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# TOLEROGENIC DENDRITIC CELLS*

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■ **Abstract** Dendritic cells (DCs) have several functions in innate and adaptive immunity. In addition, there is increasing evidence that DCs in situ induce antigen-specific unresponsiveness or tolerance in central lymphoid organs and in the periphery. In the thymus DCs generate tolerance by deleting self-reactive T cells. In peripheral lymphoid organs DCs also induce tolerance to antigens captured by receptors that mediate efficient uptake of proteins and dying cells. Uptake by these receptors leads to the constitutive presentation of antigens on major histocompatibility complex (MHC) class I and II products. In the steady state the targeting of DC antigen capture receptors with low doses of antigens leads to deletion of the corresponding T cells and unresponsiveness to antigenic rechallenge with strong adjuvants. In contrast, if a stimulus for DC maturation is coadministered with the antigen, the mice develop immunity, including interferon-$\gamma$-secreting effector T cells and memory T cells. There is also new evidence that DCs can contribute to the expansion and differentiation of T cells that regulate or suppress other immune T cells. One possibility is that distinct developmental stages and subsets of DCs and T cells can account for the different pathways to peripheral tolerance, such as deletion or suppression. We suggest that several clinical situations, including autoimmunity and certain infectious diseases, can be influenced by the antigen-specific tolerogenic role of DCs.

## INTRODUCTION

The subject of this review, dendritic cells (DCs) in T cell tolerance, may seem surprising. Prior research has emphasized the opposite outcome of DC function: strong innate and adaptive immunity to infections and other antigens in vivo (1–6). However, these two apparently incompatible functions can be reconciled in a number of ways. For example, the induction of tolerance by the deletion of naive

*Abbreviations: DCs, dendritic cells; MMR, macrophage mannose receptor; HEL, hen egg lysozyme; ovalbumin; IDO, indoleamine 2,3-dioxygenase

peripheral T cells takes place in the steady state, whereas the initiation of immunity occurs in the context of signals associated with infection and inflammation.

Infection stimulates DCs to coordinate many protective functions by immune cells, and these have been documented in vivo. Microbial products trigger DCs to produce large amounts of immune enhancing cytokines, such as interleukin-12 (IL-12) (7) and interferon-$\alpha$ (IFN-$\alpha$) (8). DCs exposed to inflammatory cytokines rapidly activate other innate protective cells such as natural killer (NK) (9) and NKT cells (10). Mature DCs initiate or prime T cell responses (11, 12), including protective immunity to infection (13) and tumors (14). Furthermore DCs are able to rapidly polarize the immune response to either Th1 or Th2 types (15–17) and to improve T cell memory (18, 19).



These functions in the control of innate and adaptive immunity require that DCs undergo terminal differentiation or maturation. Maturation is induced by numerous agents including microbial infection. In vivo, two major receptor families play prominent roles: toll-like receptors (20–22) and tumor necrosis factor (TNF)-receptors, especially CD40 (23–25). Likewise in vitro, DCs are matured by exposure to lipopolysaccharide (26), inflammatory cytokines including TNF$\alpha$ (27, 28), and CD40 ligation (29–31). Maturation results in several phenotypic changes that are linked to an enhanced ability to process antigens and activate T cells. These phenotypic changes include increased production of MHC-peptide complexes (32), increased expression of T cell binding and costimulatory molecules (29, 33), and de novo production of growth factors such as IL-2 (34) and thiols (35), chemokines (36), and cytokines (37). Therefore for DCs to serve as "nature's adjuvants" for immunity (11), they need to mature in response to stimuli inherent to the infection, vaccine, or other settings such as transplantation and contact allergy.

The expanding literature on the capacity of DCs to induce T cell tolerance in vivo originated with experiments on DCs that are not fully mature, especially those found in peripheral lymphoid tissues in the steady state. It has become possible to deliver defined antigens to specific populations of DCs in the absence of maturation stimuli (23–25) without subjecting the DCs to isolation and manipulation ex vivo, procedures that can mature the cells (11, 38, 39). The targeting of antigens to DCs in vivo involves specific uptake receptors that deliver the antigens to processing compartments for the formation of class I and II MHC-peptide complexes. Importantly, DCs within lymphoid tissues are able to form MHC-peptide complexes in the steady state without the administration of maturation stimuli. Naive T cells, after recognizing their ligands on these DCs, divide repeatedly but are then deleted, and the animal becomes tolerant. In contrast, if maturation stimuli are coadministered with antigen, immunity develops. Another strategy to dampen immune function is to prepare DCs ex vivo and expose them to antigen but not to full-maturation stimuli. These DCs, when reinfused, downregulate immunity (40–43) and can induce regulatory T cells (see below). In contrast, mature DCs are immunogenic in animals (32, 44) and humans (16, 45–48). However, the physiological counterpart of these ex vivo–derived human DCs and the induction of authentic tolerance by regulatory T cells in vivo in humans remain to be defined.

**TABLE 1** T cell tolerance: some questions

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| Experimentally, high doses of preprocessed peptides are used to tolerize animals. Can T cell tolerance be induced to low levels of intact proteins including self and environmental antigens? |
| During dendritic cell maturation a mixture of microbial, self-, and environmental antigens are captured simultaneously. How is the initiation of autoimmunity and chronic reactivity to these antigens avoided? |
| Clinically, suppression of the immune response utilizes antigen-nonspecific inhibitors. Can antigen-specific tolerance be induced to transplants, allergens, and autoantigens? |
| There are many mechanisms for tolerance: anergy, deletion, and regulatory and suppressor T cells. How are these mechanisms induced and controlled in vivo? |



This review on DCs in tolerance deals with four challenging questions (Table 1). First, is it possible to use low doses of intact antigens to silence the immune system in vivo? It seems vital that the immune system remains tolerant to intact proteins, both self and environmental, that are present in small amounts. Yet experimentally, it has been necessary to use high doses of soluble proteins and usually preprocessed peptides to induce tolerance (49–53). We review how the targeting of antigens to appropriate DCs induces tolerance in vivo with low doses of antigen and thereby more effectively controls the tolerogenic potential of the immune system. Second, when DCs are maturing in response to an infection, how do they avoid the risk of inducing autoimmunity to self-antigens and chronic reactivity to environmental proteins? It is to be expected that DCs during infection will present a mix of antigens, not just those from the microbe but also antigens from dying self-tissues and from proteins in the airway or intestine. We consider the evidence that DCs may solve this dilemma by ensuring that tolerance develops to those harmless antigens that will subsequently be processed during infection. Third, can antigen-specific tolerance be induced in clinical settings, such as transplantation, allergy, and autoimmunity? Current treatments employ antigen-nonspecific immune suppressants that globally block lymphocyte costimulation and cytokine production. DC-based tolerance offers the potential to manipulate the immune response in a more antigen-specific manner. Fourth, how are the many known mechanisms for T cell tolerance [reviewed elsewhere (54–57)] engaged and controlled in the intact animal and patient? We review examples in which antigen presentation via DCs leads to the control of specific tolerance mechanisms in vivo. The control of tolerance is in a sense analogous to the control of immunity (58) in that antigens, lymphocytes, and DCs need to operate in concert.

## ROLE OF DENDRITIC CELLS IN CENTRAL TOLERANCE

### A Role for Dendritic Cells in T Cell Deletion

The experiments of Medawer and colleagues demonstrated that the developing immune system could be actively and specifically silenced or tolerized.

Experimentally, tolerance also means antigen-specific nonresponsiveness to a challenge with antigen delivered with a strong adjuvant. They injected mice in utero with allogeneic spleen cells and induced specific transplantation tolerance (59). An early tissue culture model paralleled these experiments (60). Allogeneic DCs from spleen were added to fetal thymic organ cultures. The T cells that developed in these cultures were specifically unresponsive when rechallenged with the cells from the DC donor but were normally responsive to third-party allogeneic DCs. It was subsequently shown that DCs applied to such organ cultures enter the thymi and take up residence in their normal location, the thymic medulla (61). Thus, allogeneic DCs can redefine "self" if they are able to access the thymus prior to development of the T cell repertoire.

The function of DCs in central tolerance was taken into the realm of self-antigens with C5, the fifth component of serum complement proteins. DCs pulsed with low doses of C5 in culture deleted C5 reactive transgenic thymocytes in vitro (62). Thymic macrophages lacked this capacity, but medullary epithelium was active. In subsequent experiments the cell types presenting endogenous C5 in vivo were identified. Different cells were isolated from C5-sufficient mice and tested for their capacity to negatively select developing, C5-reactive, T cell receptor (TCR)-transgenic T cells in culture. Both DCs and epithelial cells induced deletional tolerance (63).

A less invasive approach to establishing DC function in central tolerance used the CD11c promoter to express the I-E gene selectively in the thymic DCs of C57BL/6 mice (64). This led to efficient negative selection of I-E reactive, $V\beta5^+$ and $V\beta11^+$, $CD4^+$ T cells. In contrast to their function in negative selection, DCs are neither active nor required for positive selection, which can be fully supported by cortical epithelial cells (65–67).

## Some Aspects of Mechanism of Dendritic Cell Function in the Thymus

It would be valuable to learn to manipulate central tolerance at the level of thymic DCs. However, experiments that selectively target antigens to thymic DCs, as we describe for peripheral lymphoid organs below, have yet to be carried out. It also is difficult to selectively engraft DCs into the thymus in vivo. For the most part, precursors in a total-marrow inoculum have been used (68). There is new evidence that thymic DCs themselves, not splenic DCs, home in vivo to the thymus via the intravenous route and that this can be used to prolong graft survival in a donor-specific way (69).

Two features of thymic DC function are currently apparent. First, the DCs are localized almost exclusively to the medulla (70, 71), which seems to be a major site of deletion of positively selected thymocytes (72, 73). Second, thymic DCs are presumably comparable to other sources of DCs in being efficient in antigen capture and processing. This would lead to the production of MHC-peptide complexes, including MHC class I–peptide complexes, needed to delete self-reactive T cells.

Thymic medullary epithelium also expresses high levels of antigen-presenting MHC products and should play a significant role in central tolerance, especially for many self-antigens produced by the epithelium (74, 75).

## THE NEED FOR EFFICIENT MECHANISMS OF PERIPHERAL TOLERANCE

Central tolerance is efficient, but it is also incomplete. Self-reactive T cells, especially those with a lower affinity for self-antigens, can escape negative selection (76). Other self-proteins, for which tolerance is required, may not access the thymus. This is also the case with most harmless environmental proteins, to which chronic immune reactivity must not develop. Peripheral tolerance (77, 78) is therefore necessary to supplement central tolerance. Efficient tolerance mechanisms are especially important at sites of infection, where maturing DCs process and present both self- and nonself-antigens. It has been known for some time that maturation is a control point for initiating immunity (11, 38, 39), but the concept that this carries substantial risks emerged when DCs were found to process antigens from dying cells. Examples included infected cells (79–81), tumor cells (82, 83), and allogeneic cells (84, 85). For MHC class I, presentation of influenza peptides occurred with just one dying influenza-infected monocyte per 10 DCs (79). Likewise, DCs processed trace Epstein-Barr Virus (EBV) latency antigens from apoptotic and necrotic transformed cell lines and then expanded both $CD4^+$ and $CD8^+$ EBV-specific T cells (86, 87). With dying allogeneic cells, it was possible to monitor the formation of MHC class II–peptide complexes directly with a specific antibody. When this was done, the formation of MHC-peptide complexes was $>1000$ times more efficient when DCs were given a protein as part of a dying cell relative to preprocessed peptide (84). In all of these examples, a foreign antigen is presented, but the entire cell is processed, and therefore the DCs should be loaded with MHC-self- and MHC-nonself-peptide complexes.

An additional literature shows that DCs also capture soluble proteins in the steady state, in the absence of overt infection or adjuvants (88–91). In these early experiments, which used several different routes of antigen injection, DCs were isolated from the animals and added to activated antigen-specific T cells in culture; the T cells then proliferated without further addition of antigen. More recent experiments directly visualized in vivo DC uptake of soluble proteins administered into the airway (92) and of self-components from intestinal and gastric epithelial cells (93, 94). Therefore DCs are continually capturing and presenting self- and harmless environmental proteins.

The endocytic and processing activities of DCs create a conundrum with respect to their function in innate and adaptive resistance to infection. If maturing DCs simultaneously capture a mixture of microbial antigens, self tissues, and harmless environmental proteins, how is the response limited to the microbe? To resolve this situation, it has been proposed that DCs are not immunologically quiescent

in the steady state but use their antigen-handling capacities to play a major role in peripheral tolerance (95, 96).

## LOW DOSES OF SOLUBLE ANTIGENS INDUCE PERIPHERAL TOLERANCE WHEN TARGETED TO DENDRITIC CELLS IN THE STEADY STATE

### The MMR and DEC-205, Two Multilectin Endocytic Receptors

DCs express several adsorptive endocytosis receptors, which could be used to target antigens for processing and presentation in vivo. For example, DCs express the MMR (macrophage mannose receptor) (CD206) and DEC-205 (CD205), a pair of homologous, large type-I membrane proteins. The MMR (97, 98) and DEC-205 (99, 100) have similar domain structures with an external cysteine-rich domain followed by a fibronectin II domain and several contiguous C-type lectin domains, 10 in the case of DEC-205 and 8 in the case of the MMR. The cytosolic domain of each receptor has a tyrosine-based coated pit localization sequence. These receptors localize to coated pits and are taken up into coated vesicles and endosomes (100, 101).

The ligands for the MMR include mannosyl and fucosyl residues for the C-type lectin domains and select sulfated sugars for the terminal cysteine-rich domain (102). Endogenous self-ligands for the MMR include lysosomal hydrolases and certain collagen-like peptides in serum (103). Natural ligands for DEC-205 are not yet known. Nevertheless, antibodies to DEC-205 can be used as surrogate antigens and for antigen targeting to DCs (23, 24, 100, 104).

Although both MMR and DEC-205 can be expressed by DCs, their distribution in vivo is distinct. Whereas the MMR is prominent on human monocyte–derived DCs in culture (105), this receptor has yet to be detected on DCs in the T cell areas of lymphoid organs in either mice (106) or humans (107). Instead, the MMR is found on the endothelium lining lymphatic sinuses and in macrophages of splenic red pulp and lymph node. Therefore the MMR may not provide a way to selectively target ligands to DCs in the steady state. In contrast, DEC-205 is expressed abundantly on T cell area DCs, as first shown by the development of the NLDC-145 monoclonal antibody (108), and it does provide a means to target antigens to DCs in vivo (see below).

In terms of antigen-presenting function, the only study to simultaneously compare the function of the MMR and DEC-205 involved cultured mouse bone marrow DCs, and it yielded surprising results. Rabbit antibodies to DEC-205 were presented 30–100 times more efficiently than antibodies to MMR, even though both antibodies bound comparably to the cell surface and entered the endocytic system (104). The MMR recycled quickly through cells via early endosomes, as is the case for many adsorptive endocytosis receptors. In contrast, DEC-205 localized both to early endosomes and MHC class $II^+$ late endosomes and lysosomes. An EDE sequence within the cytosolic domain of DEC-205 enabled this receptor to

target MHC II compartments. This was shown in L cells transfected with a fusion receptor formed by the external region of the CD16 Fc$\gamma$ receptor and the cytosolic tail of DEC-205. The targeting to MHC II compartments led to a marked increase in the efficiency of antigen presentation on MHC class II.

In addition to improved MHC class II presentation, ligands for DEC-205 are processed via the exogenous pathway to MHC class I in a transporters for antigenic peptides (TAP)-dependent manner (24). Although the cell biology of the exogenous pathway has not been worked out, it has been proposed that a transporter in the DC endocytic system allows macromolecules to enter the cytoplasm. According to this model, such antigens would be processed by proteasomes in the cytoplasm and transported into the endoplasmic reticulum via TAPs (109).

Finally, DEC-205 is an excellent antigen delivery vehicle because monoclonal antibodies to this receptor efficiently target DCs in vivo. When the purified anti-DEC-205 IgG is injected subcutaneously, most $CD11c^+$ DCs in the draining lymph node take up the antibody (23). Uptake is not detected in lymphocytes or macrophages, either in cell suspension or in tissue sections. In conclusion, DEC-205 is a valuable antigen-targeting receptor on DCs because antigens delivered to this receptor are processed for presentation on both MHC class I and II and because targeting is specific and efficient.



## Other Receptors for Endocytosis on Dendritic Cells

DCs express several other molecules capable of mediating adsorptive uptake. Many of these, in contrast to the MMR and DEC-205, are type II transmembrane proteins with a single external C-type lectin domain. Each of these lectins mediates uptake of its corresponding monoclonal antibody and, in some cases, presentation to mouse Ig-specific T cells. However, these monolectins have been studied primarily in human cell cultures and there is no information concerning antigen presentation in vivo, including the exogenous pathway to MHC class I. This is of some interest because the lectins are expressed by subsets of DCs. For example, Langerin or CD207 is expressed in Langerhans cells (110), the asialoglycoprotein receptor type 1 (111) and DC-SIGN or CD209 (112) in monocyte derived DCs, and the BDCA-2 molecule in plasmacytoid DCs (113).

Additional endocytic receptors are shared with other cells. Nevertheless, these receptors are distinctive because uptake into DCs leads to presentation by the exogenous pathway to MHC class I. Some examples include the Fc$\gamma$R for immune complexes (114, 114a,b,c) and the $\alpha V\beta 5$ and $\alpha V\beta 3$ integrins for dying cells (79). In summary, there are many potential ways to enhance the efficiency of antigen presentation through receptor-mediated uptake, but for most of these, there is little in vivo validation at this time.

## Delivery of Peptides Engineered into the Anti-DEC-205 Antibody

To test the idea that antibodies to DEC-205 efficiently target antigens to DCs in vivo, the heavy chain of the antibody was engineered to include a sequence

for a hen egg lysozyme (HEL) peptide presented on I-$A^k$ molecules (23). The constant regions of the rat heavy chain also were replaced with mouse C regions carrying mutations to block binding to Fc$\gamma$ receptors. Submicrogram amounts of the engineered antibody were then injected into mice that were adoptively transferred with HEL-specific TCR transgenic T cells. In spite of the low doses of antigen injected ($<1$ $\mu$g of Ig, corresponding to $<20$ ng of peptide prior to processing), efficient presentation took place on DCs in situ. All of the transgenic $CD4^+$ T cells underwent at least four to seven divisions, but then they were almost entirely deleted (23). To establish that the animal was tolerized to the peptide delivered by anti-DEC-205, the mice were rechallenged with peptide in complete Freund's adjuvant. Such mice failed to respond, indicating that the adoptively transferred T cells had been tolerized by selective presentation of antigens on DCs in the steady state. The opposite outcome developed if parallel groups of animals were given anti-DEC-205/HEL antibody plus a DC maturation stimulus, agonistic CD40 antibody. Under DC maturation conditions, the transgenic $CD4^+$ T cells produced large amounts of IFN-$\gamma$ and were not deleted (23).

These results indicate that low doses of intact soluble proteins, when targeted to DCs in the steady state, are successfully processed and presented, leading to deletional tolerance in the corresponding antigen-reactive T cells. In contrast, immunity ensues if anti-CD40 is also given to the antigen-targeted mice. It is formally possible that these two distinct outcomes represent the function of separate lineages of immunogenic and tolerogenic DCs. However, we favor the interpretation that the same DCs function in immunity and tolerance depending on their state of maturation.

## Delivery of Proteins Conjugated to the Anti-DEC-205 Antibody

A related DC-targeting approach involves the chemical conjugation of whole proteins to the anti-DEC-205 antibody (24). Following injection of $\sim$100 nanograms of ovalbumin conjugated to anti-DEC-205, an average of about $10^5$ ovalbumin molecules were selectively captured by each $CD11c^+$ lymph node DC. Plateau levels of ovalbumin were evident in the DCs for 12–48 h after subcutaneous injection. As shown for the engineered antibody (23), anti-DEC targeting with chemical conjugates did not perturb the DCs, even in the presence of antigen-reactive transgenic T cells (24). In contrast, simultaneous administration of anti-CD40 led to large increases in expression of CD40, CD80, CD86, and MHC class II (23, 24).

One valuable feature of ovalbumin as a model protein is that $CD8^+$ MHC class I–restricted TCR transgenic OT-I cells are available. Indeed, DEC-205 mediated ovalbumin presentation on MHC class I through a TAP-dependent pathway. As in the case with $CD4^+$ TCR transgenic T cells above, $CD8^+$ T cells were first driven into multiple cell cycles. Then the $CD8^+$ T cells were also deleted over 9–14 days, and the animals became tolerant to immunization with ovalbumin in Complete Freund's Adjuvant. The opposite outcome, strong immunity, developed

if the animals were given an agonistic anti-CD40 antibody together with the anti-DEC-205:ovalbumin conjugate (24).

DEC-205 is the first DC receptor to be targeted in vivo with defined antigens. The studies indicate that DCs in lymph node constitutively present antigens on both MHC class I and II products, and that receptor-mediated pathways allow very small amounts of protein to be processed successfully. T cells that recognize antigens targeted to DCs in the steady state proliferate actively but then disappear, and the animals become tolerant. All of the TCR transgenic T cells that have been evaluated for tolerance have a high affinity for the antigen in question. In this setting DCs function efficiently to maintain peripheral tolerance in the steady state.



## CELL-ASSOCIATED ANTIGENS TARGETED TO DENDRITIC CELLS IN THE STEADY STATE ALSO INDUCE DELETIONAL TOLERANCE

### Delivery of Pancreatic Islet $\beta$ Cell Antigens to Dendritic Cells

Several laboratories have produced transgenic mice using the rat insulin promoter to direct antigen expression in pancreatic islet $\beta$ cells. In each case $\beta$ cell–associated antigens were presented to T cells (both $CD8^+$ and $CD4^+$) in the steady state. However, antigen presentation was not mediated by the islet $\beta$ cells but by bone marrow–derived cells in the draining pancreatic lymph nodes. For example, when MHC class I restricted ovalbumin-specific T cells were adoptively transferred into mice expressing ovalbumin as part of the external domain of the transferrin receptor, T cell proliferation was found in the draining pancreatic lymph nodes (115, 116). In this particular transgenic line, the rat insulin promoter also drove expression of ovalbumin in renal epithelium. Accordingly, presentation to OT-I T cells took place in renal lymph nodes as well. To prove antigen presentation by bone marrow–derived cells, bone marrow chimeras were constructed such that only bone marrow–derived cells could present the relevant peptide (the $\beta$ cells expressed mutant H-$2K^b$ MHC class I molecules that could not present peptide to OT-I T cells). Only mice transferred with the restricting H-$2K^b$ bone marrow demonstrated T cell proliferation in the lymph nodes, establishing that ovalbumin antigen moved from the $\beta$ cell to bone marrow–derived antigen-presenting cells in the lymph node. Similar experiments have been performed using an influenza hemagglutin peptide expressed in $\beta$ cells and presented to $CD8^+$ T cells (117) and an islet cell autoantigen in NOD diabetes-prone mice presented to $CD4^+$ T cells (118).

DCs have been identified as the cells responsible for the presentation of islet-derived antigens. At first, the evidence was difficult to obtain. If DCs were isolated from pancreatic lymph nodes where presentation was taking place, it was difficult to detect stimulation of antigen-specific T cells in culture. An in vivo experiment was designed to address what appeared to be a sensitivity problem in detecting

relevant levels of MHC-peptide in culture. $\beta_2$-microglobulin-negative mice (MHC class I deficient) were given bone marrow from donors expressing the requisite $\beta_2$-microglobulin under the control of the CD11c promoter (119). In mice, it is known that high-level CD11c expression occurs primarily if not exclusively in DCs (70). Also, selection of cells from lymphoid tissues on the basis of CD11c expression yields highly enriched populations of $CD11c^+$ DCs and $CD11c^-$ non-DCs (120). The H-$2K^b$-expressing DCs were found to direct presentation of ovalbumin in pancreatic lymph nodes in vivo, even though the ovalbumin was expressed in MHC class I–negative $\beta$ cells.

Confirmation that DCs in lymph nodes present cell-associated antigens has also been obtained using RIP transgenic mice expressing a herpes virus glycoprotein in $\beta$ cells. In a sensitive bioassay, $CD11c^+$ pancreatic lymph node DCs from these mice presented antigen to a virus-specific, $CD8^+$ T-T hybridoma (121). When DCs from these mice were fractionated into $CD8^+$ DCs and $CD8^-$ DCs, only the $CD8^+$ fraction was active in antigen presentation. In contrast, when presentation was studied in another system (a pancreatic islet $\beta$ autoantigen for $CD4^+$ T cells), the CD11b high (also $CD8^-$) DC subset presented antigen (118). The reason for this apparent disparity is not yet evident.

Together these studies show that several different antigens in pancreatic $\beta$ cells can be presented by DCs in the draining nodes. The presentation is constitutive; that is, whenever one injects specific T cells, they begin to proliferate in the draining lymph nodes. The mechanism for antigen transfer from nonhematopoietic cells to the DCs has not been defined. In the NOD system above, transfer of antigen to DCs was increased by streptozotocin, which kills $\beta$ cells. This implies that dying cells are the vehicle for antigen transfer from the islet to lymph node DCs (118) and that in the steady state DCs capture cells that die during the normal process of $\beta$ cell turnover.

## Capture of Cell-Associated Antigens by Dendritic Cells in Other Peripheral Tissues

A subset of DCs with prominent, DNA-positive, inclusion bodies (Feulgen stain) was first found in mesenteric afferent lymph (122). These inclusion bodies are apoptotic bodies, as defined by the TUNEL staining method. The inclusions are derived from intestinal epithelial cells, because some can be stained with an antibody to an epithelial form of keratin (93). In addition, large numbers of DCs in the draining mesenteric lymph node, but not in other lymph nodes, have high levels of an esterase isoform found in the intestine but not in DCs or macrophages. Within the lamina propria, DCs marked by the OX62 mucosal integrin and expression of MHC class II also have TUNEL-positive inclusions and nonspecific esterase. Taken together, these experiments indicate that DCs in mesenteric lymph mediate a substantial flux of dying intestinal epithelial cells to the draining lymph node in the steady state. Interestingly, a subset of $CD4^-$ DCs is responsible. This subset may be analogous to the $CD8^+CD4^-$ subset of mouse spleen DCs that selectively

takes up dying cells (see below). However, it is not known whether the lymph DCs that migrate to the nodes become resident in the T cell area and present the antigen or if the migratory DCs die quickly and are then processed by other lymph node DCs, the latter being particularly efficient at forming MHC-peptide complexes (84).

Capture and processing of cell-associated antigen in the periphery has been documented using monoclonal antibodies to an authentic autoantigen, the proton pump ATPase of the gastric parietal cell (94). This autoantigen was found in $CD11c^+$ cells beneath the gastric epithelium and in the draining lymph node. Furthermore, the number of ATPase-containing DCs increased markedly during gastritis relative to the steady state. The antigen was processed to form MHC-peptide complexes in vivo because $CD11c^+$ DCs could be isolated (exclusively from the gastric lymph node), treated with chloroquine to block antigen processing upon isolation, and shown to stimulate ATPase-specific T cells (94).



## Immune Tolerance Induced by Dendritic Cells Presenting Antigens from Dying Cells

The functional consequences of the presentation of dying cells by DCs were studied with ovalbumin as a surrogate cell-associated antigen (25). In this experimental system, antigen is introduced into the dying cells by osmotic shock (123). Dead cells are then injected into mice, whereupon the cells are taken up by recipient DCs (124). Uptake is remarkably selective for the $CD8^+$ subset of splenic DCs, one of the clearest differences in the functions of the $CD8^+$ and $CD8^-$ subsets (124, 124a). These subsets may differ more in their endocytic receptors, rather than their ability to present cell-associated antigen by a TAP-dependent pathway. $CD8^+$ DCs selectively capture dying cells, but both subsets can capture virus like particles (125) and soluble proteins (K. Liu, B. Bonifaz, K. Inaba & R.M. Steinman, unpublished observations).

As in the cross presentation of islet $\beta$ cells discussed above, ovalbumin-specific T cells transferred into mice receiving ovalbumin-loaded dead cells at first proliferated actively (25). Again, submicrogram amounts of ovalbumin within the injected cells were sufficient to induce active proliferation of large numbers of $CD8^+$ T cells (25). However, most of the antigen-specific T cells were deleted from the blood, spleen, and lymph nodes after one week. Furthermore, the animals became tolerant to immunization with ovalbumin in complete Freund's adjuvant. Interestingly, if the T cells were removed from mice prior to deletion and then stimulated in culture, they exhibited changes consistent with the development of immunity rather than tolerance, that is, upregulation of CD25 IL-2 receptors and downregulation of CD62L lymph node homing receptors. Although this type of response is often designated as cross-priming, the priming is observed in vitro, whereas tolerance can be the outcome in vivo. Therefore to observe peripheral tolerance induced by DCs in situ, it is important not to perturb the DCs or the T cells from the steady state (25).

The essential role of DCs in the presentation of cell-associated antigens has recently been demonstrated by selective deletion of these cells in mice (126). The CD11c promoter was used to create transgenic mice expressing the human receptor for diptheria toxin on DCs. When a single dose of the toxin was administered to these mice, $CD11c^+$ DCs were selectively deleted for 1–2 days. During this interval the DC-depleted mice could not present ovalbumin associated with dying splenocytes (126).

Together these new experiments indicate that antigens can be transferred from many types of donor cells (endocrine cells, epithelial cells, leukocytes) to DCs constitutively in the steady state and that the outcome of antigen presentation is the deletion of naive peripheral T cells and systemic antigen-specific tolerance. This pathway may also be capable of deleting memory T cells (127).



## DENDRITIC CELLS AND THE CONTROL OF SUPPRESSOR AND REGULATORY T CELLS

### T Cells that Regulate or Suppress Other Effector T Cells

Regulatory (Tr1) and suppressor (Ts) T cells block the function of other effector $CD4^+$ and $CD8^+$ T cells (54, 128–131). As a result, immune tolerance is achieved. It is not yet clear if Tr1 and Ts are distinct in their mechanism of action, but both are unable to respond to anti-CD3 stimulation unless one adds IL-2 and possibly additional growth factors. Methods are now available to clone both Tr1 and Ts (132). The term Ts is often used for $CD4^+$ $CD25^+$ cells that are produced in the thymus, with selection taking place on the cortical epithelium (131, 133, 134). The term Tr1 is often used for cells that are generated from peripheral $CD25^-$ precursors and have the potential to produce IL-10 and/or TGF$\beta$ (131, 132). Both Ts and Tr1 suppress immune responses in vivo; for example Ts suppress autoimmune models of gastritis, thyroiditis, diabetes, and oopheritis (128), and Tr1 suppresses alloreactivity in the setting of graft versus host disease (135, 136) and blocks immune function in certain infections (137, 138). Therefore it is possible that there are two forms of actively tolerogenic T cells that are distinct in their origin and targets. It will be valuable to determine the contribution of DCs, or specific DC subsets and stages of maturation, to the formation and function of these T cells in vivo.

### Influence of Dendritic Cells on Regulatory T Cell Formation

When DCs are produced from human blood monocytes by culture in IL-4 and GM-CSF, they are weak initiators of immunity. The physiologic counterpart of these in vitro–produced immature DCs is not known, but it has been hypothesized that they correspond to monocytes beginning to differentiate into DCs during transit from tissue spaces into afferent lymphatics (139, 140). When ex vivo monocyte-derived DCs undergo maturation through toll-like receptor stimuli, CD40L, or inflammatory cytokines, they become potent stimulators of T cell proliferation

and effector cell development (27, 28). Immature DCs, however, are not necessarily inactive. Allogeneic T cells, when cultured with immature DCs, can become refractory to antigenic restimulation, even by mature DCs (141). The T cells also can inhibit other T cells from responding to mature DCs in vitro. This regulatory function requires cell contact and is also partially blocked by anti-IL-10 antibodies, leading to the interpretation that Tr1 can be induced by monocyte-derived immature DCs (141). It has been proposed that DC expression of the ILT3 immunoglobulin-like transcript also contributes to the induction of Tr/Ts (141a).

Similar populations of immature and mature monocyte-derived DCs pulsed with an influenza matrix peptide have been used to examine the $CD8^+$ immune response in healthy human volunteers primed by natural exposure to influenza. When the DCs were matured with inflammatory cytokines and injected back into the monocyte donor, they induced a clear immune response. The number of effector $CD8^+$ T cells in blood expanded (47), and memory T cells became responsive to lower doses of matrix peptide (18). In contrast, 1 week after injection with matrix peptide–pulsed immature DCs, the two volunteers showed a marked decrease in their memory IFN-$\gamma$ response and a parallel increase in matrix-peptide responsive, IL-10 producing cells (41). With time (months), the IL-10 producers waned and the IFN-$\gamma$ producers reappeared. However, when $CD8^+$ T cells from the 1-week immune samples were mixed with preimmunization or recovery samples, the IFN-$\gamma$ response was nullified (142). All the effects were specific for flu matrix peptide; that is, there were no IL-10 producing cells for EBV and cytomegalovirus (CMV) peptides, and the flu peptide did not suppress EBV and CMV responses. These experiments were interpreted to mean that DCs produced from monocytes with GM-CSF and IL-4 expand specific Tr1 cells when injected in vivo.

DCs isolated from mouse lungs after intranasal administration of ovalbumin (143), or from mouse spleen after administration of aggregated Ig (42), produce IL-10, as is the case for DCs in gut mucosal–associated lymphoid tissue (144) and lipopolysaccharide-stimulated monocyte-derived DCs from human blood (145–147). IL-10 enhances the formation of mouse (148) and human (132, 149) Tr1. The DCs isolated from lungs following intranasal ovalbumin caused cultured T cells to produce IL-10 (as well as IL-4) (143). This work has recently been extended to DCs and antigen-specific Treg in vivo, with evidence for a critical role for ICOS-ligand expression on the DCs (143a).

There are many intriguing reports that immature DCs suppress immunity following adoptive transfer into mice. Two recent examples are the transfer of antigen-specific hyporesponsiveness by ICOS-ligand expressing and IL-10 producing pulmonary DCs, in a model of airway allergy (143a), and by a subset of IL-10 producing, $CD4^+$ $CD8^-$ splenic DCs in a model of autoimmune experimental allergic encephalitis (42). Likewise the control of $CD25^+$ Ts by antigen-presenting cells in vivo remains to be investigated. This will be important because $CD25^+$ Ts are responsible for protection against a number of autoimmune syndromes. In conclusion, there is evidence that it would be worthwhile to pursue a role for DCs in inducing regulatory forms of immune tolerance.

## DISCUSSION: SOME QUESTIONS ABOUT TOLEROGENIC DENDRITIC CELLS

### What Mechanisms Underlie the Function of Dendritic Cells in Inducing Tolerance?

The T cell is likely to be a major determinant in the tolerance outcome. In the thymus engagement of the TCR on a newly generated single positive thymocyte may initiate an apoptotic program involving the bim proapoptotic pathway (150). In other words, antigen-presenting thymic DCs may not require a special capacity to kill developing self-reactive thymocytes. Analogously, peripheral tolerance may be the "default" pathway, again proceeding via bim (151) whenever T cells are stimulated to grow in the absence of further stimuli from maturing DCs. DC maturation is the critical switch that provides signals for effector T cell development and memory, diverting T cells from apoptosis to protective immune function. For example, maturation stimulates DCs to produce immune-enhancing cytokines like IL-12 or IFN-$\alpha$ and to express high levels of membrane costimulatory molecules that promote T cell survival and cytokine/cytolysin production.

A more active potential immunosuppressive pathway that needs to be evaluated in vivo involves DC production of tryptophan metabolites through the action of indoleamine 2,3-dioxygenase (IDO) (Figure 1). IDO exists in DCs within mouse (152) and human (152a) lymphoid tissues, and expression may increase during inflammation (152a). IDO activity can be induced via IFN-$\gamma$ receptors, and this leads to T cell apoptosis in culture (152). IFN-$\gamma$ receptors (CD119) are markedly downregulated as the DC matures (152–154). The IDO-catalyzed tryptophan metabolites are responsible for killing T cells, especially activated T cells (155). Recently it was shown that CTLA-4Ig triggers B7 molecules on DCs to induce IDO (155a).

More "active" roles may also need to be played if DCs prove to be important in inducing tolerance via Tr1 and Ts cells. For example, DC production of IL-10 and other immunosuppressive cytokines may be critical for the differentiation of Tr1. IL-10 production could in turn be stimulated by products in an exposed epithelium like the airway or intestine or by microbial products in the lumen. Alternatively, some suppressor cells may be programmed to suppress when they are generated in the thymus.

Even though tolerogenic DCs may not kill peripheral T cells directly, the DCs nevertheless need to carry out several sets of activities (Figure 1). The first is the capture and processing of self- and environmental antigens, including for presentation on MHC class I products. Figure 1 diagrams a number of specializations of DCs in antigen presentation, i.e., the formation of MHC-peptide complexes. These specializations include distinct endocytic receptors, the efficacy in carrying out the exogenous pathway for processing and presentation on MHC class I, and the regulation of antigen processing during responses to maturation stimuli. A second set of DC features pertains to their migration and homing properties.

These position DCs appropriately in the steady state for the capture of dying cells and environmental proteins and for interaction with T cells in lymphoid tissues. Distinct chemokine receptors can be expressed by DCs in different subsets (plasmacytoid DCs express CXCR4; monocyte-derived DCs typically express CCR5), locations (epithelial DCs express CCR6), and maturation states (mature DCs always express CCR7). Nonetheless, much needs to be learned about the origin of DCs in the T cell areas of lymphoid organs and the control of DC traffic in the steady state. Third, immature DCs can express adhesion molecules for T cells, such as DC-SIGN or CD209, a lectin that binds ICAM-3 on resting T cells (156). In the steady state, DCs in lymphoid organs also express significant amounts of CD80 and CD86, but the role of these molecules in tolerance remains to be ascertained. Likewise, many members of the TNF family can be expressed by DCs (e.g., OX40L/CD134L, 4-1BBL/CD137L), but their expression in vivo needs additional investigation. CD134L seems important for mesenteric lymph node DCs to drive colitis-inducing T cells (156a). Many of these features of DCs can change with maturation. The DCs then dampen expression of some properties and acquire new functions, including the production of growth factors like IL-2 and thiols, cytokines, and much higher levels of T cell interaction molecules like CD40, CD86, and B7-DC, which allow DCs to play active roles in initiating immunity (Figure 1).

## Will Targeting to Dendritic Cells Lead to Tolerance with Low Doses of Antigen?

As mentioned in Table 1, one of the enigmas in tolerance is how the immune system remains tolerant to small amounts of most harmless proteins that are present in the steady state, especially when DCs should be able to process and present many of these self- and environmental proteins during DC maturation in response to infection. In physiologic circumstances, DCs appear to be the predominant cells that continually take up and process antigens for presentation to T cells. Many of the markers used to identify DCs are in fact endocytic receptors that could enhance antigen uptake. We mentioned above such molecules as DEC-205, langerin, asialoglycoprotein receptor, BDCA-2, and DC-SIGN (Figure 1). Once antigen is taken up, DCs seem particularly efficient at forming and exporting MHC class II peptide complexes (32, 157, 158, 158a). For example, when a DC takes up $10^5$ I-E molecules from dying B cells, it forms several thousand MHC II/I-E peptide complexes (84). Furthermore, DCs are proficient and perhaps unique in processing nonreplicating antigens into peptides that are transported into the rough endoplasmic reticulum by TAPs and presented on MHC class I products. This exogenous pathway extends to dying cells, immune complexes, and ligands for the DEC-205 receptor (Figure 1).

Uptake and processing mechanisms are continuously active in the immature DCs located within lymphoid tissues in the steady state. These functions are further buttressed by the capacity of additional DCs to patrol peripheral tissues, picking

up proteins and dying cells continuously in the steady state (25, 93, 94). Two intriguing new studies indicate that a subset of $CD16^+$ monocytes are specialized to differentiate into DCs that move from tissues into lymph (158a), and that DCs can upregulate the CCR7 lymph node homing receptor following uptake of dying cells (158c), with uptake of dying autologous cells being a steady state function of DCs (25)(93). To date, only DCs in lymphoid organs have been shown to process antigens in the steady state, and it is possible that DCs in peripheral tissues need to receive additional stimuli to present the antigens they have captured.

Other cell types may not gain access to significant amounts of self- and environmental antigens in the steady state or may not process them to MHC class I and class II peptide complexes with the same efficiency as DCs. However, non-DCs may present MHC-peptide complexes when animals are given large amounts of antigen, especially if the antigen is given as preprocessed peptides, as is the case in several reports on the induction of peripheral tolerance. The underlying tolerance mechanism induced by non-DCs may be anergy, which is a reversible form of tolerance. Evidence for anergy induction through non-DCs was recently obtained with NKT cells from mice treated with high doses of a glycolipid, $\alpha$-galactosyl ceramide (10). This synthetic drug is presented on CD1d molecules to invariant TCRs on NKT cells. If the glycolipid was selectively targeted to DCs, a prolonged effector type of NKT response was induced, but if the drug was allowed to access non-DCs, the latter induced dominant anergy in the NKT cells.



## How Might Tolerogenic Dendritic Cells Contribute to Disease and Therapy?

If DCs continually sample self to induce peripheral tolerance, then chronic activation would vitiate their role in bringing about T cell deletion. In the autoimmune disease systemic lupus erythematosus evidence has been obtained that blood monocytes express features of stimulatory DCs (159) owing to increases in serum IFN-$\alpha$ (159). It is proposed that the more stimulatory DCs in lupus may present self-nucleoprotein complexes, resulting in autoimmunity rather than tolerance. Similarly, when CD40L is expressed as a transgene in mouse epidermis under the control of the K14 promoter, Langerhans cells are chronically mature, and systemic autoimmunity develops (160).

It is evident that many tissue antigens do not induce tolerance but are ignored by the immune system (161, 162). These ignored antigens would then have the potential to induce autoimmunity. Ignorance in cell biological terms may mean that an antigen is insufficiently processed and presented by DCs in the steady state. For example, when antigens from islet $\beta$ cells are presented by lymph node DCs in the steady state, a low level of antigen expression in the islets is associated with poor antigen presentation in the lymph node (163). During infection, ignored proteins might be processed by proteases released from microorganisms, leukocytes, or dying leukocytes. These peptides could then be presented by DCs that are maturing in response to infection, and autoimmunity could develop.

In chronic infectious diseases, the tolerogenic role of DCs might be exploited by the pathogen to reduce the protective immune response. It has been hypothesized that this situation may occur with persistent microbes that are taken up by DCs without maturing them (95). The DCs may then unwittingly induce tolerance, either deleting reactive T cells or inducing regulatory cells. HIV-1 may be such an example because it is produced in high amounts in the absence of antiretroviral drugs, and DCs in the steady state express receptors to capture virus.

In transplantation DC maturation in both donor and recipient is likely to accompany the preparation and engraftment of organ allografts (164). Whereas a block in DC maturation should reduce the initial sensitization to the transplant, at the levels of the transplant donor (the direct pathway whereby alloMHC and minor histocompatibility antigens are presented by DCs from the allograft) and recipient (the indirect pathway whereby recipient DCs present peptides from alloMHC and minor histocompatibility antigens from the graft), it is additionally possible that a block in maturation will enhance the induction of antigen-specific tolerance.

Tolerance is considered to be a potential obstacle to tumor immunotherapy because the persistent tumor cells might be regarded as self. This needs to be looked at with the new tools that are available to study specific antitumor immune responses. In the case of multiple myeloma one can use DCs to present whole tumor cells to T cells from the tumor environment. When this is done, the DCs induce strong responses in $CD8^+$ T cells from patients with progressive tumors (165), implying that if there is tolerance to myeloma, it is far from complete. Possibly the antiapoptotic processes that are vital to tumorigenesis represent the immunologic Achilles heel of tumor cells. If DCs are unable in the steady state to capture and present cancer cells in a tolerogenic mode, the antitumor T cell repertoire may not be silenced. Instead T cells would be amenable to DC-mediated active immunotherapy.

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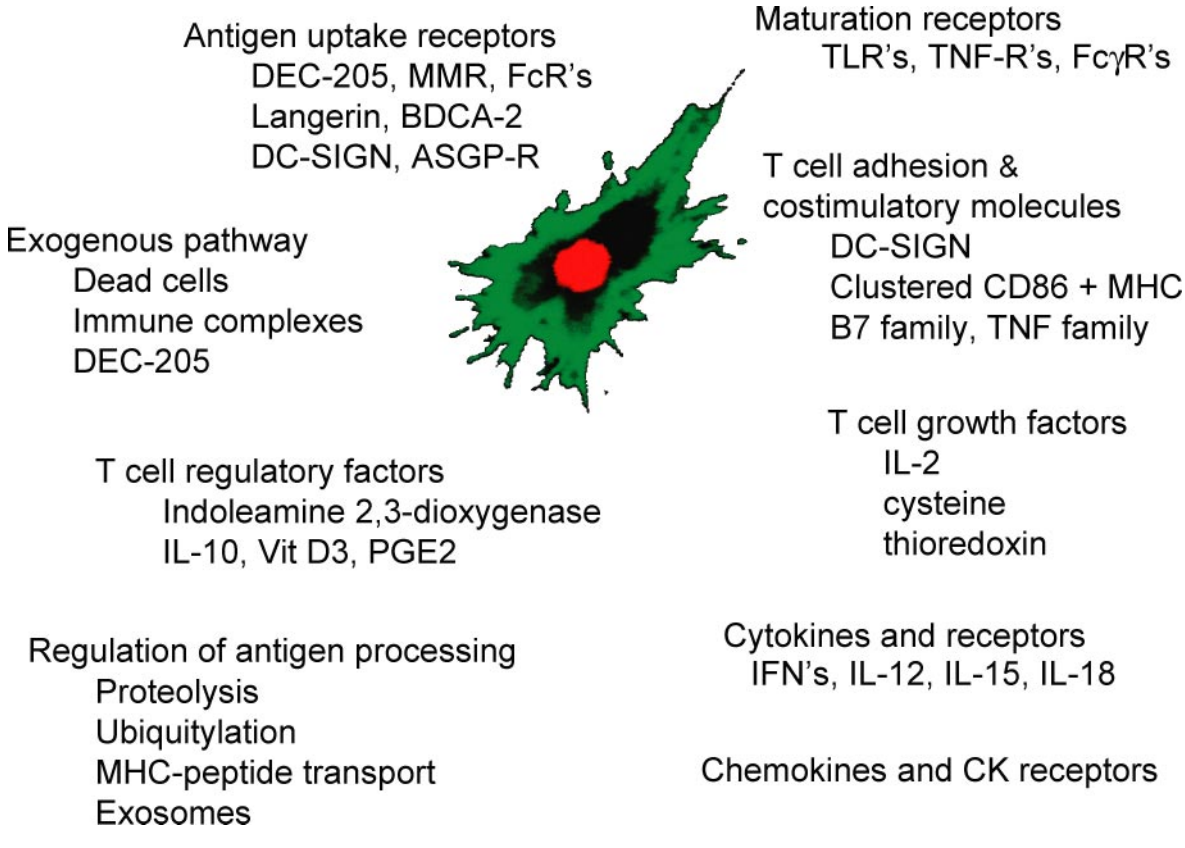


**Figure 1** Some components of immature and mature dendritic cell function (see text).

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