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Dendritic Cell Maturation Controls Adhesion, Synapse Formation, and the Duration of the Interactions with Naive T Lymphocytes

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The initiation of adaptive immune responses requires the direct interaction of dendritic cells (DCs) with naive T lymphocytes. It is well established that the maturation state of DCs has a critical impact on the outcome of the response. We show here that mature DCs form stable conjugates with naive T cells and induce the formation of organized immune synapses. Immature DCs, in contrast, form few stable conjugates with no organized immune synapses. A dynamic analysis revealed that mature DCs can form long-lasting interactions with naive T cells, even in the absence of Ag. Immature DCs, in contrast, established only short intermittent contacts, suggesting that the premature termination of the interaction prevents the formation of organized immune synapses and full T cell activation. *The Journal of Immunology, 2004, 172: 292–301.

Dendritic cells (DCs) are the only APCs that prime naive T lymphocytes and initiate immune responses efficiently. To become competent for naive T cell activation, DCs must undergo a complex developmental program called "maturation" (1, 2). Although only mature DCs prime naive T lymphocytes effectively, peripheral DCs also migrate out of peripheral tissue in the absence of strong maturation signals. Under steady-state conditions, peripheral DCs reach lymph nodes and contribute to maintaining peripheral tolerance (3). Indeed, Ag targeting to immature DCs induces deletion of CD4+ and CD8+ T cells, suggesting that immature DCs do interact with T cells in vivo (4, 5).

In lymph nodes, the relative number of DCs that display a given antigenic peptide is most likely very low. Incoming T lymphocytes, therefore, need to "scan" the surface of many DCs, mature or immature, before they find one that expresses their specific MHC peptide ligand. Upon TCR engagement on APCs expressing the right costimulatory molecules, naive T cell activation is triggered. The dynamics of interaction between mature DCs and T cells have been analyzed both in vitro and in vivo. Using in vitro a collagen three-dimensional matrix, the duration of DC-T cell interactions was found to be short-lived and Ag independent (6). Recently, dynamic imaging in intact lymph nodes showed that in the absence of Ag, T cells are highly motile (11–12 μm/min) and that DCs can scan at least 500 different T cells per hour. In the presence of Ag, the interactions became stable, with an average duration in the order of hours (7–9).

The contact zone between APCs and T lymphocytes is often referred to as the "immunological synapse" by analogy to the nervous system (10–12). The molecular structure of the immune synapse between T cells and B lymphocytes or planar artificial membranes has been extensively analyzed. At the interface between T cells and APCs, signaling and adhesion molecules often distribute in concentric rings (TCR complex in the central area, adhesion molecules in the peripheral area) defined as the central and peripheral supramolecular activation clusters (c-SMAC, p-SMAC) (13); however, other patterns can also be observed (14). Molecules involved in T cell activation such as protein kinase Cθ and linker for activation of T cells (LAT) are recruited to the c-SMAC (15, 16). SMACs form even when the MHC-peptides complexes are presented on inert planar membranes, suggesting that the role of the APC is not crucial (17). It has been suggested that this spatially organized distribution of molecules may facilitate T cell signaling by gathering together several signaling components (18). However, signaling in naive T cells occurs before SMAC formation (19).

Very little is known about the structure of the interaction between T cells and DCs. The cytoskeleton of mature DCs is important to efficiently cluster naive T cells (20) and DCs can induce signaling and synapses in a proportion of naive T cells even in the absence of Ag (21, 22). There is, however, no available information on the characteristics of the DC-T cell interactions under circumstances that determine deletion vs activation of naive T cells.

In this study, we analyze the functional consequences and the dynamics of the interactions between immature or mature DCs and naive T lymphocytes. We also examine the structure of the interaction zone, evaluating the respective contributions of DC maturation and Ag recognition to the biogenesis of the immune synapse. As expected, mature DCs induce effective naive T cell priming and robust proliferation. Interaction with immature DCs, in contrast, induces naive T cells to divide two to four times, but T cells failed to accumulate. We show that DC maturation determines the stability and duration of the initial contacts between DCs and naive T cells, as well as the formation of immune synapses.

Materials and Methods

Mice

B6 mice were obtained from IffaCredo (L’Abresle, France), their I-Aβ−/− counterparts were obtained from Centre de Développement des Technologies

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2 Abbreviations used in this paper: DC, dendritic cell; SMAC, supramolecular activation of clusters; c-SMAC, central SMAC; p-SMAC, peripheral SMAC; LAT, linker for activation of T cell; DIC, differential interference contrast; MTOC, microtubule-organizing center.
Avances (Orléans, France). Marilyn, mice, of the B6.RAG-2<sup>−/−</sup> genetic background, expressing the TCRα (V<sub>α</sub>1-J<sub>α</sub>35) and TCRβ (V<sub>β</sub>6-J<sub>β</sub>2.3) chains from Marilyn, a CD4<sup>+</sup> T cell clone specific for the complex of a male Ag (H-Y) peptide with I<sub>A</sub><b>β</b>, have been described previously (23). These mice were crossed with CD45<sup>1/2</sup> B6 mice to obtain CD45<sup>1/2</sup> Marilyn mice. The H-Y peptide (NAGFNSNRANSSRSS) was synthesized by EPTOPP (Nimes, France), purified by reverse-phase HPLC (>99%), and purity was verified by mass spectroscopy.

**Cells**

D1 is a DC line of B6 splenic origin that in the presence of growth factors is continuously maintained in the immature state (24). Primary culture of bone marrow (BM)-derived DCs from B6 mice and their I<sub>A</sub>β<sup>−/−</sup> counterparts were obtained as described elsewhere (25). For both D1 and BM-DCs, maturation of H-Y peptide, corresponding to 2-, 4-, and 8-fold the initial pE<sub>fl</sub> concentrations (30, 60, and 120 μM) with LPS (mature, D1 and BM-DCs, respectively). As a control, we added 120 or 240 μM of an irrelevant peptide (HEL 103-117) to mature and immature DCs, respectively.

**FACS analysis**

Phenotypic analysis of D1 cells and BM-DCs was performed using the following Abs: FITC-conjugated anti-mouse CD11c, I<sub>A</sub>β, CD40, CD86, and the corresponding FITC-conjugated isotype controls (BD Pharmingen, Le Pont de Claux, France). Loading of the E<sub>ex</sub> peptide was assessed by staining peptide-loaded D1 cells with 10 μg/ml of biotinylated Y-Ab followed by 5-(4,6-dichlorotiazinyl)aminofluorescein-conjugated streptavidin (ImmunoTech, Marseille, France). To measure up-regulation of CD69, immature or mature D1 cells were preincubated with dilutions of H-Y peptide for 3 h at 37°C. Free peptide was removed by three rounds of washing in complete medium. DCs were incubated with Marilyn T cells at 1:5 ratio in 96-well plates in complete IMDM (Sigma-Aldrich, St. Louis, MO). After 12 h, cells were stained for FACS analysis using FITC-conjugated anti-mouse CD4, PE-conjugated anti-mouse V<sub>β</sub>6, and biotin-conjugated anti-mouse CD69 followed by PE-conjugated streptavidin. To follow proliferation of CD4<sup>+</sup> T cells, plates were prepared as described for CD69 using CFSE-loaded T cells (1 μM; Molecular Probes, Eugene, OR). At days 2–5 of the coculture, cells were analyzed by FACS using PE-conjugated anti-mouse CD44 and Tricolor-conjugated anti-mouse V<sub>β</sub>6. All Abs were purchased from BD Pharmingen.

**Adhesion assay and FACS analysis of conjugate formation**

DCs (immature or activated by overnight treatment with 10 μg/ml LPS) pulsed or not with different doses of H-Y peptide (3 h at 37°C), were collected, washed twice with PBS, and immobilized on poly-l-lysine-coated coverslips for 20 min at room temperature (1×10<sup>5</sup> cells/cover). PBS was then removed and replaced with complete medium and the coverslips were incubated for 1 h at 37°C. The number of DCs that remained attached to the coverslips under these conditions was 1×10<sup>5</sup>. Marilyn T cells (at 1×10<sup>6</sup>/0.1 ml) in complete medium were added as a drop of 100 μl on each coverslip (ratio T:DCs = 10:1) and incubated for 1 h. After incubation, the coverslips were washed with 200 μl of PBS several times (as indicated in the figure legends), taking extreme care to ensure homogenous washing. Coverslips were then mounted onto glass slides using a Mowiol solution (Calbiochem). To quantify adhesion, each coverslip was divided into four quadrants and differential interference contrast (DIC) images of two random fields from each quadrant were acquired using a ×63 objective. For each field, we counted the total numbers of DCs, which are readily distinguishable by size and shape (around 30 cells/field and 240 cells/cover). On the same fields T cells forming clear contacts with DCs were quantified blindly (<1% of the T cells were not conjugated to DCs after the washes). Values are expressed as T cell:DC ratios, SD are referred to duplicates of coverslips or experiments performed on different days.

To quantitate conjugate formation by FACS analysis, we prestained T cells with 0.1 μM CFSE and DCs with 1 μM 5-(and-6)2-(4-chloromethyl)
FIGURE 2. Early events of activation in naive T cells stimulated by immature and mature DCs. A. Percentage of naive Marilyn T cells showing a Ca\(^{2+}\) response after coculture with immature or mature D1 treated or not with 10 nM H-Y peptide. All T-DC contacts were analyzed. Responses triggered by immature DCs (□) and by mature DCs (□). The error bars represent the SD from three independent experiments. B. Average Ca\(^{2+}\) response in 5–15 individual responding T cells interacting with immature or mature DCs loaded with 10 nM peptide. The Ca\(^{2+}\) traces were synchronized before averaging so that the shape of the average response is similar to that of (asynchronous) single-cell recording. The tiny responses obtained in the absence of peptide were too few to give a meaningful average (data not shown). C. DCs were incubated with T cells for 12 h (1:5 ratio) and expression of CD69 was assessed by FACS analysis on TCR\(^+\) CD4\(^+\) gated cells. Percentage of naive T cells positive for CD69 staining after 12 h of stimulation with immature (●) or mature (■). DCs pulsed with different doses of peptide. One experiment representative of four is shown.

DYNAMIC MARKER EXPRESSION AND CYTOKINE RELEASE

We have analyzed the interaction of immature and mature DCs with naive CD4\(^+\) T lymphocytes using either a growth factor-dependent CD line, called D1 (24), or fresh BM-DCs. Immature D1 cells grow continuously in the presence of a GM-CSF-containing conditioned medium. As shown in Fig. 1A, immature D1 cells express CD11c, intermediate levels of I-Ab and CD86, but no CD40. These cells also express ICAM-1 and LFA-1 (23). After 20 h of LPS stimulation, surface expression of these markers increase, attesting effective maturation. In parallel, I-A\(^b\) molecules are redistributed from lysosomal compartments to the plasma membrane, and cytokine and chemokine secretion is induced (data not shown). The overall morphology of DCs is also profoundly modified (24). From all these points of view, D1 cells behave exactly like BM-DCs (26). As a homogenous source of naive CD4\(^+\) T lymphocytes, we used lymph node T cells obtained from Rag\(^{−/−}\) Marilyn TCR-transgenic mice (23). The Marilyn TCR recognizes the male H-Y Ag associated to I-A\(^b\) molecules. Marilyn’s lymph nodes contain 93–98% naive (CD69\(^−\), CD44\(^−\)) Marilyn CD4\(^+\) T cells and no other T cells.

Because I-A\(^b\) molecules are 10-fold more abundant on mature than on immature DCs (Fig. 1A), we first measured their respective peptide-loading capacities. For that purpose, we used the mAb, Y-Ae, which recognizes I-A\(^b\) molecules associated to a peptide from the I-E\(_b\) chain (27). As shown in Fig. 1B, the binding of Y-Ae rises when the cells are incubated with increasing concentrations of peptide, reaching a plateau at 100 \(\mu\)M. A 2- to 3-fold difference in the concentrations of the I-E\(_b\) peptide required to

benzoi)aminetetramethylrodamine) ( Molecular Probes). T cells and DCs (pulsed or not with the H-Y peptide) were mixed at 1:1 ratio, spun for 3 min at 300 rpm (4\(^°\)C), and incubated at 37\(^°\)C for 20 min. Tubes were transferred on ice and promptly analyzed by FACS. The results are expressed as percentage of T cells that form conjugates with DCs as calculated by the ratio of two-color events to total T cells events.

Time lapse videomicroscopy and kinetic of contacts

For the dynamic analysis of conjugate formation in living cells, coverslips coated with 3 \(\times\) 10\(^{5}\) D1 cells were placed into a chamber on the microscope at 37\(^°\)C in a 5% CO\(_2\) atmosphere. DIC images were acquired using 63\(^×\) 1.32 apert ure objective and a cooled charge-coupled device camera 5 Micromax Princeton Instruments, Trenton, NJ). One minute after the addition of 3 \(\times\) 10\(^5\) T cells (\(r = 0\), we started to collect images every 10 s for 20 min. To create quick-time files, the DIC images were accelerated \(\times 60\). To quantify the duration of contacts established by individual T cells, we analyzed the fate of single T cells along the length of the movie by scrolling images one by one. Repetitive contacts were scored without taking into accounts whether they are formed with the same or with different D1.

**Immunolabeling of DC-T conjugates, quantification of clustering, three-dimensional reconstitutions**

Conjugates between D1 and CD4\(^+\) T cells were formed as described for the adhesion assay. Incubation was stopped after 30 min and coverslips were washed five times with PBS. Cells were fixed for 10 min with 3% paraformaldehyde and permeabilized with PBS, 2% BSA (Sigma-Aldrich), and 0.05% saponin (ICN Biomedicals, Costa Mesa, CA). For the “not washed” condition in the experiment shown in Fig. 6D (not washed), T cells were removed and coverslips were fixed after a gentle wash with 200 \(\mu\)l of PBS. Primary and secondary fluorescent Abs were diluted in PBS, 2% BSA, and 0.05% saponin and incubated for 1 h or 30 min, respectively. Abs used for single labeling were as follows: biotin-conjugated hamster anti-mouse CD3e (CD3-ε 145-2C11; BD Pharmingen) followed by Alexa 488-conjugated streptavidin ( Molecular Probes); anti-LAT (rabbit polyclonal IgG; Upstate Biotechnology, Lake Placid, NY) followed by Texas Red-conjugated anti-rabbit IgG (Jackson Immunoresearch Laboratories, West Grove, PA); monoclonal rat anti-mouse LFA-1 (ATCC TIB-127) followed by Cy3-conjugated anti-rat IgG (Jackson Immunoresearch Laboratories); mouse anti-tubulin (clone Ab-1; Oncogene Research Products, San Diego, CA) followed by anti-mouse Alexa 488 (Molecular Probes); and rat anti-mouse CD43 (clone S7; BD Pharmingen) followed by anti-rat Cy3.

To acquire images of conjugates, we used a Leica TCS SP2 confocal scanning microscope (Leica, Deerfield, IL) equipped with a 100\(\times\) 1.4 ap －i－ m－e－s－i－o－n－i－c－e－ o－b－ j－e－c－t－i－v－e－ objective. To quantify redistribution of molecules at the site of contact, T-DC doublets were chosen by DIC images and then scored as negative or positive by evaluating the corresponding fluorescent images along for sections on the z plane. “En face” view of the T-DC contact zone (xz) was reconstructed from series of xy sections spaced by 0.3 \(\mu\)m (Metamorph software; Universal Imaging).

**Results**

**Mature, but not immature, DCs activate naive CD4\(^+\) T lymphocytes**

We have analyzed the interaction of immature and mature DCs with naive CD4\(^+\) T lymphocytes using either a growth factor-dependent CD line, called D1 (24), or fresh BM-DCs. Immature D1 cells grow continuously in the presence of a GM-CSF-containing conditioned medium. As shown in Fig. 1A, immature D1 cells express CD11c, intermediate levels of I-A\(^b\) and CD86, but no CD40. These cells also express ICAM-1 and LFA-1 (23). After 20 h of LPS stimulation, surface expression of these markers increase, attesting effective maturation. In parallel, I-A\(^b\) molecules are redistributed from lysosomal compartments to the plasma membrane, and cytokine and chemokine secretion is induced (data not shown). The overall morphology of DCs is also profoundly modified (24). From all these points of view, D1 cells behave exactly like BM-DCs (26). As a homogenous source of naive CD4\(^+\) T lymphocytes, we used lymph node T cells obtained from Rag\(^{−/−}\) Marilyn TCR-transgenic mice (23). The Marilyn TCR recognizes the male H-Y Ag associated to I-A\(^b\) molecules. Marilyn’s lymph nodes contain 93–98% naive (CD69\(^−\), CD44\(^−\)) Marilyn CD4\(^+\) T cells and no other T cells.

Because I-A\(^b\) molecules are 10-fold more abundant on mature than on immature DCs (Fig. 1A), we first measured their respective peptide-loading capacities. For that purpose, we used the mAb, Y-Ae, which recognizes I-A\(^b\) molecules associated to a peptide from the I-E\(_b\) chain (27). As shown in Fig. 1B, the binding of Y-Ae rises when the cells are incubated with increasing concentrations of peptide, reaching a plateau at 100 \(\mu\)M. A 2- to 3-fold difference in the concentrations of the I-E\(_b\) peptide required to
induce equivalent levels of Y-Ae binding was observed between immature and mature DCs. The 3-h incubation with the peptide did not modify the surface expression of I-A\(^b\) as detected by the Y3P Ab (data not shown). Therefore, despite a 10-fold difference in the levels of I-A\(^b\) expression between immature and mature DCs (Fig. 1A), the difference in the efficacy of I-E\(^a\)/H9251 peptide loading was only 2- to 3-fold.

To evaluate the loading of the H-Y peptide on DCs (the specific peptide recognized by Marilyn T cells), we performed competition experiments. Immature or mature DCs were incubated with doses of Y-Ae peptide that give similar Y-Ae binding intensities, and Y-Ae binding was competed with increasing concentrations of the H-Y peptide. The H-Y peptide competed Y-Ae binding with similar efficiencies on immature and mature DCs. A control peptide, HEL\(_{103-117}\), which does not bind I-A\(^b\) molecules, did not compete Y-Ae binding (Fig. 1C).

We concluded that the difference in the efficacy of H-Y peptide loading between immature and mature DCs, like that of Y-Ae binding, is 2- to 3-fold. Consequently, similar levels of H-Y peptide loading are obtained using two to three times less peptide with mature than immature DCs.

We first analyzed the efficiency of peptide-pulsed immature and mature DCs to activate naive T cells by measuring calcium responses. In the absence of exogenously added peptide, Ca\(^{2+}\) responses were rarely observed in a 20-min recording. If they were, their amplitude was usually <150 nM and they did not last more than a few minutes. The fraction of T cells showing such a tiny, transient response was 1.9% with immature DCs and 6.4% with mature DCs (Fig. 2A). In the presence of 10 nM H-Y peptide, mature DCs induced Ca\(^{2+}\) responses in 63% of T cells. In contrast, immature DCs induced Ca\(^{2+}\) responses in only 12.5% of the T cells (Fig. 2A). Higher doses of peptide on immature DCs did not significantly enhance the frequency of Ca\(^{2+}\) responses (data not shown). Mature DCs also triggered more important and sustained T cell Ca\(^{2+}\) responses than immature DCs. These differences can be clearly inferred from Fig. 2B, which shows the average T cell Ca\(^{2+}\) response induced by peptide-loaded immature and mature DCs.

We next measured the up-regulation of CD69, an early marker of T cell activation that is expressed on naive T cells after TCR engagement. After 12 h of coculture with mature DCs loaded with 1 nM H-Y peptide, 20% of T cells had already up-regulated CD69, whereas up-regulation of CD69 by immature DCs was not observed (Fig. 2C). At higher peptide doses (10 nM H-Y peptide), mature DCs induced up-regulation of CD69 in around 80% of T cells. Immature DCs, in contrast, induced CD69 expression in a low proportion of the cells (around 10%) even at 10 nM H-Y peptide (Fig. 2C). Similar results were obtained after 18 h of coculture (data not shown).

We next examined T cell proliferation, using CFSE staining and FACS analysis, upon stimulation with immature or mature DCs pulsed with different doses of H-Y peptide. At day 3 of coculture, Ag-pulsed mature DCs induced robust T cell proliferation and up-regulation of CD44 (Fig. 3A, lower panels). By day 4, mature DCs pulsed with 1 nM peptide had induced virtually all naive T cells to

**FIGURE 3.** Proliferation of naive T cells stimulated by immature or mature DCs. Immature or mature D1 pulsed with different doses of peptide were cocultured for 5 days with CFSE-loaded naive T lymphocytes (1:5 ratio). **A,** Representative dot blot profile showing the loss of CFSE and the up-regulation of CD44 induced by immature (upper row) or mature (lower row) DCs loaded with different peptide doses at day 3. **B,** Histogram profile of CFSE staining on naive T cells stimulated with immature (upper row) or mature (middle row) DCs loaded with 1 nM H-Y and mature DCs loaded with 0.1 nM peptide (lower row) at days 2–5 of the coculture. **C,** Quantification of the absolute number of CD4\(^+\) T cells at the different days of coculture for immature (●) and mature (■) DCs loaded with 1 nM peptide (T cells at day 0 = 7 \(\times\) 10\(^4\)). One representative of three experiments is shown.
undergo more than six divisions (Fig. 3B, middle panels). Accordingly, the absolute number of T cells in the cultures increased (Fig. 3C). Even at 0.1 nM peptide, mature DCs induced full T cell proliferation by day 5 (Fig. 3B, lower panels).

Immature DCs induced a proportion of the T cells to enter the cell cycle at 1 and 10 nM peptide (Fig. 3, A and B, upper panels). These T cells, however, only underwent two to four division cycles. Indeed, in the presence of immature DCs, T cells never became CFSE negative, indicating that they did not proliferate extensively. In addition, the cells that had divided two to four times did not accumulate, suggesting that they had died. Accordingly, the number of T cells in the coculture wells decreased with time, and virtually no T cells survived at day 5 (Fig. 3C).

Together, these results show that, despite the presence of abundant peptide-MHC class complexes on both immature and mature DCs, mature DCs induce effective T cell activation and proliferation, whereas immature DCs induce faint T cell activation and abortive T cell proliferation.

**Adhesion of naive T cells to DC is regulated by DC maturation and Ag recognition**

Which feature of immature/mature DCs could explain the opposite effects they induce when interacting with naive T cells? Costimulation and adhesion molecules expressed on DCs are certainly involved in determining the fate of T cell responses. But how is DC maturation going to influence DC-T cell interactions?

To address this issue, we first measured adhesion of naive T cells to immature and mature DCs. Naive T cells were allowed to adhere to immature or mature DCs pulsed with different doses of peptide. After 1 h, nonadherent cells were removed by washing and the number of T cells that remained attached to DCs was counted. As shown in Fig. 4A, naive T cells adhered strongly to mature DCs in a peptide dose-dependent manner. On the contrary, adhesion to immature DCs was low and was not incremented by Ag recognition, even at high doses of peptide. Similar results were obtained when conjugate formation was assessed by FACS analysis (Fig. 4B).

Adhesion to peptide-loaded mature DCs was extremely stable: the amount of adherent T cells remained unaffected when increasing the number of washes (Fig. 4C). Analysis of adhesion at later time points confirmed that immature DCs do not form significant numbers of stable conjugates even after prolonged incubation periods (2 h, data not shown). Similar results were obtained when using primary BM-DCs (Fig. 4D). We conclude that naive T cells adhere significantly more to mature than immature DCs.

It is known that engagement of the TCR by specific MHC-peptide complexes on APCs delivers a stop signal to T cells through modification of the adhesive state of integrins (28). We therefore

**FIGURE 4.** Adhesion of T cells to immature and mature DCs. A, Briefly, $1 \times 10^5$ lymph node-purified Marilyn T cells were allowed to adhere to coverslips coated with $1 \times 10^6$ immature or mature D1 loaded with different doses of H-Y peptide (pep). After 1 h of incubation, the coverslips were washed (five times) and T cell adhesion was quantified by direct cell counting as described in Materials and Methods. Results are representative of three independent experiments. B, The formation of T-DC conjugates was assessed by FACS analysis. T cells stained in green were mixed with DCs stained in red (1:1 ratio) and green/red doublets were quantified by FACS after 20 min of interaction at 37°C. Data are expressed as percentage of T cells engaged in doublets over the total number of T cells (one of three experiments is shown). C, The adhesion assay was performed as in A. After 1 h of incubation, the coverslips were washed and T cell adhesion was quantified after the indicated number of washes. Results are representative of three independent experiments. D, Adhesion to BM-DCs. Immature and mature BM-DCs were treated or not with 10 nM H-Y peptide. Adhesion of naive CD4$^+$ T cells was quantified as in A.
asked whether the inability of naive T cells to adhere to immature DCs is due to a lack of T cell activation. To address this question, naive T lymphocytes were compared for adhesion to immature and mature D1 pulsed or not with 10 nM peptide (pep). After five rounds of washing, adhesion was quantified by manual counting as in Fig. 4. One of two experiments is shown.

Therefore, CD4+ T cells form strong interaction with mature DCs loaded with the specific Ag. On the contrary, the presence of MHC class II-peptide complexes on immature DCs is not sufficient to stabilize the DC-T interaction.

DC maturation is required for effective clustering and SMAC formation at the DC-T cell contact site

We next characterized the molecular distribution of adhesion and signaling molecules at the DC-T cells interface. As expected from the results presented thus far, the number of DC-T cell conjugates was three to four times higher for mature than for immature DCs. We found that effective clustering of CD3, LFA-1, and LAT within stable conjugates (i.e., that resisted five rounds of washes) required DC maturation. Indeed, clustering of these molecules was less frequently observed in conjugates between T cells and immature DCs. Similarly, efficient reorientation of the microtubule-organizing center (MTOC) and the tubulin network toward the DC only occurred in stable complexes with mature peptide-treated DCs (Fig. 6A).

**FIGURE 5.** Naive CD4+ T cells were activated in vivo by adoptive transfer into empty host followed by Ag (male B cells) injection in vivo. Naive and in vivo-activated T lymphocytes were compared for adhesion to immature and mature D1 pulsed or not with 10 nM peptide (pep). After five rounds of washing, adhesion was quantified by manual counting as in Fig. 4. One of two experiments is shown.

**FIGURE 6.** Efficient clustering in naive T cells requires DC maturation. Confocal images showing the distribution of CD3, LFA-1, LAT, and tubulin in T cells forming conjugates with immature or mature D1 pulsed with 10 nM H-Y peptide. Conjugates were formed for 30 min, washed five times, and fixed for immunostaining. A, One representative conjugate formed with immature DCs (upper panels) or mature DCs (lower panels) is shown for each marker. For each immunofluorescent image (right panels), a DIC image showing the two cells in contact is shown (left panels). Note that the distribution of CD3, LFA-1, and LAT is homogenous on T cells forming conjugates with immature DCs and clustered in conjugates formed with mature DCs. Similarly, the T cell MTOC (marked by an arrow) is reoriented toward the APCs in conjugates with mature, but not with immature DCs. B, Quantification of the proportion of conjugates showing clustering of CD3, LFA-1, LAT, and tubulin at the site of contact. Conjugates between naive T cells and immature or mature DCs pulsed with different doses of H-Y peptide were formed as in A. The number of conjugates presenting clustering at the contact zone was blindly quantified (see Materials and Methods) and expressed as a percentage of the total number of conjugates analyzed (percent clustering). Number of conjugates quantified is as follows: CD3, immature, n = 276; mature, n = 347; LFA-1, immature, n = 197; mature, n = 229; LAT, immature, n = 185; mature, n = 232; and tubulin, immature, n = 210; mature, n = 243.
Quantiﬁcation of these results showed that in the absence of Ag, DC maturation induced a slight increase in CD3, LFA-1, and LAT clustering at the immune synapse, but effective clustering required both DC maturation and Ag recognition. Similar results were obtained when analyzing the recruitment of protein kinase Cθ and MTOC reoration (data not shown and Fig. 6B). Interestingly, immature DCs loaded with 10 nM H-Y were less efﬁcient for inducing clustering than mature DCs loaded with 1 nM H-Y, although the extent of H-Y loading on I-Ak is stronger in immature cells than in mature cells under these conditions (see Fig. 1B). We conclude that clustering of CD3, LFA-1, and LAT as well as MTOC reorientation at the DC-T cell interface require both DC maturation and Ag recognition.

Mature immune synapses are deﬁned as structures that display a unique spatial organization into central and peripheral zones called SMACs (13). To investigate whether mature synapses could form in stable conjugates between DCs and naive CD4+ T cells, we analyzed the relative distribution of a c-SMAC molecule (CD3), a p-SMAC molecule (LFA-1), and a molecule excluded from the synapse (CD43). In immature DCs, most of the conjugates present an even distribution of the three markers which occasionally form small disperse clusters (Fig. 7A and data not shown). No large clustering or SMAC organization was detectable. In contrast, a SMAC organization with a clear-cut central enrichment of CD3, peripheral enrichment of LFA-1, and exclusion of CD43 from the interaction zone could be observed in a signiﬁcant fraction of peptide-bearing mature DCs (Fig. 7A and C, Brossard and A. Trautmann, unpublished data). Similar results were obtained with mature BM-DCs (Fig. 7B). Peptide was required for the formation of an organized structure since conjugates formed by mature DCs in the absence of H-Y peptide showed a uniform distribution of CD3 and LFA-1 molecules (Fig. 7C).

We quantiﬁed the presence of organized synapses in stable (i.e., that resisted ﬁve rounds of washing) and in total conjugates that were not selected by washing. We have scored as positive in this analysis conjugates showing segregation of CD3 and LFA-1 (or CD43) into different areas (but not necessarily as a clear bull’s eye structure). For mature DCs pulsed with peptide, we found that 48.5 and 68% of conjugates displayed organized synapses for unselected and stable conjugates, respectively (Fig. 7D). For immature DCs, we could rarely observe segregation or organized synapses, independently of the washing procedure (12% no wash, 7% after ﬁve washes). Therefore, peptide recognition and DC maturation are both required for synapse formation.

Dynamics of DC-T cell contacts

Redistribution into c-SMAC and p-SMAC in T cells that interact with APCs takes 15–20 min (17). We therefore asked whether differences in the dynamics of the interactions between immature and mature DCs with naive T cells could account for the induction

**FIGURE 7.** Immune synapse in T-DC conjugates. Conjugates of immature/mature D1 and naive T cells were formed for 30 min and stained with markers of c-SMAC and p-SMAC. A, Representative conjugates formed by naive T cells and immature (left) or mature (right) DCs pulsed with 10 nM peptide (pep). The staining with anti-CD3/LFA-1 and anti-CD3/CD43 Abs on the xy plane is shown. The corresponding DIC images of conjugates are shown. The corresponding z reconstruction of the contact zone for each labeling is shown on the right. B, As in A, a representative conjugate formed by a mature BM-DC loaded with peptide and a naive T cell. C, xy plane and z reconstruction for CD3/LFA-1 staining on a representative conjugate formed in the absence of exogenously added Ag. The two markers are evenly distributed. D, Quantification of the number of conjugates showing an organized synapse for immature and mature DCs pulsed with 10 nM peptide. Conjugates were formed for 30 min and either washed (ﬁve washes) or directly ﬁxed before staining. Conjugates were blindly chosen under transmission light. The percentage of conjugates showing an organized synapse is plotted (30 conjugates on average were quantiﬁed for each condition).
of mature immune synapses. We analyzed DC-T cell contacts using dynamic cell imaging and videomicroscopy. Immature or mature DCs were incubated for 3 h in the presence or 10 nM H-Y peptide, washed, and cocultured with freshly isolated specific naive Marilyn T cells. Sequential images were recorded during the first 20 min of coculture. As shown in Fig. 8A and web movies1 1 and 2, immature DCs were not very mobile and established multiple, sequential contacts with naive T cells. Mature DCs, in contrast, were extremely mobile, projecting membrane extensions in all directions. They actively captured, embraced T cells, and established stable contacts.

The duration and the number of individual DC-T cell contacts were quantified and classified into three categories depending on their duration: short contacts (10–100 s), intermediate contacts (100–500 s), or long contacts (500 s to 20 min). In the presence of Ag, immature DCs mainly established multiple short contacts (75%) and intermediate contacts (22%). Only 2–4% of the immature DC-T cell contacts lasted over 500 s (Fig. 8B, left panels). Mature DCs behaved quite differently. The number of very short contacts was decreased to 43% and the proportion of long contacts increased to 41%, a 5- to 10-fold increase as compared with immature DCs (Fig. 8B, right panels).

We performed the same analysis in the absence of Ag on DCs (Fig. 9A, left panel). We found that, in the absence of Ag, immature DCs formed predominantly very short contacts (74%) and few long contacts (3%). For mature DCs, even in the absence of Ag, the proportion of short contacts was of 54% and the proportion of long contacts increased to 20%. If we express these data as the percentage of T cells establishing contacts (as opposed to the percentage of contacts quantified above), we find that 50 and 65% of the T cells establish long contacts with mature DCs in the absence and in the presence of peptide, respectively. For immature DCs, these figures are 10 and 20% in the absence or presence of peptide, respectively.

These results show that naive T lymphocytes establish longer contacts with mature than with immature T cells, even in the absence of added peptide, suggesting that TCR engagement is not required for this effect.

Nevertheless, endogenous peptides expressed on mature DCs may, to some extent, engage the TCR. To ascertain that the prolongation of the contacts observed with mature DCs was independent of Ag recognition, we analyzed the dynamics of the interactions: 1) of syngeneic polyclonal T cells with immature and mature D1 cells and 2) of naive Marilyn T cells with immature and mature MHC class II-deficient BM-DCs. As shown in Fig. 9, B and C (see also web movie 3), mature DCs established longer contacts than immature DCs in both experimental systems.

We concluded that DC maturation determines the duration of initial DC-T cell contacts independently of Ag recognition. When Ag recognition occurs on mature DCs, the duration of the interactions is further prolonged.

**Discussion**

We show here that maturation of DCs dramatically modifies the physical interactions with naive CD4+ T lymphocytes. Immature DCs establish multiple very short contacts of low stability and, in the few stable conjugates formed, TCR clustering was inefficient and mature immune synapses were not observed. The presence of MHC class II molecules loaded with the specific peptide on immature DCs triggered a few cycles of proliferation of naive T cells, but failed to support accumulation of activated T lymphocytes. In contrast, when the same naive T cells interacted with mature DCs, longer contacts were observed, even in the absence of Ag. The presence of specific MHC-peptide complexes on mature DCs induced stabilization of the conjugates, formation of mature immune synapses, and effective T cell proliferation. Therefore, naive T cell priming is regulated at two critical levels: first, Ag-independent contacts between the two cell types probably determine the chances of detecting rare MHC-peptide complexes; second, once the TCRs are engaged, long-lasting interactions will allow effective immune synapse formation and T cell stimulation.

Earlier studies by Steinman’s group (29, 30) showed that DCs, but not other APCs, are capable of Ag-independent adhesion to T cells. We now extend and better define this concept, showing that this property is exclusive to mature DCs. In the absence of peptide, mature DCs establish longer contacts with naive T cells than immature DCs. The presence of Ag and thereby of efficient TCR engagement further increases the duration and the avidity of the interaction.

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1 The on-line version of this article contains supplemental material.
Interestingly, even activated T cells fail to stably adhere to immature, Ag-loaded DCs. This suggests that the increased adhesion of naive T cells to mature DCs is not an exclusive consequence of the increased ability of mature DCs to activate naive T cells. Expression of different adhesion receptors (31, 32), of chemokines (33), and changes in mobility and cytoskeleton (20) probably all concur to efficient adhesion of mature DCs to naive T cells. We previously showed that, in this model, only a 2- to 3-fold difference in the efficiency of naive T cell stimulation was found between wild-type and CD80/86-deficient mature DCs (34). Therefore, our data suggest that the modality of interaction between DCs and T cells play a role that is at least as important as differences in the expression levels of peptide complexes, adhesion, and costimulatory receptors.

We could find neither efficient clustering of signaling molecules nor SMAC formation in T cells that interact with immature DCs. Mature synapses (including SMACs) were shown to form even when the MHC class II-peptide complexes are presented on planar lipid membranes (17), which led to the notion that the APC plays a passive role in SMAC biogenesis. Our results challenge this notion. Peptide-treated immature DCs that bear abundant I-A\(^\text{b}\)/H-Y peptide complexes (Fig. 1B), as well as ICAM-1 (24), do not induce clustering efficiently. This may be due to the lack of some additional property related to the presentation of these complexes to T cells in immature DCs (costimulation, cell surface microdomain organization (35, 36). Weak TCR engagement by immature DCs may not be sufficient to induce formation of organized synapses and to stabilize APC/T cell conjugates (37). It is also interesting to speculate that immature DCs may actively interrupt the interaction with T cells. It has been shown that T cell activation can be achieved by intermittent signaling (38). For naive T cells, however, interruption of the TCR-MHC peptide complex led to only a few cell cycles of proliferation (37), a situation similar to what we observed with immature DCs.

Our results on the dynamics of initial DC-T cell contacts provide new elements to interpret recent in vivo analysis. Let us consider a naive Ag-specific CD4\(^+\) T cell arriving in the T cell zone of a lymph node. This cell will face several hundreds of thousand different cells, including other T cells, stromal cells, macrophages, as well as immature and mature DCs. This means several thousand DCs, among which, probably <1%, express the specific MHC class II-peptide complex. Furthermore, the proportion of MHC class II molecules loaded with any specific peptide probably never represents >1% of the total MHC class II molecules under physiological conditions. T cells therefore need to scan the surface of DCs in search of their specific ligand. This scanning precedes Ag recognition and is probably time-consuming. At some point, the T cells need to decide that it is not worth continuing the search. This “decision time” should be long enough to detect a minimal number of MHC-peptide complexes, but short enough to allow T cells to scan enough DCs to find those expressing their specific peptide.

The observation that DC maturation causes a prolongation in the duration of contacts suggests that in vivo T cells will spend more time scanning a mature DC than an immature DC. Thus, mature DCs would have a “better chance” to present their peptides than immature cells. This would make sense biologically as those DCs that have encountered a maturation signal are most likely the ones that have internalized a pathogen.

The duration of DC-T cell contacts has been analyzed in other experimental systems. Günzer et al. (6) showed that in the collagen matrix the median time of T cell-DC interactions is between 7 and 12 min, independently of Ag recognition (6). In vivo studies, however, showed that Ag recognition extend the duration of the contacts (7, 39). Recently, it has been shown that prolonged engagement with mature DCs loaded with Ag is required to induce IL-2 gene transcription (40). Interestingly, the work of Miller et al. (9) reports a certain percentage of transient interactions in a whole explanted lymph node. This may reflect the presence of both immature and mature DCs that, according to what we see in vitro, could establish short and long-lasting contacts, respectively.

Several recent studies show that Ags targeted to immature, steady-state DCs in lymph nodes induce peripheral tolerance (3) through Ag-specific T cell deletion (4, 5). Detailed analysis of Ag processing in vitro, however, showed that bone marrow-derived immature DCs cannot process and present Ags efficiently (41, 42). It has also been shown that DCs purified from lymphoid organs can present MHC class II-peptide complexes over a range of maturation states (43). Therefore, the nature and maturation profile of DCs that induce T cell tolerance in vivo remains controversial.

Our results show that even when the number of specific MHC-peptide complexes expressed on immature and mature DCs are similar, the modalities and functional outcome of the interactions with the
same population of naive T cells are different. Mature DCs form high-avidity stable conjugates, mature immune synapses, and effective T cell activation, whereas immature DCs establish multiple short, low-affinity contacts, no effective TCR clustering, very inefficient calcium signaling, and abortive proliferation. It is tempting to speculate that suboptimal stimulation of naive T cells by immature DCs that present less Ag and that interact only in a intermittent fashion may be responsible for peripheral tolerance induction through clonal deletion.

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