CD4+ T Cell Help Impairs CD8+ T Cell Deletion Induced by Cross-presentation of Self-Antigens and Favors Autoimmunity

By Christian Kurts,* Francis R. Carbone,‡ Megan Barnden,‡ Effrossini Blanas,* Janette Allison,* William R. Heath,* and Jacques F.A.P. Miller*

*From the *Immunology Division, The Walter and Eliza Hall Institute of Medical Research, Victoria 3050; and ‡The Department of Pathology and Immunology, Monash Medical School, Prahran 3181, Victoria, Australia*

Summary

Self-antigens expressed in extrathymic tissues such as the pancreas can be transported to draining lymph nodes and presented in a class I–restricted manner by bone marrow-derived antigen-presenting cells. Such cross-presentation of self-antigens leads to CD8+ T cell tolerance induction via deletion. In this report, we investigate the influence of CD4+ T cell help on this process. Small numbers of autoreactive OVA-specific CD8+ T cells were unable to cause diabetes when adoptively transferred into mice expressing ovalbumin in the pancreatic β cells. Coinjection of OVA-specific CD4+ helper T cells, however, led to diabetes in a large proportion of mice (68%), suggesting that provision of help favored induction of autoimmunity. Analysis of the fate of CD8+ T cells indicated that CD4+ T cell help impaired their deletion. These data indicate that control of such help is critical for the maintenance of CD8+ T cell tolerance induced by cross-presentation.

There is now considerable evidence that CD8+ T cell responses can be induced in vivo by professional APCs capable of MHC class I–restricted presentation of exogenous antigens (1–3). This mechanism is known as cross-presentation and was suggested to be instrumental in the immune response to pathogens that avoid professional APCs (2–4). However, if this pathway was only directed towards induction of immunity, cross-presentation of self-antigens to autoreactive CD8+ T cells would result in autoimmunity. Recently, in studies using transgenic mice that express a membrane-bound form of OVA under the control of the rat insulin promoter (RIP-mOVA), we have shown that this is not the case. RIP-mOVA mice express membrane-bound OVA in pancreatic islets, kidney proximal tubular cells, thymus and testis. In these mice, OVA was found to enter the class I presentation pathway of a bone marrow–derived cell population and then activate transgenic OVA-specific CD8+ T cells (OT-I cells) (3) in LNs draining the sites of antigen expression. Although this form of activation initially led to proliferation of OT-I cells, it ultimately caused their deletion (5). Thus, cross-presentation can remove autoreactive CD8+ T cells, and may tolerize the CD8+ T cell compartment to self-antigens. These studies, however, did not explain why cross-presentation of a self-antigen induced CD8+ T cell tolerance, whereas foreign antigens induced immunity (1–4, 6).

In numerous models, CD4+ T cell help has been shown to be important for the induction or maintenance of immune responses by CD8+ T cells (7–11), but such help is not always essential (12–14). CD4+ T cell help has also been shown to be important for avoiding CTL tolerance induction (15–17). In these reports, however, it was not known whether CD8+ T cells were activated by cross-presentation or by direct recognition of antigen. Thus, whether CD4+ T cell help can affect tolerance induced by cross-presentation has not been addressed. Recently, we demonstrated that cross-priming by foreign antigens requires CD4+ T cell help for induction of CTL immunity (6). In this study, we have investigated the influence of such help on 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-II (18), OT-I RAG-1 and RIP-mOVA transgenic mice (3) have been described. Bone marrow chimeras were generated as described (5).

Adoptive Transfer and Flow Cytometry. Preparation and 5,6-carboxy-succinimidyl-fluoresceine-ester (CFSE)-labeling of OT-I and OT-II cells, as well as analysis on a FACScan® (Becton Dickinson and Co., Mountain View, CA) were carried out as de-

Table 1. *Autoimmune Diabetes in RIP-mOVA Mice Depends on the Number of Adoptively Transferred OT-I Cells*

No. of OT-I cells transferred	No. mice diabetic/ total No. mice (%)	
10×10^6	3/3	(100%)
5×10^6	18/18	(100%)
2×10^6	9/10	(90%)
1×10^6	4/8	(50%)
0.5×10^6	1/12	(8%)
0.25×10^6	0/26	(0%)
0.10×10^6	0/7	(0%)

Various numbers of $V\alpha2^+$ $V\beta5^+$ $CD8^+$ cells prepared from OT-I $RAG^{-/-}$ mice were injected i.v. into unirradiated RIP-mOVA mice between 6 and 8 wk of age. These recipients were monitored daily for glucosuria. Mice that did not develop diabetes were monitored for at least 25 d and 5 recipients of 0.25×10^6 OT-I cells for 210 d. These data have been accumulated over six experiments.

scribed (3, 5, 19). The following mAbs were used for immunostaining: PE conjugated anti-CD8 (YTS 169.4) and anti-CD4 (YTS 191.1) from Caltag Labs. (San Francisco, CA); anti-Vα2 TCR (B20.1) and anti-Vβ5.1/2 TCR (MR9-4).

Immunohistology. Frozen tissue sections were stained with anti-CD8 supernatant (53.6.72 hybridoma), anti-CD4 ascites (H129.19 hybridoma) or with anti-Vα2 supernatant (B20.1), followed by anti-rat Ig-horseradish peroxidase (Chemicon, Temecula, CA) and DAB (Sigma) as described (20). Slides were counterstained with hematoxylin.

Results

High Numbers of OT-I Cells Induce Diabetes in RIP-mOVA Mice. In previous studies, OT-I cells adoptively transferred into RIP-mOVA mice were shown to be activated by cross-presentation of OVA in the draining LNs of OVA-expressing tissues (3). When a relatively large number of OT-I cells (5×10^6) was transferred, autoimmune diabetes was observed within 5–8 d (5). To investigate the dose response for induction of autoimmunity, various numbers of OT-I cells were transferred into RIP-mOVA mice (Table 1). $5–10 \times 10^6$ OT-I cells caused 100% of mice to develop diabetes. 50% of the recipients became diabetic when 1×10^6 OT-I cells were transferred, and 0.25×10^6 cells failed to cause disease in any of 26 recipients. Nevertheless, even with small numbers of OT-I cells transferred, their activation and proliferation occurred in the draining LNs (data not shown).

Histological examination revealed that on day 5 after adoptive transfer of 5×10^6 OT-I cells, the pancreatic islets of RIP-mOVA recipients were densely infiltrated with $CD8^+$ T cells (Fig. 1 *A*) and with $CD4^+$ T cells, B cells, and MAC-1–positive cells, which were of host origin (data not shown). Recipients of 1×10^6 OT-I cells that did not develop diabetes showed some $CD8^+$ T cells within the islets on day 8 (Fig. 1 *C*), whereas recipients of less OT-I cells showed only sporadic islet infiltration (data not shown).

Coinjection of OT-II Cells Can Induce Autoimmune Diabetes in RIP-mOVA Recipients of a Low Number of OT-I Cells. Both the $CD4^+$ and $CD8^+$ T cell compartments of RIP-mOVA mice were tolerant to OVA as measured by the ability to provide carrier-specific help (21), and to generate
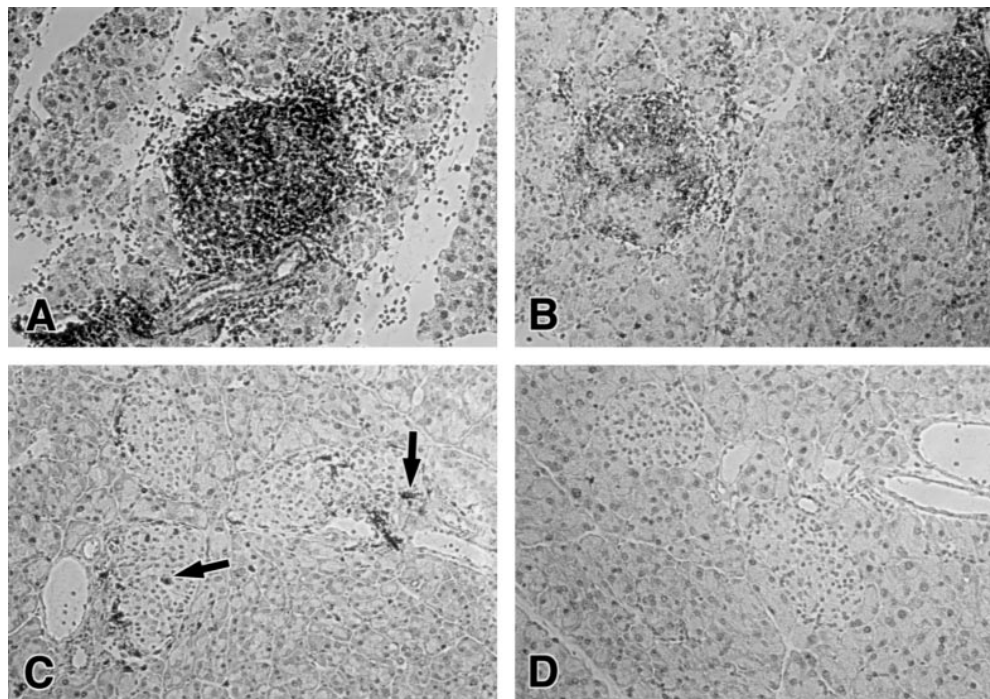
Figure 1. Islet infiltration in RIP-mOVA recipients of OT-I and OT-II cells. The following numbers of OT-I and OT-II cells were adoptively transferred into unirradiated RIP-mOVA mice. (*A*) 5×10^6 OT-I cells; (*B*) 0.25×10^6 OT-I cells + 2×10^6 OT-II cells; (*C*) 1×10^6 OT-I cells; (*D*) 10×10^6 OT-II cells. Sections of the pancreas were stained 5 (*A*, *B*, and *D*) or 8 d (*C*) after adoptive transfer for CD8 (*A–C*) or CD4 (*D*). Arrows in *C* indicate infiltrating $CD8^+$ cells.

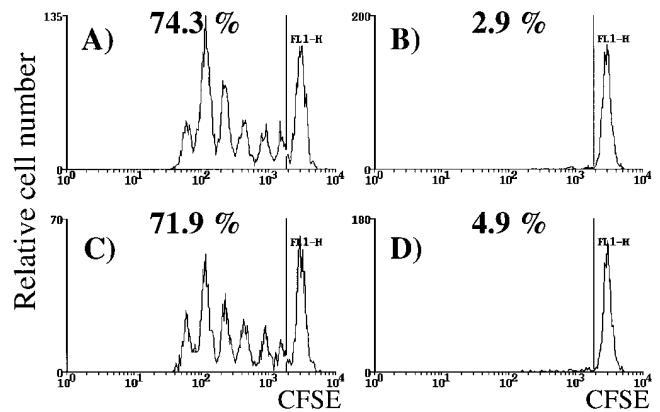**Table 2.** *Incidence of Diabetes after Cotransfer of OT-I and OT-II Cells*

No. cells transferred		No. mice diabetic/
OT-I	OT-II	total No. mice (%)
5×10^6	–	18/18 (100%)
0.25×10^6	–	0/26 (0%)
0.25×10^6	2×10^6	15/22 (68%)
–	2×10^6	0/12 (0%)
–	10×10^6	0/3 (0%)

0.25×10^6 OT-I cells were transferred either alone or together with 2×10^6 OT-II cells into sex matched RIP-mOVA mice of 6–8 wk of age. Coinjected recipients developed diabetes between day 6 and 10 after adoptive transfer and were sacrificed for histological verification of diabetes after a further 5–10 d. Mice which did not become diabetic were followed for 60–210 d. Shown are the results collected from 5 coinjection experiments with groups of 3–5 mice. The first two rows of this table were included from Table 1 for comparison.

Figure 2. OT-II cells do not enhance proliferation of OT-I cells in the draining LNs. 2×10^6 CFSE-labeled OT-I cells were adoptively transferred into littermate RIP-mOVA mice alone (*A* and *B*) or together with 2.5×10^6 OT-II cells (*C* and *D*). After 52 h, lymphocytes from the renal (*A* and *C*) and inguinal (*B* and *D*) LNs were analyzed by flow cytometry. Profiles were gated on CFSE^{+} CD8^{+} cells. The numbers indicate the percentage of OT-I cells that had proliferated in vivo. These results are representative of five experiments.

OVA-specific CTL (6), respectively (data not shown). This tolerance was most likely due to aberrant expression of mOVA in the thymus, as previously reported (3). Consequently, neither CD4^{+} nor CD8^{+} T cells specific for OVA were present in these mice prior to adoptive transfer.

To determine whether CD4^{+} T cell help could affect the response of OT-I cells, such help was provided by coinjection of CD4^{+} T cells from OT-II transgenic mice (OT-II cells), which produce H-2A[b]–restricted T helper cells specific for OVA. When RIP-mOVA mice were coinjected with 2×10^6 OT-II CD4^{+} cells and a low number of OT-I CD8^{+} cells (0.25×10^6), approximately two-thirds of the recipients developed diabetes (Table 2). 5 d after adoptive transfer, islets were densely infiltrated by CD8^{+} T cells (Fig. 1 *B*). It is important to note that OT-II cells alone, even when injected at a higher dose (10×10^6 cells), were unable to cause islet infiltration (Fig. 1 *D*) or autoimmune diabetes (Table 2). Thus, the availability of CD4^{+} T cell help enhanced the response of OT-I cells, suggesting that such help may direct a normally tolerogenic CD8^{+} T cell response (with low doses of OT-I cells) towards autoimmunity, as measured by immunopathology.

CD4^{+} T Cell Help Prolongs the Lifespan of CD8^{+} T Cells Specific for Cross-presented Autoantigens. We then investigated whether OT-II cells achieved this effect by increasing the initial expansion of OT-I cells, or by impairing their deletion. To examine the first possibility, CFSE-labeled OT-I cells were adoptively transferred into RIP-mOVA mice in the presence or absence of coinjected OT-II cells. This technique enables monitoring of proliferation by detecting cells with 2^n-fold reduced fluorescence intensity, where n is the number of cell cycles (5, 19). 2 d after adoptive transfer, we compared the CFSE fluorescence profiles of OT-I cells from the draining LNs of the kidney (Fig. 2) or pancreas

(data not shown). 2×10^6 OT-II cells did not cause an obvious change in the proliferative peaks in these sites. No changes were observed with smaller numbers of CFSE-labeled OT-I cells (0.5×10^6), or at later time points (data not shown). These results suggested that the rate of OT-I cell cycling was not affected by OT-II cell help.

An alternative possibility was that CD4^{+} T cell help increased the survival of OT-I cells. To test this, it was necessary to generate chimeric mice that would not become diabetic when given OT-I cells. This could be achieved by back-crossing RIP-mOVA mice to the bm1 haplotype, which expresses the K[bm1] molecule instead of K[b] and cannot present OVA to OT-I cells. By lethally irradiating and reconstituting these mice with B6 bone marrow, chimeric mice were generated that could present OVA on their bone marrow–derived cells, but not on peripheral tissues. These bone marrow chimeras were previously used to show that cross-presentation of self-antigen can lead to the deletion of autoreactive CD8^{+} T cells (5). To determine whether CD4^{+} T cell help could affect CD8^{+} T cell survival, these B6→RIP-mOVA.bm1 chimeric mice and B6→littermate.bm1 controls were injected with 5×10^6 OT-I cells either alone or together with 2×10^6 OT-II cells. After 6 wk, mice were sacrificed and the total number of OT-I cells in the spleen and LNs was assessed (Fig. 3). In 9 of 10 transgenic recipients of OT-II cells, this number clearly exceeded that in mice given OT-I cells alone, suggesting that CD4^{+} T cell help improved the survival of CD8^{+} T cells stimulated by cross-presentation. Islet infiltration or diabetes did not develop in B6→RIP-mOVA.bm1 chimeric mice coinjected with OT-I and OT-II cells (data not shown) indicating that induction of diabetes was dependent on class I–restricted antigen recognition on islet β cells.
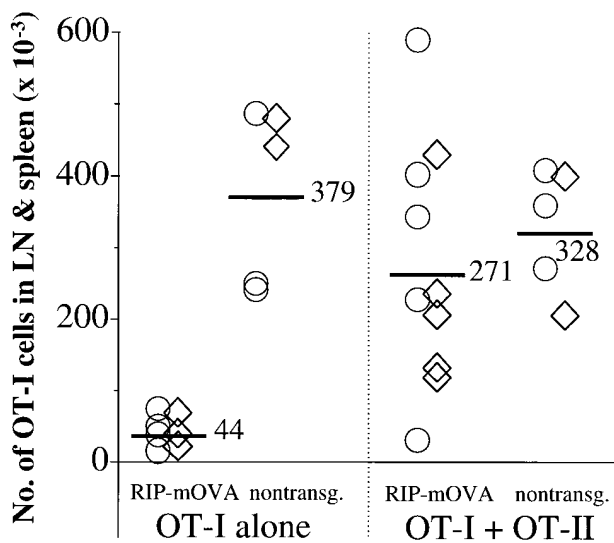
Figure 3. OT-II cells impair the deletion of OT-I cells activated by cross-presentation. Bone marrow from B6 mice was grafted into irradiated RIP-mOVA.bm1 mice and nontransgenic littermates. 12 wk later, 5×10^6 OT-I cells $\pm 2 \times 10^6$ OT-II cells were adoptively transferred, and after 6 wk the number of $V\alpha2^+$ $V\beta5^+$ $CD8^+$ cells in the LNs and spleen of the recipients was determined by flow cytometry. 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). The figure shows the results of two experiments ($\bigcirc$, $\diamondsuit$). The numbers and bars indicate the average number of OT-I cells in mice from both experiments. Injection of OT-II cells alone did not alter the proportion of host $CD4^-$ $V\alpha2^+$ $V\beta5^+$ cells.

Discussion

In this study, we have used the transgenic RIP-mOVA model to investigate the influence of $CD4^+$ T cell help on the fate of autoreactive $CD8^+$ T cells activated by cross-presentation of self-antigens. We have previously shown that naive OT-I cells, when adoptively transferred into RIP-mOVA mice, were activated by cross-presentation in LNs draining OVA-expressing tissues (3). After an initial expansion phase, this form of activation ultimately led to the deletion of OT-I cells (5), and may thus represent a mechanism that maintains $CD8^+$ T cell tolerance to self. This conclusion highlighted the difference between cross-presentation of self versus foreign antigens, the latter generally inducing immunity (1–4, 6). Here, we demonstrate that OT-I cells activated by cross-presentation of self-antigens can induce islet β cell destruction. This could occur even in the absence of $CD4^+$ T cell help, but only with a relatively large dose of OT-I cells (5×10^6). Transfer of such a high number of autoreactive $CD8^+$ T cells allowed the initial wave of expansion to generate sufficient CTL to destroy all pancreatic β cells, before the CTL could be deleted. When the number of OT-I cells was lowered, they were deleted before inducing diabetes. Thus, when precursor frequencies are low, the dynamic process of proliferation versus deletion is biased towards the latter, particularly in the absence of $CD4^+$ T cell help. Introduction of such help, provided by coin-

jecting OT-II cells, allowed low doses of OT-I cells to induce diabetes. This enhancing effect of OT-II cells was not exerted by increasing the proliferative rate of OT-I cells. Our results also argue against the interpretation that OT-I cells stimulated OT-II cells to destroy pancreatic β cells, because the induction of diabetes depended on MHC class I–restricted antigen recognition in islet cells, and because mouse islet β cells normally do not express MHC class II molecules (22). The mechanism supported by our findings is that $CD4^+$ T cell help enhanced the survival of activated $CD8^+$ T cells, allowing them more time to exert effector functions, possibly due to the induction of survival genes like *bcl-xL*. Thus, the balance between proliferation and deletion of autoreactive $CD8^+$ T cells can be shifted towards an immunogenic response if $CD4^+$ T cell help is available. This interpretation does not exclude other factors influencing this balance, such as inflammatory signals supplied by pathogens or material released from dying cells. In the present model, however, OT-II cells were able to shift the balance in favor of autoimmunity in the absence of any detectable inflammatory signals.

Previous studies investigating the influence of $CD4^+$ T cell help on CTL tolerance have used foreign antigens. For example, responses to Qa1 and H-Y (16) were tolerogenic in the absence of $CD4^+$ T cell help. Furthermore, survival of H-Y–specific transgenic T cells, which normally proliferate and then die in response to priming by male spleen cells, was increased by providing a stimulus for $CD4^+$ T cells (17). This was consistent with our current observations, suggesting that $CD4^+$ T cell help is not necessary for the initial proliferative response, but for the survival of subsequently produced $CD8^+$ T cells. Perhaps, this also explains why $CD8^+$ T cell immunity to many viral infections (11, 12, 14), but not $CD8^+$ T cell memory (10, 11) can be induced in the absence of $CD4^+$ T cell help.

The present study does not elucidate whether $CD4^+$ T cell help was provided at the site of antigen-expression, e.g., in the pancreatic islets, or in the draining LNs. However, we have recently reported that generation of OVA-specific CTL by cross-priming, in another model, required cognate $CD4^+$ T cell help (6). Thus, $CD4^+$ and $CD8^+$ T cells had to recognize antigen on the same APC in order to generate CTL immunity. In the present model it is not known whether cognate antigen recognition was required. If so, OT-II cells might influence the inductive phase of the autoreactive response by activating the cross-presenting APC such that it provided immunogenic rather than tolerogenic signals to $CD8^+$ T cells. This may be mediated by signals like CD40-L, which induces the upregulation of costimulatory molecules such as B7 (23, 24) and the synthesis of IL-12 by the APC (25). Alternatively, $CD4^+$ T cells may supply cytokines, such as IL-2, that enhance the expansion and/or survival of $CD8^+$ T cells (17). Consistent with the view that $CD4^+$ T cell help is required during priming, $CD8^+$ T cell memory to H-Y persisted in the absence of $CD4^+$ T cell help, provided such help was available during the initial priming phase (26).

The ability of antigen expressed on non-lymphoid tissues

to cross-prime naive CD8^{+} T cells in the draining LNs might provide a plausible mechanism whereby infected cells and perhaps even malignant cells that lack the molecular machinery for direct immune induction, could prime CD8^{+} T cells (1–4). This, however, raises the question of how could CD8^{+} T cells distinguish between viral antigens expressed on virus-infected tissue cells, which they must kill, and self-antigens on normal tissue cells, which they must leave intact. As the availability of CD4^{+} T cell help appears to be responsible for dictating whether the CD8^{+} T cell response is immunogenic or tolerogenic, it is possible that the immune system controls autoreactive CD8^{+} T cells by limiting the availability of CD4^{+} T cell help (16). That is, in contrast to the situation with pathogens, the CD4^{+} T cell repertoire might normally be tolerized to self-antigens. Thus, CD4^{+} T-cell help would not be available for self-reactive CD8^{+} T cell responses, resulting in the deletion of this population. In the present model, the CD4^{+} T cell compartment was tolerant to OVA because of thymic negative selection. Future studies will address mechanisms of peripheral CD4^{+} tolerance to self-antigens that are not expressed in the thymus.

In conclusion, we have shown that, in the presence of CD4^{+} T cell help, CD8^{+} T cell deletion induced by cross-presentation of self-antigens is diminished, leading to autoimmunity. Thus, by limiting such help, the immune system can maintain peripheral CD8^{+} T cell tolerance to self.

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