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Dendritic Cells Process and Present Antigens Across A Range of Maturation States

Ravi K. Veeraswamy, Marina Cella, Marco Colonna, and Emil R. Unanue

We isolated dendritic cells (DC) from lymphoid organs of mice bearing a transgene for a membrane-bound form of the model protein hen egg white lysozyme (HEL). DC from the spleen had a lower representation of costimulatory molecules and class II MHC molecules than those isolated from lymph nodes and thymus. Splenic DC were capable of further maturation by in vivo treatment of mice with LPS. The immature DC from spleen processed HEL and displayed the chemically dominant epitope as evidenced by FACS analysis. These immature DC also presented this epitope to CD4+ T cells. Splenic DC from another transgenic mouse (ML-5) containing serum HEL also showed the ability to process and present Ag despite low levels of circulating HEL. In vitro-derived DC from the bone marrow (bone marrow-derived DC) of mHEL mice also displayed immature to mature features and in both cases displayed HEL peptides as well as SDS-stable MHC class II molecules. Immature bone marrow-derived DC also processed exogenous HEL. We conclude that the DC sets normally found in tissue show a scale of maturation features but even the most immature process and present peptides by MHC class II molecules.


The interaction between APCs and CD4+ T cells is central to the adaptive immune response. This interaction is mediated via the presence of peptide-MHC class II complexes on the APC. Dendritic cells (DC) have been described as APC that sample the environment for pathogens and subsequently prime naive CD4+ T cells (1, 2). They have simultaneously been implicated in providing Ag-specific peripheral tolerance to autoreactive T cells (3–5). Thus, to fully understand how DC interact with, and influence, T cells in vivo, it is imperative to define the conditions in which they are capable of Ag processing.

DC are found with two main features. “Immature” DC have lower levels of surface MHC class II molecules and costimulatory molecules such as CD40, CD80, and CD86 while “mature” DC have higher levels of each (6, 7). Evidence has been presented, based on in vitro experiments, that immature murine DC are deficient at processing protein Ags and delivering peptide-class II complexes to the cell surface (8–11). Upon encountering inflammatory stimuli or pathogens, DC undergo maturation and generate high levels of peptide-MHC class II complexes, transport these to the cell surface, up-regulate costimulatory molecules, and become excellent APC (12–14). Recent data have challenged the view that immature DC do not transport newly synthesized MHC class II molecules to the cell surface and either retain them intracellularly or degrade them. Transport of MHC class II molecules to the surface was found to be independent of the DC maturation state (15). These data along with the findings that immature DC might tolerize T cells in an Ag-specific manner (4, 16) suggest that immature DC process Ag or present peptide-MHC class II complexes.

In this study, we characterize murine DC from various lymphoid organs and correlate their maturation state in vivo with their ability to process and present Ags to CD4+ T cells. DC isolated from spleen ex vivo have lower levels of costimulatory molecules and surface MHC class II complexes as compared with DC isolated from thymus, and splenic DC are capable of further maturation upon in vivo stimulation with bacterial products (17, 18). However, these relatively immature splenic DC are able to process Ag in vivo and present to CD4+ T cells like their mature counterparts. We also show that immature DC generated from murine bone marrow-derived DC (BMDC) transport nascent MHC class II complexes to the cell surface, assemble SDS-stable dimers, expand CD4+ T cells, and process exogenous protein Ag.

Materials and Methods

Mice

The transgenic mice expressing a membrane-bound form of hen egg lysozyme (mHEL) were generated using a cDNA gene for a chimeric HEL-L5 fusion protein under the I-Eα promoter (19). The mHEL mice express HEL tethered to the membranes of class II-positive cells in both thymus and peripheral lymphoid organs. The ML-5 HEL-transgenic mice, expressing HEL under the metallothionein promoter, were obtained from Dr. C. Goodnow (John Curtin School of Medical Research, Canberra, Australia) and backcrossed to B10.BR (H2b) mice (20). ML-5 mice were screened using a serum ELISA for HEL (21). The 3A9 mice used in this study were obtained from Dr. M. Davis (Stanford University School of Medicine, Stanford, CA) (22) and backcrossed to B10.BR (19). B10.BR mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and bred at the Washington University small animal facility (St. Louis, MO). All mice were maintained at the Washington University small animal facility.

In vitro generation of DC from BMDC

Bone marrow was flushed from the femurs of 4- to 6-wk old mice and disrupted via pipetting. Briefly, 1 × 106 bone marrow cells/well of a six-well plate were incubated in 10% FCS (HyClone Laboratories, Logan, UT)-DMEM medium supplemented with glutamine, sodium pyruvate, kanamycin, and nonessential amino acids (all from Life Technologies, Grand Island, NY) containing 1000 U/ml rGM-CSF (Schering-Plough, Kenilworth, NJ). All of the reagents used during the generation of BMDC were tested for LPS content (<10 endotoxin units/ml endotoxin). Medium with rGM-CSF was gently replaced on day 2. Cells were used on day 4.
LPS (Salmonella Abortus Equi; Sigma-Aldrich, St. Louis, MO) was added to the culture to a final concentration of 1 μg/ml to induce DC maturation. The time of exposure to LPS was 18 h except in SDS-stability experiments when the time of exposure was either 5 or 18 h as indicated in the figure legends. CD11c<sup>+</sup> cells were enriched by magnetic adsorption cell sorting (MACS; Miltenyi Biotec, Auburn, CA) per the manufacturer’s instructions using CD11c<sup>+</sup> microbeads.

Isolation of in vivo DC subsets

Single-cell suspensions from three to five spleens or thymi were prepared by mechanical disruption and treatment with collagenase D (Boehringer Mannheim, Indianapolis, IN) for 1 h at 37°C to a final concentration of 100 μg/ml (2 × 10<sup>7</sup> cells/ml). CD11c<sup>+</sup> cells were enriched by magnetic adsorption cell sorting (MACS; Miltenyi Biotec) per the manufacturer’s instructions using CD11c<sup>+</sup> microbeads. Two-color staining was then performed using anti-CD11c-allophycocyanin and anti-CD11b-FITC conjugated mAbs (BD PharMingen, San Diego, CA). Then 3 × 10<sup>5</sup>–1 × 10<sup>6</sup> cells of each subset were sorted from 10<sup>7</sup> cells on a MoFlo cytometer (Cytomation, Fort Collins, CO). To induce in vivo DC maturation, mice were injected i.p. with 100 μg LPS 18 h before use.

Abs/FACS staining

Anti-CD40, -CD80, -CD86, -I-A<sup>k</sup> Abs, streptavidin-PE, and streptavidin-allophycocyanin were purchased commercially (BD PharMingen). 1G12 mAb (IgG1 isotype) specific for the 3A9 TCR, was previously described (19). AW3.1 mAb, detecting HEL 48–62/I-A<sup>k</sup> complexes, was used.
described previously (23). Briefly, $1 \times 10^5$ cells were stained for 30 min on ice in buffer containing 0.02% azide and analyzed using FACSCalibur and CellQuest software (BD Biosciences, Mountain View, CA).

Ag presentation assays

Single-cell suspensions of two to three spleens from 3A9 mice were prepared and their CD4$^+$ T cells were purified using magnetic Dynabeads as per the manufacturer’s instructions (Dynal Biotech, Great Neck, NY). Cells were analyzed with Abs for CD4$^+$ and 1G12 clonotype and found typically to be 60% positive for the clonotype. Briefly, $8-12 \times 10^6$ CD4$^+$7G12$^+$ cells were incubated in 96-well plates with irradiated DC (3000 cGy) isolated ex vivo from HEL-transgenic mice by FACS sorting as described above. Proliferation was measured after 72 h by $[3H]$thymidine incorporation (Amersham, Arlington Heights, IL). BMDC were harvested, double stained with CD11c-allophycocyanin and CD40-FITC, and sorted to obtain pure populations of CD11c$^+$/CD40$^{low}$ and CD11c$^+$/CD40$^{high}$ cells. Cells were then gently fixed with 0.05% glutaraldehyde (Fluka, Buchs, Switzerland). The 3A9 hybridoma was previously described (24). One $\times 10^5$ hybridoma cells were added to titrated numbers of sorted, fixed DC. IL-2 generation by the hybridoma was measured by the ability to support the proliferation of the IL-2-dependent cell line CTLL.

SDS-stability experiments

This procedure has been described in detail previously (25). Briefly, $5 \times 10^6$ BMDC were suspended in LPS-free, methionine, and cysteine-free medium for 1 h. l-[35S]Methionine/cysteine (Trans-label; ICN Pharmaceuticals, Costa Mesa, CA) was added for 30 min. Cold methionine and cysteine were then added to stop the labeling and the incubation was continued for 4 h. Cells were washed, lysed, and MHC class II molecules immunoprecipitated using the anti-MHC class II mAb 40F (26) and protein-A-Sepharose (Pharmacia, Peapack, NJ). Boiled samples were run in a 12% SDS-PAGE gel. Quantitation of radioactive bands was performed using a Phosphor Imager and ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

Results

Splenic DC have a more immature phenotype than thymic DC and display peptide-MHC class II complexes at the cell surface

All CD11c$^+$ cells were enriched from the spleens and thymi of mHEL mice and characterized for their display of surface molecules. Analysis of CD40, CD80, CD86, and HEL 48–62/I-Ak complexes on CD11c$^{high}$ DC population is indicated in the right upper quadrant. The CD11c$^{low}$ DC population represents IPC, as determined by staining for CD11b and Ly6G/C (data not shown) which are not affected by LPS treatment. B, Naive 3A9 T cells proliferation to graded numbers of irradiated CD11c$^{high}$CD11b$^+$ or CD11b$^-$ DC derived from LPS-treated ( ), or untreated (○) littermates.

FIGURE 4. In vivo LPS treatment results in maturation of splenic CD11c$^{high}$ classical DC and enhances their T cell stimulatory capacity. mHEL mice were injected with LPS i.p.. Untreated littermates were used as controls. Eighteen hours later, CD11c$^+$ cells were positively enriched from the spleens and stained and sorted. A, Expression of CD40, CD80, CD86, and HEL 48–62/I-Ak complexes in treated vs untreated mice. The mean fluorescence of the CD11c$^{high}$ DC population is indicated in the right upper quadrant. The CD11c$^{low}$ population represents IPC, as determined by staining for CD11b and Ly6G/C (data not shown) which are not affected by LPS treatment. B, Naive 3A9 T cells proliferation to graded numbers of irradiated CD11c$^{high}$CD11b$^+$ or CD11b$^-$ DC derived from LPS-treated ( ) or untreated (○) littermates.
onto MHC class II. To ensure that the presence of the HEL transgene did not cause a change in the baseline features of DC, we compared levels of costimulatory molecules on DC from both mHEL and nontransgenic B10.BR mice. Equivalent levels of CD40, CD80, CD86, and MHC class II were found but the B10.BR DC did not stain with AW3.1, as expected. Thus, our data indicate that, in vivo, even relatively immature DC, such as those found in the spleen, can process Ag, load peptides onto MHC class II molecules, and display specific complexes at the cell surface.

**Ag Presentation by DC from mHEL and ML-5-transgenic mice**

To confirm the presence of surface peptide-MHC class II complexes on both thymic DC and splenic DC, we tested them for their ability to expand naive 3A9 T cells. CD11chigh CD11b/H11001 and CD11c high CD11b/H11002 DC from both spleen and thymus induced proliferation of naive 3A9 T cells. Thymic DC were slightly more efficient than splenic DC (Fig. 2).

The level of HEL in the mHEL system is high and the Ag is not necessarily taken up exogenously and processed by DC. Thus, to ensure that immature DC derived from spleen were able to process and present even when limiting amounts of Ag were provided exogenously, we analyzed splenic DC from ML-5-transgenic mice that had low doses of circulating HEL in the serum (80 ng/ml). The level of presentation of the chemically dominant 48–62/I-Ak epitope of HEL is 45-fold less in cells from ML-5 spleens than in mHEL spleen cells (28). Indeed, ML-5 CD11c/high classical splenic DC were not stained by the AW3.1 Ab, indicating that their level of the HEL 48–62/I-Ak complex was below the sensitivity of this Ab (Fig. 3a). At high numbers, ML-5 splenic DC stimulated naive 3A9 T cells (Fig. 3b). As a control, another MHC class II-positive APC, B cells, isolated from the same ML-5 spleens did not activate naive 3A9, even at high numbers (Fig. 3b). Thus, immature splenic DC show evidence of Ag presentation in the mHEL mouse or even when the amount of Ag is low and it is present extracellularly in the serum as in the ML-5 mouse.

**Immature splenic DC can undergo further maturation and acquire higher T cell stimulatory capacity when challenged with bacterial stimuli**

We next assessed the maturation state and Ag-presenting function of splenic DC from mice challenged with a bacterial product in vivo. mHEL mice were injected with LPS i.p. and their spleens were analyzed 18 h later. Levels of CD40, CD80, CD86, and AW3.1 were all increased on CD11chigh DC upon in vivo maturation (Fig. 4a). A significant proportion of CD11clow cells did not undergo activation upon in vivo LPS administration. These cells correspond to CD11clow IPC, which do not respond to LPS stimulation since they lack TLR4 expression (29). In vivo-matured splenic DC showed an ~3-fold greater Ag presentation capacity to naive 3A9 T cells than immature splenic DC (Fig. 4b).

**Immature BMDC generate SDS-stable complexes and present Ag**

To further study Ag presentation by DC in a quantitative, biochemical manner, we generated immature DC from bone marrow cells of mHEL mice by culturing with GM-CSF. After 4 days of culture, 20–30% of the cells expressed the DC marker CD11c. CD11c cells coexpressed CD11b and displayed low levels of CD40, CD80, CD86, HEL 48–62/I-Ak, and low numbers of HEL 48–62/I-Ak-specific complexes. Upon treatment with LPS, the surface expression of all of these molecules dramatically increased, as expected for mature DC (Fig. 5a) and as shown by others (9). Thus, in vitro-derived immature BMDC are a good approximation of immature splenic DC and they can both be further matured with LPS.
We measured the percentage of SDS-stable complexes generated by immature and mature DC on purified CD11c\(^+\) cells from mHEL bone marrow cultures. The formation of strong or weak peptide-MHC class II complexes for I-A\(^b\) molecules is directly proportional to their stability in SDS-PAGE under nonboiling conditions (25, 30). Experiments were performed varying the length of LPS exposure from 5 to 18 h before radiolabeling. The ratio between SDS-stable dimers to total MHC class II remained the same regardless of DC maturity, ranging from \(\sim 20\%\) for the 18-h LPS treatment to \(\sim 30\%\) for the 5-h LPS treatment (Fig. 5c). Importantly, LPS stimulation increased the number of total SDS-stable dimers by 15%. It should be emphasized that SDS-stable complexes include autologous peptides as well as one of the HEL peptides processed in APC (25).

To test for the presence of specific peptide-MHC class II complexes at the cell surface of immature DC in a functional assay, unstimulated and LPS-matured mHEL BMDC were sorted on the basis CD11c and CD40 expression to obtain pure populations of immature or mature DC (Fig. 6a). DC were then fixed and 3A9 T hybridoma cells were added. (3A9 hybridomas are much less sensitive to costimulators and their response mostly reflects the level of peptide-MHC complexes.) Both immature and mature DC had the ability to activate the T hybridoma, implying that even the most immature BMDC expressed HEL 48–62/I-A\(^a\) complexes on their surface. LPS maturation increased the DC Ag presentation capacity of \(\sim 6\)- to 9-fold as compared with immature DC.

To extend these findings, we tested whether immature BMDC generated from B10.BR mice captured and processed soluble HEL in the absence of inflammatory or pathogen-derived stimuli. B10.BR-derived BMDC, pulsed with 1 mg/ml LPS-free HEL for 5 h, and chased for additional 10 h, were analyzed for expression of costimulatory molecules to assure that the immature phenotype was maintained and for expression of HEL 48–62/I-A\(^a\) complexes. Counterstaining with CD40 indicated that no significant maturation occurred during this time frame.

**FIGURE 7.** Immature BMDC process exogenous HEL and deliver HEL 48–62/I-A\(^a\) complexes to the cell surface. Immature BMDC from B10.BR mice were cultured with 1 mg/ml LPS-free HEL for 5 h, washed, and then chased for 10 additional hours. Unpulsed or HEL-pulsed DC were analyzed for cell surface expression of HEL 48–62/I-A\(^a\) complexes. Counterstaining with CD40 indicated that no significant maturation occurred during this time frame.

**Discussion**

The concept of DC maturation is now well established and it is clear that mature DC have high levels of surface peptide-MHC complexes and are extremely potent activators of naive T cells. Our results demonstrate that immature DC, whether cultured in vitro or derived ex vivo, process protein Ag, generate peptide-MHC class II complexes, and transport these complexes to the cell surface. We therefore propose that in terms of Ag processing, the difference between the two states of DC is more quantitative than qualitative. The process of DC maturation may up-regulate the Ag-processing machinery (11), decrease the rate of cell surface recycling (15), or act through some combination of mechanisms to produce the final phenotype of a mature DC with high cell surface levels of antigenic complexes. The significance of our study is to show that immature DC are also able to generate peptide-MHC class II complexes in the absence of inflammatory stimuli.

The mHEL mouse allowed us to study a known Ag and the chemically dominant epitope, aa 48–62, from that Ag in both ex vivo and in vitro-cultured DC. By exploiting the inherent differences in maturity between DC in different organs (17, 31), we could measure the Ag-processing capacity of DC from mHEL mice without any manipulations of the in vivo system. We also had the benefit of using sensitive reagents such as the AW3.1 Ab, the 3A9 hybridoma, and, ex vivo, naive 3A9 T cells. These methods allowed us to probe for low numbers of peptide-MHC class II complexes on the surface of immature DC.

It is crucial to understand whether the immunological outcome of the DC-T cell interaction is predicated on the maturity status of
the DC. It is established that fully mature DC activate naive CD4+ T cells. At present, it is unclear whether immature DC cause T cell "anergy" or whether this occurs in an Ag-specific manner. Some recent reports have proposed that immature DC are involved in tolerizing peripheral, autoreactive CD4+ cells (4, 5, 32). Obviously, DC must be capable of presenting Ags to function in this capacity if the T cells are tolerized in an Ag-specific manner.

Conversely, the concept of a biochemical margin of safety is one that has been advanced by our laboratory (33, 34). It is possible that immature DC in specialized situations are able to present enough self-peptide-MHC complexes such that even without the benefit of costimulation, they trigger autoreactive T cells while still remaining immature. One example could be anatomic areas with localized high concentrations of self-Ags such as the peripancreatic lymph nodes in autoimmune diabetes. The onset of autoimmunity could in part then be mediated by these immature DC. Thus, the degree to which immature DC present Ags might be of relevance to both tolerance and autoimmunity. Our report establishes that immature DC process and present Ags. Further work is needed to understand the full biologic relevance of this finding.

References