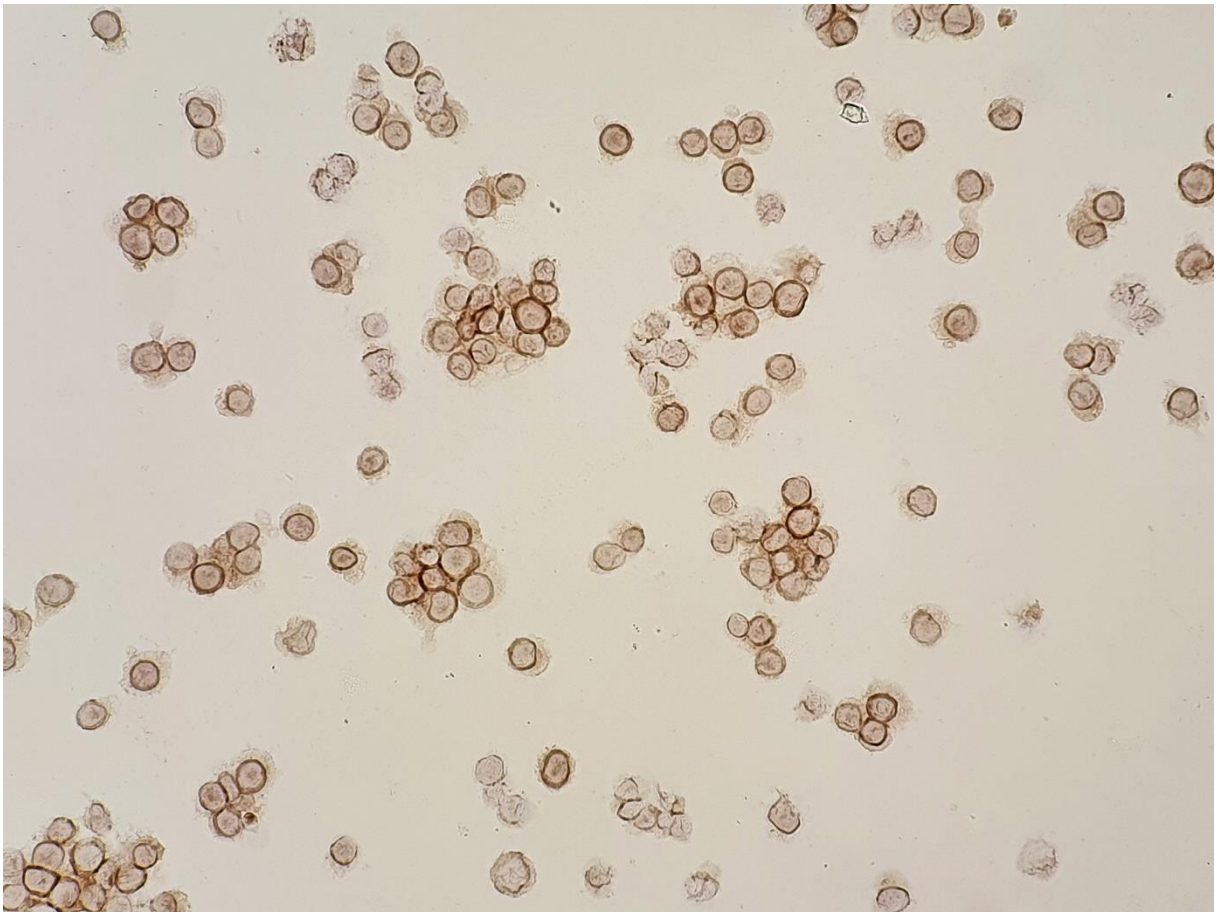

Figure 1: DAB-staining of in vitro cultured O3OD cell line dog macrophages, counterstained with hematoxylin.

Master Research Thesis

Staining O3OD cell line and primary dog macrophages by means of immunocytochemical and immunofluorescence staining using a CD14 marker to eventually compare healthy and *Leishmania* infected dogs

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18-03-2021

Abstract

Canine Leishmaniosis (CanL) is caused by the zoonotic protozoan *Leishmania infantum* and is transmitted by the bites of female phlebotomine sandflies. Via the phlebotomine sand fly, infection can be transmitted from dogs to humans, resulting in visceral or cutaneous Leishmaniasis. Due to global warming, which favors the spread of the sand fly, and the long-distance importation of dogs, CanL is expanding to new locations in Europe. Until now it is unknown how many dogs are infected with *Leishmania* and other vector-borne diseases like Ehrlichiosis and Dirofilariasis.

The target cell of *Leishmania* is the macrophage, this is where replication takes place. M1 and M2 macrophages induce a different immunological response, either beneficial or unfavorable for disease progression. Therefore, distinguishment between M1 and M2 macrophage subtypes could be important for predicting the development of Leishmaniosis. We hypothesized that there is a difference between the M1 and M2 macrophages of healthy and *Leishmania* infected dogs.

During this study we stained O3OD cell line and primary dogs macrophages by means of respectively immunocytochemical and immunofluorescence staining, using a CD14 marker. However, the initial goal of this study to compare the macrophages staining in DNAB samples obtained from healthy and *Leishmania* infected dogs was not achieved. Therefore, the drafted H_0 and H_1 hypotheses cannot yet be answered with the findings of this study and future research is necessary. These future answers can give us more insight in the role of macrophages in the pathology of *Leishmania* and may contribute to proper monitoring and treatment of the disease in dogs.

Table of contents

Introduction

Canine Leishmaniosis (CanL) is caused by the zoonotic protozoan *Leishmania infantum* and is transmitted by the bites of female phlebotomine sandflies. Non-vectorial transmission between dogs is also possible but less common. The latter can take place by transfusion of *Leishmania*-contaminated blood products, transplacental or venereal. (1–3) Below in figure 2 the transmission route of *Leishmania infantum* is displayed in detail.

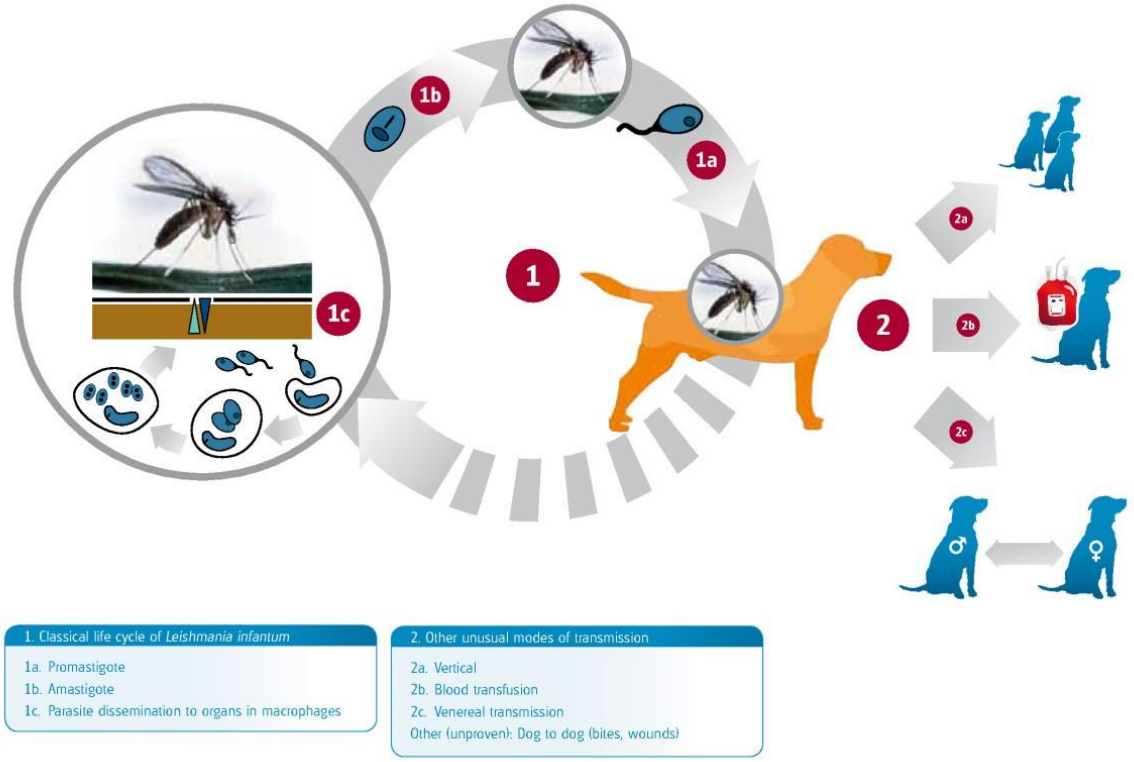


Figure 2: life cycle of *Leishmania infantum* and different transmission routes. (4)

CanL is endemic in the Mediterranean region, but due to global warming, which favors the spread of the sand fly, and the long-distance importation of dogs, it is expanding to new locations in Europe, mainly northwards. Via the phlebotomine sand fly, infection can be transmitted from dogs to humans, resulting in visceral or cutaneous Leishmaniasis, also called VL or CL. When untreated, VL can be fatal. CL however, is more benign. Therefore VL is of higher priority than CL. Furthermore, after malaria CanL is the second most important protozoan infection. (1,5–7)
Every year, thousands of dogs are imported from endemic countries to The Netherlands. (8) Furthermore, according to the study of Brigitte Menn et. al, a total of 4,226 dogs were imported to Germany from endemic regions from July, 2004 to December, 2009. (9)
Until now it is unknown how many of these dogs are infected with *Leishmania* and other vector-borne diseases like Ehrlichiosis and Dirofilariasis. Therefore, further seroprevalence research needs to be performed.

Pathology, symptoms and diagnosis

The clinical signs and underlying pathology of CanL are intrinsically related to the interaction between *Leishmania*, the arthropod vector and the host immune response. The target cell of the protozoan is the macrophage, this is where replication takes place. Settlement of infection and evolution of disease are dependent of the host's immunological responses. The infection usually persists in tissues once settled. (10,11)

Not every dog infected with *Leishmania* eventually develops clinical symptoms. This depends on the type of immunological response. Dogs will remain asymptomatic, so called resistant dogs, when they respond with a T-cell mediated immunity and Th1 cells. On the other hand, dogs that predominantly respond with a humoral immunity and Th2 cells will develop clinical signs, so called susceptible dogs. These different types of immune responses coincide with the presence of different macrophage subtypes, eventually resulting in protection against or progression of Leishmaniosis. (10,12,13)
In conclusion, both clinical and subclinical infection is possible, however subclinical infection is not necessarily permanent. Progression to clinical disease can take place under the influence of immunosuppressive conditions or simultaneous disease.
The susceptibility and resistance to CanL is strongly influenced on a genetic basis, some breeds are more susceptible to developing clinical disease than others. Furthermore, age also seems to be an important risk factor. Mainly young dogs, aging from two to four years and dogs older than seven years develop clinical disease. (10)

Canine Leishmaniosis can involve every tissue or organ and therefore comes with a wide range of clinical signs, ranging from general symptoms to skin lesions or renal disease. Skin lesions are the most frequently seen symptoms of CanL. Renal disease can progress from mild to severe and chronic renal failure is the main cause of death in canine *Leishmania* patients. (4,10)

The diagnosis of Leishmaniosis is difficult. Different methods can be used for diagnosis: parasitological (direct detection of the parasite), molecular and serological. The interpretation of the latter is the most difficult, since a positive titer does not necessarily mean the dog is currently infected with *Leishmania* and/or is clinically sick. Therefore, serological diagnosis is often compared with cytological/histological examination and possibly present clinical pathological abnormalities to confirm CanL or not. (4)

Macrophages and markers

As mentioned previously, macrophages are the main host cells of *Leishmania*. However, there are different subsets of macrophages, in particular the M1 and M2 macrophage phenotypes. These cells provide a controlled or aggravated response to infection with *Leishmania* and eventually determine whether or not infection results in clinical symptoms.
M1 and M2 macrophages have different functions. M1 is the classically activated macrophage subtype which has microbicidal properties and is pro-inflammatory. A Th1-response is coincided with presence of M1 macrophages. This induction gives a protective Th1 immune response with a high production of pro-inflammatory cytokines. M2 is the alternatively activated macrophage subtype which is related to tissue repair and resolution of inflammation and is regulatory/anti-inflammatory. In contrast to M1 macrophages, M2 macrophages coincide with a Th2-response, which is beneficial for intramacrophage growth of *Leishmania*, favoring disease progression and survival of the parasite in the contaminated macrophages. (12,13)
The study of Moreira et. al shows that the predominance of M2 macrophages favors multiplication of *Leishmania* in dogs, in organs more susceptible to infection like lymph nodes, the spleen and skin. (14)
In conclusion, distinguishment between M1 and M2 macrophage subtypes could be important for predicting the development of Leishmaniosis.

The polarization of macrophages into M1 or M2 subtypes depends on the signals coming from the microenvironment. Not only the function of M1 and M2 macrophages differs, but also the production of several mediators, such as cytokines and chemokines, and the expression of transcription factors, receptors and surface molecules. These can act as specific markers to identify the function of the macrophages. (12,13) Below in figure 3 the different markers for M1 and M2 macrophages are shown. This is the situation described for humans and mice.

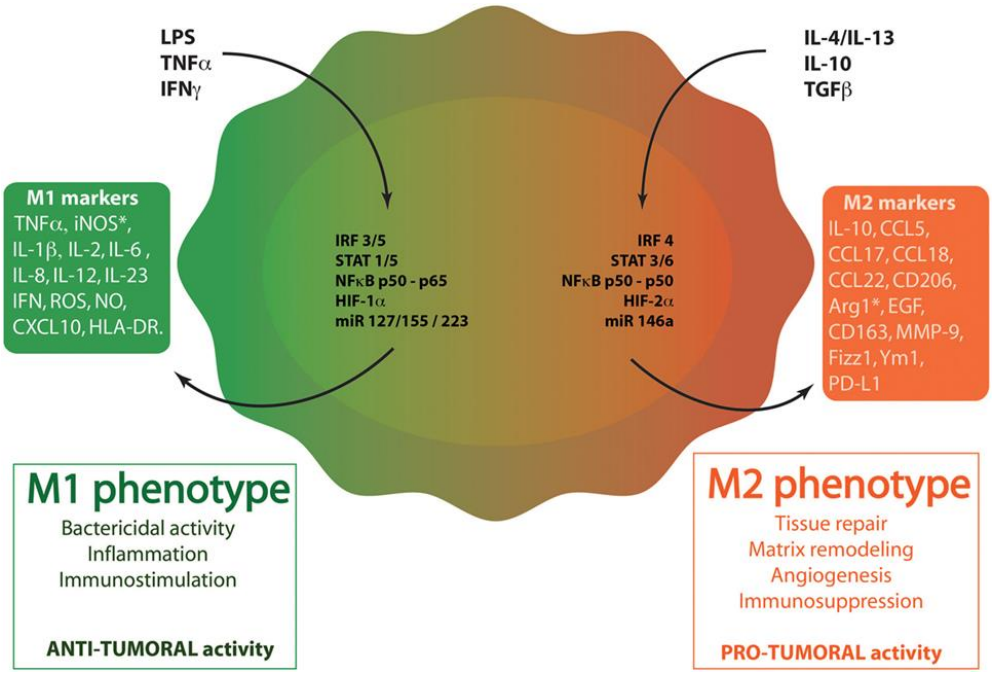


Figure 3: macrophage polarization and M1 and M2 markers. (15)

Aim of the study

The initial purpose of this study is to set up an immunocytochemical staining for cytological samples of DNABs from the peripheral lymph nodes of dogs, to identify M1 and M2 macrophages. Subsequently these macrophages will be compared between healthy and *Leishmania* infected dogs. The ratio of M1 and M2 macrophages in dog lymph nodes could be important for predicting the development of Leishmaniosis and be used as a biomarker for infection.

Research goals and hypothesis

The first part of this research will be the participation in the larger scale seroprevalence study regarding Canine *Leishmania*, *Dirofilaria* and *Ehrlichia* in dogs imported from endemic countries to the Netherlands.

The aim of the second part of this research is to develop a cytochemical staining to identify M1 and M2 macrophages, in cytological samples from peripheral lymph nodes of dogs. Once optimal staining procedures have been developed, M1 and M2 macrophages in lymph nodes, of healthy and *Leishmania* infected dogs will be compared.

For this second part of the study, the following hypothesis is formulated:

> H_0: There is no difference between the M1 and M2 macrophages of healthy and *Leishmania* infected dogs.
>
> H_1: There is a difference between the M1 and M2 macrophages of healthy and *Leishmania* infected dogs. In healthy dogs M1 macrophages occur more and in *Leishmania* infected dogs M2 macrophages occur more.

If the H_1 hypothesis is true, this information can give us more insight in the role of macrophages in the pathology of *Leishmania*, which can be used for future research.

Materials and methods

In general

This research report contains two parts. The first part of this research is a prospective study to evaluate the prevalence of Canine *Leishmania, Dirofilaria* and *Ehrlichia* in imported dogs from endemic countries in the Netherlands. Dogs, at least 6-7 months old, imported from Mediterranean areas are clinically examined at the Utrecht University Clinic of Companion Animals (UUCCA) within six weeks after arrival in the Netherlands. After their physical exam, blood will be collected for several laboratory tests: a complete haematology evaluation, an antibody titer (DAT method) for *Leishmania*, an antigen detection test for *Dirofilaria* and a Knott's test for *Dirofilaria*. Blood will be stored for *Ehrlichia* serology in the future. Nine to twelve months later, these dogs are examined again with the same protocol, because in the meantime seroconversion of the different infection diseases can have taken place.

In the second part of this research cytological samples are obtained from the lymph nodes of dogs and M1 and M2 macrophages are identified. By means of immunocytochemical-staining with specific markers, M1 and M2 macrophages are distinguished from each other.
In this research the staining possibilities for these markers will be examined in dogs. The staining protocols need to be developed.

Subsequently, we will compare the macrophages staining in DNAB samples obtained from healthy and *Leishmania* infected dogs.
Cytological specimens of normal lymph nodes will be obtained from research dogs which were humanely euthanized for other studies. *Leishmania* positive samples will be obtained from client owned dogs that are cytologically *Leishmania* positive. The collection of these samples is already part of the diagnostic process for the individual patient.

Cell culturing – O3OD cell line and primary macrophages

First of all O3OD cell line and primary dog macrophages were cultured, to eventually use them for respectively immunocytochemical and immunofluorescence staining. The protocol for culturing the primary dog macrophages can be found in appendix 2.

Antibody

For both the immunocytochemical and immunofluorescence staining a CD14 mouse anti-human antibody was used. The CD14 marker is present on both O3OD cell line as well as on all types of primary macrophages used in the experiments.

Immunocytochemical staining

An immunocytochemical staining was performed on O3OD cell line dog macrophages. The macrophages were examined using a light microscope. All pictures were taken at a 20x magnification. The staining protocol can be found in appendix 1.

Immunofluorescence staining

An immunofluorescence staining was performed on primary dog macrophages. The macrophages were examined using a confocal microscope and at last pictures of the cells were taken. All pictures were taken at a 63x magnification. The staining protocol can be found in appendix 2.

Results

Staining O3OD cell line dog macrophages using immunocytochemical staining

First of all, we stained O3OD cell line dog macrophages to develop a basic protocol for eventually staining dog tissue macrophages. During the process of optimizing the staining protocol, several extra blocking steps were added overtime, to make sure no false positive staining would occur. Furthermore different dilutions of primary and secondary antibodies were tested, to see which concentration worked best. Finally, different concentrations of H_2O_2 were used for the blocking of endogenous peroxidase activity, to see which concentration worked best. After optimizing the staining protocol, we managed to stain O3OD cell line dog macrophages. The negative controls were made to check for potential false positive staining. All pictures were taken at a 20x magnification. The pictures shown are from different attempts of the experiment, using the same protocol.

In figure 4 the negative control slides are shown. As expected, the macrophages in figure 4a, 4c and 4d are not stained. However, the macrophages in figure 4b show a false positive staining reaction.
Figure 5 shows the macrophages expected to stain, because both the antibodies were put on top. Unfortunately, in figure 5a and 5b the staining reaction is too strong, due to endogenous peroxidase activity. On the other hand, the macrophages in figure 5c are stained well; there is no endogenous peroxidase activity and an evident staining on the surface of the cells.

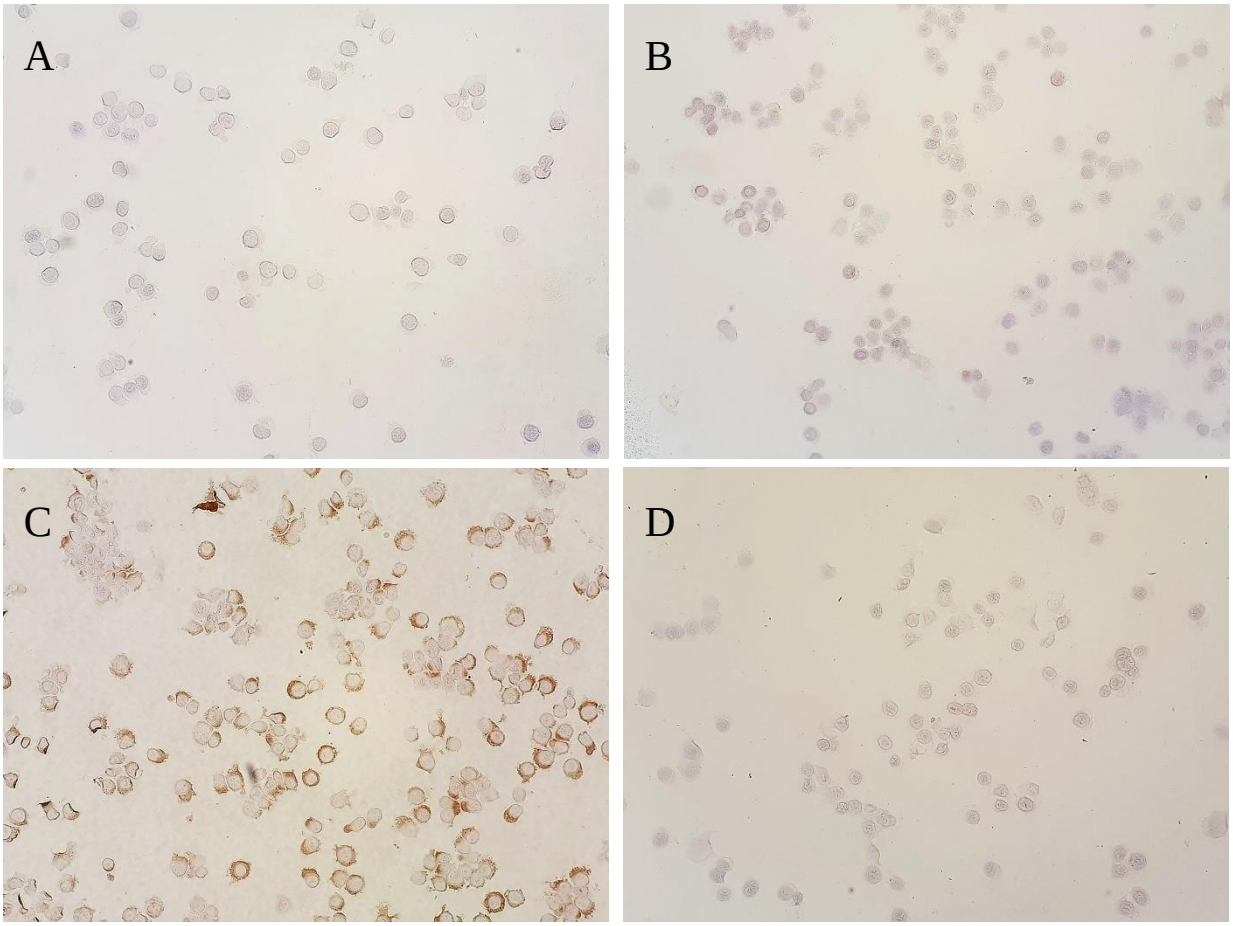
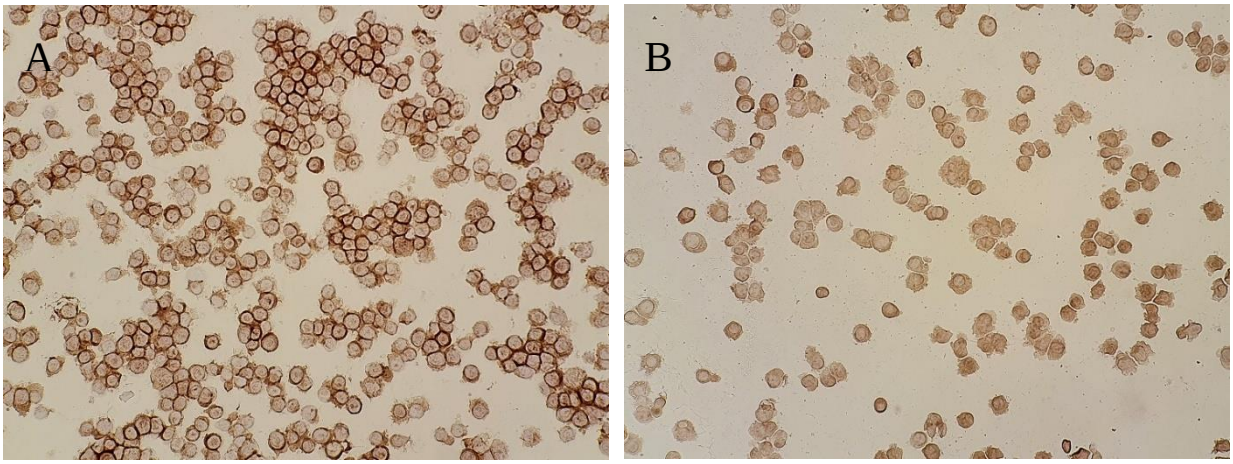


Figure 4: DAB-staining of in vitro cultured O3OD cell line dog macrophages, counterstained with hematoxylin. A: negative control; lacking the primary and secondary antibody. B: negative control; without primary antibody, not blocked for endogenous peroxidase activity (0% H_2O_2). C: negative control; without primary antibody, blocked for endogenous peroxidase activity (0,3% H_2O_2). D: negative control; without primary antibody, blocked for endogenous peroxidase activity (3% H_2O_2).



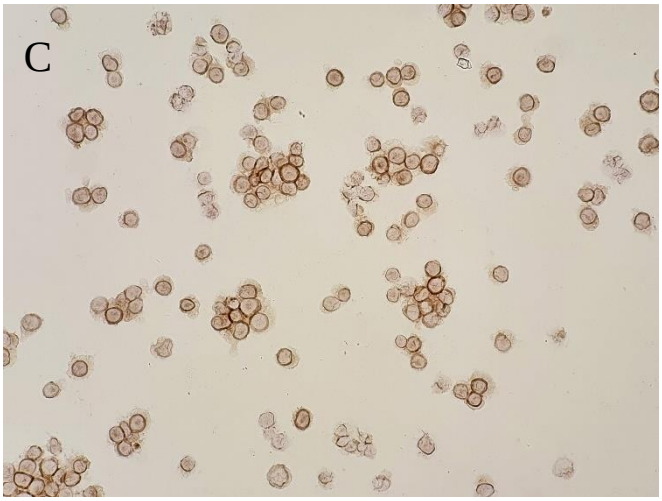


Figure 5: DAB-staining of in vitro cultured O3OD cell line dog macrophages, counterstained with hematoxylin. A: slide with both antibodies, not blocked for endogenous peroxidase activity (0% H_2O_2). B: slide with both antibodies, blocked for endogenous peroxidase activity (0,3% H_2O_2). C: slide with both antibodies, blocked for endogenous peroxidase activity (3% H_2O_2).

Staining primary dog macrophages using indirect immunofluorescence

During this study we also stained primary dog macrophages using indirect immunofluorescence and a CD14 marker antibody. Primary macrophages are highly relatable to tissue macrophages, so this staining protocol could possibly be used in the future for staining macrophages in DNAB samples obtained from lymph nodes from healthy and *Leishmania* infected dogs.

The negative controls were made to check for potential false positive staining. All pictures were taken at a 63x magnification. As expected, macrophages in all negative control slides, shown in figure 6ABC, 7ABC and 8ABC, are not stained. Furthermore, the M0, M1 and M2 macrophages are all stained, although the staining reaction is quiet weak. There is no difference in staining reaction between the different subtypes of macrophages, because the CD14 marker is present on all these macrophages. On the following pages the immunofluorescence staining of the primary dog macrophages is shown in figure 6, 7 and 8.

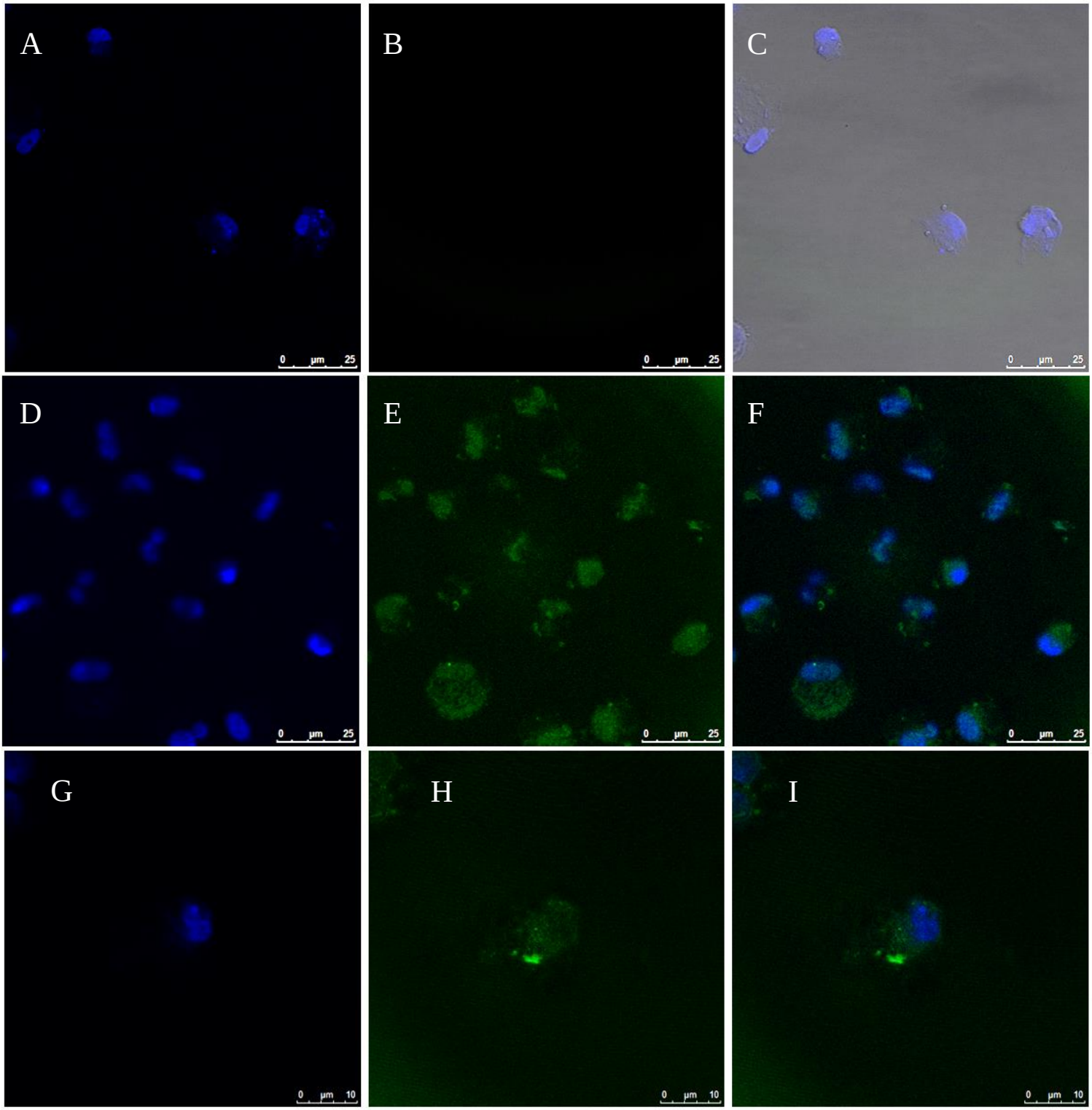


Figure 6: immunofluorescence staining of in vitro cultured primary dog M0-macrophages stained with CD14-antibody. A, B and C: negative control, lacking the primary and secondary antibody. D, E and F: with both antibodies, primary antibody at 1:100 dilution. G, H and I: with both antibodies, primary antibody at 1:50 dilution. The first column shows staining of the nuclei, the second column staining of the macrophages surfaces and the third column a merge of the nuclei and surface staining, whether or not combined with the transmission channel. The transmission channel is added in picture C to clearly show the boundaries of the cells.

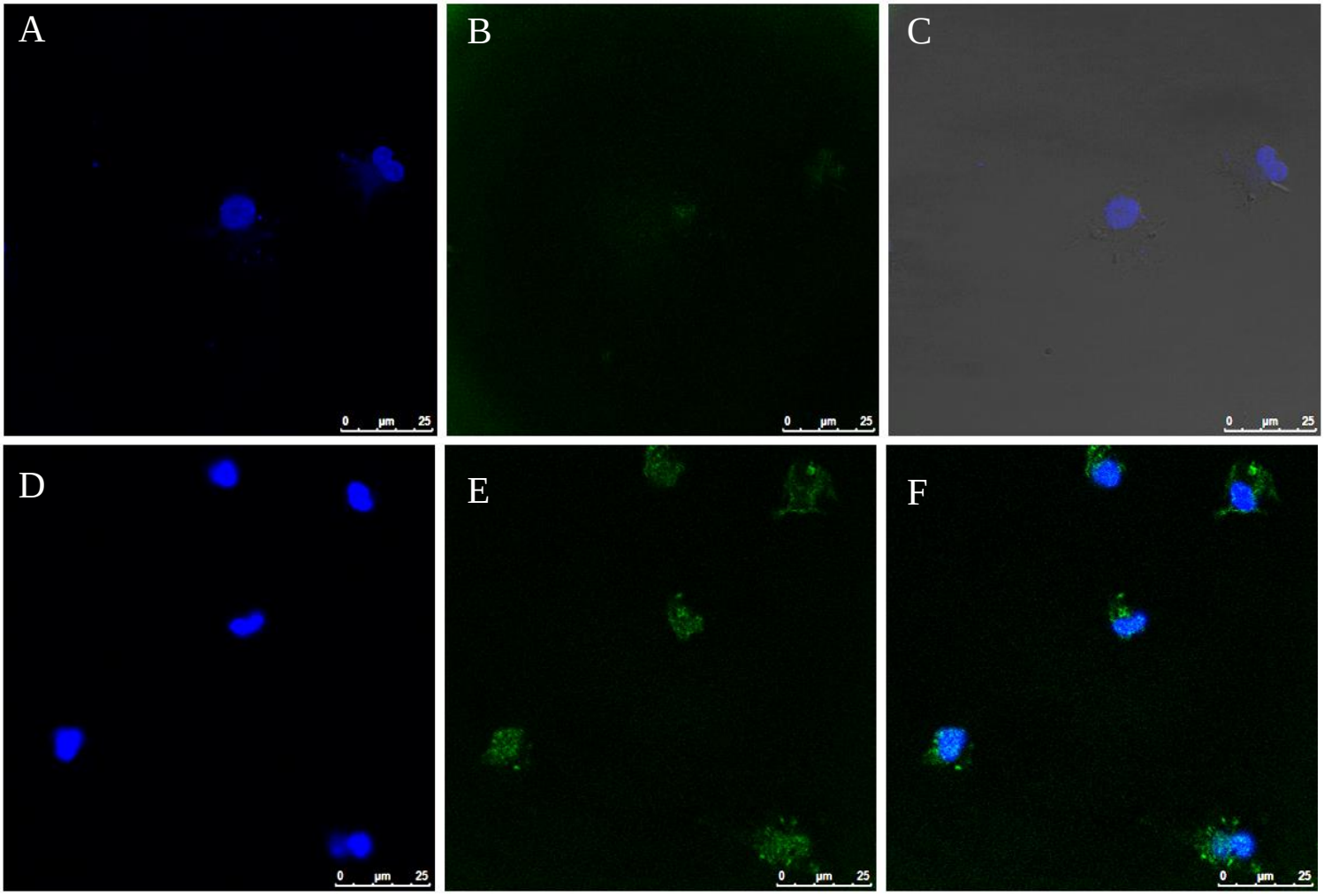


Figure 7: immunofluorescence staining of in vitro cultured primary dog M1-macrophages stained with CD14-antibody. A, B and C: negative control, lacking the primary and secondary antibody. D, E and F: with both antibodies, primary antibody at 1:100 dilution. The first column shows staining of the nuclei, the second column staining of the macrophages surfaces and the third column a merge of the nuclei and surface staining, whether or not combined with the transmission channel. The transmission channel is added in picture C to clearly show the boundaries of the cells.

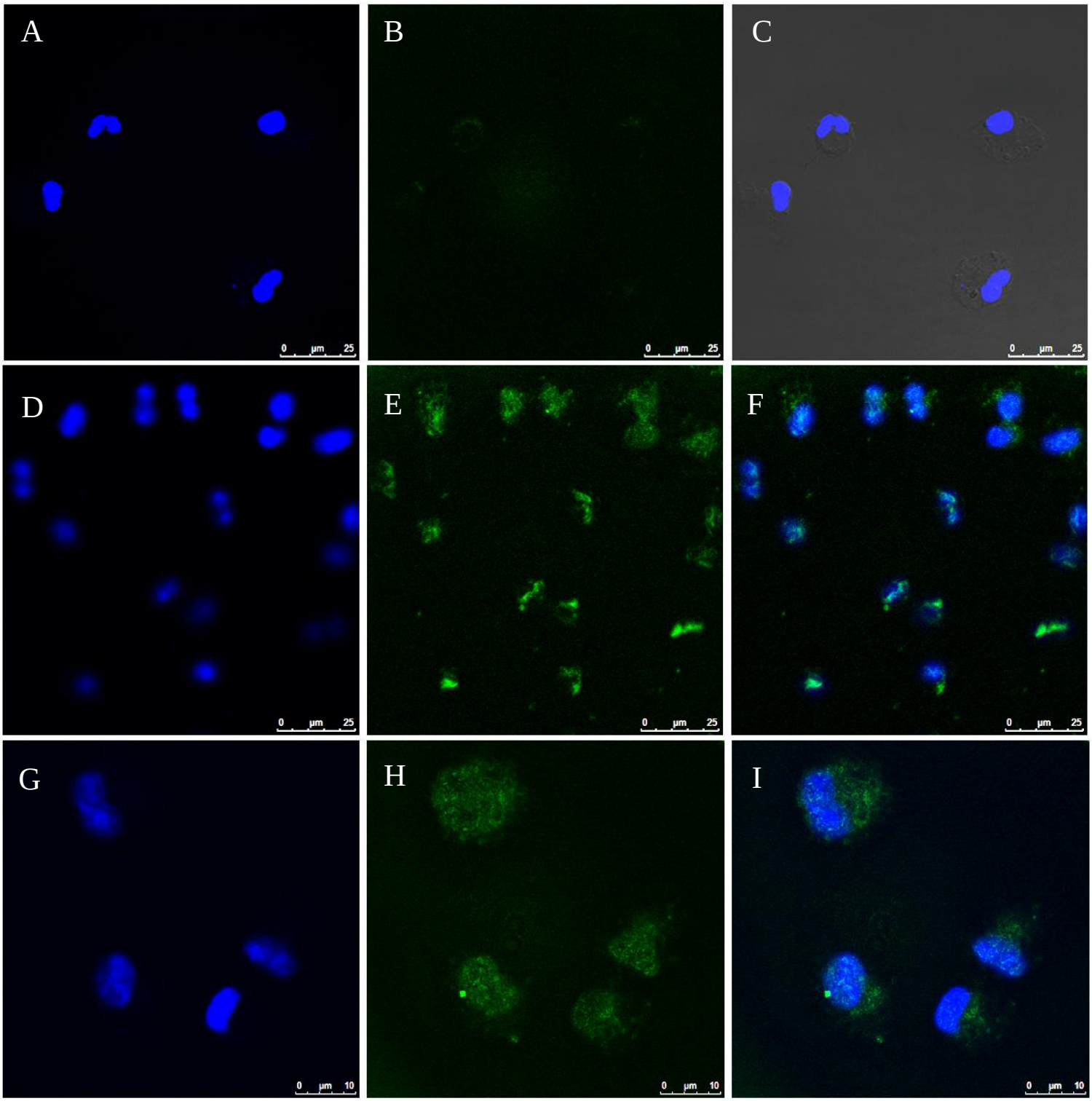


Figure 8: immunofluorescence staining of in vitro cultured primary dog M2-macrophages stained with CD14-antibody. A, B and C: negative control, without primary antibody. D, E and F: with both antibodies, primary antibody at 1:100 dilution. G, H and I: with both antibodies, primary antibody at 1:50 dilution. The first column shows staining of the nuclei, the second column staining of the macrophages surfaces and the third column a merge of the nuclei and surface staining, whether or not combined with the transmission channel. The transmission channel is added in picture C to clearly show the boundaries of the cells.

Discussion

During this study we developed a protocol for immunocytochemical and immunofluorescence staining of respectively O3OD cell line and primary dog macrophages using a CD14 marker. The results of the immunocytochemical staining show that it is best to use a 3% H_2O_2 concentration for blocking of endogenous peroxidase activity, since otherwise a false positive staining reaction is visible. Furthermore, after optimization of the staining protocol, it became clear that a primary antibody dilution of 1:50 and a secondary antibody dilution of 1:100 gave the best result, which is shown in figure 5c.
Unfortunately, staining macrophages is difficult. Therefore the initial goal of the study was not reached within time and during the experimental process the goal(s) of the study had to be altered. Eventually the aim of the study became to immunocytochemically stain O3OD cell line dog macrophages and eventually perform an immunofluorescence staining on primary dog macrophages. The initial goal of this study to compare the macrophages staining in DNAB samples obtained from healthy and *Leishmania* infected dogs was not achieved. Therefore, the drafted H_0 and H_1 hypotheses cannot yet be answered with the findings of this study and future research is necessary. However, the results and developed protocols of this study have laid the foundation for future research.

We used the CD14 marker to stain dog macrophages during this study. However, a study of Khazen et. al shows that in humans and rodents CD14 is not a macrophage-specific protein. On the other hand, CD14 was expressed in high levels on human adipocytes and mice adipose tissue. (16) This result needs to be taken into consideration, since we assumed the CD14 marker to be a dog macrophage marker. We only performed the staining protocol on cell line and primary macrophages, so this given is not a problem for this study. However, when using the protocol for staining DNAB samples in the future, we need to be sure that the CD14 marker is specific enough to only stain dog macrophages and not other cells, since these are also present in these samples.

This study consisted of ups and downs. First of all, we managed to develop a good protocol for immunocytochemical staining of O3OD cell line dog macrophages and made a design for immunofluorescence staining of primary dog macrophages.
During the immunofluorescence staining process we lost a lot of cells, due to washing of the well plate. Therefore, we could only see a few cells on the 1:50 dilution coverslips, which made it difficult to judge and capture pictures of these particular macrophages. Furthermore, we also performed a staining on M1 primary macrophages, with the primary antibody at 1:50 dilution. Unfortunately, we lost so many cells, we were not able to take pictures of these results. Due to this cell loss problem, we cannot state which primary antibody concentration is best for the immunocytochemical staining of primary dog macrophages.

Taken together, our findings demonstrate that it is possible to stain O3OD cell line and primary macrophages by means of respectively immunocytochemical an immunofluorescence staining, using a CD14 mouse anti-human antibody. To get answers to the drafted hypotheses, further research is necessary.

For future optimization of both staining protocols it would be nice to include a negative control with CD14 depleted cells, to see whether these cells show a staining reaction or not, indicating the chance of staining other cells than dog macrophages, by using a CD14 marker antibody and this specific protocol.

Fortunately, we managed to stain the primary dog macrophages by means of immunofluorescence staining. However, the protocol for the immunofluorescence staining needs to be optimized, since a lot of cells were washed away and the staining reaction of the primary macrophages was quiet weak. For optimization, the following steps in the protocol can be altered:

- The washing steps need to performed very gently, to make sure enough macrophages remain on the cover slips to make pictures of the results with the immunofluorescence or confocal microscope.
- Incubation time of the primary antibody: prolongation from 2hrs to overnight, to improve binding of the primary antibody to the CD14 marker.
- Concentration of the primary antibody: the 1:100 dilution resulted in a good staining reaction, but at the 1:50 dilution we lost many cells, so we cannot yet state that 1:100 dilution is better/worse than the 1:50 dilution, this needs to be examined while optimizing the protocol.
- The secondary antibody can also be altered. A stronger fluorochrome could be used when using a different surface marker with a lower expression rate, to make sure a good staining reaction occurs.

Eventually, when the protocol is perfectly optimized for immunofluorescence staining of primary dog macrophages, it can be used for immunofluorescence staining of dog tissue macrophage, since primary macrophages are closely relatable to tissue macrophages.
This same protocol, but using an antibody against a M1 and/or M2 specific marker, can eventually be used to specifically stain M1 and M2 macrophages.
The optimized protocol for DAB-staining of the O3OD cell line macrophages can also be used for staining primary macrophages and eventually tissue macrophages. However, with immunocytochemical staining it is more difficult to clearly distinguish between M1 and M2 macrophages, since the staining colors are less different in contrast to immunofluorescence staining. On the other hand, immunocytochemical staining can indicate *Leishmania* amastigotes, which is not possible or more difficult with immunofluorescence staining. This feature favors immunocytochemical staining, since the combination of distinguishment of M1 and M2 macrophages and the presence/absence of *Leishmania* can act as a biomarker for infection and development of Leishmaniasis.

In the future, further research is necessary to answer both hypotheses and develop a staining protocol for distinguishing M1 and M2 macrophages. The study of Heinrich et. al shows that CD206 is the only marker that can be used to distinguish between M0, M1 and M2 macrophages. The specific literature-based M1- and M2-markers like for instance iNOS and arginase-1 were inappropriate for immunophenotypic discrimination between the three macrophage subtypes. For this in vitro study blood samples of 12 healthy Beagle dogs were used and polarized dog macrophages were examined using different techniques. (17)
Using the CD206 marker as a target for specifically staining M2 macrophages is recommended for further research. Once the staining protocol is optimized, or another protocol is developed for distinguishing between M1 and M2 dog macrophages, the ratio of M1 and M2 macrophages in DNAB samples obtained from lymph nodes from healthy and *Leishmania* infected dogs needs to be examined. This last information can give us more insight in the role of macrophages in the pathology of *Leishmania* and may contribute to proper monitoring and treatment of the disease in dogs.

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Appendices

Appendix 1 - Staining O3OD cell line dog macrophages using immunocytochemical staining

Preparing the slides

1. Cultured O3OD cell line macrophages were used for this experiment. The cells were derived from the cellplate by pipetting. Subsequently the amount of cells was counted with a cell counter machine. The cells were stored on ice until cytospin.
2. The cytocentrifuge was used to make the slides. Specificially, (1) the slides are mounted with the paper pad and the cuvette in the metal holder; (2) load up 200µL of cell suspension in each cuvette; (3) spin at 800 or 900 rpm for 3min; (4) carefully detach the cuvette and the paper without damaging the fresh cytospin and dry the cell spot for 3-5min.
    - In this experiment, in total 7 slides were made:
        - Control slide
        - Three slides without primary antibody
        - Three slides with both antibodies
3. After fixating the slides with acetone for 15 min in a coplin jar the slides were dried for 3-5min, then the spots were surrounded with a circle of the DAKO-pen, so that the fluids added thereafter would not flow all over the slides.
4. Afterwards slides were rinsed 1x with PBS for 5 min to rehydrate the cells. Subsequently the slides were kept in PBS during preparation of the 0,3% and 3% H_2O_2, which was subsequently used to block for endogenous peroxidase.

Blocking for endogenous peroxidase

Sections are blocked for endogenous peroxidase by adding H_2O_2 directly on the spots on the slide for about 20 min (or when the process of bubbling has stopped) using different concentrations of H_2O_2: the control slide was blocked with 3% H_2O_2. The slides without the primary antibody were blocked with 0%, 0,3% or 3% H_2O_2. The slides with both antibodies were also blocked with 0%, 0,3% or 3% H_2O_2. Incubate until no new bubbles appear. On the slides without the H_2O_2 PBA was put on top.

1. Subsequently slides were rinsed 1x with PBS for 1 min.
2. Afterwards, an extra blocking step was added to make sure false positive staining would not occur. The slides were horizontally incubated in a closed humified box for about 30 min with 50µl dog serum per spot. Afterwards the slides were rinsed 1x for 1 min.
3. Sections are horizontally incubated with 50-300µl of appropriately diluted primary antibodies or direct conjugate. Antibodies and conjugate are diluted in PBS containing 0.5% BSA and 0.1% sodium azide (=PBA). Incubate in closed humified box either at RT for 1 hr or overnight at 4°C. From this point onwards never let the slides dry out!
    - The slides were horizontally incubated with 50µl of CD14 mouse anti-human antibody solution per slide. On the spots we used the primary antibody dilution at 1:50. On the control slide and the slides without primary antibody the PBA solution was added.
4. Slides are rinsed 3x 1 min in PBS.

5. Another extra blocking step was added to make sure false positive staining would not occur. The slides were horizontally incubated for 30 min with approximately (1 drop) 50µl horse serum per spot. Afterwards the slides were rinsed 1x for 1 min.
6. Sections are horizontally incubated with horse anti-mouse IgG-biotin (1:100) in PBA. Incubate in closed humified box at RT for about 1 hr.
    - All spots except for one (the control) were incubated with the secondary antibody solution. The control spot was incubated with PBA.
7. Slides are rinsed 3x 1 min in PBS.
8. Mix 10µl solution A and 10µl solution B in 5 ml pure PBS (10µl solution A and 10µl solution B in 500µl pure PBS was used in this experiment). This solution has to stand for 15-30 min before it can be used. Sections are horizontally incubated with this ABC solution. Incubate in closed humified box at RT for about 1 hr.
9. Slides are rinsed 3x 1 min in PBS.

Staining with DAB and counterstaining with hematoxylin

1. Dissolve 0.5 mg DAB per ml Tris buffer.
    - This was prepared beforehand, solution of the following: 100µl DAB, 900µl tris buffer and 15µl 3% H2O2.
2. A positive control should be carried out by adding the DAB-solution to the vectastain solution to see if the staining solution works (concentration 1:1).
3. Slides are incubated horizontally flooding with substrate solution (about 50µl per slide) for about 15 minutes. To ensure that no over- or understaining occurs, slides are regularly (right from the start) monitored under the microscope during the reaction.
4. Reaction is ended by transferring the slides into PBS, rinse 3x2 min with PBS.
5. Optional: slides are counter stained in hematoxylin solution for 5-10 sec and rinsed with tap water for 5min.
6. Slides are mounted in Kaiser's gelatin with a cover slip.

Appendix 2 - Staining primary dog macrophages using indirect immunofluorescence

Culturing primary macrophages

Medium

- RPMI-1640 GlutaMAX/5% FCS/1% penicillin/streptomycin
- 5 ng/ml canine recombinant GM-CSF (R&D Systems, Cat # 1546-GM-025) (17)
- 20 ng/ml recombinant canine IL-4 (R&D Systems, Cat # 754-CL-025/CF)
- 100ng/ml LPS (Cat. L2630, sigma-aldrich)
- 20 ng/ml recombinant canine IFN-γ
- 25ng/ml human M-CSF (Cat. PHC9504, gibco)

Day 1

1. Thaw 5 vials canine PBMC at 37 ºC in a water bath and keep on ice.
2. Transfer to a 50 ml tube.
3. Centrifuge for 5 min at 300x and 4 ºC and remove supernatant.
4. Resuspend the cells and isolate CD14+ cells.
5. Seed cells on a 24-well plate; 0,75×106 cells/well.

Day 2
Refresh medium for monocytes; add 5 ng/ml GM-CSF for M1 and 25 ng/ml M-CSF for M2.

Day 5
Refresh medium for monocytes; add 5ng/ml GM-CSF for M1 and 25 ng/ml M-CSF for M2.

Day 7
Refresh medium for monocytes, add 5ng/ml GM-CSF for M1 and 25 ng/ml M-CSF for M2, and add 100 ng/ml LPS and 20 ng/ml IFN-γ for M1 and 20 ng/ml IL-4 for M2.

Indirect immunofluorescence (IF)

1. Culture primary macrophages according to the protocol above.
2. Cover cells with coverslips.
3. Wash samples two times with PBS for about 1 min each.
4. Fix samples with 4% paraformaldehyde in PBS for 15 min at room temperature. (Note: paraformaldehyde is toxic, only use in fume hood).
5. Aspirate fixative, rinse two times in PBS for 1 min each.
6. Incubate samples in 10% normal dog serum in PBS for 30 min at room temperature.
7. Aspirate dog serum, incubate sections with CD14 mouse anti-human antibody at 1:50 and 1:100 dilution in PBS + 10% dog serum overnight at 4°C or 2 hours at room temperature (optimal condition should be confirmed in different laboratory).
8. Rinse three times in PBS for 1 min each.
9. Incubate samples with fluorochrome-conjugated secondary antibody at 1:400 dilution in PBS for 1 hour at room temperature in dark (optimal condition should be confirmed in different laboratory).
10. Rinse three times in PBS for 1 min each in dark.
11. Incubate samples with 1 μg/ml DAPI for 1 min.
12. Mount samples with a drop of mounting medium.
13. Examine the slides under the immunofluorescence or confocal microscope.