GENERATION OF MATURATIONALLY AND FUNCTIONALLY DIFFERENT POPULATIONS OF HUMAN DENDRITIC CELLS FROM MONOCYTES IN VITRO FOR POTENTIAL TARGETING WITH BIOCOMPATIBLE MICROSPHERES

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Dendritic cells (DC) are the most potent among the antigen–presenting cells (APC). They constitutively express the major histocompatibility complex (MHC) class II and costimulatory molecules, like CD86, and are able to increase the expression of those molecules manyfold upon stimulation. This makes them the most important cell type in the creation, maintenance and control of T cell–dependent immune response.

The first step in the exertion of DC functions is antigen endocytosis. The process enables DC to sample many and diverse antigens that they come across at their strategic locations in peripheral tissues, to process and present these antigens to naïve T cells.

When a DC engulfs an antigen connected with a danger signal (proinflammatory cytokines, microbial components, products of necrotic cells), this cell enters the path of maturation, in the course of which its morphology, function and phenotype is dramatically changed. This includes a decrease in endocytic ability and a great increase in the expression of MHC and costimulatory molecules. This is in accord with the change of the role of the maturing DC from that of sentinel cells into highly efficient APC (Banchereau et al, 1998).

Nanoparticles are artificially composed molecular structures. They are synthesized from biocompatible organic polymers, like poly(D,L–lactic–co–glycolic acid) (PLGA), through the process of auto–assembly. This process makes it possible to incorporate a desired molecule into the core of the growing nanoparticle (Newman et al, 1998; Coester et al, 2004).

Since such particles are readily endocytosed by phagocytes, including DC, they are a very good vehicle for transporting any substance into those cells. The potential benefit is obvious: an antigen within the nanoparticle, taken up by DC, is subsequently presented to T cells, followed by initiation of specific T cell immune response (Newman et al, 1998). This is particularly useful with weakly immunogenic antigens.

The purpose of this study was to compare endocytic, phenotypic and allostimulatory potential of DC generated in vitro from monocytes (MDDC) and treated with different maturational stimuli in order to use them as potential target for nanoparticle technology.

MATERIALS AND METHODS

Monocytes were obtained and purified from buffy coats of healthy volunteers by Lymphoprep gradient (Nycomed, Oslo, Norway) and plastic adherence, as previously described (Čolić et al, 2003). Monocytes were cultivated for 6 days in six–well tissue culture plates (Flow, Irvine, Scotland, UK) in 2ml of complete RPMI–1640, HEPES/sodium bicarbonate buffered medium with the addition of 10% fetal calf serum (FCS) (ICN, Costa Mesa, CA, USA), streptomycin, gentamycin, penicillin and 2–ME. Medium was supplemented with 100ng/ml of GM–CSF and 5 μg/ml of interleukin (IL)–4. After 6 days cells were fed with 1ml of fresh medium with GM–CSF and IL–4 alone or with addition of 1μg/ml LPS or a cocktail of proinflammatory mediators (10ng/ml IL–1β, 10ng/ml IL–6, 10ng/ml TNFα and 1μM PGE_2) and cultivated for additional 2 days (Čolić et al, 2003; Obermaier et al, 2003).

Phenotypic characteristics of immature and mature MDDC were tested by an immunofluorescence method and flow cytometry, using monoclonal antibodies (mAbs). The following mAbs were used: anti–CD83 and anti–CD86 uncojugated mAbs and HLA DR conjugated with fluorescein isothiocyanate (FITC) (Serotec, Oxford, London, UK). Cells incubated with unconjugated mAbs were additionally incubated with anti–mouse (Fab–2) Ig–FITC antibody (Serotec) and 5% human AB serum. Nonspecific fluorescence was assessed using irrelevant FITC–conjugated mouse Ig or PBS instead of mAb. Cells were analyzed on a flow cytometer, EPICS XL–MCL (Coulter, Krefeld, Germany).

Mannose receptor (MR)–mediated endocytosis was measured as the cellular uptake of dextran–FITC and quantified by flow cytometry after incubating cells

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with 2mg/ml dextran–FITC (mol. mass 40000; Sigma, Munich, Germany) for 60 min at 37°C or at 4°C (for control binding). At least 5000 events per sample were analyzed.

Alloreactivity was tested using classical mixed leukocyte reaction (MLR). Peripheral blood mononuclear cells (PBMNC) were isolated from buffy coats using Lymphoprep gradient and used as responders in MLR with different numbers of allogeneic MDDC as stimulators. Cells were cultivated in complete RPMI–1640 medium with 10% FCS in 96–well flat–bottomed cell culture plates for 6 days. Cells were pulsed with 1μCi/well of [^{3}H]–thymidine (Amersham, Bucks, UK) for the last 18h. Radioactivity was counted in a Beckman scintillation counter and expressed as counts per minute (cpm) ±SD of triplicates.

RESULTS AND DISCUSSION

There are different approaches in targeting DC in vitro with antigens, viral vectors, mAbs or other carriers (Fong et al, 2000). For such purposes optimization of protocols for generation of DC in vitro is needed. In a model of human DC generated in vitro from monocytes we studied endocytic, phenotypic and allostimulatory activity of these cells.

At first we compared endocytic activity of immature DC and DC induced to mature with LPS or with a cocktail of proinflammatory mediators. As presented in Fig. 1 endocytosis of dextran–FITC, a mannose receptor mediated process (Čolić et al, 2003), was higher by immature DC. Immature DC also express lower levels of costimulators (CD86) and MHC class II molecules than mature DC (Fig. 2). These results are in agreement with well–known function of immature DC related to antigen uptake and processing (Banchereau et al, 1998). Up to now a PLGA nanoparticles have not been used for studying the endocytosis of human DC. A recent paper on murine bone marrow derived DC showed that PLGA and gelatin nanoparticles were significantly taken up and localized intracellularly within DC. In contrast, albumin nanoparticles had much less uptake and concentrated mainly on DC surface (Coester et al, 2004). Although in the work published by Coester et al, 2004, the maturation state of DC has not been studied, based on obtained results it can be hypothesized that those DC were immature.

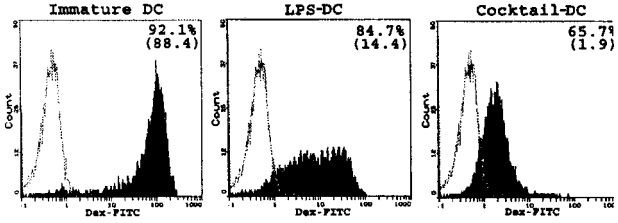

Figure 1. Endocytosis of dextran–FITC by immature and mature MDDC

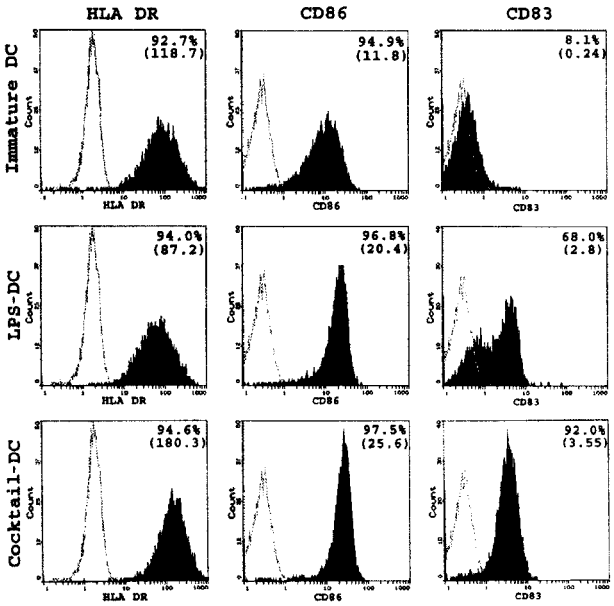

Figure 2. Phenotypic characteristics of immature and mature MDDC

In our work we showed that the cocktail of proinflammatory mediators induced better maturation of DC than LPS, as judged by greater decrease of dextran–FITC endocytosis, higher upregulation of MHC class II, CD86 and CD83 expression (Fig. 1 and Fig. 2), as well as higher stimulatory activity for proliferation of allogeneic lymphocytes (Fig. 3).

The uptake of dextran–FITC by MDDC, cultivated under different conditions, was determined by flow cytometry. Results are presented as histograms (Figure 1) of fluorescence (black), percentage of positive cells and mean fluorescence intensity (in parenthesis). Gray histograms represent negative controls. Results are from one representative experiment out of five with similar results.

Phenotypic characteristics (HLA DR, CD86 and CD83) of MDDC, cultivated under different conditions were assessed by flow cytometry. Results are presented as histograms (Figure 2) of fluorescence (black), percentage of positive cells and mean fluorescence intensity (in parenthesis). Gray histograms represent negative controls. Results are from one representative experiment out of five with similar results.

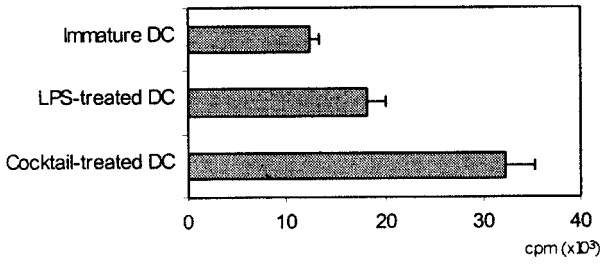

Figure 3. Allostimulatory capacity of immature and mature MDD

For testing the allostimulatory activity DC ($2.5x10^3$/well) were co–cultured with allogeneic lymphocytes ($2x10^5$ cells/well) in 96–well plates for 6 days. During the last 18h of incubation cultures were pulsed with ^{3}H–thymidine (1μCi/well). Proliferation was measured as count per minute (cpm). Results are given as mean cpm ±SD of triplicates of one representative experiment out of five with similar results (Figure 3).

Mature DC, expressing CD83, a maturation marker, and costimulatory molecules (CD80, CD86 and CD40), are the most potent APC that stimulate activation of našve T cells (Banchereau et al, 1998). Based on our results it can be concluded that proinflammatory mediators (IL–1β, IL–6, TNFα and PGE_2) are better maturational stimuli of DC than LPS alone. However, these results do not mean that DC treated with the proinflammatory mediators are better inducers of the Th1 immune response. Our unpublished data (Čolić at al; manuscript in preparation) showed that LPS–treated human DC in vitro produce more IL–12 then DC treated with the cocktail of proinflammatory mediators. It is well known that IL–12 is one of key cytokines that induces polarization of the T cell immune response towards Th1, and that induction of the Th1 immune response is needed for antitumor immunity. Therefore, many protocols for tumor immunotherapy are based on in vitro generation of DC with capability to stimulate generation of Th1 cells (Fong et al, 2000).

In vitro modulation of DC prepared for in vivo immunotherapy is also possible at the level of mature DC. Mature DC better migrate to regional lymph nodes where they activate T cells (Banchereau et al, 1998). At this stage of DC differentiation manipulation is possible using mAbs against specific DC membrane markers as carriers for different antigens or biological modifiers. An illustrative example is the use of anti–DEC205 mAb as a carrier for ovalbumin. The results showed that endocytosis and subsequent processing mechanisms mediated trough DC205, which recognizes carbohydrate structures on different antigens, are more efficient than classical processing pathways (Bonifaz et al, 2002).

CONCLUSIONS

In this study we established an in vitro method to obtain human DC from monocytes with high endocytic ability and relatively poor allostimulatory capability, as well as DC with low endocytic activity, but good allostimulatory potential. These maturationally and functionally different DC populations can be targeted differently in vitro for immunotherapy in vivo.

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