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Short communication

Phenotypic heterogeneity of peripheral monocytes in healthy dogs

Natalie Gibbons[a], Michelle R. Goulart[a], Yu-Mei Chang[b], Konstantinos Efstathiou[c], Robert Purcell[d], Ying Wu[a], Laureen M. Peters[e], Mark Turmaine[f], Balazs Szladovits[e], Oliver A. Garden[a,g,*]

[a] Immune Regulation Laboratory, Comparative Physiology and Medicine Research Group, Department of Clinical Science and Services, Royal Veterinary College, Camden Campus, London, UK
[b] Research Support Office, Royal Veterinary College, Camden Campus, London, UK
[c] Department of Medical Therapeutics, General hospital of Athens 'Alexandra', Athens, Greece
[d] Department of Comparative Biomedical Sciences, Royal Veterinary College, Camden Campus, London, UK
[e] Department of Pathology and Pathogen Biology, Royal Veterinary College, Hawkshead Campus, London, UK
[f] Division of Biosciences, University College London, London, UK
[g] Department of Clinical Sciences & Advanced Medicine, University of Pennsylvania, School of Veterinary Medicine, Matthew J Ryan Veterinary Hospital, 3900 Delancey Street, Philadelphia, PA 19104, United States

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ABSTRACT

Monocytes are key cells of the innate immune system. Their phenotypic and functional roles have been investigated in humans, mice and other animals, such as the rat, pig and cow. To date, detailed phenotypic analysis of monocytes has not been undertaken in dogs. Two important surface markers in human monocytes are CD14 and MHC class II (MHC II). By flow cytometry, we demonstrated that canine monocytes can be subdivided into three separate populations: $CD14^{pos}$MHC II^{neg}, $CD14^{pos}$MHC II^{pos} and $CD14^{neg}$MHC II^{pos}. Both light and transmission electron microscopy confirmed the monocytic identity of all three populations. The $CD14^{pos}$MHC II^{neg} population could be distinguished on an ultrastructural level by their smaller size, the presence of more numerous, larger granules, and more pseudopodia than both of the other populations.

1. Introduction

Monocytes are a heterogeneous myeloid cell population comprising 5–10% of healthy human white blood cells (Martinez, 2009). They are implicated in a number of human diseases, including diabetes mellitus (Cipolletta et al., 2005), cardiovascular disease (Ghattas et al., 2013), renal disease (Ulrich et al., 2010), Crohn disease and ulcerative colitis (Stansfield and Ingram, 2015).

Three populations of human monocytes are currently acknowledged by the Nomenclature Committee of the International Union of Immunological Societies (Ziegler-Heitbrock et al., 2010), respectively called classical ($CD14^{high}CD16^{neg}$), intermediate ($CD14^{high}CD16^{low/high}$) and non-classical ($CD14^{low}CD16^{high}$); each population is thought to give rise to the next along a linear developmental pathway from classical to non-classical.

Two murine monocyte populations have been characterised, known as $Ly6C^{high}$ (CCR2 (C-chemokine receptor type 2)$^{high}CX_3CR1^{low}$) and $Ly6C^{low}$ ($CCR2^{low}CX_3CR1^{high}$) (Gordon and Taylor, 2005). Based on CCR2 expression levels, $Ly6C^{high}$ monocytes most resemble human classical monocytes, and $Ly6C^{low}$, non-classical monocytes. However, functional disparity between the species is recognised: for example, $Ly6C^{high}$ monocytes are rapidly recruited to sites of infection in the mouse, whereas non-classical and intermediate populations show the predominant responses in certain infections in human patients (Strauss-Ayali et al., 2007, Geissmann et al., 2003). This has led to difficulties in universally extrapolating findings from one species to the other.

Delineating monocyte populations is an important first step in elucidating their role in disease. Canine monocytes represent a key, unmet study area. To date, there is a dearth of studies addressing monocyte phenotypic heterogeneity in this species; for example, Sibley et al. (2013) describe canine monocyte markers, but not individual populations. We hypothesised that multiple monocyte populations exist in healthy dogs, similar to those in humans, and used established myeloid markers to delineate the populations.

* Corresponding author at: Department of Clinical Sciences & Advanced Medicine, University of Pennsylvania, School of Veterinary Medicine, Matthew J Ryan Veterinary Hospital, 3900 Delancey Street, Philadelphia, PA 19104, United States. Tel.: 215 898 1531.
E-mail addresses: ngibbons3@rvc.ac.uk (N. Gibbons), ogarden@upenn.edu (O.A. Garden).

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2. Materials and methods

2.1. Peripheral blood mononuclear cell isolation

Blood samples were procured by jugular venepuncture from 14 healthy canine blood donors after written informed consent was granted by the owners of the dogs. This protocol has passe scrutiny by the local ethical review committee before work was allowed to commence. The health status of these dogs was ascertained by meticulous clinical history and physical examination. The blood was collected into EDTA and maintained at 4 °C for a maximum duration of 24 h. Following dilution with phosphate buffered saline (PBS) containing 2% fetal calf serum (FCS), peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation using the SepMate™ protocol (StemCell Technologies, Cambridge, UK) and Histopaque-1077 (Sigma-Aldrich). The PBMCs were washed twice at room temperature in PBS/2% FCS for 5 min at 300 *g* at 4 °C, before re-suspension in 2 mL PBS/10% FCS and counting.

2.2. Flouresence-assisted cell sorting (FACS™)

PBMCs were stained with each of the antibodies in Table 1 at the specific concentration stated, and fluorescence minus one controls, using these same concentrations, were used to determine negative gates. Analytical flow cytometry was performed using a FACS Canto II (BD, Oxford, England). Cell sorting was performed using a FACS Aria III (BD) or a FACS Aria Fusion (BD). In both analytical flow cytometry and FACS™, 200,000 PBMCs suspended in a volume of 80 µL were incubated with 20 µL antibody mix (Table 1) in PBS for 30 min on ice in the dark, before re-suspending in 100 µL PBS, washing twice at 600 *g* for 5 min at 4 °C, and re-suspending in 200 µL PBS/10% FCS. UltraComp eBeads (eBioscience) were used as compensation controls (1 µL of each antibody mixed with one drop of compensation beads, suspended in 200 µL PBS). Events were acquired using FACS Diva (BD) and data analysed using Flow-Jo (Tree Star Inc., Oregon, US). A cascaded gating approach was used: exclusion of dead cells (DAPI and FSC vs. SSC gates) → exclusion of doublets (FSC-H vs. FSC-A gate) → exclusion of lymphoid cells ($CD5^-/CD21^-$ gate) → inclusion of myeloid cells ($CD11b^+$ gate) → exclusion of neutrophils ($CADO48A^-$ gate). This gating strategy, allowing us to acquire monocytes from the isolated PBMCs, is illustrated in Supplementary Fig. 1.

2.3. Cytocentrifuge preparation and cell examination

A Shandon Cytospin 2 cytocentrifuge was used to deposit cells onto Shandon cytoslides (Thermofisher scientific) at 90 *g* for 5 min. The slides were air dried, stained using a Hematek® Stain Pak − Modified Wright's Stain (Siemens, Pennsylvania, USA), and examined with an Olympus BX50 microscope. Images were captured with an Olympus SC50 camera and edited with CellSens (Olympus, Southend-on-Sea, UK). Purity of the samples was confirmed in two ways: post-sort analyses of each of the four samples, involving re-running and gating on the sorted populations to check the percentages that were within the live population; and a 200-cell count of every sample after cytocentrifugation.

2.4. Transmission electron microscopy

Cells were fixed in 2% paraformaldehyde and 1.5% glutaraldehyde in 0.1 m sodium cacodylate for 24 h at 3 °C. They were washed in 0.1 M sodium cacodylate twice for 30 min each, then embedded in 2% low melting point agarose then fixed with 1% Oso4 (osmium tetraoxide)/ 1.5% Potassium Ferrocyanine K4Fe (CN)6) in 0.1 m cacodylate buffer. After rinsing with distilled water, specimens were dehydrated in a graded ethanol-water series, cleared in propylene oxide and infiltrated with Agar 100 resin. Representative areas were selected and ultra-thin sections were cut using a diamond knife in an Ultracut S microtome (Reichert technologies, Munich, Germany), and collected on 300 mesh grids, then stained with lead citrate and viewed with a 1010 transition electron microscope (Jeol, Massachusetts, USA). Images were recorded using an Orius CCD camera (Gatan, California, USA).

2.5. Graphs and statistics

All graphs were generated using R (R project, Aukland, New Zealand). Statistical analyses were undertaken using Prism (GraphPad software, California, US), applying the Friedman test with *post hoc* analysis (Dunn's multiple comparisons test) to determine the significance of differences in frequency between the populations.

3. Results and discussion

The objective of this study was to determine whether canine monocytes represent a heterogeneous population of cells, as in humans and mice, which we speculated could have ramifications for the pathogenesis of autoimmune and inflammatory diseases in this species (Heine et al., 2008; Ulrich et al., 2010).

Our seven-step gating strategy resulted in four apparently distinct populations of cells based on CD14 and MHC II expression, three of which had the light microscopic characteristics of monocytes: these included $CD14^{pos}MHC\ II^{neg}$, $CD14^{pos}MHC\ II^{pos}$ and $CD14^{neg}MHC\ II^{pos}$ cells (Fig. 1A). An anti-canine CD16 antibody is not commercially available.

Furthermore, Western blots revealed a lack of convincing cross-

Table 1
Flow cytometry antibodies.

Antibody target (isotype)	Clone	Fluorochrome conjugate	Concentration	Supplier	Target species
CD5 (IgG2a)	YKIX322.3	PE[a]	0.3 µg	AbD Serotec[g]	Dog
CD21 (IgG1)	CAT.1D6	PE	0.3 µg	AbD Serotec	Dog
CD11b (IgG2b, kappa)	M1/70	AF − 700[b]	0.2 µg	eBioscience[h]	Mouse[i]
CADO48 (IgG1)	CADO48A	PE-Cy7[c]	1 µg	Washington State University	Dog
MHCII (IgG2a, kappa)	YKIX334.2	APC[d]	0.3 µg	eBiosciences	Dog
CD14 (IgG2a)	TÜK4	AF − 647[e], PB[f]	0.15 µg	AbD Serotec	Human9[i]

[a] Phyocoerythrin.
[b] Alexa Fluor-700.
[c] Phycoerythrin-Cyanine7.
[d] Allophycocyanine.
[e] Alexa Fluor-647.
[f] Pacific Blue.
[g] AbD Serotec, Kidlington, UK.
[h] eBiosciences, Hatfield, UK.
[i] Recorded cross-reactivity with the canine antigen.