Major Histocompatibility Complex Class I–restricted Cross-presentation Is Biased towards High Dose Antigens and Those Released during Cellular Destruction

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Summary

Naive T cells recirculate mainly within the secondary lymphoid compartment, but once activated they can enter peripheral tissues and perform effector functions. To activate naive T cells, foreign antigens must traffic from the site of infection to the draining lymph nodes, where they can be presented by professional antigen presenting cells. For major histocompatibility complex class I–restricted presentation to CD8^{+} T cells, this can occur via the cross-presentation pathway. Here, we investigated the conditions allowing antigen access to this pathway. We show that the level of antigen expressed by peripheral tissues must be relatively high to facilitate cross-presentation to naive CD8^{+} T cells. Below this level, peripheral antigens did not stimulate by cross-presentation and were ignored by naive CD8^{+} T cells, although they could sensitize tissue cells for destruction by activated cytotoxic T lymphocytes (CTLs). Interestingly, CTL-mediated tissue destruction facilitated cross-presentation of low dose antigens for activation of naive CD8^{+} T cells. This represents the first in vivo evidence that cellular destruction can enhance access of exogenous antigens to the cross-presentation pathway. These data indicate that the cross-presentation pathway focuses on high dose antigens and those released during tissue destruction.

Key words: CD8^{+} T lymphocytes • T cell activation • antigen presentation • antigen presenting cells • apoptosis

CD8^{+} CTLs, which recognize antigens presented by class I molecules encoded by the MHC, are important for immunity to viruses and other intracellular pathogens. Access to the class I–restricted presentation pathway generally requires that protein antigens are expressed within the cytoplasmic compartment of the presenting cell (1). This pathway has been described as the endogenous pathway, contrasting it with the class II–restricted pathway, which generally presents exogenous (extracellular) antigens.

Despite the apparent demarcation between the class I– and class II–restricted pathways, Bevan demonstrated over 20 years ago that under certain circumstances exogenous antigens can access the class I–restricted pathway (2). Such "cross-presentation" has now been observed in many tolerogenic as well as immunogenic responses (2–14). It has been proposed that the role of cross-presentation is to protect against tissue-tropic viruses that, in the absence of infection of professional APCs, would not access the class I–restricted pathway of these cells, and would therefore fail to prime naive CTLs (15, 16). In this model, the advantage of the cross-priming APC is that it can capture antigens from virus-infected tissues, process them into the class I pathway, and present them to naive CTLs in the draining LNs.

Recently, we used transgenic expression of the model antigen OVA to provide direct evidence that peripheral tissue antigens can be transported to draining LNs for class I–restricted presentation by a bone marrow–derived APC (10). In this earlier study, transgenic mice expressing membrane-bound OVA (mOVA) under the control of the rat insulin promoter (RIP) in the pancreas, kidney, and thymus were injected with class I–restricted, OVA-specific, CD8^{+} T cells from the OT-I transgenic line. When responses were assessed several days later, OT-I cells had proliferated and accumulated in nodes draining OVA-expressing tissues. Furthermore, antigen was presented by a bone marrow–derived cell, implying that tissue antigens were captured and then

processed by a professional APC (the cross-presenting APC).

In this report, we examined the requirements for antigen entry into the cross-presentation pathway, using various transgenic mice expressing the model antigen OVA at different levels in the pancreas. Our studies show that only relatively high dose antigens constitutively gain effective entry into this pathway, but that cellular destruction may enhance access of low dose antigens. The implications of these findings with respect to immunity and tolerance are discussed.

Materials and Methods

Mice. All mice were bred and maintained at the Walter and Eliza Hall Institute for Medical Research. OT-I, RIP-mOVA, and RIP-OVA[lo] transgenic mice have been described previously (10, 17). Note that RIP-OVA[lo] mice were referred to previously as RIP-OVA mice (17). RIP-OVA[hi] mice were generated using the same techniques.

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 described previously (11, 18, 19). PE-conjugated anti-CD8 (YTS 169.4) was from Caltag Labs (South San Francisco, CA). Dead cells were excluded by propidium iodide staining.

Activation of OT-I Cells In Vitro. OT-I cells were activated by culturing 10^7 OT-I spleen cells for 5 d in 30 ml of medium with 10^8 irradiated (1,500 cGy) B6 spleen cells previously coated for 30 min at 5×10^7 cell/ml with 0.2 μg/ml OVA$_{257-264}$ peptide. Stimulator cells were washed once after peptide pulsing. Culture medium consisted of mouse tonicity RPMI1640, 10% fetal calf serum, 2 mM glutamine, 5×10^{-5} M 2-ME, and antibiotics.

Results

Antigen Dose Affects Cross-presentation. To examine the role of antigen dose in cross-presentation of tissue antigens, two lines of transgenic mice were produced expressing different amounts of secreted OVA in the β cells of the pancreas, under the control of the RIP. These were termed RIP-OVA[lo] and RIP-OVA[hi] mice. RIP-OVA[hi] mice expressed sufficient OVA to show weak OVA-specific staining immunohistologically, whereas OVA expression by RIP-OVA[lo] mice could not be detected by this technique (Fig. 1). However, expression of OVA by the latter line has been shown previously using alternative approaches (reference 17 and see Fig. 3).

To investigate the effect of antigen dose on the access of peripheral tissue antigens to the cross-presentation pathway, naive CD8^{+} cells from the OT-I cell line were labeled with the fluorescent dye CFSE (11, 18, 19) and injected intravenously into both RIP-OVA^{lo} and RIP-OVA[hi] mice. 3 d later, the pancreatic (draining) and inguinal (nondraining) LNs were examined for proliferating OT-I cells. When CFSE-labeled cells proliferate, their fluorescence is equally distributed between daughter cells resulting in a 2^n-fold reduction in fluorescence intensity, where n is the number of
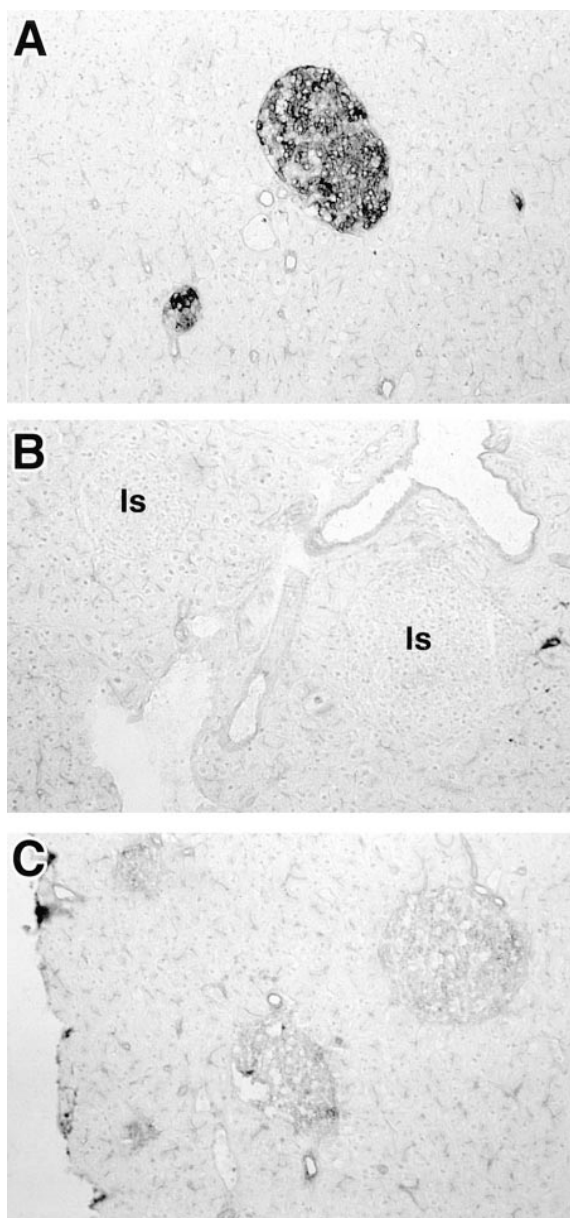

Figure 1. OVA expression by pancreatic islets of transgenic mice. RIP-mOVA mice (*A*), RIP-OVA[lo] mice (*B*), and RIP-OVA[hi] mice (*C*) were stained for OVA using a rabbit anti-OVA antiserum, obtained by immunizing rabbits with whole OVA (Grade V; Sigma Chemical Co., St. Louis, MO) and horseradish peroxidase–conjugated swine anti–rabbit Ig (DAKO, A/S, Glostrup, Denmark) as previously described (10). Note that secreted OVA was very difficult to detect immunohistologically and could only be demonstrated in the RIP-OVA[hi] line. RIP-mOVA mice, which express a membrane-bound form of OVA in their islet, were included as a positive control to show that this technique efficiently detects OVA when it is not secreted.

cell divisions. Adoptive transfer of CFSE-labeled OT-I cells revealed that only RIP-OVA[hi] mice were able to supply sufficient OVA to the cross-presentation pathway for stimulation of naive OT-I cells in the pancreatic draining LN (Fig. 2). These findings indicated that antigen expres-
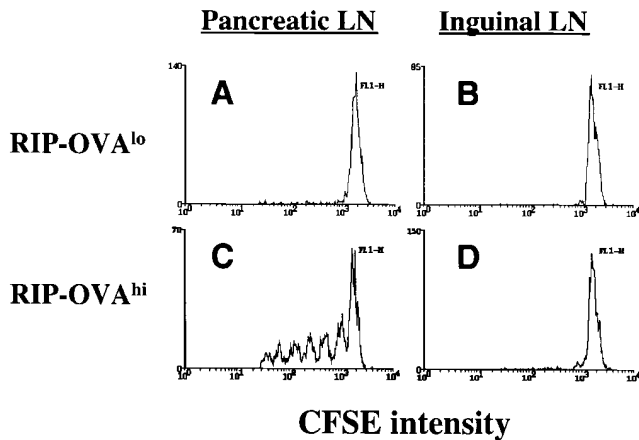
Pancreatic LN   **Inguinal LN**

Figure 2. Secreted OVA is cross-presented in the draining LNs of the pancreas when expressed at a sufficiently high dose. 5×10^6 OT-I cells were labeled with CFSE (11) and adoptively transferred intravenously into either RIP-OVA^{lo} mice (*A* and *B*), RIP-OVA^{hi} mice (*C* and *D*), or negative littermates (data not shown). 3 d later, the pancreatic (*A* and *C*) and inguinal (*B* and *D*) LN cells were analyzed by flow cytometry (11). As previously reported (11), negative littermates did not induce proliferation of OT-I cells (data not shown). Histograms were gated on CD8$^+$CFSE$^+$ cells.

Figure 3. Activated OT-I cells cause diabetes when adoptively transferred into RIP-OVA^{lo} mice or RIP-OVA^{hi} mice. OT-I cells were activated with antigen in vitro for 5 d and then 10^7 cells were adoptively transferred intravenously into adult RIP-OVA^{lo} (*solid line*) or RIP-OVA^{hi} (*dashed line*) mice. These mice were then monitored daily for induction of diabetes as measured by glucosuria. Islet destruction was confirmed by histological examination of pancreatic tissue sections from several of these mice. These data represent the compilation of five experiments.

sion above a critical level was required for antigen access to the cross-presentation pathway. Neither line of mice became diabetic when 5×10^6 naive OT-I cells were adoptively transferred (data not shown).

Lower Levels of Tissue Antigen Expression that Do Not Facilitate Cross-presentation May Still Be of Physiological Relevance. To determine whether the lower antigen dose expressed by RIP-OVA^{lo} mice was of physiological relevance, we asked whether activated CTLs, which have the capacity to enter tissues, could recognize tissue cells expressing this low dose of OVA. RIP-OVA^{lo} mice were injected with 10^7 activated OT-I cells (generated by stimulation with antigen for 5 d in vitro) and then examined for the onset of diabetes (Fig. 3). As a control, RIP-OVA^{hi} mice were also injected with activated cells. Diabetes induction in the RIP-OVA^{lo} mice indicated that the low level of OVA expressed by these mice was sufficient to target islet β cells for recognition and destruction by activated CTLs. Thus, although only high doses of OVA stimulated naive CTLs via the cross-presenting pathway, lower doses were able to target peripheral tissues for destruction by activated CTLs.

Tissue Destruction Facilitates Cross-presentation of Low Dose Antigens. If, as proposed, the cross-presentation pathway is important for virus immunity, then it makes sense to focus on high dose antigens, since virus proteins are likely to be produced in relatively large amounts during replicative infections. An alternative way to bias the immune system towards detecting virus antigens would be to preferentially present antigens released during tissue destruction. With this in mind, we asked whether destruction of β cells expressing low doses of OVA would improve antigen access to the cross-presentation pathway. RIP-OVA^{lo} mice were injected intravenously with in vitro–activated OT-I cells
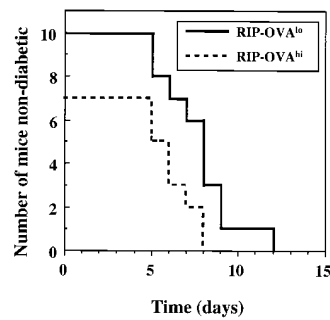
and then 4 d later with CFSE-labeled naive OT-I cells or B6 CD8$^+$ T cells. The activated OT-I cells were used to specifically cause islet destruction (as shown by the induction of diabetes in Fig. 3), and the CFSE-labeled cells were used to measure the response of naive cells to antigen (OVA) cross-presented in the draining LNs. 3 d after the transfer of CFSE-labeled cells (that is, 7 d after the transfer of activated CTLs), the LNs of these mice were recovered and examined for proliferating cells (Fig. 4). As shown, naive CFSE-labeled OT-I cells proliferated in the pancreatic (draining) LNs of RIP-OVA^{lo} mice that were previously injected with activated CTLs (Fig. 4, *C* and *D*), but not in untreated mice (Fig. 4, *A* and *B*), even if left for up to 8 d in vivo (data not shown). By contrast, CFSE-labeled B6 CD8$^+$ T cells did not proliferate in the pancreatic LNs in either the presence (Fig. 4, *E* and *F*) or absence (data not shown) of activated CTLs. Thus, an antigen-specific response of OT-I cells was observed after the destruction of β cells by activated CTLs. When proliferation was examined in bm1→RIP-mOVA.B6 bone marrow chimeras, where the bone marrow compartment expressed an inappropriate MHC haplotype for presentation of OVA to OT-I cells (10), proliferation was abolished (data not shown). This latter point indicates that a bone marrow–derived APC was responsible for capturing and cross-presenting OVA released by cellular destruction.

To examine the kinetics of cross-presentation after cellular destruction, RIP-OVA^{lo} mice were treated with activated CTLs to cause islet damage and then injected with CFSE-labeled naive OT-I cells either 1 or 4 d later. In previous studies we had shown that it takes 24 h for OT-I cells to begin proliferating after exposure to cross-presented antigen (11). Thus, we allowed the CFSE-labeled naive T cells to remain in these mice for a total of 3 d after transfer to ensure that there was enough time to adequately detect proliferation. Mice were killed and their pancreatic (draining) and inguinal (nondraining) LNs examined for proliferating OT-I cells (Fig. 4). No proliferation was seen in the pancreatic LNs from mice receiving CSFE-labeled cells on day 1 after initiation of β cell damage (Fig. 4, *G* and *H*), whereas considerable proliferation was evident in the day 4 group (Fig. 4, *C* and *D*). From these experiments, we can
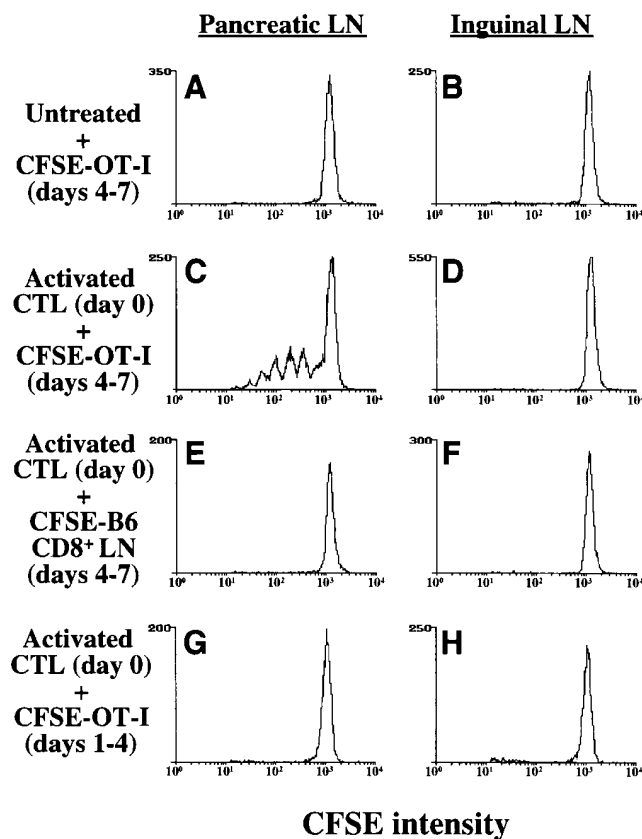
Figure 4. Tissue destruction facilitates cross-presentation of low dose antigens. RIP-OVA^{lo} mice were either left untreated (*A* and *B*) or injected with 5×10^6 OT-I cells activated as in Fig. 3 (*C–H*). 1 (*G* and *H*) or 4 d (*A–F*) later, mice were injected with CFSE-labeled cells (either 5×10^6 naive OT-I cells [*A–D, G,* and *H*] or 2×10^6 B6 CD8$^+$ LN cells [*E* and *F*]). After a further 3 d, the pancreatic (*A, C, E,* and *G*) and inguinal (*B, D, F,* and *H*) LN cells were analyzed by flow cytometry. Histograms were gated on CD8$^+$CFSE$^+$ cells.

roughly estimate the minimum time necessary for antigen to appear in LNs after OT-I cell-induced β cell damage. Simply, since it takes at least 24 h for the cells to start proliferating after exposure to cross-presented antigen (11), then antigen must reach the LNs no earlier than day 3 after induction of tissue damage based on the lack of proliferation found in Fig. 4 *G*, and no later than day 6 given the proliferation shown in Fig. 4 *C*.

Discussion

As previously reported, antigens expressed in peripheral tissues can be captured by bone marrow–derived APCs, processed via the cross-presentation pathway, and then presented in the draining LNs to naive CD8$^+$ T cells (10). In this report, we show that peripheral tissue antigens must be expressed at relatively high levels to be cross-presented constitutively. When antigens were expressed at high doses, as in RIP-OVA^{hi} mice (Fig. 2), they were cross-presented in the draining LNs and caused activation and proliferation of naive CD8$^+$ T cells. Antigen expression levels

below a certain threshold, as seen in RIP-OVA^{lo} mice, were ignored by naive CD8$^+$ T cells, which failed to proliferate in the draining LNs (Fig. 2).

Here, we also show that antigen access to the cross-presentation pathway can result after cellular destruction by CTL (Fig. 4). How CTL lysis allows antigen entry into this pathway is unknown, but it has recently been reported that apoptotic cells can be captured and presented by myeloid dendritic cells (20). This raises the possibility that apoptosis of target cells by activated CTLs may facilitate access to the cross-presentation pathway.

One of the critical observations of this report is that although lower dose antigens were unable to stimulate via the cross-presentation pathway, they still directly sensitized tissue (islet) cells, in which they were expressed, for recognition by activated CTLs. However, the direct recognition pathway is only available for recognition by activated CTLs, since only activated CTLs will have the capacity to leave the secondary lymphoid compartment and interact with these tissues. Thus, there appear to be three categories into which peripheral tissue antigens fall: category 1, those that are expressed at high levels and can be both cross-presented to naive CTLs and directly seen by activated CTLs; category 2, those that are expressed at lower concentrations and are nonstimulatory via the cross-presentation pathway, but can sensitize peripheral tissue cells for direct recognition by activated CTLs; and category 3, those antigens that can never be seen by CTLs.

The importance of these categories becomes apparent when we consider the physiological roles of the cross-presentation pathway. Recently, we provided evidence that cross-presentation of self-antigens leads to peripheral deletion of autoreactive CD8$^+$ T cells (11). We showed that adoptively transferred OT-I cells were activated by cross-presented tissue antigens in RIP-mOVA mice, leading to proliferation and then deletion of the responding OT-I cells. If, as we have shown (11), self-tolerance is a consequence of cross-presentation of self-antigens, then only those antigens expressed at levels sufficiently high to be cross-presented will induce such tolerance. Thus, the idea that peripheral antigens are ignored (21–23), may only apply to antigens that do not stimulate by cross-presentation, i.e., category 2 antigens. High dose self-antigens (category 1) would induce deletional tolerance via the cross-presentation pathway, whereas those lower dose antigens in category 2 would be ignored by naive CTLs but still able to sensitize peripheral tissues for recognition by activated CTLs. Perhaps this explains why some model self-antigens expressed by islet β cells were reported to induce tolerance (11, 24, 25), whereas others were ignored (21–23). Analysis of the effect of antigen dose on peripheral tolerance will be the subject of a later report (Kurts, C., J.F.A.P. Miller, F.R. Carbone, and W.R. Heath, manuscript in preparation).

The tolerance induced by cross-presented self-antigens contrasts with the immunity seen when foreign antigens were used to induce CTLs by cross-priming (2–5, 7–9, 13, 14). What determines whether tolerance or immunity is

induced is unclear, but CD4$^+$ T cell help appears to be important (12, 14, 26). Since the cross-presentation pathway is involved in both cross-tolerance and cross-priming, the mechanisms by which antigens gain access to this pathway is likely to affect both aspects. The focus of this pathway on high dose antigens may direct CTLs immunity towards cells producing large amounts of viral proteins, whereas the ability to recognize antigens associated with tissue destruction may be important for responses to cytopathic viruses.

In summary, this report provides the first in vivo evidence that cellular destruction can enhance access of peripheral antigens to the cross-presentation pathway. In addition, we show that high dose antigens are preferentially cross-presented, although lower doses may still be of physiological relevance by their ability to be directly presented to activated CTLs entering peripheral tissues. Together, these data lead to the conclusion that the cross-presentation pathway is focused on high dose antigens or those released during cellular destruction.

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