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Two functionally distinct myeloid dendritic cell subpopulations are present in bovine blood

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ABSTRACT

Immature myeloid (m)DCs circulating in the blood of cattle have been defined as lineage negative (Lin^-)-$MHCII^+CD11c^+CD205^+$ cells. $Lin^-MHCII^+CD11c^+CD205^+$ mDCs (0.2% blood mononuclear cells) isolated from bovine blood were heterogeneous in cell size and CD205 expression. Using highspeed cell sorting, $Lin^-MHCII^+CD11c^+CD205^+$ DCs were sorted into $CD205^{Hi}$ and $CD205^{Lo}$ subpopulations which were phenotypically distinct and differed significantly ($P < 0.01$) in TLR gene expression. $CD205^{Hi}$ and $CD205^{Lo}$ mDCs were more efficient in macropinocytosis than monocytes and expressed no or little detectable non-specific esterase activity. $CD205^{Lo}$ mDCs efficiently activated purified allogeneic T cells and the addition of TLR agonists did not significantly alter this antigen presentation capacity. T cell activation by $CD205^{Lo}$ mDCs was associated with differential up-regulation of CD40, CD80, CD86 and TGFβ1 gene expression when compared to $CD205^{Hi}$ mDCs. In conclusion, two phenotypically and functionally distinct $CD11c^+CD205^+$ mDCs were isolated from blood that had an equal capacity to acquire antigen but markedly different capacities to activate T cells.

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1. Introduction

Dendritic cells (DCs) are antigen presenting cell (APC) specialized in antigen (Ag) uptake and processing. They are the most efficient APC able to transport Ag to lymphoid tissues and present it to *naïve* T cells, providing a key link between innate and adaptive immunity (Palucka and Banchereau, 1999; Pinchuk et al., 1993; Shortman and Liu, 2002). Because DCs efficiently regulate co-stimulatory molecules and express up to 100 times more major histocompatibility complex (MHC) II–peptide than any other APC, a single DC is capable of activating up to 3000 *naïve* T cells (Banchereau and Steinman, 1998; Palucka and Banchereau, 1999; Shortman and Liu, 2002). It was accepted that once a mature DC presented Ag to T cells, in the context of co-stimulatory molecules and cytokines, the net result was induction of T cell activation and proliferation. Recent evidence indicates, however, that a mature DC phenotype is also required for the induction and maintenance of tolerance (Akbari et al., 2001; Joffre et al., 2009) which contradicts the concept that tolerance induction was the result of DCs inability to deliver proper co-stimulatory signals to T cells and not the maturation state of a DC subpopulation (Morelli and Thomson, 2007). It is presently not known if all DC subpopulations have the capacity to become tolerogenic or if this capacity is restricted to developmentally distinct lineages. Thus, it is critical to analyze the functional capacity of immature DCs circulating in blood to better understand the dual role DCs play as key regulators of acquired immune responses and tolerance.

Interest in DCs has grown since they were first described and characterized in mouse spleen (Steinman and Cohn, 1973). Since then, different DC subpopulations have been described. These subpopulations differ not only in function, phenotype and tissue distribution (Fries et al., 2011; Huang et al., 2000; Ishifune et al., 2011; Steinman and Cohn, 1973) but also in the type of T cell responses they elicit (de Jong et al., 2005; Shortman and Naik, 2007). The study of DC function has been restricted, however, by their low abundance in blood and tissues. This difficulty has been partially overcome by using a variety of methods to generate sufficient DCs *in vitro* for research and therapeutic purposes. The most widely used methods include DC differentiation from bone marrow precursors (BMDCs) and $CD14^+$ blood monocytes (MoDCs) with

Abbreviations: Ag, antigen; APC, antigen presenting cells; Lin^-, lineage negative; mAb, monoclonal antibody; MLR, mixed lymphocyte reaction; MoDC, monocyte derived dendritic cells; NSE, non-specific esterase; PBMCs, peripheral blood mononuclear cells.

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granulocyte–macrophage colony-stimulating factor (GM-CSF) and interleukin 4 (IL-4) (Inaba et al., 1992; Pinchuk et al., 2003; Sallusto and Lanzavecchia, 1994). However, important differences in the processing of Ag (McCurley and Mellman, 2010) and in the induction of T cell responses (Osugi et al., 2002) were found between human blood DCs and MoDCs. Differences in the humoral immune response induced by bovine MoDCs and BMDCs have also been reported (Pinchuk et al., 2003). These data highlight the importance of identifying and characterizing subsets of naturally occurring DCs to better understand their function *in vivo*.

Approximately 0.1–0.7% of bovine PBMCs (Renjifo et al., 1997) have been identified as DCs. Multiple DC subpopulations have been described in human blood (Dzionek et al., 2000) but their functional characterization has been limited by their scarcity. The large volume of blood required to isolate sufficient DCs for phenotype and function studies make cattle an ideal model to study blood DCs. Limited information is available, however, regarding the phenotype and function of bovine blood DCs (Gibson et al., 2012; Miyazawa et al., 2006; Renjifo et al., 1997). Methods used to purify DCs from bovine blood have been relatively inefficient and it was difficult to ascertain the purity of the resulting populations. Bovine DCs were isolated from PBMCs using immunomagnetic beads (Gibson et al., 2012; Miyazawa et al., 2006) and removal of the non-adherent fraction following *in vitro* incubations (Renjifo et al., 1997). These methods are time consuming and involve multiple cell manipulations that can alter DC activation and differentiation. Therefore, a more efficient and simple procedure is required to isolate highly purified, immature blood DCs subpopulations in sufficient numbers to analyze both phenotype and function. High-speed cell sorters provide a means to rapidly purify rare cell populations with minimum effect on cell viability and function (Ibrahim and van den Engh, 2003).

Using high-speed cell sorting, we identified two distinct $CD11c^{+}MHCII^{+}$ myeloid (m)DCs populations in bovine blood that were defined as lineage negative (Lin^{-}: $CD3^{-}$,$CD21^{-}$,$CD335^{-}$,-$CD14^{-}$) but co-expressed CD205 at either a high ($CD205^{Hi}$) or low ($CD205^{Lo}$) level. Sufficient highly purified DCs were isolated to perform a variety of phenotypic and functional analysis to determine that two functionally distinct immature mDC subpopulations are present in blood. Furthermore, we report that these two blood mDCs subpopulations have significantly different capacities to induce T cell activation which is associated with differential expression of co-stimulatory molecule and cytokine genes production.

2. Material and methods

2.1. Monoclonal antibodies

Primary monoclonal antibodies (mAbs), their specificity for bovine leukocyte antigens, isotype controls, and fluorochrome-conjugated secondary antibodies are listed in Table 1.

2.2. Peripheral blood mononuclear cells (PBMCs) isolation

All experimental protocols were reviewed and approved by University of Saskatchewan – University Committee on Animal Care and Supply and all procedures were performed following guidelines approved by the Canadian Council on Animal Care. Venous blood was collected from castrated male Holstein calves between 9 and 12 months of age, housed at the Vaccine and Infectious Disease research facility (Saskatoon, SK, Canada) using EDTA as an anticoagulant at a final concentration of 0.3%. PBMC were isolated as previously described (Arsenault et al., 2009). The single step Percoll (GE Healthcare Life Science, Quebec, CA) gradients used to isolated PBMCs were modified when purifying T cells (60% Percoll) and $CD14^{+}$ monocytes and $CD11c^{+}CD205^{+}$ DCs (55% Percoll).

2.3. Flow cytometry

PBMC were re-suspended at a final concentration of 20×10^{6} -cells/mL in phosphate buffered saline (PBS; pH 7.4) supplemented with 0.03% sodium azide (EMD Chemicals Inc. Toronto, CA) and stained as previously described (Fries et al., 2011). Labeled and fixed cells were stored at 4 °C in the dark until analyzed with a FacsCalibur (Becton–Dickinson, Franklin Lakes, NJ) using CellQuest acquisition and analysis software (version 3.3). A minimum of 10,000 events were captured for each sample analyzed. FL1 uses a 488 nm laser with a 530 nm filter to detect fluorescein isothiocyanate (FITC). FL2 uses a 488 nm laser with a 585 nm filter to detect phycoerythrin (PE). FL4 uses a 635 nm laser with a 661 nm filter to detect allophycocyanin. Isotype- and concentration matched irrelevant monoclonal antibodies (mAbs) were used to quantify non-specific labeling.

2.4. PBMC labeling for highspeed cell sorting

For sorting $Lin^{-}CD11c^{+}CD205^{Hi}$ and $CD11c^{+}CD205^{Lo}$ cells, 3×10^{8} bovine PBMC, at a final concentration of 1×10^{8} cells/mL PBS, were labeled with 10 μg anti-bovine CD3, 15 μg anti-bovine CD11c, 3 μg anti-bovine CD14, 20 μg anti-bovine CD21, 15 μg anti-bovine CD205 and 3 μg anti-bovine CD335. PBMC were incubated with mAbs for 20 min at 4 °C, with gentle mixing every 5 min. Cells were then pelleted by centrifuging at 311*g* for 8 min at 4 °C and washed twice with ice-cold PBS. The cell pellet was re-suspended in 3 mL PBS and the following fluorochrome-conjugated secondary antibodies were added: 15 μg goat anti-mouse IgG2b-FITC; 20 μg goat anti-mouse IgG1-PE; and 8 μg goat anti-mouse IgM-allophycocyanin. Cells were incubated in the dark at 4 °C for 15 min and then washed twice with ice-cold PBS. For monocyte sorting, 1×10^{8} PBMC were labeled with 4.5 μg anti-bovine CD11c and 1 μg anti-bovine CD14 mAb using the same protocol as described previously for DCs. For labeling with fluorochrome-conjugated secondary antibodies, PBMC were incubated with 5 μg goat anti-mouse IgG1-PE and 2.6 μg goat anti-mouse IgM-Allophycocyanin. For negative sorting of T cells, 1×10^{8} PBMC were labeled with 8 μg anti-bovine MHC II, 3 μg anti-bovine CD335 and 8 μg anti-bovine CD11c mAb and processed as previously described. Labeling with fluorochrome-conjugated secondary antibodies was performed by adding 5 μg goat anti-mouse IgG1-PE, 5 μg goat anti-mouse IgG2a-PE, and 5 μg goat anti-mouse IgM-PE.

2.5. High-speed cell sorting

Labeled PBMCs were adjusted to a final concentration of 1×10^{8} cells/mL in ice-cold PBS, filtered through 35 μm cell strainer capped 12 × 75 mm polystyrene round bottom tubes (BD Falcon ON, CA) to remove cell clumps. High-speed sorting was performed with a MoFlo XDP (Beckman Coulter Inc. CA, USA) equipped with a 488 argon and 633 HeNe laser. FITC, PE and Allophycocyanin fluorescence was collected through 529/25, 575/25 and 670/30 band-pass filters, respectively. Dot scatter plots of forward light scatter (FSC) and 90° light scatter (SSC) were displayed on a linear scale and used to define the first sort region (R1) which excluded dead cells and debris. Dot plots displaying FSC-Height/FSC-Width were used to define the second sort region (R2) to exclude doublets or larger cell clumps. To sort DCs, a sort region was set to include $CD11c^{+}$ and exclude Lin^{+} cells (R4). R4 was set at different positions on the CD11c log scale and samples analyzed on cytospins to identify the gate yielding the highest purity of DCs

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