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Short communication

Identification and characterization of equine blood plasmacytoid dendritic cells

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ABSTRACT

Dendritic cells (DC) are antigen-presenting cells that can be classified into three major cell subsets: conventional DC1 (cDC1), cDC2 and plasmacytoid DCs (pDC), none of which have been identified in horses. Therefore, the objective of this study was to identify and characterize DC subsets in equine peripheral blood, emphasizing on pDC. Surface marker analysis allowed distinction of putative DC subsets, according to their differential expression of CADM-1 and MHC class II. Equine pDC were found to be $Flt3^+$ $CD4^{low}$ $CD13^-$ $CD14^-$ $CD172a^-$ $CADM\text{-}1^-$ $MHCII^{low}$. The weak expression of CD4 on equine pDC contrasts with findings in several other mammals. Furthermore, pDC purified by fluorescence-activated cell sorting were found to be the only cell subset able to produce large amounts of IFN-α upon TLR9-agonist stimulation. The pDC identity was confirmed by demonstrating high-levels of *PLAC8*, *RUNX2* and *TCF4* expression, showing pDC-restricted expression in other mammals.

1. Introduction

Dendritic cells (DC) represent the professional antigen-presenting cells of the immune system and are essential regulators of immunity and tolerance (Banchereau and Steinman, 1998). They can be further categorized into conventional DC (cDC), which are responsible for antigen presentation and induction of T-cell responses, and plasmacytoid DC (pDC) representing the most potent type I interferon (IFN) producing cell, able to efficiently sense microbial nucleic acid (Liu, 2005). Conventional DC consist of at least two phenotypically distinct subsets stimulating particular T-cell responses (Schlitzer and Ginhoux, 2014). The murine $CD8\alpha^+$/human $CD141^+$ cells comprise the cDC1 subset, specialized in cross-presentation of antigens to $CD8^+$ T-cells and stimulating Th1 immunity (Bachem et al., 2010; Jongbloed et al., 2010). The cDC2 subset was identified as murine $CD11b^+$/human $CD1c^+$ cells (Haniffa et al., 2013), and is particularly capable to stimulate Th2 and Th17 immunity (Dutertre et al., 2014; Tussiwand and Gautier, 2015). Both pDC and cDC arise from a common DC progenitor in the bone marrow (Liu and Nussenzweig, 2010; Onai et al., 2007) and are dependent on the growth factor fms-like tyrosine kinase 3 ligand (Flt3L) for their development and differentiation (Karsunky et al., 2003; Schmid et al., 2010). Accordingly, all DC subsets express Flt3 receptor (CD135).

A number of reports described the presence of distinct cDC subpopulations in veterinary species, including pigs (Guzylack-Piriou et al., 2010; Maisonnasse et al., 2015; Summerfield et al., 2015; Auray et al., 2016, submitted), cattle (Howard et al., 1999; Renjifo et al., 1997) and sheep (Contreras et al., 2010; Pascale et al., 2008). Additionally, pDC have been identified in pig (Summerfield et al., 2003), in cattle (Reid et al., 2011) and in sheep (Pascale et al., 2008).

However, neither cDC nor pDC have been described in horses. Studies that have been performed to date, resorted to the use of monocyte-derived dendritic cells (MoDC) (Cavatorta et al., 2009; Mauel et al., 2006; Moyo et al., 2013). Although MoDC possess many functional attributes consistent with DC (Sallusto and Lanzavecchia, 1994), they do not represent *bona fide* DC based on their ontogeny. Nevertheless, many *in vivo* studies report MoDC as a distinct subtype of inflammatory monocytic cell, sharing some

Abbreviations: ODN, oligodeoxynucleotides; DC, dendritic cells; cDC, conventional DC; FMO, fluorescence minus one; Flt3, fms-like tyrosine kinase 3; Flt3L, Flt3 ligand; IFN, interferon; MoDC, monocyte-derived DC; pDC, plasmacytoid DC; PBMC, peripheral blood mononuclear cells.

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features with cDC in terms of antigen presentation (Guilliams et al., 2014; Schlitzer et al., 2015). Consequently, for advances in immunological research in horses, it is essential to gain more information on equine *bona fide* DC.

The aim of the present study was to phenotypically characterize equine blood DC and functionally identify pDC in healthy horses. As a first step, appropriate cell surface markers which were either equine-specific or shown to cross-react with the equine system were used to identify distinct cell subsets in equine peripheral blood mononuclear cells (PBMC) by flow cytometry. Based on the comparative approach used by Summerfield et al. (2015), a first classification of equine DC subsets is proposed. The identity of purified pDC was further confirmed by a robust IFN-α production upon TLR9-stimulation as well as by a high expression of pDC-specific transcripts.

2. Methods

2.1. Isolation of peripheral blood mononuclear cells from healthy horses

Blood samples were collected from the jugular vein of eleven healthy horses (age = 4–21 years) using sterile glass bottles supplemented with 5000 IU/ml heparin (Liquemin®, Drossapharm AG, Basel, Switzerland) or Sodium-Heparin containing vacutainers (Vacuette®; Greiner, St.Gallen, Switzerland). The study was approved by the Animal Experimental Committee of the Canton of Berne and Vaud, Switzerland (No. BE 51/13).

PBMC were isolated by density gradient centrifugation over Biocoll (ρ = 1.077 g/ml, Biochrom GmbH, Berlin, Germany) as described (Hamza et al., 2007).

2.2. Production of recombinant bovine Flt3 ligand

Bovine instead of equine Flt3L was used because the complete equine Flt3L gene sequence is still unknown. The partial equine Flt3L sequence (Genbank: XP_005596791.1) exhibits 73% amino acid sequence homology to bovine Flt3L. Bovine Flt3L (NCBI NM_181030.2) was produced as previously described (Guzylack-Piriou et al., 2010) and was originally employed for another study (Baumann et al., unpublished data). Briefly, after deletion of the stop codon, the Flt3L sequence was flanked by *Hin*dIII and *Xba*I restriction sites and was chemically synthesized in pUC57 plasmid (GenScript, Piscataway, NJ, USA). After *Hin*dIII and *Xba*I digestion, Flt3L was ligated in the pEAK8-His expression vector. The TOP10 Chemically Competent *E. coli* cells (Invitrogen, USA) were transformed with the plasmid pEAK8-His containing the bovine Flt3L. For final recombinant production of bovine Flt3L, HEK 293 cells were transfected with pEAK8-His-Flt3L using X-tremeGENE 9 following the manufacturer's instructions (Roche, Basel, Switzerland). After 5 days, supernatant was collected and expression of recombinant bovine Flt3L was assessed by western blot using an anti-his-HRP antibody (cat. no. 130-092-785, Miltenyi Biotec; Antibody Register: AB_1103231). The reaction was visualized with a WesternBright ECL Western blotting detection kit (Advansta Inc., Menlo Park, CA, USA) and a CCD-LAS3000 camera (Fuji Film) (Fig. S1).

2.3. Surface marker analysis by flow cytometry

PBMC from three horses were transferred to 5 ml FACS tubes at 3×10^6 cells per tube. First, a blocking step was performed using Chrome Pure whole mouse IgG (Jackson Immunoresearch, West Grove, PA, USA). All incubations were performed for 20 min on ice, followed by washing with PBS and centrifugation at 500×*g* for 5 min. Briefly, cells were incubated with recombinant bovine Flt3L, followed by labelling with a PE-conjugated anti-his antibody (clone GG11-8F3.5.1, cat-no 130-092-691; Miltenyi Biotec; Antibody Register: AB_1103227). Anti-human CADM-1 (cat no. CM004-3, MBL International Corporation; Antibody Registry: AB_592783), shown to specifically bind to the CADM-1 molecule in many mammalian species (Contreras et al., 2010; Dutertre et al., 2014), was detected by a secondary goat biotinylated anti-chicken antibody (cat no 103-065-155, Jackson Immunoresearch; Antibody Registry: AB_2337383), followed by labelling using Brilliant Violet (BV) 421 conjugated to Streptavidin (BD Biosciences, Franklin Lakes, NJ, USA). Other surface markers were stained using the following antibodies: anti-equine CD4 (clone CVS4; cat no MCA1078, Bio-Rad; Antibody Register AB_321274), anti-equine CD13 (clone CVS19; cat no MCA1084GA, Bio-Rad; Antibody Registry: AB_321308), anti-equine MHCII (clone CVS20; cat no MCA1085, BioRad; Antibody Register AB_321618), anti-equine CD14 (clone 105; https://courses2.cit.cornell.edu/wagnerlab/research/reagents.htm; Kabithe et al., 2010), anti-bovine CD172a (clone HR-DH59B; cat no HR-BOV2049; Monoclonal Antibody center, Washington State University; Pullman WA, USA) showing cross-reactivity with equine cells (Mérant et al., 2009). These monoclonal antibodies were labelled with mouse IgG1 Alexa Fluor 488, 647 or 700 Zenon labelling kits (Thermo Scientific, Waltham MA, USA). Appropriate isotype and fluorescence-minus-one (FMO) controls were used. Finally, cells were resuspended in phosphate buffered saline (PBS) and analysed on a LSRII flow cytometer (BD Biosciences). Automated compensation for spectral overlap of fluorochromes was calculated by the BD FACSDIVA acquisition software (BD Biosciences) based on single-stained PBMC. Data were analysed using FlowJo software 6 (Tree Star Inc. Ashland OR, USA). Gates were set to exclude doublets and lymphocytes based on forward and side scatter characteristics (Fig. 1A). Within this cell population, a further gate was set on $Flt3^+/CD14^{low}$ cells, based on isotype and fluorescence minus one (FMO) controls. This gating strategy was used for all flow cytometry experiments.

2.4. PBMC stimulation for IFN-α production

PBMC were isolated as described above and suspended in RPMI 1640 medium with HEPES and L-glutamine (Gibco, Life Technologies Ltd, Paisley UK) supplemented with 1% penicillin and streptomycin (Gibco), 1% MEM vitamins, 1% Na pyruvate, 1% Non-essential amino acids (all Biochrom GmbH) and 10% inactivated horse serum (Ziegler et al., 2016, in revision) at a density of 2×10^5 cells per 200 μl medium in a 96-well round-bottom cell culture plate (Sarstedt, Nümbrecht, Germany). Cells were cultured for 24 h in the presence of 5 μg/ml of the synthetic TLR9 agonist Type C CpG-oligodeoxynucleotides (ODN) D-SL03 (InvivoGen, San Diego, CA, USA) or of equine herpesvirus-1 (EHV-1; MOI of 0.04 $TCID_{50}$/cell, EHV1-V144/64, kindly supplied by Prof. Reto Zanoni from the Institute for Virology and Immunology, Vetsuisse Faculty, University of Berne, Switzerland). Thereafter, cell culture supernatants were collected and stored at −80 °C until used.

2.5. Enrichment of pDC by depletion of PBMC from $CD5^+$ and $CD14^+$ cells

PBMC were depleted of $CD5^+$ and $CD14^+$ cells by magnetic separation (MACS technology, Miltenyi Biotec GmbH) according to standard protocols by the manufacturer, using a monoclonal anti-equine CD5 antibody (clone CVS5; cat no MCA1079GA, Bio-Rad; Antibody Register: AB_321382) and anti-equine CD14 (clone 105, Kabithe et al., 2010). Briefly, PBMC were first incubated with anti-CD5 and anti-CD14 simultaneously and, after a washing step,