Brief Definitive Report

The CD8α$^+$ Dendritic Cell Is Responsible for Inducing Peripheral Self-Tolerance to Tissue-associated Antigens

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Abstract

We previously described a mechanism for the maintenance of peripheral self-tolerance. This involves the cross-presentation of tissue-associated antigens by a bone marrow–derived cell type that stimulates the proliferation and ultimate deletion of self-reactive CD8 T cells. This process has been referred to as cross-tolerance. Here, we characterize the elusive cell type responsible for inducing cross-tolerance as a CD8α$^+$ dendritic cell (DC). To achieve this aim, transgenic mice were generated expressing yellow fluorescent protein (YFP) linked to CTL epitopes for ovalbumin and glycoprotein B (gB) of herpes simplex virus under the rat insulin promoter (RIP). Although tracking of YFP was inconclusive, the use of a highly sensitive gB-specific hybridoma that produced β-galactosidase on encounter with antigen, enabled detection of antigen presentation by cells isolated from the pancreatic lymph node. This showed that a CD11c$^+$CD8α$^+$ cell was responsible for cross-tolerance, the same DC subset as previously implicated in cross-priming. These data indicate that CD8α$^+$ DCs play a critical role in both tolerance and immunity to cell-associated antigens, providing a potential mechanism by which cytotoxic T lymphocyte can be immunized to viral antigens while maintaining tolerance to self.

Key words: antigen presentation • cross-tolerance • CD8$^+$ T cells • dendritic cells • cross-presentation

Introduction

When CTLs develop in the thymus, autoreactive cells are deleted from the repertoire (1). However, some self-antigens are not expressed in the thymus, requiring additional tolerogenic mechanisms to operate outside the thymus (2). One way naive CTLs can be tolerized in the periphery is by the delivery of an antigenic signal in the absence of costimulation (3, 4), as would occur when most tissue cells of the body present their antigens. However, T cells recirculate primarily within the secondary lymphoid compartment (e.g., the spleen, blood, and LNs) and, while naive, would not directly encounter self-antigens on most peripheral tissues. If direct encounter were the only way to induce tolerance, an array of autoreactive T cells would not be tolerized and would be available for activation by licensed dendritic cells (DCs) carrying self-antigens together with pathogen antigens from peripheral sites of infection.

To overcome this potential driving force for autoimmunity, the immune system has evolved a mechanism by which it can induce tolerance to cellular antigens derived from tissues outside the secondary lymphoid compartment. This process has been termed cross-tolerance (5, 6). This involves a subset of specialized bone marrow–derived antigen-presenting cells that are able to capture antigens from tissue cells and present them on MHC class I molecules for recognition by naive CTL (7). Upon recognition of these self-antigens, autoreactive CTL undergo proliferation but are ultimately deleted from the peripheral T cell repertoire. While it has been clear for some years that the cell type responsible for inducing cross-tolerance is bone marrow–

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derived (7, 8), direct identification of this important cell type has yet to be achieved.

Recently, there has been some suggestion that DCs, which are normally considered the premium cell type for initiating strong immunity to foreign antigens, might also play a role in the induction of tolerance (9–12). Hawiger et al. (11) provided evidence that targeting of antigen to DEC-205, expressed primarily by DCs, led to tolerance induction. Kurts and colleagues (9) used transgenic expression of antigen-presenting molecules to implicate $CD11c^+$ DC in cross-tolerance. More recently, Hugues et al. (12) reported that islet apoptosis induced by streptozotocin could initiate presentation of antigen by a $CD11b^+$ DC subset that stimulates immunoregulatory cells in NOD mice. While these studies implicate DCs in self-tolerance, precise identification of the DC subset responsible for constitutive induction of cross-tolerance to peripheral self-antigens has not been achieved. Here, we examine the phenotype of this elusive cell type.

Materials and Methods

Mice. All mice were between 6 and 12 wk of age. Generation of OT-I and gBT-I.1 (gBT-I) mice has been described previously (13, 14).

Generation of Rat Insulin Promoter YSS Mice. Rat insulin promoter (RIP)-YSS mice were generated by cloning the RIP into the enhanced yellow fluorescent protein vector (pEYFP; CLONTECH). A polytope encoding the $CD8^+$ T cell determinants $OVA_{257–264}$ (SIINFEKL) and $gB_{498–505}$ (SSIEFARL) from OVA and Herpes simplex virus-1, respectively, was produced by annealing of complementary oligonucleotides (5′-CATGGAGAGTATAATCAACTTTGAAAAACTGACTACCTCCTCCATCGAGTTCGCCCGGCTGCAGGG-3′ and 5′-CATGCCCTGCAGCCGGGCGAACTCGATGGAGGAGGTAGTCAGTTTTTCAAAGTTGATTATACTCTC-3′). This was inserted at a NcoI/StyI site between the RIP and EYFP constructs. Vector sequences were excised by digestion at BamHI sites. DNA was microinjected into pronuclei of B6 fertilized eggs as described previously (15). DNA was prepared from tails of RIP-YSS mice and analyzed by PCR. The DNA encoding RIP-YSS was amplified by PCR using oligonucleotide primers 5′TACCTACCCCTCCTAGAGCCCTTA and 3′TGATATAGACGTTGTGGCTGTTGTAGT which cover a 550-bp fragment, or 5′GCTACCCCGACCACATGAA and 3′TGCTTGTCGGCCATGATATAG which cover a 252-bp product. Amplified DNA was detected on a 2% agarose gel containing ethidium bromide.

Analysis of T Cell Deletion. Recipients were injected with 4×10^6 gBT-I cells. After 7 wk, cells were pooled from the LNs (inguinal, brachial, axillary, sacral, cervical, and mesenteric) and spleen of individual mice. They were then stained using anti-Vβ8.1/8.2-FITC (MR 5–2), anti-Vα2-PE (B20.1), and anti-CD8α-APC (53–6.7; BD PharMingen). Live gates were set on lymphocytes by forward and side scatter profiles. Analysis was done on a FACScan™ (Becton Dickinson). 10,000–20,000 live cells were collected for analysis. gBT-I cells were identified as $V\alpha2^+$ $V\beta8^+$ $CD8^+$ cells (14). The proportion of endogenous $V\alpha2^+$ $V\beta8^+$ $CD8^+$ cells (<2%) was assessed by examining mice that did not received gBT-I cells as described previously (7). This value was subtracted from the values derived from gBT-I recipients.

DC Isolation. DCs were isolated essentially as described previously (16, 17). Antibodies used in the depletion cocktail were anti-CD3 (KT3), anti-Thy1 (T24/31.7), anti-CD19 (ID3), anti–GR-1 (RB6–8C5), and anti-erythrocyte (TER-119).

Detection of Antigen Presentation by LN DCs. The *lacZ*-inducible gB HSV-specific hybridoma (HSV-2.3.2E2; references 18 and 19) was maintained in DMEM containing 10% FCS, 50 μM 2ME, 2 mM L-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin (hybridoma media). Stimulator cells were released from the LNs by collagenase/DNase digestion for 20 min followed by a 5-min incubation with 0.099 M EDTA. Some preparations were depleted of CD11c, CD11b, or CD8α populations by incubating LN cells in predetermined optimal concentrations of N418, M1/70, or 53–6.7, respectively, for 30 min. Cells were then washed through an underlay of FCS containing EDTA, and then in balanced salt solution containing EDTA (BSS-EDTA). Finally, antibody-bound cells were removed using sheep anti–rat IgG Dynabeads and the remaining cells washed once in BSS-EDTA, once in hybridoma medium, and then resuspended in hybridoma medium. The efficiency of depletion was examined by staining depleted and undepleted preparations with goat anti–rat IgG-FITC (Caltag) for 30 min with analysis by flow cytometry. Residual specific cell populations were reduced to <0.15% of the total live LN cells (unpublished data). Depleted or undepleted lymph node cells, or purified DC subsets, were cultured with 10^5 HSV-2.3.2E2 cells for 24 h in 200 μl hybridoma media in 96-well, flat-bottomed plates (Falcon, Becton Dickinson). Then, cells were washed in PBS and fixed using 100 μl PBS containing 1% formaldehyde and 0.2% glutaraldehyde for 5 min at 4°C. The plates were washed with PBS and then overlaid with 50 μl of a solution containing 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-*D*-galactoside, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, and 2 mM $MgCl_2$ in PBS. Cultures were examined microscopically and the number of blue cells counted after 8–12 h incubation at 37°C.

Results and Discussion

Previously, we reported that expression of OVA in the pancreatic islets led to cross-presentation of OVA on a bone marrow–derived cell in the draining LNs (8), and that this cell induced the deletion of autoreactive OVA-specific CTLs (7). Despite numerous and varied attempts, we have never previously been able to isolate the bone marrow–derived cell from the draining LNs. To improve our ability to identify the cross-tolerance antigen-presenting cell, we generated transgenic mice expressing a hybrid fusion protein under the control of the rat insulin promoter (RIP). This hybrid protein consists of yellow fluorescent protein (YFP) linked to two class I epitopes: one from OVA ($OVA_{257–264}$) and one from Herpes simplex virus-1 glycoprotein B ($gB_{498–505}$). We have termed the hybrid protein YSS and the transgenic line RIP-YSS.

To determine whether YSS was presented in the draining LNs, RIP-YSS mice were injected with CFSE-labeled (7) CD8 T cells from transgenic mice specific for OVA (OT-I cells) (13) or gB (gBT-I cells) (14), and 3 d later their LN cells were examined by flow cytometry (Fig. 1). Both OT-I and gBT-I $CD8^+$ T cells proliferated in the pancreatic but not inguinal LNs of RIP-YSS mice, indicat-

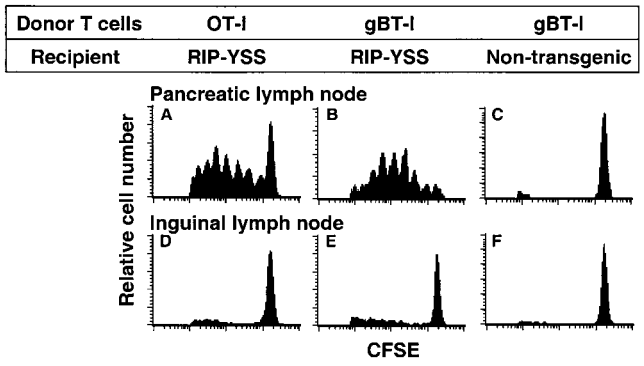

Figure 1. gB and OVA-specific T cells proliferate to YSS in the pancreatic LNs of RIP-YSS mice. 2×10^6 CFSE-labeled recombination activation gene (Rag)-$1^{-/-}$ OT-I (A and D) or Rag-$1^{-/-}$ gBT-I (B, C, E, F) $CD8^+$ T cells were adoptively transferred into RIP-YSS (A, B, D, E) or B6 (C and F) recipients. 3 d later, pancreatic (A–C), and inguinal (D–F) LNs were analyzed by flow cytometry, gating on $CD8^+CFSE^+PI^-$ cells (reference 7).

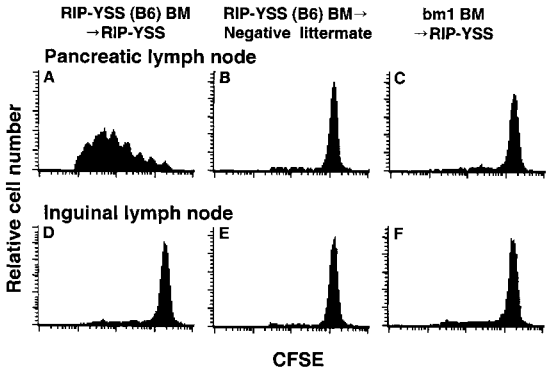

Figure 2. A bone marrow–derived cell type cross-presents YSS in RIP-YSS mice. Bone marrow chimeric mice were generated using RIP-YSS (A, C, D, F) or nontransgenic littermate (B and E) recipients. Lethally irradiated (2×550 cGray, 3 h apart) recipients were engrafted with T cell–depleted RIP-YSS ($H\text{-}2K^b$, B6 background)(A, B, D, E) or $H\text{-}2K^{bm1}$ mutant bone marrow (C and F). After 10 wk reconstitution, 2×10^6 CFSE-labeled recombination activation gene (Rag)-$1^{-/-}$ gBT-I $CD8^+$ T cells were adoptively transferred into bone marrow chimeric recipients. 3 d later, the pancreatic (A–C) and inguinal (D–F) LNs were analyzed by flow cytometry. Histograms are gated on $CD8^+CFSE^+PI^-$ cells.

ing that these MHC class I epitopes were expressed in the pancreas and effectively presented in the draining LNs.

To determine whether presentation of YSS was via cross-presentation on a bone marrow–derived antigen-presenting cell, we made use of the fact that gB is not presented by the K^{bm1} molecules of bm1 mice (unpublished data). bm1 mice are congenic to B6 mice, differing only at the H-2K locus. RIP-YSS mice of a B6 background were lethally irradiated and reconstituted with either RIP-YSS (B6; $H\text{-}2^b$) bone marrow or bm1 ($H\text{-}2^{bm1}$) bone marrow (8). As a control to exclude possible expression of YSS by bone marrow–derived cells, we also injected RIP-YSS bone marrow into irradiated B6 mice. After 10 wk, to allow reconstitution of antigen-presenting cells, chimeric mice were injected with CFSE-labeled (7) gBT-I cells. 3 d later, the pancreatic and inguinal LNs were recovered and single cells analyzed by flow cytometry (Fig. 2). Only RIP-YSS mice receiving RIP-YSS (B6, $H\text{-}2^b$) bone marrow showed proliferation of gBT-I cells, indicating that a bone marrow–derived cell was responsible for cross-presentation of islet-derived YSS in the draining LNs.

To ensure that such cross-presentation led to deletion of naive gB-specific CTLs, RIP-YSS mice or nontransgenic control mice were injected with 4×10^6 gBT-I cells and then 7 wk later examined for the presence of these cells in the spleen and LNs (Fig. 3). This showed that few gBT-I cells remained in RIP-YSS mice relative to nontransgenic controls, indicating that cross-presentation of YSS had caused the deletion of gB-specific T cells. This finding confirmed earlier studies where we have shown that OVA-specific CD8 T cells are deleted in response to cross-presented OVA in mice expressing membrane-bound or soluble OVA in the pancreas under the control of the RIP (7, 20).

Once we had established that cross-presentation of the YSS self-antigen led to peripheral deletion of autoreactive CTLs, we asked whether the cell presenting YSS in the draining LNs could be identified by fluorescence as a consequence of captured fluorescent YSS protein. Using 16–20 mice per group, we were able to isolate sufficient DCs from the pancreatic LNs of transgenic mice to examine fluorescence. This approach was inconclusive, however, with very poor detection of fluorescence in DCs of the draining LNs (unpublished data). Failure to detect fluorescence was either because the fluorescent YSS molecule was rapidly degraded after capture or because the APC responsible for presentation was not present in such preparations.

To distinguish between these possibilities, we examined antigen presentation by LN DCs using a very sensitive T cell hybridoma that produces β-galactosidase in response to stimulation with the MHC class I–restricted gB epitope (19). Previously, we had been unable to detect in vitro presentation by pancreatic LN cells from any of our transgenic lines (RIP-mOVA or RIP-OVA^{hi}) using other methods such as presentation to TCR transgenic cells (unpublished

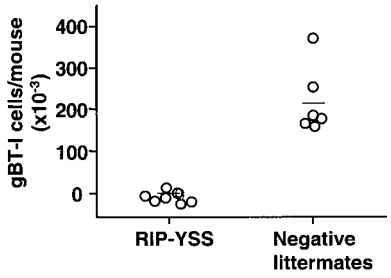

Figure 3. Cross-presentation of endogenous antigen induces deletion of antigen-specific $CD8^+$ T cells. 4×10^6 gBT-I $CD8^+$ T cells were adoptively transferred into RIP-YSS transgenic mice or nontransgenic littermates. After 7 wk, the number of gBT-I cells in the LNs and spleen was determined by flow cytometry (reference 7). Individuals (white circles) and means (—) for each group are shown.

data). In our first experiments using the β-galactosidase producing hybridoma, we removed pancreatic LNs from RIP-YSS mice or nontransgenic controls and then generated single cell suspensions by digestion with collagenase and DNase; an approach developed to minimize damage to DCs (16). After digestion, the entire cell mix derived from the pancreatic LNs was cultured for 24 h with cells from the gB-specific hybridoma. After this time, cultures were developed for β-galactosidase expression, and positive cells enumerated (Fig. 4 A). This revealed that cells capable of presenting YSS-derived gB to CD8 T cells were present in the LN cell preparation. To further characterize the cell presenting islet antigens in the pancreatic LNs, mixed cell populations were depleted of cells expressing the markers CD11c, CD8, or CD11b, and then tested for presentation of YSS (Fig. 4 B). As shown, the antigen-presenting cells were $CD11c^+$, indicating that they belonged to the DC lineage. Furthermore, this cell type was depleted by anti-CD8α, but not anti-CD11b mAb, indicating it was a $CD8\alpha^+CD11b^-$ DCs. It has recently been described that some $CD11b^+CD8\alpha^-$ DCs can convert to $CD8\alpha^+$ after antigen encounter (21). This same subset is unlikely to represent the cross-tolerance $CD8\alpha^+$ DCs which is $CD11b^-$ (Fig. 4 B) and can be found expressing CD8α within the pancreatic islets (unpublished data).

To confirm that $CD8\alpha^+$ DCs were responsible for cross-tolerance, we sorted $CD11c^+CD8\alpha^+$ and $CD11c^+CD8\alpha^-$ DCs from the pancreatic LNs of RIP-YSS mice and examined their ability to stimulate the gB-specific hybridoma (Fig. 5). This required 29 mice to recover 11,500 $CD8\alpha^+$ DCs, so we were only able to do a titration of DCs from 5,750 cells per well with a single well per dilution. In this case, however, $CD8\alpha^+$ DCs clearly stimulated gB-specific hybridoma cells in a dose-dependent manner, with no response generated by $CD8\alpha^-$ DCs.

$CD8\alpha^+$ DCs were recently shown to be responsible for cross-priming (22) when mice were immunized with foreign antigen in the form of OVA-loaded splenocytes (23). As in our example here, which involves cell-associated YSS antigen, the cross-priming observed by den Haan et al. (23) involved a cell-associated antigen, which appears to be preferred for cross-presentation (24).

There is now strong evidence that dying cells are very efficiently targeted for cross-presentation by the $CD8\alpha^+$ DC subset (25). Whether the cross-presentation associated with cross-tolerance also requires apoptotic cells or can present antigens from living cells is unclear. However, it is apparent that cross-presentation can be enhanced by causing tissue destruction (26), but whether this leads to the same sort of deletional response of autoreactive CD8 T cells as seen with constitutive cross-tolerance has not been examined. Recently, destruction of NOD islets by streptozotocin was shown to enhance islet antigen presentation to CD4 T cells by a $CD11b^+$ DC subset (12). This led to induction of immunoregulatory cells that prevented autoimmunity. The $CD11b^+$ identity of the DC subset mediating presentation in the report by Hugues et al. (12) is surprising, as a recent publication by Inaba and colleagues provides strong evidence that $CD11b^-CD8\alpha^+$ DCs are responsible for the capture and presentation of apoptotic cells in vivo (25). Perhaps this relates to a differences between NOD and B6 mice, or to differences in APCs targeted by injecting foreign apoptotic cells versus those generated by streptozotocin treatment. It is unclear why our data and Hugues et al. (12) implicate different DC subsets, but perhaps this relates to differences in class I versus class II restricted responses or constitutive versus damage-induced cross-presentation.

Given that $CD8\alpha^+$ DC subset appears to be responsible for induction of both cross-tolerance and cross-priming, what are the mechanisms that select between these opposing outcomes? One possibility is that $CD8\alpha^+$ DCs may be further subdivided into immunogenic and tolerogenic subsets. More likely, however, is that tolerance induction is the default outcome, and that additional stimuli associated with foreign antigens are responsible for their immunoge-

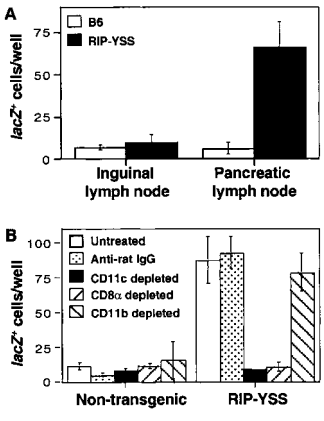

Figure 4. Identification of the cell type responsible for cross-presentation of islet antigens. LN cells were isolated from RIP-YSS mice or nontransgenic B6 controls and subjected to collagenase and DNase digestion to release DCs. The preparations were either left (A) undepleted or (B) depleted of various populations including rat Ig^+ (negative control), $CD11c^+$, $CD11b^+$, or $CD8\alpha^+$ cells using magnetic beads. These populations were then tested for their ability to stimulate lacZ production by the T cell hybridoma HSV-2.3.2E2, specific for gB (reference 19). The equivalent of one pancreatic LN ($5–8 \times 10^5$ cells) or 1/4–1/10 of an inguinal LN ($4–8 \times 10^6$ cells) was placed into each well containing 10^5 hybridoma cells and cultured for 24 h at 37°C. Error bars indicate SD of triplicate wells. The experiments shown in A and B were performed 6 and 3 times, respectively. Data were collected blind.

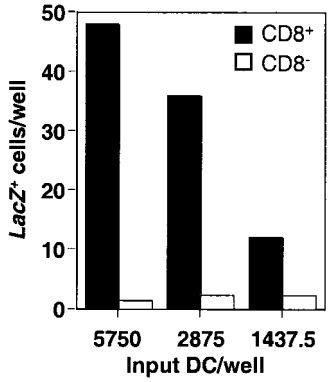

Figure 5. Isolation of the cell type responsible for cross-presentation of islet antigens. Pancreatic LNs were removed from RIP-YSS mice and digested with collagenase and DNase. The preparations were then enriched for DCs by magnetic bead depletion of other cells (Materials and Methods). Finally, DC-enriched populations were stained with anti-CD11c, anti-CD45RA, and anti-CD8α and sorted for $CD11c^+CD45RA^-$ cells that were either $CD8\alpha^+$ or $CD8\alpha^-$. CD45RA was used to exclude plasmacytoid DCs. The $CD8\alpha^+$ and $CD8\alpha^-$ DCs were titrated and used to stimulate the gB-specific hybridoma. $CD8\alpha^+$ DCs were recovered in small numbers that only allowed analysis of single wells per dilution, whereas $CD8\alpha^-$ DCs were titrated in duplicate and the mean count shown. This experiment was performed twice with similar results.

nicity (4, 27). Other studies support the notion that the CD8α$^+$ DC subset can be converted to an immunogenic form by CD40 signaling (28, 29), indicating that the immunogenicity of these DCs can be influenced by environmental signals. This dual role for a DC subset is likely to be very important in limiting autoimmunity, since under noninfectious conditions the CD8α$^+$ DCs can induce peripheral tolerance to self-antigens, deleting autoreactive T cells and leaving only pathogen-specific T cells to respond to licensed DCs during infections, when a mixture of pathogen and cellular products will be presented.

In summary, our data indicate that the CD8α$^+$ DC subset is responsible for induction of peripheral self-tolerance by their ability to capture and cross-present tissue-associated antigens to naive CTLs. These DCs appear to represent an important subset for several reasons, including their ability to capture cell–associated antigens (25), their ability to present such antigens to both CD4 and CD8 T cells (30), and their ability to induce either tolerance (shown here) or immunity (23) depending on the nature of the antigen. This knowledge greatly advances our capacity to study the signals that decide between immunity and tolerance.

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