

Department of Pathobiology
University of Veterinary Medicine Vienna
Institute of Anatomy, Histology and Embryology
(Head: Univ.-Prof. Dr. rer. nat. Armin Saalmüller)

**Immunohistochemical double staining of T cells using
primary antibodies raised in the same host species**

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Submitted by
Gregor Maurer

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Supervisor:

Priv.Doiz. Dr.med.vet. Kirsti Witter, PhD
Institute of Anatomy, Histology and Embryology
Department of Pathobiology
University of Veterinary Medicine, Vienna

Reviewer:

Dr.rer.nat. Martin Glösmann
VetImaging
VetCore Facility for Research
University of Veterinary Medicine, Vienna

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Index of Abbreviations and Companies

A. dest.....	Aqua destillata
CD	Cluster of Differentiation
DAPI.....	4', 6'-Diamidino-2-phenylindole
F(ab)	Fragment antigen binding
Fc	Fragment crystallisable
IF	Immunofluorescence
IHC	Immunohistochemistry
M.O.M. kit.....	Mouse on Mouse kit (Vector, Burlingame, CA, USA)
PBS	Phosphate buffered Saline
PALS	periarterial lymphatic sheath

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1 Introduction

Immunohistochemical (IHC) double staining is used to visualise different antigens in the same cell or in different cells in situ, i.e. in histological sections. In general primary antibodies raised in different species (e.g. rabbit and mouse) are used in order to prevent unwanted cross reactions.

In some cases, the desired combination of primary antibodies raised in different host species is not available. An example are CD (Cluster of Differentiation) markers — surface antigens of leukocytes. Most anti-porcine CD antibodies that are commonly used for FACS analysis are produced in the mouse.

Different strategies have been described for immunohistochemical double staining using two primary antibodies raised in the mouse. One possibility is the application of so-called mouse-on-mouse kits that were originally developed for the use of mouse primary antibodies in murine tissues. Another strategy is the tyramide amplification of an immunoreaction with highly diluted primary antibody, whereas the immunostaining with the second primary antibody is carried out *lege artis*. If both murine primary antibodies are immunoglobulins of different isotypes, isotype-specific secondary antibodies can be used in order to distinguish the immunoreactions of first and secondary primary antibody.

In order to provide a basis for the detection of porcine T-cell subpopulations by Immunofluorescence (IF), the following hypothesis will be tested in this study:

With a modified double staining protocol in combination with the reagents of the M.O.M. kit (Vector Laboratories, Burlingame, CA, USA) it is possible to achieve distinct and correct immunohistochemical double staining using mouse anti-porcine CD3 and mouse anti-SWC1 antibodies.

1.1 Immunohistochemistry

Immunohistochemistry (IHC) is a powerful and widely used tool for diagnosis and research in human and veterinary medicine uniting histological and immunological methods. BRANDTZAEG (1998) defined IHC as a “collective term for various methods used to recognize constituents of tissues as antigens in situ by means of corresponding colour-tagged antibodies”, usually derived from another species than the species where the tissue specimen comes from. In short, antibodies raised against a certain protein are applied to tissue sections in order to detect a specific antigen. An antibody’s paratope (antigen binding site) specifically binds to an antigen’s epitope (region binding the antibody).

Antibodies, also called immunoglobulins, are the soluble form of the B-cell receptor and consist of two identical heavy and two identical light chains. The heavy chains determine the antibody class which is important for the use of subclass specific secondary antibodies (BUCHWALOW et al., 2005). Five classes exist and can be further divided into subclasses: Ig (immunoglobulin)G, IgM, IgD, IgA, IgE. By digesting these proteins with the proteolytic enzymes pepsin or papain, different antibody portions are obtained as illustrated in Figure 1. Digestion with papain yields two F(ab) fragments and one Fc fragment. F(ab) stands for “fragment antigen binding”, as this is the antigen-binding region of the antibody. These fragments are monovalent for they only have one paratope. Fc stands for “fragment crystallisable”. Digestion with pepsin yields one F(ab’)₂-fragment which is divalent, and a digested Fc portion (MURPHY et al., 2009). It has to be kept in mind that antibody fragments have a major impact on blocking and staining procedures.

Since its beginning in the early 1940s the development of IHC has been pushed forward by the introduction of enzymes as antibody labels, the establishment of fluorescence technology and the arrival of the modern fluorescence microscope (BRANDTZAEG, 1998). According to BRANDTZAEG (1998) two labels can be distinguished: Enzymes for immunoenzymatic staining and fluorochromes for immunofluorescent staining. The former is characterised by the use of enzyme-conjugated antibodies and is recommended for double staining only when the different detected antigens are known to be located in different cell compartments or in different cells. The analysis of the coloured enzymatic products has to be done with light

microscopy. IF is more suitable when unwanted background noise from endogenous enzymes is expected. Moreover, a differentiation between the fluorescence signals in double staining is facilitated owing to the filter system of the fluorescence microscope. Besides enzymes and fluorescent dyes as a label, also the use of colloidal gold or biotin, detection of the latter by enzyme or fluorochrome conjugated avidin, have been reported (RAMOS-VARA, 2005).

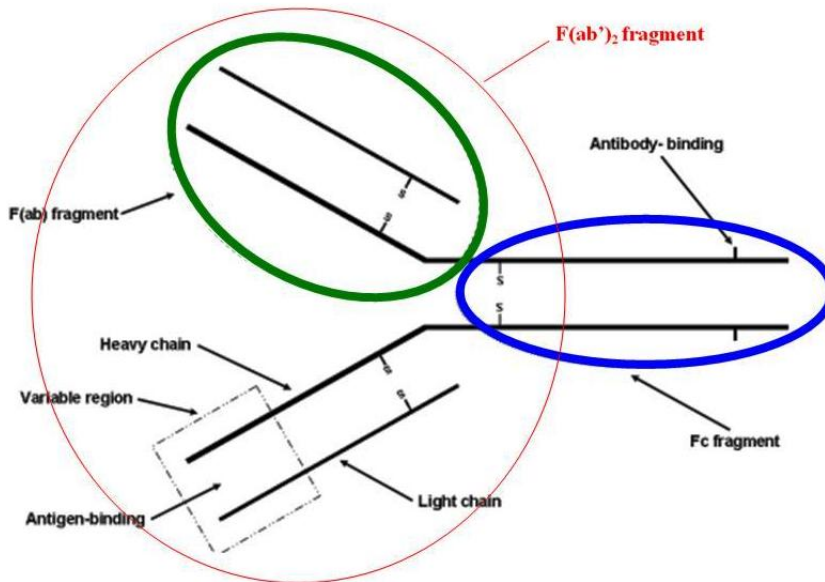


Fig. 1 Structure of an immunoglobulin molecule.

The area delimited by an oval green line corresponds to the F(ab) fragment (fragment antigen binding). The area marked by a square is the variable region (paratope). The area limited by an oval shaped blue line is the Fc fragment (fragment crystallisable). The term Antibody-binding stands for the region that binds to the T-cell receptor (modified after RAMOS-VARA, 2005).

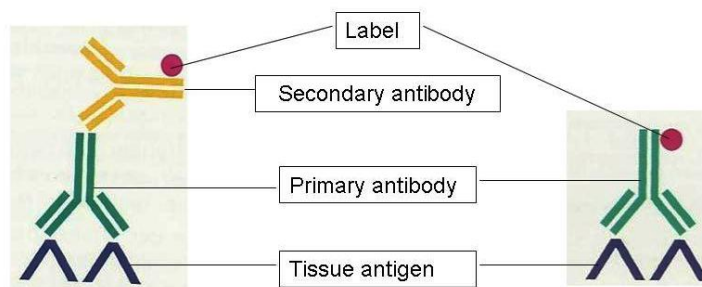


Fig. 2 Comparison of indirect (two antibody layers) and direct (one antibody layer) method of immunohistochemistry.

In the indirect method a conjugated secondary antibody specifically binds to the primary antibody directed against the tissue antigen. In the direct method a fluorochrome or enzyme labelled primary antibody recognises the tissue antigen (modified after BOENISCH, 2006).

Many IHC detection methods have been introduced so far, classified as direct and indirect staining protocols (RAMOS-VARA, 2005). Concerning the first, directly conjugated antibodies are applied to the tissue, thus avoiding possible cross reactions between the secondary antibodies. Indirect staining procedures are based on the detection of an unlabelled primary antibody by a labelled secondary antibody that is raised against the primary antibody. Figure 2 demonstrates the principle of detecting a single antigen by direct and indirect IHC. The indirect approach can also be used for multiple antigen targeting. When two or more different antigens on a single tissue specimen are visualised, the term “Co-localisation Studies” or “Double Staining Experiments” is used. The targeted antigens can be located within a single cell or within different cells. For BRANDTZAEG (1998) IF remains the best approach for testing the co-localisation of two or more antigens.

Simultaneous antigen targeting is a widely used straightforward method as primary antibodies raised in different host species are available today. In general, primary antibodies raised in different species (e.g. mouse and rabbit) are used in order to allow the secondary antibodies a clear distinction between the primary antibodies. Figure 3 illustrates an indirect two-colour IF. As primary antibodies of different species origin are applied on the tissue, the secondary antibodies (conjugated e.g. with the fluorochromes FITC and Cy3) can clearly differentiate between the mouse primary and the rabbit primary antibody. Thus no cross reactions, i.e. false positive results will be observed.

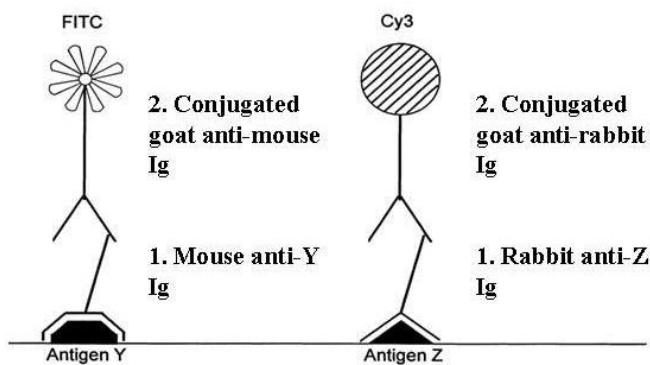


Fig. 3 Principle of an indirect double staining.

As primary antibodies raised in different species are used, no unwanted cross reactions happen: Antigen Y appears in green colour (FITC), whereas antigen Z stains red (Cy3) (modified after BRANDTZAEG, 1998).

1.1.1 Special adaptations

All IHC settings described above represent comparatively simple tasks. However there are some special cases which require adaptation of the basic protocols. One of them is single indirect IHC using primary antibodies produced in the same host species as the tissue section comes from. Most often it is the combination of mouse primary antibodies on mouse tissue.

When murine monoclonal antibodies are applied on mouse tissue, the staining results will be influenced by the high background staining (LU & PARTRIDGE, 1998). According to the M.O.M. kit instructions of the producer (Vector Laboratories, Burlingame, CA, USA), the anti-mouse secondary antibody is unable to distinguish between the mouse primary antibody and endogenous mouse immunoglobulins present in the section. The Binding to endogenous Igs is responsible for the background stain. The M.O.M. kit copes with the unspecific background staining: According to the manufacturer's instructions the kit eliminates the background by a special blocking solution applied to mouse tissue before the primary mouse antibody.

Owing to this blocking step, the enzyme-labelled or fluorochrome-labelled secondary antibody does not bind unspecifically to endogenous mouse tissue components. The contents of the blocking solution are not disclosed by the company.

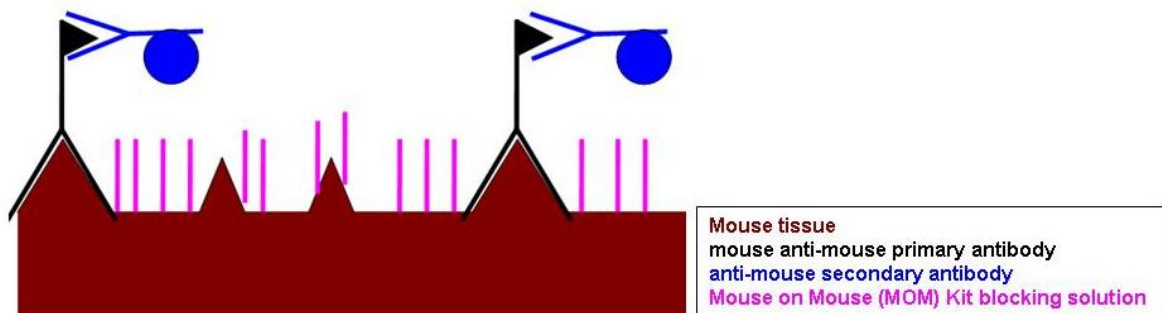


Fig. 4 Working principle of the "Mouse on Mouse" kit (Vector Laboratories, Burlingame, CA, USA).

In the first step, endogenous mouse immunoglobulins are blocked by the Mouse on Mouse kit blocking solution. Secondly, the mouse anti-mouse primary antibody is applied on the tissue and specifically binds to the targeted antigen. In the last step, the applied anti-mouse secondary antibody specifically binds to the primary antibody, thus no severe background will be observed.

Although the M.O.M. kit was not available in the 1990s, several papers have dealt with the topic of staining mouse tissue with mouse primary antibodies, often involving F(ab) fragments in the blocking steps (HIERCK et al., 1994; LEWIS CARL et al., 1993; LU & PARTRIDGE, 1998). Considering the technical principle of the M.O.M. kit, the assumption is possible that F(ab) fragments are involved in the specific blocking step.

Another special task for IHC are double staining experiments using two primary antibodies raised in the same host species, mostly mouse (LEWIS CARL et al., 1993). This is the method of choice when the desired combination of primary antibodies raised in different species is not available. An example are CD markers — surface antigens of leukocytes. Most anti-porcine CD antibodies that are commonly used for FACS analysis are produced in the mouse. In this case the staining procedure requires extensive adaptations as two primary antibodies originating in the same species are used.

NEGOESCU et al. (1994) discussed the drawbacks of double staining with primary antibodies raised in the same species. A two step protocol is obligatory. In the first step, the first antigen is visualised with the first primary and the first secondary antibody. Then a specific blocking step has to be applied to the tissue before detecting the second antigen in the second step. According to the results of NEGOESCU et al. (1994) two types of interference must be prevented, Type I interference and Type II interference.

Type I interference means that the second primary antibody (Ab II 1) is captured by paratopes of the first secondary antibody (Ab I 2). Type II interference stands for the linkage of the second secondary antibody (AbII 2) to the first primary antibody (AbI 1). These two types of cross reactions are illustrated in Figures 5 and 6.

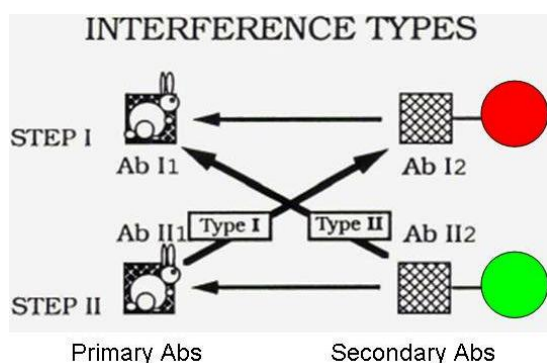
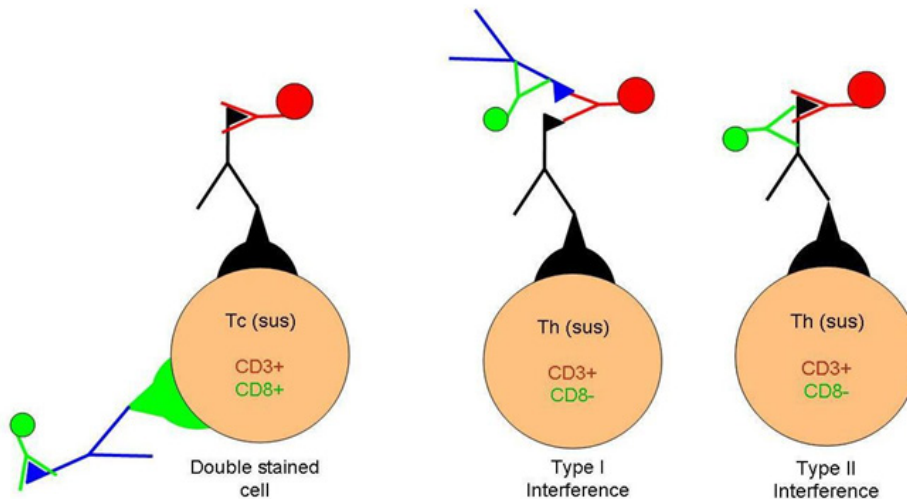


Fig. 5 Cross reactions during immunohistochemical double staining using two primary antibodies of the same species.

The situation is shown for two rabbit primary antibodies. Type I and Type II interference can be distinguished (for further explanation see text). Red and green colour stands for the different fluorochromes that are attached to the secondary antibodies. Ab, antibody (modified after NEGOESCU et al., 1994).



1st step primary antibody (Ab I 1)

1st step secondary antibody (fluorochrome labelled, Ab I 2)

2nd step primary antibody (Ab II 1)

2nd step secondary antibody (fluorochrome labelled, Ab II 2)

Fig. 6 Interference effects on double immunohistochemistry staining outcome.

In the figure above, the example of porcine lymphocytes stained for CD3 and CD8 is used. Due to the two types of interference T helper cells expressing CD3 only are turned into false double positive cells. Tc, cytotoxic T cells; Th, T helper cells (for further details see text).

1.2 Prevention of interference of secondary antibodies in double IHC

There are different strategies to prevent interferences and thus false positive double staining when two primary antibodies raised in the same host species are used: e.g. (i) application of isotype-specific secondary antibodies, if the primary antibodies are of different isotype, (ii) tyramide amplification (TERAMOTO et al., 1998) of the first immunostaining carried out with an over-diluted first primary antibody and (iii) application of F(ab) fragments (LEWIS CARL et al., 1993) or the M.O.M. kit (Vector) for blocking of Type II interference before second immunostaining.

1.2.1 Isotype-specific secondary antibodies

A possible system of IHC double staining using primary antibodies raised in the same host species makes use of isotype differences between primary monoclonal antibodies by the application of secondary antibodies directed against the corresponding isotype (BUCHWALOW et al., 2005), an approach often used in flow cytometry for the characterisation of peripheral blood leukocytes (e.g. GERNER et al., 2008).

1.2.2 Tyramide Signal Amplification

According to TERAMOTO et al. (1998) the CSA (catalyzed signal amplification) is based on the deposition of tyramide, which is mediated by a peroxidase, around the primary antibody. Due to the use of tyramide the method is also called TSA (tyramide signal amplification). The principle is illustrated in Figure 7. Originally designed to amplify a weak staining signal in a single staining experiment (MOLECULAR PROBES, 2013), various adaptations of the TSA method exist today, also for double staining experiments:

TERAMOTO et al. (1998) modified the method in order to perform a double stain with two unlabelled primary mouse monoclonal antibodies. The first primary antibody was diluted so strongly that a conventional detection was rendered impossible and was detected by tyramide amplification. The second antigen was detected conventionally.

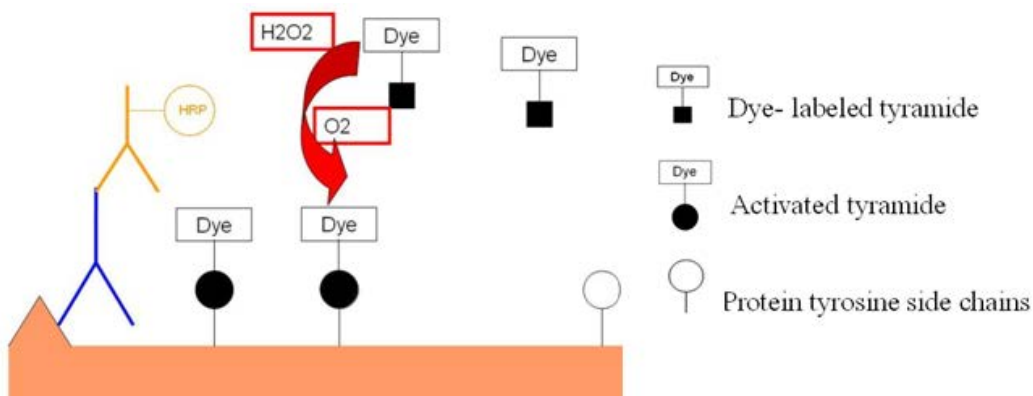


Fig. 7 Principle of the Tyramide Signal Amplification Method.

The antigen is detected by a primary antibody (blue), followed by a horseradish peroxidase-labelled secondary antibody (orange). The enzyme catalyses the transformation of the dye-labelled tyramide into activated tyramide, the radicalised form. This radicalised form is able to react with tyrosine side chains of proteins in neighbourhood to the targeted antigen. Thus, the signal around the antigen is amplified by the deposition of tyramide (MOLECULAR PROBES, 2013).

1.2.3 Mouse on Mouse (M.O.M.) kit

The M.O.M. kit has already been described above. The adaptations made for a double staining of porcine tissue, are illustrated in Figure 8. Application of the M.O.M. kit before the second immunostaining blocks Type II interference, thus making it possible to use two mouse monoclonal primary antibodies without obtaining false double positive cells.

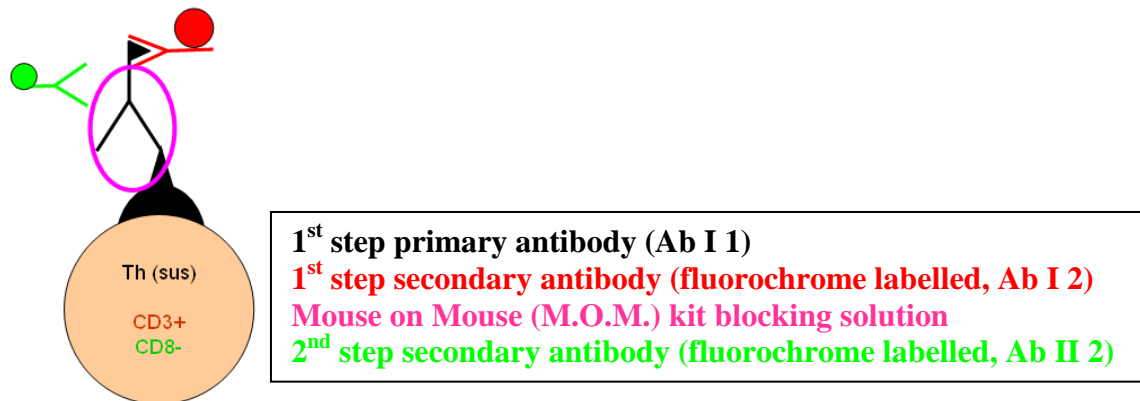


Fig. 8 Prevention of Type II interference by the application of a Mouse on Mouse blocking solution.

The first antigen is detected with the first primary antibody (black), followed by the first secondary antibody (red). Then M.O.M. blocking solution (purple) is applied on the sections to block free binding sites of the first primary antibody. Afterwards the second antigen, if present on the cell, is detected. In the case depicted, the second antigen is not present on the cell, however the blocking solution renders the linkage of the second secondary antibody (green) to the first primary antibody (black) impossible, in other words, false double labelling (Type II interference) is prevented.

1.2.4 Other approaches for double labelling with two primary antibodies raised in the same host species

Several other methods have been suggested for the prevention of cross reactions, most of them based on the use of F(ab) fragments of immunoglobulins.

Monovalent F(ab) fragments are used in different protocols to deal with the drawbacks of cross reactions:

NEGOESCU et al. (1994) suggest, in order to prevent Type II as well as Type I interference, the application of a labelled polyconal F(ab) secondary antibody for detecting the first antigen. According to their results, the F(ab) secondary antibody binds to the first primary antibody and blocks all its free epitopes. Therefore, non-specific binding of the second secondary antibody is rendered impossible, Type II interference is prevented. Due to the monovalence of the F(ab) secondary antibody, no free binding sites exist that capture the second primary antibody, precluding a Type I interference.

LEWIS CARL et al. (1993) use F(ab) antibodies in a different setup, where uncoupled F(ab) fragments are applied to block free residual binding sites of the first primary and first secondary antibody during the detection of the first antigen. The in vitro formation of primary antibody-F(ab) fragment complexes for multiple staining purposes with primary antibodies raised in the same species has been introduced as another application of F(ab) fragments (BROWN et al., 2004).

Considering the use of F(ab) fragments in the various experimental setups mentioned above, it is possible that the M.O.M. blocking solution consists of F(ab) fragments.

1.3 Microscopic anatomy of the porcine spleen

The spleen represents a suitable organ for lymphocyte staining experiments due to its clear distinguishable lymphatic structures with B- and T-cell regions. The histological structure is formed by the white pulp and red pulp. The white pulp consists of lymphatic nodules called “Malpighi bodies” (B-cell region) and periarterial lymphatic sheaths (PALS, T-cell regions) which surround the PALS-Artery. The red pulp is made up of blood sinuses, splenic cords and ellipsoids (PRESS & LANDSVERK, 2006). Figure 9 gives an overview about the red and white pulp of a porcine spleen.

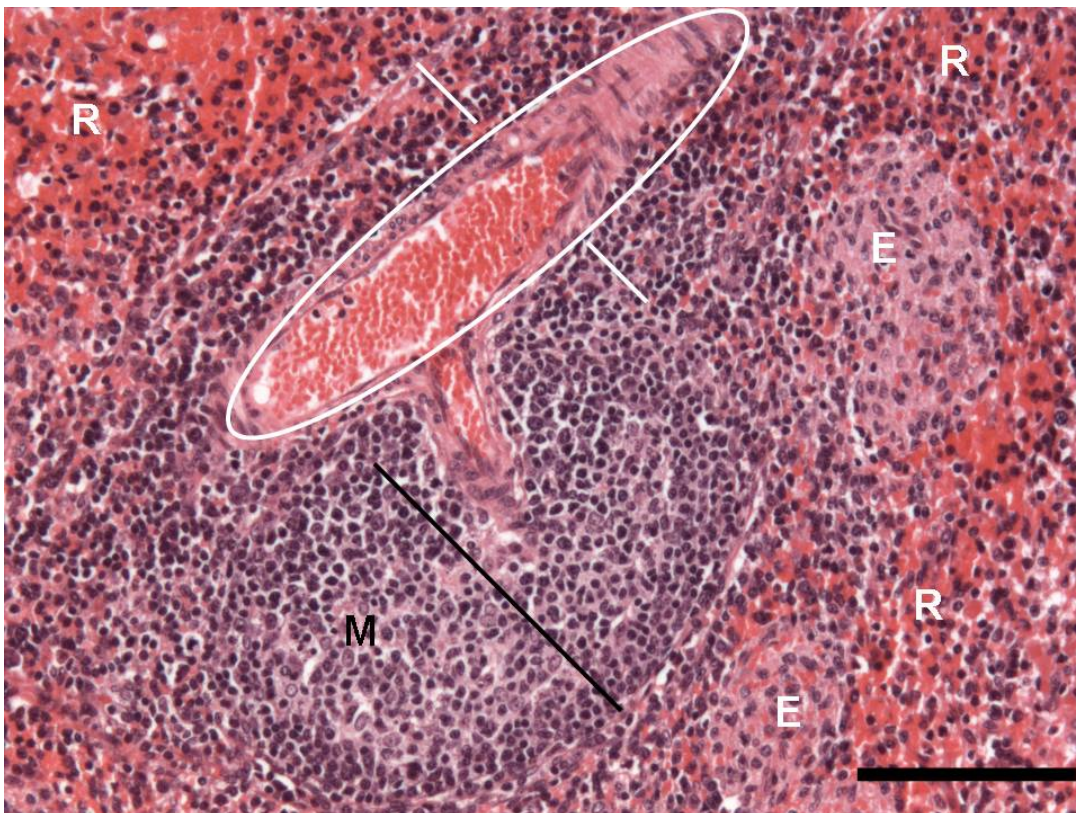


Fig. 9 Histology of the spleen.

The black line indicates the extent of the Malpighi body (lymph follicle, B-cell region); the white line the extent of the PALS (T-cell region). M: Malpighi body, E: ellipsoid, R: red pulp, white ellipse: PALS-Artery. Scale bar: 100 μ m

1.4 Aim of the study

In this study, an existing double staining protocol for primary antibodies of different host species will be modified. The modifications will be based on the reagents of the M.O.M. kit to develop a double staining protocol for primary antibodies of the same host species. The modified double staining protocol will be consequently referred to as “modified double staining protocol”.

The modified double staining protocol will be tested for immunofluorescent double staining of T-cell populations in porcine spleen tissue, using two mouse primary antibodies.

It is expected that unwanted cross reactions as described are blocked, resulting in clearly single and double positive cells.

2 *Material and Methods*

2.1 *Overview of primary and secondary antibodies*

The primary antibodies were all monoclonal and produced from cell culture supernatants at the Institute of Immunology at the University of Veterinary Medicine Vienna (Austria) with exception of the rabbit anti-CD3 antibody (clone SP7) that was bought from Thermo Scientific, CA, Fremont, USA. The secondary antibodies were bought from the commercial supplier Invitrogen, Eugene, Oregon, USA. Tables 1 and 2 list the used antibodies and their specific properties with regard to isotype, dilution, recognised antigen as well as antigen expressing cell type.

Tab. 1: Primary antibodies

Clone	Isotype	Host species	Dilution¹	Dilution for Cocktail²	Antigen	Cell Type
PPT3	IgG1	mouse	1:75	-	CD3	T cells ³
SP7	IgG	rabbit	-	1:150	CD3	T cells ³
11/305/44	IgG2a	mouse	1:40	1:20	SWC1	Granulocytes, Monocytes, quiescent T cells ⁴

¹The term "Dilution" refers to the M.O.M. stains (using primary antibodies originating from the same species and applying them sequentially).

² The Term "Dilution for Cocktail" refers to the control staining (using primary antibodies originating from different species). Before their application, the two primary/secondary antibodies with the concentrations indicated in the column "Dilution for Cocktail" were mixed 1:1 and applied to tissue as a cocktail.

³SAALMÜLLER & BRYANT (1994)

⁴SAALMÜLLER et al. (1994); LEITNER et al. (2012)

Tab. 2: Secondary antibodies

Name	Host species	Dilution ¹	Dilution for cocktail ²	Antigen
ALEXA 488 (green)	goat	1 :100	1:50	anti-mouse
ALEXA 568 (red)	goat	1 :100	1:50	anti-mouse
ALEXA 488 (green)	goat	-	1:50	anti-rabbit
ALEXA 568 (red)	goat	-	1:50	anti-rabbit

¹The term "Dilution" refers to the M.O.M. stains (using primary antibodies originating from the same species and applying them sequentially).

² The Term "Dilution for Cocktail" refers to the control staining (using primary antibodies originating from different species). Before their application, the two primary/secondary antibodies with the concentrations indicated in the column "Dilution for Cocktail" were mixed 1:1 and applied to tissue as a cocktail.

2.2 Experimental setting

For the modified double staining protocol a mouse anti-CD3 primary antibody (Clone PPT3) and a mouse anti-SWC1 primary antibody (Clone 11/305/44) were used.

The antibodies were chosen for the following reasons:

- (i) A rabbit anti-CD3 antibody working on porcine tissue is commercially available. Therefore, the staining results with two mouse primary antibodies can be controlled by classical double IF.
- (ii) CD3 and SWC1 (CD52) (LEITNER et al., 2012) are markers of different cell populations, T cells and monocytes/granulocytes, respectively. However, SWC1 is also expressed on quiescent T cells. Therefore, in histological sections of porcine lymphatic tissue, CD3 and SWC1 single positive cells as well as double positive cells should be found.
- (iii) All primary antibodies required for this study (rabbit anti-CD3, mouse anti-CD3, mouse anti-SWC1) have been proven to work on paraffin embedded tissues, after fixation with methacarn (GAISWINKLER, 2010). Therefore, standard tissue handling for histology was possible.
- (iv) The mouse anti-porcine CD3 and mouse anti-porcine SWC1 antibodies are of different isotypes. Therefore, in future studies the use of isotype-specific secondary antibodies can be tested for double IHC.

In the experiments, the modified double staining protocol was tested for IF double staining with primary antibodies from the same host species as follows:

Each primary antibody was applied as a first and as a second. Moreover, each of the two primary antibodies was visualised first by green, first by red fluorescence. As both primary antibodies originate from mouse, sequential double staining had to be carried out. Figure 10 shows the workflow of the experiments.

To control for the presence of T cells and granulocytes/monocytes in the tissue sections double IF with rabbit and mouse primary antibodies was carried out (see section 2.2.2 “Two species immunofluorescence”).

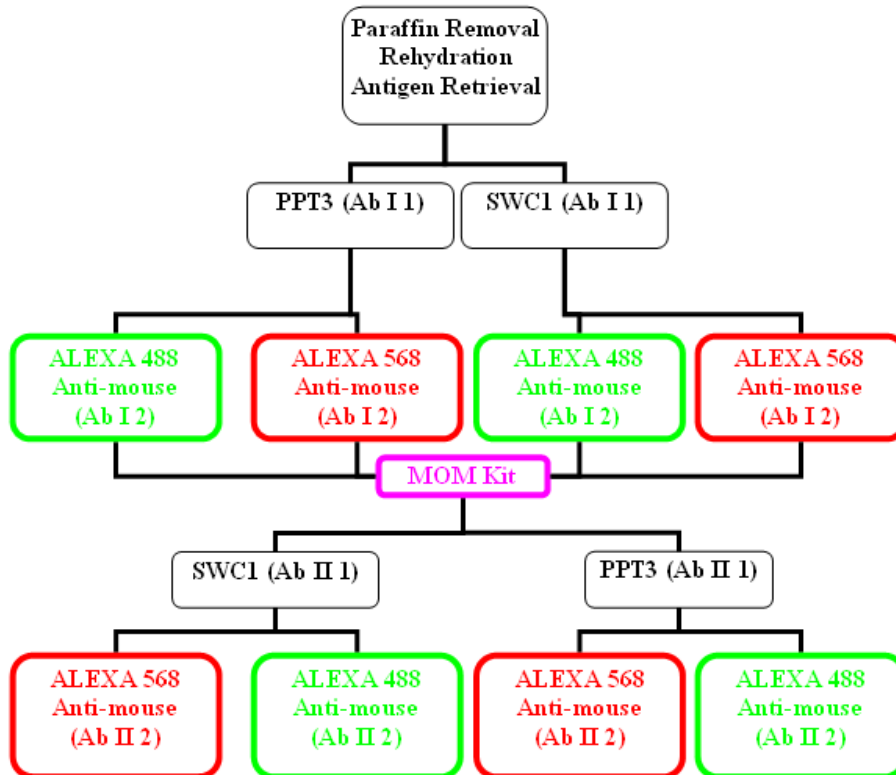


Fig. 10 Experimental paradigm of the modified double staining protocol.

Ab I 1: first primary antibody, Ab I 2: first secondary antibody, Ab II 1: second primary antibody,

Ab II 2: second secondary antibody, MOM Kit: blocking solution that was applied on the sections after the first immunostain.

2.2.1 Adaptations of the modified double staining protocol

The M.O.M. diluent working solution serves as the dilution medium for the antibodies of the second immunostain. This solution is normal horse serum (M. Rauner, Szabo-Scandic, Vienna, Austria; personal communication, 2012). It was assumed that the M.O.M. diluent is intended for the use of horse secondary antibodies.

Therefore, several adaptations of the modified double staining protocol were tested:

2.2.1.1 Modified double staining protocol

In order to perform double labelling with primary antibodies raised in the same host species, i.e. using the modified double staining protocol, sections were deparaffinised in xylene and rehydrated in a graded series of ethanol (100% to 70%). Then an antigen retrieval was applied to the sections to demask possible hidden antigens by cooking the sections in citric buffer 3 times in the microwave, each for 5 min. After blocking of non-specific binding activity with 1.5% normal goat serum (Dako Cytomation, Glostrup, Denmark) in 0.01 M phosphate-buffered saline (PBS), the sections were incubated with the first primary antibody 15h overnight at 4°C.

The next day, the sections were incubated with the first secondary antibody for 1h, followed by the M.O.M. blocking reagent for 1h. After the first step, the second primary antibody was applied to tissue and incubated for 1h, then the second secondary antibody was applied to tissue for 45min. All the staining procedures were performed in a moist chamber. The sections were counterstained with DAPI (4', 6'-Diamidino-2-phenylindole) (Invitrogen, Eugene, Oregon, USA), washed in PBS and mounted in Mowiol (see chapter 2.4.2).

2.2.2 Two species immunofluorescence

Before carrying out the modified double staining protocol, classical double IF, consequently referred to as two species IF 1 and 2, was performed. The primary antibody combination rabbit anti-CD3 (clone SP7) and mouse anti-SWC1 (clone 11/305/44) was chosen. Two stainings were carried out, detecting the primary antibodies with differently labelled secondary antibodies as illustrated in Tables 4 and 5.

In these stains, no special adaptations or blocking steps were involved as the primary antibodies originated from different species. Therefore, primary and secondary antibodies could be applied as cocktails. Two species double immunofluorescence gives a general overview of the distribution and the localisation of the cells.

Tab. 4: Two species immunofluorescence 1

Primary antibody	Dilution for cocktail (Primary Antibodies)	Detection with labelled Secondary antibody	Dilution for cocktail (Secondary Antibodies)
SP7	1:150	Alexa anti-rabbit 568, green	1:50
SWC1	1:20	Alexa anti-mouse 488, red	1:50

Tab. 5: Two species immunofluorescence 2

Primary antibody	Dilution for cocktail (Primary antibodies)	Detection with labelled Secondary antibody	Dilution for cocktail (Secondary antibodies)
SP7	1:150	Alexa anti-rabbit 488, red	1:50
SWC1	1:20	Alexa anti-mouse 568, green	1:50

2.2.2.1 Two species immunofluorescence staining protocol

The sections were deparaffinised in xylene and rehydrated in a graded series of ethanol (100% to 70%). Then an antigen retrieval was applied to the sections to demask possible hidden antigens; cooking the sections in citric buffer 3 times in the microwave, each for 5 min. After blocking unspecific binding activity with 1.5% normal goat serum in PBS, the sections were incubated 15h overnight at 4°C with a cocktail of the primary antibodies. On the next day, a cocktail of the secondary antibodies was applied to the tissue for 1h. All staining procedures were performed in a moist chamber. The sections were counterstained with DAPI, washed in PBS and mounted in Mowiol.

Negative controls and single stain controls were carried out by replacing both primary antibodies (negative controls) or only one of the primary antibodies (single stain controls) with the dilution medium and carrying out the rest of the protocol unchanged. The negative and single stain controls were performed to be able to compare the staining patterns to those obtained in the double-stained sections.

2.3 Visualisation and photodocumentation

The corresponding antigens were detected with secondary antibodies conjugated with Alexa 568 (emitting maximally in the red part of the spectrum) and Alexa 488 (emitting maximally in the green part of the spectrum). Images were obtained with a confocal laser scanning microscope (Zeiss CLSM 510 Meta, Carl Zeiss GmbH, Munich, Germany).

The three detected channels (blue for DAPI, red and green for the detected antigens) were merged by the computer software to demonstrate the overlap present in the stained sections. Thus, in the merged images yellow indicates that the two detected antigens were co-localised.

2.4 Chemicals and tissues

2.4.1 Tissue samples and histological sections

All tissues used for this study originated from the collection of the Institute of Anatomy, Histology and Embryology of the University of Veterinary Medicine Vienna (Austria).

As the aim of the study was to evaluate the different double staining protocols by the detection of T cells, macrophages and monocytes, lymphatic tissue with well preserved histological structures and without pathological changes had to be chosen for IF. The spleen fulfils these criteria. Paraffin blocks of methacarn-fixed spleen tissue of the pig were obtained from the block archive. The tissue originated from an eight-month old sow that had been slaughtered for another project before.

The paraffin blocks were sectioned at 3 µm thickness with a sliding microtome (Microm HM 440E, Histocom, Wr. Neudorf, Austria). The sections were mounted on (3-aminopropyl)triethoxysilane (Sigma-Aldrich, MO, S. Louis, USA) coated Super Frost slides (Economy Objektträger Histo Frost, Stözlze-Oberglas, Vienna, Austria). They were dried overnight in a drying cabinet. Sections were stored at room temperature until use.

2.4.2 Chemicals

The preparation of solutions used in this study is given in short protocol form in this chapter.

2.4.2.1 Phosphate Buffered Saline

Preparation: Dissolve phosphate buffered saline pH 7.4 (Sigma Aldrich, MO, S. Louis, USA) according to instructions in 1 l of distilled water (A. dest.).

2.4.2.2 Goat serum

Preparation: Add 75 µl goat serum (Dako, Glostrup, Denmark) to 5 ml PBS.

2.4.2.3 Mowiol

Preparation: Mix 3 parts solution I with 1 part solution II and store at 4°C in small bottles.

Solution I (Mounting Medium)

- 1) dissolve 20 g Mowiol 4-88 (Polysciences, Eppelheim, Germany) in 80 ml PBS, pH 7.3
- 2) stir over night
- 3) add 40 ml glycerol
- 4) stir over night
- 5) centrifuge for 1 h at 1515 g
- 6) Remove supernatant carefully, store at 4°C

Solution II (FITC-Bleach Protection)

- 1) dissolve 2,5 g n-propyl-gallat (Sigma-Aldrich, MO, S. Louis , USA) in 50 ml PBS (pH 7.0)
- 2) add 50 ml glycerin
- 3) stir over night – stock solution
- 4) store at 4°C in the dark

2.4.2.4 DAPI (staining of nuclei)

Dissolve 2 µl DAPI (4', 6'-Diamidino-2-phenylindole) (Invitrogen, Eugene, Oregon, USA) in 1 ml A. dest. and add PBS to 50 ml.

Staining:

Put the sections from PBS into DAPI solution for 3 min

wash with PBS

discard PBS and put the slides shortly in A. dest.

cover the sections with Mowiol and coverslip

store the sections at 4°C

2.4.2.5 M. O. M. TM Kit Basic BMK-2202

All chemicals given in chapter 2.4.2.5 are provided with the M.O.M. kit (Vector Laboratories, Burlingame, CA, USA).

M. O. M. mouse IgG Blocking reagent working solution

Add 2 drops of M.O.M. mouse IgG Blocking reagent stock solution to 2.5 ml of PBS.

This blocking reagent is applied to tissue after the first secondary antibody in order to block the first immunostain. It prevents the appearance of false double positive cells.

M. O. M. diluent working solution

Add 600 µl of protein concentrate stock solution to 7.5 ml of PBS.

The M.O.M. diluent working solution is the dilution medium for the antibodies of the second immunostain.

2.4.2.6 0.01M Citric buffer (pH 6.0)

Preparation: Dissolve 2,1 mg citric acid monohydrate (Merck, Darmstadt, Germany) in 900 ml A. dest., then adjust to pH 6.0 with about 13 ml 2M NaOH, fill up with A. dest. to 1000 ml.

2.4.2.7 Methacarn

Preparation: Mix 60 ml methanol (Roth, Karlsruhe, Germany), 30 ml chloroform (Merck, Darmstadt, Germany) and 10 ml glacial acetic acid (Merck, Darmstadt, Germany).

3 Results

3.1 Two species Immunofluorescence

The two classical double IF stains, “Two species IF I” and “Two species IF II”, both depicted in Figure 11a-f, showed double positive cells and CD3 single positive and SWC1 single positive cells.

The negative controls and single stain controls were carried out as described in Material & Methods, section 2.2.2.1 “Two species immunofluorescence staining protocol”. In negative controls in which both primary antibodies were omitted, no detectable fluorescence signal was recorded (data not shown). In the single stain controls, in which only one of the primary antibodies was omitted, the corresponding immunostaining was negative (data not shown).

Figures 11g-i depict a double stain with primary antibodies from the same host species that does not involve any special adaptations of the protocol. All cells were double labelled.

3.2 Double Immunofluorescence using the modified double staining protocol

When the modified double staining protocol in combination with the M.O.M. kit was used for IF double staining with two primary mouse anti-swine antibodies, different staining results were obtained.

3.2.1 Controls of the modified double staining protocol

In the negative controls of the modified double staining protocol, controls in which both primary antibodies were omitted, no fluorescence signal was detected (Data not shown).

The single stain controls, i.e. controls in which either first or second primary antibody was omitted but the modified double staining protocol was otherwise carried out completely (Table 3), were not always reliable (data not shown):

In some cases single stain controls with the first primary antibody omitted (Single Stain 1, Table 3) gave no detectable signal of the second primary antibody. However, some single stain controls in which the first primary antibody was applied to tissue and the second primary antibody was omitted (Single Stain 2, Table 3), gave not only a detectable fluorescence signal of the first primary antibody, but also of the second primary antibody.

In the double stained sections that were stained in parallel to these single stain controls mentioned above, only double positive cells could be detected.

The presence and the distribution of T cells and macrophages/monocytes in the spleen were revealed by the single stain controls (Figure 12).

CD3 positive cells were grouped around the PALS artery and in the marginal zone of the Malpighi bodies, whereas in the ellipsoid no T cells were localised (Figure 12a). SWC1 positive cells were found in the ellipsoids (Figure 12b).

3.2.2 Staining results of the modified double staining protocol

Double staining results achieved with the modified double staining protocol in combination with the M.O.M. kit were inconsistent. They are depicted in Figure 13.

The majority of the detected cells in the sections were double positive (Figure 13c, 13f, 13i, 13l).

In some cases, all cells were double-labelled (Figure 13f, 13i). In some cases, besides double positive cells, very few single positive cells were detected, CD3 single positive cells (red, 13c) or SWC1 single positive cells (red, 13l).

3.2.3 Replacement of MOM diluent with goat serum

No differences with regard to the staining outcome described in the section “Staining results of the modified double staining protocol” were observed when diluting the second primary and second secondary antibody in the various dilution media.

In one experiment M.O.M. diluent (horse serum) was used, in a second experiment it was replaced by goat serum in the standard concentration that is used for classical IF double labelling using primary antibodies of different species, i.e. 1.5%, and in a third experiment it

was replaced by goat serum in the concentration suggested by the producer, i.e. 8% (for details see Table 3).

Replacing the M.O.M. diluent by either 1.5% or 8% goat serum yielded results that were not different from those using the modified double staining protocol with the kit's M.O.M. diluent (section 3.2.2. "Staining results of the modified double staining protocol").

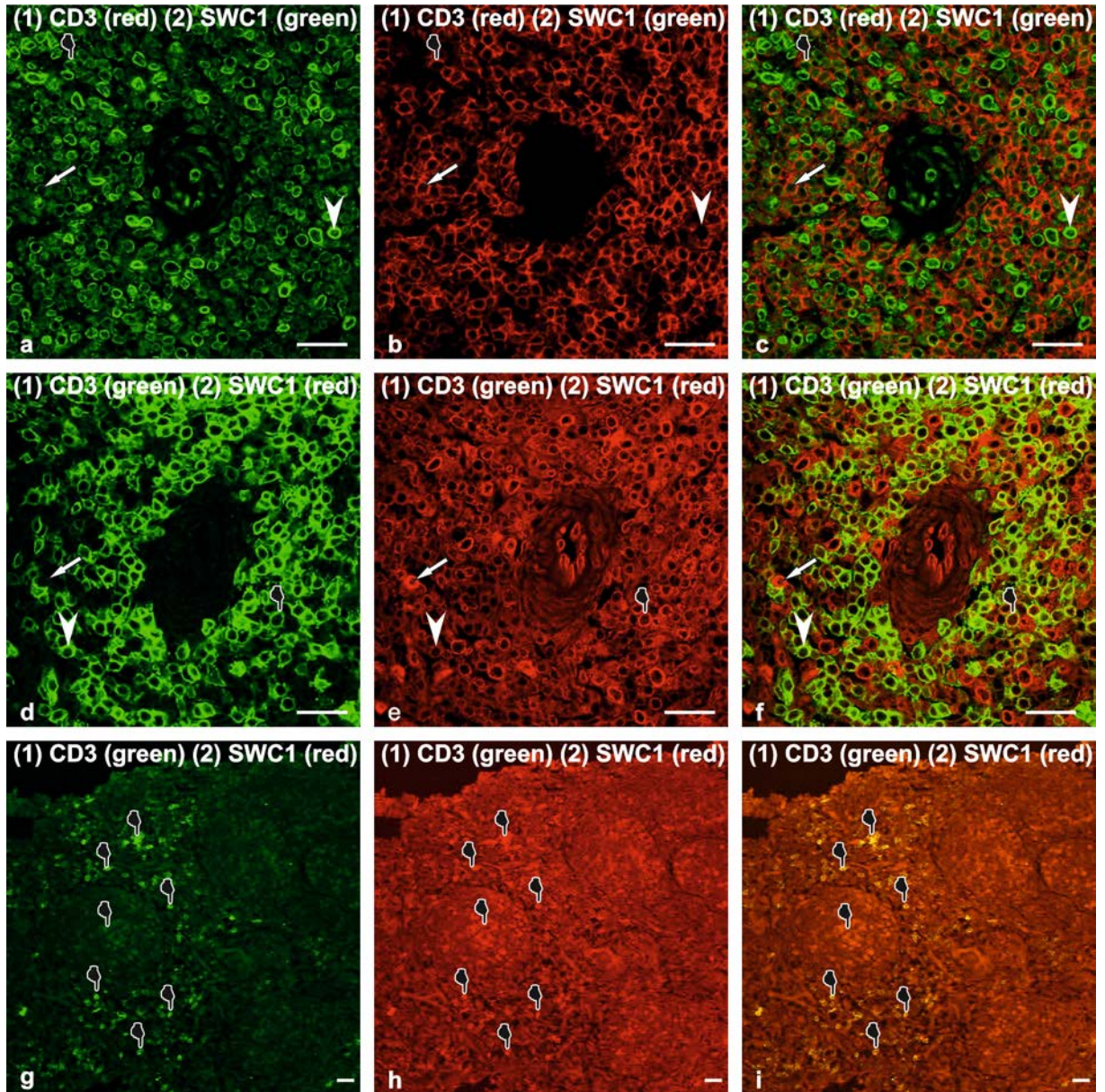


Fig. 11 Successful (classical) immunofluorescent double labelling versus unsuccessful immunofluorescent double labelling.

Figure 11a-f show the successful IF double labelling that used primary antibodies derived from different species, the classical approach (rabbit anti-CD3, mouse anti-SWC1).

However, when antibodies of the same species are applied to tissue (mouse anti-CD3, mouse anti-SWC1) and no special blocking steps are involved, only double positive cells are observed which makes an interpretation of the staining outcome impossible (Figure 11i). Two species IF I (a-c, CD3 positive cells: red, SWC1 positive cells: green), Two species IF II (d-f, CD3 positive cells: green, SWC1 positive cells: red) and stain without any special blocks (g-i).

a, d, g: green channel; b, e, h: red channel; c, f, i: overlay of green and red channel.

Arrow: red positive cells, arrowhead: green positive cells, black hand: double positive cells.

Scale bar: 20µm.

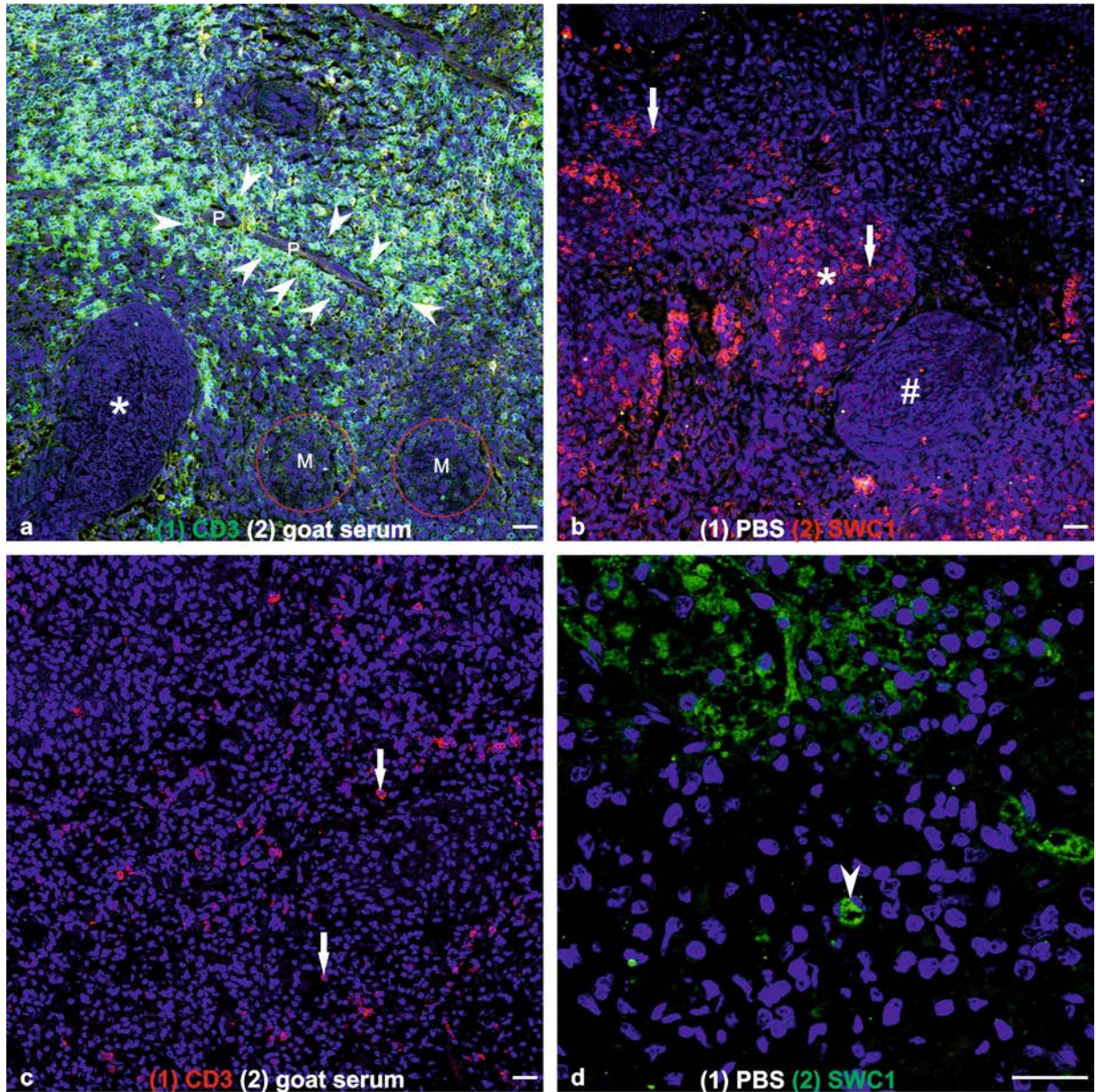


Fig. 12 Single stain controls of the modified double staining protocol.

During each single stain that was carried out one of the primary antibodies was omitted.

In (a) and (b), antigens CD3 and SWC1 were detected with Alexa 488 and Alexa 568 respectively.

In (c) and (d), antigens CD3 and SWC1 were detected with Alexa 568 and Alexa 488 respectively.

CD3 single stain: CD3 single positive cells either in green (a) or in red (c), SWC1 single stain: SWC1 single positive cells either in red (b) or in green (d).

For simplification, only the merged pictures, an overlay of the blue, green and red channel, are shown.

Arrow: red positive cells, arrowhead: green positive cells, white star: ellipsoid. Number sign: trabeculae, P: PALS artery (arrowheads around the PALS artery indicate the PALS, the periarterial lymphatic sheath), red circle & M: Malpighi bodies.

Scale bar: 20 μ m.

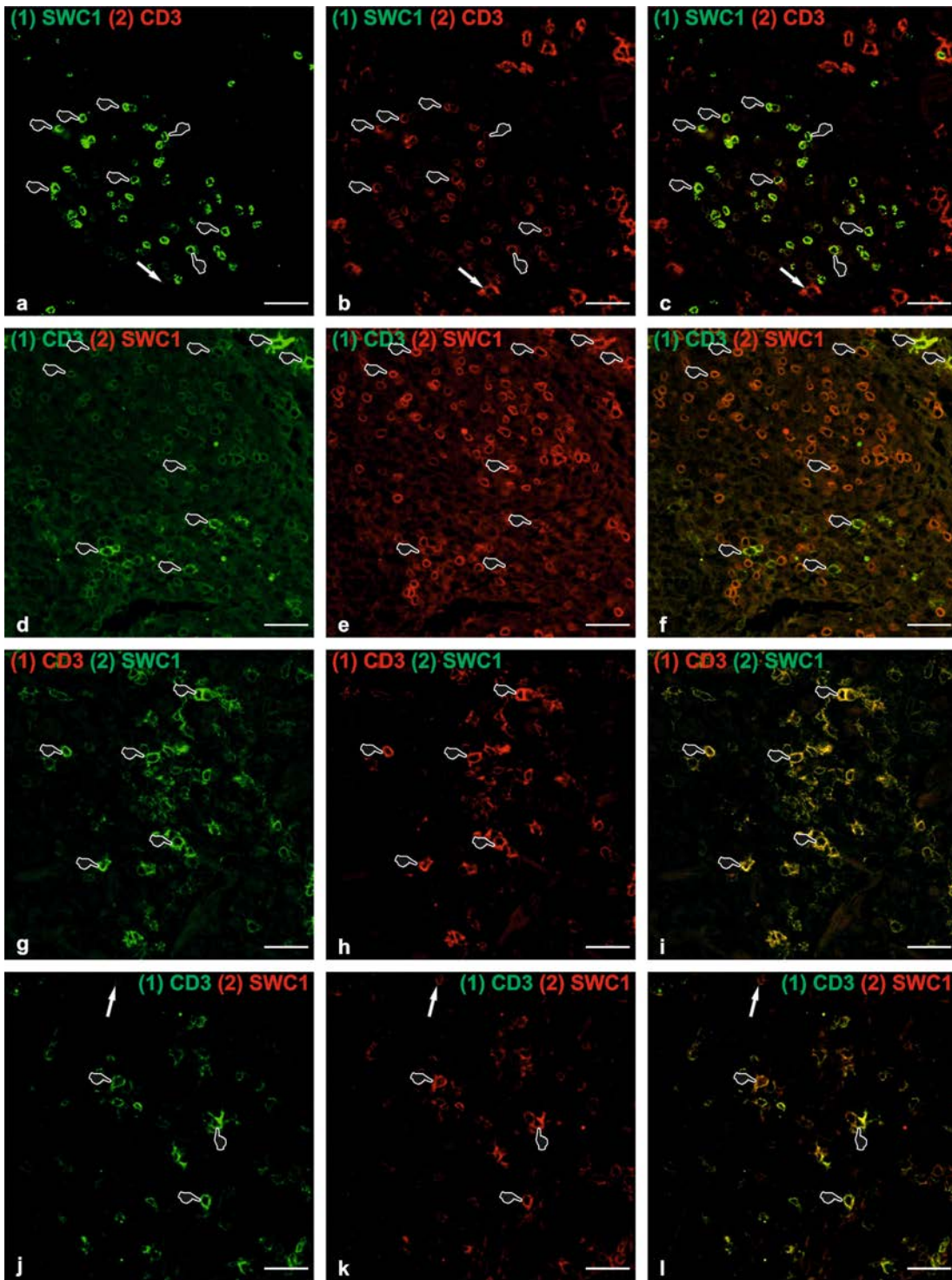


Fig. 13 CD3 and SWC1 immunostaining of porcine spleen using the modified double staining protocol.

Mouse anti-CD3 and mouse anti-SWC1 were used as primary antibodies.

Double positive cells are shown in yellow (for further explanation see text). a, d, g, j: green channel; b, e, h, k: red channel; c, f, i, l: overlay of green and red channel.

(a-c): SWC1/green as a first, CD3/red as a second immunostain; (d-f): CD3/green as a first, SWC1/red as a second immunostain;

(g-i): CD3/red as a first, SWC1/green as a second immunostain;

(j-l): CD3/green as a first, SWC1/red as a second immunostain. Arrow: red positive cells, black hand: double positive cells.

Scale bar: 20 μ m.

4 Discussion

4.1 Two species Immunofluorescence

The classical two species IF stains, “Two species IF I” and “Two species IF II” together with their single stain controls confirmed the presence of CD3 single positive cells, of SWC1 single positive cells and of cells double labelled for CD3 and SWC1 (SAALMÜLLER et al., 1994).

This staining pattern served as a reference for comparison with the staining pattern observed using the modified double staining protocol (in combination with the reagents of the M.O.M. kit) and its various adaptations (Table3).

4.2 Double Immunofluorescence using the modified double staining protocol

In this study it was shown that the modified double staining protocol in combination with the reagents of the M.O.M. kit cannot be applied for double IF using two primary antibodies raised in the mouse, as the staining results indicate.

4.2.1 Controls of the modified double staining protocol

In some cases, single stained controls, in which the first primary antibody was omitted and the second primary antibody applied (Single Stain 1, Table 3), showed no binding of the second secondary antibody. This may be a result of the incubation time for the primary antibodies. The first primary antibody was incubated for 15h overnight, this incubation time was perhaps too long.

In other cases, single stain controls, in which the first primary antibody was applied and the second primary antibody omitted (Single Stain 2, Table 3), gave fluorescence signals from both, first and second secondary antibody, indicating the occurrence of Type II interference as described by NEGOESCU et al. (1994).

Type II interference may also underlie the observation that only double positive cells were observed in those double stained sections that were stained in parallel to the controls described. In these control stainings, the second secondary antibody could have bound to the first primary antibody, yielding a false positive staining (Figure 6). This observation may be a hint that the M.O.M. blocking solution, F(ab) fragments respectively, did not block all epitopes of the first primary antibody.

4.2.2 Staining results of the modified double staining protocol

If a double stain with primary antibodies of the same host species is performed, both types of interferences, Type I and II, have to be blocked (NEGOESCU et al., 1994).

F(ab')₂ fragments and IgG molecules expose two antigen binding sites one of which still is capable of binding to the primary antibody (BORZACCHIELLO & ROPERTO, 2006). This free binding site will capture the second primary antibody and cause Type I interference. In contrast, Type II interference is caused by binding of the second secondary antibody to the Fc region of the first primary antibody (Figure 1, Figure 6).

The M.O.M. kit in its original form was designed for single indirect immunofluorescence staining using murine primary antibodies on mouse tissue. In this experimental setting, the blocking of Type I interference is not relevant.

In the employed modified double staining protocol, the blocking of Type I interference was also not considered, therefore, besides a Type II interference, it could be a possible explanation for the false double positive cells in the tissue sections.

Moreover, the fact that only very few single positive cells were detected in some double stained sections (CD3 single positive cells in Figure 13c, SWC1 single positive cells in Figure 13l) underlies the inability of the M.O.M. blocking solution to prevent false double positive double labelling when the modified double staining protocol is applied.

If the block had worked properly (within the modified double staining protocol) both, CD3 single positive cells and SWC1 single positive cells, besides double positive cells, would have been detected in each section, as cells exclusively expressing CD3 or SWC1 must be morphologically present in spleen tissue (SAALMÜLLER et al., 1994).

It was the idea of the experiments to modify an existing double staining protocol for primary antibodies of different host species. The modifications were based on the reagents of the

M.O.M. kit to develop a double staining protocol for primary antibodies of the same host species. One explanation for the inconsistent results obtained may be due to the fact that the reagents of the M.O.M. kit were not used as intended by the manufacturer.

Several differences between the manufacturer's protocol and our modified double staining protocol underlie the inconsistent staining results.

The modified double staining protocol differs from the manufacturer's protocol in the dilution media of the first primary and first secondary antibody, the detection system and the incubation times of the antibodies.

In the modified double staining protocol, the first primary and first secondary antibody were diluted in PBS instead of M.O.M. diluent, the recommendation of the manufacturer. The M.O.M. diluent could perhaps be involved in saturating free residual binding sites of the first secondary antibody, and thus prevent Type I interference, false double positive cells respectively. Due to the use of PBS as the dilution medium for the first primary and first secondary antibody, free binding sites of the first secondary antibody perhaps were not satisfactory blocked and therefore Type I interference as described in Figure 6 could have occurred.

In the modified double staining protocol, fluorochrome-conjugated secondary antibodies were used for antigen detection. In contrast, the manufacturer recommends biotinylated secondary antibodies in combination with fluorochrome-conjugated Avidin as a detection system. The complex between avidin and biotin formed during the staining could perhaps sterically cover the surface of the first secondary antibody and thus render Type I interference impossible. The fluorochrome-conjugated secondary antibodies were perhaps not able to sterically hinder unwanted interferences.

Therefore, the choice of PBS as the dilution medium and the choice of the detection system may be one of the reasons why exclusively double positive cells were detected in some cases using the modified double staining protocol.

Moreover the incubation times also differed among the two protocols. The manufacturer's protocol incubates both primary antibodies for 30 minutes and both biotinylated secondary antibodies for 10 minutes. However, the modified double staining protocol used much longer incubation times for the antibodies (see Material & Methods, section 2.2.1.1 "modified double staining protocol"). The inconsistent staining results may also be a consequence of these differences in incubation times.

4.2.3 Replacement of MOM diluent with goat serum

As the M.O.M. diluent is made up by horse serum, adaptations of the modified double staining protocol were carried out because the used secondary antibodies originated all in goat (see chapter 2.2.1).

Typically, e.g. in single IHC staining, non-specific binding activity is blocked with serum of the host species of the secondary antibody. Since in the experiments for this study goat anti-mouse secondary antibodies were used, the M.O.M. diluent (horse serum, M. Rauner, Szabo-Scandic, Vienna, Austria, personal communication, 2012) was replaced.

The fact that the staining results were similarly inconsistent using the different dilution media suggests that the M.O.M. diluent is replaceable by goat serum, but that the validity of the staining is not improved. Unsatisfactory staining results therefore do not seem to be caused by undesired binding of goat or horse immunoglobulins to tissue components or primary antibodies.

4.3 Other factors

It is known that fixation has a major impact on the staining results. The inconsistent staining results could be due to the fact that the antigenicity of the archival tissue material was not optimally preserved.

According to BRANDTZAEG (1998) frozen tissue sections are the general approach for the reliable detection of many cell surface markers and a variety of tissue antigens based on immunostaining with monoclonal antibodies. However, both mouse primary antibodies used in this study, PPT3 and SWC1, work well on paraffin sections (GAISWINKELR, 2010) and were successfully used in preliminary tests for this study (personal observation, data not shown). Moreover the necessity of cryosections precludes the use of archival tissue material. Double staining on frozen tissue sections instead of routinely fixed and paraffin embedded sections will perhaps yield stronger staining results or intensities.

4.4 Conclusion

The modified double staining protocol in combination with the reagents of the M.O.M. kit yielded inconsistent staining results. False double positive cells and very few single positive cells were detected, unwanted cross reactions could not be blocked. The validity of the staining results was not given. The hypothesis of this study must therefore be rejected.

Before new double labelling protocols for primary antibodies of the same host species are developed on basis of the M.O.M. kit, it will therefore be necessary to carry out a stain according to the manufacturer's protocol and apply the M.O.M. kit within the producer's double staining protocol first.

Another useful approach could be to block free binding sites of the first secondary antibody with non-binding mouse immunoglobulins (LEWIS CARL et al., 1993) or normal mouse serum before applying a blocking step that involves F(ab) fragments.

If a desired combination of primary antibodies is only available from a single host species, e.g. the mouse, alternative blocking methods such as the use of modified secondary antibodies (NEGOESCU et al., 1994), isotype differences between antibodies (BUCHWALOW et al., 2005; GERNER et al., 2008) or signal amplification (TERAMOTO et al., 1998) might be used.

5 Summaries

5.1 English Summary

Immunohistochemical double staining is a widely used approach for testing the co-localisation of two antigens in one tissue section. Besides the classical double labelling approach, using two primary antibodies raised in different host species, also double labelling with two primary antibodies of the same host species might be required when the desired combination of primary antibodies raised in different species is not available. An example are CD (cluster of differentiation) markers — surface antigens of leukocytes. Most anti-porcine CD antibodies that are commonly used for FACS analysis are produced in the mouse.

In order to provide a basis for the detection of porcine T-cell subpopulations by IF, the following hypothesis will be tested in this study:

With a modified double staining protocol in combination with the reagents of the M.O.M. kit (Vector Laboratories, Burlingame, CA, USA) it is possible to achieve distinct and correct immunohistochemical double staining using mouse anti-porcine CD3 and mouse anti-SWC1 antibodies.

IF double labelling was tested with the combination of primary antibodies anti-CD3 and anti-SWC1, both monoclonal, of murine origin and produced at the Institute of Immunology, University of Veterinary Medicine Vienna (Austria).

The antibodies were chosen for the following reasons:

(i) A rabbit anti-CD3 antibody working on porcine tissue is commercially available. Therefore, the staining results with two mouse primary antibodies can be controlled by a classical double IF.

(ii) CD3 and SWC1 (CD52) are markers of different cell populations, T cells and monocytes/granulocytes, respectively. However, SWC1 is also expressed on quiescent T cells. Therefore, in histological sections of porcine lymphatic tissue, CD3 and SWC1 single positive cells as well as double positive cells should be found.

(iii) All primary antibodies required for this study (rabbit anti-CD3, mouse anti-CD3, mouse anti-SWC1) have been proven to work on paraffin embedded tissues (after fixation with methacarn). Therefore, standard tissue handling for histology was possible.

(iv) The mouse anti-porcine CD3 and mouse anti-porcine SWC1 antibodies are of different isotypes. Therefore, in future studies the use of isotype-specific secondary antibodies can be tested for double IF.

Methacarn-fixed spleen samples from a slaughtered sow (8 months old) served as a specimen. As the two murine primary antibodies are applied to tissue and detected one after the other, the M.O.M. blocking reagent, applied during the modified double staining protocol, should protect and block the first immunofluorescent stain and prevent the appearance of false double positive cells. This was checked by the detection of three cell populations – the detection of single CD3 positive cells, of single SWC1 positive cells and of double positive cells in one section.

Double staining results achieved with the modified double staining protocol in combination with the reagents of the M.O.M. kit were inconsistent, false double positive cells and very few single positive cells were detected.

These findings suggest that the hypothesis must be rejected.

5.2 German Summary

Doppelmarkierungen zum Testen einer Antigenkolokalisation in einem histologischen Schnitt sind weit verbreitete immunhistochemische Färbemethoden. Neben dem klassischen Ansatz, der mit zwei Primärantikörpern aus verschiedenen Tierarten arbeitet, findet auch die Doppelmarkierung mit Primärantikörpern aus der gleichen Spezies Anwendung. Letztere ist erforderlich, wenn die gewünschte Primärantikörperkombination aus verschiedenen Spezies nicht verfügbar ist, wie dies zum Beispiel bei den CD (cluster of differentiation) Markern, den Oberflächenantigenen von Leukozyten, der Fall ist. Viele in der FACS Analyse verwendeten Antikörper gegen CD - Marker des Schweines werden in der Maus hergestellt.

Um sich mit dem Nachweis porciner T Zell Subpopulationen durch IHC näher auseinander zusetzen, sollte die folgende Hypothese im Rahmen der Studie untersucht werden:

Ein modifiziertes Doppelmarkierungsprotokoll in Kombination mit den Reagenzien des M.O.M. kit, ermöglicht korrekte Immunfluoreszenz Doppelfärbungen unter Verwendung eines Maus anti-Schwein CD3 Antikörpers sowie eines Maus anti-Schwein SWC1 Antikörpers.

Im Rahmen der Doppelmarkierung wurde die Primärantikörperkombination anti-CD3 und anti-SWC1, beide monoklonal, aus der Maus stammend und am Institut für Immunologie an der Veterinärmedizinischen Universität Wien hergestellt, angewendet.

Diese Antikörperkombination wurde aus folgenden Gründen ausgewählt:

(i) Ein Kaninchen anti-CD3 Antikörper, der auf Schweingewebe funktioniert, ist käuflich erhältlich. Die Färbeergebnisse mit zwei Primärantikörpern aus der Maus können also leicht mit der klassischen Doppel-Immunfluoreszenz überprüft werden.

(ii) CD3 und SWC1 (CD52) markieren unterschiedliche Zellpopulationen – T Zellen und Monozyten/Granulozyten. Da das SWC1 Antigen aber auch auf ruhenden T Zellen exprimiert wird, sollten in histologischen Schnitten von lymphatischem Schweinegewebe sowohl CD3 und SWC1 einfach positive Zellen, als auch doppelt positive Zellen gefunden werden.

(iii) Alle für diese Experimente notwendigen Primärantikörper (Kaninchen anti-CD3, Maus anti-CD3, Maus anti-SWC1) sind an Praffinschnitten anwendbar, wenn das Gewebe vorher mit Methacarn fixiert wurde. Eine standardisierte Handhabung der Gewebeprobe für die Histologie war somit möglich.

(iv) Da der Maus anti-Schwein CD3 und Maus anti-Schwein SWC1 Antikörper unterschiedliche Isotypen aufweisen, könnte in zukünftigen Experimenten die Verwendung isotypen-spezifischer Sekundärantikörper getestet werden.

Die Doppelfärbungen wurden an Methacarn-fixierten Milzproben einer acht Monate alten geschlachteten Sau getestet. Da Applikation und Nachweis der beiden Maus Primärantikörper nacheinander erfolgen, sollte die im Rahmen des modifizierten Doppelfärbeprotokolls verwendete M.O.M. blocking solution die Färbung des ersten Antigens schützen und abblocken sowie das Auftreten von falsch doppelt positiven Zellen verhindern. Dies wurde über den Nachweis von drei Zellpopulationen innerhalb eines Schnittes überprüft, den Nachweis von einzeln CD3 positiven Zellen, von einzeln SWC1 positiven Zellen und von doppelt positiven Zellen.

Die mit dem modifizierten Doppelfärbeprotokoll (in Kombination mit den Reagenzien des M.O.M. kit) erzielten Doppelfärbeergebnisse, waren inkonsistent, falsch doppelt positive und nur sehr vereinzelt einzeln positive Zellen wurden in den Schnitten nachgewiesen.

Die für diese Arbeit aufgestellte Hypothese muss also verworfen werden.

6 References

- BOENISCH, T. (2006): Färbemethoden-Detektionssysteme. In: BOENISCH, T. (Hrsg.): Immunchemische Färbemethoden. 3. ed., Dako, Carpinteria, CA, USA.
- BORZACCHIELLO, G., ROPERTO F. (2006): Letter to the Editor. *Vet Pathol* **43**, 83.
- BRANDTZAEG, P. (1998): The increasing power of immunohistochemistry and immunocytochemistry. *J Immunol Methods* **216**, 49-67.
- BROWN, J.K., PEMBERTON, A.D., WRIGHT, S.H., MILLER, H.R.P. (2004): Primary Antibody-Fab Fragment Complexes: A flexible alternative to traditional direct and indirect immunolabeling techniques. *J Histochem Cytochem* **52**, 1219-1230.
- BUCHWALOW, I.B., MININ, E.A., BOECKER, W. (2005): A multicolor fluorescence immunostaining technique for simultaneous antigen targeting. *Acta Histochem* **107**, 143-148.
- GABNER, S., TONAR, Z., TICHY, A., SAALMÜLLER, A., WORLICZEK, H.L., JOACHIM, A., WITTER, K. (2012): Immunohistochemical detection and quantification of T cells in the small intestine of *Isospora suis*-infected piglets – influence of fixation technique and intestinal segment. *Microsc Res Tech* **75**, 408-415.
- GAISWINKLER, C. (2010): Immunhistochemischer Nachweis von Leukozytenpopulationen in Geweben des Schweines. Thesis, University of Veterinary Medicine, Vienna.
- GERNER, W., KÄSER, T., PINTARIC, M., GROISS, S., SAALMÜLLER, A. (2008): Detection of intracellular antigens in porcine PBMC by flow cytometry: A comparison of fixation and permeabilisation reagents. *Vet Immunol Immunopathol* **121**, 251–259.
- HIERCK, B.P., IPEREN, L.V., GITTENBERGER-DE GROOT, A.C., POELMANN, R.E. (1994): Modified indirect immunodetection allows study of murine tissue with mouse monoclonal antibodies. *J Histochem Cytochem* **42**, 1499-1502.

- LEITNER, J., REUTNER, K., ESSLER, S.E., POPOWA, I., GERNER, W., STEINBERGER, P., SAALMÜLLER, A. (2012): Porcine SWC1 is CD52—Final determination by the use of a retroviral cDNA expression library. *Vet Immunol Immunopathol* **146**, 27-34.
- LEWIS CARL, S.A., GILLETE-FERGUSON, I., FERGUSON, D.G. (1993): An indirect immunofluorescence procedure for staining the same cryosection with two mouse monoclonal primary antibodies. *J Histochem Cytochem* **41**, 1273-1278.
- LU, Q. L., PARTRIDGE, T. A. (1998): A new blocking method for application of murine monoclonal antibody to mouse tissue sections. *J Histochem Cytochem* **46**, 977-983.
- MOLECULAR PROBES (2013): Principles of Tyramide Signal Amplification. <http://www.invitrogen.com/site/us/en/home/References/Molecular-Probes-The-Handbook/ ultrasensitive-detection-technology/Tyramide-Signal-Amplification-Technology.html#head1>
Accessed: 2013-05-14
- MURPHY, K., TRAVERS, P., WALPORT, M. (2009): *Janeway Immunologie*. p. 141-147; 442; 504-507)
- NEGOESCU, A., LABAT-MOLEUR, F., LORIMIER, P., LAMARCQ, L., GUILLERMET, C., CHAMBAZ, E., BRAMBILLA, E. (1994): F(ab) secondary antibodies : a general method for double immunolabeling with primary antisera from the same species. Efficiency control by chemiluminescence. *J Histochem Cytochem* **42**, 433-437.
- PRESS, C.McL., LANDSVERK, T. (2006): Immune System. In: EURELL, J.A., FRAPPIER, B.L. (Eds): *Dellmann's Textbook of Veterinary Histology*. 6. ed., Blackwell Publishing, Iowa, USA, p. 147-151.
- RAMOS VARA, J.A. (2005): Technical aspects of immunohistochemistry. *Vet. Pathol.* **42**, 405-426.
- SAALMÜLLER, A., BRYANT, J. (1994): Characteristics of porcine T lymphocytes and T-cell lines. *Vet Immunol Immunopathol* **43**, 45-52.

SAALMÜLLER, A., AASTED, B., CANALS, A., DOMINGUEZ, J., GOLDMAN T., LUNNEY, J.K., PAULY, T., PESCOVITZ, M.D., POSPISIL, R., SALMON, H., SINKORA, J., SUMMERFIELD, A., VALPOTIC, I., VIZCAINO, J.S., ZUCKERMANN, F. (1994): Analysis of mAb reactive with the porcine SWC1.

Vet Immunol Immunopathol **43**, 255-258.

TERAMOTO, N., SZEKELY, L., POKROVSKAJA, K., HU, L.F., YOSHINO, T., AKAGI, T., KLEIN, G. (1998): Simultaneous detection of two independent antigens by double staining with two mouse monoclonal antibodies.

J Virol Methods **73**, 89-97.