Dissertation

submitted to the
Combined Faculties for the Natural Sciences and for Mathematics
of the Ruperto-Carola University of Heidelberg, Germany
for the degree of
Doctor of Natural Sciences

presented by
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born in Dresden, Germany

Oral-examination: 22.07.2013

The role of Dickkopf-3 in the adaptive immune system

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Acknowledgements

This PhD thesis was accomplished in the time period from April 2010 to May 2013 in the Department of Molecular Immunology at the German Cancer Research Center (DKFZ) Heidelberg, Germany, with the invaluable support of many people.

First of all, I would like to thank my supervisor Prof. Dr. Bernd Arnold for giving me the great opportunity to work in his group on such an interesting and instructive project. Bernd, your great way of helping me to approach scientific questions has deepened my fascination for science. Thank you for sharing your vast knowledge with me. I cannot imagine a better supervisor, not only from the scientific side but also from the personal side; you are always caring about the well-being of all lab members, and this is notable in the extraordinary nice atmosphere in the lab, in which it was a pleasure to work. I also thank you for your critical reading of this PhD thesis.

In addition, I want to express my warmest thanks for the personal support of Prof. Dr. Günter Hämmerling, first referee of this thesis and first TAC member throughout the time of my PhD. Günter, I appreciate your critical understanding of science, which taught me to approach scientific problems by looking "behind the curtains" and from a different perspective. Thank you for your stimulating discussions and all your good advices throughout the time of my PhD and for the critical reading of my thesis.

Special thanks go to Prof. Dr. Hermann-Josef Gröne. Without him and some people of his lab, especially Guiseppina Federico, all the histological analysis shown in this thesis would not exist. I also thank Dr. Gernd Moldenhauer for generating the monoclonal anti-Dkk3 antibody and Prof. Christoph Niehrs for providing us with the Dkk3 deficient mice, which were established in his lab.

I am very thankful to Sabine Schmitt, the nice angel of the lab. Not only is she keeping the lab organized but also is she always offering help when needed. Thank you for your enormous help with the numerous ELISAs, without you I would not have managed to do all of them.

Furthermore, I am very grateful for the expert technical assistance with many experiments to Georg Pougialis, who helped with numerous EAE experiments, Alexandra Klevenz, who is a true expert in conducting difficult *in vitro* experiments, Sandra Prokosch, who is excellent in handling mice and doing cell culture, and Gorana Hollmann, having a lot of experience in protein biology. Thank you all for your great technical and advisory help during my PhD time.

I thank Günter Küblbeck for sharing his great interest in nature and science with me, and for always discussing experiments and outcomes. I do not know anyone, who better understands molecular biology than you.

I thank Alina Steinbach for her great help with the “Soggy-project”. It was a very nice time to have you as my Master student working with me.

I also want to express my warmest gratitude to Dr. Thilo Oelert. Your continuous ideas to the project with stimulating discussions contributed enormously to the project. I also appreciate your way of always being positive no matter which outcome results have.

Special thanks go to Anastasia Stemke and Rafael Carretero. I am very grateful, that you were always around in good and in hard times during my PhD. Thank you for being my family in Heidelberg. Of course I also thank all my other friends, my family and last but not least my partner Fabian. Without your love and great support I would not be there, where I am today.

Summary

Lymphocytes express highly specific receptors to recognize pathogens and malignant cells. At the same time, lymphocytes recognizing self-antigens also develop. These need tight regulation in order to prevent autoimmunity. The elimination or control of self-reactive cells is ensured by central and peripheral tolerance mechanisms. A detailed understanding of tolerance induction and maintenance is indispensable for the establishment of novel therapeutic strategies to treat autoimmune diseases and to prevent rejection of transplanted tissue. Recently, we identified Dickkopf 3 (Dkk3) as a novel immune-mediator in a transgenic model of peripheral T cell tolerance. To further clarify the immune-modulatory capacity of Dkk3 we investigated the influence of this secreted protein on T and B cell function.

Experimental autoimmune encephalomyelitis (EAE) is a T cell mediated mouse model of the human autoimmune disease multiple sclerosis. Dkk3 deficient mice developed more persistent symptoms of EAE with increased numbers of brain infiltrating CD4 and CD8 T cells as well as myeloid cells in comparison to wild type mice. Lack of environmental Dkk3 was responsible for the observed phenotype as shown by adoptive T cell transfers using Dkk3 deficient T cells. Disease persistence in Dkk3 deficient mice was associated with an altered local cytokine profile. Absence of Dkk3 did not change the differentiation of Th1 and Th17 T cells but led to increased levels of Th2 associated cytokines.

The role of Dkk3 on B cell function was analyzed by investigating antibody and cytokine secretion. Both assay systems showed that B1 related functions were increased in Dkk3 deficient mice, such as antibody secretion to the antigens LPS and phosphorylcholine and IL-10 production. Indeed, Dkk3 deficient mice yielded increased numbers of B1 cells. B2 cell development was supported by Dkk3 at the step from the pre- to immature B cell stage, whereas Dkk3 negatively regulated B1 cell generation. The murine MRL/LPR strain, a mouse model of human systemic lupus erythematosus (SLE), was used to investigate the role of Dkk3 in a B cell mediated autoimmune disease. Treatment of these mice with a Dkk3 neutralizing antibody led to increased numbers of B1 cells and higher pathology of SLE, including enlarged lymph nodes, higher levels of autoantibodies, increased glomerulonephritis and inflammation in pancreas and lung.

Among the Dkk protein family, only Dkk3 and Soggy share the unique sgy-domain. To address the question whether this sgy-domain may be responsible for the immune-modulatory capacity we investigated immune functions of Soggy deficient mice. Alterations in the T cell compartment were observed, such as decreased numbers of regulatory T cells in comparison to wild type mice. Furthermore, Soggy deficiency led to an earlier onset and stronger symptoms of EAE in comparison to the disease course in C57BL/6 and Dkk3$^{-/-}$ mice.

In summary, Dkk3 regulates T and B cell mediated autoimmunity. In addition, we were able to identify Soggy as a novel immune-mediator suggesting that the shared sgy-domain is contributing to the immune-suppressive capacity of Dkk3 and Soggy.

Zusammenfassung

Lymphozyten besitzen hoch spezifische Rezeptoren um Pathogene und maligne Zellen zu erkennen. Es entwickeln sich aber auch Lymphozyten, welche Selbst-Antigene erkennen. Diese müssen streng kontrolliert werden, damit keine Autoimmunität entsteht. Für die Kontrolle oder Elimination solcher autoreaktiven Zellen sorgen zentrale und periphere Toleranzmechanismen. Ein detailliertes Verständnis, wie Toleranz des Immunsystems induziert und erhalten wird, ist für die Entwicklung von neuen therapeutischen Strategien um Autoimmunerkrankungen zu behandeln und Transplantatabstoßung zu verhindern, entscheidend. Erst kürzlich identifizierten wir Dickkopf 3 (Dkk3) als ein neues immun-modulatorisches Molekül in einem transgenen Modell peripherer Toleranz. Um die immun-modulatorische Kapazität von Dkk3 weiter aufzuklären, wurde in dieser Arbeit der Einfluß von Dkk3 auf T- and B-Lymphozyten-Funktionen untersucht.

Experimentelle autoimmune Encephalitis (EAE) ist ein durch T-Zellen ausgelöstes murines Krankheitsmodell der humanen Multiplen Sklerose. Im Vergleich zu Mäusen die Dkk3 exprimieren, entwickelten Mäuse, welche kein Dkk3 besitzen, stärkere EAE Krankheitssymptome und wiesen eine erhöhte Anzahl von infiltrierenden CD4 und CD8 T-Zellen, sowie myeloide Zellen im Zentralen Nervensystem auf. Durch einen adoptiven Transfer von Dkk3-defizienten T -Zellen wurde gezeigt, dass das Dkk3 in der Umgebung entscheidend ist. Der verschlimmerte Krankheitsverlauf in Dkk3-defizienten Tieren war mit einem lokal veränderten Zytokinmileu assoziiert. Die Abwesenheit von Dkk3 veränderte die Differenzierung von Th1 und Th17 Zellen nicht, sondern führte zu bevorzugter Th2 Differenzierung.

Die Analyse von Antikörper- und Zytokinsekretion wurden genutzt, um die Rolle von Dkk3 auf B Zell Funktionen zu untersuchen. In beiden Assaysystemen zeigte sich, dass B1 Zell-Funktionen in den Mäusen, welche kein Dkk3 exprimieren, erhöht sind, wie beispielsweise LPS- und Phosphorylcholine-spezifische Antikörperantworten oder die Sekretion von IL-10. Tatsächlich fanden wir auch eine erhöhte Anzahl an B1 Zellen in Dkk3-defizienten Mäusen. Die Entwicklung von B2 Zellen wurde durch Dkk3 unterstützt, wohingegen die Generierung von B1 Zellen negativ von Dkk3 reguliert wurde. Um die Rolle von Dkk3 auf eine B Zell-vermittelte Autoimmunerkrankung zu untersuchen, benutzten wir den murinen MRL/LPR Stamm, welcher als Mausmodell von humanem systemischen Lupus Erythematosus (SLE) gilt. Die Behandlung dieser Mäuse mit einem Dkk3 neutralisierendem Antikörper erhöhte B1 Zellzahlen und führte zu einem erhöhten Grad an SLE-Pathologie. Unter anderem waren vergrößerte Lymphknoten, erhöhte Produktion von Autoantikörpern, eine stärkere Glomerulonephritis und vermehrte Entzündungen in Pancreas und Lunge zu beobachten.

Dkk3 und Soggy sind die einzigen Mitglieder innerhalb der Dkk Protein-Familie, welche die Sgy-Domäne besitzen. Um herauszufinden, ob die Sgy-Domöne für die immun-modulatorische Kapazität von Dkk3 verantwortlich ist, untersuchten wir Mäuse, welche kein Soggy exprimieren. In diesen wurde im Vergleich zu Wildtyp-Mäusen ein verändertes T Zell-Kompartiment, wie beispielsweise eine geringere

Anzahl an regulatorischen T Zellen, gefunden. Desweiteren zeigten Soggy-defiziente Mäuse ein früheren Krankheitsausbruch und verstärkte Symptome von EAE.
Zusammenfassend konnten wir zeigen, dass Dkk3 T and B Zell vermittelte Autoimmunität reguliert. Zusätzlich wurde Soggy als ein neuer Immunmediator identifiziert. Dieser Befund deutet darauf hin, dass die Sgy-Domäne zur immunhemmenden Wirkung von Dkk3 und Soggy beiträgt.

Contents

Abbreviations

ACAID	Anterior chamber associated immune deviation
AICD	Activation induced cell death
AIRE	Autoimmune regulator
ANA	Antinuclear antibody
APC	Antigen presenting cell
BCR	B cell receptor
BM	Bone marrow
BSA	Bovine serum albumin
CFA	Complete Freund's adjuvant
CFSE	5(6)-Carboxyfluorescein diacetate N-succinimidyl ester
CLP	Common lymphoid progenitor
CNS	Central nervous system
CSR	Class switch recombination
cTEC	Cortical thymic epithelial cells
CTLA-4	Cytotoxic T lymphocyte antigen-4
DC	Dendritic cell
EAE	Experimental autoimmune encephalitis
ELISA	Enzyme linked immunosorbent assay
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescent-activated cell staining
FoxP3	Forkhead box protein P3
GFP	Green luorescent protein
GM-CSF	Granulocyte monocyte colony stimulating factor
i.p.	Intraperitoneal
i.v.	Intravenous
IBD	Inflammatory bowel disease
IDO	Indolamine 2,3 dioxygenase
IFA	Incomplete Freund's adjuvant
Ig	Immunoglobulin
IL	Interleukin
iNKT cell	Invariant natural killer cell
LN	Lymph nodes(s)
LPR	Lymphoproliferation
LPS	Lipopolysaccharid
MBP	Myelin basic protein
MHC	Major histocompatibitliy complex

MLN	Mesenteric lymph nodes
MMP	Metallopproteinase
MOG	Myelin oligodendrocyte protein
mTEC	Medullary thymic epithelial cells
NK	Natural killer
NOD	Non-obese-diabetic
ON	Over night
PBS	Phosphate bufferd saline
PC	Phosphorylcholine
PC-BSA	Phosphorylcholine-BSA
PC-KLH	Phosphorylcholine-Keyhole Limpet Hemocyanin
PerC	Peritoneal cavity
PP	Peyer's patches
PT	Pertussis toxin
RT	Room temperature
s.c.	subcutaeous
SEM	Standard error of the mean
SLE	systemic lupus erythematosus
TCR	T cell receptor
TD	Thymus-dependent (T cell-dependent)
TdT	Terminal deoxynucleotidyl transferase
Tfh	Follicular helper T cells
TGF	Transforming growth factor
Th	T helper cell
TI	Thymus-independent (T cell-independent)
TLR	Toll like receptor
TNF	Tumor necrosis factor
TNP-BSA	Trinitrophenol-BSA
TNP-Ficoll	Trinitrophenol-Ficoll
TNP-LPS	Trinitrophenol-LPS
TRA	Tissue restricted antigen
Treg	Regulatory T cells
wt	Wild type

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1 Introduction

1.1 The adaptive immune system

The immune system features an extraordinary set of mechanisms working together to maintain on one hand self-tolerance and homeostasis, and on the other hand protective immunity to pathogens. It is capable of distinguishing between dangerous and non-dangerous signals and reacts subsequently with activation or tolerance [1]. If one of these mechanisms fails, exuberant infections, autoimmune or hypersensitivity reactions can develop.

The immune system can be divided into two with each other interacting branches, the innate and the adaptive immune system. Whereas the innate immune system works in a generic, non-specific way, the adaptive immune system bears highly specific receptors to recognize antigens.

The adaptive immune system consists of lymphocytes, which are subdivided into T and B cells. The development of these, and also of most of the other immune cells, takes place in primary lymphoid organs, the bone marrow (BM) and the thymus. Initiation of adaptive immune responses, which normally has to be licensed by the innate immune system, takes place in secondary lymphoid organs, which include the spleen, lymph nodes (LN) or gut associated lymphoid tissue (GALT).

The generation of the antigen-specific receptors, that both B and T cells carry, is a random process of recombination, in which different gene subunits are combined [2]. The random recombination results in a theoretical repertoire of more than 10^{11} different specificities in humans. This enables the adaptive immune system to basically recognize every possible antigen with only a small proportion of lymphocytes being reactive to one specific structure. The activation by antigen leads to an expansion of the antigen-specific lymphocytes in a process called selective clonal expansion. It ensures sufficient numbers of lymphocytes specific for a particular antigen during an immune response. Some of these clonally expanded lymphocytes differentiate into memory cells that are able to react faster and stronger to recall infections with the same pathogen. The formation of immunological memory and the high specificity to a broad range of antigens are key characteristics of the adaptive immune responses.

1.2 Mechanisms of tolerance

The broad diversity of receptors recognizing every possible antigen is required for protection against harmful pathogens. However, the random process of receptor generation also creates specific receptors recognizing self and harmless antigens. Therefore, tight regulatory mechanisms are indispensable to prevent autoimmunity and allergy.

The mechanisms ensuring the establishment of tolerance can be divided into central and peripheral tolerance. Central tolerance consists of clonal deletion or inactivation of self-reactive lymphocytes during development [3]. It takes place in the primary lymphoid organs. As B cells develop in the BM their organ of central tolerance is the BM [4]. Central tolerance of T cell takes place in the thymus [5]. As tolerance induction in these primary lymphoid organs is established during the development of B and T cells and as it is different for both lymphocyte types, it will be described more in detail in the development section of B and T cells (1.3.1 T cell development/1.4.1 B cell development).

Lymphocytes escaping the mechanisms of central tolerance can still be controlled by a multitude of mechanisms in the periphery, collectively called peripheral tolerance. They include the induction of anergy, the deletion by activation induced cell death (AICD), suppression mediated by regulatory cells and the establishment of immune privileged sites [3].

Anergy describes a state of unresponsiveness in T and B cells that is characterized by an active repression of TCR or BCR signalling resulting in the loss of proliferation [6, 7]. Auto-reactive cells which are anergic do not respond to self-antigens and stay in the anergic state, when encountering antigen (in the case of the T cells presented by antigen presenting cells (APC)) despite the presence of co-stimulatory signals, which are expressed in the presence of danger signals [8].

Another strategy of the immune system to limit overshooting responses and to eliminate self-reactive leukocytes is a mechanism called AICD. As the name implies, it is influenced by the nature of the initial activation event. The mechanism is employed for instance by tolerogenic dendritic cells (DCs) or by regulatory T cells (Tregs), which can be present during the process of activation [9, 10]. Subsequently, death is induced by death ligand - death receptor interactions leading to apoptosis signalling [11]. The most prominent subclass of death receptors is the tumor necrosis factor receptor (TNFR) family. Within this family, CD95-mediated cell death is the best studied mechanism of AICD [12]. Mice deficient for CD95, also called Fas-

receptor, (MRL.Fas$^{lpr/lpr}$) or FasL (C57BL/6.FasL$^{gld/gld}$) are prone to spontaneous autoimmunity with age [13].

A widely-used mechanism in the immune system to prevent unwanted immune responses against self is the suppression mediated by regulatory cells. There are several different cell types known, including mesenchymal stem cells (MSCs)[14], myeloid derived suppressor cells (MDSCs) [15], and the most studied Tregs, which can be divided into natural and induced Tregs (nTregs/iTregs) [16]. Suppressive cell types employ several mechanisms to inhibit immune response, such as secretion of immune-suppressive factors (e.g. IL-10, TGF-β, IL-35, IDO), contact-dependent suppression, which can lead to induction of apoptosis (e.g. CTLA-4, PD-L1), induction of anergy, cytolysis of effector cells by granzyme secretion (e.g. Granzyme A or B), or the metabolic disruption by the consumption of pro-inflammatory molecules (e.g. IL-2 or ATP consumption) [14, 15, 17, 18].

Described so far are the mechanisms to establish a systemic immunological tolerance. In addition, there are special tissues – so called immune privileged sites -, which need an immune-suppressive microenvironment in order to assure local protection. In such tissues strong immune-responses and inflammation would lead to irreparable damage. Therefore, life essential tissues or/and tissues with a limited capacity of regeneration need a special protection by immuno-regulatory mechanisms. The brain, the eye, or organs of the reproductive system for example belong to the immune privileged sites. The prevention or dampening of immune responses in these sites occurs through different mechanisms. Often immune privileged sites are anatomically separated by structural barriers [19, 20], which might present the only passive mechanisms of immune deviation. Streilein describes a mechanism taking place in the eye, called anterior chamber-associated immune deviation (ACAID), which emphasized that immune privilege is actively acquired and maintained by the immune system itself [21]. By the mechanisms of ACAID, infections of the eye result in harmless immune responses [22]. Numerous active mechanisms used commonly by immune privileged sites have been described. MHC I molecules are absent from the neurons of the brain [23], the corneal epithelium and the retina as well as from the trophoblast [24]. Additionally, a number of immunosuppressive molecules are expressed by immune privileged tissues, such as the non-classical MHC Ib molecules leading to inhibition of NK and cytotoxic T cells [25], suppressive molecules such as IDO or TGF-β [26, 27], and molecules leading to AICD and anergy, such as the CD95 or CTLA-4 [28]. Besides these soluble or membrane-bound factors the immune-privilege is also sustained by the accumulation of Tregs, which are applying their own regulatory mechanisms, as described above.

1.3 T cells

T lymphocytes (T cells) are subdivided into αβ-T cells and γδ-T cells, which refers to the T cell receptor (TCR) chains they express. αβ-T cells present the majority of T cells within the immune system, whereas γδ-T cells are a minor population residing preferentially in epithelial layers of tissues underlying surfaces of the body in contact with the external environment, such as the skin, the intestinal epithelium or the lung [29]. In this thesis only αβ-T cells have been investigated, therefore γδ-T cells are not considered in the introduction.

T cells are characterized by their antigen specific receptor, the TCR (Figure 1.1). It is a heterodimer consisting of an α and β chain. The TCR is attached to the T cell by two transmembrane regions and is not secreted. It is associated with the invariant accessory chains CD3γ, CD3δ and CD3ε, which together form the CD3 complex that is required for TCR expression and signalling. Additionally, a homodimer of two ζ-chains is bound to the complex and is responsible for intracellular signalling. Importantly, T cells can only recognize peptides presented via MHC molecules with their TCR (further described in 1.3.2 Activation of T cells) [3]. Whole unprocessed proteins or non-protein molecules do not lead to signal transduction in T cells.

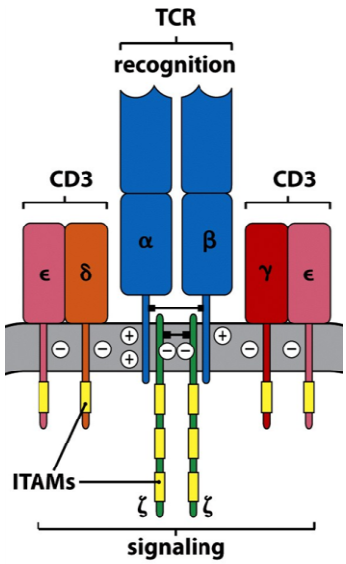


Figure 1.1 | The T cell receptor (TCR). The TCR is a heterodimer consisting of the α and β chain which assemble with the invariant accessory chains CD3γ, CD3δ and CD3ε, which together form the CD3 complex. Additionally, a homodimer of two ζ-chains is bound to the complex and is responsible for intracellular signalling by using the immunoreceptor tyrosine-based activation motifs (ITAMs / yellow segments). *Figure from Immunobiology, Janeway et al., 6th edition, 2005 Garland science Publishing.*

1.3.1 T cell development

The development of T cells takes place in a specialized organ called thymus. It is the primary lymphoid organ for the T cells. T cell progenitors are derived from pluripotent stem cells in the BM and migrate into the thymus through the blood, where the T cell maturation process, characterized by the TCR assembly, occurs. This process takes place throughout life, but is decelerated in adults, where T cell numbers are sustained by division of mature T cells outside of primary lymphoid organs [30].

T cell development in the thymus is depicted in Figure 1.2. T cell progenitor cells from the BM migrate to the thymus to enter at the cortico-medullary junction. At this time point they do not express the typical coreceptors CD4 and CD8 nor the TCR, whose genes are not yet rearranged. When the progenitor cells enter the outer cortex of the thymus and start to interact with thymic stroma cells, consisting of epithelial cells, the differentiation into the T cell lineage is initiated [31]. At the end of this phase T cells bear the first T cell specific surface markers, as Thy-1 in mice. But they are still not expressing the TCR or the correceptors CD4 and CD8. Hence, they are called "double-negative" (DN) thymocytes. The double-negative stage can be further subdivided into four developmental stages based on the expression of the adhesion molecules CD44 and CD25. First, double negative thymocytes express CD44 (DN1), in these thymocytes the genes for both chains of the TCR are still unrecombined as in the germline. When maturation of thymocytes goes on CD25 expression starts (DN2). In the next stage (DN3) CD44 is downregulated, when β-chain rearrangements start. If the rearrangements of the β-chain are not successful, cells stay in this stage and die. The cells, which successfully rearrange the β-chain, downregulate CD25 (DN4)[5]. They express the pre-TCR, in which a substitute α-chain, called pTα, interacts with the β-chain to form the receptor together with the CD3 molecules. Signalling through this pre-TCR leads to proliferation, migration towards the medulla and expression of both coreceptors CD4 and CD8. Therefore, this stage is called "double positive" (DP). Double positive cells rearrange the α-chain genes to form the mature TCR. The interaction of the TCR with self-MHC molecules expressed by cortical thymic epithelial cells (cTEC) leads to a process called positive selection [31]. Only T cells with sufficient affinity of the TCR to bind to self-MHC molecules receive survival signals. The contact leads to the development of three T cell lineages. The two major populations are CD4 and CD8 single positive T cells (CD4 T cells and CD8 T cells) and the minor population is the natural Treg, which expresses CD25. The strength and the duration of the contact between TCR and MHC molecule determine the cell type. Cells selected by MHCI will mature to a $CD8^+$ T cells, whereas cells selected by MHCII mature to $CD4^+$ T cells [32].

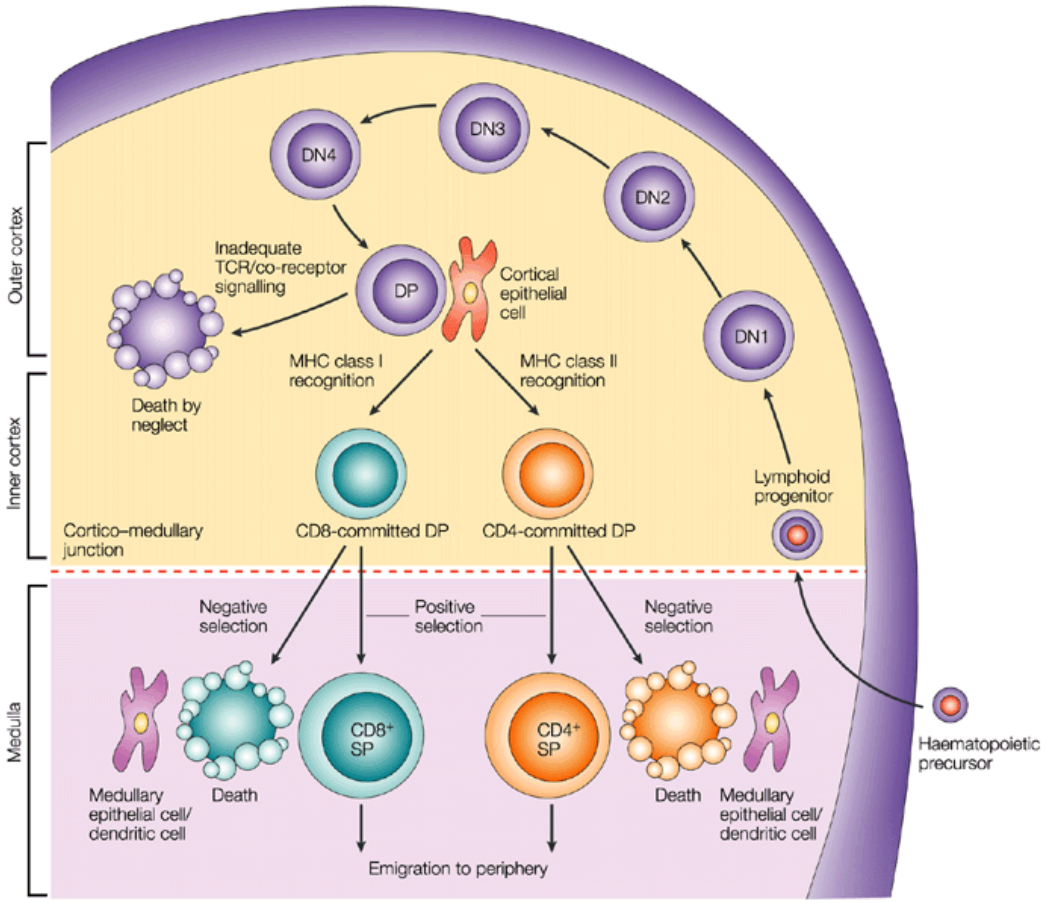


Figure 1.2 | T-cell development in the thymus. Committed lymphoid progenitors arise in the bone marrow and migrate to the thymus. Early committed T cells lack expression of T-cell receptor (TCR), CD4 and CD8, and are termed double-negative (DN) thymocytes. DN thymocytes can be further subdivided into four stages of differentiation (DN1: $CD44^{+}CD25^{-}$, DN2: $CD44^{+}CD25^{+}$, DN3: $CD44^{-}CD25^{+}$, and DN4: $CD44^{-}CD25^{-}$). Successful pre-TCR expression leads to substantial cell proliferation during the DN4 to double positive (DP) transition and replacement of the pre-TCR with the complete rearranged TCR. The DP-thymocytes then interact with cortical epithelial cells that express a high density of MHC class I and class II molecules associated with self-peptides. The fate of the DP thymocytes depends on signalling that is mediated by interaction of the TCR with these self-peptide–MHC ligands. Too little signalling results in delayed apoptosis (death by neglect). Too much signalling can promote acute apoptosis (negative selection); this is most common in the medulla on encounter with strongly activating self-ligands on haematopoietic cells, particularly dendritic cells. The appropriate, intermediate level of TCR signalling initiates effective maturation (positive selection). Thymocytes that express TCRs that bind self-peptide–MHC-class-I complexes become CD8 T cells, whereas those that express TCRs that bind self-peptide–MHC-class-II ligands become CD4 T cells; these cells are then ready for export from the medulla to peripheral lymphoid sites [5]. *Figure from Germain, R. N., 2002, Nat Rev Immunol*

The next step is one of the most important mechanisms to prevent autoimmunity. It is referred to as central T cell tolerance and leads to negative selection of T cells. In this process single positive thymocytes which have travelled to the medulla of the thymus encounter self-antigens, which are presented by medullary thymic epithelial cells (mTEC), macrophages or dendritic cells (DCs) [33].

In the thymus a ingenious mechanism called promiscuous gene expression is observed. This phenomenon builds the basis for the negative selection mechanism.

The expression of the transcription factor AIRE (autoimmune regulator) expressed in mTECs leads to the ectopic expression of almost all tissue-restricted antigens (TRAs) (Figure 1.3), which are then presented by their MHC molecules [34, 35]. The very few TRAs, which are not expressed by mTECS can be transported to the thymus via DCs or macrophages, as not all of both cell types have been arisen intrathymically [36].
When T cells carrying an auto-reactive TCR receive a strong signal through their TCR interacting with a self-peptide-MHC complex at this stage of development, they are driven into apoptosis. This ensures self-tolerance by eliminating all T cells with strong auto-immune potential. Only T cells with low affinity for self-MHC complexes survive and enter the periphery of the immune system as so called naïve CD8 and CD4 T cells [32].

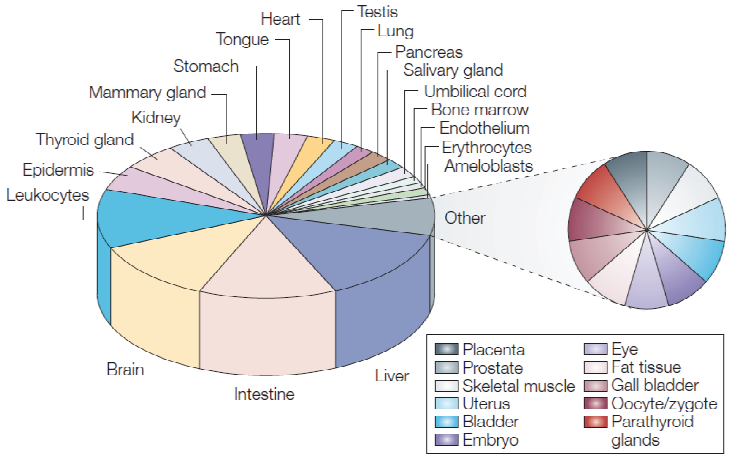


Figure 1.3 | Diverse tissues are represented by promiscuous gene expression in medullary thymic epithelial cells (mTECs). Gene chip analysis revealed that about one quarter of all mTEC-overexpressed genes could be categorized as tissue-restricted. A broad diversity of tissues is represented in mTECs and the fraction of tissue-restricted genes is probably still underestimated given their low expression levels in mTECs and the limited sensitivity of the gene array method. *Figure from Derbinski, J. et al., 2001, Nat Immunol.*

1.3.2 Activation of T cells

Naïve T cell continuously recirculate from blood to the lymphatic system, which carries them to secondary lymphoid organs, and from there back to the blood. The secondary lymphatic organs are optimally designed to facilitate the encountering of T cells with their specific antigen. Antigens are taken up by DCs in tissues, which migrate via afferent lymph streams to the draining lymph nodes (LN), where they present the antigens by MHC molecules to T cells. Depending on the maturation status of the DC, T cells become activated or anergized (tolerized), that means that

at this step an additional control mechanism allows or prevents activation of a specific T cell [3, 8]. The DC is the crucial cell in this step, in which the outcome of the TCR-peptide-MHC interaction is decided. For this decision costimulatory molecules (CD80 or CD86), expressed on the surface of the DC, are determining, as they interact with the CD28 molecule, expressed on T cells, leading to the activation of a T cell. These costimulatory molecules will only be expressed if the DC has sensed so called "danger signals" with its pattern recognition receptors (PRR), e.g. Toll-like receptors (TLRs). These danger signals are only present if pathogens have entered the body. DCs, carrying self-peptides, taken up from e.g. apoptotic tissue cells, will not express costimulatory molecules und therefore anergize those T cells, which recognize the selfpeptides presented via the MHC molecules. Therefore, auto-reactive T cells which could escape the negative selection in the process of central tolerance will be anergized in the LN due to this additional control mechanism.

1.3.3 T cell differentiation and T cell effector subsets

Besides the decision whether a T cell is activated or not, it is of importance to which effector subset cell type the T cell will differentiate. Especially for the CD4 T cell lineage this decision has to me made. CD8 T cells mainly differentiate into cytotoxic T effector cells, which have the ability to kill cells in an antigen-specific way.

To which sub cell type CD4 T cells differentiate depends on the cytokines expressed by the DC itself. But also other surrounding cells can influence this decision. Here, only the main T cell subtypes will be described.

The process of differentiation is not static, which means that T cells usually differentiate to more than only one subtype clone and T cells which have differentiated to one subtype can change their phenotype to another subset under certain conditions. Therefore, the high flexibility and plasticity of the T cell differentiation process may not be forgotten when simplifying it in a model with a monolithic view (Figure 1.4). IFN-γ and IL-12 are the classical cytokines driving a Th1 response. Th-2 cells are generated in the presence of IL-4. Whether a T cell differentiates into a Th17 or induced Treg depends on the presence of IL-6 [37]. Both cell types need TGF-β for differentiation, but only if IL-6 is present will the T cell differentiate into an effector Th17 cell. Th17 cells are further supported by the cytokine IL-23 [38-40]. During the process of differentiation, the activation via phosphorylation of STAT molecules is important [39](Figure 1.4).

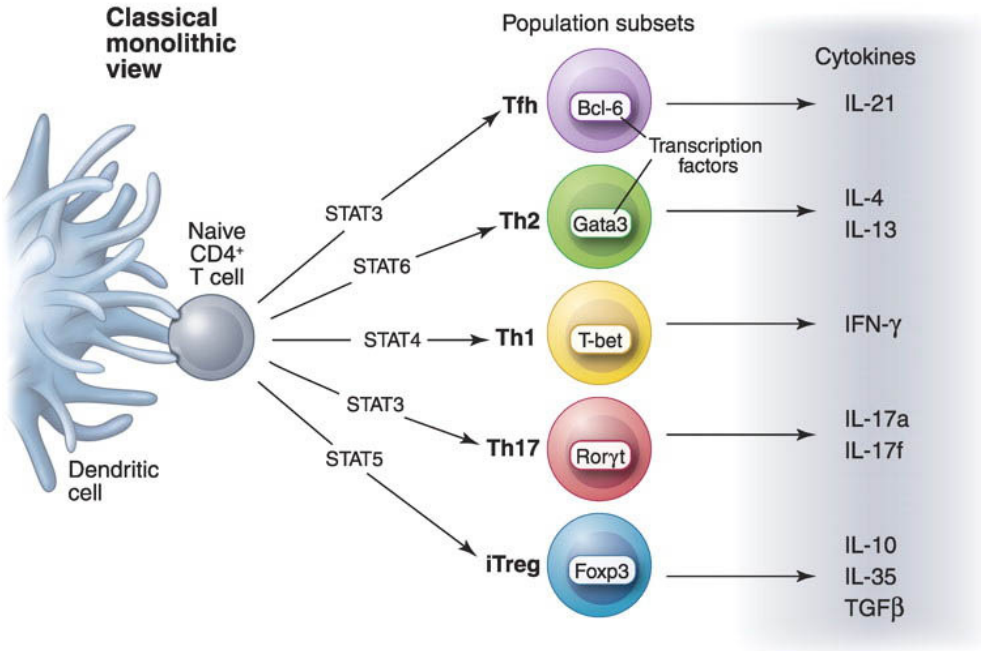


Figure 1.4 | The classical monolithic view of CD4 T cell differentiation. Upon interaction with dendritic cells, T cells differentiate to different effector subsets. These can be distinguished by specific transcription factors or cytokines they secrete. Characteristic STAT molecules are important in the activation process of each subset. *Figure from J.J. O´Shea and W.E. Paul, 2010, Science.*

Every subset can be identified by specific master regulator transcription factors or cytokines expressed by T cells (Figure 1.4). Th1 cells express T-bet and EOMES as their specific transcription factors and secrete IFN-γ and TNF-α. They are responsible for the cellular immune response and can enhance the response of cytotoxic CD8 T effector cells. Therefore, they belong to the inflammatory CD4 T cells [39]. Th1 cells also express the chemokine receptors CXCR3 and CCR5, which bind to CXCL9 , CCL3 and CCL4 which are secreted in inflammatory responses by other immune cells and attract Th1 cells to these areas [41, 42].

Th17 cells are the second group of inflammatory CD4 T cells. Their name refers to the typical cytokine IL-17 which they secrete. The master regulator transcription factor which distinguishes them from other CD4 T cell subsets is RoRγT. They express a very similar chemokine receptor pattern as Th1 cells to be attracted by inflammatory sites [42, 43]. Besides Th1 cells, Th17 have been reported to be the initiating and/or triggering cells in autoimmune diseases in a multitude of studies [44-46]. In some studies also the cytokine GM-CSF has been found to be expressed by Th17 cells and to be indispensable for their pathogenic function [47].

Th2 cells are important CD4 T cells for the extracellular also called humoral immune response (see 1.4.4 Activation of B cells by different classes of antigens). They secrete IL-4, IL-5, IL-6, IL-10, IL-13 and IL-25. IL-4 can substitute IL-2 and acts as growth

factor for these CD4 T cells, therefore these cells are independent in their proliferation and effector function from IL-2. The Th2 cytokines IL-4 and IL-13 are crucial in the development of IgE antibody responses of B cells [48]. Therefore, these cells are in most cases responsible for the development of allergic reactions. Furthermore, they inhibit the development of a Th1/Th17 response with their secreted cytokines, which can negatively effect the clearance of intracellular pathogens [39]. On the other hand, nowadays, the immune modulation from a Th1/Th17 response to a Th2 response is used therapeutically in autoimmune disease, as in this case Th2 responses are less harmful [49-52]. Th2 cells are characterized by the expression of GATA-3 as their specific transcription factor as well as the expression of the chemokine receptors CCR3 and CCR4, which bind the chemokines CCL5 (Rantes), CCL7 and CCL17 [42].

Regulatory T cells can not only be generated in the thymus (thymic or natural Tregs (nTregs)), but can also be induced in the periphery and are then called adaptive or induced Treg (iTregs). As nTregs they express the transcription factor FoxP3 and the α-chain of the IL-2 receptor CD25 [53, 54]. Besides TGF-β, also other factors can contribute to their induction, including IL-10, retinoic acid, or activation by immature DCs [55-57]. Helios has been proposed by *Thornton et al.* as a marker to distinguish natural from iTregs, as it was found predominantly on thymic Tregs [58]. But this has been questioned by others studies, which demonstrate that Helios can be a marker of T cell activation and proliferation [59, 60]. Furthermore, Helios positive Tregs have been described to be more suppressive [61].
Induced Tregs use the same mechanisms as natural thymic Tregs or other suppressive cells to inhibit immune responses. They are described in the section "1.2 Mechanisms of tolerance". The characteristic suppressive cytokines of Tregs are IL-10 and TGF-β, but as described they employ a multitude of mechanisms in order to suppress other immune cells. The highest proportion of induced regulatory T cells can be found in the GALT, where they ensure oral tolerance to food antigens as well as the tolerance to the commensal microflora [62, 63].

Whether follicular helper T cells (Tfh) are truly a CD4 T cell subset parallel to Th1, Th2, Th17, and iTregs, or a particular state of some or all of these cells is unresolved [37, 64]. Tfh cells express Bcl-6, their master regulator transcription factor, but unlike T-bet and GATA-3, Bcl-6 is a transcriptional repressor. Tfh cells have distinct properties and phenotypes (e.g. expression of CXCR5 and ICOS), however, individual Tfh cells may produce Th1 or Th2 signature cytokines, depending on their initial activation [64, 65]. Tfh cells are mostly known for their function in supporting the development of germinal centers and promoting immunoglobulin class switch recombination (CSR) and affinity maturation [37, 64, 65].

1.3.4 T cells in autoimmune diseases

The mechanisms of central and peripheral tolerance work together to prevent auto-reactive responses. However, under certain conditions, which are still intensively studied, tolerance mechanisms fail and autoimmune diseases develop.

T cells have widely been shown to be involved in the pathogenesis of autoimmune diseases [66]. Due to their specific antigen-receptor they are capable to target tissues specific proteins and lead to the destruction of the tissue either directly by cytotoxic T cells or with the help of inflammatory cytokines and chemokines that attract other immune cells, which accumulate in the affected organ and lead to a pathogenic inflammation with tissue destruction [67-69].
The simplest model explaining the activation of self-reactive T cells is that a professional APC cell, like a DC, takes up self-antigen from an apoptotic cell, which at the same time is infected with a virus or bacterium. Therefore, the APC will be activated by danger signals, mature and express costimulatory molecules while presenting self-peptides from the apoptotic cell to naïve T cells. T cells, specific for this TRA, which could escape central and peripheral tolerance would then be activated when encountering such a DC, migrate to the tissue and initiate an autoimmune reaction [3]. Fortunately, this model is not sufficient to explain the initiation of an autoimmune disease, because infections usually cause apoptosis and attraction of DCs, and every organism is exposed to several infections during lifetime without developing an autoimmune disease. Therefore, additional mechanisms have to be causal. Susceptibility studies show that autoimmune diseases are not only associated with infectious and immunological factors, but also with environmental, geographical, and genetic factors, and appear to be increasing especially in the western world [70-72]. However, the exact etiology still remains unknown.

That T cells are key players in autoimmune diseases is doubtlessly the case, as various animal models show that the transfer of auto-reactive T cells is sufficient to induce the autoimmune disease [73-81]. In these animal models Th1 and Th17 have mostly been demonstrated to be the disease initiating cells, whereas Tregs and Th2 cells are able to dampen or even prevent disease development [82-85]. Many therapies aim to increase Treg cell numbers or their activity [86-89], others aim the modulation of an inflammatory Th1/Th17 response to a less damaging Th2 response [49-52]. The crucial role that CD8 T cells play in the onset of several autoimmune diseases should also not be underestimated as shown in diabetes, multiple sclerosis or myasthenia gravis [90-92].

In the past decades, animal models of T cell mediated autoimmune disease have been generated for a multitude of autoimmune diseases including Type I diabetes [93], inflammatory bowel disease [94], autoimmune liver disease [75], autoimmune

rheumatoid arthritis [95] and multiple sclerosis [96], to just mention a few. Here, the animal model of multiple sclerosis should be described more in detail as an example.

1.3.4.1 Experimental autoimmune encephalitis as a model of human multiple sclerosis

One widely studied model for T cell mediated autoimmunity is the model of multiple sclerosis, which can be induced in mice where it is called experimental autoimmune encephalitis (EAE). In this disease auto-reactive Th1 and Th17 traffic to the central nervous system (CNS), where they recognize CNS-specific peptides of the myelin sheath with their corresponding TCR and initiate the auto-reactive immune response.
Several cytokines have been implicated to be crucial for disease initiation. Initially, IL-12 and IFN-γ were suggested, but during the past years IL-23 and IL-17 gained importance [97, 98]. Later, Becher's and Waisman's groups demonstrated that neither IL-12 nor IL-17 is necessary for disease initiation [99, 100]. Just recently, Codarri *et al.* (Becher's group) identified GM-CSF as essential factor for the effector phase of EAE [47]. But the debate still goes on by intensive research in this field.
The secretion of pathogenic cytokines leads to the attraction of further immune cells including myeloid cells and cytotoxic CD8 T cells [47, 67, 101, 102]. The immune response and the inflammatory molecules are multiplied and lead to the destruction of oligodendrocytes [103]. The consequence is demyelination and axonal loss [104]. First signs of EAE are paralysis of the tail and the legs, caused by missing signal transmission of neurons, later it can lead to death of the mice.

The disease in the model of EAE is initiated by active immunization with CNS antigens emulsified in complete Freund's adjuvant (CFA) and administration of pertussis toxin (PT), which is supposed to break the blood brain barrier [105-107] and provide access of activated auto-reactive T cells to the CNS. The most commonly used CNS-derived antigens for immunization are myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG) or proteolipid protein (PLP). Alternatively, *in vitro* activated transgenic T cells with specificities for the above mentioned antigens can be transferred for disease induction.

1.4 B cells

B lymphocytes (B cells) are characterized by their antigen specific receptor, the B cell receptor (BCR/Figure 1.5). It exists in two forms, either as a membrane bound BCR or as soluble immunoglobulin (Ig), also called antibody. The structure of the BCR receptor is identical to that of its corresponding antibody except for a small portion of the C-terminus of the heavy chain, which anchors the molecule in the membrane. The variable region recognizes the antigen and is formed by the N-terminal parts of the two identical heavy and light chains. The heavy chains, which form the major part of the constant region, determine the antibody isotype. The two light and the two heavy chains are arranged symmetrically to form the typical Y-shape of an antibody.

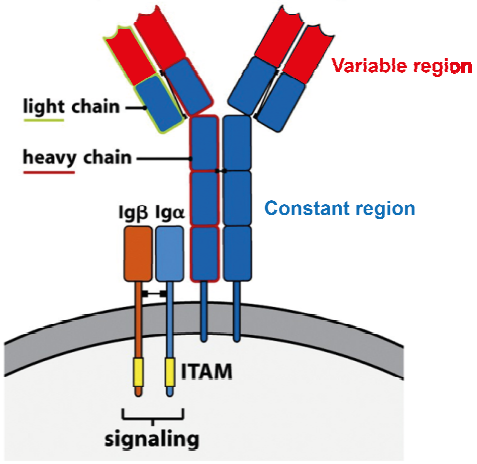


Figure 1.5 | The B cell receptor (BCR). The B cell receptor consists of two identical heavy chains and two identical light chains. They are connected by two disulfide bonds in the hinge region. Antigens are recognized by the antigen binding variable region of the BCR (red). The class of an antibody is defined by the structure of the heavy chain, which forms together with the C-terminal part of the light chain the constant region (blue). It is associated with an Igα and Igβ signalling molecule. These each have a single immunoreceptor tyrosine-based activation motif (ITAM / yellow segment), in their cytosolic tails that enables BCR signalling. Igα and Igβ form a disulfide-linked heterodimer, which is associated with the heavy chain. *Figure adapted from Immunobiology, Janeway et al., 6th edition, 2005 Garland science Publishing.*

In contrast to the TCR, the BCR is able to recognize a broad variety of antigens and does not depend on MHC molecules (further described in 1.4.4 Activation of B cells by different classes of antigens). The anchored BCR serves for antigen-specific B cell activation as it is able to transfer the event of antigen binding with the help of the associated Igα and Igβ signalling molecules to the cytoplasm. In contrast the function of soluble antibodies is to directly neutralize important structures of pathogens in order to prevent their pathogenic action or to opsonize pathogens,

which leads to the recognition and destruction of the pathogens by components of the innate immune system. Which effector function is conducted by an antibody depends on the specific isotype class (IgM, IgD, IgG, IgA, IgE).

1.4.1 B cell development

From around birth on, B cell development takes place in the bone marrow - which is known as the primary lymphoid organ of B cells - but also in peripheral lymphoid tissues such as the spleen. In comparison to T cells, B cells are generated from pluripotent hematopoietic stem cells during the whole life-time. As the development of T cells, B cell development is characterized by the stepwise assembly of the BCR.

The common lymphoid progenitor (CLP) gives rise to the earliest B-lineage cell, the pre-pro-B cell. This cell can be recognized by the expression of B220 (CD45R). CD19, a molecule whose expression characterizes all later B lineage stages [108], is still lacking on the surface (Figure 1.6) at this early stage. The commitment to the B lymphoid lineage occurs before D-J recombinations of the heavy chain start [109]. In the pro-B cells CD19 expression and heavy chain rearrangement of D to J gene segments begin. To produce a complete immunoglobulin heavy chain, the late pro-B cells proceeds with a second rearrangement of the heavy chain V gene segments to a DJ sequence. A successful rearrangement leads to the production of an intact IgM heavy chain and the cell becomes a pre-B cell. In this step, the IgM heavy chain associates with λ5 and VpreB, that together form a surrogate light chain (SLC), to form the pre-BCR [110]. This pre-BCR assembles with the Ig accessory proteins Igα and Igβ, similar to the BCR on mature B cells [111, 112]. Although it is only transiently expressed, the pre-BCR marks an important checkpoint in B cell development. Signals from the pre-BCR provide rapid feedback about the functionality of the recombined IgH gene, so that only pre-B cells that express a signalling-competent receptor can continue the process of maturation. Pre-BCR signalling induces a burst of proliferation, which serves to increase the number of cells that have successfully recombined IgH genes [113] as well as it activates the recombination of the Ig light genes [114].

After the proliferation, induced by pre-BCR signalling, the rearrangement of V to J gene segments of the light chain occurs. As soon as pre-B cells have successfully rearranged the light chain genes, it pairs with the heavy μ chain and they start to express the finished BCR as cell surface IgM. This stage is classified as immature B cells. Here, the complete BCR is first tested for tolerance to self antigens. The tolerance produced in the B-cell repertoire at this stage of development is known as

central tolerance of B cells and presents the second important checkpoint in B cell development.

Central tolerance of B cells leads to three possible fates for self-reactive immature B cells as shown by a number of studies [115-121]. These fates are: cells are eliminated by clonal deletion; cells become anergic; or cells revise their BCR to eliminate self-reactivity, known as receptor editing. It appears likely that high-affinity interactions with membrane bound antigen result in deletion, whereas low-affinity interactions and soluble antigens allow receptor editing or result in anergy [115, 116, 119, 120, 122]. Cells that pass this checkpoint, which means that they have been confirmed as non-self-reactive, are ready to emerge into peripheral lymphoid tissues. In the periphery immature B cells are called transitional B cells. They can also be divided into three different stages: T1, T2 and T3 according to the expression of IgM, IgD, CD93 and CD23 [4]. Mature B cells express only low levels of IgM and high levels of IgD, whereas immature or transitional cells express high levels of IgM and do not yet express IgD.

The different stage of B cell development can be detected by analyzing the recombination status of the heavy and light chain gene rearrangements or by surface markers, which are expressed characteristically at each B cell stage [4] (Figure 1.6).

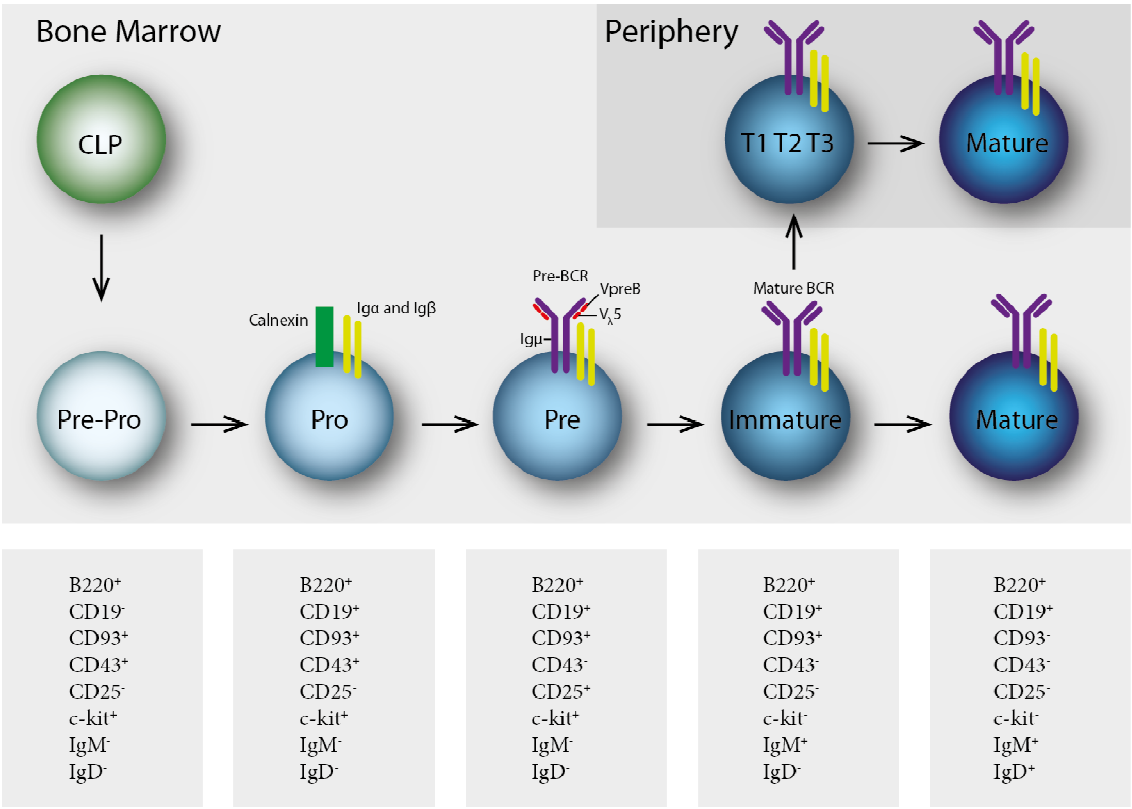


Figure 1.6 | B cell development stages are associated with the assembly of the BCR and specific cell surface markers. B cell development takes place in both the bone marrow (BM) and the peripheral lymphoid tissues. In the BM all developmental stages can be found, whereas to the periphery only immature B cells migrate to continue their maturation process. During the maturation in the BM, rearrangements at the immunoglobulin locus occur and lead first to the expression of the pre-BCR at the pre-B cell stage and later to the expression of the mature BCR on immature cells. Both, the pre-BCR and the mature BCR are able to bind antigen and to transfer this signal, which is used to first select cells which successfully rearranged the immunoglobulin genes and later to eliminate auto-reactive cells. Only cells which have successfully completed these checkpoints are released to the periphery, where the last steps of maturation take place. Different stages can be identified with specific surface expression markers (shown in the table below the corresponding cell stage). *Figure based on Cambier J.C. et al., 2007, Nature Reviews.*

1.4.2 B cell subsets

B cells can be divided into two main subsets, B1 and B2 cells. B2 cells are also often called conventional B cell as they present the majority of B cells in the secondary lymphoid organs. They can be subdivided into follicular (FO) and marginal zone (MZ) B cells (Figure 1.7). B1 cells are only found at a very low level in spleen and other peripheral lymphoid organs, they are enriched in the pleural and peritoneal cavities of the body [123]. They can be subdivided into B1a and B1b cells (Figure 1.7).

Most mature B cell are recirculating cells that home mainly to B cell follicles in secondary lymphoid organs and are known as follicular B cells. Follicles are adjacent

to T cell zones and this arrangement allows activated follicular B cells and activated T helper cells to migrate towards each other and interact at the interface between these two areas. FO B cells are therefore particularly well suited to participate in T cell dependent immune responses to protein antigens.

Marginal zone B cells are sessile cells and resided in the outer white pulp of the spleen between the marginal sinus and the red pulp. Pathogens reaching the bloodstream are efficiently trapped in the marginal zone by macrophages, and therefore MZ B cells are uniquely adapted to provide the first response to such blood borne pathogens [124, 125]. They express high levels of CD1d, which facilitate the presentation of lipid antigens to invariant natural killer (iNKT) cells and it is probable that MZ B cells are an important source of lipid-specific antibodies [126, 127]. MZ B cells are generated as naïve B cells that intrinsically have some properties that resemble those of memory cells. These include the pre-activated phenotype, the ability of self-renewal, and the survival as long as the life-span of the host [125, 128, 129]. MZ B cells may be selected during development similarly to B1 cells, as they share functional characteristics with these cells [130].

Murine B1 cells were first described in 1983 as a relatively small population of CD5 (earlier known as Ly-1) expressing B cells that spontaneously secrete high amounts of IgM and are highly increased in autoimmune prone NZB/NZW F1 mice, thereby linking these cells to autoimmunity [131]. Later, a second subset that closely resembles these CD5 expressing cells in terms of their tissue distribution, phenotype and development, but which did not express CD5, was found [132]. It was called B1b, whereas the CD5 expressing subset was called B1a. To date the functional difference between these two subsets is not clear [130].
In the pleural and peritoneal cavity B1 cells present a high proportion of the total B cells (20-70% depending on the mouse strain) [133]. The spleen and the BM contain B1 cells that spontaneously secrete large amounts of IgM, which serves to sustain the systemic level of circulating natural antibodies [134, 135]. At least 80% of serum IgM is B1 derived [136]. This steady-state production of natural IgM provides a crucial barrier against pathogens before the establishment of an adaptive immune response. Furthermore, polyspecific IgA-producing B1 cells function as a first layer of immune defense in the GALT and in the respiratory tract [137]. Due to these features, B1 cells are, besides the MZ B cells, seen as part of the innate immune system. They also share some other characteristics with MZ B cells, which make these two subsets to innate-like players of the immune system. One of these characteristics is for example the quick response to blood-borne pathogens with T cell independent antibody production [138].

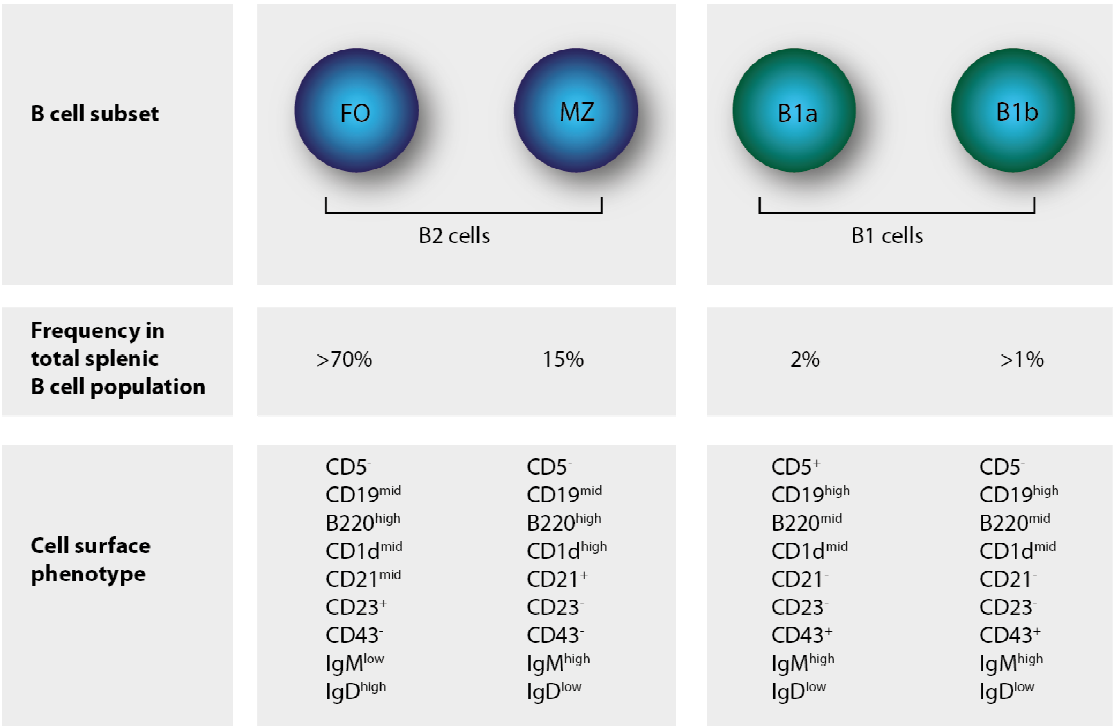


Figure 1.7 | Mature splenic B cell subsets. Four subsets of mature B cells are present in the spleen. The majority is formed by follicular (FO) and marginal zone (MZ) B cells, which together form the B2 population. They can be differentiated by the surface markers CD21, CD23, IgM and IgD. B1 B cells represent a minor population, which can be identified by the expression of CD43 and the low expression of B220. Furthermore, they do not express CD21 and CD23. B1a and B1b cells can be distinguished by the expression of CD5. *Figure based on Baumgarth, 2011, Nature Reviews.*

1.4.3 Hypotheses of B1 cell development

The term "B1-cell" has been adopted for this subset, as they develop earlier than B2 cells during ontogeny [139, 140]. They are efficiently generated before birth and during the first weeks after birth (the neonate state) in the splanchopleura region and the fetal liver [140, 141]. In contrast to the continuous *de novo* development of B2 cells in the BM, the BM does only poorly give raise to B1b cells and B1a development does almost not occur [141, 142]. Instead, B1 cells seem to be maintained by a process termed "self-renewal". In this process, they undergo limited proliferation to replace dying cells and to sustain a stable population size over time [133].

Not only do B1 cells develop at different times and locations in ontogeny compared to B2 cells, but also does the selection of B1 cells for self-reactivity seem to be contrary to existing models of tolerance induction and discrimination between self and non-self. This indicates that a distinct developmental program underlies the development of this unusual B cell subset. Studies using a natural auto-reactive BCR transgenic model revealed clearly a positive role for self-antigen in generating and

maintaining the pool of B1 cells [143], whereas B2 cells are rather negatively selected by self-antigen (as described in 1.4.1 B cell development). To explain the difference in B1 versus B2 cell origin and development two competing models were conceived.

The “selection model” proposes that the decision of a BCR expressing B cell to become B1 or B2 is driven by the response to particular antigens [132, 144]. This idea was supported by studies which demonstrated that B1 cells were preferentially generated in transgenic mice exhibiting B cells that express Ig heavy and light chains frequently used by B1 cells [144, 145]. Additionally, several studies reported that B1 cell characteristics (as e.g. CD5 expression) could be acquired by B2 cells [132].

The “lineage model” or “layered immune system model” proposes that B1 and B2 cell belong to separate lineages deriving from distinct progenitors, which emerge at different times during ontogeny [146]. This hypothesis arose when studies comparing the potential to give rise to B1 or B2 cells from various tissue donor cells demonstrated that neonatal liver efficiently reconstitutes B1 cells upon transfer into irradiated recipients, whereas BM cells did so poorly [123]. The recent identification of a precursor cell in BM and fetal liver that specifically gives raise to B1 cells is the most definite evidence in support of this hypothesis [147].

However, Baumgarth suggests that both models do not necessarily need to be mutually exclusive [133]. They can function as two coexisting developmental pathways that ensure the generation and maintenance of B1 cells. Baumgarth calls this model “the two-pathway model of B1 development” [133]. In this model the B1 cell pool is established as the lineage model hypothesizes. Nevertheless, the positive selection of B1 cells by self-antigens can still occur during this development. The B1 cell pool is then sustained by self-renewal in the adult. If steady-state conditions change (for example after whole body irradiation), the B1 cell output from the BM is increased, although the involved mechanisms are unknown. In such a situation, it is conceivable that B2 cell precursors undergo a B1 cell like selection process and contribute to the B1 cell pool.

1.4.4 Activation of B cells by different classes of antigens

In comparison to T cells, B cell activation does not rely on antigen procession followed by antigen-peptide presentation with the help of MHC molecules. Therefore, first of all, B cells have a broader spectrum of antigens to which they can respond, and secondly, they do not depend on APCs, which have to present the antigens. Instead, they can respond to membrane bound or soluble antigens. But for this

reason, control mechanisms preventing the activation of auto-reactive B cells that escaped central tolerance are indispensable.
The main control mechanism is the requirement of T cell help in order to enable naïve B cells to get fully activated [148]. The costimulatory signal provided by the T cell, which is needed in addition to the signal created by the antigen-BCR interaction to activate a B cell, is the CD40L molecule interacting with CD40 on the B cell surface [149]. CD40L is not permanently expressed on T cells but only when they are activated by DC or other APCs expressing costimulatory signals for T cells upregulated by danger signals (as described in 1.3.2 Activation of T cells). Additionally, only T cells recognizing the same antigen (or linked antigens) as B cells can provide the required help with the CD40L. This is ensured by the mechanisms that B cells take up the antigen, being specifically bound to the BCR, by internalizing the entire BCR-antigen complex, then process the antigen, and finally present peptides of the antigen in MHC molecules on their surface. The B cell is only activated if the interaction of the TCR with the presented peptides of the antigens is strong enough to also lead to the interaction of CD40L and CD40 on the B cells.
Once B cells received T cell help, they themselves transform into professional APCs expressing the B7 molecules (CD80 and CD86). With that, they provide the costimulatory signal for the T cell, which in turn upregulates CD40L, providing the T cell help to B cells, and therewith multiplying the whole immune response of T and B cells.

T cells can only control immune responses to protein antigens, but B cells can recognize a vast variety of chemical structures. Therefore, additional mechanisms are necessary which control the process of activation. One of these mechanisms is the equipment of B cells themselves with PRR (e.g. TLR) to sense danger signals. This can directly provide the costimulatory signal required in addition to the antigen-BCR interaction signal. Furthermore, other cells can substitute the CD40L signal of T cells. These cells are epithelial cells, DCs, macrophages and granulocytes, which can be activated by TLR and other PRRs leading to the secretion of BAFF and APRIL [150]. These proteins are CD40L related class switch recombination (CSR) factors, which are besides CD40L able to provide the costimulatory signal to B cells [151, 152].

Antigens, which do not elicit T cell responses are called thymus- or T cell independent (TI) antigens [153]. They can be divided into two classes, namely type I and type II antigens (Table 1.1). TI type I antigens activate B cells by binding to the BCR and giving the costiumlatory signal via a PRR. An example is LPS that binds to TLR4. In high concentrations such antigens act as mitogens and activate B cells in a polyclonal way leading to proliferation and differentiation regardless of their BCR specificity [154].

TI type II antigens consist of highly repetitive structures (e.g. long polysaccharides). They lead to extensive crosslinking of the BCR which is sufficient to activate the B cell, without any further costimulatory signals [155]. Whereas TI type I antigens can activate both immature and mature B cells, TI type II antigens can activate only mature B cells; immature B cells are negatively selected during the process of central tolerance. Table 1.1 shows examples of T cell dependent (TD) and TI antigens.

In comparison to the classification of B cell responses according the nature of the antigens, B cell responses can also be classified according to the cell type (Table 1.2). This is important when considering antigens which can not directly be classified in the first table, as for example phosphorylcholine (PC). This antigen is the most typical antigen stimulating B1 cells [132]. Structurally it belongs to the TI type II antigens class, but several studies also show that T cells can strongly enhance the response to this antigen [156]. However, type II antigens can not generally be related to B1 cells, as for example TNP-Ficoll, which is also a type II antigen, activates mainly MZ B cells [157].

Table 1.1 | Classification of B cell responses according to the type of antigen

T cell dependant (TD)	T cell independant (TI)	
	TI type I	TI type II
common antigens, which need T cell help e.g. TNP-BSA in CFA	polyclonal by mitogens e.g. LPS stimulate immature+mature B cells	long polysaccarids which lead to extensive crosslinking of the BCR e.g. TNP-Ficoll stimulate only mature B cells, immature are anergyzed

Table 1.2 | Classification of B cell responses according the cell type

Follicular B cells (FO)	Marginal zone B cells (MZ)	B1
TNP-BSA in CFA	TNP-Ficoll in PBS	Phosphorylcholine in CFA, LPS

Activated B cells proliferate and differentiate into plasma cells, which secrete large amounts of antibodies. During the differentiation into plasma cells a phenomenon called affinity maturation takes place. In this process recombined genes of antibodies gain somatic hypermutations. This leads to increased affinity of the BCR to antigens as only B cells will receive sufficient survival signals with their BCR possessing a high affinity for antigens. Additional, class switching of the antibody is induced by

recombination of the heavy chain constant region genes. Both of these processes are regulated by an enzyme called activation induced deaminase (AID) [158].

The class of an antibody determines its effector function; therefore it is an important decision which subclass of antibody will be produced by activated B cells. In earlier times it was thought that only TD antigens can induce class switch as only then CD40L-CD40 signalling would be present. Later studies, however, showed that also TI antigens can elicit CSR [159], as this is also induced by BAFF and APRIL, which are expressed by other cell types than T cells [150-152]. Besides the structure of an antigen, which seems to be important for the class switch, cytokines are pivotal factors in deciding to which isotype the B cell will switch [159, 160]. Various cytokines have been implicated in this step, including IFN-γ and GM-CSF [153, 159, 160] (leading to IgG2a and IgG3 class switch), IL-5 (leading IgA and IgE) [161], IL-4 (leading to IgG1 an IgE)[48, 162] , IL-13 (leading to IgE) [48, 163] and TGF-β (leading to IgA and IgG2b) [164]. The presence of one or more cytokine and their action together directs the CSR and therefore the specific function of an antibody.

1.4.5 B cells in autoimmune diseases

Similar to T cells, B cells also carry an antigen-receptor, which can recognize even a broader range of antigens with higher affinity than the TCR. Therefore, B cells are the other class of lymphocytes of the adaptive immune system being able to recognize self-structures therewith leading to autoimmune diseases.

As B cells can not cause direct cell killing, the damage is mainly caused by auto-antibodies, which can act directly or form immune-complexes activating the complement and FC receptor system (Figure 1.8) [165]. Additionally, secretion of inflammatory cytokines, participation in antigen presentation, and the generation of ectopic lymphogenesis are involved in B cell mediated autoimmune diseases (Figure 1.9) [165, 166]. Numerous autoimmune diseases in which B cells play important roles in pathogenesis have been described; for instance systemic lupus erythematosus (SLE), myasthenia gravis, autoimmune hematolytic anemia, sjorgens's sydrom or rheumatoid arthritis [165, 166].

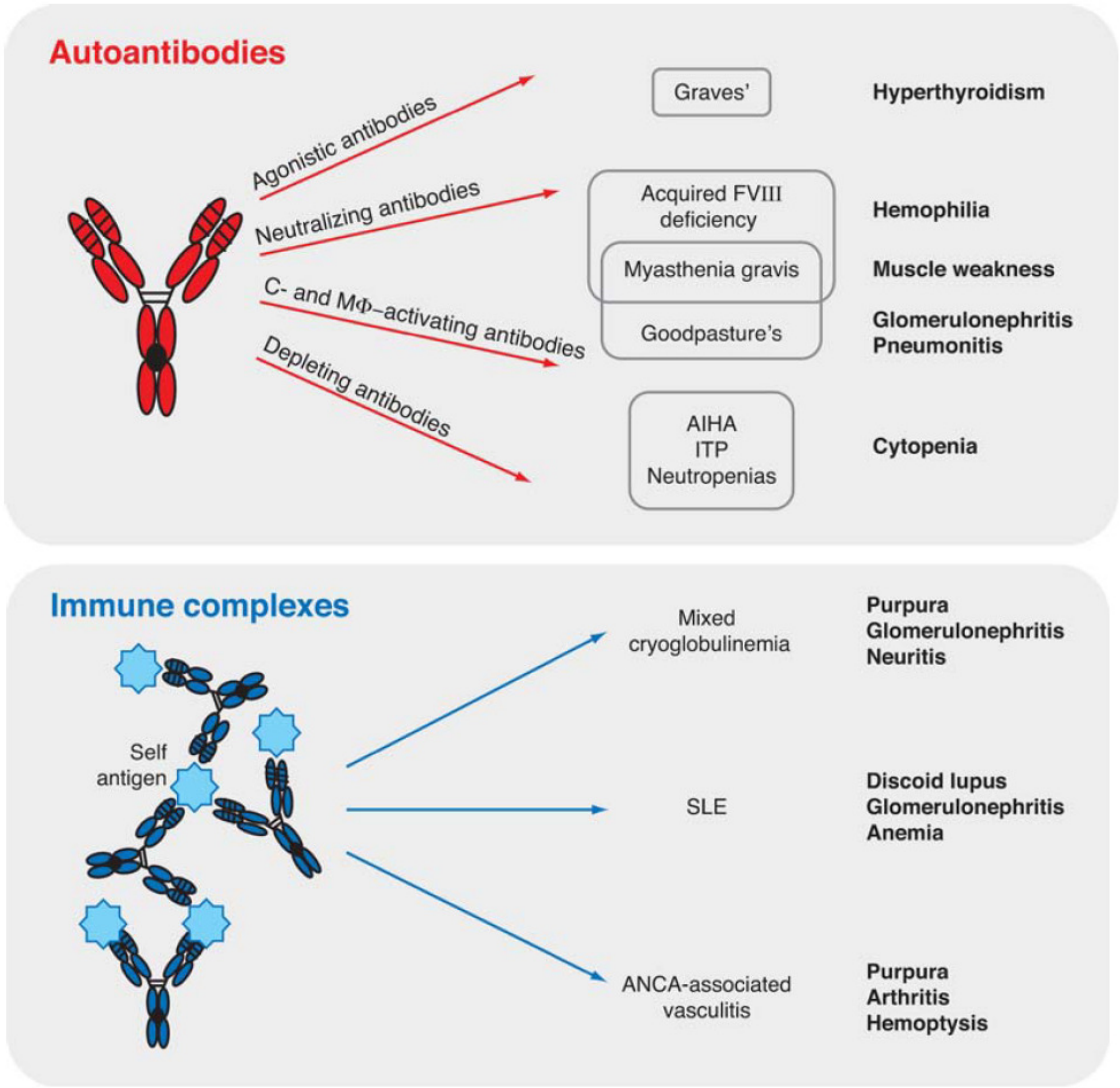


Figure 1.8 | Antibody-driven mechanisms of B cell autoimmunity. Classic antibody-dependent mechanisms are responsible either directly (top) or through immune complexes (bottom) for the pathogenesis of specific autoimmune diseases. (Abbreviations: SLE, systemic lupus erythematosus; ANCA, antineutrophil cytoplasmic antibody–associated vasculitis; AIHA, autoimmune hemolytic anemia; ITP, immune-mediated thrombocytopenia.). *Figure from Martin, F., 2006, Annu Rev Immunol.*

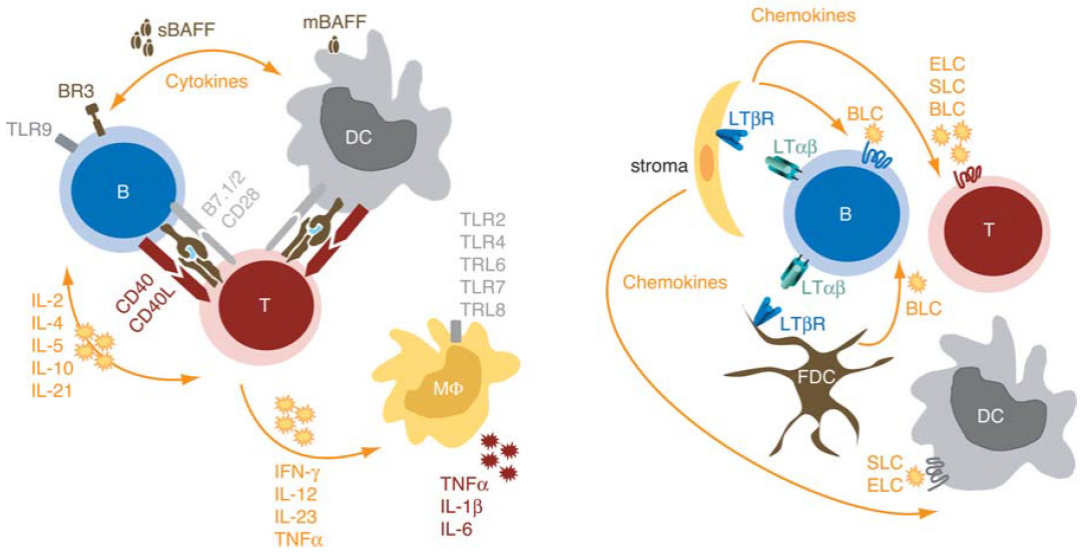


Figure 1.9 | Antibody-independent mechanisms of B cell autoimmunity. B cells can present auto-antigens by MHC molecules on their surface as well as costimulatory signals of the B7 family, thereby leading to the activation of auto-reactive T cells (left). Secreted cytokines acting on T cells, DCs and other immune cells are another mechanism how B cells can drive autoimmune diseases (left). Furthermore, B cells can contribute to autoimmunity by the ability to support de novo lymphoid tissue organization (ectopic lymphogenesis) through expression of e.g. LTα or LTβ, which interacts with LT receptors on stroma cells or follicular dendritic cells (FDCs) leading to the expression of specific chemokines (e.g. ELC, SLC and BLC) thereby attracting other immune cells, such as T cells and dendritic cells (DCs)(right). *Figure from Martin, F., 2006, Annu Rev Immunol.*

It has been shown that B1 cells are the key players in B cell mediated autoimmune diseases [132, 167]. This might be due to the fact that the selection process of B1 cells depends on self-antigens [133, 143] (as described in 1.4.3 Hypotheses of B1 cell development). Once B1 cells are recruited to germinal centers, they can undergo CSR and affinity maturation, which was found in a study of human $CD5^+$ B cells [168], leading to highly auto-reactive B cells. Elevated numbers of B1 cells have been reported in patients with Sjorgens's sydrom [169] and rheumatoid arthritis [170]. In mice, increased numbers of B1 cells have been observed in a number of naturally occurring and genetically manipulated strains that develop autoimmune manifestations [131, 171]. Even in T cell mediated autoimmune disease B1 cells promote critical steps in the development, such as in type 1 diabetes [172]. Evidence that B1 cells are not only related but causative in the development of autoimmune disease comes from several studies performed in mouse models of autoimmune hematolytic anemia, SLE or gene targeted transgenic mice [132]. Murakami *et al.* strikingly proofed that elimination of B1 cells abrogates autoimmune symptoms in autoimmune prone NZB mice [173].

Furthermore, IL-10 seems to play a crucial role in the activation of auto-reactive B1 cells. It is produced by B1 cells themselves and can activate them [132, 133, 174]. This mechanism leads to an enormous enhancement of the B1 response. Continuous

administration of anti-IL-10 antibody has been demonstrated to abrogate or delay autoimmune symptoms [175-177], while continuous administration of IL-10 accelerated disease [178].
A very recent study shows that IL-10 cooperates in the class switch to IgG when B cells are activated by the antigen CpG-DNA [179]. Since in systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA), anti-DNA IgG antibodies are highly pathogentic [180], these findings suggest that the high levels of IL-10 observed in patients of SLE and RA are connected to development of high autoantibody titers against DNA.

1.4.5.1 Animal models of systemic lupus erythematosus

In comparison to an organ-specific immune disease as multiple sclerosis, SLE is a systemic autoimmune disease, which can manifest in multiple organs. The etiology of SLE is considered to be a combination of multiple genetic and environmental factors whose amalgamation breaks the threshold of immune tolerance [165]. The disease develops through a type III hypersensitivity reaction, in which auto-antibodies form immune complexes leading to further responses (as described above). Most typical symptoms are nephritis, arthritis, pleural effusions, or heart problems.

There are numerous animal models that have long been employed in an effort to understand the cellular and genetic requirements for SLE induction [181, 182]. The most widely used ones are the NZB/NZW F1 and the MRL/lpr animal models. Both mouse strains develop the disease spontaneously and disease reflects the pathologies of human SLE, including lymph node enlargement, increased IgG levels, antinuclear antibody (ANA) production, proteinuria, and kidney failure caused by inflammation of the glomeruli [182].

The MRL/lpr model of SLE shows the most severe clinical disease of all spontaneous models. This accelerated phenotype is attributed to a recessive autosomal mutation termed lymphoproliferation (lpr). The lpr mutation, located on chromosome 19, alters transcription of the Fas receptor [183]. Due to the mutation, the Fas receptor is not functional and both T and B cells show defects in apoptosis leading to lymphoproliferation [184]. SLE symptoms attributed to Fas deficiency in MRL/lpr mice have been shown to depend on both $CD4^+$ T cells and B cells as their absence decreases autoimmunity [185, 186]. Furthermore, the MRL genetic background is important for SLE development as expression of the lpr mutation in either the C3H/HeJ and C57BL/6J strains did not result in nephritis [187]. In contrast, Fas-sufficient mice on the MRL background displayed the pathological effects of nephritis, though onset of symptoms was significantly delayed when compared to

MRL/lpr mice [187]. Therefore, the MRL background genes play an important role in the development of the disease.

1.5 The Dickkopf family

The Dickkopf (Dkk) family consists of five members, Dkk1-4 and the divergent member DkkL1, which is also called Soggy. All five members share a sequence identity of 37-50% [188, 189] and are secreted proteins, therefore containing a N-terminal signal sequence for secretion. Dkk1-4 proteins share two conserved cysteine rich domains (Figure 1.10). Each of these cysteine-rich domains exhibits 10 highly conserved residues [188, 190]. The N-terminal cysteine-rich domain (DKK_N) is unique to the Dkk protein family whereas the C-terminal cysteine-rich domain is related to the colipase fold [191]. The role of colipases in general is to facilitate the interaction of pancreatic lipases with lipid micelles. According to structure-function analysis, the colipase fold domain has been suggested to be sufficient for the biological activity of the Dkk family members 1, 2 and 4 [188]. The biological activity of these three members is to modulate the Wnt pathway, presumably with the help of the collipase fold domain interacting with lipids of the cell membrane.

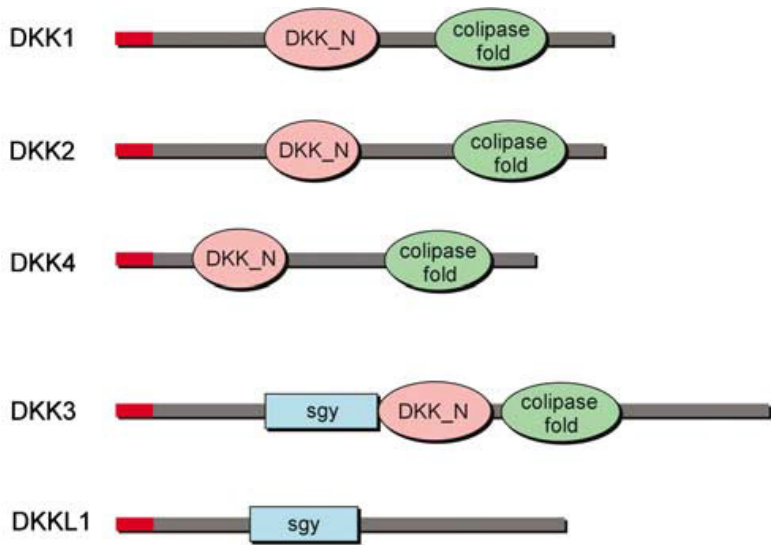


Figure 1.10 | The family of Dickkopf proteins. Domain structures of the Dkk1-4 and the distant family member DkkL1 (Soggy). Dkk1-4 share two conserved cyteine rich domains (the DKK_N and the colipase fold domain). The Soggy domain is unique to Dkk3 and DkkL1. *Figure from Niehrs, C. et al, 2006, Oncogene.*

Contrary to the conserved cysteine-rich domains, the linker region between the DKK_N domain and the colipase fold domain is highly variable between the Dkk proteins and is notably smaller in Dkk3 (12 amino acids, compared to 50-55 amino acids in Dkk1, 2 and 4). This and the additional Soggy- (sgy) domain make Dkk3 to

a structurally divergent member of the family. Soggy is the most distant member of the family as it does not contain the two cysteine-rich domains. It is most related to Dkk3, as both proteins share the conserved sgy-domain with 44% of homology in mice (NCBI database).

Furthermore, Dkk1, 3 and Soggy posses potential glycolsylation sites [188]. While Dkk1 has only a single potential glycosylation site, Dkk3 displays four, and Soggy two potential glycosylation sites. Additionally, Dkk proteins carry several potential proteolytic cleavage sites. In Dkk3, Hermann *et al.* identified a dibasic amino acid motif at position 142/143 directly at the C-terminal site of the sgy domain [192]. Notably, the presence of such dibasic residues serves as cleavage sites in prohormones like insulin. A recent study demonstrated that Dkk3 is a substrate of the matrix metalloproteinase 2 and 9 (MMP2 and MMP9) [193].

The Dkk family is known for involvement in the Wnt signalling as mentioned above. It has been shown that Dkk1, 2 and 4 bind to LRP5/6 and the Kremen receptors [191]. Whether Dkk3 plays a role in Wnt signalling is still controversial [188, 194-196]. The involvement of Soggy in this pathway has only been addressed by one study, which shows that Soggy does not influence Wnt signalling [197]. Supporting the functional difference between Dkk3 and soggy to the rest of the protein family, phylogenetical analysis also revealed that both family members diverged from other Dkk proteins already early in evolution [198]. Thus, Dkk3 and Soggy appear to be divergent members of the family, which manifest by their structural and functional differences to the other members of the family.

1.5.1 Dkk3

For a long time no function for Dkk3 had been discovered. First studies looked at the Wnt pathway as other Dkk family members are well known to influence this pathway. But no influence of Dkk3 on this pathway had been found [188, 190]. Later these findings were questioned, when Yue *et al.* found that Dkk3 prevents nuclear translocation of β-catenin and expression of the TCF-4 targets c-Myc and Cyclin D1 [195], which was confirmed in osteosarcoma and PC12 rat pheochromocytoma cells [194, 199]. Controversially, other studies reported that Dkk3 is able to act as a positive regulator of Wnt signalling [196]. So the debate if and how exactly Dkk3 acts on Wnt signalling is still ongoing.

It has been observed that the expression of Dkk3 is downregulated in several tumors including lung carcinoma [200], lymphoblastic leukemia [201], renal cell carcinoma [202], melanoma [203] and glioma [204]. Therefore, it is also called REIC, which

stands for “Reduced Expression in Immortalized Cells” and is regarded as a tumor suppressor gene. The attenuated expression of Dkk3 in tumors and tumor derived cell lines has been explained by hypermethylation of the Dkk3 promotor [195, 200]. A recent study confirmed that the methylation status of Dkk3 plays a key role in Dkk3 gene silencing in a broad range of human malignancies [205]. According to these results, it was observed that overexpression of Dkk3 leads to tumor cell death in prostate cancer [206], glioma [204], breast cancer [207], and renal cell carcinoma [208], thus suggesting Dkk3 as a therapeutic target in human cancers.
Although many studies focused on the function of Dkk3 as a tumor suppressor, there are opposing studies indicating that Dkk3 promotes tumor growth. Ketase *et al.* showed that mutation in the Dkk3 locus was associated with a better prognosis of lymph node metastasis in neck squamous cell carcinoma [209], Gregorieff *et al.* showed the overexpression of Dkk3 in adenomas [210], and Pei *et al.* the overexpression of Dkk3 in hepatoblastoma and hepatocellular carcinoma [211]. Hence, the role of Dkk3 in tumors might depend on the cell type and the particular condition which is examined.

Dkk3 is a secreted molecule being expressed in the brain, eye, heart, liver, hair follicle, uterus, embryo, placenta and pancreatic beta islet cells [188, 192, 212]. The expression pattern indicates that Dkk3 is primarily expressed in immune privileged sites. This feature lead our laboratory to the investigation of Dkk3 in the immune system, when it appeared to be highly upregulated in a differential gene analysis profile in a special tolerogenic CD8 T cell [212]. In this transgenic system a specific CD8 T cell is conferring tolerance to other T cells exhibiting the same TCR specificity [213] and Dkk3 is not only indispensable but also sufficient for the CD8 T cell mediated regulation [214].

In 2006, Dkk3 deficient mice ($Dkk3^{-/-}$) were generated and characterized [189]. $Dkk3^{-/-}$ mice are, however, viable, fertile, and do not show tumor formation or obvious signs of misled tolerance, which would manifest by symptoms of autoimmunity. Within the hematological and immunological parameters they only displayed a few alterations, which are elevated heamtokrit and hemoglobin levels, higher IgM levels and a slightly increased frequency of natural killer (NK) cells.

1.5.2 Soggy

The soggy gene is unique to mammals and its mRNA has been reported to be expressed in developing sperms, trophoblast, placenta, fetal eye, neural tissues and lymphocytes of the spleen [215, 216]. Furthermore, it has been found in several tumor cell lines including the T lymphoma EL-4 and the B cell lymphoma A20, MPC-11 and CH1 [216].

Functional analysis of the Soggy gene was in all published studies carried out in a site where Soggy was known to be abundantly expressed, the testis. Kohn *et al.* found that Soggy is first post-translationally N-glycosylated and then deglycosylated again while locating to the acrosome of the mature sperm [216]. In the acrosome, Soggy is probably modified by phosphorylation and remains sequestered in this site despite the presence of the signal sequence. In contrast, a different study showed that Soggy in the acrosome of mature sperm is truncated and only partially deglycosylated [217]. Continuing the study of the actual function of Soggy in spermatocytes, Kohn reported recently that acrosomal Soggy facilitates sperm penetration of the zona pellucida during *in vitro* fertilization [218].

To further improve the understanding of the function of Soggy, the Soggy gene was disrupted by replacing the first 45 amino acid codons with the LacZ gene [197]. Mice lacking Soggy are viable and fertile. However, Dakhova *et al.* demonstrated that Soggy regulates postpubertal spermatocyte apoptosis thereby regulating sperm counts [197]. Additionally, they found that Soggy locally leads to increased testosterone production in older animals. Molecular analysis revealed that FasL expression is induced by Soggy, which explains the decreased apoptosis of sperms in Soggy deficient mice. Therefore, Soggy is suggested to be a new pro-apoptotic factor.

1.6 Aims of this study

Protection of tissues against the devastating consequences of immune responses is essential for the integrity of the organism. Potential auto-reactive lymphocytes are either clonally deleted or controlled by mechanisms of peripheral tolerance. The search of potential mediators of peripheral tolerance led our laboratory to the protein Dkk3. Interestingly, the cells secreting the highest levels of this protein are comprised in tissues traditionally defined as immune-privileged sites, such as the brain, spinal cord and eye. Many mechanisms used by the immune system for tolerance induction and establishment of an immune privileged site are overlapping as reported for several factors like FasL, [219, 220] IDO [25, 221], or TGF-β [222, 223]. It is possible that Dkk3 is one of these molecules acting as a key player in tolerance induction and limiting immune responses in immune privileged sites.
Recently, we indeed demonstrated that Dkk3 is essential to maintain peripheral CD8 T cell tolerance in a TCR-transgenic mouse model [214]. This is the first finding showing that Dkk3 is influencing the immune system.

The aim of the thesis is to further clarify the immuno-modulatory capacity of Dkk3.
In the first part of the thesis the question will be addressed whether and how Dkk3 is contributing to the immune suppressive milieu of immune privileged sites thereby limiting auto-reactive T cell responses.

B cells are essential players of adaptive immune responses which also need to be controlled to prevent auto-reactivity. Therefore, in the second part of the thesis, main characteristics of B cells like antibody body responses and cytokine secretion will be compared between Dkk3 deficient and sufficient mice. To analyze whether Dkk3 regulates B cell mediated autoimmunity, a model of systemic lupus erythematosus will be used.

In the third part of the thesis, we will address the question which structural part of Dkk3 might be responsible for the immuno-modulatory function. Dkk3 shares the unique and homologous sgy domain with the protein Soggy. Therefore, Soggy$^{-/-}$ mice will be investigated for an immunological phenotype. This will reveal, whether Soggy exhibits a similar immuno-modulatory capacity to Dkk3.

2 Material and Methods

2.1 Material

2.1.1 Chemicals

Name	Company
2-isopropanol	Fluka
Acetic Acid ($C_2H_4O_2$)	Merck
Aceton	VWR
Acrylamide, 30%	Roth, Karlsruhe
Agar	Sigma
Agarose Ultra Pure®	Roth
Ammoniumpersulfate	Appli Chem
Amplicillin	Roth
Bacto-Agar	Beckton Dickenson, USA
Bacto-Trypton	Roth
Bromophenol Blue	Sigma
BSA	Gerbu
Calcium Chloride ($CaCl_2$)	Merck
CFSE	Sigma
Chloroform	VWR
DELFIA® Enhancement Solution	PerkinElmer
DELFIA® Eu (europium) –labeled streptavidin	PerkinElmer
Desoxyribonucelic acid, activated from calf thymus	Sigma
Dickkopf-3-IgG2 recombinant protein	DKFZ (D050)
Dimethylsulfoxid (DMSO)	Merck
Disodium carbonate (Na_2CO_3)	Roth
Disodium hydrogen phosphate (Na_2HPO_4)	Merck
dNTPs	Fermentas
Dulbecco's Modified Eagle Medium (DMEM)	Invitrogen
Dulbecco's Phosphate Buffered Saline (dPBS)	Invitrogen
EDTA disodium salt (Na_2EDTA)	Pharmacia Biotech
EGTA	Pharmacia Biotech
Ethanol	Sigma
Ethidium bromide	Appli Chem
Fetal calf serum (FCS)	Biochrom AG
Formic acid (HCO_2H)	Sigma
G418 – Geneticin	Gibco

Gelantine	Roth
Glucose	Sigma
Glutamine	Gibco
Glycerin ($C_3H_8O_3$)	Merck
Glycine	Gerbu
H_2O (30%)	Sigma
H_2SO_4	Merck
Heparin	Ratiopharm
HEPES	Roth
Hexaaminecobaltchloride	Sigma
Hydrochloric Acid (HCl)	J.T. Baker
Ketanest	Pfizer
Lipofectamine 2000 Reagent	Invitrogen
Lithiumchloride (LiCl)	Sigma
Magnesium Chloride ($MgCl_2$)	Sigma
Magnesium Sulfate (MgSO4)	Merck
Rompun	Bayer
β-Mercaptoethanol	Merck

2.1.2 Buffers and Media

Molecular cloning and DNA analysis	
DNA loading buffer 5 x	30% Glycerol
	0.1% Bromphenol Blue
	0.1% Xylenxyanol
Fast-MINIPREP pre-lysis buffer	25 mM Tris/HCl
	50 mM Glucose
	10 mM EDTA
	pH 8.0
Fast-MINIPREP lysis buffer	1 M NaOH
	1% SDS
Fast-MINIPREP neutralizing buffer	3M Potassium acetate/
	Formic acid
TAE buffer	40 mM Tris/HCl
	1 mM EDTA
	0.114% Acetic Acid
	pH 8.0
TE buffer	10 M Tris/HCl
	2 mM EDTA
	pH 8.0

Tail buffer	200 mM NaCl
	100 mM Tris/HCl
	5 mM EDTA
	0.2% SDS
	pH 8.5
Transformation buffer	10 mM Potassium acetate
	45 mM $MnCl_2$
	10 mM $CaCl_2$
	100 mM KCl
	3 mM Hexamminecobaltchloride
	10% Glycerin
	pH 7.5
Protein analysis	
ELISA carbonate buffer	50mM Na_2CO_3
	50mM $NaHC0_3$
	pH 9.6
ELISA blocking buffer	PBS
	0.2% Gelantine
	0.1% NaN_3
ELISA development buffer	0.1 M KH_2PO_4
	1 µl/ml H_2O_2
	1 mg/ml OPD
	pH 6.0
IL-2 ELISA assay buffer	50 mM Tris/HCl
	20 M EDTA
	0.9% NaCl
	0.5% BSA
	0.1% Tween20
	pH 7.8
Lysis buffer	20 mM Tris/HCl
	150 mM NaCl
	1 mM EDTA
	1 mM EGTA
	0.5% NP-40
	pH 7.6

Protein loading buffer 6x	72 mM Tris/HCl 36% Glycerol 18% SDS 9% β-Mercaptoethanol 0.24% Bromphenole Blue pH 6.8
SDS-PAGE running buffer	25 mM Tris/HCl 192 mM Glycine 0.1% SDS 0.2 pH 8.3
SDS-PAGE sample buffer 6x	62.5 mM Tris/HCl 714 mM β-Mercaptoethanol 20% Glycerin 2% SDS 0.025% Bromophenol blue pH 6.8
Separating gel buffer 4 x	1.5 M Tris/HCl pH 8.8
Sodium carbonate buffer	50 mM Na_2CO_3 50 mM $NaHCO_3$ pH 9.6
Stacking gel buffer 4 x	1 M Tris/HCl pH 6.8
Western blot blocking buffer	PBS 0.1% Tween20 5% Skim Milk Powder
Western blot transfer buffer	48 mM Tris 39 mM Glycine 1.3 mM SDS 20% Methanol pH 9-9.4
Cell biology and common laboratory buffers	
Dulbecco's PBS (dPBS)	PBS supplied with MgCl2 and CaCl2 (Invitrogen)
FACS buffer	dPBS 2% FCS 0.1% NaN3

Heparinbuffer	dPBS 2% FCS 5u/ml heparin
MACS buffer	PBS (Gibco) 2 mM EDTA 0.5% FCS pH 7.2
Phosphate buffered saline (PBS)	130 mM NaCl 2.6 mM KCl 15 mM KH2PO4 4 mM Na2HPO4 pH 7.2
Phosphate buffered saline and Tween20 (PBS-Tween)	PBS 0.05% Tween20
Cell culture media	
Cell-freezing medium	Heat-inactivated FCS with 20% DMSO
DMEM-full medium	DMEM (Invitrogen) 10 mM HEPES 2 mM L-Glutamine 10% Heat-inactivated FCS 100 units/ml Penicillin 100 g/ml Streptomycin
RPMI-full medium	RPMI 1640 (Gibco) 10 mM HEPES 2 mM L-Glutamine 0.05 mM β-Mercaptoethanol 10% Heat-inactivated FCS 100 units/ml Penicillin 100 g/ml Streptomycin

2.1.3 Kits

Name	Company
Absolute™ QPCR SYBR® Green ROX Mix	Thermo Scientific
CD19 Microbeads	Miltenyi Biotec
$CD4^+$ $CD62L^+$ T cell isolation kit	Miltenyi Biotec
CD45R Microbeads	Miltenyi Biotec
Cytometric bead array – Mouse Inflammation Kit	BD Bioscience
Cytometric bead array – Mouse Th1 Th2 Th17 Cytokine Kit	BD Bioscience
Dynal® Mouse CD4 T cell negative isolation kit	Invitrogen
Dynal® Mouse T cell negative isolation kit	Invitrogen
Foxp3 Fixation/Permeabilization Concentrate and Diluent	eBioscience
Immobilon Western Chemiluminescent HRP Substrate	Millipore
Mouse IL-10 ELISA Kit	eBioscience
Pierce BCA protein assay	Thermo Scientific
Precellys Ceramic Kit	Pequlab
ReverseAid Reverse Transcriptase kit	Thermo Scientific
RNeasy mini kit	Quiagen

2.1.4 Primers

Primers for β-*actin* cDNA template amplification	
F-Primer	TGA CAG GAT GCA GAA GGA GAT TA 3’
R-primer	AGC CAC CGA TCC ACA CAG A 3’
Primers for *HPRT* cDNA template amplification	
F-Primer	5’ ACA CCT GCT AAT TTT ACA GGC AAC A 3’
R-Primer	5’ TGG AAA AGC CAA ATA CAA AGC CTA 3’
Primers for *GAPDH* cDNA template amplification	
F-Primer	5’ CAT GGC CTT CCG TGT TCC TA 3’
R-Primer	5’ TGT CAT CAT ACT TGG CAG CTT TCT 3’
Primers for *Soggy* cDNA template amplification	
F-Primer	5’ AGG TCA CCG ACA GCT TGC A 3’
R-Primer	5’ TGG CAT CTT CAT GAT CCA GAA 3’
Primers for *Dkk3* cDNA template amplification	
F-Primer	5’ TCC CAT TGC CAC CTT TGG 3’
R-Primer	5’ CCA GTT CTC CAG CTT CAA GTA CAC 3’

2.1.5 Antibodies

2.1.5.1 Western blot and ELISA

Name	Company
Goat anti-mDKK3-Biotin	RnD Systems
Goat anti-mouse IgG1-HRP	Southern Biotech
Goat anti-mouse IgG2a-HRP	Southern Biotech
Goat anti-mouse IgG2b-HRP	Southern Biotech
Goat anti-mouse IgG3-HRP	Southern Biotech
Goat anti-mouse IgG-HRP	Jackson Immuno Research
Goat anti-mouse IgM/IgG	Jackson Immuno Research
Goat anti-mouse IgM-HRP	Southern Biotech
Goat anti-mSoggy-1-Biotin	RnD Systems
Goat anti-rabbit IgG-Peroxidase-conjugated	Jackson ImmunoResearch Laboratories Inc.
Monoclonal anti-mDKK3-4.22	DKFZ (Dr. Gerhard Moldenhauer)
Monoclonal anti-mDKK3-4.22-Biotin	DKFZ (Dr. Gerhard Moldenhauer)
Monoclonal rat anti-mouse Soggy-1	RnD Systems; Clone: 192425
Rabbit anti-mDKK3	Sino Biological Inc.
Rat anti-mouse IL-2	BioLegend; Clone: JES6-1A12
Rat anti-mouse IL-2-Biotin	BioLegend; Clone: JES6-5H4

2.1.5.2 FACS staining

Name	Company
B220 APC	eBioscience; Clone: RA3-6B2
CD19 biotin	Biolegend; Clone: MB19-1
CD19 biotin	BD Biociences; Clone: 1D3
CD21/CD35 FITC	BD Biociences; Clone: 7G6
CD23 PE-Cy7	Biolegend; Clone: B3B4
CD25 PE	BD Biosciences; Clone 3C7
CD25 PerCP-Cy5.5	BioLegend; Clone: PC61
CD3 PE	BD Biociences; Clone: 145-2C11
CD4 APC-Cy7	BD Biosciences; Clone: GK1.5
CD4 Pacific blue	BD Biosciences; Clone: RM4-5
CD43 PE	BD Biociences; Clone: S7
CD44 PE-Cy7	eBiosciences; Clone: IM7
CD5 Pacific blue	eBioscience; Clone: 53-7.3
CD62L FITC	BD Bioscience: Clone MEL-14

CD8 biotin	BD Biociences; Clone: 53-6.7
CD8 eFluor450	eBiosciences; Clone: 53-6.7
CD8a Alexa Fluour 647	BioLegend; Clone: 53-6.7
CD93 APC	Biolegend; Clone: AA4.1
Fc-Block	DKFZ, D050
Foxp3 PE	eBiosciences; Clone: FJK-16S
Helios APC	Biolegend; Clone: 22F6
IFN-γ PE-Cy7	BD Biociences; Clone: XMG1.2
IgM PerCP	BD Biociences; Clone: R6-60.2
IL-10 PE	BD Biociences; Clone: Jes5-16E3
Streptavidin APC-Cy7	BD Biociences
CD11b Pacific blue	Biolegend; Clone M1/70
IL-17 APC	Biolegend; Clone: TC11-18H10
GM-CSF PE	Biolegend; Clone: MP1-22E9
T-bet PE	eBioscience; Clone: eBio4B10
EOMES Alexa Fluour 647	eBioscience; Clone: Dan11mag
RORγt PE	eBioscience; Clone: AFKJS-9
GATA-3 PE	eBioscience; Clone: TWA-J
CD127 (c-Kit) Pe-Cy7	eBioscience; Clone: 2B8

2.1.6 Reagents used in cell simulation and differentiation assays

Name	Company
Brefeldin (Golgi-Plug)	BD Biosciences
CpG	TIB Molbiol
$F(ab')_2$ fragment goat anti-mouse IgM	Jackson Immuno Research
Ionomycin calcium salt	Sigma-Aldrich
LPS	Sigma Aldrich, L4391
Monesin (Golgi-Stop)	BD Biosciences
Monoclonal hamster anti-mouse CD28	BD Biosciences; Clone: 37.51
Monoclonal hamster anti-mouse CD3	BD Biosciences; Clone: A19-3
Monoclonal rat anti-mouse CD40	Biolegend; Clone 3/23
Monolocal rat anti-mouse IFN- γ	Biolegend; Clone: R4-6A2
Monolocal rat anti-mouse IL-17	Biolegend; Clone: TC11-18H10.1
Monolocal rat anti-mouse IL-4	Biolegend; Clone: 11B11
Phorbol 12-myristate 14-acetate (PMA)	Sigma
Polyinosinic–polycytidylic acid potassium salt (Poly I:C)	Sigma-Aldrich
Recombinant human TGF-β -1	Preprotech
Recombinant mouse IL-2	Gibco

Recombinant murine IFN-γ	Preprotech
Recombinant murine IL-12	Preprotech
Recombinant murine IL-12	R&D
Recombinant murine IL-12 (p70)	Preprotech
Recombinant murine IL-2	Gibco
Recombinant murine IL-4	Preprotech
Recombinant murine IL-6	Preprotech

2.1.7 Reagents used for *in vivo* applications

Name	Company
LPS	Sigma Aldrich, L2630
Monoclonal anti-mDKK3-4.22	DKFZ (Dr. Gerhard Moldenhauer) / BioXCell (Purification)
Monoclonal MOPC21 Isotype control (IgG1)	BioXCell
Myelin oligodendrocyte glycoprotein peptide 33-55 (MOG)	Peptide specialty laboratories Amino acid sequence: MEVGWYRSPFSRVVHLYRNGK
Phosphorylcholine-keyhole limpet hemocyanin (PC-KLH)	Biosearch Technologies
TNP-AECM-Ficoll (TNP-Ficoll)	Biosearch Technologies
TNP-BSA	Biosearch Technologies

2.1.8 Mice

Strain	Source
C57BL/6N (wt)	Charles River Laboratories
C57BL/6N Dkk3$^{-/-}$ (Dkk3$^{-/-}$)	Provided by Christof Niehr's laboratory from the DKFZ, Heidelberg (Niehrs et al., 2006)
C57BL/6N Soggy$^{-/-}$ (Soggy$^{-/-}$)	Charles River Laboratories
C57BL/6N Rag2$^{-/-}$ (wt.Rag2$^{-/-}$)	Charles River Laboratories
C57BL/6N Rag2$^{-/-}$ Dkk3$^{-/-}$ (Dkk3$^{-/-}$ Rag2$^{-/-}$)	Established by crossing of C57BL/6N Dkk3$^{-/-}$ with C57BL/6N Rag2$^{-/-}$ at the DKFZ
NOD.Rag2$^{-/-}$ Name at Jackson laboratories: NOD.129S7(B6)-*Rag1*^{tm1Mom}/J	Provided by Dr. Freurer's laboratory from the DKFZ
Bdc2.5/NOD Name at Jackson laboratories NOD.Cg-g(TcraBDC2.5,TcrbBDC2.5)	Provided by Dr. Freurer's laboratory from the DKFZ
FoxP3.LuciDTR-4 Name at Jackson laboratories: MRL/MpJ-Faslpr/J (MRL/LPR)	Established in the laboratory Jackson Laboratories
2D2.Dkk3$^{-/-}$	Established by crossing of C57BL/6N Dkk3$^{-/-}$ with 2D2 mice at the DKFZ

All mice were bred and maintained under specific pathogen-free conditions (SPF) at the animal facility of the German Cancer Research Center.

2.2 Methods

2.2.1 RNA analysis

2.2.1.1 Isolation of RNA from tissues

Organs frozen in liquid nitrogen were disrupted with Precellys Ceramic Kit 1.4mm and the FastPrep Homogenizer in buffer RLT (Quiagen) containing RNase inhibitors. Total RNA was purified from disrupted tissue using the RNeasy mini kit according to manufacturer's instructions. Additionally, RNA was desoxyribonuclease-I treated (Quiagen). RNA concentration was measured by Nanotrop and stored at -20°C.

2.2.1.2 cDNA reverse transcription

Complementary DNA (cDNA) was synthesized from messenger RNA (mRNA) using the ReverseAid Reverse Transcriptase kit. The cDNA synthesis was done according to the protocol in Table 2.1. The total volume of the synthesis reaction was 20µl. cDNA was diluted 1:50 and stored at -20°C for later use by RT-PCR.

Table 2.1 | Temperature profile and pipetting scheme of the cDNA synthesis

Component	Final amount
Purified RNA in *RNase*-free water	2 µg
dNTPs	0.5 mM (each)
Oligo dTs	5 mM
5 min, 68°C *afterwards directly chilled on ice*	
5 x RT buffer	1 x
DTT	10 mM
RiboLock *RNase* Inhibitor	2 U/µl
Reverse Transcriptase	10 U/µl
1 h, 42°C *15 min, 70°C*	

2.2.1.3 Quantitative real-time PCR

Quantitative real-time PCR was done by using the Absolute Q-PCR SYBR Green ROX Mix containing all the components necessary for the PCR with the exception of

primer and cDNA template (synthesized as described above) (Table 2.2/Table 2.3). The final volume of the reaction was 25µl. For comparative analysis, the cDNA subjected to quantitative real-time PCR was internally normalized to *β-actin* levels as a control housekeeping gene. Samples were measured in triplicates by a 7500 Real-Time PCR System and cycle threshold (Ct) values, representing the number of PCR cycles that were required to reach a given level of fluorescence, were analyzed by the Applied Biosystems Sequence Detection Software 1.4. Since SYBR green unspecifically binds DNA, the dissociation curves were analyzed for each target gene in order to control the specificity of the signal. Relative ΔCt values of the sequence of interest were calculated according to:

$$2^{-\Delta Ct} = 2^{-(Ct\ \beta\text{-actin} - Ct\ sequence\ of\ interest)}$$

Table 2.2 | Pipetting scheme of the quantitative real-time PCR

Component	Final amount
cDNA	80 ng
Forward and reverse primer	each 200 nM
Absolute Q-PCR SYBR Green ROX Mix 2 x	1 x

Table 2.3 | Temperature profile of the quantitative real-time PCR

Temperature [°C]	Time [sec]	
50	120	
95	600	
95	15	40 cycles
60	60	
95	15	
60	60	
95	15	

2.2.2 Protein analysis

2.2.2.1 Preparation of protein lysates from tissues/cells

Organs were taken, directly frozen in liquid nitrogen and stored at -80°C. Organs frozen in liquid nitrogen were disrupted in lysis buffer containing Protease Inhibitor Cocktail (Roche) with Precellys Ceramic Kit 1.4 mm and the FastPrep Homogenizer followed by 30min incubation on ice. Then, cell debris was separated by

centrifugation (16.000 x g, 10min, 4°C), the supernatant was transferred to a new tube and centrifuged again. Supernatant was again transferred to a new tube and stored on ice until usage.

2.2.2.2 Western Blot and immunodetection of proteins

Proteins that were first separated by SDS-PAGE were transferred to a PVDF membrane (Millipore) and were detected by specific antibodies. The gel and the membrane were put in direct contact to each other in a Semidry Transfer Cell (BioRad) and a righted electric field was applied. Due to their negative charge, the proteins migrated from the gel towards the anode and were fixed onto the PVDF membrane via hydrophobic interactions. By this means, the electrophoretic patterns are conserved onto the membrane. First, the PVDF membrane was activated in 100% methanol for 1min. Afterwards, the membrane, filter papers (Whatman) and gel were incubated shortly in transfer buffer and assembled to a "sandwich" Western blot. Finally, proteins were transferred at 2A and 18V for 20 min.

Non-specific binding sites on the membrane were blocked with blocking buffer containing 5% milk powder for 1h. Afterwards, the membrane was incubated with the primary biotinylated antibody (diluted to 1µg/ml in 2.5% milk in PBS-Tween) specific for the protein of interest diluted in blocking buffer containing 2.5% milk powder ON at 4°C. The membrane was washed four times with PBS-Tween for 10min. The peroxidase-conjugated streptavidin was diluted 1:40.000 in blocking buffer containing 2.5% milk powder. After 1 h of incubation, the membrane was washed four times with PBS-Tween for 10 min. This streptavidin was coupled to peroxidase that enabled the visualization of the complex by a chemiluminescence reaction. For detection of the peroxidase-conjugated streptavidin, the Immobilon Western Chemiluminescent HRP substrate was used according to the manufacturer's instructions.

2.2.2.3 ELISA

2.2.2.3.1 Immunoglobulin ELISA

Immunoglobulin ELISA was used to determine the concentration of antigen-specific or unspecific immunoglobulin isotypes in serum or in supernatants after B cell stimulation *in vitro*. For antigen-specific immunoglobulin detection, Costar 96-well polystyrene microplates (Sigma) were coated with 10µg/ml of antigen (TNP-BSA, TNP-Ficoll, TNP-LPS or PC-BSA). For the standard curve, wells on the same plate were coated with goat-anti-mouse IgM/IgG. Coating was performed in sodium

carbonate buffer at 4°C ON. Plate was then washed twice with PBS-Tween and then blocked with blocking buffer at 4°C ON or until usage. After blocking plates were washed twice with PBS-Tween and samples were mounted with different dilutions depending on the samples on the coated plates. As standard, purified immunoglobulin of the isotype of interest was used in a dilution row of 1:2 dilutions starting with 500ng/ml until 4ng/ml. Samples and standards were incubated 2 hours at RT, then washed 5 times with PBS-Tween. Afterwards, HRP-conjugated antibodies against the isotype of interest were added to the wells to detect the antigen-specific isotype and incubated 1 hour at RT. Plates were washed 5 times with PBS-Tween before development buffer was added. After the color development the reaction was stopped with 1M H_2SO_4. Absorption was measured at 492nm at the ELISA reader (Victor 1420 Multilabel counter photometer/ PerkinElmer).

2.2.2.3.2 IL-2 ELISA

The IL-2 ELISA was used to determine the IL-2 production of T cells in culture supernatants after stimulation *in vitro*. Supernatants were collected 24 hours after stimulation of T cells and stored at –20°C or directly used. Nunc F96 Micro Well™ PolySorp™ Black (Thermo Scientific) microplate were coated ON at 4°C with 1µg/ml rat anti-mouse IL-2 capture antibody in sodium carbonate buffer, washed twice with PBS-Tween and blocked with blocking buffer. Plates were washed 5 times with PBS-Tween and culture supernatants were transferred to the plates and incubated ON at 4°C. Then plates were washed five times with PBS-Tween and the IL-2 protein was detected by incubation with 0.25µg/ml rat anti-mouse IL-2-biotin detector antibody in PBS-Tween for 2h at RT. Afterwards, the plates were washed five times with PBS-Tween and 0.1µg/ml DELFIA europium (Eu)-labeled streptavidin diluted in IL-2 assay buffer was added. Plates were incubated 45 minutes at RT. The reaction was enhanced by the addition of DELFIA Enhancement solution 30 minutes later, the plate was read by the ELISA reader using 340 nm and 615 nm fluorescent filters.

2.2.2.3.3 IL-10 ELISA

The IL-10 ELISA was used to determine the IL-10 production of B cells in culture supernatants after stimulation *in vitro*. Supernatants were collected 24 hours after stimulation of B cells and stored at –20°C or directly used. IL-10 ELISA was performed using the IL-10 ELISA kit from eBioscience and Costar 96-well polystyrene microplates. The assay was performed according to manufacturer's instructions.

2.2.2.3.4 Soggy ELISA

The ELISA for Soggy was used to analyze the expression pattern of Soggy in different mouse organs and serum. Costar 96-well polystyrene microplates were coated with 3µ g/ml monoclonal rat anti-mouse Soggy-1 antibody in PBS and incubated ON at 4°C. The plates were washed twice with PBS-Tween and blocked with blocking buffer ON at 4°C. All organ lysates were adjusted to a concentration of 10mg/ml and diluted in lysis buffer containing protease inhibitors. As a standard, Soggy peptide (R&D) was diluted in lysis buffer in a dilution row of 1:2 dilutions starting 100ng/ml until 1.5ng/ml. After plates were washed 5 times with PBS-Tween samples and standards were added and incubated ON at 4°C. The plate was washed five times with PBS-Tween and incubated 1h at RT with 1µ g/ml biotinylated goat anti-mouse Soggy-1 antibody in PBS-Tween. Afterwards, the plates were washed again five times with PBS-Tween and Soggy was detected by incubation with peroxidase-conjugated streptavidin diluted 1:1000 in PBS-Tween for 1h at RT. Plates were washed 5 times with PBS-Tween before development buffer was added. The reaction was stopped immediately by adding 25µl of 1M H_2SO_4 per well and the absorption was measured at 492 nm at the ELISA reader.

2.2.2.4 Cytometric Bead Array

Cytometric Bead Arrays were used to determine cytokines secreted by B cells (CBA Inflammation Kit, BD) or to analyze T cell differentiation (CBA Th1, Th2, Th17 differentiation, BD). The assay was performed according to manufacturer's instructions and samples were measured at FACS Canto II (BD Biosciences). Data were analyzed using FCAP array software (BD Biosciences).

2.2.3 Preparation of leukocyte cell suspensions

2.2.3.1 Preparation of single cell suspensions from lymphatic tissues

Spleens, LN or BM were isolated from mice and placed into dPBS. Cells were extracted by forcing organs trough a 40µm nylon cell strainer (Falcon) using a syringe plunger. The cell strainer was then rinsed with ice-cold dPBS, cells were washed and placed into FACS buffer or RPMI depending on the experiment.

2.2.3.2 Isolation of mononuclear cells from blood

Mouse blood was taken directly into heparinbuffer and pipetted onto Lymphoprep (Axis-Shield, Oslo, Norway), which is a medium with a density of 1,077g/ml. The tubes were centrifuges 20min at 1800rpm without breaks. During centrifugation, erythrocytes and polynucleated cells pass trough the Lymphoprep due to their higher density, while mononuclear cells accumulate on top of the Lymphoprep phase due to their low density and become visible as a white band. This band is isolated and washed once with ice-cold dPBS. The cells are then resuspended in FACS buffer for FACS staining.

2.2.3.3 Isolation of peritoneal cavity cells

Skin was opened at the belly of the mice leaving the peritoneum intact and 5ml of ice-cold dPBS were injected i.p.. The belly of the mice was then massaged for about 1min. Then dPBS was sucked out with a syringe. This process was repeated to increase absolute cell numbers. Cells were washed and resuspended in RPMI, FACS buffer or MACS buffer depending on the following experiment.

2.2.3.4 Isolation of leukocytes from the brain

Mice were lethally anesthetized with 700µl of 5mg/ml Ketanest and 0,2% Rompun solution, which was injected i.p.. The pleural cavity was opened without injuring the heart. Then the right atrium was cut open and into the left ventricle 37°C warm HBSS was injected slowly. This process leads to perfusion of the liver but also the brain and the spinal cord leading to a white color.
Brain and spinal cord were isolated, placed into ice-cold dPBS and cut into small pieces. Then they were digested with 2,5mg/ml Collagenase D (Sigma-Alrich) and 1mg/ml Desoxyiribonuclease I (Sigma-Aldrich) in dPBS/10%FCS for 30min at 37°C. The digested brain and spinal cord were forced several times through a metal sieve with the help of syringe plunger, washed and resuspended in 2,5ml RPMI. To enrich the leukocyte fraction the suspension was mixed with 1ml isotonic (density of 1.13g/ml) Percoll (GE Healthcare). This was then underplayed with 1ml of Percoll with a density of 1.08g/ml. The gradient was established through centrifugation for 20min at RT at 2500 rpm without breaks. The interphase containing the leukocyte fraction was isolated washed and used for FACS staining or T cell restimulation.

2.2.4 Magnetic cell sorting

Magnetic cell sorting provides a system where specific cell types can be isolated from cell suspension with high purity. Different companies provide super-paramagnetic colloids conjugated either to monoclonal antibodies that can bind to specific surface markers, or to Streptavidin that can bind to biotinylated antibodies. After labeling the cells with the magnetic beads directly or indirectly they can be separated from unlabelled cells with the help of a magnet. In this study Dynal beads (Invitrogen) were used for negative isolation strategies (e.g. T cell negative isolation) and MACS microbeads (Milteny Biotec) were used for positive isolation strategies (e.g. $CD19^+$ or $CD45R^+$ cell isolation). The purification protocol was performed according to manufacturer's instructions.

2.2.5 Fluorescence-Activated Cell Staining (FACS)

2.2.5.1 Staining of cell surface makers

To block unspecific binding cells were incubated with FC-Block. Depending on the experiment 0,5-5 x 10^6 cells were incubated with monoclonal antibodies (listed in 2.1.5.2 Antibodies for FACS staining) in 50-100µl FACS buffer on ice for 30-60min in the dark. Cells were washed, and where relevant, biotinylated antibodies were incubated with fluorochrome-conjugated streptavidin on ice for another 30-60 min in the dark and washed again. Stained cells were resuspended in FACS buffer. FACS buffer could contain propidium iodide for dead cell exclusion (diluted 1:000) and Sphero™ Blanc calibration particles 6-6.5µm (BD) in order to determine the total number of cells per well/organ. Data were collected with FACS Canto (BD Biosciences) and analyzed using FlowJo software (Tree star). Absolute numbers of cells were determined by collecting a certain number of beads added to a specific volume of FACS buffer during FACS sample acquisition. The absolute number of a specific cell population can then be extrapolated to whole organs or culture wells with the help of the collected bead number.

2.2.5.2 Staining of intracellular cytokines

To measure intracellular cytokines cells have to be restimulated and incubated the last 4-6 hours with the relevant protein transport inhibitor Monesin or Brefeldin to prevent secretion of cytokines. Following the staining of cell surface markers (2.2.5.1 Staining of cell surface makers), 0,5-5 x 10^6 cells were washed with FACS buffer and fixed with Cytofix (BD) for 30 min at 4°C. Then cells were stained with antibodies

detecting intracellular cytokines in permeabilization buffer (BD) for 30 min at 4°C. After this step cells were washed and resuspended in FACS buffer for flow cytometric data collection.

2.2.5.3 Staining of intracellular transcription factors

Following the staining of cell surface markers (2.2.5.1 Staining of cell surface makers), cells were stained for intracellular transcription factors. 0.5-5 x 10^6 cells were incubated for 30-60 min with freshly prepared fixation solution (eBiocience)t 4°C in the dark. Cells were then washed with permeabilization buffer (eBiocience) and stained with relevant antibodies diluted in permeabilization buffer at 4°C for 30-60 min in the dark. Cells were then washed again with permeabilization buffer, before resuspending in FACS wash for flow cytometric data collection.

2.2.6 In vitro assessment of T and B cell function

2.2.6.1 In vitro (re-)stimulation of T cells

Cytokines secreted by T cells were analyzed either by using the intracellular cytokine staining protocol (2.2.5.2 Staining of intracellular cytokines) or by quantitatively analyzing the secreted cytokines in the supernatant, which was done by ELISA (2.2.2.3 ELISA) or by CBA (2.2.2.4 Cytometric Bead Array).
To (re-)stimulate T cells for cytokine analysis by ELISA or CBA, 96-well microplates were coated at 37°C ON with anti-CD3 (5µg/ml - 0.156µg/ml) and anti-CD28 (2.5µg/ml - 0.078µg/ml) antibodies diluted in dPBS. Before the T cells were added, the dPBS was removed cautiously from each well. Then, T cells resuspended in RPMI were transferred to the well plates with a density of $5x10^5$ cells/well/200µl. Where mentioned, 2µg/ml soluble α.-CD28 was added. 48h later supernatants were collected and analyzed by ELISA, CBA or stored for later analysis at -20°C.
For intracellular cytokine analysis, cells were plated on 24 well plates at a density of $1x10^6$ cells/well/ml, which were coated before with 5µg/ml anti-CD3, and restimulation was peformed for 24 hours. Monesin (Golgi-Stop/BD) or Brefeldin A (Golgi-Plug/BD) was added for the final 4-6 hours to prevent cytokine secretion. To measure intracellular IL-2, in addition to Brefeldin A, 500ng/ml ionomycin and 5ng/ml PMA cells were added for the final 4-6 hours.

2.2.6.2 In vitro differentiation of T cells

To analyze the differentiation of T cells *in vitro,* naïve T cells were positively purified using the $CD4^+$ $CD62L^+$ T cell isolation kit. Cells were plated on 24 well plates at a density of $7x10^5$ cells/well/ml in the presence of 5µg/ml soluble anti-CD3 and 5µg/ml soluble anti-CD28. Additionally, cytokines and antibodies for T cell differentiation were added as follows: 10µg/ml anti-CD4 and 10ng/ml IL-12 for Th1 conditions; 10µg/ml anti-IFN-γ and 10ng/ml IL-4 for Th2 conditions; 30ng/ml IL-6, 5ng/ml TGF-β, 50ng/ml IL-23, 10µg/ml anti-IL-4, and 10µg/ml anti-IFN-γ for Th17 conditions; 30ng/ml IL-6, 10µg/ml anti-IFN-γ, 10µg/ml anti-IL-4 and 20µg/ml anti-TGF-β for Tfh conditions. Following 2 days of incubation at 37°C T cells were analyzed for intracellular cytokines and transcription factors by FACS.

2.2.6.3 In vitro (re-)stimulation of B cells

Similar to the T cells, B cell cytokines or immunoglobulins were analyzed upon (re-) stimulation by intracellular staining, CBA or ELISA.
To measure cytokines in the supernatant, B cells were plated to 96 well plates with a density of $5x10^5$ cells/well/200µl and stimulated with 10µg/ml anti-CD40 and different concentration of LPS or CpG as described in Results. 24 hours later supernatants were collected and analyzed by ELISA, CBA or stored for later analysis at -20°C.
On the intracellular level IL-10 secretion by B cells was assessed as follows. B cells from spleen or PerC were isolated and restimulated with 10µg/ml LPS, 500µg/ml ionomycin and 5ng/ml PMA in RPMI in a 24 well plate with a cells density of $1x10^6$ cells/well/ml for 6 hours at 37°C. To block secretion of IL-10, Monesin was added during the whole time of stimulation.

2.2.6.4 In vitro proliferation analysis

To analyze proliferation of T or B cells, CFSE dilution profile was used for assessment. To this end, splenocytes, purified T cells or purified B cells were labeled with 0,5µM CFSE for 10min at 37°C. The reaction was stopped by adding pre-warmed dPBS containing 10% FCS. Cells were washed and plated in RPMI at a density of $2x10^5$ cells/well/200µl in 96 well plates and at $5x10^5$ cells/well/ml in 24 well plates.
For T cell stimulation, the 96-well microplates were coated ON at 37°C with anti-CD3 (5µg/ml - 0.156µg/ml) and anti-CD28 (2.5µg/ml - 0.078µg/ml) antibodies diluted in dPBS. Before the T cells were added, the dPBS was removed cautiously from each well. Then, T cells resuspended in RPMI were added to each well.

For B cell stimulation, cells were stimulated with 5µg/ml anti-CD40, 10µg/ml anti-IgM or 10µg/ml LPS in RPMI.
After 2 and 3 days at 37°C, both T and B cell proliferation assays were analyzed for their CFSE-fluorescence dilution, which progressively decreases with every cell division.

2.2.7 In vivo assessment of T and B cell function

2.2.7.1 Active induction of experimental autoimmune encephalitis (EAE) and EAE score definition

C57BL/6N or C57BL/6N Dkk3$^{-/-}$ mice were immunized with Myelin oligodendrocyte glycoprotein 33-55 (MOG_{33-55}) emulsified in Complete Freund´s Adjuvants (CFA). Each mouse received a subcutaneous (s.c.) injection in the flank of 100µl CFA containing 200µg MOG_{33-55}. At the time point of immunization and 48 hours later mice received 200ng of Pertussis toxin (PT) in 0,5ml PBS i.p.. The mice were observed for clinical signs and EAE was scored on a scale from 0 to 5 as follows:
0 = no overt signs of disease,
1 = limp tail or hind limb weakness (but not both),
2 = Limp tail and hind limb weakness,
3 = partial hind limb paralysis,
4 = complete hind limb paralysis,
5 = moribund state or death by EAE.

2.2.7.2 Passive induction of EAE by transfer of in vitro restimulated cells

2D2.Dkk3$^{-/-}$ mice were immunized with MOG_{33-55} in CFA. Each mouse received 100µl CFA containing 200µg MOG_{33-55} injected s.c. at two different spots of each 50µl volume in the flank. 10 days post immunization spleens and draining lymph nodes were isolated and single cell suspensions were prepared. Cells were plated in a 24 well plate at a density of $1x10^6$ cells/well/ml and restimulated with 20µg/ml MOG_{33-55} and 20ng/ml IL-12. After 3 days of restimulation, $1x10^7$ cells were transferred i.p. into C57BL/6N Rag2$^{-/-}$ or C57BL/6N Rag2$^{-/-}$ Dkk3$^{-/-}$ recipient mice, which were treated with PT as described above. The mice were observed for clinical signs and EAE scores were measured.

2.2.7.3 Diabetes induction and analysis

Naïve CD4 T cells of Bdc2.5/NOD mice were purified by negative isolation with Dynal beads. Briefly, splenocytes were labeled with a mix of biotinylated CD8, CD44, CD25, Cd11b, CD11c, CD19, B220 and CD49b antibodies. Then streptavidin conjugated magnetic beads were added to remove labeled cells (as described in 2.2.4 Magnetic cell sorting). 10.000 cells were transferred intravenous (i.v.) into NOD.RAG recipient mice. NOD.RAG mice were treated with 0,7mg/mouse of either anti-mDkk3-4.22 antibody or MOPC21 isotype control every three days, starting 6 days before cells were transferred i.v. to the mice. Glucose in the blood was measured at the beginning of the experiment every 2 days and every day once it started to increase.

2.2.7.4 Induction of inflammatory bowel disease (IBD) and analysis

For induction of colitis CD4 T cells of FoxP3.LuciDTR-4 mice were used as donors. Splenocytes were in a first step purified by CD4 negative isolation Dynal beads and in a second step, $eGFP^+$ cells, which are the FoxP3 expressing regulatory cells, were depleted by FACS sorting. $5x10^5$ cells of the regulatory T cell depleted cells were injected i.v. per mouse into either C57BL/6N $Rag2^{-/-}$ or C57BL/6N $Rag2^{-/-}$ $Dkk3^{-/-}$ recipient mice. Mouse weight was measured every 2-3 days for 5 weeks.

2.2.7.5 Immunization of mice for analysis of T cell differentiation

To analyze T cell differentiation *in vivo*, C57BL/6N and C57BL/6N $Dkk3^{-/-}$ mice were immunized with MOG_{33-55} in CFA for Th1/Th17 differentiation or with MOG_{33-55} in incomplete Freund's adjuvant (IFA) for Th2 differentiation. Each mouse received 200µg MOG_{33-55} in a volume of 100µl CFA or IFA, which was injected s.c. at the belly at two different spots of each 50µl volume. 8 days post immunization spleen and LN were taken, single cell suspensions were prepared and cells were restimulated with 5µg/ml plate-bound anti-CD3 for 48 hours. Then supernatants were collected and analyzed by CBA.

2.2.7.6 Immunization of mice for analysis of B cell responses

In this study four different antigens were used to immunize mice to assess antibody responses in C57BL/6N and C57BL/6N $Dkk3^{-/-}$ mice. TNP-BSA is applied s.c. in a 100µl emulsion of CFA containing 50µg TNP-BSA per mouse. PC-KLH is applied s.c. in a 100µl emulsion of CFA containing 100µg PC-KLH per mouse. S.c.

immunization were injected at the belly at two different spots of each 50µl volume. TNP-Ficoll (50µg/mouse) and LPS (4mg/g weight) were used in a solution with PBS and injected i.p.. Blood was taken before immunization, at day 7, day 14 and day 21. Serum was prepared from the blood and analyzed by ELISA for concentration of antigen-specific or total antibody titers of relevant isotypes.

2.2.7.7 Treatment and analysis of MRL/LPR mice

In this study it was investigated whether anti-mDKK3-4.22 or MOPC21 isotype control treatment of MRL/LPR mice leads to a different level of disease. Mice were treated with 0,7mg/mouse anti-mDkk3-4.22 antibody or MOPC21 isotype control i.p. twice a week. Treatment was started at an age of 5 weeks and lasted for 6 weeks. At an age of 12 weeks mice were sacrificed and analyzed for symptoms of systemic lupus erythematosus (SLE). Before, after 4 weeks of treatment and at the time point of scarification blood was taken to prepare serum. Analysis included organ weight measurement, T and B cell phenotype analysis by FACS, serum analysis for e.g. protein levels and kreatinines, ELISA to determine levels of autoantibodies in the serum as well as a histological examination of organs.

3 Results

3.1 The role of Dkk3 in T cell function

Previous studies of our group focused on the function of Dkk3 as a mediator of CD8 T cell tolerance [214]. These studies were carried out in a transgenic system. However, it is also of interest whether Dkk3 is able to control polyclonal T cell responses. It has been shown by our laboratory that Dkk3 is expressed in immune privileges sites [212]. The brain is one of these organs expressing high levels of Dkk3, but it also has been shown to be expressed in the crypts of the gut and in the β-islet cells of the pancreas [192, 210, 224]. Therefore, we decided to investigate T cell reactivity in the absence of Dkk3 in three different animal models: Experimental autoimmune encephalitis (EAE), inflammatory bowel disease (IBD) and diabetes type I.

3.1.1 Dkk3 regulates effector T cell subsets in experimental autoimmune encephalitis

In our laboratory the model of MOG_{35-55} induced EAE is employed. Papatriantafyllou revealed that Dkk3 deficient mice develop an exacerbated course of EAE with prolonged EAE symptoms [212], which was confirmed during the course of my PhD thesis (Figure 3.1).

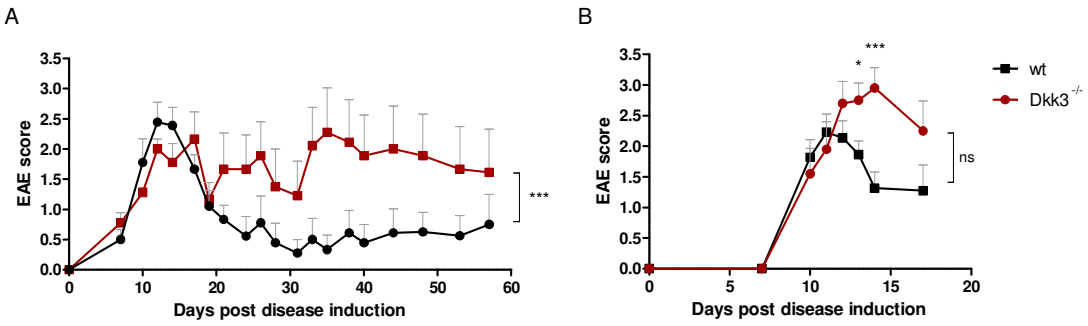


Figure 3.1 | Absence of Dkk3 results in more severe and persist EAE symptoms. C57BL/6 (wt) and Dkk3$^{-/-}$ mice were immunized with MOG_{33-55} in CFA and treated with pertussis toxin. EAE scores were determined for a period of 3 months (A) and 18 days (B) until mice were sacrificed for analysis. Mean EAE score and SEM are shown for each group (n=9). Curves were compared using 2-way ANOVA, p-values of column factors are shown, single time points were compared using student t-test (ns=not significant; *=p<0,05, ***=P<0,0005).

It could be argued that a different thymic selection of auto-reactive T cells (including MOG-specific T cells) occurs in the absence of Dkk3, which might be the reason for the higher susceptibility of Dkk3$^{-/-}$ animals to EAE. To address this point, and to

clarify whether a Dkk3 sufficient environment (without possible T cell intrinsic Dkk3) is able to regulate the pathogenic T cell response in EAE, Dkk3 deficient T cells were transferred to Rag2$^{-/-}$ mice being either sufficient or deficient for Dkk3. Rag2$^{-/-}$ mice were used as recipients to ensure that only T cells which were transferred from the same donor source and therefore feature exactly the same T cell repertoire can provoke the disease. We used T cells of transgenic 2D2.Dkk3$^{-/-}$ mice , which bear a TCR specific for the MOG peptide. In detail, cells from draining lymph nodes (LN) and spleens of MOG_{33-55} immunized 2D2.Dkk3$^{-/-}$ mice were restimulated *in vitro* with MOG_{33-55} and IL-12 and transferred to wt.Rag2$^{-/-}$ or Dkk3$^{-/-}$Rag2$^{-/-}$ recipients. Recipient mice were treated with pertussis toxin (PT) to facilitate the migration through the blood-brain barrier of MOG-specific T cells.

The onset of disease in this transfer experiment was delayed in comparison to the actively induced EAE by immunization. Interestingly, an earlier onset and more severe symptoms of EAE were observed in Dkk3$^{-/-}$Rag2$^{-/-}$ recipients compared to their Dkk3 sufficient controls. Hence, T cell responses appear to be dampened by environmental Dkk3 and a different T cell repertoire does not seem to be the reason for the increased affection of Dkk3$^{-/-}$ mice by EAE.

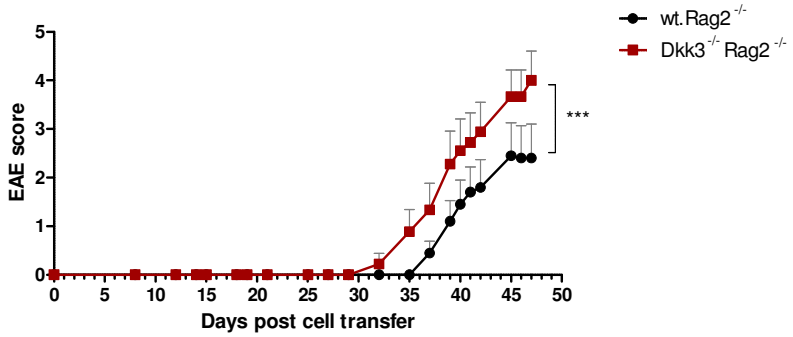


Figure 3.2 | Environmental Dkk3 is able to regulate EAE symptoms in a transfer model. 2D2.Dkk3$^{-/-}$ mice were immunized with MOG_{33-55} in CFA. 10 days post immunization cells of spleens and draining lymph nodes were prepared and restimulated *in vitro* with MOG_{33-55} and IL-12. After 3 days of restimulation, 1x10^7 cells were transferred i.p. into wt.Rag2$^{-/-}$ or Dkk3$^{-/-}$Rag2$^{-/-}$ recipient mice, which were treated with pertussis toxin. EAE scores were determined over a period of 7 weeks. One representative out of 2 independent experiments is presented. Mean EAE score and SEM are shown for each group (n=10). Curves were compared using 2-way ANOVA, p-value of column factor is shown (**=P<0,005).

To examine the cellular and molecular basis of the more severe EAE phenotype in Dkk3$^{-/-}$ mice, leukocyte infiltrates of the CNS of diseased Dkk3$^{-/-}$ and wt mice, in which EAE was directly induced (as described in Figure 3.1), were compared for parameters demonstrated to be involved in disease initiation and progression [47, 225, 226]. To this end, leukocytes from the brain and the spinal cord were isolated and either stained directly for surface marker and transcription factors or

restimulated over night, followed by a protein secretion blockage, for staining of intracellular cytokines.

The analysis of leukocyte infiltrates was carried out at two different time points. The first analyzed time point was the peak of disease or rising phase, depending on the experiment (day 11-13), characterized by no difference in disease score between wt and Dkk3$^{-/-}$ animals. The second analyzed time point was the so called recovery phase (starting around day 17), in which wt mice recover. As Dkk3$^{-/-}$ mice show a more persisent EAE, the differences in disease score at this time point were significant.

At the peak of disease/rising phase (Figure 3.3) the CNS was infiltrated by the same numbers of CD4^{+} and CD8^{+} and myeloid CD11b^{+} cells in wt and Dkk3$^{-/-}$ mice. Surprisingly, higher numbers of Tregs (CD25^{+} FoxP3^{+} CD4 T cells) were found in the CNS of Dkk3$^{-/-}$ mice. Furthermore, no difference was observed in GM-CSF or IFN-γ secreting CD4 or IFN-γ secreting CD8 T cells. However, the CNS of Dkk3$^{-/-}$ mice yielded higher numbers of IL-17 producing CD4 T cells. In the periphery (spleen and LN) T cell population were not altered. Interestingly, the number of CD11b^{+} myeloid cells was decreased in the spleen.

In the recovery phase (Figure 3.4) the Dkk3$^{-/-}$ CNS was infiltrated by higher numbers of CD4^{+}, CD8^{+} and myeloid CD11b^{+} cells in comparison to the wt CNS. Numbers of CD4 and CD8 T cells were also increased in the spleen of Dkk3$^{-/-}$ mice, whereas numbers of CD11b^{+} cells were decreased in the spleen, as also already observed at the peak of disease/recovery phase. Furthermore, numbers of Foxp3^{+} cells were decreased in the spleen of Dkk3$^{-/-}$ mice, presumably due to the selective expansion of other CD4 T cells (as described above). Cytokine analysis revealed no difference in the production of IL-17 and GM-SCF within the CD4 T cell subset, but a higher production of IFN-γ by CD4 and CD8 T cells in Dkk3$^{-/-}$ CNS. However, as the number of CD4 and CD8 T ell among the whole CNS infiltrating cells was increased in the CNS of Dkk3$^{-/-}$ mice, the number of T cells secreting any analyzed cytokine will also be higher in the Dkk3 deficient environment.

In summary, in the absence of Dkk3 more severe EAE symptoms were observed. These were associated with changes in the CNS infiltrating leukocyte populations and the local cytokine production.

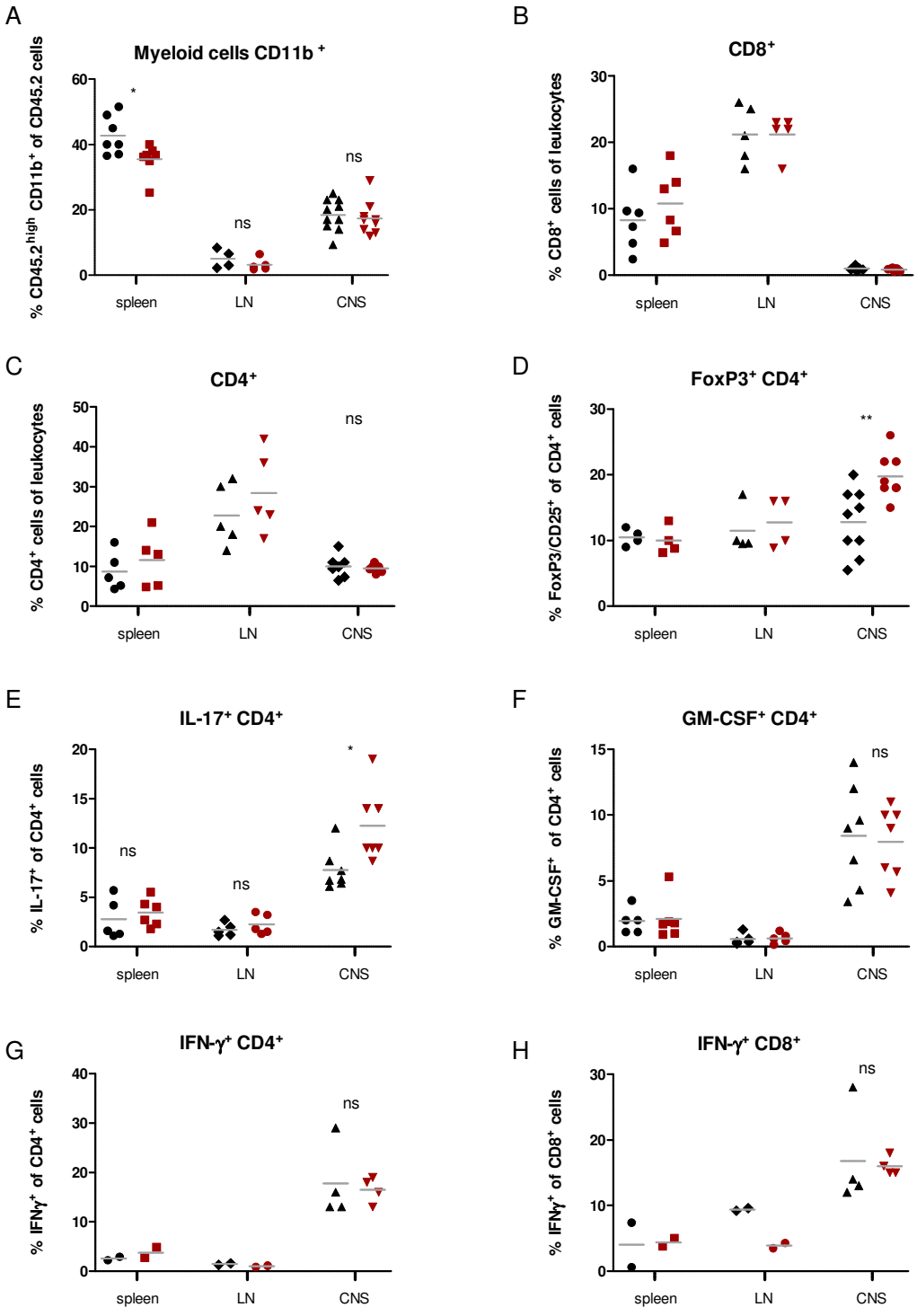


Figure 3.3 | Analysis of CNS infiltrates at the peak of disease / rising phase. At day 11 upon EAE induction, spleen, lymph node (LN) and central nervous system (CNS) infiltrating leukocytes of C57BL/6 (black symbols) and Dkk3$^{-/-}$ (red symbols) mice were isolated and stained for surface markers and transcription factors (A-D). Intracellular cytokines were stained after cells were restimulated with 5µg/ml plate-bound anti-CD3 *in vitro* for 24 hours, including the addition of protein-transport inhibitor for the final 6 hours (E-H). Summary data from 3 independent experiments are presented. One dot represents cells of 1 mouse for the spleen and the LN group and cells of 2-3 pooled mice for the CNS group. Horizontal bars represent the mean for each group. Groups were compared using student t-test (ns=not significant; *=p<0,05, **=P<0,005).

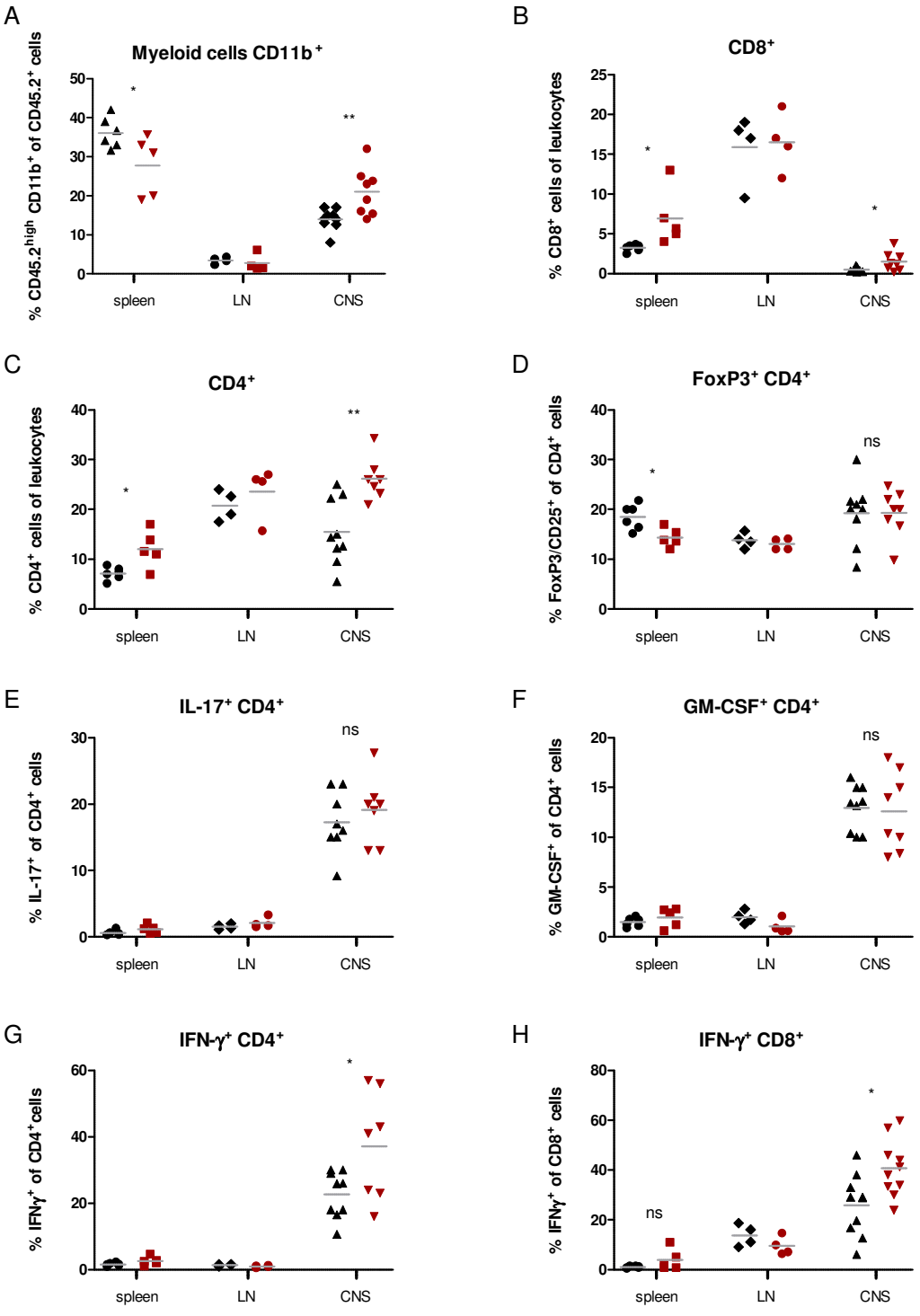


Figure 3.4 | Analysis of CNS infiltrates in the recovery phase. At day 18 upon EAE induction, spleen, lymph node (LN) and central nervous system (CNS) infiltrating leukocytes of C57BL/6 (black symbols) and Dkk3$^{-/-}$ (red symbols) mice were isolated and stained for surface markers and transcription factors (A-D). Intracellular cytokines were stained after cells were restimulated with 5µg/ml plate-bound anti-CD3 *in vitro* for 24 hours, including the addition of protein-transport inhibitor for the final 6 hours (E-H). Summary data from 3 independent experiments are presented. One dot represents cells of 1 mouse for the spleen and the LN group and cells of 2-3 pooled mice for the CNS group. Horizontal bars represent the mean for each group. Groups were compared using student t-test (ns=not significant; *=p<0,05, **=P<0,005).

3.1.2 Dkk3 does not regulate CD4 T cells in a model of inflammatory bowel disease

Dkk3 has been shown to be expressed the villus mesenchyme and myenteric plexuses of the intestine [210]. These areas of the intestine are important for tolerance induction and maintenance to oral antigens as well as to the commensal microflora of the gastrointestinal tract [227, 228]. Hence, we wanted to investigate whether Dkk3 contributes to tolerance induction in a model of IBD.

IBD is induced by CD4 T cells in the absence of Tregs [229]. Hence, we used FoxP3.LuciDTR-4 mice [230] as donors of CD4 T cells. These mice express eGFP under the FoxP3 promotor and $eGFP^+$ cells can be easily depleted by FACS sorting (Figure 3.5 A). $CD4^+$ purified T cells depleted for Tregs were transferred to either wt.$Rag2^{-/-}$ or $Dkk3^{-/-}Rag2^{-/-}$ recipient mice and weight was measured every other day for 5 weeks (Figure 3.5 B). No difference in weight change was found between Dkk3 sufficient and deficient mice. Therefore, Dkk3 produced in the gut does not seem to regulate the pathogenic CD4 T cell response in this animal model of IBD.

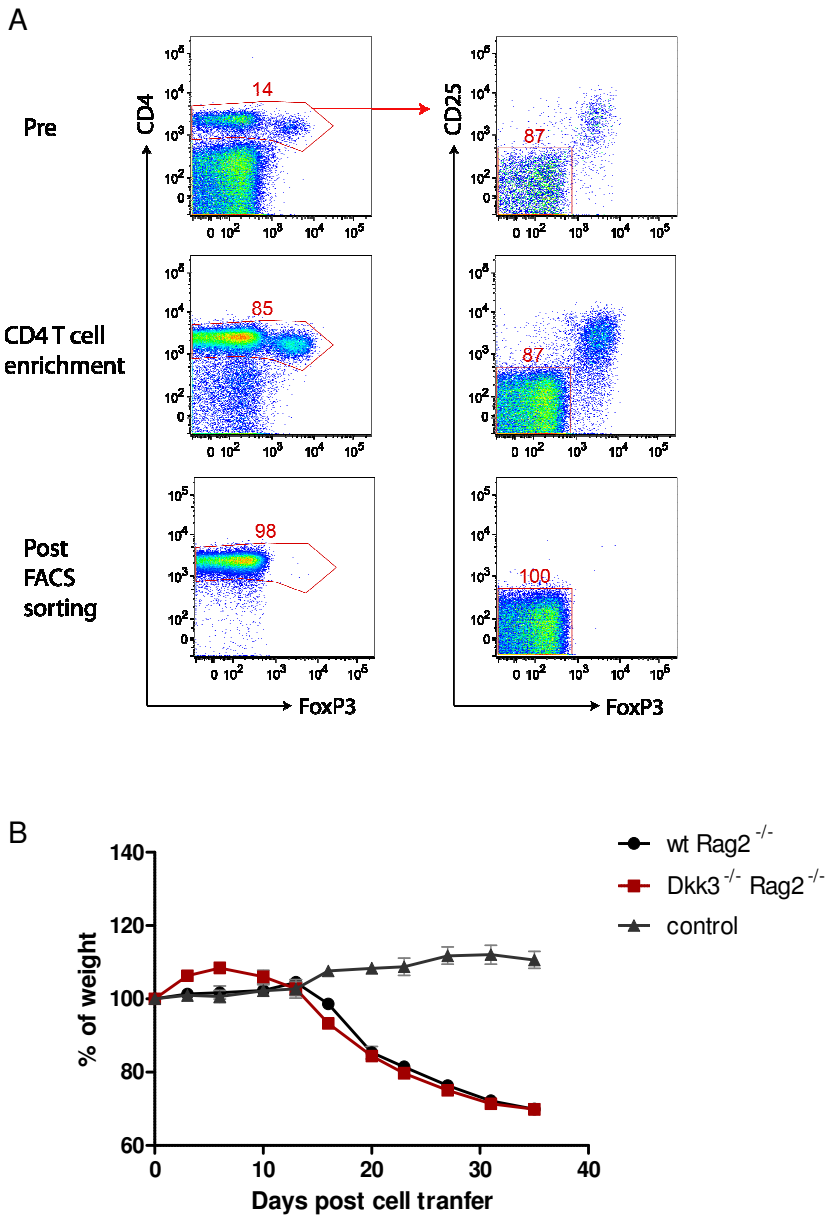


Figure 3.5 | Dkk3 does not regulate the pathogenic CD4 T cell response in a model of inflammatory bowel disease (IBD). $CD4^+$ T cells of FoxP3.LuciDTR-4 mice were first purified by magnetic cell sorting for CD4 T cell enrichment and then depleted of $eGFP^+$ ($FoxP3^+$) cells by FACS sorting (B) (Pre=before purification). Gated on leukocytes (left) and on $CD4^+$ cells (right). $5x10^5$ cells were transferred i.v. to wt.$Rag2^{-/-}$ or $Dkk3^{-/-}$ $Rag2^{-/-}$ recipient mice and weight was measured every 2-3 days for 5 weeks (B). Percentage of the mean weight of the weight on day 0 (day of cell transfer) for each group (wt, $Dkk3^{-/-}$: n=7) and SEM is shown. Control group consists of 2 wt.$Rag2^{-/-}$ and 2 $Dkk3^{-/-}$ $Rag2^{-/-}$ mice. One out of 2 representative experiments is shown.

3.1.3 Dkk3 does not regulate CD4 T cells in a transgenic model of diabetes type 1

Hermann *et al.* demonstrated that Dkk3 is expressed in subset of human pancreatic β- cells [192]. Therefore, it was intriguing to study whether Dkk3 produced in this area is able to dampen diabetes induction. We used a diabetes model with a relatively short disease induction phase. In this model CD4 T cells from Bdc2.5/NOD mice, carrying a transgenic TCR specific for a β-islet antigen [231], are transferred into NOD.Rag2$^{-/-}$ mice. These transgenic CD4 T cells were purified and depleted for memory and activated T cells as well as CD25^{+} Tregs (Figure 3.6 A) before being injected into the recipient NOD.Rag2$^{-/-}$ mice, which were either treated with isotype control or anti-Dkk3 antibody. Glucose measurement in the blood was used as the read-out system for diabetes incidence. No significant difference of disease onset or disease severity was observed (Figure 3.6 B). Therefore, Dkk3 produced in the pancreas is not able to regulate the pathogenic CD4 T cell response in the diabetes type 1 model of Bdc2.5/NOD transferred CD4 T cells into NOD.Rag2$^{-/-}$ mice.

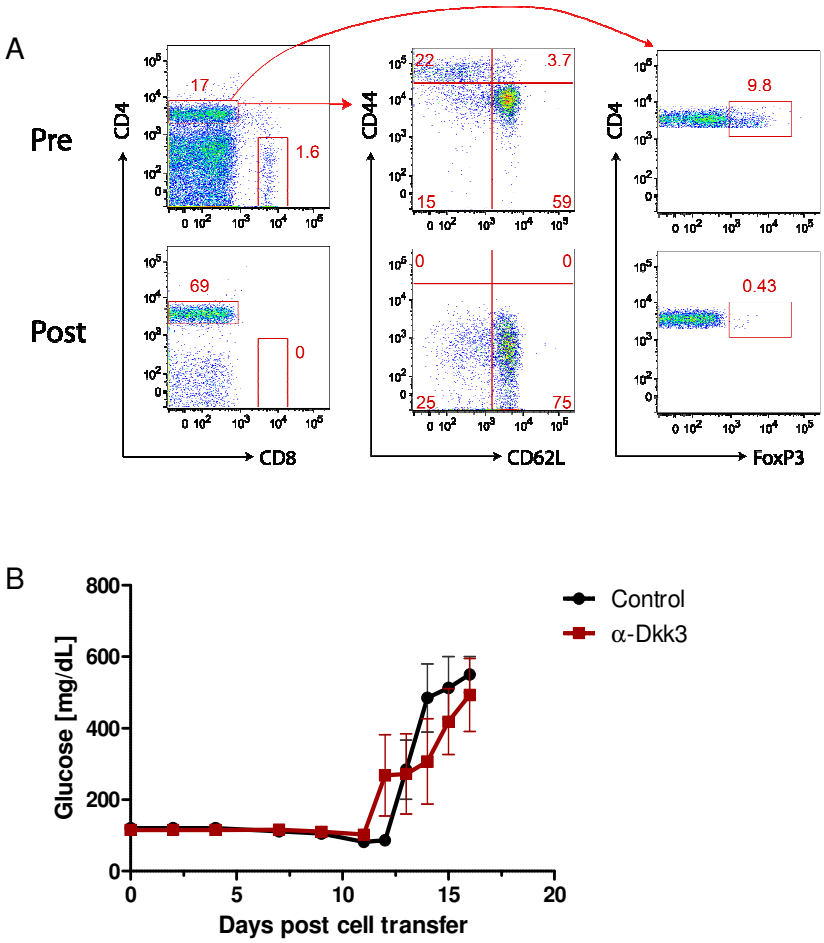


Figure 3.6 | Dkk3 does not regulate the pathogenic T cell response in a model of diabetes type 1. Naïve CD4 T cells of Bdc2.5/NOD mice were purified and activated/memory T cells and Tregs were depleted by magnetic cell sorting (A) (Pre=before purification/depletion; Post=after purification/depletion). Gated on leukocytes (left) and on $CD4^+$ cells (middle and right). 10.000 cells were transferred i.v. into NOD.RAG recipient mice. NOD.RAG mice were treated with 0,7mg/mouse of either anti-Dkk3 antibody or isotype control every three days, starting 6 days before cells were transferred to the mice. Sugar in the blood was measured at the beginning of the experiment every 2 days and every day once it started to increase (B). Mean and SEM are shown for each group (n=5).

3.1.4 Comparison of Dkk3 expression in the brain, the gut and the pancreas

Dkk3 has been demonstrated histologically to be expressed in the gut and the pancreas [192, 210, 224]. In the brain our laboratory has investigated Dkk3 expression histologically and quantitatively [212]. However, a quantitative analysis of the Dkk3 expression in the pancreas and the gut has not been conducted so far and as the brain, the gut and the pancreas are the locations were the above described T cell responses were taking place, we wanted to obtain a quantitative comparison of the expression level of Dkk3 between these tissues. Therefore, tissues were analyzed for relative mRNA expression of Dkk3 in comparison to β-actin. Strikingly, the expression of Dkk3 was 300-500 times higher in the brain and the eye (which was used as an additional positive control) compared to the organs of the gut or the pancreas. This might explain the failure of Dkk3 to regulate pathogenic T cell responses in the model of IBD and diabetes type 1.

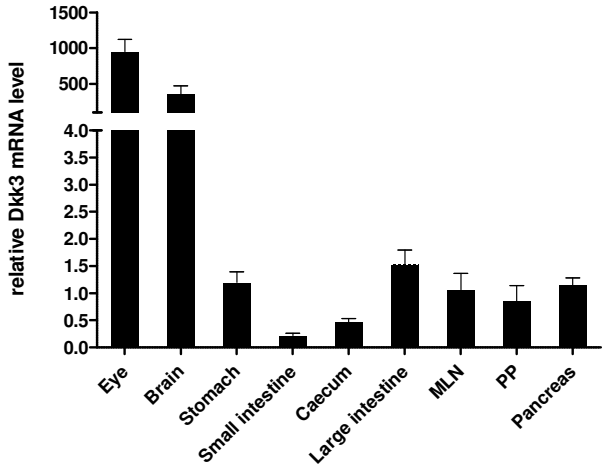


Figure 3.7 | mRNA expression profile of Dkk3 in different mouse tissues. Total RNA was purified from C57BL/6 mouse tissues, reverse transcribed to cDNA and analyzed by quantitative real-time PCR. For comparative analysis, the relative Dkk3 mRNA expression level was calculated by using β-actin mRNA levels as a control housekeeping gene. Background values of $Dkk3^{-/-}$ mice were subtracted. MLN=Mesenteric lymph nodes; PP=Peyer's patches. Mean and SEM are shown for each analyzed tissue (n=4).

3.1.5 CD4 T cells of Dkk3$^{-/-}$ mice do not show clear differences in their differentiation behavior

To investigate whether Dkk3 acts on the differentiation of CD4^{+} T cells, purified naïve CD4^{+} T cells of wt and Dkk3$^{-/-}$ mice were stimulated *in vitro* under the presence of Th1, Th2 or Th17 differentiating conditions. After 72 hours, T cells of Dkk3$^{-/-}$ showed less secretion of IFN-γ and were slightly less positive for their master regulator transcription factors T-bet and EOMES under Th1 differentiating conditions (Figure 3.8 A). In contrast, under Th2 differentiating conditions Dkk3$^{-/-}$ CD4 T cells possessed twice the number of IL-10 secreting cells and an elevated expression of their specific transcription factor GATA-3 (Figure 3.8 C). However, Th2 differentiation *in vitro* generated only very few IL-10^{+} cells, therefore it is difficult to draw a final conclusion on the Th2 differentiation behavior from these *in vitro* experiments. Nevertheless, both observations (for Th1 and Th2 conditions) were consistent throughout the four conducted experiments. Th17 conditions did not result in a consistent difference between CD4 T cells of wt and Dkk3$^{-/-}$ mice analyzed be the expression of IL-17 and the master transcription factor RoRγt (Figure 1.9 B). Therefore, it seems that CD4 T cell of Dkk3$^{-/-}$ mice exhibit a slightly increased capacity to differentiate into the Th2 phenotype *in vitro*.

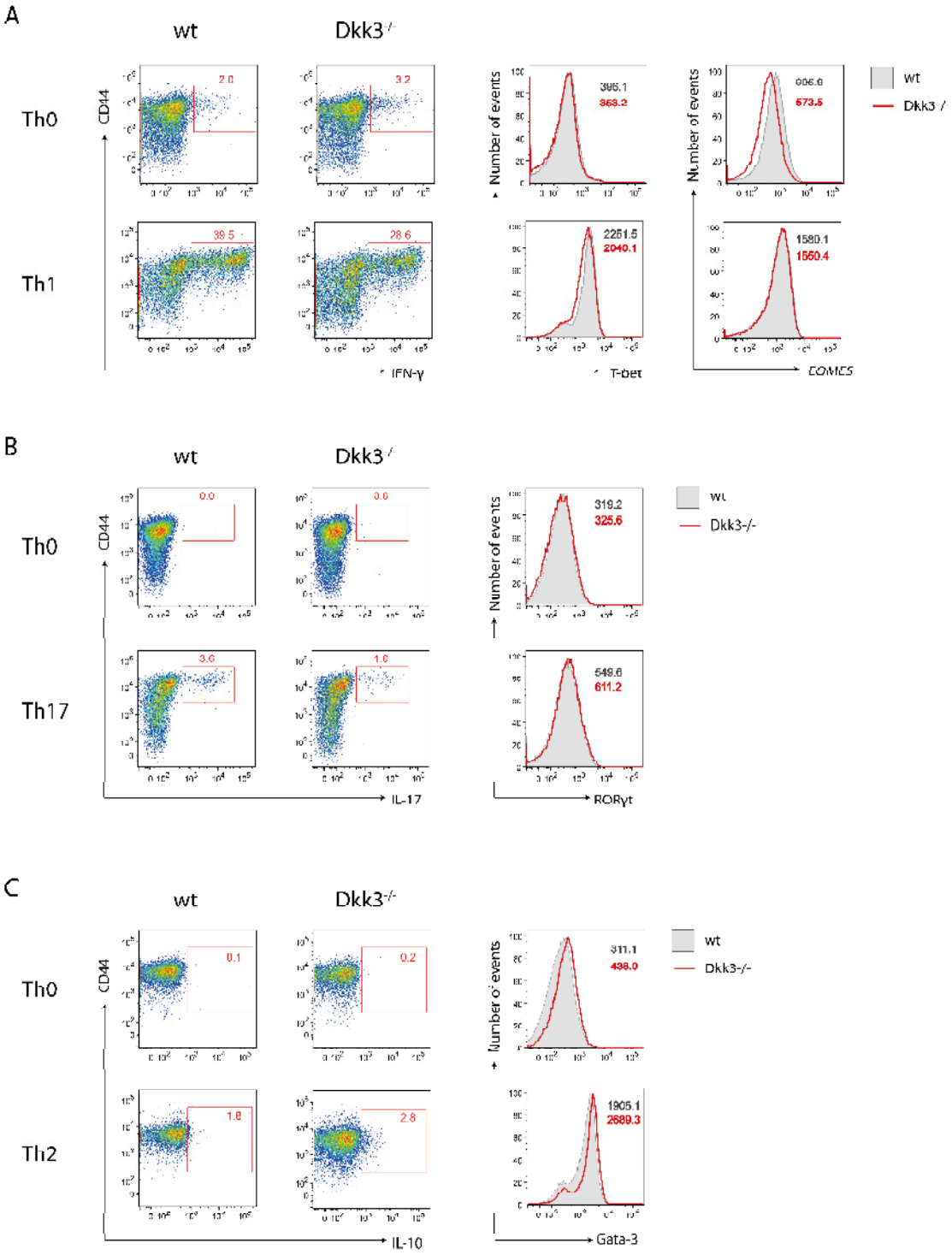


Figure 3.8 | CD4 T cells of Dkk3$^{-/-}$ mice show slightly increased Th2 differentiation *in vitro*. T cells of C57BL/6 (wt) and Dkk3$^{-/-}$ mice were cultured under Th0 (no addition of cytokines or antibodies; top of A, B and C), Th1 (A), Th17 (B) or Th2 (C) conditions for 72 hours in the presence of anti-CD3 and anti-CD28. Cells were stained for specific cytokines and transcription factors. All FACS blots are gated on CD4^{+} cells. The expression of transcription factors (right panels) are depicted as histograms and the fluorescence mean intensity (FMI) is indicated in the graph (grey=wt CD4 T cells; red=Dkk3$^{-/-}$ CD4 T cells). One representative out of 4 experiments is shown.

3.1.6 The secretion of Th2 associated cytokines is increased in Dkk3$^{-/-}$ mice *in vivo*

Dkk3 has been shown to be absent in T cells themselves [212, 214]. Therefore, the analysis of differentiation *in vitro* shows the imprint of a Dkk3 sufficient or deficient environment where cells were coming from. However, as the assay takes three days, the difference might fade as both groups were simulated in a Dkk3 deficient culture medium. Therefore, we also analyzed T cell differentiation *in vivo*, with an *ex vivo* readout system. For this purpose, wt and Dkk3$^{-/-}$ mice were immunized with the antigen MOG_{33-55} either emulsified in CFA or incomplete Freund's adjuvant (IFA). Antigens immunized in CFA have been described to lead to a strong immune response of the Th1/Th17 direction and a weak Th2 response, whereas antigens in IFA lead to increased immune response of the Th2 direction and reduced Th1/Th17 responses [232-234]. 8 days post immunization, cells of spleens and draining LN were restimulated *in vitro* with plate-bound anti-CD3 and 48 hours later supernatants were analyzed quantitatively for the concentration of Th1, Th17 and Th2 cytokines. In detail, IFN-γ, IL-17, TNF-α, and IL-2 served to detect Th1/Th17 differentiation as these cytokines are typically secreted by Th1 and Th17 cells [38, 235], whereas IL-6 (discussed in 4.3 Dkk3 limits Th2 associated cytokine secretion), IL-4 and IL-10, mostly related to Th2 cells, served to detect Th2 differentiation [38, 235, 236].

Immunization with MOG_{33-55} in CFA resulted in the secretion of substantial levels of Th1/Th17 cytokines and modest amounts of Th2 cytokines (Figure 3.9). Upon MOG_{33-55} /CFA immunization no difference between the wt and the Dkk3$^{-/-}$ group was found for IFN-γ, IL-17, TNF-α, and IL-2 secretion (Figure 3.9). Although immunization with CFA leads preferentially to Th1/Th17 responses, Th2 cytokines are still secreted and levels of IL-4 and IL-10 were found to be higher in cell supernatants of Dkk3$^{-/-}$ mice compared to wt mice.

Immunization with MOG_{33-55} in IFA resulted in decreased levels of Th1/Th17 cytokines and slightly increased levels of IL-10 compared to the immunization with MOG_{33-55} in CFA (Figure 3.10). Thus, in our experiments immunization with IFA as adjuvant decreased the Th1/Th17 response, while leaving the Th2 response unaffected.
Upon immunization with MOG_{33-55} /IFA Dkk3 deficient mice secreted decreased levels of IL-17 and increased levels of Th2 associated cytokines IL-6 and IL-10 (Figure 3.10) in comparison to wt animals (Figure 3.10). IL-4 secretion, however, was only very low upon MOG_{33-55} in IFA immunization and no difference between the two groups was revealed.

In conclusion, we found increased levels of Th2 associated cytokines independently of the kind of adjuvant, CFA or IFA, and decreased levels of IL-17 upon immunization with IFA in Dkk3$^{-/-}$ cell cultures upon *ex vivo* restimulation. Therefore, Th2 differentiation seems to be increased in Dkk3$^{-/-}$ mice, which confirms the data obtained by *in vitro* differentiation experiments.

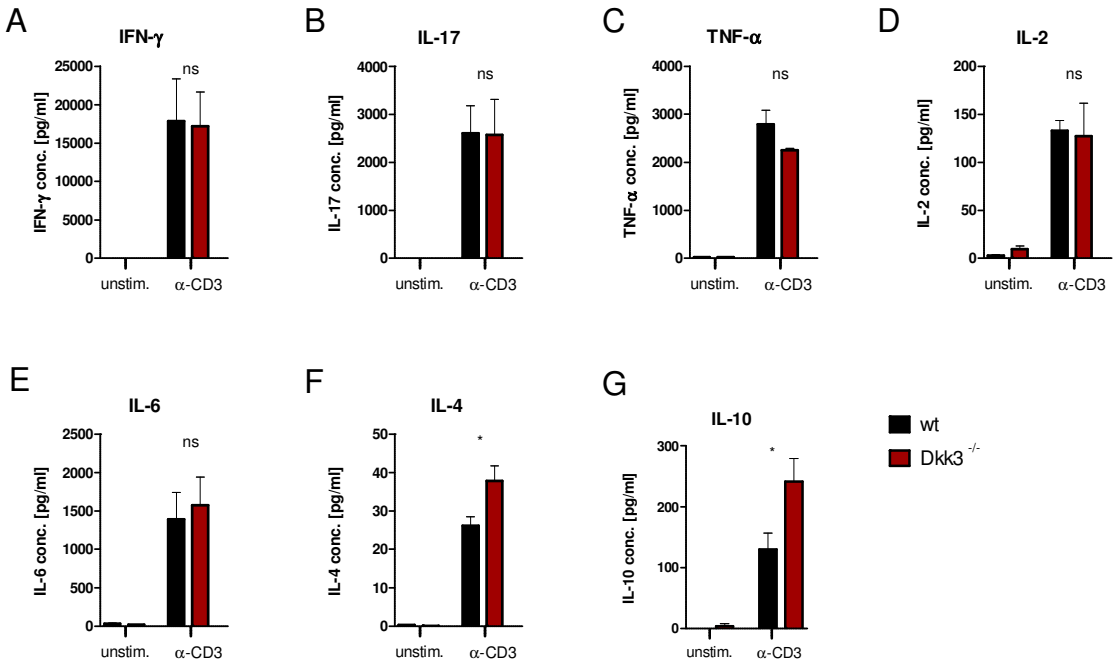


Figure 3.9 | Immunization with MOG in CFA does not lead to altered Th1/TH17 differentiation *in vivo* in Dkk3$^{-/-}$ mice. C57BL/6 (wt) and Dkk3$^{-/-}$ mice were immunized with MOG_{33-55} in CFA. 8 days post immunization, single cell suspensions of spleens were prepared and cells were restimulated with 5µg/ml plate bound anti-CD3 *in vitro*. 48 hours later supernatants were analyzed by CBA for the concentration of IFN-γ (A), IL-17 (B), TNF-α (C), IL-2 (D), IL-6 (E), IL-4 (D) and IL-10 (G). Summary data of 3 independent experiments are shown. Mean and SEM are indicated for each group. Groups were compared using student t-test (ns= not significant; *=p<0,05; **=p<0,005).

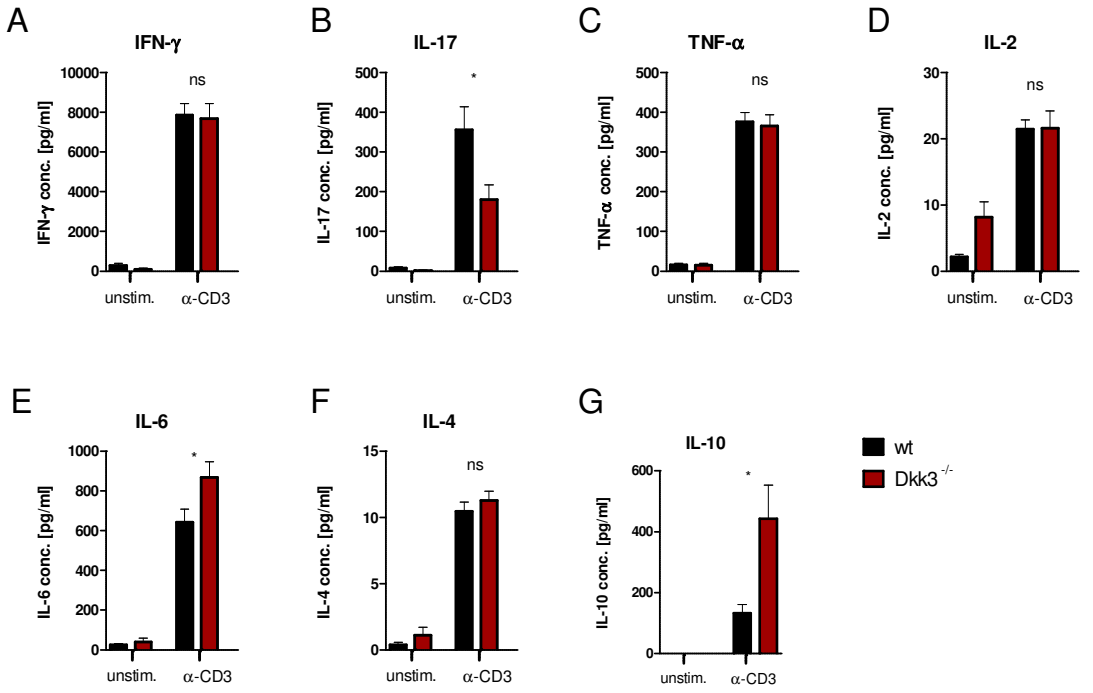


Figure 3.10 | Immunization with MOG in IFA leads to increased secretion of Th2 associated cytokines *in vivo* in Dkk3$^{-/-}$ mice. C57BL/6 (wt) and Dkk3$^{-/-}$ mice were immunized with MOG_{33-55} in IFA. 8 days post immunization, single cell suspensions of spleens were prepared and cells were restimulated with 5µg/ml plate bound anti-CD3 *in vitro*. 48 hours later supernatants were analyzed by CBA for the concentration of IFN-γ (A), IL-17 (B), TNF-α (C), IL-2 (D), IL-6 (E), IL-4 (D) and IL-10 (G). Summary data of 3 independent experiments are shown. Groups were compared using student t-test (ns= not significant; *p<0,05; **p<0,005). Mean and SEM are indicated for each group.

In summary, the experiments investigating the role of Dkk3 in T cell function showed that environmental Dkk3 is able to regulate the pathogenic T cell response in the CNS of Dkk3 deficient mice during the course of EAE. In contrast, the pathogenic T cell response in a model of IBD and diabetes type 1 was not altered in the absence of Dkk3. Thus, there is no general suppression of T cell responses in the presence of Dkk3. In vitro and in vivo analysis of T cell differentiation indicated that Dkk3 deficiency leads to an increase in Th2 associated cytokine secretion, while leaving the Th1/Th17 response mostly unaffected.

3.2 The role of Dkk3 in B cell function

B cells are besides T cells the major players of the adaptive immune system and have been described to exhibit a pathogenic role in numerous autoimmune diseases (as described in 1.4.5 B cells in autoimmune diseases). Therefore, we aimed to investigate the role of Dkk3 on B cell function, starting with the main characteristics of B cells, which are antibody production and cytokine secretion.

3.2.1 Dkk3$^{-/-}$ mice display increased antibody responses

Firstly, we investigated the antibody response to different antigens. B cell responses were analyzed by ELISA of serum samples taken before immunization and at different time points after immunization.

The first antigen we used for immunization was 2,4,6 trinitrophenol (TNP)-BSA. It is one of the most typical T cell dependent (TD) antigens used to asses the response of follicular (FO) B cells and is used in combination with complete Freund's adjuvant (CFA). It is described to elicit an IgM response between day 7 and 14, followed by class switch recombination (CSR) and IgG responses around day 14 to 21 [237]. TNP-BSA specific IgM levels were elevated under steady-state conditions and 7 days post immunization in Dkk3$^{-/-}$ mice (Figure 3.11 A, Day 0) in comparison to their wt littermate controls. On day 14 after immunization the difference was less visible. In contrary, the TNP-BSA specific antibody IgG1 response was elevated in Dkk3$^{-/-}$ animals after immunization on day 14 and day 21 (Figure 3.11 B). However, Dkk3 absence did not change the IgG2a and IgG2b response to TNP-BSA (Figure 3.11 C/D). IgG3, an isotype often related to TI responses and B1 cells similar to IgM [132], was highly elevated in Dkk3$^{-/-}$ upon immunization (Figure 3.11 E).

The second antigens we used for immunization was the T cell independent (TI) antigens TNP-Ficoll. TNP-Ficoll, an inert co-polymer of sucrose and epichlorohydrin conjugated to TNP, has been used for decades as a prototypic T independent type 2 (TI-2) antigen [238]. It has been shown to specifically activate marginal zone (MZ) B cells [157] and lead to an increase of IgM and IgG3 antibodies around day 7 to day 14 [239]. As T cell help is not required, it is injected without any adjuvant and only the described produced TI generated isotypes IgM and IgG3 (IgG3 which can be produced by CSR with the help of the costimulatory molecules APRIL and BAFF [151]) were analyzed. No difference was observed in the antibody levels specific for TNP-Ficoll upon immunization (Figure 3.12).

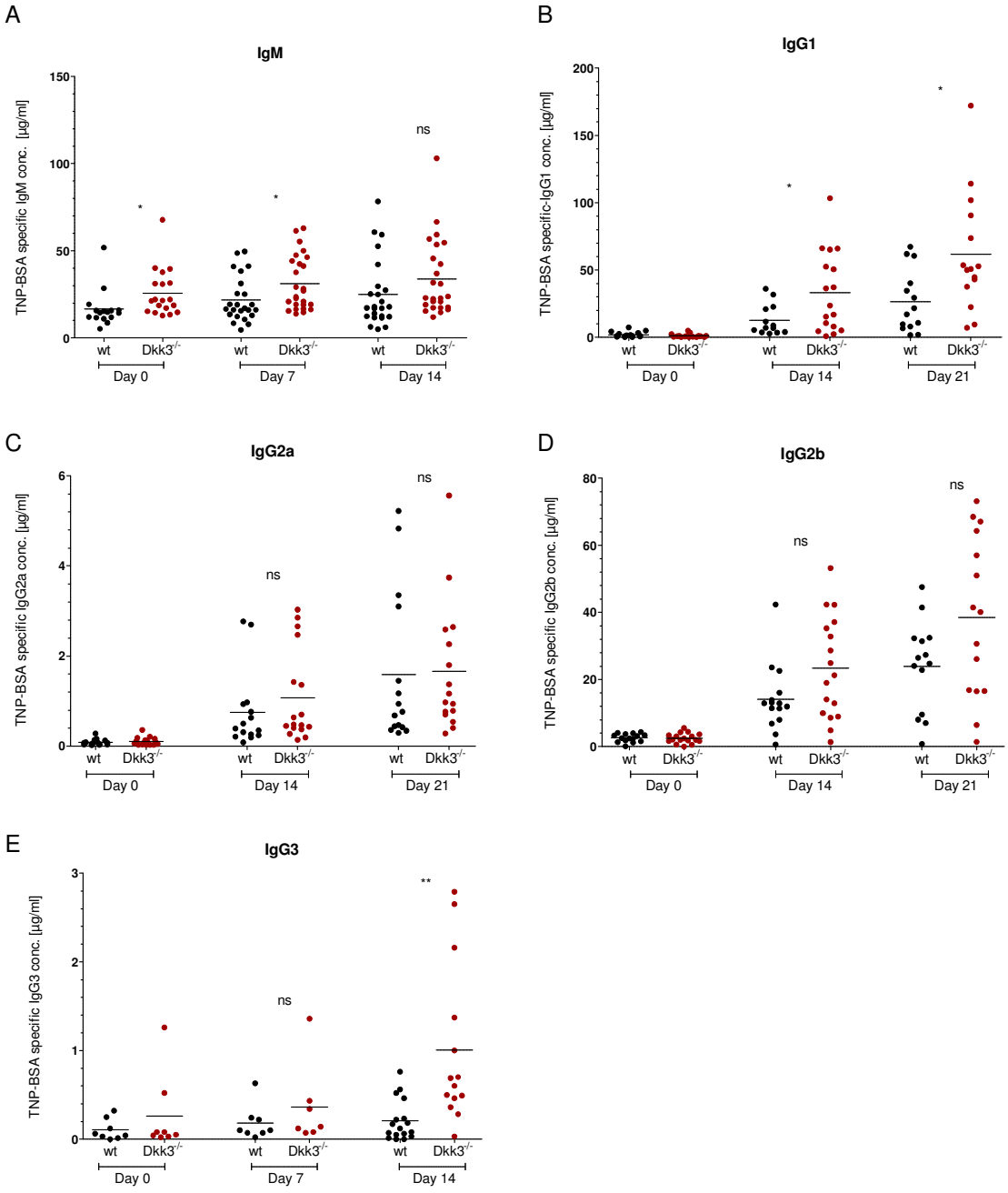


Figure 3.11 | Dkk3$^{-/-}$ mice exhibit an increased antibody response to TNP-BSA. C57BL/6 (wt) and Dkk3$^{-/-}$ mice were immunized s.c. with TNP-BSA in CFA on day 0. Serum was taken on day 0, 7, 14 and 21 and analyzed by ELISA for TNP-BSA specific antibody levels of IgM (A), IgG1 (B), IgG21 (C), IgG2b (D), and IgG3 (E). Data are pooled from two-three independent experiments and one dot represents the antibody levels of one mouse. Horizontal bars represent the mean for each group. Groups were compared using student t-test (ns= not significant; *=p<0,05).

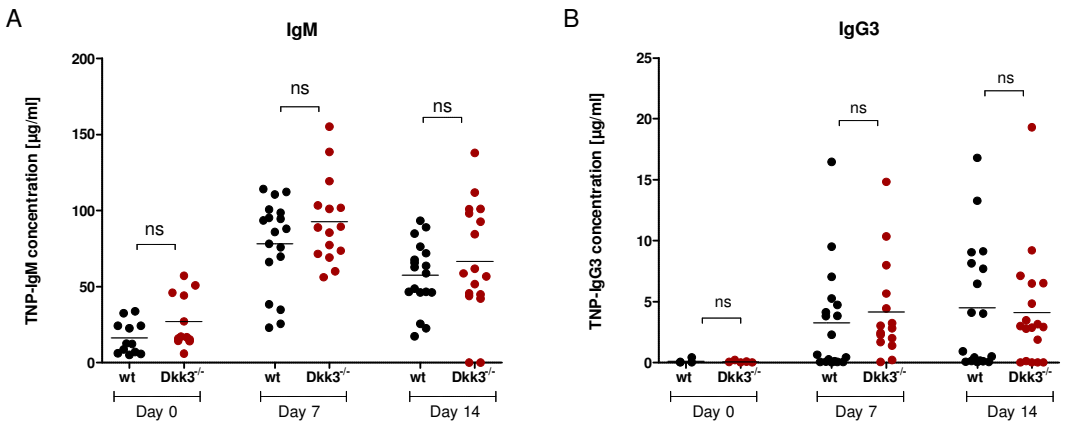


Figure 3.12 | Dkk3$^{-/-}$ mice do not show an altered antibody response to TNP-Ficoll . C57BL/6 (wt) and Dkk3$^{-/-}$ mice were immunized i.p. with TNP-BSA in PBS on day 0. Serum was taken on day 0, 7 and 14 and analyzed by ELISA for antibody levels of IgM (A) and IgG3 (B). Data are pooled from two independent experiments and one dot represents the antibody levels of one mouse. Horizontal bars represent the mean for each group. Groups were compared using student t-test (ns= not significant).

LPS has been shown to polyclonally activate all subclasses of B cells by TLRs leading to an IgM response of various specificities [154, 240]. However, B1 cells bear LPS-specific BCRs and can therefore respond to LPS with the secretion of LPS-specific IgM antibodies [241, 242]. Both total IgM and LPS-specific antibody levels were analyzed after treatment of wt and Dkk3$^{-/-}$ mice with LPS. Total IgM level in the steady state (Day0) was increased in Dkk3$^{-/-}$ mice confirming the observation of Barrantes *et al.* [189] (Figure 3.13 B). Upon immunization, strikingly, only the LPS-specific response was increased in Dkk3$^{-/-}$ mice, whereas total IgM levels were similar in wt and Dkk3$^{-/-}$ mice 7 and 14 days post immunization (Figure 3.13).

To analyze the B1 cell response with another antigen typically used to asses B1 cell responses [132], wt and Dkk3$^{-/-}$ were immunized with phosphorylcholine (PC). Although this antigen is structurally a TI type II antigen, IgG responses have been described [156]. Therefore, immunization of mice with PC is carried out in the presence of adjuvant or directly by using heat-killed PC^{+} bacteria to facilitate CSR to IgG antibodies [138, 156]. In our experiment CFA was used as adjuvant. Dkk3$^{-/-}$ mice exhibited a stronger antibody response to PC compared to wt animals. Both PC-specific IgM and IgG levels were highly significantly increased in serum levels upon immunization (Figure 3.14).

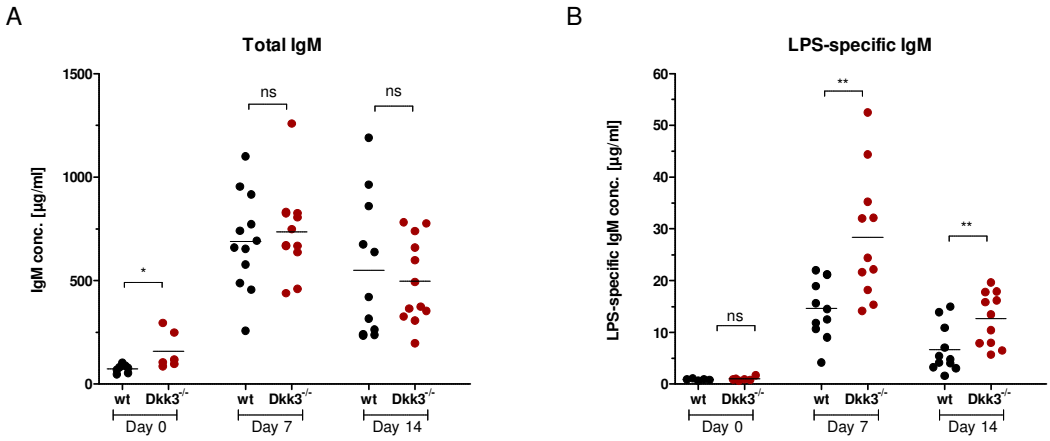


Figure 3.13 | LPS treatment leads to increased levels of LPS-specific IgM antibodies. C57BL/6 (wt) and Dkk3$^{-/-}$ mice were treated i.p. with LPS on day 0. Serum was taken on day 0, 7 and 14 and analyzed by ELISA for total (A) and LPS-specific (B) antibody levels of IgM. Data are pooled from two independent experiments and one dot represents the antibody levels of one mouse. Horizontal bars represent the mean for each group. Groups were compared using student t-test (ns=not significant; **=p<0,005).

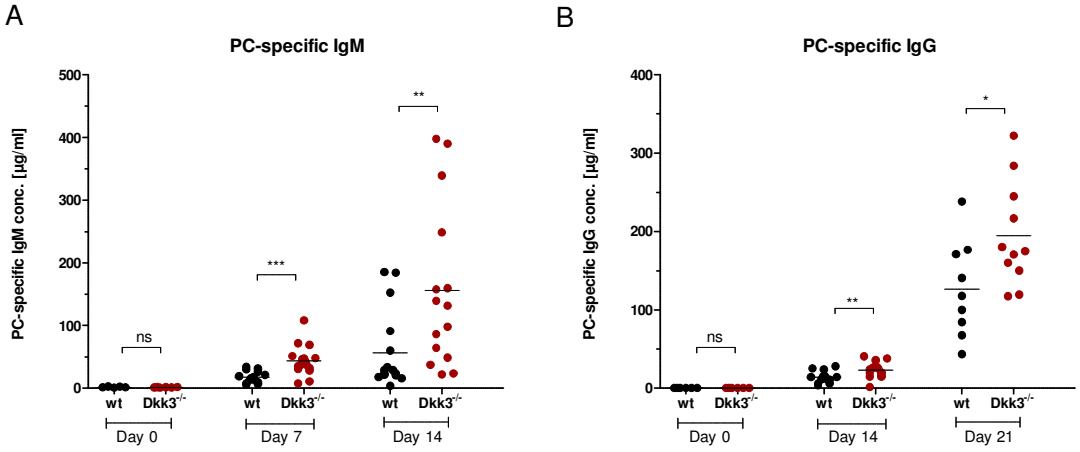


Figure 3.14 | Dkk3$^{-/-}$ mice exhibit an increased antibody response to phosphorylcholine (PC). C57BL/6 (wt) and Dkk3$^{-/-}$ mice were immunized s.c. with phosphorylcholine-KLH (PC-KLH) in CFA on day 0. Serum was taken on day 0, 7, 14 and 21 and analyzed by ELISA for PC-specific antibody levels of IgM (A) and IgG (B). Data are pooled from two independent experiments and one dot represents the antibody levels of one mouse. Horizontal bars represent the mean for each group. Groups were compared using student t-test (ns= not significant; *=p<0,05; **=p<0,005; ***=p<0,0005).

In conclusion, Dkk3 deficient mice display increased antibodies responses. This effect was observed for the FO B cell antigen TNP-BSA, where it was most prominent for the IgG3 response, an antibody subclass often related to B1 cells. The MZ B cell response to the antigen TNP-Ficoll was the only response where no alteration in

Dkk3$^{-/-}$ mice was observed. Both the LPS-specific and the PC antibody response where most significantly increased in Dkk3 deficient mice among all the analyzed antibody responses to specific antigens.

3.2.2 B cells of Dkk3$^{-/-}$ mice display increased IL-10 secretion

Secondly, we investigated whether Dkk3 deficiency leads to altered cytokine secretion by B cells. To screen for cytokines, which could be altered in the absence of Dkk3, initially, splenocytes were stimulated *in vitro* with five different stimulation conditions; anti-CD40, LPS, CpG, anti-CD40 together with LPS, and anti-CD40 together with CpG. Supernatants were quantitatively analyzed after 24 hours for 5 different cytokines involved in inflammatory responses and which have been shown to be produced by B cells [243-245]: TNF-α, IL-6, MCP-1, IL-10 and IFN-γ. IL-6 secreted by splenocytes cultures was recently reported to be mainly B cell derived [246]. No difference was found for TNF-α, IL-6 and IFN-γ (Figure 3.15 A, B, E). MCP-1, also called CCL2, was increased already in the steady state in Dkk3$^{-/-}$ mice and its secretion did not change significantly upon *in vitro* stimulation (Figure 3.15 C). IL-10 was significantly increased in supernatants of Dkk3$^{-/-}$ splenocytes upon stimulation with anti-CD40 together with LPS and CpG (Figure 3.15 D). The analysis was repeated twice with CD19^{+} purified splenocytes and results confirmed that indeed B cells of Dkk3$^{-/-}$ mice secreted higher amounts of IL-10, whereas for the other cytokines no difference was found (data not shown as detailed analysis below). The only difference which appeared using purified B cells was that MCP-1 was not detected, demonstrating that this cytokine is not expressed by B cells, and the differences observed in the assay when using splenocytes must be due to another splenic cell population besides B cells.

To analyze whether the difference between wt and Dkk3$^{-/-}$ B cells in IL-10 secretion is dose-dependent, CD19^{+} B cells were purified and stimulated with different concentrations of LPS together with anti-CD40 and CpG. Additionally, peritoneal cavity (PerC) B cells were included in the assay, as it was described in the literature, that this compartment possesses the highest numbers of IL-10 secreting B cells within the body [174]. Analysis of IL-10 concentration in supernatants of splenic B cells showed that B cells from Dkk3$^{-/-}$ mice secrete increased levels of IL-10, when stimulated with sufficient amounts of LPS or CpG (Figure 3.16 A/B). B cells purified from the PerC, however, showed that IL-10 secretion is already increased in Dkk3$^{-/-}$ B cells in the steady state without any stimulation (Figure 3.16 C). Upon stimulation, PerC B cells secreted 10-fold higher levels of IL-10 compared to splenic B cells and the difference between wt and Dkk3$^{-/-}$ group was more pronounced in the PerC compartment.

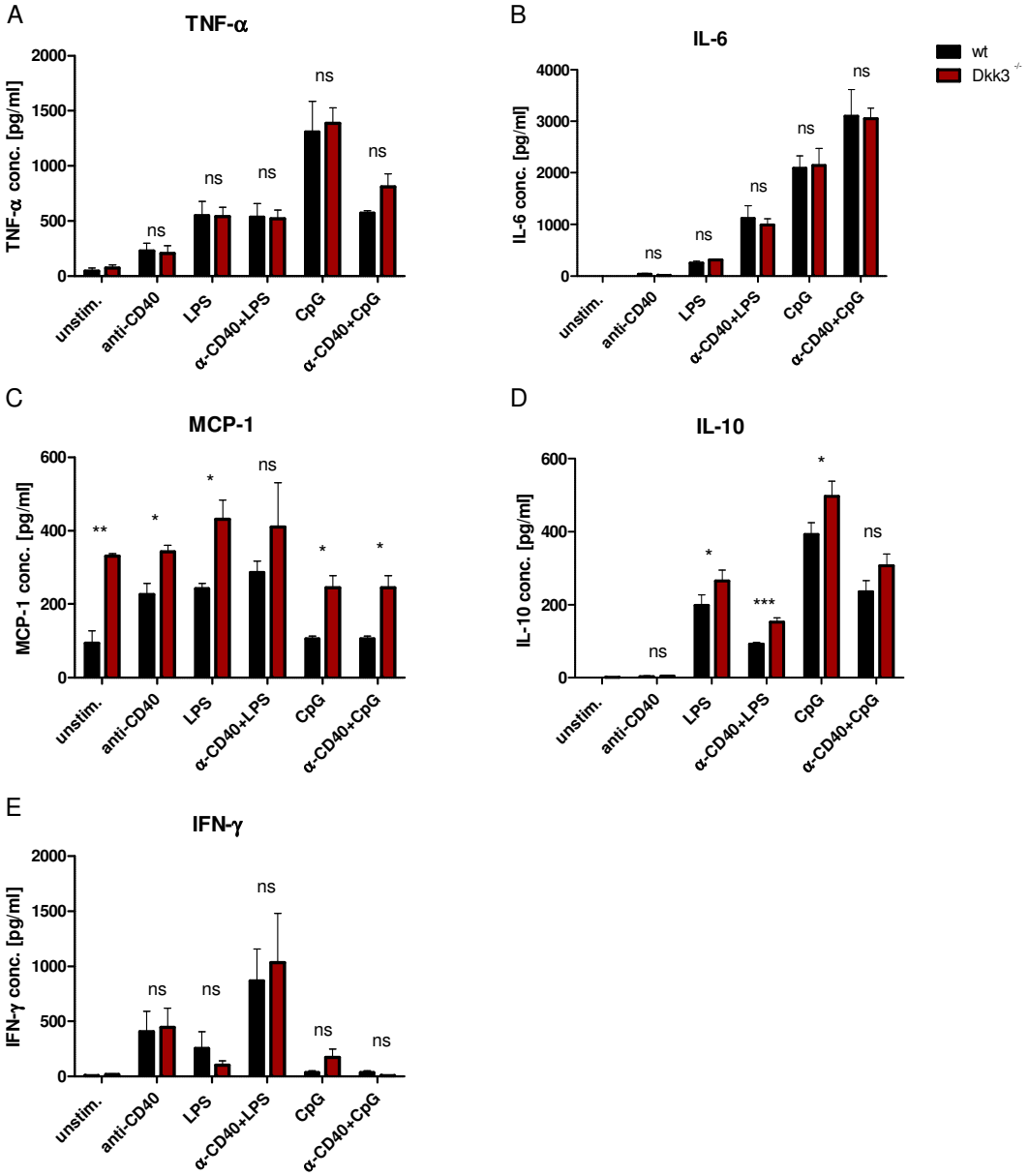


Figure 3.15 | Splenocytes of Dkk3$^{-/-}$ mice secrete increased amounts of MCP-1 and IL-10. Splenocytes of C57BL/6 (wt) and Dkk3$^{-/-}$ mice were cultures with 10µg/ml anti-CD40, 5µg/ml LPS, 2µg/ml CpG or a combination of anti-CD40 with LPS or CpG for 24 hours. Supernatants were analyzed by cytometric bead array (CBA) for TNF-α (A), IL-6 (B), MCP-1 (C), IL-10 (D), IFN-γ (E), and IL-12 (not detected). Results show pooled data from 2 independent experiments out of three experiments (n=8). Mean and SEM are indicated for each group. Groups were compared using student t-test (ns= not significant; *=p<0,05; **=p<0,005; ***=p<0,0005).

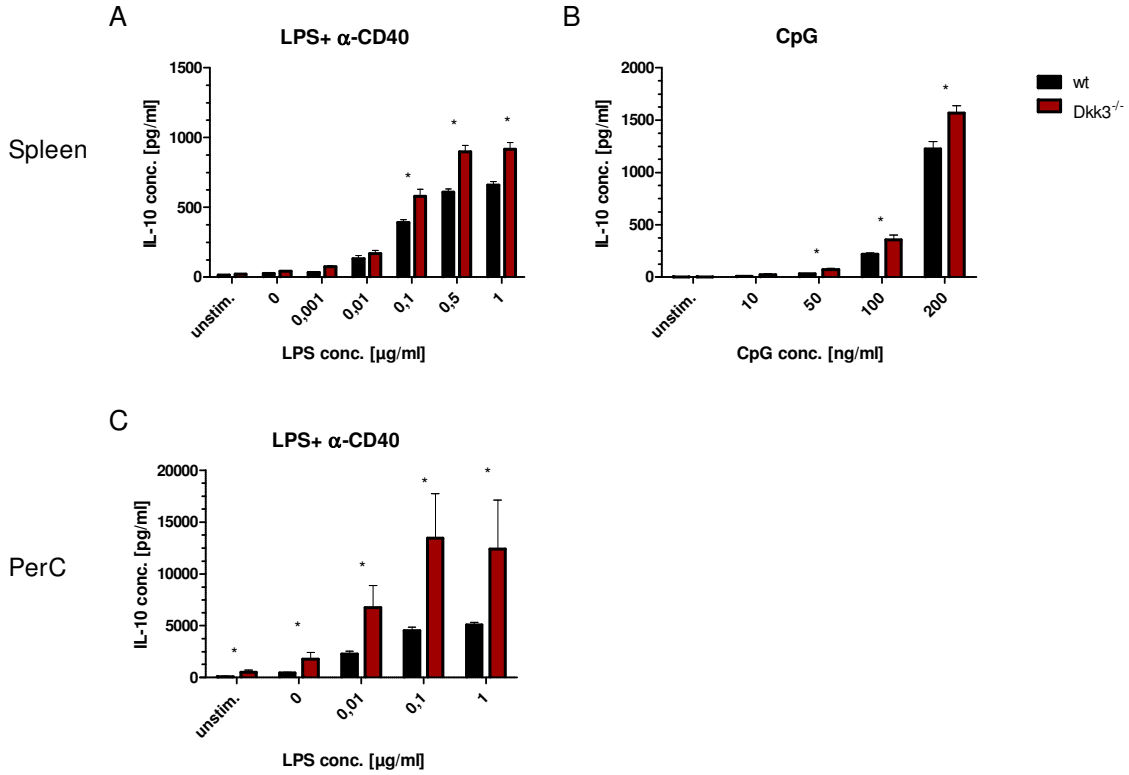


Figure 3.16 | B cells of spleen and peritoneal cavity secrete increased levels of IL-10. $CD19^+$ purified B cells from spleens (A+B) or peritoneal cavity (PerC/C) of C57BL/6 (wt) and $Dkk3^{-/-}$ mice were cultured with different concentrations of LPS together with 10µg/ml anti-CD40 (A+C) or CpG (B) for 24 hours. IL-10 concentration in supernatants was determined by ELISA. Results show one out of 2 independent experiments (n=4). Mean and SEM are indicated for each group. Groups were compared using student t-test (ns= not significant; *p<0,05).

3.2.3 A higher proportion of B1 cells secretes IL-10 in $Dkk3^{-/-}$ mice

As higher levels of IL-10 were observed in supernatants of cultured PerC B cells, and as the PerC is the compartment where B1 cell numbers are very high in comparison to other areas of the body, we addressed the question whether the increased levels of IL-10, observed in $Dkk3^{-/-}$ B cell cultures, are due to higher numbers of IL-10 secreting B1 cells, or due to a general IL-10 overproduction by B cells of $Dkk3^{-/-}$ mice. For this purpose, IL-10 secretion was analyzed 6 hours upon stimulation with LPS *in vitro* by intracellular FACS analysis enabling us to tract IL-10 secretion on a single cell level.

Upon stimulation, $B220^{low}$ $CD19^+$ B1 cells showed a drastic upregulation of IL-10, whereas only 1-2 % of $B220^{high}$ $CD19^+$ B2 cells upregulated IL-10 (Figure 3.17). In the PerC this effect was even clearer visible as this compartment yields high numbers of B1 cells. Splenic and PerC B1 cells coming from $Dkk3^{-/-}$ mice exhibited higher numbers of IL-10 secreting cells compared to wt mice (Figure 3.17 C+D). These results demonstrate that the difference observed in IL-10 secretion between B cells

from wt and Dkk3$^{-/-}$ mice is not due to a general IL-10 overproduction of the B cell compartment, but rather a specific property of Dkk3$^{-/-}$ B1 cells. However, the higher proportion of IL-10 secreting cells within the B1 cell subset does not seem the only reason for increased IL-10 levels. Additionally, it appeared that total B1 cell numbers are increased in Dkk3$^{-/-}$ animals, which would contribute to the observed effect. This lead us to a detailed analysis of B cell subsets, described in the next chapter.

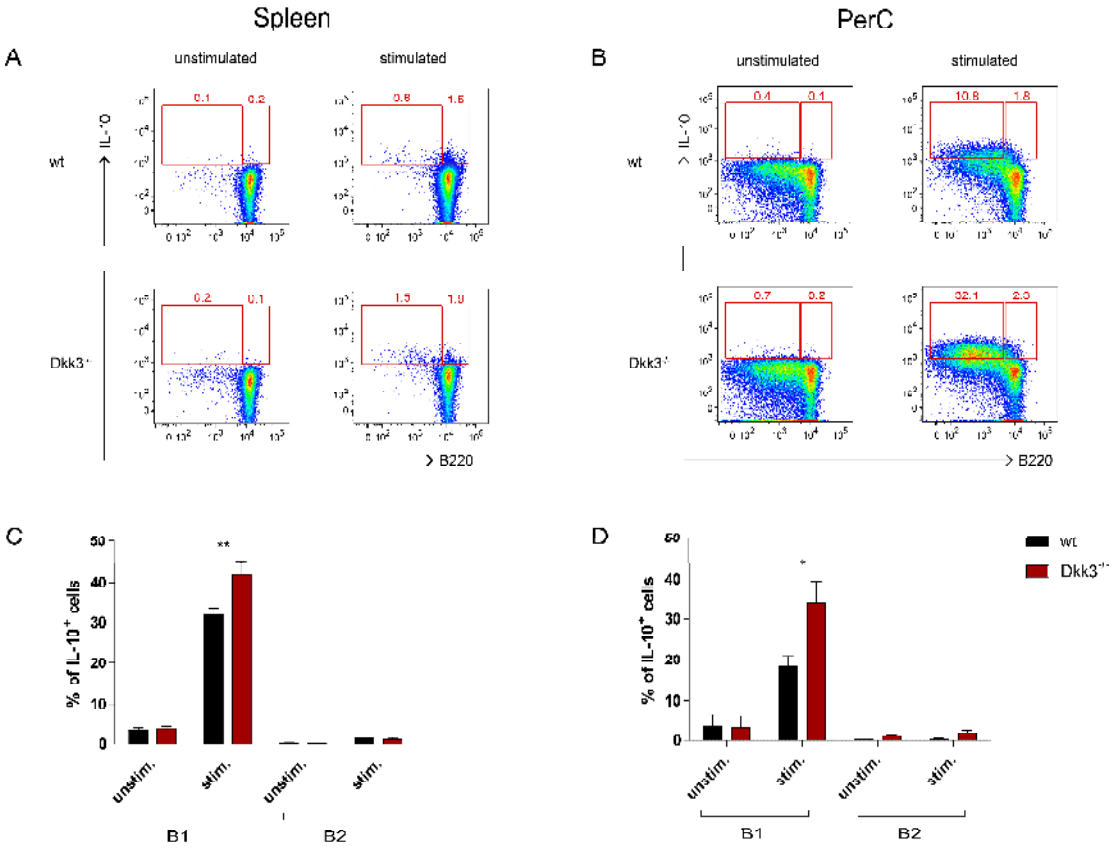


Figure 3.17 | A higher proportion of B1 cells secretes IL-10 in Dkk3$^{-/-}$ mice. Splenocytes and peritoneal cavity (PerC) cells were isolated and stimulated with LPS, ionomycin and PMA in the presence of monesin for 6 hours. Cells were stained for cell surface markers and intracellular IL-10. Dot blots show gated CD19^{+} lymphocytes from the spleen (A) and the peritoneal cavity (B) comparing unstimulated with stimulated and C57BL/6 (wt) with Dkk3$^{-/-}$ cells. B1 cells express lower levels of B220 and can be distinguished from B2 cells via this molecule. Lower panels show summary data of the frequency of unstimulated (unstim.) and stimulated (stim.) IL-10 secreting B cells within the pregated B1 or B2 population of spleen (C) and PerC (D) cells. Results show one out of 2 independent experiments (n=3). Mean and SEM are indicated for each group. Groups were compared using student t-test (ns= not significant; *p<0,05; **p<0,005).

3.2.4 Dkk3$^{-/-}$ exhibit higher numbers of B1 cells and lower numbers of B2 cells

As described in the introduction, two major B cell populations can be distinguished, B1 and B2 cells. To investigate whether the numbers of these populations are altered in the absence of Dkk3, different peripheral lymphoid tissues as well as cells of the PerC were analyzed for their B cell subpopulations in wt and Dkk3$^{-/-}$ mice by FACS. The first noticeable observation was that the frequency of total B cell within the leukocyte population was reduced in Dkk3$^{-/-}$ mice in the spleen, the MLN and the PerC (Figure 3.18, left FACS dot blots, and numbers on the top). This was due to reduced numbers of B2 cells (Figure 3.18). In contrast the frequency of B1 cells within the leukocyte population (and B cell population) was increased in the spleen, the LN and the PerC of Dkk3$^{-/-}$ mice (Figure 3.18). In all analyzed locations the ratio of B1:B1 was shifted towards B1 cells in Dkk3$^{-/-}$ mice.

B1 cells present only a minor population in the lymphatic organs. Therefore, the absolute number of B1 cells was determined. This would clarify whether the increased frequency of B1 cells in Dkk3$^{-/-}$ deficient mice is due to decreased levels of B2 cells, or whether this population is indeed present at higher numbers.
The absolute number of B1 cells was increased in the same locations in which higher numbers of B1 cells were found by frequency analysis: the spleen, LN and PerC (Figure 3.19). In the MLN, no difference in B1 cell numbers was found.

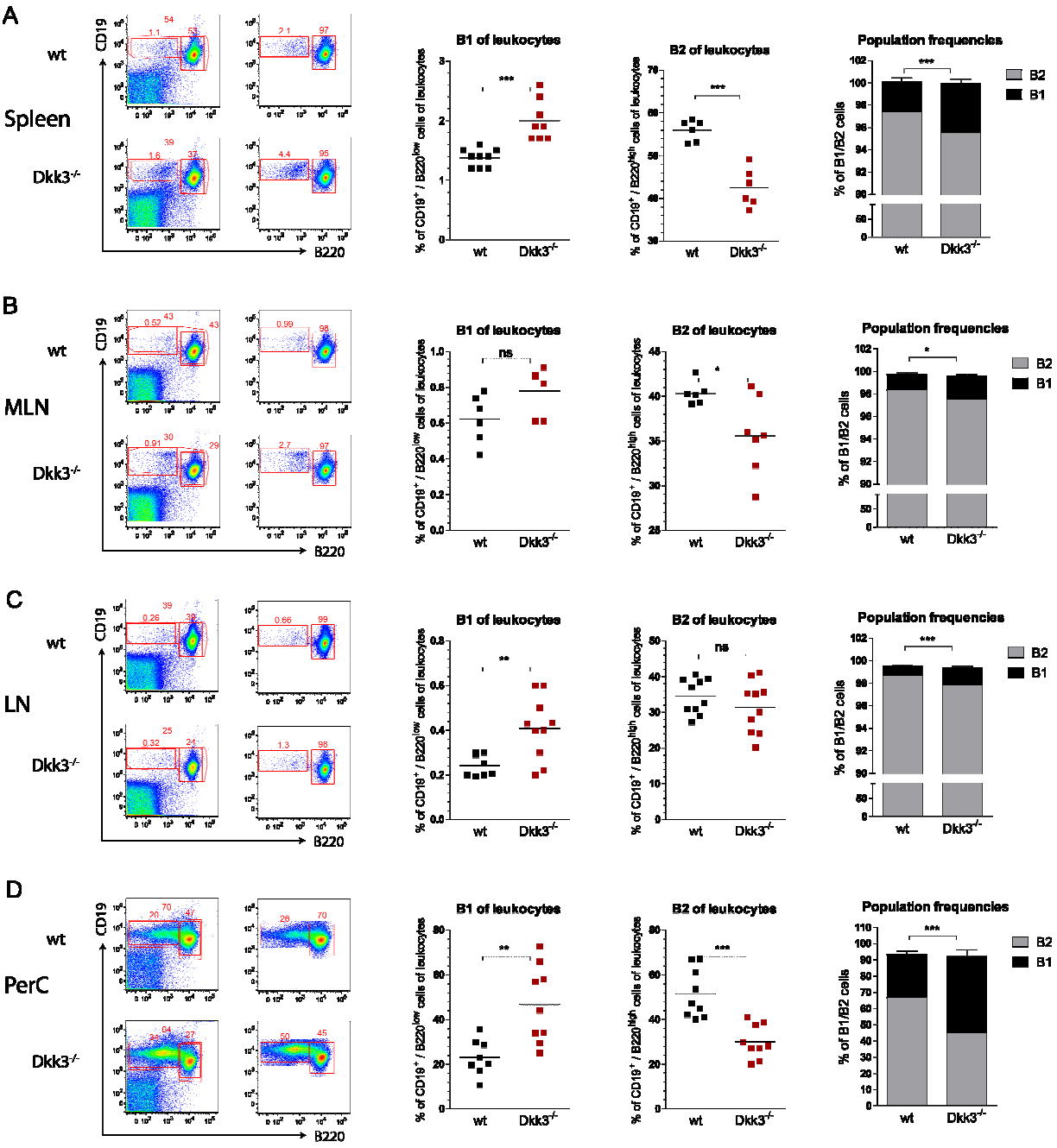


Figure 3.18 | The ratio of B2:B1 cells is shifted towards B1 cells in Dkk3$^{-/-}$ mice. Cells were isolated from spleen (A), mesenteric lymph nodes (MLN/B), lymph nodes (LN/C) and peritoneal cavity (PerC/D) of C57BL/6 (wt) and Dkk3$^{-/-}$ mice and analyzed by FACS for their B1/B2 phenotype via B220 and CD19 surface markers. Dot plots on the left show frequencies of total B cells (number on top), B1 cells (B220^{low}) and B2 cells (B220^{high}) within the leukocyte populations which was gated. Dot plots on the right are gated on all B cells and show percentage of B1 and B2 cells within the B cell population. The panels in the middle are summary data of the FACS dot blots on the left and show numbers of B1 and B2 cells within the leukocyte populations. One dot represents cell numbers of one mouse. Horizontal bars represent the mean for each group. The panels on the right are summary data of the FACS dot blots on the right and show the ration of B2:B1 within the B cell population (n=9). Mean and SEM are indicated for each group. Groups were compared using student t-test (ns= not significant; *=p<0,05; **=p<0,005; ***=p<0,0005).

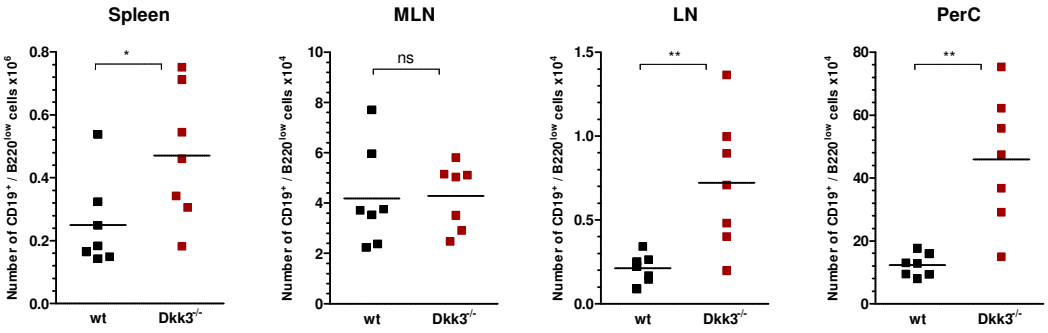


Figure 3.19 | Dkk3 deficiency leads to increased absolute numbers of B1 cells. Cells were isolated from spleen, mesenteric lymph nodes (MLN), lymph nodes (LN) and peritoneal cavity (PerC) of C57BL/6 (wt) and Dkk3$^{-/-}$ mice and analyzed by FACS for their B1 phenotype via B220 and CD19 surface markers. Absolute numbers of B1 cells per organ/site were determined using Sphero Blank Calibration beads during sample acquisition. Mean and SEM are indicated for each group. Groups were compared using student t-test (ns= not significant; *=p<0,05; **=p<0,005; ***=p<0,0005).

3.2.5 The reduction of B2 cells in Dkk3$^{-/-}$ mice is due to decreased numbers of follicular B cells

B2 cells can be further subdivided into FO and MZ B cells. As the name tells, MZ B cells are primarily found in the marginal zone of the spleen. Therefore, to analyze whether the reduction in B2 cell numbers in Dkk3$^{-/-}$ mice is due to decreased levels of FO or MZ B cells, or both, the proportion of these populations was determined within the leukocyte population of splenic cells. FO and MZ B cells were distinguished by the surface markers CD21/CD35 and CD23. Clearly, the reduction in B2 levels was due to decreased numbers of FO B cells (Figure 3.20). MZ B cells were present at similar numbers in wt and Dkk3$^{-/-}$ mice.

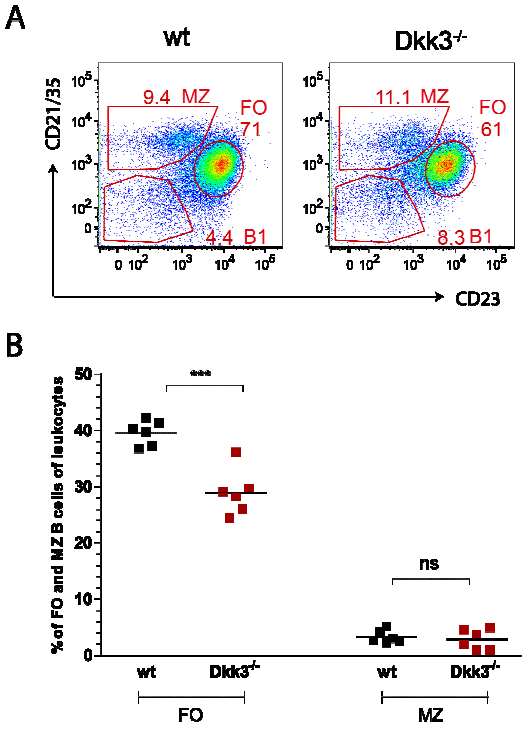


Figure 3.20 | Dkk3$^{-/-}$ mice exhibit reduced levels of follicular B cells. Cells were isolated from spleen of C57BL/6 (wt) and Dkk3$^{-/-}$ mice and analyzed by FACS for their FO and MZ phenotype. The identification of FO, MZ and B1 cells by the surface markers CD21 and CD21/CD35 is shown in A for cells of wt and Dkk3$^{-/-}$ mice. FACS dot plots were gated on CD19^{+} lymphocytes. Summary data of two independent experiments are presented in B showing resulting percentages of FO and MZ B cells which were back calculated to frequencies of leukocytes. One dot represents cell numbers of one mouse. Horizontal bars represent the mean for each group. Groups were compared using student t-test (ns= not significant; ***=p<0,0005).

3.2.6 Both B1a and B1b cell are increased in Dkk3$^{-/-}$ mice

B1 cells are further subdivided into B1a and B1b cells. To analyze whether the increased B1 cell numbers are due to an augmentation in one of these two subsets, levels of B1a and B1b cells from different locations in wt and Dkk3$^{-/-}$ mice were compared. In this analysis B1 cells were identified by using several markers (CD19^{+} B220^{low}, CD21/CD35^{-}, CD23^{-}, IgM^{+}, CD43^{+}), this way combining different gating strategies described in the literature [132, 133], followed by the discrimination of B1a and B1b cells via the expression of CD5 (Figure 3.21 A). Numbers of B1a and B1b cells were calculated as percentages of total B cells. Results demonstrated that both B1a and B1b cells were increased in the spleen, MLN, LN and PerC of Dkk3$^{-/-}$ mice (Figure 3.21).

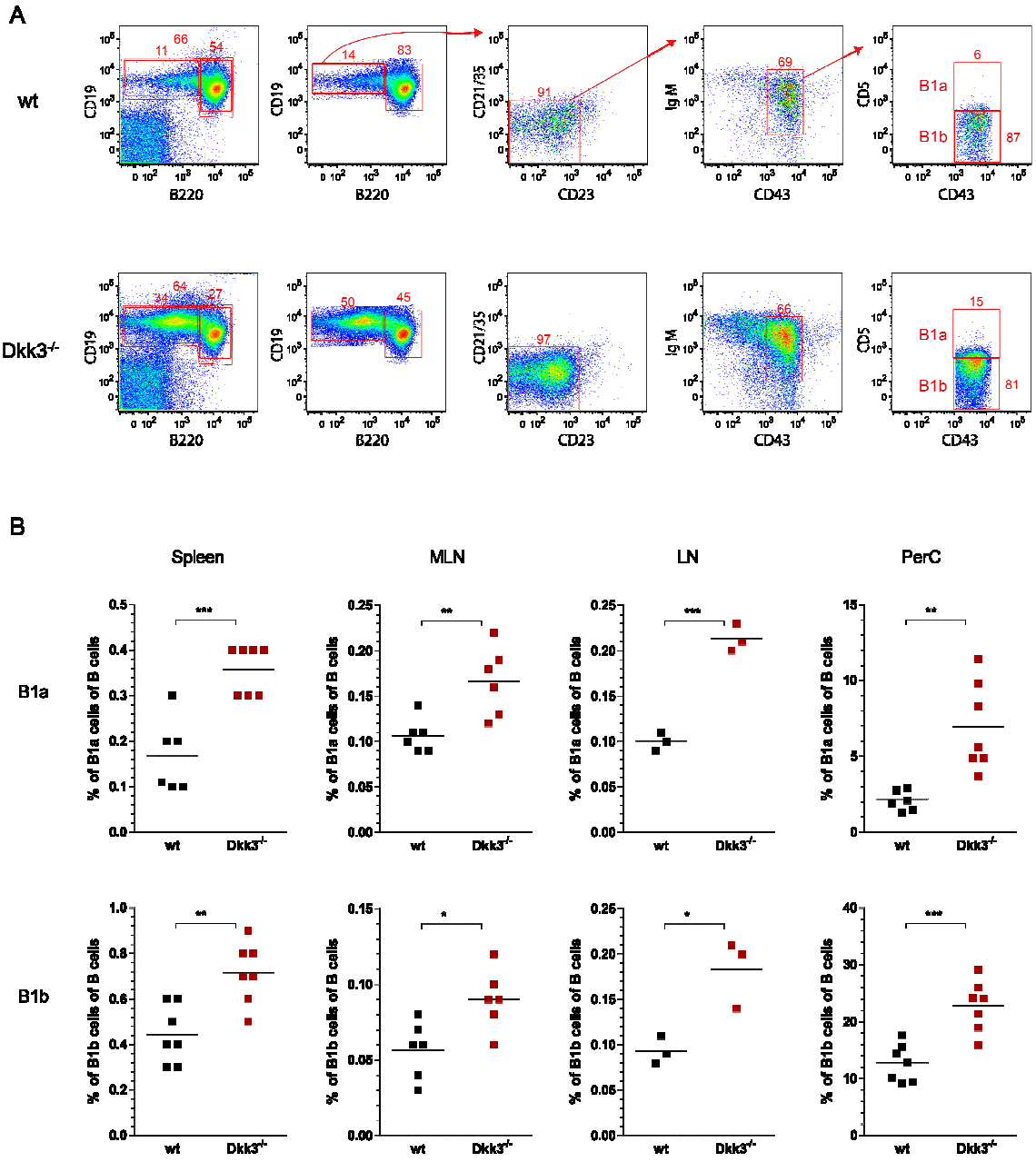


Figure 3.21 | B1a and B1b cells are increased in Dkk3$^{-/-}$ mice. Cells were isolated from spleen, mesenteric lymph nodes (MLN), lymph nodes (LN) and peritoneal cavity (PerC) of C57BL/6 (wt) and Dkk3$^{-/-}$ mice and analyzed by FACS for their B1a/B1b phenotype. The gating strategy is shown in A as an example for wt (top) and Dkk3$^{-/-}$ mice (below) PerC cells. First FACS dot blot (left) is gated on leukocytes. Summary data of two independent experiments are presented in B. One dot represents cell numbers of one mouse. Horizontal bars represent the mean for each group. Groups were compared using student t-test (ns= not significant; *=p<0,05; **=p<0,005; ***=p<0,0005).

3.2.7 B cell development in neonatal Dkk3$^{-/-}$ mice is impaired

B1 cell development takes mainly place in the fetus and in the neonate. There, the splanchopleura and the liver have been described to posses B1 precursor cells [140, 141]. However, final steps of B1 cell development occur in the neonatal spleen [139]. In the spleen immature (in peripheral organs also called transitional and mature B cells can be distinguished in general by the expression of CD93 (AA4.1) and the substages of transitional cells can be identified by different CD23 expression levels (Figure 3.22 A) [4]. Unfortunately, no marker has been found so far to distinguish B1 and B2 transitional cells. Therefore, we can only talk about transitional cells in general without knowing to which cells they would differentiate in the process of maturation. To solve this problem, Montecino-Rodriguez *et al.* suggested that PerC cells could indicate which transitional cells (B1 or B2) are present in the spleen, as this compartment exhibits the matured B cells immigrated from the spleen [247]. Therefore, we decided to look at the distribution of transitional and mature B cells in neonatal spleen and PerC of wt and Dkk3$^{-/-}$ mice.

Surprisingly, similar numbers of CD93^{+} transitional B cells were found in the PerC and the spleen of 5 days old neonatal mice (Figure 3.22 B), thus the analysis of the PerC was not able to tell us which cell fate will transitional B cells from the spleen take. However, already at this early time of B cell development total B cell numbers were drastically decreased in Dkk3$^{-/-}$ animals, which was due to decreased numbers of transitional B cells (Figure 3.22). In contrast to adult mice, at this stage of B cell development no difference in FO B cell numbers was found. B1 cells were increased in the spleen similar to adults. Interestingly, besides B1 cells, MZ B cells were also increased in neonatal mice, which could be due to the similar selection process of MZ B cells to B1 B cells.

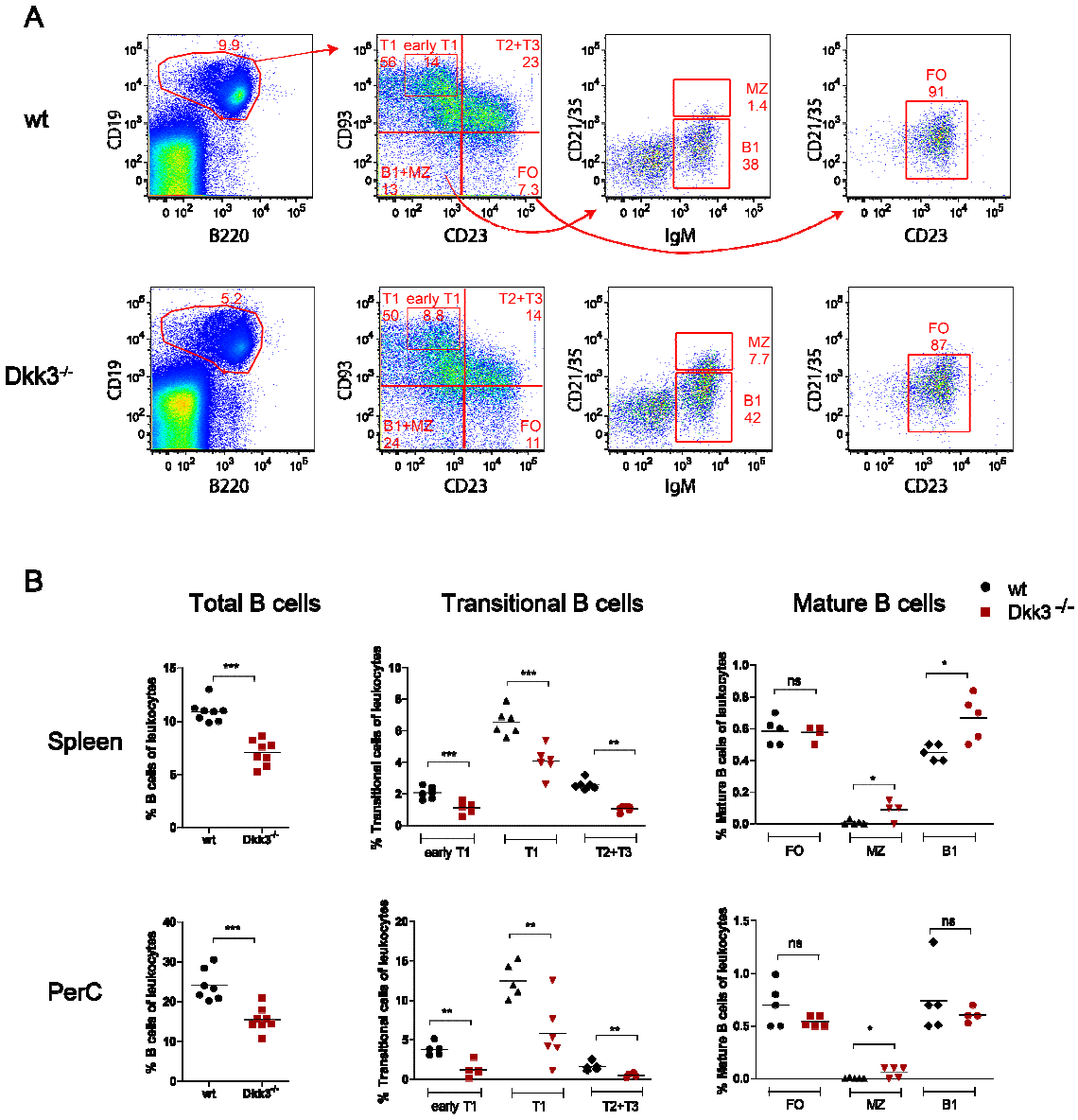


Figure 3.22 | B cell development in neonatal Dkk3$^{-/-}$ mice is impaired. Cells were isolated from spleen and peritoneal cavity (PerC) of C57BL/6 (wt) and Dkk3$^{-/-}$ 5 days old neonatal mice and analyzed by FACS for their B cell phenotype. The gating strategy is shown in A as an example for wt (top) and Dkk3$^{-/-}$ mice (below) splenic cells. First FACS dot blot is gated on leukocytes. Summary data from two out of three independent experiments are shown in B. One dot represents cell numbers of one mouse. Horizontal bars represent the mean for each group. Groups were compared using student t-test (ns= not significant; *=p<0,05; **p<0,005; ***=p<0,0005). Abbreviations: T=transitional B cells; FO= follicular B cells, MZ= marginal zone B cells.

3.2.8 B cell development in adult bone marrow is impaired in Dkk3$^{-/-}$ mice

Analysis of neonatal spleen only allows the investigation of the late steps of B cell maturation. Although adult BM has been shown to poorly generate B1 cells [141, 142], it is still generating the pool of B2 cells and also in this population a developmental alteration was observed. In order to investigate full B cells development with all B cell developmental stages, BM of wt and Dkk3$^{-/-}$ mice was analyzed.

In contrast to peripheral organs in adult and neonatal Dkk3$^{-/-}$ mice, total B cell numbers were not impaired in adult bone marrow of Dkk3$^{-/-}$ mice (Figure 3.23). The first B cell stages, which are the prepro- and the pro- B cells (B220^{+} CD93^{+} CD25^{-} c-kit^{+} CD43^{+} cells), were increased in Dkk3$^{-/-}$ BM. Pre- B cell numbers (B220^{+} CD93^{+} CD25^{+} cells) were unchanged. Similar to the analysis in neonatal mice, numbers of immature B cells (B220^{+} CD93^{+} CD25^{-} c-kit^{-} CD43^{-} IgM^{+} cells) were decreased, whereas numbers of mature FO B cells (B220^{+} CD93^{-} CD21/CD35^{+} CD23^{+} cells) were unaltered in the BM of Dkk3 deficient mice in contrast to the periphery (which will be discussed in chapter 4.5 Dkk3 regulates B cell development and maintenance). These results demonstrate that B cell development at the step from the pre-B cells to the immature B cells seems to be impaired in Dkk3$^{-/-}$ mice. B cells which do not continue the maturation from the pre-B cell stage might accumulate to a low level already in the prepro/pro stage (perhaps by a feedback mechanism) or disappear by apoptosis. B1 cells (B220^{+} CD93^{-} CD21/CD35^{-} CD23^{-} cells) were also in the adult BM observed to be increased in Dkk3$^{-/-}$ mice, which could be due to an increased generation of B1 cells in the BM but also due to migration from peripheral tissues to the BM, in which a part of mature B cells rests. Why total B cell numbers did not show an alteration in Dkk3$^{-/-}$ and wt mice similar to the neonatal mice can be explained by the low representation (~5-10%) of immature B cells in adult BM and the additional compensation by B1 and prepro/pro B cells.

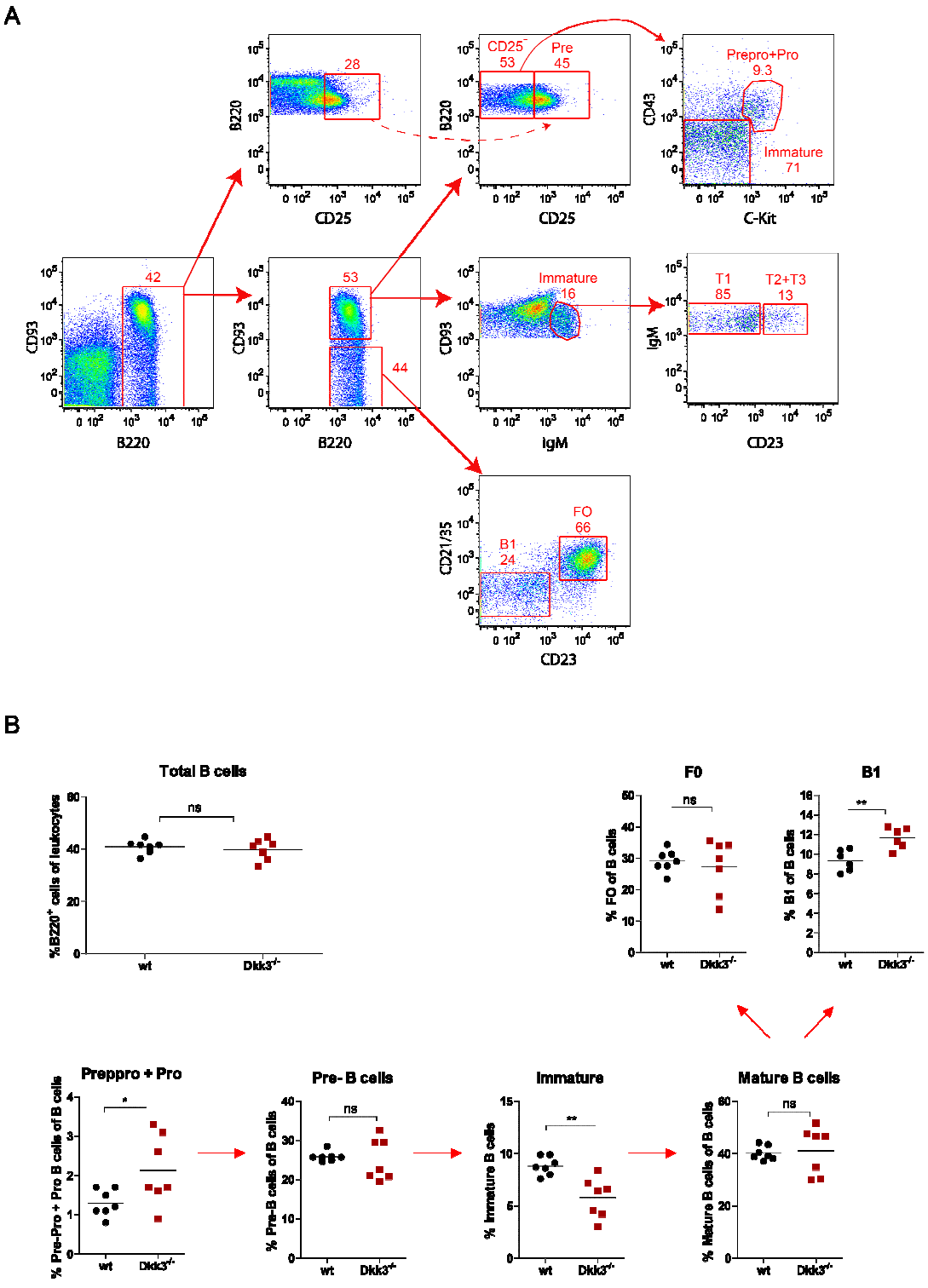


Figure 3.23 | B cell development in adult bone marrow of Dkk3$^{-/-}$ mice is impaired. Cells from bone marrow of adult C57BL/6 (wt) and Dkk3$^{-/-}$ mice were isolated and analyzed by FACS for their developmental B cell stage. The gating strategy is shown in A. First FACS dot blot is gated on leukocytes. Dashed line indicated where gates were dragged to subgates in order to determine gate borders. In B summary data from two out of three independent experiments are presented and one dot represents cell numbers of one mouse. Horizontal bars represent the mean for each group. Groups were compared using student t-test (ns= not significant; *=p<0,05; **=p<0,005).

3.2.9 The self-renewal capacity of B1 cells is increased in Dkk3$^{-/-}$ mice

B1 cell numbers in adults are mainly maintained by self-renewal. Therefore, there are two possibilities why increased B1 cell number appear in Dkk3$^{-/-}$ mice. Either B1 cells possess a selection advantage during the development in Dkk3$^{-/-}$ mice or they receive increased survival signals and therefore have an increased self-renewal capacity.

As the first hypothesis is presently not testable because immature (transitional) B1 cells can not be distinguished from immature B2 cells, but this is the cell stage which would indicate an altered developmental process, we decided to test whether the self-renewal capacity of B1 cells is increased. CD19^{+} B cells of spleens from adult mice were purified (Figure 3.24 A) and transferred to either Dkk3$^{-/-}$Rag2$^{-/-}$ or wt.Rag2$^{-/-}$ mice. To additionally answer the question whether environmental or intrinsic Dkk3 leads to the observed effect the experiment was performed with donor cells from wt and Dkk3$^{-/-}$ mice.

The adult spleen contains mainly mature B cells, therefore *de novo* generation of B cells can not take place and the cell numbers after a longer time of incubation reflect the self-renewal/survival capacity of B cells. We decided to give the transferred cells 4 weeks to expand and maintain themselves by self-renewal before being analyzed.

After 4 weeks higher numbers of B cells were detected in recipient spleen and PerC in the groups of either B cells transferred to a Dkk3 deficient environment or cells coming from Dkk3$^{-/-}$ deficient mice (Figure 3.24 B). This was due to increased B1 cell numbers in spleen and PerC (Figure 3.24 C). B1 cell numbers exceeded 5 times the numbers of B2 cells, even in spleens, where normally B1 cells are only a minor population within the B cell subsets, confirming that B1 cells have a higher capacity of self-renewal/survival than B2 cells.

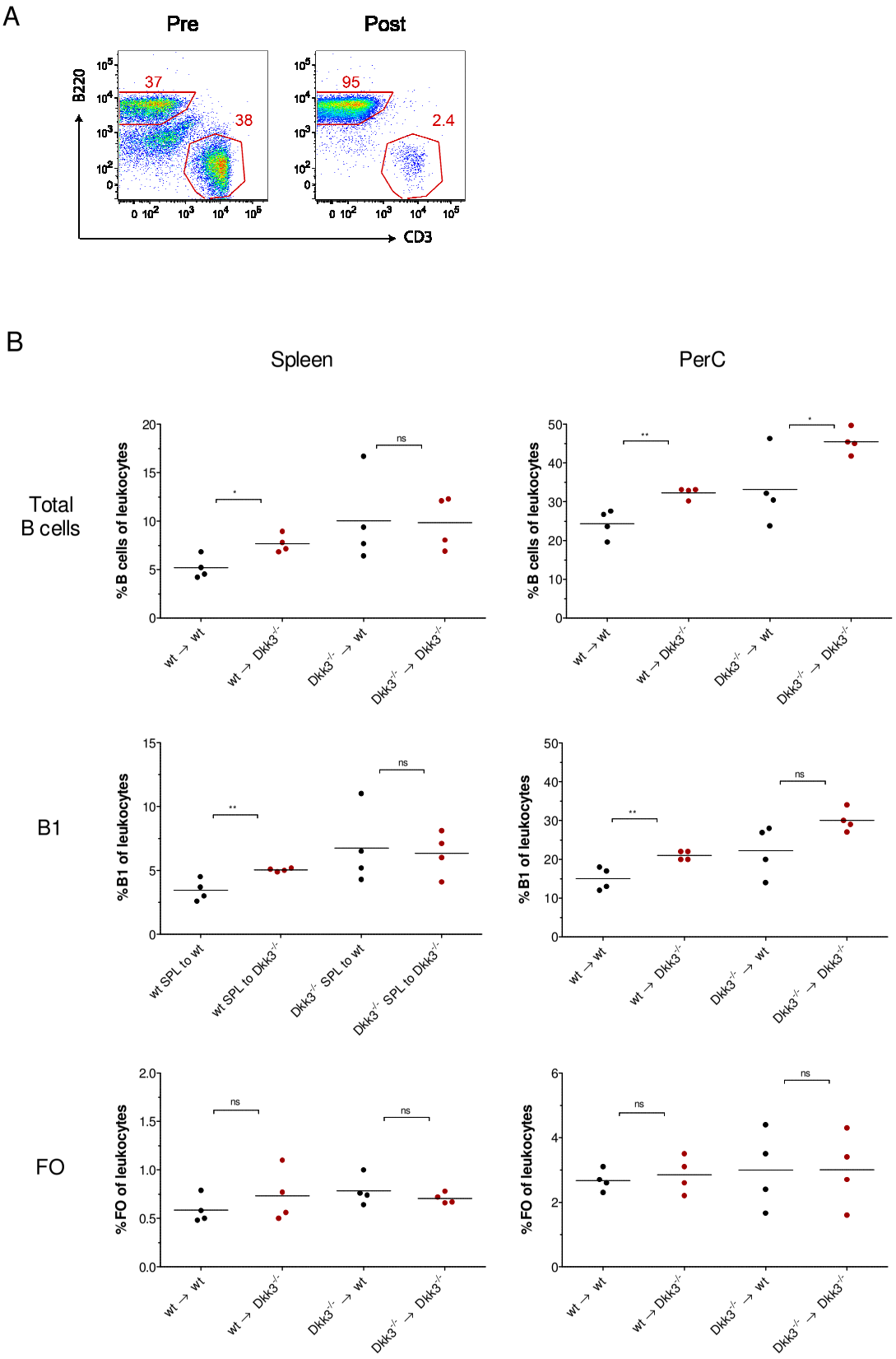
FO cell numbers were also determined in this experiment, to analyze whether decreased numbers observed in the periphery of adult Dkk3$^{-/-}$ mice are due to a lower self-renal/survival rate or due to impaired B cell development (as indicated by the experiment described in the last chapter). No difference between the four groups was found in FO cells numbers (Figure 3.24 D). This demonstrates that the reduction of FO B cells in the absence of Dkk3 is due to a developmental impairment and not due to differences in self-renewal/survival.

Furthermore, serum IgM levels were analyzed 4 weeks after cell transfer. As B1 cells are the major producer of natural IgM in serum and this has also been demonstrated for immunodeficient mice in which B cells were transferred [156], this function can be used as readout system for B1 cell numbers and their IgM secreting activity. Indeed,

total IgM levels in serum of the four groups mirrored B1 cell numbers detected by FACS (Figure 3.24 C). Cells transferred to a Dkk3 deficient environment generated higher IgM levels, than the cells transferred to a Dkk3 sufficient environment. However, cells of a $Dkk3^{-/-}$ origin generated even higher IgM levels in serum in comparison to wt control cells, similar to the increased B1 cell numbers detected by FACS.

These observations were additionally confirmed by *ex vivo* IL-10 secretion analysis of splenocytes (Figure 3.24 D), as IL-10 was shown to be selectively expressed by B1 cells (3.2.3 A higher proportion of B1 cells secretes IL-10 in $Dkk3^{-/-}$ mice).

Thus, the results of all three readout-systems suggest that the increased numbers of B1 cells found in $Dkk3^{-/-}$ mice are due to increased self-renewal capacity of B1 cells. How environmental and intrinsic Dkk3 contributes to this effect is unclear.


A
Pre
Post
B220
CD3
37
38
95
2.4
B
Spleen
PerC
Total B cells
B1
FO
% B cells of leukocytes
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% FO of leukocytes
wt → wt
wt → Dkk3-/-
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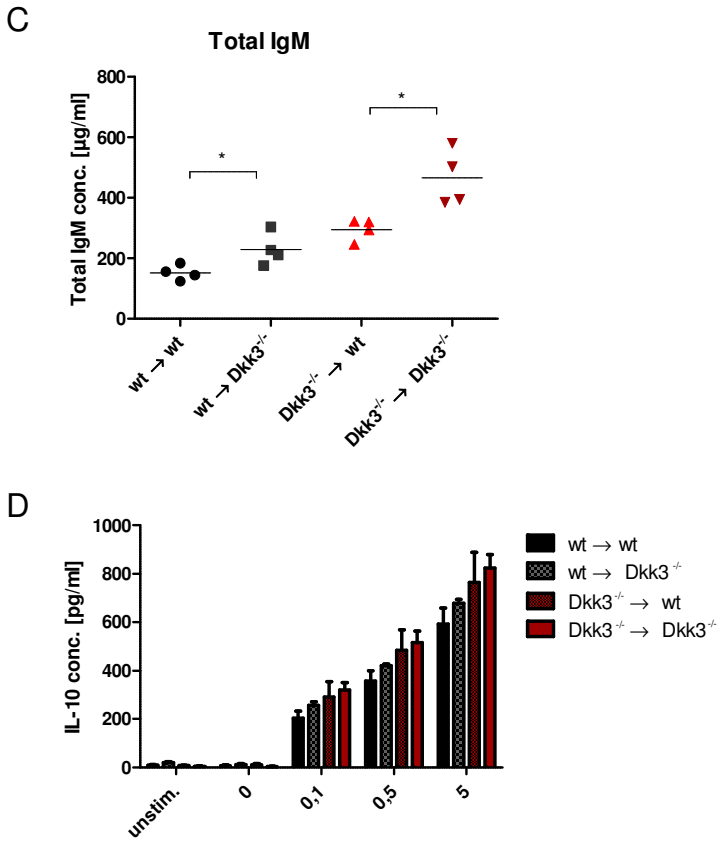


Figure 3.24 | B1 cells from Dkk3$^{-/-}$ mice and B1 cells transferred to a Dkk3 deficient environment display increased self-renewal capacity. CD19^{+} B cells were purified from spleens of C57BL/6 (wt) or Dkk3$^{-/-}$ mice (A; Pre= Before purification; Post= After purification) and 7x10^{6} cells were transferred to either wt.Rag2$^{-/-}$ (wt) or Dkk3$^{-/-}$Rag2$^{-/-}$ (Dkk3$^{-/-}$) mice. 4 weeks after transfer spleens and peritoneal cavity cells (PerC) of recipient mice were analyzed for numbers of total B cells, B1 cells and follicular B cells (FO) within the leukocyte population by FACS analysis (B). 4 weeks after transfer total IgM in serum was determined by ELISA (C). One dot represents cell numbers/ IgM serum concentration of one mouse and horizontal bars represent the mean for each group (B/C). Furthermore, 4 weeks after transfer, splenocytes were cultured with different concentrations of LPS together with 10µg/ml anti-CD40 for 24 hours. IL-10 concentration in supernatants was determined by ELISA (D). Mean and SEM are indicated for each group (D). Results show summary data from two independent experiments. Groups were compared using student t-test (ns= not significant; *=p<0,05; **=p<0,005).

3.2.10 Dkk3 is not expressed by splenic or peritoneal cavity B cells

It has been shown by our group, that Dkk3 is not detected in the spleen on the protein level [212]. However, it is possible that low amounts of Dkk3 are expressed by B cells, and that this can not be detected when using the complete tissue of the spleen. To additionally have a comparison between B1 and B2 cells, CD19^{+} cells of spleen (yielding mainly B2 cells) and PerC (yielding high numbers of B1 cells) were purified and RNA was extracted to analyze the Dkk3 expression level specifically in B cells. The tissue of the eye was used as a positive control, whereas Dkk3$^{-/-}$ samples

served as background controls. RNA expression analysis revealed that Dkk3 is not expressed by mature splenic or PerC B cells (Figure 3.25). The detected expression levels were in the range of the background levels of Dkk3$^{-/-}$ mice.

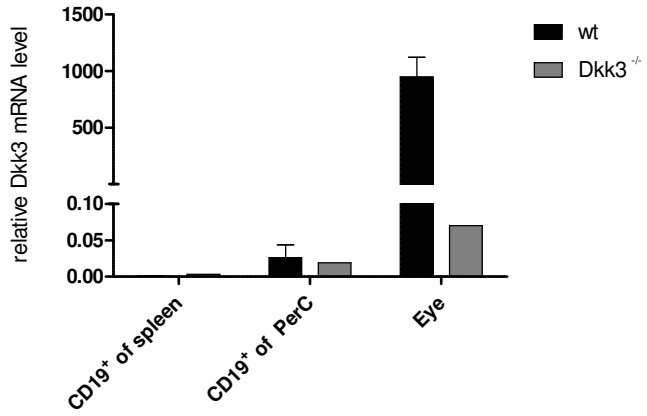


Figure 3.25 | Dkk3 is not expressed by splenic and peritoneal B cells. Total RNA was purified from C57BL/6 (wt) and Dkk3$^{-/-}$ CD19^{+} purified B cells of spleen, peritoneal cavity (PerC) and from the tissue of the eye as comparison, reverse transcribed to cDNA and analyzed by quantitative real-time PCR. For comparative analysis, the relative Dkk3 mRNA expression level was calculated by using β-actin mRNA levels as a control housekeeping gene. Mean and SEM are shown (n=4).

3.2.11 Dkk3 neutralization leads to increased symptoms of lupus erythematosus in MRL/LPR mice

B1 cells have been described to play important roles in autoimmune diseases, especially in systemic lupus erythematosus (SLE) [132, 167]. In the previous chapters it was demonstrated that, Dkk3$^{-/-}$ mice yield increased numbers of B1 cells, show increased B1 cell antibody responses and display increased IL-10 secretion, a B1 derived cytokine which was shown to play a crucial role in lupus erythematosus [175-178]. Thus, we aimed to analyze whether Dkk3 neutralization would result in deteriorated autoimmune symptoms in a model of SLE. For our studies we used the MRL/LPR mouse model of SLE, as disease onset (at 5-8 weeks of age) and progression in this strain occurs in a suitable time frame [248] for Dkk3 antibody treatment. Anti-Dkk3 antibody or isotype control treatment was started when mice had an age of 5 weeks, just shortly before first SLE symptoms appear. Treatment was carried out twice a week for a time period of 6 weeks. Serum was taken at several time points to allow the determination of autoantibodies. Mice were sacrificed at an age of 12 weeks and analyzed for multiple parameters, including weight of organs, phenotype of lymphocytes, levels of autoantibodies, as well as the inflammatory status of organs by histological analysis.

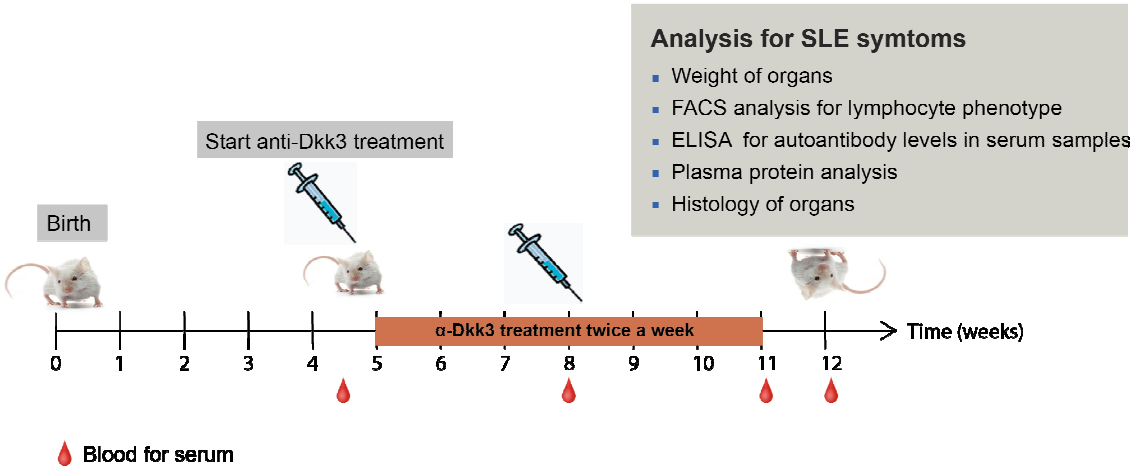


Figure 3.26 | Experimental setup for anti-Dkk3 antibody treatment of MRL/LPR mice. Anti-Dkk3 antibody or isotype control treatment of MRL/LPR mice was started at an age of 5 weeks. Mice were treated twice a week with 0,7mg anti-Dkk3 antibody or isotype control for 6 weeks. At an age of 12 weeks mice were analyzed for symptoms of SLE. Blood was collected several times for serum extraction.

3.2.11.1 Anti-Dkk3 antibody treatment results in enlarged lymph nodes and kidneys in MRL/LPR mice

MRL/LPR mice suffer from enlarged organs, caused by the failure of lymphocytes to go into apoptosis leading to organ infiltration and inflammation [181]. Therefore, when mice were sacrificed weight of different organs was measured. The spleen was huge in both, the isotype control and the anti-Dkk3 antibody treated, groups. The heart and liver also had a similar weight in both groups and can therefore serve as control organs. Interestingly, all measured LN, axillary, inguinal and mesenteric LN, were increased to a higher level in anti-Dkk3 antibody treated MRL/LPR mice. Furthermore, kidneys, the typical inflamed organ in SLE patients, of anti-Dkk3 antibody treated mice possessed a significantly higher weight than the control treated mice.

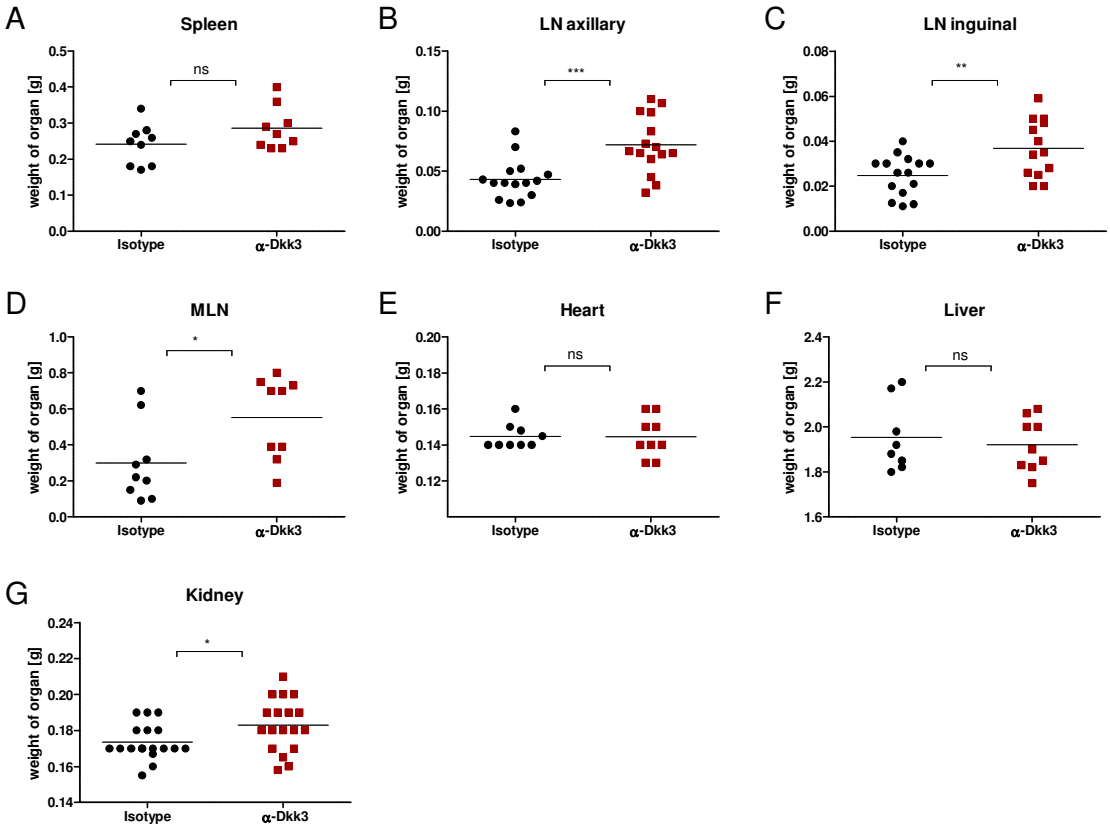


Figure 3.27 | Anti-Dkk3 antibody treatment results in enlarged lymph nodes and kidneys in MRL/LPR mice. MRL/LPR mice were treated with anti-Dkk3 antibody or isotype control for 6 weeks as described in Figure 3.26. One week after treatment was finished, weight of spleen (A), inguinal LN (B), axillary LN (C), mestenteric lymph nodes (MLN/D), heart (E), liver (F), and kidney (G) was determined. Summary data of 3 independent experiments are shown. One dot represents weight of one organ. Horizontal bars represent the mean for each group. Groups were compared using student t-test (ns= not significant; *=p<0,05; **=p<0,005; ***=p<0,0005).

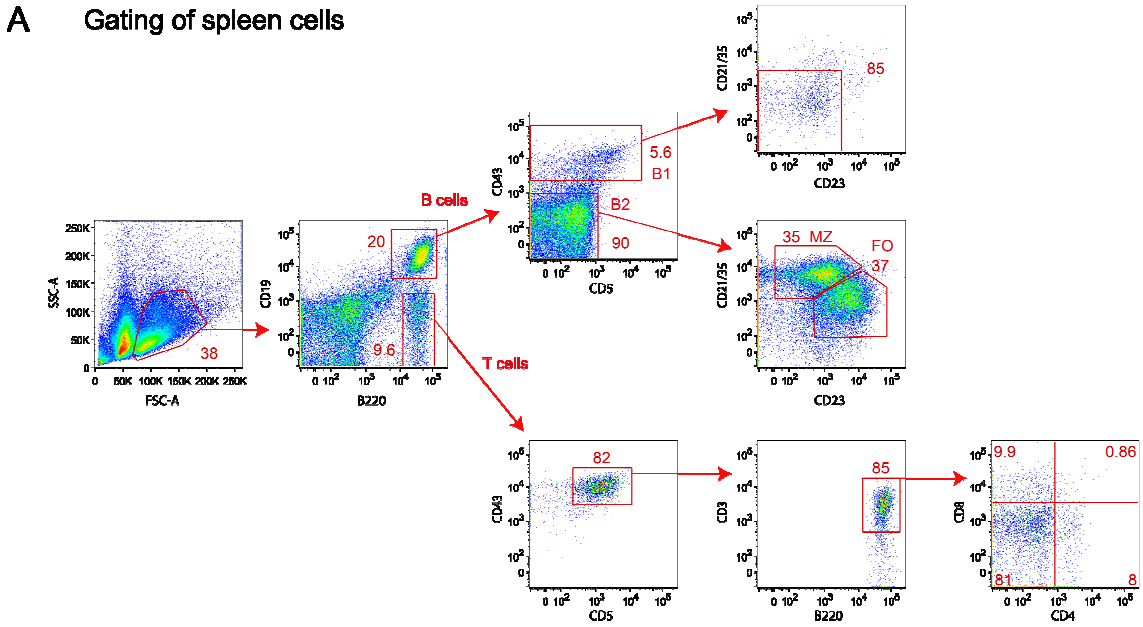
3.2.11.2 Anti-Dkk3 antibody treatment results in higher numbers of B1 cells in PerC and spleen of MRL/LPR mice

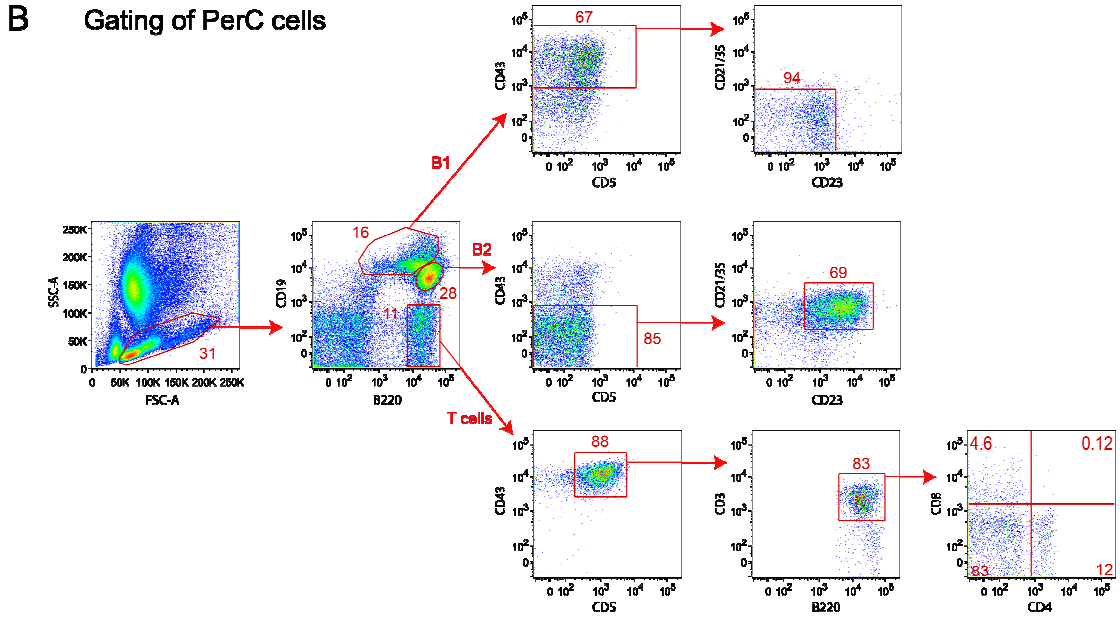
The disease of MRL/LPR mice is caused by increased numbers of lymphocytes, which do not undergo apoptosis, proliferate, infiltrate organs, and produce numerous inflammatory stimuli as well as auto-reactive antibodies. B1 cells are especially known for the capacity to secrete auto-reactive antibodies as well as disease exacerbating IL-10 [132]. Therefore, we were interested whether the phenotype of lymphocytes was differing between isotype control and anti-Dkk3 antibody treated MRL/LPR mice. Spleen, LN and PerC cells were isolated and analyzed for different B and T cell markers by FACS.

The distribution of B and T cell populations was abnormal in both treatment groups (Figure 3.28) compared to C57BL/6 mice (e.g. Figure 3.18). B cells consisted of

enormous high numbers of B1 cells, as defined here as $B220^{+}$, $CD19^{+}$ $CD43^{+}$, $CD5^{+/-}$, $CD21/CD35^{-}$ and $CD23^{-}$. In the spleen the frequency of B1 cells was 2-3 fold increased, but in lymph nodes the frequency exceeded 10-20 times the normal number of B1 cells found in C57BL/6 wt mice. T cells did not only exhibit an abnormal high frequency in lymph nodes but also did they show an abnormal phenotype. They highly expressed B220 and were mainly negative for the coreceptors CD4 and CD8.

The number of T cells and B cells was similar between isotype control and anti-Dkk3 antibody treated mice in the spleen and the LN (Figure 3.28 C). However, the PerC of anti-Dkk3 antibody treated mice exhibited higher number of B cells than the control group, which was due to increased numbers of B1 cells. In the spleen, the frequency of B cells was the same in both groups. However, within the B cells of the spleen the ratio of B2:B1 changed towards increased numbers of B1 cells in the anti-Dkk3 antibody treated group. In the lymph nodes, the ratio of B2:B1 was the same in the isotype control and anti-Dkk3 antibody treated group. But nevertheless, the weight of LN was significantly higher in anti-Dkk3 antibody treated mice; therefore increased numbers of all cells were present in the LN, including B1 cells.


A
Gating of spleen cells
SSC-A
FSC-A
38
CD19
B220
20
9.6
B cells
T cells
CD43
CD5
5.6
B1
B2
90
CD21/35
CD23
85
35 MZ
FO
37
82
CD3
85
CD8
CD4
9.9
0.86
81
8


B
Gating of PerC cells
SSC-A
FSC-A
31
CD19
B220
16
28
11
B1
B2
T cells
CD43
CD5
67
CD21/35
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94
85
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88
CD3
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CD8
CD4
4.6
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12

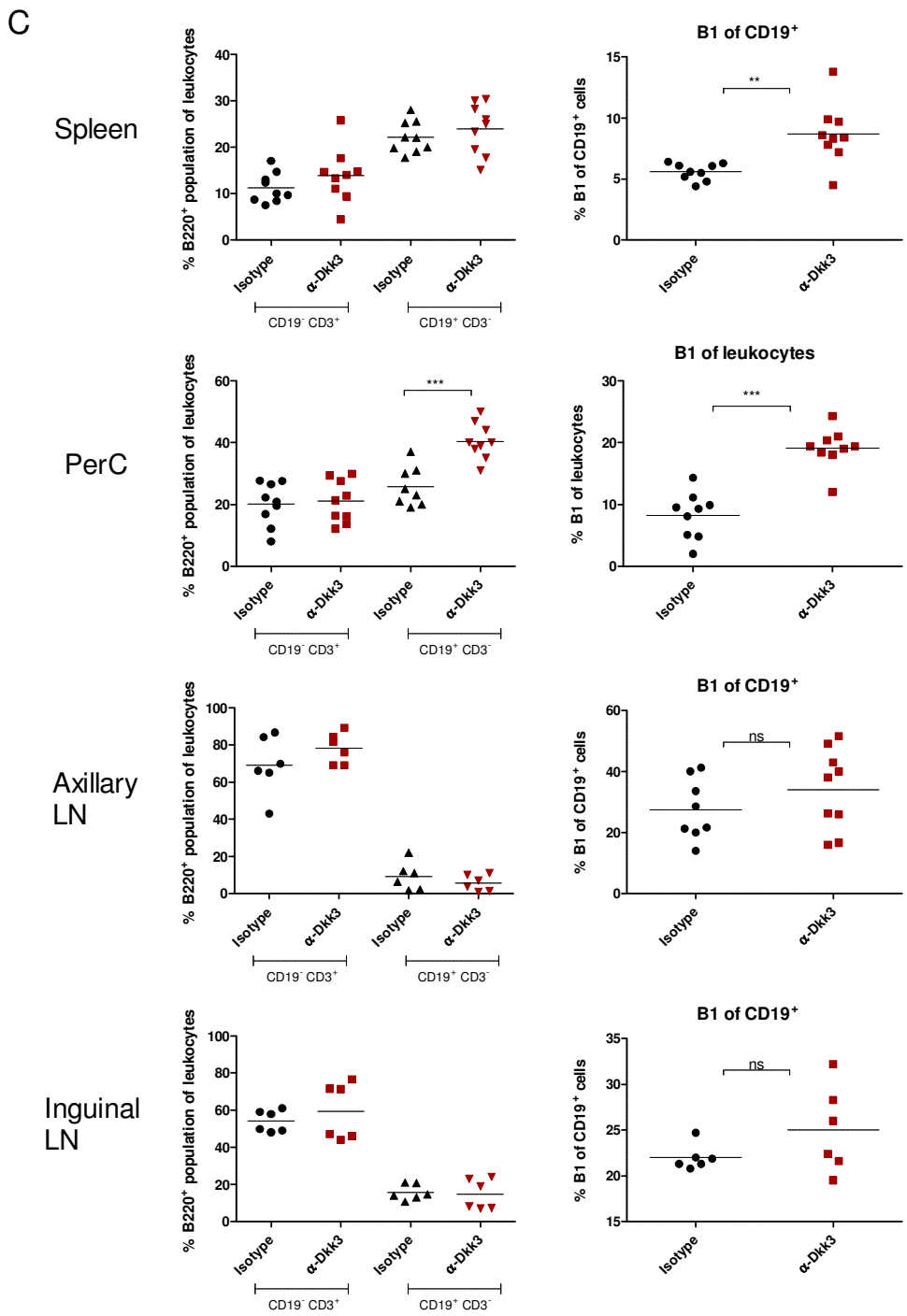


Figure 3.28 | MRL/LPR mice treated with anti-Dkk3 antibody exhibit increased numbers of B1 cells. MRL/LPR mice were treated with anti-Dkk3 antibody or isotype control for 6 weeks as described in Figure 3.26. One week after treatment was finished, cells of spleen, axillary lymph nodes (LN), inguinal LN and peritoneal cavity (PerC) were stained for different T and B cell markers by FACS. The gating strategy of T cells, B1 and B2 cells for spleen, axillary LN and inguinal LN cells is shown in A as an example of spleen cells. The gating stategy for PerC cells is shown in B. In C summary data of 3 independent experiments are presented. Left panels show B220$^+$ T (CD19$^-$CD3$^+$) and B (CD19$^+$ CD3$^-$) cells as gated in A and B. Right panels shows analysis of B1 cells in the different lymphatic sites. One dot represents cell numbers of one mouse. Horizontal bars represent the mean for each group. Groups were compared using student t-test (ns= not significant; **=p<0,005; ***=p<0,0005).

3.2.11.3 Anti-Dkk3 antibody treatment leads to increased levels of autoantibodies in the serum

One of the hallmarks of SLE is the high level of autoantibodies in serum which contributes to organ destruction [186]. It is assumed that these autoantibodies are secreted especially by B1 cells, which are recruited to germinal centers, undergo CSR and produce high affinity antibodies. However, this process makes them hard to be distinguished from B2 cells, which is the reason why it is still controversial whether in deed B1 cells are solely responsible for autoantibody production [132, 249, 250]. As B1 cell numbers were increased in Dkk3 deficient and MRL/LPR mice treated with a Dkk3 neutralizing antibody, we were interested in the level of autoantibodies in serum of isotype control treated and anti-Dkk3 antibody treated MRL/LPR mice.

Four different autoantibody specificities were tested for IgM and IgG concentration in serum of MRL/LPR mice. Myelin oligodendrocyte glycoprotein (MOG) and myelin basic protein (MPB) specific immunoglobulins are directed to CNS antigens and are observed to be increased is patients of SLE and multiple sclerosis [251-253]. Antibodies against single and double strand (ss and ds) DNA are most typically observed to be increased in patients of SLE and are used as diagnostic parameter for SLE [254, 255]. The immunoglobulins of all four analyzed specificities were highly increased in MRL/LPR mice at the age of 12 weeks in comparison to 5 weeks old MRL/LPR mice or C57BL/6 control mice (Figure 3.29 to Figure 3.32). No difference was found in anti-MBP IgM and IgG antibody levels between the isotype control and anti-Dkk3 antibody treated group (Figure 3.29). Anti-MOG IgM and IgG antibodies were significantly increased in MRL/LPR mice treated with anti-Dkk3 antibody (Figure 3.30). Also ss-DNA and ds-DNA IgG antibodies were significantly increased in anti-Dkk3 antibody treated mice (Figure 3.31 and Figure 3.32). Therefore, we conclude, that Dkk3 neutralization leads to increased numbers of auto-reactive antibodies to different target structures which may contribute to the tissue destruction in the pathogenesis of SLE in the model of MRL/LPR mice.

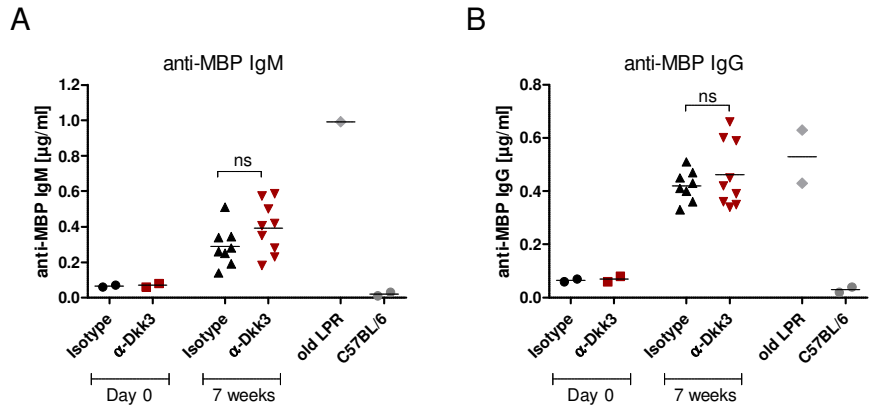


Figure 3.29 | MRL/LPR mice treated with anti-Dkk3 antibody do not exhibit higher levels of anti-MBP antibodies. MRL/LPR mice were treated with anti-Dkk3 antibody or isotype control for 6 weeks as described in Figure 3.26. Concentration of anti-MBP specific IgM (A) and IgG (B) antibodies was determined in sera taken at the start of treatment (Day0) and one week after treatment was finished (7 weeks) by ELISA. Serum of 4-5 months old MRL/LPR mice (old LPR) and 2 months old C57BL/6 mice served as positive and negative controls. One dot represents antibody concentration in serum of one mouse. Horizontal bars represent the mean for each group. Groups were compared using student t-test (ns= not significant).

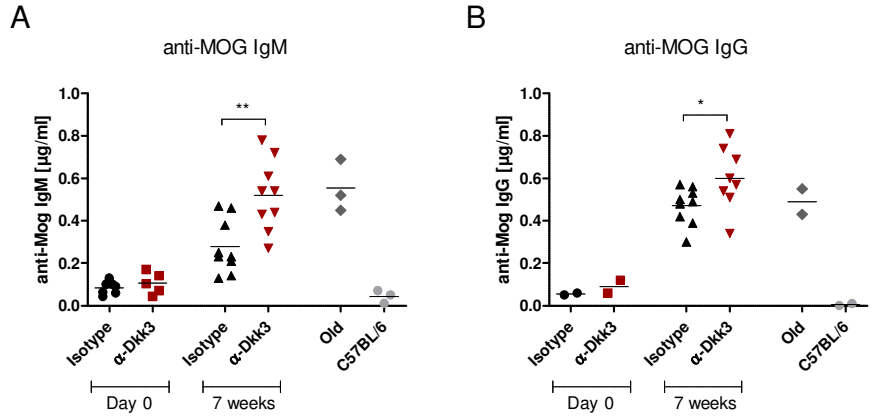


Figure 3.30 | MRL/LPR mice treated with anti-Dkk3 antibody exhibit higher levels of anti-MOG antibodies. MRL/LPR mice were treated with anti-Dkk3 antibody or isotype control for 6 weeks as described in Figure 3.26. Concentration of anti-MOG_{33-55} specific IgM (A) and IgG (B) antibodies was determined in sera taken at the start of treatment (Day0) and one week after treatment was finished (7 weeks) by ELISA. Serum of 4-5 months old MRL/LPR mice (old LPR) and 2 months old C57BL/6 mice served as positive and negative controls. One dot represents antibody concentration in serum of one mouse. Horizontal bars represent the mean for each group. Groups were compared using student t-test (ns= not significant; *=p<0,05; **=p<0,005).

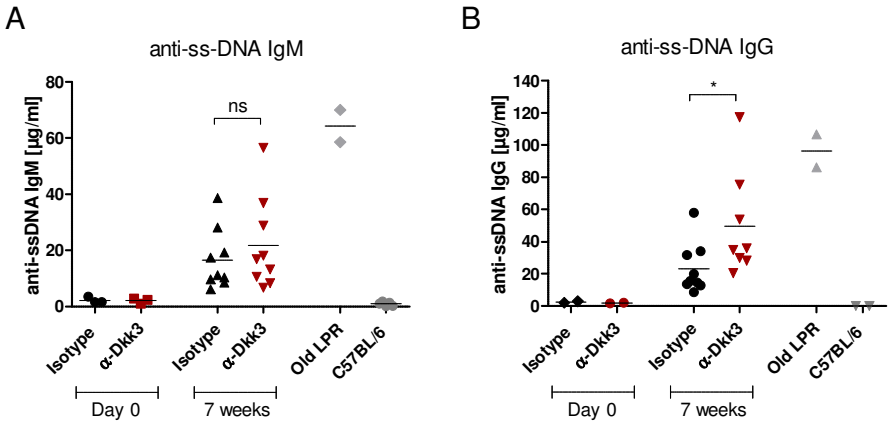


Figure 3.31 | MRL/LPR mice treated with anti-Dkk3 antibody exhibit higher levels of anti-ss-DNA IgG antibodies. MRL/LPR mice were treated with anti-Dkk3 antibody or isotype control for 6 weeks as described in Figure 3.26. Concentration of anti-single strand (ss) - DNA specific IgM (A) and IgG (B) antibodies was determined in sera taken at the start of treatment (Day0) and one week after treatment was finished (7 weeks) by ELISA. Serum of 4-5 months old MRL/LPR mice (old LPR) and 2 months old C57BL/6 mice served as positive and negative controls. One dot represents antibody concentration in serum of one mouse. Horizontal bars represent the mean for each group. Groups were compared using student t-test (ns= not significant; *=p<0,05).

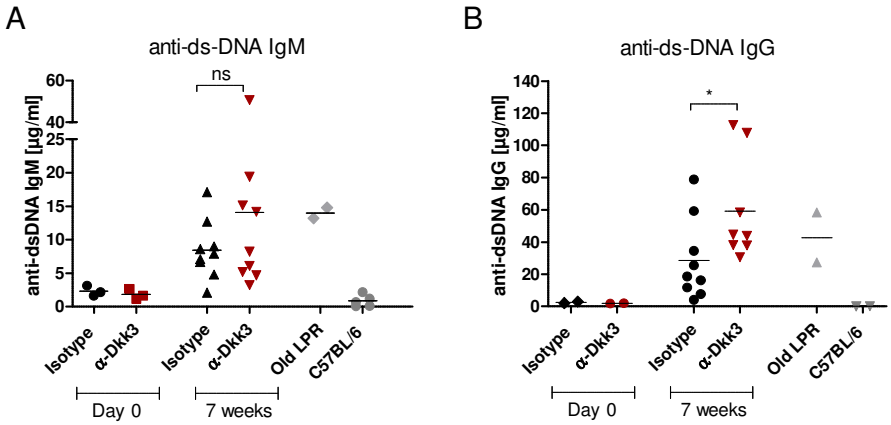


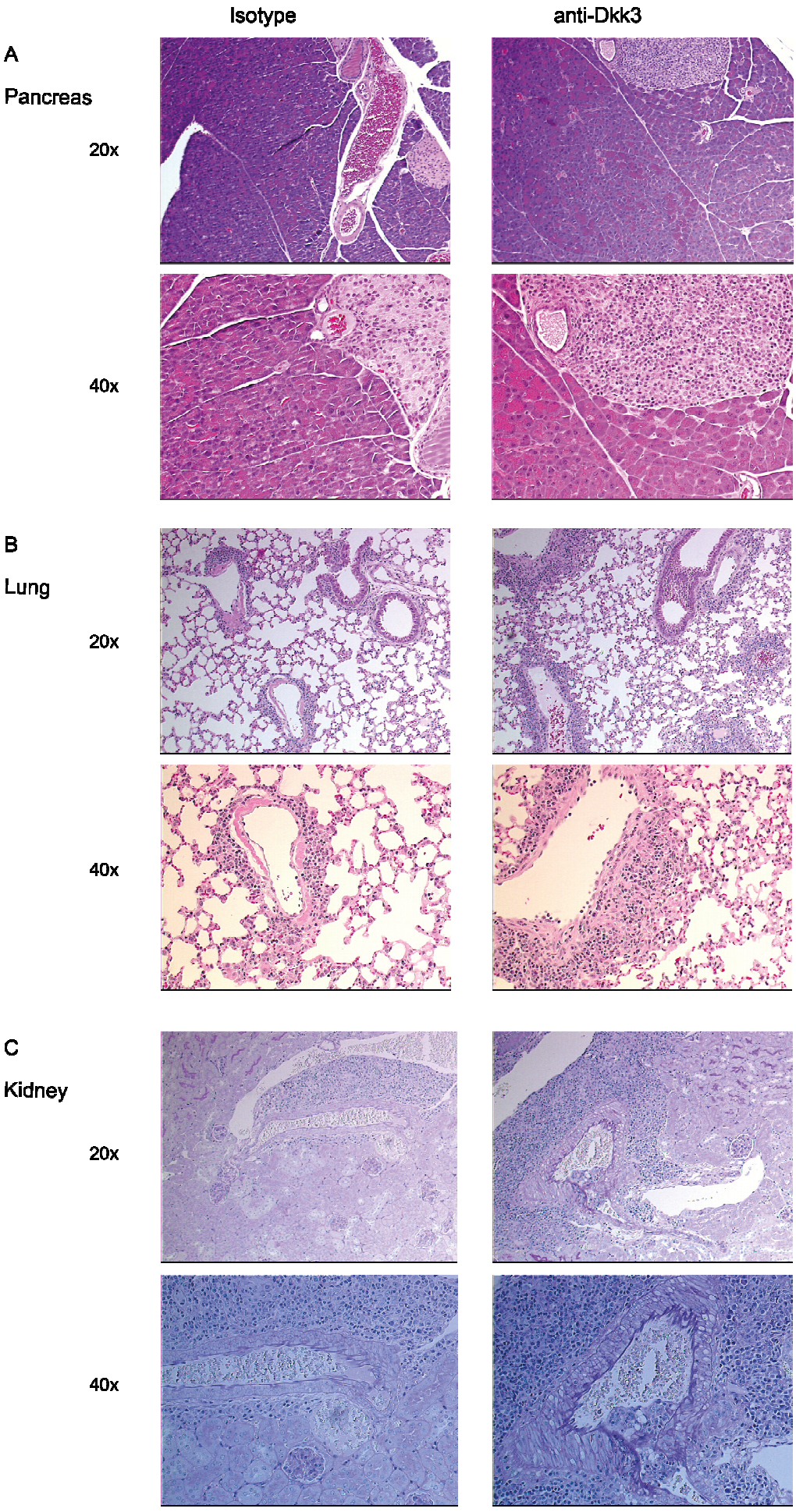
Figure 3.32 | MRL/LPR mice treated with anti-Dkk3 antibody exhibit higher levels of anti-ds-DNA IgG antibodies. MRL/LPR mice were treated with anti-Dkk3 antibody or isotype control for 6 weeks as described in Figure 3.26. Concentration of anti-double strand (ds) - DNA specific IgM (A) and IgG (B) antibodies was determined in sera taken at the start of treatment (Day0) and one week after treatment was finished (7 weeks) by ELISA. Serum of 4-5 months old MRL/LPR mice (old LPR) and 2 months old C57BL/6 mice served as positive and negative controls. One dot represents antibody concentration in serum of one mouse. Horizontal bars represent the mean for each group. Groups were compared using student t-test (ns= not significant; *=p<0,05).

3.2.11.4 Anti-Dkk3 antibody treatment results in kidney, lung and pancreas inflammation

The accumulation of immune complexes and infiltration of lymphocytes in organs of SLE patients leads to a strong inflammatory response of the immune system which can lead to organ failure [165, 166]. Especially kidneys and lungs are mostly observed to be damaged in SLE patients. Also in MRL/LPR mice, glomerulonephritis and progress to end-stage renal failure is a typical consequence of disease progression [181, 182]. To investigate, whether anti-Dkk3 antibody treatment leads to aggravated inflammation of organs, lungs, pancreas and kidneys of isotype control and anti-Dkk3 treated MRL/LPR mice were histologically examined.

Pancreata, lungs and kidneys of anti-Dkk3 antibody treated mice exhibited increased inflammatory foci and higher numbers of infiltrating leukocytes compared to mice treated with isotype control (Figure 3.33). The tissues of anti-Dkk3 antibody treated mice exhibited severe tissue damage. In future experiments further immunohistochemistry will be added to distinguish different leukocytes such as macrophages, B and T cells and to examine tissue damage more specifically by e.g. collagen staining.

Figure 3.33 | MRL/LPR mice treated with anti-Dkk3 antibody exhibit higher levels of infiltrating leukocytes. MRL/LPR mice were treated with anti-Dkk3 antibody (n=12) or isotype control antibody (n=13) for 6 weeks as described in Figure 3.26. Mice were sacrificed and tissues collected in formalin. Paraffin tissue sections of pancreas (A), lung (B) and kidney (C) were prepared and stained with hematoxylin and eosin. Upper pictures of each tissue are at 20x magnification, lower pictures at 40x magnification. Representative pictures for each tissue and treatment group are depicted. Violet dots are infiltrated lymphocytes. Stronger leukocyte infiltration in anti-Dkk3 antibody treated mice as shown in this figure was observed in 8/12 mice in pancreas tissue. 6/12 mice in lung tissue and 9/12 mice in kidney tissue.



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The increased inflammation observed in the pancreata of anti-Dkk3 antibody treated mice was confirmed by increased levels of blood sugar in these mice in comparison to isotype treated mice.

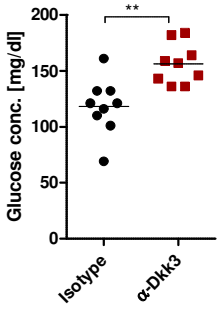

Figure 3.34 | MRL/LPR mice treated with anti-Dkk3 antibody exhibit higher levels of blood sugar. MRL/LPR mice were treated with anti-Dkk3 antibody or isotype control for 6 weeks as described in Figure 3.26. Concentration of Glucose was determined after treatment was finished (7 weeks) in blood plasma. One dot represents Glucose concentration in plasma of one mouse. Horizontal bars represent the mean for each group. Groups were compared using student t-test (**=p<0,005).

Concluding, we found an increased pathology in MRL/LPR mice treated with anti-Dkk3 antibody, which was asserted on different levels of disease assessment. Firstly, lymph nodes and kidneys were enlarged; secondly levels of autoantibodies were increased in the serum, and thirdly increased infiltration of organs by leukocytes and severe tissue damage in these organs was observed in anti-Dkk3 antibody treated mice. These observations were associated with increased numbers of B1 cells in these mice compared to isotype treated mice.

In summary the experiments investigating the role of Dkk3 in B cell function revealed that Dkk3 deficient mice exhibit increased B1 cell numbers whereas numbers of B2 cells are decreased. Therefore, antigen responses to B1 antigens as well as B cell derived IL-10, the typical cytokine secreted by B1 cells, were increased in Dkk3$^{-/-}$ mice. B2 cell function was not found to be reduced by Dkk3 deficiency. B cell development and maintenance analysis showed that B2 development is impaired in the bone marrow of Dkk3$^{-/-}$ mice. In contrast the self-renewal capacity of B1 cells was increased in Dkk3$^{-/-}$ mice as demonstrated by transfer experiments. Analyzing the role of Dkk3 in a model of SLE, we found that neutralization of Dkk3 leads to higher numbers of B1 cells, increased autoantibody production and more severe tissue damage in MRL/LPR mice.

3.3 Search for an immunological phenotype in Soggy$^{-/-}$ mice

Soggy is the most distant member of the Dkk protein family. The sgy domain is solely present in the proteins Soggy and Dkk3 [191]. As this domain distinguishes Dkk3 from the other members it is tempting to speculate that this domain confers the immune-modulatory property to Dkk3. To address this question, the immunological phenotype of Soggy$^{-/-}$ mice was explored. This will tell us, whether Soggy also exhibits immune-modulatory properties and it will give hints whether the sgy domain is important in conferring this feature to Dkk3 and Soggy. (Experiments in chapter were performed together with A. Steinbach and also contributed to her master thesis.)

3.3.1 Expression of Soggy on the mRNA and protein level

Dkk3 was described to be expressed primarily in immune privileged sites [212]. We were interested whether soggy displays a similar expression pattern, which would provide a first indication whether Soggy may be relevant as an immune regulatory candidate similar to Dkk3. Furthermore, the expression pattern of Soggy was analyzed in wt and Dkk3$^{-/-}$ mice revealing whether genetic deletion of Dkk3 has an effect on the Soggy expression level, because based on their homology, (sgy domain has 41% homology in Dkk3 and Soggy (Figure 3.35)) they may complement their functions. To address these questions, the expression of Soggy was assessed on the mRNA and on the protein level.

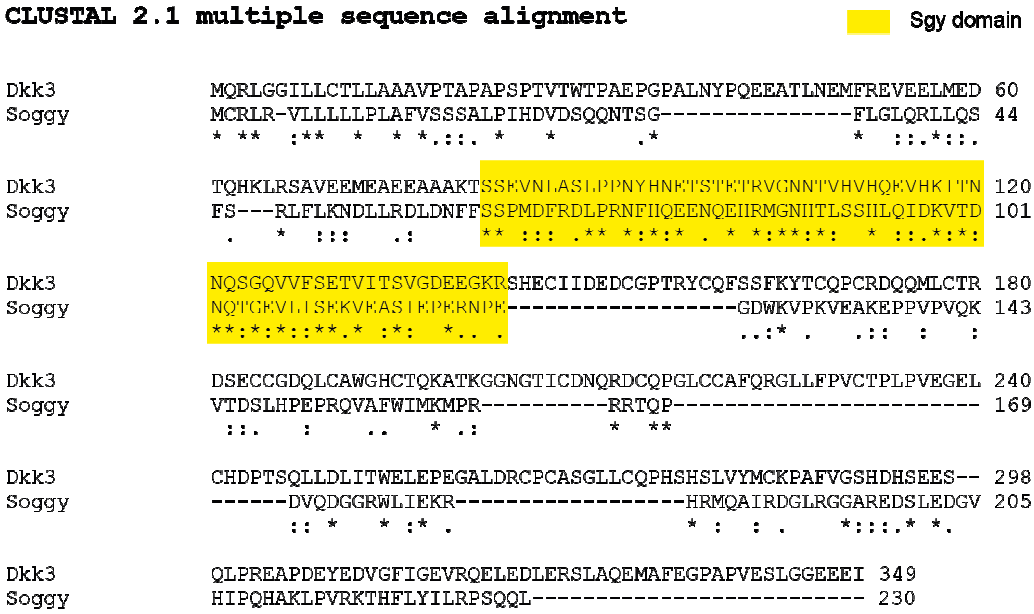

Figure 3.35 | The sgy domain of Dkk3 and Soggy exhibit 41% homology. Multiple sequence alignment was carried out with ClustalW algorithm of murine Dkk3 and Soggy protein sequences found in the NCBI database. The location of the sgy domain (as described by Niehrs [191]) is indicated by the yellow shaded box. Stars (*) indicate homologue amino acids. The sgy domain exhibits 24 homologue amino acids out of 58 total amino acids in Dkk3 and Soggy, counting up to 41% domain identity. The overall sequence identity of the Soggy and Dkk3 protein is 22%.

The analysis of mRNA and protein expression of Soggy revealed that Soggy is highly expressed in the testis and to a lower level in the brain and the skin. Interestingly, brain, testis and the proximal hair follicle epithelium in the skin have been described to belong to immune privileged sites [25, 28, 256]. Additionally, Soggy was also detected to a very low level in the stomach. In all other analyzed tissues, eye, pancreas, atrium, ventricle, liver, kidney, spleen, lymph nodes, thymus, lung, small intestine, caecum, large intestine and bone marrow, it was absent. In $Dkk3^{-/-}$ mice the expression of Soggy was not significantly altered, demonstrating that Dkk3 deficiency does not lead to a compensatory upregulation of Soggy expression.

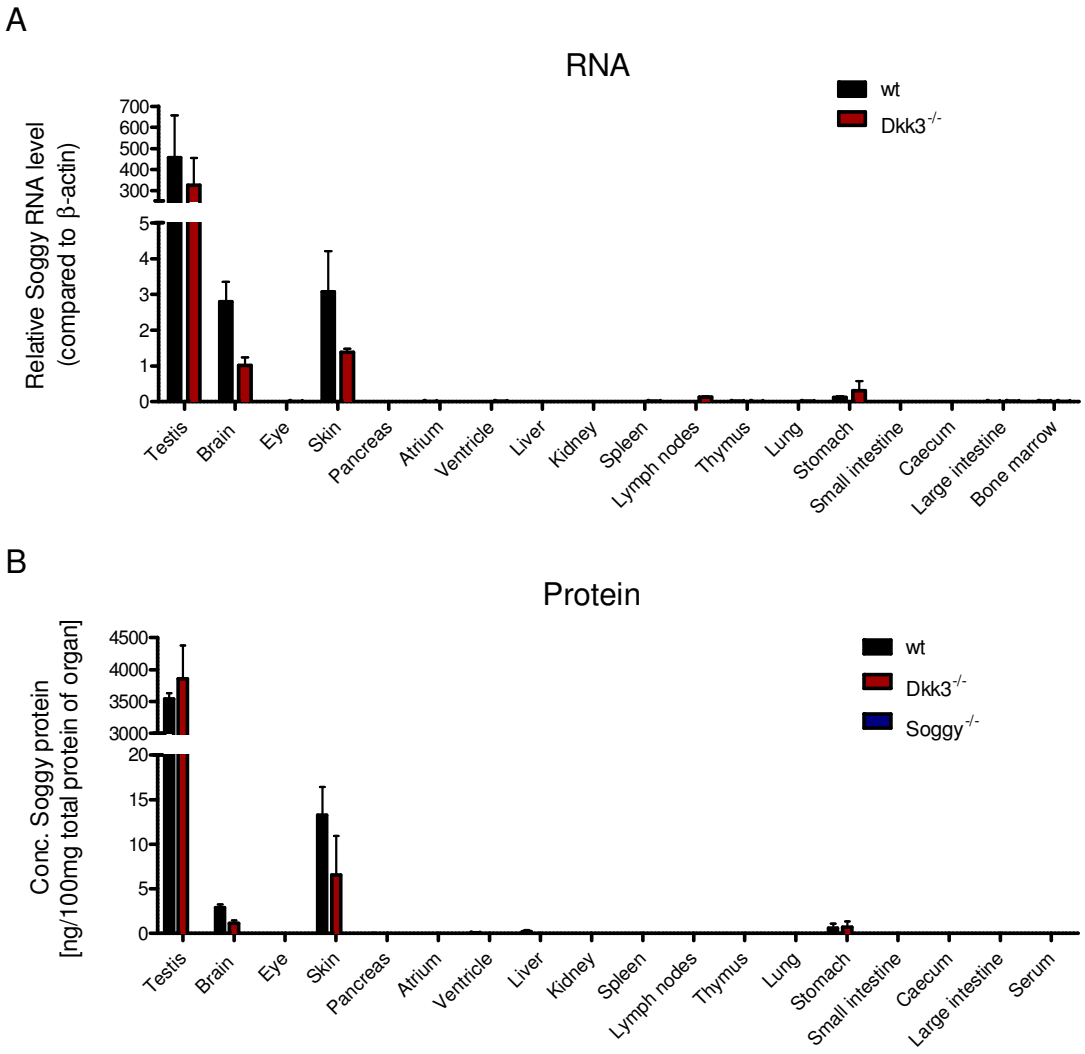

Figure 3.36 | Soggy is expressed in testis, brain and skin. To determine the expression of Soggy on the mRNA level (A), total RNA was purified from mouse tissues of C57BL/6 (wt), Dkk3$^{-/-}$ and Soggy$^{-/-}$ mice, reverse transcribed to cDNA and analyzed by quantitative real-time PCR. For comparative analysis, the relative Soggy mRNA expression level was calculated by using β-actin mRNA levels as a control housekeeping gene. Mean and SEM are shown (n=5). To determine the expression of Soggy on the protein level (B), total protein was extracted from mouse tissues of C57BL/6 (wt) and Dkk3$^{-/-}$ mice, adjusted to a protein concentration of 10 mg/ml and analyzed by ELISA. Organ lysates from Soggy$^{-/-}$ mice were negative for Soggy protein, confirming that Soggy antibodies do not crossreact with Dkk3 protein. Mean and SEM are shown (n=6).

3.3.2 Soggy deficiency does not alter the numbers of mature T cells in the thymus

In order to examine, whether T cell development is altered in Soggy$^{-/-}$ mice, thymocytes were analyzed for their phenotypical maturation status.

Soggy deficient mice exhibited changes in the first developmental stages. Slightly but significantly increased CD8/CD4 double negative (DN) cells and slightly reduced numbers of CD8/CD4 double positive (DP) cells were detected (Figure 3.37). Analyzing the DN stage for substages, revealed that the number of CD44^{+}CD25^{-} (DN1) cells was reduced in Soggy deficient mice. The other DN stages (DN12-4) were not affected by Soggy deficiency. Although differences in the first developmental stages were detected, mature single positive CD4 and CD8 T cells were present at similar numbers in the thymus of wt and Soggy$^{-/-}$ mice.

Tregs detected by the expression of CD4 and FoxP3 were Helios positive (Figure 3.38 A), confirming the observation by Thornton *et al.*, which claimed Helios to be a marker for thymic Tregs [58]. No difference in Treg numbers in the thymus was found in Soggy$^{-/-}$ mice compared to wt controls (Figure 3.38 B).

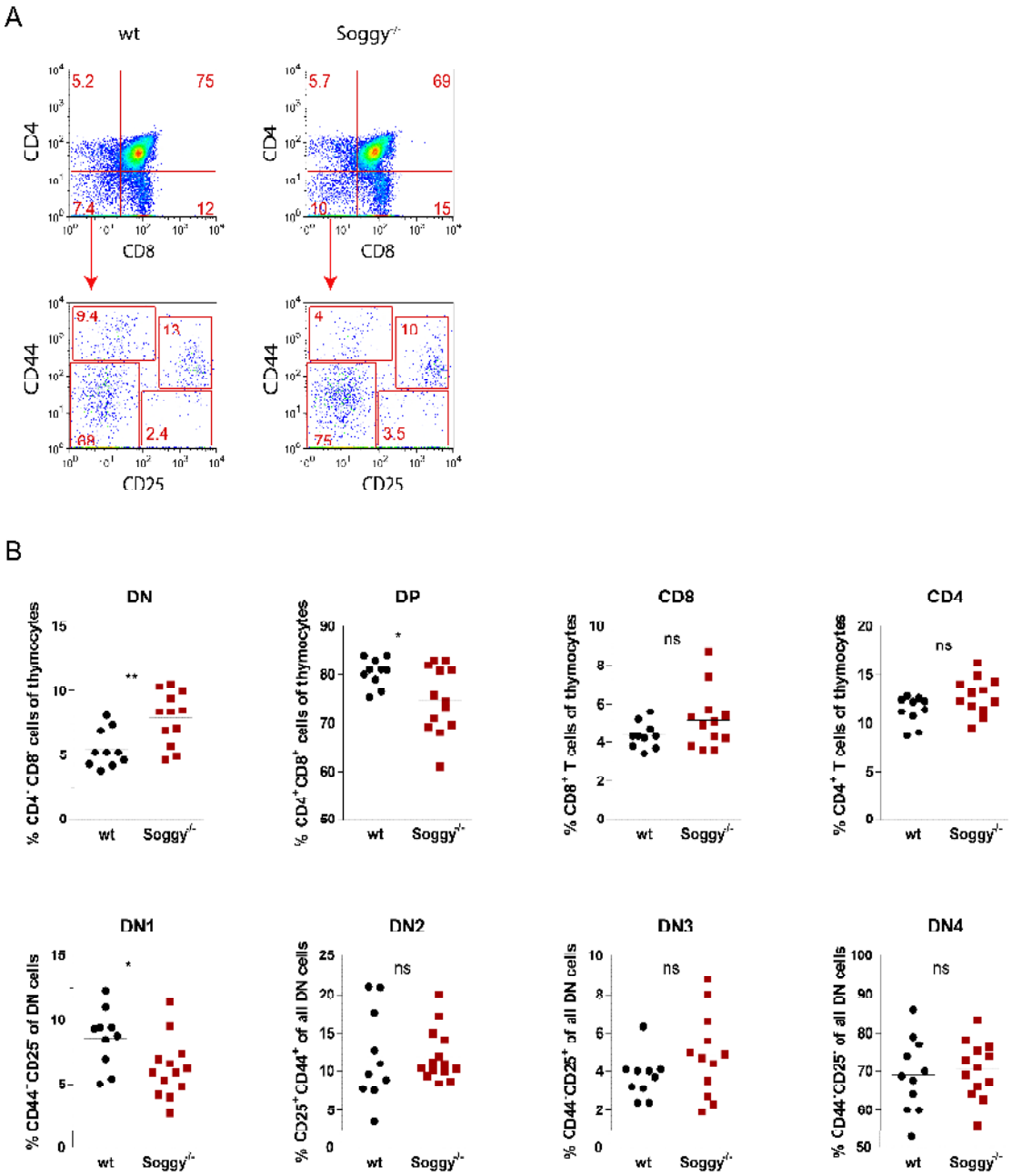

Figure 3.37 | T cell development in the thymus in Soggy deficient mice. Cells were isolated from the thymus of C57BL/6 (wt) and Soggy$^{-/-}$ mice. Population frequencies of the different stages of CD4 and CD8 T cell development were analyzed by FACS. The gating strategy is shown in A for wt and Soggy$^{-/-}$ mice. Upper FACS dot blot are gated on thymocytes. Summary data of three independent experiments are presented in B. One dot represents cell numbers of one mouse. Horizontal bars represent the mean for each group. Groups were compared using student t-test (ns= not significant; *=p<0,05; **=p<0,005).

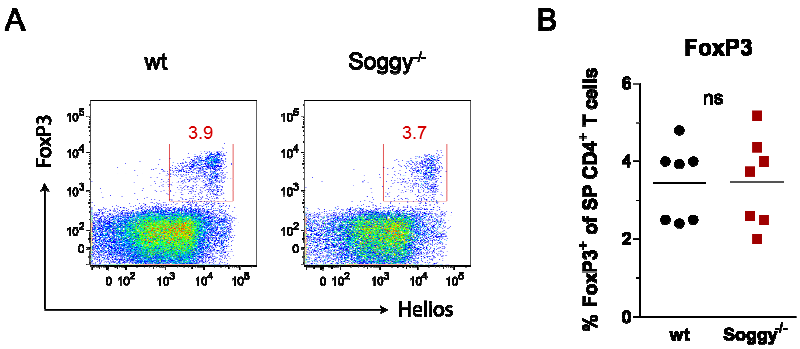

Figure 3.38 | Numbers of regulatory T cells are not altered in the thymus of Soggy$^{-/-}$ mice. Cells were isolated from the thymus of C57BL/6 (wt) and Soggy$^{-/-}$ mice. The number of FoxP3^{+} regulatory T cells was determined by FACS analysis. The FACS dot blots in A are gated on single positive CD4 T cells and shows the gating of Helios^{+} FoxP3^{+} cells-. Summary data of two independent experiments are presented in B. One dot represents cell numbers of one mouse. Horizontal bars represent the mean for each group. Groups were compared using student t-test (ns= not significant).

3.3.3 T cell subpopulations in peripheral tissues are altered in Soggy$^{-/-}$ mice

To investigate, whether peripheral T cell populations are altered in Soggy$^{-/-}$ mice, CD4 and CD8 T cells were analyzed in wt, Dkk3$^{-/-}$ and Soggy$^{-/-}$ mice. Cells were stained for the surface molecules CD44 and CD62L commonly used to determine the activation status of a T cell and allowing the determination of effector and memory status. CD44 is upregulated upon T cell stimulation and expression is sustained on memory T cells [257], whereas CD62L is expressed on naïve T cells promoting homing to secondary lymphoid organs and its expression is downregulated upon stimulation allowing the migration to sites of infection outside of lymphoid organs [258-260]. Central memory T cells home to secondary lymphoid organs and therefore express CD62L, whereas effector memory cells reside mainly in specific tissues and therefore do not express CD62L. Additionally, cells were stained for FoxP3 and CD25 to identify Tregs. Helios has been described to distinguish natural from iTregs, as it was found predominantly on thymic Tregs [58]. Therefore, we used this marker to get an impression, whether we find differences in Treg origin in Soggy$^{-/-}$ mice compared to wt and Dkk3$^{-/-}$ mice.

T cell analysis of splenic cells (Figure 3.39) revealed, that the spleen in Soggy$^{-/-}$ exhibits slightly but significantly less CD8 T cells. Furthermore, memory cells (CD44^{high} cells) were reduced among CD4 and CD8 T cell subsets, in Soggy$^{-/-}$ spleens. In the CD4 T cell subset this was due to a reduction of both central and effector memory T cells, whereas in the CD8 T cell subset this was due to a reduction of only central memory T cells. Strikingly, Treg cell numbers were decreased in Soggy$^{-/-}$ spleens as both FoxP3 and CD25 positive cells were decreased. Interestingly, in the

spleen higher numbers of Helios^{low} cells within the Treg cell population were found, pointing to an increased number of induced Tregs in Soggy$^{-/-}$ spleens.

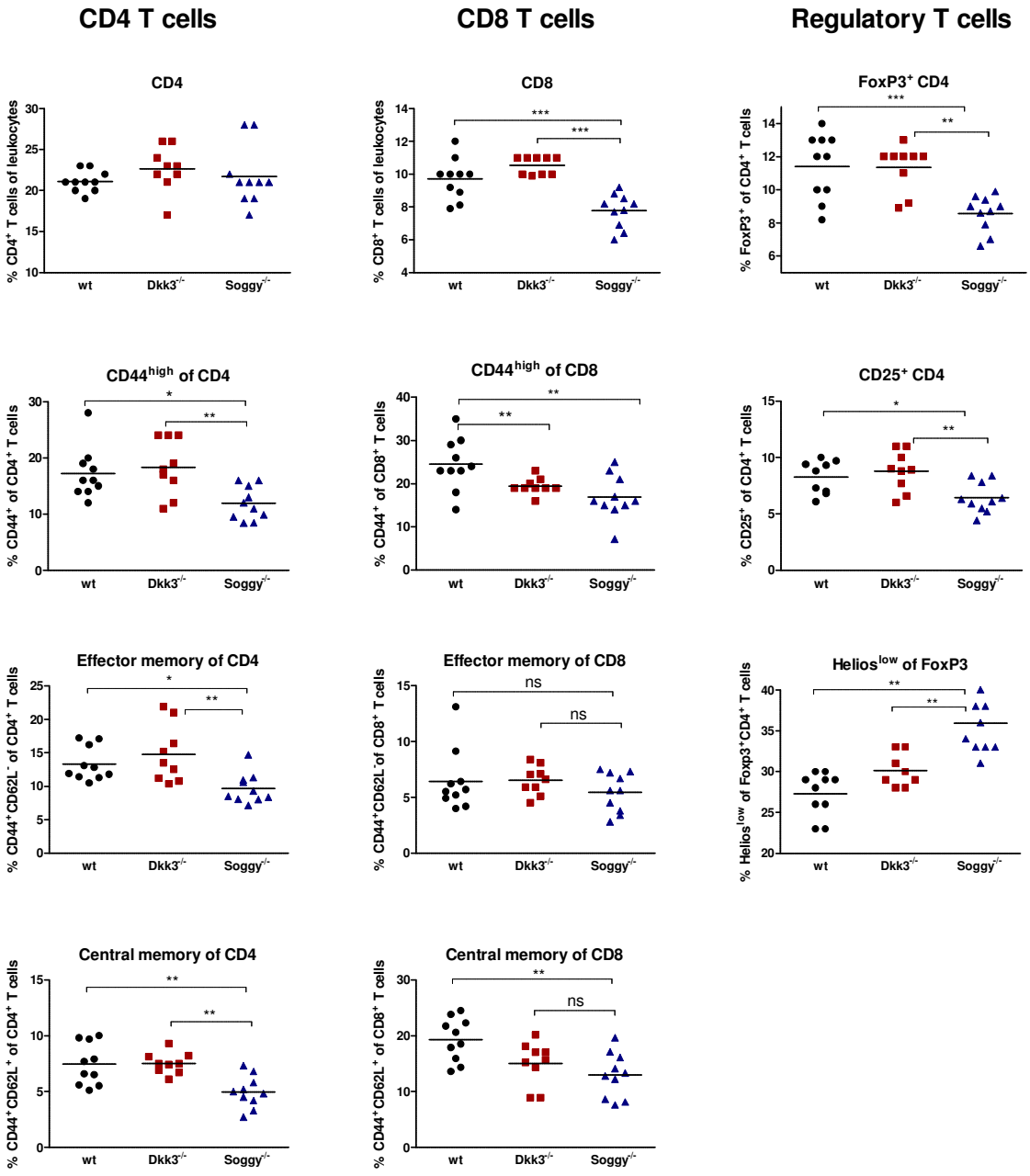

Figure 3.39 | Soggy$^{-/-}$ mice display decreased levels of memory and regulatory T cells in the spleen. Cells were isolated from spleen of C57BL/6 (wt), Dkk3$^{-/-}$ and Soggy$^{-/-}$ mice and CD4, CD8 and regulatory T cells were analyzed by FACS for their numbers and expression of different activation/memory markers. Summary data of four independent experiments are presented. One dot represents cell numbers of one mouse. Horizontal bars represent the mean for each group. Groups were compared using student t-test (ns= not significant; *=p<0,05; **=p<0,005; ***=p<0,0005).

In the MLN a similar T cell distribution compared to the spleen was observed for Soggy$^{-/-}$ mice (Figure 3.40). Numbers of CD8 T cells were equal in wt, Dkk3$^{-/-}$ and Soggy$^{-/-}$ mice. But still the ratio of CD8/CD4 T cells was shifted towards CD4 in Soggy$^{-/-}$ MLN, as higher numbers of CD4 T cells were present in Soggy$^{-/-}$ MLN. The expression of CD44 was reduced in both CD4 and CD8 T cells also in the MLN of Soggy$^{-/-}$ mice and, exactly as in the spleen, in the CD4 T cell subset this was due to the reduction of both central and effector memory T cells, whereas for the CD8 T cells this was due to only the reduction of central memory T cells. The number of regulatory T cells was also in the MLN found to be reduced. However, in contrast to the spleen, the proportion of induced Tregs (Helios^{low} Tregs) was lower in Soggy$^{-/-}$ MLN compared to wt and Dkk3$^{-/-}$ MLN.

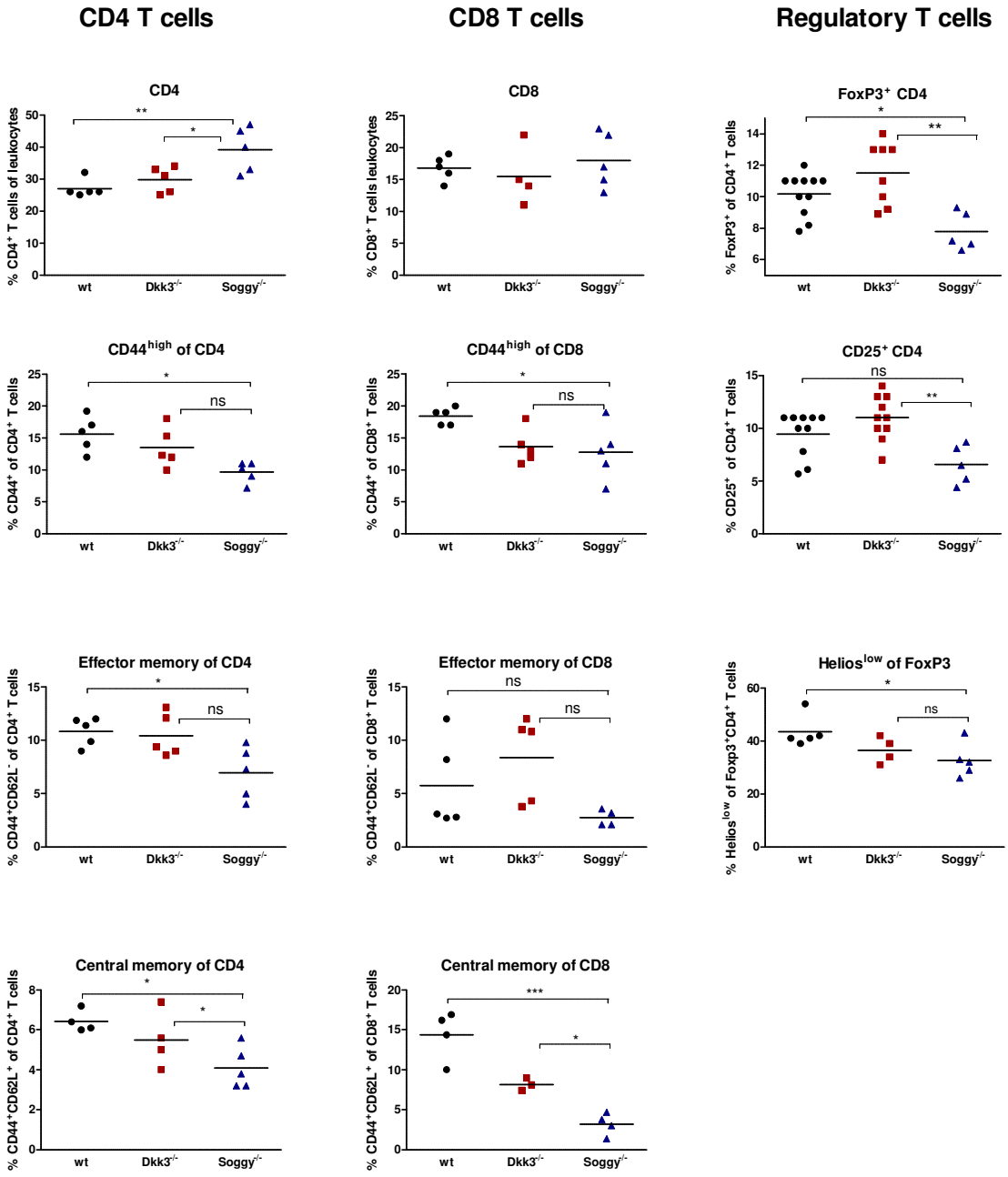

Figure 3.40 | Soggy$^{-/-}$ mice display decreased levels of memory and regulatory T cells in the mesenteric lymph nodes. Cells were isolated from mesenteric lymph nodes of C57BL/6 (wt), Dkk3$^{-/-}$ and Soggy$^{-/-}$ mice and CD4, CD8 and regulatory T cells were analyzed by FACS for their numbers and expression of different activation/memory markers. Summary data of two independent experiments are presented. One dot represents cell numbers of one mouse. Horizontal bars represent the mean for each group. Groups were compared using student t-test (ns= not significant; *=p<0,05; **=p<0,005; ***=p<0,0005).

3.3.4 Soggy deficiency leads to increased proliferation of CD4 T cells, while proliferation of CD8 T cells is not affected

The expressions of Soggy in immune privileged sites as well as the altered numbers of T cell populations in peripheral lymphoid organs indicate that Soggy is a candidate that may regulate the immune system. Therefore, it is of great interest whether T cell reactivity is affected in Soggy deficient mice. T cells from Dkk3$^{-/-}$ mice have been shown by our laboratory to possess an increased proliferation capacity under suboptimal stimulation conditions compared to T cells from wt mice [214]. In order to investigate, whether the proliferation of T cells of Soggy$^{-/-}$ mice is similarly altered, splenic T cells of wt, Dkk3$^{-/-}$ and Soggy$^{-/-}$ mice were purified, CFSE labeled and stimulated with different concentrations of plate bound anti-CD3 and anti-CD28. 48 hours after stimulation, proliferation was assed by analyzing the progressive halving of CFSE fluorescence upon cell divisions by FACS.

CD4 T cells of Soggy$^{-/-}$ mice exhibited a strongly increased proliferation rate than T cells of wt and Dkk3$^{-/-}$ mice (Figure 3.41). Also in this assay, even after T cell stimulation the lower CD44 expression level in CD4 T cells of Soggy$^{-/-}$ mice was noticeable (Figure 3.41 A) as investigated in the previous chapter.

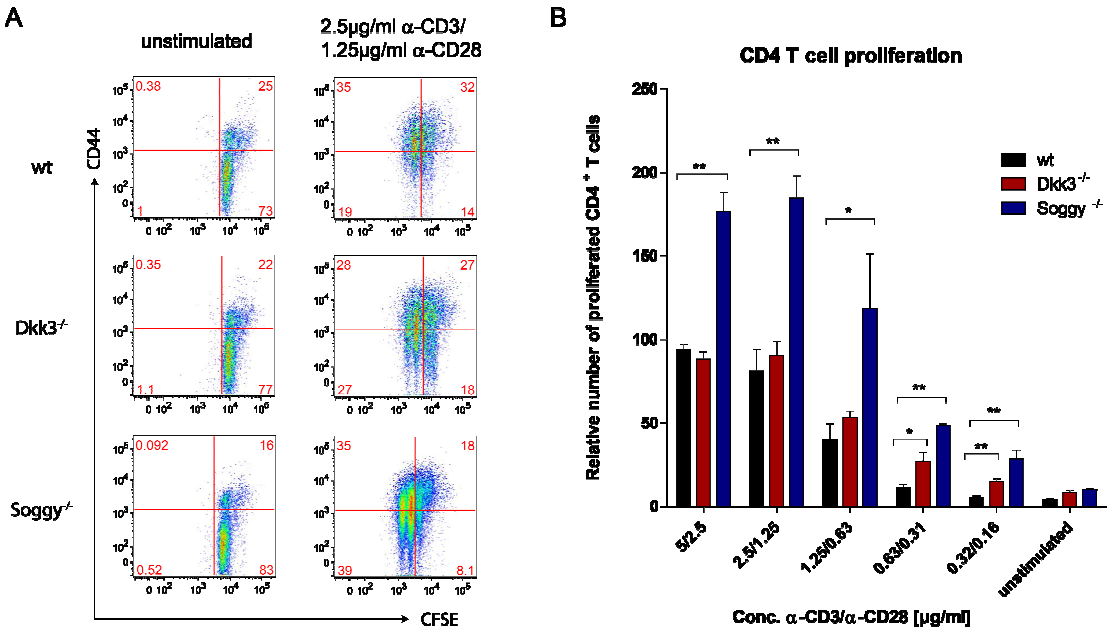

Figure 3.41 | CD4 T cells of Soggy$^{-/-}$ mice display increased proliferation *in vitro*. T cell were purified from spleens of C57BL/6 (wt), Dkk3$^{-/-}$, and Soggy$^{-/-}$ mice, labeled with CFSE and stimulated with titrated concentrations of anti-CD3 and anti-CD28. 48 hours later proliferation was measured by FACS analysis. Representative FACS blots are gated on CD4^{+} T cells and display CD44 expression against CFSE dilution (A). Numbers in the upper and lower left-hand quadrants indicate percentages of proliferated cells. Summary data show proliferated CD4^{+} T cell numbers per well (cells with a lower CFSE fluorescence that unstimulated cells), which were measured by FACS using Sphero Blank Calibration beads (B). In order to pool individual experiments, the highest cell number of proliferated wt CD4 T cells was set to 100 and all other cell numbers were calculated in relation. Summary data from 2 out of 4 independent experiments (n=6 per group) are presented. Mean and SEM are indicated for each group. Groups were compared using student t-test (*=p<0,05; **=p<0,005).

CD8 T cells of Soggy$^{-/-}$ mice did not show an altered proliferation rate compared to T cells of wt and Dkk3$^{-/-}$ mice (Figure 3.42). Similar to the CD4 T cells the lower CD44 expression level in Soggy$^{-/-}$ CD8 T cells was noticeable (Figure 3.42 A) as investigated in the previous chapter.

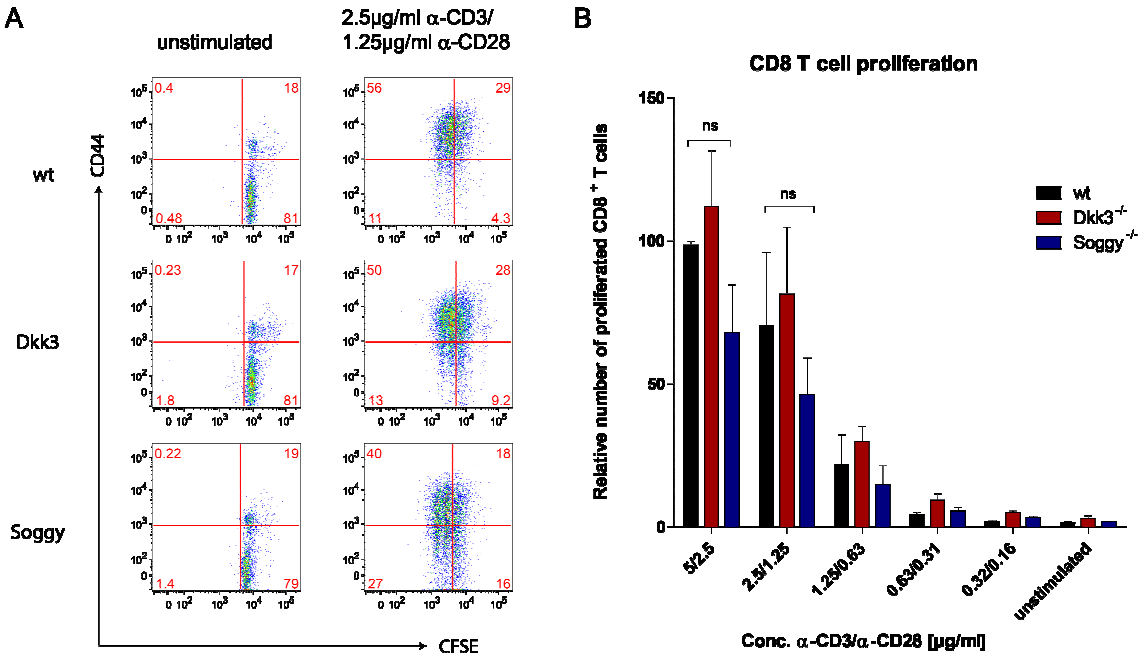

Figure 3.42 | Soggy deficiency does not affect CD8 T cell proliferation. T cell were purified from spleens of C57BL/6 (wt), Dkk3$^{-/-}$, and Soggy$^{-/-}$ mice, labeled with CFSE and stimulated with titrated concentrations of anti-CD3 and anti-CD28. 48 hours later proliferation was measured by FACS analysis. Representative FACS blots are gated on CD8^{+} T cells and display CD44 expression against CFSE dilution (A). Numbers in the upper and lower left-hand quadrants indicate percentages of proliferated cells. Summary data are depicted in B. Proliferated CD8^{+} T cells numbers per well (cells with a lower CFSE fluorescence that unstimulated cells) were measured by FACS using Sphero Blank Calibration beads. In order to pool individual experiments, the highest cell number of proliferated wt CD8 T cells was set to 100 and all other cell numbers were calculated in relation. Summary data from 2 out of 4 independent experiments are presented (n=6 per group). Mean and SEM are indicated for each group.

3.3.5 Soggy deficiency leads to increased IL-2 secretion of CD4 T cells

IL-2 is a cytokine commonly secreted upon T cell stimulation and acts as growth factor. Besides the increased proliferation capacity Dkk3$^{-/-}$ T cells have been described to secrete higher levels of levels of IL-2 compared to wt T cells [212]. In order to analyze whether this is also true for T cells from Soggy deficient mice, splenic T cells of wt, Dkk3$^{-/-}$ and Soggy$^{-/-}$ mice were purified and stimulated with different concentrations of plate bound anti-CD3 and anti-CD28. 24 hours later supernatants were analyzed for IL-2 levels by ELISA. T cells of Dkk3$^{-/-}$ mice produced approximately 1,5 fold higher levels of IL-2 compared to T cells of wt mice (Figure 3.43). Remarkably, the IL-2 production of T cells from Soggy$^{-/-}$ mice even exceeded the production of IL-2 by Dkk3$^{-/-}$ T cells. Soggy$^{-/-}$ T cells secreted approximately 2,4 fold higher levels of IL-2 than wt T cells (Figure 3.43).

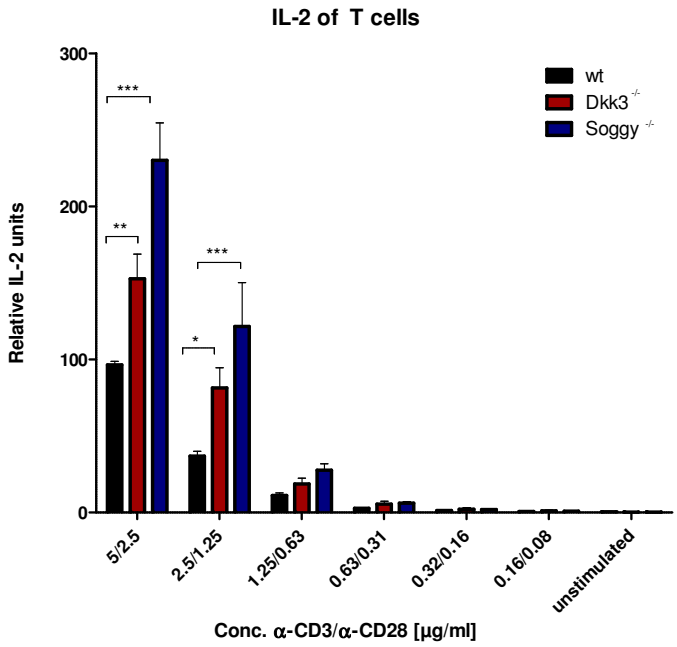

Figure 3.43 | T cells of Soggy$^{-/-}$ mice secrete higher levels of IL-2. T cell were purified from spleens of C57BL/6 (wt), Dkk3$^{-/-}$, and Soggy$^{-/-}$ mice and stimulated with titrated concentrations of anti-CD3 and anti-CD28. 24 hours later IL-2 was measured in supernatants by ELISA. In order to pool individual experiments, the highest IL-2 value of wt T cells was set to 100 and all other values were calculated in relation. Summary data from 3 independent experiments (n=6 per group) are presented. Mean and SEM are indicated for each group. Groups were compared using student t-test (*=p<0,05; **=p<0,005; ***=p<0,0005).

Soggy deficiency resulted in a significant reduction of CD8 T cell numbers in the spleen. In this assay purified T cells of spleens were used. CD4 T cells - not CD8 T cells - are mainly responsible for IL-2 secretion [261]. Hence, it may be argued that after T cell isolation, purified T cell cultures of Soggy$^{-/-}$ mice contained higher numbers of CD4 T cells compared to purified T cells from wt and Dkk3$^{-/-}$ spleens which might have lead to the observed effect. To exclude, that the higher secretion of IL-2 by Soggy$^{-/-}$ T cells is due to higher numbers of CD4 T cells being present in the stimulation assay, the assay was performed with purified CD4 T cells from wt and Soggy$^{-/-}$ mice.

Higher IL-2 secretion by CD4 T cells from Soggy$^{-/-}$ mice was still observable, when exactly same numbers of purified wt and Soggy$^{-/-}$ CD4 T cells were plated (Figure 3.44). Also here, CD4 T cells of Soggy$^{-/-}$ mice secreted more than twice the amounts of IL-2. Therefore, the increased production of IL-2 in T cell cultures of Soggy$^{-/-}$ mice was not due to higher numbers of CD4 T cells.

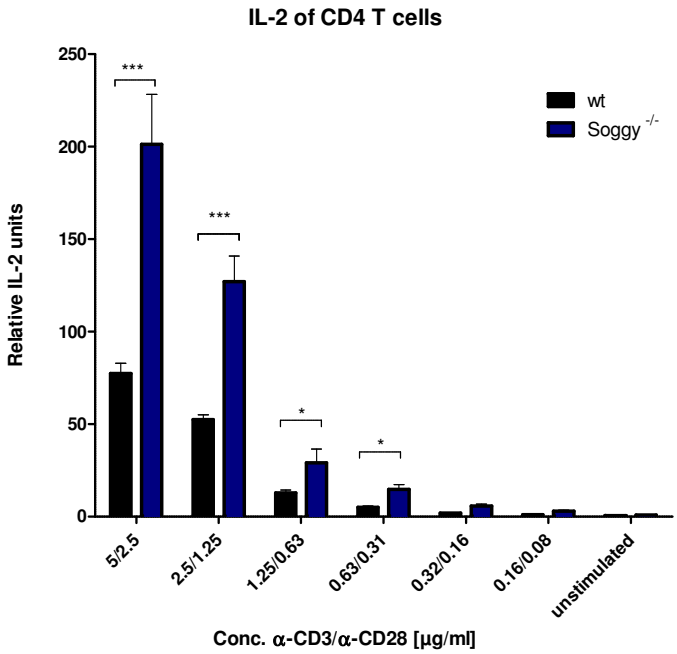

Figure 3.44 | Increased secretion of IL-2 in T cell cultures of Soggy$^{-/-}$ mice is not due to higher numbers of CD4 T cells. CD4 T cell were purified from spleens of C57BL/6 (wt) and Soggy$^{-/-}$ mice and stimulated with titrated concentrations of anti-CD3 and anti-CD28. 24 hours later IL-2 was measured in supernatants by ELISA. In order to pool individual experiments, the highest IL-2 value of wt T cells was set to 100 and all other values were calculated in relation. Summary data from 2 out of 3 independent experiments (n=9 per group) are presented. Mean and SEM are indicated for each group. Groups were compared using student t-test (*=p<0,05; **=p<0,005; ***=p<0,0005).

3.3.6 Increased IL-2 secretion of CD4 T cells might be due to decreased levels of regulatory T cells in Soggy$^{-/-}$ mice

Genetic deletion of Soggy resulted in significantly decreased numbers of Tregs in peripheral immune organs including the spleen. Tregs have been shown on one hand to inhibit IL-2 secretion of CD4 T cells and on the other hand to consume large amounts of IL-2 [262, 263]. Therefore, it is reasonable to investigate whether the observed effect of increased IL-2 secretion in T cell cultures of Soggy$^{-/-}$ mice is due to decreased numbers of Tregs being present in the stimulation assay. In order to analyze this, CD4 T cells were first purified of wt and Soggy$^{-/-}$ spleen suspensions and then depleted of CD25^{+} cells by MACS. Through this procedure, besides the small population of CD25^{+} effector T cells, mainly Tregs were depleted.

After depletion of CD25^{+} cells, CD4 T cells were stimulated and the resulting effector T cells of Soggy$^{-/-}$ mice produced the same amount of IL-2 as effector CD4 T cells of wt mice (Figure 3.45). Therefore, the effect of increased IL-2 secretion in T cell cultures of Soggy$^{-/-}$ mice seems to be due to decreased numbers of Tregs in the T cell cultures.

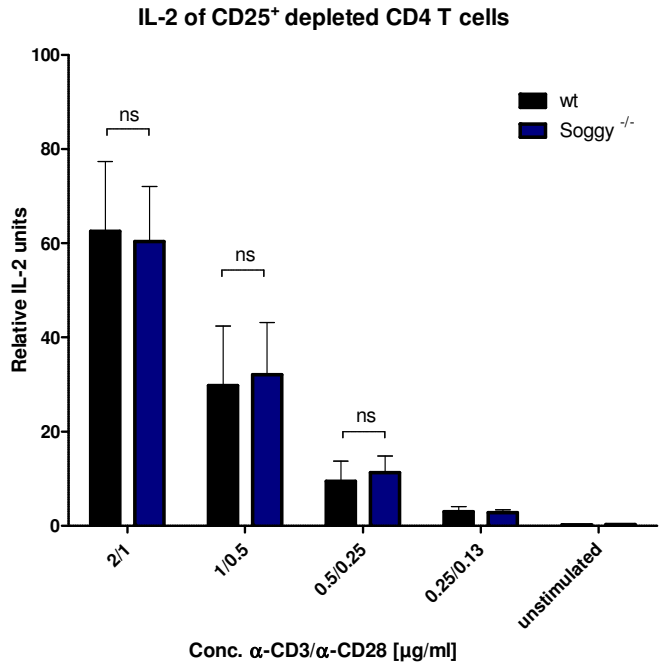

Figure 3.45 | Increased secretion of IL-2 in T cell cultures of Soggy$^{-/-}$ mice is due to lower numbers of regulatory T cells. CD4 T cell were purified from spleens of C57BL/6 (wt) and Soggy$^{-/-}$ mice depleted for CD25^{+} cells and stimulated with titrated concentrations of anti-CD3 and anti-CD28. 24 hours later IL-2 was measured in supernatants by ELISA. In order to pool individual experiments, the highest IL-2 value of wt T cells was set to 100 and all other values were calculated in relation. Summary data from 2 out of 3 independent experiments (n=6 per group) are presented. Mean and SEM are indicated for each group. Groups were compared using student t-test (*=p<0,05; **=p<0,005; ***=p<0,0005).

3.3.7 Soggy$^{-/-}$ mice display increased EAE symptoms

Above we showed that Soggy is expressed in the brain (3.3.1 Expression of Soggy on the mRNA and protein level). Dkk3 the structurally similar member of the Dkk protein family, also expressed in the brain, was shown to regulate EAE (3.1.1 Dkk3 regulates effector T cell subsets in experimental autoimmune encephalitis). Additionally, Tregs widely been shown to regulate autoimmune disease, are reduced in Soggy$^{-/-}$ mice. Therefore, these mice might be more prone to the development of autoimmune diseases including EAE as wt mice. For these reasons it was of great interest, whether Soggy is able to regulate the course of EAE similarly to Dkk3.

Wt, Dkk3$^{-/-}$ and Soggy$^{-/-}$ mice were immunized with MOG$_{33-55}$, treated with pertussis toxin and EAE was measured for 3 weeks. Strikingly, Soggy$^{-/-}$ displayed an earlier onset and increased symptoms of EAE (Figure 3.46) compared to wt. EAE symptoms were even stronger in Soggy$^{-/-}$ mice than in Dkk3$^{-/-}$ mice.

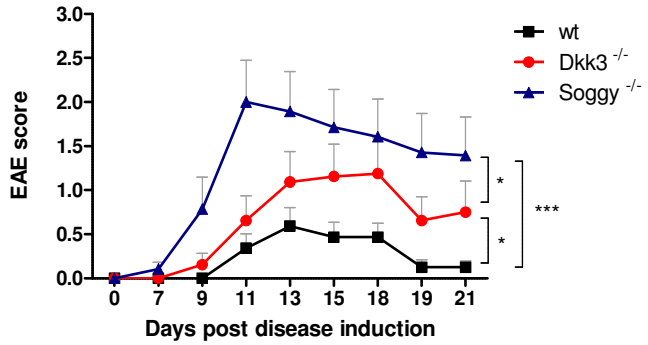

Figure 3.46 | Soggy$^{-/-}$ mice display increased symptoms of EAE. C57BL/6 (wt), Dkk3$^{-/-}$ and Soggy$^{-/-}$ mice were immunized with MOG_{33-55} in CFA and treated with pertussis toxin. EAE symptoms were measured in for 21 days. Summarized data of two out of three independent experiments with similar results are shown. Mean EAE score and SEM are shown for each group (n=16). Curves were compared using 2-way ANOVA, p-value of column factor is shown (*=p<0,05, ***=P<0,0005).

In summary the experiments investigating the role of Soggy in the immune system, revealed that Soggy$^{-/-}$ mice have an immunological phenotype. The expression of Soggy is restricted to only few tissues, the testis, the brain and the skin, which belong to the immune privileged sites. Furthermore, T cells in the periphery of Soggy deficient mice were altered. Most strikingly expression of CD44 was downregulated and numbers of Tregs were reduced. Analyzing T cell function, we found increased proliferation of CD4 T cells as well as increased secretion of IL-2 in T cell cultures of Soggy$^{-/-}$ mice, which appears to be due to decreased numbers of CD25^{+} cells. Furthermore, Soggy$^{-/-}$ mice displayed increased symptoms of EAE compared to wt and Dkk3$^{-/-}$ mice.

4 Discussion

Dkk3 has previously been shown by our laboratory to be necessary for peripheral T cell tolerance mediated by regulatory CD8 T cells in a transgenic model [214]. In the first two parts of this thesis, the function of Dkk3 on the two lymphocyte populations - T and B cells - of the adaptive immune system was studied. As the failure of tolerance induction and/or maintenance might lead to autoimmunity, we investigated models of T and B cell mediated autoimmune disorders in the absence of Dkk3 in a polyclonal T and B cell repertoire. In the last part of the thesis, we addressed the question whether the sgy domain might confer the immune-modulatory function to Dkk3. Our interpretations and the significance of the findings made in the three parts of the thesis will be discussed below.

4.1 Dkk3 regulates T cell mediated autoimmunity in experimental autoimmune encephalitis

Experimental autoimmune encephalitis (EAE) is a T cell mediated autoimmune disease resembling human multiple sclerosis. It is initiated in mice upon priming of T cells with central nervous system (CNS)-derived antigens in the presence of an adjuvant [264]. In our studies, a peptide containing the amino acids 33-55 of myelin oligodendrocyte protein (MOG_{33-55}) emulsified in complete Freund's adjuvant (CFA) was used for active disease induction. Alternatively to this actively-induced EAE, the disease can be passively induced by transferring T cells which were activated *in vitro* with CNS-derived antigens [264]. We used *in vitro* restimulated transgenic T cells with specificity for MOG (2D2 T cells) for passive EAE induction. In both strategies of EAE induction mice were treated with pertussis toxin (PT), which facilitates the entry of activated MOG-specific T cells into the brain through the neurovasculature juncture, also called blood-brain barrier [265]. There, the T cells release cytokines, which lead to the activation of microglia secreting high amounts of cytokines and chemokines [266]. As a result of the inflammatory process the blood-brain barrier will be disrupted and a second wave of leukocytes including blood-borne macrophages, dendritic cells (DCs) and granulocytes are recruited to the brain. These cells contribute to the pathogenic T cell response by presenting myelin antigens to T cells and secreting further proinflammatory cytokines triggering the cascade. Eventually, neurotoxic molecules, such as reactive oxygen species (ROS), are secreted by microglia and infiltrated leukocytes, and lead to acute focal inflammatory demyelination and axonal loss [72, 267].

Studies of the EAE model have helped to subdivide the sequence of these immunopathological events into two major phases; the initial T-cell priming/activation phase and the subsequent recruitment and effector phase [267]. However, it is difficult to define the time frame of these phases by only outwardly looking at the EAE scores, as both, the activation phase and the recruitment/effector phase take place before disease is peaking. Additionally, in contrast to human multiple sclerosis, EAE in mice is mostly self-limiting. Therefore, a third phase of disease can be defined, the recovery phase. It is characterized by the control of inflammation, remyelination and recovery from EAE. The terms of the different phases as used in this thesis can be found in Figure 4.1.

The comparison between actively immunized Dkk3 sufficient and deficient mice revealed that lack of Dkk3 leads to more severe and persistent EAE symptoms in the later stages (Figure 3.1). The initiation phase of EAE did not seem to be affected by Dkk3 as the onset of EAE was occurring at the same time point in Dkk3 deficient and sufficient animals. Therefore, we assumed that the recruitment/effector phase and/or the recovery phase are regulated by Dkk3. It is not clear why the onset of disease was taking place at an earlier time point when EAE was passively induced in $Dkk3^{-/-}$ $Rag2^{-/-}$ mice compared to wt.$Rag2^{-/-}$ mice. Importantly, the passive induction of EAE in Dkk3 sufficient and deficient $Rag2^{-/-}$ mice demonstrated that environmental Dkk3 alone is able to dampen disease symptoms. This supports the idea that the difference between wt and $Dkk3^{-/-}$ mice observed when EAE was actively induced is not due to a different T cell pool initiating the disease but due to the regulation of the immune response by environmental Dkk3 present in the CNS.

To investigate in detail how Dkk3 contributes to the control of EAE, leukocytes in the CNS and in the periphery (spleen and lymph nodes (LN)) of actively EAE induced mice were analyzed at the peak of disease/rising phase and in the recovery phase. At the peak of disease/rising phase, mice of both groups were equally affected by the disease, whereas in the recovery phase Dkk3 deficient mice were clearly suffering from more severe EAE symptoms. In our analysis we were interested in different factors which have been demonstrated to play a role in the immunopathology of EAE.

Myelin-reactive CD4 T cells are widely accepted to be the key players in the development of EAE [267]. The role of CD8 T cells still remains controversial [268, 269]. While several groups clearly demonstrate the contribution of CD8 T cells to the pathology of the disease [91, 270, 271], other groups report that CD8 T cells regulate the disease [272-274].
At the peak of disease/rising phase no difference in numbers of CD4 and CD8 T cells was found in the CNS and in the periphery (Figure 3.3), which was not surprising as

EAE scores were similar in wt and Dkk3$^{-/-}$ mice. In the recovery phase, in which both groups clearly differed in EAE symptoms, higher numbers of CD4 and CD8 T cells were detected in the CNS, but also in the spleen (Figure 3.4). This finding suggests that Dkk3 either controls survival by actively inducing apoptosis or that it inhibits the proliferation of T cells. Both ways of action would lead to increased numbers of CD4 and CD8 T cells when Dkk3 is absent, and increased numbers of T cells most likely contribute to the persistent symptoms of EAE in Dkk3 deficient animals.

The activity of Tregs is now recognized as a strong regulatory mechanism in the control of autoimmune diseases [89, 275]. Analyzing the numbers of Tregs in our experimental system surprisingly revealed higher numbers of Tregs at the peak of disease/rising phase in the CNS of Dkk3$^{-/-}$ mice, which was not observed for the recovery phase (Figure 3.3/Figure 3.4).
It is difficult to imagine, why higher numbers of Tregs were observed at the peak of disease/rising phase in Dkk3$^{-/-}$ mice, although these are the mice, which will eventually not recover from EAE symptoms as fast as the control mice.
One possible explanation could be that Tregs are expanded in the CNS of Dkk3$^{-/-}$ mice as a compensatory mechanism to prevent the stronger disease progression observed in these animals. However, this mechanism might be effective only in the rising phase, as no difference in EAE scores were observed between the two groups of mice in this phase. In the recovery phase then, Dkk3$^{-/-}$ mice suffered from more severe symptoms of EAE compared to wt mice. In that phase no compensation of EAE pathology by Tregs might take place in Dkk3$^{-/-}$ mice as numbers of Tregs were similar in Dkk3$^{-/-}$ and wt animals.
Alternatively, Tregs might be increased in Dkk3$^{-/-}$ mice due to a lack of regulation by Dkk3. This hypothesis is raised by the finding that also IL-17 expressing CD4 T cells were present at higher numbers only at the peak of disease/rising phase in the CNS and not in the recovery phase of Dkk3 deficient mice. It has been shown that TGF-β is necessary for the differentiation of both cell types, Tregs and Th17 T cells [276]. It is possible that Dkk3 acts on TGF-β availability or activity. Indeed, Pinho *et al.* and Hsu *et al.* showed a connection between the TGF-β pathway and Dkk3 in xenopus and zebrafish development [277, 278]. Supporting the importance of TGF-β for disease induction, Veldhoen clearly demonstrated that TGF-β signalling is required for initiation of EAE, as mice with defective TGF-β signalling neither develop the Th17 subset nor succumbed to EAE [279]. Furthermore, Tregs have been shown *in vitro* to be a sufficient source of TGF-β for the induction of Th17 cells [279, 280] and just recently it was shown that also *in vivo* Tregs promote Th17 cell development [262]. Therefore, it would be interesting to explore in future studies whether the increased level of Tregs in Dkk3$^{-/-}$ mice is connected to the increased level of Th17 cells and whether and how exactly Dkk3 acts on TGF-β signalling in T cells.

Th17 cells have clearly been demonstrated to be important for the development of autoimmune diseases, such as multiple sclerosis and EAE [281-284]. Whether the increased numbers of Th17 cells in the CNS of Dkk3$^{-/-}$ mice are causative for the severe and prolonged EAE symptoms in our model is unclear. It is possible that they contribute to the severity of the symptoms in the recruitment/effector phase, as there (at the peak/rising phase) the number of Th17 cells was specifically increased within the CD4 T cell population in Dkk3$^{-/-}$ mice (Figure 3.3). In the recovery phase, no difference in population frequency within the CD4 T cell subset was observed (Figure 3.4). However, as the number of CD4 T cells was increased in Dkk3$^{-/-}$ brains in the recovery phase, also IL17^{+} and other cytokine secreting cells will be present in higher numbers and may contribute to the damage.

IFN-γ is regarded traditionally as the signature cytokine of Th1 immune responses and as these cells were found to be implicated in the pathogenesis of autoimmune diseases [285-288], it was believed to be inflammatory and therewith destructive [289]. Early evidence however indicated an opposing, protective role for IFN-γ. In these studies IFN-γ deficient mice were found to be more susceptible to EAE than IFN-γ sufficient mice [290, 291]. Therefore, the function of IFN-γ in autoimmune disease is still controversial.
In this thesis, IFN-γ expressing T cells were found at equal numbers in wt and Dkk3$^{-/-}$ mice in the periphery and the CNS at the peak of disease/rising phase (Figure 3.3). In the recovery phase higher numbers of both CD8 and CD4 T cells secreting IFN-γ were found in the CNS of Dkk3$^{-/-}$ mice (Figure 3.4). Two scenarios can be imagined to explain the increased number of IFN-γ secreting T cells in the CNS of Dkk3$^{-/-}$ mice in this phase. In the first scenario Dkk3 directly controls IFN-γ production, leading to increased levels of destructive IFN-γ in Dkk3 deficient mice, which leads to increases symptoms of EAE. In the second scenario IFN-γ acts as a regulating factor and is upregulated in the late phase of EAE as a compensatory mechanism to dampen the stronger EAE progression in Dkk3$^{-/-}$ mice. Feuerer *et al.* showed that IFN-γ acts as a proinflammatory cytokine in the early phase of disease, whereas in the late phase IFN-γ controls antigen-specific immune responses [292]. As in our model the increased expression of IFN-γ in the absence of Dkk3 was observed during the late phase of EAE, the second hypothesis is favored in our laboratory.

GM-CSF has recently been reported by Bechers group to be necessary for disease induction [47]. Autoreactive helper T cells lacking GM-CSF failed to initiate neuroinflammation despite expression of IL-17A or IFN-γ, whereas GM-CSF secretion by IFN-γ/IL-17 negative CD4 T cells was sufficient to induce EAE. The expression of GM-CSF was driven by the Th17 cell supporting cytokine IL-23 and the Th17 master transcription factor RoRγt. Therefore, it can be assumed that these GM-CSF secreting cells in the initiation phase of EAE are a special form of Th17

cells. In our model of EAE, no difference was found in CD4 T cells expressing the cytokine GM-CSF at both analyzed time points between wt and Dkk3$^{-/-}$ mice. Hence, a mechanism employed by Dkk3 involving the regulation of GM-CSF expression by T cells can be ruled out in the control of EAE.

It is unlikely that T cells are directly responsible for the demyelination and axonal loss. Instead, myeloid cells are supposed to be the damaging cell type in EAE due to their arsenal of toxic molecules, which they are able to secrete [266]. Indeed, Codarri *et al.* demonstrated that neuroinflammation was sustained via CD11b^{+} myeloid cells by GM-CSF secreting T cells [47]. Therefore, numbers of myeloid cells in diseased CNS and in the periphery were compared between Dkk3 sufficient and deficient mice. Dkk3$^{-/-}$ animals, suffering from more severe EAE symptoms in the recovery phase, displayed increased numbers of CD11b^{+} cells in the CNS (Figure 3.4). In the spleen significantly reduced numbers of CD11b^{+} cells at both analyzed time points were present in Dkk3$^{-/-}$ mice (Figure 3.3/Figure 3.4), indicating the migration of myeloid cells from the periphery to the brain. Most probably, these cells are contributing to increased and prolonged symptoms of EAE in Dkk3$^{-/-}$ mice. However, we can not distinguish, whether these myeloid cells are directly affected by Dkk3 or whether their increased migration into the brain is a consequence of the T cell triggered cascade, which was also found to be aggravated in Dkk3$^{-/-}$ mice.

Concluding, the following scenario can be imagined: MOG specific T cells are activated in the periphery by immunization. They migrate through the blood-brain barrier and enter the brain, thereby inducing the disease. In Dkk3 sufficient animals environmental Dkk3 affects survival or proliferation of T cells in the CNS. This eventually leads to lower EAE scores and a faster recovery from EAE of wt animals.
As Dkk3 deficiency leads to increased numbers of CD4 and CD8 T cells in the CNS in general in the recovery phase, higher numbers of IL-17, IFN-γ and GM-CSF producing T cells will be present. These may contribute to aggravated disease in the late phase. In this late phase only the proportion of IFN-γ secreting T cells was selectively increased within the CD4 T cells, indicating the existence of a connection between Dkk3 and this cytokine.
The increased T cell response in Dkk3 deficient mice will attract myeloid cells from the periphery to the CNS contributing to increased EAE symptoms in Dkk3 deficient mice. Alternatively, Dkk3 could also directly influence the migration of these cells.
Whether Dkk3 acts directly or indirectly on the discussed T cells *in vivo* remains to be determined by future studies. The main findings made in this chapter are summarized in a model of Dkk3 action on the immune response in the different phases of EAE (Figure 4.1).

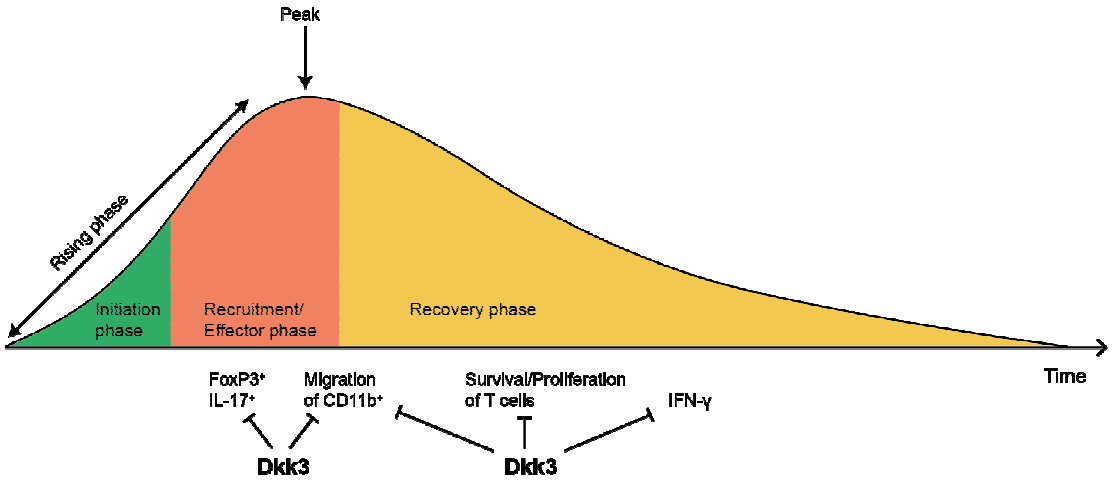

Figure 4.1 | Model of Dkk3 action on the immune response in the different phases of EAE. The curve demonstrates the typical course of EAE observed in Dkk3 sufficient mice. The terms used in this thesis for the different phases are indicated. Dkk3 limits the number of FoxP3$^+$ and IL-17$^+$ cells in the recruitment/effector phase of EAE. Furthermore, it inhibits the migration of CD11b$^+$ myeloid cells from the periphery into the brain, which starts in the recruitment phase/effector phase and continuous until the recovery phase. The T cell response is dampened by Dkk3 in the recovery phase by decreasing the proliferation and IFN-γ secretion of T cells.

4.2 Dkk3 does not regulate the pathogenic T cell response in a model of inflammatory bowel disease and diabetes type 1

The analysis of EAE in Dkk3 deficient mice showed that Dkk3 is able to regulate pathogenic T cells. Hence, it was interesting to explore, whether this is also true for other autoimmune diseases. As Dkk3 was shown to be expressed in the intestine [210] and in the β-islet cells of the pancreas [192], we decided to analyze the function of Dkk3 on T cells in a model of inflammatory bowel disease (IBD) and a model of diabetes type 1.

The term IBD covers a group of disorders in which the intestine is attacked by autoimmune T cells. However, it is not yet clear whether these T cells are responsible for the initiation of tissue destruction or whether their contribution is a consequence of tissue inflammation caused by other factors [293]. To address immune-regulatory mechanisms in the gut, an adoptive transfer model, which employs immune deficient hosts, has been developed and is widely used [294]. In this

model the disease is T cell initiated. Flavell's group furthermore showed that colitis induced by $CD4^+CD45RB^{high}$ cells exhibits a higher Th1 response compared with Treg depleted $CD4^+CD45^{RBlow}$ cells, which showed an increased Th17 response [295]. We decided to not discriminate between CD45RB and to use CD4 T cells depleted of Tregs in order to allow both types of T cell responses to take place.

Diabetes type 1, also called nonobese diabetes, is an autoimmune disease, in which cellular infiltration and immune-mediated destruction of the insulin-producing islets of Langerhans precedes the onset the disease [296]. Different animal models have evolved in the past years. The best studied models of human autoimmune diabetes involve mice of the non-obese-diabetic (NOD) background [296]. We decided to use an adoptive transfer model, in which naïve CD4 T cells with TCR specificity to ß-islets (T cells from Bdc2.5 mice) were Treg depleted and transferred to immunodeficient mice in the NOD background.

Neither in the mouse model of IBD nor in the model of diabetes type 1, absence of Dkk3 led to increased disease progression (Figure 3.5/Figure 3.6). Therefore, we concluded that Dkk3 is not able to regulate the pathogenic T cell response in these models. One explanation could be the relatively low expression level of Dkk3 found in the intestine and the pancreas (Figure 3.7) in comparison to the brain, where Dkk3 was found to be expressed 300-500 times higher. Lack of increased disease symptoms in $Dkk3^{-/-}$ mice could also be explained by the very robust T cell response already taking place in Dkk3 sufficient animals in these models. This robust T cell response might not be controllable by Dkk3 and therefore the absence of Dkk3 would not lead to increased disease symptoms. We tried to decrease the disease threshold in the diabetes type 1 model by transferring fewer cells (data not shown). However, only a delay of disease onset was achieved. We can not rule out, that in other models of diabetes or IBD, where disease development takes longer or/and disease symptoms are rather moderate, disease would be increased in a Dkk3 deficient environment.

4.3 Dkk3 limits Th2 associated cytokine secretion

Increased numbers of IFN-γ and IL-17 positive cells were found in the CNS of Dkk3 deficient mice. IFN-γ is the signature cytokine of Th1 immune responses and IL-17 the signature cytokine of the Th17 immune response [38]. To address the question, whether Dkk3 influences the differentiation of T cells, two different approaches were started.

In the first approach, we investigated whether T cells coming from a Dkk3 deficient environment would differentiate into higher or lower numbers of specific T subsets *in*

vitro under specific differentiating conditions (Figure 3.8). Surprisingly, under Th1 differentiating conditions, we found lower numbers of IFN-γ positive Th1 cells when T cells were coming from Dkk3 deficient mice. Conversely, under Th2 differentiating conditions, T cells from Dkk3 deficient mice exhibited slightly increased numbers of IL-10 secreting Th2 cells, although the number of IL-10 positive T cells was very low in our assay. The differentiation of Th17 cells was not found to be altered by Dkk3.

As Dkk3 has been shown not to be expressed by T cells themselves [212], we found it important to also analyze T cell differentiation *in vivo* in a second approach. For this purpose we made use of the properties of complete and incomplete Freund's adjuvant (CFA and IFA). Antigens immunized in CFA have been described to preferentially lead to an immune response of the Th1/Th17 direction, whereas antigens in IFA lead to a reduced Th1 response and an increased immune response of the Th2 direction [232-234]. Confirming the *in vitro* data, analysis of *in vivo* differentiation in $Dkk3^{-/-}$ deficient mice also revealed that Th2 responses might be increased in the absence of Dkk3. This was indicated by increased levels of IL-10 and IL-4 independent of immunization, and decreased levels of IL-17, when immunized with MOG_{33-55} in IFA (Figure 3.9/Figure 3.10). However, no difference was found in Th1/Th17 differentiation between $Dkk3^{-/-}$ and wt mice when mice were immunized with antigen in CFA, as levels of IFN-γ and IL-17 were similar (Figure 3.9).

Interestingly, the cytokine IL-6 was increased in $Dkk3^{-/-}$ mice when mice were immunized with antigen in CFA (Figure 3.10). IL-6 is a cytokine produced by a number of cell types including fibroblasts, macrophages, dendritic cells, T and B lymphocytes, endothelial cells, and keratinocytes in response to a variety of external stimuli (e.g. IL-1, TNF, and PDGF) [236]. It has been shown to shift the Th1/Th2 balance towards the Th2 direction by promoting IL-4 production and Th2 differentiation, and by inhibiting IFN-γ production and Th1 differentiation [236]. However, other studies demonstrated that IL-6 is a cytokine important for the differentiation of Th17 cells [297, 298]. In our system the Th2 response was increased and the Th17 response decreased in Dkk3 deficient mice immunized with antigen in IFA. Therefore, IL-6 seems to rather contribute to the Th2 response in $Dkk3^{-/-}$ mice.

In summary, our data suggest that Dkk3 might influence T cell differentiation by limiting Th2 responses. As in EAE the Th1 and Th17 response was increased in the absence of Dkk3, we can conclude that this seems not to be due to an increased differentiation of Th1/Th17 cells in a Dkk3 deficient environment.

4.4 Dkk3 regulates B1 cell numbers and function

B cells are subdivided into different subsets, which exhibit different functions in the immune system. Interestingly, Dkk3 deficiency led to approximately twice the number of B1 cells found in wt mice, and a reduction of B2 cells of approximately 30% (Figure 3.18). The B1 subsets consist of B1a and B1b cells, which both were found to be increased in Dkk3$^{-/-}$ mice (Figure 3.21). The reduction of B2 cells was due to decreased numbers of follicular (FO) B cells, whereas numbers of marginal zone (MZ) B cells were similar in Dkk3 deficient and sufficient mice (Figure 3.20).

These findings explain why we observed increased B cell antibody responses to B1 antigens in Dkk3$^{-/-}$ compared to wt mice. One of the most typical B1 antigens is phosphorycholine (PC), an autoantigen being part of many phospholipids within cell membranes [132, 299]. Immunization with this antigen led to highly significantly increased IgM and IgG levels in Dkk3$^{-/-}$ mice (Figure 3.14) demonstrating clearly the increased response to the B1 cell antigen in Dkk3 deficient mice. In order to analyze the response to LPS, total IgM and LPS-specific IgM levels were determined after LPS injection, as LPS has the ability to activate B cells polyclonally by TLR stimulation and specifically by binding to LPS-specific BCRs. The polyclonal stimulation by LPS activates all B cells polyclonally independent of BCR and subset [154, 240], whereas LPS-specific BCRs are mainly found in the B1 subset [241]. Strikingly, in this experiment the LPS-specific IgM response was significantly increased in Dkk3$^{-/-}$ mice while the polyclonal response was not affected by Dkk3 deficiency (Figure 3.13). This confirms the finding that B1 specific antibody responses are stronger in the absence of Dkk3.

Barrantes *et al.* described that total IgM levels of Dkk3 deficient mice are increased in the steady state [189]. This observation was confirmed in our studies (Figure 3.13, A, Day0). As B1 cells are responsible for the steady state level of natural IgM [133], the increased IgM levels of Dkk3$^{-/-}$ mice is explainable by the increased B1 cell numbers in Dkk3$^{-/-}$ mice. This is further supported by the findings that IgM levels of transferred B cells into immunodeficient hosts correlate perfectly with B1 cell numbers (Figure 3.24).

The response to TNP-Ficoll, an antigen which has been demonstrated to activate MZ B cells [157], was not altered in Dkk3$^{-/-}$ mice (Figure 3.12). This is not surprising, as we found no differences in MZ B cell numbers between Dkk3 sufficient and deficient mice (Figure 3.20).

TNP-BSA is regarded as a thymus dependent (TD) B cell antigen activating FO B cells [300, 301]. We observed increased TNP-BSA specific IgM levels in Dkk3

deficient mice already in the steady state (Figure 3.11, A, Day0). How can we explain this phenomenon, since FO B cell numbers were decreased? When looking at the antigen TNP-BSA more in detail, it can be recognized that the TD response will be due to the BSA part, which is able to be processed and presented by MHCII molecules to T cells. However, the TNP part might activate B1 B cells leading to TNP-specific antibodies, which could not be distinguished from BSA-specific antibodies in our analysis. This argument is supported by early studies showing that TNP-specific B cells arise early in ontogeny [302, 303], a hallmark of B1 cells [133], and that anti-TNP antibodies display characteristic features of naturally occurring autoantibodies [304], whose secretion is another hallmark of B1 cells [132, 133]. Hence, it is likely that higher numbers of TNP-BSA specific IgM levels in the steady state are due to the same reason why total IgM levels were increased (as described above). As the IgM response to this antigen was weak, IgM levels did not change drastically on day 7 post immunization and the difference was still observed between wt and Dkk3$^{-/-}$ mice. On day 14 no significant difference between Dkk3 sufficient and deficient mice was observed.
Furthermore, TNP-BSA specific IgG1 and IgG3 levels were significantly increased in Dkk3 deficient animals upon immunization, whereas the TNP-BSA specific IgG2a and IgG2b response was not affected by Dkk3 (Figure 3.11). IgG3 is an antibody typically produced by B1 cells after class switch recombination (CSR) [132]. Therefore, this finding is most likely due to the increased B1 cell numbers. Also IgG1 antibodies have been reported to be expressed by B1 cells, and in the same study B1 cells were implicated to be enhanced by T cell help [156]. This could also account for B1 cell secreted IgG1. However, we can not rule out that FO B cells lead to increased TNP-BSA specific antibody levels in Dkk3 deficient mice, as TNP-BSA activates not preferentially B1 cells. Nevertheless, we can conclude that despite the decreased numbers of FO cells present in Dkk3$^{-/-}$ mice the response to TD antigens is not impaired. Thus, the reduction of FO B cell numbers in Dkk3$^{-/-}$ mice does not seem to have a dramatic consequence for immune responses. On the contrary, the approximately double number of B1 cells present in Dkk3$^{-/-}$ mice influences B1 related immune responses.

The next B cell function we explored to be dependent on Dkk3 was cytokine secretion. We started this analysis with performing a small screen, in which we found that MCP-1 (CCL2) and IL-10 are increased in supernatants of stimulated splenocytes from Dkk3$^{-/-}$ mice compared to wt mice (Figure 3.15). MCP-1 turned out not to be secreted by B cells in our assay system, as later experiments conducted with purified B cells showed no detection of MCP-1 in supernatants. Nevertheless, it is interesting that this chemokine is increased in supernatants of Dkk3$^{-/-}$ splenocytes, since it has been shown to promote Th2 differentiation [305], which we in fact observed to be increased in Dkk3$^{-/-}$ mice detected by T cell differentiation analysis

(as described above). Also the cytokine IL-10 is known to drive Th2 differentiation [306, 307], which might as well contribute to the increased Th2 phenotype in Dkk3 deficient mice.

The increased IL-10 level in supernatants of Dkk3$^{-/-}$ splenocytes was confirmed to be B cell derived (Figure 3.16). When comparing IL-10 levels in supernatants of purified B cells from spleen with peritoneal cavity (PerC), we found that PerC B cells secreted enormously higher amounts of IL-10 (Figure 3.16/Figure 3.17). In the literature B1 cells have been shown to secrete large amounts of IL-10 [174], and as the PerC is besides the pleural cavity the compartment with the largest numbers of B1 cells [133], it is not surprising, that we detected such high levels of IL-10 in supernatants of PerC B cells. The difference observed between wt and Dkk3$^{-/-}$ B cell derived IL-10 levels was more pronounced when using PerC B cells as when using splenic B cells in the assay (Figure 3.16/Figure 3.17). This fits with the finding that also the increased number of B1 cells in Dkk3$^{-/-}$ mice was more pronounced within the PerC compartment compared to the spleen (Figure 3.18). That the secreted IL-10 was in deed B1 cell derived in our system was demonstrated by analyzing its expression on a single cell level (Figure 3.17). Only B1 cells expressed the cytokine IL-10. Additionally, this analysis showed that that the increased IL-10 levels detected in B cell supernatants of Dkk3$^{-/-}$ mice are not only due to higher numbers of B1 cells present in Dkk3$^{-/-}$ mice, but also due to higher numbers of IL-10 secreting cells within the B1 cell subset in Dkk3$^{-/-}$ mice. Therefore, this analysis indicated that not only the number of B1 cells is regulated by Dkk3 but also their threshold of activation.

Concluding, Dkk3 deficiency leads to decreased numbers of FO B cells and higher numbers of B1 cells. So far no defects of B cell function were found due to the decreased number of FO B cells in Dkk3$^{-/-}$ mice. Presumably, the remaining approximately 75% of FO B cells are enough to compensate the reduction. However, the approximately double number of B1 cells in Dkk3 deficient mice leads to an increased B1 cell function demonstrated by the increased antibody response to B1 cell specific antigens and the increased production of B1 derived IL-10.

4.5 Dkk3 regulates B cell development and maintenance

In order to answer the question why numbers of B cells were altered in Dkk3$^{-/-}$ mice in comparison to wt mice, B cell development and maintenance was investigated. FO B cells are generated during the whole lifetime from the BM, sustaining the pool of peripheral B cells, whereas B1 cell development occurs early in ontogeny and does poorly take place in the adult BM [141, 142]. In adult mice B1 cells are maintained by continuous self-renewal taking place in the periphery [133].

The investigation of B cell development was started by analyzing neonatal spleen, as there the final steps of B1 cell development are still visible [139]. Interestingly, we found decreased numbers of immature cells (in the periphery called transitional) in the spleen as well as in the PerC of Dkk3$^{-/-}$ mice (Figure 3.22). Although the PerC was supposed to mainly contain mature cells [247], this was not confirmed in our experiment, as large B cell numbers expressed the molecule CD93, a surface marker commonly expressed by B cell progenitors [4]. Mature cells were found as a minor population in spleen and PerC. There, the B1 cell population was already increased in Dkk3$^{-/-}$ mice, whereas the FO B cell population was not yet decreased at this time in ontogeny in Dkk3$^{-/-}$ mice (Figure 3.22). This indicates that B cell development might be impaired due to Dkk3 deficiency before or at the step to immature B cell generation and not at a later step, as FO B cell numbers would then be decreased directly, which was not the case. As already higher numbers of B1 cells were found in neonatal Dkk3$^{-/-}$ spleens, B1 cell development does not seem to be impaired. It indicates that the impairment of B cell development lies within the FO B cell subset.

To further investigate the impairment of B cell development due to a deficiency of Dkk3, BM of adult mice was analyzed. The investigation of adult BM has the advantage that the all developmental stages of B cells can be found in this site, whereas in the neonatal spleen only immature until mature stages can be found.
Similar to the neonatal spleen reduced numbers of immature B cells were found in Dkk3$^{-/-}$ BM and numbers of mature FO B cell were not altered in comparison to wt BM (Figure 3.23). Also, the number of B1 cells was found to be increased in the BM of Dkk3 deficient mice. Interestingly, numbers of pre-B cells were equal in Dkk3 sufficient and deficient mice, demonstrating that the impairment of B cell development observed in Dkk3$^{-/-}$ mice occurs after this developmental stage. In addition, we even found higher numbers of prepro- and pro-B cells is Dkk3$^{-/-}$ mice, which could be caused by the failure of pre- B cells to further continue their maturation program, leading to an accumulation of prepro- and pro-B cells is Dkk3$^{-/-}$ mice. These data indicate that B cell development is impaired at the maturation step from pre- to immature B cells.
The finding that numbers of mature FO B cells were not different between wt and Dkk3$^{-/-}$ mice in sites of development (neonatal spleen/adult BM) demonstrates that the maturation step from immature to mature B cells is not impaired. However, it raises the question why numbers of mature FO B cells are then decreased in the periphery in adult Dkk3$^{-/-}$ mice. One possible explanation could be that the BM yields mechanisms to release B cells to the periphery only after a certain number of mature B cells is reached. As B cell development is impaired in Dkk3$^{-/-}$ mice, this process would be delayed and less mature FO B cells would be released to the periphery.

BM B cell development in adult mice mainly mirrors B2 development. Therefore, the analysis of adult BM indicated that in the absence of Dkk3 FO B cell generation is impaired without dampening B1 cell development. However, it was still unclear, which mechanism is responsible for the increased B1 cell numbers in Dkk3 deficient mice. Is Dkk3 actively regulating B1 cell numbers?

This question can not easily be investigated, since the mechanisms of B1 cell development are not yet fully understood. As presented in the introduction two main hypotheses have evolved: the selection model and the lineage model, which nevertheless do not need to be mutually exclusive as proposed by Baumgarth [133]. B1 cells arise early in ontogeny and are then maintained by self-renewal [139]. Hence, in $Dkk3^{-/-}$ mice B1 cells either possess a selection advantage during the development or they receive increased survival/proliferation signals and as a result have an increased self-renewal capacity, or both. As B1 and B2 transitional cells can currently not be distinguished, the question whether Dkk3 affects B1 cell development could not be fully answered by the analysis of neonatal spleens. Therefore, we decided to investigate in another experiment whether self-renewal of B1 cells is influenced by Dkk3. Purified B cells from wt or $Dkk3^{-/-}$ mice were transferred into immunodeficient ($Rag2^{-/-}$) mice sufficient or deficient for Dkk3. We used cells from wt and $Dkk3^{-/-}$ mice to additionally answer the question whether potential intrinsic Dkk3 or only environmental Dkk3 plays a role in self-renewal of B cells.

As the spleen does not contain progenitor cells in adult mice, the resulting B cell numbers after a period of incubation in the host mice represent the self-renewal capacity of the B cells, which in turn depends on survival and proliferation. Clearly, numbers of B1 cells were increased in mice with a deficiency in environmental Dkk3, but also in mice where Dkk3 was present in the environment but absent in the donor B cells, which was associated with higher levels of serum IgM and IL-10 secretion (Figure 3.24). This demonstrated that the self-renewal capacity of B1 cells is increased in $Dkk3^{-/-}$ mice and that this mechanism is contributing (or even responsible) for the increased B1 cell numbers in Dkk3 deficient animals.

The surprising finding in this experiment was that not only environmental Dkk3 deficiency but also intrinsic Dkk3 deficiency of donor cells increased B1 cell numbers. There are several options of how this could be explained. One explanation could be that the higher proportion of B1 cells in purified B cell samples of $Dkk3^{-/-}$ spleens compared to wt spleens was sustained throughout the time from transfer until analysis. A second explanation could be that B1 cells are irreversibly “primed” in $Dkk3^{-/-}$ mice. Another explanation is, as B cell purification never leads to 100% of purity, that contaminating other splenocytes created a Dkk3 deficient environment. A last explanation, which should not be forgotten, although thought to be unlikely, is that Dkk3 expressed by the B cells themselves and by the environment act

together to regulate B1 cell numbers. Then deficiency in either the environmental or the intrinsic Dkk3 production leads to higher levels of B1 cells.
To address the last assumption, Dkk3 expression in purified B cells from the spleen and the PerC was analyzed. However, neither in splenic B cells nor in PerC B cells yielding high numbers of B1 cells, mRNA transcripts for Dkk3 could be detected (Figure 3.25). Therefore, it is unlikely that B cells themselves express Dkk3, contributing to the regulation of the self-renewal capacity of B1 cells. However, this can not be completely ruled out, as the analysis could possibly not be sensitive enough.
Although we can not yet answer which of the hypotheses is right and how Dkk3 exactly regulates B1 cell numbers, we can conclude that an increased self-renewal capacity of B1 cells in Dkk3$^{-/-}$ mice contributes to higher numbers of these cells.

The number of FO B cells was also analyzed in this experiment. No difference was found between the different groups in FO B cell numbers (Figure 3.24 A). This demonstrates that neither environmental nor intrinsic Dkk3 influences the self-renewal capacity of FO B cells. As they continuously develop from the BM in contrast to B1 cells self-renewal is anyway not an important feature of FO B cells. Nevertheless, the finding confirms our hypothesis that the decreased FO B cell numbers in Dkk3$^{-/-}$ mice are due to a developmental defect (as described above).

In summary, two different mechanisms have been demonstrated to contribute to the observation that the absence of Dkk3 shifted the B1/B2 balance towards the B1 direction: The impaired development of FO B cells and the increased self-renewal capacity of B1 cells. However, there are still remaining questions. As the development of FO cells is impaired at the important step of B cell development where negative selection occurs, we may hypothesize whether Dkk3 presence is necessary for the positive selection of non-self-reactive B cells (FO B cells), and for negative selection of self-reactive cells (B1 cells). This would mean that Dkk3 is an important molecule in deciding whether a cell is harmful and needs to be deleted. Findings supporting this hypothesis are that in the neonatal spleen and in the adult BM of Dkk3$^{-/-}$ mice increased numbers of mature B1 cells were found. This indicates that B1 cell development could be favored while B2 development is impaired in Dkk3$^{-/-}$ mice. Furthermore, MZ numbers were besides B1 cell numbers increased in the neonatal spleen of Dkk3$^{-/-}$ mice (Figure 3.22). As MZ B cells have been described to exhibit a similar selection process to B1 cells [125, 308, 309], this additionally strengthens the hypothesis. It is also possible that similar mechanisms are used by Dkk3 to act on self-renewal, which we demonstrated to be increased in the absence of Dkk3, and on survival of B1 cells during development. Furthermore, it remains to be determined whether the observed modulation of B cell development and maintenance is a direct effect of Dkk3 or whether other cells and molecules are important

intermediates. A model of Dkk3 action on B cell development and maintenance is depicted in Figure 4.2.

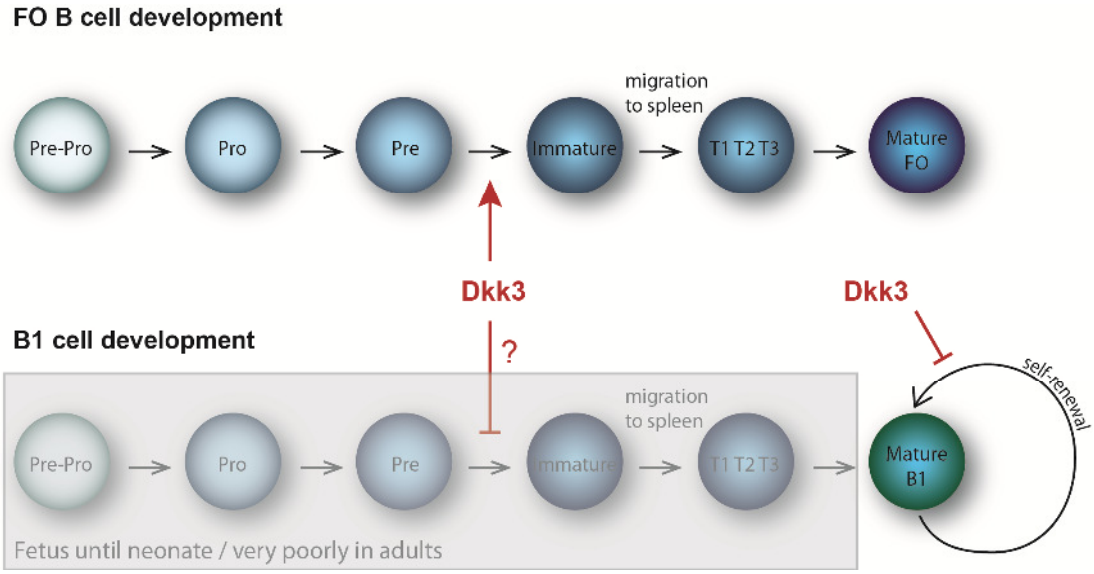

Figure 4.2 | Model of Dkk3 action on B cell development and maintenance. The figure illustrates the FO B cell development, which takes place during the whole life-span of a mouse, and the B1 cell development taking place mainly in the fetal until neonatal stage. In adult mice B1 cells are maintained by self-renewal. Dkk3 was shown in this thesis to support FO B cell development at the step to immature FO B cells, whereas B1 cell development could be inhibited at the same step. In adult mice Dkk3 was demonstrated to inhibit the self-renewal of B1 cells. Here, B1 and FO B cell development is illustrated as two separate lineages, this is however still controversial. Dkk3 might influence the process of positive/negative selection of B cells in general taking place at the immature step, where the mature B cell receptor is expressed for the first time, thereby supporting FO B cell development and decreasing B1 development.

4.6 Dkk3 regulates B cell mediated autoimmunity

The investigation of the B cell compartment in Dkk3$^{-/-}$ mice revealed that Dkk3 deficiency leads to increased B1 cell numbers associated with increased B1 related antibody responses and IL-10 secretion. As B1 cells are the B cell subset mostly considered to be responsible for auto-reactivity [132, 133, 167] and also B1 derived, IL-10 has been shown to exacerbate autoimmune disease [175-178[175-178], it is intriguing to explore whether Dkk3 deficiency leads to increased B cell mediated autoimmunity.

We used the LPR/MRL mouse strain, which is a model of human systemic lupus erythematosus (SLE) [182], and treated the mice with a neutralizing anti-Dkk3 antibody in order to eliminate Dkk3 function. It has been demonstrated by our group

that the anti-Dkk3 antibody abolishes Dkk3 function [214]. Control mice were treated with an isotype control antibody.

After 6 weeks of treatment (as described in Figure 3.26), LN and kidneys of anti-Dkk3 antibody treated mice possessed an increased weight compared to isotype control treated mice (Figure 3.27), suggesting an increased number of accumulated leukocytes and inflammation within the organs. The analysis of T and B cell populations demonstrated that B1 cells were selectively expanded in anti-Dkk3 antibody treated mice in the spleen and the PerC (Figure 3.28). The selective expansion of B1 cells in the absence of Dkk3 in these mice confirms the results obtained in C57BL/6 mice deficient for Dkk3 in a different mouse strain, in which Dkk3 function was abrogated by another method than genetic manipulation. The increased weight of the analyzed LN (inguinal, axillary and MLN) in anti-Dkk3 antibody treated mice together with the population analysis showed that in contrast to the spleen and the PerC in these sites of the body numbers of all cells were increased in anti-Dkk3 antibody treated mice. The relative proportion between the cell subsets did not change in these areas by abrogation of Dkk3 function.
Furthermore, levels of autoantibodies were determined in serum of MRL/LPR mice treated with anti-Dkk3 antibody or isotype control antibody. Higher levels of anti-MOG IgM and IgG, anti-ss-DNA IgG and anti-ds-DNA IgG were detected in anti-Dkk3 antibody treated mice (Figure 3.30/Figure 3.31/Figure 3.32). The histological examination analysis of organs revealed that anti-Dkk3 antibody treated mice suffered from more severe tissue damage (Figure 3.33). This was presumably due to the increased amounts of autoantibodies as well as the increased number of infiltrated leukocytes in pancreas, lung and kidney found in anti-Dkk3 antibody treated mice (Figure 3.33).

Taken together, the treatment of MRL/LPR mice with an antibody abrogating Dkk3 function, lead to increased pathology of the disease. This was associated with increased numbers of B1 cells.

In summary, Dkk3 seems to be an important molecule to control the development and maintenance of different B cell subsets. In the absence of Dkk3, higher numbers of auto-reactive B1 cells arise, which can lead to autoimmunity under specific genetical and environmental conditions (as here shown by using the MRL/LPR mouse strain). It would be interesting to explore, whether human patients of SLE show defects in Dkk3 expression or related pathways.

4.7 Identification of Soggy as a novel immune modulator

Soggy shares the unique sgy domain with Dkk3. To investigate whether the sgy domain might be responsible for conferring the immune-modulatory function to Dkk3, Soggy$^{-/-}$ mice were analyzed.

One hallmark feature of Dkk3 is the expression in immune privileged sites. Interestingly, Soggy was also found to be expressed in immune privileged sites although its expression was restricted to fewer tissues. In the literature it has already been described to be expressed in the testis, the placenta, the trophoblast, fetal eye and fetal neural tissue, as well as various tumors in mice and humans [188, 215, 216, 218]. We detected RNA and protein expression of Soggy in the testis, confirming the reports in the literature but interestingly also in the adult brain and the skin (Figure 3.36). Both the brain and the hair follicles of the skin belong to the immune privileged sites. In future studies, a histological analysis could clarify in which regions and by which cell types Soggy is produced in these areas.
We could not detect Soggy in lymphoid tissues. This is important if we assume Soggy to be an immuno-suppressive molecule, since in these sites immune responses need to take place. In contrast to Dkk3, it was not found in the serum. A genetic complementation by Soggy in Dkk3$^{-/-}$ mice could not be revealed by our analysis as Dkk3 deficient mice did not show increased expression of Soggy (Figure 3.36).
In conclusion, the expression analysis was the first hint that Soggy might be an immune-modulatory molecule similar to Dkk3 contributing to the protection of immune-privileged sites.

Dkk3 has been shown not to influence T cell populations in the thymus and the periphery [212, 214]. However, mice deficient for Soggy exhibited altered numbers of T cell populations in the spleen and the MLN (Figure 3.39/Figure 3.40). There, the ratio of CD4:CD8 T cells was shifted towards CD4 T cells. Furthermore, CD44 expressing cells were decreased. This could be due to an impaired upregulation of CD44 or due to decreased development or survival of memory T cells in Soggy$^{-/-}$ mice. As CD44 is generally used as a marker to identify memory T cells, we analyzed whether the reduction would be due to a reduction of central or effector memory cells. In both analyzed sites the reduction of CD4 memory T cell was due to a reduction of both, central and effector memory T cells, whereas the reduction of CD8 memory T cells was due to a reduction of central memory T cells alone.
The most striking observation in Soggy$^{-/-}$ mice was the reduced number of Tregs (Figure 3.39/Figure 3.40). In order to clarify whether thymic or induced Tregs are affected by the absence of Soggy, Tregs were analyzed for the expression of Helios. FoxP3 cells exhibiting low expression of Helios were claimed to be Tregs induced in the periphery [58]. Also in our analysis, Tregs within the thymus were mainly Helios

positive (Figure 3.38). However, conflicting results were obtained in the two analyzed lymphoid sites. Splenic Tregs displayed increased numbers of Helios^{low} cells, whereas Tregs in the MLN showed slightly decreased Helios^{low} cells in Soggy$^{-/-}$ mice. The finding in the spleen could indicate that higher numbers of Tregs are induced in the periphery in order to compensate the reduced number of Tregs in Sogyy$^{-/-}$ mice. But as the MLN belongs to the system of the GALT, where high numbers of Tregs are commonly induced within the body [310], and Soggy$^{-/-}$ mice do not show increased Helios^{+} cells at this site, the argument is invalidated. Therefore, the analysis of Tregs for Helios expression could not give a conclusive explanation, which kind of Tregs are decreased in Soggy deficient mice.

The analysis of T cell development in the thymus showed some minor changes within the immature T cell population. The number of double negative (DN) cells was decreased, whereas the number of double positive (DP) cells was increased in Soggy$^{-/-}$ mice compared to wt mice (Figure 3.37). Within the DN population CD44^{+} expressing cells were decreased as observed in the periphery. This was due to CD44^{high}CD25^{-} cells. As Soggy deficiency seems to affect CD44 expression in all lymphoid tissues including the thymus, where CD44 cells present immature cells, not memory cells, the suggestion is raised that Soggy regulates the expression level of this surface marker rather than the survival of memory T cells. This would be interesting to clarify in future studies.
Furthermore, the same number of mature single positive CD4, CD8 and Tregs were present in the thymus of wt and Soggy$^{-/-}$ mice (Figure 3.37/Figure 3.38). These are the cells eventually released to the periphery to build the pool of peripheral T cells. Therefore, an altered T cell development in Soggy deficient mice can not be responsible for altered frequencies of T cells in the periphery of Soggy$^{-/-}$ mice. Changes in the number of CD4, CD8 and Tregs must be due to a regulation in the periphery.

To analyze whether T cell reactivity is affected in Soggy$^{-/-}$ mice, we investigated two important features of T cells; the ability to proliferate and the ability to secrete IL-2. Whereas Dkk3$^{-/-}$ T cells have been demonstrated to exhibit a higher proliferation rate under suboptimal stimulation concentration compared to wt T cells *in vitro* ([214] and Figure 3.41), Soggy$^{-/-}$ CD4 T cells exhibited even an increased proliferation rate also at optimal stimulation concentrations (Figure 3.41). The higher proliferation of CD4 T cell of Soggy$^{-/-}$ mice could explain why we observed increased numbers of CD4 T cells in Soggy$^{-/-}$ mice, at least it might contribute to this phenomenon. The proliferation of CD8 T cells, however, was not affected by Soggy deficiency (Figure 3.42).
The secretion of IL-2 was strongly increased in T cells cultures of Soggy$^{-/-}$ mice (Figure 3.43). After depletion of CD25^{+} T cells, CD4 T cells of Soggy$^{-/-}$ mice did not

secrete higher levels of IL-2 in comparison to wt T cells (Figure 3.45). $CD25^+$ depletion eliminates both, recently activated $CD25^+$ effector T cells and Tregs.
As anti-CD3/anti-CD28 stimulation leads to the activation of T cells, CD25 is upregulated and new effector T cells expressing CD25 and secreting IL-2 will be generated. Therefore, the depletion of these cells would presumably not influence IL-2 secretion drastically. Nevertheless, we can not exclude, that the effect was due to these cells. This needs to be clarified by future studies.
Tregs have been demonstrated to decrease IL-2 secretion of T cells by consumption of IL-2 and suppression of T cells via different other molecules (as described in the introduction) [17, 18]. We found decreased numbers of Tregs in $Soggy^{-/-}$ mice in comparison to wt and $Dkk3^{-/-}$ mice (Figure 3.39/Figure 3.40). When this difference was eliminated by using CD4 T cells devoid of $CD25^+$ Tregs, the effect of higher IL-2 secretion of $Soggy^{-/-}$ T cells was abrogated. Therefore, $Soggy^{-/-}$ T cells might secrete increased levels of IL-2 due to lower numbers of Tregs present in the T cell cultures. This would imply that the increased secretion of IL-2 of $Soggy^{-/-}$ T cells is not a feature of the T cells *per se* to secrete higher levels of IL-2 or to possess a stronger activation status, but an indirect feature due to less suppression of Tregs.

To finally investigate whether the observed immunological changes in Soggy deficient mice affect autoimmunity, the course of EAE was analyzed in $Soggy^{-/-}$ mice and compared to wt and $Dkk3^{-/-}$ mice. Very strikingly, the absence of Soggy led to an earlier onset of EAE and increased severity of symptoms (Figure 3.46). This might be due to a decreased level of regulatory T cells in $Soggy^{-/-}$ mice, presumably also present at lower numbers within the CNS, as they have been shown to be able to regulate the pathogenesis of EAE [225]. Future studies should analyze whether indeed lower numbers of Tregs can be found in the brain of $Soggy^{-/-}$ mice affected by EAE. Furthermore, it needs to be clarified whether Soggy produced locally within the brain is important for the regulation of EAE, as this is assumed for Dkk3. Cell infiltrates of the brain could be compared to those of $Dkk3^{-/-}$ mice, giving initial hints. Moreover, transfer experiments could help clarifying this issue, since there are no tissue specific knockout mice for the Soggy gene available.

Given the high abundance of Soggy in the testis, we analyzed whether Soggy expression in the testis affects T cell populations in the periphery, T cell proliferation, IL-2 secretion or EAE. Therefore, T cells of male and female $Soggy^{-/-}$ mice were compared in the relevant assays. However, the expression of Soggy in the testis did not affect any analyzed parameters. Therefore, male and female mice were pooled for experimental presentation in the figures.
It is unclear where the molecule Soggy might meet the immune system to lead to the observed immunological phenotype of $Soggy^{-/-}$ mice. In addition, we do not know the molecular mechanisms how Soggy acts on immune cells. It could act directly or

indirectly via other cell types or molecules. These questions will be addressed in future studies.

In conclusion, we identified Soggy as a novel immune-mediator. Soggy exhibits several similarities to Dkk3 but also differences as listed briefly in Table 4.1. Like Dkk3, Soggy is expressed in immune-privileged tissues, although its expression was restricted to fewer tissues compared to Dkk3. Furthermore, similar to T cells from $Dkk3^{-/-}$ mice, $Soggy^{-/-}$ CD4 T cells exhibited an increased proliferation and IL-2 secretion. EAE was found to be even stronger regulated by Soggy than by Dkk3. These findings suggest that the shared sgy-domain is contributing to the immune-suppressive capacity of Dkk3 and Soggy. The sgy-domain of Dkk3 and Soggy exhibit 41% homology, and the entire Dkk3 and Soggy protein only 22% homology (Figure 3.35). Therefore, it is not surprising that also differences in Soggy and Dkk3 action on the immune system were found, such as the decreased number of Tregs and $CD44^{+}$ cells in the absence of Soggy but not in the absence of Dkk3. Future studies may clarify whether the sgy-domain is indeed responsible for the immune-suppressive capacity of both molecules and which part of the sgy domain or the rest of the protein contributes to the specific function distinguishing Dkk3 and Soggy.

Table 4.1 | Similarities and differences of Dkk3 and Soggy function in the immune system

Similarities	Differences
▪ Expression in immune-privileged sites	▪ Broader expression of Dkk3 than Soggy
▪ Regulation of CD4 T cell proliferation	▪ Dkk3 regulates CD8 T cell proliferation in addition
▪ Regulation of IL-2 secretion	▪ Soggy regulates numbers of Tregs, and this mechanism might be used to regulate IL-2 secretion. Dkk3 does not affect Treg numbers and might use a different mechanism to act on IL-2 secretion
▪ Regulation of EAE	▪ Soggy regulates numbers of $CD44^{+}$ T cells

4.8 Conclusion and outlook

Dkk3 and Soggy, both proteins belonging to the Dkk family and sharing the unique sgy domain, have been demonstrated in this thesis to act as modulators of the adaptive immune system. Dkk3 affects both, T and B cell function, whereas for Soggy a regulation of T cell responses was so far revealed. The functional regulation of T and B cells included the dampening of autoimmune responses mediated by both kinds of lymphocytes.

Many interesting questions remain about the molecular function of Dkk3 and Soggy. One of the most important questions which needs to be addressed is the to date unknown receptor for both molecules. It could be a classical membrane receptor but as Dkk3 and Soggy are secreted molecules, they could also be internalized by cells and interact with intracellular signalling partners. We presently favor the second possibility because Dkk3 has been suggested to interact with βTrCP in the cytoplasm, thereby modulating β-catenin signalling [195, 311]. The identification of the receptor(s) or intracellular signalling partners would help characterizing the molecular pathway triggered by Dkk3 and Soggy leading to the observed effects. It would furthermore tell us whether the effects on T and B cells are directly or indirectly caused by Dkk3 and Soggy. In case they are caused indirectly, it has to be analyzed which cell types or intermediate molecules would be involved. As Dkk3 has been shown to be posttranslationally processed in a similar way to proinsulin [193], it would also be of interest to investigate whether these posttranslational changes alter the activity of Dkk3. The processing of Dkk3 could possibly be used by the immune system to control Dkk3 activity.

Dkk3 and Soggy are both molecules which we just recently identified to act on the immune system. The effects appear to be broad and to influence several different cell types and pathways. Therefore, the interesting journey to explore the mechanistic action of these molecules is just starting and might lead us to therapeutic applications in the field of autoimmune diseases and transplantation.

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