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

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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.*lpr* mice (The Jackson Laboratory, Bar Harbor, ME) were crossed to OT-I mice. TNFR2 gene disruption and the presence of the *lpr* mutation were confirmed by PCR.

Adoptive Transfer and FACS® Analysis. Preparation and adoptive transfer of OT-I cells, 5,6-carboxy-succinimidyl-fluorescein-ester (CFSE)-labeling, and analysis on a FACScan® (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-Vα2 (B20.1), PE-conjugated CD8 (Caltag Labs., So. San Francisco, CA), and anti-$V\beta5^+$ biotin-conjugated (MR9-40) revealed with Streptavidin-Tricolor (Caltag Labs.). An average of 1.4% of $CD8^+$ cells were $V\alpha2^+V\beta5^+$ in uninjected mice. The total number of OT-I cells was derived using the formula: (% $V\alpha2^+V\beta5^+$ cells in the $CD8^+$ cells -1.4%) $\times$ (% $CD8^+$ T cells in live cells) $\times$ (number of live cells) as previously described (4). Anti-Vα2 TCR (B20.1) and anti-Vβ5.1/2 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.bm1) 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) ascites intraperitoneally. As $TNFR2^{-/-}$ mice were generated in 129.SV-Ter (129) mice, RIP-mOVA.bm1 mice were reconstituted with fetal liver cells from (B6 $\times$ 129)F1 embryos to prevent rejection of adoptively transferred OT-I.$TNFR2^{-/-}$ 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 β 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.$TNFR2^{-/-}$ 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.$TNFR2^{-/-}$ 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 vitro 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 either CD95 or TNFR2. There are two main possibilities to account for the lack of deletion of OT-I.*lpr* cells in RIP-mOVA mice: either CD95 was required directly for the deletion signal, or else it participated in the initial

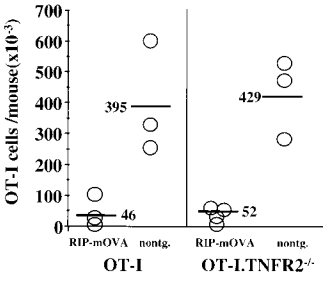

Figure 1. The deletion of OT-I cells induced by cross-presentation is independent of TNFR2. Fetal liver cells from (B6 $\times$ 129)F1 embryos were grafted into irradiated RIP-mOVA.bm1 mice and nontransgenic littermates. 6 wk later, 6 $\times$ 10^6 OT-I cells or OT-I.$TNFR2^{-/-}$ cells were adoptively 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.

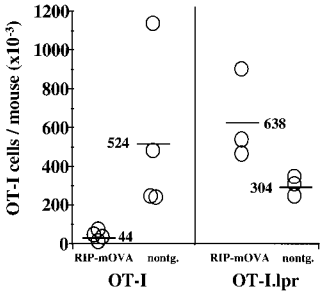

Figure 2. The deletion of OT-I cells induced by cross-presentation is mediated by CD95. Bone marrow from B6 mice was grafted into irradiated RIP-mOVA.bm1 mice and nontransgenic littermates. 22 wk later, 5×10^6 OT-I cells or OT-I.*lpr* cells were adoptively transferred, and after a further 6 wk the number 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$^{-/-}$ 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$^{-/-}$ 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).

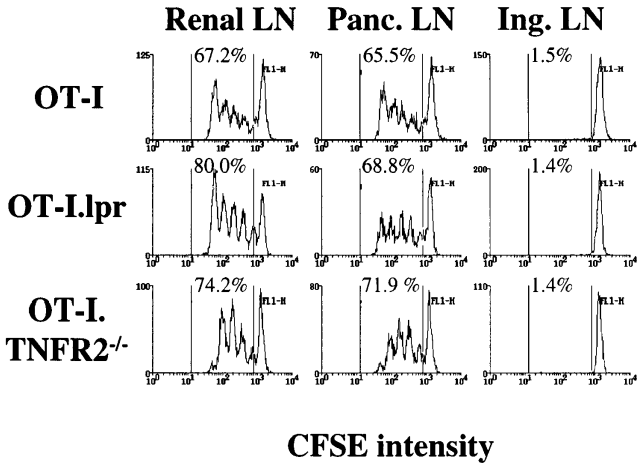

Figure 3. Influence of CD95 and TNFR2 on the proliferation of OT-I cells in vivo. 2×10^6 CFSE-labeled OT-I (*top row*), OT-I.*lpr* (*center row*), and OT-I.TNFR2$^{-/-}$ (*bottom row*) cells were adoptively transferred into RIP-mOVA mice. After 52 h, lymphocytes from the renal (*left column*), pancreatic (*center column*), and nondraining inguinal LNs (*right column*) were analyzed by flow cytometry. Profiles were gated on CFSE$^+$ CD8$^+$PI$^-$ cells. The numbers indicate the percentage of OT-I cells that had proliferated in vivo. These results are representative of four such experiments.

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

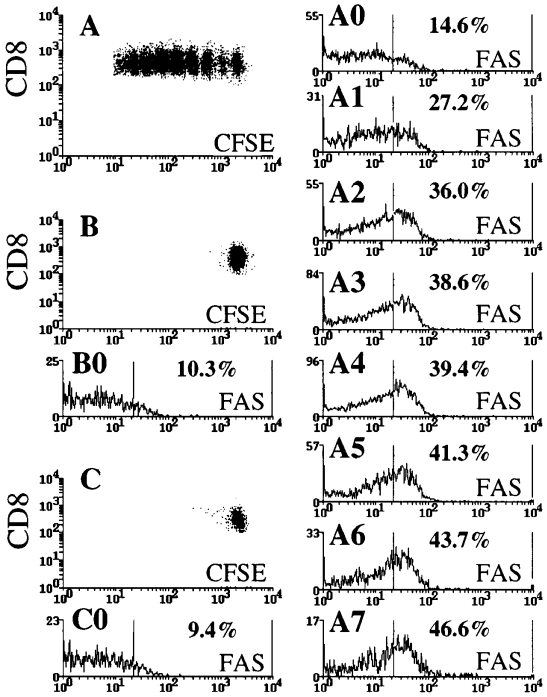

Figure 4. Upregulation of CD95 on OT-I cells activated by cross-presentation. 3×10^6 CFSE-labeled OT-I RAG$^{-/-}$ cells were injected into RIP-mOVA mice. After 3 d, the levels of CD95 expression on OT-I cells that had undergone different numbers of cell cycles in the renal LN was determined. These were identified in a FACS® dot-plot showing CFSE versus CD8-PE fluorescence (*A*). The histograms show the expression of CD95 on the undivided cells (*A0*), cells that had divided once (*A1*), cells that had undergone 2 divisions (*A2*), and so on up to seven divisions (*A7*). A marker was set arbitrarily to allow comparison of CD95 expression. The numbers indicate the percentage of OT-I cells that expressed a CD95 level higher than this marker. Dot-plot *B* shows undivided OT-I cells from the inguinal LN of the same RIP-mOVA recipient and histogram *B0* shows their CD95 expression. Dot-plot *C* shows OT-I cells in the renal LN of a nontransgenic recipient, and histogram *C0* shows their CD95 expression. These results are representative of three such experiments.

genes, incapacitating these molecules (13, 14). In vitro studies further demonstrated that CD95 can transduce a death signal to activated T cells (26, 27). Activated $CD4^+$ T cells or T cell hybridomas were resistant to anti-CD3 or superantigen-induced AICD when CD95 was blocked or when T cells from *lpr* mice were used (8–12). These results were verified in vivo by showing that superantigen-induced T cell apoptosis was severely retarded in *lpr* mice (5, 28). The crucial role of CD95 in controlling autoreactive $CD4^+$ T cells was demonstrated in TCR transgenic models. In these studies, lack of CD95-function prevented AICD of TCR transgenic $CD4^+$ T cells in the cytochrome *c* model (6, 20) or when hen egg lysozyme was expressed as a model autoantigen (21). However, in mice expressing a hemagglutinin-specific TCR, blocking of TNF together with CD95 prevented deletion induced by antigen injection more profoundly than did blocking of CD95 alone (18). This suggested an additional role for TNF in mediating peripheral deletion of $CD4^+$ T cells. For $CD8^+$ T cells, in vitro studies suggested that TNF, primarily via TNFR2, played a more important role than CD95 for inducing apoptosis (19, 29). Consistent with this idea, deletion induced in vivo by viral infections was CD95 independent (22, 23), and peptide-induced deletion of transgenic $CD8^+$ T cells was diminished in TNFR1 knockout mice (30).

A possible role for TNFR2 in the peripheral deletion of T cells had originally been considered unlikely, since TNFR2 lacks the death-inducing domain present in TNFR1 and CD95, and because TNFR2-knockout mice did not show lymphoproliferative disease (24), which had been reported to be MHC class I dependent (31). Furthermore, there was in vitro evidence for a costimulatory role of TNFR2 signaling in the activation of T cells (32, 33).

The roles of CD95 and TNFR2 in the peripheral deletion of autoreactive $CD8^+$ T cells had not been extensively investigated in vivo. We addressed this question using the transgenic RIP-mOVA model in which cross-presentation of a self-antigen induces deletion of autoreactive $CD8^+$ T cells. Our results showed that this form of peripheral tolerance was mediated by CD95, and not by TNFR2. These results demonstrate that CD95 can control autoreactive $CD8^+$ T cells, unveiling a new role for CD95 in the maintenance of peripheral T cell tolerance. Furthermore, the CD95 molecule itself was upregulated on the surface of OT-I cells in vivo, suggesting that this may be linked to rendering OT-I cells more receptive to CD95-induced AICD. This in vivo upregulation of CD95 confirms previous in vitro studies demonstrating TCR-mediated induction of CD95-expression on the surface of $CD4^+$ T cell hybridomas or lines (8–12).

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 downregulation of T cells numbers at the end of a response to foreign antigens is controlled by mechanisms other than CD95, such as expression of genes of the *bcl-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 *bcl-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.

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