Mechanisms of dendritic cell-induced T cell proliferation in the primary MLR assay

Irina L. Tourkova, Zoya R. Yurkovetsky, Michael R. Shurin, Galina V. Shurin *

Department of Pathology, Clinical Immunopathology—CLSI, University of Pittsburgh Medical Center and University of Pittsburgh Cancer Institute, 5725 CHP MT, 200 Lothrop Street, Pittsburgh, PA 15213, USA

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Abstract

DC are unique antigen presenting cells, and their ability to induce proliferation of T cells in a mixed leukocyte reaction (MLR) assay is commonly used for the evaluation of their function. To determine the mechanisms involved in DC-induced T cell activation in a primary MLR assay, a variety of different agents were examined in this study that interfere with DNA synthesis, membrane organization, protein synthesis, and maturation induced by bacterial products. While only live DC were able to induce T cell proliferation in the MLR assay, irradiation of DC did not influence their stimulatory capacity. Fixation of DC membrane with paraformaldehyde resulted in a loss of DC capacity to induce T cell proliferation demonstrating that physical organization of the plasma membranes plays an important role in the induction of T cell activation. In addition, the pretreatment of DC with cycloheximide revealed that protein synthesis was not critical for the ability of DC to activate T cells. Finally, *Staphylococcus aureus*-mediated activation of DC significantly increased T cell proliferation and this effect was not dependent on IL-12 production of DC since DC generated from IL-12 knockout mice were not different from wild type DC. In summary, these data suggest that DC membrane structures are responsible for the antigen presentation and co-stimulation and play a key role in T cell recognition and activation by DC. © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

Dendritic cells (DC) are potent antigen presenting cells (APC), which are highly efficient in antigen presentation and stimulation of T lymphocytes [1,2]. The strong T cell stimulatory activity of DC can be demonstrated in the primary mixed leukocyte reaction (MLR) [3]. In the MLR assay, DC and T lymphocytes form multiple aggregates upon co-incubation in cultures. The aggregates from the primary MLR are represent large numbers of sensitized allospecific T lymphoblast clusters. In a secondary MLR assay, blast-transformed T cells could be stimulated not only by DC but also by allogeneic macrophages, B cells and B lymphoblasts [4–6].

The ability of DC to induce proliferation of T cells in the primary MLR is commonly used for the evaluation of their function [7,8]. However, the mechanisms of the induction of T cell proliferation by DC are still not completely resolved. It has been demonstrated that specific properties of DC are critical for the induction of T cell activation. For instance, it has been reported that live splenic DC are required for induction of the primary MLR [6]. Recently it has been reported, that DC, obtained by culturing of plastic-adherent monocytes gave rise to two types of DC with the opposite properties: stimulatory and inhibitory [9]. The stimulatory DC expressed high levels of costimulatory molecules, produced interleukin-12 (IL-12), and efficiently activated naive allogeneic T cells in the MLR assay. The inhibitory DC, in contrast, expressed low levels of costimulatory molecules, produced large amounts of interleukin 10 (IL-10), and were ineffective in an allogeneic MLR. Neutralization of the endoge-
nously derived IL-10 in DC cultures repolarizes the inhibitory DC toward the stimulatory phenotype. It has also been shown that FasL and B7 expression on DC provide additional counter-regulatory signals for T cell survival and proliferation and increased expression of costimulatory molecules on activated DC might result in increased antigen presenting cell (APC) activity [10–12].

Up-regulation of the accessory activity of DC is mediated by the activation of protein kinase C (PKC) and increased levels of intracellular Ca$^{2+}$, as observed in murine splenic DC, stimulated with calcium ionophore A23187 and PKC activator phorbol myristate acetate in the primary MLR assay [13]. Apri le et al. reported that the cluster formation of canine DC and lymphocytes is a Ca$^{2+}$-dependent process and cannot be inhibited by cyclosporine [14]. It was also shown that the addition of N-acetyl-L-cysteine, an antioxidant molecule endowed with immunomodulatory properties, to primary human MLR cultures resulted in a profound inhibition of alloreactive responses through its inhibitory action on DC [15].

Finally, the physical organization of APC membrane is important for T cell activation. For instance, Barisas and colleagues reported that antigen presentation by MHC class II molecules could be enhanced by paraformaldehyde fixation of APC [16]. In that study, cells from mouse B cell lymphoma-derived cell line A2 were used as APC. In contrast, Inaba and Steinman have demonstrated that glutaraldehyde and formaldehyde treatment decreased the ability of splenic DC to induce proliferation of T cells [6]. Thus, it is not clear which physical properties of DC play a crucial role in T cell activation. Only a few studies focused on different aspects of this problem using bone marrow-derived DC, a commonly used population of ex vivo generated myeloid DC. The stimulatory capacity of DC, generated from mouse bone marrow precursors and treated with paraformaldehyde has not been studied yet. It is still unknown whether the fixation of DC surface structures or protein synthesis inhibition is critical for accessory function of these cells. Furthermore, while it is a general thought that gamma-irradiation of APC does not influence their ability to activate T cell proliferation, the data are controversial. For example, using splenic DC, Tachash et al. have shown that DC required irradiation for optimal T cell stimulatory activity [17]. In contrast, using PBMC derived DC, Anton et al. have demonstrated that irradiated DC are less efficient in antigen presentation and stimulation of T cells than non-irradiated DC [18]. Thus, it is not clear whether irradiation affects the stimulatory capacity of DC generated from mouse bone marrow precursors. Moreover, it has not been evaluated in a murine system whether activation of DC induced by bacterial products affects the activation of naive allogeneic T cells in the MLR assay and if IL-12 is involved in this effect. The aim of this study was to evaluate physical and biochemical properties of DC that might be important for T cell activation in the primary MLR assay.

2. Materials and methods

2.1. Animals

Male C57BL/6 and BALB/c mice, 6–8 weeks old, were obtained from Taconic (Germantown, NY). IL-12-knockout mice of C57BL/6 origin were a gift from Dr Maurice Gately (Hoffman-La Roche, Nutley, NJ) and were bred at the University of Pittsburgh Central Animal Facility. Animals were housed in a pathogen-free facility under controlled temperature, humidity, and a 12-h light:dark cycle with food and water available ad libitum. All animals were acclimatized for at least 2 weeks prior to the experiments. All experimental protocols were reviewed and approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

2.2. Dendritic cell cultures

Murine DC were generated from hematopoietic progenitors isolated from bone marrow. Bone marrow cells were collected from tibias and femurs of C57BL/6 mice, passed through a nylon cell strainer to remove pieces of bones and debris. Bone marrow cells were then depleted of red blood cells with lysing buffer for 2–3 min. The single-cell suspensions were then incubated with anti-mouse B220, CD4 and CD8 antibodies for 1 h at 4 °C followed by incubation with rabbit complement for 30 min at 37 °C to deplete B and T lymphocytes. The cells were cultured (37 °C, 5% CO$_2$) in a complete medium (CM) (RPMI-1640, 2 mM L-glutamine, 50 µg/ml gentamicin sulfate, 10 mM HEPES, 10% FBS, 10 mM non-essential amino-acids, 1 mM sodium pyruvate, 1 µg/ml indomethacin, and 50 µM N-methyl-L-arginine (NMLA) in 6-well plates (10$^6$ cells/ml) overnight. Then the non-adherent cells were collected by gentle pipetting, resuspended in a CM supplement with murine GM-CSF (1000 units/ml) and IL-4 (1000 units/ml) and cultured in 6-well plates (2 $\times$ 10$^6$ cells/ml) at 37 °C, 5% CO$_2$ for 7 days. DC were harvested, counted and used for further analysis.

The DC obtained in this manner contained consistently 80–90% live cells. To separate live and dead cells, cell suspensions were loaded onto the NycoPrep solution (1:1 mixed 1.068 and 1.077 g/ml, Nycomed Pharma AS, Oslo, Norway) and centrifuged for 20 min at 400 × g. The cells from the interface exhibited a viability of >98% (trypan blue-negative or ‘live’ DC), and the cells from the bottom exhibited a viability of <2% (trypan blue-positive or ‘dead’ DC).
2.3. Treatment of cultured DC

Several different approaches were used to modulate cultured DC, including (i) irradiation (3000 rad from a $^{137}$Cs source), (ii) treatment with 2% paraformaldehyde for 10 min at 10 ºC, (iii) pre-incubation with cycloheximide (1 µg/ml) for 30 min at 37 ºC, and (iv) activation with S. aureus (0.1% v/v of essentially non-viable S. aureus cell suspension; Sigma) (20 µl per well/1 x 10^6 DC) for 24 h at 37 ºC.

2.4. Mixed leukocyte reaction (MLR)

Functional activity of DC was determined in the primary allogeneic MLR assay, using mouse T lymphocytes as responder cells. Allogeneic T cells were obtained from spleen cell suspensions by passage through the nylon wool columns after the lysing of red blood cells. The MLR assays were carried out in round-bottomed 96-well plates to ensure efficient DC/T cell contact. DC were added in triplicates in graded doses (10^2–10^6 cells per well) to T cells (1 x 10^5 cells per well) in a total volume of 200 µl of CM (without indomethacin and NMLA) per well. Proliferation of T cells was measured by uptake of ³H-thymidine (1 µCi/well, 5 Ci/mmol; DuPont-NEN, Boston, MA) pulsed for 16–18 h after 3 days in culture. The cultures were harvested on GF/C glass fiber filter paper (Whatman Intl. Ltd., Maidstone, UK) using a MACH III microwell harvester (Tomtec, Hamden, CT). Incorporation of ³H-thymidine was determined on a MicroBeta TRILUX liquid scintillation counter (WALLAC, Gaithersburg, MD). The counts were expressed as a count per minute (cpm).

2.5. Flow cytometry analysis

Harvested cells were washed in FACS medium (HBSS containing 0.1% BSA and 0.1% NaN₃) and stained with appropriately diluted antibodies directly conjugated with FITC or PE according to the standard procedure, and followed by fixation in 2% paraformaldehyde. Antibodies used for FACS staining were the following: FITC-labeled anti-mouse MHC class II, CD86, CD80, CD40, CD11b, and PE-labeled CD11c (PharMingen, San Diego, CA). Fluorescence was measured using a FACSScan flow cytometer (Becton Dickinson) and data analysis was performed using the Cell Quest Software (Becton Dickinson, San Diego, CA).

2.6. Reagents

RPMI 1640, L-glutamine, gentamicin, HEPES, FBS, non-essential amino-acids, sodium pyruvate and rabbit complement were purchased from Grand Island Biolog-ical Company (Gibco, Grand Island, NY); RBC lysing buffer, indomethacin, NMLA, paraformaldehyde, cycloheximide, S. aureus obtained from Sigma (St Louis, MO); GM-CSF and IL-4 were a gift from Schering-Plough Research Institute (Kenilworth, NJ).

2.7. Statistical analysis

Statistical significance was determined using the Student t-test and the non-parametric Mann–Whitney test. For all statistical analysis, the level of significance was set at a probability of 0.05 to be considered significant. Data are presented as the Mean ± S.E.M.

3. Results

3.1. Comparison of ‘live’ and ‘dead’ DC in the MLR assay

To determine whether intact APC are required for the MLR, we first compared and contrasted the effects of equal numbers of ‘live’, ‘dead’ and non-separated (mixed) DC on T cell proliferation (Fig. 1A). The maximum T cell proliferation was observed at 1:10 DC/T ratio: 115,262 ± 2912 and 105,710 ± 5713 cpm, for ‘live’ and mixed DC, respectively. There was no significant difference between the stimulatory capacity of ‘live’ and mixed DC (P > 0.1). In contrast, DC which were trypan blue-positive did not induce T cell proliferation in the MLR assay when DC/T cell ratio varied from 1:100 to 1:3. However, we have observed a low stimulatory effect when 1:1 DC/T were used: 29,915 ± 2860 cpm. It is possible that these cell suspensions contain small numbers (< 2%) of ‘live’ cells. As an additional control for evaluation of stimulatory capacity of ‘dead’ DC in MLR assay, we have used DC treated with 10⁻⁵–10⁻³ M corticosterone. This treatment induced cell death (70–90% trypan blue-positive cells) and decreased allostimulatory activity of DC up to 90% (data not shown). Thus, the comparison of effects of ‘live’ and ‘dead’ DC on T cell proliferation in the MLR assay showed a significant difference between the DC populations (P < 0.01), suggesting that live DC are required for the primary MLR whereas dead DC do not induce T cell proliferation in the MLR assay.

3.2. Effect of irradiation on DC stimulatory activity

To evaluate whether active DNA synthesis in DC is necessary for antigen presentation, we have examined whether DC-induced T cell proliferation is depended on DC irradiation. The stimulating effects of irradiated (3000 rad) DC and control DC were not different (Fig. 1B). For instance, the maximum stimulation of T cell proliferation was observed at DC/T cell ratio 1:10:
149,164 ± 12,149 versus 154,544 ± 13,653 cpm for irradiated and not irradiated DC, respectively (P > 0.05). Thus, these results suggest that irradiation of DC did not influence the ability of DC to induce T cell proliferation in MLR.

3.3. Effect of paraformaldehyde treatment on DC

To determine the effect of fixation of the DC-surface structures on the stimulatory capacity of DC, the cells were treated with paraformaldehyde prior to the MLR assay. The results of these experiments (Fig. 1C) indicated that paraformaldehyde-treated DC lost their capacity to induce T cell proliferation. For instance, the maximum stimulation of T cell proliferation was observed at DC/T cell ratio 1:10: 162,476 ± 18,387 and 21,443 ± 5120 cpm for control and paraformaldehyde-treated DC, respectively (P < 0.01).

Next, to examine whether paraformaldehyde treatment of DC affects the expression of surface-marker molecules, flow cytometric analysis of MHC class II, CD86, CD80 and CD40, CD11c molecules was performed. The analysis of the results demonstrated the similar high levels of expression of these markers both on the control and treated DC (P > 0.05).

Together, these data suggest that the intact membrane, its permeability and possible reorganization of the DC-surface structure molecules is important for activation of T cell proliferation. Since paraformaldehyde treatment of DC does not change the expression of their surface-marker molecules (MHC class II, CD86, CD80, CD40, CD11c), it is likely that rearrangement of DC surface structure is essential for an effective antigen presentation.

3.4. Effect of cycloheximide treatment on DC

To evaluate the effect of inhibition of protein synthesis on the stimulatory capacity of DC in MLR, the cells were treated with cycloheximide (1 µg/ml) prior to the assay. The results of these experiments (Fig. 1D) indicated that cycloheximide-treated DC induced either the same (at DC/T cell ratio 1:30–1:1) or even slightly higher (1:100 and 1:1 DC/T cell ratio) proliferative response when compared with control non-treated DC. For instance, the maximum stimulation of T cell proliferation was observed at DC/T cell ratio 1:10: 162,476 ± 18,387 and 175,314 ± 17,326 cpm for control and cycloheximide-treated DC, respectively (P > 0.05).

Fig. 1. Stimulatory ability of DC in the MLR assay after different treatments. The MLR assay was performed as described in Section 2. DC used as stimulators (H-2K b), were generated from bone marrow progenitors. Allogeneic T cells (H-2K d), obtained from the spleens, served as responders. DC were added in graded doses (10^3–10^5 cells/well) to T cells (1 × 10^5 cells/well) and proliferation of T cells was measured by uptake of ^3^H-thymidine. Data represent the mean ± S.E.M. of triplicate measurements from three independent experiments. (A). Live DC are required for the efficient induction of T cell proliferation. The comparison of functional activity of ‘live’ (gray triangular), ‘dead’ (black diamond) and non-separated mixed DC (white circle) in a primary MLR assay was performed as described in Section 2. Cell suspensions were loaded onto the NycoPrep solution to isolate live and dead cells: cells from the interface exhibited a viability of > 97% (‘live’ DC) and cells from the bottom exhibited a viability of < 3% (‘dead’ DC). (B). The irradiation of DC did not influence their ability to induce T cell proliferation. Irradiated (black triangular) (3000 rad from a 137Cs source) or non-irradiated (white circle) control DC were added in graded doses to T cells and proliferation of T cells was measured by uptake of ^3^H-thymidine. (C). Paraformaldehyde-treated DC (black circle) lost their stimulatory capacity to activate T cell proliferation. Prior to the assay DC were treated with 2% paraformaldehyde for 10 min at 10 °C. (D). Block of protein synthesis did not affect ability of DC to induce T cell proliferation. Cycloheximide-treated DC (black triangular) induced either the same or even slightly higher proliferative response than control DC (white circle) in the MLR assay. Prior to the assay, DC were treated with cycloheximide (1 µg/ml) for 30 min at 37 °C.
Next, to test whether cycloheximide treatment changes the expression of surface-marker molecules of DC, flow cytometric analysis of MHC class II, CD86, CD80, CD40 and CD11c molecules was performed. No significant differences were detectable between control and cycloheximide-treated DC (data not shown).

Thus, the results showed that acute inhibition of protein synthesis in DC changed neither the levels of T cell proliferation in the MLR assay nor the expression of DC surface-marker molecules (MHC class II, CD86, CD80, CD40 and CD11c). This suggests that blockage of protein synthesis in mature DC is not critical for the ability of DC to induce T cell proliferation in MLR assay.

3.5. Stimulatory effect of S. aureus on DC-function in MLR was not IL-12 dependent

Since it has been reported that S. aureus activates DC to produce cytokines [19], in the next series of experiments we have determined whether S. aureus-induced activation of DC influences their ability to induce T cell proliferation. Day 5 and 6 cultured DC (1 × 10^6 cells/ml) were exposed to heat-inactivated S. aureus for 24 h and then used as effector cells in the MLR assay. The results of these experiments demonstrated that S. aureus significantly increased the ability of DC to stimulate T cell proliferative response when compared to control non-activated DC (Fig. 2). The highest difference (up to 160%) between S. aureus-treated and non-treated DC was observed with Day 5 DC. For 6-day old DC, the high index of stimulation of cell proliferation (664.2 ± 88.7 and 519.0 ± 30.6, for S. aureus-treated and non-treated DC, respectively) and about 30% difference between the levels of T cell proliferation were also observed. These data suggest that S. aureus activates not only DC cytokine production but also the stimulatory capacity of DC.

In order to examine whether stimulatory effect of S. aureus depends on IL-12 production, we carried out similar experiments with DC generated from IL-12-deficient mice. DC were generated from bone marrow of IL-12 knockout mice and treated with S. aureus. The DC stimulated T cell proliferation at the same levels as DC generated from bone marrow of control mice treated with S. aureus (Fig. 2). We have shown earlier that S. aureus-treated DC from IL-12 knockout mice do not produce IL-12, whereas S. aureus-treated DC from control mice express high levels of IL-12 [20]. Taken together, these data suggest that the mechanism of stimulatory effects of S. aureus on DC-induced T cell proliferation in primary MLR assay is not mediated by an increased IL-12 production in DC.

Next, to determine whether activation of DC with S. aureus influences expression of DC surface molecules, the flow cytometry analysis of MHC class II, CD86, CD80, CD40, CD11c and CD11b molecules was performed. FACScan analysis revealed a significant increase of cell surface molecule expression on S. aureus-treated DC in comparison to control DC both in wild type and IL-12 knockout mice (Fig. 3). The differences between the levels of S. aureus-treated and non-treated DC expression were the following: CD11c-up to 34%, MHC class II-up to 40%, CD86-up to 98%, CD80-up to 110%, and CD40-up to 137%. It is necessary to note here that levels of CD11b expression were different in wild type and IL-12 knockout mice: 52.4 and 30.5%, respectively. It is not clear why only expression of CD11b molecules was different, on DC generated from IL-12 deficient mice in comparison to wild type mice. Nevertheless, stimulation of DC with S. aureus also increased CD11b expression up to 69.1 and 37.1%, respectively for wild type mice and IL-12 knockout mice. These results showed that DC activation with S. aureus increases the DC surface molecule expression and the ability of DC to stimulate T cell proliferation in MLR both for wild type and IL-12 knockout mice. This suggests that such activation does not depend on IL-12 production by DC, and the mechanisms of stimulatory effects of S. aureus on different functions of DC are also different.
Fig. 3. *S. aureus* significantly altered expression of surface molecules on DC. DC were generated as described in Fig. 1 legend. *S. aureus*-activated DC generated from IL-12 deficient mice or wild type mice have increased expression of surface markers when compared with control DC generated from IL-12-knockout mice or wild type mice. DC were treated with *S. aureus* for 24 h at 37 °C and then FACScan analysis was performed using double-staining with FITC-labeled anti-mouse MHC class II, CD86, CD80, CD40, CD11b, and PE-labeled CD11c antibodies. The expression levels of the MHC class II, CD86, CD80, CD40, CD11b, and CD11c molecules are shown by filled histograms and open histograms show background staining with isotype-matched control antibody. Data are represented as log mean fluorescence intensity (MFI) on the X-axis and number of events on the Y-axis.
4. Discussion

The results of the MLR assay illustrate a key role of DC as important accessory cells for the induction of a primary immune response. DC are the most potent APC and MLR stimulators [2,21]. We have determined here which physical, biochemical and phenotypic properties of DC are important for the induction of T cell activation in a primary MLR assay.

First, we have demonstrated that only live DC are able to stimulate T cell proliferation. This might be significant for practical application since most of published DC-based clinical protocols do not include isolation of live DC after their ex vivo generation, harvesting, pulsing with antigenic peptides or dead tumor cells. These procedures, as well as genetic modifications of DC, result in a marked decrease in DC survival and liability. Dead DC do not induce T cell proliferation but might compete with live DC for interaction with T cell. Since we have observed a low stimulatory effect at high doses of ‘dead’ DC (10⁶ cells per well), it is possible that these cell populations contained small numbers (< 2%) of ‘live’ cells. While only live DC were able to induce T cell proliferation in the MLR assay, gamma-irradiation of DC (3000 rad) did not influence their stimulatory capacity. This suggests that the active DNA synthesis in DC is not necessary for their properties, which are responsible for induction of T cell proliferation in an MLR assay.

To answer the question whether DC-surface membrane structures or protein synthesis are involved in the regulation of T cell proliferation, DC were treated with paraformaldehyde or cycloheximide and then used in an MLR assay. We have demonstrated that the DC-surface structures are important for the stimulatory capacity of DC in the primary MLR assay. We observed that paraformaldehyde-treated DC lost their capacity to induce T cell proliferation. However, Barisas and colleagues showed that paraformaldehyde fixation of APC may enhance antigen presentation by MHC class II molecules [16]. These authors propose that paraformaldehyde treatment might result in the aggregation of membrane proteins and thus stabilization of and strengthening of transient protein–protein interactions involved in intercellular cooperation. In that study, cells from mouse B cell lymphoma-derived cell line A2 were used as APC and their ability to present antigen (OVA), but not their stimulatory activity for T cell proliferation in the primary MLR after paraformaldehyde fixation were evaluated. Moreover, in another study, it was shown that B cells fixed in paraformaldehyde, did not stimulate T cell in MLR [22]. Thus, while the physical organization of the plasma membrane of APC might be involved in the regulation of antigen presentation and induction of T cell proliferation via different pathways, it is extremely and equally important for both functions.

In contrast, the treatment of DC with cycloheximide prior to the MLR assay showed that blockage of protein synthesis in mature 7-day-old DC is not critical for the ability of DC to induce T cell proliferation. This suggests that 7-day-old DC generated from mouse bone marrow precursors have all the properties necessary for the induction of efficient T cell proliferation and the additional protein synthesis is not required. However, we have demonstrated here that the stimulatory capacity of DC might be further increased by additional activation with S. aureus. Treatment of DC with S. aureus caused enhanced expression of surface-marker molecules on DC, including MHC class II, CD86, CD80, CD40, and CD11c molecules. The mechanisms of this activation are still not completely resolved. Interestingly, this phenomenon was not mediated by IL-12. Similar effect of bacterial lipopolysaccharide on human monocyte-derived DC was also reported [23]. Using the addition of anti-IL-12 antibody, authors have shown that IL-12 neutralization did not affect T cell proliferation. In the murine system, we have demonstrated here, that DC isolated from IL-12 knockout mice and treated with S. aureus did not produce IL-12, but enhanced expression of surface-marker molecules on DC and stimulated T cells at the same level as control DC treated with S. aureus. Taken together these data suggest that stimulatory effect of S. aureus on DC-functions in MLR and expression of surface-marker molecules do not depend on IL-12 production by DC.

In summary, we have demonstrated that induction of T cell proliferation by DC requires an intact cell membrane and does not depend on IL-12 production and protein synthesis in DC. Taken together, these data bring new insights in our understanding of immunobiology of DC and emphasize the importance of DC membrane organization which is most critical for their functional activity in the primary MLR assay.

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