Synaptic Clusters of MHC Class II Molecules Induced on DCs by Adhesion Molecule–mediated Initial T-Cell Scanning

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Submitted January 4, 2005; Revised March 29, 2005; Accepted April 22, 2005
Monitoring Editor: Mark Ginsberg

Initial adhesive contacts between T lymphocytes and dendritic cells (DCs) facilitate recognition of peptide–MHC complexes by the TCR. In this report, we studied the dynamic behavior of adhesion and Ag receptors on DCs during initial contacts with T-cells. Adhesion molecules LFA-1- and ICAM-1,3-GFP as well as MHC class II-GFP molecules were very rapidly concentrated at the DC contact area. Binding of ICAM-3, and ICAM-1 to a lesser extent, to LFA-1 expressed by mature but not immature DC, induced MHC-II clustering into the immune synapse. Also, ICAM-3 binding to DC induced the activation of the Vav1-Rac1 axis, a regulatory pathway involved in actin cytoskeleton reorganization, which was essential for MHC-II clustering on DCs. Our results support a model in which ICAM-mediated MHC-II clustering on DC constitutes a priming mechanism to enhance antigen presentation to T-cells.

INTRODUCTION

Bone marrow–derived dendritic cells (DCs) are the most efficient Ag-presenting cells (APC), capturing and processing Ags and migrating to the paracortical region of lymph nodes, where they interact with naïve T-cells (Banchereau and Steinman, 1998). The interaction of T lymphocytes with APC involves different and sequential cellular events. Initially, the T-cell adheres transiently to the APC, scanning its surface for the presence of specific peptide-MHC complexes, which is independent of Ag recognition. This creates an initial contact interface that evolves into an Ag-dependent interaction and is accompanied by the reorganization of different molecules at T-cell-APC interface (Grakoui et al., 1999; Montoya et al., 2002b). Ag, adhesion, and costimulatory receptors as well as signaling intermediates are organized in supramolecular activation clusters (SMACs), comprising a central SMAC involved in Ag-dependent responses, in which the TCR, CD28, CD3, CD2, protein kinase C-θ, Lck, and Fyn molecules have been located surrounded by a peripheral SMAC containing adhesion-related components, such as the LFA-1 integrin and talin (Monks et al., 1998; Grakoui et al., 1999).

Initial T-cell-DC adhesion-mediated scanning facilitates recognition of Ag-MHC complexes by the TCR. Several pairs of adhesion molecules are involved in the initial adhesive interactions such as CD2/LFA-3, LFA-1/ICAM-1, -3, or ICAM-3/DC-SIGN. LFA-1 ligands include ICAM-1, -2, -3, and JAM-1 (Montoya et al., 2002b; Ostermann et al., 2002). ICAM-1 and ICAM-3 provide costimulatory signals to T lymphocytes (Van Seventer et al., 1990; Campanero et al., 1993; Juan et al., 1994), and both have been localized at the T-cell-APC interface (Grakoui et al., 1999; Montoya et al., 2002a). Although LFA-1 binds to ICAM-3 with low affinity compared with ICAM-1 (Bleijs et al., 2000), blocking antibodies directed against ICAM-3 have been shown to inhibit T-cell proliferation in DC-stimulated mixed leukocyte reactions (Starling et al., 1995). These facts together with the high expression of ICAM-3 on naïve T lymphocytes support its role in initial transient contacts between T lymphocytes and APC (Montoya et al., 2002a).

Although DCs are the most important APC, relatively little is known on the nature of the immune synapse (IS) formed between T-cells and DCs. An active role of DC actin cytoskeleton during IS formation has been reported (Alwan et al., 2003). The relative density of a given MHC-epitope complex on DC surface is quite low. Thus, cooperative mechanisms to enhance Ag presentation based on changes of surface density of MHC-II molecules may exist (Banchereau and Steinman, 1998).

We describe herein the organization and dynamics of adhesion and MHC class II molecules at the initial steps
of DC interaction with T lymphocytes, as well as the role of ICAMs on the induction of MHC-II clustering at the DC- T lymphocyte contact area. Such clustering is induced by triggering of the LFA-1 integrin on mature DC and requires signaling involved in actin cytoskeleton reorganization.

MATERIALS AND METHODS

Cells
Human monocyte-derived DCs were obtained as described (Sallusto and Lanzavecchia, 1994). At day 6, maturation of DCs was induced by LPS (10 ng/ml, Sigma Chemical Co., St. Louis, MO). CD4+ T-cell clone 5385B (S3) were generated as described (Montoya et al., 2002a). PPL-1, a Jurkat cell line variant deficient for ICAM-3 surface expression was provided by F. Lozano (Hospital Clinic I Provincial de Barcelona; Lozano et al., 1993), and PPL-1 stably transfected with ICAM-3 (PPL-1-ICAM-3) was generated by J. M. Ser- rador (Servicio de Inmunologia, Hospital de La Princesa, Madrid, Spain). Human CD4+ CD45RA+ CD45RO+ naïve Th-cells were isolated from human PBMC using negative selection CD4/CD45RO columns kit (R&D Systems, Minneapolis, MN).

Antibodies and Reagents
We used the following mouse anti-human mAbs: DC51/21 (anti-MHC-II; Mittelbrunn et al., 2002), HP2/19 (anti-ICAM-3), Hu5/3 (anti-ICAM-1), TPI/40 (anti-ol. integrin chain), D3/9 (anti-CD45), anti-CD14, Tbi (anti-CD3), anti-CD81 (E32.22), FN1 (tetraspan associated anti-MHC-II), 44B (anti- TCR, anti-CD79a, anti-CD79b, anti-α-tubulin, monoclonal, anti-β-tubulin, monoclonal, and chain), and HP2/1 (anti-VLA-4). MR1 monoclonal antibody (mAb; anti-DC-SIGN) was from Dr. A. L. Corbi (Centro de Investigaciones Biológicas, Madrid, Spain), and the blocking anti-DC-SIGN (AZN-D1) was from Dr. Y. van Kooyk (University Medical Center, Amsterdam, The Netherlands). An additional blocking anti-DC-SIGN mAb was obtained from R&D (clone 120507). Blocking anti-CD11d (2401) was from Dr. D. E. Stanton (ICOS, Bothell, WA). peptide against phosphorylated (Ytr 174) Vav1 was kindly provided by Dr. X. R. Bustelo (CIC, Salamanca, Spain). pAb against total Vav1 was purchased from Upstate Biotechnology (Lake Placid, NY). Antibody against Rac1 was from Transduction Laboratories (Becton Dickinson, Mountain View, CA). ICAM-1-Fc, consisting of the entire extracellular portion of human ICAM-1 fused to human Fcγ Fc was generated and, ICAM-3-Fc was kindly provided by Dr. M. Robinson (Celltech, Cambridge, UK). Recombinant human fibronectin, poly(I):lysine (PLL), and SEB were from Sigma. Human recombinant IL-2 (Hoffmann-La Roche Nutley, NJ) was from the NIH AIDS Research and Reference Reagent program. CM-TMR (chloromethylbezo- yaminotetrametil-Rhodamine) and CMAC (chloromethyl derivative of amin- ocoumarin) fluorescent cell tracker colors were from Molecular Probes (Eugene, OR). The antagonist of LFA-1,R(5)-4 (bromobenzyl)-3 (3,5-dichlorophenyl)-1,5-dimethylimidazolidine-2,4-dione (BIRT 377) was kindly provided by T. A. Kelly, Boehringer Ingelheim Pharmaceuticals, Ridgefield, CT). CS3 (control peptide) was kindly provided by Dr. A. Garcia-Pardo (Centro de Investigaciones Biologicas, Madrid, Spain). D(+)mannose, Latrunculin A (LaA), taxol, and glycoforphin were from Sigma.

Conjugate Formation and Immunofluorescence Assays
RESULTS

Adhesion Molecules in DC-T-cell Synapses

ICAM-3 is essential in the initial scanning of the APC surface by T-cells, clustering at the contact area during the first stages of the T-APC contact (Montoya et al., 2002a). Such clustering at the T-cell side of the IS prompted us to investigate the distribution of their two main counterreceptors, DC-SIGN and LFA-1. First, we studied the localization of LFA-1, ICAM-3, ICAM-1, and DC-SIGN in conjugates formed between SEB specific CD4⁺ T lymphocytes (S3 T-cells) and SEB-loaded DC (Figure 1A). To recognize mature IS, only those conjugates in which T-cells showed the specific clustering of CD3 or CD3-associated ζ chain were analyzed. ICAM-3, ICAM-1, and LFA-1 were detected at the contact area, forming a clear peripheral ring (Figure 1, A and B). Although DC-SIGN has been described as an important counterreceptor for ICAM-3 (Geijtenbeek et al., 2000), it did not concentrate at the interface of DC-T-cell contacts (Figure 1A). CD11d is another integrin described as counterreceptor for ICAM-3 (Van der Vieren et al., 1995), this molecule is weakly expressed on human monocyte derived-DC and failed to redistribute at the IS (unpublished data).

The dynamic behavior of LFA-1, in addition to that of ICAMs, was studied in primary DC transiently transfected with GFP constructs by time-lapse fluorescence confocal microscopy during DC interactions with SEB-specific T lymphocytes (Figure 1C). LFA-1 redistributed to the contact area; the first clusters were found 4 min after the cellular interaction (Figure 1C and Supplementary Video 1). These clusters were small at the beginning, but evolved into well-organized clusters, which redistributed to the peripheral ring upon conjugate stabilization. Both ICAM-3 and ICAM-1 also redistributed rapidly to the DC contact area with the T lymphocyte (Figure 1C). ICAM-3 was the first molecule that localizes at the DC site of interaction after contact with T lymphocyte. We observed the presence of small transient clusters of ICAM-3 within the first 45 s (Figure 1C). On conjugate stabilization, ICAM-3 was accumulated at the outer zone of the cell-cell contact (zoom Figure 1C). ICAM-1 redistributed later to the contact area (Figure 1C). Like
ICAM-3, we observed that upon conjugate stabilization ICAM-1 was distributed at the outer zone, forming a peripheral ring. As control, no relocation of CD44 was observed during the process of DC-T-cell conjugate formation (Supplementary Video 2).

Early MHC-II Clustering on DC-T-cell Conjugates
MHC-II localization at DC-T-cell conjugates was analyzed. The presence of MHC-II on DC at the contact area was observed in conjugates with either peripheral blood naïve T-cells with DC in the absence of antigen or S3 T-cells with DC pulsed with SEB (Figure 2A). Confocal microscopy studies showed the colocalization of clusters of MHC-II molecules on DC with CD3 at T-cell side (Figure 2B). Dynamic videomicroscopy assays revealed the unexpected finding of a rapid clustering of MHC class II molecules at the contact area, forming discrete transient clusters 1.5 min after conjugate formation, which were more evident 12–30 min later and suggest a possible role for this molecule during the first stages of synapse formation (Figure 2C and Supplementary Video 3).

ICAMs Trigger MHC-II Clustering in DC
To investigate whether LFA-1 engagement influences MHC class II redistribution on membrane DC, we studied the reorganization of LFA-1 and MHC-II in DCs upon conjugation with latex microspheres coated with human recombinant ICAM-1-Fc, ICAM-3-Fc, or both. LFA-1 clustering was markedly enhanced at the area of cell contact with ICAM-1-latex microspheres, whereas ICAM-3 induced a lower clustering. Interestingly, ICAM-3-microspheres binding, induced formation of MHC-II clusters at the contact area with DC in a large percent of conjugates (Figure 3A). In contrast with LFA-1 clustering, ICAM-3 tended to be a more efficient inductor of MHC-II clustering than ICAM-1. We also found that microspheres coated with both ICAM-1 and ICAM-3 exerted an additive effect on MHC-II relocation (Figure 3A). The fluorescence intensity of MHC-II in the contact area
with ICAMs was twofold higher than the rest of the DC membrane fluorescence (Table 1). This phenomenon was specific of MHC-II since the leukocyte-specific molecule CD45 was not clustered under the same experimental conditions (Figure 3A and B, and Table 1).

Interestingly, clustering of MHC class II molecules at the contact area seems to be intrinsic to mature DC, whereas immature DCs and other APCs analyzed such as Raji and LG-2 B cells were unable to redistribute MHC-II in the presence of ICAM-coated latex microspheres (Figure 3C, and unpublished data).

DC-SIGN and CD11d are other counterreceptors for ICAM-3 besides LFA-1 (Geijtenbeek et al., 2000). To assess the role of these molecules in MHC-II clustering, DCs were incubated with blocking reagents against LFA-1, DC-SIGN, and CD11d before the addition of ICAMs latex microspheres. BIRT 377 (Kelly et al., 1999; Woska et al., 2003), and the blocking anti-α chain mAb (TS1/11), were used to impair function of LFA-1; D(+) mannose, and two mAb (AZN-DI and clone 120507) were used to block DC-SIGN and blocking anti-CD11d (240I) to inhibit CD11d. Anti-VLA-4 mAb was used as control. BIRT 377 prevented clus-

Table 1. Quantification of fluorescence intensity at the contact area between DCs and ICAMs-coated latex microspheres

<table>
<thead>
<tr>
<th>Staining</th>
<th>ICAM-1+ICAM-3</th>
<th>Anti-Fc</th>
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<tr>
<td></td>
<td>Contact area</td>
<td>DC membrane</td>
</tr>
<tr>
<td>MHCI-I</td>
<td>179.5 ± 30.6</td>
<td>86.5 ± 43.8</td>
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<tr>
<td>CD45</td>
<td>160.4 ± 40.02</td>
<td>158.8 ± 34.2</td>
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Mean arithmetic ± SD are shown, n = 10, *p < 0.05.
* Data correspond to mean fluorescence intensity.
tering of LFA-1 induced by ICAM-1 or ICAM-1/H11001ICAM-3 (unpublished data). Remarkably, clustering of MHC-II induced by ICAM-3 or ICAM-1/H11001ICAM-3 was also significantly reduced by LFA-1 blockade (BIRT 377 and mAb TS1/11), whereas no effect was observed with inhibitors of DC-SIGN, CD11d, or VLA-4 (Figure 4 and unpublished data). These results point to LFA-1 as the major DC counterreceptor for ICAM-3 and ICAM-1 involved in MHC-II clustering.

The local density of MHC-II molecules loaded with Ag peptides, along with the presence of costimulatory molecules, determine the avidity of APC-T-cell interactions (Reay et al., 2000). It has been described that a subset of di- or oligomerized MHC-II molecules localizes to tetraspanin-based microdomains (Kropshofer et al., 2001). We have found that ICAM-3 is also able to induce the clustering of CD81 (unpublished data), a member of tetraspanin family. Moreover, we have detected MHC class II clustering using the FN1 mAb (unpublished data), which specifically recognizes tetraspanin-associated MHC-II molecules (Drbal et al., 1999).

To further confirm the role of ICAM-3 in MHC-II clustering, DC-T-cell conjugates were formed either with ICAM-3–deficient PPL1 T-cells or with PPL-1 cells stably transfected with ICAM-3 (PPL1-ICAM-3). Both PPL1 and PPL1-ICAM-3 displayed comparable levels of ICAM-1 (Figure 5A). MHC-II clustering was detected in a large percent of DCs in conjugates with PPL1-ICAM-3 compared with a lower proportion of PPL1 cells (Figure 5, B and C; 58.13% ± 9.9 vs. 24.8% ± 6.7). In addition, DCs in conjugates with PPL1-ICAM-3 cells also showed a clear relocation of the costimulatory molecule CD86 to the contact area (Figure 5, B and C), which postulates a role for ICAMs not only in MHC-II clustering but in relocation of costimulatory molecules for efficient Ag pre-

Figure 4. MHC-II relocation induced by ICAMs is LFA-1 dependent. Relocation of MHC-II molecules at the contact area between DC and ICAM-coated microspheres was analyzed in the presence of two different anti-DC-SIGN–blocking antibodies (AZN-DI and clone 120507, 10 μg/ml), D (+) mannosae (100 mM), BIRT 377 (20 μM), anti-αL TS1/11 (10 μg/ml), anti-CD11d 2401 (40 μg/ml), CS3 (control peptide), or anti-VLA-4 (10 μg/ml) as isotype control. Bars represent the fold induction of MHC-II relocation induced by ICAMs relative to that induced by glycoporphin-coated latex microspheres of three independent experiments. *p < 0.05.

Figure 5. ICAM-3 induces clustering of MHC-II and CD86 to DC-T-cell synapses. (A) Flow cytometry analysis of ICAM-1 and ICAM-3 expression on PPL-1 cells. (B) MHC-II and CD86 on DC conjugates with PPL1 or PPL1-ICAM-3. (C) Mature DCs were mixed with PPL-1 (Jurkat T-cells ICAM-3 negative) or PPL-1 stably transfected with ICAM-3. Conjugates were fixed and stained with anti-MHC-II or anti-CD86. Data are mean ± SEM of three independent experiments. Bars represent the mean relocation of MHC-II or CD86 relative to number of DC-PPL-1 conjugates. Arrowheads show molecular clustering at DC-PPL-1 contact.
sentation. Thus, ICAM-3 binding to LFA-1 on DC rearranges the Ag presentation and costimulatory molecular machinery at the DC interface with the T-cell.

**Actin Cytoskeleton on MHC-II Clustering Induced by ICAMS**

The actin cytoskeleton is critical for the assembly of signaling components and the formation of the IS (Dustin and Cooper, 2000). We found that ICAM-3 and ICAM-1 were able to induce actin reorganization at the contact area of DC with ICAM-3-ICAM-3-coated latex microspheres. Our results show not only that ICAMs binding triggers actin reorganization on the interacting area of DC (Figure 6, A and B), but also that the disruption of actin cytoskeleton impairs MHC-II clustering. In this regard, clustering of MHC-II induced by ICAMs was blocked by Latrunculin A, an inhibitor of actin polymerization, but no significant effect was exerted by tubulin disrupting drugs such as taxol (Figure 6C). These data support the active role of the DC actin cytoskeleton during IS formation. In contrast, DC microtubular network and MTOC positioning remain unaffected by ICAM-mediated initial cellular interactions (Figure 6A).

To assess whether the actin cytoskeleton-associated signaling machinery is triggered by ICAMs engagement on DC, we analyzed the activation of Vav1 and GTPase Rac, two key molecules in actin cytoskeleton rearrangement. Phosphorylation of Vav1 in Tyr 174, which is essential for Vav1 activation, was studied in DC stimulated with latex microspheres coated with ICAM-1, ICAM-3, or both. ICAM-3, and ICAM-1 to a lower extent, induced Vav1 phosphorylation at this residue with a kinetics profile that peaked at 10 min (Figure 6D). Consistent with Vav1 activation, Rac1 activation was also observed in DC treated with ICAMS (Figure 6E). The kinetics of Rac1 activation correlated with that of Vav1 phosphorylation, suggesting that the activation of Vav1 and Rac1 are causally related (Figure 6E).

**DISCUSSION**

Until now, most efforts to explore the regulation of the IS have been aimed to T-cells. Furthermore, most studies performed with B-cells as APCs have assigned a passive role to the APC in IS formation. Although DCs are the most important APCs in vivo, relatively little is known on the nature of their IS with T lymphocytes. Here, we describe the localization and dynamics of cell surface molecules involved in IS formation using human monocyte-derived DCs and a CD4+ T-cell clone specific for SEB. Dynamic videomicroscopy assays revealed the unexpected finding of a rapid clustering of MHC class II molecules at the contact area and suggest a
possible role for this molecule during the first stages of synapse formation.

During initial contacts, low-affinity adhesive interactions facilitate the exploration of the DCs surface by T-cells. ICAM-3 is expressed by all leukocytes, particularly by naive T lymphocytes, and is essential in the initial scanning of the APC surface by T-cells, clustering at the contact area during the first stages of the T-APC contact (Montoya et al., 2002a). Interestingly, ICAM-3 binding to LFA-1 on DCs induces the redistribution of MHC-II, which suggests the existence of an adhesion-dependent component in MHC-II lateral mobility on the membrane of the DC. Thus, our data support a key role for ICAM-3 and, to a lower extent ICAM-1, in the initial contact with DCs, mediating cell adhesion and facilitating the priming of T-cells by inducing an enhancement of the density of MHC-II and the costimulatory molecule CD86 on DC-T-cell contact area prior to TCR Ag recognition. These data are further supported by the relocation on DC of MHC class II at the contact area with naive T-cells in the absence of Ag. Previously it has been described that immunological synapses are formed between DCs and polyclonal naive T-cells in the absence of antigen (Revy et al., 2001). The clustering of MHC class II induced by ICAMs is observed in mature but not immature DCs. In this regard, recent findings have described the presence of organized IS in conjugates of mature, but not immature, DCs with naive T lymphocytes (Benvenuti et al., 2004b).

Our data based on the use of both peptide and antibody blocking reagents demonstrate that LFA-1 is the ICAM-3 counterreceptor implicated in the induction of MHC class II clustering on DCs. It has been previously described that some B lymphoma APC supported CD4 clustering in the absence of antigen or during presentation of null peptides, and this CD4 recruitment was dependent on MHC class II, and LFA-1 on the APC (Zal et al., 2002). LFA-1 is relocalized to the contact area within the first 5 min during the process of IS formation, later than MHC-II molecules. This suggests that an organized cluster of LFA-1 is not required for its role in MHC class II clustering. Also, the apparent temporal dissociation between clustering of MHC-II and of LFA-1 would rule out the simplest explanation of the findings, i.e., that a cluster of MHC-II is induced by ICAM-3 through a lateral association with LFA-1.

ICAM-3-mediated MHC-II clustering may be related to the inclusion of the latter into lipid (rafts) or protein (tetraspanin-based) membrane microdomains (Anderson et al., 2000; Kropshofer et al., 2001; Hemsler, 2003). We found that MHC-II molecules clustered by interaction with ICAM-coated microspheres were associated with tetraspanins as demonstrated by CD81 clustering and detection of MHC-II clusters with the mAb FN1. Although it has been reported that MHC-II molecules on B-cells seem to cluster into detergent-resistant membrane microdomains (Anderson et al., 2000), recent evidence indicates that rafts exhibit a random distribution on the T-cell during IS formation (Glebov and Nichols, 2004).

The enrichment of MHC-II at the synapse could be due at least in part to an enhanced polarized transport of intracellular MHC class II to the synapse. In this regard, it has been described that cytochalasin D treatment leads to a delayed appearance of stable forms of class II molecules and a reduced presentation efficiency of Ag determinant in B-cells (Barois et al., 1998). Boes et al. (2002) have observed that interaction of Ag-loaded DCs with Ag-specific CD4 T-cells induces the formation of long tubular class II MHC-positive compartments that polarize toward the interacting T-cell. Under our experimental conditions we did not observe those class II MHC compartments. These apparent discrepancies could be attributed to specific characteristics of T-cells and APCs used in the different experimental systems. Thus, in our work, DCs were loaded with SEB, which does not require endosomal processing, and it has been described that endosomal tubulation requires acquisition of peptide in endosomal compartments (Bertho et al., 2003). Our results show not only that ICAM-3 binding triggers actin rearrangement on the interacting area of DC, but also that the disruption of actin cytoskeleton impairs MHC-II clustering. These data that support the active role of the DC cytoskeleton during IS formation complement those previously described by other group (Al-Alwan et al., 2001, 2003). Our data clearly demonstrate that cytoskeletal reorganization is required for ICAM-driven MHC-II clustering.

ICAMs binding to DC triggers activation of Vav1 and GTPase Rac1, in accordance with the promotion of actin rearrangement. Vav1 regulates integrin clustering on the T-cell during Ag presentation (Krawczyk et al., 2002), being involved in TCR-p2 integrin cross-talk during this process (Ardouin et al., 2003). Despite being expressed on DC and B-cells, the role of Vav in supramolecular activation on the APC side of the IS has not yet been addressed. Recently, it has been described the role of both Rac1 and Rac2 in the initial phases of DC-T-cell interactions and in T-cell priming. Rac1/2-/-/- DCs have an impaired ability to interact with naive T-cells, as well as in priming T-cells (Benvenuti et al., 2004a). Our data provide a mechanism to Rac activation in DCs by naive T-cells at early phases of synapse formation, linking cytoskeletal rearrangements with clustering of plasma receptors such as LFA-1 and MHC-II molecules in the DC area in close contact with T-cells.

In summary, our results demonstrate that the initial binding mediated by ICAM-3 at the T-cell side, to LFA-1 on the DC side triggers the clustering of MHC class II and other costimulatory molecules toward DC synapse area, before Ag recognition by TCR. This provides a mechanism for a more efficacious presentation of peptide-MHC complexes by DCs.

ACKNOWLEDGMENTS

We are grateful to Dr. R. Gonzalez Amaro and Dr. A. L. Corbi for the critical reading of this manuscript. This work was supported by grants BMC02-00536 and Ayuda a la Investigación Básica Juan March 2002 to F. Sánchez-Madrid. M.M. is supported by FPU AP2000-0279 from the Ministerio de Educación, Cultura y Deporte.

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