Enhanced ex vivo Maturation of Peripheral Blood Monocyte-derived Dendritic Cells Pre-treated with Muramyl Dipeptide Analog [MDP-Lys(L18)]

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ABSTRACT

Recently, immune therapy that uses dendritic cells (DCs) has been attempted in many facilities. The results, however, has not been satisfactory. Therefore, DC therapy in which systemic administration of several cytokines (IL-2, etc.) is also used has been attempted. However, systemic administration of cytokines has limitations due to their side effects. Therefore, we decided to focus on romurtide (muramyl dipeptide-Lys, Nopia™) which is a glycopeptide of the Mycobacterium tuberculosis cell wall skeleton that has a potential enhancement anti-tumor effects. It is well known that muramyl dipeptide modulates the functions of monocyte/macrophages, but its effects on DCs are poorly documented. To determine whether romurtide enhances DC maturation. We examined the effects of romurtide on the expression surface molecules, cytokine secretion, and antigen-presenting function of human monocyte-derived immature DCs (iDCs). We found that romurtide markedly up-regulated the expression of CD83 and CD86 but not human leukocyte antigen-DR (HLA-DR), and stimulated the production of tumor necrosis factor-alpha (TNF-α), and IL-12 by human DCs in a dose-dependent manner. In an allogeneic mixed lymphocyte reaction (MLR), romurtide-treated DCs enhanced antigen-presenting function compared with untreated DCs. Similar results were obtained for DCs from cancer-bearing patients. Altogether, our results demonstrate that romurtide triggers the maturation and activation of monocyte-derived iDCs. As this immunomodulator has been approved for use in humans, it could be a useful adjunct to boost the efficacy of DC-based vaccines designed against tumors.

Introduction

Dendritic cells (DCs) are potent antigen presenting cells that can elicit primary and boost secondary immune responses to foreign antigens [1]. In a variety of settings, these activated antigen presenting cells (APCs) can induce both the generation and proliferation of specific cytotoxic T lymphocytes (CTLs) and T helper cells via antigen presentation by major histocompatibility complex (MHC) class I and class II molecules.

Recently, several human trials which DCs were initiated using tumor lysate [2-4], peptide-loaded
MATERIALS AND METHODS

[5, 6] or protein-loaded have been tried against malignant tumors [7, 8]. However, those clinical responses were not fully satisfactory. Attention is therefore being focused on identifying agents to further enhance the reactivity of CTLs against tumors in DC-based immunotherapy.

Under in vivo conditions, the maturation process of DCs is efficiently regulated and controlled by a complex array of signals in the DC microenvironment [9]. A number of cytokines have been proposed to promote DC growth and differentiation [10]. Inflammatory stimuli such as interferon-γ (IFN-γ), tumor necrosis factor (TNF), interleukin (IL)-1, and IL-6 are known to further activate DCs, resulting in maturation of DCs with strong T cell stimulatory potential [11]. Lipopolysaccharide (LPS) from Escherichia coli strain 026:B6 and MDP were purchased from Sigma (St. Louis, MO).

Generation of DCs. DCs were isolated from the human PBMCs as described previously [8][25] with some modifications [26, 27]. Briefly, PBMCs were obtained from 50 ml of leukocyte-enricheduffy coat from healthy donors (HLA A24+) by centrifugation with Ficoll-Hypaque (Pharmacia Biotech, Uppsala, Sweden), and the light density fraction from the 42.5-50% interface was recovered. The cells were resuspended in culture medium, collected, and selected with anti-CD14 monoclonal antibody (mAb)-conjugated immunomagnetic beads (Dynal, Oslo, Norway) and MACS Separation columns (Miltenyi Biotec GmbH, Germany). The resulting cells (>95% CD14+ cells) were used as monocytes and cultured for 7 days in 3 ml of medium supplemented with GM-CSF (800 U/ml) and IL-4 (500 U/ml). During the final 24-h incubation period, the tested cells were treated with the indicated concentrations of LPS, MDP or RM. After differentiation, cells were harvested, washed, and used for subsequent experiments as immature DCs. These cell populations exhibited typical DC morphology and phenotype (>95% pure as indicated by anti-CD1a mAb, anti-CD11c mAb, and anti-CD4-DR staining, and <1% CD3+ cells, CD19+ cells, CD14+ cells [26, 27]). Mature DCs were obtained by incubating the cells for an additional 24 h with tumor lysate (TL) of HT29 cells, which was prepared by 5 freeze (on liquid nitrogen) and thaw (37°C hot water) cycles as we described previously [28].

Phenotype analyses of DCs. Expression of surface molecules was quantified by flow cytometry using an Epics Elite flow cytometer (Beckman Coulter, Miami, FL) and CellQuest software (Becton Dickinson, Mountain view, CA) with 5,000-30,000 events acquired for each sample. Antibodies used for immunolabeling were phycoerythrin (PE)-conjugated anti-CD45-DR (Becton Dickinson), anti-CD83 (Serotec, Raleigh, NC), and anti-CD86 (PharMingen, San Diego, CA). Immature DCs were generated from healthy donors’ PBMCs by a 7-day differentiation in
medium supplemented with GM-CSF and IL-4. During the final 24-h incubation, tested DCs were stimulated with 1.0 µg/ml of LPS, 10 µg/ml of MDP, or 10 µg/ml of RM. Maturation was induced by pulsing immature DCs for an additional 24 h with HT29 TL at the concentration of 100 µg/ml. The expression of HLA-DR, an MHC class II molecule, CD86, a B7-2 co-stimulatory molecule required for T-cell activation [26], and CD83, the most specific differentiation marker of mature DCs [31], was measured by flow cytometry.

**Phagocytosis.** Phagocytic activity was measured by monitoring the incorporation of fluorescein isothiocyanate (FITC)-conjugated dextran (Molecular Probes, Leiden, The Netherlands) as reported previously [29]. In brief, DCs generated by treatment with LPS, MDP, or RM, and subsequently pulsed with HT29 TL were incubated for 1 h at 37°C in the presence of 1 mg/ml FITC-dextran and analyzed for phagocytic activity as judged by the appearance of many FITC-positive cells with high mean fluorescence intensity.

**Allogenic MLR.** Allogenic CD3+ T cells were prepared from an unrelated donor by negative depletion of non-adherent PBMCs with nylon wool fiber columns (Polyscience, Inc. Warrington, PA) according to the manufacturer's instructions. For MLR, immature DCs incubated with medium alone or with medium containing LPS, MDP or RM were seeded at graded numbers (5 x 10^2 - 1 x 10^4) per well in a 96-well plate. The allogenic CD3+ T cells were then added at 1 x 10^4 cells in a final volume of 200 µl per well. Cells were co-cultured for 4 days, and then incubated with 5.0 µCi/ml of [3H]thymidine for an additional 18 h. The cells were harvested onto a glass fiber filter plate and cell-bound radioactivity was counted in a liquid scintillation counter (Packard Japan, Tokyo, Japan).

**Cytotoxicity.** Immature DCs incubated with medium alone or with medium containing RM were pulsed for 24 h with HT29 TL at the concentration of 100 µg/ml. The cells were then seeded at 5 x 10^4 per well in a 96-well plate, and the autologous CD3+ T cells prepared as described above were then added at 1 x 10^5 cells in a final volume of 200 µl per well. Subsequently, the cells were co-cultured with target HT29 cells (1 x 10^3) for up to 72 h. After fixation with 25% glutaraldehyde, the plates were washed 3 times by PBS and stained with methylene blue. The viability of target cells was determined by measuring the absorbance of the wells at 600 nm [30].

**Detection of cytokines.** The production of cytokines by mature DCs was determined by ELISA. Immature DCs incubated with medium alone or with medium containing LPS, MDP or RM were pulsed for 24 h with HT29 TL, and seeded at 1x10^5 cells per well in 6-well culture plates. After 24 h of culture, cell-free supernatants were collected for detection of the cytokines IL-12, and TNF-α (Ab pairs; PharMingen San Diego, CA).

**Generation and maturation of DCs from a tumor-bearing patient.** DCs were isolated from the PBMCs of a patient with breast cancer whose clinical stage was determined to be stage IV of T4 N2 M1 according to the classification of the Union International Contre le Cancer, as described above. Maturation of the patient’s DCs was performed by incubating the cells for an additional 24 h with TL made from surgically resected tumor specimens by 5 freeze and thaw cycles. Informed consent was obtained from the patient, and the experimental protocol was approved by the ethics committee of Osaka Medical College.

**Statistical analysis.** The values were expressed as mean ± SD. The significance was analyzed by the unpaired Student’s t-test (two-tailed). P-values less than 0.05 were regarded as statistically significant.

**RESULTS**

**Upregulation of maturation-related dendritic cell-surface markers.** In the first set of experiments, we investigated the changes in cell surface expression of maturation-related molecules following treatment with RM and other immunomodulators. The results are summarized in Table 1 and representative flow cytometry are depicted in Figure 1A. Before pulsing, DCs showed a characteristic surface phenotype of HLA-DR^low, CD86^low, CD83^low. In control cultures without addition of any stimulants, maturation with TL induced a characteristic phenotype of mature DCs, including HLA-DR^high, CD86^high, CD83^high. Stimulation of differentiating DCs with RM resulted in significant increases in the number of cells expressing CD86 and CD83. The changes in the expression of maturation-related cell surface...
Table 1. Surface phenotype of DC generated under different culture conditions

<table>
<thead>
<tr>
<th>stimulant</th>
<th>TL</th>
<th>HLA-DR</th>
<th>CD86</th>
<th>CD83</th>
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</thead>
<tbody>
<tr>
<td>-</td>
<td>-</td>
<td>36.6 ± 5.7</td>
<td>5.2 ± 1.4</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>-</td>
<td>+</td>
<td>38.3 ± 5.3</td>
<td>5.3 ± 1.8</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>LPS</td>
<td>+</td>
<td>41.0 ± 2.5</td>
<td>7.4 ± 3.1</td>
<td>1.2 ± 0.7</td>
</tr>
<tr>
<td>MDP</td>
<td>+</td>
<td>40.6 ± 8.2</td>
<td>6.2 ± 3.0</td>
<td>0.6 ± 0.2</td>
</tr>
<tr>
<td>RM</td>
<td>+</td>
<td>41.9 ± 8.2</td>
<td>6.3 ± 2.8</td>
<td>0.5 ± 0.1</td>
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</tbody>
</table>

The data were presented as mean LFI of cell surface marker of DC generated under different culture conditions. DCs were cultured in medium containing different DC stimulation factors as described in Materials and Methods.

Fig 1.A. Phenotypic modifications of untreated (□), TL-pulsed mature (shaded) and TL+Stimulant pulsed mature (■) DC gene-rated under different culture conditions. CD14+ PBMCs were differentiated to DC in the medium containing GM-CSF and IL-4. The expression of surface molecules was analyzed by flow cytometry. TL, tumor lysate (100 μg/ml). LPS (1.0 μg/ml), MDP and RM (10 μg/ml).

Fig 1.B. Phagocytotic activity of DC matured under different culture conditions. Phagocytotic capacity was judged by incorporation of FITC-conjugated dextran using flow cytometry. The histograms (□) indicate the control DC. The shaded histograms were TL-pulsed mature DC. The histograms (■) were TL+Stimulant pulsed mature DC.
markers following RM treatment were similar to those observed following stimulation with LPS. In contrast, stimulation with MDP at the same concentration did not increase expression of any cell surface markers.

**Attenuation of phagocytic activity.** Although taking up tumor antigens is an important function of differentiating immature DCs in their role as antigen presenting cells, mature DCs no longer possess phagocytic activity. Therefore, we next examined whether stimulation with RM could modulate the phagocytic activity of TL-pulsed mature DCs. The results are shown in Figure 1B. Maturation induced by pulsing with TL produced a nearly 20% decrease in FITC-dextran incorporation as compared with that of untreated immature DCs. Furthermore, pre-stimulation with RM resulted in reduction by 22% of FITC-dextran incorporation in TL-pulsed DCs. This attenuation of phagocytic capacity was not observed in TL-pulsed DCs treated with LPS or MDP.

**Proliferation of allogenic T cells stimulated by DCs.** Since DCs are potent stimulators of allogenic T lymphocytes, we next examined the effect of RM on the MLR. Graded numbers \((5 \times 10^2 - 1 \times 10^3)\) of DCs incubated with or without immunomodulators were co-cultured with \(1 \times 10^5\) allogenic CD3+ T cells (DC:T-cell ratios, 1:10 to 1:200), and the proliferation of T cells was analyzed by a [3H]thymidine incorporation assay. The results are shown in Figure 2. Untreated DCs did not affect the MLR. Consistent with previous reports, LPS markedly increased the ability of DCs to stimulate the proliferation of allogenic CD3+ T cells. Stimulation with RM during DC differentiation resulted in an increase in the proliferation of allogenic CD3+ T cells at DC to T cell ratios between 1:20 to 1:100 in a dose-dependent manner, although the effect was not equivalent to that of LPS.

**Enhancement of CTL-mediated Cytotoxicity.** PBMC-derived DCs posses the ability to inhibit the growth of tumor cells via activation of CTLs. Therefore, we next examined whether the treatment with RM of TL-pulsed DCs could enhance the CTL-mediated cytotoxicity. DCs treated with or without RM \((10 \mu g/ml)\) were pulsed with HT29 TL for immunization, and incubated with autologous CD3+ T cells (DC:T cell ratio of 1:2) and target HT-29 cells for up to 72 h. The proliferation curves of target cells as measured by methylene blue staining are shown in Figure 3. Co-culturing with untreated DCs did not inhibit the proliferation of target cells. In contrast, TL-pulsed DCs were effective in inducing CTL-mediated cytotoxicity. This cytotoxic effect was significantly enhanced by using DCs which were pre-stimulated with RM before pulsing.

Fig 2. The levels of blastogenesis were significantly higher in allogenic MLR containing DCs treated with RM compared with allogenic MLR containing DCs cultured with media containing no stimulant. In addition, the levels of RM treated DCs were higher than MDP treated DCs and as well as LPS treated DCs.

Fig 3. CTL-mediated cytotoxicity against HT29 human colon cancer cells. DCs treated under different culture conditions were pulsed with the lysate of HT29 cells, and incubated with autologous CD3+ T cells and target HT29 cells for up to 72h. The viability of target cells was determined by methylene blue staining using spectrophotometry.

\(*, **\) \(P<0.05\) as compared with control DC.
**Enhanced maturation of tumor-bearing patient-derived DCs.** Several reports have described the defective function of DCs in patients with malignancies [28]. Therefore, in the final set of experiments, we examined whether treatment with RM could modulate the production of cytokines by DCs. Immature DCs were differentiated from PBMCs of a patient with breast cancer. Before inducing maturation by pulsing with TL made from surgically resected tumor specimens, the tested DCs were stimulated with 10 μg/ml of RM. A flow cytometry shown in Figure 5 demonstrated that pre-stimulation with RM resulted in a significant increase in the number of CD83+ but not CD86+ or HLA-DR+ cell populations, and in reduction by 45.4% of FITC-dextran incorporation in TL-pulsed DCs.

**Increased release of cytokines by DCs.** Several cytokines produced by activated DCs are indispensable for stimulation of antigen-specific T-cell responses. In the next set of experiments, we examined whether treatment with RM could modulate the production of cytokines by DCs. Supernatants from TL-pulsed mature DCs were collected and analyzed for TNF-α and IL-12 contents using ELISA assays. The results are shown in Table 2 and Figure 4. After treatment with LPS, there was a significant increase in the release of TNF-α and IL-12. The increase in cytokine production was also observed in DCs which were pre-stimulated with RM before pulsing, although the levels of these cytokines were not equivalent to those induced by LPS.

**Table 2** Production of cytokines by mature DC generated under distinct culture conditions.

<table>
<thead>
<tr>
<th></th>
<th>untreated</th>
<th>LPS</th>
<th>MDP</th>
<th>RM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1.0</td>
<td>10</td>
<td>1.0</td>
<td>10</td>
</tr>
<tr>
<td>TNF-α</td>
<td>0.59 ± 1.03</td>
<td>336.89 ± 57.92</td>
<td>450.61 ± 13.75</td>
<td>40.67 ± 1.43</td>
</tr>
<tr>
<td>IL-12</td>
<td>0.89 ± 1.54</td>
<td>84.44 ± 3.15</td>
<td>114.0 ± 13.33</td>
<td>17.80 ± 25.25</td>
</tr>
</tbody>
</table>

**Discussion**

Since the discovery of DCs by Steinman in 1973, significant amount of evidences from many studies has shown that DCs are able to modulate the immune response in animals and humans [32]. Now DCs are well known for their important role in stimulating the adaptive immune system, due to their capacity for efficient presentation of foreign antigens to naive T cells [9]. This event induces the formation of effector lymphocytes which either kill target cells directly or produce various cytokines.

Recently, DC-based immunotherapy which pulsed with autologous tumor lysate [2], tumor RNA or DCs-fused with cancer cells [33, 34] have been tried against human and animal malignant
Muramyl dipeptide analog could enhance the maturation of peripheral blood monocyte-derived DCs

Fig 5. RM induces a phenotypic maturation of tumor bearing patient-derived DCs. Changes in cell surface marker expression following a 24-hr stimulation with RM. Phenotypic modifications of immature (□), TL-pulsed mature (shaded) and TL + RM pulsed mature (■) DC generated under different culture conditions. The expression of surface molecules were analyzed by flow cytometry. Tumor lysate(100 μg/ml) and RM(10 μg/ml).

Phagocytic capacity was judged by incorporation of FITC-conjugated dextran using flow cytometry. The histograms(□) indicate the control iDC. The shaded histograms were TL-pulsed mature DC. The histograms(■) were TL + romurtide treated mature DC.

This study demonstrated that DCs pulsed with RM triggers the maturation of DCs, and induces a distinct pattern of cytokine release. We first addressed the question of whether RM had any effect on the spectrum of molecules expressed at the surface of iDCs. Our results clearly showed that this muramyl dipeptide increases the expression of CD83 and CD86, which are considered to be markers of DC maturation and activation, respectively [3, 36].

In the maturation process, iDCs treated with RM have a decreased capacity to take up dextran, a surrogate marker of endocytosis. This decrease has been observed in maturing DCs following stimulation with inflammatory mediators [9].

MLR was used to investigate the antigen-presenting ability of potential APCs. In this reaction, and similarly to the interactions believed to occur in vivo, maximal T cell responses require effective antigen presentation plus costimulatory signals provided by the APCs. The enhancement of T cell stimulation following RM treatment may be explained by the transient increase in antigen uptake immediately following the addition of some maturation-promoting agents, and also by the increased expression of CD86, CD83 and co-stimulatory molecules at the cell surface of RM-treated iDCs.

We provided preliminary evidence here showing that RM-activated DCs have a greater capacity than unstimulated DCs to inhibit the proliferation of the tumor cell line HT29. A similar effect has been previously reported for LPS [37]. Those authors also showed that the continuous presence of LPS in the co-culture medium was dispensable, as LPS-pretreated DCs are as effective in blocking the growth of various tumor cells. It remains to be determined whether RM-treated DCs are active toward other tumor cell lines, and whether this tumoristatic activity is cytokine-mediated or requires cell contact, as is the case for LPS.

Of interest are the findings that the production of cytokines (IL-12 and TNF-α) by in vitro cultured human DCs can be induced by stimulation with agents such as RM and LPS. It was previously demonstrated that LPS induces the production of cytokines [18]. Monocyte-derived DCs produce a large amount of IL-12 when they are stimulated with CD40L and the Th1 reaction is
induced [38]. Hilkens et al. [39] reported that when DCs are activated by exogenous IL-12-inducing factors such as bacteria or their constituents, they lead to Th1 development through the release of IL-12. RM is also a constituent of the organism that causes tuberculosis and is thought to induce the production of IL-12 via a similar mechanism to LPS.

MHC class II molecule is an important factor which presents antigen to CD4+ T cells and activates T cells. MHC class II molecule can accumulate in conventional late endocytotic compartments (MIIC) or in vesicles related to but distinct from early endosomes (CIIVs) [39]. DCs are remarkable in that at different stages of development, they show localization of MHC class II molecules first in MIIC (lysosomes), then in CIIVs, and finally on the cell surface [40]. In immature DCs, class II molecules targeted to lysosomes (MIIC) exhibit relatively short half-lives and are poorly expressed at the cell surface. Thus, immature DCs can accumulate but cannot present antigen until they are induced to mature by inflammatory mediators (e.g., LPS and TNF-α) and redirect MHC class II molecules to the plasma membrane [40]. Thus, it seems that this enhancement of DC maturation results in the activation of the Th1 pathway by IL-12 and the activation of MHC class II molecules by TNF-α, both of which are produced by DCs.

Similar results were obtained in the second set of experiment with use of the DCs from cancer bearing patients. Altogether, these results suggest that the immunoadjuvant activity of MDP-Lys in vivo is mediated, in part, by its stimulation of DC function.

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