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Increased Expression of Intracellular HLA-DM but Not on the Surface of Blood Monocyte-derived Dendritic Cells During Maturation

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Cutaneous dendritic cells (DCs), Langerhans cells (LCs) and dermal dendritic cells (DDCs), are present in an immature state. The maturation of DCs is crucial for initiating an immune response. Since HLA-DM has an important role for antigen presentation, an increase in HLA-DM expression according to the maturation of blood monocyte-derived dendritic cells (MoDCs), which have similar characteristics with DDCs, is expected. Therefore, the aim of this study was to determine whether or not HLA-DM expression in MoDCs is related to maturation at each culture day (from day 0 to day 13) by flow cytometry. This was compared with the functional changes related to the maturation of MoDCs. MoDCs were generated by culturing human peripheral blood monocytes in the presence of GM-CSF and IL-4 for 7 days, which were followed by subsequent treatment with a cytokine cocktail (GM-CSF, IL-4, IL-1β, TNF-α, IL-6 and PGE2) for the maturation of MoDCs. The intracellular HLA-DM was expressed in the immature MoDC. A sudden 3 to 8 fold increase in the intracellular HLA-DM expression was observed after treatment with a cytokine cocktail. HLA-DM was weakly expressed on the surface of the immature MoDC, but it seemed to be decreased with maturation. This study indicated that the intracellular HLA-DM expression increased, but not on the MoDC surface during maturation. This was despite the fact that HLA-DM expression was noted not only on the surface but also in the intracellular in the MoDC.

Key Words: Antigen presentation, MHC classs II molecules, HLA-DM, monocyte-derived dendritic cell

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INTRODUCTION

Cutaneous antigen presenting cells (APCs) are members of the dendritic cell (DC) family[1] and include CD1a+ Langerhans cells (LCs)[2] and dermal dendritic cells (DDCs), which express factor X111a.[3,4] CD1a+ LCs are found mainly in the epidermis, but smaller numbers are seen within the dermis. DDCs are normally found in the dermis, predominantly around microvasculature, and in a loose network arrangement within the superficial dermis just beneath the epidermal basement membrane.[5] DCs are a system of highly efficient APCs that initiate the primary immune response. They are more potent APCs than peripheral blood monocytes or B cells.[6] DCs are also critically involved in autoimmune disease, graft rejection, and human immunodeficiency virus infection.[7,8] They capture and process antigens in the non-lymphoid tissues, then migrate to the T-cell dependent areas of the secondary lymphoid organs through the afferent lymph or the blood stream to prime naive T-cells and initiate the immune response.[9] During this process, the DCs lose their antigen-capturing/processing ability as they differentiate into mature, fully stimulatory APCs.[10]

In the most non-lymphoid tissues like in the skin, DCs are present in a so-called 'immature' state, and unable to stimulate T cells. Although these immature DCs lack the necessary accessory signals for T-cell activation, such as CD40, CD54 and CD86, they are extremely well equipped to capture antigens. These antigens are able to induce full maturation and mobilization of the

DCs.[11] Immature DCs have several features that allow them to capture antigens. They can take up particles and microbes by phagocytosis, the extracellular fluid and solutes by macropinocytosis. In addition, they express receptors that mediate adsorptive endocytosis, including C-type lectin receptors like the macrophage mannose receptor[12] and DEC-205,[13] as well as the Fcγ and Fcε receptors.[14]

DC maturation is crucial for initiating immunity. It can be influenced by a variety of factors, notably microbial and inflammatory products. Whole bacteria,[15] the microbial cell-wall component LPS,[14] and cytokines like IL-1, GM-CSF and TNF-α, all stimulate DC maturation, whereas IL-10 blocks it.[16] During DC maturation, the molecules that capture and process antigens, like the Fc receptor, and the intracellular MHC class II molecule, are decreased but molecules that initiate the immune response, like the surface MHC class II molecules, and accessory molecules, are increased.[11]

Two methods for generating human DCs from hematopoietic precursor cells in the peripheral blood have been established. One approach utilizes the rare CD34+ precursor and GM-CSF plus TNF-α,[17-19] which has similar characteristics of LCs.[20] The other makes use of the more abundant CD14+ population and GM-CSF plus IL-4,[14,21,22] which has similar characteristics of DDCs.[23] However, the GM-CSF and IL-4 treated MoDCs are not stable mature dendritic cells. To generate fully mature and stable DCs, a monocyte-conditioned medium or a cytokine cocktail is required. This is achieved by adding a 1-2 day maturation culture to the initial 7 day culture in the presence of GM-CSF and IL-4.[24]

HLA-DM is a non-classical MHC class II molecules and acts as a chaperone that facilitates antigen loading on MHC class II molecules.[25] HLA-DM is largely absent from the APC cell surface,[25] accumulating instead in the intracellular compartments, including the MHC class II compartment.[26,27] Since HLA-DM has an important role for antigen presentation, it is expected that an increase of HLA-DM acccording to the maturation of MoDCs.

Therefore, the aim of this study was to determine whether or not HLA-DM expression in MoDCs may be related to the maturation at each culture day (from day 0 to day 13) by flow cytometry. This was compared with the functional changes related to the maturation of MoDCs.

MATERIALS AND METHODS

Media and cytokines

X-vivo (BioWhittaker, Walkersville, Maryland, USA) was supplemented with 2 uM L-glutamine (Gibco Laboratories, Grand Island, NY, USA), 100 IU/ml penicillin (Gibco), 100 ug/ml streptomycin (Gibco), 5×10^{-5} M β-mercaptoethanol (Merk, Munchen, Germany), and 1% heat-inactivated autologous plasma.

The following human recombinant cytokines were used: 800 U/ml of the granulocyte-macrophage colony stimulating factor (GM-CSF) (Novartis, Frimley, UK) and 1000 U/ml IL-4 (PBH, Hannover, Germany), 1000 U/ml IL-6 (PBH), 10 ng/ml IL-1β (PBH), 10 ng/ml TNF-α (PBH), 1 ug/ml prostaglandin E2 (PGE2) (Sigma Chemical Co., St. Louis, MO, USA)

Generation of DCs from peripheral blood mononuclear cells (PBMCs)

Whole blood from normal donors was used for preparing the PBMC isolates using Ficoll-Hypaque. The PBMCs were plated in 6-well plates for 1 hour. The supernatant and floating cells were discarded. The attatched cells were cultured in X-vivo complete medium containing GM-CSF and IL-4 for 7 days. The cells were fed every other day with fresh complete medium ($\pm$ 1 ml) containing GM-CSF and IL-4. At day 7, the non-adherent cells were harvested, washed once, and transferred to fresh 6-well plates in X-vivo complete medium containing GM-CSF and IL-4 then stimulated with a cytokine cocktail (GM-CSF, IL-4, IL-1β, TNF-α, IL-6, and PGE2).[28] Subsequently, they were further cultured in the same media without adding any extra cytokines.

Flow cytometry analysis

For intracellular HLA-DM staining, the DCs

were treated with Perm Sol. (Becton Dickinson, Mountain View, CA, USA) for cell fixation and permeabilization according to the manufacturer's instruction, then incubated with the PE-conjugated monoclonal anti HLA-DM antibodies for 20 min at 20℃. Analysis was performed with a FACSCalibur (Becton Dickinson). To determine the DC surface expression molecule, monoclonal antibodies (mAb), toward the following antigens; HLA-DM (Pharmingen, San Diego, CA, USA), CD1a (Becton Dickinson), CD14 (Becton Dickinson), CD19 (Becton Dickinson), CD86 (Pharmingen), HLA-DR (Becton Dickinson), CD83 (Serotec, Oxford, England) were used. The isotype controls were run in parallel. As a secondary antibody, FITC-conjugated affinity pure F(ab')2 goat anti mouse Ig's (Biosource, Camarillo, CA, USA) was used. The marker expression was evaluated as a percentage of positive cells in the relevant population defined by the forward scatter/side scatter characteristics. The expression levels were evaluated by assessing the mean fluorescence intensity (MFI) indexes calculated by relating the MFI noted with the relevant mAb to that with the control mAb for samples labeled in parallel and acquired using the same setting.

Endocytosis assay

FITC-conjugated Dextran (Sigma) was added to the cultured DCs to a final concentration of 1 mg/ml and incubated for 60 min at 37℃ or 4℃. After incubation, DCs were washed 3 times with ice-cold PBS and analyzed by flow cytometry.

Allogeneic MLR

T cells (1×10^5/well), enriched using nylon wool columns as described,[29] were stimulated with immature or cytokine cocktail induced mature DCs. The cells were cultured in a flat-bottom microtiter plate to a final volume of 200 μl/well in RPMI with 5% FCS for 4 days and an additional 16 hr in the presence of [^{3}H] thymidine (1 μ Ci/well=37 kBq/well, Amersham, braunschweig, Geramny). [^{3}H] thymidine incorporation was measured using a liquid scintillation counter (Beckman, Palo Alto, CA, USA).

RESULTS

Intracellular HLA-DM is highly expressed in the MoDC and suddenly increased after treatment with a cytokine cocktail. The intracellular HLA-DM was expressed in the MoDC at the initial culture day. They increased slowly from the first culture day to day 7. At culture day 7, the MoDCs were treated with a cytokine cocktail for maturation. The intracellular HLA-DM expression rapidly increased 2 to 8 fold and then was remained unchanged for a few days (Fig. 1).

HLA- DM is weakly expressed at the MoDCs surface and decreased after treatment with a cytokine cocktail. The HLA-DM was weakly expressed on the MoDC surface, but was decreased with maturation (Fig. 2).

The endocytic capacity of the MoDCs using FITC-dextran reached a peak at culture day 7, then rapidly decreased after treatment with a cytokine cocktail. The MoDCs endocytic capacity from culture day 0 to day 7 slowly increased, and reached a peak at culture day 7 by more than 10 times compared with the MoDCs at culture day 0. The endocytic capacity was rapidly reduced after treatment with a cytokine cocktail, then reached the baseline by culture day 11 (Fig. 3).

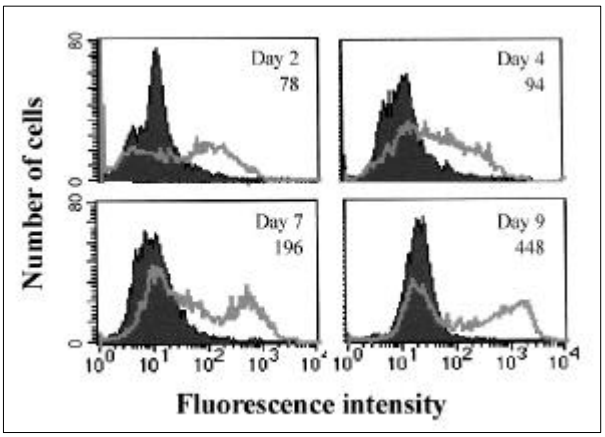

Fig. 1. Change of intracellular HLA-DM expression in MoDCs during maturation. The MFIs of immature MoDCs at day 2, day 4 and at day 7 are 78, 94 and 196, respectively. The MFI of mature MoDCs at day 9 is 448. Mature MoDCs more highly expressed than immature MoDCs and there is a sudden increase in expression after treatment with a cytokine cocktail at culture day 7. The gray line is HLA-DM; the black line is isotype control. The numbers indicate MFI.

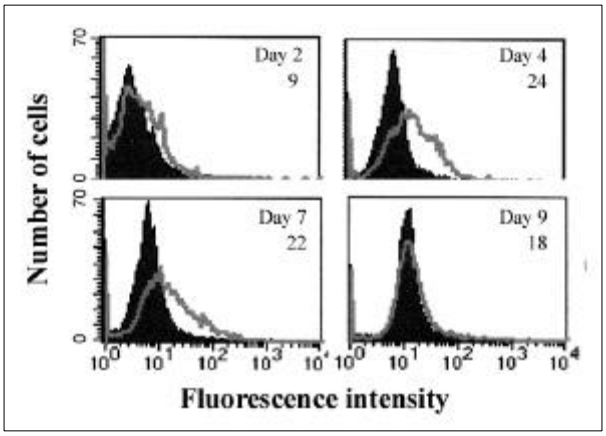

Fig. 2. HLA-DM expression at the MoDC surface. HLA-DM is weakly expressed at the MoDC surface and slightly decreased after treatment with a cytokine cocktail. The gray line is HLA-DM; the black line is isotype control.

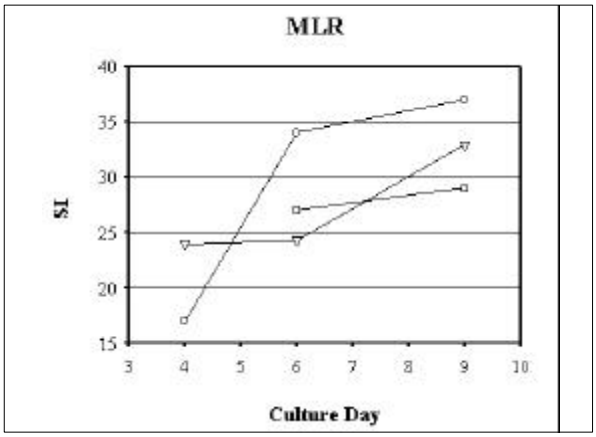

Fig. 4. Change in MoDC stimulatory activities during maturation. The T cell stimulatory activity of MoDC increased with time. However, an abrupt increment in the T cell stimulatory activity of mature MoDC is not observed. SI: stimulation index=uptake of [^{3}H] thymidine of allogenic MLR/uptake of [^{3}H] thymidine of control.

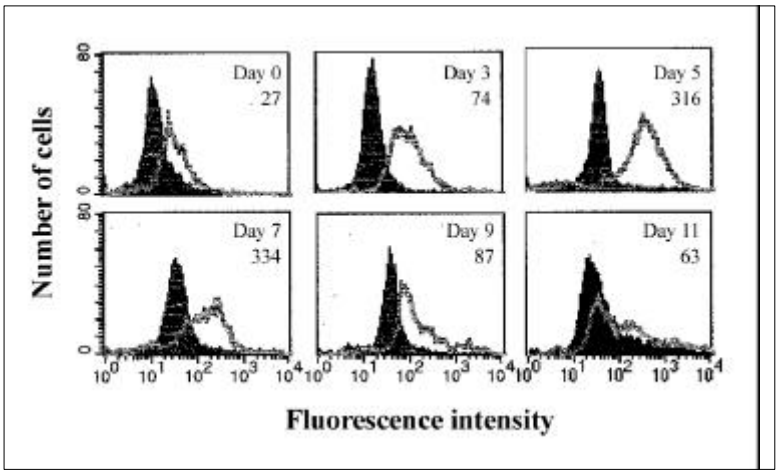

Fig. 3. Change in MoDC endocytic capacity using FITC-dextran during maturation. The endocytic capacity increased with time and reached a peak at culture day 7. Subsequently, it rapidly decreased after treatment with a cytokine cocktail. The gray line is FITC-dextran; the black line is isotype control. The numbers indicate MFI.

Stimulatory activities of mature MoDC (at culture day 9) using mixed lymphocyte reactions were slightly higher compared with immature MoDCs (at culture day 6). The stimulatory activities of MoDC at day 9 were higher compared with MoDC at day 4 and 6 (Fig. 4).

DISCUSSION

LCs and DDCs, cutaneous DCs, are present in an immature state. Immature DCs are efficient in antigen uptake but need to mature and migrate into the lymphoid organ before acquiring the capacity to efficiently prime T cells.[11] Thus, DC maturation is a key event for inducing immunity. Whole bacteria,[15] cell-wall component lipopolysaccharide,[12] GM-CSF, TNF-α,[16] IL-13,[30] ceramide[31] and CD40L,[32] all of them can promote differentiation of immature DC in vitro, resulting in irreversible morphologic changes, upregulation of MHC products and adhesion/costimulatory molecules, down-regulation of antigen uptake, and processing capacity. This can result in enhanced T cell stimulatory capacity. MoDCs cultured with GM-CSF and IL-4 for 7 days are still in an immature state. Thus, the MoDCs need to be maturated in order for them to present antigens to T lymphocytes.

The differences in the function, morphology,

and surface molecules between immature and mature DC are well known. However, the change in HLA-DM expression during the maturation in MoDCs is not known. This study has shown that immature DCs exhibit a relatively high intracellular HLA-DM expression level and the expression of intracellular HLA-DM slowly increased with time before being treated with a cytokine cocktail. After such treatment, a strong maturation signal is observed, and HLA-DM expression increases rapidly 2 to 8 times. Because HLA-DM is an important molecule for effective antigen presenting, the rapid increase in the HLA-DM level suggests that there is a marked increase in the antigen presenting capacity of MoDC. During maturation, increased expression of HLA-DM develops in the cytoplasm of DC compared to the MHC class II expression, which is increased on the surface of DC.

The MoDC antigen presenting capacity was investigated using a mixed lymphocyte reaction. The antigen presenting capacity using a mixed lymphocyte reaction increased with increasing time, and this occurred despite the fact that a rapid increase in the antigen presenting capacity was not observed after treatment with the cytokine cocktail, which is contrary to that observed for HLA-DM. The endocytic capacity increased slowly from day 1 to day 7 during the immature state of MoDC. This result is not consistent with previous reports showing that the MoDC endocytic capacity decreased with time.[11] However, a dramatic decrease in endocytic capacity after treatment with the cytokine cocktail was observed. This result correlated with the change in HLA-DM expression well in when considering the abrupt change developed after treatment with the cytokine cocktail.

These results suggest that maturation is an abrupt change, maybe occurring within a few hours, but it is not slow when an adequate maturational signal, such as cytokine cocktail, is given. The intracellular HLA-DM is largely expressed in the APC. However, in this study, HLA-DM is also expressed at the MoDC surface,[33] although not quite as intense. Although the role of HLA-DM at the MoDC surface is unknown, the presence of HLA-DM at the MoDC surface may be related to the extracellular processing pathway[33] or due to an accidental location during antigen presentation.

In conclusion, HLA-DM is a marker for the maturation of MoDC. MoDC maturation is a rapid process in view of the functional and phenotypic changes because intracellular HLA-DM and MoDC endocytosis are rapidly changed in response to maturational signals such as the cytokine cocktail, although the T cell stimulation capacity is not.

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