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Modulation of the T_H1/T_H2 differentiation by B cells, C5aR1 and CD154 in antigen dose dependent mouse models

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II List of abbreviations

AA	amino acids
AAM	alternatively activated macrophage
AF647	alexa fluorescent dye (maximum emission at 647 nm)
APC	antigen presenting cell
BCR	B cell receptor
BCZ	B cell zone
BDNF	Brain-Derived Neurotropic Factor (housekeeping gene)
bp	base pairs
° C	degree Celsius
C3, C5	complement factor 3 and 5
C3b	large subunit and fission product of C3
C5a	anaphylatoxin, fission product from C5
C5aR1	C5a receptor (CD88)
CAM	classically activated macrophage
CD	Cluster of Differentiation
cDNA	complementary DNA
CDR	complementary determining region
CFSE	5(6) Carboxylfluorescein diacetate N-succinimidyl ester
C region	constant region of the T cell receptor gene segment
ct	cycle of threshold
cTEC	cortical thymic epithelial cell
d	days
DC	dendritic cell
dLN	draining lymph node
DNA	deoxyribonucleic acid
DNase	deoxy ribonuclease
D region	diversity region of the T cell receptor gene segment
DTH	delayed type hypersensitivity
FACS	fluorescence activated cell sorting (Flow cytometry)
FCS	fetal calf serum
FDC	follicular dendritic cell
Fig.	figure
FITC	Fluoresceinisothiocyanat (maximum emission of 488 nm)
FSC	forward scatter
g	gravity
GC	germinal centre
GFP	green fluorescent protein
gp63	*Leishmania major* surface protease
h	hour
HD	high dose
HEC	highly-expanded clonotype
iC3b	inactive form of C3b
IFN	interferon
IgG	immunoglobulinin
IL	interleukin
i.v.	intravenous
J region	joining region of the T cell receptor gene segment
kDa	kilo-Dalton
Ki-67	Kiel-67 (proliferation marker)
LD	low dose
L. major	*Leishmania major*

LN	lymph node
MACS	magnetic adhesion cell sorting
MD	medium dose
mDC	myeloid dendritic cell
MFI	medium fluorescence intensity
MHC	major histocompatibility complex
min	minute
mg	milligram
ml	milliliter
µl	microliter
MLN	Metastatic Lymph Node Gene 51 (housekeeping gene)
mm	millimeter
µm	micrometer
mRNA	messenger RNA
ND	not detectable
ng	nanogram
nm	nanometer
OVA	Ovalbumin
PBS	Phosphate buffered saline
PCR	polymerase chain reaction
PE	Phycoeryrthrin (maximum emission of 575 nm)
pMHC	peptide loaded and surface expressed major histocompatibility complex
qRT-PCR	quantitative real-time reverse transcription PCR
RBC	red blood cells
RNA	ribonucleic acid
RNase	ribonuclease
RPMI	Roswell Park Memorial Institute
RT	room temperature
s	second
s.c.	subcutaneous
SDS	Sodium Dodecyl Sulfate
sec.	secondary
SEM	standard error of the mean
SRBC	sheep red blood cells
SSC	sideward scatter
Tab.	table
TCR	T cell receptor
TCRR	T cell receptor repertoire
TCZ	T cell zone
T_H cell	T helper cell ($CD4^+$ cell)
U	unit
V region	variable region of the T cell receptor gene segment
WT	wild type
$X^{-/-}$	knockout of gene X

1 Introduction

$CD4^+$ T helper cells (T_H cells) are an important cell type of the human immune system because they orchestrate an immune response by interacting and influencing many other immune cells. The broad spectrum of functions is achieved by the differentiation of T_H cells into one of several subsets. Therefore, differentiation into a certain T_H cell subset is of critical importance for the overall disease progression. This differentiation influences the success of vaccination and the progress of allergies and infections. The best investigated subsets are T_H1 and T_H2, which polarize the immune response into almost antagonistic directions. Although important factors influencing T_H cell differentiation into one or the other subset are described, differentiation *in vivo* is still not fully predictable, and requires further research. This thesis focusses on three mechanisms which force the differentiation into T_H1 and T_H2 cells *in vivo*.

1.1 T cells

Naïve T cells have a diameter of about 7.5 µm and fulfil a huge variety of roles within the immune response. They are defined by their specific receptors (T cell receptor (TCR)), of which each T cell expresses about 30.000 on their surface, with which they can recognize foreign peptides. On a single T cell, all TCRs are identical, consisting of the same amino-acid (AA) sequence. This T cell receptor AA sequence defines the individual T cell clonotype. One clonotype consists of all T cells with the same T cell receptor. A human body contains $\approx 10^{11}$ different T cells which represent 2.5×10^7 different clonotypes (Zarnitsyna et al. 2013). The advantage of having such a high diversity of cells is that they can recognize all kinds of different antigen. However, this leads to the disadvantage that only a minor portion of the T cells are able to react to a current pathogenic encounter. Therefore, the human body needs to increase the number of reacting T cells, leading to a delay of a few days in fighting the pathogen.
This diversity of T cells is generated early in T cell development, during the process of TCR assembly, by the recombination of different genomic regions.

1.1.1 T cell receptor development

T cells are part of the lymphoid lineage and develop in the bone marrow. Having no TCR yet, pre-T cells leave the bone marrow and migrate into the thymus, where they start to develop a TCR. It consists of two chains (α and β), with the β chain being assembled first. These chains are assembled out of different genomic regions, which are called variable (V), diversity (D),

joining (J) and constant (C) region. The recombination of the human TCRβ chain is started with the recombination of one Vβ (of 52) and one Dβ (of 2) gene. During this process random nucleotides (1-9) are inserted in between these gene segments, extending the maximal achievable diversity. After that process, binding of one Jβ (of 13) region to the V/D segment is catalysed, again inserting random nucleotides in between these regions in the same fashion. Finally one Cβ (of 2) region is added to complete the β chain. After the assembly of the TCRβ chain, the TCRα chain is assembled in similar fashion, though missing the D region. Therefore, one Vα (of 70) and one of Jα (of 61) region are recombined to the Cα region in order to complete the TCR.

The TCR contains six specific regions, which are called Complementary Determining Regions (CDR). The CDR1β, 1α, 2β and 2α are genomically encoded on the Vβ and the Vα chain, respectively. These CDRs are predominantly used to bind to the Major Histocompatibility Complex (MHC) of antigen presenting cells (APCs) and bring the CDR3 in close proximity to the presented peptide. The CDR3 region has the highest diversity of all TCR parts, since it consists at least in some part of the V, J and D region of the respective chain, as well as the randomly inserted nucleotides. The tertiary structure of the CDR3β and the CDR3α form a loop which can bind the peptide, making it to the signature region of a TCR clonotype.

The difference between theoretically 10^{20} possible T cell clonotypes and $2.5x10^{7}$ actual clonotypes in a human organism (Zarnitsyna et al. 2013) is the result of the positive and negative selection of T cells in the thymus (Klein et al. 2014; Starr et al. 2003). Since T cells should not recognize parts of the own organism (self-antigens), a tightly regulated mechanism has to select T cells for a T cell receptor repertoire (TCRR), which recognizes as much foreign antigens as possible while still maintaining tolerance to self-antigens. After the selection process only T cells with TCRs, which maintain the tolerance to self-antigens are able to mature and leave the thymus through the blood, whereas the other T cells die by apoptosis.

After leaving the thymus, the naïve T cells become part of the adaptive immune system. In this function T cells migrate towards secondary lymphatic organs, ready to encounter antigen which is presented on MHCs by APCs.

1.1.3 T cells interact with MHCs on the surface of APCs

The MHC gene loci are the most polymorphic genes known in the human population. For some of the MHCs more than 500 alternative MHC alleles are distributed in the human population (Garrigan and Hedrick 2003; Mungall et al. 2003). MHCs are polygenic, meaning that each human being expresses multiple different MHC alleles, defining an individual MHC haplotype. Since the MHC-molecules present antigen to T cells, the MHC haplotypes strongly influence the T cells which are selected in the thymus, initiating a unique TCRR for each individual organism.

There are two classes of MHCs (I and II), each of them reacting with one of the two T cell types, namely cytotoxic T cells and T helper cells. These types are defined by the expression of the common co-receptors CD8 and CD4, respectively. Whereas pre-T cells have none of them (double negative stage), T cells start to express both of them (double positive stage) during the development within the thymus. Later the affinity of the TCR to the type of MHC defines which co-receptor stays on a certain T cell, and which one is suppressed.

Cytotoxic T cells express the co-receptor CD8 on their surface ("$CD8^+$ T cells"), which allows them to bind MHC-I on the surface of other cells. MHC-I is expressed on almost all cells and presents parts of the cells interior. If a $CD8^+$ T cell recognizes a foreign antigen on MHC-I, or a cell without MHC-I, it will kill these cells. T helper cells carry the co-receptor CD4 on their surface, determining the naming of these cells as "T_H cells" or "$CD4^+$ T cells". The CD4 co-receptor allows the T_H cells to bind MHC-II, which presents short peptides (12-22 AA long) and is expressed only by professional APCs such as dendritic cells (DCs), macrophages, B cells and to a lesser extend neutrophils. Since this thesis focusses on T_H cells, the antigen encounter will be described in regard to the TCR-MHC-II interactions.

In the periphery and in the secondary lymphoid organs it is important that MHC-II expression is tightly regulated because it impacts the maintenance of an immune response (Otten et al. 2003) and especially the T_H cell activation (Janeway et al. 1984).

APCs take up antigens by different ways, including endocytosis, phagocytosis and autophagy (Kamphorst et al. 2010; Lanzavecchia 1996). The antigen is then dissected within a vesicle, which merges with MHC-II carrying vesicles. MHC-II is loaded in the newly formed vesicle with a peptide. This peptide is folded into the binding grove of the MHC-II (pMHC-II), exposing some parts for recognition by the TCR. pMHC-II is then transported to the cell surface for antigen presentation. This process increases the pMHC-II density on the surface of the APC. The more

pMHC-II is available for TCR recognition the more possible T_H cell interactions with pMHC-II can occur (Huang et al. 2010), influencing the activation status of the T_H cells (Bajenoff et al. 2002).

1.2 Two antigen encounters are necessary to induce a cytokine secreting effector T_H cell

T_H cells migrate through the periphery and into secondary lymphatic organs (Mandl et al. 2012) constantly interacting with pMHC-II on APCs. Most of these interactions are of minor intensity, causing no reaction of T_H cells. However, when they interact with a foreign peptide on MHC-II which their TCR can recognize, called relevant peptide, contact is intensified (Huang et al. 2010). Multiple TCR-pMHC-II interactions between the T cell and the APC presenting the relevant peptide occur. During this process the TCRs on the surface of the T cell transmit signals intracellularly, increasing the TCR dependent signalling to the T cell. These TCR signals are called Signal 1.

Importantly, Signal 1 alone would induce a functional inactivity of the T cell, called anergy, and no activation (Schwartz 1990; Gimmi et al. 1993). Therefore, the T cell activation requires a second signal, called Signal 2 (Bretscher 1992). Signal 2 is mediated by the interaction of other T cell surface receptors with their ligands on APCs. These interactions occur between surface molecules such as CD28-CD80/CD86 (Harris and Ronchese 1999) or CD134/CD252 (Gramaglia et al. 1998). Signal 1 and 2 account for the first encounter a T cell receives, and activate the T cell. As a consequence, T cell growth factor Interleukin-2 (IL-2) is secreted to induce T cell proliferation (Fig. 1) (Schorle et al. 1991). The proliferation leads to the expansion of activated T cell clonotypes, which is called clonal expansion. The strength of the proliferation depends on the overall signalling strength a T cell receives from the accumulation of all Signals (1 and 2) during the first encounter (Jenkins et al. 1991). High signalling strength can induce highly expanded clonotypes (HECs) by inducing many circles of proliferation for some T cell clonotypes. These HECs emerge within an ongoing immune response and are considered to be a fundamental part of it. Therefore the appearance of HECs is of importance to assess the immune response.

Additionally to the proliferation, T cells start to express surface receptors like CD154, which are required for further T cell activation. In order to develop from a naïve T_H cell into a cytokine secreting effector T_H cell a second encounter from the TCR with a relevant pMHC-II is

necessary (Bretscher 1992). T cells become eligible to receive a second TCR-pMHC-II encounter after they received the first encounter, proliferated (Bajenoff et al. 2002) and increased the expression of the surface receptor CD154 (Wu et al. 1995). The second encounter induces the effector cytokine production of e.g. Interferon-γ (IFN-γ) or IL-4. Furthermore, it is not known whether the second encounter contributes to the clonal expansion by providing TCR interactions only to some T cells. Otherwise the clonal expansion would depend solely on the signalling strength during the first encounter.

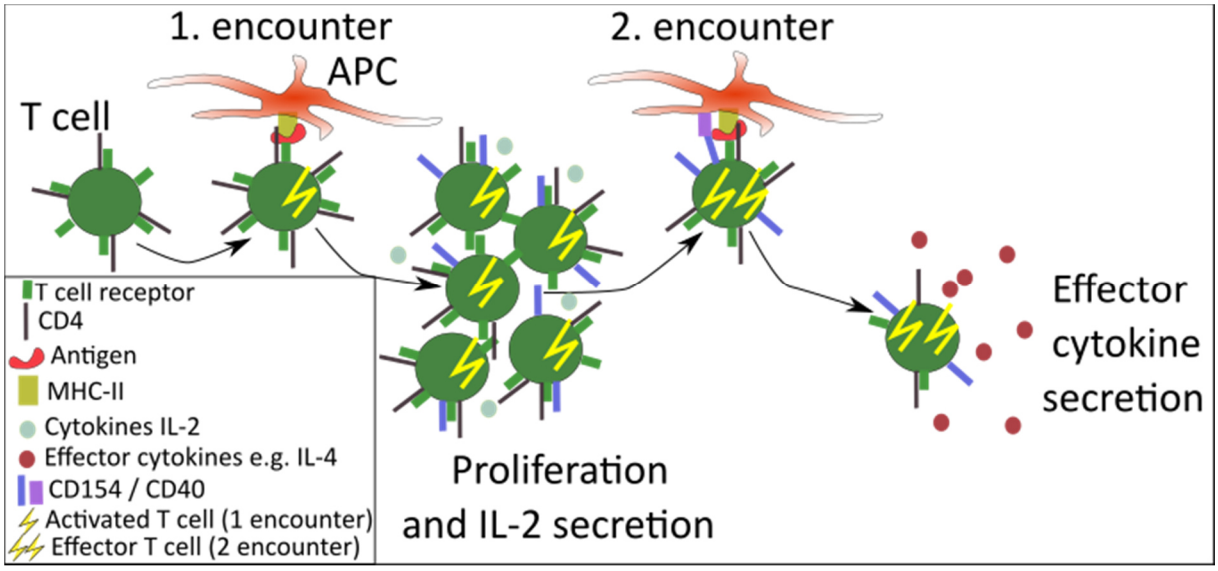

Fig. 1: T_H cell differentiation requires two TCR-pMHC-II encounters with a relevant peptide carrying APC.
$CD4^+$ T cells interact with APCs (e.g. DCs), which carry a relevant peptide (antigen) on their surface. This induces IL-2 secretion, T cell proliferation and CD154 upregulation. With the appearance of a second TCR-pMHC-II encounter, the T cells become cytokine producing effector T cells.

1.3 T_H cells differentiate into one of several T_H subsets during the activation process

During the two encounters for T_H cell activation, the T_H cell differentiates into a T_H cell subset. There are seven $CD4^+$ T_H cell subsets known, which are T_H1, T_H2, T_H9, T_H17, T_H21, T_{reg} and T_{FH} cells (Fig. 2). The induction of a specific subset is of high importance for the development of the ongoing immune response.

The best investigated factor modulating the appearing T_H cell subset is the cytokine milieu that is present at the time of T_H cell activation (O'Garra and Murphy 1996). Early in research, its investigation lead to the knowledge which cytokine induces which T_H cell subset (Fig. 2). Furthermore it is known that the cytokines have to be present to maintain the T_H cell subset during the immune response.

These T_H subsets are very heterogeneous and account for different roles within the immune system. They recruit and activate other immune cells orchestrating the immune response by cytokine secretion and cell-to-cell interactions (Zhu and Paul 2010). This makes the appearing T_H subset crucial for the development of an adaptive immune response.

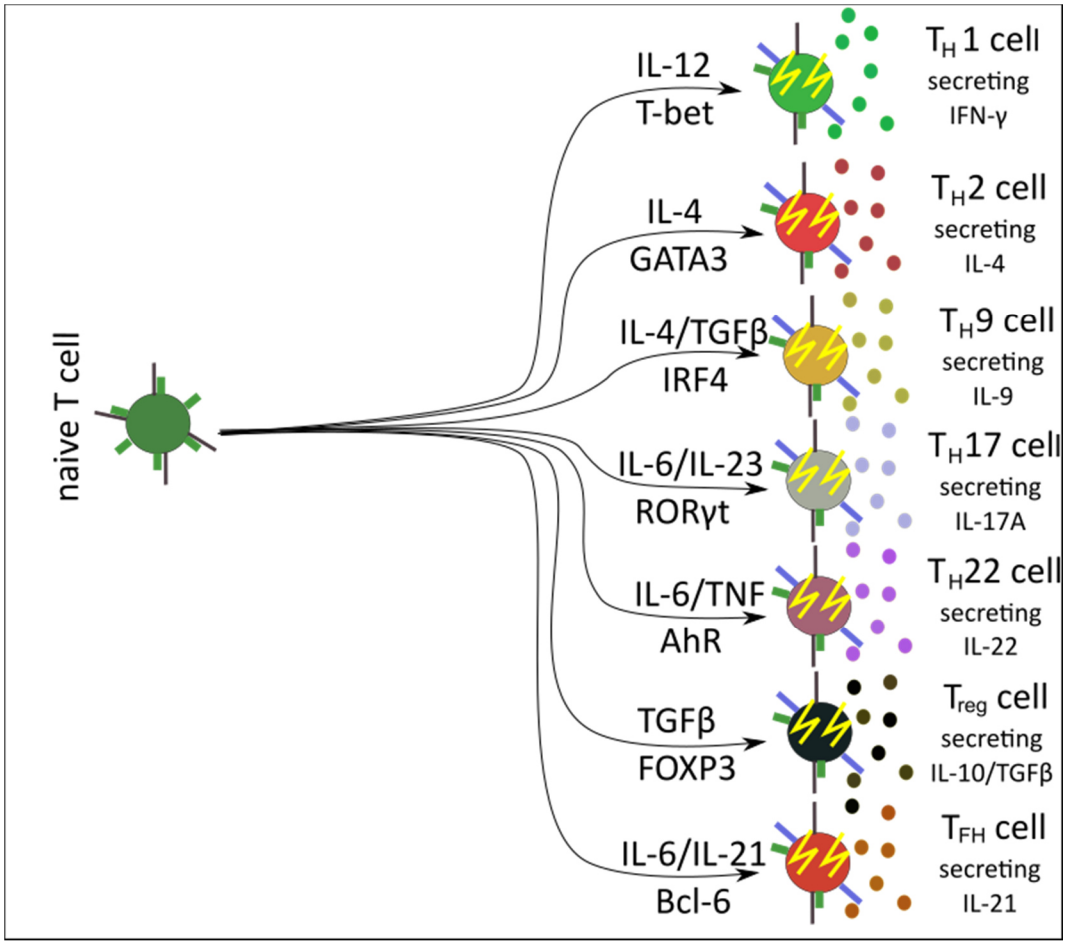

Fig. 2: T_H cell differentiation into one of serval T_H cell subsets.
Differentiation of naïve T_H cells into T_H cell subsets. Signature cytokines to induce the subset (above black arrow) and signature transcription factor (below black arrow) are marked. Modified after Tripathi and Lahesmaa (Tripathi and Lahesmaa 2014).

1.3.1 The T_H cell subsets T_H1 and T_H2

The focus of this study lays on the subsets T_H1 and T_H2, which are the main T_H cell subsets, being investigated in depth first and having clearly distinct functions that mutually exclude each other (Mosmann et al. 1986).

T_H1 cells secrete the pro-inflammatory cytokine IFN-γ and develop in an IL-12 dominated milieu. The effector function of these cells is predominantly the activation of macrophages, guiding them to active digestion of intracellular pathogens, which reside in their vesicles. In summary, T_H1 cells are required for an effective immune response against infections and intracellular pathogens. T_H2 cells on the other hand secrete the anti-inflammatory cytokines

IL-4 and IL-10. These cells provide cognate help to B cells, activating them, initiating the production of antibodies in the germinal centre reaction and leading to a humoral immune response. Both subsets fulfil an antagonistic role, blocking the development of each other with their appearance (Ohmori and Hamilton 1997; Parronchi et al. 1992).

Since the T_H cell subsets fulfil so different roles, it is of high importance to induce the most effective one, when the immune system is challenged by a pathogen. On the one hand, T_H1 development is required to control intracellular infections from protozoa like *Leishmania major* (Heinzel et al. 1993; Sacks and Noben-Trauth 2002), bacteria like *Mycobacterium leprae (Sasaki et al. 2001)* and virus like HIV (Maggi et al. 1994). On the other hand, T_H2 cells are necessary to control infection by helminths (Chen et al. 2012). Therefore, ample work has been executed to identify and analyse factors influencing the decision, whether T_H1 or T_H2 cells are generated (Rogers and Croft 1999).

During the process of T_H cell activation multiple factors influence the T_H cell to differentiate into T_H1 or T_H2 (Fig. 3). There are three major factors namely the cytokine milieu, the TCR avidity to the pMHC-II and the antigen dose (Rogers and Croft 1999), which will be presented individually.

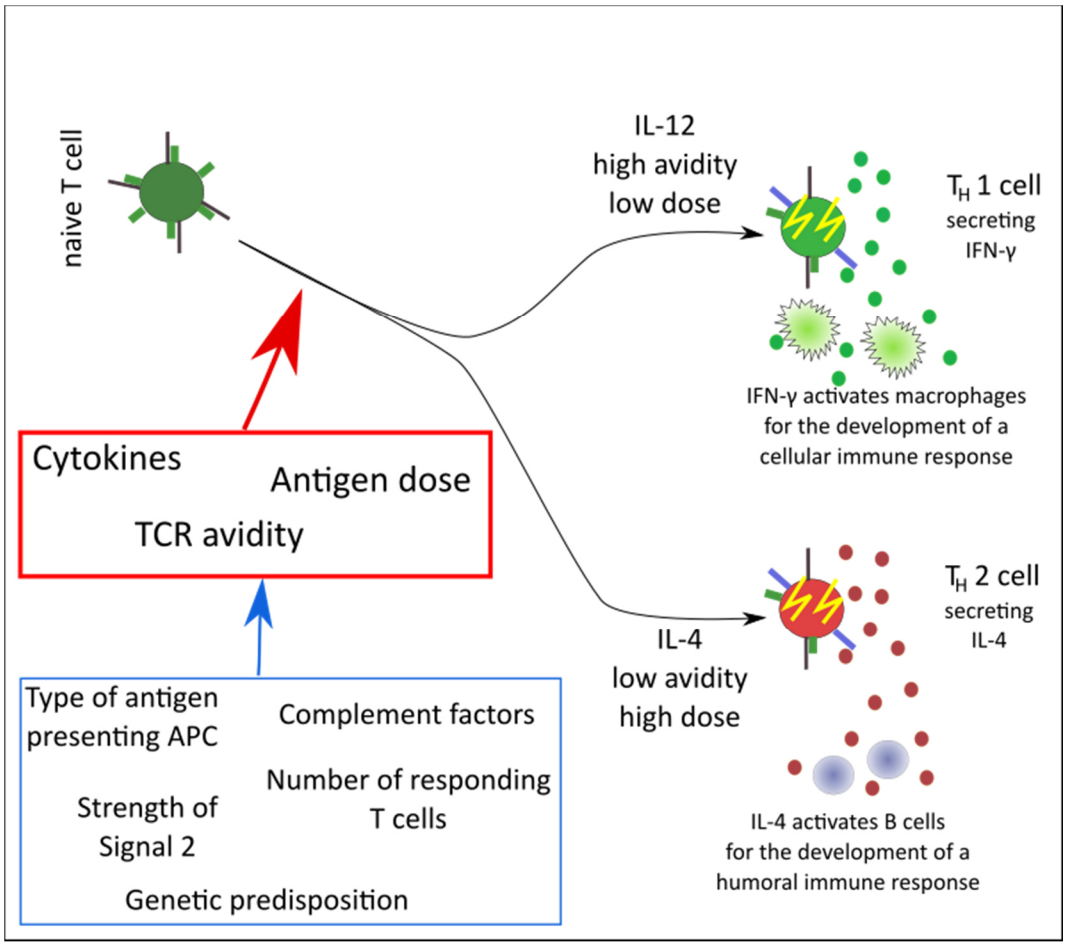

Fig. 3: : T_H cell differentiation into T_H1 and T_H2 cells is dependent on the 3 major factors "cytokines", "TCR avidity" and "antigen dose".
Those three factors are influenced by other modulating elements (blue box). Signature cytokines to induce the subset, relative TCR avidity and antigen dose are marked alongside the arrows.

1.4 The major factors that determine the T_H1/T_H2 differentiation

1.4.1 The influence of the cytokine milieu on the T_H1/T_H2 differentiation

The first studies provided evidence that T_H cell differentiation is solely impacted by the cytokine milieu, present at the time of T cell activation (Fiorentino et al. 1989; O'Garra and Murphy 1996). Consequently, it has been proven that the appearance of IL-12 tends to polarize the T cell response towards T_H1 (Hsieh et al. 1993), whereas IL-4 induces T_H2 cells (Chatelain et al. 1992). Generally, the cytokine milieu depends on the activated APCs, which secrete cytokines in response to the antigen uptake. Therefore, the APC dependent cytokine secretion is closely correlated with the nature of the antigen, since the initial route of antigen uptake drives APCs to a certain cytokine secretion pattern (Reis e Sousa et al. 2001). However, following strictly this theory, all T_H cells would develop in the same direction, since all experience the same cytokine milieu in a given environment at a given time. In consequence

the TCR signal would only decide if the T cell activates or not. Although it is widely accepted that the cytokine milieu establishes a certain T_H cell response (Fig. 2), it is often overlooked, that the initial T_H cell polarization has to be cytokine independent (Tubo et al. 2013), since some of the necessary cytokine receptors are not expressed on resting $CD4^+$ T cells (Desai et al. 1992).

1.4.2 The influence of the TCR avidity on the T_H1/T_H2 differentiation

The second major factor influencing T_H1/T_H2 development is the avidity of the TCR to the pMHC-molecule on the APC. The avidity depends on the signal quality (affinity) (Jenkins et al. 2009), the quantity (duration of interaction) (Tubo et al. 2013; Kalergis et al. 2001) and also the number of recognizing TCR molecules and co-receptor binding (Stone et al. 2009).

In general, a high avidity, generated from a high affinity and longer dwell times of interaction, results in an increased TCR signalling strength (Tubo et al. 2013). Compared to T_H2 cells, T_H1 cells develop from T cells bearing a TCR with a higher avidity (Tao et al. 1997; Keck et al. 2014) (Fig. 4). This has been proven experimentally, showing that the reduction of the TCR avidity induces the shift from the T_H1 response towards a T_H2 response (Blander et al. 2000).

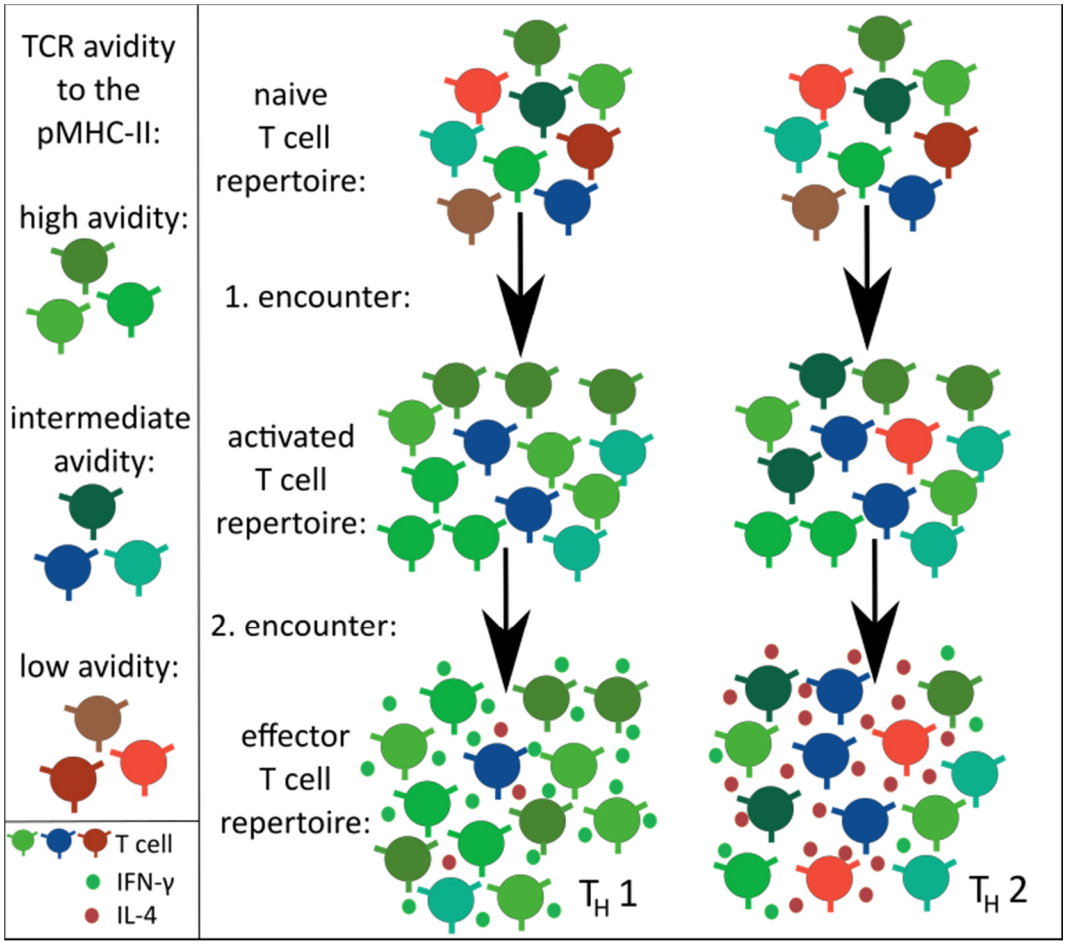

Figure 4: Influence of the TCR avidity on the development of T_H1 and T_H2 cells.
The selection of T_H cells into an immune response depends on the TCR avidity to the pMHC-II. Whereas high avidity clonotypes (green) develop predominantly into T_H1 cells, intermediate (blue) and low (red) avidity clonotypes develop into T_H2 cells.

The recruitment of T_H cells into an immune response leads to their proliferation and increases their abundance due to clonal expansion. The clonal expansion of T_H cells induces thereby a shift in the composition of all appearing clonotypes. This composition is called clonality.
This recruitment of T cell clonotypes bearing a relevant TCR is mainly dependent on the TCR avidity to the pMHC-II (Knowlden and Sant 2016). In regard to a given antigen, it is suggested that each clonotype may be predominantly conducive for a specific T_H cell fate (Corse et al. 2011). Nevertheless, the cytokine milieu necessary to sustain a specific T_H1/T_H2 response is defined by the entity of differentiations from all recruited clonotypes (Fig. 4). Therefore, the recruitment of T_H cell clonotypes into an immune response is an important factor for T_H1/T_H2 differentiation, closely related to the factor TCR-pMHC-II avidity.

1.4.3 The influence of the antigen dose on the T_H1/T_H2 differentiation

Interestingly, both of these factors (cytokine milieu and TCR avidity) depend on the nature of the antigen itself. Specific antigens are ingested on defined routes by specific APCs (e.g. via different Pattern Recognition Receptors). These routes normally predetermine the induced T_H cell differentiation by intracellular mechanisms in the APC. This leads to a specific cytokine secretion pattern and/or specific processed antigen epitopes that decide the T cell recruitment (TCR avidity).
Nevertheless, it is often overlooked that a T_H cell response can be modulated, solely by varying the amount of antigen. Therefore the antigen dose (Hosken et al. 1995) is the third major factor to influence the T_H cell differentiation. It describes an effect where a development of T_H2 cells after high antigen dose administration and T_H1 cells after low antigen dose administration can be observed (Cho et al. 2000; Keck et al. 2014). The impact of the antigen dose on the T_H1/T_H2 cell fate can even outcompete the effect of other factors like cytokines. This was shown experimentally by inducing antigen dose dependently T_H1 and T_H2 cells in a similar cytokine milieu, outcompeting the effect of T_H1 and T_H2 polarizing adjuvants (van Panhuys et al. 2014). This implies that the antigen dose is one important factor for T_H1/T_H2 differentiation. But to date, it is investigated poorly.

To elaborate the effect of the antigen dose on the T_H1/T_H2 development, multiple mouse models were established. Two of the best characterized are the Sheep red blood cell (SRBC) mouse model in C57Bl/6 mice and the *Leishmania major* infection model in Balb/c mice.

1.4.3.1 The antigen dose dependent SRBC model

Heterologous erythrocytes can induce T_H1 and T_H2 cells dependent on the injected antigen dose. This is best elaborated in the SRBC mouse model (Lagrange et al. 1974a; Kaufmann and Hahn 1979; Hurtrel et al. 1992). In this model, SRBC reflects a blood borne antigen, which does not reproduce and is degraded after a few hours (Stamm et al. 2013). Therefore, the antigen dose can be defined precisely due to the non-replicative nature of SRBCs. In contrast to many other models, this model does not require adjuvants to develop T_H1 or T_H2 cells, allowing the exclusion of the cytokine influence during early T cell activation. The peak of the splenic immune response is reached on day three, allowing the relatively fast analysis of mechanistic links between antigen dose and T_H1/T_H2 differentiation.

In this model, SRBCs are injected into the tail vein of mice. By injecting a high dose (HD) of SRBCs, splenic T cells are activated, proliferate and become T_H2 cells. This is detectable due to a high increase of IL-4 at the third day post immunization (Stamm et al. 2013). Therefore it is assumed that both TCR-pMHC-II encounters occur in the spleen, producing effector T_H cells (Ismail and Bretscher 2001) (Fig. 1).

After low dose (LD) immunization T_H cells are neither secreting T_H2 nor T_H1 cytokines in the spleen, although the immunization induces T cell proliferation. Therefore it is assumed that LD immunization provides only the first TCR-pMHC-II encounter. However, after subcutaneous challenge, LD immunized mice develop a T_H1 dependent delayed type hypersensitivity reaction (DTH) type IV (Cher and Mosmann 1987). Therefore the current understanding is that the challenge with SRBCs in the footpad provides the second encounter, inducing T_H1 effector cells (Ismail and Bretscher 2001).

1.4.3.2 The antigen dose dependent *Leishmania major* infection model

The *Leishmania major* (*L. major*) mouse model is the second antigen dose dependent T_H1/T_H2 model (Bretscher et al. 1992). *Leishmania* is a single cell parasite, which is distributed by the sand fly *phlebotominae spp.* (Ribeiro-de-Jesus et al. 1998; Lainson and Shaw 1978). One of the most common species is *Leishmania major* which induces cutaneous *leishmaniasis*. The infection severity of *leishmaniasis* ranges from local self-healing skin lesions (cutaneous *leishmaniasis*) to diffuse lesions (diffuse cutaneous *leishmaniasis*). While the self-healing infection progression requires a T_H1 response and IFN-γ secretion, a T_H2 response leads to diffuse cutaneous *leishmaniasis* (Ribeiro-de-Jesus et al. 1998).

The importance of the T_H1/T_H2 differentiation can be studied in the *leishmaniasis* progression of C57Bl/6 mice, which develop a T_H1 response and are resistant to a *L. major* infection, and Balb/c mice, which develop a T_H2 response and are susceptible to a *L. major* infection (Sacks and Noben-Trauth 2002).

When anti-IFN-γ-antibodies are administered to a resistant mouse strain to suppress T_H1 cells, the strain cannot control the infection thereafter (Belosevic et al. 1989). Balb/c mice meanwhile become more resistant to *L. major* infection after applying anti-IL-4-antibodies (Sadick et al. 1991; Chatelain et al. 1992).

Interestingly, Balb/c mice are not generally prone to severe *L. major* infection. It has been shown that the antigen dose can overcome this fate: Whereas high amounts of parasites (>100.000 parasites) induce a severe infection and lead to death of the animals within 13-15 weeks, low amounts of parasites (<10.000 parasites) induce only minor symptoms and the mice are protected from severe infection (Bretscher et al. 1992). These different infection progressions correlated with a shift from predominantly *L. major* specific IgG1 antibodies (after HD infection) to mainly IgG2a antibodies (after LD infection) (Bretscher et al. 1992). The development of these antibodies correlates closely with the appearance of T_H2 and T_H1 cells, respectively (Mountford et al. 1994).

1.5 Modulation or blockage of T_H1/T_H2 differentiation by B cells, C5aR1 or CD154 deficiency

The cytokines, the TCR avidity to the pMHC-II and the antigen dose are the three major factors that influence T_H1/T_H2 differentiation. Nevertheless, in addition other modulators are known, which influence these factors in their effect on T_H1/T_H2 differentiation (Fig. 3 (blue box)). Three of these factors are analysed in this study in the context of an antigen dose dependent environment.

1.5.1 B cells as potential modulator of the T_H1/T_H2 differentiation

One modulator of the T_H1/T_H2 differentiation is the type of APC that presents the antigen to the T_H cell. It has been suggested that T_H2 development requires the antigen presentation by B cells. The importance of B cells for the T_H2 response was reported in different models (Drake et al. 2015; Linton et al. 2003).

In the before mentioned SRBC model early cytokine secretion in the splenic B cell zone (BCZ) was detected after the T_H2 inducing HD immunization, but not after LD immunization (Stamm et al. 2013). This indicated the activation of B cells for the T_H2 response in this model and is a hint that the B cells are important for the T_H2 response.

In the *Leishmania* mouse model a T_H2 response is detrimental. Consequently, B cell deficient mice were shown to be resistant against the infection (Smelt et al. 2000).

1.5.2 C5aR1 can modulate the T_H1/T_H2 differentiation

Another modulator of the T_H1/T_H2 differentiation is the Complement System. Its impact on the adaptive immune system is appreciated more and more in recent years. Especially the T_H cell differentiation is in the focus of these studies. However, the molecular basis is not understood, yet.

The Complement System is the first line of defence against invading pathogens, consisting of more than 30 serum proteins and cell surface receptors. Its predominant task is the lysis of pathogens, although more and more studies have shown its impact on the adaptive immune system (Carroll 2004; Heeger and Kemper 2012; Kolev et al. 2013). Most of these studies focus on the complement anaphylatoxins C3a and C5a, as well as their respective receptors (Hawlisch et al. 2004).

C5a, the fragment of the complement component C5, is a strong chemoattractant and a major factor for the recruitment of inflammatory cells and APCs to tissue where currently the complement system is ongoing (Price et al. 2015; Guo and Ward 2005). The best investigated receptor for C5a is C5aR1, which is expressed on neutrophils, DCs, macrophages and B cells (Karsten et al. 2015).

The influence of C5aR1 on the T_H cell differentiation is described in many reports (Johswich et al. 2009; Sabio et al. 2017; Wenderfer et al. 2005). Unfortunately, these reports present conflicting data, making it impossible to identify the mechanism behind this effect. These conflicting results can be categorized in three groups:

[1] In the *L. major* and the *M. tuberculosis* infection model, C5aR1$^{-/-}$ mice showed an increased T_H1 response, which supported the parasite control (Hawlisch et al. 2005; Sabio et al. 2017). The ensuing investigations in *in vitro* cultures identified increased IL-12 in C5aR1$^{-/-}$ macrophages as the functional basis of this result. These data were supported by investigations in the asthma model: This model showed less susceptibility to T_H2-dependent asthma in C5aR1$^{-/-}$ mice (Engelke et al. 2014), due to lower numbers of T_H2 effector cells in the lungs.

[2] In contrast to these models, sepsis and systemic *lupus erythematosus* models showed increased T_H2 responses in C5aR1$^{-/-}$ mice (Wenderfer et al. 2005; Ma et al. 2013). Both reports showed suppressed IL-12 secretion, correlating with a reduced DC migration (Ma et al. 2013).

[3] Furthermore in a model of inflammatory bowel disease both T_H cell subsets required C5aR1 signalling for full effector functions (Johswich et al. 2009). Therefore the C5aR1$^{-/-}$ mice showed impaired T_H1 and T_H2 responses.

Although these publications demonstrate opposing views, the recurring theme is the impaired development of at least one T_H cell subset. In most of the before mentioned publications the switch from one T_H cell subset to the other occurs due to the impairment of one of them. In this context, multiple papers showed heavily impaired effector T_H cell responses due to C5aR1$^{-/-}$ (Weaver et al. 2010; Johswich et al. 2009). One possible explanation for these discrepancies is that C5aR1 regulates the availability of antigen in the secondary lymphoid organs by either APC recruitment or disturbed expression of MHC-II. As a result, impaired antigen presentation might result in reduced TCR-pMHC-II interactions and suppressed T_H cell responses.

1.5.3 CD154$^{-/-}$ blocks the T_H1/T_H2 differentiation

The recruitment of T_H cells into an immune response defines the clonality within an immune response. In 1.4.2 it was introduced that the entity of all TCR-pMHC-II avidities from recruited T cells within an immune response is a major factor for T_H cell differentiation. Therefore, the recruitment of T_H cells and the resulting clonality is a modulator for the developing T_H cell subset (Fig. 4) (Corse et al. 2011; Rogers and Croft 1999). Whereas the recruitment of T_H cells is decided by the first encounter a T_H cell receives, the clonality is defined by the overall clonal expansion of all recruited T_H cells. However, so far it is not known if the clonal expansion is determined solely by the first encounter, or if the second encounter also modulates the clonality.

One possibility to analyse the effect of the second encounter, is the blockage of this encounter, which can be done by CD154 deficiency. CD154 is expressed on activated T cells after the first encounter. Its expression is required for the T_H cell to become eligible to receive a second encounter and become a cytokine secreting effector T_H cell (Al-Ojali et al. 2012; Blair et al. 2000; van Essen et al. 1995).

CD154 binds to CD40 on the surface of other cells, such as APCs (Alderson et al. 1993; Armitage et al. 1992) or endothelial cells (Hollenbaugh et al. 1995). CD154/CD40 interaction is best known for T and B cell interactions because it is a required signal to allow B cells to present antigen to T cells, to start the germinal centre reaction and to induce the antibody class switch (Grewal et al. 1995; Kawabe et al. 1994). Furthermore, this signal is also required for the T cell activation mediated by DCs and macrophages (Cella et al. 1996; Kennedy et al. 1996) or interactions with $CD8^+$ T cells (Bourgeois et al. 2002).

In a CD154$^{-/-}$ immune response, it is not possible to study T_H1 and T_H2 differentiation directly, since none of the T_H cells release effector cytokines. However it is possible to compare an immune response with the occurrence of only one encounter (in CD154$^{-/-}$ mice) to an immune response after both encounters (in WT mice).

The second encounter defines which T_H cells start to produce effector cytokines and continue to proliferate. No data exist to date, whether and how the second encounter impacts the clonal expansion of individual T_H cell clones. It is possible that the second encounter provides additional TCR signals to some T_H cells, guiding them to further expansion and allowing them to outcompete other activated T_H cells. Therefore the second encounter might change the clonality and modulate the number of HECs within an immune response.

1.6 Aim of this study

Three major factors, which are the cytokine milieu, the avidity of the TCR to the pMHC-II and the antigen dose, have been described to influence the T_H1/T_H2 differentiation. This study focusses on the TCR-pMHC-II interactions and their influence on the T_H cell differentiation. In this context, three modulators are analysed in antigen dose dependent models.
To investigate the influence of the B cells, the C5aR1 signalling and the T cell clonality on the T_H1/T_H2 differentiation, three aims were investigated:

1) In the SRBC model it has been suggested that T_H2 induction after HD immunization depends on the activation of B cells. The question arises whether B cells contribute as APCs to the T_H2 induction after HD application in the SRBC model. Answering this question would unravel a checkpoint to control T_H2 induction in the future.

2) C5aR1 has been described as a decisive factor for T_H1/T_H2 development in multiple models. However, the mechanism behind this is not well understood. The question arises whether C5aR1 signaling modulates the T_H2 differentiation in the spleen after HD SRBC application or the draining lymph node (dLN) after *L. major* infection by affecting pMHC-II antigen presentation. Answering this question could help to unravel the underlying mechanism behind the influence of C5aR1 on the T_H cell differentiation in the secondary lymphoid organs.

3) The second TCR-pMHC-II encounter requires the interaction of CD154/CD40 and induces T_H2 cells in the spleen after HD SRBC immunization. The question arises whether the lack of CD154/CD40 signaling changes the number of HECs within a T_H2 response to a HD of SRBCs. By answering this it would be possible to explain how a high dose of antigen modulates the clonotype selection into an immune response.

2 Material & Methods
2.1 Materials

2.1.1 Mice

BALB/cAnNCrl	Charles River Laboratories, Research Models and Services, Germany GmbH, Sulzfeld
C57BL/6J	Charles River Laboratories, Research Models and Services, Germany GmbH, Sulzfeld
C5aR1$^{-/-}$ C57Bl/6	Kindly provided by Prof. Köhl, own inbred, C57BL/6J background
C5aR1$^{-/-}$ Balb/c	Kindly provided by Prof. Köhl, own inbred, BALB/cAnNCrl background
JHT	own inbred, C57BL/6J background
CD154$^{-/-}$	own inbred, C57BL/6J background

All mice were maintained in a pathogen-free animal facility of the University of Lübeck for the duration of the experiments. BALB/cAnNCrl and B57BL/6NCrl mice were purchased and kept in the facility for at least 2 weeks before starting experiments, C5aR1$^{-/-}$ C57Bl/6, C5aR1$^{-/-}$ Balb/c, JHT and CD154$^{-/-}$ mice were bred in the facility. Mice were used at 8 to 12 weeks of age. Food and water was available at all time, day-night rhythm was 12 and 12 hours, respectively. Animal care was provided by the GTH (Gemeinsame Tierhaltung, University of Lübeck) in accordance with German law. Studies were approved by the Schleswig-Holstein state authorities:

v 242-7224 122-1 (120-8/13)	T cell response in the spleen
v 242-7224 122-1 (34-4/14)	Influence of C5a-signaling on disease progression of murine *leishmaniasis*
v 242-7224.122-1 (112-9/14)	Influence of antigen dose on T cell activation in spleen and lymph node

2.1.2 Reagents

Acetone	C. Roth GmbH & Co. KG, Karlsruhe
Anaesthesia	10 mg Ketamine, 1.5 mg Xylazin per ml H_2O
Aquatex®, mounting medium	Merck KGaA, Darmstadt
B-mercaptoethanol	Merck KGaA, Darmstadt
Bovine serum albumin (BSA)	Sigma-Aldrich Chemie GmbH, Taufkirchen
CFSE	Sigma-Aldrich GmbH, Steinheim
DAB	DAKO, Glostrup, DK
Diethylpolycarbonate (DEPC)	Sigma-Aldrich GmbH, Steinheim
DNAse-buffer	200 mM TRIS-HCl, 20 mM $MgCl_2$ in H_2O at pH 8.3
dNTP-mix (10 mM)	PEQLAB Biotechnology GmbH, Erlangen

EDTA	Serva GmbH, Heidelberg
Tissue-Tek®, mounting medium	Leica Instruments GmbH, Nussloch
Ethanol (70 % or 100 %)	C. Roth GmbH & Co. KG, Karlsruhe
ExtrAvidin®-alkaline phosphatase	Sigma-Aldrich Chemie GmbH, Taufkirchen
FACS-Clean	BD Bioscience Pharmingen, Heidelberg
FACS-Flow	BD Bioscience Pharmingen, Heidelberg
FACS-Rinse	BD Bioscience Pharmingen, Heidelberg
Fast Blue RR	Sigma-Aldrich Chemie GmbH, Taufkirchen
Fast Red TR	Sigma-Aldrich Chemie GmbH, Taufkirchen
FACS-buffer	PBS, 0.5 % Bovine serum albumin
FcγR-block solution	1 ‰ anti-CD16/32 antibody diluted in FACS-buffer
First Strand buffer 5x	Invitrogen GmbH, Karlsruhe
Formaldehydlösung 37 %	Sigma-Aldrich Chemie GmbH, Taufkirchen
Glycerin	Merck KGaA, Darmstadt
Ketanest®S (25 mg/ml)	Pfizer Pharma GmbH, Berlin
L-gluthamine	PAA Laboratories GmbH, Cölbe
MACS-buffer	PBS, 2 mM EDTA, 0.5 % Bovine serum albumin
MACS® Cell separation Columns (LS)	Miltenyi Biotec GmbH, Bergisch Gladbach
Methanol	C. Roth GmbH & Co. KG, Karlsruhe
Moviol® 4-88	Polyscience Inc., Warrington, PA, USA
NaCl	Merck KGaA, Darmstadt
NaOH	Merck KGaA, Darmstadt
Paraformaldehyd (PFA)	Merck KGaA, Darmstadt
PBS	PAA Laboratories GmbH, Cölbe
Penicilline (10000 U/ml)	PAA Laboratories GmbH, Cölbe
Random hexamer primer	MWG Biotech AG, Ebersberg
RBC lysis buffer	155 mM NH_4Cl, 0.05 mM EDTA
Reverse Transcriptase (BD PowerScript™)	BD Bioscience Pharmingen, Heidelberg
Rompun® (20 mg/ml Xylazin)	Bayer HealthCare, Leverkusen
Roswell Park Memorial Institute Medium (RPMI 1640)	PAA Laboratories GmbH, Cölbe
RPMI	RPMI 1640, 10 % FCS, 2 mM L-glutamin, 100 U/ml penicilline, 100 U/ml streptomycine, 50 µM β-mercaptoethanol
Sodium Dodecyl Sulfate (SDS)	C. Roth GmbH & Co. KG, Karlsruhe

Sheep-Erythrocytes (E-410) (in 50 % Alsever buffer)	Labor Dr. Merk, Ochsenhausen
Staining buffer	PBS containing 1 % Bovine serum albumin and 0.1 % NaN_3
Streptomicine (10 mg/ml)	PAA Laboratories GmbH, Cölbe
TBS	0.05 M TRIS, 0.8 % NaCl in H_2O at pH 7.6
TBS-Tween	0.05 M TRIS, 0.05 % Tween® 20, 0.8 % NaCl in H_2O at pH 7.6
Tissue freezing medium	Leica Microsystems, Wetzlar
Toluidine Blue	Waldeck GmbH&Co, Münster
Toluidine Blue solution	Water containing 0.1 % Toluidine Blue and 1 % methanol (15 %)
TRIS(hydroxymthyl)amino-methan	Serva GmbH, Heidelberg
Trypan Blue solution (0,4 %)	Thermo Scientific, Waltham, MA, USA
Tween®20	Serva GmbH, Heidelberg

2.1.3 Antibodies

Antibodies used for flow cytometry:

Protein	Fluorescent dye	Concentration	Isotype	Clone	Company
B220	PerCP-Cy5.5	1:300	Rat IgG2a, κ	RA3-6B2	eBioscience
CD4	PerCP-eFluor® 710	1:300	Rat IgG2a, κ	RM4-5	eBioscience
CD4	APC	1:400	Rat IgG2a, κ	GK1.5	eBioscience
CD8a	Alexa Fluor® 647	1:400	Rat IgG2a, κ	53-6.7	Biolegend
CD11b	FITC	1:100	Rat IgG2a, κ	M1/70	eBioscience
CD11c	APC	1:100	Armenien Hamster IgG	N418	eBioscience
CD16/32 (FCγR-block)	-	1:000	Rat IgG2a, λ	93	eBioscience
CD19	Alexa Flour 647	1:300	Rat IgG2a, κ	1D3	BD Bioscience
CD44	FITC	1:400	Rat IgG2b, κ	IM7	Biolegend
CD69	PE	1:300	Armenien Hamster IgG	H1.2F3	eBioscience
Ly6g	PE-Cy7	1:100	Rat IgG2b, κ	RB6-8C5	eBioscience
MHC-II	PE	1:100	Rat IgG2b, κ	M5/114.15.2	eBioscience
NK1.1	APC	1:400	Rat IgG2a, κ	PK136	Biolegend

Antibodies used for immunohistochemically staining:

Protein	Concentration	Isotype	Clone	Company
B220	1:100	Rat IgG2a, κ	RA3-6B2	BD Bioscience
Biotin	1:150	Polyclonal Rabbit IgG	-	Biozol
Ki-67	1:100	Goat IgG	Poly4054	Biolegend
TCRβ	1:2000	Armenian Hamster IgG2 λ	H57-597	BD Bioscience

Antibodies were purchased from the following companies: BD Bioscience Pharmingen (Heidelberg), Biolegend (London, GB), Biozol Diagnostica GmbH (Eching), eBioscience (Thermo Scientific, Waltham, MA, USA), Celtag Laboratories (Buckingham, GB)

2.1.4 Primer

The primers were purchased from MWG biomers.net GmbH (Ulm). Taq-Man probes were coupled to the fluorophore FAM (emission at 520 nm) and TAMRA.

Gene	Primers and probes	Sequence	Gene accession number
CCL3	CCL3 for	5´-CAC TCT GCA ACC AAG TCT TC-3´	NM_011337.2
	CCL3 rev	5´-CAC CTG GCT GGG AGC AAA G3´	
GR-1	GR-1 for	5' GCG TTG CTC TGG AGA TAG AAG	XM_909927.2
	GR-1 rev	5' CTT CAC GTT GAC AGC ATT ACC	
IFN-γ	mIFN-γ for	5’ GCAAGGCGAAAAAGGATGC	NM_008337.2
	mIFN-γ rev	5’ GACCACTCGGATGAGCTCATTG	
	mIFN-γ probe	5’ TGCCAAGTTTGAGGTCAACAACCCACAG	
IL-4	mIL-4 for	5’ GAG ACT CTT TCG GGC TTT TCG	NM_021283.1
	mIL-4 rev	5’ AGG CTT TCC AGG AAG TCT TTC AG	
	mIL-4 probe	5’ CCTGGATTCATCGATAAGCTGCACCATG	
IL-10	mIL-10 for	5’ TCCCTGGGTGAGAAGCTGAAG	NM_010548.1
	mIL-10 rev	5’ CACCTGCTCCACTGCCTTG	
	mIL-10 probe	5’ CTGAGGCGCTGTCATCGATTTCTCCC	
IL-12	mIL-12 for	5’ GAGCACTCCCCATTCCTACTTCTC	NM_008352.2
	mIL-12 rev	5’ TGCATTGGACTTCGGTAGATGTC	
	mIL-12 probe	5’ CTCTACGAGGAACGCACCTTTCTGGTTACAC	

MLN	mMLN51 for	5’ CCAAGCCAGCCTTCATTCTTG	NM_138660.2
	mMLN51 rev	5’ TAACGCTTAGCTCGACCACTCTG	
	mMLN51 probe	5’ CACGGGAACTTCGAGGTGTGCCTAAC	
Illumina Primer	iRep 2 for	5’ AATGATACGGCGACCACCGAGATCTA CACTCTTTCCCTACACGACGCTCTTCCGATCT	
	iRep 2 rev	5’ CAAGCAGAAGACGGCATACGAGATCGGT CTCGGCATTCCTGCTGAACCGCTCTTCCGATCT	
	P5 Primer	5’ AATGATACGGCGACCACCGA	
	P7 Primer	5’ CAAGCAGAAGACGGCATACGAGAT	
	Seq. Primer for	5’ ACACTCTTTCCCTACACGACGCTCTTCCGATCT	
	Seq. Primer rev	5’ CGGTCTCGGCATTCCTGCTGAACCGCT CTTCCGATCT	

2.1.5 Kits

B cell MACS isolation Kit	Miltenyi Biotec GmbH, Bergisch Gladbach
GeneJet genomic DNA purification Kit	Thermo Scientific, Waltham, MA, USA
MinElute Gel extraction Kit	Qiagen, Venlo, NL
One-Step-RT-PCR	Qiagen, Venlo, NL
PerfeCta NGS Quantification Kit	Quantabio, Beverly, MA, USA
innuPREP RNA MINI direct Kit	Analytik Jena AG, Jena

2.1.6 Devices

BD Accuri C6 flow cytometer	BD Bioscience Pharmingen, Heidelberg
Centrifuges:	
Centrifuge 5702	Eppendorf AG, Hamburg
Megafuge 1.0R Heraeus	Kendro Laboratory Products GmbH, Hanau
MiniSpin®Plus	Eppendorf AG, Hamburg
Vacuumcentrifuge (Concentrator 5301)	Eppendorf AG, Hamburg
Kryostat HYRAX C50	Carl Zeiss Microscopy GmbH, Göttingen
Microscopes	
Axiovert 200	Carl Zeiss MicroImaging LLC, Göttingen
Wilovert®	Will, Wetzlar
Microdissection PALM®	P.A.L.M. Microlaser Technologies AG, Bernried

Miseq™	Illumina, San Diego, CA, USA
Neubauer improved (counting chamber)	Glaswarenfabrik Karl Hecht GmbH&Co.KG, Sondheim
Primus 96^{Plus} (PCR machine)	MWG Biotech AG, Ebersberg
Quadro MACS™ Seperator	Miltenyi Biotec GmbH, Bergisch Gladbach
Quantus Fluorometer	Promega, Madison, WI, USA
ABI PRISM® 7900 (RT Sequence detection)	Applied Biosystems, Waltham, MA, USA

2.1.7 Software

Adobe® Acrobat® 8.0, Adobe Systems Inc., San Jose, CA, USA

Adobe® Illustrator CS, Adobe Systems Inc., San Jose, CA, USA

Adobe® Photoshop® 6.0, Adobe Systems Inc., San Jose, CA, USA

Axiovert 4.2 software, Carl Zeiss MikroImaging LLC, Göttingen

EndNote X7.2, Thomson Reuter, New York City, NY, USA

GraphPad Prism 5.03, GraphPad Software, Inc., La Jolla, CA, USA

Microsoft® Excel 2013, Microsoft Cooperation, Mountain View, CA, USA

Microsoft® Word 2013, Microsoft Cooperation, Mountain View, CA, USA

PALM® Robo, P.A.L.M. Microlaser Technologies AG, Bernried

SDS 2.2.1 RQ, Applied Biosystems, Darmstadt

2.2 Methods

2.2.1 The SRBC model

2.2.1.1 Immunization with SRBCs

SRBCs were purchased from Labor Merk in solution with 50 % Alveser buffer, and prepared directly prior to immunization (max. 1 h). SRBCs were carefully mixed and 400 µl per mouse (HD immunized) was transferred into a 50 ml tube, adding PBS up to the total volume of 30 ml. Tubes were centrifuged in the Centrifuge 5702 (Eppendorf) for 10 min with 700 x g. The supernatant was discarded and the pellet was diluted in 30 ml of PBS for a second time. After centrifugation (10 min with 700 x g) the supernatant was discarded and the pellet was transferred into a 2 ml reaction tube. 10 µl RBCs were diluted 990 µl PBS and counted using Trypan Blue solution and a Neubauer counting chamber at the microscope Wilovert® (Will). 5 inner squares were counted and the RBC concentration was calculated:

$$RBCs\ per\ \mu l = \frac{\sum 5\ squares\ \ x\ 2\ x\ 100}{0.02}$$

According to the calculation RBCs were diluted with PBS to 5 mio RBCs/µl. This dilution was used for the HD injection (10^9 SRBCs in 200 µl). For MD injection (10^7 SRBCs in 200 µl), 10 µl of the HD were diluted with 990 µl PBS. For the LD injection (10^5 SRBCs in 200 µl) 10 µl of the MD were diluted with 990 µl PBS. All SRBC solutions were kept on ice and immediately used for immunization. Mice were transferred one after the other in a small cage and set under a red light to slightly increase blood pressure and visualize the tail vein. Mice were monitored during the whole procedure and behavioural boldness or coughing led to immediate cancellation of the procedure. After a 2-3 minutes mice were immobilized in a specialized support and 200 µl of the respective antigen dose (or PBS) was injected i.v. into the tail vein.

2.2.1.2 Subcutaneous challenge and DTH measurements

On day 5 after immunization (2.2.1.1) the mice were challenged into the right hind footpad. Therefore SRBCs were prepared as described above (2.2.2.1), but finally diluted with PBS to a concentration of 20 mio SRBCs/µl. SRBCs are kept on ice and were immediately used for injection. Therefore mice were immobilized and 50 µl SRBCs (containing 10^9 SRBCs) were injected into the right hind footpad sole of all mice.

Measurement of the DTH was started before challenge, measuring the right hind footpad before challenging. DTH was further measured on the next 5 days at the same time of the day

(±1 h). Footpad swelling was calculated by the difference from the measured footpad to the footpad thickness before the challenge was applied.

2.2.1.3 Immunization with CFSE labelled SRBCs

For the immunization with CFSE labelled SRBCs, SRBCs were prepared as described above (2.2.2.1) and diluted to a concentration of 5 mio SRBCs/µl (HD). Afterwards the HD was diluted to receive a LD (see 2.2.2.1). SRBCs (and a PBS control) were transferred into a light protected reaction tube (either covered with foil or specialized UV protected tubes) and CFSE and PBS was added to get to a final concentration of 1 µl CFSE per 200 µl SRBCs in a volume of 2 ml. The solution was mixed by inverting and incubated for 30 min (20° C). After incubation SRBCs were centrifuged with a Centrifuge 5702 (Eppendorf) for 8 min at 700 x g. The supernatant was discarded and PBS was added to the pellet up to 2 ml. Another centrifugation step was performed (8 min at 700 x g) and the supernatant was discarded. All SRBCs were kept on ice under exclusion of light and were immediately injected into the tail vein of the mice as described above (2.2.2.1).

When CFSE is taken up by APCs it loses its fluorescent colour during phagocytosis. Therefore an early point of time for CFSE measurement was chosen, by analysing $CFSE^+$ cell population after 3 hours. Two controls were used to measure the amount of $CFSE^+$ B cells. The first control were splenic B cells from a naïve mice. Since fluorescent CFSE emission is measured at a wavelength of 520 nm, which intervenes with auto-fluorescence background of cells, naïve B cells were used to exclude auto-fluorescence signals. Unspecific surface binding of CFSE was excluded by using T cells, which cannot take up the antigen directly and $CFSE^+$ T cells would indicate the unspecific CFSE signal. $CFSE^+$ B cells are calculated of the percentage by which the population exceeded the two controls (indicated by the Δ).

2.2.1.4 B cell transfer into JHT mice in the SRBC model

C57BL/6 WT mice were sacrificed and the spleens were removed and stored in 1 ml RPMI on ice. Cells were isolated according to the protocol (2.2.3).

For isolation of B cells from the cell suspension the MACS B cell isolation Kit (Miltenyi Biotec) was used according to the user manual. The cells were counted (2.2.3) and centrifuged for 10 min at 500 x g. After the supernatant was discarded, the pellet was resolved in 40 µl MACS

buffer and 10 µl antibodies (provided) per 10^7 cells in the original pellet. After 10 min of incubation at 4° C, another 30 µl of MACS buffer and 20 µl Microbeads (provided) were added to each sample per 10^7 cells. During the following 5 min incubation time, the MACS® Cell separation columns (Miltenyi Biotec) were fixed in the Quadro MACS™ Seperator (Miltenyi Biotec). The columns were washed with 1 ml MACS buffer three times in a row. After the third washing step a collection tube was arranged under the column and the incubated sample was transferred onto the column. The flow through contained the B cells. This was repeated with the other samples on a new separation column. Finally all samples were pooled to one B cell suspension. The cells in this suspension were counted (2.2.3). Afterwards, the suspension was centrifuged (10 min at 500 x g), the supernatant was discarded and the pellet was resolved in 1 ml PBS. After an additional washing step (centrifugation for 10 min at 500 x g, supernatant discarded) the pellet was resolved with PBS to a concentration of $2.5x10^8$ cells/ml. $5x10^7$ B cells were injected in 200 µl PBS into the tail vein of JHT mice (as described in 2.2.2.1) two days prior to starting the SRBC mouse model (2.2.2.1).

2.2.1.5 Organ removal

Animals were sacrificed after 3 hours (when immunized with CFSE labelled SRBCs) or on days 3, 7 or 10 after immunization between 8 and 10 AM on the respective day. Therefore, mice were narcotized with CO_2 and sacrificed with oxygen revocation. Spleen was carefully cut in half. One half was used for cryosection (2.2.5) and frozen in liquid nitrogen. The other half was used for cell isolation (2.2.3) and stored in 500 µl RPMI medium. Animals sacrificed on days 7 or 10, the sole of the hind footpads was additionally removed from the bone and frozen in liquid nitrogen. All cryosections were stored in -80° C, spleen in RPMI was directly used for cell isolation (2.2.3). All procedures were done using freshly autoclaved and with SDS 1 % cleaned scalpels, scissors and tweezers.

2.2.1 The *L. major* model

2.2.1.1 Subcutaneous injection of *L. major* promastigotes

Leishmania major promastigotes were kindly provided by Prof. Dr. Laskay (Institute of Microbiology, University of Lübeck). *L. major* promastigotes (strain MHOM/IL/8I/FEBNI) were cultured on microtiter plates on rabbit or sheep blood agar at 26° C in humidified atmosphere

containing 5 % CO_2 for 7 days. At day 7, parasites were collected from the plate and transferred in a 50 ml tube, filled up with PBS, centrifuged and re-suspended in medium.

For footpad injection, the following concentrations of *L. major* promastigotes were prepared, and 50 µl were injected into the right hind footpad of each mouse:

High dose (HD)	$2x10^7$ *L. major* promastigotes per ml	resulted in 10^6 per mouse
Medium dose (MD)	$6.6x10^5$ *L. major* promastigotes per ml	resulted in $3.3x10^5$ per mouse
Low dose (LD)	$2x10^4$ *Leishmania* promastigotes per ml	resulted in 10^3 per mouse

The definition of the HD, MD and LD number of *L. major* promastigotes was selected according to a publication (Bretscher et al. 1992). High dose corresponded to the highest dose used in this paper. Medium dose corresponded to the lowest dosage, which was able to induced a severe *leishmaniasis* in the WT mice. Low dose corresponded to a dose, where WT mice controlled the infection.

For the ear infection model mice were narcotized with 100-125 µl Ketamin/Xylazin corresponding to 35 mg per kg bodyweight. 10 µl of *L. major* promastigotes in media were injected in the right ear dermis. High dose injection was adapted from a publication (Hawlisch et al. 2005), using 10 µl of a 10^7 *L. major* per ml solution, corresponding with 10^5 promastigotes per mouse.

2.2.1.2 Footpad measurements

Footpad and ear swelling was measured twice a week. For the footpad measurement the diameter of the infected and the control hind footpad was determined. The difference was defined as the footpad swelling.

The ear model was scored with an adapted scoring method (Schuster et al. 2014) taking redness, necrosis and tissue loss into consideration by scoring the infected area in regard to its size with a score from 1 (redness) to 8 (tissue destruction).

2.2.1.3 Organ removal

Animals were taken between 8 and 10 AM on the respective day. Therefore, mice were narcotized with CO_2 and sacrificed with oxygen revocation. Spleen and LNs were prepared and directly frozen in liquid nitrogen for cryosection. Draining LNs were carefully cut in half. One half was used for cryosection and frozen in liquid nitrogen. The other half was used for cell

isolation (2.2.3) and stored in 500 µl RPMI medium. The sole of the infected and uninfected hind footpad was removed from the bone and frozen in liquid nitrogen for cryosection. All cryosection samples were stored in -80° C. The draining LNs in RPMI were directly used for cell isolation (2.2.3). All procedures were done using freshly autoclaved and with SDS 1 % cleaned scalpels, scissors and tweezers.

2.2.3 Cell isolation from the spleen or the dLN

Spleens and draining LNs were covered with RPMI and crushed between two frozen glass slides to get a single cell suspension. The suspension was then filtered through a 70 µm filter into a 50 ml tube. Two additional filter washes were performed, leading to a final volume of 15 ml of cell suspension in each tube. Afterwards, the suspension was centrifuged for 10 min at 500 x g. In case of spleens the supernatant was discarded and the pellet was dissolved in 3 ml RBC lysis buffer and incubated for 3 min. Lysis was stopped by adding RPMI up to a total volume of 15 ml and centrifugation for 10 min at 500 x g. The supernatants of spleen and LN suspensions were discarded, the pellet was diluted in 2 ml RPMI. 10 µl of the suspension was diluted with 190 µl of RPMI and counted using Trypan blue solution and a Neubauer counting chamber. 4 outer squares were counted and the cell concentration was calculated:

$$Cells\ per\ ml = \emptyset 4\ squares\ x\ 2\ x\ 20\ x\ 10^4$$

2.2.4 Fluorescence staining and flow cytometry measurements

For flow cytometric measurements 1-2 mio cells per sample and panel were stained. Therefore the calculated amount of cells from 2.2.3 was transferred into a 2 ml reaction tube and centrifuged for 4 min at 600 x g. The supernatant was discarded and the pellet was resolved in 300 µl FACS-Buffer. After another centrifugation step (4 min at 600 x g) the supernatant was discarded and the pellet diluted in 100 µl FcγR-block solution. After an incubation of 10 min the cells were centrifuged (4 min at 600 x g), the supernatant discarded and the pellet diluted in 100 µl FACS-buffer. Corresponding to the colour-panel, the samples were mixed with the respective mastermix and incubated for 40 min at 4° C.

After 40 min 200 µl of FACS-buffer was added to the staining solution and centrifuged (4 min at 600 x g). The supernatant was discarded and the pellet was resolved in 300 µl FACS-buffer and centrifuged again (4 min at 600 x g). After discarding the supernatant the sample was diluted in 300 µl FACS-buffer and measured in the flow cytometer.

Tab. 1: Flow cytometric staining.
Used flow cytometric colour-panels with name (column 1), antibodies (column 2-5) and final volume per sample (column 6). "Def*" included antibodies for CD4 (APC), CD8a, CD11c, CD19 and NK1.1.

Panel	Fluorescence 1 (FITC)	Fluorescence 2 (PE)	Fluorescence 3 (PECy7,PerCP)	Fluorescence 4 (APC, Alexa Fluor 647)	µl MM per sample
B cell	-	MHC-II	B220	CD19	2.66
MyP	CD11b	MHC-II	Ly6G	Def*	5
DCs	-	MHC-II	B220	CD11c	2.33
T 1	CD44	CD69	CD4 (PerCP eFluor@ 710)	CD8a	1.25

Three gating strategies were used to distinguish between the major APC populations (Fig. 6A). Macrophages and neutrophils were gated according to an established gating strategy relying on CD11b and Ly6G expression (Rose et al. 2012). This strategy was adopted towards a four-colour staining instead of the published six-colour staining. Live/dead staining as well as Ly6C were excluded from the analysis, because it was not needed to identify macrophages and neutrophils and reduced flow cytometric compensation issues. DCs were gated by a $CD11c^+$ population, because in current knowledge all murine splenic DCs, but none of the macrophage populations, express CD11c (Hey and O'Neill 2012). B cells were defined as the $CD19^+$ and $B220^+$ population. T_H cells and cytotoxic T cells were defined by their expression of CD4 and CD8, respectively. Additionally, T cells were characterized with certain activation markers (CD44, CD62L, CD69, CD127).

Control samples were used to determine the compensation of the panels. Therefore the mastermixes of the 4 colour panels were prepared 4 additional times with exception of one of the four antibodies in each mastermix (Fluorescence-minus-one-control). The samples were analysed in the flow cytometer. Although the antibody is missing, cells showing positive signals in a channel due to auto-fluorescence or intervening in wavelength to other antibodies. These false-positive signals were excluded by fluorescence compensation.

During this study pMHC-II surface expression on APCs was flow cytometrically assessed. Therefore MHC-II was stained with a PE-labelled antibody in absence of intracellular staining,

and quantified. This method was preferred, since it detects the actual protein on the surface and not unloaded MHC-II protein in the vesicles or the endoplasmic reticulum.

2.2.5 Cryosection

For cryosection samples were frozen in liquid nitrogen (spleen, LN or skin). For DNA (2.2.6) and RNA isolation (2.2.7), immunohistochemically staining (2.2.9) and laser microdissection (2.2.10) cryo samples were incorporated in Tissue freezing medium (Leica) and dissected into 12 µm thick sections. For immunohistochemically staining (2.2.9) and laser microdissection (2.2.10) the sections were transferred onto glass slides and incubated for 2 hours, and eventually stored in -20° C till further proceeding with staining (2.2.9).

2.2.6 DNA extraction

For DNA extraction the GeneJet genomic DNA Kit (Thermo Scientific) was used. 5 cryosections of LNs or spleens or 10 cryosections of footpad skin (2.2.5) were used for the extraction according to the instruction manual: Samples were diluted in 180 µl lysis solution and 20 µl proteinase K, and incubated for 1 h at 56° C. 20 µl RNAse A enzyme was added, followed by a 10 min incubation step at 20° C. Finally 200 µl lysis solution and 400 µl 70 % ethanol were added and mixed. The prepared lysate was then transferred on a purification column and centrifuged for 1 min at 6.000 x g. The flow through was discarded and 500 µl washing buffer I was added. After another centrifugation (1 min at 8.000 x g), the flow through was discarded and 500 µl washing buffer II was added. After another centrifugation step (3 min at 12.000 x g), the column was transferred on a 1.5 ml reaction tube and DNA was eluted with two elution steps (adding 50 µl of water, incubating 2 min and centrifuging for 1 min at 8.000 x g). DNA was directly used (2.2.8) or stored at -20° C.

2.2.7 RNA isolation and cDNA synthesis for qRT-PCR

For RNA isolation the innuPREP RNA MINI direct Kit (Analytik Jena) was used according to the instruction manual. In case of spleens and LNs 5 cryosected sections, in case of skin 10 sections (2.2.5) were used and diluted with 700 µl lysis solution. Samples were mixed for 45 sec and further disrupted using a syringe sucking it up 10 times. Afterwards samples were mixed with 700 µl 70 % ethanol. 700 µl of the solution was transferred to the filter column and centrifuged 1 min at 10.000 x g. The flow through was discarded and the other 700 µl were transferred on

the filter column. Following centrifugation (1 min at 10.000 x g), the flow through was discarded and 500 µl of the washing solution HS is added on the filter. After another centrifugation (1 min at 10.000 x g), the flow through was discarded and 700 µl washing solution LS was added on the filter, followed by another centrifugation step (1 min at 10.000 x g). After discarding the flow through the filter column was again centrifuged (2 min at 10.000 x g) to get it dry. Afterwards the collection tube was discarded and the filter column was transferred on a 1.5 ml reaction tube. 40 µl of RNAse-free water was added and incubated on the filter for 1 min. After centrifugation (1 min at 6.000 x g) the flow through was transferred back on the filter, incubated for another min and centrifuged (1 min at 6.000 x g). Filter column was discarded and RNA used for cDNA synthesis or stored at -80° C.

For cDNA synthesis RNA samples were vacuum centrifuged for 15 min. This led to the evaporation of the liquid phase in the reaction tube. RNA was diluted in 10 µl DNAse mix containing 8 µl RNAse-free water, 1 µl 10x DNAse buffer and 1µl DNAse enzyme (1 U/µl). Samples were incubated 15 min at 20°C before adding 1.5 µl Stopp solution. After carefully mixing, the samples were incubated 10 min at 70° C. Afterwards, 8.5 µl cDNA mastermix were added, containing 4 µl 5x RT buffer, 2 µl RNAse-free water, 1 µl dNTPs (10 µM), 1 µl random oligo primer (0.5 µM) and 0.5 µl reverse transcriptase enzyme (100 U/µl). The samples were incubated for 10 min at 20° C, for 1 h at 42° C and finally for 10 min at 70°C. cDNA was stored at -20° C until further proceeding with qRT-PCR (2.2.8).

2.2.8 qRT-PCR and qPCR

For qRT-PCR the Taq-Man detection system was used if possible. For some primers no Taq-Man probes were available, therefore SYBR-green fluorescence intensity was measured. One PCR reaction contains 10 µl 2x Taq-Man mix or SYBR-Green mix, as well as 7 µl H_2O, 1 µl cDNA (from 2.2.7) and 2 µl of primer mix. The primer mix for Taq-Man PCRs contained 0.18 µl forward primer, 0.18 µl reverse primer, 0.04 µl Taq-Man probe and 1.6 µl water. The primer mix for SYBR-green PCRs contained different amounts of primer, which are described below. Three different primer concentrations were used:

0.5 µM	0,1 µl primer (forward and reverse)	1,8 µl H_2O (used for CCL-3 Primer)
0.25 µM	0,05 µl primer (forward and reverse)	1,9 µl H_2O (used for GR-1 Primer)
0.125 µM	0,025 µl primer (forward and reverse)	1,95µl H_2O (used for MLN Primer)

For qPCR the DNA isolated in step 2.2.5 was used. qPCR was performed with Taq-Man PCR only, according to the protocol listed above for qRT-PCR.

Depending on the fluorescence detection system, two protocols were used.

Taq-Man PCR:

	Repeats	Temperature	Time
1.		95° C	10 min
2.	50 cycles	95° C	45 sec
		60° C	1 min
3.		72° C	10 min

SYBR-Green PCR:

	Repeats	Temperature	Time
1.		95° C	10 min
2.	50 cycles	95° C	45 sec
		60° C	1 min
3.	in 0.5° steps	95° C	15 sec
		60° - 90° C	15 sec
4.		72° C	10 in

2.2.9 Immunohistochemically staining

For immunohistochemically staining of spleen sections, antibodies were diluted with TBS-Tween. All washing steps were performed with TBS-Tween. Therefore the liquid phase was discarded and the slices (2.2.5) were covered with TBS-Tween, followed by an incubation of 5 min. All incubation steps were performed in a dark chamber.

2.2.9.1 TCRβ/B220

For this immunohistochemically staining glass slides (2.2.5) were incubated at 20° C for 10 min. At first, methanol and acetone were added, followed by a 10 min incubation. Afterwards, two washing steps were performed before the anti-TCRβ antibody was diluted in staining buffer to the final concentration (2.1.3) and added onto the slides for a 60 min incubation.

After two washing steps, ExtrAvidin®-alkaline (Sigma Aldrich) (1:100) is added and the slides were incubated for another 30 min. Two washing steps were performed, followed by the addition of DAB (DAKO) and a 5 min incubation. After another two washing steps B220 was stained on the same slides. Therefore the anti-B220-antibody was diluted in staining buffer to the final concentration (2.1.3) and incubated for 60 min. Two washing steps were performed, ExtrAvidin®-alkaline was added and the slides were incubated for 30 min. After two additional washing steps Fast Blue was added to the slides and incubated for 25 min. Finally, two washing steps were performed and the slides were covered with Aquatex® (Merck).

2.2.9.2 Ki-67/B220

For the determination of proliferating cells, anti-Ki-67 antibody was used to stain splenic sections. Ki-67 is a marker which is expressed during all phases of a cell cycle, but not in resting T cells (G_0) (Scholzen and Gerdes 2000), marking cells which are proliferating. Therefore glass slides (2.2.5) were incubated at 20° C for 10 min. At first, chloroform was added onto the slides until they were completely covered. After a 10 min incubation step acetone was added, followed by another 10 min incubation. Afterwards, three washing steps were performed before paraformaldehyde (4 %) is added and incubated for at 4° C for 45 min. Finally the anti-Ki-67 antibody was diluted in staining buffer to the final concentration (2.1.3) and added onto the slides for over-night incubation.

On the following day, a washing step was performed followed by the addition of the biotin antibody. After an incubation at 20° C for 30 min and two washing steps, ExtrAvidin®-alkaline is added and the slides were incubated for another 30 min. Another two washing steps were performed, followed by the addition of Fast Red (Sigma-Aldrich) and a 25 min incubation. After two washing steps B220 was stained on the same slides (2.2.9.1) before they were covered with Aquatex® (Merck).

2.2.9.3 Toluidine Blue (Laser microdissection)

For laser microdissection glass slides (2.2.5) were stained with Toluidine Blue solution. Therefore, the glass slides were incubated at 20° C for 10 min, covered with ethanol (75 %) and incubated for 2 min. After two washing steps with water (instead of TBS-Tween), Toluidine Blue solution was added and incubated for 10 min. Afterwards the liquid phase was discarded

and twice rinsed with water and 15 sec incubated in ethanol. Finally the slides were dried for 10 min and stored at -80° C until proceeding with laser microdissection (2.2.10.1).

2.2.10 Sequencing of the T cell receptor repertoire

2.2.10.1 Laser microdissection

For laser microdissection immunohistochemically stained spleens (2.2.9.4) were taken from -80° C into a glass cuvette with silica gel. After a 15 minute incubation sections were processed. 2.8-6 mio µm² of TCZ were selected with PALM® Robo software and dissected with the microdissection PALM®. Dissected TCZs were collected with a tweezer, mixed with 350 µl of RNA Lysis buffer and incubated for 30 min at 20° C. Afterwards TCZs were stored at -20° C until proceeding with RNA isolation (2.2.10.2).

By immunohistochemically staining of serial spleen sections with TCRβ/B220 (2.2.9.1) it was ensured that none of the selected TCZs were connected with each other.

2.2.10.2 RNA isolation and cDNA synthesis for sequencing

For RNA extraction from dissected TCZs (2.2.10.1) the innuPREP RNA MINI direct Kit (Analytik Jena) was used according to the instruction manual (described in 2.2.7). The DNA was digested as described above (2.2.7). After isolation, RNA was quantified with the Quantus Fluorometer (Promega). Therefore, 1 µl RNA was diluted with 99 µl 1xTE buffer and 100 µl 1xQuantiFloor® dye (provided). After carefully mixing, the solution was incubated for 5 min and measured. RNA concentration is calculated according to measured standard samples (provided).

cDNA synthesis was performed using the Quiagen-One-Step-RT-PCR Kit according to manual (from iRepertoire). Beside RNA and RNAse-free water, all used substances were provided.

5 µl of RNA (10-100 ng) were carefully mixed with 9 µl RNAse-free water, 5 µl 5x RT buffer, 1µl dNTPs (10 µM), 4 µl “MTBI” Primer and 1 µl enzyme mix. The “MTBI” primers contain individual identifier sequences. Using different “MTBI” primers for different samples provided the opportunity to differentiate between the samples afterwards when they were sequenced within the same sequencing run (2.2.10.4). Samples were amplified using a Primus 96^{plus} PCR machine (MWG Biotech) with the following program:

	Repeats	Temperature	Time
1.		50° C	40 min
2.		95° C	15 min
3.	15 cycles	94° C	30 sec
		60° C	2 min
		72° C	30 sec
4.	10 cycles	94° C	30 sec
		72° C	30 sec
5.		72° C	10 min

Afterwards, 1 µl of the PCR product were used to perform a second PCR amplification by adding 9 µl RNAse-free water, 2.5 µl commersal Primer and 12.5 µl mastermix (provided). This was a Multiplex PCR, meaning that the primer pairs for the second PCR different from the primers of the first PCR and are located on the amplified sequence from the first PCR. This leads to a higher specificity of the final PCR product.

	Repeats	Temperature	Time
1.		95° C	15 min
2.	40 cycles	94° C	30 sec
		55° C	30 sec
		72° C	30 sec
3.		72° C	4 min

Afterwards, samples were stored at -20° C until proceeding with the library preparation (2.2.10.3).

2.2.10.3 Library preparation for sequencing

TCZ samples (2.2.10.2) were incubated at room temperature for 3 min and carefully mixed with 5 µl of gel loading buffer. Mixture was added in multiple slots of a 2 % agarose gel. Gel was incubated with 100 V for 30 min. Gel band with a length of ≈300 bp contained the product and was extracted from the gel, using a MinElute Gel Extraction Kit (Qiagen). The kit was used according to protocol and contained the following steps: cDNA band was carefully dissected out of the gel (≈300 bp of size). Afterwards gel slice was weighed. Gel slices were used up to

a maximum of 400 mg per gel slice. The steps performed during gel extraction were dependent on the weight of the gel slice and were adapted to each individual sample.

At first, gel slice were diluted in buffer “QG”, using three-times the volume of the gel slice (example: A 100 mg gel slice was diluted in 300 µl of buffer “QG”). The mixture was incubated for 10 min at 50° C. Afterwards one volume (depending on the gel weight) of isopropanol was added and carefully mixed. The mixture was transferred on a spin column (provided) (to a maximum of 700 µl) and centrifuged for 1 min at 13.000 x g. After discarding the flow through, 500 µl buffer “QG” was added and samples were centrifuged again (1 min at 13.000 x g). After discarding again the flow through, 750 µl buffer “PE” was added and incubated for 3 min. Following centrifugation (1 min at 13.000 x g), the flow through was discarded. After another centrifugation step (1 min at 13.000 x g), the filter was transferred on a new reaction tube. Samples were diluted with 10 µl of buffer “EB” on the filter. After a 1 min incubation a final centrifugation step (1 min at 13.000 x g) was performed.

For the quantification of the cDNA within the gel eluate PerfeCta NGS Quantification Kit (Quantabio) was used according to the manual. Therefore 4 µl of diluted (1:10.000) gel eluate was mixed with 5.4 µl water, 0.6 µl Illumina Primer Mix (provided) and 10 µl PerfeCta Sybr Green SuperMix (provided). Additionally, standard samples (provided) were used instead of the RNA for calculating the absolute amount of cDNA after amplification.

	Repeats	Temperature	Time
1.		95° C	5 min
2.	35 cycles	95° C	20 sec
		60° C	30 sec
		72° C	45 sec

2.2.10.4 Sequencing

For sequencing the gel eluate is diluted to a concentration of 2 nM cDNA. Afterwards a denaturation step is performed by mixing 5 µl of the diluted cDNA with 5 µl of 0.2 M of NaOH and brief centrifugation for 1 min at 280 x g. Afterwards 5 min incubation denature DNA into single strands. The mixture is afterwards further diluted with 990 µl of dilution buffer HT1 (provided), generating a concentration of 10 pM cDNA. For these experiments a 7 pM cDNA concentration was used. Therefore, the cDNA was further diluted by mixing 420 µl of 10 pM cDNA with 180 µl HT1. Additionally to the sample library, 15 % PhiX is used as a control.

Therefore 2 µl of 10 nM PhiX solution is diluted with 3 µl of 10 nM Tris-Cl. This mixture is used for the denaturation step as described above. Finally 375 µl of 20 pM denatured PhiX is diluted with 225 µl of HT1 to a final concentration of 12.5 pM. 15% of this PhiX library is used for sequencing. Sequencing is done with an Illumina MiSeq™ V2, using 150 bp paired end sequencing, which means that all nucleotide strands are sequenced from both ends for 150 bp.

2.2.10.5 Sequencing analysis

The data were analyzed according to an established workflow. Six different samples were sequenced within one sequencing run. The total reads (raw reads) were separated via the individual identifiers within the "MTBI" primer sequences of each sample. The reverse read (paired end sequencing) was allocated to the forwards read (called pearing). This is possible since all reads are located by x/y coordinates on the flow cell. The reads with the same x/y coordinates were peared to become the completed PEAR reads. These PEAR reads were compared to the imgt database (http://www.imgt.org) to allocate the used V, D and J region to each CDR3. This was done by using the R package "MiTCR" (Bolotin et al. 2013).

2.2.11 Statistics

For all groups a Grubbs' test (ESD method (extreme studentized deviate)) was performed to identify significant outlier. Gaussian distribution was tested for all groups exceeding n=6 with the D'Agostinio and Pearsons omnibus normality test. For groups which passed the D'Agostinio and Pearsons test students t-test (compare two groups) or One-way ANOVA (with Turkey posttest) was used to identify significant differences. For all other groups Mann-Whitney test, Kruskal-Wallis test (with Dunns posttest) or Two-way ANOVA was performed. Significances were marked with a star, according to the level of significance: $p<0.05$ =*, $p<0.01$ =** and $p<0.001$ =***. All statistical tests were performed with GraphPad Prism 5.03 and GraphPad QuickCalcs online tool.

3 Results

The cytokine milieu, the TCR avidity to the pMHC-II and the antigen dose are the three major factors to modulate the T_H1/T_H2 differentiation. Beside those three, the modulation by other factors like the type of APC presenting the antigen or the complement receptors such as C5aR1 are appreciated more and more in recent years.

Nevertheless, the T_H cell differentiation is also dependent on the T_H cell clonality within the immune response. This clonality is dependent on the clonal expansion of recruited T_H cells, making the T_H cell recruitment a critical important step for the developing immune response. This study analysis in the following three parts different aspects of (1) the influence of the type of APC presenting the antigen, (2) the expression of C5aR1 on antigen presentation and (3) the T_H cell recruitment on the T_H1/T_H2 differentiation, in antigen dose dependent mouse models.

3.1 T_H1/T_H2 differentiation can be modulated by the antigen presentation from B cells

A HD of antigen induces T_H2 cells, whereas a LD of antigen induces T_H1 cells. The underlying mechanism is not known, but this study hypothesizes that a HD induces T_H2 cells by increasing the antigen presentation. The antigen presentation can be increased by an increase of (1) the antigen presenting APCs and/or (2) the pMHC-II density on antigen presenting APCs. The sum of the pMHC-II generated this way, is termed in this work the pMHC-II availability. Additionally to the amount of antigen presentation, the type of APC providing the antigen presentation is a potential modulator of T_H1/T_H2 differentiation. In case of T_H2 cells, some reports suggest the predominant role of B cells as inducing APCs (Drake et al. 2015).

3.1.1 Establishment of the antigen dose dependent SRBC mouse model

The SRBC mouse model was established in C57Bl/6 mice (Kaufmann and Hahn 1979; Lagrange et al. 1974a) , and reproduced in this study in accordance to the literature.

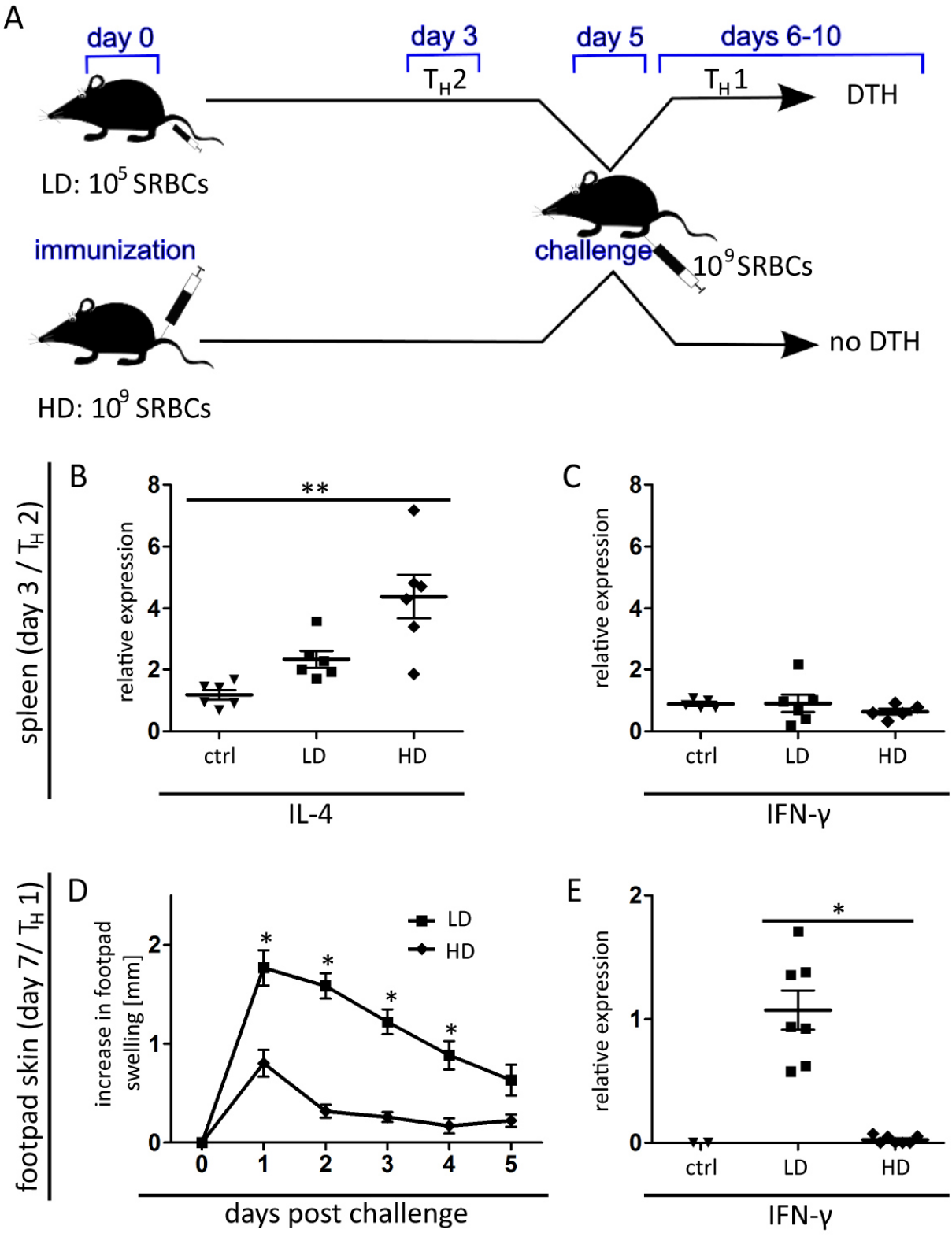

Fig. 5: Establishment of the SRBC mouse model in WT C57Bl/6 mice.
Schematic figure of the SRBC model (A): Different antigen doses were used for immunization. A T_H2 response develops in the spleen 3 days after immunization. A T_H1 response develops in the footpad as a DTH reaction around day 7, two days after challenge (day 5) with 10^9 SRBCs. Relative quantification of mRNA expression of IL-4 (B) and IFN-γ (C) in spleen, 3 days post immunization with PBS (ctrl), 10^5 (LD) or 10^9 (HD) SRBCs, horizontal lines represent mean ±SEM (Kruskal-Wallis test), n=5-6. Increase of footpad swelling after challenge (D) was calculated as the difference between measured footpad thickness and footpad thickness on day 5 (before challenge was applied), dots represent mean ±SEM

(Two-way ANOVA), n=6-8. Relative mRNA expression of IFN-γ in skin on day 7 post immunization (E), horizontal lines represent mean ±SEM (Kruskal-Wallis test), n=5-6$^{\$}$, *p<0.05, **p<0.01. $^{\$}$Fig. 1E contains 5 control mice, but 3 showed no detectable IFN-γ expression.

In this model the T_H2 response develops in the spleen on day 3, whereas the T_H1 response develops in the footpad as a DTH dependent swelling on day 7. Therefore T_H cell differentiations was analysed by the investigation of immunized mice 3 and 7 days post immunization.

Figure 5A depicts the course of the SRBC model: Mice were immunized on day 0 with either a LD (10^5) or a HD (10^9) of SRBCs (Fig. 5A). Cytokine mRNA expression in the spleen was measured 3 days post immunization. On day 5, mice were challenged with 10^9 SRBCs in the hind footpad, and the swelling of the footpad was used as a read out for the ongoing immune response as a DTH. This immune response was observed from day 5 to 10, peaking at day 7 (2 days post challenge) (Stamm et al. 2013).

On day 3, LD immunized mice did neither show significant amounts of IL-4 (Fig. 5B) nor an increased IFN-γ expression (Fig. 5C) in the spleen. In contrast, HD immunized mice showed a significant increase in IL-4 expression compared to control mice (Fig. 5B). The challenge of both groups in the footpad at day 5 resulted in a significant swelling of the footpads in LD immunized mice over four days (day 6-9) (Fig. 5D), whereas HD immunized mice showed only an initial swelling one day after challenge. Furthermore, the IFN-γ mRNA expression was significantly increased in the footpads of LD, compared to HD immunized mice (Fig. 5E).

These data confirmed earlier results (Lagrange et al. 1974a), showing a T_H2 response in the spleen of HD immunized mice on day 3, and a T_H1 response after LD immunization and a challenge on day 7.

3.1.2 The HD increases the antigen presentation by inducing presentation by B cells

In the SRBC model, a T_H1 response can appear only when a T_H2 response in the spleen has not occurred earlier (Stamm et al. 2013). This converts the T_H2 induction in the spleen into an crucial checkpoint for the T_H1/T_H2 cell fate.

This study suggests that a HD immunization increases the pMHC-II availability from B cells, compared to a LD immunization, in order to induce T_H2 cells in the spleen. To test this, pMHC-II availability was quantified on the classical T_H cell activating APCs (macrophages, DCs and B cells) with special focus on B cells, under different antigen dose conditions using flow cytometry.

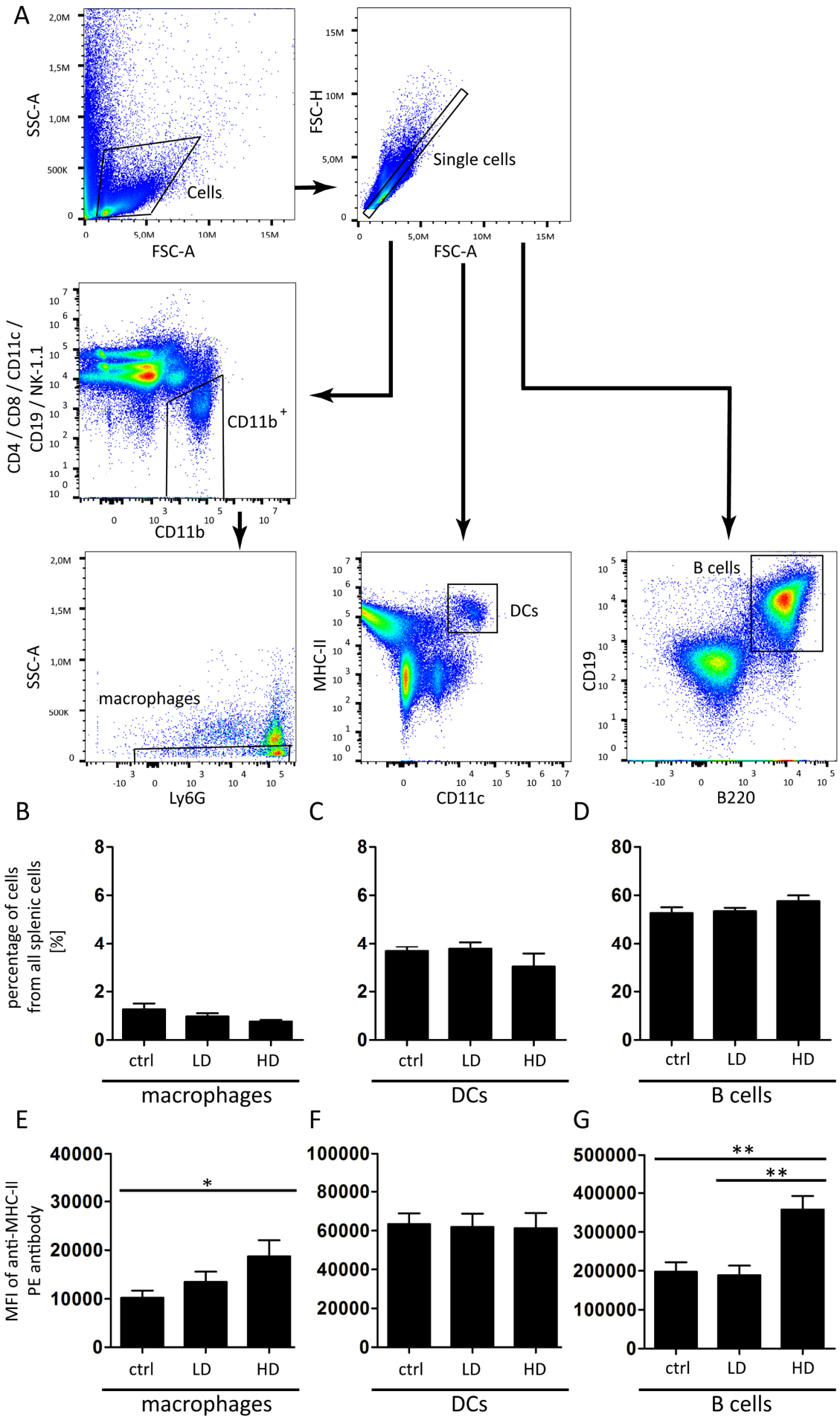
A
SSC-A
FSC-A
Cells
FSC-H
FSC-A
Single cells
CD4 / CD8 / CD11c /
CD19 / NK-1.1
CD11b
CD11b+
SSC-A
Ly6G
macrophages
MHC-II
CD11c
DCs
CD19
B220
B cells
B
C
D
percentage of cells
from all splenic cells
[%]
ctrl
LD
HD
macrophages
DCs
B cells
E
F
G
MFI of anti-MHC-II
PE antibody
*
**
**
ctrl
LD
HD
macrophages
DCs
B cells

Fig. 6: HD immunization increased pMHC-II availability by increasing MHC-II density on B cells.
Gating strategy (A) and flow cytometric measurement of cellular composition (B, C, D) and MHC-II on the surface of (E, F, G) splenic macrophages ($CD11b^+$, $Ly6G^{low}$, SSC^{low}) (B, E), DCs ($CD11c^+$ Gate) (C, F) and B cells ($CD19^+$, $B220^+$) (D, G), 3 days post immunization with PBS (ctrl) or SRBCs (LD/HD). Bars represent mean ±SEM, n=6, *p<0.05, **p<0.01 (Kruskal-Wallis test).

The cellular composition and the MHC-II surface expression of the classical APCs were measured in the spleen with three established gating strategies (Fig. 6A; Methods 2.2.4). Splenic cells are composed amongst others of ≈1 % macrophages (Fig. 6B), ≈4 % DCs (Fig. 6C) and ≈55 % B cells (Fig. 6D). None of the cell populations showed significant changes in cellular abundance due to the immunizations.

Macrophages showed slightly increased MHC-II expression from control to LD immunized mice and significantly increased expression in HD immunized mice (Fig. 6E). Initially DCs showed higher MHC-II surface expression than macrophages, which did not change based on the treatment with SRBCs (Fig. 6F). LD immunization did not affect the MHC-II level on B cells, but HD led to a significant increase of the MHC-II surface expression (Fig. 6G).

Even though it is possible that macrophages and DCs increased their presentation of SRBC derived epitopes without elevating the levels of MHC-II, the highest difference was clearly found in B cells. These data demonstrated that antigen dose has an influence on pMHC-II availability and the HD immunization significantly increased the B cell MHC-II expression compared to the LD immunization. In consequence, further work aimed to characterize the impact of B cells on the T_H2 development.

3.1.3 B cells take up SRBCs only after HD injection

To test if B cells interact with the antigen, SRBCs were labelled with CFSE and injected i.v. into the tail vain. After 3 hours, splenic B and T cells were isolated and $\Delta CFSE^+$ B cells were calculated.

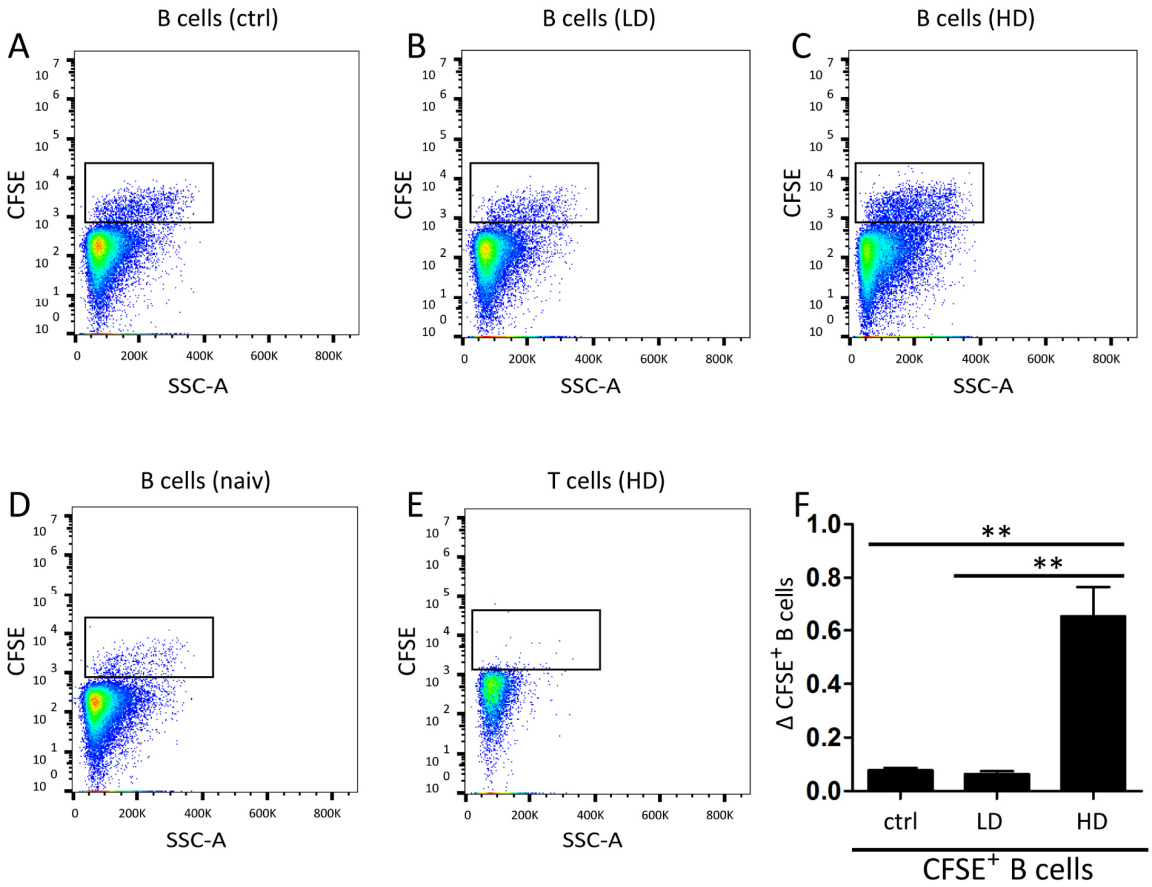

Fig. 7: Splenic B cells took up SRBCs only after HD immunization.
Flow cytometric measurement of CFSE positive B cells ($CD19^+$, $B220^+$) (A, B, C, D) and T cells ($TCR\beta^+$, $CD4^+$) (E) as control, 3 hours after different immunizations. $CFSE^+$ B cells after PBS (ctrl), LD or HD immunization were quantified as the difference to naïve B cells (to exclude auto-fluorescence) and T cells (to exclude unspecific surface CFSE) (F). Bars represent mean ±SEM (Kruskal-Wallis test), n=6, **p<0.01.

PBS and LD immunized B cell populations were comparable to B cells from naïve mice in regard to the $CFSE^+$ gate (Fig. 7A, B, D). The percentage of $CFSE^+$ B cell population increased in response to the HD immunization (Fig. 7C). Only very few $CFSE^+$ T cells were detected (Fig. 7E). Calculation of $\Delta CFSE^+$ B cells showed that only HD immunization increased the $CFSE^+$ B cell population significantly above the average control staining (Fig. 7F).

The increase of the $CFSE^+$ B cell population after HD immunization confirmed the assumption that B cells are a major cell population in the immune response after HD immunization.

3.1.4 B cells are required for a substantial T_H2 response after HD immunization

To test whether B cell pMHC-II is required for a T_H2 response, B cell deficient JHT mice were used. These mice lack the B cell receptor J_{heavy} chain and cannot develop B cells (Gu et al. 1993). The development of T_H2 cells after HD immunization was measured by the appearance of IL-4.

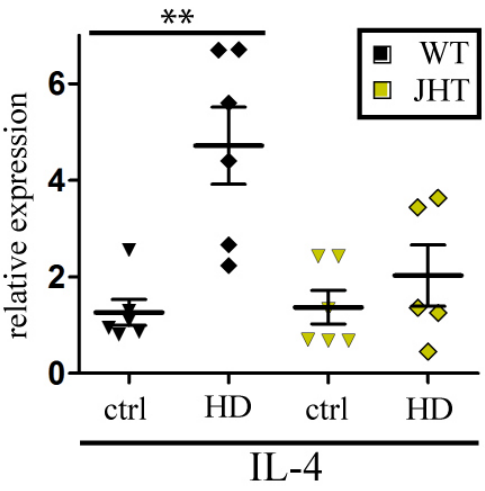

Fig. 8: IL-4 expression was induced only in the presence of B cells.
Relative mRNA expression of IL-4 in the spleen compared to the internal control on day 3 post immunization with HD or PBS (ctrl). Horizontal lines represent mean ±SEM (Kruskal-Wallis test), n=5-6, **p<0.01.

HD immunized WT mice significantly increased IL-4 compared to the control mice (Fig. 8). In contrast, JHT mice did not develop a significant increase in IL-4 expression in the 3 days post HD immunization.

The lack of IL-4 in HD immunized JHT mice was hypothesized to be the result of the loss of B cells. Therefore B cells were transferred into JHT mice, two days prior to the immunization with a HD of SRBCs in order to increase the IL-4 expression.

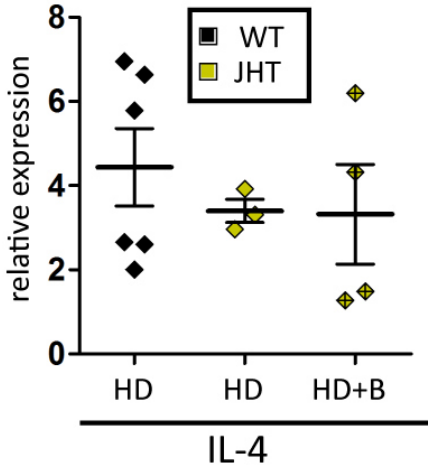

Fig. 9: B cell transfer into JHT mice did not increase IL-4 expression.
Relative mRNA expression of IL-4 in the spleen of WT and JHT mice with and without B cell transfer (50 mio B cells transferred on day -2) on day 3 post HD immunization. Horizontal lines represent mean ±SEM (Kruskal-Wallis test), n=3-6.

IL-4 mRNA expression in the spleen of JHT mice revealed no significant increase due to the transfer of B cells (Fig. 9).

The transfer was done according to a published protocol (Hoerauf et al. 1996), aiming to restore at least 20 % of splenic B cells in JHT mice. It was hypothesized that this amount is necessary to induce a significant effect on the T_H cell differentiation. To test whether this goal was reached immunohistochemically stained spleens from JHT mice of the immunization day were compared to control spleens with an intact splenic microarchitecture and normal B cell numbers (Fig. 10).

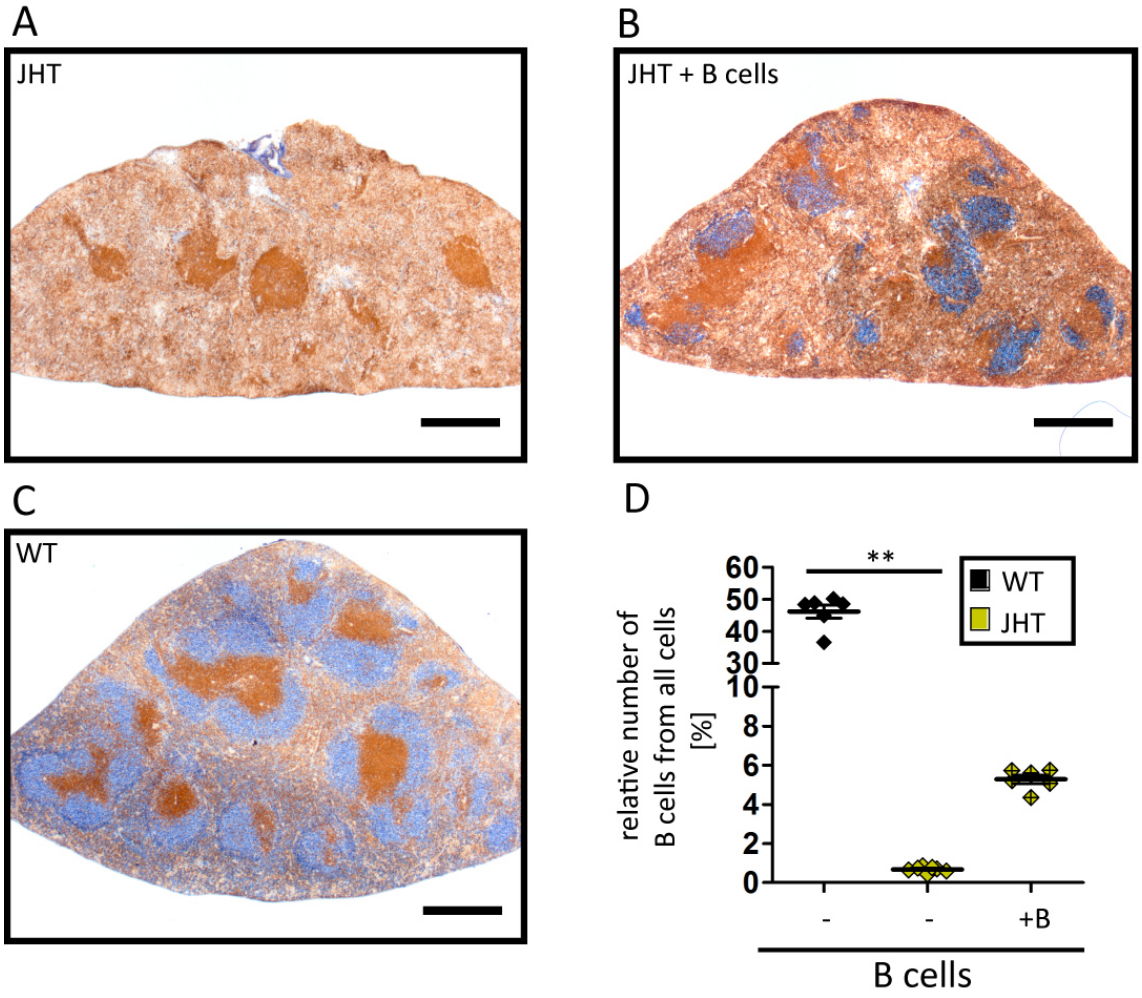

Fig. 10: B cell transfer into JHT mice led to clustering of B cells around T cell zones.
Immunohistochemically staining of splenic sections from JHT mice without (A) and with (B) B cell transfer 2 days prior, and a WT control spleen with intact microarchitecture (C). Scale bar represents 500 µm, TCRβ (brown), B220 (blue). Flow cytometric quantification of B cells ($CD19^+$, $B220^+$) in the spleen of WT and JHT mice without and with B cell transfer (D). Horizontal lines represent mean ±SEM (Kruskal-Wallis test), n=6, **p<0.01.

Naïve JHT mice showed areas where T cells (stained with TCRβ (brown)) accumulated, forming TCZs (Fig 10A). B cells (stained with B220 (blue)) were detected, accumulating around these TCZs after B cell transfer into JHT mice (Fig. 10B). Nevertheless, JHT mice with transferred B

cells showed significantly less B cells than control spleens (Fig. 10C). This was quantified by flow cytometric measurement of B cells in the spleens of these mice (Fig. 10D), revealing only 5 % of B cells in JHT spleens after the transfer.
In consequence, the number of transferred B cells reaching the spleen was significantly below the target number of 20 % and was rated too low to reliably investigate the influence of the transferred B cells.

In conclusion, it was shown that a T_H2 inducing HD immunization recruited B cells into the immune response. The importance of this recruitment was further confirmed by the absence of T_H2 development in B cell deficient mice.

3.2 T_H1/T_H2 differentiation can be modulated by C5aR1

Beside the three major regulators of T_H cell differentiation (cytokine milieu, TCR avidity to the pMHC-II and antigen dose), complement receptors such as C5aR1 have been shown to modulate the outcome of the differentiation. However the mechanism behind this is not known so far. Some studies suggested an impairment of one T_H cell subset. Additionally, some publications showed reduced pMHC-II availability by C5aR1$^{-/-}$ APCs (Peng et al. 2009; Weaver et al. 2010). This present study has shown that B cell dependent pMHC-II availability is a crucial checkpoint to induce T_H2 cells (3.1). Therefore, it is suggested that the C5aR1$^{-/-}$ dependent suppression of the pMHC-II availability by B cells suppresses the T_H2 development.

3.2.1 C5aR1 deficiency reduces the SRBC induced T_H2 response in the spleen

It was shown that B cell dependent pMHC-II availability is required to induce T_H2 differentiation (3.1.4). C5aR1 is expressed on B cells and C5aR1$^{-/-}$ B cells express significantly less surface MHC-II (Weaver et al. 2010). Therefore, C5aR1$^{-/-}$ mice were analysed in order to confirm a reduced B cell dependent pMHC-II availability, which could result in a reduced T_H2 response after HD immunization. In order to analyse the pMHC-II availability by B cells, the number of B cells and their MHC-II density was measured.

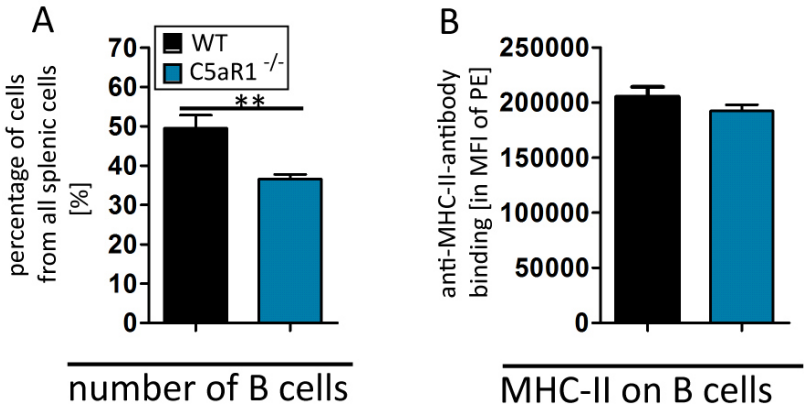

Fig. 11: C5aR1$^{-/-}$ showed decreased B cell numbers but no significant reduction of surface MHC-II. Quantification of B cells (CD19$^+$, B220$^+$) (A) and their MHC-II surface expression (B) from cells of spleens from WT and C5aR1$^{-/-}$ mice, using flow cytometry. Bars represent mean ±SEM (Kruskal-Wallis test), n=6, **p<0.01.

The number of B cells was significantly reduced to 37 % of all splenic cells in C5aR1$^{-/-}$ compared to 50 % in WT spleens (Fig. 11A). The MHC-II expression was slightly reduced on B cells of C5aR1$^{-/-}$ mice, without being significantly different (Fig 11B).

Although the pMHC-II density was not significantly reduced on B cells, as was previously observed (Weaver et al. 2010), the pMHC-II availability was reduced due to a significant

reduction of B cells in the spleens of C5aR1$^{-/-}$ mice. In consequence a reduced T_H2 development in C5aR1$^{-/-}$ mice after HD immunization was expected. This was tested by evaluating IL-4 mRNA expression 3 days after HD immunization.

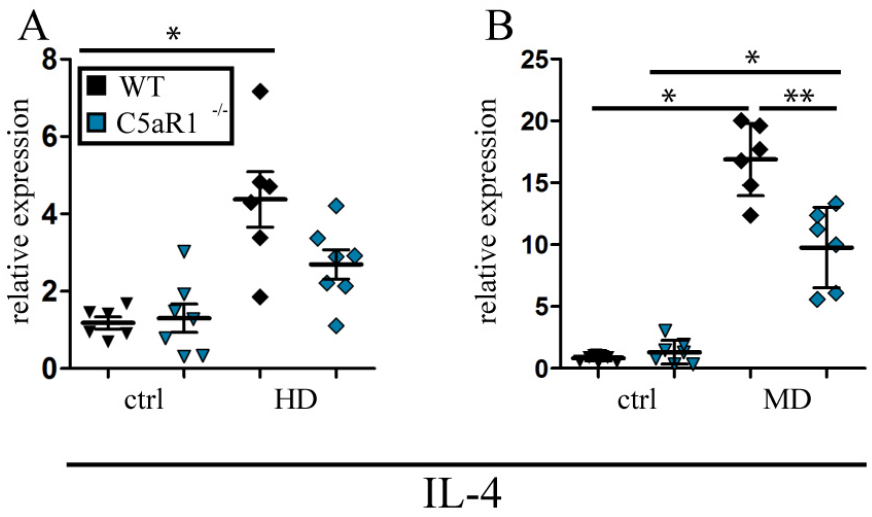

Fig. 12: IL-4 expression in the spleen of C5aR1$^{-/-}$ mice was reduced after HD and MD immunization. Relative mRNA expression of IL-4 in spleens on day 3 post immunization with PBS (ctrl) or 10^9 SRBCs (HD) (A) or PBS (ctrl) or 10^7 SRBCs (MD) (B). Horizontal lines represent mean ±SEM (Kruskal-Wallis test and Mann-Whitney test), *p<0.05, **p<0.01.

The IL-4 mRNA expression in the spleen of WT mice was significantly increased after HD immunization compared to the control mice (Fig. 12A). As hypothesized, C5aR1$^{-/-}$ mice developed less IL-4 mRNA after HD immunization, just trending towards significance (p=0.05). HD immunization induces a strong T_H2 response. Since the modulatory effect of C5aR1$^{-/-}$ on the pMHC-II availability is weaker than expected, it was assumed that the C5aR1 dependent modulation could be more prominent during a weaker T_H2 response. As the T_H2 response to SRBCs can be weakened by reducing the antigen dose, a medium dose (MD) of SRBCs was applied. The MD immunization induced a significant increase of IL-4 expression in both mouse groups compared to the control (Fig. 12B). Furthermore, IL-4 expression was significantly reduced in MD immunized WT mice compared to MD immunized C5aR1$^{-/-}$ mice.

These data show that C5aR1$^{-/-}$ mice develop weaker T_H2 responses. This decrease in the T_H2 response is even more pronounced after the application of a MD of antigen. This shows that a HD of antigen can overcome the effect of C5aR1$^{-/-}$. Furthermore, it is indicated that lower numbers of B cells in the spleen of C5aR1$^{-/-}$ mice reduced the pMHC-II availability.

3.2.2 C5aR1 deficiency modulated the T_H cell differentiation in the *L. major* model

The *L. major* infection model in Balb/c mice is another antigen dose dependent model. However, it is a more complex model than SRBCs, having separated locations for T cell activation (dLN) and T cell effector phase (skin). Therefore, infection control requires the T_H1 dependent IFN-γ secretion in the dLN as well as in the skin. In the next section, this second antigen dose model was established. Afterwards, it was assessed whether the effect of C5aR1 on the T_H1/T_H2 differentiation, which has been shown in the SRBC model, can be applied also in this more complex infection model.

3.2.2.1 Establishment of the antigen dose dependent *L. major* model

The influence of the antigen doses on the infection progression and the T_H1/T_H2 development in the *L. major* model was monitored in response to three different antigen doses.

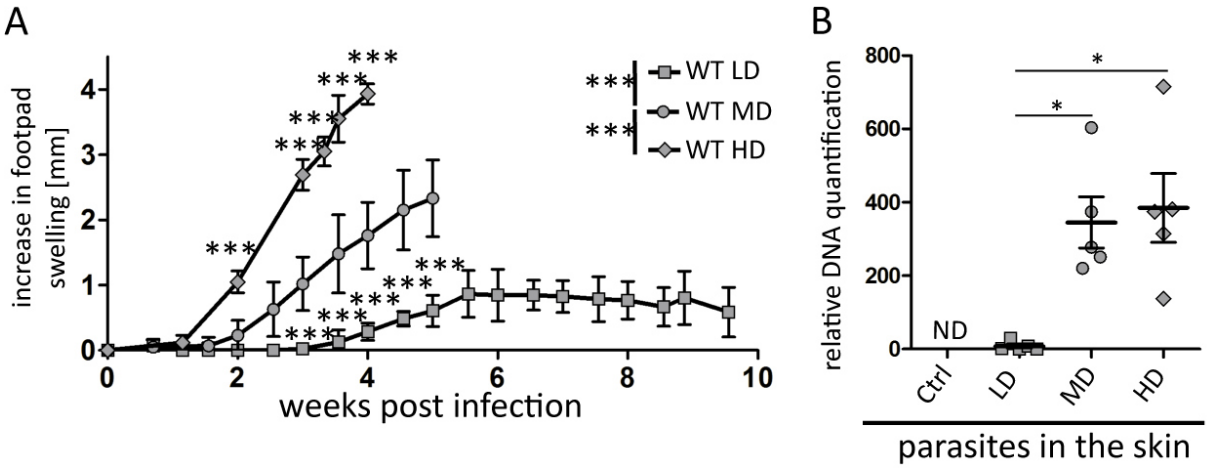

Fig. 13: Footpad swelling and parasite burden were dependent on the *L. major* antigen dose.
Balb/c mice were infected with 1000 (LD), 33000 (MD) or 1 mio *L. major* promastigotes in the right hind footpad. The infection induced footpad swelling, which was measured twice a week. Increase in footpad swelling was determined as the measured difference between infected footpad and control (left hind) footpad (A). LD mice were observed until week 10, whereas MD and HD infected mice were sacrificed after 5 and 4 weeks, respectively, due to severe wounds. Dots represent mean ±SEM (Two-way ANOVA), in graph asterisks mark significant differences to same dpi MD infected mice (significance of LD to HD not shown), legend asterisks mark overall infection severity differences n=5-15 (LD and HD-MD). Relative quantification of *L. major* polymerase POLA to BNDF in footpad skin (B) 10 weeks (LD mice), 5 weeks (MD mice) or 4 weeks (HD mice) post infection with the respective number of *L. major* promastigotes. Horizontal lines represent mean ±SEM (Kruskal-Wallis test), n= 5-10, *p<0.05, ***p<0.001.

Balb/c mice were infected with 1000 (LD), 33000 (MD) or 1 mio (HD) *L. major* promastigotes in the right hind footpad on day 0. The footpad swelling was used as a readout for the infection progression over time. LD infected mice developed only minor footpad swelling (Fig. 13A), without severe infection symptoms. MD infected mice developed footpad swelling from week

2 onwards. These mice developed severe infections, and were sacrificed 5 weeks post infection, due to infection symptoms (open wounds). HD infection led to the fastest and most severe onset of the footpad swelling. These mice were sacrificed at week 4 due to severe infection symptoms such as open wounds and loss of tissue. The parasite burden correlated with the footpad swelling, showing significantly reduced parasite numbers in LD infected mice compared to MD and HD infected mice (Fig. 13B). No difference in parasite burden between MD and HD infection was detectable.

In conclusion LD infected mice were able to control the infection whereas MD and HD infections led to the development of a severe infection phenotype requiring the sacrifice of the mice after week 5 and 4, respectively.

3.2.2.2 The influence of the antigen dose on the T_H1/T_H2 differentiation in the dLN

The different infection progressions were correlated with the T_H1/T_H2 response by measuring IFN-γ and IL-4 in the dLN. The T_H1/T_H2 response in the dLN is thought to be a major check point for the infection control in this model (Nicolas et al. 2000).

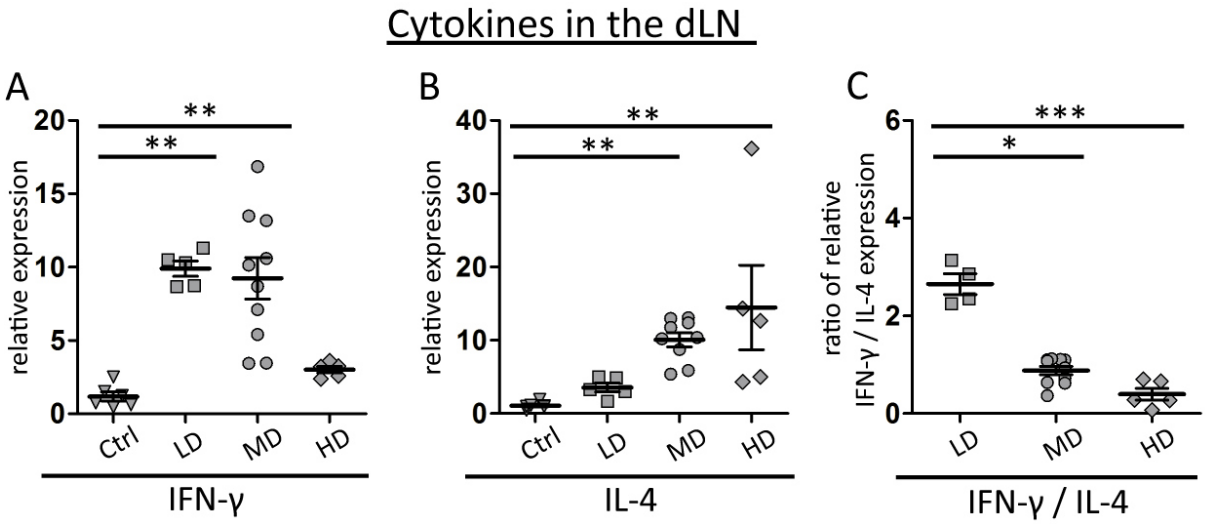

Fig. 14: The T_H1/T_H2 ratio in the dLN is significantly modulated by the antigen dose.
Relative mRNA expression of IFN-γ (A) and IL-4 (B) in the dLN, 10 weeks (LD mice), 5 weeks (MD mice) or 4 weeks (HD mice) post infection with the respective number of *L. major* promastigotes. Results from (A) and (B) depicted in a ratio of IFN-γ divided by IL-4 expression (C). Horizontal lines represent mean ±SEM (Kruskal-Wallis test), n= 5-10, *p<0.05, **p<0.01, ***p<0.001.

IFN-γ expression in the dLN of LD and MD infected mice is significantly increased compared to control mice and ≈3-fold higher compared to HD infected mice (Fig. 14A). Regarding IL-4 mRNA expression, LD infected mice did not increase the expression significantly compared to control mice, whereas MD and HD infected mice did, expressing 3-4 fold more IL-4 mRNA than LD

infected mice (Fig. 14B). These results were used to calculate a ratio of IFN-γ divided by IL-4 expression. This ratio showed that LD infected mice developed a high IFN-γ/IL-4-ratio (≈4:1), which was significantly reduced in MD infected mice (≈1:1) and even more diminished in HD infected mice (<1:1) (Fig. 14C).

3.2.2.3 The influence of the antigen dose on the T_H1/T_H2 differentiation in the infected skin

L. major infection control requires the T_H1 dependent IFN-γ secretion also in the skin. Consequently the skin immune response was analysed by measuring IFN-γ and IL-4.

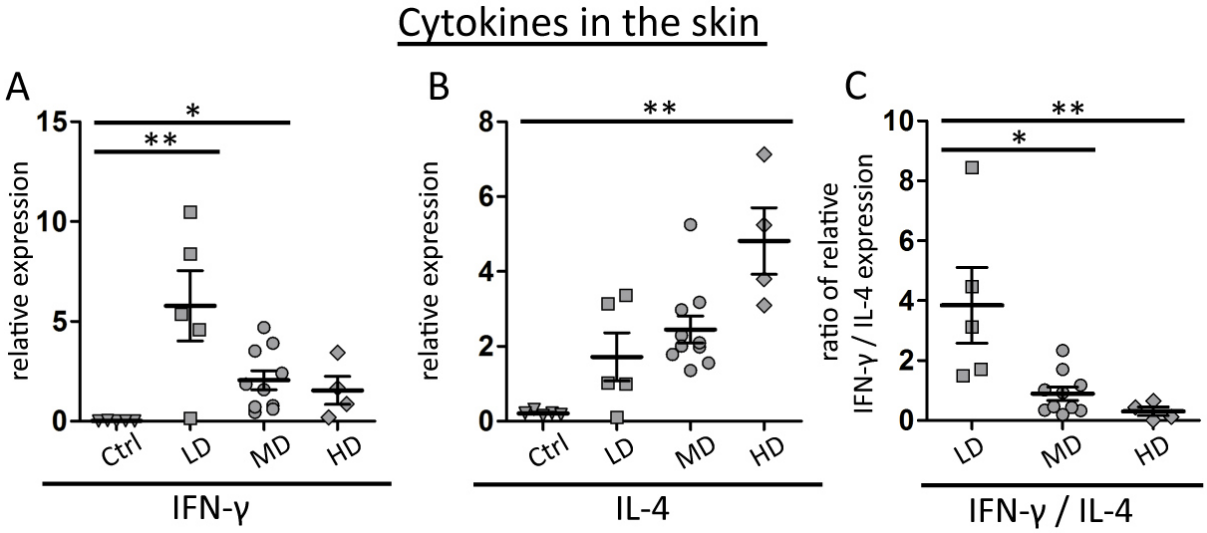

Fig. 15: Clear distinction of LD from MD/HD infected mice by the IFN-γ/IL-4 ratio in infected skin. Relative mRNA expression of IFN-γ (A) and IL-4 (B) in footpad skin, 10 weeks (LD mice), 5 weeks (MD mice) or 4 weeks (HD mice) post infection with the respective number of *L. major* promastigotes. Results from (A) and (B) depicted in a ratio of IFN-γ divided by IL-4 expression (C). Horizontal lines represent mean ±SEM (Kruskal-Wallis test), n=4-10, *p<0.05, **p<0.01.

The cytokine mRNA expression patterns in the infected skin samples showed a close correlation to the dLN samples. IFN-γ mRNA was significantly higher expressed compared to control mice only in footpads of LD and MD, but not in HD infected mice (Fig. 15A). LD infected mice showed the highest IFN-γ expression. IL-4 mRNA was expressed slightly higher in LD and MD, and significantly higher in HD infected mice, compared to control mice (Fig. 15B). As shown for the dLN, the IFN-γ/IL-4 ratio was calculated, resulting in a significantly higher ratio in LD samples (≈4:1) compared to MD (≈1:1) and HD (<1:1) (Fig. 15C).

As expected, reducing the antigen dose led to a significant decrease in footpad swelling and parasite burden. LD infected mice were able to control the parasite burden by developing a

high IFN-γ/IL-4 ratio in the dLN and the skin. MD and HD infected mice could not control infection, developing significantly lower ratios.

These data confirmed that the antigen dose modulates T_H1/T_H2 differentiation in the *L. major* model in order to control the infection severity. Therefore, this antigen dose dependent model was further used for the assessment of the effect of C5aR1 on the T_H1/T_H2 differentiation.

3.2.2.4 The effect of C5aR1 deficiency on the development of *L. major* infection

C5aR1$^{-/-}$ modulated T_H1/T_H2 differentiation in an antigen dose dependent manner in the SRBC model, correlating with reduced pMHC-II availability by B cells (3.2.1). Therefore, it was investigated whether C5aR1 dependent reduction of the B cell dependent pMHC-II availability can modulate the T_H cell response in this model too. It has been described before that C5aR1$^{-/-}$ Balb/c mice have less MHC-II on B cells (Weaver et al. 2010), which could give an indication for a comparable outcome in the *L. major* model.

3.2.2.4.1 The influence of C5aR1 deficiency on the pMHC-II availability of B cells

At first it was tested whether C5aR1$^{-/-}$ mice have significantly reduced pMHC-II availability from B cells in their lymph node (LN). Therefore the pMHC-II availability was assessed by quantifying B cells and their MHC-II expression in the dLN.

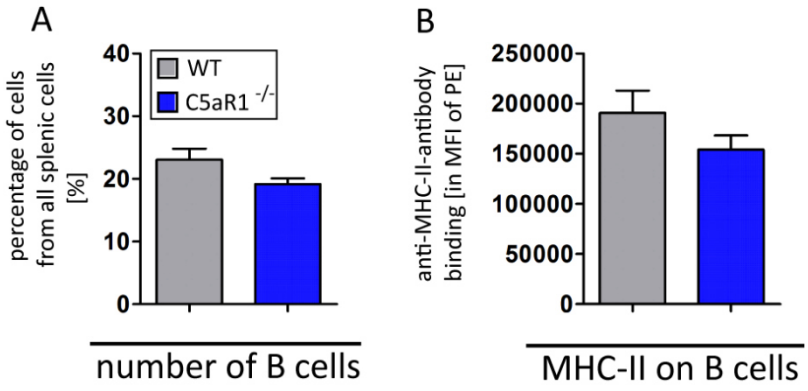

Fig. 16: C5aR1$^{-/-}$ has no influence on pMHC-II availability in Balb/c mice.
Quantification of B cells (A) and their MHC-II surface expression (B) with flow cytometry from cells of control LNs. Bars represent mean ±SEM (Kruskal-Wallis test), n=10-11.

Flow cytometric quantification of B cells within the dLN cellularity showed ≈20 % B cells in WT and C5aR1$^{-/-}$ mice (Fig. 16A). pMHC-II density was analysed on the surface of these cells, revealing no difference due to C5aR1$^{-/-}$ (Fig. 16B).

Unexpectedly, these results could not demonstrate the previously described significant reduction of pMHC-II availability on B cells by C5aR1$^{-/-}$.

3.2.2.4.2 The influence of C5aR1 deficiency on the footpad swelling phenotype

The infection severity in C5aR1$^{-/-}$ Balb/c mice was characterized by measuring the dose dependent footpad swelling in comparison to WT mice. Since no difference in pMHC-II availability by B cells was detected, no impaired T_H cell differentiation in the LN and consequently no altered phenotype was expected.

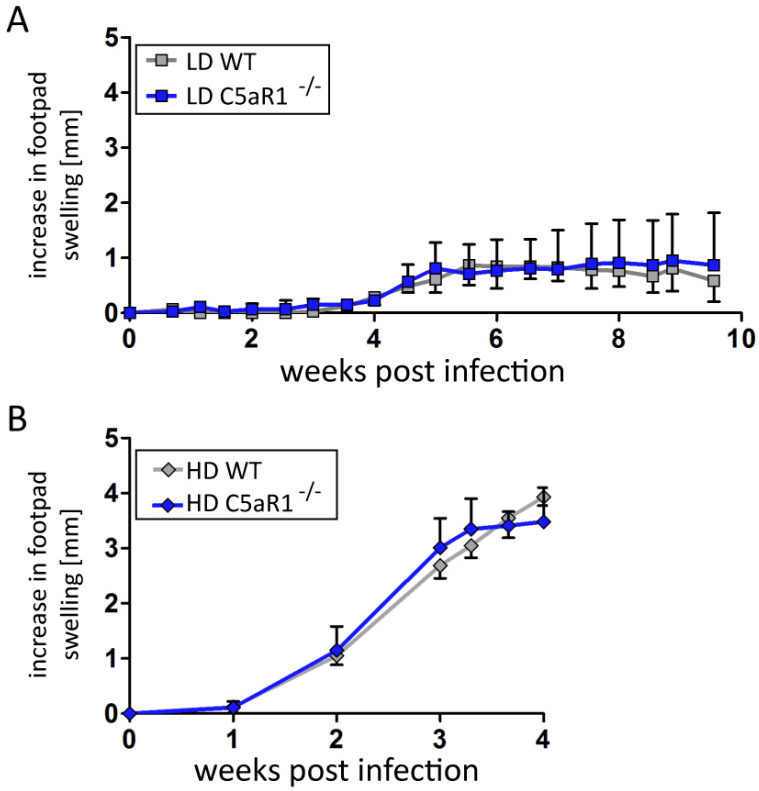

Fig. 17: C5aR1$^{-/-}$ showed no difference in footpad swelling in LD or HD infected mice compared to WT mice.
The increase in footpad swelling was determined as the detected difference between infected (right hind) footpad and control (left hind) footpad, after infection with 1.000 (LD; A) or 1.000.000 (HD; B) *L. major* promastigotes. Dots represent mean ±SEM (Two-way ANOVA), n=5.

Indeed, LD infected WT and C5aR1$^{-/-}$ mice did not develop severe lesions during *L. major* infection. Increased footpad thickness was measured around week 4, but the values were below 1 mm of increase due to swelling (Fig. 17A). In the HD infection model both mouse groups developed severe lesions. The beginning of the footpad swelling was detected around week 2 (Fig. 17B). 3 weeks post infection severe symptoms appeared with the first crusted areas on the skin. After 4 weeks mice were sacrificed due to high percentage of necrotic areas at some footpads. For both antigen dose infections, no modulation of infection severity due to C5aR1$^{-/-}$ was detected.

Since C5aR1$^{-/-}$ showed a dose dependent modulation of the T_H cell differentiation in the SRBC model, the *L. major* infection model was investigated in response to a MD infection too.

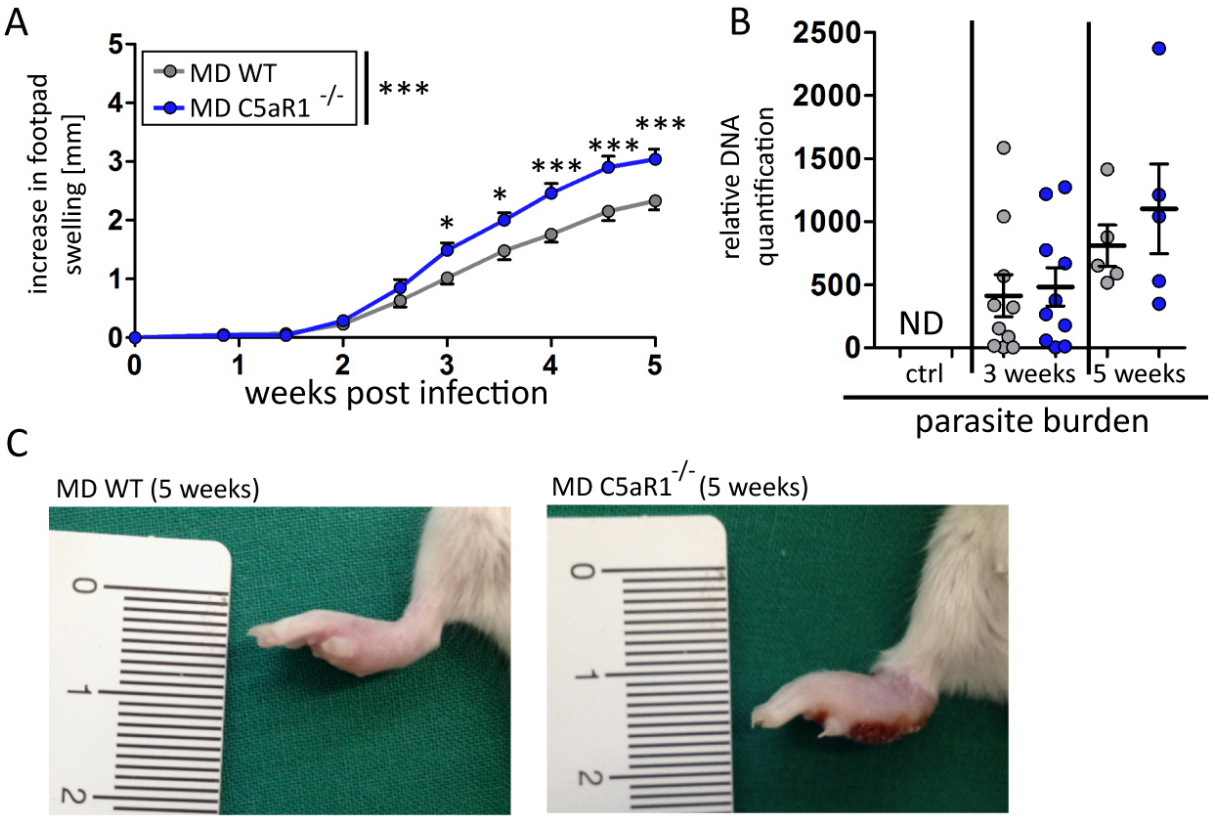

Fig. 18: MD infected C5aR1$^{-/-}$ mice developed significantly more severe lesions compared to WT mice. Increase in footpad swelling was determined as the detected difference between infected (right hind) footpad and control (left hind) footpad, after infection with 33.000 (MD) *L. major* promastigotes (A). Dots represent mean ±SEM (Two-way ANOVA), asterisks at legend indicate significant differences in overall disease progression, n=10. Relative quantification of parasites with *L. major* polymerase POLA in control mice and 3 and 5 weeks post infection with a MD of parasites (B). Horizontal lines represent mean ±SEM (Mann-Whitney test), n=5-10. Representative pictures from infected hind footpads of WT and C5aR1$^{-/-}$ mice, 5 weeks post MD infection (C). *p<0.05, ***p<0.001, ND = not detectable.

After MD infection, both groups developed footpad swelling around week 2 post infection. Unexpectedly, C5aR1$^{-/-}$ mice showed more severe lesions, starting with swelling at day 18, turning significant from week 3 onwards till the end of the experiments (Fig. 18A). This also included more necrotic areas and open wounds after 5 weeks (Fig. 18C). The overall infection progression differed with p<0.001 (Two-way ANOVA). Due to this difference in infection progression, the parasite burden was quantified after week 3, when the first significant differences appeared between the mouse groups, and week 5, at the end of the experiment. However no significant difference in parasite burden was detectable between WT and C5aR1$^{-/-}$ mice (Fig. 18B)

3.2.2.4.3 The influence of C5aR1 deficiency on the T_H1/T_H2 differentiation in the dLN

To investigate whether increased footpad swelling in MD infected C5aR1$^{-/-}$ mice correlates with the T_H cell response in the secondary lymphoid organ, IFN-γ and IL-4 was measured in the dLN.

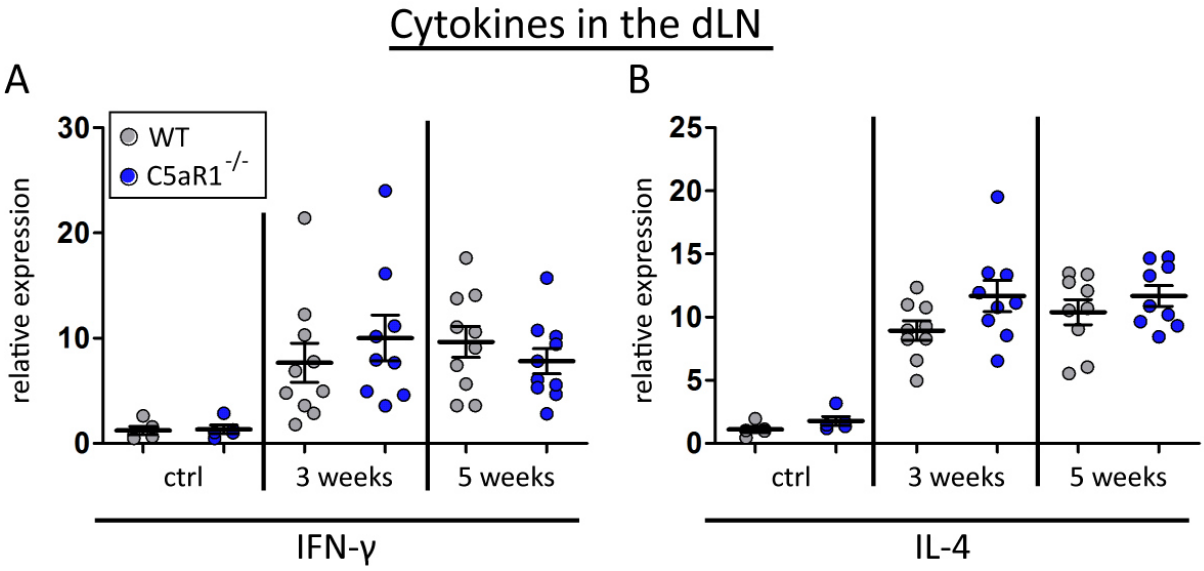

Fig. 19: IFN-γ and IL-4 expression were not impaired in dLN of C5aR1$^{-/-}$ mice compared to WT mice. Relative mRNA expression of IFN-γ (A) and IL-4 (B) in the dLN of control mice, or 3 weeks and 5 weeks post infection with 33.000 (MD) *L. major* promastigotes. Horizontal lines represent mean ±SEM (Mann-Whitney test), n=5-10.

The *L. major* infection model was monitored over the duration of 5 weeks. To analyse the development of the cytokine pattern in MD infected WT and C5aR1$^{-/-}$ mice, three important stages were selected. First, control mice were used to analyse the cytokine expression in naïve LNs. Second, 3 weeks post infection states were used because it is has been shown that at this point the T_H cell fate decision appears strongly (Barthelmann et al. 2012). And third, 5 weeks post infection represented the end point of the experiment, when all the mice were sacrificed due to severe infection symptoms in C5aR1$^{-/-}$ mice.

The IFN-γ mRNA expression in the dLN is significantly increased after infection (≈8-10 fold; significance not shown), however neither significant differences between week 3 and week 5 post infection, nor between WT and C5aR1$^{-/-}$ mice could be detected (Fig. 19A). IL-4 mRNA measurements showed the same pattern, revealing no difference in IL-4 mRNA expression due to C5aR1$^{-/-}$ (Fig. 19B).

Analyses of the dLN revealed no modulation of pMHC-II availability (Fig. 16), consequently T_H cell differentiation was not impaired in the dLN (Fig. 19). Nevertheless, it remains unclear, why MD infected C5aR1$^{-/-}$ mice develop a more severe phenotype.

3.2.2.4.4 The influence of C5aR1 deficiency on the T_H1/T_H2 differentiation in infected skin

WT and C5aR1$^{-/-}$ mice showed no difference in T_H1/T_H2 differentiation in the dLN. However, MD infection induced a more severe infection progression in the skin of C5aR1$^{-/-}$ mice. Therefore, the IFN-γ and IL-4 expression was measured in the footpad skin to assess the T_H1/T_H2 response. Taking increased footpad swelling into account, less IFN-γ expression was expected in the skin of MD infected C5aR1$^{-/-}$ mice, although the dLNs did not show this pattern.

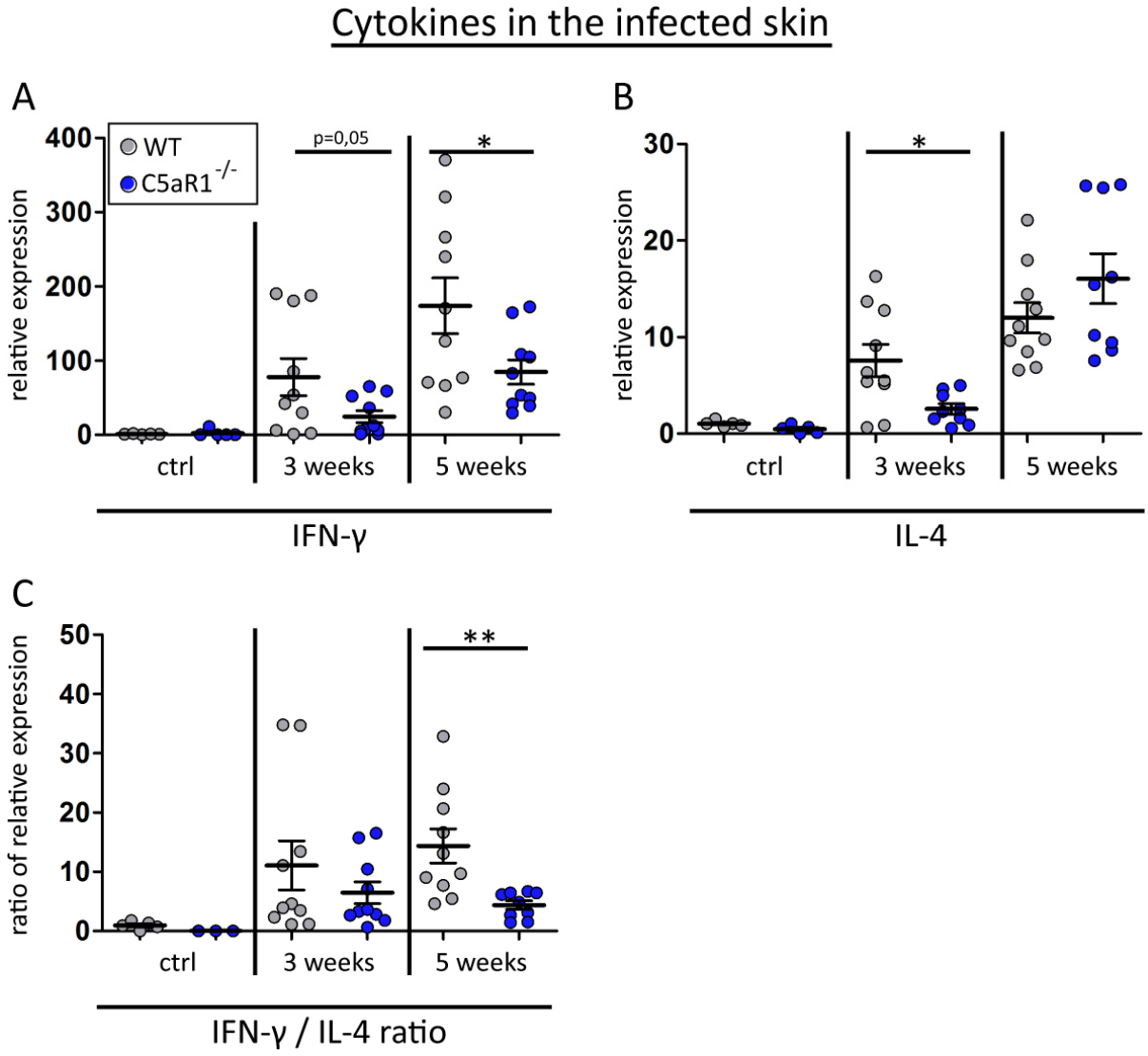

Fig. 20: C5aR1$^{-/-}$ skin samples expressed almost no IFN-γ or IL-4 after 3 weeks and significantly less IFN-γ after 5 weeks.
Relative mRNA expression of IFN-γ (A), IL-4 (B) in footpad skin of control mice or 3 weeks and 5 weeks post infection with 33.000 (MD) *L. major* promastigotes. Results from (A) and (B) are depicted in a ratio of IFN-γ divided by IL-4 expression (C). Horizontal lines represent mean ±SEM (t test and Mann-Whitney test), n=5$^{\$}$-10, *p<0.05, **p<0.01. $^{\$}$Two samples of C5aR1$^{-/-}$ control mice showed no detectable IL-4 expression. Therefore, IFN-γ/IL-4 ratio could not been calculated, although IL-4 measurement was valid. Therefore only n=3 are depicted in C5aR1$^{-/-}$ ctrl of (C).

3 weeks post infection, WT mice revealed 3-fold higher IFN-γ mRNA expression than C5aR1$^{-/-}$ mice (Fig. 20A). This resulted in a statistical p-value of 0.05, which is almost significant. 5 weeks

post infection this difference became significant. IL-4 expression was also significantly increased in WT mice after 3 weeks, compared to C5aR1$^{-/-}$ mice (Fig. 20B). Although not significant, C5aR1$^{-/-}$ mice developed slightly more IL-4 expression compared to WT mice, 5 weeks post infection. As previously done, a ratio was calculated from these data, revealing a ≈10:1 IFN-γ/IL-4 ratio in both mouse groups at week 3 (Fig. 20C). This ratio increased within the next two weeks in WT mice, up to a 13:1 ratio. In contrast, the ratio decreased in this timespan to a 3:1 ratio in C5aR1$^{-/-}$ mice. This was significantly different to WT mice.

These results demonstrated that although footpad swelling was significantly more severe in C5aR1$^{-/-}$ mice after 3 weeks, almost no T cell cytokine response was detectable in the skin of these mice, showing expression levels close to the detection minimum. These expression patterns were different to the dLN patterns. In order to visualize this unexpected difference, the data shown above were depicted within one graph, showing all significant differences between WT and C5aR1$^{-/-}$ mice on the side of the infected skin, but not on the side of the dLN (Fig. 21). These data indicated that C5aR1 signalling has no effect on the cellularity or the pMHC-II availability in the dLN, but is important for a protective skin reaction to *L. major*. This lead to the hypothesis that the impaired skin reaction of C5aR1$^{-/-}$ mice is the reason for the more severe lesions in these mice.

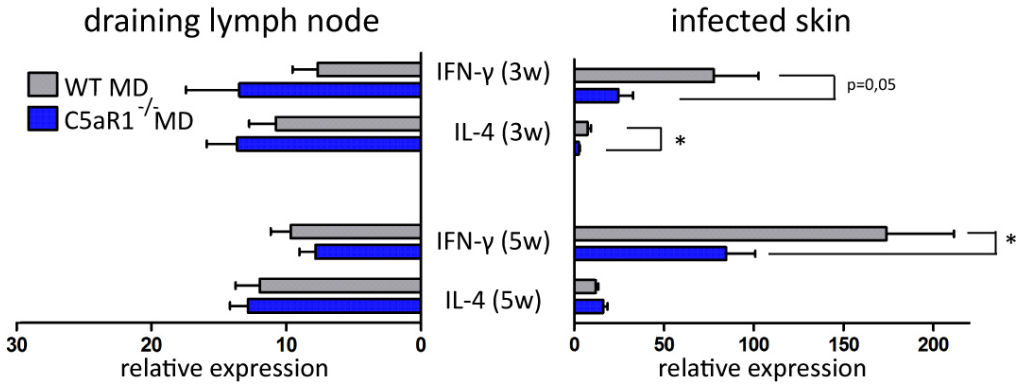

Fig. 21: C5aR1$^{-/-}$ dependent IFN-γ or IL-4 expression differences to the WT were detectable only in skin samples.
Different depiction of above shown results. Relative mRNA expression of IFN-γ and IL-4 in the dLN (left side) and footpad skin (right side) of mice 3 weeks (3w) and 5 weeks (5w) post infection with 33.000 *L. major* promastigotes. Bars represent mean ±SEM (t test and Mann-Whitney test), n=5-10, *p<0.05.

C5aR1 has been shown to modulate the T_H1/T_H2 differentiation differently in multiple models. This study hypothesized that in antigen dose dependent T_H1/T_H2 models the C5aR1 dependent pMHC-II availability provides the mechanism to explain the experienced differences of decreased T_H2 responses. In the SRBC mouse model the reduction of B cell dependent pMHC-

II availability was able to supress the T_H2 response after MD immunization in the spleen. This showed that C5aR1 modulation was tightly regulated and is overruled by the effect of the antigen dose outside of a small range of antigen doses.

In the *L. major* model, C5aR1$^{-/-}$ mice fail to modulate the pMHC-II availability. Consequently, the T_H cell response was not modulated in the dLN. Nevertheless, C5aR1$^{-/-}$ mice developed more severe skin lesions after MD infection, which were considered to be the result of an impaired skin immune reaction. This shows that the effect of C5aR1$^{-/-}$ is dependent on the antigen dose.

Therefore, it is concluded that C5aR1$^{-/-}$ dependent modulation of T_H1/T_H2 differentiation in secondary lymphoid organs in these antigen dose dependent models, depends on the influence of C5aR1 on the pMHC-II availability. However, migratory effects become important in subcutaneous T_H1/T_H2 differentiation. Furthermore the influence of C5aR1 on the pMHC-II availability was different between C57Bl/6 and Balb/c mice. Therefore the influence seems to be affected by the genetic background of the mouse strain.

3.3 The influence of the second TCR-pMHC-II encounter on the number of HECs after HD SRBC immunization

The T cell clonality within an immune response affects the T_H1/T_H2 differentiation (Rudulier et al. 2014). One known factor to influence the T_H cell clonality is the antigen dose (Obst et al. 2005). This study has shown that increased pMHC-II availability by B cells after HD SRBC immunization led to the development of T_H2 cells in the spleen (3.1), demonstrating that HD immunization induced the second T_H cell encounter in the spleen. Combining those facts, it is hypothesized that the HD induced second T_H cell encounter induces the observed shift in T_H cell clonality.

In order to assess the shift, the number of HEC clonotypes, as well as their individual abundance in the clonality of an immune response was measured.

3.3.1 CD154 is necessary to induce T_H cell effector functions

To confirm that CD154 is necessary for the second encounter which induces T_H cell effector functions (van Essen et al. 1995) such as cytokine release or germinal centre formation, WT and $CD154^{-/-}$ mice were analysed after HD immunization in regard to those two effector functions. Due to the disabled second encounter, no germinal centre formation and effector cytokine release was expected in $CD154^{-/-}$ mice.

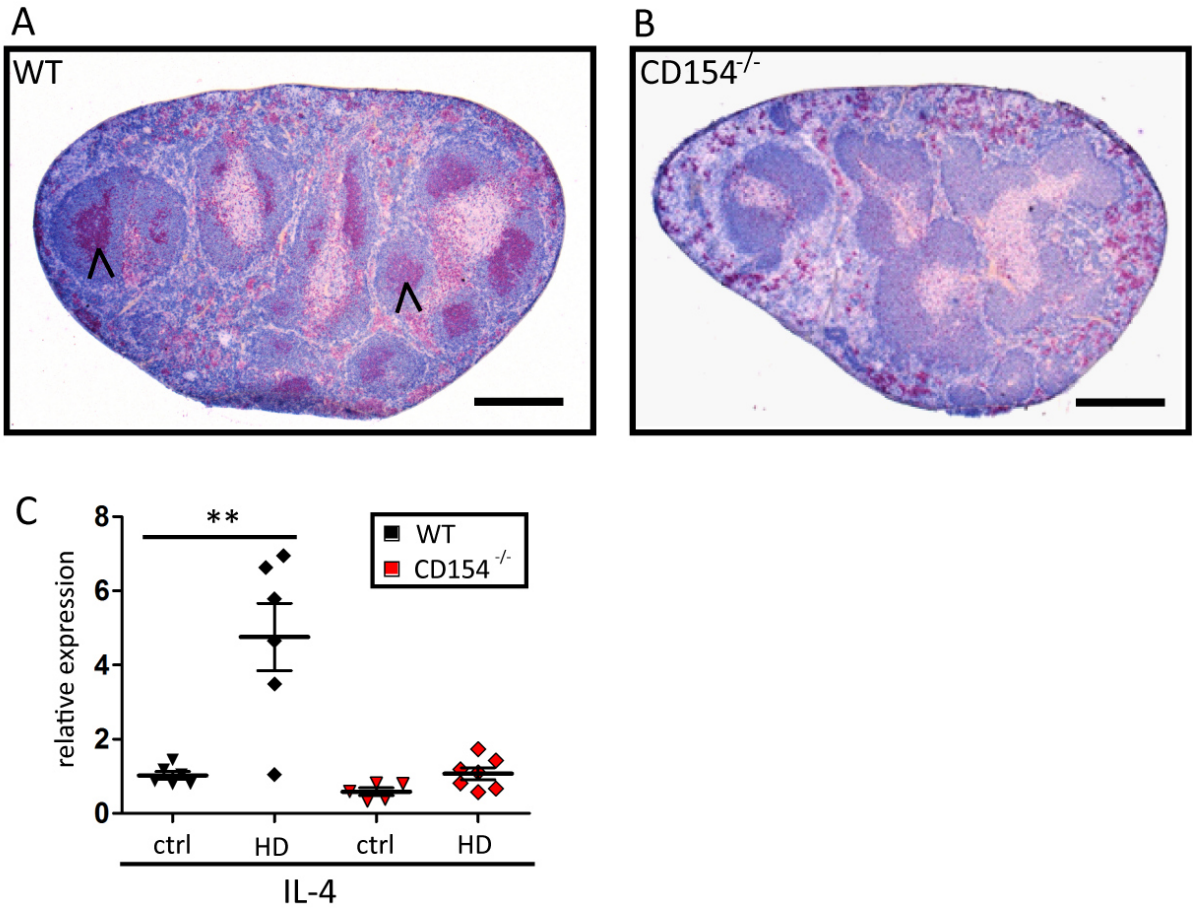

Fig. 22: CD154$^{-/-}$ mice show no IL-4 mRNA after HD immunization.
Immunohistochemically staining of splenic sections from WT (A) and CD154$^{-/-}$ (B) mice, 10 days post HD immunization with Ki-67 (red), B220 (blue), scale bar represents 500 µm, arrows point on germinal centres. Relative expression of IL-4 mRNA from splenic sections, 3 days post HD immunization. Horizontal lines represent mean ±SEM (Kruskal-Wallis test), n=6-7 **p<0.01.

To test this, splenic sections of WT (Fig. 22A) and CD154$^{-/-}$ mice (Fig. 22B) were stained 10 days post HD SRBC immunization with Ki-67 and B220. As expected, WT mice developed germinal centres, whereas CD154$^{-/-}$ mice did not. IL-4 mRNA expression was measured 3 days post HD immunization to analyse the appearance of effector cytokines. WT spleens showed increased IL-4 mRNA expression after HD immunization, whereas CD154$^{-/-}$ spleens did not (Fig. 22C). This demonstrated that no second encounter for T_H cells occurs in the spleen of CD154$^{-/-}$ mice. Consequently, no cytokine producing effector T_H cells appear.

3.3.2 CD154 deficiency does not impair splenic microarchitecture

To exclude that the lack of T_H2 differentiation was caused by an impaired splenic microarchitecture due to the CD154 deficiency, splenic compartments were quantified in detail. Therefore, the naïve spleens of WT and CD154$^{-/-}$ mice were immunohistochemically

stained and structural elements such as white pulp to red pulp ratio and TCZ and BCZ area were quantified.

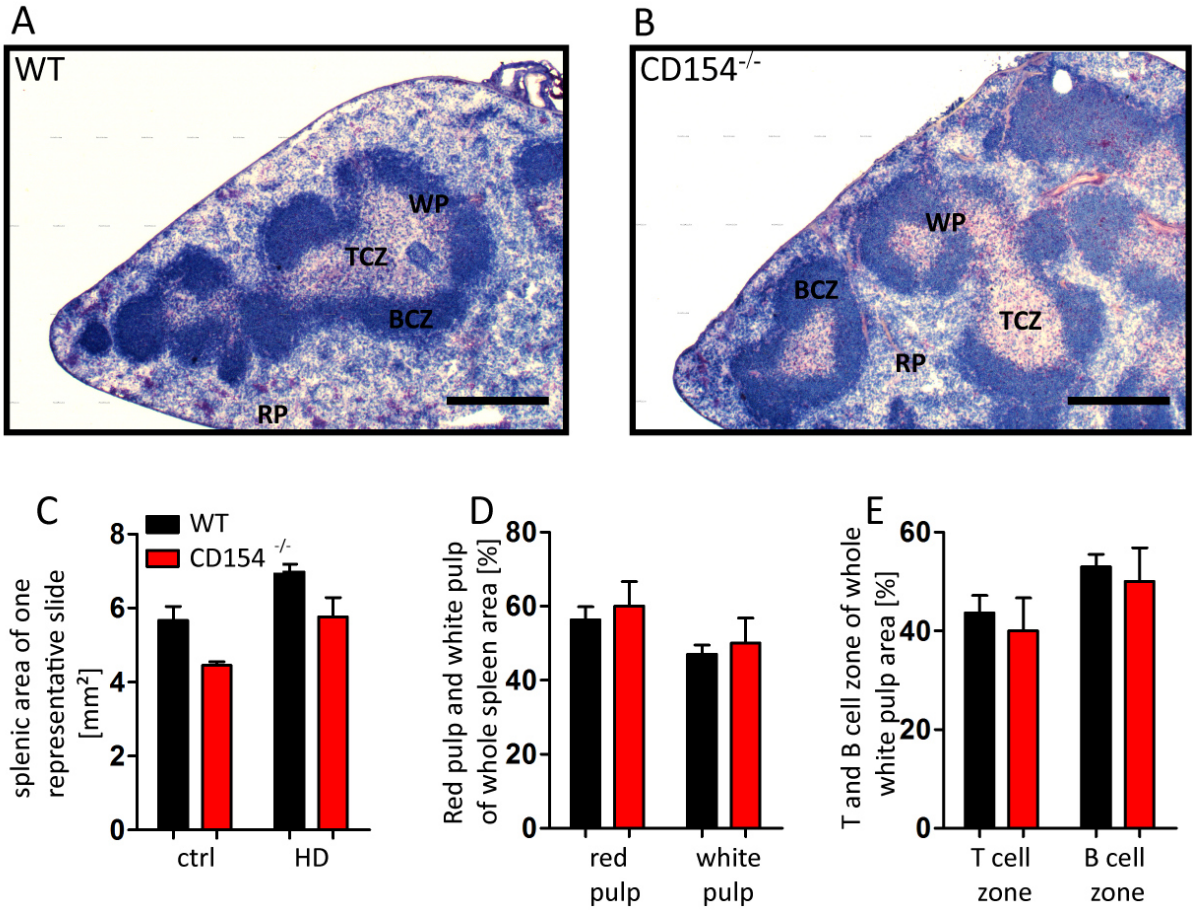

Fig. 23: The splenic structure is not impaired due to CD154$^{-/-}$.
Immunohistochemically staining of splenic sections from naïve WT (A) and CD154$^{-/-}$ (B) mice, with Ki-67 (red), B220 (blue), scale bar represents 500 µm. Quantification of splenic area to measure splenic size (C) in control and HD immunized mice and insurance of an intact splenic structure by measuring red pulp (RP)/white pulp (WP) (D), T and B cell zone (E). Bars represent mean ±SEM (Mann-Whitney test), n=3.

Immunohistochemically staining of naïve splenic sections showed no impaired microarchitecture in CD154$^{-/-}$ mice, revealing normal development of T and BCZs (Fig. 23A; B). Neither the size of spleen sections in naïve (4.4-5.6 mm²) or HD immunized (5.8-7 mm²) mice (Fig. 23C), nor the distribution of structural elements within the spleen (ratio of red pulp to white pulp) or within the white pulp (ratio of TCZ to BCZ) were significantly impaired by the CD154$^{-/-}$ (Fig. 23D; E).

These data demonstrated that the splenic microarchitecture is intact. It was concluded that the first T cell encounter could occur normally. Therefore this model provided a basis to analyse the influence of the second encounter on the T_H cell clonality.

3.3.3 CD154 deficiency does not affect the primary clonal expansion of T cells

To analyse whether CD154 modulates the overall clonal expansion of T cells in the TCZ, the T cell proliferation in CD154$^{-/-}$ mice was compared to WT mice after HD SRBC immunization.

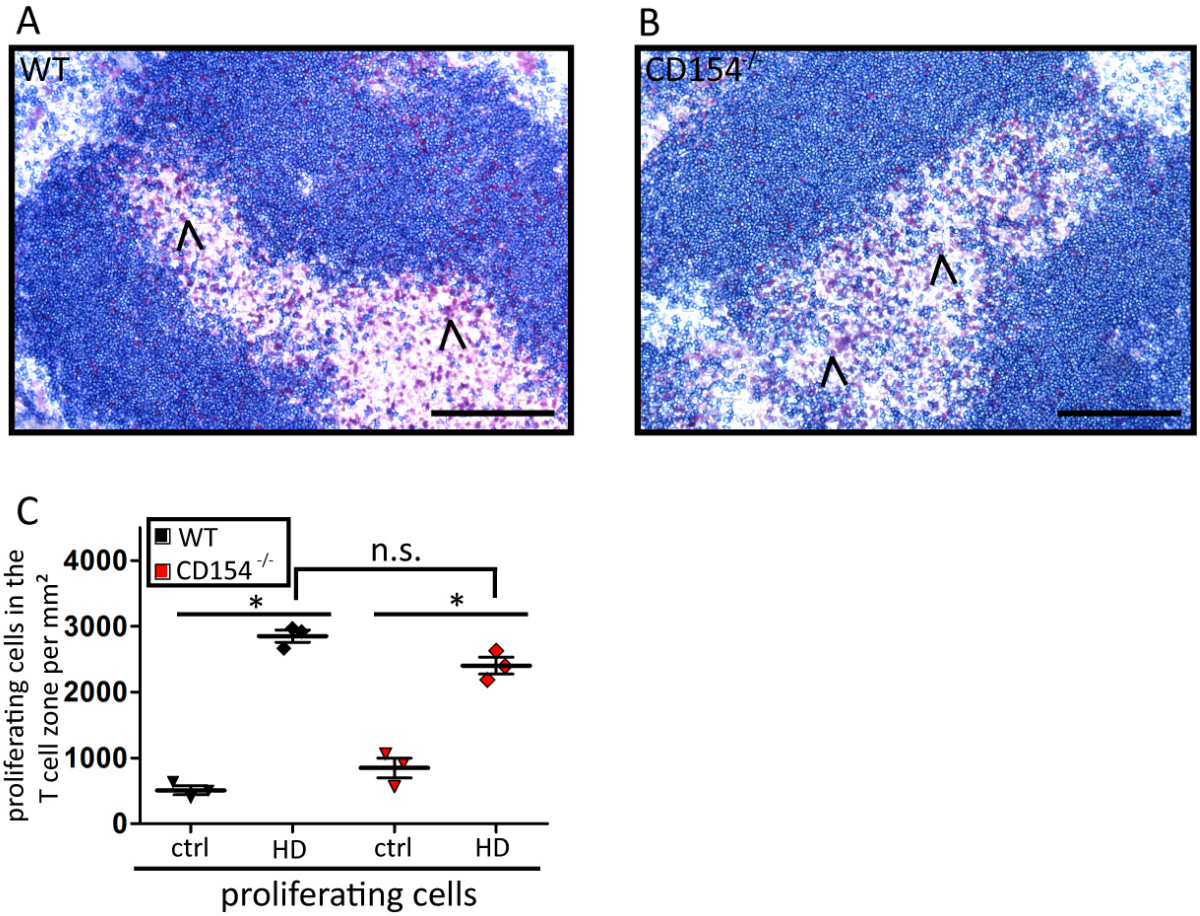

Fig. 24: The T cell proliferation is not impaired due to CD154$^{-/-}$.
Immunohistochemically staining of splenic TCZs from WT (A) and CD154$^{-/-}$ (B) mice, 3 days post HD immunization with Ki-67 (red), B220 (blue), scale bar represents 250 µm, arrows point on proliferating cells in the TCZ. Quantification of Ki-67^{+} cells per mm² of TCZ area on immunohistochemically stained splenic sections. Horizontal lines represent mean ±SEM (Kruskal-Wallis test), n=3, *p<0.05.

T cell proliferation in CD154$^{-/-}$ mice was investigated by immunohistochemically staining of splenic TCZs from WT (Fig. 24A) and CD154$^{-/-}$ mice (Fig. 24B) 3 days post HD immunization with Ki-67 and B220. Afterwards, Ki67^{+} cells within the T cell zones were quantified. TCZs of both naïve mouse groups showed about 500-850 Ki67^{+} cells per mm². After HD immunization, Ki67^{+} cell count increased to 2400-2800 cells per mm² (Fig. 24C).

This result demonstrated that CD154$^{-/-}$ T cells proliferate normally and overall clonal expansion is not altered due to CD154 deficiency.

3.3.4 CD154 deficiency mice showed less TCR reads in TCZ samples

SRBCs are multiepitopic antigens, guiding many different T cell clonotypes to activation and proliferation. Preliminary data have shown that a HD immunization increases the number of proliferating T cells over a LD immunization (Stamm, Dissertation, unpublished).

Since the clonal expansion is not detectable by simple analysis of proliferating T cells, individual T cell clonotypes have to be analysed. This is possible by using the technique of Next Generation Sequencing (NGS). The TCRβs were chosen instead of the TCRα, because TCRβ sequences have a higher variability and in consequence more influence on the final TCR. By sequencing their individual CDR3 regions, it was aimed to determine the TCRβ sequences and define the HECs. A naïve murine spleen contains more than 30 mio T cells. It is technically not possible to analyse all TCRβ clonotypes at once and follow their clonal expansion. Therefore two individual samples of each spleen were analysed by isolation of single TCZs. To avoid any kind of unwanted bias during T cell isolation, T cell zones were isolated directly from cryosection by laser microdissection and analysed.

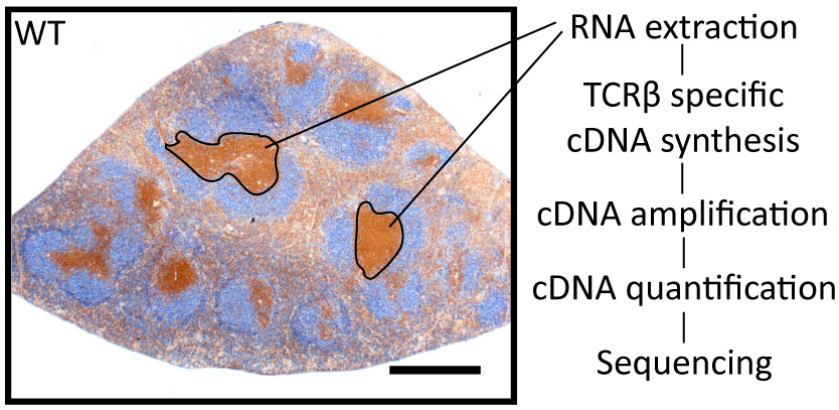

Fig. 25: Workflow for the sequencing of two individual TCZs from one mouse.
Two different splenic TCZs from one mouse were dissected with the laser microdissection from serial spleen sections. RNA was extracted from each individual sample, and TCRβ specific primers were used for reverse transcriptase cDNA synthesis. The cDNA was amplified and normalized to 2 pM cDNA per sample on a sequencing flow cell. Scale bar represents 500 µm.

The workflow is described in 2.10 but a brief summary of the steps is depicted in figure 25. The results of the intermediate steps as well as the sequencing procedures are listed below (Tab. 2).

Tab. 2: List of sequenced T cell zones with results.
Source of TCZ (animal and its treatment), microdissected area in mm² (TCZ size) and results from the sequencing run (reads; intact TCRβ-CDR3 reads "after PEAR" analysis; number of distinct clonotypes in each sample) are listed in the columns.

Animal	Treatment	TCZs	TCZ Size	Reads	After PEAR	Clonotypes
WT 1	naïve	1	5.1 mio	1.0 mio	1.00 mio (98%)	54652
WT 1	naïve	2	2.4 mio	2.4 mio	2,05 mio (85%)	21016
WT 2	naïve	1	5.0 mio	1.8 mio	1,69 mio (90%)	36628
WT 2	naïve	2	3.0 mio	2.2 mio	2,04 mio (93%)	36196
WT 3	naïve	1	2.0 mio	1.5 mio	1,00 mio (68%)	26190
WT 3	naïve	2	2.0 mio	1.6 mio	1,00 mio (65%)	16477
CD154$^{-/-}$ 1	naïve	1	2.8 mio	1.3 mio	0,77 mio (61%)	12729
CD154$^{-/-}$ 1	naïve	2	2.7 mio	1.3 mio	0,85 mio (64%)	16780
CD154$^{-/-}$ 2	naïve	1	2.1 mio	1.5 mio	1,06 mio (71%)	8280
CD154$^{-/-}$ 2	naïve	2	1.9 mio	1.3 mio	0,80 mio (62%)	8475
CD154$^{-/-}$ 3	naïve	1	2.8 mio	1.0 mio	0,78 mio (82%)	25737
CD154$^{-/-}$ 3	naïve	2	2.8 mio	1.2 mio	0,79 mio (69%)	11660
CD154$^{-/-}$ 3	naïve	3	2.8 mio	1.0 mio	0,84 mio (81%)	24302
WT 1	HD 3 days	1	5.0 mio	3.4 mio	3,03 mio (88%)	34926
WT 1	HD 3 days	2	6.0 mio	3.7 mio	3,41 mio (92%)	30141
WT 2	HD 3 days	1	6.0 mio	2.8 mio	2,43 mio (87%)	23253
WT 2	HD 3 days	2	6.0 mio	2.8 mio	2,25 mio (80%)	30200
WT 3	HD 3 days	1	5.0 mio	3.1 mio	3,05 mio (98%)	65299
WT 3	HD 3 days	2	6.0 mio	1.4 mio	1,30 mio (92%)	28173
CD154$^{-/-}$ 1	HD 3 days	1	3.4 mio	1.2 mio	0,94 mio (81%)	27368
CD154$^{-/-}$ 1	HD 3 days	2	3.2 mio	1.1 mio	0,32 mio (24%)	12371
CD154$^{-/-}$ 2	HD 3 days	1	2.4 mio	1.5 mio	0,86 mio (57%)	6347
CD154$^{-/-}$ 2	HD 3 days	2	2.5 mio	1.6 mio	0,89 mio (54%)	7225
CD154$^{-/-}$ 3	HD 3 days	1	3.0 mio	1.3 mio	1,15 mio (89%)	25260
CD154$^{-/-}$ 3	HD 3 days	2	3.0 mio	1.2 mio	0,74 mio (61%)	12349

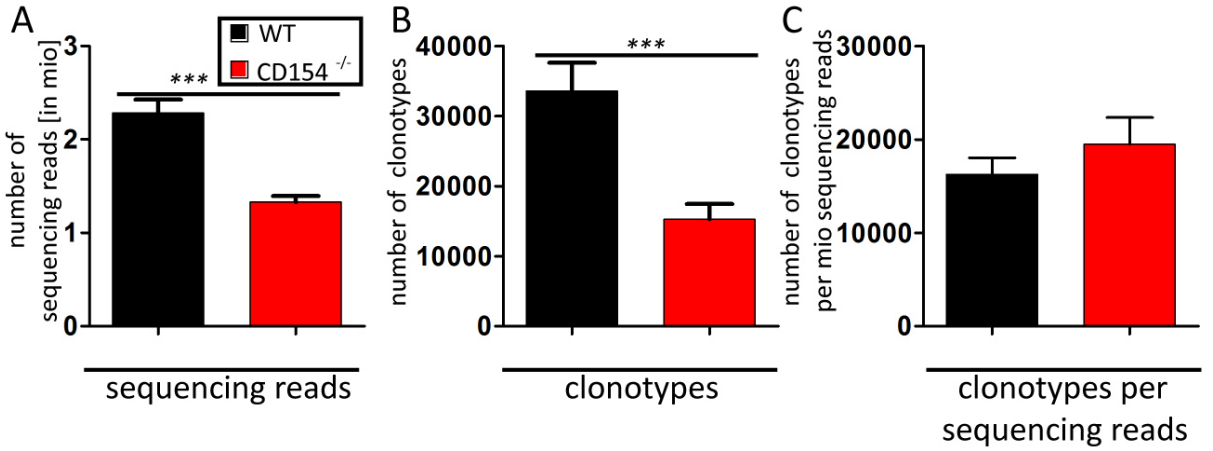

Fig. 26: Depiction of sequencing results and clonal diversity within TCZs.
Graphical depiction of results described in table 2 column 5 (A) and column 7 (B). Due to the significant difference in sequencing reads and clonotype numbers, the clonal diversity was determined in relation to the number of sequencing reads (C).

The TCZs were prepared for sequencing and normalized according to the described procedure (2.2.10.3). Unexpectedly, CD154$^{-/-}$ samples showed significantly less reads than WT mice (Tab. 2; column 5 and Fig. 26A). This resulted in reduced reads after PEAR-quality correction (Tab.

2; column 6). The absolute numbers showed that WT mice had significantly more clonotypes within each T cell zone (Tab. 2; column 7 and Fig. 26B). However, taking into account that this number of clonotypes was based on significantly more reads, a relative declaration revealed that there is no difference between WT and CD154$^{-/-}$ clonal diversity per TCZ (Fig. 26C).

3.3.5 The clonality shift due to HD immunization is not affected by CD154$^{-/-}$

An immune repertoire is characterized by the vast majority of small clonotypes and a few medium abundant ones (Freeman et al. 2009; Robins et al. 2009). Both groups are summed as low expanded clonotypes (LECs). Only very few T cell clonotypes are rated as HECs. HECs proliferated the most since they are considered to have the highest avidity to the relevant pMHC-II, so their appearance has a major influence on the clonality. So far it is not known if these HECs are induced solely by the first encounter, or if the second T cell encounter modulates the number of HECs.

For the quantitative analysis of these HECs, they were defined by the average abundance of the 10th most abundant clonotype in naïve mice. In consequence, both naïve groups revealed on average 10 HECs. With this calculation, the increase of the HECs due to the HD immunization was measured. To visualize the HECs in the repertoire two dot plots depict all sequenced samples (one sample per column) (Fig. 27, green). Each clonotype is depicted by a single dot, according to its size.

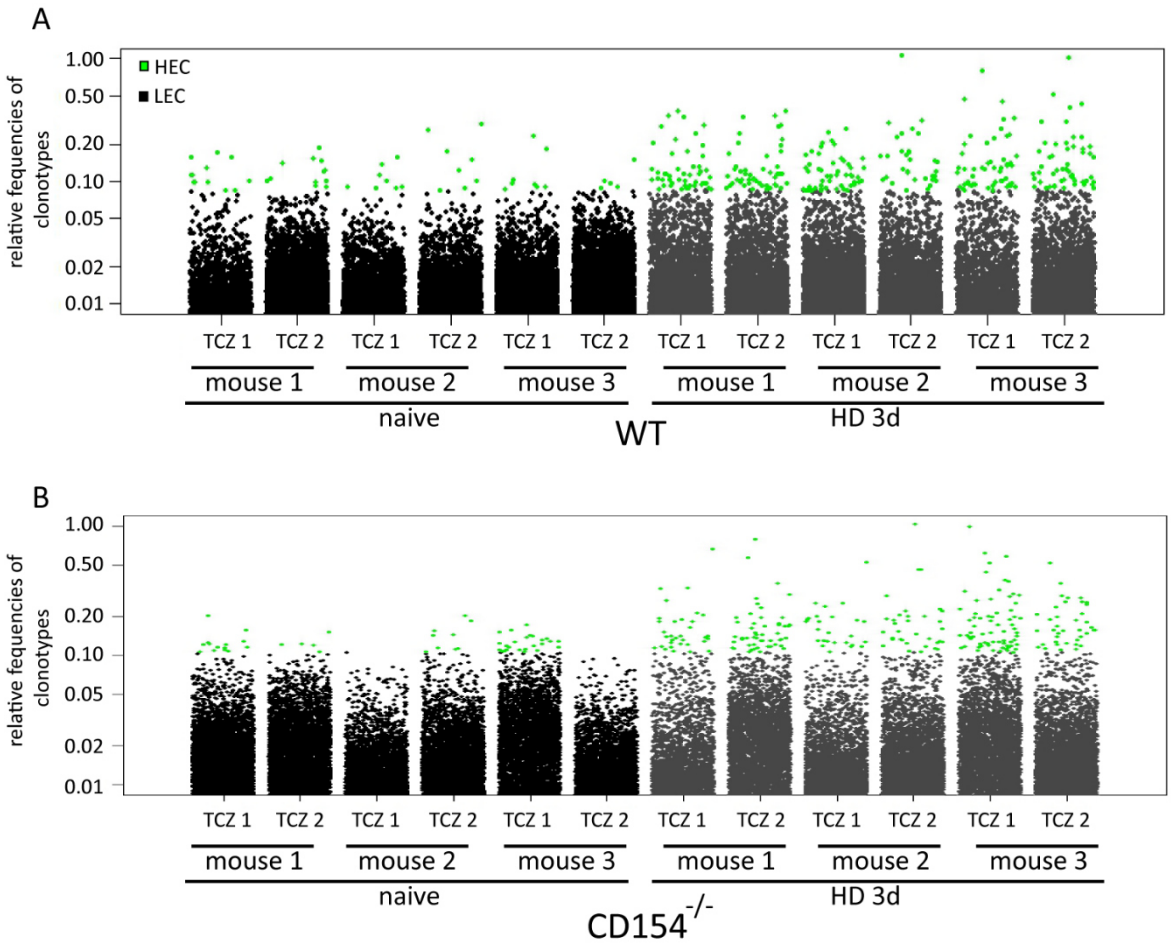

Fig. 27: CD154$^{-/-}$ did not change number of HECs recruited into the immune response.
Relative frequencies of clonotypes from naïve and HD immunized (3 days) WT (A) and CD154$^{-/-}$ (B) mice. Two splenic TCZs per mouse, with 3 mice per group are depicted in different columns (one for each sample). Y-axis shows percentage (up to 100%) a given clonotype occupies in whole repertoire. The baseline for a HEC (green) is defined for each plot individually, in respect to the naïve measurements.

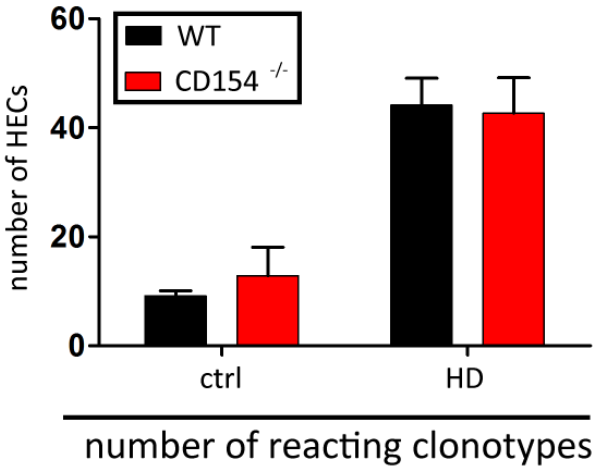

Fig. 28: The second TCR-pMHC-II encounter did not modulate the number of HECs.
The average number of HECs per TCZ were calculated. An increase of HECs due to immunization is significant for both groups, but not depicted. Bars represent mean ±SEM (mouse groups within each treatment were compared with Mann-Whitney test), n=6-7.

Quantification of the HECs depicted in figure 27 (labelled in green), showed that TCZs of HD immunized mice developed on average 4-fold more HECs than control mice (Fig. 28). This was true for both mouse groups, revealing no influence of CD154$^{-/-}$.

Although the second TCR-pMHC-II encounter did not modulate the number of HECs, it is possible that it modulates the number of cell divisions from these top clonotypes. This would result in an increased abundance of the top clonotypes, although the number of responding clonotypes was not changed.

To measure the abundance of these top clonotypes, the top clonotypes were summed up and compared to the overall repertoire. Since the comparison of HEC clonotypes would result in the comparison of 10 naïve clonotypes against ≈40 immunized clonotypes, it was preferred to use a different normalization system. The top 0.1 % of most abundant clonotypes were used to assess the clonal space which was occupied. In order to show the clonal expansion of these clonotypes the naïve sum was set to 1.

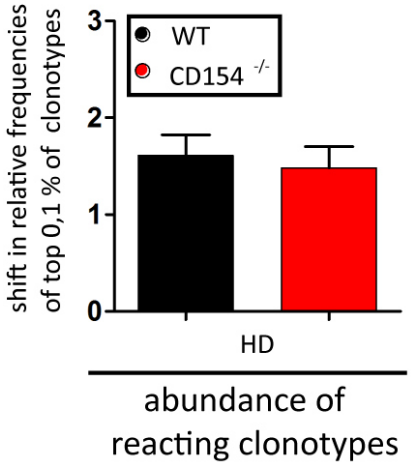

Fig. 29: The second TCR-pMHC-II encounter did not modulate the abundance of top clonotypes.
The increase of the sum of relative frequencies from the most abundant 0.1% of clonotypes from naïve (set to 1) to HD immunized mice. Bars represent mean ±SEM (Mann-Whitney test), n=6-7. Increase from naïve to HD immunization was significant for both groups (not shown).

Due to the immunization, abundance of the top clonotypes increased to a 1.6-fold abundance compared to naïve mice in both groups. This increase was significant in WT and CD154$^{-/-}$ TCZs (not shown). However, no difference in clonal expansion of HECs was detectable between WT and CD154$^{-/-}$ mice.

In conclusion, this study showed that the number of proliferating cells and the number of HECs is not modulated by the second TCR-pMHC-II encounter. These data indicate that T_H cell differentiation into T_H2 cells by CD154/CD40 interaction does not select T cell clonotypes for additional proliferation. Therefore, it was concluded that the second T cell encounter did not modulate the clonality shift during an immune response and does not serve as an additional checkpoint for recruitment of antigen specific T_H cell clones.

4 Discussion

The antigen dose is a major factor for the T_H1/T_H2 differentiation, although influence can be affected by different other factors as modulators. Discussed here are the analyses of two of these modulators that affect pMHC-II availability, first the type of APC presenting the antigen and second the C5aR1 expression on APC.

This study shows for the first time that pMHC-II availability is a major checkpoint when an antigen dose effects the T_H1/T_H2 differentiation. Furthermore, it could be shown that the blockage of the T_H cell differentiation, by the second T cell encounter, does not modulate the clonal expansion.

4.1 The antigen dose modulated the pMHC-II availability by B cells to induce T_H2 cells

In the first part (3.1) it was aimed to show that B cells modulate the T_H cell differentiation by increasing their pMHC-II availability after high antigen dose immunization. It was hypothesized that this extension is an important checkpoint to induce T_H2 cells. This was analysed in the SRBC mouse model (Liew et al. 1990; Lagrange et al. 1974a), which was established according to published results (Fig. 5) (Hurtrel et al. 1992; Kaufmann and Hahn 1979; Lagrange et al. 1974b).

4.1.1 The HD of antigen increases pMHC-II availability from B cells

The pMHC-II availability was determined by measuring cellularity and MHC-II density on the surface of macrophages, DCs and B cells. Regarding cellularity, none of these cell populations had significant increases or decreases in cellularity due to the immunization (Fig. 6B-D). This correlates with recent data (Carr et al. 2016), suggesting an elastic but robust cellular composition against antigen challenge in secondary lymphoid organs.

Regarding the MHC-II density, it was observed that the density increases on macrophages, leading to the interpretation that macrophages take part in the antigen presentation of SRBCs to T cells (Fig. 6E). This was expected, since the blood born antigen is taken up in the marginal zone, where the marginal zone macrophages are the first cells to ingest the foreign antigen (den Haan and Kraal 2012).

However, in contrast to macrophages, DCs did not show changes in MHC-II expression due to the immunizations (Fig. 6F). This was unexpected, since they are the most common activators

of $CD4^+$ T cells in secondary lymphoid organs (Hey and O'Neill 2012). However, it is possible that immunized DCs present SRBCs although they did not upregulate MHC-II on the surface. Naïve DCs already express MHC-II (Muhlethaler-Mottet et al. 1997) on which they can present self-peptides. Presenting self-peptides on MHC-II is a process named autophagy, which is described for DCs and epithelial cells (Schmid et al. 2007; Cooney et al. 2010). When DCs take up SRBCs, it is possible that they present SRBCs instead of self-peptides on their MHC-II, leading to no changes in overall MHC-II surface expression. Furthermore, this work did not detect a correlation between MHC-II density and antigen presentation by DCs (Fig. 6F). This is in agreement with former data, showing that MHC-II density on DCs is mainly a result of the maturation state of DCs (Mellman and Steinman 2001), rather than the antigen load.

B cells did not increase their MHC-II density in response to a LD immunization (Fig. 6G), although they are prone to take up small amounts of antigen due to their high affinity BCR (Rock et al. 1984; Lanzavecchia 1985). An explanation for this result could be that the shuttling of a blood borne antigen from the marginal zone to the follicle, where B cells would take up the antigen, takes too much time. In order to get to the follicular B cells, an antigen passes different stages including uptake by marginal zone macrophages and shuttling to follicular dendritic cells (Mebius and Kraal 2005; den Haan and Kraal 2012; Kraal 1992). This process may take too much time to efficiently be part of the immune response against an antigen which is gone after 1 hour (Stamm et al. 2013). In contrast to the LD immunization, in the HD immunization a significant increase in MHC-II expression on B cells was observed. This is a clear indication that B cells present antigen in response to a HD of SRBCs. In conclusion, it was assumed that there is a threshold of the antigen dose to activate B cells and induce their antigen presentation to induce T_H2 cells.

For further investigation, it was studied, whether B cells take up the antigen, and if they do it merely under HD conditions. The data from the CFSE-uptake assay (Fig. 7F) showed an increased $CFSE^+$ B cell populations only after HD immunization, indicating again a dose dependency of B cell activation. The acquired data supported the assumption that B cells are an important part of the T_H2 response.

To confirm these findings, B cell deficient mice were used to reveal whether B cell dependent pMHC-II availability is required to polarize a response towards T_H2 after HD immunization

4.1.2 B cells are necessary to induce substantial T_H2 development

The absence of a T_H2 response in B cell-deficient JHT mice (Fig. 8) demonstrated that B cells are necessary for the T_H2 development. This confirmed the hypothesis about the necessity of B cell dependent pMHC-II availability for the T_H2 response as well as it reflects published data from other models (Drake et al. 2015; Linton et al. 2003; Harris et al. 2005; Moulin et al. 2000).

Besides the importance of B cells for the T_H2 response, no publications indicated the importance of B cells for a T_H1 response. To reveal their possible role for a T_H1 response, B cell deficient mice were challenged in the footpad to use the DTH as a read out for an ongoing T_H1 response. After challenge, HD immunized JHT mice developed a T_H1 dependent DTH reaction equal to LD immunized WT (Supplement; Fig. S1A; S1B) as well as LD immunized JHT mice (Supplement; Fig. S1A). These results lead to the conclusion that a T_H1 response proceeds independently of B cells in the SRBC model, and can be induced in absence of B cells, instead of a dose dependent T_H2 response. This leads in return to the conclusion that the activation of B cells decides about the T_H cell differentiation in this model.

4.1.3 Antigen presentation by B cells requires an intact splenic microarchitecture

If B cells are depleted, the splenic microarchitecture is lost (Fig. 10A). This is disadvantageous, as the microarchitecture of the spleen is important for the establishment of a T_H2 response (Franzoso et al. 1998). Therefore the question arose whether the observed lack of a T_H2 response is the result of missing B cells. To confirm this, JHT mice were reconstituted with B cells, however far less B cells than expected were detectable. Consequently, due to the low amount of B cells, neither a T_H2 response was induced (Fig. 9) nor was the DTH after HD immunization suppressed (Supplement; Fig. S1C).

In conclusion, the number of B cells transferred was too low to significantly influence the T_H cell development. Therefore the importance of B cells could not be confirmed but it could not be ruled out either. This indicates that there is a threshold for the number of B cells in order to influence the T_H cell differentiation.

Overall it is difficult to verify that the lack of a T_H2 response in JHT mice is the result of absent B cells, as the lack of B cells always induces impaired splenic microarchitecture. Especially the marginal zone is missing, where the SRBCs can be “trapped” (Aichele et al. 2003) before being distributed for efficient antigen presentation within the white pulp (Cerutti et al. 2013; Kraal

1992). Therefore, in order to grant the success of this experimental setup, the complex shuttling mechanism of a blood borne antigen to follicular B cells has to be circumvented (Cerutti et al. 2013; Cyster 2010), or a model has to be used where B cells are present, but present no antigen.

An approach which would fit these criteria is an experiment where SRBC pulsed B cells are transferred, in order to induce a T_H2 response (Lapointe et al. 2003), evading the complex shuttling. Alternatively, C57Bl/6 mice with MHC-II deficient B cells could be used in the future (Molnarfi et al. 2013). These mice have an intact splenic structure and B cells can take up antigen in the spleen, however the antigen presentation to T_H cells is blocked.

To summarize, this part of the thesis proved that the antigen dose can modulate the pMHC-II availability within an immune response (Fig. 6). Furthermore it highlights the modulatory capacity of B cells in an antigen dose dependent model: Their pMHC-II availability is necessary to induce T_H2. Therefore, their absence impairs a T_H2 response and shifts the response towards T_H1.

In conclusion, an increasing antigen dose shifts a T_H cell response towards T_H2, when the antigen dose is high enough to be recognized by the B cells and induce their antigen presentation. Therefore the exclusion of B cells could be a versatile target to avoid unwanted T_H2 responses.

4.2 C5aR1 deficiency modulates T_H2 development by reduction of the pMHC-II availability

The antigen dose effect on the T_H1/T_H2 differentiation can be modulated by C5aR1. This modulation was assumed to be dependent on the modulation of the pMHC-II availability. Since the first part of this work showed the importance of B cells for the development of a T_H2 response, the second part focussed on whether C5aR1 can modulate the pMHC-II availability from B cells to influence the T_H2 development.

4.2.1 C5aR1 deficiency reduces the B cell dependent pMHC-II availability and suppresses the T_H2 response in the SRBC model

$C5aR1^{-/-}$ reduced the pMHC-II availability in C57Bl/6 mice, via a B cell reduction of 15 %, decreasing their availability for antigen presentation (Fig. 11). This reduction is clearly weaker than the previously described reduction by 40 %, due to the decrease of pMHC-II density on B cells (Weaver et al. 2010). Based on this, only a minor suppression of T_H2 cells was expected. In accordance with this expectation, the influence of $C5aR1^{-/-}$ on the T_H2 response was weaker than initially hypothesized, and overshadowed by the effect of the antigen dose. The results show a prominent T_H2 response after HD immunization which could not be modulated by $C5aR1^{-/-}$, while the weaker T_H2 response after MD immunization was modulated (Fig. 12).
This leads to the conclusion that lower B cell numbers in dLNs of $C5aR1^{-/-}$ mice result in reduced pMHC-II availability by B cells, thus suppressing the T_H2 development. This suppression is strong enough to significantly influence a MD response, but is not able to overcome the antigen dose dependent T_H2 development after HD immunization. Therefore the modulatory effect of C5aR1 is antigen dose dependent in the SRBC model.

4.2.2 C5aR1 deficiency impairs the T_H cell differentiation in the *L. major* infection model

Following the results from the SRBC model, it was tested whether a $C5aR1^{-/-}$ dependent reduction of the B cell antigen presentation can also supress the T_H2 response in a model of persistent infection, like the *L. major* infection model in Balb/c mice.

4.2.2.1 Establishment of the *L. major* antigen dose model

The *L. major* model was established, developing three significantly different infection progressions due to three different antigen doses. The results were in accordance to the

previously described results (Bretscher et al. 1992), and confirmed that severe *L. major* infection can be suppressed by a reduction of the antigen dose.

The analysis of the cytokine pattern showed a change in the IFN-γ/IL-4 ratio in the dLN as well as in the skin from T_H2 development after HD infection towards T_H1 development after LD infection (Fig. 14C; 15C). This development was supported by changing expression patterns of IL-12 and IL-10 (Supplement; Fig. S2). Overall, these data demonstrate a close correlation between the antigen dose and the T_H cell development, which fits previous reports from infection models of other *Leishmania* strains (Oliveira et al. 2012). It is concluded that the antigen dose modulates the infection progression phenotype by the T_H1/T_H2 differentiation, revealing that susceptible Balb/c mice can control the infection after LD infection.

To further characterize the phenotype of the infection in the future, it would be of interest to assess the parasite distribution within the body. Whereas C57Bl/6 mice can trap parasites locally in the skin and control their appearance in other organs, in Balb/c mice the parasites are distributed into liver and spleen (Chen et al. 1994). This raises the question whether the T_H1 response after LD infection prevented the infection of these organs in Balb/c mice.

The presented results confirmed the expected shift from a T_H2 response during severe infection to a T_H1 response after LD infection. Due to this experimental conformation it is possible to establish the *L. major* model as an antigen dose dependent T_H1/T_H2 model, which can be used for the investigation of C5aR1$^{-/-}$ and its pMHC-II availability dependent influence on the T_H1/T_H2 differentiation.

4.2.2.2 The effect of C5aR1 deficiency in the *L. major* antigen dose model

In the SRBC model, C5aR1$^{-/-}$ C57Bl/6 mice had less dLN B cells and thereby less pMHC-II antigen presentation, which resulted in a suppressed T_H2 development (4.2.1). This suppression could be of great value for the modulation of the *L. major* progression, since a T_H2 response is associated with susceptibility to the infection.

C5aR1$^{-/-}$ Balb/c mice had no altered pMHC-II availability compared to WT mice (Fig. 16). This is unexpected since previous results showed only 1/3 of MHC-II on the surface of C5aR1$^{-/-}$ B cells, compared to WT B cells (Weaver et al. 2010). Based on the initial hypothesis that the modulatory effect of C5aR1 is mediated by the pMHC-II availability, no altered infection

progression would be expected, relying on the presented results of no altered pMHC-II availability.

In accordance with this hypothesis, the progressions of LD and HD infections were not modulated by C5aR1$^{-/-}$ (Fig. 17). Unexpectedly, the MD infection progressed significantly more severe in C5aR1$^{-/-}$ mice (Fig. 18A). Obviously, C5aR1$^{-/-}$ was not sufficient to modulate a HD (and LD) infection progression, but the MD progression. This is not unexpected, taking into account how susceptible the respective infection progressions are to minor changes in the respective antigen dose: A HD infection contains 10^6 parasites. A difference of 10^5-10^6 parasites does not modulate the phenotype considerably (Bretscher et al. 1992). However, in the range of a MD ($3{,}3x10^4$) a slight reduction from $3.3x10^4$ to $1x10^4$ can modulate the outcome from severe infection to complete resistance. Therefore, the modulatory effect of C5aR1$^{-/-}$ was expected to be the same in HD and MD infection. However, due to the stable condition after HD infection, it is detectable only after MD infection.

Nevertheless, it is unclear why this altered infection severity appears. The pMHC-II availability was shown to be no factor for the observed difference in the *L. major* model, since it is not affected by C5aR1$^{-/-}$. Therefore, it was assumed that the T_H cell differentiation in the dLN is not impaired due to the C5aR1$^{-/-}$ in Balb/c mice. In accordance to this assumption, the T_H1/T_H2 differentiation did not reveal any significant differences at any time point due to C5aR1$^{-/-}$ (Fig. 19). It was concluded that the initial hypothesis of an effect of C5aR1 on pMHC-II availability is correct in regard to the T_H cell differentiation in the dLN. Nevertheless, this data showed that the infection progression is modulated by another, unknown factor. Previously a significant reduction of T cell numbers (Hawlisch et al. 2005) and the severe impairment of T cell activation (Weaver et al. 2010) was reported in C5aR1$^{-/-}$ mice. In order to exclude this as possible explanations, the cellular composition, the MHC-II availability of other APCs and the T_H cell activation in the dLN were investigated. However, none of these measurements showed detectable alterations due to C5aR1$^{-/-}$ (Supplement; Fig. S4; S5; S6). In conclusion, these data showed that C5aR1 had no impact on the immune response to *L. major* in the dLN.

The control of a *L. major* infection requires also T_H1 cells in the skin, being the location where the parasite persists. In contrast to the results in the dLN, several significant differences were measured comparing the skin of WT and C5aR1$^{-/-}$ mice (Fig. 20). The detected impaired T_H1

development after an infection could explain the observed more severe phenotype, as well as the significantly increased T_H2 response in C5aR1$^{-/-}$ mice.

Although the most significant difference in IFN-γ/IL-4 ratio in the skin between C5aR1$^{-/-}$ and WT mice was detected at week 5, the most conspicuous effect was detected after 3 weeks. At this time, C5aR1$^{-/-}$ skin showed no significant cytokine expression regarding IFN-γ, IL-4, IL-12 or IL-10 (Supplement; Fig. S6), although these mice suffered from a significantly more severe infection progression. This demonstrated that C5aR1$^{-/-}$ skin did not develop any significant T_H cell response. Therefore, it was concluded that the lack of an effector T_H response, especially IFN-γ secretion, provides the parasite with an opportunity to evade the phagocytosis by the innate immune system, and thereby to survive and multiply. This could increase the parasite burden, lead to an increased infection severity, which could result in the observed more severe lesions in C5aR1$^{-/-}$ mice.

It was further concluded that the significant increase of the IFN-γ/IL-4 ratio after 5 weeks is the result of a more efficient immune response in WT skin after 3 weeks. Comparing the expression patterns from the skin to the patterns of the dLN, where none of these significant differences appeared, it can be concluded that the communication between LN and skin is severely interrupted in C5aR1$^{-/-}$ mice (Fig. 21). This is an unexpected result and its molecular origin might base on migratory effects of C5aR1. As this is not the focus of this section, it is further discussed in an additional section (4.5).

C5aR1 has been shown to modulate the T_H1/T_H2 differentiation in diverse models differently (Johswich et al. 2009; Sabio et al. 2017; Wenderfer et al. 2005). This study hypothesized that the antigen dose dependent T_H1/T_H2 modulation by C5aR1 is dependent on the modulation of the pMHC-II availability. It was suggested that the decreased pMHC-II availability, which was previously reported for C5aR1$^{-/-}$ mice impairs T_H2 responses in favour of the T_H1 development.

In the SRBC mouse model this hypothesis was supported and the reduction of B cell dependent pMHC-II availability was able to supress the T_H2 response after MD immunization in the spleen of C5aR1$^{-/-}$ mice. This proved that C5aR1 was able to modulate T_H1/T_H2 differentiation but is effective only within a small range of antigen dose.

In the *L. major* model, C5aR1$^{-/-}$ failed to modulate pMHC-II availability. In accordance to the hypothesis, the T_H cell response was not modulated in the dLN. Nevertheless, the skin showed

significantly increased T_H2 responses, depicting a contradictory development compared to the T_H cell development in the SRBC model. In the *L. major* model the migratory effect of C5aR1 was suspected to be the reason for the stronger T_H2 response, while in the SRBC model the reduced pMHC-II availability was the reason for the suppressed T_H2 response.

C5aR1 has converse effects on the T_H cell differentiation, depending on the model. This work aimed to characterize the effect of C5aR1 in antigen dose models. Even in these related models, two converse effects of C5aR1 were found, giving an indication why C5aR1 effects are described so diverse in literature (For further discussion see 4.5).

4.3 The second TCR-pMHC-II encounter does not modulate the T cell clonality

The T cell clonality within an immune response, which is affected by the antigen dose (Obst et al. 2005), modulates the T_H1/T_H2 differentiation (Rudulier et al. 2014). In the previous part of this study it was shown that the increased pMHC-II availability by B cells after HD SRBC immunization induced a second T_H cell encounter and a T_H2 differentiation in the spleen (3.1). To elucidate whether the second encounter modulates the clonality, the clonality was assessed by focussing on HECs, as signature clonotypes of an immune response.

$CD154^{-/-}$ was established for blocking the second encounter in the spleen. This succeeded, which was confirmed by the lack of T_H2 effector cells after HD SRBC immunization (Fig. 22). Nevertheless, the splenic microarchitecture was not affected (Fig. 23), which fits previous reports (Blair et al. 2000; Banczyk et al. 2014; Xu et al. 1994).

The influence of the second encounter on the clonal expansion of T_H cells was assessed by measuring the T cell proliferation. As no difference was detected, it leads to the conclusion that clonal expansion is determined by the first encounter, in which CD154 has no influence (Matthies et al. 2006; Roy et al. 1993). These analyses (3.3.1, 3.3.2 and 3.3.3) demonstrated that $CD154^{-/-}$ provides a model where the first encounter develops normally, whereas the second encounter is completely abolished.

Although overall T_H cell proliferation is not modulated by the second encounter, the development of individual HECs was determined with NGS. Unexpectedly, significantly less clonotypes were measured in $CD154^{-/-}$ compared to WT samples (Tab. 2; Fig. 26B). It was assumed that this was a result of the lower numbers of raw reads (Fig. 26A), which could be confirmed by showing identical clonotype diversity (Fig. 26C). The only reason for the lower number of raw reads can be an overestimation of the $CD154^{-/-}$ cDNA molecules within our quantification and calibration procedure before sequencing. Therefore the samples were compared in regard to quality and RNA amount. Interestingly the sequencing results revealed significantly lower quality of $CD154^{-/-}$ sequences (Tab2; Supplement; Fig. S7A). This effect could be based on significantly reduced RNA amounts (Supplement; Fig. S7B). Reduced RNA content in the samples could be caused by a lower activation of the $CD154^{-/-}$ cells in the spleen: CD154 is expressed on APCs and provides costimulatory signals (Schonbeck and Libby 2001). Without these signals it is possible that the average activation and RNA expression of the cells

is lower, compared to the WT. The effect of CD154 signalling on cell activation was to date only reported for monocytes (Alderson et al. 1993) but various influences on the expression of other molecules have been reported recently (Seijkens et al. 2010). It was concluded that CD154$^{-/-}$ does not impair the clonal diversity of T_H cells within a TCZ, but it supresses the overall activation of splenic cells. Nevertheless the model provides an opportunity to assess the effect of the second encounter on the HECs.

The HECs were defined by an average frequency of the 10th most abundant clonotype in naïve controls (Fig. 27), because the different clonotype numbers between CD154$^{-/-}$ and WT samples did not allow to use the relative frequencies for determination of HECs, which was established previously (Tong et al. 2016).

The HD immunization resulted in increased HEC numbers (Fig. 28) and abundance (Fig. 29), but revealed no impact of the second encounter. This demonstrated that the second TCR-pMHC-II encounter does not modulate the development of HECs within an immune response, which leads to the conclusion that the second encounter does not affect the T cell clonality. Nevertheless, previous observations confirmed that the clonality is influenced by the antigen dose (Obst et al. 2005; Stamm et al. 2013). In conclusion, this influence must be based on the first TCR-pMHC-II encounter. This would mean that the T_H cell recruitment and clonal expansion are determined solely by the first encounter. This is supported by previous reports (Bajenoff et al. 2002), but contradicts results showing that sustained antigen availability affects clonal expansion (Obst et al. 2005).

Bringing this into the context of the major factor “TCR avidity to the pMHC”, which exerts its influence via the recruitment of T_H cells into an immune response, it has to be concluded that this factor can affect only the first T_H cell encounter and loses its influence during the second encounter. Additionally, both major factors, the “TCR avidity to the pMHC-II” and the “antigen dose” influence the T cell clonality during the first encounter, before effector cytokines are induced. To assess the importance of those two factors, the question arises whether the activated T_H cell repertoire in CD154$^{-/-}$ mice is able to develop into a T_H1 response. Previous results suggest that this is not the case (van Panhuys et al. 2014). If this is correct, these two factors determine the T_H cell differentiation cytokine independently, revealing the role of cytokines as a factor to sustain a specific T_H cell fate throughout an immune response, rather than inducing the initial differentiation in antigen dose dependent models.

4.4 A model of T_H cell activation and differentiation in antigen dose models

4.4.1 Two encounters are necessary to induce a cytokine producing effector T_H cell

Multiple models for T cell activation have been published (Bretscher 1992; Bajenoff et al. 2002; Mohrs et al. 2005). For all of them it is accepted that T cells need two TCR-pMHC-II encounters (also called “2 Hits”) to become a cytokine producing effector cell. This was supported by the presented work, by showing T cell proliferation and effector cytokine release after HD immunization in the spleen of WT mice (T cells receive two encounters), and only T cell proliferation in the spleen of $CD154^{-/-}$ mice (T cells receive only one encounter).

In these models prevails accordance that the first encounter is given to naïve T cells (without CD154), thus leading to proliferation, but not to effector cytokine secretion. This is confirmed in this thesis, since $CD154^{-/-}$ T cells activated normally, did not show an impaired proliferation, but did not secrete effector cytokines.

Importantly, SRBC activated T cells did not express cytokine mRNA, which contradicts some previous reports (Mohrs et al. 2005). Instead, the second TCR-pMHC-II encounter induced cytokine mRNA production and secretion. The major difference to the published data lays in the way of measuring cytokine mRNA directly on day 3 in this study, whereas the group of Mohrs et al. (2005) drew their conclusion from GFP^+ cells (co-expressed with IL-4 in GFP receptor mice) in the periphery, 2 weeks post immunization.

4.4.2 The antigen dose modulates the pMHC-II availability to influence the T_H1/T_H2 differentiation

This thesis identifies the importance of the pMHC-II availability as one major checkpoint for the antigen dose effects on T_H cell fate *in vivo*.

For the aim to connect the presented results of this study with current knowledge in literature, the following model for the influence of the antigen dose on the T_H2 differentiation after HD immunization is proposed:

The high antigen dose increases the pMHC-II availability in the secondary lymphoid organ (3.1). Supported by other studies (Drake et al. 2015; Maddur et al. 2014), this thesis highlights and demonstrates the importance of pMHC-II availability by B cells for the T_H2 differentiation (3.1.3) (Fig. 30 (A)) and shows that T_H cells differentiate into T_H1 cells, in the absence of B cells (4.1).

The importance of the modulation of the pMHC-II availability on B cells was confirmed within the second part of this thesis, analysing the pMHC-II availability modulation by C5aR1 in regard to the T_H1/T_H2 differentiation (4.2):
(i) C5aR1$^{-/-}$ C57Bl/6 mice showed reduced pMHC-II availability on B cells, consequently suppressing the T_H2 response in the spleen. (ii) C5aR1$^{-/-}$ Balb/c mice showed no reduction of the pMHC-II availability on B cells, consequently C5aR1$^{-/-}$ did not modulate the T_H2 response in the dLN.

Former studies demonstrated that pMHC-II availability can additionally influence the recruitment of T_H cells into an immune response (Baumgartner et al. 2010). In this context, it is well documented that increasing numbers of recruited T_H cells *in vitro* tend to develop into a T_H2 response (Rudulier et al. 2014) (Fig. 30 (B)). Therefore, it is likely that the increased number of recruited T cells after HD immunization (Obst et al. 2005), relays on increased pMHC-II availability and supports a trend towards T_H2 development. Nevertheless, B cells are required to induce T_H2 instead of T_H1 (Fig. 30 (C)), which was shown by using B cell deficient mice in the presented study (4.1).

However, none of the before mentioned reports suggested whether the increased clonal expansion after HD immunization results from the first or the second encounter of T_H cell activation. This thesis could exclude that the second TCR-pMHC-II encounter is responsible for this increase by measuring the appearance of signature HEC clonotypes (4.3). In consequence, it is very likely that it is caused by the pMHC-II availability guiding the T cell clonality within the first TCR-pMHC-II encounter (Fig. 30 (D)). The increased pMHC-II availability after HD immunization could perhaps recruit more clonotypes, containing also low avidity clonotypes, into the immune response (Baumgartner et al. 2010; Blander et al. 2000), which forces the T_H cell development into a T_H2 response (Rudulier et al. 2014) (Fig. 30 (B)). This model suggests a mechanism how the antigen dose can apply an effect on the T_H cell differentiation: The antigen dose affects the number of recruited T_H cell clonotypes and the antigen presentation by B cells. A HD of antigen induces high numbers of recruited clonotypes and B cell antigen presentation. Both are needed to induce T_H2 cells.
It could be expected that the increased number of reacting clonotypes after HD contained also many low affinity T_H cell clonotypes. This would connect the factor antigen dose with another

factor, which is the TCR affinity to pMHC-II. Thereby suggesting that a HD immunization lowers the average TCR affinity to the pMHC-II within an immune response and further supports T_H2 development.

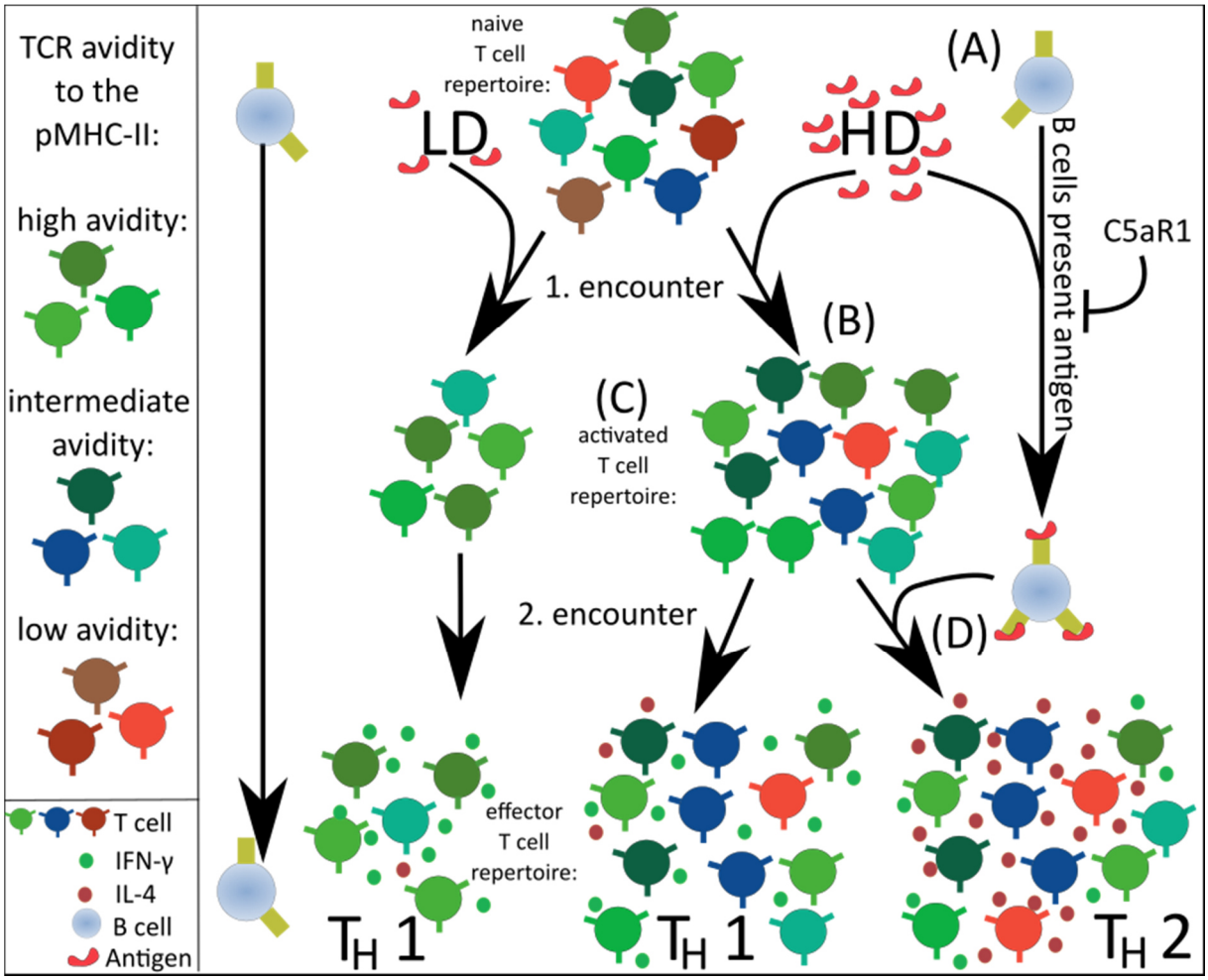

Fig. 30: A summary: How pMHC-II availability by B cells induces T_H2 development in secondary lymphoid organs in antigen dose models.
The figure depicts the conclusion from the results and recent publications. The HD induces antigen uptake and presentation by B cells (A). Additionally, it increases the number of recruited T cells in the immune response (B). In contrast to the small activated T_H cell repertoire after LD immunization, the broader repertoire after HD immunization (C) (Obst et al. 2005) predominantly supports T_H2 development (Rudulier et al. 2014). Nevertheless, the antigen presentation by B cells is necessary to induce T_H2 (D).

4.4.3 Antigen availability is necessary to allow a T_H2 response after HD SRBC immunization

In an attempt to apply the above described model to the SRBC antigen presentation, it has to be considered that the SRBC antigen is gone shortly after immunization. Therefore a specific focus lays on the efficient antigen transport into and within the splenic structure.

After a LD injection, SRBCs accumulate in the marginal zone and are taken up by macrophages (3.1) and probably DCs (4.1). These APCs present the antigen to T_H cells inducing their activation and proliferation (Fig. 31, left panel). Due to the low pMHC-II availability most T cells do not receive enough TCR contact for significant TCR signalling and activation. Thus only high avidity clonotypes receive activation signals. However, after activation, proliferation and upregulation of CD154, no pMHC-II is available in the spleen. Thereby the induction of the effector functions for high avidity clonotypes is impeded, because the antigen is gone after one hour (Stamm et al. 2013).

In contrast to the LD injection, after HD injection SRBCs are taken up by macrophages and are shuttled efficiently to follicular B cells (Fig. 32, right panel). First the macrophages present antigen, thus again inducing only high avidity T cell clonotypes, and providing IL-12 secretion for a T_H1 development within the first hours (Stamm et al. 2013). It has been shown that B cells are not required for this initial activation (Epstein et al. 1995). However, the HD of antigen increases the pMHC-II availability, leading to the activation of significantly more T cells compared to the state after LD immunization (Stamm et al. 2013; Obst et al. 2005).Thereby also low avidity clonotypes are recruited into the immune response, which tend to develop into T_H2 cells (Blander et al. 2000). When B cells become activated, an additional cell type presents antigen, causing the T cells to develop into T_H2 cells (Rudulier et al. 2014), and thereby expressing the antigen dose effect on the T_H cell differentiation.

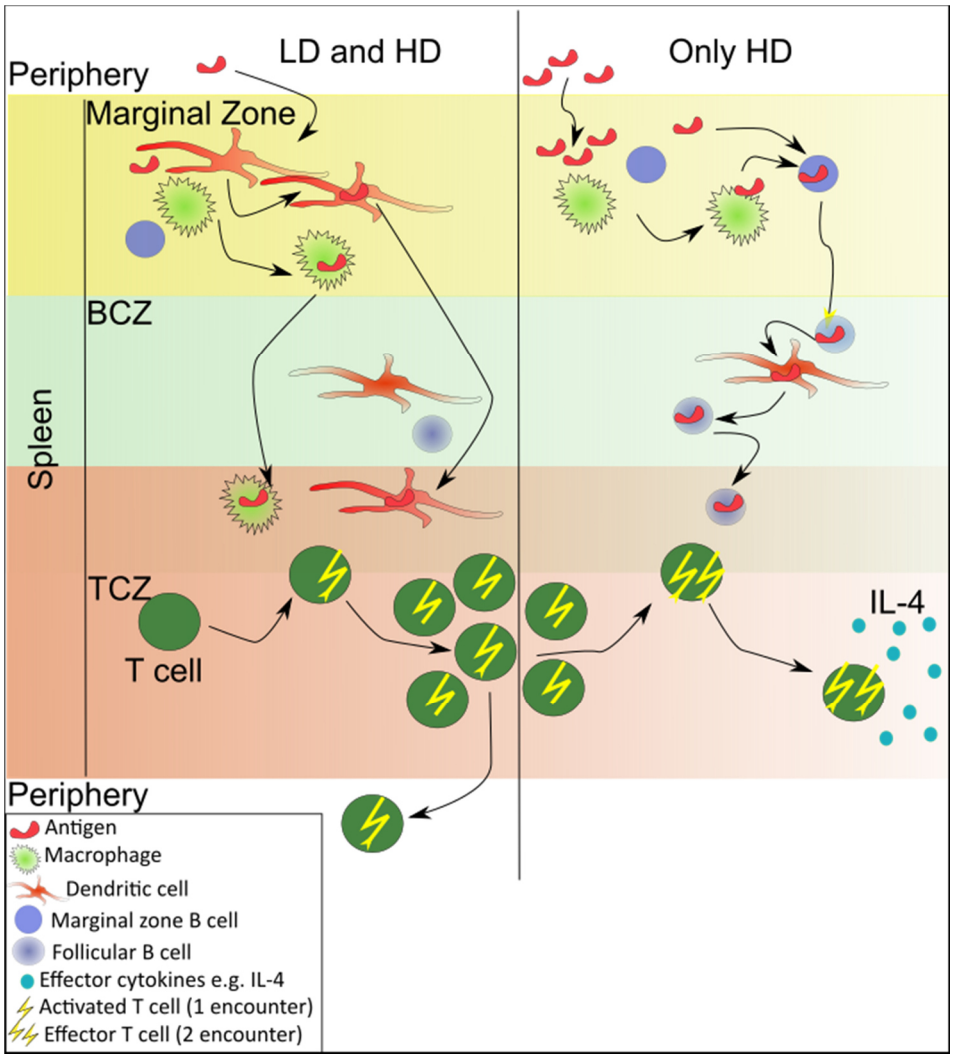

Fig. 31: SRBC antigen presentation in the spleen: B cell activation is the signature checkpoint to induce T_H2 cells.

Both antigen doses induce a first TCR-pMHC-II encounter by activating macrophages and DCs, which are actively migrating to the border between TCZ and BCZ. However, only HD immunization induces shuttling from the blood borne antigen via marginal zone macrophages and marginal zone B cells to FDCs, and finally to follicular B cells. This additionally induces B cell activation and provides a second TCR-pMHC-II encounter to drive T_H2 effector cell development.

4.5 C5aR1 modulates T_H1/T_H2 differentiation in multiple ways, into different directions

4.5.1 The impaired skin immune reaction of C5aR1 deficient mice to *L. major* infection is a result of the migratory effects of C5aR1

Although C5aR1$^{-/-}$ Balb/c mice were reported to be resistant against *L. major* infection (Hawlisch et al. 2005), footpad swelling resulted in unexpectedly more severe lesions in MD infected C5aR1$^{-/-}$ mice compared to WT. Furthermore, in these experiments no influence of C5aR1 was detectable on the cellularity or the cytokine milieu within the dLN (Discussed above (4.2.2)).

The major differences between WT and C5aR1$^{-/-}$ mice were detected in T_H1/T_H2 associated cytokines after 3 weeks of infection in the skin, revealing major differences between the T_H cell response in the dLN and the skin (Fig. 21). Although this thesis focusses on the T_H cell development within the secondary lymphoid organs, the impaired communication between dLN and skin in C5aR1$^{-/-}$ mice is discussed briefly:

It was shown that T cells do not express C5aR1 (Karsten et al. 2015). Therefore the reason for an impaired skin response of T_H cells bases on an indirect influence on T_H cells. Therefore transcripts of macrophages, neutrophils and DCs, which directly interact with the parasite (Liew et al. 1990; Lima et al. 1998; Woelbing et al. 2006) and express C5aR1 in WT mice were analysed in the footpad skin (Supplement; Fig. S2). However regarding these gene expression patterns, no significant difference could be related to C5aR1$^{-/-}$, indicating no influence on cell migration in week 3 or 5. The most likely assumption is that modulation by C5aR1 signalling appeared even earlier in the immune response. This is likely since the complement is active early during an immune response, peaking at 24 hours post an incision (Clark et al. 2006). Since no T cell effector cytokines were measured in the skin of C5aR1$^{-/-}$ mice after 3 weeks, it was hypothesized that C5aR1 is necessary for chemoattraction of cells into the skin to allow a substantial T_H cell response.

One type of cells requiring C5aR1 for efficient migration into the skin are neutrophils (Heit et al. 2002). For these, it has been shown that without C5aR1 significantly less cells reach the skin early (Grant et al. 2002).

Within the last years more and more reports suggest a double sided role of neutrophils in *L. major* infection (Ribeiro-Gomes et al. 2004). Beside the detrimental effect of neutrophils, providing a host cell for parasite survival (Afonso et al. 2008; Ritter et al. 2009), beneficial

effects of early neutrophil appearance have been shown (Charmoy et al. 2010; Akhzari et al. 2016). This is supported by demonstrating that resistant C57Bl/6 and susceptible Balb/c mice recruit neutrophils early in the immune response against *L. major* (Beil et al. 1992), indicating that no harmful effect is related to early migration.

One beneficial effect of early neutrophil immigration into the skin is the CCL3 secretion, a chemokine which induces DC recruitment and mDC maturation (Charmoy et al. 2010). These mDCs are needed to secret IL-12 to polarize T cells into the T_H1 direction (Schleicher et al. 2007). Based on these data, it was hypothesized that C5aR1$^{-/-}$ mice have an impaired migration of neutrophils into the skin, within the first days post infection. Although neutrophils appear within the next weeks without C5aR1, the early induction of the immune response by neutrophils might be missing in the C5aR1$^{-/-}$ mice, which could delay the T_H1 response, IL-12 and IFN-γ secretion (Leon et al. 2007; Schleicher et al. 2007).

To test this hypothesis early neutrophil recruitment and CCL3 expression was analysed by isolating cells from the footpad skin of *L. major* infected mice, 24 hours post infection (Supplement; Fig. S9). Although these results are not significant, they indicated less neutrophils in the skin of C5aR1$^{-/-}$ mice, supporting the hypothesis. For further indications the measurements have to be repeated, testing also other points of time post infection, and including also early appearance of mDCs, which have been proven beneficial for T_H1 development (Schleicher et al. 2007).

4.5.2 The impaired skin immune reaction of C5aR1 deficient mice to *L. major* infection also appears in the ear infection model

MD *L. major* infection resulted in unexpectedly more severe lesions in C5aR1$^{-/-}$ mice compared to WT. To our knowledge, the only publication dealing with this question *in vivo* used the ear infection model of *L. major* (Hawlisch et al. 2005), demonstrating that C5aR1$^{-/-}$ mice are resistant to *L. major* infection. To compare the data of this thesis with the published data, the ear infection model was performed as described (Hawlisch et al. 2005) (Supplement; Fig. S10). This model confirmed the data from the footpad model, showing higher scores, more severe infection progression in the skin of in C5aR1$^{-/-}$ mice compared to the WT. These results excluded that the difference of the here shown *L. major* results to previously published results depend on the used *L. major* infection model.

4.5.3 C5aR1 dependent modulation of the T_H1/T_H2 differentiation is influenced by the genetic background and environmental factors

This study could show that the C5aR1$^{-/-}$ dependent modulation of T_H1/T_H2 differentiation in the secondary lymphoid organs, depends on the influence of C5aR1 on the pMHC-II availability. The comparison of C57Bl/6 and Balb/c mice revealed that C5aR1 has different modulatory capacities on the pMHC-II availability (Fig. 11; 16). Therefore the analysis of the pMHC-II availability is an important factor to compare different C5aR1$^{-/-}$ mouse strains and their T_H1/T_H2 development.

Contrasting results from C5aR1 dependent modulation of the T_H cell differentiation were observed previously (Engelke et al. 2014; Hawlisch et al. 2005; Johswich et al. 2009; Ma et al. 2013; Wenderfer et al. 2005). This study focussed especially on the influence of C5aR1 on antigen dose dependent models. And even in those models, C5aR1$^{-/-}$ induced different results. T_H2 development was suppressed in the SRBC response (Fig. 12), while it was increased in the skin of *L. major* infected mice (Fig 20).

In this study it was demonstrated that C5aR1$^{-/-}$ has a different influence on the pMHC-II availability in C57Bl/6 mice compared to Balb/c mice (Fig. 11; 16).

- In C57Bl/6 mice the pMHC-II availability is severely impaired. In the systemic model of SRBCs, where migration to secondary lymphoid organs is of minor importance, the pMHC-II availability modulation supresses the T_H2 development.
- In Balb/c mice, pMHC-II availability is not impaired. However, the *L. major* infection model requires dLN-skin interplay, including efficient cell migration into the skin. Without C5aR1, this migration is impaired, and T_H1 development is suppressed in the skin.

Therefore, one reason for different effects of C5aR1$^{-/-}$ in different studies could be the genetic background of the mice, impairing pMHC-II availability or not, and thereby explaining the contradicting results.

Additionally the environment can affect the C5aR1$^{-/-}$ effect. This is supported by the difference of the presented results to the results from Weaver et al. (2010). The mice which were used in this study originate from the same laboratory as did Weaver et al.'s (2010). However the breeding was transferred to Lübeck a few years ago. Even though both mouse strains originate from the same laboratory they nevertheless reveal significant differences in regard to their pMHC-II availability.

These discrepancies are overshadowed by the fundamental question which cells do express C5aR1. For example, some groups could clearly exclude C5aR1 expression on $CD4^+$ T cells (Karsten et al. 2015; Dunkelberger et al. 2012). However, other groups have confirmed its occurrence on T cells (Lalli et al. 2008; Strainic et al. 2008; Nataf et al. 1999). In conclusion, this factor could be the relevant reason for the discrepancies in literature and further research is required in the future to evaluate the molecular and/or environmental basis for the diverse effects of C5aR1 on the T_H cell differentiation.

5 Summary

5.1 Summary (English)

$CD4^+$ T_H cells fulfil diverse functions by developing into one of several T_H cell subsets with specific roles, such as subsets T_H1 and T_H2. Since the T_H cell subsets have so versatile roles, it is important to induce the most effective one, when the immune system encounters a pathogen. However, how T_H cell differentiation into one subset or the other works is not fully understood.

Three major factors are known to control the T_H1/T_H2 differentiation: 1. The cytokine milieu; 2. The binding affinity of the T cell receptor to the presented peptide; and 3. the antigen dose, where over the latter only limited knowledge exists.

In this study it is hypothesized that a high antigen dose increases the available peptide loaded MHC-II complexes (pMHC-II) in the secondary lymphoid organ, thereby increasing the antigen presentation and the number of T cell receptor-pMHC-II interaction. This could be an explanation why a HD of antigen induces a T_H2 response. Three different elements modulating the T_H cell differentiation by affecting pMHC-II were analysed:

- The quantity of pMHC-II complexes and the ***type of antigen presenting cell***. This was analysed by focussing on B cells as these induce predominantly T_H2 cells.
- The reduction of the available pMHC-II by ***Complement receptor C5aR1*** deficiency. This was analysed by measuring the effect of the C5aR1 dependent reduction of the antigen presentation on the development of the T_H2 response.
- The Impact of the ***differentiation into T_H2 cells on the clonal expansion*** within an immune response. This was analysed by characterization of the recruitment of T_H cell clonotypes into an immune response after (i) activation and (ii) differentiation into T_H2 cells after activation.

Regarding the pMHCII, the results obtained in this study show that B cells are the most important APCs in antigen dose models. They increased their pMHCII expression after the injection of a HD of SRBC. Therefore it is suggested that the antigen presentation by B cells is of critical importance to induce T_H2 cells. The T_H2 differentiation was completely abolished in B cell deficient mice, confirming this hypothesis. Instead, these mice developed a T_H1 response, independently of the antigen dose. This proves that B cell antigen presentation is necessary to get an antigen dose effect.

As C5aR1 is known to modulate the T_H cell differentiation, this was analysed, in the SRBC and the *L. major* model. In the SRBC model only mild reduction of pMHC-II availability was detectable due to C5aR1$^{-/-}$. Consequently, the strong T_H2 response after HD SRBCs was not modulated. However, by decreasing the dose of administered SRBCs the T_H2 response was weaker, and was significantly suppressed in C5aR1$^{-/-}$ mice. In the *L. major* model, pMHC-II availability was not modulated. Consequently T_H cell differentiation in the draining lymph node, as well as infection severity after LD and HD infection was not influenced. However, medium dose infection led to a significantly more severe infection progression. The results showed an impaired communication between the draining lymph node (lymphoid organ) and the infected skin (peripheral tissue), indicating the higher importance of C5aR$^{-/-}$ signalling in peripheral tissue than in lymphoid organs. However, this can be overridden by the antigen dose effect.

By blockage of CD154/CD40 interaction, T_H cell differentiation into cytokine producing effector T_H cells is abolished. By quantitative measurements of single T cell clonotypes with Next Generation Sequencing it was determined whether the T_H cell differentiation affects the clonal expansion of effector T_H cells. Analysis of the number and sizes of T cell receptor β sequences revealed that the injection of a HD of SRBC induce a similar number of highly expanded T cell clones in WT and CD154 deficient mice. This data show that CD154/CD40 signalling impacts only T_H1/T_H2 differentiation but not the expansion of individual T cell clonotypes.

5.2 Summary (German)

Die $CD4^+$ T_H Zellen haben eine Vielzahl von Funktionen für die Entwicklung einer Immunantwort. Diese Funktionen werden von verschiedenen T_H Zell Untergruppen (Subsets) übernommen, wie zum Beispiel T_H1 und T_H2. Obwohl die Entwicklung der T_H Zellen entscheidend für die induzierte Immunantwort ist, sind die beeinflussenden Faktoren für die noch nicht vollständig erforscht.

Die drei wichtigen Faktoren die die T_H1/T_H2 Differenzierung bestimmen sind (i) das Zytokinmilieu während der T_H Zell Aktivierung, (ii) die Bindungsstärke des T Zell Rezeptors zum präsentierten Antigens und (iii) die Antigen Dosis, mit der das Antigen im Organismus auftritt. Über Letzteres ist bisher nur wenig bekannt.

Die Hypothese dieser Arbeit ist, dass eine hohe Antigen Dosis die Menge der MHC-II Antigen Präsentation erhöht, und diese Erhöhung der Grund ist, warum eine hohe Antigen Dosis eine T_H2 Antwort einleiten kann. Dieser Zusammenhang wurde anhand von drei einzelnen Elementen untersucht, die die T_H Zell Differenzierung beeinflussen können, und in Zusammenhang mit der MHC-II Antigen Präsentation stehen:

- Die Menge der MHC-II Antigen Präsentation und der ***Typ der Antigenpräsentierenden Zellen***. Dies wurde untersucht mit speziellem Fokus auf B Zellen die eine T_H2 Differenzierung unterstützen können. Darum wurde die Relevanz von B Zellen für die T_H2 Antwort untersucht.
- Die Reduktion der pMHC-II Antigenpräsentation durch ***Complement Rezeptor C5aR1***. Daher wurde der Einfluss einer durch C5aR1 verringerten Antigen Präsentation auf die T_H2 Entwicklung untersucht.
- Die Einfluss der ***T_H Zell Differenzierung in T_H2 Zellen auf die klonale Expansion*** innerhalb einer Immunantwort. Daher wurde untersucht ob die Initiierung einen direkten Einfluss auf die klonale Expansion einzelner T_H Zell Klonotypen nimmt.

In dieser Arbeit wurde vermutet dass Antigen Präsentation von B Zellen nach einer HD Immunisierung eine T_H2 Antwort induziert. Es wurde gezeigt, dass B Zellen nur nach Hochdosis Immunisierung Antigen präsentieren, und ihre Anwesenheit für die Entwicklung einer T_H2 Antwort unablässig ist. Folglich können B Zell defiziente Mäuse, Antigen Dosis unabhängig, keine T_H2 Antwort einleiten und entwickeln immer eine T_H1 Antwort. Dies bewies das B Zell Antigenpräsentation notwendig ist um einen Antigen Dosis Effekt zu induzieren.

Vorhergehende Publikationen beschreiben eine verringerte Antigen Präsentation von C5aR1 defizienten B Zellen, dessen Auswirkungen auf die T_H2 Antwort untersucht werden sollte. Im SRBC Modell konnte lediglich ein geringe Verringerung der Antigen Präsentation von B Zellen in C5aR1 defiziente Mäusen gezeigt werden. Folgerichtig konnte die starke T_H2 Antwort nach Hochdosis Immunisierung nicht supprimiert werden. Dennoch konnte eine schwache T_H2 Antwort nach mittlerer Antigendosis signifikant verringert werden. Im *Leishmania major* Modell konnte keine Verringerung der Antigen Präsentation gefunden werden. Konsequenterweise hatte C5aR1 Defizienz keinen Einfluss auf die Entwicklung der T_H Differenzierung im Lymphknoten. Erneut konnten keine Unterschiede im Infektionsverlauf nach Niedrig- und Hochdosis Infektion gezeigt werden, jedoch entwickelte sich unerwarteter Weise eine stärkere Fußschwellung nach mittlerer Antigen Dosis Injektion. Dieser Unterschied lässt sich auf eine gestörte Kommunikation zwischen Lymphknoten und Haut zurückführen.

Durch Blockade von CD154/CD40 Interaktion konnte die Differenzierung von T_H Zellen in Zytokin produzierende Effektor Zellen verhindert werden. Bisher ist nicht bekannt, ob dieser Differenzierungsschritt einen Einfluss auf die Klonale Expansion von T_H Zellen hat, oder ob er nur die Differenzierung bestimmt. Durch quantitative Bestimmung von einzelnen Klonen durch „Next Generation Sequencing“ konnte gezeigt werden, dass die Differenzierung die Klonale Expansion nicht moduliert.

6 Literature

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7. Figures and Tables

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IV Supplement

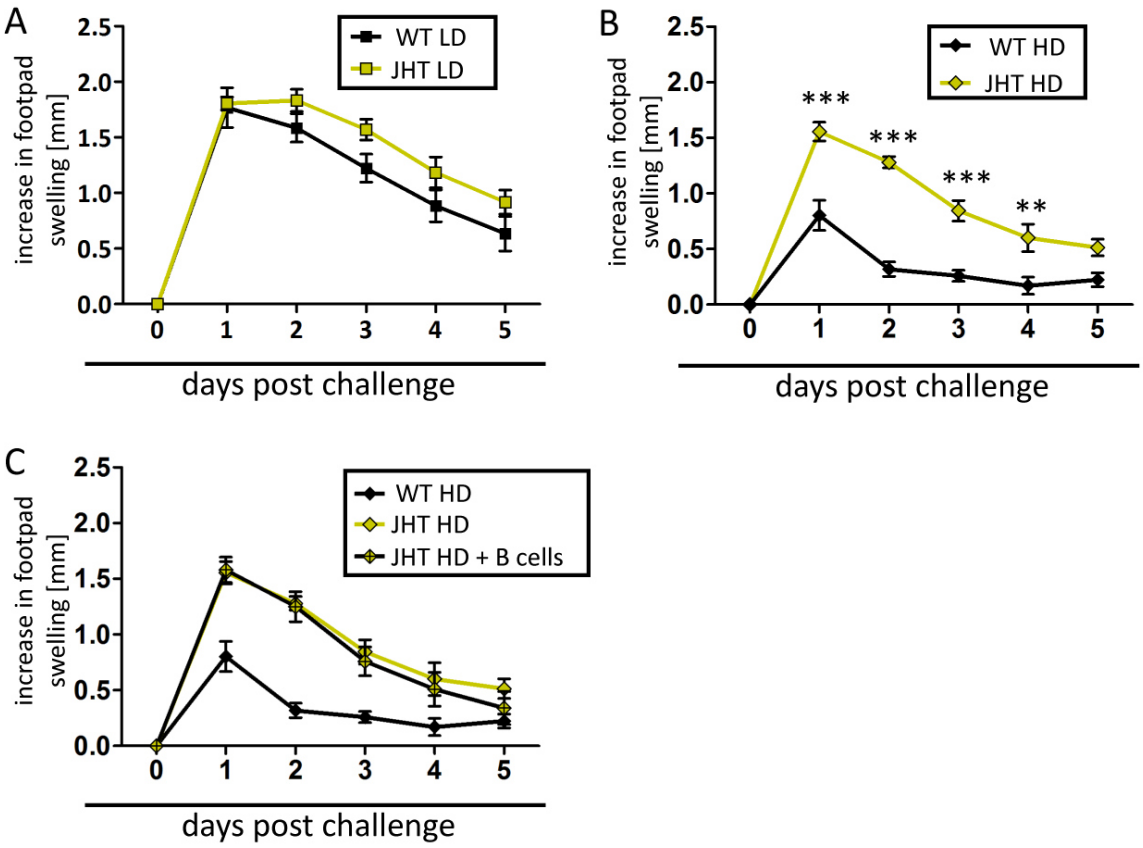

Fig. S1: SRBC induced DTH reactions in WT and JHT mice.
Increase of footpad swelling after challenge with 10^9 SRBCs in the right hind footpad was calculated as the difference between measured footpad thickness and footpad thickness on day 5 (before challenge was applied). JHT mice were compared after LD (A) and HD (B) immunization, as well as after B cell transfer (C) with the respective WT. Dots represent mean ±SEM (Two-way ANOVA), n=5-8, **p<0,01, ***p<0,001.

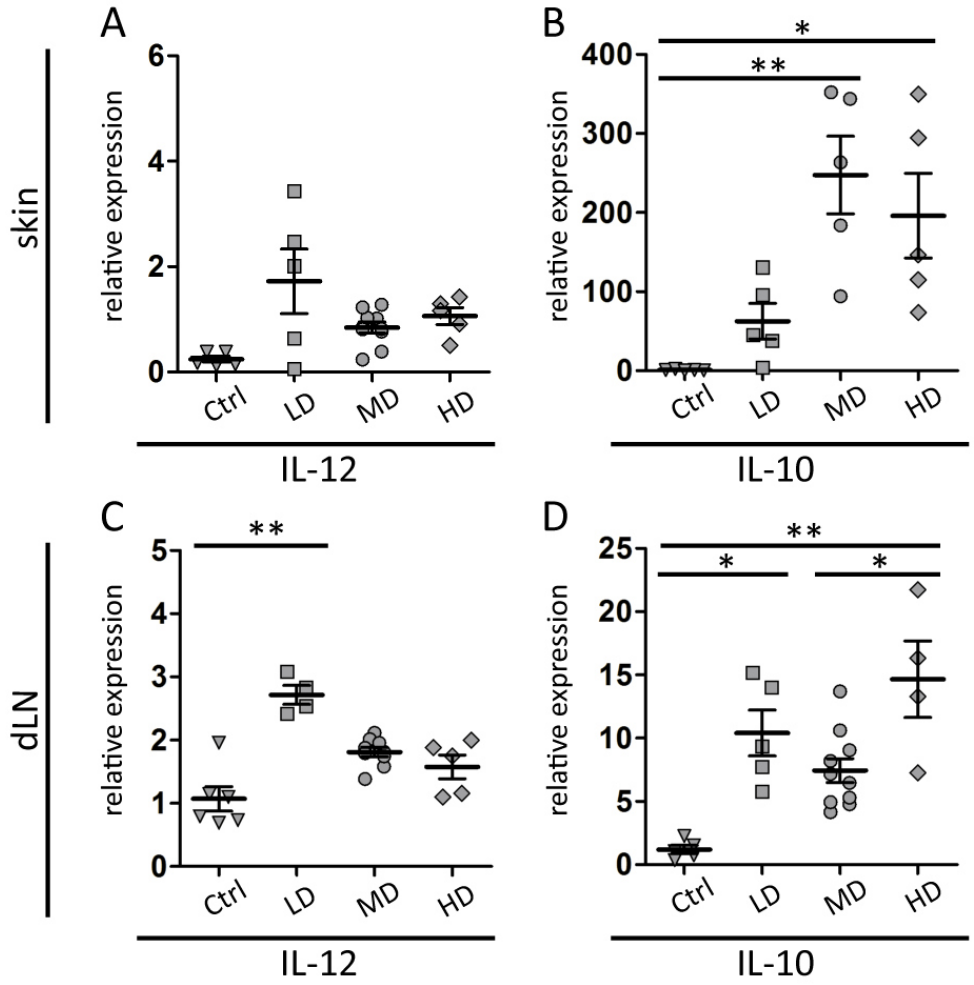

Fig. S2: Low levels of IL-12 but high levels of IL-10 expression in skin and dLN characterize the susceptible Balb/c mice.

Relative mRNA expression of IL-12 (A, C) and IL-10 (B, D) in footpad skin (A,B) and dLNs (C, D), 10 weeks (LD infected mice), 5 weeks (MD infected mice) or 4 weeks (HD infected mice) post infection with the respective number of *L. major* promastigotes. Horizontal lines represent mean ±SEM (Kruskal-Wallis test), *p<0,05, **p<0,01.

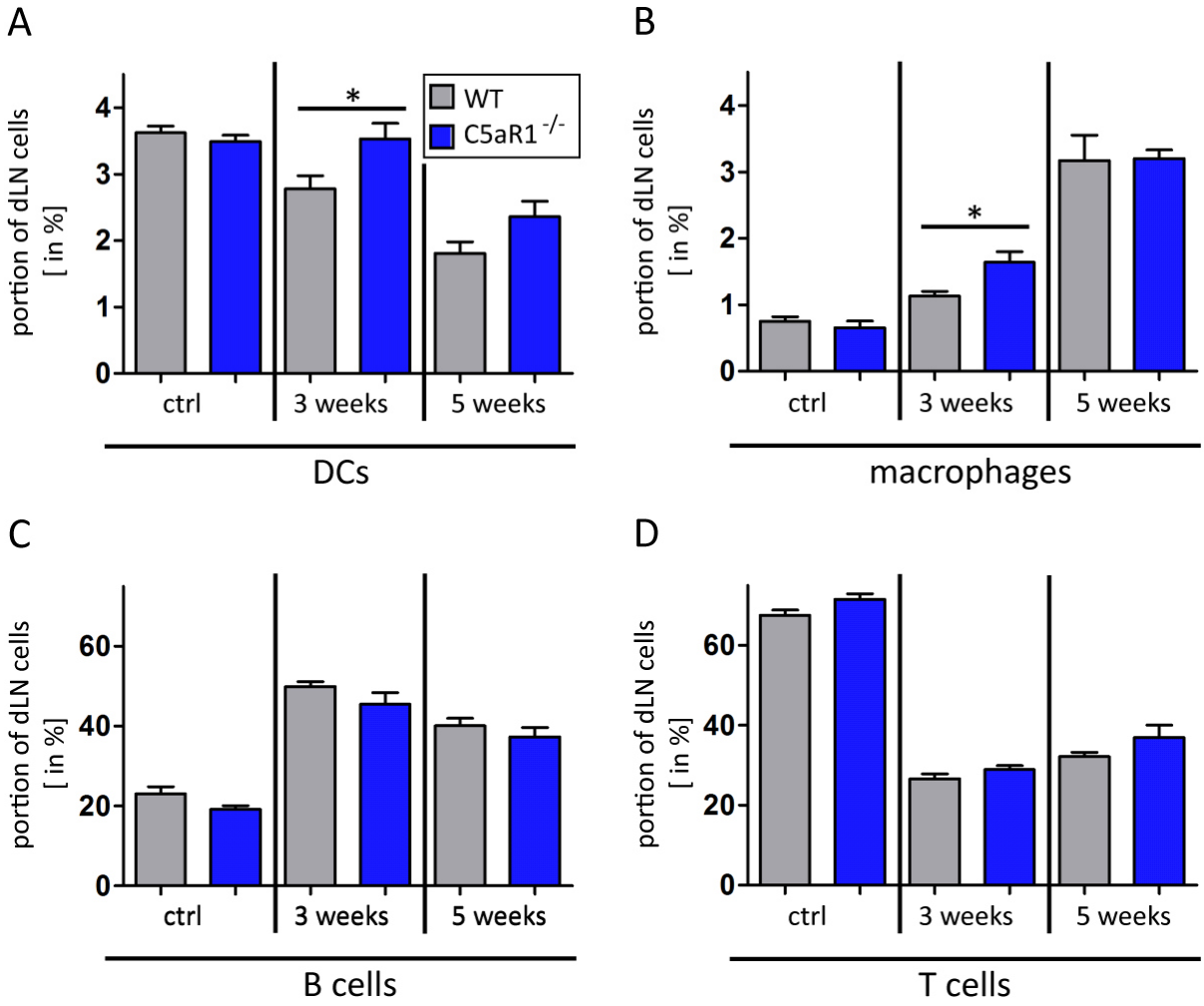

Fig. S3: C5aR1$^{-/-}$ mice showed only minor differences in the dLN cellularity.
Quantification of DCs (A), macrophages (B), B cells (C) and T cells (D) with flow cytometry from cells of control LNs, 3 weeks and 5 weeks post infection with 33.000 *L. major* promastigotes. Bars represent mean ±SEM (Mann-Whitney test), n=10-12, *p<0,05.

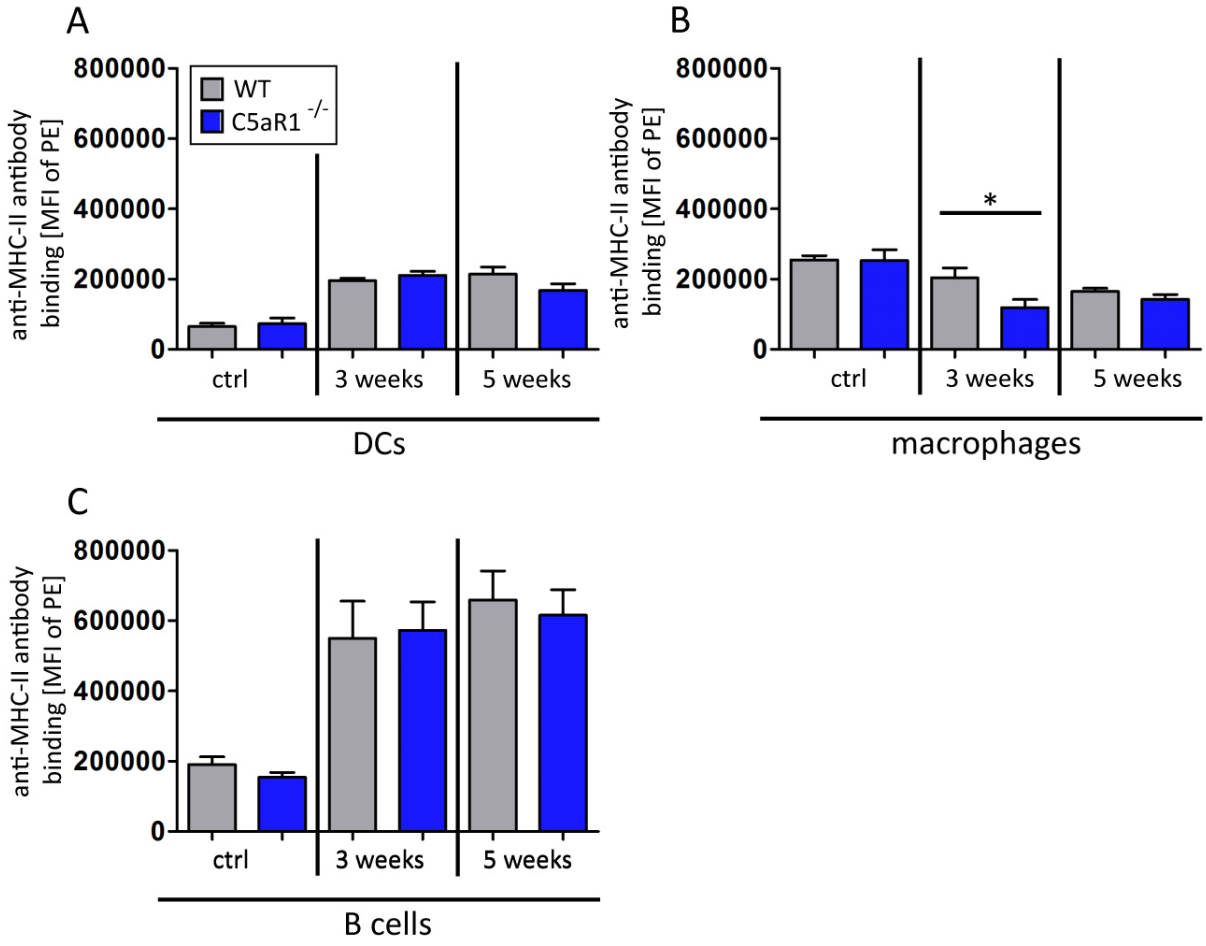

Fig. S4: C5aR1$^{-/-}$ did not impair MHC-II surface expression substantially.
Quantification of MHC-II surface expression on DCs (A), macrophages (B) and B cells (C) with flow cytometry from cells of control LNs, 3 weeks and 5 weeks post infection with 33.000 *L. major* promastigotes. Bars represent mean ±SEM (Mann-Whitney test), n=5-9, *p<0,05.

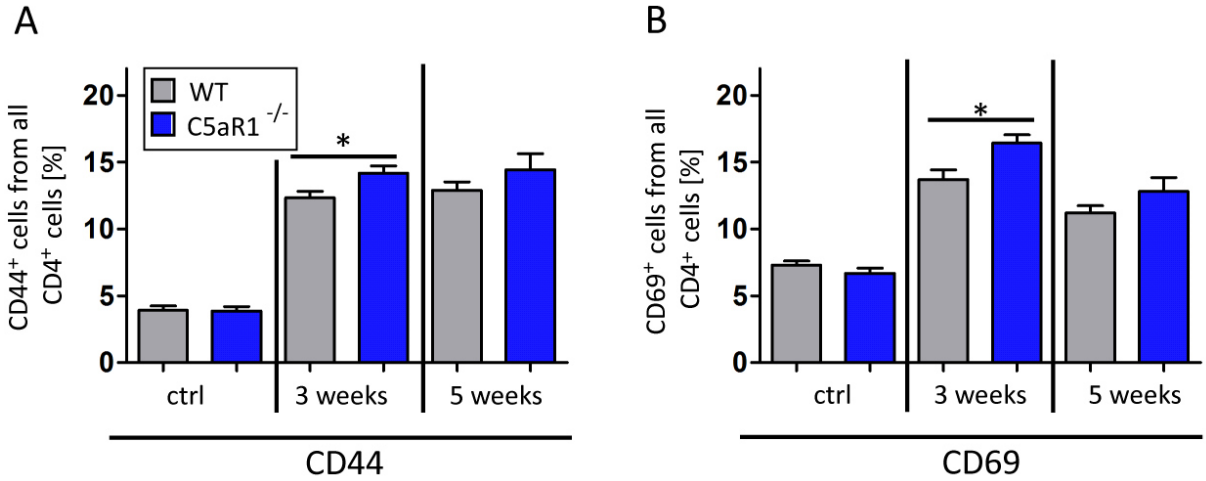

Fig. S5: C5aR1$^{-/-}$ mice showed no substantially impaired T cell activation in the dLN.
Quantification of CD44^{+} (A) and CD69^{+} cells (B) from a CD4^{+} population with flow cytometry from cells of control LNs, 3 weeks and 5 weeks post infection with 33.000 *L. major* promastigotes. Bars represent mean ±SEM (Mann-Whitney test), n=5-9, *p<0,05.

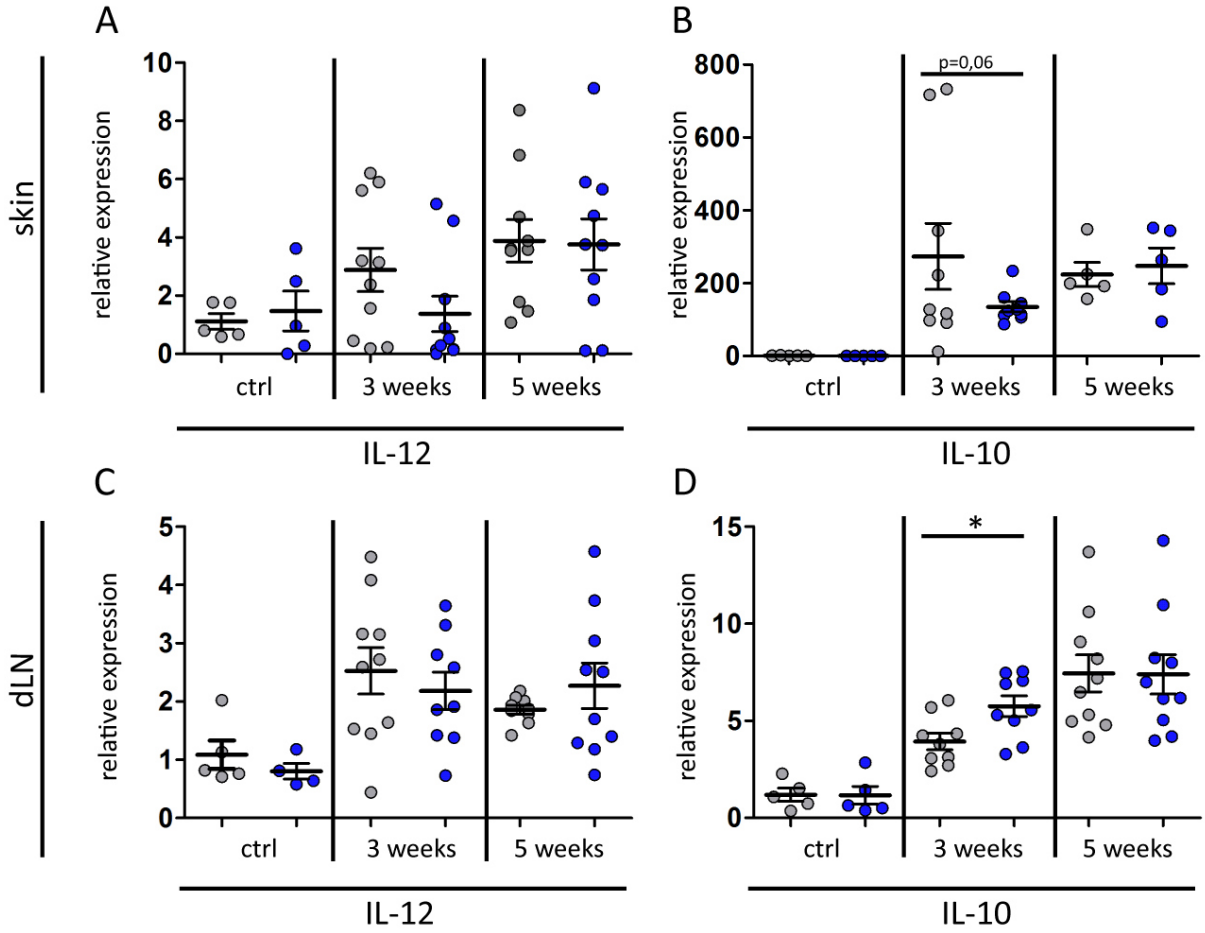

Fig. S6: C5aR1$^{-/-}$ did neither modulate IL-12 nor IL-10 expression in the skin or the dLN.
Relative mRNA expression of IL-12 (A, C) and IL-10 (B, D) in footpad skin (A,B) and dLNs (C, D) of control mice, or 3 weeks and 5 weeks post infection with 33.000 *L. major* promastigotes. Horizontal lines represent mean ±SEM (Mann-Whitney test), *p<0,05.

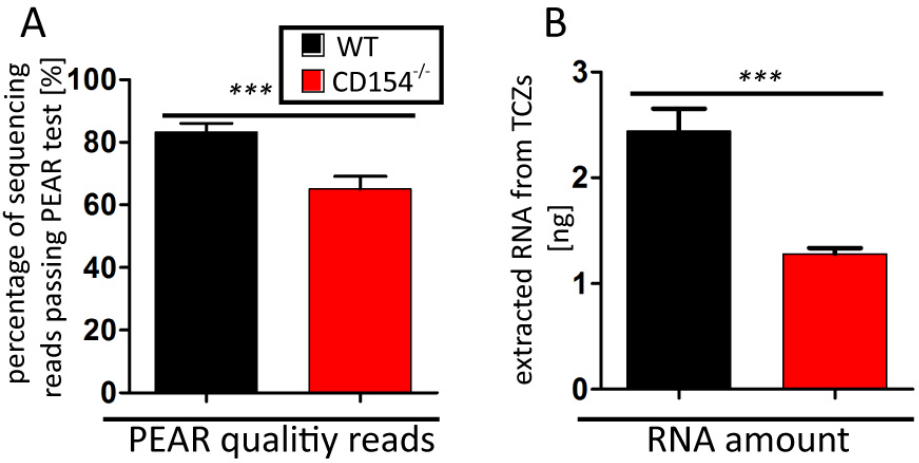

Fig. S7: CD154$^{-/-}$ samples showed significantly less PEAR quality and RNA amount.
Sequencing samples were Peared, adjusting the forward to the reverse read. The percentage of raw reads that could be effectively Peared assign for the PEAR quality (A). The amount of extracted RNA from TCZ samples (B). Bars represent mean ±SEM (Mann-Whitney test), n=12-15.

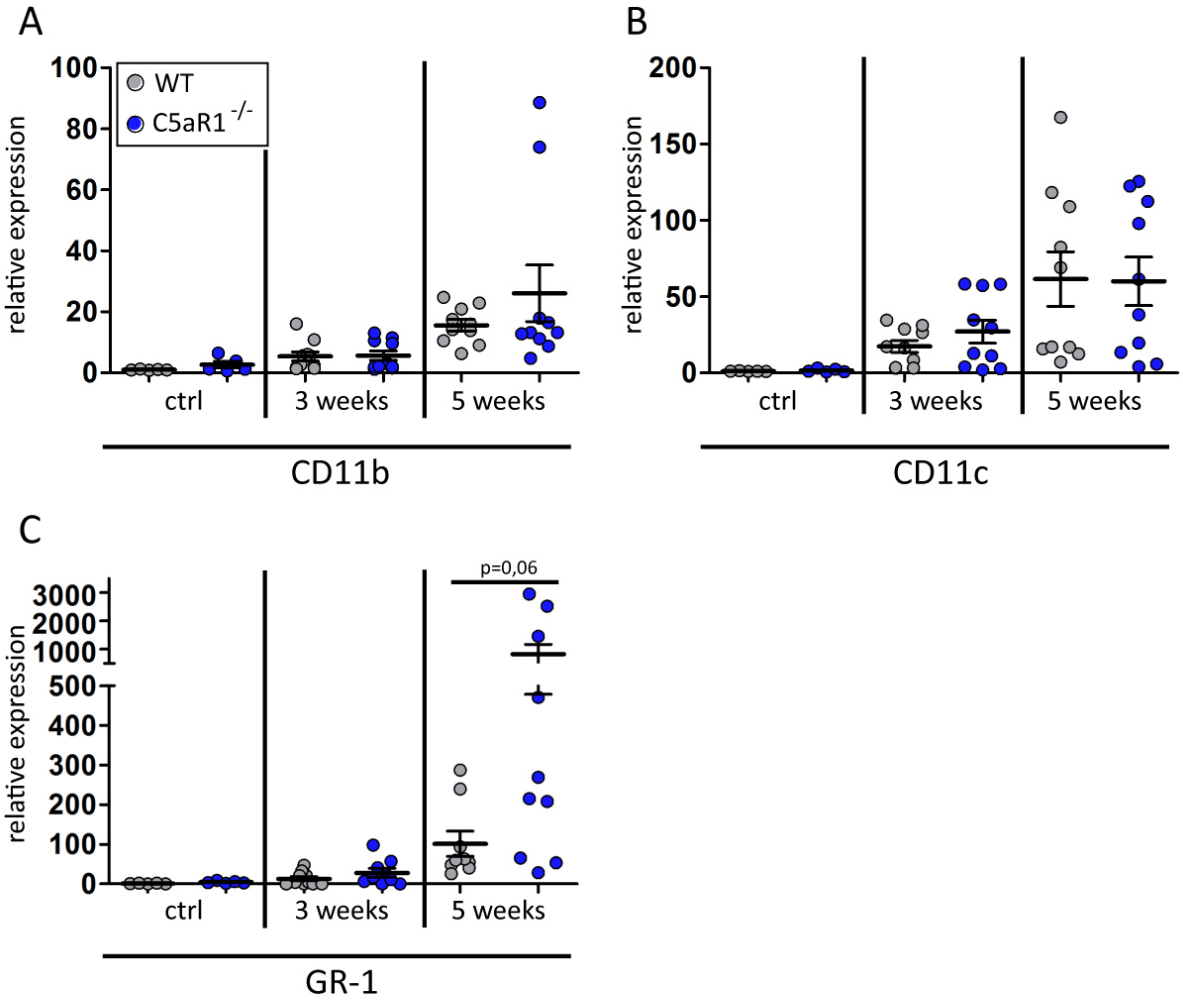

Fig. S8: C5aR1$^{-/-}$ did not modulate APC migration into the skin after 3 or 5 weeks post infection. Relative mRNA expression of CD11b (A), CD11c (B) and GR-1 (C) in footpad skin of control mice, or 3 weeks and 5 weeks post infection with 33.000 *L. major* promastigotes. Horizontal lines represent mean ±SEM (Mann-Whitney test), *p<0,05.

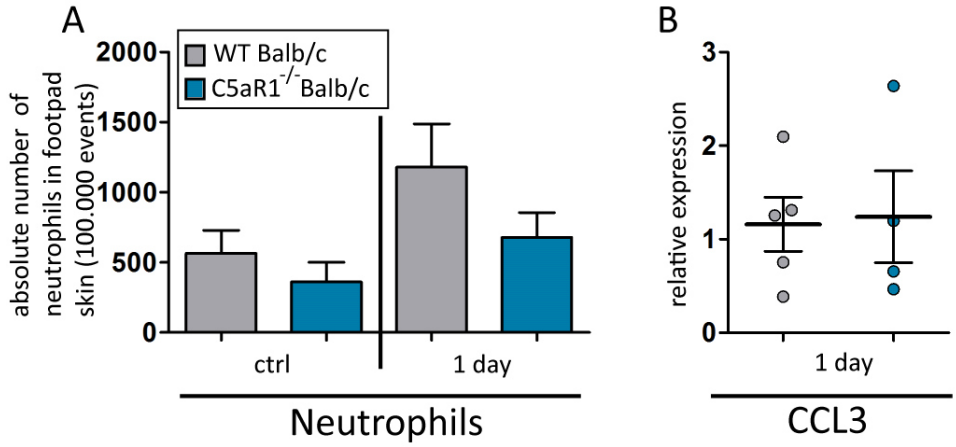

Fig. S9: C5aR1$^{-/-}$ decreased neutrophil migration into footpad slightly, but did not influence CCL3 expression.
Neutrophils were stained and quantified via flow cytometry (Fig. 2A;(Rose et al. 2012)) in the footpad skin of WT and C5aR1$^{-/-}$ mice (A) and relative expression of CCL3 in footpad skin (B) was determined,

1 day post infection with 33.000 *L. major* promastigotes. Bars and horizontal lines represent mean ±SEM (Mann-Whitney test and Kruskal-Wallis test), n=2-8.

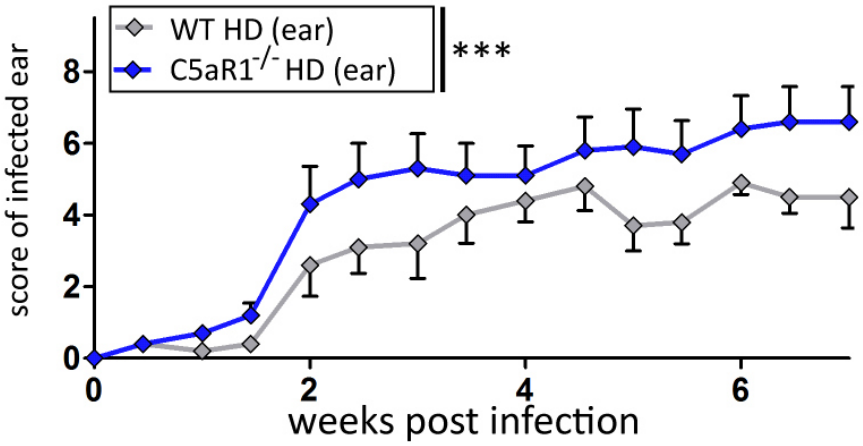

Fig. S10: C5aR1$^{-/-}$ mice developed significantly more severe lesions in the ear infection model. The score of ear, after infection with 100.000 *L. major* promastigotes, was determined by measuring lesion size and scoring severity after Schuster et al. (Schuster et al. 2014). Dots represent mean ±SEM (Two-Way ANOVA), asterisks at legend indicate significantly different infection severity over time, n=5, ***p<0,001.