TITLE: Asymptomatic Hypertrophic Cardiomyopathy: Diagnosis and Therapy

AUTHORS: Gibbons, N; Goulart, M R; Chang, Y M; Efstathiou, K; Purcell, R; Wu, Y; Peters, L M; Turmaine, M; Szladovits, B; Garden, O A

JOURNAL: Veterinary Immunology and Immunopathology

PUBLISHER: Elsevier

PUBLICATION DATE: 28 June 2017

DOI: 10.1016/j.vetimm.2017.06.007

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] (**corresponding author**).

[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 Studies, Philadelphia, University of Pennsylvania, School of veterinary medicine.

Highlights

- Heterogeneity of canine monocytes was demonstrated on the basis of differential expression of CD14 and MHC class II

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.

Key words

Monocyte; Innate immunity; CD14; CD16; MHC class II; Subpopulation

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 $Ly6C^{high}$ ($CCR2^{high}CX_3CR1^{low}$) 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.

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^{o}C for a maximum duration of 24 hours. 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 minutes at 300*g* at 4°C, before re-suspension in 2mL PBS/10% FCS and counting.

2.2 Flouresence-assisted cell sorting (FACS^{TM})

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 80µL were incubated with 20µL antibody mix (Table 1) in PBS for 30 minutes on ice in the dark, before re-suspending in 100µL PBS, washing twice at 600*g* for 5 minutes at 4°C, and re-suspending in 200µL PBS/10% FCS. UltraComp eBeads (eBioscience) were used as compensation controls (1µL of each antibody mixed with one

drop of compensation beads, suspended in 200µL 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 Figure 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 minutes. 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 0.1m sodium cacodylate for 24 hours at 3^{o}C. They were washed in 0.1M sodium cacodylate twice for 30 minutes each, then embedded in 2% low melting point agarose then fixed with 1% Oso4 (osmium tetraoxide) / 1.5% Potassium Ferrocyanine K4Fe (CN)6) in 0.1m 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 (Figure 1A). An anti-canine CD16 antibody is not commercially available.

Furthermore, Western blots revealed a lack of convincing cross-reactivity of an anti-human CD16 antibody (clone LNK16) with canine PBMCs. Densitometry revealed that 10^6 human PBMCs yielded a CD16 band that was 103% of the beta actin control band, yet the same number of canine PBMCs yielded a band only 20% that of the respective beta actin control (human PBMCs n=1, canine PBMCs n=2; data not shown) and multiple attempts to use the antibody in the setting of flow cytometry also failed to yield reliable staining. We were therefore unable to interrogate CD16 expression of the

three monocyte populations, precluding direct comparisons of the canine subsets with those of humans, which are predominantly defined by their CD14 and CD16 expression. Nevertheless, we were able to make speculative inferences based on the known MHC II^{high} status of CD16^{pos} human monocytes (Kim et al., 2010; Abeles et al., 2012; Gordon and Taylor, 2005) prompting us to hypothesise that the canine CD14^{pos}MHC II^{neg} cells are equivalent to human classical monocytes, that the canine CD14^{pos}MHC II^{pos} cells are equivalent to human intermediate monocytes, and that the canine CD14^{neg}MHC II^{pos} cells are equivalent to human non-classical monocytes.

Classical monocytes comprise 80% or more of the peripheral monocyte pool in healthy humans (Wong et al., 2012, Cros et al., 2010), but species differences are known to exist. In mice, classical monocytes comprise approximately 50% of peripheral monocytes, whereas in rats they account for less than 20%, the non-classical population being the dominant in this latter species (Strauss-Ayali et al., 2007). Cows also exhibit the same three populations (Corripio-Miyar et al., 2015). Our data showed a predominance of the CD14^{pos} population in dogs, but of these the MHC II^{pos} cells, which we speculate represent intermediate monocytes, appeared to predominate (Figure 1B), raising interesting questions about their function in the dog. Moreover, we note that some studies of human monocytes suggest that the intermediate population expresses higher levels of MHC II than the non-classical population, an observation that is at variance with our speculative designations of monocyte populations (Wong et al., 2011; Abeles et al., 2012). The reason these observations and ours do not coincide, is that the mean and median MHC II MFI of our CD14^{neg}MHC II^{pos} canine monocytes are higher than those of our CD14^{pos}MHC II^{pos} canine monocytes (data not shown). This again emphasises that further studies, ideally with an anti-dog CD16 antibody, are required to make more confident comparisons of canine monocyte populations with those of other species. Of interest, the CD14^{neg}MHC II^{neg} population appeared to comprise eosinophils, thus yielding a novel sorting strategy for the enrichment of canine eosinophils for downstream analysis. The mean eosinophil percentage as a fraction of total PBMCs was 1.0071%, with a minimum of 0.04% and a maximum of 4.1%. The purity of these eosinophils, determined by both post-sort analyses and a 200-

cell count of the samples after cytocentrifugation, exceeded 75% in every case, with a maximum value of 100% and a mean value of 93.9%. Similarly, the monocyte purity ranged from 79.2% to 100% with a mean value of 95%.

We were intrigued by the presence of apparently $CD14^{neg}$ monocytes, which comprised from 2.76% to 30.95% of total monocytes, according with previous studies that have demonstrated similar percentages of $CD14^{neg}$ monocytes in dogs (Jacobsen et al., 1993). The median $CD14^{neg}$ percentage was 9.79% and the median $CD14^{pos}$ percentage was 90.21%. Whether these are truly monocytes that fail to express CD14 or monocytes with lower CD14 expression (i.e. $CD14^{low}$) undetected by the cross-reactive antibody, whose affinity for canine CD14 has never been formally assessed, remains unclear. Indeed, various studies of human monocytes appear not to make a distinction between $CD14^{low}$ and apparently $CD14^{neg}$ monocytes (Abeles et al., 2012), suggesting that this may be a point of semantics rather than a biologically important phenomenon.

Transmission electron microscopy of the monocyte populations (Figure 1C) supported observations made at the light microscopic level, as well as aligning with TEM literature in humans and other species. For example, the modest numbers of mitochondria we observed are common to the guinea pig (Daems and Brederoo, 1973) and bird (Sutton and Weiss, 1966). The $CD14^{pos}MHCII^{neg}$ population, which we speculated were equivalent to human classical monocytes, appeared to be smaller and to possess more numerous and larger granules, as well as more vacuoles and pseudopodia, than the other two populations, possibly reflecting functional differences between the populations such as greater phagocytic activity (Grage-Griebenow et al., 2000, Nichols et al., 1971, Sutton and Weiss, 1966). However, these observations remain preliminary until images from a larger number of dogs have been analysed, and further work would need to be undertaken to investigate functional differences between the canine monocyte populations and any correlations with monocyte populations in the mouse, rat and human.

In conclusion, canine monocytes display phenotypic heterogeneity and may be divided into three populations based on CD14 and MHC II expression. Functional and transcriptomic studies will be necessary to further define these myeloid populations in dogs and their similarities to those in other species. The dog is rapidly gaining traction as a spontaneous, large animal model of a number of human diseases (Davis and Ostrander, 2014, Rowell et al., 2011), superior in several regards to induced murine models (Gordon et al., 2009, Wilbe et al., 2015). Detailed characterisation of myeloid populations, which play a key role in a number of autoimmune, inflammatory and neoplastic diseases, is a key step in further elucidating the translational merit of this species.

4. Acknowledgements

The authors are grateful for funding from the Kennel Club (supported by Vernon and Shirley Hill of Metro Bank) in the form of an Undergraduate Student Award to NG. Additional funding for this work in OAG's laboratory is gratefully acknowledged from the Petplan Charitable Trust. Many thanks also to Dr Rebecca Pike, QMUL for her flow cytometry help and expertise, and to Dr Charlotte Lawson (RVC) for her procurement of blood samples for Western blotting.

5. References

ABELES, R. D., MCPHAIL, M. J., SOWTER, D., ANTONIADES, C. G., VERGIS, N., VIJAY, G. K., XYSTRAKIS, E., KHAMRI, W., SHAWCROSS, D. L., MA, Y., WENDON, J. A. & VERGANI, D. 2012. CD14, CD16 and HLA-DR reliably identifies human monocytes and their subsets in the context of pathologically reduced HLA-DR expression by CD14(hi) /CD16(neg) monocytes: Expansion of CD14(hi) /CD16(pos) and contraction of CD14(lo) /CD16(pos) monocytes in acute liver failure. *Cytometry A,* 81**,** 823-34.

CIPOLLETTA, C., RYAN, K. E., HANNA, E. V. & TRIMBLE, E. R. 2005. Activation of peripheral blood CD14+ monocytes occurs in diabetes. *Diabetes,* 54**,** 2779-86.

CORRIPIO-MIYAR, Y., Hope, J., MCINNES, C., WATTEGEDERA, S., JENSEN, K., PANG, Y., ENTRICAN, G. & GLASS, E. (2015). Phenotypic and functional analysis of monocyte populations in cattle peripheral blood identifies a subset with high endocytic and allogeneic T-cell stimulatory capacity. *Veterinary Research*, 46(1).

CROS, J., CAGNARD, N., WOOLLARD, K., PATEY, N., ZHANG, S. Y., SENECHAL, B., PUEL, A., BISWAS, S. K., MOSHOUS, D., PICARD, C., JAIS, J. P., D'CRUZ, D., CASANOVA, J. L., TROUILLET, C. & GEISSMANN, F. 2010. Human CD14dim monocytes patrol and sense nucleic acids and viruses via TLR7 and TLR8 receptors. *Immunity,* 33**,** 375-86.

DAEMS, W. T. & BREDEROO, P. 1973. Electron microscopical studies on the structure, phagocytic properties, and peroxidatic activity of resident and exudate peritoneal macrophages in the guinea pig. *Z Zellforsch Mikrosk Anat,* 144**,** 247-97.

DAVIS, B. W. & OSTRANDER, E. A. 2014. Domestic dogs and cancer research: a breed-based genomics approach. *ILAR J,* 55**,** 59-68.

GEISSMANN, F., JUNG, S. & LITTMAN, D. R. 2003. Blood monocytes consist of two principal subsets with distinct migratory properties. *Immunity,* 19**,** 71-82.

GHATTAS, A., GRIFFITHS, H. R., DEVITT, A., LIP, G. Y. & SHANTSILA, E. 2013. Monocytes in coronary artery disease and atherosclerosis: where are we now? *J Am Coll Cardiol,* 62**,** 1541-51.

GORDON, I., PAOLONI, M., MAZCKO, C. & KHANNA, C. 2009. The Comparative Oncology Trials Consortium: using spontaneously occurring cancers in dogs to inform the cancer drug development pathway. *PLoS Med,* 6**,** e1000161.

GORDON, S. & TAYLOR, P. R. 2005. Monocyte and macrophage heterogeneity. *Nat Rev Immunol,* 5**,** 953-64.

GRAGE-GRIEBENOW, E., FLAD, H. D., ERNST, M., BZOWSKA, M., SKRZECZYNSKA, J. & PRYJMA, J. 2000. Human MO subsets as defined by expression of CD64 and CD16 differ in phagocytic activity and generation of oxygen intermediates. *Immunobiology,* 202**,** 42-50.

HEINE, G. H., ULRICH, C., SEIBERT, E., SEILER, S., MARELL, J., REICHART, B., KRAUSE, M., SCHLITT, A., KOHLER, H. & GIRNDT, M. 2008. CD14(++)CD16+ monocytes but not total monocyte numbers predict cardiovascular events in dialysis patients. *Kidney Int,* 73**,** 622-9.

JACOBSEN, C. N., AASTED, B., BROE, M. K. & PETERSEN, J. L. 1993. Reactivities of 20 anti-human monoclonal antibodies with leucocytes from ten different animal species. *Vet Immunol Immunopathol,* 39**,** 461-6.

KIM, O. Y., MONSEL, A., BERTRAND, M., CORIAT, P., CAVAILLON, J. M. & ADIB-CONQUY, M. 2010. Differential down-regulation of HLA-DR on monocyte subpopulations during systemic inflammation. *Crit Care,* 14**,** R61.

MARTINEZ, F. O. 2009. The transcriptome of human monocyte subsets begins to emerge. *J Biol,* 8**,** 99.

NICHOLS, B. A., BAINTON, D. F. & FARQUHAR, M. G. 1971. Differentiation of monocytes. Origin, nature, and fate of their azurophil granules. *J Cell Biol,* 50**,** 498-515.

ROWELL, J. L., MCCARTHY, D. O. & ALVAREZ, C. E. 2011. Dog models of naturally occurring cancer. *Trends Mol Med*, 380-388.

SIBLEY, T. A., MILLER, M. M. & FOGLE, J. E. 2013. Human intravenous immunoglobulin (hIVIG) inhibits anti-CD32 antibody binding to canine DH82 cells and canine monocytes in vitro. *Vet Immunol Immunopathol,* 151, 229-34.

STANSFIELD, B. K. & INGRAM, D. A. 2015. Clinical significance of monocyte heterogeneity. *Clin Transl Med,* 4, 5.

STRAUSS-AYALI, D., CONRAD, S. M. & MOSSER, D. M. 2007. Monocyte subpopulations and their differentiation patterns during infection. *J Leukoc Biol,* 82, 244-52.

SUTTON, J. S. & WEISS, L. 1966. Transformation of monocytes in tissue culture into macrophages, epithelioid cells, and multinucleated giant cells. An electron microscope study. *J Cell Biol,* 28, 303-32.

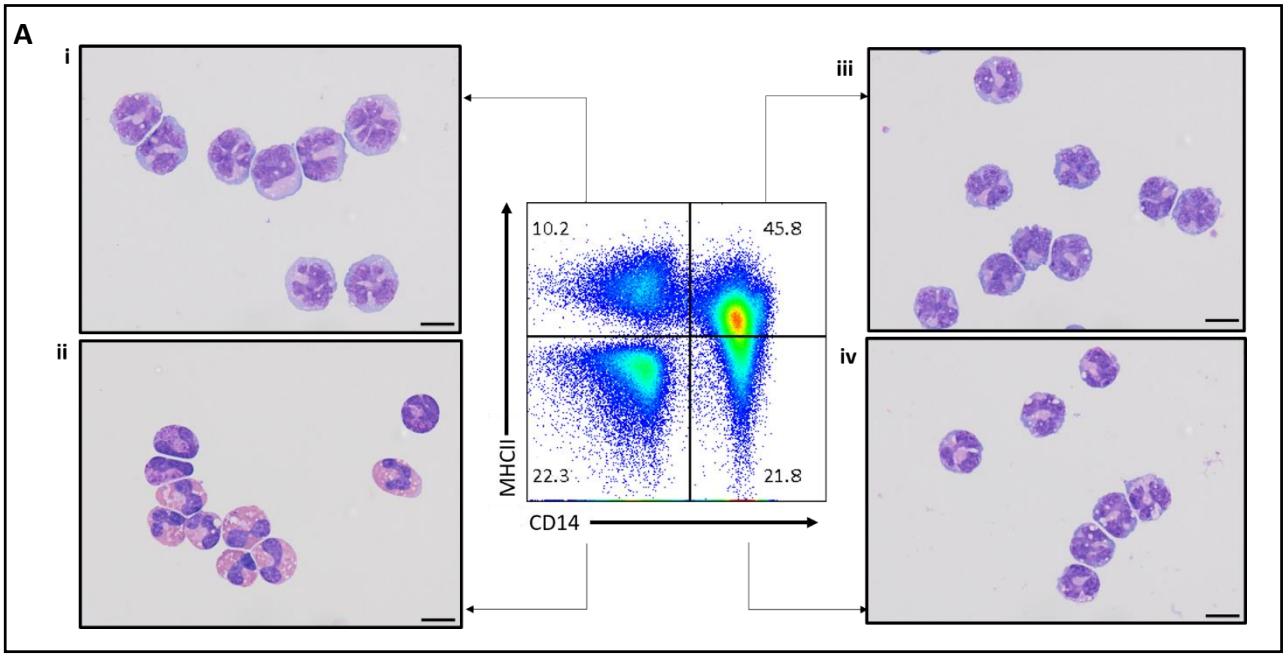
ULRICH, C., HEINE, G. H., SEIBERT, E., FLISER, D. & GIRNDT, M. 2010. Circulating monocyte subpopulations with high expression of angiotensin-converting enzyme predict mortality in patients with end-stage renal disease. *Nephrol Dial Transplant,* 25, 2265-72.

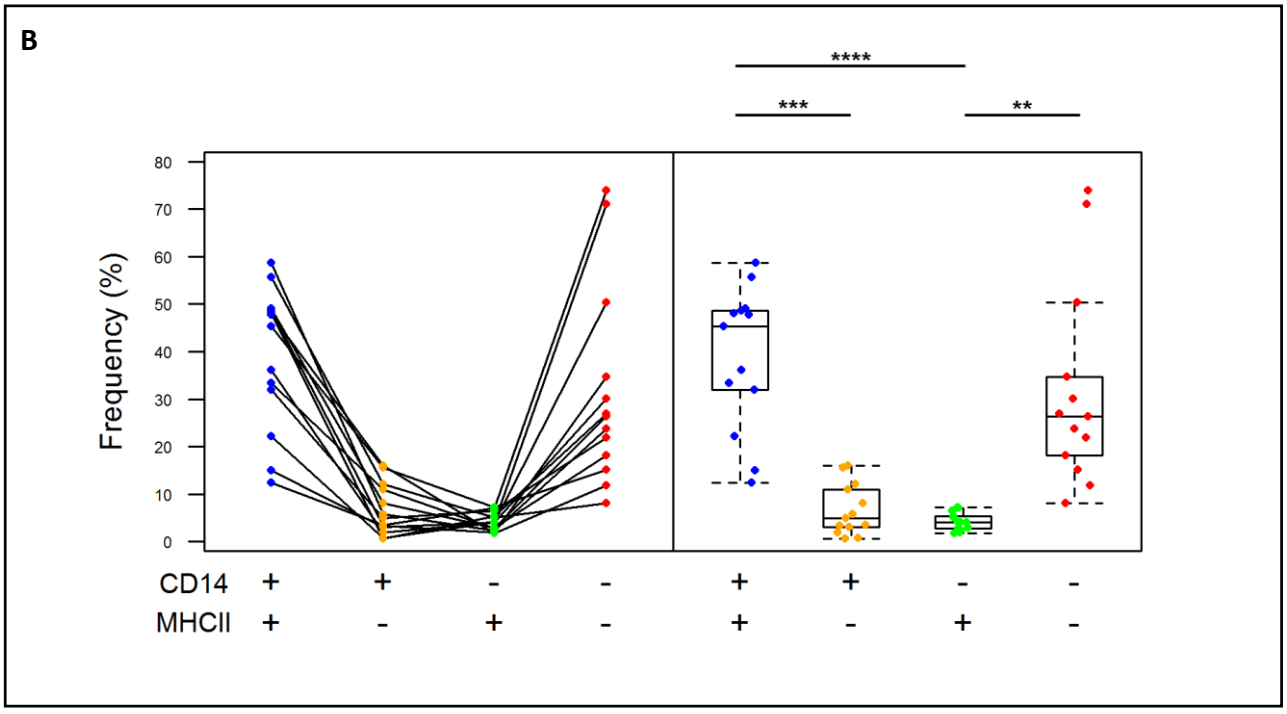
WILBE, M., KOZYREV, S. V., FARIAS, F. H., BREMER, H. D., HEDLUND, A., PIELBERG, G. R., SEPPALA, E. H., GUSTAFSON, U., LOHI, H., CARLBORG, O., ANDERSSON, G., HANSSON-HAMLIN, H. & LINDBLAD-TOH, K. 2015. Multiple Changes of Gene Expression and Function Reveal Genomic and Phenotypic Complexity in SLE-like Disease. *PLoS Genet,* 11, e1005248.

WONG, K. L., YEAP, W. H., TAI, J. J., ONG, S. M., DANG, T. M. & WONG, S. C. 2012. The three human monocyte subsets: implications for health and disease. *Immunol Res,* 53, 41-57.

WONG, K. L., TAI, J. J., WONG, W., HAN, H., SEM, X., YEAP, W., KOURILSKY, P. AND WONG, S. (2011) Gene expression profiling reveals the defining features of the classical, intermediate, and nonclassical human monocyte subsets. Blood, 118(5), 16-31.

ZIEGLER-HEITBROCK, L., ANCUTA, P., CROWE, S., DALOD, M., GRAU, V., HART, D. N., LEENEN, P. J., LIU, Y. J., MACPHERSON, G., RANDOLPH, G. J., SCHERBERICH, J., SCHMITZ, J., SHORTMAN, K., SOZZANI, S., STROBL, H., ZEMBALA, M., AUSTYN, J. M. & LUTZ, M. B. 2010. Nomenclature of monocytes and dendritic cells in blood. *Blood,* 116, e74-80.





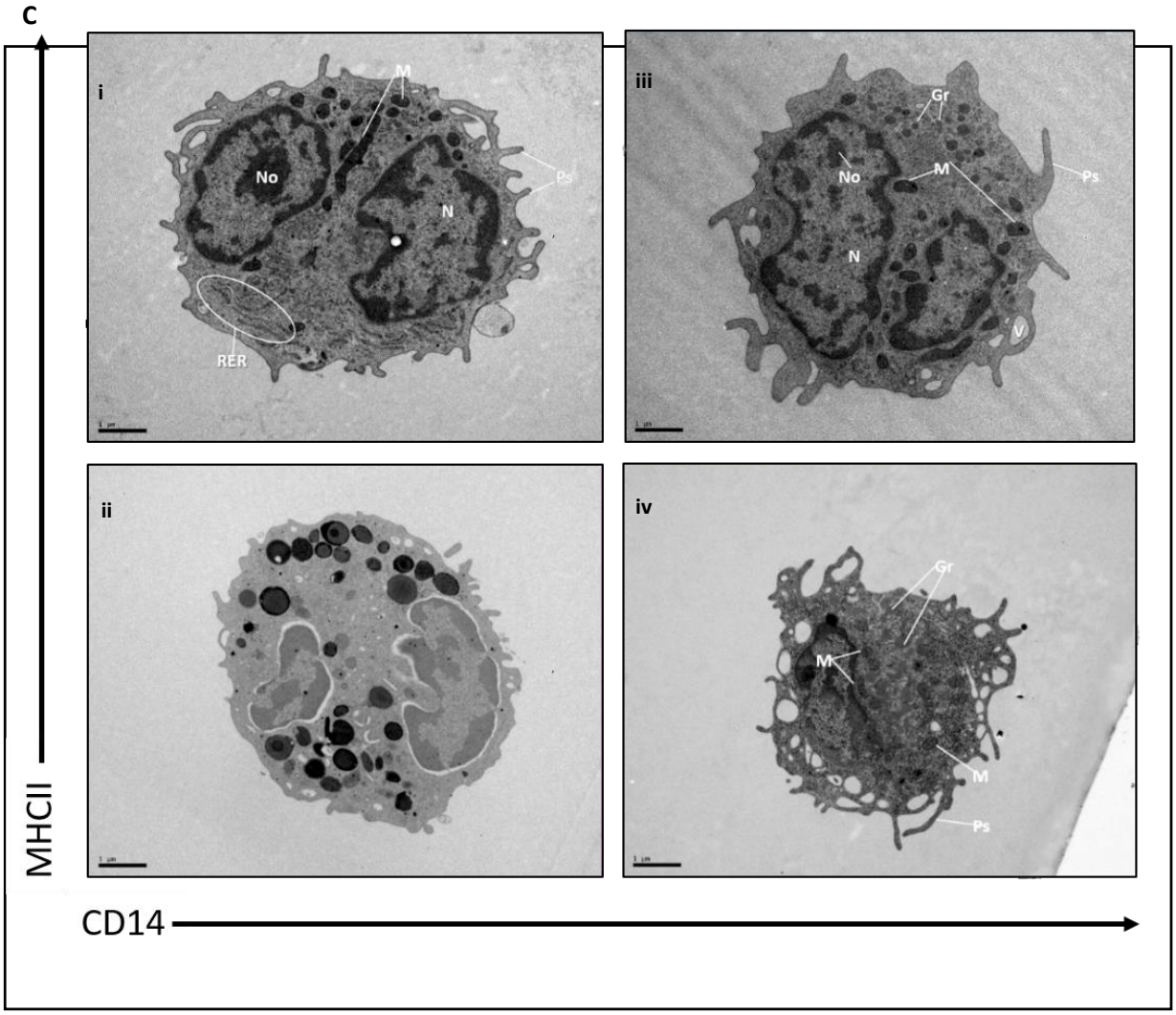


Figure 1: *Differential expression of CD14 and MHC II by monocyte populations in healthy dogs. (A) Representative cytological images (cytocentrifuge preparations, modified Wright's stain) of four myeloid populations defined by CD14 and MHC II expression in healthy dogs (n=5). The scale bars all represent* 10*µm. The populations were delineated by means of a cascaded gating strategy using FMO controls (Supplementary Figure 1). Cells in panels i, iii and iv most resemble monocytes, while those in panel ii resemble eosinophils. (B) Graphical representation of the frequency, expressed as a percentage of all cells in the CD14/MHC II plot, of each of the four populations from each dog (n=13). Significant differences in frequencies were apparent (** p≤0.01; *** p≤0.001; **** p≤0.0001). (C) Representative transmission electron microscopy images of cells from each of the four myeloid populations in healthy dogs (n=3). These confirm the monocytic and eosinophilic identities of the cells as established in (A). Overall, CD14^{pos}MHC II^{neg} cells appeared smaller, with more numerous, larger granules, and more pseudopodia than the other two monocyte subpopulations. All scale bars represent* 1*µm. Abbreviations: Gr=granules, M=mitochondria, N=nucleus, No=nucleolus, Ps=pseudopodia, R=free ribosomes, RER=rough endoplasmic reticulum, and V=vacuoles.*

Table 1: Flow cytometry antibodies

Antibody target (isotype)	Clone	Fluorochrome conjugate	Concentration	Supplier	Target species
CD5 (IgG2a)	YKIX322.3	PE[1]	0.3µg	AbD Serotec[7]	Dog
CD21 (IgG1)	CAT.1D6	PE	0.3µg	AbD Serotec	Dog
CD11b (IgG2b, kappa)	M1/70	AF-700[2]	0.2µg	eBioscience[8]	Mouse[9]
CADO48 (IgG1)	CADO48A	PE-Cy7[3]	1µg	Washington State University	Dog
MHCII (IgG2a, kappa)	YKIX334.2	APC[4]	0.3µg	eBiosciences	Dog
CD14 (IgG2a)	TÜK4	AF-647[5], PB[6]	0.15µg	AbD Serotec	Human[10]

[1]Phyocoerythrin; [2]Alexa Fluor-700; [3]Phycoerythrin-Cyanine7; [4]Allophycocyanine; [5]Alexa Fluor-647; [6]Pacific Blue; [7]AbD Serotec, Kidlington, UK; [8]eBiosciences, Hatfield, UK. [9,10] Recorded cross-reactivity with the canine antigen.