. Consequently, one possible explanation for the results in Fig. 2A was that the CD8+ T cells primed in the absence of CD4+ T cell help suppress any subsequent immune response through the expression of TRAIL. To examine this possibility, mice were injected with haptenated apoptotic cells and then immunized 7 days later. Cells from tolerant or control immune mice were examined for expression of several markers of “helped” vs “helpless” CTL (Fig. 2B). Although immune CD8+ T cells showed elevated levels of c-FLIP and Bcl-xL, CD8+ T cells from mice pretreated with haptenated apoptotic cells (tolerant mice) showed elevated levels of TRAIL mRNA. The gene expression pattern in the T cells from mice injected with haptenated apoptotic cells and then immunized resembled the pattern described for stimulated helpless CTL in that TRAIL mRNA was elevated (28). Therefore, we examined the expression of TRAIL in mice injected with haptenated apoptotic or necrotic cells in the presence or absence of CD4+ T cells. Mice were injected with apoptotic or necrotic cells and 7 days later they were immunized with TNBS. Twenty-four hours after immunization, CD8+ T cells were isolated and the level of TRAIL mRNA determined. Mice injected with apoptotic cells (CD4-depleted or intact) and CD4+ T cell-depleted mice injected with haptenated necrotic cells possessed CD8+ T cells with elevated levels of TRAIL mRNA (Fig. 2C). Other cell types from the spleen of tolerant mice (e.g., CD4+ T cells, B cells, etc.) did not show TRAIL production (data not shown). Even though a variety of cells in the immune system can be induced to express TRAIL under a number of stimulatory conditions (29–31), these cells do not produce TRAIL under these tolerogenic conditions. We conclude that the generation of TRAIL-expressing CD8+ T cells after i.v. immunization with apoptotic cells is similar to that seen with necrotic cells in the absence of CD4+ T cell help, resulting in the activation of cells resembling helpless CTL in both cases.

CTL stimulated in the absence of help have normal killing function following primary stimulus; however, upon restimulation with cognate Ag, the cells produce TRAIL and undergo AICD (28). Therefore, we examined our system for the requirement for restimulation to induce TRAIL production. Mice were injected with apoptotic cells and 7 days later mice were immunized with TNBS or left untreated. One day later, the CD8+ T cells were removed and TRAIL mRNA expression was determined. Fig. 2D shows that CD8+ T cells from tolerized mice (apoptotic cells/immunize) increased TRAIL mRNA, while mice that were immunized (no apoptotic cells) or injected with apoptotic cells (not immunized) did not produce TRAIL. These data show that CD8+ T cells do not produce TRAIL from just a single encounter with apoptotic cells, but do so after a subsequent encounter with Ag (i.e., immunization).

Tolerant mice display TRAIL-mediated cellular cytotoxicity

Our data thus far suggest that apoptotic cells generate CD8+ T cells that regulate immunity and these cells resemble helpless CTL based on production of TRAIL. CD8+ T cells that regulate immunity are generated when apoptotic cells pass through the cross-priming pathway of the DC (12). Similarly cross-priming in the CD8+ CTL compartment requires that Ags associated with apoptotic cells pass through the same class I pathway of the DC (4, 5, 32). Because we observed the generation of TRAIL-producing CD8+ T cells, it is possible that tolerant mice contain CTL that mediate cytotoxicity via TRAIL. We examined this using an in vivo killing system that can sensibly measure death (33). This assay monitored eradication of an adoptively transferred target population (TNP-spl) in tolerant and nontolerant mice. Recipient C57BL/6 or TRAIL−/− mice were injected with haptenated apoptotic cells and immunized 1 wk later. Three days later, these mice were infused with haptenated syngeneic spleen cells labeled with a high concentration of CFSE (target cells) and unmodified syngeneic spleen cells labeled with low CFSE (reference population) at a 1:1 ratio. Eighteen hours later, the remaining haptenated spleen cells were enumerated and their numbers compared with the reference population. As controls, mice were untreated, immunized only, or given an injection of stimulatory anti-CD40 at the time of injection of apoptotic cells as this treatment overcomes tolerance in this system (12). These data are summarized in Table I and presented as percent (%) cytotoxicity. Immunization of C57BL/6 or TRAIL−/− mice did not result in detectable cytotoxicity (group A), while injection of haptenated apoptotic cells along with anti-CD40 generated significant cytotoxicity in both strains (group D). However, mice rendered tolerant with apoptotic cells followed by immunization demonstrated cytotoxicity in C57BL/6 mice but not in TRAIL−/− mice (group C). Interestingly, mice injected with haptenated apoptotic cells and not immunized (group B) displayed some cytotoxicity in TRAIL−/− mice, suggesting some influence of reencounter with Ag (see Fig. 2D). Thus, the same procedure that generates tolerance also activates TRAIL-mediated cytotoxicity. These data show that apoptotic cells induce cellular cytotoxicity mediated by TRAIL and further support the idea that apoptotic cells generate CD8+ T cells with a helpless CTL phenotype.

TRAIL is critical to tolerance induction

To confirm that TRAIL production was critical to the tolerance (i.e., immune suppression) mediated by “helpless” CD8+ T cells, we examined the generation of these cells in wild-type C57BL/6 and TRAIL−/− mice. First, wild-type C57BL/6 mice or TRAIL−/− mice were injected with haptenated apoptotic cells. Animals were immunized 2 days later, the mice were challenged after 4 days and the immune response was analyzed the next day. Fig. 3A shows that TRAIL−/− mice were not rendered tolerant with apoptotic cells. Similarly, only CD8+ T cells obtained from tolerized C57BL/6 (TRAIL+/+) mice could transfer tolerance to naive mice (Fig. 3B). CD8+ T cells from

Table I. In vivo killing of CFSE-labeled TNP-spl

<table>
<thead>
<tr>
<th>Group</th>
<th>TNP-spl*</th>
<th>Anti-CD40b</th>
<th>Immune*c</th>
<th>% Cytotoxicity4</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>+</td>
<td>+</td>
<td>0</td>
<td>0</td>
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<tr>
<td>B</td>
<td>+</td>
<td>+</td>
<td>26 ± 4</td>
<td>12 ± 2</td>
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<td>C</td>
<td>+</td>
<td>+</td>
<td>29 ± 3</td>
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<td>E</td>
<td>+</td>
<td>+</td>
<td>100 ± 10</td>
<td>100 ± 14</td>
</tr>
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</table>

* TNBS coupled spleen cells injected i.v.

b Mice received a single injection of 300 μg of F9G45 (anti-CD40) at the time of TNP-spl injection.

c Mice were immunized with 0.1 ml of 10 mM TNBS s.c. 7 days following TNP-spl.

4 Determined 3 days following infusion of CFSE-labeled cells. Values represent the mean of five animals ± SE.
TRAIL−/− mice did not have this function. We conclude from these results that TRAIL-producing CD8+ T cells mediate tolerance in this system.

The importance of TRAIL in our model system was further addressed by testing whether TRAIL might be secreted by the CD8+ T cells after injection of apoptotic cells. Mice were tolerized by injection of haptenated apoptotic cells and then immunized with TNBS after 7 days. Spleen cells were then isolated 1 day later, cultured in vitro for 48 h, and supernatants collected. Supernatants were then mixed with 4-day immune CD4+ T cells for 1 h. After washing, the CD4+ T cells were transferred to naive mice that were challenged immediately. In some groups, 1 μg of anti-TRAIL Ab (N2B2) or 1 μg of isotype control IgG2a was added to the tolerant supernatant. One group of immune CD4+ T cells was incubated with 1 μg/ml rTRAIL before transfer. Mice were challenged immediately with 0.033 ml of 10 mM TNBS in the right footpad and 0.033 ml of PBS in the left footpad. Measurements (micrometer ± SE) were taken 24 h later and represent the difference between right footpad (Ag challenge) and left footpad (PBS challenge). Background (Bkg) values represent the difference between challenged and unchallenged sites in naive mice. *, Significantly different from the immune control.
warming, the CD4+ T cells were transferred to naive mice that were challenged immediately. When immunity was measured 24 h later, we found that supernatants obtained from the spleens of tolerant mice (tolerant supernatant) prevented the transfer of immunity (Fig. 4A). This effect was reversed when a neutralizing anti-TRAIL Ab (N2B2) was added to the supernatant before incubation with immune CD4+ T cells. Supernatants from immune cells (immune supernatant) did not have this function. In addition, when immune cells were treated with recombinant mouse TRAIL (rTRAIL) the transfer of immunity was completely eliminated.

TRAIL is a well-known cytotoxic member of the TNF family that induces caspase-dependent apoptosis in target cells (22). It is possible that CD8+ T cells activated by haptenated apoptotic cells could use TRAIL to kill the CD4+ T cells that mediate the immune response (as was suggested in Table I). We tested this by performing the experiment shown in Fig. 4B. Immune CD4+ T cells (4 day immune) were treated with rTRAIL in presence or absence of zVAD-fmk, a caspase inhibitor known to block apoptotic cells death. Treated CD4+ T cells were then transferred to naive recipients and the mice challenged simultaneously. These results show that the potent inhibition of adoptive immunity by rTRAIL was reversed by treatment of the cells with the pan-caspase inhibitor. Thus, one function of TRAIL in this system is to induce apoptosis in immune CD4+ T cells. Together, the results in Fig. 4 compliment the data in Figs. 1–3 demonstrating that TRAIL-expressing (rTRAIL) the immune system. Our results demonstrate that the outcome of an encounter with dead cells depends on the availability of CD4+ T cell help; 2) necrotic cells do not activate CD8+ T cells; and 3) apoptotic cells stimulate a

whereas immunity was induced by haptenated necrotic cells in both cases. Therefore, the failure of haptenated apoptotic cells to prime a CD4+ T cell-dependent immune response is independent of the production of TRAIL by CD8+ T cells. This is consistent with the results in Fig. 1 where apoptotic cells did not stimulate immunity, even when CD8+ T cells (the source of TRAIL) were absent.

Finally, we tested whether the loss TRAIL altered the susceptibility of these mice to TRAIL regulation. C57BL/6 or TRAIL−/− mice were injected with haptenated apoptotic cells and CD8+ T cells were isolated from the spleen 7 days later. These cells were transferred to naive TRAIL−/− mice that were immunized and assessed for tolerance. Fig. 6 shows that CD8+ T cells obtained from TRAIL−/+ mice (but not TRAIL−/− mice) transferred tolerance to TRAIL−/− mice. Thus, TRAIL−/− mice are susceptible to regulation by TRAIL-producing CD8+ T cells activated by apoptotic cells.

Discussion

The interaction between the immune system and dead cells is continuous throughout the life of the organism. Because dead cells can modulate immunity, mechanisms are in place to control aberrant responses that can modulate protective immunity or lead to autoimmunity. Using a classical system for inducing immune unresponsiveness (34), we have examined presentation of dead cells to the immune system. Our results demonstrate that the outcome of an encounter with dead cells depends on the availability of CD4+ T cell priming (i.e., help). The major findings of this work are: 1) apoptotic cells induce CD8+ T cells without activating CD4+ T cell help; 2) necrotic cells do not activate CD8+ T cells unless CD4+ T cell help is removed; and 3) apoptotic cells stimulate a
helpless CD8\(^+\) T cell phenotype that can function as CTL and regulate CD4\(^+\) T cell-mediated immunity by the secretion of TRAIL. Our results provide a molecular mechanism for infectious tolerance mediated by CD8\(^+\) T cells and suggest how apoptosis in the immune system can promote immune tolerance.

The induction of immunological unresponsiveness by apoptotic cells has been attributed to a number of mechanisms. These include the induction of immunosuppressive cytokines from phagocytic cells (35), the production of inhibitors from the dead cell itself (36, 37), and effects on the maturation of the DC (4, 5). We have observed that apoptotic cells do not prime CD4\(^+\) T cells, but do stimulate CD8\(^+\) cells. In recent years, a number of reports have highlighted the pivotal role that CD4\(^+\) T cells play in the induction of CD8\(^+\) T cell responses (4, 25, 28, 38). Although naive CD8\(^+\) T cells can be directly activated to proliferate in the absence of CD4\(^+\) T cells, the amount of IL-2 produced by CD8\(^+\) T cells is ~10-fold less than CD4\(^+\) T cells, an amount that may be insufficient to sustain a response (39). Thus, most CD8\(^+\) T cell-mediated responses depend on concomitant priming of CD4\(^+\) T cells to be effective. In other studies, CD8\(^+\) T cell priming in the absence of CD4\(^+\) T cell help leads to their deletion from the periphery, an effect that can be overcome by supplying help during the initial priming phase (9). Recently, it was shown that the priming of CD8\(^+\) T cells in the absence of CD4\(^+\) T cell help alters the programming of these cells, which was only revealed upon secondary stimulation. Specifically, CD8\(^+\) T cells activated without help (i.e., helpless) produce TRAIL and undergo AICD upon secondary antigenic stimulation (28). We have observed a similar phenomenon following encounter with apoptotic cells, i.e., the apoptotic cells do not prime CD4\(^+\) cells, and the CD8\(^+\) T cells acquire a helpless phenotype upon second stimulation. These cells mediate cytotoxicity and suppress the response of CD4\(^+\) T cells that mediate delayed-type hypersensitivity. Thus, helpless CTL and CD8\(^+\) T cells that regulate immunity (i.e., Ts cells) may be the same cells.

Although the role of TRAIL in the concept of helpless CTL is an important observation when considering immunologic memory (28), the participation of TRAIL in activation-induced T cell death was first reported nearly 10 years ago by several laboratories (40, 41) (42). Consequently, the immunoregulatory TRAIL-expressing CD8\(^+\) T cells activated in response to hapten identified in our system may also act upon other CD8\(^+\) T cells that would be involved in any subsequent Ag-specific immune response. CD8\(^+\) T cells are more sensitive to TRAIL-mediated regulation compared with CD4\(^+\) T cells (31), which supports this possibility. However, since CD8\(^+\) T cells are not involved in the transfer of immunity in the present system, TRAIL effects on CD8\(^+\) T cells may not influence the immune response. Interestingly, an effect on CD8\(^+\) T cells might lead to the elimination of the induced Ts cells following TRAIL secretion.

One interesting finding from our studies was that haptenated necrotic cells, which normally prime for immunity when given i.v., can also generate a “helpless CTL” phenotype if CD4\(^+\) T cells are first eliminated. It has been thought that what determines the outcome of the immune response to dead cells is influenced by surface receptors displayed by the dying cells. Apoptotic cells express unique markers, such as CD36 and phosphatidylycerine, and are efficiently phagocytized (43, 44). These markers help guide the apoptotic cells through the Ag-processing machinery leading to tolerance by deletion and/or the production of immunosuppressive cytokines, such as IL-10 or TGF-β (36, 37). Conversely, necrotic cells do not display such markers and are thus thought to not enter the cross tolerance pathway. Necrotic cells can further stimulate immunity by the release proinflammatory molecules, such as high mobility group box protein 1 (6), uric acid (7), and/or heat shock protein 70 (8), which prime CD4\(^+\) T cell help and stimulate DC maturation. Our data show that in the absence of CD4\(^+\) T cell help, necrotic cells perform the same function as apoptotic cells, i.e., induce immunologic tolerance and promote the development of TRAIL-expressing CD8\(^+\) Ts cells. Thus, necrotic cells must be handled similarly to apoptotic cells by APC, even though they can release proinflammatory molecules that can result in DC maturation. Perhaps more importantly, necrotic cells, even if they mature the DC, can induce immunologic unresponsiveness should there be inadequate CD4\(^+\) T cell help. CD4\(^+\) T cell help is, therefore, a key determinant in the immunological outcome after the encounter with dead cells.

TRAIL is becoming an increasingly important member of the TNF family of death-inducing ligands (22). Originally thought to be a tumor-specific killer, recent studies have shown that TRAIL is functional in a variety of other physiological systems (28, 45, 46). Although the TRAIL\(^{-/-}\) mice do not have an overt phenotype (24), studies with infectious agents and autoimmune models show that TRAIL can play an important role in controlling the extent of the autoimmune reaction, as well as be an effector molecule in certain disease states (47, 48). In the experimental autoimmune encephalomyelitis model, for example, the lack of TRAIL exacerbates autoimmunity. Recent studies with TRAIL\(^{-/-}\) mice in a model of multiple sclerosis have suggested an important role for suppressor CD8\(^+\) T cells protecting against disease recurrence and exacerbation (49). In the NOD mouse, which is prone to autoimmune diabetes, TRAIL blockade similarly exacerbates the onset of type 1 diabetes (50). Also, treatment of mice undergoing experimental autoimmune thyroiditis with rTRAIL ameliorated the disease (51). In our system, the failure of apoptotic cells to prime CD4\(^+\) T cells results in CD8\(^+\) cells that produce TRAIL, suggesting an important immunoregulatory role for TRAIL in the control of potentially dangerous anti-self responses that could be induced with dead cells. TRAIL appears to function in our tolerance model by inhibiting Ag-reactive CD4\(^+\) cells through the induction of apoptosis, which is consistent with previous reports of TRAIL in regulating T cell homeostasis (40, 42). Consistent with this is the finding that functional TRAILR has been reported on mouse (52) and human (53) activated T cells, but not on resting T cells. Together, these results suggest that TRAIL may play a critical role in controlling immune reactions.

TRAIL can also induce apoptosis in a number of other cells in the immune system, including plasma cells, neutrophils, and DCs (53) (54) (55). Although we only examined the ability of the TRAIL-expressing CD8\(^+\) Ts cells induced in our system to regulate hapten-specific CD4\(^+\) T cells, it is also possible that the TRAIL could be modulating the function of other cells, such as APCs. TRAIL, or more specifically TRAILR, has been recently reported to act as a negative regulator of innate immune cell responses (56). Using TRAILR-deficient mice, it was demonstrated that these mice displayed enhanced innate immune responses after bacterial or viral challenge. Further investigation revealed that stimulation of TRAILR-deficient macrophages with various TLR agonists led to enhanced cytokine production compared with wild type, suggesting that the TRAIL-TRAILR system has the capacity to regulate cytokine production and the resultant immune response. Similar findings were reported for Listeria-induced hepatitis (48) and for Listeria infections (47) in TRAIL-deficient mice. In both systems, the loss of TRAIL led to enhanced innate immunity. Thus, while the regulatory activity of TRAIL-expressing CD8\(^+\) T cells may be beneficial for protection in autoimmunity, their presence may be detrimental during times of infection.
Some of the earliest descriptions of Treg examined the function of CD8+ T cells (13–15), which could regulate immune responses through the secretion of a soluble factor that suppressed immunity by inhibiting T cell function (called T suppressor factor) (57, 58). Although our results are not intended to explain all observations with T suppressor factor, it is interesting to speculate that at least some of the suppressor activity in the supernatants of CD8+ T cells was TRAIL. It is important to note that T cells that suppress immune responses may not belong to a unique T cell subset with specialized function, but may instead represent regulatory activities of otherwise normal helper and cytotoxic CD8+ T cells. Our data showing that the CD8+ Treg is a helpless CTL support this idea. This may explain why it was previously so difficult to identify unique cell surface markers that identified the Tcs cells as a functional population (and one of the main issues that fueled the “suppressor cell” controversy years ago) (59). Perhaps our results also demonstrate why it was so difficult to clone Ts cells, i.e., when generated they produced TRAIL and underwent AICD.

Disclosures

The authors have no financial conflict of interest.

References


