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An improved method to generate equine dendritic cells from peripheral blood mononuclear cells: Divergent maturation programs by IL-4 and LPS

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Abstract

Equine dendritic cells (eqDC) can be generated from peripheral blood monocytes by propagation in GM-CSF and IL-4. Despite similarities with the generation of human DC, we found significant improvements for eqDC generation and functional influences on eqDC maturation. The fractionation of peripheral blood mononuclear cells (PBMC) by two subsequent gradients at densities of 1.090 and 1.077 as well as an adherence step in AIM V® medium on dishes coated with extracellular matrix components (Primaria®) improved the purity and yield of DC. After 3 days, eqDC cultures with GM-CSF alone developed into three subsets of (i) MHC II^{neg} cells, (ii) MHC II^{low} immature, endocytic cells and (iii) MHC II^{high} spontaneously mature, non-endocytic DC. The immature DC fraction of the GM-CSF cultures matured, as detected by MHC II up-regulation, upon LPS exposure overnight. DC cultures in GM-CSF plus IL-4 resulted in higher cell yields, a loss of the immature MHC II^{low} population but increased mature MHC II^{high} DC, suggesting maturation. However, the MHC II^{high} DC fraction was still endocytically active and did not lose their endocytic function after LPS treatment. They marginally up-regulated MHC II expression but this did not result in an enhanced stimulation of an allogeneic mixed lymphocyte reaction. However, LPS treatment clearly induced mRNA for IL-12p35 and p40, which was not observed by addition of IL-4 alone. Together our data indicate that IL-4 and LPS induce two different maturation programs. IL-4 induces a semi-maturation where the cells are still endocytic, which can be further matured to secrete cytokines in a second step by LPS.

Keywords: Culture method; Dendritic cell; Horse; Maturation

Introduction

Dendritic cells (DC) represent sentinel cells of the innate immune system, which can, upon migration into lymph nodes, communicate with adaptive immune cells (Steinman and Hemmi, 2006). The outcome of this

Abbreviations: DC, dendritic cells; Eq, equine; PBMC, peripheral blood mononuclear cells.

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crosstalk may be immunity in the case of infection or tolerance in the case of self-antigen presentation in the steady state (Tarbell et al., 2006; Lutz and Schuler, 2002). The presence of epidermal Langerhans cells in horses as a representative of the DC family was first described in 1992 (Hamada et al., 1992). Initial attempts used an adherence step and a Metrizamide gradient to culture monocytes without growth factors (Siedek et al., 1997). With this method only a few DC could be identified among the macrophages dominating the cultures. The identification of equine (eq) cytokines allowed the study of eq immunology in more detail and also shed more light on eqDC (Steinbach et al., 2002, 2005). The generation of eqDC from monocytes (eqMoDC) succeeded with human GM-CSF and eqIL-4 (Hammond et al., 1999). Finally, eqDC generation was performed exclusively with eq cytokines (Mauel et al., 2006).

The understanding of eqDC biology combined with techniques for their efficient generation at defined immature and mature stages may allow cellular immunotherapy with eqDC as a therapeutic tool in the future (Vecchione et al., 2002). Here, we present an improved method to generate eqMoDC by performing the adherence step in a specific medium and on specific culture plates. We describe differences in eqDC generation by GM-CSF alone or together with IL-4. When IL-4 was used to generate DC, the cells appeared mature regarding their surface marker expression and this could not be further induced by LPS. However, these IL-4-cultured eqDC further matured by induction of IL-12 mRNA production.

Materials and methods

Blood preparation and DC generation

Horses of various blood lines and both sexes between 6 and 16 years of age from the "Reitstall Behr" in Buttenheim and the "Pferdeklinik" Dr. Vornberger in Speikern, Germany, were used to collect blood. Peripheral blood from healthy horses was taken from the jugular vein and collected in heparinized syringes. After centrifugation for 15 min at RT at 750*g* the plasma was carefully removed. Cells were resuspended in 50 ml PBS and 30 ml of this was layered on 10 ml of a first Biocoll® gradient (Biomol, Berlin, Germany, density 1.090) and centrifuged at RT for 20 min at 500*g*. The interphase consisting of leukocytes was washed in PBS/2% EDTA and resuspended in 30 ml PBS. This fraction was layered on a second gradient of 10 ml Lymphoprep® (Fresenius, Bad Homburg, Germany), density 1.077 and centrifuged at RT for 20 min at 500*g*. The resulting interphase contained mostly lymphocytes and monocytes, while granulocytes were pelleted. The interphase was washed twice in PBS before cells were resuspended in an adherence medium. As adherence medium either RPMI-1640 (Bio-Whitaker, Cambrex, New Jersey, USA) containing 10% heat-inactivated fetal calf serum (PAA, Cölbe, Germany), 292 μg/ml L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin or AIM V® medium was used (R10) (Gibco, Paisley, UK). Cells were plated into 10-cm-petri dishes of standard quality (Falcon, #3003) or Primaria® quality (Falcon) in 10 ml at a density of $5–6 \times 10^7$ cells per plate for 45 min in a 5% CO_2/37 °C incubator. After removal of the non-adherent fraction the adherent cells were cultured in R10 medium with human GM-CSF (Tebu, Offenbach, Germany) and eq IL-4 (R&D Systems, Minneapolis, USA) as indicated in the figure legends or at standard doses of 2000 U/ml huGM-CSF and 10 ng/ml eqIL-4 at day 0, respectively. Cells were fed by adding 10 ml R10 medium containing 500 U/ml GM-CSF and 10 ng/ml IL-4 at days 2, 4 and 6. As a standard, cells were harvested at day 3 or 4 if not indicated otherwise. DC maturation was performed with indicated amounts of LPS (*Pseudomonas aeruginosa* from horse, Sigma L9143).

Endocytosis and FACS analysis

The uptake of FITC-conjugated ovalbumin (Molecular Probes, Invitrogen) by eqDC for 30 min at 4 °C or at 37 °C was described for murine DC previously (Lutz et al., 1999). FACS analysis was performed essentially as described in detail earlier (Lutz et al., 1999). The following murine antibodies against eq or human antigens were used: eqMHC I, eqMHC II, eqCD4, eqCD8, (Serotec), huCD83 (clone HB-15e, BD Pharmingen), huCD86 (clone IT2.2, BD Pharmingen) and the appropriate isotype controls. Monoclonal antibodies in hybridoma cell supernatants, which do detect the markers EqWC1 (CZ 1.1), EqWC2 (CZ 1.5) and EqWCCD11a/CD18 (CZ1.6)(Zhang et al., 1998), were kindly provided by D. F. Antczak (James A. Baker Institute for Animal Health, Cornell University Ithaka, NY, USA) and detected with goat-anti-mouse IgG or IgM phycoerythrin-conjugated antibodies. Cross-reactivity of human markers for eq cells has been described before (Saalmuller et al., 2005).

Cytospins

A total content of $5–20 \times 10^4$ DC were centrifuged in a Cytospin-3 (Shandon) for 3 min at RT at 500 rpm onto glass slides, air dried and stained with May–Grünwald–Giemsa dye according to standard protocols. Photographs were taken with a Nikon Coolpix 990 camera.