Evidence for lipopolysaccharide-induced differentiation of RAW264·7 murine macrophage cell line into dendritic like cells

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Effect of lipopolysaccharide (LPS) on RAW264·7 macrophage cell line was studied. LPS-treated RAW264·7 cells increased in cell size and acquired distinct dendritic morphology. At the optimal dose of LPS (1 μg/ml), almost 70% RAW264·7 cells acquired dendritic morphology. Flow cytometric studies indicate that the cell surface markers known to be expressed on dendritic cells and involved in antigen presentation and T cell activation (B7·1, B7·2, CD40, MHC class II antigens and CD1d) were also markedly upregulated on LPS-treated RAW264·7 cells. Our results suggest the possibility that LPS by itself could constitute a sufficient signal for differentiation of macrophages into DC-like cells.

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1. Introduction

Dendritic cells (DCs) comprise a class of leukocytes that perform the crucial function of antigen presentation to naive T cells in a primary immune response. While exclusively DC colony forming units have been shown to exist in bone marrow, DCs in general are considered to be heterogeneous with regards to their origin and may have a myeloid as well as a lymphoid origin (Steinman and Inaba 1999; Lane and Brocker 1999; Banchereau *et al* 2000; Grabbe *et al* 2000). Myeloid precursor cells can differentiate into DCs in the presence of GM-CSF, followed by further maturation in presence of bacterial lipopolysaccharide (LPS) or other stimuli such as signalling through CD40 molecules (Inaba *et al* 1992; Lane and Brocker 1999; Banchereau *et al* 2000). Migration through endothelium could also induce differentiation of monocytes into DCs through a pathway, which appears to be cytokine independent (Randolph *et al* 1998). As differentiation of immature to mature DCs takes place, the cells down regulate their phagocytic activity and upregulate the expression of molecules like CD40 and CD80/CD86 coreceptors, and MHC class II molecules, required for antigen presentation (Palucka and Banchereau 1999; Banchereau *et al* 2000). The capability of mature DCs to (i) process and present antigenic peptides associated with MHC class I or class II antigens and (ii) impart a costimulatory signal makes them highly efficient antigen presenting cells for naive T cells. Macrophages as well as DCs belong to the class of professional antigen presenting cells having a common lineage (Peters *et al* 1996). Exact placement of macrophages, monocytes and DCs in myeloid differentiation pathway however remains unsettled. While monocytes may clearly differentiate into macrophages as well as DCs, whether mature macrophages and DCs can freely interconvert is not known.

Bacterial lipopolysaccharides, which are potent inducers of DC maturation, have been shown to bind to a variety of immune cells via CD14 and a soluble LPS binding protein (LBP) in serum (Fenton and Golenbock 1998).

Keywords. Dendritic cells; differentiation; flow cytometry; lipopolysaccharide

Abbreviations used: DCs, Dendritic cells; LBP, lipopolysaccharide binding protein; LPS, lipopolysaccharide.

Downstream signalling of the CD14-LBP complex occurs through recently discovered toll-like receptors expressed on a variety of LPS-sensitive cells (Beutler 2000; Schuster and Nelson 2000). RAW264·7, a murine macrophage cell line initially derived from Balb/c mice infected with Abelson leukemia virus, is extremely sensitive to LPS (Raschke *et al* 1978). While studying the interaction of RAW264·7 cells with LPS, we observed that a large proportion of LPS-treated RAW264·7 cells acquire dendritic morphology. In view of these morphological changes, we hypothesized that LPS could be inducing the differentiation of RAW264·7 cells into dendritic cells. To test this hypothesis, we carried out flow cytometric studies to see if surface phenotypic markers associated with dendritic cells were expressed on LPS-treated RAW264·7 cells. The present study documents these findings. In addition, we have shown that a variety of cell surface molecules involved in antigen presentation are also markedly upregulated in LPS-treated RAW264·7 cells. These results support the possibility that macrophages can be induced to differentiate into DC-like cells by LPS.

2. Materials and methods

2.1 *Reagents*

LPS (from *Escherchia coli* strain 055 : 85) was obtained from Difco Laboratories, Detroit, MI, USA. All monoclonal antibodies (CD16/32, clone 2·4G2; CD1d-FITC, clone 1B1; CD80-PE, clone 16-10A1; anti-Ia^d-FITC, clone AMS-32·1 and the corresponding isotype control antibodies) were obtained from Pharmingen/Becton Dickinson (San Diego, CA, USA).

2.2 *Cell culture*

RAW264·7 cells were obtained from the American Type Culture Collection and maintained in RPMI1640 culture medium containing glutamine (2·0 mM), penicillin (100 U/ml), and 10% heat-inactivated fetal bovine serum (defined FBS, Hyclone Laboratories, Logan, UT, USA). For harvesting the cells, monolayers of RAW264·7 cells were washed with PBS two times and a non-enzymic cell dissociation solution from Sigma (Cat. No. C5789, Sigma, St Louis, MO, USA) was added. After 20 min incubation at room temperature, cells were detached by vigorous mixing with a Pasteur pipette and harvested. For microscopic studies, cells were stained using Leukostat solution I [eosin Y (0·1% W/V) and formaldehyde (0·1% W/V) in phosphate buffer] and counterstained with solution II [methylene blue (0·047% W/V) and Azure A (0·044% W/V) in phosphate buffer], according to the procedure recommended by the manufacturer (Fisher Diagnostics, Pittsburgh, PA, USA).

2.3 *Flow cytometry*

For staining with fluorochrome-tagged antibodies, cells ($2·5 \times 10^5$) were distributed in deep-welled microtest plate (96 wells per plate, capacity 1 ml/well, Fisher Scientific, Pittsburgh, PA, USA). Cells were washed twice with staining buffer (1% FBS and 0·1% sodium azide in PBS, 1600 rpm for 7 min) in an Eppendorf centrifuge with a micro-plate centrifugation rotor, and suspended in 20 µl of staining buffer containing 1 µg Fc-block antibody (anti CD16/32 mab). After 10 min of incubation, staining antibody or the corresponding isotype control antibody, were added (1 µg in 20 µl staining buffer) followed by incubation for 30 min at room temperature. Cells were then washed twice with staining buffer, fixed in 0·4% paraformaldehyde and analysed on a Becton Dickinson, Faxcaliber flow cytometer.

3. Results

3.1 *LPS induced morphological changes in RAW264·7 cells*

Proliferative activity of RAW264·7, a mouse macrophage-like cell line, is strongly inhibited by LPS (Raschke *et al* 1978). While confirming this observation (results not shown), we also found that RAW264·7 cells cultured with LPS underwent a remarkable morphological transformation. Control RAW264·7 cells had macrophage-like morphology with deeply stained nuclei and finely granulated cytoplasm. Cells were generally elongated with two short cytoplasmic processes (figure 1A, B). A morphological transformation of RAW264·7 cells from macrophage-like cells into dendritic-like cells was clearly observed at all doses of LPS. Dendritic morphology was characterized by multiple prominent cytoplasmic processes (figure 1B, D). LPS-treated RAW264·7 cells also acquired larger nuclei, prominent nucleoli and relatively prominent cytoplasm with increased granularity (figure 1B, D). Increase in size of LPS-treated RAW264·7 cells was quantitatively assessed by studying cell size distribution on a Coulter particle size analyser. Results in figure 2 show relative cell size distribution of control and LPS-treated RAW264·7 cells. A dose dependent shift in cell size was clearly observed (figure 2). Degree of transformation of RAW264·7 cell morphology in response to different doses of LPS was assessed by fixing and staining the cells and counting the number of cells with transformed morphology under a microscope. Considering that cells with dendritic-like morphology were non-existent in control RAW264·7 cell cultures, results in figure 3 indicate that a significant morphological transformation occurred at all test doses of LPS ($P < 0·01$ at all doses),

and that maximal morphological transformation (70%) occurred at 1 μg/ml dose of LPS.

3.2 *Phenotypic changes in LPS-treated RAW264·7 cells*

Results of LPS induced morphological changes in RAW264·7 cells suggest that LPS may induce differentiation of these cells to DCs. To further explore this possibility, we looked for phenotypic changes which occur in LPS stimulated RAW264·7 cells. Molecules involved in antigen presentation, such as B7·1 (CD80), CD40 and MHC class II antigens, are expressed on DCs (Randolph *et al* 1998; Banchereau *et al* 2000). The effect of LPS on the expression of these markers on RAW264·7 cells was studied. Results in figure 3A show the effect of LPS on B7·1 (CD80) and B7·2 (CD86) expression on RAW264·7 cells. There was little basal expression of B7·1/B7·2 molecules on RAW264·7 cells but almost all cells became B7·1 positive after two days of LPS treatment. B7·2 expression also went up in response to LPS, but was restricted to about 40% of the RAW264·7 cells (figure 4A). Likewise, there was no expression of MHC class II antigens and CD40 molecules on control RAW264·7 cells, but LPS treatment rendered about 70% of the cells positive for these two markers (figure 4B, C). Time kinetics studies indicate that LPS induced a marked upregulation of B7·1, CD40, MHC class II, and CD1d markers on RAW264·7 cells (figure 5). CD1d expression was examined because this marker has been shown to be involved with presentation of certain mycobacterial anti-

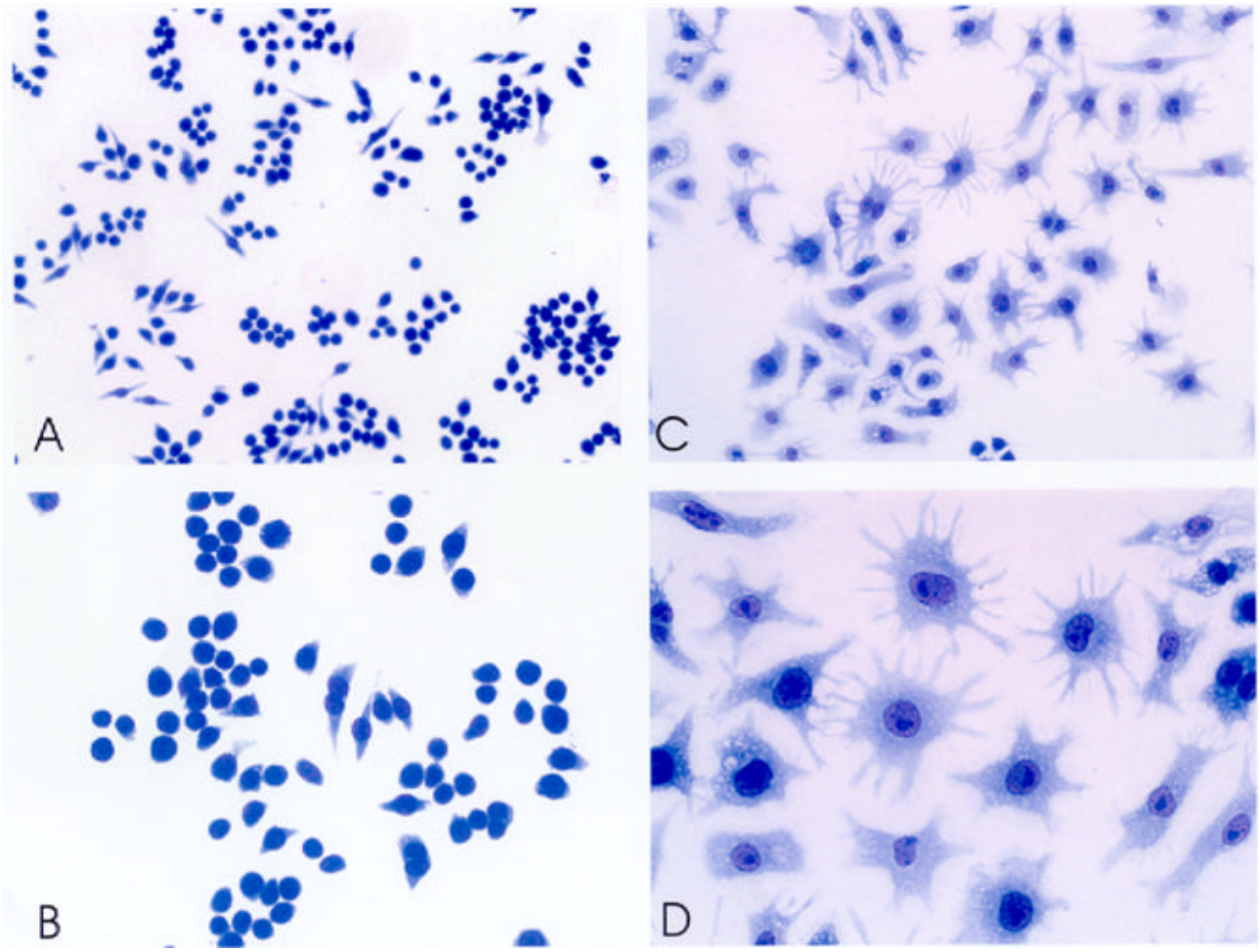

Figure 1. LPS-induced morphological changes in RAW264·7 cells. RAW264·7 cells were cultured on glass slides in a slide culture chamber. LPS (1 and 61549; g/ml) was added to medium in some slide chambers. After two days, glass slides with control (**A, B**) and LPS-treated (**C, D**) cells were removed from the slide chambers and fixed in ice-cold methanol for 30 min. Slides were then washed and stained with Leukostat solutions I and II, as described in § 2. Slides were observed under microscope with a digital camera and pictures taken at 100 X (**A, C**) and 200 X (**B, D**) magnifications.

gens (Moody *et al* 1999). Time kinetics studies also indicated that maximal level of CD80 and CD40 expression was reached one day after treatment with LPS. Upregulation of class II MHC and CD1d expression was relatively slower and the peak of expression occurred two days after LPS treatment followed by some decline at three day time point (figure 5). Taken together, the results of morphological changes as well as phenotypic changes in membrane expression of several relevant markers on LPS-treated RAW264·7 cells support the hypothesis that LPS alone may induce differentiation of RAW264·7 cells to dendritic-like cells.

4. Discussion

While DCs can be derived from lymphoid as well as myeloid precursors (Peters *et al* 1996; Banchereu *et al* 2000), factors involved in differentiation of precursor cells into DCs are not completely understood. GM-CSF induces the differentiation of myeloid precursors/ monocytes into immature DCs (Inaba *et al* 1992). Immature DCs have high macropinocytic activity but are poor expressers of molecules such as B7·1, CD40 and MHC class II antigens, which are required for antigen presentation. At this stage the cells can concentrate the antigens but cannot efficiently present them to T cells. At the site of bacterial infection, bacterial products, such as LPS, induce maturation of DCs, a process that is accompanied with a decreased antigen uptake activity, but with an efficient presentation of the antigen-derived peptides to naïve T cells. A common myeloid lineage precursor, such as the monocyte, may differentiate into macrophages or to DCs depending upon the cytokines present and other

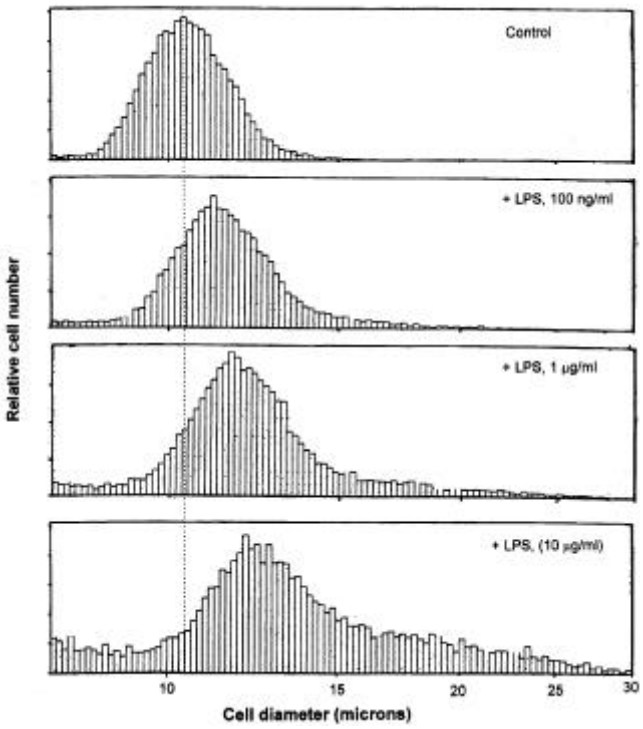

Figure 2. Dose-dependent effect of LPS on the cell size distribution of RAW264·7 cells. RAW264·7 cells were cultured in the presence of various given doses of LPS. After two days, cells were detached and analysed on a Coulter cell size analyser. Relative cell size distribution patterns of control and LPS-treated cell preparations are shown.

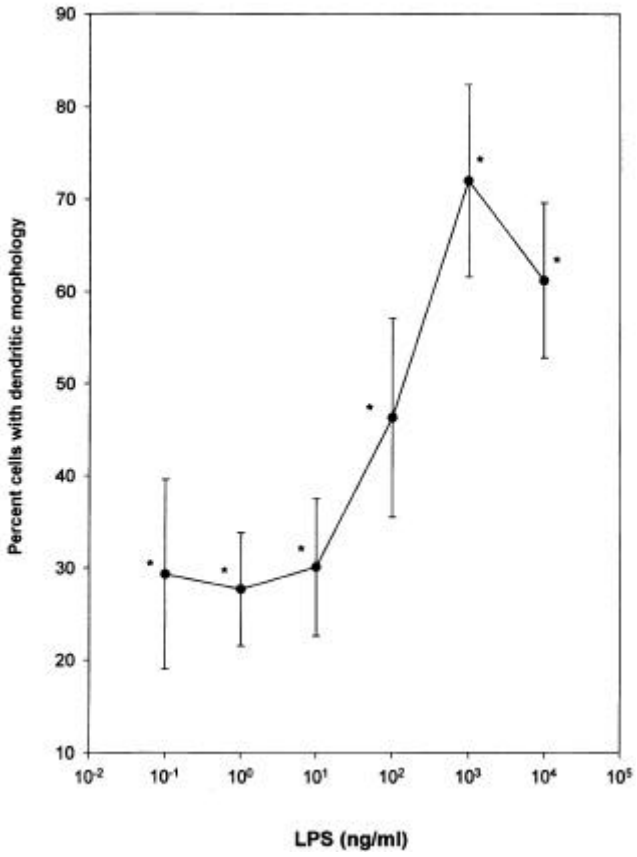

Figure 3. Dose-dependent morphological transformation of RAW264·7 cells by LPS. RAW264·7 cells were cultured on glass slides in a slide chamber in the presence of various given doses of LPS. After two days, slides were fixed and stained as described in § 2. Slides were observed under a microscope with a digital camera and images of 9–12 different fields were captured for control cells as well as cells treated with different concentrations of LPS. Printouts of images were used to count cells with normal and dendritic morphology. Cells with dendritic morphology in the view field were calculated as percent of all cells observed. Results denote mean ± SD of percentage of cells with dendritic morphology in 9 to 12 different slides for each concentration of LPS. In control cultures, < 1% of cells had dendritic morphology.

*Effect of LPS is significant at all doses of LPS ($P < 0{\cdot}01$ by Dunnett's multiple comparison tests).

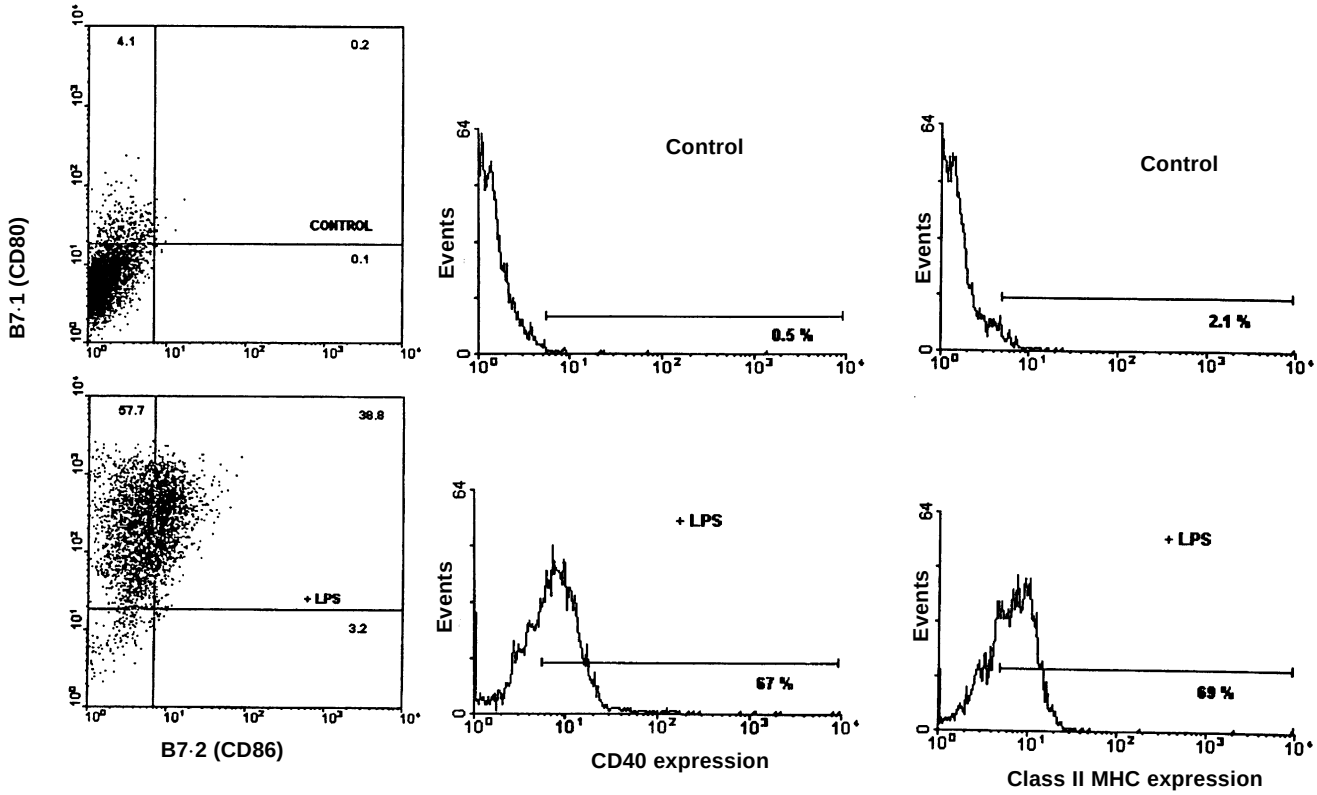

Figure 4. LPS-induced expression of B7·1, B7·2, CD40 and MHC class II molecules on RAW264·7 cells. RAW264·7 cells were cultured ± LPS (1 μg/ml) for 2 days, harvested, stained with different antibodies, and analysed on a flow cytometer. Left panels (2 colour plots) show the expression of B7·1 and B7·2 markers on control and LPS-treated RAW264·7 cells. Middle and right panels show the expression of CD40 and class II MHC markers respectively on control and LPS-treated RAW264·7 cells. The results are representative of four experiments with similar results.

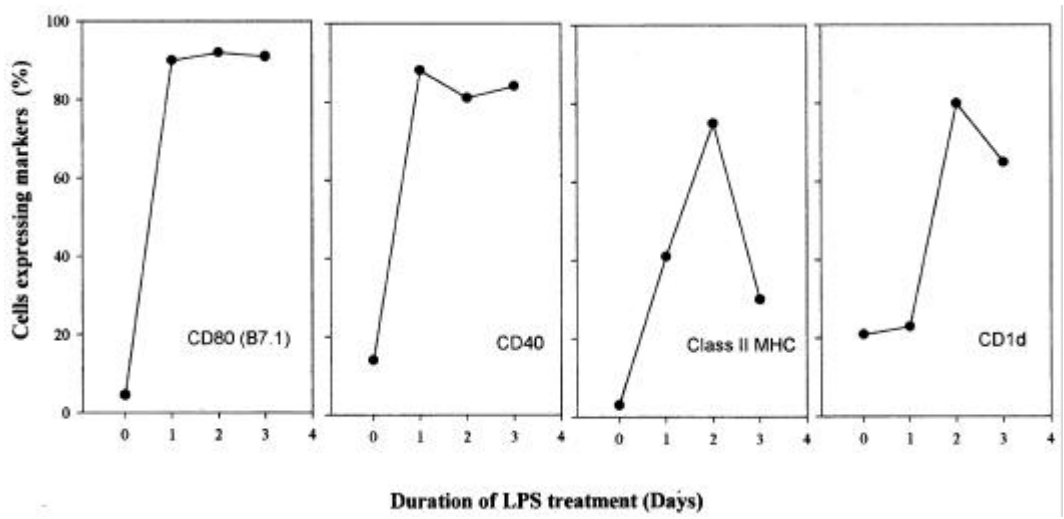

Figure 5. Time kinetics of the effect of LPS treatment on expression of various phenotypic markers on RAW264·7 cells. RAW264·7 cells were cultured in tissue culture flasks + LPS (1 μg/ml). At given time points, cells were stained with various antibodies and analysed on a flow cytometer. Zero day points in all curves represent the expression of respective markers on control cells not exposed to LPS. Results are representative of three time kinetics experiments with similar results.

environmental signals (Peters *et al* 1996; Banchereu *et al* 2000). However it is not clear if mature macrophages themselves can convert to DCs.

In the present study we have found that a murine macrophage cell line RAW264·7, appears to differentiate into dendritic-like cells upon treatment with bacterial LPS. The RAW264 cell line was initially derived from Balb/c mice injected with Abelson murine lukemia virus (Raschke *et al* 1978). This cell line which secretes lysozymes, phagocytizes zymosan or latex beads and antibody coated erythrocytes, expresses Fc receptors for immunoglobulins, and was designated as macrophage cell line (Raschke *et al* 1978). Subsequently, this cell line has extensively been used to study macrophage functions. Sensitivity of proliferative activity of RAW264·7 cells to LPS is well known and was confirmed in the present study. In addition, we observed marked morphological changes in LPS-treated RAW264·7 cells, which acquired dendritic-like morphology. Under optimal conditions not only did the majority of RAW264·7 cells acquire dendritic morphology but also started expressing the molecules (B7·1, CD40, MHC class II and CD1d) involved in antigen presentation and which are known to be associated with DCs (Banchereau *et al* 2000). After attaining peak MHC II and CD1d expression on day 2 of LPS treatment, expression of these two markers on RAW264·7 cells tended to decline. Reason for this decline are not clear but may be related to lack of certain crucial secondary factors in the culture system. LPS has generally been considered to induce maturation of immature DCs (Peters *et al* 1996; Banchereu *et al* 2000). Our present results support the proposition that under certain circumstances, LPS may provide a sufficient signal for generation of DCs from cells of the macrophage lineage. This proposition is supported by our recent experiments in which some cells with dendritic morphology were observed in LPS-treated cultures of mouse spleen cells (unpublished results). Recently, similar LPS-induced changes in human macrophages have also been suggested (Lyakh *et al* 2000). Macrophages are considered to be end-differentiated cells. If RAW264·7 cells represent mature macrophages, as is generally believed, their differentiation into DCs may point to a mechanism through which tissue macrophages may be recruited to become DCs during localized bacterial infection. Such a conversion would be beneficial for mounting an effective immune response. Whether the morphological and phenotypic changes seen in RAW264·7 cells in response to LPS were mediated solely by signalling through the LPS receptor system or required additional signals in the form of cytokines or molecules induced in response to LPS needs to be further elucidated.

Macrophages and DCs can be clearly identified on basis of morphological differences. However, the cells representing intermediate stages of differentiation pathways, leading to macrophages and DCs, are defined only on the basis of a variety of morphological, functional and phenotypic properties and, as such, no unique markers exist for these cells. While our results, based upon the study of some selected markers, suggest that LPS may induce differentiation of macrophages into DCs, further work is required to consolidate this suggestion and fit it into an overall scheme of myeloid differentiation lineage involving DCs and macrophages.

Acknowledgement

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