Dendritic Cells Induce Peripheral T Cell Unresponsiveness Under Steady State Conditions In Vivo

Daniel Hawiger, Kayo Inaba, Yair Dorsett, Ming Guo, Karsten Mahnke, Miguel Rivera, Jeffrey V. Ravetch, Ralph M. Steinman, and Michel C. Nussenzweig

1Laboratory of Molecular Immunology, 2Howard Hughes Medical Institute, 3Laboratory of Cellular Physiology and Immunology, and 4Laboratory of Molecular Genetics and Immunology, The Rockefeller University, New York, NY 10021
5Laboratory of Immunobiology, Graduate School of Biostudies, Kyoto University, Kyoto 606-8502, Japan

Abstract

Dendritic cells (DCs) have the capacity to initiate immune responses, but it has been postulated that they may also be involved in inducing peripheral tolerance. To examine the function of DCs in the steady state we devised an antigen delivery system targeting these specialized antigen presenting cells in vivo using a monoclonal antibody to a DC-restricted endocytic receptor, DEC-205. Our experiments show that this route of antigen delivery to DCs is several orders of magnitude more efficient than free peptide in complete Freund’s adjuvant (CFA) in inducing T cell activation and cell division. However, T cells activated by antigen delivered to DCs are not polarized to produce T helper type 1 cytokine interferon γ and the activation response is not sustained. Within 7 d the number of antigen-specific T cells is severely reduced, and the residual T cells become unresponsive to systemic challenge with antigen in CFA. Coinjection of the DC–targeted antigen and anti-CD40 agonistic antibody changes the outcome from tolerance to prolonged T cell activation and immunity. We conclude that in the absence of additional stimuli DCs induce transient antigen–specific T cell activation followed by T cell deletion and unresponsiveness.

Key words: antigen delivery • DEC 205 • dendritic cells • peripheral T cell tolerance • CD40

Introduction

Dendritic cells (DCs)* are uniquely potent inducers of primary immune responses in vitro and in vivo (1, 2). In tissue culture experiments, DCs are typically two orders of magnitude more effective as APCs than B cells or macrophages (3, 4). In addition, purified antigen-bearing DCs injected into mice or humans migrate to lymphoid tissues and efficiently induce specific immune responses (5–7). Likewise, DCs migrate from peripheral tissues to lymphoid organs during contact allergy (8, 9) and transplantation (10), two of the most powerful known stimuli of T cell immunity in vivo. Based on these and similar experiments, it has been proposed that the principal function of DCs is to initiate T cell–mediated immunity (1). However, nearly all of these experiments involved DC purification or culture in vitro, or some perturbations in vivo that induce major alterations in DC maturation and function. Thus, the physiologic function of DCs in the steady state has not been determined (6, 11).

There is indirect evidence from a number of different laboratories suggesting that DCs may play a role in maintaining peripheral tolerance (summarized in reference 12). For example, injection of mice with 33D1, a rat monoclonal antibody to an unknown DC antigen, appeared to induce T cell unresponsiveness to the rat IgG (13). However, the specificity of antigen delivery was uncertain and the relevant T cell responses could not be analyzed directly. In addition, peripheral tolerance to ovalbumin and hemagglutinin expressed in pancreatic islets was found to be induced by bone marrow–derived APCs (14–16), but...
the identity of these antigen presenting cells has not been determined (17).

Materials and Methods

Mice. 6–8-wk-old females were used in all experiments and were maintained under specific pathogen free conditions. B10.BR, B6.SJL (CD45.1), and B6/MRL (Fls lpr) mice were purchased from The Jackson Laboratory. 3A9 transgenic mice were maintained by crossing with B10.BR mice. To obtain CD45.1 3A9 or 3A9/lpr T cells, B6.SJL or B6/MRL mice were crossed extensively with 3A9 mice and tested for CD45.1 and I-A<sup>+</sup>, by flow cytometry. Fls lpr mutation was tested by PCR. Mice were injected subcutaneously with peptide in CFA and subcutaneously or intravenously with chimeric antibodies. All experiments with mice were performed in accordance with National Institutes of Health guidelines.

Flow Cytometry and Antibodies Used for Staining. CD4<sup>-</sup> (L3T4), MHC II<sup>-</sup> (10-3.6), CD11c<sup>-</sup> (HL3), CD11c<sup>-</sup> (HL3), B220<sup>-</sup> (RA3-6B2), or CD3<sup>-</sup> (145-2C11), CD80(B7-1)<sup>-</sup> (16-10A1) I-A<sup>+</sup> (10-3.6), CD45.1 (A20), II-2 (JES6-5H4), IFN-γ (XM16.2), CD40<sup>-</sup> (HM40-3-FITC), CD86(B7-2)<sup>-</sup> (GL1) specific antibodies were from BD Pharmingen. Rat IgG-PE (goat anti-rat IgG) specific antibody was from Serotec. 3A9 T cell receptor (1G12) specific antibody was a gift from Dr. Emil Unanue, Washington University, St. Louis, MO (18).

For visualization of rat IgGs on surface of mononuclear cells, lymphoid cells were purified from peripheral LNs 14 h after antibody injection and stained with anti-rat IgG-RPE (goat anti-rat IgG-RPE; Serotec) to visualize surface bound NLDC145 and GL117 antibodies. The cells were then incubated in mouse serum to block nonspecific binding and stained with FITC anti-CD11c (HL3), or -B220 (RA3-6B2), or -CD11c (145-2C11).

For intracellular cytokine staining, lymphocytes were stimulated in vitro for 4 h with leukocyte activation cocktail (BD Pharmingen) according to the manufacturer’s manual. Cells were fixed and permeabilized using cytotox/ cytoperm buffer from BD Pharmingen.

Immunohistology. Popliteal LNs were removed from antibody injected mice and 5-μm cryosections (Microm; ZEISS) were generated using deconvolution software (Metamorph).

For T cell proliferation assays in adoptive transfer recipients, 9 × 10<sup>4</sup> of the same irradiated CD11c<sup>-</sup> cells isolated from spleens of wild-type B10.BR mice were cultured in 96-well plates with 3 × 10<sup>5</sup> T cells from each experimental group. Synthetic HEL peptide was provided by the Howard Hughes Medical Institute Keck Biotechnology Resource Center.

Adoptive Transfer. CD4 cells from 3A9 mice were enriched by depletion as described above, washed 3× with PBS, and 5 × 10<sup>6</sup> cells injected intravenously per mouse. Alternatively, before depletion total cells were labeled with 2 μM 5-(6)-carboxyfluorescein diacetate succinimidyl diester (CFSE) in 5% FCS RPMI (Molecular Probes) at 37°C for 20 min and washed twice.

Results

To examine the function of DCs in vivo, we devised a means of delivering antigens to DCs in situ. We used NLDC145 (19), a monoclonal antibody specific for DEC-
205, an endocytic receptor that is a member of a family of multiligand receptors including the macrophage mannose receptor (MMR) (22, 23). Like MMR, DEC-205 displays an NH2-terminal cysteine-rich domain, a fibronectin type II domain, and multiple C-type lectin domains (22). However, the tissue distribution of DEC-205 and the MMR differ in that DEC-205 is highly expressed by DCs within the T cell areas of lymphoid tissues, particularly on CD8+ DCs that have been implicated in cross-priming (24), whereas the MMR is expressed by some tissue macrophages (25, 26). We chose DEC-205 for targeting antigens to DCs because the cytoplasmic domain of DEC-205 orchestrates a distinct endocytic pathway that enhances antigen presentation (23). DEC-205 recycles through late endosomes or lysosomes rich in MHC II, and antigens delivered to these compartments by DEC-205 are efficiently processed and presented to T cells (23).

To determine whether the NLDC145 antibody targets DCs in vivo, we injected mice subcutaneously with purified NLDC145 or GL117, a nonspecific isotype-matched rat monoclonal antibody control, and visualized the injected antibody in tissue sections 24 h after injection. NLDC145 was found localized to scattered large dendritic profiles in the T cell areas of LNs and spleen while uptake of control GL117 was undetectable (Fig. 1 A, left and middle). This pattern was similar to the pattern found when the antibody was applied to sections directly (Fig. 1 A, right). The NLDC145-targeted cells were negative for B220 and CD4, markers for B cells and T cells, respectively, but positive for characteristic DC markers including MHC II and CD11c (Fig. 1 B). Thus, subcutaneously injected NLDC145 targets specifically to CD11c+MHC II+ DCs in lymphoid tissues in vivo.

To further characterize the lymphoid cells that were targeted by NLDC145 in vivo, we stained lymphoid cell suspensions from antibody injected mice with anti-rat Ig and examined the cells by multiparameter flow cytometry (Fig. 1 C). High levels of injected NLDC145 were found on the surface of most CD11c+ DCs but not on the surface of B220+ B cells or CD3+ T cells (Fig. 1 C). We conclude that when NLDC145 is injected into mice it binds efficiently and directly to DCs but not to other lymphoid cells.

To deliver antigens to DCs in vivo, we produced fusion proteins with amino acids 46–61 of HEL added to the COOH terminus of cloned NLDC145 (αDEC/HEL) and GL117 (GL117/HEL) control antibody (Fig. 1 D). To minimize antibody binding to Fc (FcR) receptors and further ensure the specificity of antigen targeting, the rat IgG2a constant regions of the original antibodies were replaced with mouse IgG1 constant regions that carry point mutations interfering with FcR binding (20). The hybrid antibodies and control Igs without the terminal HEL peptide (αDEC and GL117) were produced by transient transfection in 293 cells (Fig. 1 E).

To determine whether antigens delivered by αDEC/HEL were processed by DCs in vivo, we injected mice with the hybrid antibodies and controls and tested CD11c+ DCs, CD19+ B cells and CD11c−CD19− mononuclear cells for their capacity to present HEL peptide to naive HEL-specific T cells from 3A9 TCR transgenic mice (27). DCs isolated from antibody-injected mice expressed levels of CD80 and MHC II similar to those found on PBS controls and thus showed no signs of increased maturation, in contrast to what occurs when DCs are stimulated with microbial products like bacterial LPS and CpG deoxyoligonucleotides (28, 29; Fig. 2 A). Nevertheless DCs from mice injected with αDEC/HEL induced strong T cell proliferative responses, whereas DCs isolated from PBS-injected mice or mice injected with the control antibodies had no effect (Fig. 2 B). DC isolated 3 d after αDEC/HEL injection showed reduced antigen-presenting activity (data not shown). In contrast to DCs, B cells and bulk CD11c−CD19− mononuclear cells purified from the same mice showed little antigen-presenting activity (Fig. 2 B). We conclude that antigens can be selectively and efficiently delivered to DC by αDEC/HEL in vivo, and the targeted DCs successfully process and load the peptides onto MHC II.

As DC isolation leads to activation, we performed adoptive transfer experiments with HEL-specific transgenic T cells to follow the response of these T cells to otherwise unmanipulated, antigen-targeted DCs in vivo. CD4+ 3A9 T cells were transferred into B10.BR recipients and 24 h later hybrid antibodies were injected subcutaneously. To measure T cell responses, CD4+ cells were isolated from the draining LNs of the injected mice and cultured in vitro in the presence or absence of added HEL peptide. T cell responses were measured by [3H]thymidine incorporation and are shown as proliferation indices normalized to the PBS control (this index facilitates comparison between experiments, see Materials and Methods). In addition to αDEC/HEL, GL117/HEL, αDEC, and GL117 antibodies, we included 100 μg of HEL peptide in CFA as a positive control.

As described in previous reports (30, 31), CD4+ T cells isolated 2 d after challenge with 100 μg of HEL peptide in CFA showed strong proliferative responses to antigen when compared with PBS controls (Fig. 3 A). Similar responses were obtained from mice injected with as little as 0.2 μg of αDEC/HEL (i.e., ∼4 ng peptide per mouse) but not from mice injected with up to 1μg of αDEC, GL117, or GL117/HEL controls (Fig. 3 A, and not shown). We conclude that antigen delivered to DCs in vivo by αDEC/HEL efficiently induces activation of specific T cells.

To determine whether antigen delivered to DCs in vivo induces persistent T cell activation, we measured T cell responses to antigen 7 d after the administration of αDEC/HEL. CD4 T cells continued to show heightened responses to antigen when purified from LNs 7 d after injection with 100 μg of HEL peptide in CFA (30, 31; Fig. 3 B). In contrast, T cells isolated from mice 7 d after injection with αDEC/HEL were no longer activated when compared with PBS controls (Fig. 3 B). Thus, T cell activation by antigen delivered to DCs by αDEC/HEL in vivo is transient, readily detected at 2 but not 7 d. This transient activation resembles the CD4 T cell response to large doses...
Figure 1. NLDC-145 targets DCs in vivo. (A) Biotinylated NLDC-145 (scNLDC145, left) or rat IgG (scRatIgG, middle) was injected into the hind footpads (50 μg/footpad) and inguinal LNs harvested 24 h later. Sections were stained with Streptavidin Cy3. Control sections from uninjected mice were stained using biotinylated NLDC145 and streptavidin Cy3 (NLDC145, right). (B) Two-color immunofluorescence. Mice were injected with biotinylated NLDC145 as in panel A. Sections were stained with streptavidin FITC (green) and PE-labeled antibodies (red) to B220 as indicated. Specimens were analyzed by deconvolution microscopy. Double labeling is indicated by the yellow color. (C) FACS® analysis of lymphoid cells 14 h after injection with NLDC145 and control GL117 antibody. Histograms show staining with anti-rat IgG on gated populations of CD11c⁺ DCs, B220⁺ B cells, and CD3⁺ T cells. (D) Diagrammatic representation of hybrid antibodies. (E) Hybrid antibodies. GL117, GL117/HEL, αDEC, and αDEC/HEL antibodies analyzed by PAGE under reducing conditions, molecular weights in kD are indicated.
of peptide in the absence of adjuvant, or the response to self-antigens presented by bone marrow–derived antigen-presenting cells in the periphery (15, 16, 30–32). To determine whether the absence of persistent T cell activation in mice injected with αDEC/HEL is due to clearance of the injected antigen, multiple doses of αDEC/HEL were administered. Repeated injection of αDEC/HEL at 3-d intervals failed to induce prolonged T cell activation (Fig. 3C). In addition, after 7 or 20 d, T cells initially activated by αDEC/HEL could not be reactivated when the mice were challenged with 100 μg of αDEC/HEL or GL117/HEL or αDEC or PBS as indicated. CD11c+, CD19+, and CD11c−CD19− cells were isolated from draining LNs 24 h after antibody injection and assayed for antigen processing and presentation to purified 3A9 T cells in vitro. T cell proliferation was measured by [3H]thymidine incorporation and is expressed as a proliferation index relative to PBS controls. The results are means of triplicate cultures from one of four similar experiments.

To examine the fate of 3A9 T cells after exposure to antigen presented by DCs in vivo, we performed adoptive transfer experiments with CD45.1+ 3A9 T cells labeled with CFSE, a reporter dye for cell division. As described previously, T cells challenged with peptide in CFA divide, upregulate CD69 but not CD25, and produce IL-2 and IFN-γ but not IL-4 or IL-10. These cells are therefore considered to be Th1 polarized (30, 31; Fig. 4, A and B, and not shown). A burst of cell division and increase of CD69 but not CD25 expression was also seen after injection with 0.2 μg αDEC/HEL but not with GL117/HEL. Only clonotype positive CD4 cells showed these effects (Fig. 4, A and C, and not shown). However, 3A9 cells activated by antigen presented on αDEC/HEL targeted DCs produced only IL-2 but not IFN-γ, IL-4, or IL-10 at the time of the assay and thus were not polarized to Th1 or Th2 phenotype 3 d after antigen challenge. (Fig. 4 B, and not shown). Therefore, 3A9 cells proliferate in response to αDEC/HEL targeted DCs in vivo, but the T cells do not produce a normal effector cell cytokine profile.

Although there was persistent expansion of 3A9 T cells in regional LNs and spleen 7 and 20 d after challenge with HEL peptide in CFA (Fig. 4 C, spleen not shown), few 3A9 T cells survived in the LNs or spleen after exposure to
antigen delivered by αDEC/HEL. Surviving cells appeared to be anergic as they could not be stimulated in vivo by HEL peptide in CFA (Fig. 3 D). The loss of 3A9 T cells was Fas independent as it also occurred with 3A9/lpr T cells (Fig. 4 C, and not shown). Thus, the initial expansion of T cells in response to antigen presented by DCs in vivo is not sustained, and most of the initial responding T cells disappear from lymphoid organs by day 7. These cells are either deleted or persist in extravascular sites (33). If they do persist outside lymphoid tissues they must be anergic, because they cannot be activated by further exposure to antigen, including peptide in CFA (Fig. 3 D).

DCs can be stimulated to increase their antigen-presenting activity and their immunogenic potential by exposure to bacterial products or CD40L (34–36), a TNF family member expressed on activated CD4 T cells, platelets, and mast cells (37). To determine whether the combination of costimulators and antigen delivery to DCs produces persistent T cell activation, mice were injected with αDEC/HEL, GL117/HEL, or PBS and rechallenged 7 or 20 d later with 100 µg of HEL peptide in CFA (Figs. 3 B and 5 B). The level of T cell activation seen with αDEC/HEL and FGK 45 at day 7 was comparable to HEL peptide in CFA at day 2 and 7 (compare Figs. 3 B and 5 B). To determine whether anti-CD40 treatment altered 3A9 T cell numbers in αDEC/HEL-treated mice, we performed adoptive transfer experiments.
with CD45.1 allotype-marked T cells and assayed by flow cytometry. Whereas FGK 45 alone showed no effect on the number of 3A9 T cells in LNs at day 7, the combination of FGK 45 and αDE/HEL induced persistent 8–10-fold expansion of 3A9 T cells, an increase similar to that seen with HEL peptide in CFA at day 7 (Figs. 5 A and 4). We conclude that persistent T cell responses can be induced by antigen delivered to DCs in vivo if an additional activation signal such as CD40 ligation is provided.

To determine if CD40 ligation induced detectable phenotypic changes on DCs in our system, we analyzed DCs from mice transferred with 3A9 cells and injected with FGK 45 and αDE/HEL. Consistent with work by others we found that those DCs upregulated their surface expression of CD40 and CD86 (39; Fig. 5 C). This increase was more pronounced in the presence of antigen-specific T cells suggesting a positive feedback mechanism between activated DCs and T cells (Fig. 5 C).

**Discussion**

**Targeting Antigens to DCs In Situ through DEC-205.** Our results establish that antigens can be selectively delivered to DCs in vivo via the DEC-205 adsorptive endocytosis receptor. DEC-205, originally identified as an antigen recognized by the monoclonal antibody NLDC-145, offers several advantages as a receptor that will mediate antigen targeting to DCs in situ for purposes of antigen presentation. DEC-205 is expressed in abundance on DCs in the T cell area (19, 40) and antibodies bound to DEC-205 are efficiently internalized and delivered to antigen processing compartments (22, 23). When compared with the MMR, a closely related receptor, DEC-205 was at least 30 times more effective in antigen delivery to processing compartments (23). In vivo, anti-DEC-205 monoclonal antibody targets to DCs very efficiently, a dose of <1 μg of antibody (20 ng of HEL peptide) leading to presentation by DCs that...
was comparable to 100 μg of peptide in CFA (Fig. 3 A). It thus appears that the use of DEC-205 increases the efficiency of DC presentation by at least 100–1,000-fold relative to current adjuvants.

**DCs Induce T Cell Tolerance in the Periphery.** Remarkably, when DEC-205 targeting is used to charge DCs with antigen in the steady state, these MHC II rich cells induce extensive T cell proliferation but do not induce normal Th-subset polarization or prolonged T cell expansion and activation. Instead, the T cells exposed to antigen on DCs in vivo either disappear or become anergic to antigenic re-stimulation. An initial burst of T cell proliferation followed by deletion was reported when ovalbumin or hemagglutinin were expressed as transgenes in the beta cells of the pancreatic islets (15, 16). These, antigens were presented not by pancreatic beta cells but by bone marrow–derived APCs in the draining LNs. Our studies, using direct DC targeting of antigen, suggest that DCs are likely to be the tolerogenic APCs for self-antigens in vivo (for a review, see reference 17). Thus, in the steady state, the primary function of DCs is to maintain peripheral tolerance (Fig. 3, C and D). While it has been proposed that immature antigen-bearing DCs can induce tolerance to transplantation antigens and contact allergens (41, 42), the DEC-205 approach reveals the capacity of DCs to induce profound antigen-specific tolerance in vivo and in the steady state.

**Figure 5.** CD40 ligation prolongs T cell activation in response to antigens delivered to DCs and induces upregulation of costimulatory molecules on DCs. (A) CD40 ligation induces persistent expansion of 3A9 cells in response to antigens delivered to DCs. CD45.1" 3A9 T cells were transferred into B10.BR mice and 24 h later the recipients were injected subcutaneously in the footpads with 0.2 μg of αDEC/HEL alone or 90 μg of FGK45 or both or PBS. CD4" T cells were purified by negative selection from regional LNs 7 d after challenge with antigen and analyzed by flow cytometry using antibodies specific for CD45.1 and CD4. The numbers indicate the percentages of CD4"CD45.1" cells in LNs. (B) CD40 ligation prolongs T cell activation. 3A9 T cells were transferred into B10.BR mice and 24 h later, recipients were injected subcutaneously in the footpads with 0.2 μg of αDEC/HEL alone or 90 μg of FGK45 or both or PBS. After 2 or 7 d, CD4 T cells were isolated from the draining LNs and cultured in vitro with irradiated B10.BR CD11c" cells in presence or absence of HEL peptide. T cell proliferation was measured by [3H]thymidine incorporation. The results represent triplicate cultures from two independent experiments. (C) CD40 ligation induces costimulatory molecules on DCs. B10.BR mice with or without 3A9 cell transfer were injected with 90 μg FGK45 plus 0.2 μg αDEC/HEL or αDEC/HEL or PBS. 3 d later DCs were isolated as in Fig. 2 and analyzed by flow cytometry using antibodies specific for CD11c, B220, CD86, and CD40. Histograms show staining with anti-CD40 and anti-CD86 on gated populations of DCs. Thick lines indicate control with PBS, which was same as αDEC/HEL alone.
establishing tolerance to self and nonpathogenic environment. During inflammation or infection, the steady state versus inflammation, are not in conflict because they are elicited under distinct circumstances, the steady state versus inflammation, isothiocyanate. Functional and morphological studies. Dendritic cells pulsed with protein antigens in vitro can prime antigen-specific, MHC-restricted T cells in situ. J. Exp. Med. 172:631–640.

Our experiments are consistent with the notion that self-antigens such as serum components and apoptotic cells captured and presented to T cells by DCs under physiological conditions induce tolerance. In contrast, antigens taken up by DCs in the context of activation stimuli such as those found during inflammation or tissue destruction induce prolonged T cell activation. These two functions of DCs, maintaining tolerance to self and inducing immunity, are not in conflict because they are elicited under distinct circumstances, the steady state versus inflammation and infection. Moreover, the steady state tolerizing function of DCs may be essential for their subsequent role in eliciting immunity. During inflammation or infection, DCs present self-antigens simultaneously with non-self. By establishing tolerance to self and nonpathogenic environmental proteins before challenge with pathogens, DCs can focus the adaptive immune system entirely on the pathogen, thereby avoiding autoimmunity. The ability to target antigens to DCs and control their function in vivo has significant implications for development of vaccines and therapies for autoimmunity.

The authors thank Dr. Hitoshi Nagaoka for help with surgical procedures, Dr. Mark Davis for 3A9 mice, Dr. Fritz Melchers for FGK 45 hybridoma, Dr. Emil Unanue for 1G12 hybridoma, and Dr. Richard J. Nokes for the GL117 hybridoma. The authors also thank Dr. Eva Besmer for critical review of the manuscript.

This work was supported in part by Human Frontier Science (HFS) and grants to M.C. Nussenzweig and R.M. Steinman from the National Institute of Allergy and Infectious Diseases to R.M. Steinman and National Institutes of Health to M.C. Nussenzweig and AI13013, DK program project grant to M.C. Nussenzweig, R.M. Steinman, and J.V. Ravetch. M.C. Nussenzweig is a Howard Hughes Medical Institute investigator.

References


