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# Requirements for T Cell-Polarized Tubulation of Class II<sup>+</sup> Compartments in Dendritic Cells<sup>1</sup>

Nicolas Bertho,\* Jan Cerny,<sup>†‡§</sup> You-Me Kim,\* Edda Fiebiger,\* Hidde Ploegh,<sup>2\*</sup> and Marianne Boes\*

Activation of naive CD4 T cells by dendritic cells requires the sequential interaction of many TCR molecules with peptide-class II complexes of the appropriate specificity. Such interaction results in morphological transformation of class II MHC-containing endosomal compartments. In this study, we analyze the requirements for long tubular endosomal structures that polarize toward T cell contact sites using dendritic cells from I-A<sup>b</sup> class II-enhanced green fluorescent protein knock-in mice and I-A<sup>b</sup>-restricted CD4 T cells specific for OVA. Clustering of membrane proteins and ligation of T cell adhesion molecules LFA-1 and CD2 are involved in induction of endosomal tubulation. Activation of T cells increases their ability to induce class II-enhanced green fluorescent protein-positive tubules in dendritic cells, in part through up-regulation of CD40 ligand. Remarkably, and in stark contrast with the result obtained with dendritic cells loaded with intact OVA, OVA peptide added to dendritic cells failed to evoke T cell-polarized endosomal tubulation even though both conditions allowed T cell stimulation. These results suggest the existence of microdomains on the membrane of dendritic cells that allow Ag-specific T cells to evoke tubulation in the dendritic cell. *The Journal of Immunology*, 2003, 171: 5689–5696.

Antigen presentation is the process whereby intact protein Ags are converted into peptide-MHC complexes suitable for interaction with Ag-specific receptors on T cells. The functional dichotomy of CD4 and CD8 T cells reflects the different types of MHC products they interact with, class II and class I MHC molecules, respectively. The intracellular transport routes of MHC molecules help explain the distinction between the class I- and class II MHC-restricted pathways of Ag presentation. Assembly of MHC molecules occurs in the endoplasmic reticulum (ER),<sup>3</sup> from where they travel to their final destination. Whereas MHC molecules acquire peptide in the ER shortly after their assembly, class II MHC molecules follow a more circuitous route that takes them via the endocytic pathway to the cell surface (1, 2). In the ER, class II MHC molecules interact with a chaperone, the invariant chain Ii, which fulfills an essential role during assembly and is believed to prevent premature peptide loading of class II MHC molecules. Furthermore, Ii contains all of the information required to distribute class II MHC molecules over the appropriate

endocytic compartments, either by targeting of class II MHC molecules directly or by retrieval from the cell surface (3).

A mouse strain in which all I-A<sup>b</sup> molecules are replaced by I-A<sup>b</sup> fused at the C terminus of the  $\beta$ -chain with green fluorescent protein has been described recently (4). Such animals are phenotypically normal with respect to their immune system. We generated dendritic cells (DCs) by culture of bone marrow cells as described elsewhere (4, 5). class II-enhanced green fluorescent protein (eGFP) molecules in bone marrow-derived DCs are located in late endocytic compartments concentrated around the microtubule organizing center in vesicles as well as in short tubules. Upon contact with an Ag-specific T cell, class II-eGFP molecules are rapidly recruited to the DC/T cell contact zone by polarization of the tubulovesicular endosomes, but only when cognate Ag is available (4). Fusion of class II-eGFP-positive endosomes with the plasma membrane was demonstrated in live DCs (6), consistent with the possibility that such polarized endosomes may transport specific peptide-loaded class II MHC molecules to the cell surface. An APC thus responds to the encounter with an Ag-specific T cell in a manner that may help sustain the chain of events leading to full activation of the T cell.

When a TCR on an Ag-specific T cell is ligated by the correct peptide-class II MHC complex, T cells respond by the formation of specialized structures, referred to as supramolecular activation clusters (SMACs) or immunological synapses (7, 8). The serial engagement of several T cell receptors is apparently required to surpass a threshold required for activation of the responding T cell (9–12). Molecules such as TCR, CD2, CD3, CD28, and protein kinase C- $\theta$ , lck, and fyn cocluster at and near the T cell surface (7, 8, 13–16). A central SMAC is surrounded by the peripheral SMAC in which the integrin LFA-1 and the cytoskeletal protein talin are concentrated (8). The involvement of T cell surface molecules TCR, CD2, CD28, and LFA-1 in the induction of polarized endosomal tubulation in the DCs must therefore be explored.

We here describe cell surface requirements of DCs and T cells for T cell-polarized class II-positive tubular endosomes in DCs. In addition to the Ag-specific interaction between class II MHC and

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<sup>3</sup> Abbreviations used in this paper: ER, endoplasmic reticulum; DC, dendritic cell; eGFP, enhanced green fluorescent protein; SMAC, supramolecular activation cluster; CHX, cycloheximide; CD40L, CD40 ligand; RT, room temperature; ON, overnight; TfR, transferrin receptor.

TCR, requirements for endosomal tubulation include the mobility of proteins in the lipid bilayer of the plasma membrane, the ligation of cell adhesion molecules, and induced protein expression on the surface of activated T cells. An unexpected result is the observation that a T cell can distinguish between peptide-loaded class II MHC complexes depending on their mode of peptide acquisition. Whereas T cell ligation of endosome-derived peptide/class II MHC allowed endosomal tubulation, DCs loaded with exogenously added peptide induced no morphological transformation of endosomal compartments upon interaction with Ag-specific T cells. We propose that the microenvironment of class II MHC molecules that acquired their antigenic cargo in endosomes may be important for polarized tubulation of class II -positive compartments. Activation of naive T cells could benefit from such spatial and temporal conservation of peptide-loaded class II MHC molecules on the surface of the APC.

## Materials and Methods

### Mice

I-A<sup>b</sup>-eGFP knock-in mice (4) and transgenic mice that express an OVA-specific TCR (OTII) (17) have been described. Mice were housed in a barrier facility and studies were performed according to institutional guidelines for animal use and care.

### Cell preparation and culture

DCs were generated as described previously (4, 5). Briefly, bone marrow cells from class II-eGFP mice were flushed from the femurs and tibia and resuspended in DMEM (Life Technologies, Grand Island, NY) supplemented with 10% FCS, 10 ng/ml GM-CSF (PeproTech, Rocky Hill, NJ) and 1 ng/ml IL-4 (Boehringer Mannheim, Indianapolis, IN). Cells were cultured on 25-mm circular coverslips in six-well plates ( $5 \times 10^6$  cells/well), with changes of medium every second day; nonadherent cells were removed by gentle washing. Naive T cells were obtained either directly from lymph nodes of OTII mice in which CD4 T cells represent 25% of all of the cells or purified from spleen and/or lymph node using a T cell purification kit (Miltenyi Biotec, Auburn, CA), resulting in >95% pure T cell preparation. OTII cells were stained with nuclear dye Hoechst 33258 (Molecular Probes, Eugene, OR).

### Flow cytometry

mAbs used for cell typing as well as for T cell activation and blocking experiments were the following: anti-CD69-PE (Armenian hamster clone H1.2F3), anti-CD4-allophycocyanin (rat clone L3T4), anti-TCR $\beta$ -biotin (hamster clone H57-597), anti-TCR-V $\beta$ 5.1/5.2-FITC (mouse clone MR9-4), anti-CD71-biotin (rat clone C2), anti-CD2-biotin (rat clone RM2-5), anti-LFA-1-biotin (rat clone 2D7), anti-CD40 ligand (CD40L)-biotin (Armenian hamster clone MR1), and anti-CD28-biotin (Syrian hamster clone 37.51) and were obtained from BD Biosciences (Mountain View, CA). Biotin-conjugated Abs were combined with PE-conjugated streptavidin for flow cytometry. Cells were analyzed on a BD Biosciences FACScan instrument.

### Fixation of T cells

In some experiments, OTII cells were fixed using mild conditions:  $2 \times 10^6$  cells/ml were incubated at room temperature (RT), 30 s in 0.05% glutaraldehyde, then 0.2 M glycine was added for 90 s before stopping the fixation reaction by addition of 10 vol of cold PBS. Robust fixation of OTII cells was performed by incubating cells in 3% paraformaldehyde ( $2 \times 10^6$  cells/ml, 10 min, RT). For both fixation conditions, cells were washed twice in PBS and once in medium before addition to DC cultures. To arrest processing of Ag and loading of peptide on class II MHC molecules in live DCs, cells were incubated at  $2 \times 10^6$  cells/ml in 0.05% glutaraldehyde (30 s)/0.2 M glycine (90 s). Before the addition of T cells, DCs were washed twice in PBS and once in medium.

### Cycloheximide (CHX) treatment and inhibition of cell surface molecule ligation using blocking mAbs

To block T cell surface molecules LFA-1 and CD2, T cells were incubated on ice 1 h with 10  $\mu$ g/ml of the relevant mAb before washing and adding to the DCs. To block newly expressed CD40L and CD28, the relevant mAb was also included in the DC/T cocultures (also 10  $\mu$ g/ml). For CHX treat-

ment, OTII cells were incubated at 37°C in the presence of 25  $\mu$ g/ml CHX for 1 h and washed three times before addition to DCs. For experiments with activated T cells, OTII cells were activated overnight with PMA (1.7  $\mu$ g/ml) and ionomycin (114  $\mu$ g/ml) or with plate-bound anti-TCR $\beta$  Abs. Plates were coated by incubation with anti-TCR $\beta$  Abs (5  $\mu$ g/ml in 0.1 M NaHCO<sub>3</sub>/Na<sub>2</sub>CO<sub>3</sub>, pH 9.5, overnight (ON)).

### Peptide

The OVA-derived OTII epitope ISQAVHAAHAEINAGR (OVA<sub>323-339</sub>, ISQ in short) (18) was synthesized on an advanced ChemTech 40 channel peptide synthesizer using standard F-moc chemistry. Its molecular mass was confirmed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry.

### Ag presentation and real-time imaging of class II-eGFP in DCs

At day 5 of culture, OVA (40  $\mu$ M; Sigma-Aldrich, St. Louis, MO) or ISQ peptide (20  $\mu$ M) was added to DCs. OVA uptake and processing was allowed for 4 h at 37°C, after which DCs were washed and  $1 \times 10^6$  OTII T cells were added. Plates were centrifuged (1200 rpm, 1 min at RT) to induce rapid contact of T cells with DCs. When using ISQ peptide as Ag, binding of peptide to surface-exposed empty class II MHC was allowed for 1 h at RT. DCs were then washed three times with medium. Naive OTII T cells were obtained from lymph nodes of OTII-transgenic mice. T cells ( $1 \times 10^6$ ) were labeled with the nuclear dye Hoechst 33258 (Molecular Probes), washed, and added to each coverslip containing DC. Conjugates of DCs and T cells were analyzed for the induction of class II-eGFP-positive tubular compartments between 1 and 2 h of T cell addition using an inverted Zeiss 200M microscope equipped with a  $\times 63$  objective (1.4 aperture PanApo) supplied with a 37°C open perfusion temperature-controlled chamber (20/20 Technology, Wilmington, NC). We used Slidebook (Intelligent Imaging Innovation, Denver, CO) for image acquisition and data processing.

At least 100 DCs were analyzed for the induction of tubulation and were scored independently by two individuals.

### Activation of T cells by culture with Ag-presenting DCs

DCs were allowed to endocytose different concentrations of crystalline OVA for 2 h and were washed. Lymph node cells from naive OTII mice were added ( $1 \times 10^6$ ) and were incubated for 6 h (in duplicate experiments). Activation of OTII T cells was gauged by the increase in cell surface expression of the early activation marker CD69 (by flow cytometry). For the time course of OVA presentation by DCs, DCs were allowed to endocytose 40  $\mu$ M OVA for 20 min to 6 h at 37°C, fixed by addition of 0.05% glutaraldehyde (30 s, RT) and 0.2 M glycine (90 s, RT), and were washed three times. OTII T cells ( $1 \times 10^6$ ) were then added to DCs and activation of T cells was assessed as above after 24 h.

## Results

### Culture of DCs from class II-eGFP knock-in mice for microscopy analysis

To visualize the intracellular distribution of class II MHC molecules, we made use of I-A<sup>b</sup>-eGFP knock-in mice. We generated DCs from bone marrow by culturing them with GM-CSF and IL4 as described previously (4). DCs are generally cultured in plastic tissue culture dishes, but for epifluorescence microscopy, bone marrow precursors were grown on glass coverslips contained in regular plastic dishes. The culture of DCs on a modified surface may affect DC differentiation from bone marrow precursors or produce a change in activation status of DCs once fully developed. Therefore, we examined DCs that were cultured in plastic tissue culture dishes, on glass coverslips, or on poly-L-lysine-coated coverslips for DC yield and activation status, as judged by direct visualization of class II-eGFP and staining for CD11c and CD86 (our unpublished observations). We did not observe a significant difference in the development of CD11c/class II double-positive cells grown on different surfaces. After 5 days of culture, a third of the cells expressed class II-eGFP, at least 85% of which expressed the DC marker CD11c and were therefore considered DCs. The majority of DCs, whether grown on plastic or glass, exhibited low levels of CD86 (our unpublished observations) and we consider DCs from these cultures early/intermediate-type DCs (19).



*Ag loading requirement for tubulation of class II-containing endosomal compartments in DCs*

Before addressing the signals required for the induction of T cell-polarized endosomal tubulation, we examined the time required of effective Ag loading and presentation. DCs were allowed to endocytose OVA (40  $\mu$ M, 37°C) for periods of 20 min to 6 h and were then fixed to arrest all further processing of Ag. Naive T cells from transgenic mice that express an I-A<sup>b</sup>-restricted TCR specific for OVA (OTII) were purified from lymph nodes and added to the fixed DCs. Activation of T cells was gauged by the increase in CD69 expression after 24 h of further incubation.

Two hours of OVA uptake suffices for display of peptide-loaded class II MHC molecules to activate 30% of OTII cells (CD69<sup>high</sup>), with a further increase to 50% after 4 h and 80% after 6 h (Fig. 1*a*). In all subsequent Ag presentation experiments, DCs were allowed to endocytose OVA for 4 h before confrontation with T cells.

In our earlier experiments, DCs were allowed to endocytose OVA at a high concentration of 300  $\mu$ M (4). We determined the minimal concentration of OVA that still induces T cell-directed tubulation in DCs. DCs were allowed to endocytose OVA (between 0.4 and 400  $\mu$ M) for 4 h, washed, and then OTII T cells were added. A 1-min centrifugation step (1000 rpm, RT) was used to ensure T cell contact with DCs. DCs were monitored for the formation of class II-eGFP-positive tubules between 1 and 2 h after T cell addition by epifluorescence microscopy on our instru-

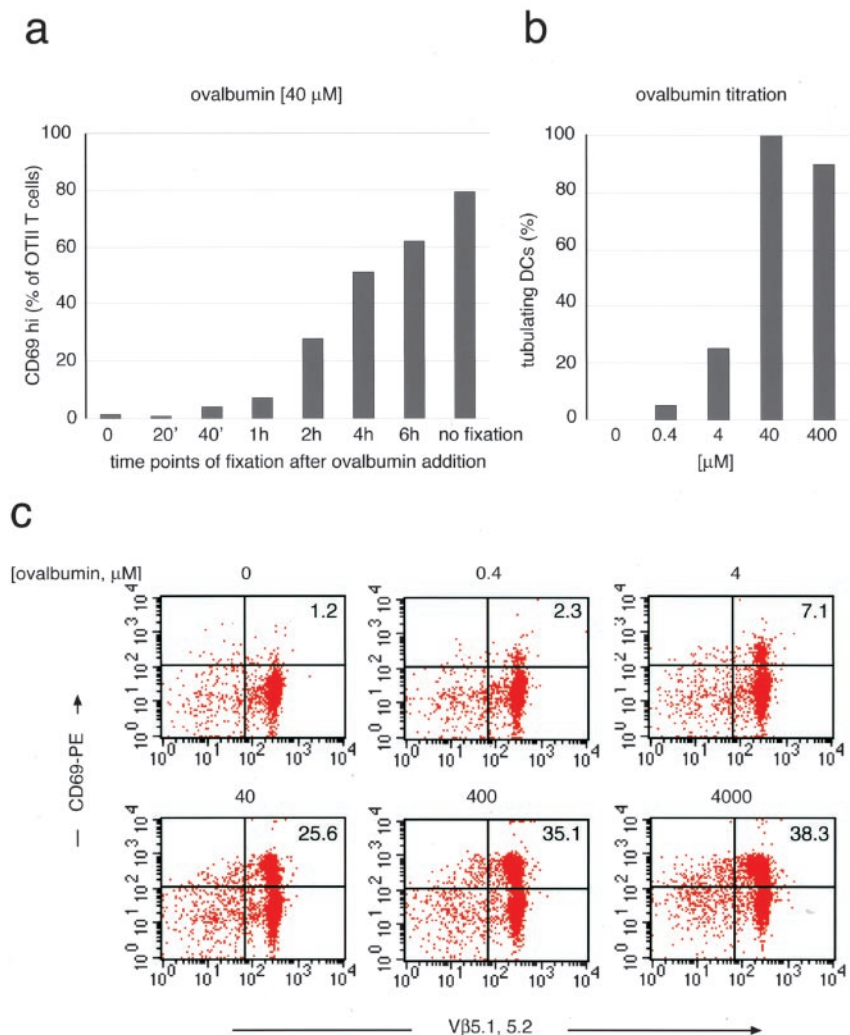
ment equipped with a 37°C open perfusion temperature-controlled chamber.

Because of the number of different parameters examined here, we present the data as the percentage of DCs that express tubulating endosomes, calculated based on observation of at least 100 DCs. With 40  $\mu$ M OVA, 40–60% of all of the DCs examined had class II -positive tubular endosomes. The percentage of DCs that express tubular endosomes was normalized by setting the maximum response as 100%. We analyzed DCs after engagement of Ag-specific T cells by analysis of images rather than movies, and therefore this study focuses on the conditions required for tubulation rather than dynamic endosomal transport. For representative images of DCs that express tubular endosomes, we refer to Boes et al. (4).

We observed that T cells evoke class II-eGFP tubules in DCs in an Ag concentration-dependent manner. As little as 0.4  $\mu$ M OVA triggers formation of tubules in some DCs, the maximal response being observed at 40  $\mu$ M OVA (Fig. 1*b*). We have no estimate of the success of conversion of intact OVA into the relevant peptide-MHC complex.

Is there a correlation between the concentration of OVA required for endosomal tubulation in DCs and the minimal dose necessary to activate naive T cells? The activation status of OTII T cells was analyzed after 6 h of culture with OVA-loaded DCs. The Ag dose that induces T cell-directed endosomal tubules in

**FIGURE 1.** Activation of OTII T cells after coculture with DCs pulsed with OVA before fixation. *a*, Day 5 DCs were cultured from class II-eGFP<sup>+/+</sup> bone marrow and were allowed to endocytose a graded amount of OVA (40  $\mu$ M) for 20 min to 6 h. They were then fixed in 0.05% glutaraldehyde to stop processing and transport of class II MHC. Naive T cells ( $1 \times 10^6$  cells) were extracted from lymph nodes of OTII-transgenic mice and were cocultured with DCs (24 h, 37°C). Activation of OTII T cells was analyzed the next day by flow cytometry (cell surface staining) for V $\beta$ 5, CD4, and CD69. *b*, DCs were allowed to endocytose a graded amount of OVA between 0.4 and 400  $\mu$ M for 4 h, washed, and OTII T cells were added. Formation of endosomal tubulation in class II-eGFP<sup>+/+</sup> DCs was visualized at 1–2 h after T cell addition. *c*, DCs were allowed to endocytose a graded amount of OVA between 0.4 and 400  $\mu$ M for 2 h, washed, and OTII T cells were added. After 6 h of coculture (or 8 h after Ag addition), OTII T cells were analyzed by flow cytometry by cell surface staining for V $\beta$ 5, CD4, and CD69.



DCs correlates well with the Ag dose required to activate naive T cells, as judged from up-regulation of the early activation marker CD69 (Fig. 1c). Little up-regulation of CD69 was observed when OTII T cells were cocultured with DCs loaded with OVA at a concentration of 0.4  $\mu$ M, but a significant number of activated T cells was present after coculture with DCs that had endocytosed 40  $\mu$ M OVA. Subsequent experiments were thus performed using 40  $\mu$ M OVA endocytosed by DCs for 4 h.

*Mild fixation compromises the ability of T cells to induce tubular endosomes in DCs*

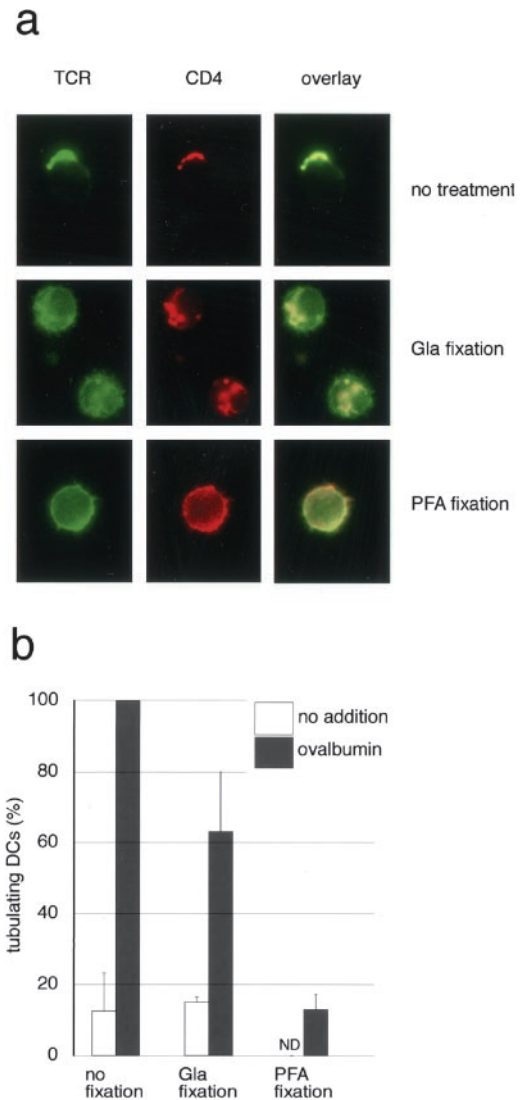
Cytoskeleton-driven clustering of T cell membrane proteins at the T cell/APC interface plays an important role in T cell activation (7, 8, 13). Are there analogous requirements to induce polarized endosomal rearrangement in DCs upon contact with Ag-specific T cells? To interfere with rearrangements of surface proteins, OTII T cells were fixed using 0.05% glutaraldehyde or using a stronger fixative (3% paraformaldehyde). To assess the effect of these fixations on membrane protein clustering, naive purified OTII T cells were labeled with fluorescein-coupled anti-TCR Ab and PE-coupled anti-CD4 Ab (20). In untreated naive T cells, a 30-min incubation at 37°C induces complete colocalization of TCR and CD4 (Fig. 2a, top panels). When cells were fixed using 0.05% glutaraldehyde, some clusters of TCR and CD4 developed, but no full cap was produced (Fig. 2a, middle panels). Strong fixation using 3% paraformaldehyde completely blocked the ability of TCR and CD4 to cluster in the membrane (Fig. 2a, bottom panels). Of note, the conditions of fixation of OTII T cells were verified by examining up-regulation of CD69, when fixed T cells were exposed to DCs loaded with OVA peptide. Expression of CD69 was not induced in T cells that were fixed using either cross-linking reagent (data not shown).

Is there a necessity for clustering of membrane proteins in T cells to induce polarized endosomal tubulation in DCs? OTII T cells were fixed using glutaraldehyde or paraformaldehyde, washed extensively, and then added to DCs loaded with OVA (40  $\mu$ M, 4 h). DCs were not harmed by release of either fixative from the T cells, as they retained the ability to induce activation of OTII T cells that had not been previously fixed (data not shown). Over the ensuing 2 h, DCs were analyzed by microscopy for the induction of endosomal tubulation (Fig. 2b).

Fixation of OTII T cells using paraformaldehyde blocked 90% of polarized endosomal tubulation in DCs (Fig. 2b). Thus, the maintenance of normal membrane dynamics appears to be essential to induce tubulation. T cell fixation using glutaraldehyde resulted in 40% reduction in tubulation. Glutaraldehyde fixation, but not paraformaldehyde fixation, allowed for some clustering of surface glycoproteins TCR and CD4 (see Fig. 2a), which therefore appears sufficient for the induction of tubulation in some DCs. Thus, the mobility of proteins in the T cell membrane is essential for their ability to induce tubulation.

*Adhesion requirements between DCs and Ag-specific T cells to induce tubular endosomes*

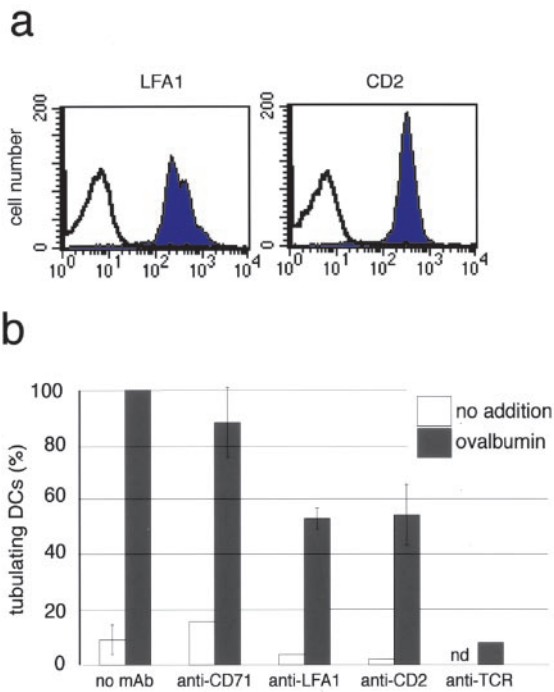
To examine whether adhesion molecule-mediated binding is important for induction of endosomal tubulation, we blocked these interactions using mAbs to adhesion molecules LFA-1 and CD2. Both LFA-1 and CD2 are present at the interface between APC and T cells during Ag-specific contact (21). First, the blocking assay was optimized using mAb to the TCR as a positive control and mAb to transferrin receptor (TfR, or CD71) as a negative control Ab unlikely to affect tubulation. Inhibition of interaction of TCR using anti-TCR $\beta$  mAb blocked 90% of tubulation. Thus, in this system, endosomal tubulation is dependent on the interaction



**FIGURE 2.** Clustering of T cell membrane proteins is required to induce endosomal tubulation in DCs. *a*, Naive T cells were extracted from lymph nodes of OTII-transgenic mice and were labeled with fluorescein-coupled anti-TCR mAb and PE-coupled anti-CD4 mAb. Cells were either left untreated, or fixed using 0.05% glutaraldehyde or fixed using 3% paraformaldehyde. Cells were then incubated for 30 min at 37°C and analyzed by epifluorescence microscopy for cap formation. *b*, OTII T cells were left untreated or fixed using 0.05% glutaraldehyde or fixed using 3% paraformaldehyde, were washed three times, and added to class II-eGFP<sup>+/+</sup> DCs. Development of endosomal tubulation in DCs was visualized by epifluorescence microscopy.

with Ag-specific TCR, as demonstrated previously (4). Ab-mediated blocking of TfR interaction did not affect tubulation significantly compared with untreated DC/T cell conjugates (Fig. 3a).

Must DCs ligate adhesion molecules on T cells to allow formation of polarized tubular endosomes? To confirm expression of LFA-1 and CD2 on naive OTII T cells, their level was assessed by flow cytometry. The OTII T cell surface levels of LFA-1 and CD2 are high (Fig. 3a). Pretreatment of purified OTII T cells with mAb to LFA-1 to block binding with ICAM-1, -2, or -3 on the DCs reduced the number of DCs that express polarized tubulation by 50% (Fig. 3b). Similarly, mAb-mediated blocking of T cell-expressed CD2 and LFA-3 on the DCs reduced the number of DCs that express endosomal tubulation by 50% (Fig. 3b). The simultaneous inclusion of mAb to CD2 and LFA-1 did not show an



**FIGURE 3.** Adhesion molecule-mediated binding to OTII T cells stimulates endosomal tubulation in DCs. *a*, Expression levels of LFA-1 and CD2 on naive OTII T cells was determined by flow cytometry (filled histograms). Isotype-matched Abs were included as controls (open histograms). *b*, Naive T cells were purified from lymph node cells of OTII-transgenic mice and were incubated with mAb to TCR, Tfr (CD71), LFA-1, or CD2 (10  $\mu$ g/ml in PBS, 1 h, on ice). T cells were washed three times with cold PBS and were then added to class II-eGFP<sup>+/+</sup> DC cultures by a brief centrifugation (1200 rpm, RT). Over the next 2 h, development of tubulation in DCs was analyzed by epifluorescence microscopy.

additive effect: we observed 50% inhibition of tubulation in DCs (data not shown). Thus, inhibition using mAb to either adhesion molecule disturbs the contact zone between APC and T cell sufficiently to decrease endosomal tubulation in the interacting DCs, although we have as yet been unable to obtain full inhibition using Abs as blocking agents. The simultaneous addition of anti-LFA-1 and anti-CD2 Abs did not result in stronger inhibition of endosomal tubulation than seen with either Ab alone (data not shown). Taken together, inhibition of interaction between LFA-1 on T cells and ICAM-1, -2, or -3 or inhibition of binding of CD2 on T cells to LFA-3 diminishes the ability of naive Ag-specific T cells to induce tubular endosomes in DCs.

#### Activated T cells induce more endosomal tubulation in DCs

Alterations in surface expression of proteins on the T cell plasma membrane during activation may enhance their ability to induce extended endosomes in interacting DCs. Do activated Ag-specific T cells stimulate more endosomal tubulation in DCs compared with their naive counterparts? Purified OTII cells were preactivated with PMA/ionomycin or with plate-bound anti-TCR Abs (ON) and were then added to OVA-loaded DCs. Both methods induce strong activation of T cells as verified by increased CD69 expression (data not shown). Induction of tubulation by activated OTII T cells remained strictly dependent on the presence of Ag, as was the case with naive Ag-specific T cells. Both methods of activation increased the ability of OTII T cells to induce polarized class II-eGFP-positive tubules in DCs (Fig. 4*a*). OTII T cells that were activated using PMA/ionomycin increased DCs that express tubulating endosomes by 28%, whereas anti-TCR mAb used as

activating agents increased the number of DCs that express extended endosomes by 68% compared with naive OTII T cells.

#### CD40L expression on activated T cells stimulates endosomal tubulation in DCs

A candidate ligand induced upon T cell activation is CD40L. We therefore assayed the possible involvement of CD40L in inducing the rearrangement of endosomal compartments in DCs through ligation of CD40 on the DC. First, is CD40L induced at the cell surface during T cell activation? Naive OTII T cells were purified from lymph nodes and were activated using plate-bound anti-TCR Abs. Activation-induced cell surface expression of CD40L on the OTII plasma membrane, with and without pretreatment with CHX to block de novo synthesis of proteins, was measured over a 6-h culture period. Expression of CD40L on OTII T cells increased as soon as 2 h after activation (Fig. 4*b*). The increased ability of activated T cells to induce endosomal tubulation in DCs therefore correlates with induced expression of CD40L on the T cells.

We then examined whether the induction of CD40L on activated T cells stimulates tubulation of endosomal compartments in interacting DCs. Blocking mAb to CD40L (10  $\mu$ g/ml) was added simultaneously with naive OTII lymph node cells to Ag-loaded DC cultures (40  $\mu$ M OVA for 4 h), and mAb were kept in the cultures throughout the experiment. DCs were then monitored for the formation of class II-positive tubules between 3 and 4 h after T cell addition by epifluorescence microscopy.

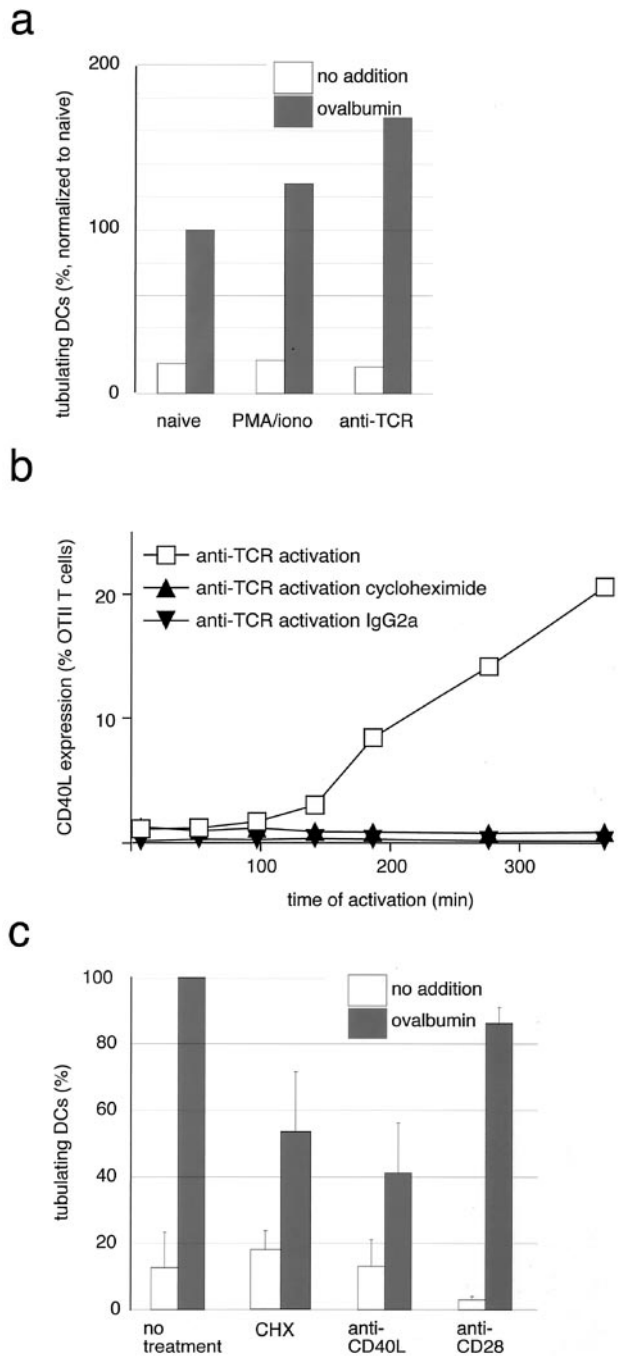
Although inhibition of CD28 ligation using anti-CD28 mAb did not significantly reduce tubulation, treatment with mAb to CD40L resulted in a 60% reduction of endosomal tubulation compared with untreated T cells (Fig. 4*c*). Pretreatment of T cells with CHX also resulted in inhibition of tubulation comparable to anti-CD40L mAb treatment. We conclude that the CD40-CD40L interaction is involved in the induction of tubular endosomes during T cell activation, although other molecules must play a role as well and on a whole the process is likely to be complex. The involvement of CD40-CD40L interaction in endosomal extensions is probably limited to interaction with newly activated T cells, but is irrelevant for initiation of the response.

#### DC membrane requirements to induce tubular endosomes in DCs

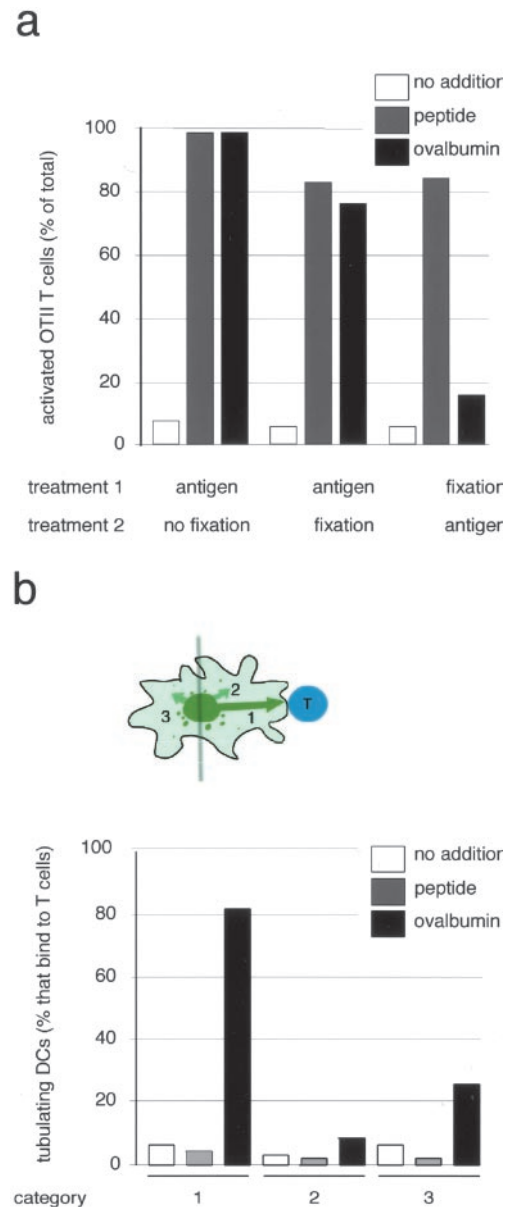
Most surface class II MHC segregate constitutively into cholesterol- and glycosphingolipid-enriched plasma membrane microdomains (22), and reorganization of proteins into microdomains at the interface of a T cell and an APC plays an important role in T cell activation (21–23). In APC, class II-positive microdomains at the plasma membrane may be a manifestation of the transient preservation of the membrane composition of transport vesicles that recently inserted in the plasma membrane. Do T cells normally encounter peptide-loaded class II MHC in a spatial configuration that is enriched for Ag-loaded class II molecules and costimulatory molecules? The presence of surface-displayed empty class II MHC or self-peptide-loaded class II MHC is probably not enhanced in tetraspan-expressing microdomains (23). How would T cells respond to peptide-loaded class II MHC, when the cognate epitope was administered in the form of a synthetic peptide from the outside?

DCs were treated with synthetic peptide or with intact OVA Ag before or after fixation using glutaraldehyde. DCs were then washed and OTII lymph node cells were cocultured with the DCs for 24 h (37°C). Both routes of peptide acquisition result in equally effective stimulation of naive T cells, as judged from the ensuing increase in expression of the activation marker CD69 by unfixed DCs (Fig. 5*a*). Loading of synthetic peptide onto class II MHC





**FIGURE 4.** Up-regulation of CD40L on activated OTII T cells stimulates endosomal tubulation. *a*, Naive T cells were purified from lymph node cells of OTII-transgenic mice and were activated with PMA/ionomycin or with plate-bound anti-TCR $\beta$  mAb (ON, 37°C). T cells were washed three times and added to DC cultures by a 1-min centrifugation (1200 rpm, RT). DCs were analyzed for tubulation by microscopy. *b*, T cells were purified from lymph nodes of OTII-transgenic mice and were activated by culture on plate-bound anti-TCR Abs in the presence or absence of CHX. The up-regulation of CD40L on the plasma membrane during 6 h of culture was measured by flow cytometry. An isotype control for the anti-CD40L mAb was included at all time points. In T cells that were not activated by plate-bound anti-TCR Abs, CD40L expression was found in 4% of T cells and was unaffected by CHX treatment (data not shown). *c*, Naive OTII lymph node cells were incubated with CHX (1 h, 37°C), with anti-CD40L mAb or were left untreated (on ice, 1 h). Cells were harvested and resuspended in DC medium for addition to DC cultures. Only in the case of CHX treatment, OTII T cells were washed (three times) before addition to class II-eGFP<sup>+/+</sup> DC cultures. DCs were analyzed for induction of endosomal tubulation after 3–4 h of coculture by epifluorescence microscopy.



**FIGURE 5.** Endosomal tubulation requires acquisition of peptide in endosomal compartments. *a*, DCs were allowed to endocytose intact OVA for 4 h (40  $\mu$ M, 37°C) and were washed. Alternatively, DCs were incubated with synthetic peptide that carries the OTII epitope (OVA<sub>323–339</sub>; 20  $\mu$ M). When indicated, DCs were fixed using glutaraldehyde (see *Materials and Methods*) to stop Ag processing and intracellular transport of class II MHC. DCs were washed with medium three times and OTII lymph node cells were added. After 24 h of coculture, OTII T cells were analyzed for activation (as gauged by up-regulation of CD69) by flow cytometry. *b*, DCs were treated with OVA, OVA peptide, or were left untreated (see *a*). OTII lymph node cells were added (by a 1-min centrifugation) to class II-eGFP<sup>+/+</sup> DC cultures. DCs were analyzed by epifluorescence microscopy after 1–2 h of coculture for the presence of endosomal tubulation. Class II-positive tubules were classified according to their direction toward the T cell in each DC/T cell conjugate; those tubules that extend directly toward the interacting T cell are classified as 1, tubules with a vector in the direction of, but not directly pointing at the T cell are classified as 2, and tubules pointing away from the interacting T cell are classified as 3 (4).

does not require endosomal processing and should therefore be largely unaffected by prior fixation of DCs, whereas endosomal processing requires enzymatic activity of live DCs, abrogated by prior fixation of DCs (24, 25). Indeed, up-regulation of CD69 by



OTII T cells was severely compromised by fixation of the DCs before addition of intact OVA Ag, but not when DCs loaded with synthetic peptide were used after fixation. Class II MHC molecules that capture synthetic peptide from the outside can activate naive T cells even when the DCs are fixed.

Analysis of 100 images of DC/T cell clusters acquired 2 h after T cell addition showed that 80% of DCs that had endocytosed intact OVA exhibited endosomal tubular extensions polarized toward T cells. When DCs were loaded with synthetic peptide, <10% of DCs showed signs of endosomal tubulation (Fig. 5*b*). Thus, Ag-specific T cells that interact with DCs induce a focused response to the DCs, depending on the site of Ag acquisition by class II MHC.

## Discussion

DCs, as opposed to B cells, actively contribute to the formation of the immunological synapse (26). In B/T cell synapses, the class II MHC is believed to be held at the interface by interactions with the T cell (27). In DCs on the other hand, active endosomal transport of peptide-loaded class II MHC to the T cell contact zone may stabilize the DC/T cell interaction and stimulate T cell activation. The availability of mice that express eGFP-tagged class II MHC molecules has allowed us to visualize the trafficking behavior of class II MHC molecules in live cells (4). When DCs interact with T cells, tubular endosomes that contain abundant class II molecules are aimed directly at the interacting T cell in a strictly Ag-dependent manner. There may well be a link between the ligation of class II molecules at the surface, as brought about by contact with an Ag-specific T cell, and the ensuing polarization toward the contact interface. For such signaling to be possible, some small number of the appropriate peptide-MHC complexes would have to be present. Further recruitment of class II molecules from intracellular stores would follow upon T cell contact. But does such tubulation rely on membrane remodeling on the T cell surface, the DC surface, or both?

We describe here signals exchanged between DCs and T cells that are likely involved in polarized endosomal tubulation. Because of the large number of conditions tested, we relied on still images of DCs that were cocultured with Ag-specific T cells rather than time-lapse movies. In the current study, we therefore refrain from statements regarding the transport of class II MHC to the cell surface and focus on the requirements to generate polarized endosomal tubulation toward the Ag-specific T cell.

Endosomal tubulation that occurs in DCs after engagement of Ag-specific T cells relies on the rearrangement of membrane proteins on the T cell surface. A partial reduction of the mobility of membrane proteins, as effectuated by glutaraldehyde fixation of T cells, correlates with diminished tubulation, whereas the harsher paraformaldehyde fixation results in a complete block in T cell-polarized tubulation. Therefore, we conclude that rearrangement of proteins on the T cell surface is required for Ag-specific T cells to induce tubular endosomes in DCs.

The initial interaction between APC and T cells is probably mediated by low-affinity interactions, thus allowing the serial scanning for Ag on the surface of different APC (28). Such initial contacts between APC and T cells are probably mediated by adhesion molecules with long extracellular domains (28). The adhesion molecules LFA-1 and CD2 are involved in both the stabilization of DC-T cell interaction as well as the initiation of intracellular signaling (29, 30). We show here that the induction of tubular endosomes in DCs relies on the interaction of DCs with T cells, mediated at least in part by adhesion molecules LFA-1 and CD2 as inferred from Ab inhibition experiments (Fig. 3), although we were unable to achieve complete inhibition.

The activation of T cells induces cell surface expression of molecules that may influence their ability to induce rearrangement of endosomal compartments in DCs (31). OTII T cells were activated by treatment with either PMA/ionomycin or plate-bound anti-TCR mAb. Compared with naive OTII T cells, activated OTII T cells induced more endosomal tubulation in DCs. We show that in OTII T cells, cell surface expression of CD40L emerges after 2 h of coculture with DCs that were allowed to endocytose intact OVA. A reduction of tubulation was found in DCs that were cocultured with anti-CD40L mAb-treated OTII T cells, whereas anti-CD28 mAb only had a minimal effect. Thus, T cell surface expression of CD40L does stimulate tubulation of class II-eGFP-positive compartments in DCs.

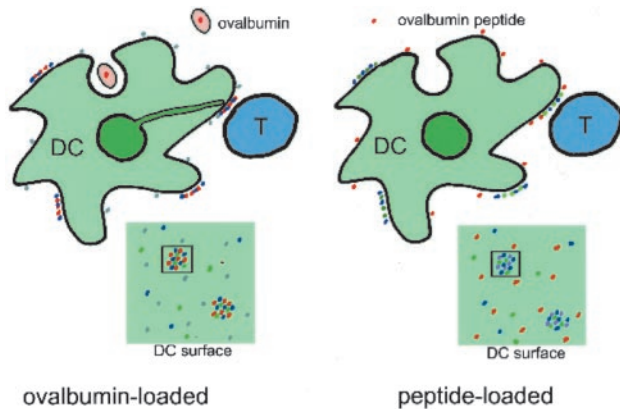
In this regard, it may be of interest to point out that class II MHC-positive compartments in B cells are also subject to rapid remodeling, in response to ligation of the B cell receptor (32). We consider it likely that the ability of class II-positive endosomal compartments to undergo such structural rearrangements may be a more general property of APC and may have a beneficial effect on the proper display of class II MHC-peptide complexes on the surface of the APC.

APC can regulate the distribution of their surface class II MHC within the plane of the membrane to microdomains before contact with CD4 T cells (33, 34). Specialization is achieved through the microdomains that contain members of the tetraspanin family, such as CD9, CD81, and CD82. These tetraspanins form two-dimensional networks in certain areas of the plasma membrane (35, 36). Approximately 10% of human surface class II MHC molecules are found in such tetraspanin-positive domains, and in human cells can be differentiated phenotypically from other surface class II MHC using a mAb, FN1, that probably recognizes clusters of at least two class II MHC molecules (23, 33, 34, 37).

In the endosomal pathway, where protein-lipid sorting occurs, structural and functional microdomains are already formed (38). Moreover, the segregation of specific peptide-loaded class II MHC molecules into microdomains was demonstrated in endosomes as well as on the plasma membrane (23). Class II MHC recently loaded with antigenic peptide may be enriched in microdomains, whereas empty class II MHC or pre-existing peptide-loaded class II MHC may be present in a more even distribution in the lipid membrane (23).

The activation of naive or resting T cells generally requires co-engagement of costimulatory molecules between the APC and T cells. Upon arrival of endosomal vesicles at the cell surface, costimulatory molecules such as CD86 remain clustered with class II MHC (39). Our results suggest that the clustered presence of peptide-loaded class II MHC and costimulatory molecules may be essential for T cell-polarized tubulation, a behavior that may be important early on in the DC-T cell interaction (21, 23). In contrast, T cell activation after 24 h of coculture with DCs (as judged by up-regulation of CD69) occurs irrespectively of the initial gathered presence of CD86 with specific peptide-loaded class II MHC.

Loading the DC surface with exogenously provided peptide necessarily results in a local density of class II MHC complexes and of the auxiliary molecules required for tubulation that is distinct from that found at new insertion sites (see model, Fig. 6). This would explain the differences in the tubulation response to T cells observed for intact protein- vs peptide-loaded DCs (Fig. 5). DCs display large numbers of peptide-receptive class II MHC molecules at their surface, generated presumably through loss of bound peptide. If the formation of polarized tubules is essential to activate naive T cells in the lymph node, then inadvertent activation of T cells by DCs with passively acquired peptides would be less likely. This aspect of T cell activation may help avoid unwanted T cell



**FIGURE 6.** Rendering of the APC surface after loading with intact OVA (*left*) or after addition of synthetic OVA peptide (*right*). MHC molecules loaded with the proper antigenic OVA-derived peptide are shown in red, peptide-receptive (“empty”) MHC molecules in gray, and irrelevant MHC peptide combinations in different shades of green. Auxiliary (adhesion; costimulation; other) molecules required for T cell activation are rendered in blue. Presentation of intact OVA via endocytosis, proteolysis, and subsequent surface display yields patches rich in OVA peptide-loaded molecules at sites of new membrane insertion (*left box*). Addition of synthetic OVA peptide results in loading of empty surface-disposed class II molecules (*right box*). For the identical number of antigenic MHC peptide combinations (red dots,  $n = 12$ ), the spatial arrangement of OVA peptide-MHC combinations is very different for that obtained by endocytic processing, as opposed to loading through addition of synthetic peptide (*box*). Tubulation is evoked only when the proper distribution requirements can be sensed by a T cell, as on the *left*.

responses and contribute to establish peripheral tolerance. Seen from that perspective, it is not only the maturation or activation status of a DC that helps determine its immunogenic or tolerogenic properties: the local distribution of surface proteins is an additional factor to be taken into account.

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