Constitutive Class I–restricted Exogenous Presentation of Self Antigens In Vivo
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Summary
Ovalbumin (OVA)-specific CD8+ T cells from the T cell receptor–transgenic line OT-I (OT-I cells) were injected into unirradiated transgenic RIP-mOVA mice, which express a membrane-bound form of OVA (mOVA) in the pancreatic islet β cells and the renal proximal tubular cells. OT-I cells accumulated in the draining lymph nodes (LN) of the kidneys and pancreas and in no other LN. They displayed an activated phenotype and a proportion entered cell cycle. Unilateral nephrectomy 7–13 d before inoculation of OT-I cells into RIP-mOVA mice allowed the injected T cells to home only to the regional LN of the remaining kidney (and pancreas), but when the operation was performed 4 h before injecting the T cells, homing to the LN of the excised kidney was evident. When the bone marrow of RIP-mOVA mice was replaced with one of a major histocompatibility haplotype incapable of presenting OVA to OT-I cells, no homing or activation was detectable. Therefore, OT-I cells were activated by OVA presented by short-lived antigen-presenting cells of bone marrow origin present in the draining LN of OVA-expressing tissue. These results provide the first evidence that tissue-associated “self” antigens can be presented in the context of class I via an exogenous processing pathway. This offers a constitutive mechanism whereby T cells can be primed to antigens that are present in nonlymphoid tissues, which are not normally surveyed by recirculating naive T cells.

Antigens presented in association with MHC-encoded class I molecules are generally derived from intracellularly synthesized proteins (1). In contrast, exogenous antigens are taken up by APC and presented with MHC class II molecules. Under some circumstances, however, exogenous antigens have been reported to enter the class I presentation pathway in vivo. This was first suggested by cross-priming experiments, where MHC-mismatched grafts could prime recipients for a cytotoxic CD8+ T lymphocyte response specific for minor histocompatibility antigen (HA) (2), H-Y antigen (3), or SV-40 antigen (4). Presentation of the donor minor antigens in the context of host class I molecules required antigen processing and presentation by recipient APC, violating the separation between the two antigen-processing pathways. Such cross-priming was later shown for the induction of CTL to the more conventional antigens OVA and β-gal, where mice were primed by the intravenous injection of antigen-coated spleen cells (5).

Furthermore, certain nonself antigens, such as tumor antigens (6) or antigens carried by microorganisms (7), have been reported to access the class I pathway. Tolerance to donor minor HA in nude mice that received thymus grafts expressing both minor and major HA incompatibility suggested that this pathway operates in the thymus (8).

Attempts to identify an exogenous class I presentation pathway in vitro remain contradictory. In these studies, soluble antigens (9, 10) or antigens carried by microorganisms (11) or beads (12, 13) have been incubated with various APC that were then shown to present antigen to class I-restricted T cell hybridomas. Some reports have attributed such presentation to specialized APC that shunt exogenous antigens into the class I pathway (13–15). The relevance of these findings has been questioned, however, by the observation that particle overloading of the phagosomal compartment may cause mechanical disruption resulting in antigen escaping into the cytosol (16).

Collectively, recent work on the exogenous presentation of class I–restricted antigens has focused on the introduction of nonself antigens into this pathway. The report presented here provides evidence that self antigens expressed by healthy tissues can be processed by a constitutively acting exogenous pathway for class I presentation. This may
be important in the surveillance of nonlymphoid tissues for infection or malignancy.

Materials and Methods

Mice. All mice were bred and maintained at the Walter and Eliza Hall Institute for Medical Research. Female mice between 8 and 16 wk old were used.

RIP-mOVA Mice. The membrane-bound form of OVA (mOVA) consists of a fusion protein made up of the first 118 residues of the human transferrin receptor, which includes the cytoplasmic tail and signal-anchor domain and is linked to residues 139-385 from mature OVA. The cDNA encoding the mOVA fusion protein was excised from the pSVT7-OVA/TIR plasmid (17) by digestion with HindIII after converting the BgIII site situated 39 to the coding region to a HindIII site using a synthetic linker (Promega, Madison, WI). This cDNA fragment was then cloned into the unique HindIII site of the pBlueRIP vector (18), which brings it under the control of the rat insulin promoter (RIP). Vector sequences were excised by digestion with Apal, NotI, and PvuII. The purified DNA was microinjected into the pronuclei of C57BL/6 fertilized eggs as described (19).

Screening for the Presence of the OVA Transgene in RIP-mOVA Mice. DNA from blood of RIP-mOVA mice was prepared using Instagene-Matrix (BioRad Laboratories, Hercules, CA). DNA encoding OVA was amplified by PCR using the primers 5' CAA-GCACATGCAGCTGAAGCTT and 3' GCAATTGCCTTGTCAGCAT, which cover a 480-bp fragment. As positive controls, primers specific for mouse insulin were used. PCR reagents were purchased from Promega. After 35 cycles (60 s 95°C, 60 s 55°C, 60 s 72°C), amplified DNA was detected on a 1.2% agarose gel containing ethidium bromide.

Preparation of OVA-specific class I-restricted T Cells (OT-I) Cells for Adoptive Transfer. OT-I mice producing OVA-specific CD8+ T cells (20) were crossed to a RAG-1-/- background to prevent endogenous rearrangement of TCRs and to avoid generation of CD4+ T cells and B cells. To remove erythrocytes and macrophages, the LN and spleen cells of OT-I mice were first incubated with anti-HSA (112 ([21]) control supernatant, followed by treatment with rabbit complement. Debris was removed by filtering the cell suspension through a 100-μm mesh. 5×10^6 cells were injected intravenously into irradiated recipient mice.

FACS® Analysis. For three-color FACS® (Becton Dickinson & Co., Mountain View, CA) analysis, cells from single LN or spleen were stained as described before (22) using the following mAbs: PE-conjugated anti-CD8 (YTS 169.4), anti-Vα2 (MR9-4), anti-Vβ2 (SAVP-Tricolor), anti-Thyl.1 (OX-7), anti-Thyl.2 (2D7), and FITC-conjugated anti-CD44, anti-CD45, and anti-Thyl.1 (OX-8). These antibodies were used to stain the LN and spleen cells of OT-I mice. OT-I cells were stained with anti-Vα2, followed by SAVP-Tricolor, as described above. In this study, donor cells were stained with FITC-conjugated anti-CD44, anti-CD69 (H1.SF3), and anti-thy1.1 (OX-7).

Expression of OVA in RIP-mOVA Mice. Immunohistochemical examination revealed OVA expression in pancreatic β cells and kidney proximal tubular cells (Fig. 1), but not in the liver, intestine, stomach, spleen, LN, thymus, salivary glands, adrenal gland, lung, heart, connective tissue, and ovaries (data not shown). Low level expression was found in the testes (data not shown). The experiments presented in this paper use only female RIP-mOVA mice. When RIP-mOVA mice were crossed to OT-I mice, which produce OVA-specific CD8+ T cells, double transgenic mice
showed thymic deletion of OT-I cells (data not shown). This suggested that mOVA was present in low amounts at this site.

Homing of OT-I Cells to the Draining LN of OVA-expressing Tissues in RIP-mOVA Mice. To examine the fate of OVA-specific CD8+ T cells after antigen encounter in vivo, these cells were injected intravenously into unirradiated adult female RIP-mOVA mice. After 3 d, the LN and spleen of the recipient mice were analyzed for the presence of OT-I cells (Fig. 2). In the draining LN of OVA-expressing tissues, namely the kidneys and the pancreas, the proportion of OT-I CD8+ cells among the total CD8+ cells was two- to sixfold higher in the kidney and pancreatic LN than in the pyloric, mesenteric, inguinal, or cervical LN, or in the spleen. No such accumulation or "homing" was detected in nontransgenic littermate controls. The total cell number in the two renal LN was not statistically different in RIP-mOVA and nontransgenic littermate controls. The total cell number in the two renal LN was not statistically different in RIP-mOVA and nontransgenic littermate mice (1.8 ± 0.5 × 10^6 cells vs. 1.5 ± 0.3 × 10^6 cells, mean of 15 homing experiments). No homing was detected in axillary, brachial, gastric, or popliteal LN (data not shown).

To assess the kinetics of homing, draining LN were analyzed 22, 30, 42, 68, and 160 h after T cell transfer (Fig. 3). Homing was maximal after 3 d and had decreased by day 7. A slight increase in OT-I cells was also detected in control LN between 22 and 30 h, but this is likely to reflect injected OT-I cells joining the pool of recirculating CD8+ cells.

Homing OT-I Cells Are Activated In Vivo. To determine whether OT-I cells were activated upon homing, the surface activation markers CD44, CD69, or L-selectin were examined. Three control cell populations were used: (a) non–OVA-specific CD8+ cells present in the same LN (CD8+ Vα2− cells); (b) OT-I cells prepared from a LN that

**Figure 1.** Expression of OVA in RIP-mOVA mice. Immunohistochemistry performed on kidney (A) and pancreas (B) sections. The arrow in A indicates a proximal tubule originating from a glomerulus.

**Figure 2.** Homing of OT-I cells to the draining lymph nodes of OVA expressing tissues in RIP-mOVA mice. 3 d after intravenous injection of 7.5 × 10^6 OT-I cells into RIP-mOVA mice and nontransgenic littermate mice, OVA-specific CD8+ cells were detected by FACS® analysis for CD8+, Vα2+, and Vβ5+ cells. The horizontal line at 1.4% represents the proportion of Vα2+ and Vβ5+ cells among the CD8+ cells in noninjected RIP-mOVA mice. The LN draining particular organs such as the kidney or the pancreas had been mapped by injecting Fount India ink (Pelikan, Hannover, Germany) into the organ of interest and identifying 24 h later those LN that had accumulated ink particles. Experiments were performed at least four times. Representative results are given here. ■, RIP-mOVA mice; □, nontransgenic littermates.

**Figure 3.** Kinetics of accumulation of OT-I cells. RIP-mOVA and nontransgenic littermate mice were analyzed at various timepoints after injection of 7.5 × 10^6 OT-I cells, and the proportion of OT-I cells per CD8+ cells was determined. ◊, Renal LN, RIP-mOVA; □, inguinal LN, RIP-mOVA; ■, renal LN, nontransgenic littermate.
does not drain OVA-expressing tissues in RIP-mOVA mice, such as the inguinal LN; (c) OT-I cells prepared from the renal LN of a nontransgenic littermate mouse.

In contrast to control populations, OT-I cells that homed to the renal LN of the RIP-mOVA mouse upregulated CD44 and CD69 and downregulated L-selectin (Fig. 4).

To determine whether activation led to the proliferation of OT-I cells, the recipient mice were injected with BrdU 24 h
Figure 6. Restimulation of OT-I cells in vitro. RIP-mOVA and nontransgenic littermate mice were injected intravenously with 6 × 10^6 OT-I cells. After 3 d, cells from the renal (——) or the inguinal (····) LN of RIP-mOVA recipients or from the renal lymph node of a nontransgenic recipient (----) were cocultured in different dilutions with 5 × 10^6 irradiated syngeneic spleen cells pulsed or not pulsed with the OVA peptide. The proportion of OT-I cells in LN cells was determined by FACS® analysis. 18 and 42 h after restimulation, incorporation of [3H]thymidine over 8 h was determined. Mixed lymphocyte reactions were done in duplicate and the response to nonpulsed stimulators was subtracted. This response to nonpulsed stimulator was 529 cpm on day 1 and

978 cpm on day 2 for the highest number of responder cells from the renal LN of RIP-mOVA mice. Comparable background responses were found for stimulators from control LN. Error bars indicate standard errors. The results are representative of two such experiments.

before analysis (Fig. 5). At least 50% of OT-I cells (Vα2+ CD8+ cells) in the kidney and pancreatic LN had incorporated BrdU, whereas little incorporation was seen for nonantigen-specific cells or OT-I cells present in LN that did not drain OVA-expressing tissues. When homing OT-I cells from the renal LN of RIP-mOVA mice were restimulated in vitro with antigen, they showed antigen-specific proliferation within 1 d, whereas OT-I cells from control LN responded to antigen later (Fig. 6).

CD4+ help was not required for OT-I cell activation in our model because homing and CD69 expression were not abolished by depleting CD4+ cells in RIP-mOVA recipients, and CD4+ cells in RIP-mOVA mice were specifically tolerant of OVA (data not shown).

The BrdU incorporation by OT-I cells in the renal LN at day 3 indicated that their accumulation was not only caused by strict homing, but also by proliferation. For simplicity, however, OT-I cells that have accumulated in the renal LN of RIP-mOVA mice will be referred to as homing OT-I cells.

OT-I Cells Proliferate in the Renal LN after Nephrectomy. If OT-I cells were activated after encountering antigen on kidney tubular cells, neither homing nor signs of activation should be detected in the renal LN draining the site of an excised kidney. Therefore, left nephrectomy was performed before the injection of OT-I cells, leaving the right kidney and the draining LN of the left kidney in situ. 3 d after OT-I cell transfer, homing to the left and right renal LN were compared. When nephrectomy was performed 4 h before transfer, homing of OT-I cells to the left renal LN was comparable to homing to the right renal node. When the recipient mice were nephrectomized 7, 10, or 13 d before, however, no homing to the left renal LN was detected, whereas OT-I cells still homed to the right renal node (Fig. 7) and to the pancreatic node (data not shown). A similar result was obtained after removal of the right kidney (data not shown). No homing was detected in nephrectomized littermate mice. These findings demonstrate that antigen is derived specifically from the kidney, and they rule out nonspecific accumulation of OT-I cells in the LN that drain the operation site.

When transgenic CD8+ T cells specific for an antigen other than OVA, such as H-Y–specific T cells (28), were injected into nephrectomized female RIP-mOVA mice, no selective homing was detected (data not shown).

OT-I cells that had homed to the left renal LN of RIP-mOVA mice nephrectomized 4 h before T cell transfer had

Figure 7. Homing of OT-I cells after nephrectomy. 4 h or 10 d after removal of the left kidney, 5 × 10^6 OT-I cells were injected intravenously into RIP-mOVA mice or nontransgenic littermates. 3 d after injection, homing OT-I cells were identified by staining for CD8+ Vα2+ Thy1.1+ cells, and the proportion of these cells in all CD8+ cells found in the LN was calculated. Each group consisted of two mice. The results shown here are representative of five such experiments. ‡, Left renal LN; ■, right renal LN, □ inguinal LN.
a phenotype similar to that displayed by OT-I cells in non-nephrectomized RIP-mOVA mice. They expressed surface activation markers (data not shown), and a proportion incorporated BrdU (Fig. 8).

Homing of OT-I Cells Depends on a Bone Marrow-derived Cell. The above results indicated that antigen was presented to OT-I cells in the renal LN, rather than in the kidney itself. To determine the origin of the APC and to formally rule out the migration of tubular cells or MHC-peptide complexes, we lethally irradiated RIP-mOVA mice and reconstituted them twice with bone marrow of either a RIP-mOVA B6, a nontransgenic B6, or a nontransgenic bm8 donor and 4 mo before analysis. 3 d after injection, the presence of BrdU was detected in CD8+ Thy1.1+ cells in the renal and the inguinal lymph nodes. Recipient mice had been injected with 1.5 mg BrdU in two doses 24 and 12 h before analysis. $\exists$, Left renal LN; $\Box$, right renal LN, $\|$ inguinal LN.

Discussion

Presentation of exogenous antigens by MHC class I molecules has been demonstrated in several in vitro studies (9-11, 13, 15, 29, 30). However, doubt has been cast recently on whether this form of processing or cross-presentation represents some truly novel exogenous pathway, and its relevance to general immune surveillance has been questioned. In particular, as shown by Reis e Sousa and Germain (16), in vitro class I processing and presentation of certain particulate forms of exogenous antigens can result from their escape into the cytosol as a consequence of disruption of phagosomal membrane integrity. While this might be important for class I-restricted T cell priming by certain intracellular pathogens replicating within endosomal compartments, it may prove too inefficient for the presentation of the wider class of exogenous antigens.

In contrast to the demonstration of cross-presentation in vitro, cross-priming (2-5), and cross-tolerance (8) experiments provide in vivo evidence for access to the exogenous class I presentation pathway. In the early studies of Bevan (2), immunization with antigens expressed on allogeneic cells allowed efficient generation of minor histocompatibility antigen-specific CTL that were restricted to the host's class I MHC. Host APCs must therefore have been able to shunt what was essentially exogenous antigen into the class I processing and presentation pathway for effective T cell activation.

Previous studies showing in vivo presentation of exogenous class I-restricted antigens have all involved the deliberate introduction of some foreign agent into the host (1, 31-33). By contrast, the experiments described in this report show that the antigen involved in cross-presentation in vivo can be derived from constitutively expressed proteins. The specific homing of the OT-I cells in the transgenic mice expressing mOVA in the islet $\beta$ cells and the kidney proximal tubules provided means of assessing organ-specific T cell recognition of peripheral antigen in vivo. The OT-I cells homed exclusively to LN draining
phenotype. This homing and activation was clearly evident even after nephrectomy, as long as OT-I cells were injected shortly after surgery. Consequently, the class I-restricted T cells recognized antigen that was presented within the draining LN and not in the kidney itself.

Until now, the APC responsible for in vivo cross-presentation has remained elusive. The problem with earlier studies was the lack of knowledge of the site of antigen presentation. The advantage of our RIP-mOVA model is that this site is well defined, and experiments designed to identify the APC can proceed with the knowledge that this cell is found within the LN that are targeted by homing OT-I cells. The APC involved are of bone marrow origin, since reconstitution with bm mutant bone marrow, but not with C57BL/6, abrogated OT-I cell homing, further reinforcing the notion that antigen recognition is not taking place within the kidney itself.

As yet, it is not known how and where the antigen is introduced into the APC, but we can envisage several possibilities. mOVA might have been shed from tubular cells to be taken up and processed by APC that are present in the kidney or in the draining LN. Another possibility is that heat shock proteins (HSP) carrying mOVA-derived peptides might have been released from tubular cells to be picked up by APC. Recent studies have suggested that HSP might play a role in cross-priming (34). In particular, macrophages in vitro were shown to interact with HSP96 and present associated peptides in the context of class I (35). Furthermore, HSP96 purified from tumors could prime CTLs in mice of allogeneic MHC haplotypes (31). The HSP pathway may, however, constitute an inducible mechanism that is important when active intervention occurs, such as during tissue transplantation.

mOVA molecules may follow an unusual presentation route, since they contain the transmembrane domain of the transferrin receptor. Thus, a signal delivered by this region may have been instrumental in internalizing mOVA shed from tubular cells into APC. Internalized transferrin receptors, however, are confined to the lysosomal compartment and are thus unlikely to enter the class I pathway (36, 37). Alternatively, mOVA molecules may have been degraded extracellularly to peptides that directly bind class I molecules on APC. This is likely to be a rather inefficient mechanism, however, since the SIINFEKL peptide would be one of many peptides generated by extracellular degradation of proteins; these peptides would be diluted in the extracellular fluid, degraded by serum peptidases, and capable of binding only that minor subset of empty class I molecules on the surface of APC. We therefore favor the alternative possibility of some form of direct uptake and processing of antigen by an APC, such as a dendritic cell in the kidney, after which this cell migrates to the LN and is involved in T cell activation.

Regardless of the method of antigen uptake and processing in our model, the results we have presented provide the first evidence that self antigens expressed by healthy tissues can be efficiently processed via an exogenous class I-restricted pathway for presentation to CD8+ T cells. They also provide a plausible answer to a question that has perplexed immunologists for a long time: how can naive CD8+ T cells recognize foreign antigens expressed in nonlymphoid tissues through which they do not recirculate (38) and whose cells express few class I molecules, no class II molecules, and no costimulator molecules? If a pathogen, like a virus, has tropism for nonlymphoid tissue cells and cannot infect APC, it would be advantageous if the antigen expressed by this tissue could proceed to the regional node, where it could stimulate a protective CD8+ T cell response. Our model therefore offers a mechanism by which the antigen expressed in infected cells, and perhaps even in malignant cells, could activate naive CD8+ T cells.

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