Comparative Medicine

Messerli Research Institute Veterinary University of Vienna

Head of department: Univ.-Prof. Dr.med.univ. Jensen-Jarolim Erika

# Impact of iron deficiency on canine DH-82 macrophages

Diploma Thesis

Veterinary University Vienna Yvonne Mayer Vienna, June 2024 Betreuerin: Dr.rer.nat. Priv.-Doz. Franziska Roth-Walter Begutachterin : Dipl. ECVD. Dr. med. vet. Lucia Panakova

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# Abstract

**Background:** Iron-deficiency is the most common deficiency world-wide and is associated with increased all-cause morbidity and mortality. It is closely linked to inflammation as the major cell responsible for iron recycling and distribution are regulatory macrophages. As such, in this diploma thesis the impact of iron deficiency on canine DH82 macrophages were assessed.

**Methods:** DH82 cells were stimulated under iron-deprived (using the iron-chelator DFO) or sated conditions (by adding FCS into the culture) with and without PMA for 18 hours, before analyzing CD14 expression and the labile iron content. Moreover, supernatant of stimulated cells were analyzed for IL-6 by sandwich ELISA.

**Results:** Culturing DH82 cells with the iron -chelator DFO resulting in moderate lowering of the labile iron content and improved CD14 expression. In contrast, PMA-stimulation of DH82 cells lowered CD14+expression and markedly decrease the labile iron content indicating immune activation. However, within 18h stimulation IL6 were not secreted in the supernatants

**Conclusion:** DFO participates in iron mobilization and promoted rather an anti-inflammatory phenotype in DH82 cells, whereas PMA rather promoted an inflammatory phenotype in DH82 canine macrophages.

# Abstrakt:

*Hintergrund*: Eisenmangel ist weltweit der häufigste Mangel und geht mit einer erhöhten Gesamtmorbidität und Mortalität einher. Dieser steht in enger Verbindung mit Entzündungen, da die wichtigsten Zellen, die für die Eisenrecycling- und -verteilung verantwortlich sind, regulatorische Makrophagen sind. Somit kam es im Zuge dieser Diplomarbeit zur Untersuchung der Auswirkungen von Eisenmangel auf canine DH82-Makrophagen.

*Methoden*: DH82-Zellen wurden unter eisenarmen (unter Verwendung des Eisenchelators DFO) oder gesättigten Bedingungen (durch Zugabe von FCS zur Kultur) mit und ohne PMA 18 Stunden lang stimuliert, bevor die CD14-Expression und der labile Eisengehalt analysiert wurden. Darüber hinaus wurde der Überstand stimulierter Zellen mittels Sandwich-ELISA auf IL-6 analysiert.

*Ergebnisse*: Die Kultivierung von DH82-Zellen mit dem Eisen-Chelator DFO führte zu einer moderaten Senkung des labilen Eisengehalts und einer verbesserten CD14-Expression. Im Gegensatz dazu verringerte die PMA-Stimulation von DH82-Zellen die CD14+-Expression und den Gehalt an labilem Eisen deutlich, was auf eine Immunaktivierung hinweist. Allerdings wurde innerhalb der 18-stündigen Stimulation kein IL6 in den Überständen sezerniert

*Schlussfolgerung*: DFO ist an der Eisenmobilisierung beteiligt und fördert eher einen anti- inflammatorischen Phänotyp in DH82-Zellen, wohingegen PMA eher einen inflammatorischen Phänotyp in DH82-Hundemakrophagen fördert.

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# **1** Introduction

#### 1.1 Defining iron

Iron is a micronutrient and a trace element, therefor naturally present in certain foods and exists primarily in two forms heme- and nonheme- iron.<sup>1</sup> Heme- iron is predominantly found in meat and fish, whereas nonheme- iron comes from legumes, fruits, grains and cocoa.<sup>1</sup> However, about half of the iron content in meat is present as non- heme iron and opposed to that, hemeiron is also present in certain plants such as soy named leghemoglobin.<sup>1</sup> Iron uptake is always an active process, with the absorption of heme- iron, which is five times more effective and occurs through receptors.<sup>1</sup> Nonheme-iron, has to be reduced to its ferrous form first, before receptor-mediated uptake, which is facilitated by vitamin C.<sup>2</sup> The main site for iron absorption is the duodenum, which has an acidic pH range from four to five, compared to the other sections of intestines, which are possessing a pH range from seven to nine.<sup>1</sup> Nonheme- iron is transported across the apical membrane of the enterocytes by the divalent metal transporter one, followed across the basolateral membrane of the enterocytes by ferroportin, bound to transferrin in the plasma and transported to the target organs either for instant use or storage.<sup>3</sup> Despite the important role of iron in our bodies, many animals and people are affected by iron deficiency.<sup>1</sup> Some of the main reasons include blood loss, chronic lack of dietary iron intake and impaired iron absorption due to immune activation.<sup>3</sup>

But sufficiency of iron and other micronutrients such as zinc and various vitamins in our body, contribute to immune resilience, promotion of regulatory cells and tolerance induction.<sup>2</sup> Due to iron's high affinity for oxygen, access to which is tightly controlled and requires that iron is always present in a complexed and/or protein-bound state. <sup>1</sup> Otherwise reactive oxygen species are formed with all its harmful consequences.<sup>1</sup> According to that iron presents itself throughout our immune system in two forms, either a reduced ferrous form (Fe2+, electron donor) or an oxidative ferric form (Fe3+, electron acceptor).<sup>1,3</sup> In anaerobic environments the ferrous form prevails, whereas in oxygen- rich environments it is the ferric form.<sup>1</sup> This form is hazardous, because of its high affinity of binding oxygen.<sup>1</sup> As a result it is able to damage tissue by building oxygen radicals which attack cellular membranes, proteins and the DNA.<sup>1</sup>

Heme-iron is a component of hemoglobin, a protein contained in erythrocytes, that functions in carrying and subsequently transferring oxygen from the lungs to the tissues.<sup>2</sup> Hemoglobin contains a large amount of heme- iron, smaller amounts are found in myoglobin.<sup>3</sup> Therefor iron is a component of myoglobin, a protein providing oxygen and supporting muscle metabolism as well as healthy connective tissue.<sup>1</sup> The next biggest iron stores are the macrophages, being the main responsible cells for recycling iron.<sup>1</sup> As these cells are also considered immune cells, iron is tightly linked to the immune system with iron turnover in these cells determining whether these cells are part of the inflammatory type (with an iron retention phenotype) or the regulatory type and actively partaking in distributing iron.<sup>1,2</sup> A significant amount of allergens such as lipocalins and pathogenesis- related proteins are able to bind iron and sequester or supply it from antigen- presenting immune cells, resulting in initiating presentation or activation.<sup>1</sup> Iron deficiency promotes Th2- cell survival, immunoglobulin class switching and plays a contributing role in the effector phase, as a lack of iron stimulates mast cell degranulation.<sup>1,2</sup> Th1 cells are more sensitive to iron deprivation, therefore the milieu is favoring Th2 cells.<sup>1</sup>

Under iron deficient conditions the AID enzyme (activation-induced cytidine deaminase) in B cells, which is responsible for antibody production, initiates class-switching and affinity maturation.<sup>1</sup>

Mast cells are able to degranulate and release inflammatory cytokines under poor iron conditions.<sup>2</sup> In contrast providing mast cells with iron saturated with transferrin, lactoferrin and betalactoglobulin inhibits their ability of mast cell degranulation.<sup>2</sup>

#### **1.2 Iron Homeostasis**

The storage of iron is precisely regulated so that enough iron is available to meet the needs of the body without causing toxicity due to excess.<sup>3</sup> Since the bodies only mechanism to excrete excessive iron is blood loss, the maintenance of an appropriate iron level is tightly controlled by limiting enteric iron uptake through impaired efflux from enterocytes, erythropoiesis, recycling from senescent RBCs and storage.<sup>3</sup> The key regulator of iron homeostasis was discovered in 2001 as the hormone hepcidin, which is only 25 amino acids long and is mainly synthesized in the liver.<sup>1</sup> It is mainly secreted by the liver in response to inflammation or iron overload, and to a lesser extent by the parietal cells of the stomach and macrophages, which synthesize it

themselves.<sup>1</sup> The increased synthesis is stimulated by cytokines of which interleukin 6 has the greatest importance.<sup>2</sup> To suppress hepcidin production during anaemia, erythropoietic activity is required because it synthesizes growth differentiation factor 15, which inhibits hepcidin at high concentrations.<sup>4</sup>

Hepcidin is responsible for the reduction of iron in plasma by binding to ferroportin and its subsequent destruction.<sup>3,4</sup> Consequently, absorbed dietary iron remains in the enterocyte, where it is lost through enterocyte shedding.<sup>3</sup> When iron levels are low, hepcidin production and secretion are suppressed leading to an increased dietary iron absorption and iron efflux from enterocytes into the blood.<sup>3</sup> During inflammation, iron overload and hepcidin deficiency, hepcidin is upregulated.<sup>5</sup> It also acts as the key mediator of anemia of chronic disease and anemia of inflammation.<sup>5</sup> Due to functioning as an acute phase reactant it is responsible for the removal of iron from circulation along with iron binding proteins for instance lactoferrin, hap-toglobulin, hemopexin, lipocalin2 and ferritin.<sup>1,5</sup>

Because of its multiple roles in iron regulation, hepcidin levels are indicative of ongoing inflammation as well as iron requirements when iron stores are still adequate.<sup>1</sup> However, when iron stores are empty, as in severe anemia, hepcidin levels remain low despite inflammation.<sup>1</sup> Tight homeostasis is essential, as excessive iron accumulation leading to the presence of "free" iron in hepatocytes leads to pathological damage, called hemochromatosis, followed by fibrosis and cirrhosis.<sup>3</sup> Hemochromatosis is the result of genetic mutations that cause too much iron to be absorbed, or is the result of blood transfusions and intravenous iron administration.<sup>3</sup> However, iron depletion and ultimately iron deficiency anemia affects about one fourth of the world's entire population.<sup>3</sup>

### 1.3. Iron deficiency in dogs

Iron deficiency is either caused by insufficient dietary intake or chronic external non- resorptive blood loss.<sup>3,6</sup> The recommended dietary iron intake for adult dogs and cats is estimated at 80mg/kg dry matter and higher in puppies and kittens due to their rapid growth.<sup>3</sup> Insufficient dietary iron intake occurs seldom when fed with commercial pet foods, but can occur when fed home-cooked or vegetarian options without recommended iron supplementation.<sup>3</sup>

Iron deficiency has many different causes, particularly often it is attributed to chronic blood loss.<sup>5,6</sup> In contrast during acute blood loss, body iron stores are normally sufficient for accelerated erythropoiesis and subsequent iron uptake is adequate for iron homeostasis.<sup>3,6</sup> Iron deficiency anemia develops over a larger period of time usually several weeks to months including chronic or recurrent blood loss.<sup>3</sup> Reasons for such severe ongoing blood loss include ectoparasites, endoparasites, hematuria, epistaxis, hemorrhagic skin pathology, coagulopathy, thrombocytopenia, thrombocytopathia and gastrointestinal hemorrhage.<sup>3,6</sup> Blood loss through the gastrointestinal tract is one of the main causes.<sup>5,6</sup> The blood loss can stem from malign or benign tumors, gastric ulcers, inflammatory bowel disease, parasites, ulcerogenic drugs or secondary to systemic diseases such as renal or hepatic diseases, bleeding disorders and hypoadrenocorticism.<sup>3,6</sup>

Iron deficiency anemia is characterized in three stages: storage iron deficiency, iron deficient erythropoiesis and iron deficiency anemia.<sup>3,6</sup> Primarily during blood loss iron body stores are predominantly used for accelerated erythropoiesis.<sup>3,6</sup> When the iron reserves are exhausted, erythropoiesis and production of iron depending proteins such as hemoglobin and myoglobin become limited, leading to an overt iron deficiency anemia.<sup>3</sup> Anemia exaggerates due to the fact that erythrocytes produced under iron insufficient conditions are prone to shorter survival and contain a third of hemoglobin compared to healthy erythrocytes.<sup>3</sup> Consequently , this may result in mild hemolysis further facilitating iron-deficiency, but also affecting measurements of serum iron levels.<sup>3,7</sup> These erythrocytes distinguish themselves from normal ones by hypochromasia and microcytosis.<sup>7</sup>

Especially prone to iron deficiency anemia are nursing animals, due to lower capacity for iron storage, larger requirements and decreased intake due to milk based diet.<sup>3</sup> Severe iron deficiency is characterized by a microcytic, hypochromic, low serum ferritin concentration with potentially severe anemia with a varying regenerative response.<sup>6,7</sup>

#### 1.4. Immune resilience and iron

Micronutrients and the immune system work closely together and develop strategies against pathogens for instance by starving invading pathogens by withholding and depriving them of micronutrients.2

Due to the fact that, immune cells need micronutrients themselves for proper growth and function, a lack thereof may signal an imbalance throughout the immune system and leads to their priming and activation.<sup>2</sup> If it is mild it appears to have a positive effect on parasitic infections and acts protective against their infestation.<sup>2</sup> On the contrary, if withholding the pathogens of micronutrients fails, the pathogens and the defense mechanisms such as blocking micronutrient uptake exacerbate the situation and may lead to anemia and chronic inflammation.<sup>2</sup> Even if ferritin levels are normal there may be an underlying iron deficiency present.<sup>2</sup> Despite the adequate body iron levels, the iron supply and incorporation into erythroid precursors are lacking.<sup>2</sup> As a result iron is metabolically inactive, implying it is stored within ferritin in reticuloendothelial cells which embody primarily macrophages and monocytes and therefor is unavailable for instantaneous use.<sup>2</sup> This "functional iron deficiency" is seen in infectious, inflammatory and malignant diseases such as anemia of chronic disease.<sup>2</sup>

# 1.5. The effect of iron on macrophages

Macrophages have two well-known and important functions, the surveillance followed by the recognition of pathogens and the phagocytosis of apoptotic and senescent cells.<sup>1</sup> In addition to that other functions have been revealed recently, for one the distribution of iron throughout the body and the location of nutritional demand of the adjacent tissue.<sup>1</sup>

Macrophages appear in different subtypes, therefor are two main types the pro- inflammatory and anti- inflammatory ones.<sup>1</sup> They differentiate through their iron-handling capacity.<sup>2</sup> M1/ pro inflammatory macrophages neither partake in iron sequestration nor export.<sup>2</sup> Intracellular their labile and metabolic active iron levels are low, as the available iron is bound to ferritin, making it inaccessible for pathogens as well as nutritional supply.<sup>2</sup> On the contrary M2/ anti- inflammatory macrophages, which have a high expression of CD163 (hemoglobin/haptoglopin) receptors are essential for heme iron import, hold a large labile iron pool, that represents metabolic active iron within the cell.<sup>2</sup> These M2 macrophages only have a small amount of iron contained in ferritin.<sup>2</sup> When iron levels are low, less iron is delivered to the macrophage, which means that iron turnover is reduced, leading to a decrease in metabolically active iron.<sup>2</sup> Thus, the classical characteristics of the anti-inflammatory macrophage, with a large labile iron pool and a

high turnover rate, are altered to a more pro-inflammatory phenotype.<sup>2</sup> Resulting in the fact that nutritional iron deficiency leads to low grade inflammation.<sup>2</sup>

# 1.6. Background information on FACS

FACS (fluorescence activated cell sorting) is used to count various cells in suspension while measuring their physical and molecular traits.<sup>8</sup> This technology allows an extensive analysis of high amounts of cells in suspension in a short period of time.<sup>8</sup> The principle behind FACS is to lead a single stream of cells in fluid through a laser beam to measure its physical and chemical traits.<sup>8</sup> While the cells pass through the laser beam, they emit, retract and absorb various amounts of light, which is measured and analyzed by detectors.<sup>8</sup> Light which is retracted in forward direction provides information about the size and refractive index of the cell.<sup>8</sup> If the cell has an uneven surface due to granules or organelles the light gets retracted to the side.<sup>8</sup> These side scatter provides insight about the complexity of the cells surface.<sup>8</sup> Due to some cells distinct size and complexity, FSC and SCC provide enough information to fully identify them such as red blood cells, platelets, monocytes, neutrophils, eosinophils and basophils.<sup>8</sup> The different subsets of lymphocytes are too similar to be differentiated based on their FSC and SCC alone.<sup>8</sup> In order to differentiate these cell subsets as well as the maturation state of monocytes, these cells can be stained with fluorescent- labelled antibodies against cell- specific markers to identify specific immune cell subsets.<sup>8</sup> The laser beam stimulates these fluorophores, which subsequently emit light at various wavelengths for detection.<sup>8</sup>

# 1.7. Background information on ELISA

The direct ELISA was invented by two different research teams at the same time, the first team consisting of Engvall and Perlman, and the second of Van Weemen and Schuurs.<sup>9</sup> It was developed after an alteration of the radioimmunoassay, by exchanging the radioactive iodine 125 with conjugated tagged antigens and antibodies with enzymes.<sup>9</sup> Nowadays it has become a routine laboratory research and diagnostic tool around the world.<sup>9</sup>

Enzyme immunoassays are able to use the catalytic qualities of enzymes to identify as well as quantify immunological reactions in bodily fluids.<sup>9,10</sup> Enzyme-linked immunosorbant assay, ELISA, acts as a heterogeneous EIA technique.<sup>9</sup> This mediator analysis functions as followed by binding one of the reaction components covalently to the surface of a solid phase, such as a 96 well plate.<sup>9,10</sup> This connection differentiates between bound and free-labeled reactants.<sup>9</sup>

ELISA works as followed adding a sample that embodies the antigen in question and letting it bind with a solid- phase antibody.<sup>9</sup> This antibody is called capture antibody and is specific in binding only to the antigen in question.<sup>9,10</sup> It is immobilized on the surface of a 96 well plate and captures the antigen.<sup>9,10</sup> After washing, which is important to minimize the possible back-ground signals, by removing the loosely attached antibodies, a blocking buffer is supplemented.<sup>9,10</sup> Wash again and add an enzyme labeled antibody which is building the "sandwich complex".<sup>9,10</sup> This antibody is called detection antibody, its purpose is to target and bind the primary capture antibody.<sup>9,10</sup> The coating is followed by a washing step and further detection, which is done by adding a specific solution to generate a color change visible for the eye.<sup>9,10</sup> HRP horseradish peroxidase is commonly used in combination with hydrogen peroxide and produces at first a blue color change and after adding the stopping solution which is commonly an acid the color intensifies and changes to a yellow.<sup>9</sup>

# 2. Used stimulating agents and staining compounds

#### 2.1. Deferoxamine

The three main iron chelators currently used are deferoxamine (DFO), deferasirox and deferiprone, because they are very selective for iron and appear to have no effect on levels of calcium, lead, copper, phosphate or magnesium.<sup>11</sup>

In patients with iron overload diseases such as haemochromatosis, DFO is used as a first-line treatment.<sup>11</sup> It was first utilized during the 1960s in clinical practices and is now a commonly used first line medication.<sup>11</sup>

DFO works by chelating small amounts of unbound iron, providing antioxidant protection and inducing the hypoxia-inducible factor (HIF)-1 protein, which then modulates gene expression.<sup>11</sup> This is thought to be the primary mechanism by which DFO achieves its neuroprotective effects.<sup>11</sup>

It has been shown that DFO is able to reduce hepatic lipid peroxidation and oxidative stress and has an ameliorative effect on hepatic steatosis in rodents.<sup>11</sup> In addition, deferoxamine increases the activity of the insulin receptor and the signaling pathway in hepatocytes both in vivo and in vitro.<sup>11</sup> Despite these beneficial traits of DFO the precise mechanisms of function are still not fully elucidated.<sup>11</sup>

#### 2.2. PMA

Phorbol-12-myristat-13-acetat is a diester of phorbol and is able to activate the signal transduction enzyme protein kinase C (PKC).<sup>12,13</sup> It functions as a potent cell activator, through promoting cell division especially in B cells.<sup>12,13</sup> In moderate concentrations PMA is able to induce apoptosis in macrophages.<sup>12,13</sup>

#### 2.3. Calcein

Calcein was used as a fluorescent probe for cellular iron and is able to reflect the nutritional status of iron in mammalian cells.<sup>14</sup> Calcein functions as a good chemo sensor for iron (III) in cells and biological fluids but not for Fe (II).<sup>14</sup> It is able to measure the labile iron pool and the concentration of cellular free iron.<sup>14</sup>

This is of importance, because numerous cells show symptoms of iron deficiency as their "chelatable" or "labile iron pool" is low, although containing large quantities of iron.<sup>14</sup> This pool functions as source of cellular iron transport, and can regulate iron regulatory genes and the activity of iron containing proteins.<sup>14</sup> Studies have shown, that Calcein is dynamically sensitive to metabolic changes of iron pools under different nutritional conditions.<sup>14</sup> Therefor Calcein is claimed to reflect also the ferric iron pool.<sup>14</sup>

### 2.4. CD14

This glycoprotein receptor is biologically active, either as a monomeric protein attached to the cell membrane via a glycosylphosphatidylinositol (GPI) anchor or as a secreted soluble protein (sCD14).<sup>15</sup> In response to lipopolysaccharides or oxidized lipids it is able to stimulate endothelial and epithelial cells.<sup>15</sup> CD14 is a membrane receptor on macrophages, It is also able to directly bind Toll- like receptors (TLRs) and transferring LPS to them.<sup>15</sup> As such, it acts as pattern -recognition receptor in innate immunity capable to identify pathogen-associated molecular pattern on bacteria and viruses.<sup>15</sup> If needed, they induce pro inflammatory signals, that promote transcription factors to activate adaptive immunecells.<sup>15</sup> CD14 is also detected in neutrophils, dendritic cells and tissue- resident macrophages such as the Kupffer cells in the liver.<sup>15</sup> Apart from immune cells, their expression has also been proven in enterocytes, hepatocytes and pancreatic islet beta cells.<sup>15</sup>

#### 2.5. IL6

IL-6 is a soluble pro-inflammatory interleukin and acts as a signal messenger for the immune system.<sup>16</sup> It has a key role in the innate immune defense mechanism, by an instant and short-term stimulation of acute phase proteins, hematopoiesis and immune reactions.<sup>16</sup> IL-6 functions

through its ability to induce differentiation of activated B cells into antibody-producing cells, the synthesis of acute phase proteins in hepatocytes and its IFN antiviral activity.<sup>16</sup> Its expression is tightly controlled by transcriptional and posttranscriptional mechanisms, as constant synthesis contributes to chronic inflammation and autoimmunity.<sup>16</sup>

Acute inflammation induces the production of IL-6, which is transported through the bloodstream to the liver to activate acute phase reactants, such as CRP, SAA, fibrinogen, haptoglobin and  $\alpha$ 1- antichymotrypsin.<sup>16</sup> It also increases the production of fibronectin, albumin and transferrin and regulates circulating serum iron and zinc levels via controlling their transporters.<sup>16</sup> In regard to serum iron, IL6 induces hepcidin production, which degrades the cellular iron exporter ferroportin 1 on enterocytes and macrophages resulting in lower serum iron levels.<sup>16</sup> As such, the IL 6 hepcidin axis is responsible for hypoferremia and anemia associated with chronic inflammation.<sup>16</sup>

# 3. Aim of the study

We hypothesized, that PMA, a known inflammation trigger, will be able to promote the differentiation of monocytes into M1 macrophages under iron deficient conditions. Deferoxamine is able to bind to the iron present in the cell and may reduce the labile iron pool. DH 82 cells are able, under stimulation with PMA, to produce a higher Interleukin 6 signal. The aim of the study was to investigate the behavior of DH 82 macrophages, which are derived from a ten year old Labrador with malignant histiocytosis. Therefor the cells behave differently than macrophages derived from healthy dogs.

# 4. Materials and Methods

# 4.1 DH-82 macrophages

In this study canine DH-82 cells were used. These cells originate from dogs showing canine macrophage physiology and are capable of consuming latex particles. The cells were derived

from a ten year old male golden retriever with malignant histiocytosis. They are positive for Fcgamma receptors and are negative for Fc-mu and C3b receptors. In addition to that, they are not capable to produce Il-1. The cells are semi adherent, implying that most of the cells attach to the surface of the flask and flatten, while some grow floating in suspension.

#### 4.2 Thawing DH-82 macrophages



Picture 1: Microscopic view of DH-82 cells

In order to thaw the cell line the culture medium for DH-82, which is Dulbecco's Modified

Eagle's Medium with 15 % of fetal calf serum FCS had to be preheated to 37°C. The tube with the cells rest in a water bath at 37°C until fully thawed. 7ml of the new created medium and 500  $\mu$ l FBS/FCS plus the cells are going to be pipetted in a 25cm<sup>2</sup> flask. Due to the fact that these DH-82 cells were frozen in the presence of DMSO, which acts as a cryoprotective agent in order to prevent the formation of ice crystals whilst frozen, since it would lead to cell destruction. After thawing the cells DMSO has to be removed from the freshly thawed cells, by removing the old media and adding fresh medium.

The culture medium for optimal cultivation has additional 10 % of heat inactivated fetal bovine serum, 1 % non-essential amino acids, 1 % penicillin- streptomycin and 1 % L-glutamine. The cells are grown at 37°C. Subculturing should be carried out, whenever 75 % confluency of the cells are reached.

#### Materials used in this section of the experiment:

> DMEM low glucose, 1000mg/L, L-glutamine, sodium bicarbonate, liquid, sterile

filtered, (+)10 % FBS/FCS, (+) 1 % PenStrep, (+) 1 % NonEssentialAminoAcids, (+)

1% L-Glutamine (Sigma-Aldrich, St. Louis, Missouri, USA)

- FCS (Thermo Fisher Scientific, Waltham, Massachusetts, USA)
- ▶ Pipettes (100-1000µl, 20-200µl, 10-100µl, 2-20µl) (Eppendorf, Hamburg, Germany)
- Flasks 25cm<sup>2</sup> Falcon

#### 4.3 Culturing cells and medium change

75 % of the cells should be attached to the flask before changing the medium. At first the used medium should be removed with a suction pump. After that the cells should be washed once with 10ml of 0.90 % NaCl until residuals are removed, solution should be sucked off, before 1-2 ml of Trypsin-EDTA solution should be added until cells are detached from the flask. After eight minutes of incubation, the cells should be detached, before five ml of fresh medium are added to block the enzymatic action of Trypsin-EDTA. The flask should be held in your hands and slapped on both sides five to six times so the cells are able to fully detach themselves from the flask. As for the next step, cells should be pipetted into a 50 ml tube and centrifuged for 5

minutes at 600g. After the supernatant is removed with the suction pump, a cell pellet should be visible at the bottom of the tube. The cells should be resuspended with 10 ml of new medium and transferred into a new flask. The flask should be labeled with date and new passage number.



Picture 2: Counting of DH\_82 cells via cell Drop

#### Materials used in this section of the experiment:

- ➢ Flasks 75cm<sup>2</sup> Falcon, REF: 353135
- > 0.9 % NaCl (BRAUN, Maria Enzersdorf, Austria)
- Trypsin- EDTA (Thermo Fisher Scientific, Waltham, Massachusetts, USA)
- Pipettes (100-1000µl, 20-200µl, 10-100µl, 2-20µl) (Eppendorf, Hamburg, Germany)

- DMEM low glucose, 1000mg/L, L-glutamine, sodium bicarbonate, liquid, sterile filtered, (+)10 % FBS/FCS, (+) 1 % PenStrep, (+) 1 % NonEssentialAminoAcids, (Sigma-Aldrich, St. Louis, Missouri,USA)
- Microscope Primo Vert (Zeiss, Baden- Württemberg, Germany)
- CellDrop FL (DeNovix, Wilmington, Delaware, USA)

# 4.4 FACS

#### 4.4.1 Protocol for FACS analysis

Cells had to be cultured and incubated under the conditions described below, before staining them further for flow cytometric analysis. 48 well plates were set up for the following experiment. The solutions which were used remained the same throughout the eight repetitions, though slight variations occurred during the individual experiments as can be revisited in the attached protocols in the appendix. Cells were incubated under the following conditions:

- DMEM without iron as the control value.
- Dilution series including 1mg DFO/ml DMEM, 10µg DFO/ml DMEM, 10µg DFO/ml DMEM, 1µg DFO/ml DMEM.
- ο 10µl of 1mg PMA/ml DMEM, 10µg of 1mg PMA/ ml of 10µg DFO/ml DMEM.
- ο FCS diluted in DMEM, FCS diluted in 10µg DFO/ml DMEM.

#### 4.4.2 Execution of the experiment

- $\circ$  Detach cells with Trypsin-EDTA from the 75cm<sup>2</sup> flask and transfer them to a 50ml tube.
- Centrifuge cells at 600g for 5 minutes.
- Remove the used medium.
- Resuspend the cell pellet in iron free medium (=Dulbecco's Modified Eagle's Mediumlow glucose without any additives), count the cells and dilute them to 1 million cells per milliter.
- Add 300 000 cells in a in a 48 well plate.
- $\circ$  Add 300µl/well of the stimulants according to scheme and incubate cells for 18h at 37 °C.

- Collect the supernatants, (about 400µl are transferred in 2ml Eppendorfer tubes) and freeze them for later mediator analysis.
- $\circ$  Pipette the remaining cells in 200µl media in the FACS tubes.
- Stain the FACS tubes adding 1µl/sample from 5µM Calcein stock and anti- CD14-APC (2µl/tube).
- Incubate them for 30 minutes protected from light.
- o Before flow cytometric measurements, briefly resuspend cells.
- Extract the mean fluorescent intensity of the Calcein signal from the gated cell population and analyze them using prism.

#### Materials used during FACS:

- Gibco DMEM, (+)1g/L D –Glucose, (+)Pyruvate, (-) L-Glutamine, (-)Phenol Red (Sigma-Aldrich, St. Louis, Missouri,USA)
- > DFO Deferoxamin mesylate salt (Sigma-Aldrich, St. Louis, Missouri, USA)
- Img/ml DMSO PMA (Sigma-Aldrich, St. Louis, Missouri, USA)
- > FCS (Thermo Fisher Scientific, Waltham, Massachusetts, USA)
- > Trypsin- EDTA (Thermo Fisher Scientific, Waltham, Massachusetts, USA)
- ➢ Flasks 75cm<sup>2</sup> Falcon, REF: 353135
- Centrifuge-Hettich, Rotana 460R (Hettich Lab., Tuttlingen, Germany)
- ➤ 48-well plate flat bottom Falcon, REF: 353078
- > 2ml Reaktionsgefäße (Eppendorf, Hamburg, Germany)
- IgG2a Clone: MOPC 173 (Biolegend, San Diego, California, USA)
- PE/Cyanine 7: Clone MOPC-21 (Biolegend, San Diego, California, USA)
- ➤ IgG2bK Clone: eBMG2b (Thermo Fisher Scientific, Waltham, Massachusetts, USA)
- ➢ Pipettes (100-1000µl, 20-200µl, 10-100µl, 2-20µl) (Eppendorf, Hamburg, Germany)
- FACS tubes (Sigma-Aldrich, St. Louis, Missouri, USA)

FACS Canto II (BD Biosciences, Franklin Lakes, New Jersey, USA)

# 4.5 ELISA

#### 4.5.1 Protocol for ELISA

The aim of this experiment was to assess IL6 cytokines in the supernatant of DH82 cells incubated in iron-poor or iron-rich conditions for 18h. Supernatants of the 8 different experiments were collected and stored at -20 °C prior assessment.

#### **Procedure:**

- ο Add 90µl/well of 1:250 diluted capture antibody, seal plate and incubate at 4 °C.
- $\circ\,$  Wash once with 250µl/well wash solution.
- ο Add 200µl blocking buffer and incubate for a minimum of 1h at room temperature.
- Wash twice with 250µl/well wash solution, remove any remaining wash buffer by blotting it against paper towels.
- Add 60µl sample (singlets when many) or standard in duplicates according to scheme, seal plate and incubate for a minimum of 2h at room temperature.
- Wash thrice with 250µl/well wash solution, remove any remaining wash buffer by blotting it against paper towels.
- $\circ$  Add 60µl/well 1:250 diluted detection antibody in blocking buffer, seal plate and incubate for 2 h at 4 °C.
- Wash thrice with 250µl/well wash solution, remove any remaining wash buffer by blotting it against paper towels.
- Add 60µl/well 1:200 diluted Streptavidin-HRP- in blocking buffer for 20 min, protect from light.
- Wash thrice with 250µl/well wash solution, remove any remaining wash buffer by blotting it against paper towels.
- Add 60µl TMB/well, incubate for 15min before stopping the reaction with 40µl stop solution.
- o Measure OD at 450nm.

#### Materials and solutions used during IL6 - Elisa:

- Samples: Supernatants of stimulated DH82 cells
- ➢ Pipettes (100-1000µl, 20-200µl, 10-100µl, 2-20µl) (Eppendorf, Hamburg, Germany)
- Canine IL6 Elisa, DuoSet DY1609 (R&D systems, Minneapolis, USA)
- Canine IL6 capture antibody #841719: 144µg reconstitute in 720µl 0.9 % NaCl to generate a 200µg/ml solution. (Thermo Fisher Scientific, Waltham, Massachusetts, USA)
  - → working solution dilute 1:250 (4µl/ml=800ng/ml) in 0.9 % NaCl: (40µl in 10ml 0.9 % NaCl/plate).
- > 0.9 % NaCl (BRAUN, Maria Enzersdorf, Austria)
- Blocking buffer: 1 %BSA/0.9 % NaCl (Sigma-Aldrich, St. Louis, Missouri,USA)
- Canine IL6 detection antibody #841720: 9µg reconstitute in 720µl <u>blocking buffer</u> to generate a 12.5µg/ml solution. (Thermo Fisher Scientific, Waltham, Massachusetts, USA)

 $\rightarrow$  dilute 1:250 (4µl/ml = 50ng/ml) in blocking buffer.

- Canine IL6 standard #841721: 55ng reconstitute in 550µl <u>blocking buffer</u> to generate a 100 ng/ml solution. (Thermo Fisher Scientific, Waltham, Massachusetts, USA)
- → Highest standard: 4ng/ml (1:25 = 20µl in 500 blocking buffer.)
- Streptavidin HRP #890803: dilute 1:200 (R&D systems, Minneapolis, USA)
- TMB substrate solution Ref: 00-4201-56 (Thermo Fisher Scientific, Waltham, Massachusetts, USA)
- Stop solution : 2N H2SO4 (R&D systems, Minneapolis, USA)

# 4.6 Statistical analysis

During the statistical evaluation of the experiments, One- Way ANOVA was used.<sup>17</sup> This statistical method is an advanced version of the t-test, which evaluates a significant difference between two means of two different groups.<sup>17</sup>

One-way ANOVA is used in order to assess whether more than two means are significantly different from each other.<sup>17</sup> This method requires numerical variables, which were the samples in our experiments, two or more factors that have a potential influence on the samples, which were the characteristics we wanted to study in our thesis, and the mean of the samples.<sup>17</sup>

In addition to the One-Way ANOVA method we used the Geisser- Greenhouse correction, which is used for the adjustment in case for lack of sphericity.<sup>17</sup>

The uncorrected Fisher's LSD, which calculates the pooled significant difference from all groups, whereas the t-test only compares the pooled significant difference from two groups.<sup>17</sup> Tukey's multi comparison test was used as well and compares every mean with every other mean.<sup>17</sup>

	1	2	3	4	5	6	7	8	9	10	11	12
Α	0	0	1µg	10µg	100µg	PMA	FCS	FCS	PMA+FCS	PMA+FCS	4000	4000
В	0	0	1µg	1µg	10µg	10µg	100µg	100µg	1mg	1mg	2000	2000
С	FCS	FCS	PMA	PMA	FCS+10µg	FCS+10µg	PMA+10µg	PMA+10µg	0	0	1000	1000
D	1µg	1µg	10µg	10µg	100µg	100µg	1ml	1ml	FCS	FCS	500	500
E	PMA	PMA	FCS+10µg	FCS+10µg	PMA+10µg	PMA+10µg	0	0	0	0	250	250
F	1µg	1µg	1μg	1µg	10µg	10µg	10µg	10µg	100µg	100µg	125	125
G	100µg	100µg	1mg	1mg	1mg	1mg	FCS	FCS	FCS	FCS	63	63
Н	PMA	PMA	PMA	PMA	FCS+10µg	FCS+10µg	FCS+10µg	FCS+10µg	PMA+10µg	PMA+10µg	0	0

#### Plate 2

	1	2	3	4	5	6	7	8	9	10	11	12
Α	0	0	0	0	1µg	1µg	1µg	1µg	10µg	10µg	4000	4000
В	10µg	10µg	100µg	100µg	100µg	lmg	1mg	1mg	lmg	FCS	2000	2000
С	FCS	FCS	PMA	PMA	PMA	PMA	FCS	FCS	FCS	FCS	1000	1000
							+10µg	+10µg	+10µg	+10µg		
D	PMA	PMA+10µg	PMA	PMA	0	0	0	0	1µg	1µg	500	500
	+10µg		+10µg	+10µg								
E	1µg	lμg	10µg	10µg	100µg	100µg	100µg	100µg	lmg	lmg	250	250
F	lmg	lmg	FCS	FCS+10µg	FCS	FCS	РМА	РМА	PMA	PMA	125	125
G	FCS	FCS+10µg	FCS+	FCS	PMA	PMA	PMA+10µg	PMA+10µg	0	0	63	63
	+10µg		10µg		+10µg	+10µg						
Н	0	0	0	0	0	0	1µg	lμg	1µg	1µg	0	0

Plate 3

	1	2	3	4	5	6	7	8	9	10	11	12
Α	1µg	10µg	10µg	10µg	10µg	10µg	100µg	100µg	100µg	100µg	4000	4000
B	100µg	1mg	lmg	lmg	lmg	lmg	FCS	FCS	FCS	FCS	2000	2000
С	FCS	PMA	PMA	PMA	PMA	PMA	FCS	FCS	FCS+	FCS	1000	1000
							+10µg	+10µg	10µg	+10µg		
D	FCS	PMA	PMA	PMA	PMA	PMA	0	0	0	0	500	500
	+10µg	+10µg	+10µg	+10µg	+10µg	+10µg						
E	0	0	0	0	1μg	1µg	1μg	1µg	1μg	10µg	250	250
F	10µg	10µg	10µg	10µg	100µg	100µg	100µg	100µg	100µg	1mg	125	125
G	1mg	1mg	1mg	1mg	FCS	FCS	FCS	FCS	PMA	PMA	63	63
Η	PMA	PMA	FCS	FCS	FCS	FCS	PMA	PMA	PMA	PMA	0	0
			+10µg									

Color codes: Experiment 1, Experiment 2, Experiment 3, Experiment 4, Experiment 5, Experiment 6 ,Experiment 7, Experiment 8, Standards in pg/ml

# 5. Results

#### 5.1 Mildly lower LIP upon DFO exposure in DH82 cells

Only the summary of the flow cytometry analyses is presented here (individual experiments and data table are added in the appendix of this thesis). In total eight experiments were performed. However, presumably an expired Calcein-AM batch was chosen for experiments two-five rendering a very low Calcein signal, making a direct comparison nearly impossible. As such, the Calcein signal was normed to experiment six. Here the medium control (n=eight) served as the norm.



Figure 1. DFO and PMA decrease the labile iron content in DH82 cells. Flowcytometric analyses of DH82 cells stimulated with medium, DFO, PMA, FCS or combinations hereto. Calcein mean fluorescence intensity. Statistical analysis were performed with mixed-effect analysis and the Geisser-Greenhouse correction, followed by uncorrected Fisher's LSD. \* p < 0.05; \*\* p < 0.01, \*\*\*\* p < 0.001

	0	1µgDFO	10µgDFO	100µgDFO	lmgDFO	PMA	PMA+10µgDFO	FCS	FCS+10µgDFO
DH82_1	10052,9	5521,7	5595,5	5199,8	/	6066,4	/	7428,2	/
DH82_1	/	6091,2	6264,5	5670,2	/	5645,5	/	4803,6	/
DH82_1	/	6759,7	5076	5348,3	/	6982,5	/	6710,2	/
DH82_2	7708	9020	7872	9184	9020	10988	10004	6068	6888
DH82_2	8036	8856	6068	8364	8036	10496	10660	7052	7872
DH82_2	7216	7872	9020	7544	8364	10496	13284	5740	6396
DH82_2	7544	8036	8036	7544	8528	7216	10660	6396	8036
DH82_4	11512	4229	4464	2467	3642	9633	9985	6461	5404
DH82_4	8928	3289	4699	4816	4581	9280	8928	5521	6461
DH82_4	5404	3407	3994	3759	3759	11160	9280	6343	5873
DH82_4	5051	/	/	5639	3524	10455	/	6931	4699
DH82_4	4464	/	/	/	/	/	/	/	/
DH82_4	4229	/	/	/	/	/	/	/	/
DH82_4	4816	/	/	/	/	/	/	/	/
DH82_4	6696	/	/	/	/	/	/	/	/
DH82_5	3172	5002	7382	5673	4270	21657	11774	7382	5490
DH82_5	/	6833	8724	6039	3782	18789	/	6161	3538
DH82_5	/	/	6161	2562	5856	15190	/	6039	5185
DH82_5	/	/	/	/	/	/	/	5673	/
DH82_6	12356	21533	15487	19954	8796	33504	34738	5504	9545
DH82_6	9487	19451	10154	13688	11696	28435	36934	7124	11061
DH82_6	17107	20928	/	42040	14993	32893	27215	/	13532
DH82_6	/	9148	/	21211	/	/	/	/	6619
DH82_6	/	/	/	14471	/	/	/	/	/

DH82_7	3040	2990	3324	2473	3436	5250	3000	5503	8523
DH82_7	2777	2483	2665	2696	3882	3649	1368	4885	10875
DH82_7	3132	2311	2777	3314	3162	3344	6699	7956	7358
DH82_7	3466	2949	2463	2838	3111	/	16783	6912	6912
DH82_7	2787	/	2848	/	/	/	/	/	/
DH82_7	2838	/	/	/	/	/	/	/	/
DH82_7	3061	/	/	/	/	/	/	/	/
DH82_8	8281	11877	19020	17406	11351	24855	9284	7926	6886
DH82_8	6507	8819	13797	6153	20256	19950	8269	4991	7229
DH82_8	11999	10898	8012	12330	16439	17124	/	6030	5651
DH82_8	12036	10348	12990	7486	18825	16892	/	7474	8501
DH82_8	/	8244	9847	/	/	17112	/	5150	6519
DH82_8	/	/	/	/	/	18629	/	/	/

 Table 1: Normalized data of the mean fluorescence intensity of the Calcein signal

 Unit: MFI= mean fluorescence intensity

As the Calcein- staining did not always work in the same extent, we normalized the Calceinsignal of the different experiments using the average Calcein signal in the media controls n=8of experiment 6 as the normative. Here we could demonstrate that the iron-chelator, DFO led to a significant increase p < 0.05, of the Calcein signal and thus lowered the labile iron content of these cells. PMA as an activator of the cytokine signaling chain, inducer of reactive oxygen species and thus being an immune activator, further significantly depleted the labile iron content of DH82 cells. In contrast, addition of iron in form of FCS to the cells, improved the labile iron content, with small addition of DFO again able to reduce the cytosolic iron content.

#### 5.2. Lower CD14 expression upon DFO exposure in DH82 cells

CD14 is a cell surface antigen that primarily is described to act as a co-receptor for toll-like receptors (TLRs) to activate innate immunity responses to pathogens and tissue injury in mac-rophages and monocytes. CD14 expression has been reported to be up regulated by LPS, due

to its anti-apoptotic effect of LPS on monocytes. In contrast, IL-4 has been reported to downregulate monocyte CD14 due to its apoptotic effect on monocytes.

Interestingly, the summary of all the experiments revealed that DFO rather stabilized CD14 expression in the canine macrophage-cell line similar as FCS, while PMA and medium (with no iron and nutrient) rather decreased its expression. This suggest that DFO similarly as LPS or FCS seems to exert an anti-apoptotic in DH82 cells, while medium without any nutrients or PMA rather decreased its expression, which may be due to increased apoptosis.



Figure 2. DFO and PMA decrease the labile iron content in DH82 cells. Flowcytometric analyses of DH82 cells stimulated with medium, DFO, PMA, FCS or combinations hereto. Calcein mean fluorescence intensity. Statistical analysis were performed with mixed-effect analysis and the Geisser-Greenhouse correction, followed by uncorrected Fisher's LSD. . \*p < 0.05; \*\*p < 0.01

				10		- 00 10µ6D10
DH82_1	10052.9	5595.9	6066.4	/	7428.2	/
DH82_1	/	6264.5	5645.5	/	4803.6	/
DH82_1	/	5076.0	6982.5	/	6710.2	/
DH82_2	7708.0	7872.0	10988.0	10004.0	6068.0	6888.0
DH82_2	8036	6068	10496.0	10660.0	7052.0	7872.0
DH82_2	7216	9020	10496.0	13284.0	5740.0	6396.0
DH82_2	7544	8036	7216.0	10660.0	6396.0	8036.0
DH82_4	11512	4464	9633.0	9985	6461	5404
DH82_4	8928	4699	9280	8928	5521	6461
DH82_4	5404	3994	11160	9280	6343	5873
DH82_4	5051	/	10455	/	6931	4699
DH82_4	4464	/	/	/	/	/
DH82_4	4229	/	/	/	/	/
DH82_4	4816	/	/	/	/	/
DH82_4	6696	/	/	/	/	/
DH82_5	3172	7382	21657	11774	7382	5490
DH82_5	/	8724	18789	/	6161	3538
DH82_5	/	6161	15190	/	6039	5185
DH82_5	/	/	/	/	5673	/
DH82_6	12356	15487	33504	34738	5504.	9545
DH82_6	9487	10154	28435	36934	7124	11061
DH82_6	17107	/	32893	27215	/	13532
DH82_6	/	/	/	/	/	6619
DH82_7	3040	3324	5250	3000	5503	8523
DH82_7	2777	2665	3649	1368	4885	10875
DH82_7	3132	2777	3344	6699	7956	7358
DH82_7	3466	2463	/	16783	6912	6912
DH82_7	2787	2848	/	/	/	/
DH82_7	2838	/	/	/	/	/
DH82_7	3061	/	/	/	/	/
DH82_8	24855	19020	8281	9284	7926	6886
DH82_8	19950	13797	6507	8269	4991	6886
DH82_8	17124	8012	11999	/	6030	5651
DH82_8	16892	12990	12036	/	7474	8501
DH82_8	17112	9847	/	/	5150	6519
DH82_8	18629	/	/	/	/	/
DH82_8	10715	/	/	/	/	/
DH82_8	9565	/	/	/	/	/

Table 2: Data of the mean fluorescence intensity of the Calcein signal comparing 10µgDFO, PMA, PMA+10µgDFO, FCS and FCS+10µgDFO Unit: MFI= Mean fluorescence intensity





Figure 3. DFO and PMA decrease the labile iron content in DH82 cells. Flowcytometric analyses of DH82 cells stimulated with medium, DFO, PMA, FCS or combinations thereof. Calcein mean fluorescence intensity. Statistical analysis were performed with mixed-effect analysis and the Geisser-Greenhouse correction, followed by uncorrected Fisher's LSD

	0	1µgDFO	10µgDFO	100µgDFO	1mgDFO	PMA	PMA+10µgDFO	FCS	FCS+10µgDFO
DH82_1	1,88	2,12	27,37	-0,25	/	-0,25	/	-1,25	/
DH82_1	-1,80	/	/	/	/	/	/	-3,62	/
DH82_2	7,12	24	11	-0,63	46,50	26,63	10,87	67,62	85,25
DH82_2	2,25	42,25	19,88	10,5	19,5	51	9	53,50	67,25
DH82_3	24,12	12,62	15,87	41,12	23	13,75	34,25	40,88	17,75
DH82_3	37,13	62	53,63	22	30	39	11,63	38,37	18
DH82_4	28,75	5,87	27	8,88	12,5	-6,5	4	11,37	27,37
DH82_4	34,88	23,38	3,87	10,25	20,5	13,38	-7,13	4,25	-1,25
DH82_4	22,63	8,12	18,38	131,75	20,63	-0,5	/	5,37	6,62
DH82_4	16,87	19,5	20,25	20	2	4,37	/	5,63	-5,87
DH82_5	12,88	7,12	5,88	10	32,63	10,13	14,13	16,25	14,25
DH82_5	37,37	28,88	18,75	9,75	11,5	9,13	39	8,12	16,5

$\begin{array}{c c c c c c c c c c c c c c c c c c c $	DH82_5	10,25	12,5	11,62	8,12	8	9,5	14,87	9,5	10
$\begin{array}{c c c c c c c c c c c c c c c c c c c $	DH82_5	11,62	8,75	40,62	/	8,87	9,37	114,13	/	16,5
$\begin{array}{c c c c c c c c c c c c c c c c c c c $	DH82_6	12,12	12,38	5,5	46,38	5,5	8,75	5,62	10	7,62
$\begin{array}{c c c c c c c c c c c c c c c c c c c $	DH82_6	8,87	14,37	5,88	9,75	8,5	17,62	29,25	8,5	30
$\begin{array}{c c c c c c c c c c c c c c c c c c c $	DH82_6	12	6,25	/	9,37	7,25	7,62	4,63	17,5	8,87
$\begin{array}{c c c c c c c c c c c c c c c c c c c $	DH82_6	7,12	32,25	/	5,5	20,37	7,25	4,63	64,87	11
$\begin{array}{c c c c c c c c c c c c c c c c c c c $	DH82_7	3,37	3,5	3,14	-0,86	4,43	3,57	2,14	4,29	3,43
DH82_7         8         3,13         1,14         -1,14         3,71         4,71         4,86         4,14         3,29           DH82_7         12,88         25,25         2,43         1         1         1,57         5,71         4         5,57           DH82_7         4,5         0,71         0,14         3         1,29         1,71         6,86         4,71         8,14           DH82_7         5,75         /	DH82_7	4	22,5	0,71	0,71	6,14	2,57	3,57	5,43	4,14
$\begin{array}{c c c c c c c c c c c c c c c c c c c $	DH82_7	8	3,13	1,14	-1,14	3,71	4,71	4,86	4,14	3,29
DH82_7         4,5         0,71         0,14         3         1,29         1,71         6,86         4,71         8,14           DH82_7         5,75         /	DH82_7	12,88	25,25	2,43	1	1	1,57	5,71	4	5,57
DH82_7       5,75       /	DH82_7	4,5	0,71	0,14	3	1,29	1,71	6,86	4,71	8,14
DH82_7       32,63       /	DH82_7	5,75	/	/	/	/	/	/	/	/
DH82_7       7,88       /	DH82_7	32,63	/	/	/	/	/	/	/	/
DH82_8         3,86         3,71         5,43         4         2,57         6,86         3,43         4,43         5,86           DH82_8         4,43         8,14         4,86         1,57         4,86         5,86         6,43         9,86         27,57           DH82_8         4,86         5,29         2         5,57         4,14         3,57         1,29         6         3,71           DH82_8         12,57         2         9,71         1,43         20,29         3,14         6,14         6,86         9,14           DH82_8         5,29         2,86         3,71         3,86         4,14         /         /         /         /         /           DH82_8         5,29         2,86         3,71         3,86         4,14         /<	DH82_7	7,88	/	/	/	/	/	/	/	/
DH82_8         4,43         8,14         4,86         1,57         4,86         5,86         6,43         9,86         27,57           DH82_8         4,86         5,29         2         5,57         4,14         3,57         1,29         6         3,71           DH82_8         12,57         2         9,71         1,43         20,29         3,14         6,14         6,86         9,14           DH82_8         5,29         2,86         3,71         3,86         4,14         /         /         /         /         /           DH82_8         5,29         2,86         3,71         3,86         4,14         /	DH82_8	3,86	3,71	5,43	4	2,57	6,86	3,43	4,43	5,86
DH82_8         4,86         5,29         2         5,57         4,14         3,57         1,29         6         3,71           DH82_8         12,57         2         9,71         1,43         20,29         3,14         6,14         6,86         9,14           DH82_8         5,29         2,86         3,71         3,86         4,14         /         /         /         /         /         /           DH82_8         11         /<	DH82_8	4,43	8,14	4,86	1,57	4,86	5,86	6,43	9,86	27,57
DH82_8         12,57         2         9,71         1,43         20,29         3,14         6,14         6,86         9,14           DH82_8         5,29         2,86         3,71         3,86         4,14         /         /         /         /         /         /           DH82_8         11         /         /         /         /         /         /         /         /         /           DH82_8         3         / <td>DH82_8</td> <td>4,86</td> <td>5,29</td> <td>2</td> <td>5,57</td> <td>4,14</td> <td>3,57</td> <td>1,29</td> <td>6</td> <td>3,71</td>	DH82_8	4,86	5,29	2	5,57	4,14	3,57	1,29	6	3,71
DH82_8         5,29         2,86         3,71         3,86         4,14         /	DH82_8	12,57	2	9,71	1,43	20,29	3,14	6,14	6,86	9,14
DH82_8         11         / </td <td>DH82_8</td> <td>5,29</td> <td>2,86</td> <td>3,71</td> <td>3,86</td> <td>4,14</td> <td>/</td> <td>/</td> <td>/</td> <td>/</td>	DH82_8	5,29	2,86	3,71	3,86	4,14	/	/	/	/
DH82_8         3         / <td>DH82_8</td> <td>11</td> <td>/</td> <td>/</td> <td>/</td> <td>/</td> <td>/</td> <td>/</td> <td>/</td> <td>/</td>	DH82_8	11	/	/	/	/	/	/	/	/
DH82_8 2,2 / / / / / / / / / /	DH82_8	3	/	/	/	/	/	/	/	/
							1			

 Table 3: Data of the mean fluorescence intensity of the Calcein signal stimulated with IL6

 Unit pg/ml

Blue data points were excluded from the diagram due to implausibility

In a next step we assessed the IL6 content of the supernatants of DH82 cells incubated for 18h with nutrient-deprived medium alone or in combination with DFO, PMA, FCS. Interleukin 6 is a pro inflammatory cytokine, which contributes to host defense through the stimulation of acute phase and immune reaction. Data show a concentration dependent bell-shaped decrease in the IL6 content upon addition of DFO up to  $100\mu$ g/ml, which then again increased. This is in line with the CD14 expression-data showing similarly a stabilizing effect by DFO.



DH82\_IL6\_DFO vs FCS

**solutions** Figure 4. Flowcytometric analyses of DH82 cells stimulated with medium, DFO and FCS. Calcein mean fluorescence intensity. Statistical analysis were performed with mixed-effect analysis and the Geisser-Greenhouse correction, followed by uncorrected Fisher's LSD. . \*p < 0.05; \*\*p < 0.01, \*\*\*p < 0.001

	0	1µgDFO	10µgDFO	100µgDFO	1mgDFO	FCS
DH82_1	1,88	2,12	27,37	-0,25	/	-1,25
DH82_1	-1,80	/	/	/	/	-3,62
DH82_2	7,12	24	11	-0,63	46,50	67,62
DH82_2	2,25	42,25	19,88	10,5	19,5	53,50

DH82_3	24,12	12,62	15,87	41,12	23	40,88
DH82_3	37,13	62	53,63	22	30	38,37
DH82_4	28,75	5,87	27	8,88	12,5	11,37
DH82_4	34,88	23,38	3,87	10,25	20,5	4,25
DH82_4	22,63	8,12	18,38	131,75	20,63	5,37
DH82_4	16,87	19,5	20,25	20	2	5,63
DH82_5	12,88	7,12	5,88	10	32,63	16,25
DH82_5	37,37	28,88	18,75	9,75	11,5	8,12
DH82_5	10,25	12,5	11,62	8,12	8	9,5
DH82_5	11,62	8,75	40,62	/	8,87	/
DH82_6	12,12	12,38	5,5	46,38	5,5	10
DH82_6	8,87	14,37	5,88	9,75	8,5	8,5
DH82_6	12	6,25	/	9,37	7,25	17,5
DH82_6	7,12	32,25	/	5,5	20,37	64,87
DH82_7	3,37	3,5	3,14	-0,86	4,43	4,29
DH82_7	4	22,5	0,71	0,71	6,14	5,43
DH82_7	8	3,13	1,14	-1,14	3,71	4,14
DH82_7	12,88	25,25	2,43	1	1	4
DH82_7	4,5	0,71	0,14	3	1,29	4,71
DH82_7	5,75	/	/	/	/	/
DH82_7	32,63	/	/	/	/	/
DH82_7	7,88	/	/	/	/	/
DH82_8	3,86	3,71	5,43	4	2,57	4,43
DH82_8	4,43	8,14	4,86	1,57	4,86	9,86
DH82_8	4,86	5,29	2	5,57	4,14	6
DH82_8	12,57	2	9,71	1,43	20,29	6,86
DH82_8	5,29	2,86	3,71	3,86	4,14	/
DH82_8	11	/	/	/	/	/
DH82_8	3	/	/	/	/	/
DH82_8	2,2	/	/	/	/	/

Table 4: Data of the mean fluorescence intensity of the Calcein signal stimulated with IL6 only comparing data of the DFO dilution seriesand FCSUnit pg/ml

# 6. Discussion

Similar as in the literature, also our data indicate that the activation status of canine macrophages is strongly dependent on their labile iron content and thus iron-poor or rich conditions.<sup>18</sup> The differences in CD14+ expression can be explained by its relation to the function and differentiation of monocytes.<sup>18</sup> Lipopolysaccharides are able to up-regulate monocyte CD-14+ expression, which is compatible with the anti- apoptotic effect, whereas IL-6 is able to down-regulate it, related to its apoptotic effect.<sup>19</sup>

PMA is a potent cell activator, able to differentiate THP-1 monocytes into mature macrophages and can polarize monocytes with the addition of cytokines into either proinflammatory macrophages M1 or anti-inflammatory macrophages M2.<sup>16</sup> The samples, which were under iron deficient conditions, stimulated with PMA for 18h, had lower CD14+expression, while the Calcein signal was higher. Both factors, indicating immune activation and a low labile iron pool.<sup>20</sup> These characteristics indicate the differentiation process of monocytes into proinflammatory M1 macrophages, due to the addition of PMA.<sup>20</sup> We hypothesized that PMA would be able to promote the differentiation of monocytes into M1 macrophages under iron-deficient conditions, and our experiments showed results indicating that this hypothesis is valid. M1 proinflammatory macrophages neither partake in iron sequestration nor export. Their labile and metabolic active iron levels are low, as the available iron is bound to ferritin, making it inaccessible for pathogens as well as nutritional supply. These results show that dogs with an iron deficiency tend to have an ongoing underlying low grade inflammation.

Deferoxamine is used to treat hemochromatosis, due to its ability to bind iron and therefor inhibit excessive iron accumulation leading to free iron in hepatocytes and ultimately pathological damage. We assumed that Deferoxamine is able to bind to the iron present in the cell and may reduce the labile iron pool. Contrary to literature in our hands, DFO had a stabilized effect on CD 14 expression, which indicates no maturation of DH 82 cells. As reported in literature, and also shown in our experiment. DFO was able to penetrate the cell, but our results indicate that the cells were able to utilize the iron provided from the DFO, maybe due to their malignant origin. Therefore, they have a higher LIP. We believe that these different behavioral characteristics are due to the fact that the DH 82 cells used in our experiments were taken from a ten year old golden retriever with malignant histiocytosis. Consequently more experiments with these cells are needed to investigate their behavioral pattern.

Interestingly, also PMA stimulation decreased in our hand the IL6 signal. There was almost no IL6 signal to be measured. The results are opposite our expectations. We assumed that DH 82 cells, under stimulation with PMA, are able to produce a higher Interleukin 6 signal. Based on literature, IL6 signals are higher in proinflammatory macrophages, which have additionally a low labile iron pool.<sup>18</sup> PMA is known to be able to increase the IL-6 secretion further. Thus while expression and labile iron content fitted in PMA-stimulated DH82 cells with an inflammatory state, we were not able to detect an increase in IL6 signals. Here, a bigger amount of samples and more experiments are needed. Moreover the protocols could be revised and adjusted in order to work straight away with the supernatant of the cells, rather than freeze them. The results, may be explainable for DFO, which functions as an iron chelator and is able to inhibit the formation of reactive oxygen species, which may decrease the capacity of these cells to synthesize and secrete IL6. There is also the possibility that the time-frame of 18h is too short to detect relevant level of these cytokine and that other cytokines such as TNF alpha would have served as better marker to detect an inflammatory setting.

Furthermore DFO, while decreased significantly the LIP- though to a lower extent than PMA-, had a stabilizing effect on CD14 expression and this was accompanied by less IL6 secretion. Thus, these results suggest that DFO participates in iron mobilization and promotes rather M2 macrophages.

For the statistical evaluation of the results obtained from the experiments, we used One-Way ANOVA and Two- Way ANOVA. In order to determine the significant differences between the mean of one experiment and the means between all experiments combined. In combination with the two forms of ANOVA, we used the Geisser- Greenhouse correction, uncorrected Fisher's LSD and the Tukey's multi comparison test to receive the most significant results.

In conclusion, PMA is able to differentiate monocytes into proinflammatory macrophages under iron deficient conditions, DFO had a rather stabilizing impact on DH82 cells, while the labile iron content could be somewhat diminished by DFO, which rather promoted iron mobilization and thus an anti-inflammatory phenotype in DH82 cells. A heightened secretion of Interleukin- 6 in cells stimulated with PMA could not be detected.

# 7. Literature

1. Roth-Walter F. Iron-Deficiency in Atopic Diseases: Innate Immune Priming by Allergens and Siderophores. Front Allergy 2022; 3:859922.

2. Peroni DG, Hufnagl K, Comberiati P, Roth-Walter F. Lack of iron, zinc, and vitamins as a contributor to the etiology of atopic diseases. Front Nutr 2022; 9:1032481.

3. Naigamwalla DZ, Webb JA, Giger U. Iron deficiency anemia. Can Vet J 2012; 53(3):250–6.

4. Fry MM, Kirk CA, Liggett JL, Daniel GB, Baek SJ, Gouffon JS et al. Changes in hepatic gene expression in dogs with experimentally induced nutritional iron deficiency. Vet Clin Pathol 2009; 38(1):13–9.

5. Zaldívar-López S, Iazbik MC, Marín LM, Couto CG. Iron Status in Blood Donor Dogs. J Vet Intern Med 2013; 28(1):211–4.

6. Weiser G, O'Grady M. Erythrocyte volume distribution analysis and hematologic changes in dogs with iron deficiency anemia. Vet Pathol 1983; 20(2):230–41.

7. Steinberg JD, Olver CS. Hematologic and biochemical abnormalities indicating iron deficiency are associated with decreased reticulocyte hemoglobin content (CHr) and reticulocyte volume (rMCV) in dogs. Vet Clin Pathol 2005; 34(1):23–7.

8. Brestoff JR. Full spectrum flow cytometry in the clinical laboratory. Int J Lab Hematol 2023; 45 Suppl 2(Suppl 2):44–9.

9. Alhajj M, Zubair M, Farhana A. StatPearls: Enzyme Linked Immunosorbent Assay. Treasure Island (FL); 2024.

10. The enzyme-linked immunosorbent assay (ELISA). Bull World Health Organ 1976; 54(2):129–39.

11. Feng Y, Jia L, Ma W, Tian C, Du H. Iron Chelator Deferoxamine Alleviates Progression of Diabetic Nephropathy by Relieving Inflammation and Fibrosis in Rats. Biomolecules 2023; 13(8).

12. Thompson CB, Lindsten T, Ledbetter JA, Kunkel SL, Young HA, Emerson SG et al. CD28 activation pathway regulates the production of multiple T-cell-derived lymphokines/cyto- kines. Proc Natl Acad Sci U S A 1989; 86(4):1333–7.

13. Pai D, Gruber M, Pfaehler S-M, Bredthauer A, Lehle K, Trabold B. Polymorphonuclear Cell Chemotaxis and Suicidal NETosis: Simultaneous Observation Using fMLP, PMA, H7, and Live Cell Imaging. J Immunol Res 2020; 2020:1415947. 14. Thomas F, Serratrice G, Béguin C, Aman ES, Pierre JL, Fontecave M et al. Calcein as a fluorescent probe for ferric iron. Application to iron nutrition in plant cells. J Biol Chem 1999; 274(19):13375–83.

15. Sharygin D, Koniaris LG, Wells C, Zimmers TA, Hamidi T. Role of CD14 in human dis- ease. Immunology 2023; 169(3):260–70.

16. Tanaka T, Narazaki M, Kishimoto T. IL-6 in inflammation, immunity, and disease. Cold Spring Harb Perspect Biol 2014; 6(10):a016295.

17. GraphPad. <u>https://www.graphpad.com/guides/prism/latest/statistics/stat\_how\_to\_one-way\_anova.htm</u> (Zugriff 1.9.2024)

18. Liu E, Tu W, Law HK, Lau YL. Changes of CD14 and CD1a expression in response to IL-4 and granulocyte-macrophage colony-stimulating factor are different in cord blood and adult blood monocytes. Pediatr Res 2001; 50(2):184–9.

19. Daigneault M, Preston JA, Marriott HM, Whyte MKB, Dockrell DH. The identification of markers of macrophage differentiation in PMA-stimulated THP-1 cells and monocyte-derived macrophages. PLoS One 2010; 5(1):e8668.

20. Genin M, Clement F, Fattaccioli A, Raes M, Michiels C. M1 and M2 macrophages derived from THP-1 cells differentially modulate the response of cancer cells to etoposide. BMC Cancer 2015; 15:577.

# 8. Appendix

# DH82\_1 Calcein signal in CD14 positive cells



Figure 5. Calcein signal in CD14 positive cells experiment 1. Flowcytometric analyses of DH82 cells stimulated with medium, DFO, PMA, FCS or combinations hereto. Calcein mean fluorescence intensity. Statistical analysis were performed with mixed-effect analysis and the Geisser-Greenhouse correction, followed by Tukey's multiple comparisons test, with individual variances computed for each other.

	0	1µgDFO	10µgDFO	100µgDFO	PMA	PMA+FCS	FCS
DH82_1_1	90,5	72,4	69,3	73,2	84,1	77,6	94,4
DH82_1_2	90,5	75,1	72,8	61,9	84,5	78,2	95,7
DH82_1_3		73,6	68,2	67,2		79	92,6

Table 5: DH_82_1 raw data of the mean fluorescence intensity of the Calcein s	signal
Unit MFI=Mean fluorescence intensity	

In comparison with other acquired data one can assume, that 0 was contaminated, due to its high results. An elevation of intracellular iron upon addition of DFO was observed, while DFO

is assumed to bind iron and being able to penetrate the cell, while including iron in it as well. Therefor an increase in the labile iron pool, while concentration dependently was detected. PMA stimulation, depleted further intracellular iron of DH82 cells. It became apparent that stimulation with PMA, which is a potent activator of cells, had an important impact on DH82 cells, decreasing the labile iron content of these cells. Adding iron in form of FCS decreased the iron-content of these cells, which is contrary to all our other results.

#### DH82\_2 Calcein signal in CD14 positive cells



Figure 6. Calcein signal in CD14 positive cells experiment 1. Flowcytometric analyses of DH82 cells stimulated with medium, DFO, PMA, FCS or combinations hereto. Calcein mean fluorescence intensity. Statistical analysis were performed with Ordinary one-way ANOVA.

	0	1µgDFO	10µgDFO	100µgDFO	lmgDFO	PMA	PMA+10µgDFO	FCS	FCS+10µgDFO
DH82_2_1	47	55	48	56	55	67	61	37	42
DH82_2_2	49	54	37	51	49	64	65	43	48
DH82_2_3	44	48	55	46	51	64	81	35	39
DH82_2_4	46	49	49	46	52	44	65	39	49

 Table 6: DH\_82\_2 raw data of the mean fluorescence intensity of the Calcein signal Unit MFI=Mean fluorescence intensity

This experiment shows throughout very low signals which could be a sign of cell death, before the FACS measurement. Nearly no or very low levels of Calcein could be measured, though in with PMA stimulated cells, a higher signal by the addition of the iron-chelator DFO could be measured, whereas the culture of same cells in iron-rich conditions in media containing FCS significantly reduced the signal, showing a better intracellular iron-status in these cells.

# DH82\_3 Calcein signal in CD14 positive cells

During this attempt, there could not be any results acquired, due to lack of living cells after the incubation in the 48 well plate.



DH82\_4 Calcein signal in CD14 positive cells

Figure 7. Calcein signal in CD14 positive cells experiment 1. Flowcytometric analyses of DH82 cells stimulated with medium, DFO, PMA, FCS or combinations hereto. Calcein mean fluorescence intensity. Statistical analysis were performed with Ordinary one-way ANOVA.

	0	1µgDFO	10µgDFO	100µgDFO	1mgDFO	PMA	PMA+10µgDFO	FCS	FCS+10µgDFO
DH82_3_1	98	36	38	21	31	82	85	55	46
DH82_3_2	76	28	40	41	39	79	76	47	55
DH82_3_3	46	29	34	32	32	95	79	54	50
DH82_3_4	43			48	30	89		59	40
DH82_3_5	38								
DH82_3_6	36								
DH82_3_7	41								
DH82_3_8	57								

Table 7: DH\_82\_4 raw data of the mean fluorescence intensity of the Calcein signal Unit MFI=Mean fluorescence intensity

Similarly, as with DH82\_2, nearly no or very low levels of Calcein could be measured. Still, it became apparent that stimulation with PMA, had a profound impact on DH82 cells, decreasing the labile iron content of these cells, which is associated with inflammatory cells. In addition to that, DFO was capable of elevating the intracellular iron-content a little bit, while the addition of FCS significantly improved the iron-status of these cells and even further adding DFO to it.

# DH82\_5 Calcein signal in CD14 positive cells



Figure 8. Calcein signal in CD14 positive cells experiment 1. Flowcytometric analyses of DH82 cells stimulated with medium, DFO, PMA, FCS or combinations hereto. Calcein mean fluorescence intensity. Statistical analysis were performed with Ordinary one-way ANOVA.

	0	1μgDF Ο	10µgDFO	100µgDFO	1mgDFO	PMA	PMA+10µgDFO	FCS	FCS+10µgDFO
DH82_5_1	336	397	488	341	441	772	632	395	484
DH82_5_2		407	467	485	414	658		344	428
DH82_5_3			455	394	542	249		422	556
DH82_5_4									1111

 

 Table 8: DH\_82\_5 raw data of the mean fluorescence intensity of the Calcein signal Unit MFI=Mean fluorescence intensity

In DH82\_5, a new batch of Calcein was used, rendering adequate Calcein signals to measure. The addition of increasing concentration of DFO in otherwise unstimulated cells increased concentration-dependently the Calcein-signal, which reached by about 15nM a plateau. PMA stimulation depleted further intracellular iron of DH82 cells, with the addition of DFO to PMA being able to further decrease the iron-pool. As such, the stimulation with PMA, a potent activator of cells, had a huge impact on DH82 cells, decreasing the labile iron status of these cells. FCS significantly improved the iron status in these cells, but also here the addition of  $10\mu$ g/ml DFO led to an intracellular decrease of the iron status.



Figure 9. Calcein signal in CD14 positive cells experiment 1. Flowcytometric analyses of DH82 cells stimulated with medium, DFO, PMA, FCS or combinations hereto. Calcein mean fluorescence intensity. Statistical analysis were performed with Ordinary one-way ANOVA.

	0	1µgDFO	10µgDFO	100µgDFO	1mgDFO	PMA	PMA+10µgDFO	FCS	FCS+10µgDFO
DH82_6_1	12356	21533	15487	19954	34251	33504	20910	5504	9545
DH82_6_2	9487	19451	10154	13688	8796	28435	34738	7124	11061
DH82_6_3	17107	20928		42040	11696	32893	36934		13532
DH82_6_4		9148		/	14993		27215		6619
DH82_6_5				21211					
DH82_6_6				14471					

 Table 9: DH\_82\_6 raw data of the mean fluorescence intensity of the Calcein signal

 Unit MFI=Mean fluorescence intensity

In DH82\_6, the Calcein staining worked very well. In addition the increase in concentration of DFO in otherwise unstimulated cells showed again a trend in which till a certain degree the labile iron content was increased. PMA stimulation further depleted intracellular iron of DH82 cells and further addition of DFO did elevate the iron-pool a little. In iron-sated conditions, the labile iron content was the highest and adding 15nM DFO was able to reduce it again. Adding FCS to it, improved the labile iron status, while adding 15nM DFO to FCS decreasing the iron content again and resulted in a higher Calcein-signal.

#### DH82\_7 Calcein signal in CD14 positive cells



Figure 10. Calcein signal in CD14 positive cells experiment 1. Flowcytometric analyses of DH82 cells stimulated with medium, DFO, PMA, FCS or combinations hereto. Calcein mean fluorescence intensity. Statistical analysis were performed with Ordinary one-way ANOVA.

	0	1µgDFO	10µgDFO	100µgDFO	1mgDFO	PMA	PMA+10µgDFO	FCS	FCS+10µgDFO
DH82_7_1	300	295	328	244	339	518	296	543	841
DH82_7_2	274	245	263	266	383	360	135	482	1073
DH82_7_3	309	228	274	327	312	330	661	785	726
DH82_7_4	342	291	243	280	307		1656	682	682
DH82_7_5	275		281						
DH82_7_6	280								
DH82_7_7	302								

 Table 10: DH\_82\_7 raw data of the mean fluorescence intensity of the Calcein signal Unit MFI=Mean fluorescence intensity

In DH82\_7 was similar as the previous results, though not as clear with some outliers making the interpretation more difficult. There was a weak concentration-dependency though the PMAand FCS-data had very high signal to begin with. All in all, DH82\_7 was similar to experiment 6, 5 and 1.



DH 82 8

Figure 11. Calcein signal in CD14 positive cells experiment 1. Flowcytometric analyses of DH82 cells stimulated with medium, DFO, PMA, FCS or combinations hereto. Calcein mean fluorescence intensity. Statistical analysis were performed with Ordinary one-way ANOVA.

	0	1µgDFO	10µgDFO	100µgDFO	1mgDFO	PMA	PMA+10µgDFO	FCS	FCS+10µgDFO
DH82_8_1	677	971	1555	1423	928	2032	759	648	563
DH82_8_2	532	721	1128	503	1656	1631	676	408	591
DH82_8_3	981	891	655	1008	1344	1400		493	462
DH82_8_4	984	846	1062	612	1539	1381		611	695
DH82_8_5		674	805			1399		421	533
DH82_8_6						1523			
DH82_8_7						876			
DH82_8_8						782			

Table 11: DH\_82\_8 raw data of the mean fluorescence intensity of the Calcein signal Unit MFI=Mean fluorescence intensity In DH82\_8, the Calcein staining worked very well. Also here principally, a decrease of intracellular iron upon addition of DFO was observed. PMA worked well, due to its reduction of intracellular iron and the addition of 10µg/ml DFO decreased again cytosolic iron.

# Summary of the normalized Calcein MFI signal of CD14 positive cells (including experiments 1,5,6,7 and 8)





Figure 12. Calcein signal in CD14 positive cells experiment 1. Flowcytometric analyses of DH82 cells stimulated with medium, DFO, PMA, FCS or combinations hereto. Calcein mean fluorescence intensity. Statistical analysis were performed with Mixed-effects analysis, with the Geisser-Greenhouse correction, followed by an uncorrected Fisher's LSD, with individual variances computed for each comparison.

	0	1µgDFO	10µgDFO	100µgDFO	1mgDFO	PMA	PMA+10µgDFO	FCS	FCS+10µgDFO
DH82_1	10052,9	5521,7	5595,5	5199,8	/	6066,4	/	7428,2	/
DH82_1	/	6091,2	6264,5	5670,2	/	5645,5	/	4803,6	/
DH82_1	/	6759,7	5076	5348,3	/	6982,5	/	6710,2	/
DH82_5	3172	5002	7382	5673	4270	21657	11774	7382	5490
DH82_5	/	6833	8724	6039	3782	18789	/	6161	3538
DH82_5	/	/	6161	2562	5856	15190	/	6039	5185
DH82_5	/	/	/	/	/	/	/	5673	/
DH82_6	12356	21533	15487	19954	8796	33504	34738	5504	9545
DH82_6	9487	19451	10154	13688	11696	28435	36934	7124	11061
DH82_6	17107	20928	/	42040	14993	32893	27215	/	13532
DH82_6	/	9148	/	21211	/	/	/	/	6619
DH82_6	/	/	/	14471	/	/	/	/	/
DH82_7	3040	2990	3324	2473	3436	5250	3000	5503	8523
DH82_7	2777	2483	2665	2696	3882	3649	1368	4885	10875
DH82_7	3132	2311	2777	3314	3162	3344	6699	7956	7358
DH82_7	3466	2949	2463	2838	3111	/	16783	6912	6912
DH82_7	2787	/	2848	/	/	/	/	/	/
DH82_7	2838	/	/	/	/	/	/	/	/
DH82_7	3061	/	/	/	/	/	/	/	/
DH82_8	8281	11877	19020	17406	11351	24855	9284	7926	6886
DH82_8	6507	8819	13797	6153	20256	19950	8269	4991	7229
DH82_8	11999	10898	8012	12330	16439	17124	/	6030	5651
DH82_8	12036	10348	12990	7486	18825	16892	/	7474	8501
DH82_8	/	8244	9847	/	/	17112	/	5150	6519
DH82_8	/	/	/	/	/	18629	/	/	/

 Table 12: Normalized data of the mean fluorescence intensity of the Calcein signal from the experiments 1,5,6,7 and 8

 Unit MFI=Mean fluorescence intensity

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