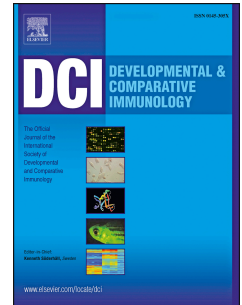


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Canine macrophages can like human macrophages be *in vitro* activated toward the M2a subtype relevant in allergy

Ina Herrmann^{a,b*}, Jelena Gotovina^a, Judit Fazekas-Singer^{a,e}, Michael B. Fischer^{c,d}, Karin Hufnagl^a, Rodolfo Bianchini^a, Erika Jensen-Jarolim^{a,e}

a. Comparative Medicine, The interuniversity Messerli Research Institute, University of Veterinary Medicine Vienna, Medical University Vienna and University Vienna, Veterinärplatz 1, 1210, Vienna, Austria

b. Department for Companion Animals and Horses, University of Veterinary Medicine Vienna, Veterinärplatz 1, 1210, Vienna, Austria

c. Department of Blood Group Serology and Transfusion Medicine, Medical University of Vienna, Sensengasse 2a, 1090, Vienna, Austria

d. Center for Biomedical Technology, Krems Danube University Krems, Dr.-Karl-Dorrek-Straße 30, 3500, Krems, Austria

e. Institute of Pathophysiology and Allergy Research, Center for Pathophysiology, Infectiology and Immunology, Medical University Vienna, Währinger Gürtel 18-20, 1090, Vienna, Austria

Correspondence

Prof. Erika Jensen-Jarolim, MD
Inst. of Pathophysiology and Allergy Research
Center of Pathophysiology, Infectiology and Immunology,
Medical University Vienna, Spitalgasse 23, 1090 Vienna, Austria
Email: erika.jensen-jarolim@meduniwien.ac.at

Abbreviations¹

¹ Abbreviations: dendritic cells- DCs; fetal bovine serum- FBS; granulocyte macrophage stimulating-factor- GM-CSF; immunoglobulin- Ig; Interleukin- IL; macrophage colony-stimulating factor- M-CSF; melting temperature- T_m; monocyte-derived macrophages- MDMs; no template control- NTC; phorbol 12-myristate 13-acetate- PMA; phosphate buffered saline- PBS; recombinant- r; room temperature- RT

Abstract

The M2a subtype of macrophages plays an important role in human immunoglobulin E (IgE-mediated allergies) and other Th2 type immune reactions. In contrast, very little is known about these cells in the dog. Here we describe an *in-vitro* method to activate canine histiocytic DH82 cells and primary canine monocyte-derived macrophages (MDMs) toward the M2a macrophages using human cytokines. For a side-by-side comparison, we compared the canine cells to human MDMs, and the human monocytic cell line U937 activated towards M1- and M2a cells on the cellular and molecular level. In analogy to activated human M2a cells, canine M2a, differentiated from both DH82 and MDMs, showed an increase in CD206 surface receptor expression compared to M1. Interestingly, canine M2a, but not M1 derived from MDM, upregulated the high-affinity IgE receptor (FcεRI). Transcription levels of M2a-associated genes (IL10, CCL22, TGFβ, CD163) showed a diverse pattern between the human and dog species, whereas M1 genes (IDO1, CXCL11, IL6, TNFα) were similarly upregulated in canine and human M1-cells (cell lines and MDMs). We suggest that our novel *in vitro* method will be suitable in comparative allergology studies focussing on macrophages.

Key Words

allergy; canine; comparative; DH82; M1 macrophage; M2a macrophage; polarization;

1. Introduction

Macrophages are cells of the innate immune system displaying essential functions in defense against pathogens, tissue repair and cytokine production. The cells are known for their high phenotypic plasticity and heterogeneity, and respond to microenvironmental cues like growth factors, cytokines and chemokines (Mantovani et al., 2004). Macrophages are very plastic cells that differentiate into distinct functional subpopulation define by the paradigm M1/M2 to mirror the Th1 Th2 paradigm for the T cells.

M1 cells are characterized by the production of pro-inflammatory cytokines, an ability to mediate resistance to pathogens, active microbicidal properties, high production of reactive nitrogen and oxygen intermediates, and promotion of Th1 responses. M1 macrophages are polarized by granulocyte-macrophage colony stimulating factor (GM-CSF), IFN-gamma and TNF- alpha or LPS. Their function is to help the Th1/Th17 response by secreting pro-inflammatory cytokines such as IL-1-beta, tumor necrosis factor (TNF), IL-12, IL-18 and IL-23 and expressing high levels of major histocompatibility complex class II (MHC II), and co-stimulatory molecules CD80 and CD86. Moreover, they are implicated in the activation of iNOS production and consequent NO release secreted from L-arginase (Arnold et al., 2014; MacMicking et al., 1997).

On the other hand, M2 macrophages are involved in parasite control, tissue remodeling, immune regulation, allergy, tumor promotion and efficient phagocytic activity. M2 macrophages are polarized by macrophage colony stimulating factor (M-CSF), IL-4, IL-13, IL-10 and TGF-beta. Their function is to help the Th2 response. Recently, this population of M2 macrophages has been divided into three distinct sub-populations defined as M2a, M2b and M2c macrophages (Mantovani et al., 2004; Martinez and Gordon, 2014; Röszer, 2015). The M2a subtype is promoted by IL-4, IL-13 or fungal and helminth infections; M2b subtype is supported by IL-1 receptor ligands or LPS plus immune complexes; M2c subtype is promoted by IL-10, TGF-beta and glucocorticoids. The three subtypes are also defined as alternatively activated macrophages (M2a), type 2 macrophages or immune-regulatory macrophages (M2b), and deactivated macrophages (M2c) (Röszer T 2015). M2b are characterized by the expression of IL-10, IL-6, TNF-a and the chemokine CCL1. M2c are characterized by the expression of high levels of IL-10 and TGF-b and express high levels of CD163 and CD206.

M2a macrophages enhance the Th2 response by secreting IL-10, TGF-b and IL-1ra and the chemokines CCL22 and CCL17. They express CD163, CD206, CD86, TGM2 and, in mouse Arg-1, Fizz1 and Ym1/2 (Ferrante and Leibovich, 2012). In contrast, in particular conditions, M2a macrophages can contribute to pathologies, like promoting neoplasm growth or participating in chronic allergic inflammation (Röszer, 2015; Jensen-Jarolim et al, 2017).

Due to the interest of our research group in comparative aspects in allergy, we aimed to explore the M2a properties of canine macrophages. The dog displays a spontaneous model of atopic dermatitis and the considerable immunological homology between humans and dogs drives comparative and translational research (Jensen-Jarolim, 2017). To obtain canine primary cells an ethical approval is required, the amount of blood is limited and the interindividual variants are unpredictable. To reduce these limitations, we also included the canine histiocytic cell line DH82 in our research. DH82 is a well-characterized cell line used mainly for infectious translational studies and canine macrophage functional studies (Barnes et al., 2000; Heinrich et al., 2015; Mendonça et al., 2017; Yu et al., 2011).

The purpose of this study was to develop a protocol for canine macrophage differentiation for side-by-side comparison to human macrophage activation.

For this purpose, we stimulate DH82 cells and primary canine monocyte-derived macrophages (MDMs) toward the classically activated M1 and the alternatively activated M2a macrophages, and compared their *in vitro* activation potentials to that of human histiocytic lymphoma cells U937 and human MDMs on the cellular and molecular level.

2. Material and methods

2.1 Cell lines

Canine histiocytic cell line DH82 (ATCC CRL-10389) obtained from Public Health England was used between passage number 11 and 19.

The comparative experiments on human cells were performed with the human histiocytic lymphoma cells U-937, passage 10-16 (ATCC CRL-1593.2) obtained from ATCC. Maintaining medium and materials are listed in Table 1.

2.2 Blood drawing from healthy humans and dogs

Eight ml of blood was collected by vein puncture from thirteen healthy, privately owned dogs without any current diseases or any treatment, besides ecto- and endoparasitic prevention. A written consensus was obtained from the dog owners. The intervention was approved by the institutional ethics committee of the University of Veterinary Medicine Vienna, Austria, and the national authority according to the Law for Animal Experiments Austria (TVG 2012) (BMVFW-68.205/0186-WF/V/3b/2015).

For comparison, four healthy human volunteers donated 32 ml blood. The blood drawing was approved by the institutional ethics committee of the Medical University of Vienna (ECS2007/2016) and conducted in accordance with the Helsinki Declaration of 1975. All subjects gave their written informed consent.

In addition, PBMCs from two donors originated from a leukocyte reduction system (LRS) chambers of a TrimaAccel® blood collector in the process of the production of a single donor platelet concentrate.

The use of LRS chambers as medical waste for scientific purpose was approved by the local ethics committee of the Medical University Vienna, Austria (ECS2177/2013).

2.3 Isolation and culture of monocyte-derived macrophages (MDMs)

Fresh lithium-heparinized whole blood from human and canine donors was diluted 1:3 in Hank's Balanced Salt Solution (HBSS) (GE healthcare, Solingen, Germany) and layered over a Ficoll-Paque density gradient (1077g/ml) using the recommended protocol (GE Healthcare, Solingen, Germany). Isolated peripheral blood mononuclear cells (PBMCs) were seeded in 6 well plates (Falcon, Corning, NY) and non-adherent cells were removed by washing after 2 hours. Cells were maintained over 7-9 days in complete RPMI medium enriched with 20ng/ml recombinant-human (rh)-GM-CSF and rh-M-CSF for human MDMs and 20ng/ml rh-GM-CSF and 40ng/ml rh-M-CSF for canine MDMs in order to differentiate them into macrophages. Half of the medium was replaced every 2-3 days and fresh growth factors were added. After 7 days in culture, canine MDMs acquired a macrophage-like morphology and stained CD14⁺; CD3⁻, CD19⁻ cells in >98% of the cell population (Supplementary Fig. S1) (Debey et al., 2004; Goto-Koshino et al., 2011).

2.4 Activation of MDMs to M1 and M2a macrophages

The guidelines on macrophage activation propose using terms M (LPS+IFN- γ) and M (IL4+IL13) instead of M1 and M2a respectively (Murray et al., 2014), but for the purpose of easier reading, we refer here to M1 and M2a for (LPS+IFN- γ) and (IL4+IL13), respectively. On day 7-9 MDMs were detached using 2.5mM EDTA (VWR, Radnor, PA) in ice-cold Dulbecco modified PBS w/o Mg² and Ca² (ThermoScientific, Waltham, MA) and their activation was performed. The activation scheme is illustrated in Fig. 1A and concentration and source of reagents are summarized and listed in Table 1. In need of priming the monocytes towards a pre-state of anti-or pro-inflammatory macrophages, GM-CSF or M-CSF was used (Vogel et al., 2014). In canine and human MDMs, we introduced four conditions, two negative controls GM-CSF and M-CSF and two activated M1 (LPS+INF) and M2a (IL4+IL13) cell types. Human cytokines were chosen to stimulate canine cells based on their high sequence similarity but a higher dose was

required to activate canine cells in comparison to human MDMs based on their morphology appearance (data not shown). Canine M2a cells required 40 ng/ml compared to 20 ng/ml of M-CSF, IL4 and IL13 in human MDMs (Tab. 1).

2.5 Activation of U937 and DH82 toward M1 and M2a macrophage phenotype

The concentration of reagents of U937 and DH82 activation are summarized and listed in Table 1. U937 were activated according to a slightly modified protocol described (Taniguchi et al. 2015) (Fig. 1B). PMA treatment for 24 h was required to stimulate 95% of the cells from round, non-adherent, monocytic cells into more mature, adherent, phenotypically macrophage-like cells. The cells were washed twice with medium and activated (Taniguchi et al., 2015).

DH82 are macrophage-like adherent cells with round to elongated appearance and therefore did not require stimulation before activation. Cells were detached with trypsin-EDTA (Sigma-Aldrich St. Louis, MO) for 5-10 min resuspended in fresh medium, seeded in 24 well plates and immediately activated. For both cell lines medium controls were included and named M0.

All further experiments were performed with adherent macrophages only, floating cells were washed away before processing.

2.6 Morphological characterization and quantification

After 72 h of activation with M1 or M2a stimuli the morphology of activated cells and controls were assessed, according to the criteria adopted and modified from Heinrich et al. (2017). Briefly, 100 cells were randomly counted and classified as small and round (diameter $\leq 20\mu\text{m}$), amoeboid (diameter $\geq 20\mu\text{m}$ with cell membrane protrusion), spindle-shaped (more than twice the ratio of major axis to minor axis) or large and flat (diameter $\geq 80\mu\text{m}$). Images of cells were acquired using Primo Vert Microscope (Carl Zeiss, Germany) and the ToupCam camera system (ToupTek, Zhejiang, China).

For acquisition of stained pictures cells were grown and activated on glass cover slides diameter 12 mm (Thermo Scientific, Waltham, Massachusetts) in a 24 well plate. After 72 h the cells attached to glass slides were stained with DiffQuick and mounted on slides. Pictures were taken using TissueFAXS imaging system (TissueGnostics GmbH, Vienna, Austria) equipped with a Zeiss Axio Imager Z1 fitted with a PCO USB camera.

4.1 Flow Cytometry

For the flow cytometry analysis, 1.5×10^5 cells per tube (Falcon, Corning, NY) were detached after 72 h activation using either ice-cold 2.5 mM EDTA/ PBS (30 min, 4 °C and mechanic detachment through pipetting) in MDMs or Accutase (20-30 min, 37 °C) (Sigma-Aldrich, St. Louis, MO) for cell lines. After two washing steps with PBS+10% FBS, monoclonal antibodies against surface markers (Tab. 2) and isotype controls at a dilution 1:100 were added and incubated at 4 °C for 30 min. After two washing steps, 10.000 single cell events were recorded using dual laser FACSCanto II equipped with FACSDiva Software (Becton Dickinson). 7AAD (Biolegend, San Diego, CA) was included as a live/dead discriminator and SSC-W vs. SSC-H analysis was used to identify single cells. Cell-based compensation excluded spill over in the multicolor assays. Acquired data were analyzed with the software FlowJo version 10.1 (Flowjo, LLC, Ashland, OR). Delta median fluorescence intensity (ΔMFI) values were calculated from the median MFI of the target antibody by subtracting MFI of the isotype control (Alvarez et al., 2010).

2.8 RNA isolation and reverse transcription-quantitative PCR (RT-qPCR)

After 48 h of activation, cells were detached with ice-cold 2,5 mM EDTA in PBS, washed once with PBS, pelleted and lysed in Trizol (Sigma-Aldrich, St. Louis, MO). RNA was isolated using Direct-zol RNA MiniPrep column system (Zymo Research, Irvine, CA) including a DNase I digestion step (15 min, RT). The RNA quantity was measured by NanoDrop (Implen). In addition, 10% of randomly selected RNA samples were tested in the Agilent TapeStation

(Agilent Technologies) to check RNA integrity number (RIN) (all samples RIN > 8.5) and to crosscheck the concentration with those measured by NanoDrop. The cDNA synthesis was performed according to the manufacturer's recommendations using 200 ng RNA for DH82, U937 and human MDMs and 100 ng for canine MDMs using iScript cDNA Synthesis Kit (BioRad, Hercules, CA). For each donor, RNA from all conditions was pooled and used for the reverse transcriptase negative control (RT-). RT-qPCR was performed on a QuantStudio™12K Flex system (Applied Biosystems, 45 cycles; Tm 59-61 °C; 96 well plate and plate cover (Applied Biosystems) using a Solis Biodyne Supermix (Solis Biodyne, Tartu, Estonia). Target genes were selected according to human literature, concerning a strong discrimination between M1- and M2a macrophages (Martinez et al., 2006; Röszer, 2015). The primers (Supplementary Tab.S1) were either selected based on the recent literature or designed using the NCBI Blast and the Oligo Analyzer Tool (Thornton and Basu, 2011). Every plate included pooled RT- per donor and NTC per gene controls. M0 for DH82 and U937 M1 and M2a, GM-CSF for M1 MDMs and M-CSF for M2a MDMs were used as controls. A standard curve was generated and amplification efficiency (E) was calculated from the slope s of this curve ($E = 10^{(-1/s)} - 1$) using the QuantStudio 12k Flex Software v1.2.3 (Applied Biosystems). Quantification cycle (Cq) values were corrected by the term $Cq \times \log_{10}(E + 1)/\log_{10}(2)$ (Robledo et al., 2014). For evaluating reference genes in canine macrophages, we tested ten candidates and chose ornithine decarboxylase antizyme (OAZ1) based on a stable expression profile over all activation conditions (data not shown). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as reference gene for human cells based on Martinez et al. (2006). After normalizing the data with the reference gene (OAZ1 for DH82 and canine MDMs; GAPDH for human MDMs and U937). $\Delta\Delta Cq$ levels were calculated and presented as log2 fold change for M2a- versus M1 macrophages. The mean fold change expression was calculated for M1/M2a versus M0 in DH82 and U937 and M1 versus GM-CSF, M2a versus M-CSF for MDMs, in case of a negative value the reciprocal value is presented.

2.9 Statistical Analysis

The morphological data were analyzed using a one-way ANOVA followed by the Tukey's multiple comparisons test to compare M0 controls to M1 and M2a in U937 and DH82 and GM-CSF, M-CSF controls respectively to activated M1- and M2a macrophages in MDMs. For analyzing the flow cytometry data a t-test (MDMs) or ANOVA (DH82; U937) corrected for multiple comparisons using Bonferroni Post Hoc Test were performed. Statistical significance was defined as * = $p \leq 0.05$, ** indicated $p \leq 0.01$, *** = $p \leq 0.001$ and **** = $p \leq 0.0001$.

3. Results

3.1 Comparative analysis of the morphology: interspecies variation

To compare human and canine M1- and M2a macrophages we first assessed the morphology (Fig. 2A) based on four defined cell shapes (Fig. 2B). Activation of DH82 and U937 cells caused a significant reduction in small round cell morphology ($\geq 20\mu m$) in M1 and M2a macrophages compared to the medium control M0 (both $p < 0.0001$). DH82 cells showed a tendency toward developing an amoeboid and large/flat morphology after activation with no significant difference between M1 and M2a cells (Fig. 3). In contrast, U937 M2a cells had a higher number of amoeboid cells, but lower amount of spindle shaped macrophages compared to M1 cells ($p = 0.0003$ and $p < 0.0001$), which is in agreement with previous reports (Taniguchi et al., 2015). In canine MDMs, the M1 cells showed predominantly a round and small morphology in contrast to M2a macrophages ($p = 0.0001$). M2a cells presented a higher percentage of large/flat cells compared with M1 macrophages ($p = 0.0062$). In human MDMs no clear difference in morphology could be observed between M1- and M2a cells, but the large diversity in each of three experiments rather confirmed the finding by Porcheray et al. (2005) that activated human MDMs do not seem to display a particular morphology. Spindle shaped cells were predominantly seen in all conditions of human MDMs, as in Vogel et al. (2014), but rarely in canine MDMs. Taken together the activated canine MDMs, as well as activated DH82 cells showed increased

numbers of amoeboid and large/flat cells while the spindle shaped morphology was more abundant in human MDMs and U937.

These facts are in contrast to canine MDM morphology. Our findings that canine MDMs display a prominent amoeboid (M1 and M2a) and large/flat morphology (M2a) compared to human cells are in agreement with the findings of Heinrich et al. (2017). The large and flat morphology was not seen in M1 cells and might be helpful to distinguish M1 and M2a subsets. In contrast to Heinrich et al. (2017) that used canine r-IL4, we successfully activated canine MDMs using human r-IL4 and r-IL13. Both cytokines were selected based on their high sequence similarity and used in a slightly higher concentration as in human cells, suggesting human cytokines as efficient activators of canine macrophages. However, as the human IFN- γ did not show any inflammatory cytokine responses in the canine MDM (data not shown), canine r-IFN- γ was used in combination with LPS to activate canine cells toward M1 phenotype.

The morphology analysis pointed towards clear species-specific variations between canine and human activated macrophages, potentially influenced by distinct cytoskeletal arrangements. Such actin cytoskeleton diversities were described between murine M1- and M2a macrophages, influencing their distinct migration and motility capacity, with M2a cells showing an elongated morphology (Vereyken et al., 2011). In contrast, human macrophages showed more round morphology after activation with IL4, whereas activation by LPS and IFN- γ resulted in increased numbers of elongated macrophages inverse to the mouse cells (Porcheray et al., 2005). In contrast to these studies, we could not detect a significant phenotype to discriminate M1- and M2a macrophages in DH82 and canine MDMs, neither in human cells. Among all cells U937 M1- and M2a macrophages showed the most distinct phenotype, with an amoeboid type in M2a- versus more spindle-shaped M1 cells. Here we show that canine activated M1- and M2a macrophages, derived from canine MDMs or DH82 cells, show a prominent amoeboid and large/flat phenotype. Overall, even if the morphological analysis is not specific to determine M1- and M2a macrophages, it shows a clear activation from M0 cells; therefore, further characterization of canine M1- and M2a cells is worthwhile.

3.2 Comparison of surface markers between human and canine primary cells and cell lines points toward both similarities and differences

After 72 h of activation, the cells were stained with monoclonal antibodies against surface receptors (Tab. 2) and samples were run through flow cytometry. The delta MFI (Δ MFI) value was calculated by subtraction between the MFI value from the stained sample and the isotype control. The Δ MFI levels of the T-cell activation co-receptors CD80 (Fig. 4A) and CD86 (Fig. 4B) were increased in M1-compared to M2a cells, respectively CD80 in DH82 ($p=0.029$) and CD86 in human MDMs ($p=0.028$). A trend towards an increase of Δ MFI expression was observed in M1 cells for CD80 in human MDMs and U937. In contrast, a preliminary experiment with 2 donors in canine MDMs showed a higher expression of the CD80 in M2a cells compared to the M1 cells (Supplementary Fig. S2). However these results differed not significantly between M1- and M2a cells. As previously discussed, upregulation of CD80 and CD86 is described in literature as characteristics of the human M1 MDMs (Mantovani et al., 2004). In contrast, U937 M1 cells showed an identical Δ MFI of CD86 compared to M2a, but in both conditions values were increased compared with the M0 cells. These findings mirror recently published data (Abdulahdi, 2014; Chimal-Ramirez et al., 2016), where CD86 was shown to be a marker for macrophage activation in general rather than in differently expressed M1- versus M2a macrophages. In contrast, our preliminary experiment in canine MDMs showed a higher expression of CD80 in M2a. Further investigations are required to define the true value of this finding.

The typical M2 marker mannose receptor CD206 was non-significantly upregulated in M2a- compared to M1 cells in canine MDMs ($p=0.0508$) and in human MDMs ($p=0.086$). Also in DH82 and U937 no significant difference in CD206 (Fig. 4C) expression between M2a and M1 was found, we only observed a trend toward an increase of CD206 Δ MFI in M2a macrophages. To enhance an upregulation of CD206, the DH82 and U937 were also primed with

combinations of GM-CSF, LPS and IFN- γ or M-CSF, IL4 and IL13 (*marked concentration see Tab. 1), instead of only LPS+ IFN- γ or IL4+ IL13, but no increase of CD206 was achieved compared to the standard protocol (data not shown). Interestingly, the Δ MFI of the high affinity IgE receptor Fc ϵ RI of canine MDMs (Fig. 4D) showed significantly higher expression in M2a- compared to M1 macrophages ($p=0.022$), confirming previous transcriptional data (Heinrich et al. 2017). These data could support a role of the M2a subtype in the interplay with IgE at least in the dog. Human monocytes on the other hand lose their Fc ϵ RI expression during differentiation into macrophages (Segura et al., 2013). Anyhow the canine IgE/Fc ϵ RI biology appears diverse compared to humans. Dogs have significant higher total IgE-values (approximately 100 times more) compared to non-atopic humans without parasitic infections (Hill et al., 1995; Ledin et al., 2006), possibly due to a higher parasitic burden in dogs. Furthermore, IgE itself is shown to upregulate Fc ϵ RI expression in human basophils and monocytes of atopic patients and the absence leads to downregulation (Novak et al., 2002; Saini and MacGlashan, 2002) hypothesizing that high IgE levels in dogs could lead to a Fc ϵ RI expression in macrophages. In addition to IgE, also IL4 was shown to upregulate Fc ϵ RI on human mast cells (Toru et al., 1996). This mechanism could hold true to explain the significant higher Fc ϵ RI expression in canine IL4 and IL13 treated MDMs compared to M1 cells. Interestingly Fc ϵ RI crosslinking in human monocytes and DC is documented to influence the immune response. In the literature controversial results are published. Kraft et al. (2002) showed that crosslinking of Fc ϵ RI leads to TNF- α and IL6 production in human monocytes and DCs in contrast to an increase in IL10 and suppression of T-cell proliferation *in vitro* (Novak et al., (2001). These results could indicate an immunological function of the Fc ϵ RI in canine M2a macrophages, but further investigation on the functional level are required to confirm this hypothesis. In contrast to canine MDMs, DH82 derived M2a macrophages did not show an increase in Fc ϵ RI expression, but DH82 cells expressed the Fc ϵ RI. To summarize, we identified CD80 in canine and human cells, CD86 in human MDMs only, as strong M1 marker. Importantly, the Fc ϵ RI emerged as a highly upregulated marker in M2a macrophage subtype of the canine MDMs, confirming previous transcriptional results (Heinrich, 2017).

3.3 Transcriptional profile reflecting M1- and M2a activated macrophages

The mRNA expression of nine candidate genes was tested in RT-qPCR for DH82, U937 and MDMs and results were normalized against the reference genes. Fig. 5A-D shows the up/downregulation of genes in a log2 fold change of M2a- against M1 macrophages. In general, the expression of M1 genes including IDO1, IL6, CXCL11 and TNF α showed similar patterns between all tested cells. These genes were downregulated in M2a- compared to M1 macrophages. The pro-inflammatory function of macrophages may be regarded as a preserved part of the innate immunity, as it is found in invertebrates and fish (Buchmann, 2014; Lu et al., 2017). Therefore a high similarity between canine to murine and human M1 cells was expected based on previous reports (Martinez et al., 2013) and is presented in supplementary Table S2. IDO1, in our hands, was the most distinctive gene between M1- and M2a macrophages, with the highest levels found in M1 treated U937, confirming previous findings (Godin-Ethier et al., 2011). Beside IDO1, also IL6 and CXCL11 mRNA levels were clearly downregulated in M2a- versus M1 macrophages, confirming them as genes, strongly discriminating between M1- and M2a macrophages in human and murine macrophages (Jablonski et al., 2015; Jaguin et al., 2013; Martinez et al., 2006) and U937 (Xie et al., 2016). In canine MDMs IL6 was only expressed in M1-, but not in M2a macrophages and therefore no fold change could be calculated. In a similar manner TNF α was also downregulated, but with less distinct negative fold change compared to the other M1 genes in all tested cell types. These findings reflect published results on less prominent TNF α gene expression compared to strong markers like IDO1 and CXCL11 (Taniguchi et al., 2015; Tarique et al., 2015). M2a related genes showed less prominent effects and more variability between the four tested cell types. This finding reflects the current research, where more and more different M2a genetic marker are published, but are highly variable between protocols and cell origin. In addition, these simplified in-vitro results are only a short glance at highly plastic cells and the in-vitro conditions do not reflect the true in-vivo microenvironment (Mantovani, et al., 2005). A

strong M2 marker, like arginase-1 in mice, has not been detected in humans or dogs (Heinrich et al., 2017; Martinez et al., 2013).

In contrast to M1 genes, M2a related genes showed less prominent effects and more variability between the four tested cell types. IL10 mRNA was elevated in every tested cell type, but CCL22 showed the highest upregulation in U937, slight upregulation in human MDMs and downregulation in canine MDM and DH82. CD163 was upregulated in M2a- versus M1 macrophages in canine MDMs and DH82, reflecting results of higher CD163 expression in human M2a MDMs (Ambarus et al., 2012). Interestingly, LCN2, a gene encoding lipocalin-2, a protein essential in innate immune responses, was upregulated in canine MDMs and U937 in M2a macrophages, but downregulated in DH82 M2a and not expressed in any of the human MDMs conditions. Heinrich et al. (2017) reported a downregulation of LCN2 mRNA in M1 and M2a compared to M0, using a different activation protocol and M0 as control cells for M1- and M2a macrophages, whereas an upregulation of LCN2 after 4 h stimulation with IL10 was described in human macrophages (Jung et al., 2012). To the authors' knowledge, no data are available in regard of LCN2 transcription in U937 and DH82 cells, but our results in U937 and canine MDMs are in line with the upregulation of LCN2 in human M2a macrophages. In the human MDMs, no LCN2 transcription could be detected after 24 h. That could be explained by the decline of mRNA transcription after a 4 h peak reported in Jung et al. (2012). TGF β showed no benefit in discriminating M1- and M2a cells. This finding is in agreement with Vogel et al. (2014) where TGF β protein production was increased in activated human MDMs, with no detectable differences between subtypes.

5. Summary

We report here an *in-vitro* protocol to activate canine macrophages (histiocytotic DH82 cells and primary MDMs) using human cytokines, into an alternatively activated M2a phenotype. M2a cells showed similar transcription and surface marker profiles in comparison to activated human macrophages (U937 and MDMs). With surface receptor staining and the transcriptional analysis we confirmed activation of canine MDMs and DH82 into M2a macrophages. Variations between the species concerned more prominently upregulated CD163 expression and IL10 secretion in canine M2a than in human MDMs, and the usefulness of CCL22 as a marker in the human U937- in contrast to canine M2a cells.

The described novel method allows *in-vitro* activation of canine macrophages towards the M2a phenotype as a basis for comparative studies on the pathophysiological mechanism in humans versus dogs, with specific impact on immune mechanisms in oncology and allergy pathogenesis. In addition, we identified the high affinity IgE receptor Fc ϵ RI as a new marker for canine M2a macrophages. With our work we believe to contribute to a better understanding of the canine immune system for comparative trials, and to generate knowledge to tackle IgE mediated diseases also in the dog, which like the human allergies, are on the rise.

Conflict of Interest

None.

Acknowledgment

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Legends

Figure 1: Schematic presentation of macrophage differentiation. A) Monocytes from canine and human PBMCs were purified by Ficoll separation and brought into culture using either GM-CSF or M-CSF for M0 generation. For M1 generation, GM-CSF, LPS and INF-gamma was added and experiments were performed two or three days after stimulation. M2a were stimulated using M-CSF, IL-4 and IL-13.

B) In the same fashion, human monocytic cell line U937, or canine histiocytic cell line DH82 were differentiated *in vitro* using medium for M0, LPS and IFG-gamma for M1 and IL-4 and IL-13 for M2a. *) PMA treatment was only used in U937 for activating the monocyte/macrophage phenotype.

Figure 2: Morphological analysis and quantification of differently activated macrophages, A) using cell lines DH82 or U937, or B) primary macrophages from canine or human origin. X-axis indicated the phenotypic characteristics; y-axis represents the relative percentage of 100 randomly counted cells. The bars indicate the range and the line in the bar is the mean over three experiments. A one-way ANOVA and Tukey's multiple comparisons test was used. ** indicated $p \leq 0.01$, *** = $p \leq 0.001$ and **** = $p \leq 0.0001$. White boxes: M0, grey: M1, black: M2a macrophages. C) Typical light microscopic pictures of the different morphologies observed *in vitro*.

Figure 3: Light microscopic characteristics of the canine DH82 differentiated macrophages (M0, M1 and M2a) in DiffQuick stain. M0 cells appear in a small round shape in contrast to bigger and more dendritic-like M1 cells. M2a cells display large, flat, sometimes multinucleated cells. Scale bar presents 50 μm .

Figure 4: Flow cytometric analysis of surface markers expressed by *in vitro* differentiated canine cell line DH82 or canine cMDMs (canine monocyte-derived macrophages), as compared to human U937 cell line or hMDMs (human monocyte-derived macrophages). Each graph presents mean and SD of three independent experiments. Statistical analysis was performed by one-way ANOVA plus multiple comparisons for DH82/U937 and unpaired t-test for MDMs (* = $p \leq 0.05$). White boxes: M0, grey: M1, black: M2a macrophages. A) CD80, B) CD86, C) CD206, D) Fc ϵ RI expression relative to staining with isotype controls (ΔMFI).

Figure 5: Transcriptional profiling of M2a genes versus M1 genes in differentiated A) canine cell line DH82, or B) cMDMs (canine monocyte-derived macrophages), as compared to C) human U937 cell line, or D) hMDMs (human monocyte-derived macrophages). X-axis: target genes; Y-axis: the up- or downregulation of genes in log2 fold change of expression; Exact numbers in Table 3; n.d.: not determined.

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Table 1 Cell culture reagents and final concentrations used for activation of human (U937 and MDMs) and canine cells (DH82 and MDMs); * these reagents were used in control experiments to check for a potential increase of surface protein expression (specifically mentioned in the text) and are not part of routinely used protocol in presented experiments. DMEM, Dulbecco's Modified Eagle's medium; RPMI, Roswell Park Memorial Institute; v/v, volume per volume

Material	Supplier	Concentration ng/ml			
		U937	DH82	Human MDMs	Canine MDMs
Phorbol 12-myristate 13-acetate (PMA)	Sigma-Aldrich, St. Louis, MO	100nM	/	/	/
Recombinant human GM-CSF	ImmunoTools, Friesoythe, Germany	20*	20*	20	20
Lipopolysaccharide from <i>E.coli</i> E055:B55	Sigma-Aldrich, St. Louis, MO	100	100	50	50
Recombinant human IFN γ	ImmunoTools, Friesoythe, Germany	100	/	20	/
Recombinant canine IFN γ	R&D Systems, Minneapolis, MN	/	100	/	20
Recombinant human M-CSF	Biolegend, San Diego, CA	20*	40*	20	40
Recombinant human IL4	ImmunoTools, Friesoythe, Germany	20	40	20	40
Recombinant human IL13	ImmunoTools, Friesoythe, Germany	20	40	20	40
Concentration of cells for Activation (cells/ml)		2.5×10^5	2.5×10^5	3×10^5	3×10^5
Medium	RPMI, DMEM, Penicillin-Streptomycin (Pen-Strep), non-essential amino acid solution (NEAA): all Sigma-Aldrich, St. Louis, MO fetal bovine serum (FBS) Life Technologies, Carlsbad, CA	Complete RPMI + FBS + 1%Pen-Strep	DMEM low glucose + 10% (v/v) FBS + 1% (v/v) NEAA + 1%(v/v) Pen-Strep	Complete RPMI see U937	Complete RPMI see U937

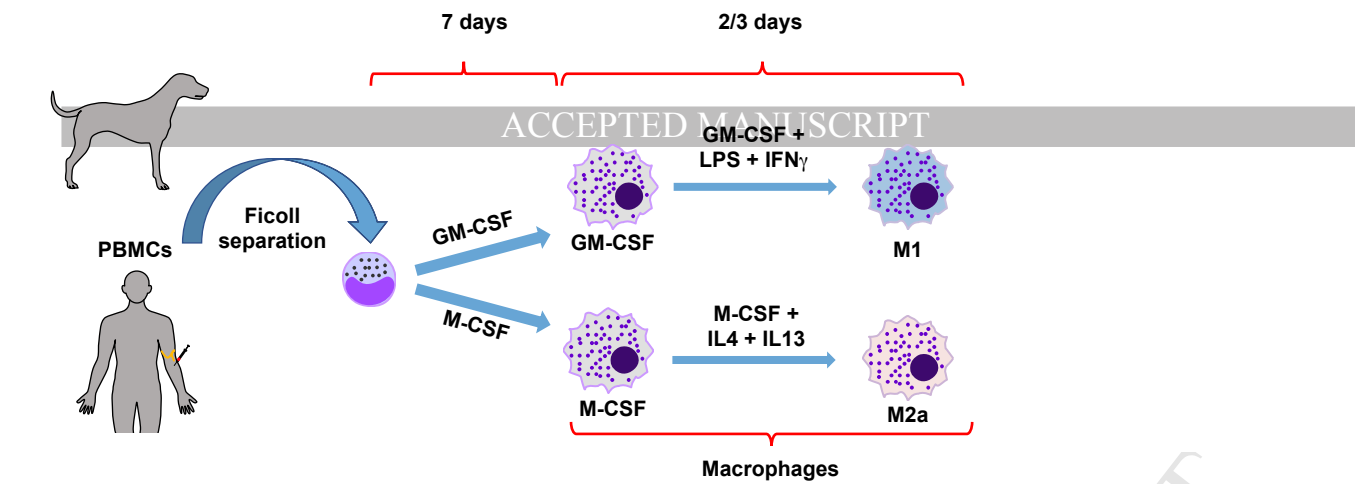
Table 2 Antibody list

Cells	Reactivity	Target	Clone	Company	Isotype	Label	NIF Antibody ID
DH82; U937	Anti-human	CD14	M5E2	Biologend	mouse-IgG2a, κ	APC/Cy7	AB_493695
DH82; U937; ca MDMs; hu MDMs	Anti-human	CD206	15-2	Biologend	mouse-IgG1, κ	APC/Cy7	AB_2144930
U937; hu MDMs	Anti-human	CD86	IT2.2	Ebioscience	mouse-IgG2b, κ	PE	AB_10732345
U937; hu MDMs	Anti-human	CD80	2D10	Biologend	mouse-IgG1, κ	PE/Cy7	AB_2076148
DH82; ca MDMs	Anti-mouse	CD80	16-10A1	Biologend	armenian hamster IgG	PE	AB_313129
DH82; ca MDMs	Anti-mouse	FcεRI	MAR-1	Ebioscience	armenian hamster IgG	PE	AB_466029

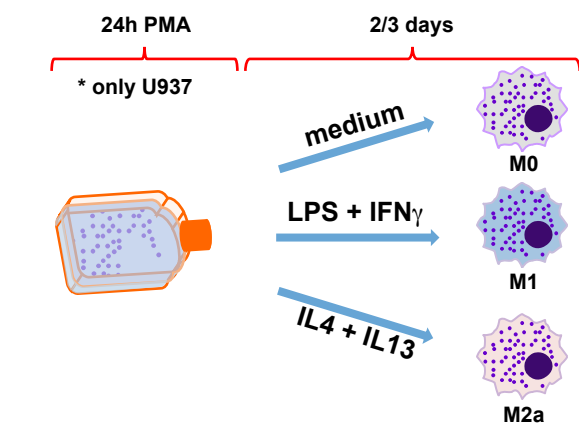
Table 3 Fold change of mRNA expression evaluated for all tested cells is shown. Results present the mean and standard deviation of three experiments each.

Gene Name	Gene Symbol	DH82			U937			Canine MDMs			Human MDMs
		M1 vs. M0	M2 vs. M0	M2 vs. M1	M1 vs. M0	M2 vs. M0	M2 vs. M1	M1 vs. GMCSF	M2 vs. MCSF	M2 vs. M1	M2 vs. M1
Tumor necrosis factor alpha	TNF-a	14.94 ± 7.75	-1.21 ± 0.86	-19.79 ± 4.14	-1.31 ± 0.1	-4.25 ± 2.36	-3.12 ± 1.9	31.64 ± 17.84	-4.0 ± 4.00	-17.13 ± 8.34	-4.37 ± 4.1
Interleukin 6	IL-6	221.70 ± 46.1	-6.46 ± 2.92	-1495.20 ± 513.85	3.56 ± 4.34	-49.63 ± 28.06	-24.84 ± 18.11	**)	**)	**))	-44.66 ± 39.0
Indoleamine 2,3-dioxygenase	IDO1	417.71 ± 164.21	-1.55 ± 0.13	-514.77 ± 266.14	337.74 ± 138.55	-14.18 ± 2.2	-3991.87 ± 1731.7	939E+05 ± 0.01	7.98 ± 0.43	-308.06 ± 317.20	-442.6 ± 293.55
C-X-C motif chemokine 11	CXCL11	139.90 ± 66.96	-2.88 ± 2.08	-446.55 ± 153.0	33.06 ± 36.64	-1.07 ± 0.644	-13.34 ± 10.57	*	-90.73 ± 49.36	-191.75 ± 163.42	-23.95 ± 22.84
Transforming growth factor beta 1	TGF-b1	-1.0 ± 0.75	-2.1 ± 1.16	-1.71 ± 0.43	-1.31 ± 0.58	-1.03 ± 0.42	1.36 ± 0.17	-2.63 ± 0.54	1.29 ± 0.24	1.87 ± 1.0	-0.59 ± 0.54
Interleukin 10	IL-10	-2.06 ± 1.55	1.72 ± 0.75	4.71 ± 2.16	-1.95 ± 2.23	-1.07 ± 0.28	6.35 ± 3.73	3.04 ± 1.41	3.32 ± 1.89	21.96 ± 25.4	1.25 ± 0.8
C-C motif chemokine 22	CCL22	***)	***)	-2.83 ± 1.48	-5.00 ± 2.29	108.30 ± 95.10	695.47 ± 709.33	3004.43 ± 2538.21	123.58 ± 50.05	1.29 ± 0.79	1.31 ± 0.01
Lipocalin 2	LCN2	2.34 ± 0.77	1.26 ± 0.73	-1.44 ± 0.49	1.24 ± 0.99	3.72 ± 3.46	6.02 ± 2.18	*)	33.97 ± 32.62	1.16 ± 1.06	***)
Cluster of Differentiation 163	CD163	-13.37 ± 8.91	-1.05 ± 0.7	13.39 ± 2.98	n.d	n.d	n.d	5.99 ± 6.33	14.84 ± 15.46	11.35 ± 4.75	n.d.

*) not detectable in GMCSF control; **) only detectable in M1; ***) not detected; n.d. not determined



A cMDMs / hMDMs

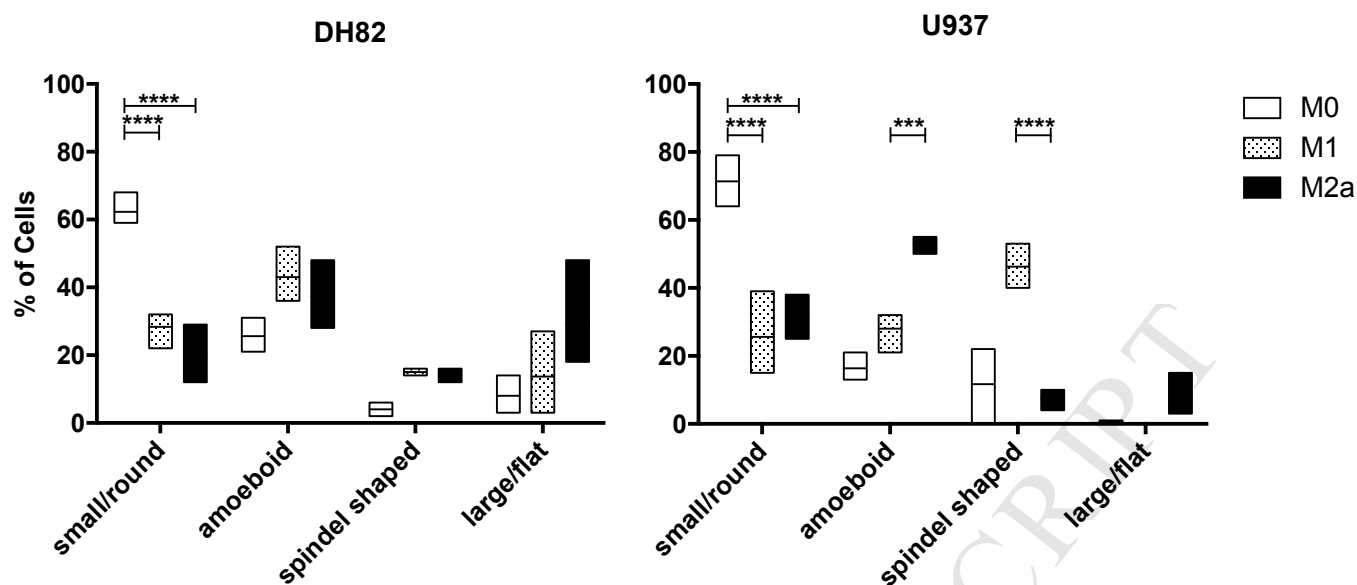


B DH82 / U937

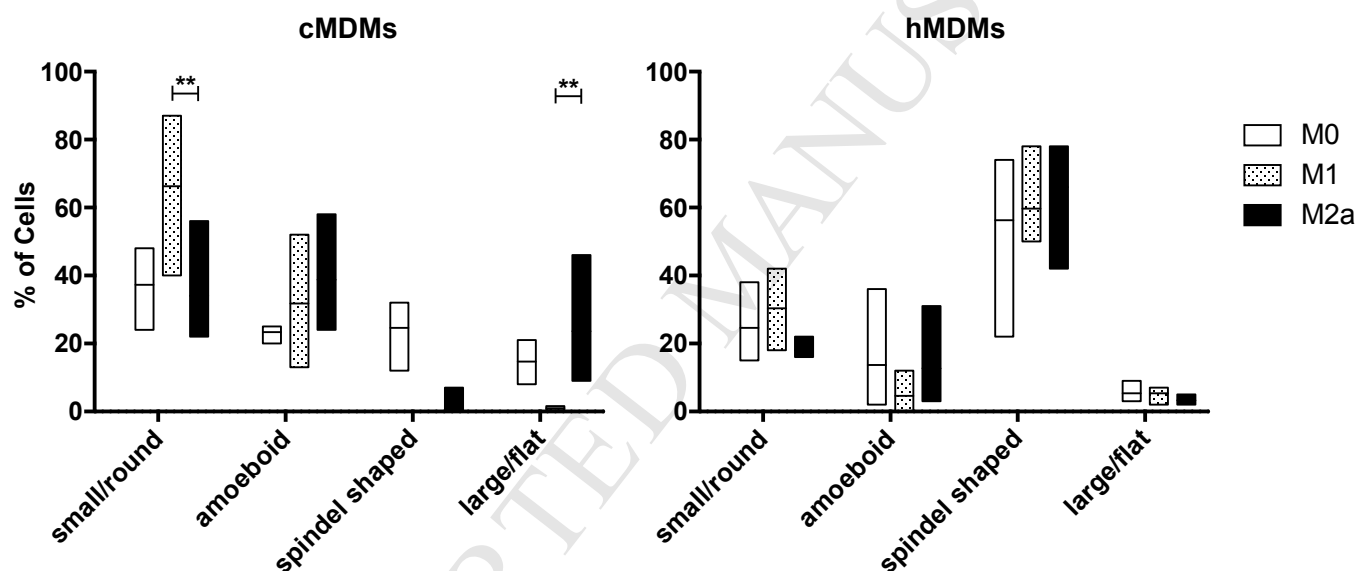
Figure 1

A

ACCEPTED MANUSCRIPT



B



C



Figure 2

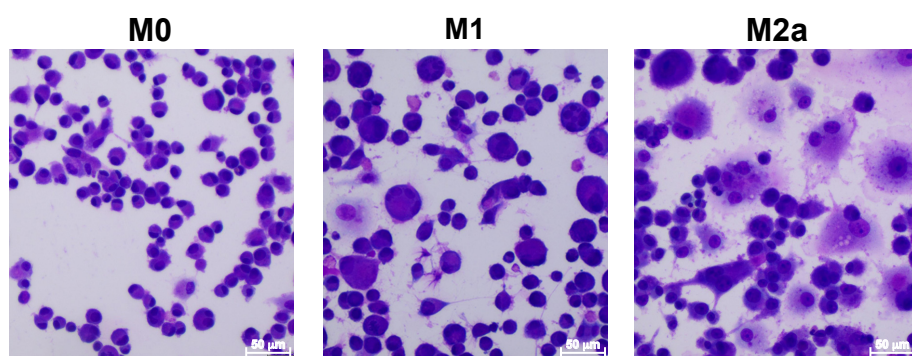
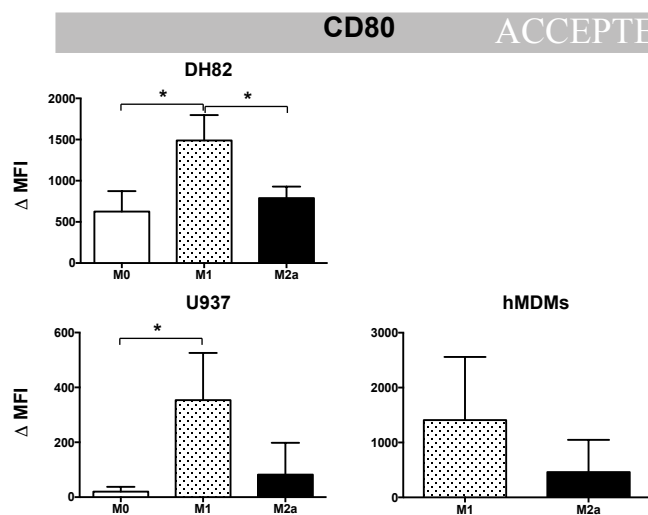
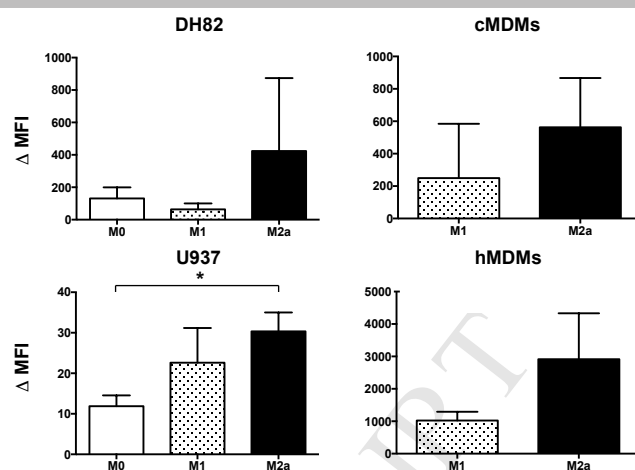


Figure 3

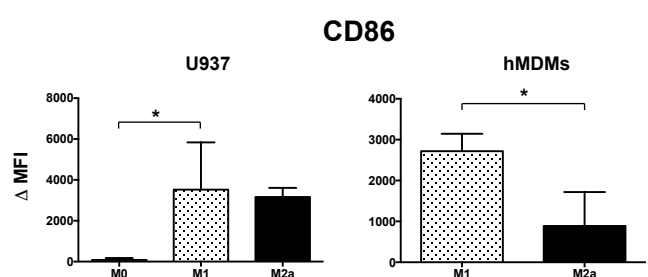
A



C



B



D

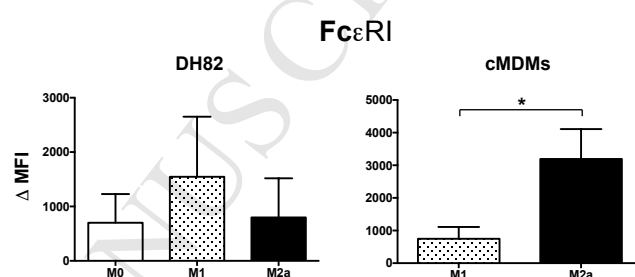
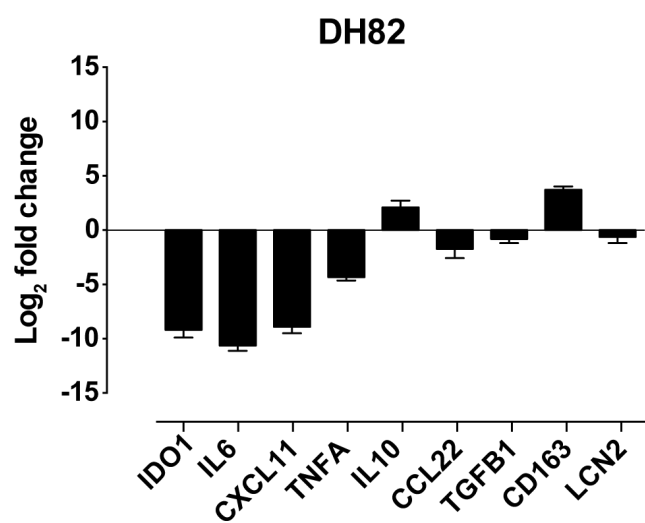
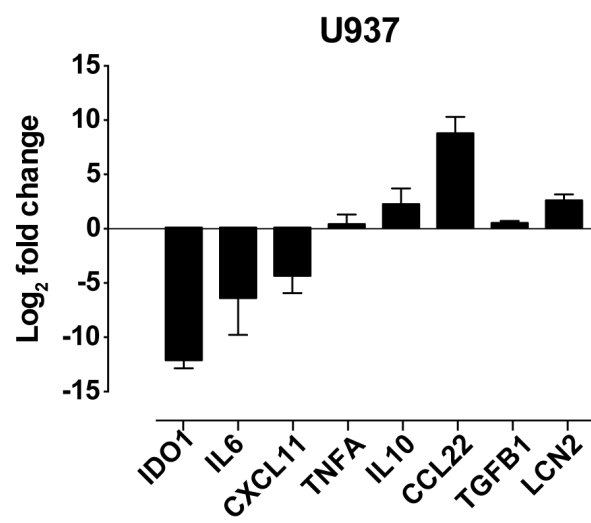
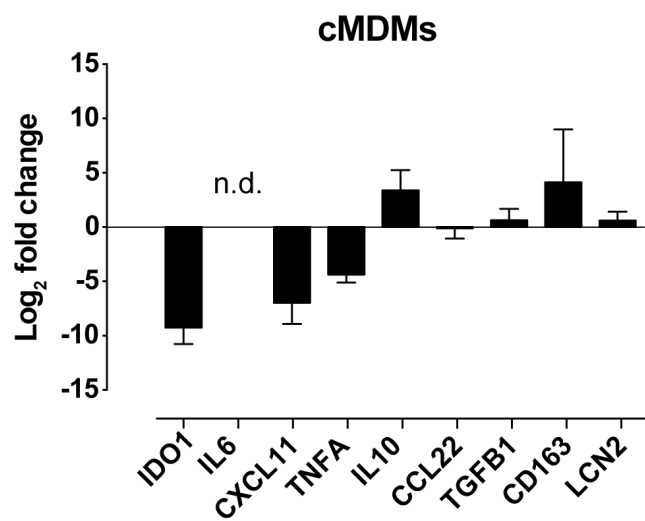
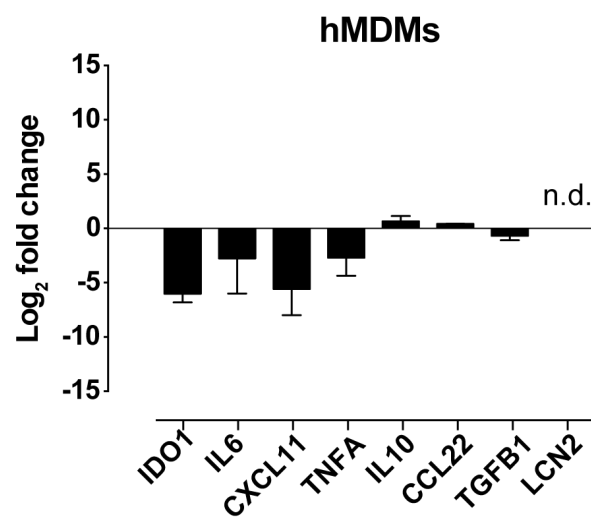


Figure 4

A**C****B****D****Figure 5**

Highlights

- By a novel *in-vitro* activation method now also canine macrophages can be differentiated to M2a subtype.
- This can be done with canine primary monocyte-derived macrophages as well as cell line DH82 histiocytotic cells.
- The high affinity IgE receptor FcεRI is a specific marker for canine, but not human M2a macrophages.
- Characteristics of canine and human macrophages were systematically compared side-by-side, rendering a catalogue of similarities and differences.

Supplementary Table S1 Canine and human primers for RT-qPCR

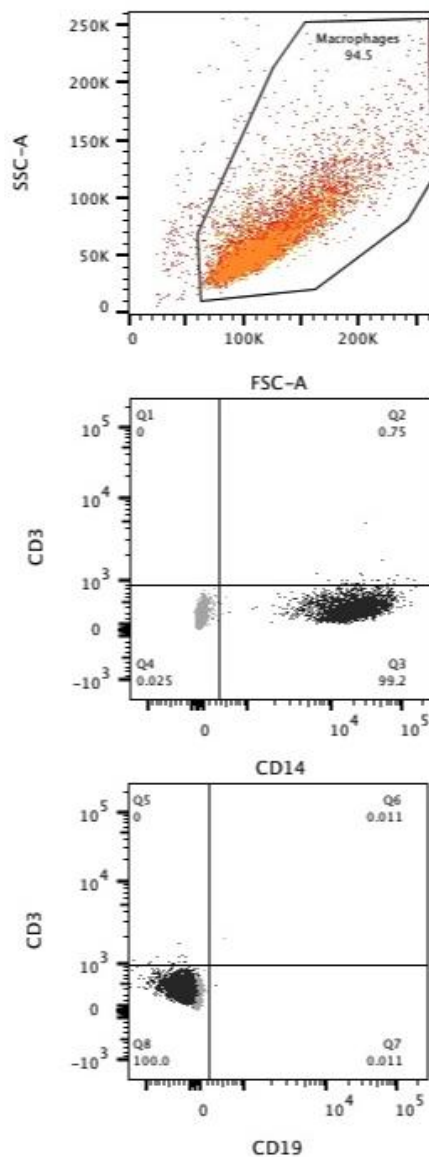
Canine Primers									
Name	Gene	Sequence (5'→3') forward primer reverse primer	Product length	Efficiency	R ²	Reference No. NCBI	Start	Stop	Reference
Ornithine decarboxylase antizyme	OAZ1	CACGGGCTCCAAACACATTA	180	0.99	0.994	NM_00112 7234.1	433	452	
		TCATCGCGGTTCTTATGGAA					612	593	
Tumor necrosis factor alpha	TNFA	ACCACACTCTTCTGCCTGCT	137	1.10	0.996	NM_00100 3244.4	133	152	
		GCTACTGGCTTGCTCACTTGGG					269	249	
Interleukin 6	IL6	CTCTCCACAAGCGCCTTCTC	102	0.84	0.996	NM_00100 3301.1	67	86	doi:10.1016/j.vetimm.2004.1 1.007
		TGAAGTGGCATCATCCTTGG					168	149	
Indoleamine 2,3- dioxygenase	IDO1	GCACCGAGCCCATAAAGAGTT	102	0.89	0.992	XM_53279 3.5	758	778	doi: 10.4049/jimmunol.177.10.73 03
		GAGTTGCCTTTCCAACCAGAC					859	839	
C-X-C motif chemokine 11	CXCL11	AGTGTGAAGGGCATGTTACA	77	0.90	0.986	XM_00364 0114.2	7	27	doi: 10.4049/jimmunol.177.10.73 03
		CCTTTGAACATGGGGAAATCTTG					83	61	
Transforming growth factor beta 1	TGFB1	GATTCTTGGCGCTACCTCAG	199	0.97	0.995	NM_00100 3309.1	586	605	
		GGCTGGAAGTGAACCCGTTA					784	765	
Interleukin 10	IL10	CGACCCAGACATCAAGAACC	101	1.10	0.998	NM_00100 3077.1	307	326	doi:10.1016/j.vetimm.2004.1 1.007
		CACAGGGAAGAAATCGGTGA					407	388	
Cluster of Differentiation 163 (transcript variant 1)	CD163	GCGGCTTACAGTTTCCTTGAG	160	1.08	0.998	NM_00104 8020.1	3222	3242	doi: 10.1128/JVI.00513-07
		AGACACAGAAATTAGCCCAGCA					3381	3360	
C-C motif chemokine 22	CCL22	ACTACATCCGTCACCCCTCTG	96	1.05	0.985	XM_00343 3778.2	137	156	
		TGACAGTTAGGAAGACCACGC					232	212	
Lipocalin 2	LCN2	AGCTGAAAGATGACCAGAGCTACAA	140	1.00	0.994	XM_54844 1.5	304	328	
		TACTCCAGGGTAAAGCTGAATGTCTG					443	419	
Human Primers									
Glyceraldehyde 3- phosphate dehydrogenase (transcript variant 1)	GAPDH	TTTTGCGTCGCCAGCCGAG	115	1.02	0.986	NM_00204 6.5	152	170	
		ACCAGAGTTAAAAGCAGCCCTGG					266	244	
Tumor necrosis factor alpha	TNFA	CAGGCGGTGCTTGTTCTCTCA	183	1.07	0.986	NM_00059 4.3	256	275	
		GGCTTGCTACTCGGGGTTCTG					438	419	
Interleukin 6 (transcript variant 1)	IL6	ATCCTCGACGGCATCTCAGC	164	0.97	0.987	NM_00060 0.4	299	318	
		TTCACCAGGCAAGTCTCCTCA					462	442	
Indoleamine 2,3- dioxygenase	IDO1	GGCAAAGGTCATGGAGATGT	203	1.02	0.986	NM_00216 4.5	391	410	doi: 10.1016/j.cellimm.2014.02.00 5
		CTGCAGTCTCCATCACGAAA					593	574	
C-X-C motif chemokine 11	CXCL1 1	GTTCAAGGCTTCCCCATGTTC	198	1.02	0.994	NM_00540 9.4	265	285	
		AAGCCTTGCTTGCTTCGAT					462	444	
Transforming growth factor beta 1	TGFB	GTTGTGCGGCAGTG GTTGAG	114	1.10	0.996	NM_00066 0.6	1446	1465	
		AGTGAACCCGTTGATGTCCACT					1559	1538	
Interleukin 10	IL10	GCCTAACATGCTTCGAGATC	206	1.04	0.998	NM_00057 2.2	189	170	
		TGATGTCTGGGTCTTG GTTC					356	375	
C-C motif chemokine 22	CCL22	TCCGTTACCGTCTGCCCTG	124	1.03	0.997	NM_00299 0.4	156	175	
		GGCACTCTGGGATCGGCACA					279	260	
Lipocalin 2	LCN2	ACCCTCTACGGGAGAACCAAGG	131	1.09	0.986	NM_00556 4.4	562	583	
		CAGCCGTCGATACACTGGTCTG					692	672	

Supplementary Table S2: Comparison of murine, canine and human M1 and M2a markers.

Adapted from Heinrich, 2017 (a); Jablonski, 2015 (b); Mantovani, 2004 (c); Martinez, 2006 (d); Martinez, 2013 (e); Rószler, 2015 (f); Ying, 2013 (g). Arg-1, arginase-1; Egr2, early growth response protein 2; F13A1, coagulation factor XIII, A1 polypeptide; FCER1A, Fc fragment of IgE, high affinity I receptor; FIZZ1, resistin-like molecule- α ; Fpr2, formyl peptide receptor 2; Gpr18; G-protein coupled receptor 18; IDO1, indoleamine-pyrrole 2,3 dioxygenase; IL1-ra, IL-1 receptor antagonist; iNOS, inducible nitric oxide synthase; LIF, leukocyte inhibitory factor; LYVE1, lymphatic vessel endothelial hyaluronan receptor 1 succinate; MMR, mannose receptor (CD206); P2RY14, purinergic receptor P2Y, G-protein coupled, 14; PI3, peptidase inhibitor 3, skin-derived; SOCS3, suppressor of cytokine signaling 3; SUCNR1, succinate receptor 1; TGF- β , transforming growth factor- β ; TGM2, transglutaminase 2; TNF- α , tumor necrosis factor α ; Ym1, chitinase-3-like protein-3

	M1			M2a		
	Mouse	Dog ^a	Human	Mouse	Dog ^a	Human
Marker expression	CD86 ^g CD38 ^b	SUCNR1 P2RY14	CD86 ^c CD80 ^c MHC II ^c IL1R ^c	MMR/CD206 ^e Ym1/2 ^f Arg-1 ^{b,g,f} Fizz1 ^f Erg2 ^b TGM2 ^e	MMR/CD206 MHC II FCER1A LYVE1	MMR/CD206 ^{c,e,f} MHC II ^f CD163 ^f TGM2 ^{e,f} Erg2 ^f
Cytokines secretion and receptors	TNF- α ^g IL6 ^b IL1 β ^b	TNF- α IL6	TNF- α ^c IL6 ^d IL1 β ^c IL12 ^c IL23 ^c	IL10 ^g	IL13RA2 TGFB2	IL10 ^a TGFB ^f IL1RA ^f
Chemokine secretion and receptors	CXCL9 ^b CXCL3 ^b CXCL10 ^b	CCL22 CXCL12	CXCL11 ^d CXCL10 ^c CCL5 ^c	CCL22 ^b CCL17 ^b	CCL17 CCL24	CCL22 ^c CCL17 ^f CCL24 ^c
Miscellaneous	Gpr18 ^b Fpr2 ^b LCN2 ^b	PI3	IDO1 ^d	c-Myc ^b	F13A1	

Supplementary Figure S1: Three colour assay of canine monocytes cultured during 7 days with M-CSF/GM-CSF. For confirmation of a clear macrophage population, stainings with antibodies to CD14, CD3 and CD19 were performed. The cell population was gated with forward and side scatter. CD3 vs CD14 analysis revealed a population with 99.2% of cells positive for CD14 and negative for CD3. This population was further analysed for CD19 and was CD19 and CD3 negative.



Supplementary Figure S2: Pilot experiment with canine MDM M1 versus M2 macrophages in flow cytometry; y-axis: CD80 expression relative to staining with isotype controls (Δ MFI). Data in the M1 experiment were obtained from three canine patients, M2a from two donors.

