Brief Definitive Report

The Peripheral Deletion of Autoreactive CD8\(^+\) T Cells Induced by Cross-presentation of Self-antigens Involves Signaling through CD95 (Fas, Apo-1)

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Summary

Recently, we demonstrated that major histocompatibility complex class I–restricted cross-presentation of exogenous self-antigens can induce peripheral T cell tolerance by deletion of autoreactive CD8\(^+\) T cells. In these studies, naive ovalbumin (OVA)-specific CD8\(^+\) T cells from the transgenic line OT-I were injected into transgenic mice expressing membrane-bound OVA (mOVA) under the control of the rat insulin promoter (RIP) in pancreatic islets, kidney proximal tubules, and the thymus. Cross-presentation of tissue-derived OVA in the renal and pancreatic lymph nodes resulted in activation, proliferation, and then the deletion of OT-I cells. In this report, we investigated the molecular mechanisms underlying this form of T cell deletion. OT-I mice were crossed to tumor necrosis factor receptor 2 (TNFR2) knockout mice and to CD95 (Fas, Apo-1) deficient mutant \(^{lpr}\) mice. Wild-type and TNFR2-deficient OT-I cells were activated and then deleted when transferred into RIP-mOVA mice, whereas CD95-deficient OT-I cells were not susceptible to deletion by cross-presentation. Furthermore, cross-presentation led to upregulation of the CD95 molecule on the surface of wild-type OT-I cells in vivo, consistent with the idea that this is linked to rendering autoreactive T cells susceptible to CD95-mediated signaling. This study represents the first evidence that CD95 is involved in the deletion of autoreactive CD8\(^+\) T cells in the whole animal.

Key words: CD8\(^+\) T lymphocytes • T cell tolerance • apoptosis • CD95 • tumor necrosis factor receptor 2

Exogenous antigens derived from nonlymphoid tissues can be presented by professional APCs to naive CD8\(^+\) T cells by a mechanism termed cross-presentation. This may be important for the induction of immunity to pathogens that avoid professional APCs (1, 2). Using transgenic mice expressing membrane-bound OVA (mOVA) under the control of the rat insulin promoter (RIP), we have demonstrated that self-antigens can also gain access to the cross-presentation pathway and activate autoreactive CD8\(^+\) T cells in vivo (3). When transgenic OVA-specific CD8\(^+\) T cells were injected into RIP-mOVA mice, which expressed OVA in pancreatic islets, kidney proximal tubules, and the thymus, they were activated in the renal and pancreatic LNs. This form of activation initially led to proliferation and then to the deletion of transgenic OVA-specific class I–restricted CD8\(^+\) T (OT-I) cells (4). Thus, cross-presentation can induce peripheral tolerance by deleting autoreactive CD8\(^+\) T cells. The molecular mechanisms underlying this deletion have not been addressed in previous studies.

Programmed death of activated T cells can result either passively from lack of survival factors such as IL-2 (death-by-neglect) or actively by activation-induced cell death (AICD), which is mediated by molecules of the TNFR superfamily (5–7). CD95 (Fas, Apo-1), a member of this family, is upregulated on the surface of T cells upon antigen-induced activation, and induces apoptosis of activated T cells when ligated in vitro (8–12). A mutation of the CD95 gene causes the \(^{lpr}\) (lymphoproliferation) mutation in mice characterized by lymphadenopathy and accumulation of nonfunctional CD4\(^-\)CD8\(^-\)B220\(^+\)TCR\(^+\) cells, and by autoimmune diseases such as immune-complex nephritis (13). A similar pathology is seen in \(^{gld}\) (generalized lymphoproliferative disease) mice, which carry a point mutation in the CD95 ligand gene rendering this protein functionless (14). Mutations in the human CD95 gene lead to a related clinical picture, referred to as autoimmune lymphoproliferative syndrome (15–17). Since thymic-negative selection does not require functional CD95 (6, 18), these symptoms are
thought to be caused by defects in the peripheral deletion of activated T cells (7). Whereas in vitro studies have shown a critical role of CD95 in the deletion of mature CD4\(^+\) T cells, TNFR2 was suggested to mediate CD95-independent deletion of CD8\(^+\) T cells (19). In vivo studies using TCR transgenic mice have confirmed the key role of CD95 in AICD of CD4\(^+\) T cells (6, 18, 20, 21). The roles of CD95 and TNFR2 in the peripheral deletion of CD8\(^+\) T cells have not been extensively investigated, but there is evidence that CD95 does not participate in peripheral deletion associated with virus infection (22, 23). In this study, we investigated the role of CD95 and TNFR2 in the deletion of CD8\(^+\) T cells induced by cross-presentation of self-antigens.

**Materials and Methods**

**Mice** All mice were bred and maintained at the Walter and Eliza Hall Institute for Medical Research. OT-I and RIP-mOVA transgenic mice have been described previously (3). TNFR2-deficient mice on a C57BL/6 (B6) background (24) and B6.Tg mice (The Jackson Laboratory, Bar Harbor, ME) were crossed to OT-I mice. TNFR2 gene disruption and the presence of the wild-type allele was confirmed by PCR. A adoptive T transfer and FACScan\textsuperscript{a} analysis. Preparation and adoptive transfer of OT-I cells, 5,6-carboxy-succinimidyl-fluorescein-ester (CFSE)-labeling, and analysis on a FACScan\textsuperscript{a} (Becton Dickinson, Mountain View, CA) were carried out as previously described (4). In adoptive transfer experiments, OT-I cells were identified in recipient mice by staining with FITC-conjugated anti-V\textsubscript{a}2 (B20.1), PE-conjugated CD8 (Caltag Labs., San Francisco, CA), and anti-V\textsubscript{b}5 (b) biotin-conjugated (MR9-40) reagents and analyzed with Streptavidin-Tricolor (Caltag Labs.). An average of 1.4% of CD8\(^+\) cells were V\textsubscript{a}2\(-\)V\textsubscript{b}5\(+\) in uninjectected mice. The total number of OT-I cells was derived using the formula: (V\textsubscript{a}2\(-\)V\textsubscript{b}5\(+\) cells in the CD8\(^+\) cells – 1.4%) \(\times\) (CD8\(^+\) cells in live cells) \(\times\) (number of live cells) as previously described (4). Anti-V\textsubscript{a}2 TCR (B20.1) and anti-V\textsubscript{b}5.1/2 TCR mAbs were prepared from hybridoma supernatants and conjugated to biotin or to FITC using standard protocols. Dead cells were excluded by propidium iodide. Biotinylated anti-CD95 (Jo2) was from Pharmingen (San Diego, CA).

Bone marrow chimeras. RIP-mOVA mice expressing the MHC class I molecule H-2K\(^b\) on bone marrow–derived cells, and H-2K\(^bm1\) on non–bone marrow–derived tissue cells (B6–RIP-mOVA mice) were generated by injecting 1–4 \(\times\) 10\(^6\) fetal liver cells from B6 embryos at days 14–16 after gestation into 900 cGy irradiated RIP-mOVA mice backcrossed to the bm1 haplotype. The next day, radioresistant T cells were depleted with T24 (anti-Thy-1) antibodies intraperitoneally. As TNFR2 \(2/2\) mice were generated in 129.SV-Tcr (129) mice, RIP-mOVA bm1 mice were reconstituted with fetal liver cells from B6 \(\times\) 129F1 embryos to prevent rejection of adoptively transferred OT-I.TNFR2 \(2/2\) cells carrying murine strain 129 minor histocompatibility determinants.

**Results**

Deletion of OT-I Cells Is Mediated by CD95 but Not TNFR2. We have previously shown that when OVA-specific CD8\(^+\) T cells from the OT-I transgenic line (OT-I cells) were adoptively transferred into RIP-mOVA mice, which express OVA in the pancreatic islets and other tissues, these cells were activated and proliferated in the draining lymph nodes of OVA-expressing tissues (3), but were deleted as a result of this process (4). The deletion of CD8\(^+\) T cells induced by cross-presentation had originally been demonstrated by following the fate of OT-I cells adoptively transferred into B6→RIP-mOVA.bm1 bone marrow chimeras (4). These chimeras expressed the MHC class I molecule K\(^b\) on their bone marrow compartment, and K\(^bm1\) on all other tissues. This provided the advantage that only the bone marrow compartment could present OVA to CD8\(^+\) T cells, allowing examination of the effect of cross-presentation in the absence of direct presentation by tissue cells expressing this antigen. Also, it allowed for the transfer of large numbers of OT-I cells, which was necessary for monitoring their survival by flow cytometry but would otherwise have led to diabetes if islet \(\beta\) cells were able to directly present OVA (4).

To explore the roles of CD95 and TNFR2 in the deletion of CD8\(^+\) T cells, OT-I mice were crossed to either CD95-deficient B6.lpr mice, or to mice lacking TNFR2 (24). Survival of these cells was examined 6 wk after adoptive transfer into B6→RIP-mOVA.bm1 chimeras. For OT-I.TNFR2 \(2/2\) cells, it was necessary to use (B6 \(\times\) 129)F1 bone marrow, since TNFR2-deficient mice contained minor antigens of 129 origin that would otherwise lead to rejection of OT-I.TNFR2 \(2/2\) cells. These experiments revealed that deletion of OT-I cells did not require TNFR2, since TNFR2-deficient OT-I cells, like wild-type OT-I cells, were effectively deleted (Fig. 1). In contrast, CD95 signaling was necessary for the deletion process, since OT-I.lpr cells were not deleted but increased in numbers relative to those transferred into nontransgenic littermates (Fig. 2). These cells generated effective CTLs in vivo in response to antigen, indicating that they were not anergized in vivo (data not shown). These results led to the conclusion that CD95 signaling was necessary for the deletion of autoreactive CD8\(^+\) T cells.

The Initial Proliferation of OVA-specific CD8\(^+\) T Cells (OT-I Cells) Induced by Cross-presentation Is Not Affected by a Deficiency in CD95. Figure 1. The deletion of OT-I cells induced by cross-presentation is independent of TNFR2. Fetal liver cells from B6 \(\times\) 129F1 embryos were grafted into irradiated RIP-mOVA bm1 mice and nontransgenic littermates. 6 wk later, 6 \(\times\) 10\(^4\) OT-I cells or OT-I.TNFR2 \(2/2\) cells were adoptively transferred, and after a further 6 wk the number of remaining OT-I cells in the LNs and spleen was determined by flow cytometry. These results are representative of two such experiments.
activation of OT-I cells preceding their deletion. To investigate whether CD95 or TNFR2 affected the proliferation of OT-I cells induced by cross-presentation, CFSE-labeled OT-I, OT-I.lpr, or OT-I.TNFR2^2/2 cells were adoptively transferred into RIP-mOVA mice. CFSE-labeling allows visualization of cellular proliferation by detecting dilution of the fluorescent dye by flow-cytometry, with each cell cycle resulting in a halving of fluorescence intensity. This technique has been used to compare the in vivo proliferative responses of T cells under different conditions (25). The CFSE profiles of OT-I.lpr and OT-I.TNFR2^2/2 cells in the renal LNs of RIP-mOVA mice were similar to those of wild-type OT-I cells (Fig. 3), demonstrating an equivalent proliferative response.

Upregulation of CD95 on OT-I Cells Activated by Cross-presentation. These results suggested that the deletion of OT-I cells induced by cross-presentation was mediated by CD95. This molecule is constitutively expressed on CD8^+^ T cells but is upregulated upon TCR-mediated activation in vitro (8–12). To investigate whether CD95 is also upregulated after activation by cross-presentation in vivo, the kinetics of CD95-expression on OT-I cells was investigated on OT-I cells proliferating in the renal LNs of RIP-mOVA mice. This was achieved by comparing CD95 expression on CFSE-labeled OT-I cells that had undergone 0–7 cell cycles, separately. Indeed, CD95 expression increased with the number of cell cycles completed (Fig. 4), suggesting that one way cross-presentation renders CD8^+^ T cells susceptible to CD95-mediated signals is through upregulation of this receptor. Such upregulation of CD95 was not seen on OT-I.lpr cells under the same conditions (data not shown).

Discussion

The key role of CD95 in T cell tolerance became evident from the lymphoproliferative disease in lpr and gld mice, which carry mutations of the CD95 and CD95L...
CD95 has been shown to play a pivotal role in the deletion of autoreactive B cells (34) and CD4^+ T cells (6, 18, 20, 21), and now we demonstrate a role for this molecule in the control of autoreactive CD8^+ T cells. This contrasts with the observation that deletion of CD8^+ T cells during viral infection did not depend on CD95 (22, 23), suggesting that a different homeostatic mechanism operates under these conditions. Likewise, after challenge with a foreign antigen, deletion of transgenic CD4^+ T cells was not dependent on CD95 (21). This latter study concluded that in contrast to the control of autoreactive T cells, the down-regulation of T cell numbers at the end of a response to foreign antigens is controlled by mechanisms other than CD95, such as expression of genes of the b2-2 family. Survival genes of this family are downregulated when activated T cells cease to receive antigenic or costimulatory signals (35, 36), resulting in death by neglect. This demarcation between b2-2-mediated “passive” downregulation after clearing foreign antigens and autoantigen-driven CD95-mediated “active” control of autoreactive T cells is supported by studies showing that these two molecules are involved in different intracellular apoptotic mechanisms (5).

At present, although foreign and self-antigens have been suggested to induce sensitivity to different death pathways (21), it is unclear what antigenic property is responsible for this switch. Given that only a few antigens have been tested thus far, it remains possible that the alternative outcomes could simply be due to quantitative differences in antigen dose or location (rather than to foreign versus self). However, the lack of CD95-sensitivity in response to ubiquitous antigens derived from a viral pathogen (22, 23) versus the sensitivity to this pathway when ubiquitous self-antigen is available (21) (at least for CD4^+ T cells) suggests that qualitative differences may be important. One such difference may be the availability of inflammatory signals associated with infections, which may induce resistance to CD95-mediated signaling (37).

In conclusion, we have demonstrated that CD95 plays an important role in the deletion of autoreactive CD8^+ T cells induced by cross-presentation of self-antigens, demonstrating a further role for CD95 in the maintenance of self-tolerance.

We thank Dr. Mark Moore (Genentech, South San Francisco, CA) for the use of his TNFR2 knockout mice; Dr. Andreas Strasser and Dr. Michael Lenardo for helpful discussions; and Tatiana Banjanin and Paula Nathan for their technical assistance.

C. Kurts is supported by a fellowship from the Deutsche Forschungsgemeinschaft (Grant Ku1063/1-2). This work was funded by the National Institutes of Health grant AI-29385 and grants from the National Health and Medical Research Council of Australia and the Australian Research Council.

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