Protective effect of dendritic cells in mice infected by *Klebsiella pneumoniae*

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Abstract

Protective effect of dendritic cells (DC) was studied in CBA mice infected by K. pneumoniae K_2. Lipopolysaccharide K. pneumoniae, K. pneumoniae K_2 lysate and polycomponent vaccine Immunovak-VP-4 of conditionally pathogenic microorganisms were used as maturation inductors for DCs generated from murine bone marrow. Immature DCs, as well as mature cells, regardless the type of stimulating agent injected intraperitoneally demonstrated protective effect of 83.3 up to 100% in mice infected by K. pneumoniae (100LD50). DCs generate a range of cytokines that might activate complex mechanisms of inter-cellular relations. Immature protective DC activity against K. pneumoniae may be associated with the following induction of cytokine synthesis in vivo stimulated both by DCs and microorganism cells. The results of the study lead to a conclusion that DCs can have additional functions besides their high ability to stimulate proliferation of syngeneic mononuclear leukocytes that is due to DC involvement in reactions of inter-cellular interaction while non-specific immune response is being formed.

Development of DC-based vaccines for adoptive immunotherapy suggests perspective way for prophylactics and therapy of infectious diseases.

Key words: *dendritic cells, TNF-α, Immunovak VP-4, lipopolisaccharide K. pneumoniae, immunophenotype.*

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Introduction

Adoptive immunotherapy has demonstrated promising results in experimental studies and a number of clinical trials. Biotherapy methods are based on the functions of immune system. Recent studies showed effectiveness of biotherapy approaches in managing cancer [1, 4, 23] and infectious diseases [21]. One of the ways to improve anti-tumor immunotherapy effect includes interlukine-2/lymphokine--activated killer cells therapy (IL-2/LAK therapy) after radical surgery of the malignant lesion [2, 18]. Another approach involves dendritic cells (DCs) loaded *ex vivo* with various antigens, which then are capable of priming naïve T-cells *in vivo* and induce protective immune response against different tumor antigens and bacterial infections [14, 20, 22]. Application of DCs challenged with the antigen was rather effective in initiating anti-bacterial protective immune response against intracellular pathogens (*Leishmania donovani*, *Mycobacterium tuberculosis*) where Th1 T-cell dominant response was most important in regulation of the infectious process [12, 16]. In the studies on experimental animals infected by *Bordetella pertussis* DCs enhanced specific immune response characterized by immunoglobulin IgG and IgA synthesis in lungs [13, 24].

New approaches for generating vaccines against certain bacterial pathogens such as *Chlamidia trachomatis* and *Leishmania donovani* include induction of local mucosal Th1 response with IFN-γ release and increase of activating markers on lymphocyte surface. The development of treatment strategies involving anti-infectious vaccines is based on DC functions to present an antigen with paracrine release of active regulatory cytokines such as IL-2 at the local infection site [8].

Dendritic cells are professional antigen-presenting cells (APC) that have unique potential of initiating primary

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immune response [5, 9, 11]. DCs can prime naïve T-cells, cytotoxic T-lymphocytes (CTL) and activate memory cells as well. Besides that, DCs can also activate natural killers (NK) and NKT-cells and induce B-cell differentiation into IgM-producing cells [10, 15].

Nowadays a steady increase in mutation-associated resistance of both pathogenic and conditionally pathogenic microorganisms to anti-bacterial agents is registered at a global level. Due to this fact it may have promising results to generate DC-vaccines on the base of bacterial antigens against opportunistic and conditionally pathogenic infections, which include *K. pneumoniae*.

The aim of the present study was to evaluate protective effect of DCs in CBA mice infected by *K. pneumoniae* K_2 strain.

Materials and Methods

Dendritic cells were generated from bone marrow cells of 30 CBA mice. The animals were withdrawn at the end of the study by ether according to "The guidelines of conducting studies with the use of experimental animals".

DC generation

DCs were generated from bone marrow cells of CBA mice. Murine bone marrow was homogenized in RPMI 1640 medium (Sigma, USA), centrifuged three times (250 g × 5 min) and re-suspended in enriched culturing medium (1×10^6 cells in 1 ml RPMI 1640 with 100 µg/ml gentamicin sulphate and 10% fetal bovine serum) containing 80 ng/ml recombinant GM-CSF and 20 ng/ml IL-4 (Biosource, USA). On day 6 of incubation at 37°C in 4% CO_2 the medium was changed and either *K. pneumoniae* K_2 lysate (50 µl/ml), or lipopolisaccharide (LPS) *K. pneumoniae* (0.125 mg/ml) (Sigma, USA), or polycomponent vaccine VP-4 (50 µg/ml) was added to induce DC maturation. Three days later DCs were washed from the medium and administered in mice.

Isolation of mononuclear leukocytes (MLs)

Murine spleen was homogenized in 199 medium (Institute of Poliomyelitis and Viral Encephalitis RAMS, Moscow) and centrifuged three times. Suspension of spleenocytes was centrifuged at 400 g for 30 min at Fikoll-Urografin gradient, density 1.077 g/cm^3 ("Pharmacia", USA). MLs of the interphase ring were collected and washed in 199 medium three times. The cells were centrifuged at 200 g in 10-fold medium volume after each washing.

To obtain lymphocytes ML suspension in RPMI 1640 was placed into flasks and non-adhesive cells were then transferred into culturing medium (1×10^6 cells in 1 ml of enriched RPMI 1640).

Mice treatment

DC suspension ($1.5\text{-}3.0 \times 10^6$) was injected in mice intraperitoneally in the left side in 0.5 ml of 0.9% NaCl solution. Then, immediately 500 cells of *K. pneumoniae* K_2 (100 LD50) were injected intraperitoneally in the right side in 0.5 ml of 0.9% NaCl solution.

Agents and vaccines

The following preparations were used: lipopolisaccharide (LPS) *K. pneumoniae* (0.125 mg/ml) (Sigma, USA), *K. pneumoniae* K_2 lysate (10^7 bact. cells/ml) and polycomponent vaccine Immunovac VP-4 (50 µg/ml) containing antigens of conditionally pathogenic microorganisms (I.I. Mechnikov RIVS, RAMS), LPS associated with the protein of the external membrane of gram-negative microorganisms, peptidoglycan, teichoic acids and labile protein components of staphylococcus. DC maturation was achieved by commercial TNF-α (Biosource, USA).

Preparation of bacterial lysate of *K. pneumoniae* K_2

Bacterial culture was exposed to impulse ultra-violet irradiation using "Alfa-1" device for 15 min. Then the bacterial culture was diluted to the concentration of 10^7 cells/ml and treated twice by freezing and melting. Complete inactivation of the cells was tested by seeding into Petri dishes with nutrient agar. The lysate was squeezed through the filter (d=0.25) and 50 µl/ml introduced into the medium with DCs.

DC phenotype analysis

DC phenotype was analyzed by monoclonal antibodies (mAb) (Caltag Laboratories, USA) against corresponding antigens. The cells were washed by cold phosphate buffer (PBS) and stained by FITC and PE – conjugated mAb according to the manufacturer's instruction and analyzed by flow cytometry (FacsCalibur, Becton Dickinson, USA). Expression rate of the following surface antigens of DCs generated from murine bone marrow cells was measured: F4/80, CD34, CD38, CD40, CD80, CD86, MHC I and MHC II. 10000 cells were collected for each test. Statistical analysis was performed by WINMDI 2.8 software.

Cytokine production

Cytokine production was measured with the use of ELISA (Biosource, USA).

DC phagocytosis activity

DC phagocytosis activity was assessed by engulfing activity towards latex particles 2.7 µm diameter (DIAeM, Russia). Phagocytosis index (PhI) – percentage of cells interacting in phagocytosis and phagocytosis number (PhN) – average number of intracellular bacteria (the result of the division of total engulfed bacteria number by the number of cells interacting in phagocytosis) were determined. Both parameters were evaluated on slides obtained after 30 and 90 minutes of incubation (i.e. total 120 minutes).

Table 1. Effect of agents – maturation inductors of dendritic cells generated from murine bone marrow cells (n=15) on expression rate of surface molecules

NN	Concentration of components in the DC culture medium				Expression of surface molecules (%)							
	lysate (µl/ml)	LPS (µg/ml)	VP-4 (µg/ml)	TNF-α (ng/ml)	CD34	CD38	CD40	CD80	CD86	MHC I	MHC II	F4/80
1	0	0	0	0	15.9±1.2	5.0±0.1	0.5±0.1	8.1±0.4	5.3±0.4	9.3±2.3	9.1±2.1	58.9±2.2
2	0	0	0	20	6.9±3.5	22.6±2.7*	23.2±3.4*	65.9±0.9*	61.2±2.8*	31.0±4.5*	32.3±4.2*	4.6±0.7**
3	0	0	50	0	5.8±2.3*	13.5±1.2*	36.8±2.3*	66.3±0.8*	30.3±3.5*	18.1±3.8*	18.3±3.4*	36.5±2.5
4	0	0.125	0	0	6.2±1.5*	10.8±1.7*	10.6±3.5*	64.3±1.5*	59.1±8.5*	22.1±4.7*	17.5±2.5*	45.3±7.7
5	50	0	0	0	6.2±2.3*	12.3±1.2*	10.2±2.8*	63.1±1.6*	48.4±5.2*	21.6±0.7	14.7±0.8*	32.1±5.1
6	initial phenotype of bone marrow cells				28.2±3.7	0.3±1.5	0.3±0.1	0	0	0	0	0.5±0.1

*Reliable difference between groups: * – p_1 and $p_{2, 3, 4, 5}$ <0,05.*

Evaluation of murine DC effect on ML proliferation

Proliferation activity of MLs obtained from murine spleen was measured by colorimetric test with vital stain AlmarBlue (Biosource, USA) under sterile conditions. DCs were set in enriched RPMI 1640 into 96-well plates in the number of 10×10^3 cells/well. Suspension of syngeneic MLs 200×10^3 were added into these wells and mixed cultures were incubated for 4 days under standard culturing conditions. At the end of the incubation period 10% AlmarBlue was added to the cell culture. Fluorescence was measured after a four-hour incubation at 37°C, 5%CO_2 on Versa Fluor (Vtocal) fluorimeter at the excitation wave 530-560 nm, emission wave 590 nm; the obtained values presented units of fluorescence. Stimulation index (SI) was calculated as the ratio of ML proliferation stimulated by mature DCs to ML proliferation stimulated by immature DCs.

Statistical processing of the data was performed by Student's t-criterion with software Windows 98 (StatSoft 5.5).

Results

DCs generated from bone marrow cells in the presence of GM-CSF and IL-4 displayed phenotype of immature cells ($CD34^+$, $CD80^-$, $CD86^-$) (table 1). DC incubation in the presence of *K. pneumoniae* K_2 lysate, *K. pneumoniae* LPS, VP-4 vaccine and TNF-α as maturation inducing agents led to generation of mature DCs with typical morphological features and phenotype $CD34^-$, $CD38^+$, $CD40^+$, $CD80^+$, $CD86^+$, MHC I^+, MHC II^+ and $F4/80^{-/+}$ (figure 1). Expression of macrophage marker F4/80 (receptor to LPS) on DC membrane stimulated by the studied bacterial agents is associated with the presence of bacterial LPS in these DCs. These results comply with the earlier received data showing that DCs generated in the presence of LPS can express not only antigens of mature DCs, but also receptors to LPS [7]. The obtained results demonstrate that all studied agents stimulated DC maturation. The generated DCs expressed co-stimulating molecules (CD40, CD80, CD86) and MHC I and MHC II molecules as well, the highest expression rate (almost 8-fold higher as compared to that of immature DCs) was shown by CD80 differentiation molecule.

The DCs generated under the described conditions were registered by phase-contrast microscopy as star-like or veiled cells with typical cytoplasmic extensions, that complies with DC description by other researchers [19]. DCs regardless of the type of stimulating agent (LPS, *K. pneumoniae* lysate or VP-4 vaccine) or immature DCs (not pulsed by an agent) when injected intraperitoneally had protective effect of 83.3 to 100% in mice infected by *K. pneumoniae* K_2 (100LD50) (table 2). While control animals, which did not receive any DC injections, died within 3 days after infection of *K. pneumoniae* K_2 (100LD50, i.e. 500 bacteria cells; LD50

Table 2. Non-specific protective activity of murine DCs generated from bone marrow of mice infected by strain K_2 *K. pneumoniae* in the dose of 100LD50 (n=12)

Group	DC maturation inductor for DC-treated mice	Percentage of survived mice (%), day 14 M±m
1	lysate of *K. pneumoniae* K_{16}	100.0
2	lysate of *K. pneumoniae* K_2	83.3±15.2*
3	LPS *K. pneumoniae*	100.0
4	VP-4	100.0
5	no inductor	100.0
6 (control)	no DC treatment	0**

** – the data were registered on day 4 and remained unchanged; ** – the data were registered on day 3.*
100LD50 corresponds to the infection dose of 500 K. pneumoniae K_2 cells.

Fig. 1a. Histograms show expression of surface molecules on mature DCs generated in the presence of Immunovak VP-4 (50 ng/ml)

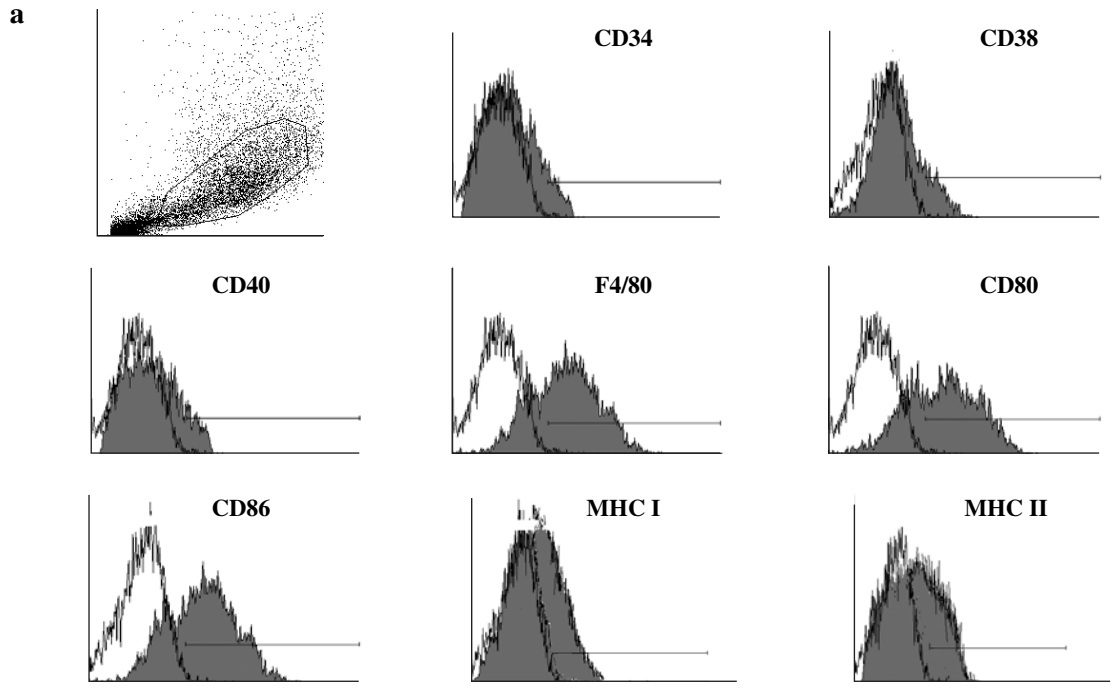

Fig. 1b. Histograms show expression of surface molecules on mature DCs generated in the presence of *K. pneumoniae* K_2 lysate

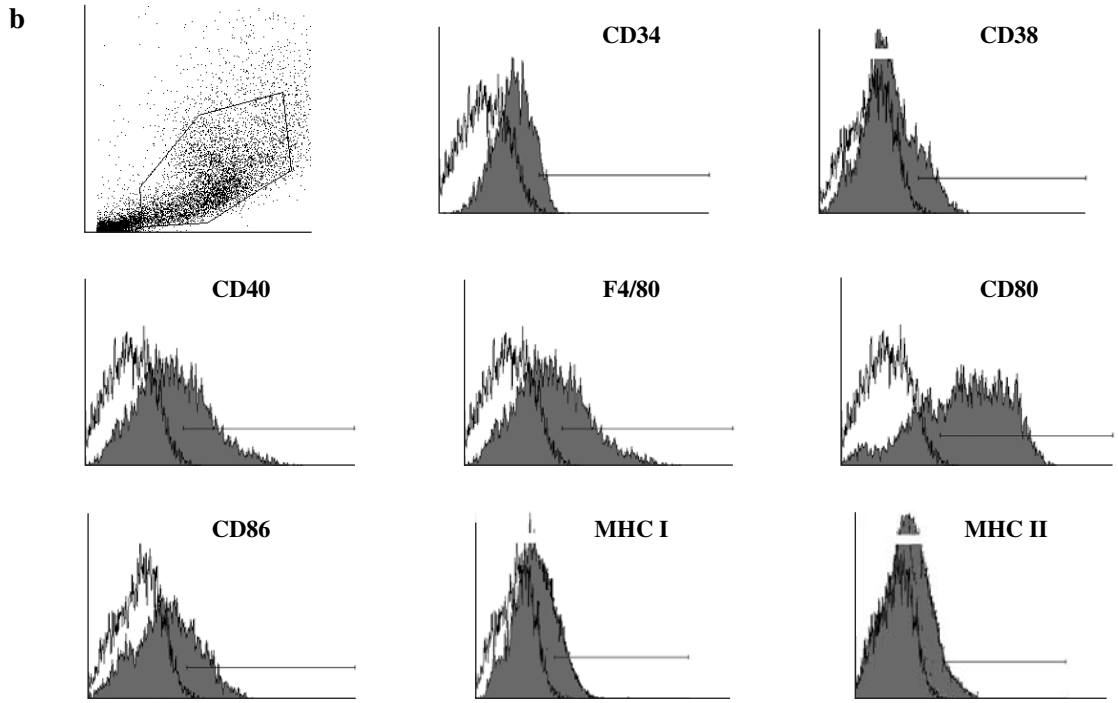

Fig. 1c. Histograms show expression of surface molecules on mature DCs generated in the presence of recombinant TNF-α (20 ng/ml)

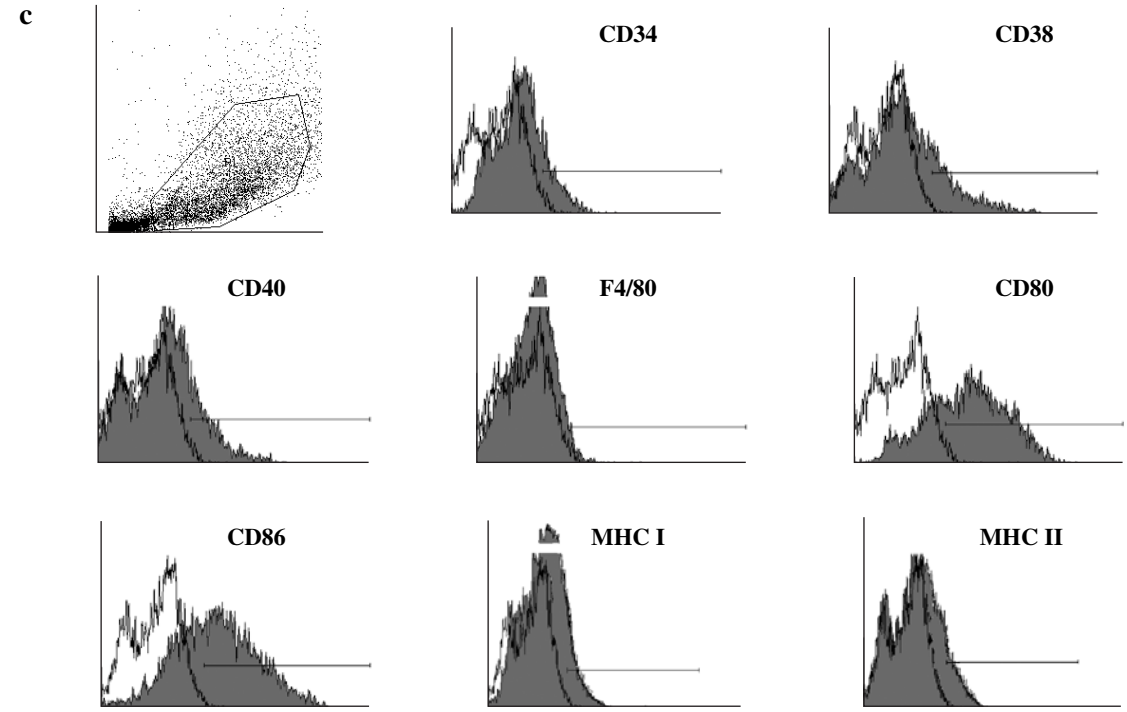

Fig. 1. Histograms show expression of surface molecules on mature DCs generated in the presence of: a) Immunovak VP-4 (50 ng/ml), b) *K. pneumoniae* K_2 lysate (50 µl/ml), c) recombinant TNF-α (20 ng/ml)

Upper line, dotplot: light dispersion (gated mature DC population), histograms: left peak shows autofluorescence of cells stained with isotopic control, right peak – fluorescence of cells stained with corresponding antibodies (FITC and R-PE conjugated).

y-axis – number of cells, x-axis – fluorescence (relative units); CD – differentiation antigen; MHC I, II – molecules of major histocompatability complex, class I and II.

corresponds to 5 cell dose infection). The follow up continued 2 weeks with no deaths among DC-treated animals.

DCs had phagocytic activity against various conditionally--pathogenic microorganisms and phagocyted latex particles. Phagocytic index (PI_{30}) of immature DCs was 95.12±1.56%, phagocytic number (PN_{30}) accounted for 15.31±1.2%, while ability for phagocytosis decreased along with maturation rate. Pulsed DC PI_{120} was 44.3±2.5%, PN_{120} – 6.7±0.88% ($p<0.05$).

Cytokine rate (IL-1β, IL-6, IL-12, INF-γ and TNF-α) increased significantly in DC culture medium as compared with immature DCs, the highest concentrations were those of IL-6 (566.2±10.3 pg/ml), IL-12 (152.1±7.2 pg/ml), TNF-α (733.2±12.8 pg/ml) and INF-γ (159.8±6.4 pg/ml) (table 3). The rate of IL-2 and IL-10 did not change, while IL-4 production by mature DCs decreased significantly compared to that of immature cells (table 3).

Table 3. Induction of cytokines by DCs

DC	IL-1β	IL-2	IL-4	IL-6	IL-10	IL-12	TNF-α	IFN-γ
immature	1.03±0.05	13.6±1.5	44.9±5.3	90.9±4.8	101.4±5.9	5.0±0.03	77.2±5.6	18.42±2.1
mature	26.6±1.7**	13.6±1.7	19.1±1.8*	566.2±10.3**	109.6±6.2	152.1±7.2**	733.2±12.8**	159.8±6.4**

*Reliable difference * – p<0,05; ** – p<0,001.*

Table 4. Ability of dendritic cells to stimulate proliferation of syngeneic mononuclear leukocytes

Group	Concentration of components in cultural medium						Proliferative activity of ML (relative units)	
	ML	DC	DC maturation inductors					
			VP-4 µl/ml	lysate µg/ml	LPS µg/ml	TNF-α ng/ml	Optical density	Stimulation index (SI)
1	10^6	0	0	0	0	0	0.650±0.073	–
2	10^6	5×10^4	0	0	0	0	0.666±0.054	–
3	10^6	5×10^4	0	0	0	100	1.458±0.074*	2.2
4	10^6	5×10^4	50	0	0	0	1.592±0.080*	2.3
5	10^6	5×10^4	0	50	0	0	1.564±0.090*	2.3
6	10^6	5×10^4	0	0	0.125	0	1.348±0.033*	2.0

*SI – stimulation index is the ratio of ML proliferative activity stimulated by mature DCs to ML proliferative activity stimulated by immature DCs (group 2). Reliable difference between groups: * – p_2 and $p_{3, 4, 5, 6} < 0,05$.*

The results of the proliferative activity test of the MLs generated from murine bone marrow precursors revealed DC effect increasing syngeneic ML blast-transformation (table 4). Spontaneous ML proliferative activity in this study accounted for 0.650±0.073 relative units (group 1). MLs cultured in the presence of mature DCs pulsed by bacterial agents and TNF-α showed increased proliferative activity ($p<0.05$) and furthermore, all the studied agents enhanced DC stimulating activity equally. On the other hand, immature DCs had no marked effect on ML proliferation.

Discussion

Mature DCs have a number of special features that enable them to present an antigen in the most effective way as compared with the immature cells [5, 8]. Only mature DCs express high rate of co-stimulating molecules that mediate formation of necessary signals for lymphocyte activation, as well as high rate of MHC I and MHC II that make effective antigen presentation possible [13]. Thus the results of present studies comply well with the data of other authors [11, 12].

High rate of dendritic cell phagocytic activity suggests their possible implication for DC-vaccine generation for biotherapy of infectious diseases [14, 24]. Significant increase in IL-6, IL-12 and TNF-α production by mature DCs generated from murine bone marrow precursors and cultured in the presence of the studied agents indicates essential role of DCs in immune cell activation. IL-6 is a powerful factor of B- and T-lymphocyte differentiation; in particular, it is the main inducing factor of the final B-cell and macrophage maturation as well as cytotoxic lymphocyte differentiation. IL-6 and IL-3 synergic effect triggers differentiation of bone marrow stem cells. TNF-α enhanced concentration leads to increase of IL-6 production [3].

The increase of IL-12 in the medium of the generated cell culture induces INF-γ production by T-cells that consequently stimulates T-lymphocytes to acquire Th1 characteristics [5]. Therefore the key role of IL-12 is stimulating precursor cell differentiation into Th1-cells, activating naïve $CD8^+$ T-cell differentiation into functionally active cytotoxic T-lymphocytes and enhancing NK cytolytic activity and proliferation. DCs probably produce a number of cytokines that may promote activation of complex inter--cellular interaction mechanisms. Protective activity of immature DCs against *K. pneumoniae* infection may be associated with the following induction of cytokine synthesis by DCs *in vivo* resulting from the bacteria effect. It is well known that pathogens can modify DC function. In particular, *M. tuberculosis* interacts with DCs and deactivates these cells. *P. gingivalis* can exist in DC precursors ($CD4^+$ cells) for over 24 hours because of the lack of lysosomal system in DCs, while monocytes can destroy the pathogen within 60 minutes [5]. However immature DCs in the experimental study could enhance innate immunity mechanisms via TLRs (Toll like receptors), in particular, macrophage and NK activity that can lyse cells with any disorders and do not involve any specific receptors or MHC molecules in this process. Thus the obtained results suggest that DCs play the key part in inter-cellular interactions when non-specific immune response is being formed.

Generation of DC-based vaccines for adoptive immunotherapy can be a perspective approach of prevention and treatment of infectious diseases. It is important to consider the effect of different factors, namely, pathogen specificity and DC effect on immune response type, when generating vaccines on the base of dendritic cells.

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