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Cell-Associated Ovalbumin Is Cross-Presented Much More Efficiently than Soluble Ovalbumin In Vivo

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To better understand the antigenic requirements for cross-presentation, we compared the in vivo efficiency of presentation of cell-associated vs soluble OVA with the OT-I (CD8) and OT-II (CD4) TCR transgenic lines. Cross-presentation of cell-associated OVA was very efficient, requiring as little as 21 ng of OVA to activate OT-II cells and 100-fold less to activate OT-I cells. In contrast, soluble OVA was presented inefficiently, requiring at least 10,000 ng OVA for activation of either T cell subset. Thus, cell-associated OVA was presented 500-fold more efficiently than soluble OVA to CD4 T cells and 50,000-fold more efficiently to CD8 T cells. These data, which represent the first quantitative in vivo analysis of cross-presentation, show that cell-associated OVA is very efficiently presented via the class I pathway.


While most cells of the body cannot present exogenous Ags in their class I presentation pathway, there exist specialized APCs that are capable of capturing such Ags and presenting them on both class I and class II MHC molecules (1–3). The capacity to capture and indirectly present class I- and class II-restricted Ags from other cells has been referred to as cross-presentation. This pathway is accessible to various cell-associated Ags, and efficiently presents apoptotic cellular material (4–6). Cross-presentation has been reported to be involved in the induction of CTL immunity to various Ags, including minor histocompatibility Ags tumor-associated Ags, and viral Ags, a process referred to as cross-priming (1). Cross-presentation has also been reported to be responsible for presentation of self Ags, leading to tolerance induction by deletion or anergy (called cross-tolerance) (7–9). We and others have shown that like cross-priming, cross-tolerance is mediated by a bone marrow-derived APC that is able to capture exogenous cellular Ags and present them in the class I and class II pathways (9–11). Recently, the cross-presentation of cell-associated Ags has been reported to belong to the CD8+ subset of splenic dendritic cells (12). Whether this same subset is responsible for cross-tolerance is yet to be determined.

To examine the efficiency of cross-presentation of self Ags to CD4 and CD8 T cells, we generated several transgenic lines expressing different amounts of OVA under the control of the rat insulin promoter (RIP)3 (13, 14). Comparison of the activation of OVA-specific transgenic CD8 T cells (OT-I cells) and CD4 T cells (OT-II cells) revealed that it was somewhat more difficult to activate OT-II cells by OVA expressed in the pancreas. This raised the question of whether there was a bias for presentation of cell-associated vs soluble Ags in the class I and class II pathways. This study examines the efficiency of presentation of soluble vs cell-associated Ag in the class I and class II presentation pathways to OT-I and OT-II cells, respectively. We provide evidence that cell-associated Ags are very efficiently presented in both pathways. The implications of these findings with respect to presentation of self Ags are discussed.

Materials and Methods

Mice

All mice were used between 6 and 12 wk old and were bred and maintained at the Walter and Eliza Hall Institute for Medical Research. OT-II mice of the 425-2 line (15), and Rag-1−/− OT-I mice (16) have been previously described. Because OT-II mice possess very few T cells when crossed to Rag-1−/− mice, these mice were used on a Rag-1 wild-type background. I-mOVA mice on a C57BL/6 (B6) background were generated as described (G. M. Davey, M. Li, C. Hirst, F. R. Carbone, and W. R. Heath, manuscript in preparation). Briefly, I-mOVA transgenic mice express a membrane-anchored form of OVA under the control of the class I promoter. The TIR.OVA fragment encoding a fusion protein consisting of the transmembrane anchor of the transferrin receptor and residues 149–385 from OVA was isolated from the pSVT7-TfR.OVA plasmid (17) by EcoRI digestion and cloned into the BamHI site of the pES4 vector (18) by blunt end ligation. Vector sequence was removed from the resultant plasmid (pES4-TIR.OVA) by Clal/NotI digestion, and the purified insert was injected into B1o oocyte to generate the I-mOVA transgenic line. These mice were then crossed to B6 mice twice to generate H-2b mice.

Priming of mice

For priming mice with cell-associated OVA (19), syngeneic bm1 spleen cells (1–2 × 106/ml) were incubated with 10 mg/ml (or other concentrations, as stated in Results) OVA for 10 min at 37°C. Cells were then washed twice, irradiated 1000 cGy, and injected into mice. Usually, 20 × 106 cells were injected, but in some cases this number was varied, as indicated in Results. bm1 spleen cells were used to ensure that there was no direct class I-restricted presentation of OVA, but similar results were obtained with B6 spleen cells. For priming mice with soluble protein, OVA was dissolved in PBS and then injected (0.5 ml/mouse).

Abbreviations used in this paper: RIP, rat insulin promoter; mOVA, membrane-anchored OVA; int, intermediate.
Preparation of responder T cells

OT-I and OT-II cells were prepared as described (10, 11). Briefly, OT-I cells were derived from the spleen and lymph nodes of Rag-1-deficient OT-I mice. Cell suspensions were treated with RL172 (anti-CD4) and J11d (anti-HSA) for 30 min on ice, centrifuged, and then depleted by treatment with rabbit complement for 25–30 min at 37°C. For OT-II cells, lymph nodes were removed from OT-II mice and single-cell suspensions were treated with 3.168 (anti-CD8) and J11d and then complement as above. A total of $2 \times 10^6 \text{CD}^4^+ \text{V}o\text{a}^2^+ \text{(OT-II) or CD}^8^- \text{V}o\text{a}^2^+ \text{(OT-I) cells was adoptively transferred into each recipient. Purified cell populations were 70}^-85\% \text{CD}^8^- \text{V}o\text{a}^2^+ \text{from OT-I mice and 60}^-70\% \text{CD}^4^- \text{V}o\text{a}^2^+ \text{from OT-II mice.}

Fluorescent labeling of T cells

5-(and 4-carboxyfluorescein diacetate, succinimidyl ester (CFSE) labeling was performed as previously described (20). Briefly, semipurified T cells (OT-I or OT-II) were resuspended in PBS containing 0.1% BSA (Sigma, St. Louis, MO) at $10^7$ cells/ml. For fluorescence labeling, 1 μl of a CFSE (Molecular Probes, Eugene, OR) stock solution (5 mM in DMSO) was incubated with $10^7$ cells for 10 min at 37°C.

Flow cytometry

Before adoptive transfer, or culture in vitro, OT-I and OT-II cell preparations were analyzed by flow cytometry using a FACScan (Becton Dickinson, Mountain View, CA), as previously described (10, 11).

Quantitation of OVA on OVA-coated spleen cells and in islet β cells

Quantitation of OVA per mg of islet β cell protein was achieved as previously described (13).

The amount of OVA associated with OVA-coated spleen cells was quantitated by capture ELISA. Briefly, after OVA coating at $2 \times 10^6$ cells/ml, spleen cells were washed three times in HEM, then lysed in buffer containing 1% Triton X-100 and proteinase inhibitors (21). The soluble fraction was recovered after centrifugation at 15,000 × g. For the ELISA, microtiter plates (Dynatech, Chantilly, VA) were coated with protein A-purified rabbit anti-OVA antiserum (22) at 4 μg/ml in PBS overnight at 4°C, and washed six times with PBS/0.05% Tween 20. Serial dilutions of test lysates were incubated on the plates overnight at 4°C. Similarly, serial dilutions of OVA protein (Sigma) were used as standards. Plates were washed again, then mouse anti-OVA antiserum (23) at 1:1000 in PBS/5% skim milk was added as the secondary antiserum for 3 h at room temperature. Plates were washed and incubated for 3 h at room temperature with HRP-conjugated rabbit anti-mouse IgG (Southern Biotechnology, Birmingham, AL), then washed before addition of a 3,3',5,5'-tetramethylbenzidine substrate solution. The reaction was stopped with 0.5 M H$_2$SO$_4$ and the OD read at 450 nm. Data were analyzed using KC Jr. software (Biotek Instruments, Winooski, VT).

Results

Response of OT-I to cell-associated OVA in vivo

Intravenous injection of spleen cells coated with OVA for 10 min at 37°C can induce an OVA-specific CTL response (19). This response is CD4 T cell dependent, and requires cross-presentation of donor Ags on host APC (19, 24). To determine the amount of Ag required to activate CD8 T cells by OVA-coated spleen cells, CFSE-labeled transgenic OT-I (CD8$^+$) cells were transferred into B6 mice that were primed 1 day later with spleen cells coated with different amounts of OVA. Three days after priming, recipients were sacrificed and their spleen cells were analyzed by flow cytometry (Fig. 1, top row). This revealed that OT-I cells could proliferate in response to cell-associated OVA when the coating concentration was as low as 0.01 mg/ml.

Quantitation of OVA on OVA-coated spleen cells

To determine the amount of OVA associated with the injected spleen cells, protein was isolated from Triton X-100-solubilized cells and quantitated by ELISA. In duplicate samples, coating with OVA at 10 mg/ml yielded 160 and 162 ng OVA per $2 \times 10^7$ cells (average, 161 ng), and coating with OVA at 1 mg/ml yielded 18 and 24 ng OVA per $2 \times 10^5$ cells (average, 21 ng). This meant that the minimum amount of cell-associated OVA required to activate OT-I cells was ~0.2 ng/mouse.

Soluble OVA is not presented as efficiently as cell-associated OVA for recognition by OT-I cells

The above data indicated that very small amounts of cell-associated OVA were able to stimulate OT-I cells. This observation contrasts the generally held belief that exogenous proteins have poor access to the class I pathway. Perhaps this was related to the fact that we used cell-associated OVA, which may behave very differently from traditionally examined soluble protein Ags (19). To address this issue, mice were injected with soluble OVA i.v. at various doses and then the proliferation of CFSE-labeled OT-I cells was examined (Fig. 2, top row). While soluble OVA was able to stimulate OT-I cells, it required a minimum dose of 10 μg per mouse, 50,000-fold more Ag than needed to stimulate when in a cell-associated form (Fig. 1, top row).

One explanation for the poor response to soluble OVA relative to cell-associated OVA could be that the former did not supply any type of inflammatory stimulus. Thus, the difference in the sensitivity of proliferative responses may reflect differences in the co-stimulatory state of the APC. To test this possibility, various doses of soluble OVA were injected i.v. with 1 μg of LPS, and then the

![Figure 1: Response of OT-I and OT-II cells to spleen cells coated with various concentrations of soluble OVA.](http://www.jimmunol.org/)

![Figure 2: Response of OT-I and OT-II cells to soluble OVA injected i.v.](http://www.jimmunol.org/)
proliferative response of OT-I cells was examined. The presence of LPS did not enhance the dose response of OT-I cells (data not shown).

**Response of OT-II cells to cell-associated or soluble OVA in vivo**

To date, we had examined the class I-restricted response to cell-associated vs soluble OVA. To gain understanding of the class II-restricted response to these forms of OVA, CFSE-labeled transgenic OT-II (CD4+ T cells were transferred into B6 mice that were primed the next day with titrated amounts of OVA-coated spleen cells (Fig. 1, bottom row) or soluble OVA (Fig. 2, bottom row). Three days after priming, recipients were sacrificed and their spleen cells were analyzed by flow cytometry. This revealed OT-II cells required a minimum concentration of 1 mg/ml OVA during spleen cells were analyzed by flow cytometry. This revealed OT-II row. Three days after priming, recipients were sacrificed and their spleen cells were analyzed by flow cytometry. This revealed OT-II cells required a minimum concentration of 1 mg/ml OVA during spleen cell coating (Fig. 1, bottom row), which translates to approximately 21 ng OVA associated with the injected cells. This was 500-fold less than the 10 μg of soluble OVA required to activate OT-II cells (Fig. 2, bottom row).

These data indicated that both OT-I and OT-II cells were more sensitive to cell-associated OVA than to soluble OVA, with a 50,000-fold increase in sensitivity for OT-I cells and a 500-fold increase in sensitivity for OT-II cells. Furthermore, they indicated that while soluble OVA equally stimulated OT-I and OT-II cells, cell-associated OVA was approximately 100-fold more efficient at activating OT-I cells.

We have previously reported that cross-priming with cell-associated OVA requires CD4 T cell help (24), raising the question of why OT-I cells were able to proliferate to doses of cell-associated OVA that were too low to stimulate OT-II help. This is most likely explained by the observation that high frequencies of CD8 T cells, such as those used in our adoptive transfer experiments, can be cross-primed in the absence of CD4 T cell help (unpublished data).

**Are the sensitivities of OT-I and OT-II cells similar?**

One explanation for the greater sensitivity of OT-I cells to cross-presented cell-associated OVA could be that these cells are of greater sensitivity to Ag than OT-II cells. In an attempt to compare their sensitivities, we examined the proliferative response of OT-I and OT-II cells to peptide in vivo and in vitro. In vivo, OT-II cells required only a little more peptide than OT-I cells for activation (Fig. 3). Interestingly, however, when examined in proliferative responses in vitro, OT-I cells were approximately 500-fold more responsive than the OT-II cells to peptide stimulation (data not shown). The differences between these two conditions highlight the limitations in comparing these two T cell subsets.

**Are OT-I cells more sensitive than OT-II cells to other forms of cell-associated OVA?**

To determine whether the biased sensitivity of OT-I cells over OT-II cells in response to in vivo stimulation with OVA-coated spleen cells (Fig. 1) was a general outcome when cell-associated OVA was used, we examined the response of OT-I and OT-II cells to spleen cells transgenically expressing OVA. To do this, mice expressing membrane-bound form of OVA (mOVA) under the control of the class I promoter were generated (I-mOVA mice). Irradiated spleen cells from these mice were then used as a source of cell-associated OVA to stimulate mice containing CFSE-labeled OT-I or OT-II cells (Fig. 4). While OT-I cells proliferated in response to I-mOVA spleen cells, OT-II cells were unresponsive to this level of transgenically expressed mOVA. Thus, even when OVA was expressed as a cellular Ag, it still preferentially stimulated OT-I cells. To check that the I-mOVA mice produced the class II-restricted determinant, CFSE-labeled OT-II cells were injected into these mice, and then proliferation was examined 2 days later. This showed strong proliferation of OT-II cells, indicating the presence of the class II determinant in these mice (data not shown).

**Presentation of self Ags to the T cell subsets**

The data outlined above suggested that soluble OVA was presented much less efficiently than cell-associated OVA (to both OT-I and OT-II cells). Furthermore, they indicated that while soluble OVA was presented equivalently to OT-I and OT-II cells (Fig. 2), cell-associated OVA was more efficiently presented to OT-I cells (Fig. 1). Knowledge of this pattern of relative responses to different forms of Ag allowed us to ask about the form of cross-presented Ag when soluble OVA was expressed as a model self Ag in the pancreas. If OVA was simply secreted and presented in the draining node, then it should equally stimulate both OT-I and OT-II. If, however, it was captured in a cell-associated form, then we might expect to see biased presentation to OT-I cells. To address this issue, we examined the response of OT-I and OT-II cells in transgenic lines expressing OVA under the control of the RIP. We have three transgenic lines that express different amounts of secreted OVA: RIP-OVAhigh mice, which express 1 μg/mg islet tissue (13); RIP-OVAint mice, which express 0.04 μg/mg islet tissue; and RIP-OVAlow mice, which express less than 0.03 μg/mg (13).

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**FIGURE 3.** Response of OT-I and OT-II cells to different amounts of their cognate peptide in vivo. Mice were injected with CFSE-labeled transgenic OT-I or OT-II T cells, and then 1 day later primed i.v. with the appropriate peptide: OVA257-264 for OT-I cells and OVA323-339 for OT-II cells. Three days later, these mice were sacrificed, and their spleen cells were analyzed by flow cytometry. Profiles are gated on CD8+ or CD4+, CFSE+, propidium iodide-negative cells.

**FIGURE 4.** Response of OT-I and OT-II cells to priming with spleen cells expressing mOVA. Spleen cells from mice expressing mOVA under the control of the class I promoter (I-mOVA mice) were irradiated and used to prime B6 mice injected 1 day prior with CFSE-labeled OT-I or OT-II cells. Three days later, these mice were sacrificed, and their spleen cells were analyzed by flow cytometry. Profiles are gated on CD8+ or CD4+, CFSE+, propidium iodide-negative cells.
These mice were injected with CFSE-labeled OT-I or OT-II T cells, and then 3 days later their pancreatic lymph nodes were harvested and single cells were examined by flow cytometry (Fig. 5). This revealed that OT-I cells proliferated in both the RIP-OVA-high and RIP-OVA-int lines, whereas OT-II cells proliferated only in the RIP-OVA-high line. Thus, cross-presentation of transgenically expressed tissue-specific OVA favored the activation of OT-I cells.

Discussion

Although numerous mechanisms have been proposed for the entry of exogenous Ags into the class I pathway (reviewed in Ref. 3), it is unclear how cell-associated Ags gain access during cross-presentation. Perhaps the most exciting prospect is that apoptotic cells are targeted by the cross-presenting APC (5, 6, 14, 25). Since not all cases of cross-presentation are associated with obvious apoptosis however (7), alternative routes are likely. In support of this idea, necrotic cells were shown to be cross-presented on class II molecules (4), indicating that apoptotic cells are not the only targets.

In this study, we examined the efficiency of cross-presentation of cell-associated OVA by using OT-I and OT-II transgenic T cells to detect class I- and class II-restricted presentation, respectively. We provided evidence that cell-associated OVA was presented very efficiently into both the class I and class II pathways. Compared with free soluble OVA, cell-associated OVA was presented 500-fold more efficiently to CD4 T cells and 50,000-fold more efficiently to CD8 T cells.

It should be stressed that we have no way to determine whether the response of OT-I or OT-II cells reflects the strength of the response, or the ability to prime normal naive OVA-specific CD8 or CD4 T cells. Thus, we cannot draw general conclusions about the comparative response of normal CD4 versus CD8 T cells from our data. What can be compared is the relative efficiency of class I-restricted presentation for the different forms of Ag (cell associated, soluble, and pancreatic), and the same applies to class II-restricted presentation. Thus, while a 100-fold difference in the comparative response of normal CD4 versus CD8 T cells is unclear how cell-associated Ags gain access during cross-presentation in vitro, in which dendritic cells were shown to efficiently present cell-associated class II molecules (4), but our study represents the first quantitative analysis in vivo for either pathway. These data support the idea that cell-associated Ags are specifically targeted for presentation by the immune system. This suggests that the immune system is dedicated to capturing and presenting cellular material, presumably because CTL immunity is only important for responses to such Ags.

We also examined the responsiveness of OT-I and OT-II cells to tissue-specific OVA expressed in the pancreatic islet β cells under the control of the RIP. In these experiments, we had three lines of mice expressing whole, secreted OVA at different concentrations. This allowed us to show that cross-presentation of tissue-expressed OVA was biased toward activation of OT-I cells over OT-II cells. It is important to stress that even though the transgenically expressed OVA was a secreted form, the phenotype of the T cell responses was similar to that seen for the cell-associated OVA of OVA-coated spleen cells, which was biased toward activation of OT-I cells over OT-II cells. Given that soluble OVA was presented equally well to both OT-I and OT-II cells (Fig. 2), these data imply that even though OVA was expressed in its secreted form in the pancreas of RIP-OVA mice, it was presented as if it were cell associated with biased activation of OT-I cells over OT-II cells. This favors the idea that APCs capture transgenically expressed OVA directly from the islets when it is cell associated, and traffic to the draining lymph nodes, where they present it to T cells. It is possible, however, that cellular material carrying OVA, e.g., heat-shock proteins, traffic to the draining lymph nodes and is captured by resident APC. Clearly, the source of Ag in the pancreatic lymph nodes is not secreted soluble OVA, which would be expected to better activate OT-II cells.

In summary, the data presented in this work show that cell-associated OVA is presented much more efficiently than soluble OVA in vivo, especially for class I-restricted presentation. Such favorable cross-presentation of cell-associated OVA suggests that the immune system is particularly concerned with responding to cellular Ags, presumably to monitor intracellular pathogens such as viruses and some bacteria. At present, we do not know whether other Ags will have a similarly biased presentation into the class I pathway, but if this is the case, it would be tempting to speculate that cross-presentation is designed specifically to make it difficult to stimulate CD4 T cell help, thus favoring CTL immunity to Ags expressed in abundance during infection.

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References


