

The Formation of Immunogenic Major Histocompatibility Complex Class II–Peptide Ligands in Lysosomal Compartments of Dendritic Cells Is Regulated by Inflammatory Stimuli

By Kayo Inaba,* Shannon Turley,[†] Tomonori Iyoda,* Fumiya Yamaide,* Susumu Shimoyama,* Caetano Reis e Sousa,[§] Ronald N. Germain,[§] Ira Mellman,[‡] and Ralph M. Steinman^{||}

From the *Laboratory of Immunobiology, Graduate School of Biostudies, Kyoto University, Kyoto 606-8502, Japan; the [†]Department of Cell Biology, Yale University Medical School, New Haven, Connecticut 06520-8002; the [§]Laboratory of Immunology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20892-1892; and the ^{||}Laboratory of Cellular Physiology and Immunology, The Rockefeller University, New York, New York 10021-6399

Abstract

During their final differentiation or maturation, dendritic cells (DCs) redistribute their major histocompatibility complex (MHC) class II products from intracellular compartments to the plasma membrane. Using cells arrested in the immature state, we now find that DCs also regulate the initial intracellular formation of immunogenic MHC class II–peptide complexes. Immature DCs internalize the protein antigen, hen egg lysozyme (HEL), into late endosomes and lysosomes rich in MHC class II molecules. There, despite extensive colocalization of HEL protein and MHC class II products, MHC class II–peptide complexes do not form unless the DCs are exposed to inflammatory mediators such as tumor necrosis factor α , CD40 ligand, or lipopolysaccharide. The control of T cell receptor (TCR) ligand formation was observed using the C4H3 monoclonal antibody to detect MHC class II–HEL peptide complexes by flow cytometry and confocal microscopy, and with HEL-specific 3A9 transgenic T cells to detect downregulation of the TCR upon MHC–peptide encounter. Even the binding of preprocessed HEL peptide to MHC class II is blocked in immature DCs, including the formation of C4H3 epitope in MHC class II compartments, suggesting an arrest to antigen presentation at the peptide-loading step, rather than an enhanced degradation of MHC class II–peptide complexes at the cell surface, as described in previous work. Therefore, the capacity of late endosomes and lysosomes to produce MHC class II–peptide complexes can be strictly controlled during DC differentiation, helping to coordinate antigen acquisition and inflammatory stimuli with formation of TCR ligands. The increased ability of maturing DCs to load MHC class II molecules with antigenic cargo contributes to the >100-fold enhancement of the subsequent primary immune response observed when immature and mature DCs are compared as immune adjuvants in culture and in mice.

Key words: dendritic cell • maturation • MHC class II–peptide complex • lysosome • inflammation

Introduction

Dendritic cells (DCs),¹ perhaps the most potent and versatile of APCs, capture foreign antigens encountered in peripheral tissues, process the antigens into peptides bound to MHC molecules, and migrate to lymphoid organs for presentation of the MHC–peptide complexes to T lympho-

cytes (1, 2). In keeping with this critical role in stimulating antigenically naive T cells, the immunostimulatory activity of DCs is carefully regulated, with the cells exhibiting at

C. Reis e Sousa's present address is Immunobiology Laboratory, Imperial Cancer Research Fund, London WC2A 3PX, United Kingdom.

Address correspondence to Ralph M. Steinman, Laboratory of Cell Physiology and Immunology, The Rockefeller University, 405 Bronk Bldg., 1230 York Ave., New York, NY 10021-6399. Phone: 212-327-8106; Fax: 212-327-8875; E-mail: steinma@rockvax.rockefeller.edu

¹Abbreviations used in this paper: CD40L, CD40 ligand; DC, dendritic cell; HEL, hen egg lysozyme; ³H-TdR, [³H]thymidine; Ii chain, MHC class II-associated invariant chain; MHCs, MHC class II compartments.

least two functionally distinct states. Resting or immature DCs, corresponding to those found in the periphery, generally have a high capacity for endocytosis and thus for antigen uptake (3–6), but a low capacity for binding and stimulating T cells. Mature DCs, on the other hand, are generated from immature cells by exposure to proinflammatory agents (TNF, LPS), physical trauma, or T cell surface molecules (CD40 ligand [CD40L]; references 7–10). Mature DCs exhibit a limited capacity for endocytosis, but they are exceptional at stimulating T cells (3–10), typically presenting immunogenic peptides derived from previously encountered antigens. The capacity of mature DCs to activate naive T cells reflects several developmentally regulated changes. These include upregulation of CD40, CD80, and CD86 costimulatory molecules (11, 12), release of cytokines like IL-12 (13, 14), resistance to IL-10-mediated immunosuppression (15), and expression of new chemokine receptors like CCR7 that guide DCs to the T cell areas (16–19). Importantly, mature DCs upregulate surface MHC class II and functional MHC class II-peptide complexes that engage the TCR (20, 21).

This increased expression of MHC class II molecules is known to reflect at least two alterations (20, 21). First, newly synthesized MHC class II molecules become re-routed intracellularly. Instead of being transported to lysosomes for sequestration or degradation, the MHC class II is targeted to the plasma membrane. One underlying mechanism involves the activation of the APC-restricted protease cathepsin S, which in turn mediates the post-Golgi complex cleavage of the MHC class II-associated invariant (Ii) chain. These events facilitate the early release of Ii chain from MHC class II α/β dimers, allowing surface transport rather than intracellular retention due to lysosomal targeting/endocytosis signals found in the Ii chain cytoplasmic domain (22). The cleavage of Ii could also free the MHC class II binding site for antigen binding (23, 24), but this level of regulation has not yet been demonstrated in maturing DCs. Second, because of the downregulation of endocytosis that occurs concomitant with maturation, there is an increase in the surface residence time, half-life, and thus overall expression of any MHC class II-peptide complexes that do reach the plasma membrane (20, 21).

To further delineate the mechanism underlying the critical conversion of DCs from immature sentinels to mature immunostimulatory cells, we made use of a monoclonal antibody, C4H3, that allows the direct visualization of a peptide-MHC class II complex formed from a hen egg lysozyme (HEL) peptide and an I-A^k α/β dimer (25, 26). We also identified conditions that allow the in vitro maintenance and rapid manipulation of immature bone marrow-derived DCs. Together, these approaches revealed an additional and more fundamental level at which the development of antigen presentation capacity is controlled during DC maturation, the actual formation of MHC class II-peptide complexes within MHC class II-rich lysosomal compartments. We show that this maturation is a major control for T cell priming by DCs in vivo, a new finding that impacts upon their optimal use for immunotherapy in humans (27–30).

Materials and Methods

Mice. CBA/J and lipid A-unresponsive C3H/HeJ female mice were purchased from The Jackson Laboratory or from Japan SLC, and were used at 6–8 wk of age.

Dendritic Cells. DCs were grown in RPMI 1640 containing 5% FCS from bone marrow progenitors with mouse rGM-CSF (31) at 10 ng/ml (gift of Dr. Y. Yamaguchi, Kirin, Maebashi, Japan), or the supernatant (3% vol/vol) from J558L cells transduced with murine (m)GM-CSF (from Dr. A. Lanzavecchia, Basel Institute, Basel, Switzerland). At day 6, the cultures contained many aggregates of immature DCs loosely attached to the monolayer. To mature the DCs, we added either TNF (100–500 U/ml; Dainippon Pharmaceutical Co.), CD40L (a baculovirus preparation provided by Drs. M. Nussenzweig and Y. Choi, The Rockefeller University, New York, NY), or LPS (1–10 ng/ml, *Escherichia coli* type 0111.B4; Sigma Chemical Co.), or we simply transferred the cells to a fresh vessel at $<5 \times 10^6$ cells/ml (31).

Antigen Administration. HEL (Sigma Chemical Co.) was added to immature bone marrow DCs at 30–3,000 $\mu\text{g/ml}$ for 0.5–24 h. Also, explants of ear skin (epidermis and dermis) were bathed in 3,000 $\mu\text{g/ml}$, after which the DCs were examined within epidermal sheets or as cells that had emigrated from the explants (32, 33). The dominant HEL 46-61 peptide for I-A^k was synthesized at Yale University Medical School. OVA was used as control protein. HEL protein uptake was visualized with 1B12 monoclonal IgG2b anti-HEL antibody, provided by Dr. P. Allen (Washington University, St. Louis, MO), and MHC class II-HEL peptide complexes visualized with C4H3 rat IgG2b monoclonal antibody (25, 26). Antibody staining, including that with isotype controls (PharMingen), was assessed on a FACScanTM (Becton Dickinson) or by immunofluorescence confocal microscopy. DCs were identified by labeling for the I-E MHC II product (14-4-4S antibody), and mature DCs by high expression of CD86 (GL-1; PharMingen).

Removal of Endotoxin Activity. To deplete much of the endotoxin activity (limulus amoebocyte assay; BioWhittaker) in HEL preparations, HEL at 10 mg/ml was adsorbed with tachyplesin III-conjugated Sepharose CL (34; KutsucleanTM; Maruha Corp.) according to the manufacturer's instructions.

Antigen Presentation Assays. Presentation of HEL to T cells was monitored using purified CD4⁺ T cells from 3A9 TCR transgenic mice (35) provided by Dr. M. Davis (Stanford University, Palo Alto, CA). These T cells are specific for the same MHC class II-peptide complex recognized by the C4H3 antibody. Graded doses of DCs that were exposed to HEL minus or plus a maturation stimulus were applied to 250,000 CD4⁺ transgenic T cells in 96-well flat-bottomed microtest plates in RPMI 1640 containing 5% FCS. The DCs were fixed beforehand in 0.75% paraformaldehyde for 30 min on ice. CD4⁺ T cells were enriched by negative selection from spleen and lymph node suspensions by coating other cells with antibodies (TIB 120 anti-MHC class II, TIB 207 anti-CD8, HB198 F4/80 anti-macrophage, 6B2 anti-B220, and NK1.1) and depleting them with sheep anti-rat Ig Dynabeads[®] M-450 (No. 110.08; Dynal). T cell responses were monitored at 5 h by a decrease in TCR (V β 8) or increase in CD69 (PharMingen antibodies), or at 30–42 h by [³H]thymidine (³H-TdR) uptake at 1 $\mu\text{Ci/ml}$. Data are from triplicate cultures with SE <10% of the mean. For presentation studies in vivo with adoptively transferred DCs, immature cells were cultured with graded doses of HEL overnight with or without CD40L or LPS as a maturation stimulus. The DCs were harvested, washed, and injected subcutaneously at a dose of 200,000 DCs per paw of non-transgenic mice. 5 d later, the draining lymph nodes were re-

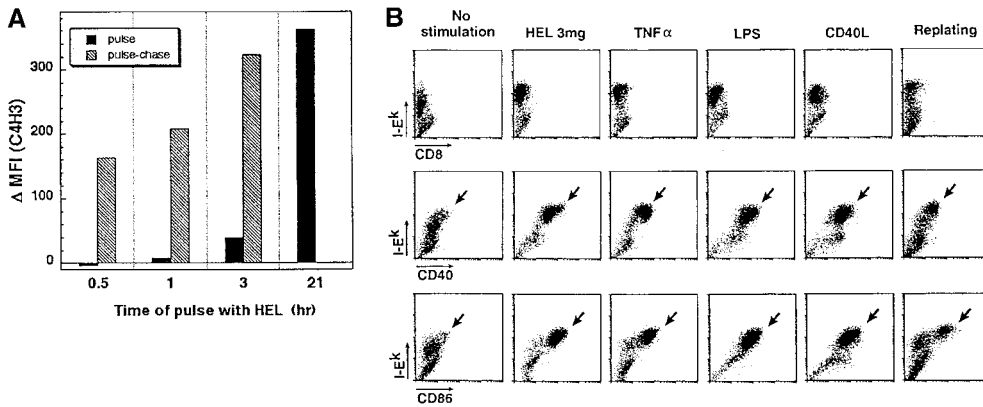


Figure 1. Formation of MHC-peptide complexes by bone marrow-derived DCs. (A) DCs in bone marrow cultures were pulsed with 3,000 $\mu\text{g/ml}$ of HEL for the times indicated on the x-axis, washed, and assayed immediately for C4H3 staining (pulse) or chased until 21 h of total culture (pulse-chase). The y-axis displays increases in mean fluorescence index (MFI) for I-E⁺ DCs relative to cultures in the absence of HEL protein. (B) A maturation stimulus is present in commercial preparations of HEL. Immature 6-d marrow cultures were main-

tained for one additional day in the absence of another stimulus or in the presence of the stimuli listed on the top, including transfer to a new vessel (right). Maturation was monitored at the level of surface MHC class II expression (y-axis) and surface CD40 and CD86 (x-axis), with anti-CD8 antibody used as a nonreactive isotype control.

moved, dissociated into single cell suspensions, and cultured at 300,000 cells per flat-bottomed microtest well in Click's medium with 0.75% mouse serum and graded doses of HEL. ³H-TdR uptake was measured at 52–64 h to document the extent of CD4⁺ T cell priming.

Results

Synergistic Effects between the Exposure to Antigen and a Maturation Stimulus in the Formation of MHC Class II–Peptide Complexes. Immature DCs from day 6 GM-CSF-stimulated marrow cultures (31) were exposed for various periods of time to increasing concentrations of HEL. The formation of the C4H3 epitope, i.e., MHC class II–HEL peptide complexes, was monitored by FACS[®]. Strong signals were not seen at the DC surface after a 3-h exposure to HEL, but were present after 18–24 h (pulse data; Fig. 1 A). The formation of C4H3 was proportional to the added dose of HEL; signals became detectable at 30 $\mu\text{g/ml}$ (not shown). Immature DCs only had to be pulsed a short time with

HEL to develop strong C4H3 signals 1 d later (pulse-chase data; Fig. 1 A). For example, a 3-h pulse of 3,000 $\mu\text{g/ml}$ HEL followed by an 18-h chase in its absence gave comparable C4H3 staining to continuous culture in HEL.

However, the addition of HEL to immature DCs not only led to the appearance of C4H3 epitope, but also to maturation. Most MHC class II-positive DCs began to express high levels of CD40 and CD86 and higher levels of surface MHC class II compared with cultures not incubated with HEL (Fig. 1 B). Although initially surprising, HEL-induced maturation could be explained by the fact that 1 mg/ml HEL contained high endotoxin activity, 1–10 ng/ml in the limulus endotoxin assay, as reported (36). Likewise, simple transfer of the immature DCs to a fresh culture dish, a procedure that is used to enhance the expansion of residual proliferating cells in the culture, enhanced maturation (Fig. 1 B; references 21 and 31). Therefore, in all subsequent experiments, a new invertebrate adsorbent termed Kuttsuclean[™] was used to remove most endotoxin (34) without decreasing HEL content, and we

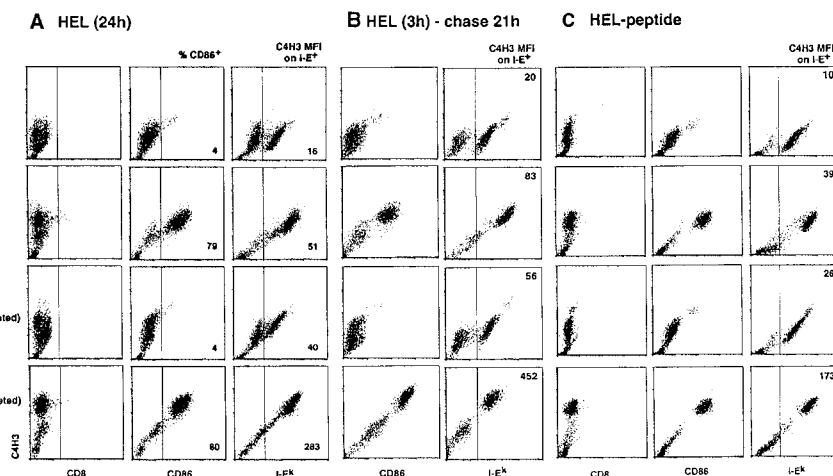


Figure 2. Antigen and a maturation stimulus synergize to form MHC-peptide complexes. (A) Day 6 bone marrow cultures from CBA/J mice were cultured for 24 h in the absence of HEL or LPS, with either HEL (1,000 $\mu\text{g/ml}$) or LPS (5 ng/ml), or with both. The HEL had been passed over a Kuttsuclean[™] adsorbent. Left: anti-CD8 isotype control. Middle: CD86 marker for DC maturation, with the percentage of mature CD86-rich cells indicated. Right: mean fluorescent index (MFI) of the C4H3 signal on I-E⁺ DCs. Similar results were obtained using C3H/HeJ DCs matured with CD40L in three experiments. (B) Cells were pulsed with HEL protein for 3 h, and then washed before adding the maturation stimulus where indicated for a chase period of 21 h. (C) Cells were pulsed with preprocessed HEL peptide for 3 h, and then washed before adding LPS where indicated for a chase period of 21 h.

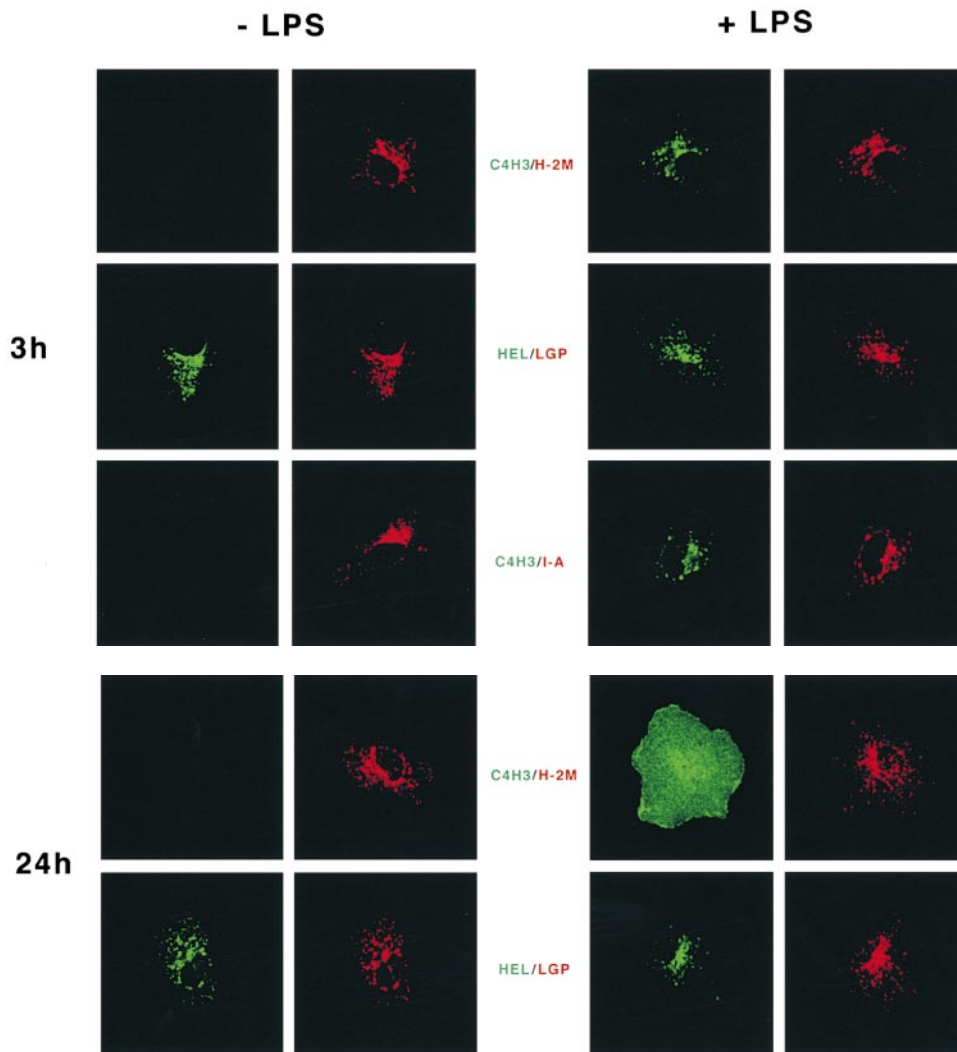


Figure 3. Localization of HEL protein and MHC class II-peptide complexes in DCs. The procedure was the same as described in the legend to Fig. 2, but here the formation of MHC class II-peptide was monitored at 3 and 24 h by immunofluorescence confocal microscopy. On the left are CBA DCs cultured in HEL only, and on the right are cells cultured with HEL + LPS. Representative cells are shown following double labeling in green for the HEL antigen, either HEL protein with 1B12 antibody or I-A^k/HEL MHC-peptide complexes with C4H3. Red stain identifies the lysosomal membrane glycoprotein lysosomal-associated membrane protein 2. Identical red staining is seen with H-2M or MHC class II. The results are representative of >10 experiments, and of experiments with both HEL protein and preprocessed HEL peptide. The maturation-induced C4H3 signal at 24 h is shown for a well-spread cell, but in optical sections through thicker cells, C4H3 stain is almost entirely along the cell perimeter.

compared immature and maturing cells in cultures that were not subcultured from their initial wells.

The addition of KuttsucleanTM-treated HEL did not induce DC maturation, as indicated by a lack of generation of CD86⁺ cells or the increased expression of surface MHC class II (I-E and I-A). Importantly, only small amounts of C4H3 epitope appeared at the plasma membrane (Fig. 2 A). DCs incubated under these conditions could be kept in the immature state for at least 3–4 d. In prior work (20, 21), immature DCs exhibited a slow but spontaneous rate of maturation, and some formation of the SDS stable dimers suggestive of MHC-peptide complex formation. The maturation stimuli (LPS, TNF, CD40L, and transfer to a new culture vessel) induced an increase in CD86⁺ cells, and some increase in background C4H3 staining (Fig. 2 A), presumably because the higher levels of I-A on mature DCs presented self-peptides that cross-reacted with HEL peptide (25, 26). Nevertheless, the combination of HEL and a maturation stimulus was markedly synergistic, with very high C4H3 levels being observed on most DCs in >10 similar experiments (Fig. 2 A).

Mechanisms Underlying the Synergism of Antigen and a Maturation Stimulus in Controlling MHC Class II-Peptide Complex Formation. To establish that the maturation stimulus was not merely increasing uptake of HEL, we applied the antigen as a pulse for 3 h, and then washed the cells before the addition of LPS or CD40L. The synergistic effect of the maturation stimulus remained apparent in all three experiments (Fig. 2 B), indicating that maturation functioned to enhance HEL processing and/or formation of C4H3-reactive MHC class II-HEL peptide complexes. In a companion study (37), we found that the HEL-pulsed DCs can be cultured for at least 48 h before adding a maturation stimulus, but the cells still display C4H3 at levels >50% of that seen when the addition of LPS or CD40L is not delayed. Therefore, maturation stimuli increase the formation of TCR ligands, and do not simply reduce their degradation.

To determine if maturation was acting only at the level of antigen processing/proteolysis, we pulsed DCs for 3 h with preprocessed HEL peptide (that is able to bind directly to I-A^k), washed the cells, and then cultured without or with a maturation stimulus. The latter was again required for MHC-

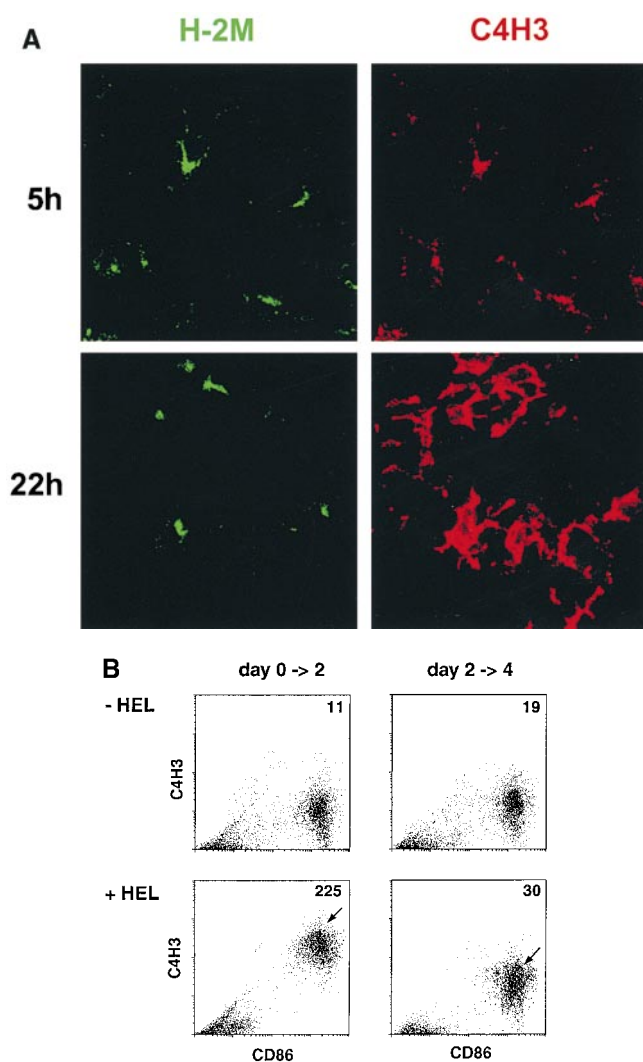


Figure 4. Formation of MHC-peptide complexes in immature DCs in situ. (A) Explants of CBA ear skin were bathed in 3,000 $\mu\text{g}/\text{ml}$ LPS containing HEL for 5 or 22 h. At each time point, epidermal sheets were prepared and double labeled for the H-2M marker of MIICs (green) and for C4H3 epitope (red). The sheets were examined by two-color immunofluorescence confocal microscopy. The effect of a maturation stimulus could not be examined in vivo, because simple injection of PBS could induce some DCs to mature. (B) Explants of ear skin were cultured in the absence or presence of HEL for 48 h, and then the emigrated cells were double labeled for CD86 to identify the DCs (arrows) and C4H3. Some of the cells that emigrated in the absence of HEL were then cultured for 2 d in the presence of HEL before similar FACS[®] studies. The MFI for CD86⁺ cells is given in each panel.

peptide complex formation (Fig. 2 C), indicating that peptide access to the MHC class II binding site was controlled by the maturation process.

Conceivably, maturation might control C4H3 epitope expression by controlling the delivery of HEL to the MHC class II compartments (MIICs) that are thought to be critical for MHC-peptide complex formation. Therefore, we performed immunofluorescence confocal microscopy to localize C4H3 in the absence or presence of a maturation stimulus. As shown in Fig. 3 (left), immature DCs accumulated endotoxin-free HEL protein in intracellular compartments,

as detected with 1B12 antibody to intact HEL. These structures were positive for MHC class II, Ii chain, and the peptide editing H-2M product, and were judged to be late endosomes and lysosomes by staining for the lysosomal membrane glycoprotein, Igp-B (or lysosomal-associated membrane protein [LAMP]-2; Fig. 3). Despite the fact that HEL was delivered to MIICs, no C4H3 staining was observed even after incubations of up to 24 h in HEL (Fig. 3) or preprocessed HEL peptide (not shown). Also, no C4H3 staining was found when only a maturation stimulus was given (LPS or CD40L; not shown), in spite of the weak signals on the FACS[®] (Fig. 2).

Quite a different result was obtained in cells exposed to HEL together with a maturation stimulus (Fig. 3, right). As before, HEL protein was found in MIICs, but in all the DCs, abundant C4H3 stain could also be observed within MIICs. After 3 h or less in HEL, C4H3 staining was only observed within MIICs (Fig. 3). At 24 h, most of the MHC-peptide complexes were on the cell surface (Fig. 3), as described previously for the distribution of MHC class II products in maturing DCs (20, 21). Similar findings were made in pulse-chase experiments, carried out as in Fig. 2 B; when a maturation stimulus was applied after the pulse with HEL antigen, MHC-peptide complexes began to form in MIICs within 3 h, and were later found on the cell surface.

MIICs as a Site for MHC-Peptide Complex Formation in the Epidermis. To visualize the intracellular sites for MHC class II-peptide formation in DCs in intact tissue, we studied epidermal DCs that mature when they migrate from skin (32, 33) via afferent lymphatics (38, 39). Endotoxin-containing HEL was injected into the ear dermis or added to skin explants in vitro. Epidermal sheets and the emigrated DCs were then examined. 5 h after injection, clear cut colabeling for C4H3 and the lysosomal marker H-2M was observed within all DCs in the epidermal sheets (Fig. 4 A), but not in DCs given control OVA protein. At 22 h, the C4H3 was on the surface of most DCs (Fig. 4 A). DCs that emigrated from explants into the medium during 2 d of culture had a mature CD86⁺⁺ phenotype (Fig. 4 B) and stained strongly for C4H3 if HEL had been added to the explant. If HEL was added to mature DCs after emigration, C4H3 staining was not seen (Fig. 4 B). Thus, immature endocytic epidermal DCs form MHC-peptide complexes in MIICs in vivo, but mature DCs are no longer able to do so.

Functional Consequences of the Synergism between Antigen and a Maturation Stimulus: In Vitro Studies of Antigen Presentation to Naive TCR Transgenic T Cells. In vitro antigen presentation assays were used to assess the functional consequences of DC maturation and increased amounts of MHC-peptide complexes. We used naive 3A9 TCR transgenic T cells (35) specific for the same I-A^k-HEL peptide complex recognized by C4H3 antibody. Immature, marrow-derived DCs were exposed for 1 d to HEL in graded doses, to CD40L or LPS, or to both HEL and the maturation stimulus. The cells were washed, fixed in paraformaldehyde, and added in graded doses to TCR transgenic T cells (Fig. 5 A). Fixation ensured that the DCs did not mature during the culture with T cells. HEL-pulsed imma-

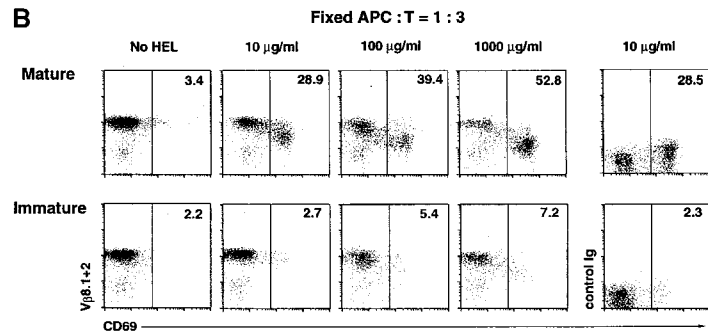
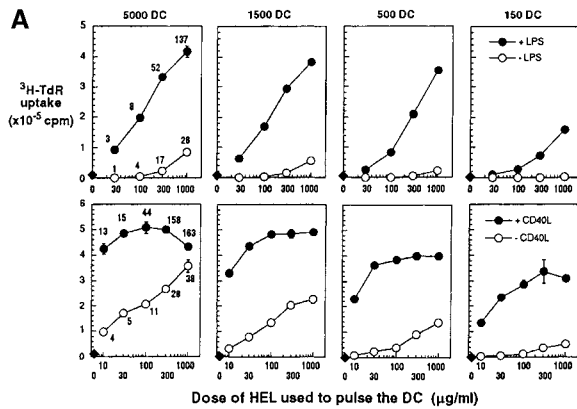


Figure 5. Antigen presenting activity of DCs in vitro after culture with graded doses of HEL, minus or plus LPS or CD40L as a maturation stimulus. (A) Day 6 bone marrow DCs from CBA mice (top) or C3H/HeJ mice (bottom) were cultured for 20 h with graded doses of HEL (x-axis) that had been endotoxin depleted with Kuttsuclean™. One group of cultures was matured by simultaneous addition of LPS or CD40L (closed symbols), and the other was unstimulated (open symbols). After 20 h, the cells were harvested, washed, and fixed in paraformaldehyde to block further processing and maturation. MHC-peptide complexes were quantified in terms of C4H3 staining, and are shown as mean fluorescence indices on the left. The fixed DCs were added in graded doses (top) to 250,000 CD4⁺ T cells from 3A9 TCR transgenic mice (specific for the same complex of I-A^k + HEL peptide as C4H3 antibody). ³H-TdR uptake was measured at 30–42 h. One of three similar experiments. (B) The display of TCR ligands by fixed DCs was monitored at 5 h on T cells (gated away from DCs by light scattering) by the criteria of TCR downregulation (anti-Vβ8, y-axis) and CD69 upregulation (x-axis). The DC/T cell ratio was 1:3 in the data that are shown, and the percentage of CD69⁺ cells is indicated in each dot plot (upper right).

ture DCs only weakly stimulated the TCR transgenic T cells, but when the DCs had been exposed to both HEL and CD40L or LPS, strong T cell stimulation ensued (Fig. 5 A). With mature DCs, the DNA synthesis (³H-TdR uptake) that we observed early in the response (day 2) with low DC to T cell ratios (1:60–1:2,000) was $>2 \times 10^5$ cpm per well under standard assay conditions, and it was proportional to the level of MHC class II-peptide on the DCs (Fig. 5 A).

The poor T cell-stimulatory activity of immature DCs could reflect a lack of either TCR ligand (“signal one”) or costimulatory molecules such as CD86 (“signal two”). The use of the C4H3 antibody for the detection of signal one was likely to be insensitive relative to that of the TCR. For example, an exposure of DCs to 30 μg/ml of HEL was necessary to detect MHC-peptide complex formation with C4H3 antibody, whereas an exposure to 10 μg/ml was sufficient to saturate T cell stimulation at a DC/T cell ratio of 1:60 (Fig. 5 A, left). To assess MHC class II-peptide complexes at the level of the TCR, we used the criterion of TCR downregulation. Prior studies have shown that the TCR is cleared from the cell surface upon encounter of MHC-peptide complexes; this downregulation appears not to require a special type of APC or the presence of costimulatory molecules on that APC (40, 41). Using staining with anti-Vβ8 antibody, we monitored expression of the TCR on 3A9 cells cultured for 5 h with mature or immature HEL-pulsed DCs, prepared exactly as described in the legend to Fig. 5 A. At all doses of HEL studied (10–1,000 μg/ml), mature DCs quickly activated a sizable fraction of the T cells, as indicated by de novo expression of the CD69 T cell activation marker. TCR downregulation was evident on all CD69⁺ cells. In contrast, immature DCs induced little increase in CD69 or

decrease in TCR (Fig. 5A). We conclude that a maturation stimulus is needed for DCs to form MHC class II-peptide complexes, as assessed with B cell (C4H3 antibody) or T cell (the 3A9 TCR) receptors.

Functional Consequences of the Synergism between Antigen and a Maturation Stimulus: In Vivo Studies of Antigen Presentation to Naive Mice. The above data establish a relationship between maturation and the formation of immunogenic MHC-peptide complexes in vitro, but the relevance of DC maturation to immunogenicity in vivo has yet to be tested. To this end, immature DCs were incubated in graded doses of endotoxin-free HEL with or without a

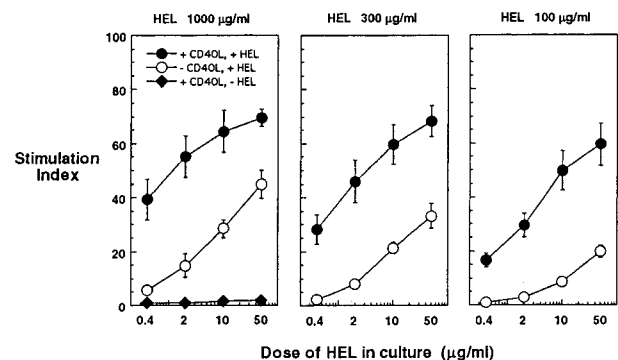


Figure 6. Antigen presenting activity of DCs in vivo after culture with graded doses of HEL, minus (open symbols) or plus (closed symbols) CD40L as a maturation stimulus. As described in the legend to Fig. 5, graded doses of antigen (top) were used to pulse C3H/He DCs overnight. The washed, unfixed cells were injected subcutaneously into syngeneic mice, 2×10^5 per paw. 5 d later, cultured draining lymph node cells were challenged with different doses of HEL protein (x-axis), and ³H-TdR uptake measured at 42–48 h (y-axis). Data are shown as stimulation relative to the background cpm in lymph nodes cultured without antigen. One of three similar experiments.

maturation stimulus, CD40L. Unfixed DCs were then injected subcutaneously into naive syngeneic mice. The mature cells were far more powerful immunogens compared with immature cells, and priming was proportional to the dose of HEL and MHC peptide on the DCs (Fig. 6). When we tested T cells isolated from lymph nodes of mice injected with mature HEL-loaded DCs, the proliferation was >100-fold more sensitive to a given dose of antigen. If 25 μ g of HEL was injected without DCs, T cell priming was not seen (not shown). Therefore, maturation markedly increases the adjuvant capacity of adoptively transferred DCs that have been previously pulsed with antigens *ex vivo*.

Discussion

As introduced above, maturation changes DCs in many ways that explain their potency in initiating immunity. Maturing cells express surface molecules like CD40 and CD86 required for T cell expansion and cytokine synthesis (11, 12), produce high levels of IL-12 (13, 14), resist suppression by IL-10 (15), and upregulate chemokine receptors (16–19) that recognize chemokines produced in lymphoid organs (42, 43). Here, a new feature of maturation has emerged, an intensified capacity to convert endocytosed antigens to MHC class II–peptide complexes.

There is evidence with human monocyte–derived DCs that maturation can prolong the life span of MHC class II–peptide complexes on the cell surface (20). However, direct visualization of these complexes now shows that the maturation process can regulate an earlier and more critical step, the formation of TCR ligands in MIICs. The results in a companion study (37), in which antigen–pulsed DCs can be cultured for 48 h before forming MHC–peptide complexes in response to a maturation stimulus, nullifies the possibility that immature DCs are simply degrading these complexes.

MHC class II products are designed to capture antigenic peptides from pathogens internalized by endocytosis (44, 45). Through the chaperone function of the Ii chain (46, 47) or by dileucine motifs within the cytoplasmic domain of the class II β chain (48), MHC class II molecules are targeted to acidic compartments termed MIICs (49–51). These lysosomes contain proteases for antigen processing, as well as HLA-DM or H-2M molecules for enhancing peptide loading to MHC class II (52, 53). Nevertheless, there has been little direct visualization of newly formed MHC–peptide complexes in physiological APCs. A key control is likely to be the uptake and delivery of antigens into MIICs. DCs are actively endocytic at the immature stage of development (3, 4), when they also contain numerous MIICs (21, 54, 55). Both endocytosis and the number of MIICs are decreased, and even extinguished, upon further differentiation or maturation in response to bacteria and TNF family members. Here, we have studied DCs frozen at the immature stage (Materials and Methods). These DCs internalize antigens into MIICs, but surprisingly, MHC class II–peptide complexes fail to form efficiently until an inflammatory stimulus is applied.

Because cathepsin S controls Ii chain proteolysis (56) and the availability of MHC class II molecules for peptide loading, one of the possible mechanisms explaining these data is that maturation downregulates the cathepsin S inhibitor, cystatin C (22). Consistent with this, we observed a striking inability to load the MHC class II products of immature DCs with peptide, until a maturation stimulus was applied. Similar events could take place *in vivo*, because when the maturation stimulus LPS in HEL preparations is given to mice, there is an accumulation of DCs with high levels of C4H3 in T cell areas (36). The control of MHC class II–peptide formation could also be exerted at other levels, e.g., the acidity of the endocytic system and the function of additional proteases.

In companion studies, we show that MHC class II–peptide complexes, newly formed in the late endosomes or lysosomes of DCs, selectively accumulate, together with CD86 costimulators, into specialized vesicular carriers before their delivery to the plasma membrane (37). We term these MHC class II–positive lysosome–negative vesicles “CIIVs”, for class II vesicles. Remarkably, the MHC class II–peptide complexes and CD86 costimulators remain in clusters at the cell surface. This juxtaposition may explain the potent naive T cell stimulation of DCs that we now observe, even when the cells are fixed in a low dose of formaldehyde (Fig. 5).

Our findings, especially the *in vivo* priming results in Fig. 6, are relevant to the optimal use of DCs for immunotherapy in humans (27–30). It is not only valuable to deliver antigens to DCs, but also to mature the cells to increase successful antigen processing and to provide other valuable physiological features cited above. The data also relate to the potential of DCs to control the alternative outcome of antigen deposition, tolerance rather than immunity. Antigen-bearing immature DCs may induce non-responsiveness, according to views that tolerance develops if T cells recognize antigen in the absence of costimulation (57, 58). We would question whether immature DCs, at least the cells that we have generated here from bone marrow progenitors, can directly tolerize, since requisite MHC class II–peptides do not form efficiently. Instead, the recently described transfer of antigen (59) from immature peripheral DCs to resident lymph node–processing DCs may generate the MHC class II peptide complexes needed by tolerizing APCs that are found in lymph nodes (60). The mature cells that we generate *ex vivo* and that efficiently prime T cells *in vivo* (Fig. 6) express high levels of CD86 costimulators. We are intrigued by the fact that in the apparent absence of maturation stimuli, lymph node DCs that are implicated in tolerance (60) can express high levels of MHC–peptide but relatively low levels of CD86 (61).

We are grateful to many colleagues who provided vital reagents for this work: Dr. Mark Davis for 3A9 TCR transgenic T cells, Dr. E. Unanue for 1G12 anti-clonotype antibody, and Dr. P. Allen for 1B12 anti-HEL antibody.

This work was supported by grants to R.M. Steinman from the National Institute of Allergy and Infectious Diseases (AI-13013 and

AI-39672) and the Yamanouchi Fund, to I. Mellman from the National Institutes of Health (AI-34098 and GM-33904), and to K. Inaba from the Ministry of Education, Science and Culture of Japan (Nos. 08282104, 10153226, and 10044268).

Submitted: 6 January 2000

Revised: 8 February 2000

Accepted: 11 February 2000

Released online: 13 March 2000

References

- Hart, D.N. 1997. Dendritic cells: unique leukocyte populations which control the primary immune response. *Blood*. 90: 3245–3287.
- Banchereau, J., and R.M. Steinman. 1998. Dendritic cells and the control of immunity. *Nature*. 392:245–252.
- Inaba, K., M. Inaba, M. Naito, and R.M. Steinman. 1993. Dendritic cell progenitors phagocytose particulates, including bacillus Calmette-Guerin organisms, and sensitize mice to mycobacterial antigens in vivo. *J. Exp. Med.* 178:479–488.
- Reis e Sousa, C., P.D. Stahl, and J.M. Austyn. 1993. Phagocytosis of antigens by Langerhans cells in vitro. *J. Exp. Med.* 178:509–519.
- Romani, N., S. Koide, M. Crowley, M. Witmer-Pack, A.M. Livingstone, C.G. Fathman, K. Inaba, and R.M. Steinman. 1989. Presentation of exogenous protein antigens by dendritic cells to T cell clones: intact protein is presented best by immature, epidermal Langerhans cells. *J. Exp. Med.* 169: 1169–1178.
- Pure, E., K. Inaba, M.T. Crowley, L. Tardelli, M.D. Witmer-Pack, G. Ruberti, G. Fathman, and R.M. Steinman. 1990. Antigen processing by epidermal Langerhans cells correlates with the level of biosynthesis of major histocompatibility complex class II molecules and expression of invariant chain. *J. Exp. Med.* 172:1459–1469.
- De Smedt, T., B. Pajak, E. Muraille, L. Lespagnard, E. Heinen, P. De Baetselier, J. Urbain, O. Leo, and M. Moser. 1996. Regulation of dendritic cell numbers and maturation by lipopolysaccharide in vivo. *J. Exp. Med.* 184:1413–1424.
- Cella, M., F. Sallusto, and A. Lanzavecchia. 1997. Origin, maturation and antigen presenting function of dendritic cells. *Curr. Opin. Immunol.* 9:10–16.
- Caux, C., C. Massacrier, B. Vanbervliet, B. Dubois, C. Van Kooten, I. Durand, and J. Banchereau. 1994. Activation of human dendritic cells through CD40 cross-linking. *J. Exp. Med.* 180:1263–1272.
- Winzler, C., P. Rovere, M. Rescigno, F. Granucci, G. Penna, L. Adorini, V.S. Zimmermann, J. Davoust, and P. Ricciardi-Castagnoli. 1997. Maturation stages of mouse dendritic cells in growth factor-dependent long-term cultures. *J. Exp. Med.* 185:317–328.
- Inaba, K., M. Witmer-Pack, M. Inaba, K.S. Hathcock, H. Sakuta, M. Azuma, H. Yagita, K. Okumura, P.S. Linsley, S. Ikehara, et al. 1994. The tissue distribution of the B7-2 costimulator in mice: abundant expression on dendritic cells in situ and during maturation in vitro. *J. Exp. Med.* 180:1849–1860.
- Caux, C., B. Vanbervliet, C. Massacrier, M. Azuma, K. Okumura, L.L. Lanier, and J. Banchereau. 1994. B70/B7-2 is identical to CD86 and is the major functional ligand for CD28 expressed on human dendritic cells. *J. Exp. Med.* 180: 1841–1847.
- Cella, M., D. Scheidegger, K. Palmer-Lehmann, P. Lane, A. Lanzavecchia, and G. Alber. 1996. Ligation of CD40 on dendritic cells triggers production of high levels of interleukin-12 and enhances T cell stimulatory capacity: T-T help via APC activation. *J. Exp. Med.* 184:747–752.
- Koch, F., U. Stanzl, P. Jennewien, K. Janke, C. Heufler, E. Kampgen, N. Romani, and G. Schuler. 1996. High level IL-12 production by murine dendritic cells: upregulation via MHC class II and CD40 molecules and downregulation by IL-4 and IL-10. *J. Exp. Med.* 184:741–746.
- Thurner, B., C. Roder, D. Dieckmann, M. Heuer, M. Kruse, A. Glaser, P. Keikavoussi, E. Kampgen, A. Bender, and G. Schuler. 1999. Generation of large numbers of fully mature and stable dendritic cells from leukapheresis products for clinical application. *J. Immunol. Methods*. 223:1–15.
- Dieu, M.-C., B. Vanbervliet, A. Vicari, J.-M. Bridon, E. Oldham, S. Ait-Yahia, F. Briere, A. Zlotnik, S. Lebecque, and C. Caux. 1998. Selective recruitment of immature and mature dendritic cells by distinct chemokines expressed in different anatomic sites. *J. Exp. Med.* 188:373–386.
- Yanagihara, S., E. Komura, J. Nagafune, H. Watarai, and Y. Yamaguchi. 1998. EB11/CCR7 is a new member of dendritic cell chemokine receptor that is upregulated upon maturation. *J. Immunol.* 161:3096–3102.
- Sallusto, F., P. Schaerli, P. Loetscher, C. Schaniel, D. Lenig, C.R. Mackay, S. Qin, and A. Lanzavecchia. 1998. Rapid and coordinated switch in chemokine receptor expression during dendritic cell maturation. *Eur. J. Immunol.* 28:2760–2769.
- Tang, H.L., and J.G. Cyster. 1999. Chemokine up-regulation and activated T cell attraction by maturing dendritic cells. *Science*. 284:819–822.
- Cella, M., A. Engering, V. Pinet, J. Pieters, and A. Lanzavecchia. 1997. Inflammatory stimuli induce accumulation of MHC class II complexes on dendritic cells. *Nature*. 388:782–787.
- Pierre, P., S.J. Turley, E. Gatti, M. Hull, J. Meltzer, A. Mirza, K. Inaba, R.M. Steinman, and I. Mellman. 1997. Developmental regulation of MHC class II transport in mouse dendritic cells. *Nature*. 388:787–792.
- Pierre, P., and I. Mellman. 1998. Developmental regulation of invariant chain proteolysis controls MHC class II trafficking in mouse dendritic cells. *Cell*. 93:1135–1145.
- Roche, P.A., and P. Cresswell. 1990. Invariant chain association with HLA-DR molecules inhibits immunogenic peptide binding. *Nature*. 345:615–618.
- Romagnoli, P., and R.N. Germain. 1994. The CLIP region of invariant chain plays a critical role in regulating major histocompatibility complex class II folding, transport, and peptide occupancy. *J. Exp. Med.* 180:1107–1113.
- Zhong, G., C. Reis e Sousa, and R.N. Germain. 1997. Production, specificity, and functionality of monoclonal antibodies to specific peptide-major histocompatibility complex class II complexes formed by processing of exogenous protein. *Proc. Natl. Acad. Sci. USA*. 94:13856–13861.
- Zhong, G., C. Reis e Sousa, and R.N. Germain. 1997. Antigen-unspecific B cells and lymphoid dendritic cells both show extensive surface expression of processed antigen-major histocompatibility class II complexes after soluble protein exposure in vivo or in vitro. *J. Exp. Med.* 186:673–682.
- Schuler, G., and R.M. Steinman. 1997. Dendritic cells as adjuvants for immune-mediated resistance to tumors. *J. Exp. Med.* 186:1183–1187.
- Nestle, F.O., S. Aljagic, M. Gilliet, Y. Sun, S. Grabbe, R.

- Dummer, G. Burg, and D. Schadendorf. 1998. Vaccination of melanoma patients with peptide- or tumor lysate-pulsed dendritic cells. *Nat. Med.* 4:328–332.
29. Dhodopkar, M., R.M. Steinman, M. Sapp, H. Desai, C. Fossella, J. Krasovsky, S.M. Donahoe, P.R. Dunbar, V. Cerundolo, D.F. Nixon, and N. Bhardwaj. 1999. Rapid generation of broad T-cell immunity in humans after single injection of mature dendritic cells. *J. Clin. Invest.* 104:173–180.
 30. Thurner, B., I. Haendle, C. Roder, D. Dieckmann, P. Keikavoussi, H. Jonuleit, A. Bender, C. Maczek, D. Schreiner, P. von den Driesch, et al. 1999. Vaccination with Mage-3A1 peptide-pulsed mature, monocyte-derived dendritic cells expands specific cytotoxic T cells and induces regression of some metastases in advanced stage IV melanoma. *J. Exp. Med.* 190:1669–1678.
 31. Inaba, K., M. Inaba, N. Romani, H. Aya, M. Deguchi, S. Ikehara, S. Muramatsu, and R.M. Steinman. 1992. Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. *J. Exp. Med.* 176:1693–1702.
 32. Larsen, C.P., R.M. Steinman, M. Witmer-Pack, D.F. Hankins, P.J. Morris, and J.M. Austyn. 1990. Migration and maturation of Langerhans cells in skin transplants and explants. *J. Exp. Med.* 172:1483–1493.
 33. Pope, M., M.G. Betjes, H. Hirmand, L. Hoffman, and R.M. Steinman. 1995. Both dendritic cells and memory T lymphocytes emigrate from organ cultures of human skin and form distinctive dendritic-T-cell conjugates. *J. Invest. Dermatol.* 104:11–17.
 34. Muta, T., T. Fugimoto, H. Nakajima, and S. Iwanaga. 1990. Tachyplesins isolated from hemocytes of Southeast Asian horseshoe crabs (*Carcinoscorpius rotundicauda* and *Tachyplesius gigas*): identification of a new tachyplesin, tachyplesin III, and a processing intermediate of its precursor. *J. Biochem.* 108:261–266.
 35. Ho, W.Y., M.P. Cooke, C.C. Goodnow, and M.M. Davis. 1994. Resting and anergic B cells are defective in CD28-dependent costimulation of naive CD4⁺ T cells. *J. Exp. Med.* 179:1539–1549.
 36. Reis e Sousa, C., and R.N. Germain. 1999. Analysis of adjuvant function by direct visualization of antigen presentation in vivo: endotoxin promotes accumulation of antigen-bearing dendritic cells in the T cell areas of lymphoid tissue. *J. Immunol.* 162:6552–6561.
 37. Turley, S.J., K. Inaba, W.S. Garrett, Jr., M. Ebersold, J. Unternaehrer, R.M. Steinman, and I. Mellman. 2000. Transport of peptide-MHC class II complexes in developing dendritic cells. *Science*. In press.
 38. Ortner, U., K. Inaba, F. Koch, M. Heine, M. Miwa, G. Schuler, and N. Romani. 1996. An improved isolation method for murine migratory cutaneous dendritic cells. *J. Immunol. Methods.* 193:71–79.
 39. Weinlich, G., M. Heine, H. Stossel, M. Zanella, P. Stoitzner, U. Ortner, J. Smolle, F. Koch, N.T. Sepp, G. Schuler, and N. Romani. 1998. Entry into afferent lymphatics and maturation in situ of migrating murine cutaneous dendritic cells. *J. Invest. Dermatol.* 110:441–448.
 40. Cai, Z., H. Kishimoto, A. Brunmark, M.R. Jackson, P.A. Peterson, and J. Sprent. 1997. Requirements for peptide-induced T cell receptor downregulation on naive CD8⁺ T cells. *J. Exp. Med.* 185:641–651.
 41. Iezzi, G., K. Karjalainen, and A. Lanzavecchia. 1998. The duration of antigenic stimulation determines the fate of naive and effector T cells. *Immunity.* 8:89–95.
 42. Willmann, K., D.F. Legler, M. Loetscher, R.S. Roos, M.B. Delgado, I. Clark-Lewis, M. Baggiolini, and B. Moser. 1998. The chemokine SLC is expressed in T cell areas of lymph nodes and mucosal lymphoid tissues and attracts activated T cells via CCR7. *Eur. J. Immunol.* 28:2025–2034.
 43. Gunn, M.D., S. Kyuwa, C. Tam, T. Kakiuchi, A. Matsuzawa, L.T. Williams, and H. Nakano. 1999. Mice lacking expression of secondary lymphoid organ chemokine have defects in lymphocyte homing and dendritic cell localization. *J. Exp. Med.* 189:451–460.
 44. Wolf, P.R., and H.L. Ploegh. 1995. How MHC class II molecules acquire peptide cargo: biosynthesis and trafficking through the endocytic pathway. *Annu. Rev. Cell Dev. Biol.* 11:267–306.
 45. Watts, C. 1997. Capture and processing of exogenous antigens for presentation on MHC molecules. *Annu. Rev. Immunol.* 15:821–850.
 46. Bakke, O., and B. Dobberstein. 1990. MHC class II-associated invariant chain contains a sorting signal for endosomal compartments. *Cell.* 63:707–716.
 47. Elliot, E.A., J.R. Drake, S. Amigorena, J. Elsemore, P. Webster, I. Mellman, and R.A. Flavell. 1994. The invariant chain is required for intracellular transport and function of major histocompatibility complex class II molecules. *J. Exp. Med.* 179:681–694.
 48. Zhong, G., P. Romagnoli, and R.N. Germain. 1997. Related leucine-based cytoplasmic targeting signals in invariant chain and major histocompatibility complex class II molecules control endocytic presentation of distinct determinants in a single protein. *J. Exp. Med.* 185:429–438.
 49. Guagliardi, L.E., B. Koppelman, J.S. Blum, M.S. Marks, P. Cresswell, and F.M. Brodsky. 1990. Co-localization of molecules involved in antigen processing and presentation in an early endocytic compartment. *Nature.* 343:133–139.
 50. Tulp, A., D. Verwoerd, B. Dobberstein, H.L. Ploegh, and J. Pieters. 1994. Isolation and characterization of the intracellular MHC class II compartment. *Nature.* 369:120–126.
 51. Amigorena, S., J.R. Drake, P. Webster, and I. Mellman. 1994. Transient accumulation of new class II MHC molecules in a novel endocytic compartment in B lymphocytes. *Nature.* 369:113–120.
 52. Sanderson, F., M.J. Kleijmeer, A. Kelly, D. Verwoerd, A. Tulp, J.J. Neefjes, H.J. Geuze, and J. Trowsdale. 1994. Accumulation of HLA-DM, a regulator of antigen presentation, in MHC class II compartments. *Science.* 266:1566–1569.
 53. Pierre, P., L.K. Denzin, C. Hammond, J.R. Drake, S. Amigorena, P. Cresswell, and I. Mellman. 1996. HLA-DM is localized to conventional and unconventional MHC class II-containing endocytic compartments. *Immunity.* 4:229–239.
 54. Sallusto, F., M. Cella, C. Danieli, and A. Lanzavecchia. 1995. Dendritic cells use macropinocytosis and the mannose receptor to concentrate antigen in the major histocompatibility class II compartment. Downregulation by cytokines and bacterial products. *J. Exp. Med.* 182:389–400.
 55. Nijman, H.W., M.J. Kleijmeer, M.A. Ossevoort, V.M. Oorschot, M.P. Vierboom, M. van de Keur, P. Kenemans, W.M. Kast, H.J. Geuze, and C.J. Melief. 1995. Antigen capture and MHC class II compartments of freshly isolated and cultured human blood dendritic cells. *J. Exp. Med.* 182:163–174.
 56. Riese, R.J., P.R. Wolf, D. Bromme, L.R. Natkin, J.A. Villadangos, H.L. Ploegh, and H.A. Chapman. 1996. Essential

- role for cathepsin S in MHC class II-associated invariant chain processing and peptide loading. *Immunity*. 4:357–366.
57. Schwartz, R.H. 1990. A cell culture model for T lymphocyte clonal anergy. *Science*. 248:1349–1356.
58. Boussiotis, A., G.J. Freeman, G. Gray, J. Gribben, and L.M. Nadler. 1993. B7 but not intercellular adhesion molecule-1 costimulation prevents the induction of human alloantigen-specific tolerance. *J. Exp. Med.* 178:1753–1763.
59. Inaba, K., S. Turley, F. Yamaide, T. Iyoda, K. Mahnke, M. Inaba, M. Pack, M. Subklewe, B. Sauter, D. Sheff, et al. 1998. Efficient presentation of phagocytosed cellular fragments on the MHC class II products of dendritic cells. *J. Exp. Med.* 188:2163–2173.
60. Kurts, C., H. Kosaka, F.R. Carbone, J.F. Miller, and W.R. Heath. 1997. Class I-restricted cross-presentation of exogenous self-antigens leads to deletion of autoreactive CD8⁺ T cells. *J. Exp. Med.* 186:239–245.
61. Inaba, K., M. Pack, M. Inaba, H. Sakuta, F. Isdell, and R.M. Steinman. 1997. High levels of a major histocompatibility complex II-self-peptide complex on dendritic cells from the T cell areas of lymph nodes. *J. Exp. Med.* 186:665–672.