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

Dipl.-Biochem. **Michael Meister**

born in Buchen, Germany

Oral-examination: 28.03.2014

The role of Dickkopf-3 in tissue mediated immune modulation

Referees:

Prof. Dr. Hans-Reimer Rodewald

Prof. Dr. Bernd Arnold

Summary

The immune system protects our organism from harmful environmental insults. Pathogens induce innate and adaptive immune responses that resolve infection and clear damaged cells. A tight balance between efficient elimination of the initial insult and the containment of the immune response, assures protection of the organism as well as the preservation of organ integrity. Disregulation of this system can lead to devastating effects like tissue damage and organ failure caused by excessive inflammation. Tissue components play a pivotal role in the regulation of immune responses. In order to develop novel therapeutic strategies for the treatment of inflammatory diseases, their properties have to be incorporated. Hence, a detailed understanding of mechanisms by which tissue cells influence immune responses is indispensable. Recently, our lab identified Dickkopf-3 (Dkk3) as an immune modulator mainly expressed by tissue cells. Thus we aimed to uncover the contribution of Dkk3 to tissue mediated immune modulation during inflammation.

With the help of a transgenic mouse model we investigated how antigen-presenting keratinocytes in the inflamed skin modulate T cell reactivity. In these mice, keratinocytes present a myelin basic protein (MBP) peptide only upon skin inflammation. In the absence of systemic MBP immunization acute skin inflammation resulted in keratinocyte-mediated activation of MBP-specific $CD4^+$ T cells that were encephalitogenic. However, chronic skin inflammation limited the encephalitogenic potential of systemically primed MBP-specific T cells. In this setting Dickkopf-3 was indispensable for the limitation of CD4 T cell reactivity.

We successfully generated a transgenic Dkk3 reporter mouse that reliably indicates sites of Dkk3 expression. Furthermore, *in vitro* and *in vivo* studies identified interferon-γ (IFNγ) as a potent regulator of Dkk3 expression in tissue cell.

Finally, we found that Dkk3 promotes the development of renal fibrosis in 2 different mouse models, indicated by decreased severity of fibrosis in Dkk3 deficient mice. Furthermore, we observed that Dkk3 expression is induced in tubular epithelial cells in the course of fibrosis development. Less fibrosis in Dkk3 deficient mice was accompanied by elevated levels of pro-inflammatory cytokines and increased T cell infiltration in the respective kidneys. Additionally, we observed an altered polarization of infiltrating CD4 T cells towards a Th1/Treg phenotype in *dkk3*$^{-/-}$ kidneys, which came along with decreased expression of Wnt target genes in these cells.

In conclusion, Dkk3 contributes to tissue mediated immune modulation by regulation of T cell responses.

Zusammenfassung

Das Immunsystem schützt unseren Organismus vor schädlichen Einflüssen. Pathogene induzieren Antworten des angeborenen und des adaptiven Immunsystems, welche die Infektion eindämmen und beschädigte Zellen beseitigen. Ein ausgewogenes Gleichgewicht zwischen der effizienten Beseitigung des Erregers auf der einen Seite und der Begrenzung der Immunantwort auf der anderen Seite, gewährleistet den Schutz des Organismus und die Erhaltung der Organintegrität. Dysregulation dieses Systems kann zu verheerenden Auswirkungen, wie Gewebszerstörung und Organversagen durch eine ausufernde Entzündung, führen. Gewebskomponenten spielen bei der Regulation von Immunantworten eine entscheidende Rolle. Bei der Entwicklung neuer therapeutischer Strategien für die Behandlung von entzündlichen Erkrankungen müssen deren Eigenschaften mit berücksichtigt werden. Deswegen ist das Verständnis der Mechanismen, mit deren Hilfe Gewebszellen Immunantworten beeinflussen, unabdingbar. Vor kurzem identifizierte unser Labor Dickkopf-3 (Dkk3) als einen Immunmodulator, der von Gewebszellen produziert wird. Daher war es unser Ziel, den Beitrag von Dkk3 zur gewebsvermittelten Regulation von Immunantworten aufzudecken.

Mit Hilfe eines transgenen Mausmodels haben wir untersucht wie Antigen-präsentierende Keratinozyten in der entzündeten Haut T-Zell Reaktivität modulieren können. In diesen Mäusen präsentieren Keratinocyten unter Entzündungseinfluss ein Peptid des Myelin Basisches Proteins (MBP). In Abwesenheit einer systemischen MBP Immunisierung, führte akute Hautentzündung zu einer Keratinozyten-vermittelten Aktivierung MBP-spezifischer, enzephalitischer CD4+ T-Zellen. Eine chronische Entzündung führte jedoch zu einer Limitierung des enzephalitischen Potentials systemisch aktivierter, MBP-spezifischer T-Zellen. In diesem Rahmen war Dickkopf-3 unverzichtbar für die Begrenzung der T-Zell Reaktivität.

Wir generierten erfolgreich eine Dkk3 Reportermaus, die verlässlich Stellen der Dkk3 Expression anzeigte. Des Weiteren identifizierten *in vitro* als auch *in vivo* Studien Interferon-γ (IFNγ) als potenten Regulator der Dkk3 Expression in Gewebszellen. Schließlich haben wir herausgefunden, dass Dkk3 die Bildung von Nierenfibrose in zwei Mausmodellen fördert. Das wurde durch eine verringertes Maß an Fibrose in Dkk3 defizienten Mäusen angezeigt. Darüberhinaus haben wir beobachtet, dass die Dkk3 Expression im Verlauf der Fibrose in Tubulusepithelzellen induziert wurde. Geringere Fibrose in Dkk3 defizienten Mäusen ging mit einem erhöhten Level an pro-inflammatorischen Zytokinen und einer erhöhten T-Zellinfiltration in die entsprechenden Nieren, einher. Zusätzlich beobachteten wir in *dkk3-/-* Nieren eine veränderte T-Zell Polarisation, hin zu einem Th1/Treg Phänotypen. Dies wurde von einer verringerten Expression von Wnt Zielgenen in den entsprechenden T-Zellen begleitet. Zusammenfassend trägt Dkk3 zur Gewebs-vermittelten Regulation von Immunantworten durch die Beeinflussung von T-Zellantworten,bei.

Content

1 Introduction

1.1 The adaptive immune system

Our body is constantly exposed to many different pathogens, such as viruses, bacteria or fungi. Yet, it evolved an entire system of organs and cellular and soluble components, that is able to potently fight these threats. The most important organs being part of the immune system are the bone marrow, the spleen, the thymus and lymph nodes, all together called lymphoid tissues. All cells of the immune system originate in the bone marrow and arise from a common precursor, a specialized hematopoietic stem cells (HSC).

The immune system can be divided in two branches, an innate and an adaptive arm. While the innate immune system works in a generic, non-specific way, the adaptive immune system only acts in an antigen specific manner. These distinct properties are based on the type of recognition receptor on the surface of the respective cells.

The adaptive immune system is mainly comprised of two different types of lymphocytes, the T and the B cells and is characterized by their highly specific receptors for antigen recognition. These antigen specific receptors are generated by random gene recombination, called VDJ joining. This process results in a theoretical repertoire of more than 10^{11} specificities. Hence, the adaptive immune system is able to recognize virtually every possible antigen.

The antigen specific receptor on T lymphocytes is called the T cell receptor (TCR). This is a heterodimeric complex comprised of an α and β chain. It is associated with the invariant accessory molecule CD3 and a homodimer of two TCR ζ-chains. According to the expression of the TCR co-receptors CD4 and CD8, T cells can be divided in two different subtypes. CD8 positive T cells are so called cytotoxic T cells. Their function is to kill cells that are infected by viruses or other intracellular pathogens. CD4 positive T cells, however, are called helper T cells and mainly provide essential additional signals for the activation of macrophages and B cells.

Antigen recognition via the TCR is only possible when antigens are presented as peptides by specialized major histocompatibility complex (MHC) molecules. These are highly polymorphic cell surface glycoproteins with a peptide-binding groove. The MHC molecules

bind an antigen in an intracellular location and deliver it to the cell surface where the combined ligand can be recognized by a T cell. $CD4^+$ and $CD8^+$ T cell subsets recognize antigens presented by different classes of MHC molecules. While CD8 T cell exclusively recognize peptides bound to MHC class I molecules, MHC class II can only present peptides to CD4 T cells. The MHC class I consists of two polypeptide chains. The α-chain spans the membrane and contains the peptide-binding groove. This α-chain is non-covalently linked to the smaller β2-microglobulin. These MHC class I complexes bind peptide-antigens originating from intracellular pathogens in the cytosol. The MHC class II complex consists of a non-covalent complex of two chains, α and β, both of which span the membrane. The peptide-binding cleft is formed by both chains (Figure 1.1). These complexes bind peptides derived from extracellular pathogens that were generated by degradation via the proteasome. While MHC class I molecules can be found on virtually every cell type in the body, MHC class II expression is mainly found on specialized antigen presenting cells (APCs). T cell receptor engagement, via interaction with an MHC-peptide complex, leads to activation, proliferation and differentiation of T cells in order to conduct their function (Delves and Roitt 2000). These processes will be further explained in the following chapters.

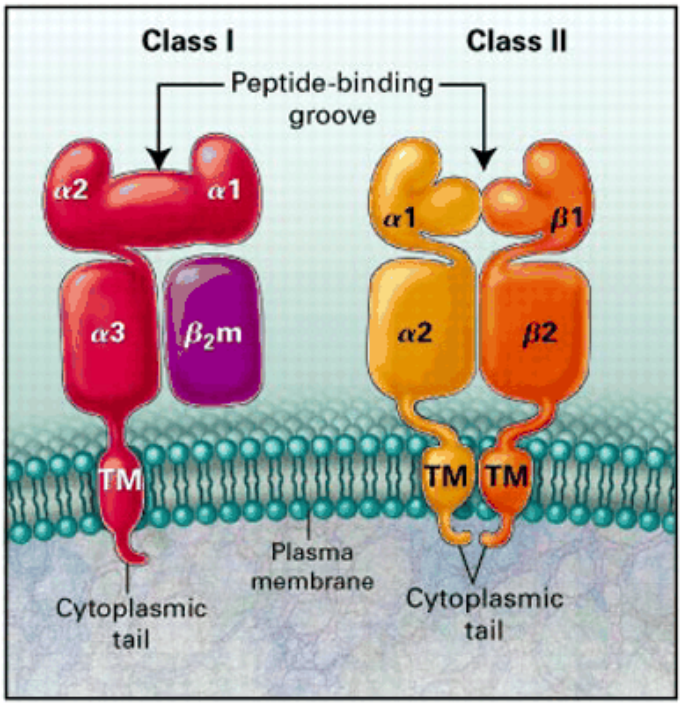

Figure 1.1 Structural composition of MHC class I and II complexes. The MHC class I complex consists of a membrane spaning a-chain and a non-covalently linked ß2 microglobulin. The petide binding groove is located on the a-chain. The MhC class II complex consists of a non-covalent complex of two chains, α and β both of which span the membrane and together form the peptide binding cleft (Neurology, Multiple Sclerosis Edition; Living Medical eTextbook, 2013).

1.1.1 T cell development

T cell precursors, derived from hematopoietic stem cells in the bone marrow migrate, via the blood, into the thymus. There, the development into mature T cells takes place (Figure 1.2). Progenitor T cells, lacking expression of CD4 and CD8 (described as double negative, DN), enter the cortex of the thymus at the cortico-medullary junction. Initially, the TCRβ and, after entering the double positive ($CD4^+CD8^+$, DP) stage, the TCRα genes undergo random recombination mediated by an enzymatic complex of the recombination activation genes 1 and 2 (RAG1/RAG2).

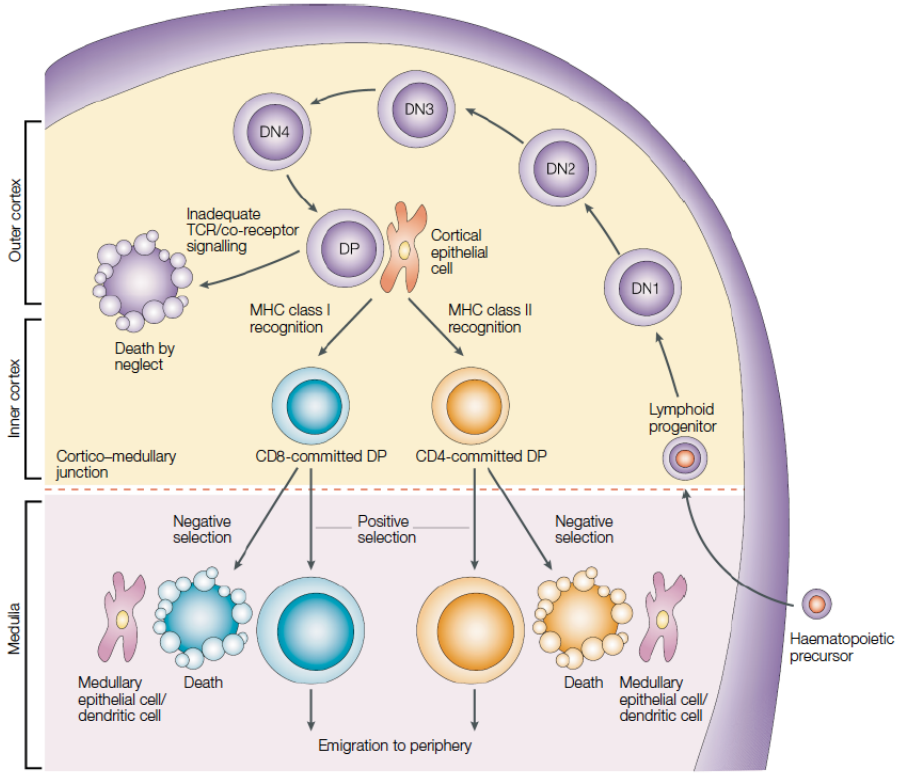

Figure 1.2 T-cell development in the thymus. Committed lymphoid progenitors arise in the bone marrow and migrate to the thymus. Early committed T cells lack expression of T-cell receptor (TCR), CD4 and CD8, and are termed double-negative (DN) thymocytes. DN thymocytes can be further subdivided into four 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 selfpeptide–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 periphery (Germain 2002).

The randomly generated TCRs are next tested for their ability to recognize the host's MHC molecules, in a process called positive selection (Anderson, Owen et al. 1994). Only the T cells with a proper TCR, able to recognize self-MHC molecules, get a survival signal and are positively selected (Zinkernagel and Doherty 1974, Kisielow 1988). An estimated 90-95% of DP T cells that are not able to bind to the available MHC complexes undergo death by neglect (Huesmann, Scott et al. 1991).

Random recombination also generates TCRs with affinities for antigens present in the own body, so called self-antigens. T cells bearing such TCRs (auto-reactive) are deleted before they escape the thymus to avoid self-destructive T cell responses in the periphery. This process is described as negative selection or clonal deletion and takes place in the thymic medulla. Clonal deletion occurs upon self-antigen presentation by the medullary thymic epithelial cells (mTECs) (Burkly, Degermann et al. 1993) and the thymic dendritic cells (DCs) (Brocker, Riedinger et al. 1997). mTECs promiscuously express a variety of tissue restricted antigens (TRAs), under the transcriptional regulation of Autoimmune Regulator (AIRE). Self-antigens expressed by mTECs represent virtually all the parenchymal organs, thereby mirroring the peripheral self (Derbinski, Schulte et al. 2001). The MHC-self antigen recognition by a TCR usually leads to the deletion of the respective T cell. Even though this process is highly efficient, since approximately 50-70% of the positively selected T cells are thought to be negative selected (van Meerwijk, Marguerat et al. 1997), not all auto-reactive T cells are eliminated by clonal deletion (Kappler, Roehm et al. 1987). After surviving negative selection, naïve T cells emigrate from medulla to the periphery.

1.1.2 CD4 T cell activation and differentiation

Upon its maturation in the thymus, naïve T cells continuously recirculate from blood to the lymphatic system and back. The secondary lymphatic organs 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. There T cells can become activated or anergized, depending on the activation status of the dendritic cell. Anergy has been described as a long lasting cell intrinsic state of T cell unresponsiveness that is induced by suboptimal stimulation (Chappert and Schwartz 2010). Proper activation of T cells requires, beside the TCR-MHC interaction, co-stimulatory signals. In this process, co-stimulatory molecules like CD80 and CD86 on APCs interact with CD28 on the T cell surface and provide essential contributions to TCR signaling.
Upon TCR dependent activation, naive CD4 T cells not only proliferate but also differentiate into different T helper subsets. The microenvironment present during CD4 T cell priming determines its fate. This is mainly influenced by the priming DCs but can also be influenced by other cells, including tissue cells like for example keratinocytes in the skin (Soumelis, Reche et al. 2002). Differentiation into different effector subsets is accompanied by the expression of different transcription factors, the master regulators of linage commitment.
In the following, the most important subtypes are discussed. In a simplified model, interferon-γ (IFNγ) and interleukin-12 (IL-12) are the classical cytokines driving a Th1 response. Th2 cells are generated in the presence of IL-4. Whether a T cell differentiates into a Th17 or induced regulatory T cells depends on the presence of IL-6. Both cell types need transforming growth factor-β (TGF-β) for their differentiation, but only if IL-6 is present the T cell will differentiate into an effector Th17 cell. Th17 cells are further supported by the cytokine IL-23. During the process of differentiation, signaling via STAT molecules is important (Figure 1.2). However, these differentiation processes are characterized by high flexibility and plasticity. T cells, which have differentiated into a one particular subtype, can change their phenotype to another subset again under the influence of certain cytokines (Figure 1.3) (Zhu and Paul 2010, Magombedze, Reddy et al. 2013).

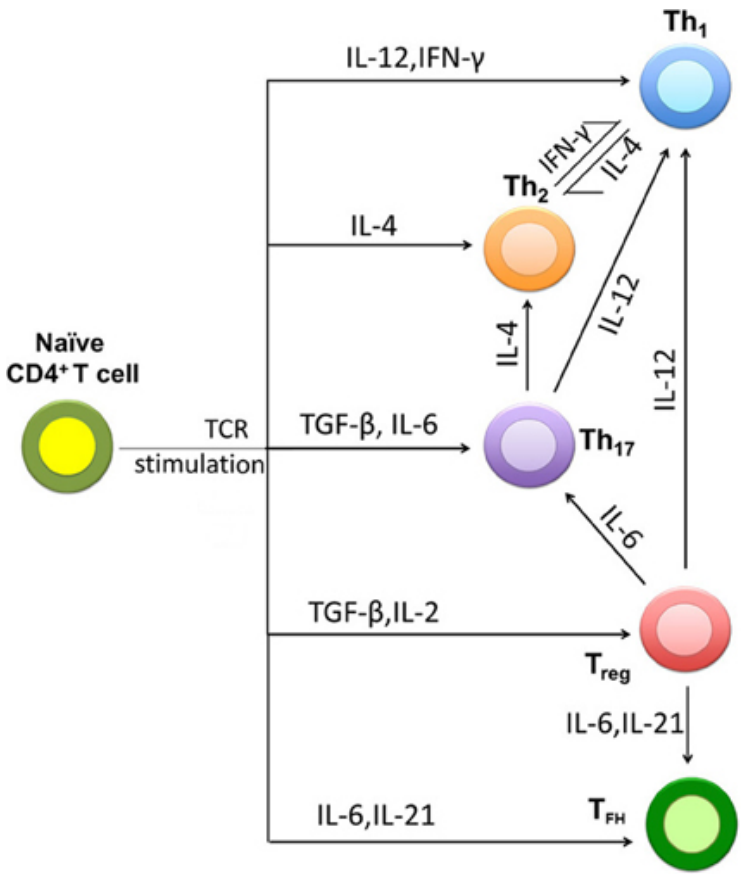

Figure 1.3 Major pathways of naïve CD4+ T cell differentiation into effectors. Upon encountering the antigens presented by the professional antigen-presentingcells (APCs) naïve CD4+ T cells differentiate into Th1, Th2, or Th17 effector cells. Cytokines present in the environment during differentiation play the major role in determining the phenotype that the CD4+ T cell will acquire. Two other CD4+ T cell subsets include regulatory T cells (Treg) and T follicular helpercells (Tfh). Due to cellular plasticity differentiated effector CD4+ T cells may convert from one type into another. For example, Th17 cells under strong polarizing conditions (e.g., high concentrations of IL-12) may convert into Th1 cells (Magombedze, Reddy et al. 2013).

Th1 effectors express the transcription factors T-bet and EOMES and secrete the cytokines IFNγ and tumor necrosis factor α (TNFα). These cells play an essential role in enhancing the cytotoxic effect of CD8 T cells and thus inhibit replication of intracellular pathogens such as viruses. Additionally, they play an important role in the induction of autoimmune diseases (Magombedze, Reddy et al. 2013) .

Th2 cells express the transcription factor GATA-3 and secrete the cytokines IL-4, IL-5, and IL-13. IL-4 functions as autocrine driver of GATA 3 expression and thus stabilizes linage commitment. These cells are critical during infection by extracellular pathogens such as extracellular bacteria and helminthes. IL-4 and IL-13 are crucial in the development of IgE antibody responses (Poulsen and Hummelshoj 2007) and IL-5 activates eosinophiles. Therefore these cells are mainly responsable for the development of allergic reactions. In addition, they play a crucial role in driving fibrosis upon chronic organ inflammation (Wick, Grundtman et al. 2013).

Th17 cells express the transcription factor RORγT and produce the cytokines IL-17 and granulocyte macrophage colony-stimulating factor (GM-CSF) (Pantelyushin, Haak et al. 2012). These cells are important for the control of certain bacterial and fungal infections. In addition, similar to Th1 cells, Th17s have been reported to be important for the initiation of autoimmune diseases (Haak, Gyulveszi et al. 2009).

Regulatory T cells express the transcription factor FoxP3. These cells secrete anti-inflammatory cytokines like TGF-β and IL-10. Tregs maintain immune homeostasis by limiting the magnitude of immune response against pathogens and control inflammatory reactions (Sakaguchi 2004).

T follicular helper cells (Tfh) express the transcription factor Bcl-6 and these cells are essential for the production of high affinity IgG antibodies (Crotty 2011).

1.1.3 T cells in autoimmunity

Negative selection of auto-reactive T cells in the thymus as well thymic generation of regulatory T cells work together to build up central tolerance, one mechanism to avoid the induction of auto-reactive T cell responses. However, since not all auto-reactive T cells are eliminated by clonal deletion, potentially harmful T cells arrive in the periphery. Usually, these cells are controlled by mechanisms of peripheral tolerance. They include the induction of anergy, deletion by activation induced cell death (ACID) and suppression by regulatory T cells and tissue cells (Arnold, Schonrich et al. 1993). Even though, there is a high degree of redundancy in the system, autoimmune diseases develop. If initiated, autoimmune responses lead to the destruction of tissue. This is mediated 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 (Pierson, Simmons et al. 2012).

How autoimmune disorders are initiated remains so far unclear. It was suggested that auto-reactive T cells are stimulated by DCs that took up self-antigen from apoptotic cells. In parallel, this DC would have to be infected with a virus or bacterium in order to be activated by danger signals and to be able to stimulate the auto-reactive T cell and to initiate an autoimmune reaction (Tan and O'Neill 2005). However, susceptibility studies showed that

autoimmune diseases are not only associated with infections and immunological factors, but also with environmental, geographical, and genetic factors, and appear to be increasing especially in the western world (Willer and Ebers 2000). The exact etiology still remains unknown.

Research over the last decades revealed that Th1 and Th17 cells probably play a pivotal role in initiating autoimmune diseases, whereas regulatory T cells and Th2 cells are able to dampen or even prevent disease development (Barcala Tabarrozzi, Castro et al. 2013). Therefore, many therapies aim to increase Treg cell numbers or their activity (Haque, Lei et al. 2012), or redirect Th1/Th17 responses to a less damaging Th2 response (Mouzaki, Deraos et al. 2005, Hassan, Sliem et al. 2012). A lot of this insight was gained by studying animal models. Such animal models of T cell mediated autoimmune disease have been generated for a multitude of autoimmune diseases including Type I diabetes (Boitard 2012), autoimmune rheumatoid arthritis (Komatsu and Takayanagi 2012) and multiple sclerosis (Furlan, Cuomo et al. 2009)

Experimental autoimmune encephalitis (EAE) is a frequently used experimental model for T cell mediated autoimmunity. This mouse model reflects the human disease of multiple sclerosis. In this model activated, auto-reactive CD4 T cells traffic to the central nervous system (CNS) where they initiate a tissue destructive immune response. EAE is initiated by active immunization with CNS derived self-antigens emulsified in complete Freund's adjuvant (CFA) and administration of pertussis toxin (PT). PT is supposed to break the blood brain barrier (Juhler, Barry et al. 1984) and provide access of activated auto-reactive T cells to the CNS. The most commonly used CNS-derived antigens for immunization are peptides of the myelin basic protein (MBP) and myelin oligodendrocyte glycoprotein (MOG). Alternatively, in vitro activated transgenic T cells with specificities for the above mentioned antigens can be transferred for disease induction. After priming of auto-reactive CD4 T cells by immunization, effector cells traffic to the CNS where they are re-primed by tissue resident APCs and subsequently conduct their effector function (Kivisakk, Imitola et al. 2009). Main effector cells in this pathological situation are Th1 und Th17 cells. Cytokines related to these T cell linages have been implicated to be crucial for disease initiation. Besides IL-12 and IFNγ, IL-23 and IL-17 were shown to be important (Becher, Durell et al. 2002, Aranami and Yamamura 2008)[97, 98]. Additionally, GM-CSF was identified to be an essential factor for the effector phase of EAE (Codarri, Gyulveszi et al. 2011). The secretion

of these cytokines leads to the attraction of further immune cells including myeloid cells and cytotoxic CD8 T cells (Gold, Linington et al. 2006, Codarri, Gyulveszi et al. 2011).

Therefore, the immune response is enforced and leads to the destruction of oligodendrocytes (Hartung and Rieckmann 1997). The consequence is demyelination and axonal loss (Slavin, Kelly-Modis et al. 2010). An obvious signs of this tissue destruction is paralysis of the tail and limbs. Accordingly, the severity of disease is described by these symptoms in the EAE score.

1.2 Immune regulation by tissue cells

The efficacy and the self-containment of inflammatory responses are essential to protect against the primary insult and to preserve organ integrity. For a long time, the immune system was regarded as an isolated, self-regulating system that independently assures the balance between these two essential requirements. Tissues were considered only as passive victims, building up the framework for immunological processes.

However, in the last few years it became clear that tissue cells play an active role in designating the course of immune responses via a tightly regulated crosstalk with cells of the innate and adaptive immune system. The observation that the type and strength of immune responses depend on the local microenvironment of a given tissue (Alferink, Aigner et al. 1999, Matzinger 2007) has fostered investigations on the contribution of various tissue-specific components to the resulting immune response. For example, hepatocytes in the liver (Bottcher, Knolle et al. 2011) as well as oligodendrocytes in the brain (Balabanov, Strand et al. 2007, Na, Hermann et al. 2012) were shown to influence immune responses in the respective organs, contributing to the balance between tolerance and immunity. Moreover, there is growing awareness of immunologists about the regulatory capacity of distinct local commensal niches particularly at barrier tissues, such as the skin, the lung and the gastrointestinal tract (Grice, Kong et al. 2009, Belkaid and Naik 2013).

1.2.1 Immune modulatory capacity of keratinocytes

Keratinocytes represent the majority of epidermal cells and provide the structural integrity and barrier function of the epidermis. By virtue of their anatomical location, they are the first cells which come in contact with external stimuli and thus build up a first line of defence against environmental insults like pathogens, UV irradiation, chemical agents or mechanical stress. In the last few years, it has become clear that they play an important role in the initiation and perpetuation of innate and adaptive immune responses in the skin (Banerjee, Damodaran et al. 2004). This immune-modulatory capacity was shown to be mediated by numerous molecular mechanisms.

Keratinocytes are able to trigger the early phase of immune responses by attraction and activation of key innate immune cells. In order to sense their environment for barrier

compromise, keratinocytes express several surface receptors. For example, they are known to express Fc receptors such as FcgRI, FcgRII, and FcgRIII (Tigalonowa, Bjerke et al. 1990, Cauza, Grassauer et al. 2002) as well as complement receptors, mannose receptors (Szolnoky, Bata-Csorgo et al. 2001) and other molecules that potentially contribute to the process of internalization such as the α5ß1 fibronectin–binding integrin (Pellegrini, De Luca et al. 1992). Binding to these receptors results in effective endocytosis and subsequent killing of bacteria, fungi, and viruses, as documented for a variety of pathogens (Csato, Bozoky et al. 1986, Szolnoky, Bata-Csorgo et al. 2001, Kisich, Howell et al. 2007). In addition, various studies reported that keratinocytes express Toll-like receptors (TLRs) 1–6 and 10 (Baker, Ovigne et al. 2003, Mempel, Voelcker et al. 2003, Flacher, Bouschbacher et al. 2006, Lebre, van der Aar et al. 2007) as well as TLR4 associated CD14 and MD-2 proteins (Kollisch, Kalali et al. 2005). In addition, some studies showed the presence of TLR7, 8, and 9 in these cells. ((Mempel, Voelcker et al. 2003, Miller, Sorensen et al. 2005, Flacher, Bouschbacher et al. 2006, Lebre, van der Aar et al. 2007). TLR expression can be further up-regulated by certain pathogens and pathogen derived products (Pivarcsi, Bodai et al. 2003). Additionally, it has been documented that keratinocytes express other intracellular receptors that recognize conserved molecular patterns, like nucleotide oligomerization domain (NOD)-like receptors. Both NOD 1 and 2 seem to be constitutively present in these cells (Harder and Nunez 2009). C-type lectins comprise another group of such receptors that may have a role in epidermal recognition of microbes (Lee, Shin et al. 2009, de Koning, Rodijk-Olthuis et al. 2010). Expression of family members of the RIG-like helicase receptors that are able to detect viral single-stranded RNA and double-stranded RNA, was detected on keratinocytes (Kalali, Kollisch et al. 2008). All these findings indicated a role of keratinocytes in early detection of pathogens characterized by the presence of conserved molecular patterns, such as viral nucleic acids, lipopolysaccharides, or flagellin.

Sensing incoming threats by these receptors, enables the epidermis to react by producing chemotactic mediators, such as IL-8, CCL2, CCL20, CCL27, CXCL16, CXCL9, and CXCL10 (Pivarcsi, Bodai et al. 2003, Kollisch, Kalali et al. 2005, Miller, Sorensen et al. 2005, Lebre, van der Aar et al. 2007, Lee, Shin et al. 2009, Niebuhr, Baumert et al. 2010) as well as cytokines like tumor TNFα, type I interferons, IL-1α/ß, IL-6, IL-18, defensins, cathelicidin and thymic stromal lymphopoietin (TSLP) upon contact with conserved molecular patterns (Lebre, van der Aar et al. 2007, Prens, Kant et al. 2008, Kinoshita, Takai et al. 2009, Kobayashi, Yoshiki et

al. 2009). Secretion of these molecules by keratinocytes promotes recruitment of neutrophils, monocytes, dendritic cells and various subsets of lymphocytes to the epidermis and their activation (Gutowska-Owsiak and Ogg 2012). In addition to the production of soluble mediators, it was demonstrated that keratinocytes are able to activate skin resident innate lymphocytes in a cell contact dependent manner, after barrier disruption. In response to mechanical stress, keratinocytes were shown to up-regulate the expression of Rae-1, a membrane bound NKG2D ligand, which in turn activates cytokine production by γδ T cells (Strid, Tigelaar et al. 2009).

Additive to their role as immune sentinels of the skin and their properties in modulating early innate responses, keratinocytes were shown to be able to influence adaptive immune responses by various means by affecting professional APCs in the skin (Figure 1.1). As already mentioned before, the activation of pattern recognition pathways by stimulation with pathogenic products results in the induction of chemokines and cytokines, which influence APCs. For example, it was documented that keratinocyte derived GM-CSF promotes DC differentiation and their ability to stimulate naïve T cells (Pastore, Fanales-Belasio et al. 1997). Furthermore, murine models imply that IL-1α produced by keratinocytes modulates class II expression and function of Langerhans cells (Lundqvist and Back 1990). Keratinocytes also express TNFα, which seems to be further induced by exposure to lipopolysaccharide (Kock, Schwarz et al. 1990). Additionally, an effect of keratinocyte derived TNFα on induction of Langerhans cell migration and their accumulation in the lymph nodes was reported (Cumberbatch and Kimber 1995). This cytokine has been showed to be involved in the generation of immature Langerhans cells from their precursors (Arrighi, Soulas et al. 2003). Moreover, keratinocytes that sense the presence of viral products are able to induce DC maturation in a TNFα/IL-1ß dependent mode (Lebre, Antons et al. 2003).

By conditioning APCs, keratinocytes were shown to be able to influence the linage commitment of T cells. For example, the secretion of thymic stromal lymphopoetin by keratinocytes instructs DCs to induce T helper 2 (Th2) cell differentiation thereby promoting allergic responses (Soumelis, Reche et al. 2002). These findings were confirmed by other studies, implying a general role of keratinocyte derived TSLP as a Th2 promoting cytokine in the skin. (Ito, Wang et al. 2005, Allakhverdi, Comeau et al. 2007, Ebner, Nguyen et al. 2007). Another epidermis-derived cytokine that influences T cell differentiation is IL-25, a member of the IL-17 family (IL-17E). Keratinocytes constitutively express IL-25 transcript and protein

(Corrigan, Wang et al. 2011). APC stimulation with this cytokine was documented to result in the induction of Th2 responses (Fort, Cheung et al. 2001). In addition, keratinocyte derived IL-33 was also reported to mediate Th2 promoting effects (Schmitz, Owyang et al. 2005).

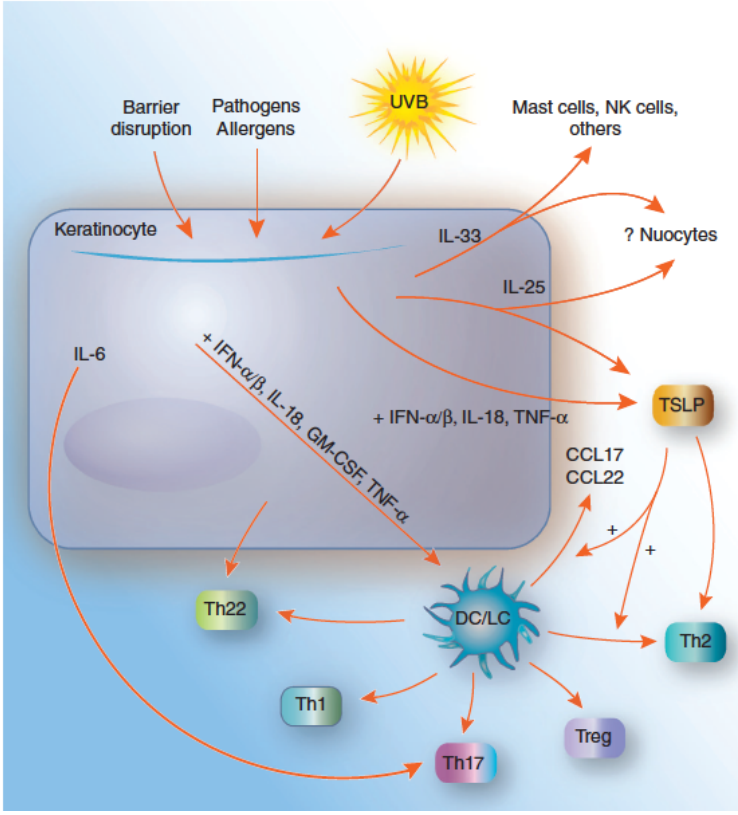

Figure 1.4 Direct and indirect effects of keratinocytes on innate and adaptive immune responses. After sensing of danger signals, keratinocytes influence imunne responses by the secretion of several cytokines and chemokines. **(Gutowska-Owsiak and Ogg 2012).**

In contrast, keratinocytes are also capable of directing T cell differentiation toward a Th1 phenotype, by inducing IFNγ release from T cells, monocytes and macrophages. For example, IL-18 which is constitutively expressed by keratinocytes of the basal epidermal layer mediates this function (Kohka, Yoshino et al. 1998, Naik, Cannon et al. 1999). Moreover, this cytokine has been reported to influence Langerhans cell migration in mouse models of contact hypersensitivity (Cumberbatch, Dearman et al. 2001, Plitz, Saint-Mezard et al. 2003). Additionally, the presence of type I interferons and IL-18 in the supernatants of keratinocytes, stimulated with double-stranded RNA, promoted IL-12p70 secretion from immature DCs, which in turn induced a strong Th1 response (Lebre, Antons et al. 2003).

More than 30 years ago, it has been demonstrated that under inflammatory conditions, keratinocytes can up-regulate both, major histocompatibility complex class I and II molecules (Basham, Nickoloff et al. 1985, Gaspari and Katz 1988) as well as various molecules required for effective MHC peptide loading (Albanesi, Cavani et al. 1998). Moreover, they were shown to express co-stimulatory molecules like B7.1 (Nengwen, Li et al. 2009), ICAM and CD80 upon inflammatory stimulation (Little, Metcalfe et al. 1998, Black, Ardern-Jones et al. 2007). Accordingly, it was suggested that keratinocytes may activate T cells by direct antigen presentation. Early *in vitro* studies have indicated that class II–mediated presentation of antigens by keratinocytes only results in tolerance or anergy of naive T cells (Gaspari, Jenkins et al. 1988, Bal, McIndoe et al. 1990, Otten, Bor et al. 1996), probably due to missing co-stimulation (Black, Ardern-Jones et al. 2007). However, further studies reported that keratinocytes pre-treated with IFNγ can efficiently stimulate $CD4^+$ antigen–specific T cells, that have already encountered their cognate antigen (Mutis, De Bueger et al. 1993, Ardern-Jones, Black et al. 2007, Black, Ardern-Jones et al. 2007).

The mechanisms that allow keratinocytes to directly stimulate T cells have been investigated in the context of S. aureus exposure (Strange, Skov et al. 1994, Ardern-Jones, Black et al. 2007). It was observed that keratinocyte-mediated activation of allogeneic T cells in the presence of IFNγ and staphylo–enterotoxin B was reduced by the addition of HLA-DR antibodies (Strange, Skov et al. 1994).

In an other experimental system, the capacity of keratinocytes to present house dust mite antigen Der p1 to specific CD4+ T cells was significantly increased, when they were first pre-treated with supernatant of staphylo–enterotoxin B exposed peripheral blood mononuclear cell cultures (Ardern-Jones, Black et al. 2007). The study showed that soluble factors, particularly IFNγ, released from immune cells following exposure to the bacteria, could greatly enhance the T-cell stimulation capabilities of keratinocytes and induced adaptive immune response to unrelated antigens (Black, Ardern-Jones et al. 2007). Thus keratinocytes seems to be able to either activate or tolerize T cells by direct antigen presentation, depending on their activation status.

Moreover, there are reports indicating that, when keratinocytes are involved in stimulating T cells, a bias towards differential T-helper effector function can be observed. For example differences in cytokine secretion patterns between T cells stimulated by IFNγ exposed keratinocytes or peripheral blood mononuclear cells were observed. (Goodman, Nestle et al.

1994). *In vivo*, both, the T cell stimulating and tolerizing capacity of keratinocytes could be detected. For example, keratinocytes have been reported to directly activate T cells in transgenic mice that constitutively express peptide–MHC conjugates exclusively in keratinocytes. Inflammatory skin disease was observed in 20-40% of mice that expressed the MHC class II I-A^b β chain under the keratin 14 regulatory sequences and a corresponding autoreactive T cell receptor (TCR) on $CD4^+$ T cells (Fan, Busser et al. 2003). Moreover, our lab has previously shown that $CD8^+$ T cells with a transgenic MHC class I K^b-specific TCR become tolerant following encounter of the K^b antigen on keratinocytes (Alferink, Tafuri et al. 1998). As in both animal models the respective TCR recognized only the intact MHC molecule it was proposed that T cell stimulation occurred through direct contact with keratinocytes and was not based on antigen processing and presentation by professional antigen presenting cells (APC). Direct contact between naïve T cells and keratinocytes occurs mainly during the neonatal phase when extensive trafficking of T cells through tissues including the skin is observed (Kimpton, Washington et al. 1995). In contrast, T cell migration into the healthy skin is limited during adulthood (Mackay 1993), and T cells can only infiltrate inflammatory sites (Hou and Doherty 1993, Tietz, Allemand et al. 1998, Wang, Fujita et al. 2010).

So far, it is unknown whether keratinocytes presenting self-antigens during inflammation can modulate auto-reactive T cell responses.

1.3 Dickopf-3

Dickkopf-3 (Dkk3) is mainly expressed by tissue cells in immune-privileged sites, including the hair follicle in the skin (Ohyama, Terunuma et al. 2006). Our group has recently identified Dkk3 as a novel immune modulator that is capable to restrict T cell responses and participates in mediation of immunological tolerance (Papatriantafyllou, 2008). Thus it is possible that Dkk3 is acting as an immune modulator in the skin. In the following the biology of Dickkopf proteins and in particular of Dkk3 is described.

1.3.1 The Dickkopf protein Family

Dickkopf genes comprise an evolutionary conserved small gene family of four members, *dkk 1-4* and a unique *dkk3*-related gene *dkkl1* or *sgy* (Niehrs 2006). They encode 5 secreted glycoproteins of 250 - 350 amino acids, which share a sequence identity of 37-50% (Krupnik, Sharp et al. 1999, Barrantes Idel, Montero-Pedrazuela et al. 2006). The Dkk family members contain a N-terminal signal peptide and share two conserved cysteine-rich domains separated by a linker section of distinct length (Figure 1.5).

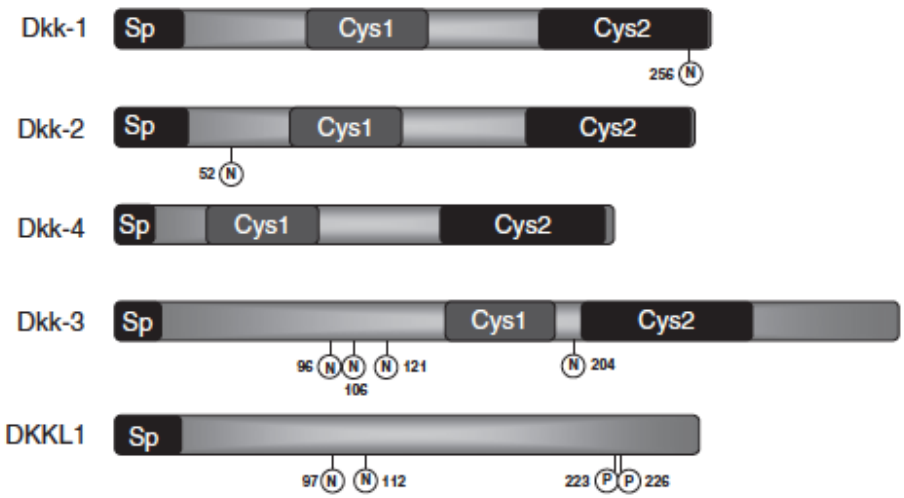

Figure 1.5 Structural homology of the Dickkopf protein family. Dkk proteins contain two highly conserved cysteine-rich domains (Cys1 and Cys2), which are separated by a nonconserved linker region of variable length. Within the Dkk family, the structural diversity of Dkk-3 is pronounced by its larger protein size, a shorter linker region and a high N-terminal sequence similarity with DKKL1 but not with Dkk-1, Dkk-2 or Dkk-4.Dkk-3 possesses four putative N-glycosylation sites, whereas Dkk-1 and Dkk-2 harbor one site. Numbers refer to the modified amino acid residue. In theC-terminal region of DKKL1 two serine residues are phosphorylated. (Veeck and Dahl 2012).

Both cysteine-rich domains possess 10 conserved cysteine residues. The N-terminal DKK_N domain is unique to the Dkk family while the C-terminal (formerly called Cys-2) Colipase Fold domain has a pattern of 10 cysteine residues related to that in proteins of the colipase family (Aravind and Koonin 1998). Additionally, structural analysis shows that Dkks and colipases have the same disulfide-bonding pattern and a similar fold. The structure consists of short ß-strands connected by loops and is stabilized by the disulfide bonds, resulting in finger like structures that may serve as interactive surface (Niehrs 2006).

Structure-function analysis of vertebrate Dkk1, 2 and 4 exhibited that the colipase fold domain alone is sufficient for their biological activity, the Wnt pathway modulation. Colipases usually facilitate the interaction of pancreatic lipases with lipid micelles, therefore, it has been suggested that the Cys2 domain may enable Dkk proteins to interact with lipids in order to regulate Wnt signalling (Krupnik, Sharp et al. 1999). As already mentioned, the linker region between the highly conserved domains Dkk_N and colipase fold (Cys-2) is greatly variable between Dkks. It is remarkably smaller in Dkk3 (12 amino acids) when compared to Dkk 1 ,2 and 4 (50-55 amino acids).The dickkopf proteins exclusively carry N-glycosylations. The mammalian Dkk1 possesses one potential glycosylation site located close to the C-terminus of the protein. Chicken and mammalian Dkk3 display four potential glycosylation sites which are not conserved among the other Dkks (Krupnik, Sharp et al. 1999). In addition, each Dkk harbours various dibasic cleavage sites (Niehrs 2006) which display targets for potential posttranslational proteolytic modifications by furin type proteases (Nakayama 1997).

Soggy exhibits a sequence similarity to Dkk3 but not other Dkks. Dkk3 and Soggy share a N-terminal signal peptide, which enables the secretion of both proteins. Sequence homology between these two proteins is most pronounced within the N-terminal region of Dkk3, displayed as Sgy domain, although significant amino-acid identities are seen beyond this domain that extend into the cysteine rich domains of Dkk3. The residues shared by Sgy and Dkk3 are poorly conserved in other Dkks. Thus the two proteins are uniquely related (Krupnik, Sharp et al. 1999).

By a number of criteria *dkk3* appears to be a divergent member of the Dkk family. First of all, DNA sequence comparisons show that vertebrate *dkk1, 2* and *4* are more related to each other than they are to *dkk3* (Glinka, Wu et al. 1998). Second, Sgy, the distant member of the Dkk family, shares sequence similarity with Dkk3 but not with other Dkk family members

(Krupnik, Sharp et al. 1999). Due to the fact, that this similarity is most pronounced outside the two conserved cysteine rich domains (Niehrs 2006) it is possible that the Sgy gene arose from an ancestral Dkk3 precursor Cnidaria. Hydra and Nematostella, have two *dkk* genes only, one related to vertebrate *dkk1, 2* and *4* (Guder, Pinho et al. 2006) and one related to vertebrate *dkk3* (Fedders, Augustin et al. 2004). Human *dkk1, 2* and *4* are located within the well-characterized chromosome 4/5/8/10 paralogy group (*dkk1* maps to 10q11, *dkk2* to 4q25 and *dkk4* to 8p11). Genes within this paralogy region were duplicated early in vertebrate evolution (Pollard and Holland 2000). In contrast, human *dkk3* maps to 11p15.3, which is not part of the same paralogous chromosome group.

Taken together, these observations suggest a deep split into *dkk3* and *dkk1/2/4* gene families during early metazoan evolution, and more recent gene duplications accompanied by functional divergence of the two *dkk* subfamilies (Guder, Pinho et al. 2006).

1.3.2 Function and biological role of the Dickkopf protein family

The name Dickkopf was assigned to this protein family since its founding member Dkk1 induced head formation in Xenopus embryos by its ability to inhibit Wnt signaling during early embryogenesis (Glinka, Wu et al. 1998).

The Wnt pathway regulates a wide range of essential processes during embryonic development and adult tissue homeostasis (Clevers 2006, De Ferrari and Moon 2006). It has been reported to regulate cell proliferation, motility, cell polarity and cell fate in adulthood (Etheridge, Spencer et al. 2004). The so far known 19 secreted Wnt glycoproteins trigger at least two, possibly three, pathways that employ 10 Wnt receptors of the frizzled seven transmembrane class. These are (1) the canonical Wnt or Wnt/β-catenin pathway (Cadigan and Liu 2006), (2) the planar cell polarity pathway (PCP), which does not involve β-catenin but recruits small GTPases of the rho/cdc42 family to activate JNK and (3) the Wnt/Ca2+ cascade that is still controversial and may be partly overlapping with the PCP pathway (Kohn and Moon 2005)

Dickkopf proteins specifically modulate the well characterized and highly conserved canonical Wnt pathway. Aberrant activation of this Wnt/β-catenin signaling, due to

mutations in one of its components, is closely connected to human disease including cancer, osteoporosis, aging and degenerative disorders (Moon, Kohn et al. 2004, Clevers 2006).

Independent from Wnt/ß-catenin signaling, most of the cellular β-catenin is bound to cadherins and α-catenin, forming intercellular adherent junctions (Jamora and Fuchs 2002). In the inactive state of the canocial Wnt pathway, thus in absence of Wnt ligands, free cytoplasmic β-catenin is bound to its destruction complex consisting of casein kinase 1 (CK1), the tumor suppressor gene product adenomatous polyposis coli (APC), Axin and/or its homologue Axin2 (also termed conductin), the glycogen synthase kinase 3β (GSK3β) and other auxiliary proteins. Priming phosphorylation of β-catenin at Ser 45 by CK1 allows subsequent phosphorylation at Thr 41, Ser 37 and Ser 33 throughout GSK3β. Phosphorylation of the two latter amino acids triggers β-catenin ubiquitination by the β-transducin repeat containing protein (β-TrCP) and therefore its proteasomal degradation, thus keeping the cytoplasmic level of β-catenin low (Figure 1-6, left panel).

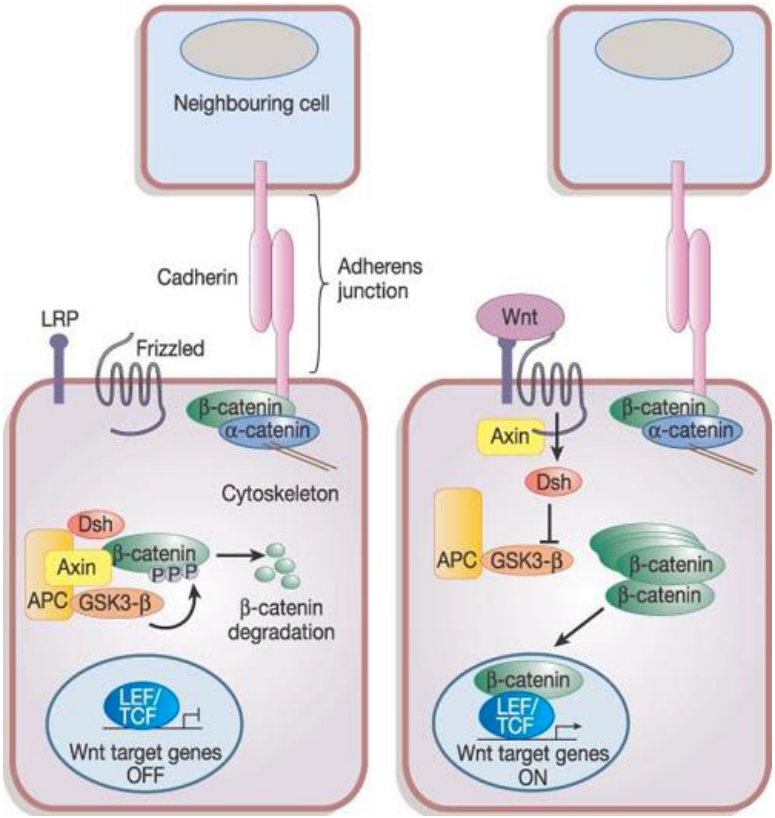

Figure 1.6 Canonical Wnt signaling pathway. In the absence of Wnt signalling (left panel), β-catenin is in a complex with axin, APC and GSK3-, and gets phosphorylated and targeted for degradation. β-Catenin also exists in a cadherin-bound form and regulates cell–cell adhesion. In the presence of Wnt signalling (right panel), β-catenin is uncoupled from the degradation complex and translocates to the nucleus, where its binds Lef/Tcf transcription factors, thus activating target genes (Reya and Clevers 2005).

Binding of Wnt ligands to Frizzled receptors and essential co-receptors of the LDL receptor-related family (LRP 5/6) leads to formation of a ternary complex and subsequently to phosphorylation of Dishevelled (Dsh). The activated Dsh in turn destabilizes the multi-protein destruction complex, impeding β-catenin phosphorylation and therefore enabling its cytosolic accumulation. Stabilized β-catenin translocates to the nucleus, where it replaces transcriptional repressors, such as groucho, from transcription factors of the lymphoid enhancer-binding factor/T-cell factor (LEF/TCF) family, allowing transcriptional activation of Wnt/β-catenin target genes, such as c-myc and cyclin D1 (Figure 1.6, right panel) (Lustig and Behrens 2003).

Dkk-1 and Dkk-4 share the ability to inhibit canonical Wnt/β-catenin signaling via binding on LRP5/6 and Kremen co-receptors. Several mechanisms for inhibition of canonical Wnt-pathway by Dkk1 have been proposed. In the absence of Kremen co-receptors (Krm) Dkk1 is able to bind to LPR6 and thus blocking directly the formation of the Wnt-LRP5/6-Frizzled ternary complex (Figure 1.7 b) (Semenov, Tamai et al. 2001). Krm1/2 greatly potentiates the ability of Dkk1 to block Wnt signaling. Krm2 forms a ternary complex with Dkk1 and LRP6, and induces rapid endocytosis and removal of LRP6 from the plasma membrane thus preventing indirectly Wnt-LRP5/6-Frizzled ternary complex formation and further signaling (Figure 1.7 c) (Mao, Wu et al. 2002). While Dkk1 acts as a pure inhibitor of Wnt/b-catenin signaling, Dkk2 can either activate or inhibit the pathway, depending on the cellular context. At low LRP6 doses Dkk2 would compete with Wnt and thus lower the signaling output, while at high dose Dkk2-LRP6 signaling can overcompensate for lack of Wnt-Lrp5/6-Frzzeld interaction and further amplify signaling (Figure 1.7d) (Mao and Niehrs 2003).

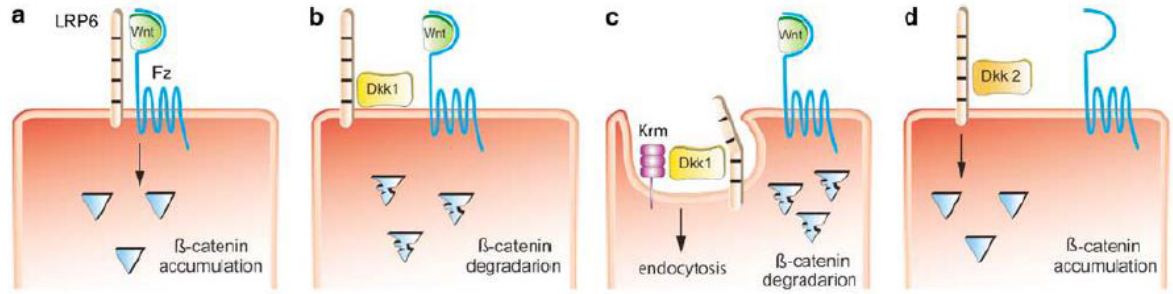

Figure 1.7 Model of Dkk interactions with the Wnt/b-catenin pathway. (a) Wnt forms a ternary complex with Frizzled and LRP6, which promotes stabilization of b-catenin, thereby activating the pathway. (b, c) Dkk1 binding to Lrp6 blocks signal transduction by preventing Wnt-Frizzled binding (b) and/or LRP6 endocytosis in the presence of the Dkk1 coreceptor Kremen (c). (d) Dkk2 can activate LRP6 (Niehrs 2006).

Besides their role in Wnt/ß-Catenin pathway modulation, Dkk proteins show distinct and elevated expression patterns in tissues that mediate mesenchyme-epithelial transitions, indicating that they may participate in the development of the heart, tooth, hair follicles, limb and bone (Monaghan, Kioschis et al. 1999). In Xenopus embryos Dkk1 is expressed in the Spemann organizer region and is essential for head development (Glinka, Wu et al. 1998). In rodents Dkk1 is essential for head development and is involved in distal limb patterning as well as (Mukhopadhyay, Shtrom et al. 2001) osteoblast and cardiac myocyte proliferation (Kawano and Kypta 2003).

Little is known about Soggy (also called DkkL1), other than that the protein is associated with spermatogenesis, and that it localizes to the acrosome (Kohn, Kaneko et al. 2005).

1.3.3 Functions of Dkk3

As well as the other members of the Dickkopf family, Dkk3 is expressed during vertebrate development in many organs. Additionally, it has been reported to be significantly expressed in adult rodents. Prominent Dkk3 mRNA expression was observed in the brain, spinal cord, eye, lung, heart, colon, ovary, uterus (Niehrs 2006) hair follicle (Ohyama, Terunuma et al. 2006) and gastric crypt bases (Byun, Karimi et al. 2005). In the eye, Müller glial cells were identified to be the main source of Dkk3 (Nakamura, Hunter et al. 2007).

However, Dkk3 knockout mice are viable and fertile. Dkk3 deficient mice only display increased lung ventilation and hyperactivity as well as minor changes in the frequency of NK cells, immunoglobulin M, hemoglobin and hematocrite levels (Barrantes Idel, Montero-Pedrazuela et al. 2006).

In addition, there are several reports identifying Dkk3 expression in human cells. For example, a study using human pancreatic tissue revealed Dkk3 protein expression in a subpopulation of the human pancreatic beta cells. There, Dkk3 is stored in cytoplasmatic vesicles and co-localized with insulin (Hermann, Pirkebner et al. 2007). Additionally, Dkk3 protein was found in the zona glomerulosa of the human adrenal cortex (Suwa, Chen et al. 2003). Furthermore, Dkk3 is shown to be strongly expressed in tumor endothelial cells where it supports capillary formation as well as in endothelial colony-forming cells (ECFC) where it is co-localized with the von Willebrand Factor in Eibel Palade Bodies. In these cells, Dkk3 secretion is shown to be up-regulated in response to stimulation with the pro-inflammatory cytokine TNFα (Untergasser, Steurer et al. 2008).

In addition, megacaryocytes and platlets are shown to express Dkk3. Intracellularly, it is colocalized with the vascular endothelial growth factor (VEGF) in α-granules. This expression is exhibited to be increased in patients with myeloproliferative neoplasems (MPN) (Medinger, Tzankov et al. 2010). Furthermore, human mesenchymal stem cells were reported to express Dkk3. In these cells Dkk3 production could be further elevated by TNFα stimulation (Lee, Yoon et al. 2012). Soluble dkk3 is also found in human serum (Jiang, Huang et al. 2010) and cerebrospinal fluid (Zenzmaier, Marksteiner et al. 2009).

Contrary results have been published concerning the role of Dkk3 in the Wnt/β-catenin pathway. While early reports point out that Dkk3 does not influence Wnt/ß-Catenin signalling and does not interfere with the LRP5/6 as well as the Kremen (Krm) receptors (Mao, Wu et al. 2002), later studies revealed that Dkk3 is an ligand for the Kremen receptors Krm1 and Krm2 and potentiates Wnt signaling via this interaction (Nakamura and Hackam 2010). Additionally, Dkk3 may have distinct roles in regulating Wnt pathway depending on the cell types examined. For example, Dkk3 potentiates Wnt signaling in human Müller Glia Cells (MIOM1), HEK293 cell lines hunter (Nakamura, Hunter et al. 2007) and SH-SY5Y cells (Nakamura and Hackam 2010) but inhibits Wnt signaling in PC12 and osteocarcinoma Saos-2 cells (Caricasole, Ferraro et al. 2003, Hoang, Kubo et al. 2004) as well as in Dkk3 transfected HeLa cervical cancer cells through blocking the nuclear translocation of β-catenin (Lee, Jo et al. 2009). In line with that, more recent studies suggest a suppressive role of Dkk3 in Wnt/ßcatenin signalling during spermatogenesis (Das, Wadhwa et al. 2013) as well as in apopstosis regulation in glioma cells (Li, Shen et al. 2013) and breast carcinogenesis (Xiang, Li et al. 2013). However, the precise role of Dkk3 in modulation of the Wnt/β-catenin pathway remains still elusive.

Dkk3 is also known as ”REIC” (Reduced Expression in Immortalized Cells) (Tsuji, Miyazaki et al. 2000), since it has been proposed to act as tumor suppressor. Dkk3 has been claimed to be down-regulated in a broad range of tumors, including hepatocellular carcinoma, lymphoblastic leukemia, prostate cancer, renal cell carcinoma, lung carcinoma, melanoma, ovarian cancer and neuroblastoma (Lodygin, Epanchintsev et al. 2005, Koppen, Ait-Aissa et al. 2008). Lack of Dkk3 expression in tumor cells has been attributed to hypermethylation of CpG islands in the Dkk3 promoter (Roman-Gomez, Jimenez-Velasco et al. 2004). Dkk3 overexpression suppresses tumor cell proliferation and induces apoptosis via a wide range of mechanisms (Abarzua, Sakaguchi et al. 2005, Abarzua, Sakaguchi et al. 2007, Abarzua, Kashiwakura et al. 2008, Mizobuchi, Matsuzaki et al. 2008, Chen, Watanabe et al. 2009, Kawasaki, Watanabe et al. 2009, Lee, Jo et al. 2009, Sakaguchi, Kataoka et al. 2009).

Although hypermethylation of Dkk3 correlates with certain cancers (Roman-Gomez, Jimenez-Velasco et al. 2004, Lodygin, Epanchintsev et al. 2005), the physiological relevance of altered Dkk3 expression in tumors and its potential growth inhibitory effect are unknown. In contrast to reports considering Dkk3 as tumorsupressor, Dkk3 deficient mice show no enhanced tumorigenesis (Barrantes Idel, Montero-Pedrazuela et al. 2006). Additionally it has been shown, that knockdown of Dkk3 induces apoptosis in lung adenocarcinoma (Jung, Kang et al. 2010) and alterations at the dkk3 gene locus is related with lower lymph node metastasis and better prognosis in head and neck squamous cell carcinomas (Katase, Gunduz et al. 2008). These findings rather indicate a function of Dkk3 in tumor protection.

1.3.4 Dkk3 in immunology

Our group showed just recently that Dkk3 was expressed in a CD8 regulatory T cell population (but not in naïve and activated T- cells) that was crucial for the mediation of peripheral tolerance. This observation was made in a system in which the MHC-I antigen Kb was expressed on keratinocytes in mice transgenic for a Kb-specific Des-T cell receptor inducing a state of peripheral tolerance (Alferink, Tafuri et al. 1998). Remarkably after introduction of a null mutation of Dkk3, Des.KerKb mice were no longer tolerant (Papatriantafyllou, Moldenhauer et al. 2012)

Additionally, after genetic deletion or blocking of Dkk3 by a monoclonal antibody, mice bearing induced autoimmune encephalomyelitis (EAE) were shown to develop a more exacerbated and chronic disease than WT controls, indicating an immunosuppressive function of Dkk3. Dkk3 deficient mice bearing a chronic EAE displayed an increased amount of IFNɣ-producing T cells in the brain while this effect could not be observed in spleen and lymph nodes, indicating a locally restricted effect of Dkk3 (Papatriantafyllou, 2008).

Thus we can assume that Dkk3 is part of the control of T cell responses in inflamed tissues

1.4 Renal fibrosis

Tissue injury provokes inflammation by creating pro-inflammatory niches in which the concentration gradients of chemotactic cytokines provide a directional signal for guiding the infiltration of inflammatory cells to the injured sites (Chung and Lan 2011). Renal fibrosis is now discussed as example of tissue damage, which is almost always preceded by the infiltration of inflammatory cells, including lymphocytes, monocytes/macrophages, dendritic cells and mast cells.

1.4.1 Basic kidney anatomy and physiology

Kidneys are the filter units of our body. They purify toxic metabolic waste products from the blood in hundred thousands of functionally independent units called nephrons. A nephron consists of one glomerulus and one double hairpin-shaped tubule that drains the filtrate into the renal pelvis. The glomeruli located in the kidney cortex are bordered by the Bowman's capsule. They are lined with parietal epithelial cells and contain the mesangium with many capillaries to filter the blood. The glomerular filtration barrier consists of endothelial cells, the glomerular basement membrane and visceral epithelial cells (also known as podocytes).

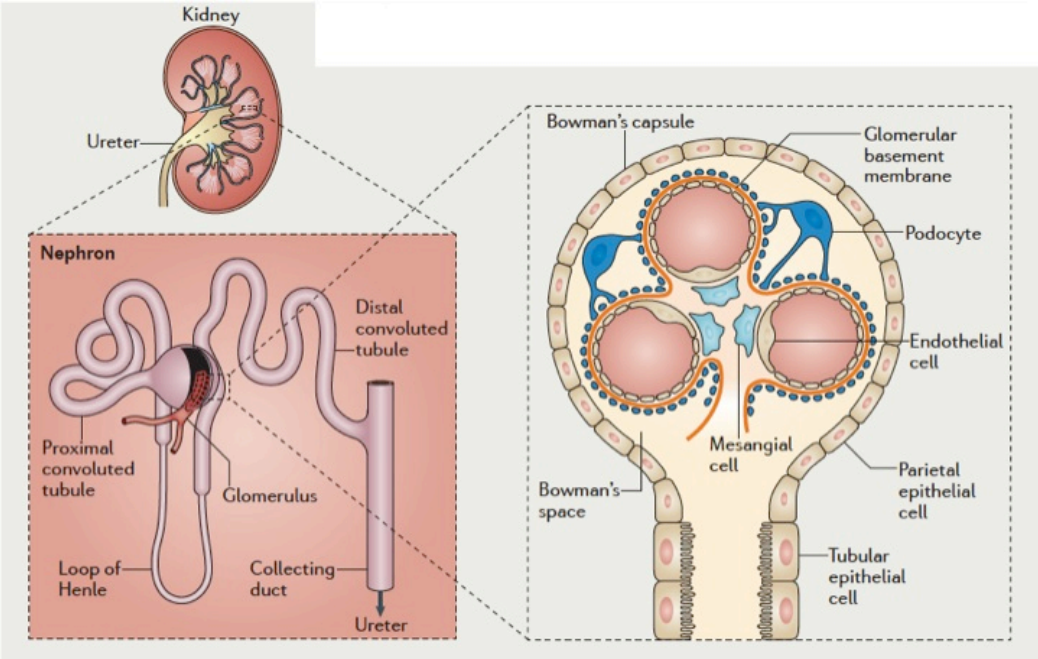

Figure 1.8 Shematic illustration of basic kidney anatomy. Whole kidney (upper left), nephron (lower left) and glomerulus (right) composition (Kurts, Panzer et al. 2013).

All molecules below the molecular size of albumin (that is, 68 kDa) pass the filter and enter the tubule, which consists of the proximal convoluted tubule, the loop of Henle and the distal convoluted tubule. An intricate countercurrent system forms a high osmotic gradient in the renal medulla that concentrates the filtrate. The tubular epithelial cells reabsorb water, small proteins, amino acids, carbohydrates and electrolytes, thereby regulating plasma osmolality, extracellular volume, blood pressure and acid–base and electrolyte balance. Non-reabsorbed compounds pass from the tubular system into the collecting ducts to form urine. The space between the tubules is called the interstitium (Figure 1.8) (Kurts, Panzer et al. 2013).

1.4.2 Cellular and molecular mechanisms of renal fibrosis

Renal fibrosis is the common final outcome of almost all progressive chronic kidney diseases (CKD) irrespective of the initial cause. It is considered to be a failed wound healing process that leads to the loss of kidney function and requires life long dialysis or kidney transplantation. Major cellular events after initial insults include infiltration of inflammatory cells, fibroblast activation and expansion from various sources, production and deposition of a large amount of extracellular matrix (ECM) components, which finally causes microvascular rarefaction, hypoxia, tubular atrophy and apoptosis (Figure 1.9). Together, they constitute a core set of fibrogenic events that result in the ultimate destruction and loss of renal parenchyma and end-stage renal failure.

As already pointed out, renal fibrogenesis is considered, to be a failed wound healing process that occurs after the initial insults of various injuries (Wynn 2008), for example caused by infection, autoimmune diseases, foreign bodies, mechanical stress or tumors (Wick, Grundtman et al. 2013). In this regard, the cellular and molecular responses in the injured kidneys are largely dictated by evolutionarily conserved defence programs of wound healing in an attempt to repair and recover from damage (Liu 2011). Almost all the cell types in the kidneys, including fibroblasts, tubular epithelial cells, pericytes, endothelial cells, vascular smooth muscle cells, mesangial cells and podocytes, as well as the infiltrated cells such as lymphocytes, macrophages and fibrocytes, are involved and participate in some way

in the pathogenesis of renal fibrosis, which illustrates the immense complexity of this process (Boor, Ostendorf et al. 2010).

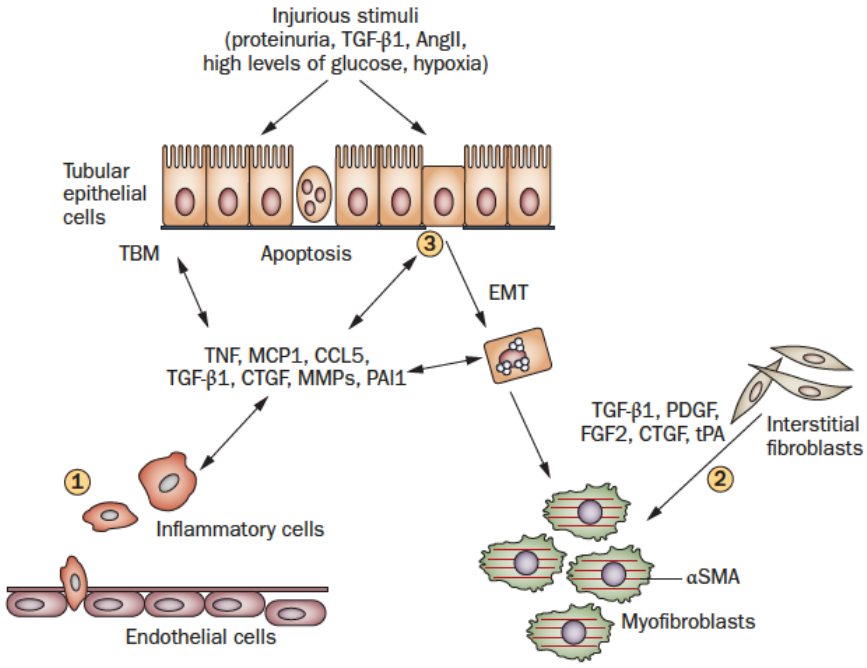

Figure 1.9 Major events in renal interstitial fibrogenesis. (1) Peritubular infiltration of inflammatory cells, particularly T cells and macrophages, is an early event that sets up a fibrogenic stage. (2) Myofibroblast activation and expansion from various sources. The majority of the matrix-producing myofibroblasts are probably generated from local activation of interstitial fibroblasts. (3) Tubular cell apoptosis and EMT, leading to tubular atrophy (Liu 2011).

1.4.3 Chronic inflammation in renal fibrosis

Although inflammation is an integral part of the host defence mechanisms in response to injury, non-resolving, chronic inflammation is a major driving force in the development of fibrotic diseases (Nathan and Ding 2010). Following injury, infiltrated inflammatory cells become activated, produce molecules that damage tissues and induce the production of fibrogenic cytokines and growth factors (Duffield 2010, Vernon, Mylonas et al. 2010). This series of events builds up sustained pro-fibrotic cytokine pressure within the local microenvironment and primes fibroblasts and tubular epithelial cells to undergo phenotypic activation or transition and to produce a large amount of ECM components. Therefore, inflammation after a sustained injury serves as a primer that sets up the fibrogenic stage and triggers tissue fibrogenesis (Liu 2011). Best characterized immunological players in this complex process are T lymphocytes and macrophages. In the early phase of renal injury

chemoattractants released by damaged and infiltrating immune cells, direct the migration of activated T lymphocytes into the injured tissue. In this process CC (RANTES/CCL5), CXC (IP-10/CXCL10) chemokines, macrophage inflammatory proteins (MIPs), lipid mediators (leukotriene) and anaphylatoxic complement fragments play a pivotal role (Kuroiwa, Schlimgen et al. 2000). Functionally, the degree of tubulointerstitial fibrosis is related to the number of infiltrating T cell. It was shown that mature B and T lymphocytes owing to a deficiency of RAG1, are protected against fibrosis after obstructive injury (Tapmeier, Fearn et al. 2010). Similarly, depletion of $CD4^+$ T cells in wild-type mice considerably reduced the extent of interstitial fibrosis, whereas reconstitution with purified $CD4^+$ T cells in RAG1 deficient mice restores fibrogenesis after injury (Tapmeier, Fearn et al. 2010).

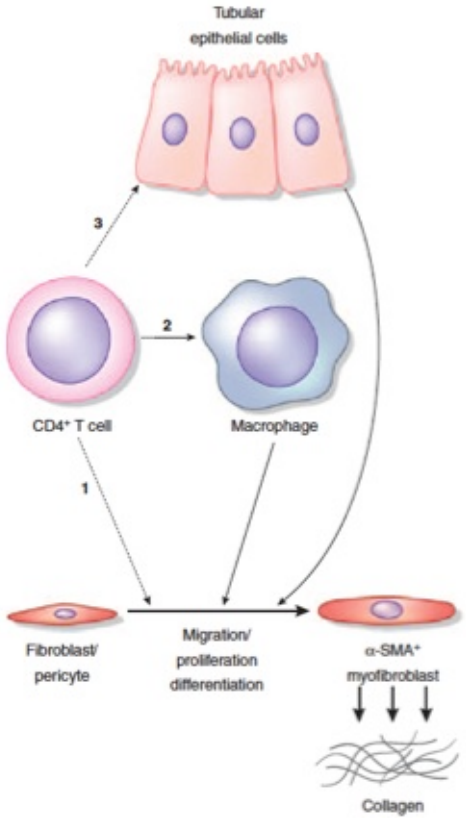

Figure 1.10 Schematic diagram illustrating the potential role of T cells in interstitial fibrosis. It is now evident that virtually all of the collagen-producing, a-smooth muscle actin-positive myofibroblasts in the interstitium of the obstructed kidney are derived through proliferation and differentiation of resident pericytes and fibroblast populations. This process may be modulated by T cells by at least three non-exclusive mechanisms. (1) T cells may act directly on renal fibroblasts and pericytes to promote their migration, proliferation, and differentiation, resulting in the accumulation of a-SMA+ myofibroblasts, which synthesize and deposit interstitial matrix. (2) T cells may induce a profibrotic phenotype in the infiltrating macrophage population, which, in turn, secrete pro-proliferative and profibrotic cytokines and growth factors that induce fibroblast migration, proliferation, and differentiation. (3) T cells may act directly on tubular epithelial cells to induce secretion of cytokines and growth factors that, in turn, act on fibroblasts (Nikolic-Paterson 2010).

These findings clearly establish a critical role for lymphocytes, specifically CD4+ T cells, in the onset of renal fibrosis. However, despite theses findings, the precise mechanisms by which $CD4^+$ T cells may promote renal fibrosis remain elusive. Possible target cells for T cells are tubular epithelial cells, macrophages or myofibroblast precursors itself (Fig 1.10).

Moreover, more recent studies implicate that not only the number of infiltrating T cells but mainly their phenotype determine the outcome of renal fibrosis (Figure 1.11). For example, it has been shown that especially $CD4^+$ cells of the Th2 linage play a pivotal role in the development of renal fibrosis. Cells with this phenotype were reported to be the predominant T cell subset in later stages of renal fibrosis after unilateral ureteral obstruction (UUO). Furthermore, Th2 $CD4^+$ T cells were shown to potently restore experimental renal fibrosis when transferred into Nu/Nu mice, while TH1 $CD4^+$ T cells failed to do so (Liu, Kou et al. 2012). Additionally, another study revealed that $CD4^+$ T cells of the Th2 linage promote renal fibrosis mediated by the secretion of IL-4, which in turn activates macrophages (Braga, Correa-Costa et al. 2012). These findings in models of kidney fibrosis are supported by a number of studies showing the pro-fibrotic properties of Th2 cells and their associated cytokines IL-4, IL-5 and IL-13 in several models of experimental fibrosis in other organs. For example, neutralization of IL-4 resulted in reduced development of experimental fibrosis in liver and skin (Cheever, Williams et al. 1994, Ong, Wong et al. 1998). As one mode of action, IL-4 was shown to directly induce synthesis of extracellular matrix proteins, typ I and II collagens and fibronectin in mouse and human fibroblasts in vitro (Sempowski, Beckmann et al. 1994, Doucet, Brouty-Boye et al. 1998) thereby being twice as effective as TGF-β (Fertin, Nicolas et al. 1991). Similarly, by using antibody blockade or genetic deletion, IL-13 was identified as the dominant effector cytokine in several experimental models of fibrosis (Kumar, Herbert et al. 2002, Kolodsick, Toews et al. 2004). IL-5 was reported to be responsible for the differentiation, activation and recruitment of eosinophils. These cells are an important source of fibrogenic cytokines including TGF-β and IL-13 (Le Moine, Flamand et al. 1999).In contrast, the Th1 cytokine IFNγ was shown to inhibit experimental renal fibrosis. For example, administration of recombinant IFNγ significantly improved disease outcome in rats (Oldroyd, Thomas et al. 1999, Yao, Zhang et al. 2011). Additionally, several experimental models of fibrosis in other organs have documented potent anti-fibrotic activities for the Th1-associated cytokines IFNγ and IL-12 (Gurujeyalakshmi and Giri 1995, Oldroyd, Thomas et al. 1999).

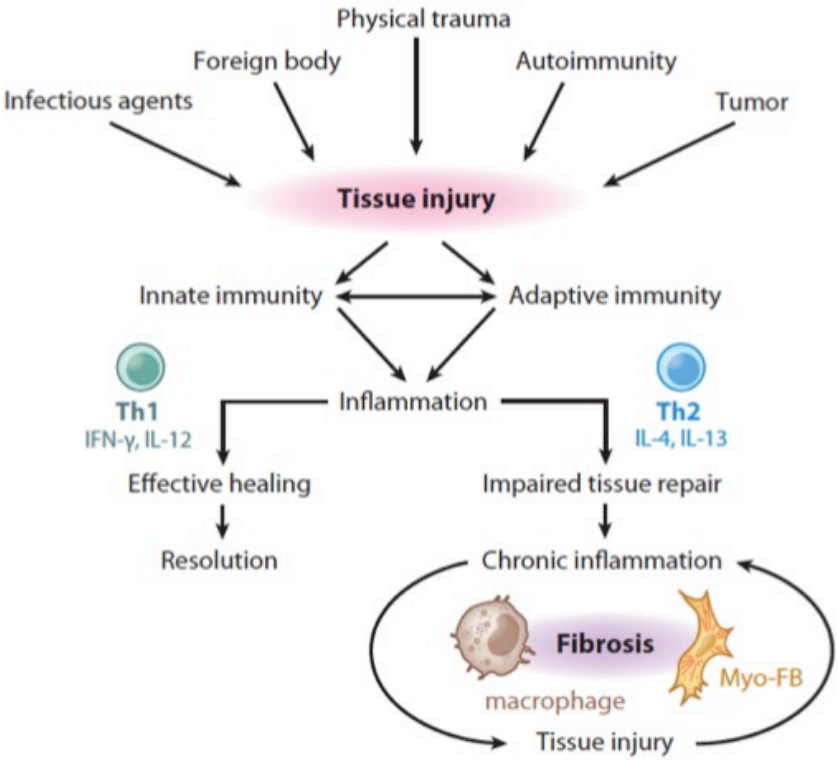

Figure 1.11 Pathogenesis of fibrosis: Tissue injuries caused by infection, foreign bodies, mechanical stress, autoimmune reactions, or neoplasia activate the immune system and repair mechanisms. Effective healing is usually characterized by adominant Th1 response, whereas a predominant Th2 response lead to chronic inflammation, which can ultimately result in fibrosis (Wick, Grundtman et al. 2013).

Only little is known about the role of regulatory T cells in renal fibrosis. However, data from experimental fibrosis models in other organs as well as clinical studies revealed a contradictory role for Tregs in fibrosis development. This divergent impact on fibrosis outcome may be explained by the (im)balance between the degree of inflammatory response suppression and production of pro-fibrotic factors (Wick, Grundtman et al. 2013). On the one hand, Tregs produce large amounts of IL-10, shown to down-regulate chronic inflammation trough many mechanisms (Moore, de Waal Malefyt et al. 2001). Consistent with its role as suppressive cytokine, IL-10 has been shown to inhibit fibrosis in numerous models. IL-10 treated mice develop significantly less liver, lung and pancreatic fibrosis (Louis, Van Laethem et al. 1998, Thompson, Maltby et al. 1998, Arai, Abe et al. 2000), while IL-10 deficiency leads to a higher susceptibility of fibrosis in these models. Furthermore, IL-10 was shown to suppress the synthesis of type I collagens in human fibroblasts indicating that it

can directly inhibit fibrosis (Wangoo, Laban et al. 1997). However, on the other hand Tregs are also potent producers of pro-fibrotic TGF-β, which drives fibrosis development.

Cells of the monocyte/macrophage lineage are always present and are the predominant infiltrating cell type both in experimental models and in human chronic kidney diseases. Macrophages produce a wide variety of different cytokines, chemokines and growth factors, reactive oxygen and nitrogen species, matrix metalloproteinases and components of the extracellular matrix. Thus, the presence of macrophages often correlates with the degree of fibrosis. Infiltrated macrophages has been considered to be key effector cells by modulating inflammatory response and subsequent proliferation of myofibroblasts, extracellular matrix deposition and other fibrotic processes (Eddy 1995). However, a significant number of reports noted an inverse correlation between the number of interstitial macrophages and the degree of fibrosis, especially at the later stage of CKD. Therefore macrophages are also assumed to have a role in the repair processes and protection of the injured kidney (Cochrane, Kett et al. 2005).

Macrophages can be functionally distinguished into two broad phenotypes based on cell surface markers and cytokine profile with different roles in inflammation and fibrosis (Duffield 2003, Lupher and Gallatin 2006). Classically activated (M1) macrophages are induced by the Th-1 cytokine IFNγ, bacterial and fungal cell wall components or degraded matrix. In contrast, alternatively activated (M2) macrophages are induced by Th-2 cytokines such as IL-4, IL-10, IL-13 and TGF-ß, anti-inflammatory agents such as corticosteroids as well as the phagocytosis of apoptotic cells (Goerdt and Orfanos 1999). M1 and M2 macrophages mediate contrasting and complementary functions in tissue fibrosis. For example, M1 macrophages may induce apoptosis of host tissue cells through the release of pro-inflammatory mediators such as TNFα (Duffield, Ware et al. 2001) and nitric oxide (NO) (Kipari and Hughes 2002) thereby inducing tissue injury. Tubular epithelial cells represent a target of macrophage-induced apoptosis in the obstructed kidney (Lange-Sperandio, Cachat et al. 2002). Apoptotic cells are rapidly recognized and phagocytosed by macrophages with this process promoting differentiation into M2 macrophages (Duffield, Ware et al. 2001). Macrophages reprogrammed by apoptotic cell ingestion or exposure to anti-inflammatory cytokines act to dampen inflammation by the secretion of cytokines such as IL-10 and TGF-β and appear to be highly pro-fibrogenic and contribute to tissue remodeling where increased ECM deposition predominates. Indeed, M2 macrophages produce large amounts of TGF-β

(Fadok, Bratton et al. 1998) and can induce myofibroblast proliferation (Song, Ouyang et al. 2000). In contrast, M1 macrophages produce matrix metalloproteinases (MMPs) (Song, Ouyang et al. 2000) and can also induce myofibroblasts to produce MMPs (Mariani, Sandefur et al. 1998) that promote ECM degradation and facilitates the resolution of fibrosis. Furthermore, MMPs may contribute to the resolution of fibrosis by stimulating myofibroblast apoptosis (Iredale, Benyon et al. 1998). *Upar*$^{-/-}$ mice, which lack the scavenger receptor urokinase-type plasminogen activator receptor, exhibit increased myofibroblast accumulation and fibrosis that is accompanied with decreased macrophage infiltration at a late stage after UUO (Zhang, Kim et al. 2003), suggesting that infiltrating macrophages may exert anti-fibrotic actions by affecting the size of the interstitial myofibroblast population. On the other hand, MMPs may induce basement membrane injury and promote renal fibrosis (Cheng and Lovett 2003). It is of interest that early inhibition of MMP2 activity in experimental allograft nephropathy ameliorated fibrosis whereas MMP2 inhibition in the setting of established fibrosis resulted in more severe allograft nephropathy (Lutz, Yao et al. 2005). Thus, stimulation of the production of MMPs by M1 macrophages during the later stages of fibrosis may shift the equilibrium towards degradation and play an important anti-fibrotic role. Macrophage depletion during progressive inflammatory fibrosis ameliorated scarring and reduced myofibroblast numbers whilst macrophage depletion during the recovery phase led to a failure of matrix degradation and persistent scarring (Nishida and Hamaoka 2008). The contribution of these two phenotypes of macrophages at each stage of the fibrotic process still remains to be elucidated.

1.4.4 Sources of myofibroblasts in renal fibrosis

The increase of pro-fibrotic cytokine concentration in the inflamed microenvironment after kidney injury leads to the activation of matrix-producing cells, which is a central event in renal fibrogenesis. Although many cell types in the tubulointerstitium such as fibroblasts, tubular epithelial cells, vascular smooth muscle cells and a subset of macrophages, are capable of producing ECM, fibroblasts are commonly regarded as the principal matrix-producing cells that generate a large amount of interstitial matrix components, including fibronectin and type I and type III collagens. Activated fibroblasts in diseased kidneys often express α smooth muscle actin (αSMA), and are also referred to as myofibroblasts (Liu 2011). In this context, one of the fundamental issues in the field is to delineate the origin, activation and regulation of these matrix-producing myofibroblasts (Grande and Lopez-Novoa 2009, Meran and Steadman 2011).

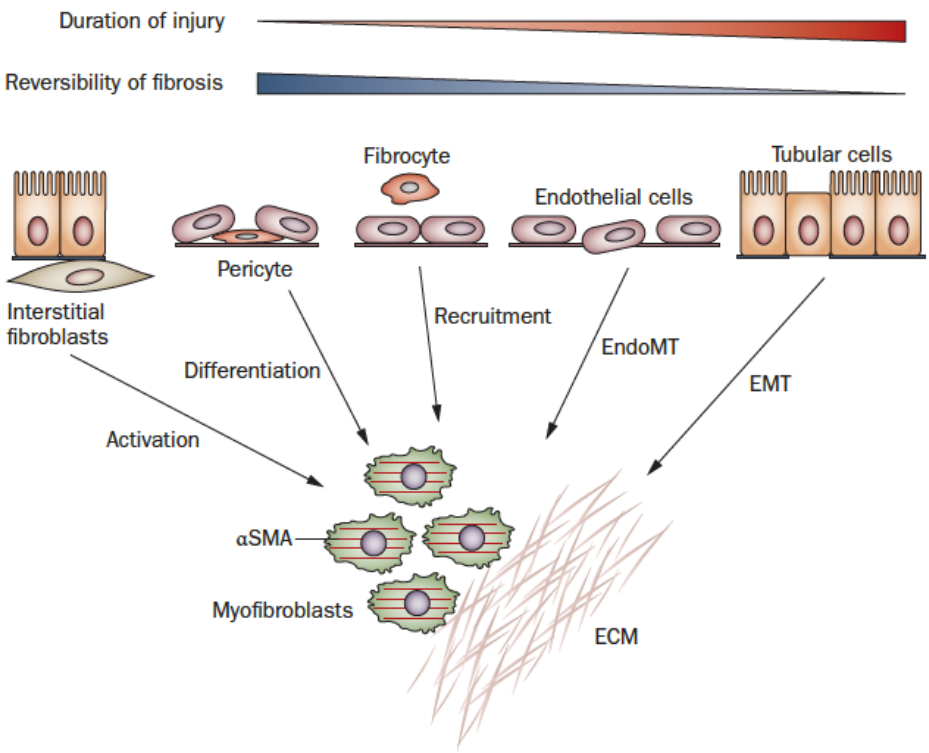

Figure 1.12 Multiple origins of myofibroblasts have been proposed in renal fibrosis. Myofibroblasts can be derived from at least five different sources through various mechanisms: phenotypic activation from interstitial fibroblasts; differentiation from vascular pericytes; recruitment from circulating fibrocytes; capillary EndoMT; and tubular EMT. The relative contribution of each source to the myofibroblast pool in renal fibrosis is controversial. Conceivably, local activation of resident fibroblasts remains the major route for the generation of myofibroblasts in diseased kidneys, at least in the early stage. By contrast, EMT could be a late event, and contribute to the irreversible progression of fibrosis (Liu 2011)

At least five different sources, with diverse mechanisms, have been proposed as contributors to the myofibroblast pool in diseased kidneys (Figure 1.12). These include activation of interstitial fibroblasts, differentiation of pericytes, phenotypic conversion of tubular epithelial cells and endothelial cells and recruitment of circulating fibrocytes (Barnes and Gorin 2011). The relative contribution, and even the existence, of each particular myofibroblast-generating pathway to renal fibrosis is a matter of debate and is highly controversial (Liu 2011).

Historically, matrix-producing myofibroblasts were assumed to derive from resident fibroblasts by activation after renal injury (Hewitson 2009). Although this concept has been challenged, it remains largely authenticated (Strutz and Muller 2006, Grande and Lopez-Novoa 2009). The majority of myofibroblasts could conceivably originate from local activation and proliferation of interstitial fibroblasts in the injured kidneys. In normal adult kidneys, fibroblasts are situated in the interstitial space between the capillaries and the epithelia and form a network throughout the renal parenchyma, thereby stabilizing tissue architecture (Kaissling and Le Hir 2008). Morphologically, these cells are stellate shaped and exhibit abundant rough endoplasmic reticulum, collagen-secreting granules and actin filaments. They possess multiple cell processes, which connect them to the tubular and capillary basement membranes (Kaissling and Le Hir 2008). Upon activation by profibrotic cytokines and mechanical stress, fibroblasts acquire a myofibroblast phenotype by expressing αSMA and producing a large amount of ECM components. In many respects, myofibroblasts resemble both activated fibroblasts and smooth muscle cells because of their excessive production of matrix components and their expression of αSMA and contractility, respectively (Liu 2011). Both fibroblasts and myofibroblasts have the capacity to proliferate in response to cytokines, which lead to expansion of the fibroblast population and interstitial space in diseased kidneys Several fibrogenic growth factors, including platelet derived growth factor (PDGF), TGF-β, fibroblast growth factor (FGF2) and connective tissue growth factor (CTGF), are well-known mitogens for fibroblasts (Strutz, Zeisberg et al. 2000, Bottinger 2007, Hewitson 2009, Phanish, Winn et al. 2010, Boor and Floege 2011, Ostendorf, Eitner et al. 2012).

Secondly, it is suggested that vascular pericytes are a major source of myofibroblasts in fibrotic kidneys (Schrimpf and Duffield 2011). Pericytes are a subset of stromal cells that

partially cover capillary walls, thereby stabilizing the endothelium. Following kidney injury, pericytes are detached from the endothelium, undergo migration and proliferation, and differentiate into myofibroblasts (Lin, Kisseleva et al. 2008, Humphreys, Lin et al. 2010). Pericyte detachment and differentiation into myofibroblasts under pathological conditions not only results in destabilization of the microvasculature, but also contributes to myofibroblast activation, which leads to interstitial fibrosis (Kaissling and Le Hir 2008).

Another source of matrix-producing cells could be tubular epithelium that undergoes EMT, a cell phenotypic conversion process that occurs during embryonic development, tumor metastasis and organ fibrosis (Kalluri and Weinberg 2009, Thiery, Acloque et al. 2009). Broad agreement exists that tubular epithelial cells *in vitro* can undergo EMT, characterized by loss of epithelial features and acquisition of mesenchymal markers, under the influence of various pro-fibrotic cytokines, particularly TGF-β (Yang and Liu 2001, Humphreys, Lin et al. 2010). However, whether this transition mainly represents an *in vitro* artefact or does occur *in vivo* is at the centre of the argument (Liu 2011).

Fibrocytes are a subset of bone marrow-derived, circulating monocytes with fibroblast-like features in the peripheral blood (Herzog and Bucala 2010). They are spindle-shaped cells that express the hematopoietic cell marker CD45 and are capable of producing type I collagen (Wada, Sakai et al. 2011). Fibrocytes also express certain chemokine receptors, such as CCR7. In response to kidney injury, fibrocytes mobilize, infiltrate renal parenchyma and participate in fibrogenesis. The differentiation of fibrocytes is modulated by other inflammatory cells, such as $CD4^+$ T cells, through secreted cytokines (Niedermeier, Reich et al. 2009). Profibrotic cytokines IL-4 and IL-13 promote fibrocyte differentiation, whereas antifibrotic cytokines IFNγ and IL-12 inhibit this process, suggesting that an inflamed milieu that contains a complex mixture of cytokines is a major determinant of fibrocyte differentiation (Shao, Suresh et al. 2008). The relative importance of fibrocytes in renal fibrogenesis is controversial. As specific markers for these cells are lacking, clear discrimination of fibrocytes from monocytes, macrophages, fibroblasts and myofibroblasts is rather difficult. Therefore, results from experimental studies on the involvement of fibrocytes in renal fibrosis are inconsistent (Niedermeier, Reich et al. 2009).

The relative contribution of each lineage to the myofibroblast pool might depend on the disease model and specific stage of the disease. Regardless of their different origins, activated fibroblasts from all sources produce a large amount of ECM components, leading

to excessive accumulation and deposition of interstitial matrix and formation of collagenous fibers that predominantly contain type I and type III collagens and fibronectin (Liu 2011). The expression and synthesis of ECM proteins by the matrix-producing cells is primarily controlled at the level of gene transcription in response to various extracellular fibrogenic cues. Key fibrogenic factors include TGF-β1, PDGF, FGF2, CTGF and angiotensin II, whereas hepatocyte growth factor and bone morphogenetic protein 7 inhibit the production of matrix components primarily by antagonizing TGF-ß1 activation (Yang, Dai et al. 2003, Schnaper, Jandeska et al. 2009, Luo, Phillips et al. 2010, Boor and Floege 2011).

1.4.5 Causes of renal failure

The concept that renal fibrosis is solely the result of excessive accumulation of matrix components is simplistic and inaccurate. Whether fibrosis itself actually causes kidney failure remains an open question; after all, it is the normal early response to wound healing. Many cellular and molecular events beyond the production of matrix components promote the progression of fibrotic injury and are actually responsible for the progressive loss of kidney function. In many ways, the events that occur in the progression phase determine the reversibility and ultimate outcome of renal fibrogenesis (Liu 2011). Tubular epithelial cells comprise the bulk of renal parenchyma and are the primary target of a variety of metabolic, immunologic, ischemic and toxic insults. Depending on the severity and duration of injury, tubular cells exhibit a wide range of responses, such as proliferation, autophagy, growth arrest, EMT and apoptosis. In patients with CKD, the histopathological presentation of tubular damage is often characterized as tubular atrophy. The potential involvement of tubular epithelia in renal fibrosis is illustrated by the preferential activation of major inflammatory and fibrogenic signaling in renal tubules after injury. In response to cell stress or injuries, NFκB signaling is activated in renal tubular epithelium, which triggers the production and release of inflammatory cytokines, and initiates peritubular inflammation. Tubular epithelial cells are susceptible to TGF-β1, produced by both the injured tubule and the infiltrating cells, and instigate a fibrogenic program. In tubular epithelial cells, sustained injury also activates β-catenin (He, Dai et al. 2009, He, Kang et al. 2011), Notch1 (Bielesz, Sirin et al. 2010) and hypoxia-inducible factor 1 (HIF1) (Higgins, Kimura et al. 2007) all of

which are key intracellular signaling mediators implicated in renal fibrogenesis. Damage to tubular epithelia often induces protective and regenerative responses such as autophagy and cell proliferation. Autophagy, a process of cell 'self-eating', is regarded as a mechanism of cell survival and protection that mediates adaptation to calorie restriction or hypoxia in renal tubules *in vivo* (Jiang, Liu et al. 2010). However, excessive autophagy is proposed as the underlying mechanism that leads to total decomposition of tubular cells (Koesters, Kaissling et al. 2010). Tubular injury and atrophy undoubtedly impair tubular function and diminish the effectiveness of its endogenous protective mechanisms (Liu 2011).

Additionally, renal fibrosis is typically associated with peritubular microvascular contraction, particularly in the advanced stage. Multiple mechanisms could account for the loss of peritubular capillaries in the fibrotic kidneys. Peritubular vasculature is susceptible to damage induced by apoptosis of endothelial cells in advanced CKD. Ischemia and oxidant stress are the known apoptotic stimuli for endothelial cells, which could be a potential mechanism that leads to rarefaction of peritubular capillaries. Likewise, the integrity of peritubular capillaries could be impaired by endothelial dysfunction (Kelly, Burford et al. 2009, Venkatachalam, Griffin et al. 2010).

Chronic hypoxia in renal interstitium is characteristic of advanced CKD, and is a common pathology in patients with this condition (Fine and Norman 2008, Mimura and Nangaku 2010). Several mechanisms lead to chronic hypoxia in diseased kidneys, including microvascular constriction, decreased oxygen diffusion as a result of fibrosis and increased metabolic demands of tubular cells. Hypoxia can lead to tubular EMT or apoptosis, activate resident fibroblasts and impair peritubular capillaries, thereby creating a cycle of chronic hypoxia and progressive kidney failure. HIF1 is a key regulator of cell responses to hypoxia (Gunaratnam and Bonventre 2009). Chronic hypoxia often coexists with increased oxidative stress and generation of reactive oxygen species (ROS), which incites damage to biologically important macromolecules, including proteins, lipids and carbohydrates, and causes them to undergo structural modifications, leading to generation of advanced glycation end products, advanced oxidation protein products and advanced lipoperoxidation end products (Negre-Salvayre, Coatrieux et al. 2008). These modified macromolecules are not a reliable surrogate marker for estimating the degree of oxidant-mediated damage in CKD, they also affect various kidney cells as pathogenic mediators and trigger renal inflammation and fibrogenic responses, thereby promoting the progression of kidney injury and renal fibrosis (Shi, Hou et

al. 2008, Zhou, Hou et al. 2009). In addition, the hypoxic and fibrotic microenvironment might lead to perturbed cellular signaling and induce epigenetic modifications, which could contribute to increased fibroblast proliferation, activation and fibrogenesis (Bechtel, McGoohan et al. 2010).

1.4.6 Wnt signaling in renal fibrosis

Wnt/ßcatenin signaling has been shown to play a role in kidney development and diseases. For example, Wnt4 and Wnt9 were shown to be highly expressed in the early stage during kidney development and are functionally important for kidney formation (Carroll, Park et al. 2005). While, in adult kidney, Wnt signaling seems to be silenced (Iglesias, Hueber et al. 2007), experimental induction of kidney diseases leads to overexpression of diverse Wnt proteins and induction of ß-catenin signalling (He, Dai et al. 2009). Several studies provided that hyperactive Wnt/ß-catenin signaling is detrimental in the glomeruli and tubules resulting in CKD (Pulkkinen, Murugan et al. 2008). These studies suggest, that Wnt proteins may potentially work together in a coordinated fashion to trigger interstitial fibrosis, respectively.

Systematic analysis of Wnt signaling in normal kidney compared to fibrotic kidneys showed that all members of Wnt except Wnt5b, Wnt8b, and Wnt9b were up-regulated in the fibrotic kidneys with different dynamics. Additionally, most FZD receptors and Wnt regulators including Dkk1, 2, 3 and 4 were induced in the fibrotic kidneys. Along with this, accumulation of ß-catenin and up-regulation of target genes including c-Myc, Twist and fibronectin was observed. In addition, delivery of Dkk1 effectively inhibited ß-catenin accumulation, induction of target genes, and myfibroblast activation (He, Dai et al. 2009). Wnt4 was suggested to play a role in the pathogenesis of renal fibrosis. Wnt4 expression was induced throughout the collecting ducts in 4 murine models of renal tubulointerstitial fibrosis. Additionally, presence of the highest cellular Wnt4 expression coincided with interstitial fibroblasts overexpressing collagen I and α-SMA mRNA in the fibrotic lesions. Furthermore, Wnt4 induced stabilization of cytosolic ß-catenin in a cultured myofibroblast cell line and transplantation of fibroblasts expressing Wnt4 under the renal capsule induced lesions with tubular epithelial destruction in mice (Surendran, McCaul et al. 2002). The Wnt4/ß-catenin

pathway may also regulate the regeneration of renal tubules in acute renal failure. (Terada, Tanaka et al. 2003).

The pro-fibrotic role of ß-catenin signalling was further supported by a study showing that transgenic mice expressing an oncogenic form of ß-catenin suffer from severe polycystic lesions in the glomeruli, proximal tubules, distal tubules, and collecting ducts soon after birth (Saadi-Kheddouci, Berrebi et al. 2001).

Collectively, the Wnt pathway seems to be activated in the fibrotic kidney. Furthermore, its activation in fibrosis has been reported in other tissues, including lung, liver and skin (Lam and Gottardi 2011). Therefore, chemical inhibitors of the Wnt pathway may be promising for clinical therapy for patients with fibrotic disease. However, the detailed mechanisms of Wnt pathway activation in renal fibrosis remain to be studied (He, Dai et al. 2009).

1.4.7 Chronic kidney disease in the clinic

CKD has become a major public health problem on global scale, indicated by the fact that an estimated 13% oft he adult population in the USA has some degree of CKD (Andl, Reddy et al. 2002, Coresh, Selvin et al. 2007) and a considerable proportion of cases eventually progress to end-stage renal failure. Numerous epidemiological studies indicate that the prevalence of patients with end-stage renal disease is increasing worldwide (Andl, Reddy et al. 2002, Coresh, Selvin et al. 2007). However, current therapeutic options for CKD in the clinical setting are scarce and often ineffective. An approved treatment specifically targeted to renal fibrosis is almost non-existent.

In this context, an improved understanding of the cellular and molecular mechanisms of renal fibrosis is paramount and essential, not only for gaining novel insights into the pathogenesis of the process, but also for developing rational strategies to treat patients with fibrotic kidney disorders (Liu 2011).

1.6 Aim of the study

In recent years, the idea that tissue cells contribute to the regulation of innate and adaptive immune responses has been affirmed in numerous studies. The crosstalk between cells of the tissue and the infiltrating cells of the immune system is essential to establish the right balance between efficacy and the self-containment of the inflammatory response within a particular tissue.

Therefore, the aim of this study is to identify new mechanisms by which tissue cells can influence adaptive immune responses in general and how Dickkopf-3 contributes to this tissue mediated immune modulation in particular.

In the first part of the thesis we will address the question whether keratinocytes in the skin are capable to present self-antigen to CD4 T cells and thus influence following systemic autoimmune responses. Additionally, we aim to investigate the role of Dkk3 in this process.

Due to its immune modulatory capacity, the expression pattern of Dkk3 is of particular interest. Therefore, in the second part, we will analyse the capacity of a newly generated Dkk3 reporter mouse to identify sites of Dkk3 expression. In addition, the influence of cytokines on Dkk3 production will be examined.

Finally, we will ask whether Dkk3 influences the development of kidney fibrosis and examine the underlying mechanisms. These investigations will be conducted in two different mouse models of renal fibrosis.

2 Materials and Methods

2.1 Materials

2.1.1 Chemicals/Reagents

Chemicals	Company
2-isopropanol	Fluka
Acetic acid	Merck
Aceton	Fluka
Acrylamid	Roth
Bromphemol Blue	Sigma
Bovine Serum Albumine (BSA)	Gerbu
CFSE	Molecular Probes
Chloroform	VWR
Dimethylsulfoxid (DMSO)	Merck
Disodium Carbonate	Roth
Disodium hydrogen phosphate	Merck
EDTA	Pharmacia
Ethanol	Sigma
Ethidium Bromide	Appli Chem
Fetal Calf Serum (FCS)	Biochrom
Dulbecco´s Phosphate Buffered Saline (dPBS)	Invitrogen
Glutamine	Gibco
Glycerine	Merck
Glycine	Gerbu
Hydrogen Peroxid	Sigma
Sulfuric Acid	Merck
Heparin	Ratiopharm
HEPES	Roth
Hydrochloric Acid	J.T. Baker
Magnesium Chloride	Sigma
Magnesium Sulfate	Sigma
Sodium Hydroxide	J.T. Baker
Sodium Chloride	Roth
ß-Mercaptoethanol	Merck
Dithiothreitol (DTT)	Roth
Retionic Acid	Sigma
Lipopolysaccharide (LPS)	Sigma
Dulbecco´s Modified Eagle Medium (DMEM)	Invitrogen
RPMI 1640	Gibco
Diphenylcyclopropenone	Sigma
Oxazolone (4-Ethoxymethylene-2-phenyl-2-oxazolin-5-one)	Sigma

Succrose	Roth
Paraformaldehyde	Sigma
Tris/HCl	Roth
Sodium Azide	Roth
Sodium Disulfate (SDS)	Sigma
TEMED	Roth
Ammoniumpersulfate	Appli Chem
D-Luciferine	Synchem
Tween 20	Sigma
Trypsin	Gibco
L-Glutamine	Gibco
Penicilin/Streptomycin	Gibco
Cell/Tissue Lysis Buffer	Cell Signaling
Methanol	Sigma
Höchst 33342	Sigma
DAPI	Life Technologies
O.C.T Compound	Tissue Tek
Percoll	GE Healthcare
Sepharose-Protein G Beads	Millipore
Brefeeldin A (Golgi Plug)	BD Bioscience
Monesin (Golgi Stop)	BD Bioscience
Proease inhibitor Cocktail (PIC)	Roche
Phospho Stop	Roche
Pertussis toxin	Sigma
Incomplete freunds adjuvant	Thomas Gayer
Mycobacterium tuberculosis	Thomas Gayer
Flouromount	Southern Biotech
Milk powder	Roth
Donkey Serum	Sigma
Isoflurane	Baxter
D-Luciferine	Synchem

2.1.2 Media

Name	Ingredients
DMEM Full Medium	Dulbeccos Modified Eagle Medium 10% FCS (heat inactivated) 10 mM HEPES 2 mM L-Glutamine 1 mM Sodium Pyruvate 0.05 mM 2-Mercaptoethanol 100 units/ml Penicillin 100 mg/ml Streptomycin
RPMI Full Medium	RPMI 1640 10% FCS (heat inactivated) 10 mM HEPES 2 mM L-Glutamine 1 mM Sodium Pyruvate 0.05 mM 2-Mercaptoethanol 100 units/ml Penicillin 100 mg/ml Streptomycin
Cell Freezing Medium	FCS (heat inactivated) 20% (v/v) DMSO

2.1.3 Buffers

Buffer	Ingredients
Phosphate Buffered Saline (PBS)	130 mM NaCl 2.6 mM KCl 15 mM KH2PO4 4 mM Na2HPO4
ELISA Carbonate Buffer	50mM Na2CO3 50mM NaHCO3 pH 9.6
ELISA Blocking Buffer	PBS 0.2% Gelantine 0.1% NaN3
ELISA Development Buffer	0.1M KH2PO4 1mg/ml OPD 0.1% H2O2 pH 6.0
SDS-PAGE Sample Buffer	150 mM Tris pH 6.8 6% SDS 30% Glycerin

	0.15% Bromphenol blue 0.3 M DTT
SDS-PAGE Running Buffer	25 mM Tris pH 8.3 192 mM Glycine 0.1 % SDS (w/v)
Westen Blot Transfer Buffer	48 mM Tris 39 mM Glycine 1.3 mM SDS 20% Methanol pH 9.2
Western Blot Blocking Buffer	PBS 0.05% Tween 20 5% Milk Powder
Dulbecco´s Posphate Buffered Saline (dPBS)	PBS MgCl2 CaCl2
Western/ELISA Washing Buffer	PBS 0.05% Tween 20
Heparine Buffer	dPBS 2% FCS 5u/ml Heparin
MACS Buffer	2mM EDTA 0.5% FCS pH 7.2
FACS Buffer	dPBS 0.5% FCS 0.05% NaN3
Trypsin Buffer	PBS 0.25% Trypsin 2.5mM EDTA
Cell Lysis Buffer (Cell Signaling)	20 mM Tris-HCl (pH 7.5) 150 mM NaCl 1 mM Na_2EDTA 1 mM EGTA 1% Triton 2.5 mM sodium pyrophosphate 1 mM beta-glycerophosphate 1 mM Na_3VO_4 1 µg/ml leupeptin
Rehydrat Buffer	PBS 1% BSA 0.1% NaN_3 10% Donkey Serum
Blocking Buffer	PBS 1% BSA 0.1% NaN_3 10% Donkey Serum 0.1mg/ml mouse IgG

2.1.4 Kits

Kit	Company
Absolute QPCR SYBR Green ROX Mix	Thermo Scientific
Cytofix/Cytoperm Kit	BD Bioscience
Foxp3 staining Kit	eBioscience
RNeasy mini Kit	Quiagen
RNAzol	Wack Chemie
Maxima Reverse Transcriptase Kit	Thermo Scientific
Immobilion Western HRP Substrate	Millipore
Dynal mouse T cell negative Isolation Kit	Invitrogen
CD4+ CD62L+ T cell isolation kit	Milteny Biotec
Cell Tracker	Molecular Probes
Percelly Keramik kit	Peqlab
CD5 microbeads / MS columns	Milteny Biotec

2.1.5 Antibodies

2.1.5.1 Flow Cytometry

Antigen	Source	Conjugate	Clone	Company
CD4	Rat	PB	RM4-5	Biolegend
CD8	Rat	FITC	53-6.7	BD Bioscinece
F23.1	Rat	Bio	F23.1	Self-generated
CD44	Rat	PE-Cy7	IM7	eBioscience
CD25	Rat	PerCP-Cy5.5	PC61	Biolegend
CD62L	Rat	PE	MEL-14	BD Bioscience
IFNγ	Rat	eFlour660	XMG1.2	eBioscience
GATA3	Rat	eFlour660	TWAJ	eBioscience
Tbet	Rat	eFlour660	eBio4B10	eBioscience
Foxp3	Rat	APC	FJK-16s	eBioscience
IFNγ	Rat	APC	XMG1.2	BD Bioscience
TNF	Rat	PE	Mab1	BD Bioscience
IgG1 Isotype	Rat	eFlour660	eBRG1	eBioscience
IgG2b Isotype	Rat	eFlour660	eB149/10H5	eBioscience
IgG1 Isotype	Rat	PE	eBRG1	eBioscinece

2.1.5.2 WesternBlot / ELISA

Antigen	Source	Conjugate	Clone	Company
Dkk3	Mouse	Biotin	4.22	-
Dkk3	Mouse	-	4.22	-
Dkk3	Rabbit	-	Polyclonal	Sino Biological Inc.
Anti-Rabbit IgG	Goat	HRP	Polyclonal	Dianova
Beta-Actin	Mouse	-	Polyclonal	Sigma
Anti-Mouse IgG	Rabbit	HRP	Polyclonal	Dianova

2.1.5.3 Immunohistology

Antigen	Source	Conjugate	Clone	Company
mCherry	Goat	-	Polyclonal	Acris
Keratin15	Rabbit	-	Polyclonal	Sigma Aldrich
MBP1-10	Rabbit	-	Polyclonal	Self-generated
Anti-Goat IgG	Donkey	Alexa Flour488	Polyclonal	Life Technologies
Anti-rabbit IgG	Mouse	FITC	Polyclonal	Life Technologies

2.1.6 Primer

Gene	Fw: 5´- 3´	Rv: 5´- 3`
Actb	TGACAGGATGCAGAAGGAGATTA	AGCCACCGATCCACACAGA
Gapdh	CATGGCCTTCCGTGTTCCTA	TGTCATCATACTTGGCAGGTTTCT
Foxp3	CACAACCTGAGCCTGCACAA	TCAAATTCATCTACGGTCCACACT
Tgfb1	TGGAGCAACATGTGGAACTC	CAGCAGCCGGTTACCAAG
Il10	CCAGCCTGAGCCTAGAATTCA	TGGCTTCAAACCACACATAGGA
Pdl1	TGGACAAACAGTGACCACCAA	TGTCCGGGAAGTGGTGACA
Ctla4	ACTCATGTACCCACCGCCATA	TGGTTCTGGATCAATGACATAAAT
Dkk3	TCCCATTGCCACCTTTGG	CCAGTTCTCCAGCTTCAAGTACAC
H2-Aa	TCTCCCTCCTGTGATCAACA	CACCGTCTGCGACTGACTTG
Cxcl9	GTTCGAGGAACCCTAGTGATAAGG	CCTCGGCTGGTGCTGATG
Cxcl10	GACGGTCCGCTGCAACTG	CCCTATGGCCCTCATTCTCA
Ccl2	AGCAGGTGTCCCAAAGAAGCT	GGGTCAGCACAGACCTCTCTCT
IFNR1	ACCCTGAAGTCGTTGTGAATGG	GGTGCTCCCGTCACCAAA
IL-6	ATGAACAACGATGATGCACTTG	TATCCAGTTTGGTAGCATCCAT
IFNg	GATGCATTCATGAGTATTGCCAAGT	GTGGACCACTCGGATGAGCTC
IL-1ß	TCGTGCTGTCGGACCCATA	TGTTGGTTGATATTCTGTCCATTGA
Gata3	AGAACCGGCCCCTTATGAA	AGTTCGCAGGATGTCC
Tbet	CAACAACCCCTTTGCCAAAG	TCCCCCAAGCAGTTGACAGT
CB99 Luciferase	GGAGTCATGCAGACCCATCA	CGTGGATCGAGAGCATGGA
TCF7	AGCGCTGCCATCAACCA	TGGCCTGCTCTTCTCGAGAT
LEF1	CAACCAGATCCTGGGCAGAA	GTTCATAGTATTTGGCCTGCTCTTC
Nlk	TTTCCATCTCTTGGATCTGTTACTGA	GAGCCGGATAGACCAATTGG
cJun	ACTCCGAGCTGGCATCCA	TGGTTCATGACTTTCTGCTTAAGC
Fzd1	TGGCGCCTCTCTTCGTTTA	AAACCGGCCAGCAGGAA
Fzd7	TACCTGCCAGACCCACCTTT	AGCGGCCTCTGCCATCT

All primers were purchased from Sigma-Aldrich.

2.1.7 Recombinant Proteins /Peptides

Protein	Company
Murine IFNγ	Peprotech
Murine IL-1β	Peprotech
Murine IL-4	Peprotech
Murine IL-7	Peprotech
Murine IL-10	Peprotech
Murine IL-12p40	Peprotech
Murine IL-13	Peprotech
Mouse Dkk3	Peprotech
MBP 1-10 peptide (Ac-ASQKRPSQRS)	PSL
MOG 33-55 peptide (MEVGWYRSPFSRVVHLYRNGK)	PSL
Human TGF-β	Peprotech

2.1.8 Mice

Strain	Source
C57BL/6N	Charles River Laboratories
B6.B10/PL	Charles River Laboratories
Dkk3-/-	C. Niehrs (Barrantes Idel, Montero-Pedrazuela et al. 2006)
IFN1R-/-	T. Schüler (Sercan, Stoycheva et al. 2010)
Tg4	D. Wraith (Liu, Fairchild et al. 1995)
Ker-MBP	Self generated
Dkk3-LCh	Self generated

Mice were maintained under specific pathogen free (SPF) conditions at the German Cancer Research Center. Animal care was in concordance with the instructions of the regulating authorities.

2.1.9 Electronic devices

Device	Company
7500 Real Time PCR System	Applied Biosystems
FACS Canto II	BD Bioscience
IVIS 100	Xenogen
1420 Multilable Counter Victor	PerkinElmer
Zeiss Cell Observer	Zeiss
Trans Blot SD Semi Dry Tranfer Cell	BioRad
FastPrep 120 Tissue Homogenizer	Thermo
Cryostat CM 1860	Leica

2.1.10 Software

Software	Source
Adobe Illustrater	Adobe
Adobe Photoshop	Adobe
Endnote	Thomson Reuters
Excel	Microsoft
FACS DIVA	BD
FlowJo 9.	FlowJo
ImageJ	Fiji
Living Image 2.50	PerkinElmer
PowerPoint	Microsoft
Prism	Graphpad
Word	Microsoft
Zen	Zeiss

2.2 Methods

2.2.1 Generation of transgenic mice

2.2.1.1 Ker-MBP mice

For keratinocyte-specific expression the 2.4 kb fragment of the Keratin IV promoter was used (Alferink, Tafuri et al. 1998). The introduction of the signal sequence and the MBP1-10-peptide into the I-Au β-chain cDNA has been described (Kurschus, Oelert et al. 2006). To assure appropriate cleavage of the signal sequence the amino acid sequence GDS was introduced N-terminal to the MBP-sequence. As a substitute of natural acetylation a glycin residue was inserted between the GDS sequence and the MBP1-10 sequence. We replaced the cDNA after exon 2 with the genomic DNA of the I-Ab β-chain (obtained from RZPD; identical with the β-chain I-Au coding sequence). The DNA construct was linearized and the purified fragment was injected into C57BL/6 x DBA/2 F2 oocytes. Founders and litters were tested by southern blot. Transgenic mice were backcrossed more than 10 times to B10.PL and were crossed to Tg4 mice.

2.2.1.2 Dkk3-LCh mice

In order to identify Dkk3 expressing tissues and cells a BAC transgenic mouse expressing the reporter genes CB99 Luciferase and mCherry under regulatory elements of the Dkk3 gene was generated. To allow the stochiometric co-expression of the 2 reporter genes CB99Luciferase and mCherry were linked via a viral 2A peptide consensus motif. The generation of this CB99Luciferase-2A-mCherry construct has been described (Miloud, Henrich et al. 2007). The CB99Luciferase-2A-mCherry cassette was inserted into the ATG of the Dkk3 gene within a BAC, containing the entire Dkk3 gene and its regulatory elements (BAC clone ID: RP23-12M6) After removal of the excessive vector, the DNA construct was purified and injected into C57BL/6 F2 oocytes which were transplanted to super-ovulated C57B6 females.

2.2.2 Primary Cell Cultures

2.2.2.1 Dermal Fibroblats

Primary dermal fibroblasts were isolated from ears of WT (C57B6/J) *IFNγ*$^{-/-}$ and *dkk3*$^{-/-}$ mice. Ears were mechanically disrupted and incubated for one day in DMEM full medium with 3x Pen/Strep and 1mg/ml Collagenase I at 37°C and 5% CO_2 in. At day 1 ear fragments were further disrupted by pipetting, washed and resuspended in DMEM full medium. Further culturing was accomplished in DMEM full medium.

2.2.2.2 Keratinocytes

Primary epidermal keratinocytes were isolated from the skin of new born C57BL/6 mice as described previously (Lichti, Anders et al. 2008). Briefly, isolated cells were resuspended in complete Keratinocyte Growth Medium 2 containing 0,05mM CaCl2 and 2x10^6 cells were seeded into 6-well plates. Primary keratinocytes were cultured until confluent (37°C, 5% CO_2). The medium was changed every 3 days. The culture supernatants for western blot were collected 72h after the previous medium exchange.

2.2.3 RNA Analysis

2.2.3.1 RNA isolation

To isolate RNA from tissues, organs were disrupted in RLT Buffer of the RNeasy mini Kit with Precellys Ceramic Kit 1.4mm in a FastPrep Homogenizer. Total RNA was extracted from disrupted tissue using the RNeasy mini kit according to manufacturer's instructions. Total RNA isolation from cells suspensions was performed by QiaSchredder and the RNeasy Mini Kit. Additionally, on column DNA digestion was performed using DNAseI. RNA concentrations were determined by a Nanodrop photometer.

2.2.3.2 cDNA Synthesis

2µg of isolated total RNA was reverse transcribed into cDNA using oligo dT(12-18) together with the Maxima Reverse Transcriptase Kit according to maufactural instructions.

2.2.3.3 Quantitative Real Time PCR

Quantitative Real-time PCR was performed using Absolute qPCR SYBR Green ROX Mix with a final primer concentration of 200nM in 25µl reaction volume. PCR products were detected by a 7500 Real Time PCR System from Applied Biosystems during 40 cycles at 60°C (Temperature profile see table below). Data were calculated relative to the housekeeping gene Actb by using the $2^{-\Delta\Delta CT}$ method.

2.2.4 Lymphocyte isolation for flow cytometry / in vitro assays

2.2.4.1 Lymphoid tissue

Single cell suspensions of thymus, spleen and lymph nodes were prepared by processing the organs with a 40µm nylon mesh. For further isolation of distinct Lymphocyte populations the indicated Kits were used according to manufactural instructions.

2.2.4.2 Skin

Skin tissue was dissociated mechanically before incubation in 1 mg/ml collagenase D + 0.02 mg/ml DNAseI in PBS containing 5% FCS for 30 min at 37°C. Leukocytes were further isolated using a Percoll (GE Healthcare) gradient and used for in vitro restimulation experiments.

2.2.4.3 Kidney

Kidneys were incubated in RPMI containing 2% Collagenase I (Sigma) and 2% DNAseI Type IV for 30 min. Tissue was homogenized by pipetting up and down every 10min. After washing with MACS Buffer, CD5 positive cells were isolated using CD5 microbeads and MS columns (Milteny Biotec). Extracted $CD5^+$ cells were used for in vitro restimulation and flow cytometric analysis. Alternatively, CD4 positive cells were isolated using the Dynabeads $CD4^+$ T cell isolation Kit. Extracted $CD4^+$ cells were used for RNA isolation and subsequent qRT-PCR.

2.2.5 Flow Cytometry (FACS)

2.2.5.1 Surface Staining

1-2 x 10^6 cells were suspended in 100µl FACS buffer containing fluorochrome-conjugated antibodies in a concentration of 2µg/ml each. Cells were incubated with the antibody mixture for 20 min at 4°C and then washed with FACS buffer. If primary antibody was biotin conjugated, an additional incubation step with fluorescence labeled streptavidin (2µg/ml) for 20 min at 4°C was accomplished. Cell pellets were resuspended with 0.1 ml ice-cold FACS buffer. Flow cytometry was performed on a FACS Canto II with the FACS Diva software (BD Biosciences). The FACS data was analyzed with the FlowJo software.

2.2.5.2 Intracellular Cytokine Staining

After restimulation, cells were stained for surface markers, and then permeabilized using the cytofix/cytoperm kit. Cells were gently resuspended in the cytofix/cytoperm solution for 30 min at 4°C, washed with the cytofix/cytoperm wash buffer and stained with fluorochrome-conjugated antibodies (2µg/ml) for intracellular antigens in 0.1ml cytoperm/wash buffer for 30 min at 4°C. Cells were then washed with the cytoperm/wash buffer and resuspended in FACS buffer.

2.2.5.3 Intracellular transcriptionfactor staining

Following the staining of cell surface markers, cells were stained for intracellular transcription factors using the Foxp3 staining kit. Cells were incubated for 30 min with freshly prepared fixation solution at 4°C in the dark. Cells were then washed with permeabilization buffer and stained with the appropriate transcriptionfactor antibodies diluted in permeabilization buffer at 4°C for 30 min. Cells were then washed again with permeabilization buffer, before resuspending in FACS buffer.

2.2.6 Enzyme-linked immunosorbent assay (ELISA)

2.2.6.1 Tissue lysis

Fresh or frozen tissue was disrupted in Cell Lysis Buffer containing Protease Inhibitor Cocktail and Phosphatse Inhibitor using Precellys Ceramic Kit 1.4 mm in a FastPrep Homogenizer. After 20 min of incubation on ice, cell debris was removed by centrifugation (16.000 x g, 10min, 4°C) and the supernatant was used for the appropriate assay.

2.2.6.2 Dkk3 ELISA

A Flexible Assay Plate (BD Biosciences) was coated with anti-Dkk3-4.22 antibody (3µg/ml) over night at 4°C and blocked with ELISA blocking buffer for 1h at 37°C. Cell culture supernatant or tissue lysate was applied to pre-coated plates and incubated for 1h at RT. Incubation with detection antibody (1µg/ml rabbit anti-mouse Dkk3 antibody; Sino Biological) for 1h at RT was followed by application of HRP conjugated goat anti-rabbit antibody (1h, RT). Each step was followed by extensive washing with washing buffer. Afterwards, ELISA Development buffer containing HRP substrate was applied. The reaction was stopped with 2 M sulphuric acid. Plates were photometrically analyzed by Victor plate reader (PerkinElmer). As standard, dilutions of a recombinant Dkk3-IgG fusion protein were used on each plate.

2.2.7 Biochemical protein analysis

2.2.7.1 Immunoprecipitation

Cell culture supernatants or tissue lysates were incubated with 30 µl of Sepharose-Protein G Beads in PBS-Tween and 5 µg/ml capture antibody for at least 12 hours at 4°C. Subsequently supernatant was removed and beads were washed 3 times with Washing Buffer. In order to elute precipitated proteins, beads were diluted in 15 µl of SDS Loading Buffer and the whole sample was boiled for 10 min at 95°C.

2.2.7.2 Western Blotting

Samples were applied to a 10 % SDS-polyacrylamid gel. The proteins were separated at 140V and transferred on PVDF membranes (Millipore) via Trans Blot SD Semi Dry Tranfer Cell (BioRad). Previously, the membranes had been incubated in methanol (30 sec), destilled water (2 min) and transfer buffer (5 min). After being blocked 1 hour with blocking buffer, the membrane was incubated with biotinylated primary antibody (table x) in 0.5 x Blocking Buffer overnight at 4°C. Subsequently, the membranes were washed 4 times in PBS-Tween and incubated in peroxidase conjugated streptavidin for 1 hour at room temperature. After 1 min incubation in Immobilon Western HRP substrate (Milipore) membranes were imaged in a Lumiimager (Roche Boehringer Diagnostics).

2.2.8 Histology

Skin and brain tissue was fixed in a zinc-based fixative (Dako), embedded in paraffin, and routinely stained with H&E. Sections were stained with rat anti-mouse CD4 or rat anti-mouse MAC-3 followed by a biotinylated rabbit anti-rat IgG and development with the LSAB-AP kit (Dako).

2.2.9 Immunofluorescence staining

2.2.9.1 Skin

Immunofluorescence was performed on formalin (4%) fixed skin sections with rabbit polyclonal Abs specific for MBP_{1-10} or Keratin-15 followed by fluorochrome–conjugated secondary Abs. Counterstaining was performed with DAPI.

2.2.9.2 Whole Embryo

Dkk3-LCh Embryos were harvested 14 days after vaginal plaque detection and fixed in paraformaldehyde (4%) for 24h followed by 24h in 30% sucrose. After freezing embryos in Tissue Tek O.C.T. compound 5µm slides of the whole embryo were cut using a cryostat. After rehydration and blocking, anti-mCherry staining was performed over night at 4°C, followed by anti-goat-Alexa488 staining for 1h at RT and 5 min of Hoechst 33342 staining.

2.2.10 Experimental Autoimmune Encephalomyelitis (EAE)

2.2.10.1 EAE induction in C57B6 mice

Mice with a C57B6/J Background were immunized with the myelin oligodendrocyte glycoprotein 33-55 peptide (MOG_{33-55}: MEVGWYRSPFSRVVHLYRNGK) emulsified in Complete Freund´s Adjuvants (CFA) containing 2mg/ml Mycobacterium tubercolosis. Each mouse received a subcutaneous (s.c.) injection of 100µl CFA containing 200µg MOG_{33-55}. At day 1 and 2 after immunization mice received 200ng of Pertussis toxin (PT).

2.2.10.2 EAE induction in B10.PL mice

EAE was induced by injecting 200 µg the myelin basic protein peptide (MBP_{1-10}: Ac-ASQKRPSQRS) emulsified in 100µl complete freund´s adjuvant containing 200µg Mycobacterium tuberculosis (Thomas Geyer) s.c. in mice with a B10.PL background. At day 1 and 2 after immunization each mouse recieved 200 ng pertussis toxin i.p.

2.2.10.3 EAE transfer in B10.PL

10 days after B10.PL mice were immunized with $MBP_{1-!0}$ in CFA spleens were taken out and single cell suspensions using an 40µm nylon mesh were prepared. Spleenocytes were cultured for 3 days in RPMI containing MBP_{1-10} peptide (20µg/ml) and IL-12 (20ng/ml) at 37°C and 5% CO_2. $2x10^7$ of these cells were injected i.p. into the respective recipient mice followed by two applications of pertussis toxin i.p. (200ng). N-terminal acetylated MBP_{1-10} peptide Ac-ASQKRPSQRS was synthesized in house using standard Fmoc chemistry and was HPLC purified.

2.2.10.4 Clinical symptom assessment

Clinical symptoms were scored as follows: 0, normal; 1, limp tail or hind limb weakness; 2, limp tail and hind limb weakness; 3, partial hind limp paralysis; 4, complete hind limb paralysis; 5, dead or moribund, killed by investigator.

2.2.11 T cells Analysis

2.2.11.1 In vitro T cell restimulation for FACS analysis

Spleenocytes or isolated lymphocytes were resuspended in RPMI full medium. Cells were then stimulated with either 50 µg/ml MBP1-10 peptide or with a combination of PMA (0.5µg/ml) and Ionomycin(1µg/ml) in the presence of Golgi Stop (Monensin) for 6 h at 37°C and 5% CO2. Subsequently, cells were fixed, stained for intracellular cytokines analysed by flow cytometry.

2.2.11.2 In vivo T cell proliferation

Single-cell suspensions of spleens were prepared from donor mice of the indicated strains. For CFSE labeling, total spleen cells were incubated with 5 µM CFSE in DPBS for 15 min at 37°C. Cells were washed twice with ice-cold DPBS and were finally resuspended in DPBS before injection. $5x10^7$ Cells were injected into the tail vein in a volume of 200 µl. Five days later, proliferation of T cells in spleen, lymph nodes and skin was analyzed by flow cytometry.

2.2.12 DTH

7 days after (last) abdominal contact sensitizer treatment, 20µl of a 1% oxazolone solution in aceton was applied to the right ear. Ear thickness was monitored for the following 3 days by a calliper.

2.2.13 DC migration assay

Green fluorescent CellTracker was used to monitor inflammation-induced migration of skin DC into the draining lymph nodes. 400 µl CellTracker (1/20 in ethanol) were applied onto the skin at the site of skin irritation. Twenty-four hours later, single cell suspensions from draining lymph nodes were prepared by treatment in RPMI (Gibco) supplemented with 5% FCS and 100µg/ml collagenase D for 30 min and analyzed by flow cytometry.

2.2.14 Bioluminescence Imaging

2.2.14.1 *In vivo* Bioluminescence Imaging

Mice were injected intraperitoneally (i.p.) at a dose of 150 mg/kg D-Luciferin dissolved in PBS before measurement of luminescence in an IVIS bioluminescence imaging sytstem 100. General anesthesia was induced with 5% isoflurane and continued during the procedure with 1.5% isoflurane. After acquiring of photographic images, luminescent images were acquired with 5min exposure time. Resulting data was analysed using the Living Image 2.5 software.

2.2.14.2 Ex vivo Bioluminescence Imaging

After intraperitoneal injection with a dose of 150 mg/kg D-Luciferin dissolved in PBS mice were sacrificed. Organs were taken out and incubated in a solution of D-Luciferin in PBS (1mg/ml) for 5min at 37°C. Subsequently, photographic images and luminescent images, with an exposure time of 5 min, were acquired.

2.2.15 Statistical analysis

Student's *t*-test was used unless indicated otherwise to test significant numerical differences between groups. The mean disease score of EAE was calculated as the mean of all individual daily scores. Differences in EAE progression in between two groups was analysed using the repeated measures ANOVA test. Dead mice due to EAE were scored 5 until the end of the study. *P* values of less than 0.05 were regarded as statistically significant (**P* <0.05; ***P* < 0.01; ****P* < 0.001).

3 Results

3.1 Self-antigen presented by keratinocytes upon inflammation modulates T cell auto-reactivity at a distal site

Self-antigen specific activation and tolerization of CD4 T cells in the periphery is thought to occur in the secondary lymphoid organs, mediated by professional APC´s. However, the contribution of parenchymal cells in general and keratinocytes in particular to these processes remains to be elucidated in detail.

To gain further insight into this, our lab generated a transgenic mouse model, in which the influence of self-antigen presentation by keratinocytes in the inflamed, adult skin on systemic auto-reactive T cell responses could be studied in the absence of antigen presentation by professional APCs in the draining lymph nodes.

In this transgenic mouse a silent antigenic determinant, the encephalitogenic myelin basic protein (MBP_{1-10}) peptide, linked to the MHC class II A^u β-chain is expressed exclusively in keratinocytes. Cell surface expression of this transgene product requires co-expression of the endogenous MHC II α-chain, which is absent from keratinocytes in the healthy skin but is induced under inflammatory conditions (Nickoloff and Turka 1994). In this setting, skin inflammation facilitates CD4 T cells to encounter the MBP determinant by (i) inducing cell surface presentation of the transgene and (ii) enabling tissue access to T cells.

This model system allowed us to study the contribution of keratinocytes to T cell activation in the inflamed skin and to the associated T cell auto-reactivity at distal sites in the context of experimental autoimmune encephalomyelitis (EAE). The central nervous system (CNS) derived peptide MBP_{1-10} was chosen as a model antigen because auto-antigens of the brain can also be found in the skin under physiological conditions. As melanocytes originate from neural crest they share self-antigens with neurons and glia cells in the peripheral and central nervous system (Kormos, Belso et al. 2011).

3.1.1 Generation and characterization of transgenic Ker-MBP mice

To generate a mouse model for conditional induction of a self-antigen in the inflamed skin of adult mice, a cDNA encoding the MBP_{1-10} peptide and a glycine-serine linker were fused to the genomic DNA of the I-A^u β chain. This transgene was expressed under the control of a keratin IV promoter element that is known to direct expression exclusively into keratinocytes of the hair follicle (Figure 3.1B).

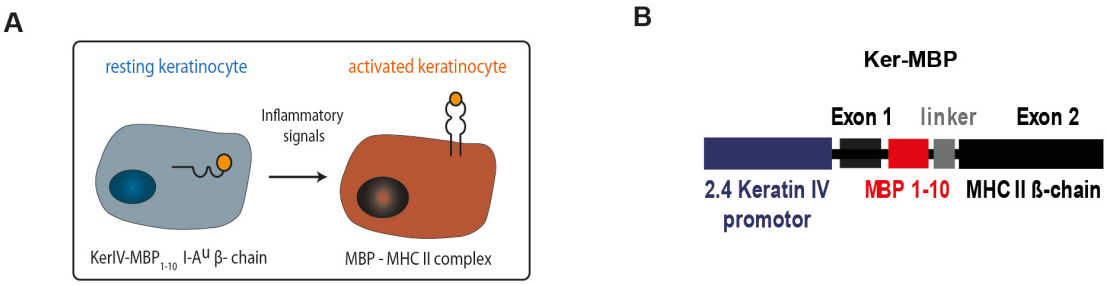

Figure 3.1Ker-MBP Mouse model. (A) The transgenic MBP-MHC class II β-chain construct is constitutively expressed in keratinocytes. However, cell surface expression of the transgene requires co-expression of the endogenous MHC II α-chain which is absent in keratinocytes in the healthy skin but induced under inflammatory conditions. (B) MBP-MHC class II β-chain transgenic construct: The cDNA encoding for the MBP1-10 peptide (ASQKRPSQRS) and a linker sequence was cloned between exon 1 and 2 of the ß-chain of I-A^u. For organ-specific expression the 2400 bp KeratinIV promoter element was used (Alferink, Tafuri et al. 1998).

As a first step tissue-specific expression of the transgenic product was confirmed by RT-PCR using RNA extracted from the respective tissues. As shown in Figure 3.2A this analysis revealed skin specificity of Ker-MBP expression. Furthermore, the keratinocytes specific protein expression of the transgenic construct was analysed by immunohistochemistry of the skin using a MBP_{1-10}-specific rabbit anti-serum and an anti-Keratin15 antibody as a keratinocyte marker. In Ker-MBP mice MBP-positive cells in the hair follicle of the skin (white arrows) co-stained with Keratin15 are detectable whereas they are completely absent in wild type controls (Figure 3.2B). This reveals the presence of the protein product of the Ker-MBP transgene exclusively in keratinocytes of the skin.

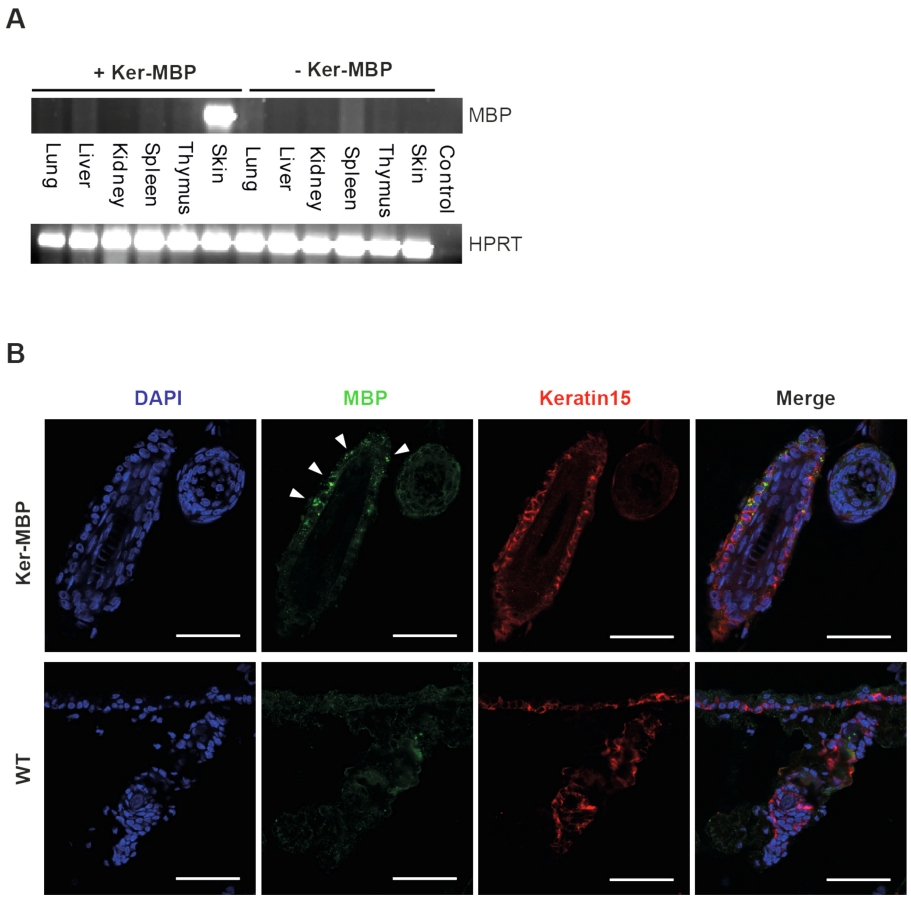

Figure 3.2 Keratinocyte specific expression of the transgenic ß-chain. (A) RNA was extracted from the respective organs of transgenic Ker-MBP and control mice. QRT-PCR identified skin-specific expression of the transgenic β-chain. One representative experiment out of 3 is shown. (B) Keratinocyte specific MBP1-10 peptide expression in the skin of Ker-MBP mice was determined by immuno-histochemistry with a MBP1-10-specific (green) and a Keratin15-specific (red) rabbit anti-serum. Transgene-negative mice were used to confirm the specificity of the staining (Scale Bar: 50µm). Depicted is one representative out of 3 experiments.

In order to increase the proportion of MBP specific T cells in our system, Ker-MBP mice were crossed with MBP-specific TCR transgenic Tg4 mice (Liu, Fairchild et al. 1995). In these mice the fate of the antigen-specific T cells was followed. Thymocyte subsets (Figure 3.3A) and percentages of MBP-specific T cells in lymph nodes (Figure 3.3B) were comparable in Tg4 and Tg4 x Ker-MBP mice excluding negative T cell selection in the thymus and indicating normal T cell development in both mouse strains. Moreover, no differences were observed between Tg4 and Tg4 x Ker-MBP mice in the expression levels of CD62L and CD44 and thus in the activation state of MBP-specific T cells (Figure 3.3C). These results suggest, that T cell homeostasis was not perturbed in the newly generated mouse model. To investigate whether MBP-specific T cells were aware of the transgenic MBP peptide, EAE was induced in Ker-MBP positive and negative Tg4 transgenic mice by immunization with the MBP peptide

in CFA and treatment with pertussis toxin. Both types of mice showed similar levels of EAE (Figure 3.3D). Thus, the intracellular transgenic MBP peptide-MHC class II A^u β-chain is apparently not recognized by T cells and therefore does not induce tolerance in the steady state.

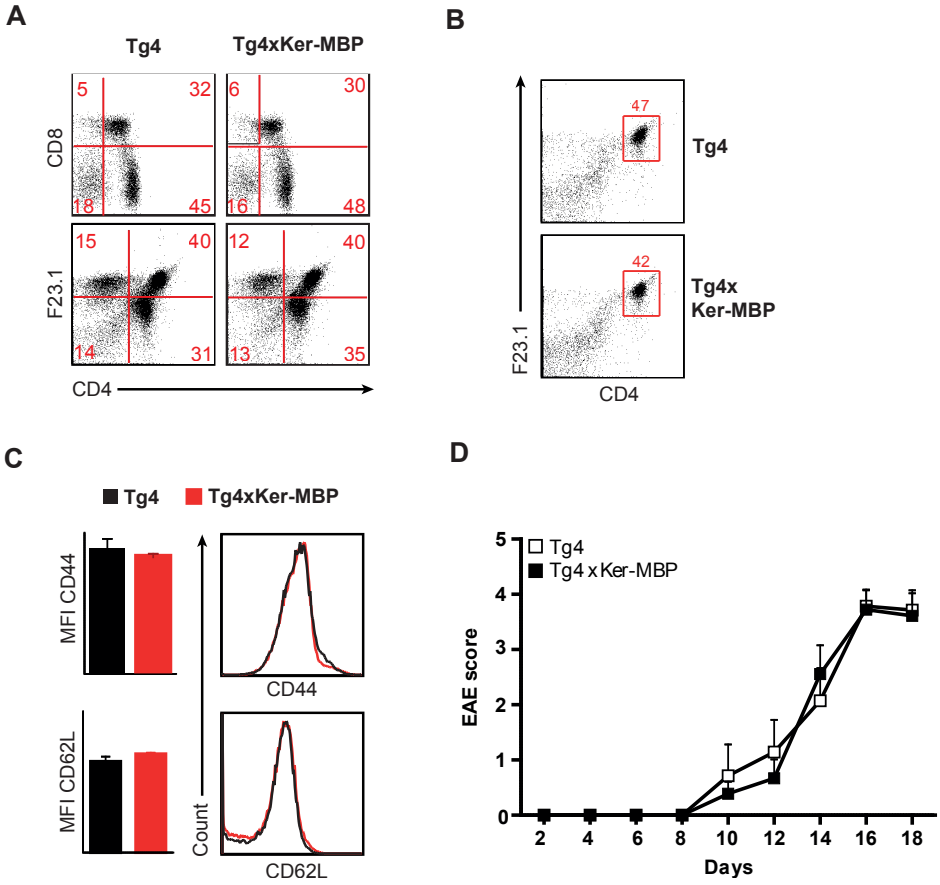

Figure 3.3 Normal T cell development in transgenic Tg4xKer-MBP mice. (A) Thymocytes of the indicated strains were analyzed by flow cytometry. The percentage of $CD4^+$ and $CD4^+/F23.1^+$ (Vβ8.2) or $CD8^+$ T cells are presented. One representative experiment out of three is shown. (B+C) Peripheral lymph node cells of naive Ker-MBPxTg4 and Tg4 mice were analyzed by flow cytometry. (B) The percentage of $CD4^+/F23.1^+$ (Vβ8.2) and (C) the mean fluorescence intensity (MFI) of the activation markers CD44 and CD62L on CD4 T cells are shown. Depicted is one representative experiment out of 3. (D) MBP1-10/CFA induced EAE is not altered in Tg4xKer-MBP mice. Tg4xKer-MBP and Tg4 were immunized by MBP1-10/CFA s.c. injection followed by two applications of pertussis toxin (200 ng) and EAE score was monitored. Mean clinical score ± SEM is plotted over time. Depicted is one representative experiment out of 3 (n=8, n.s. repeated measures ANOVA).

This mouse model was established by former members of our laboratory and the so far presented results (Figure 3.2 and Figure 3.3) were provided by them.

3.1.2 Functional MBP presentation in the skin following inflammation

Surface expression and functional presentation of the transgenic MBP peptide-MHC class II A^u β-chain by keratinocytes requires co-expression of the endogenous MHC II α-chain which occurs only under inflammatory conditions (Nickoloff and Turka 1994). Therefore, we induced acute local skin inflammation by applying the contact sensitizers oxazolone or diphenylcyclopropenone (DPCP) to the shaved abdomen of Ker-MBP mice. To investigate whether the MBP peptide could be recognized by MBP-specific T cells, Tg4 T cells were labelled with CFSE and transferred into Ker-MBP mice one day before skin irritation. T cells were isolated from draining lymph nodes, spleen and treated skin and analyzed by flow cytometry 5 days after transfer (Figure 3.4A). Proliferating Tg4 T cells could be observed in the inflamed skin of Ker-MBP mice, but were undetectable in the spleen and draining lymph nodes. In contrast, strong proliferation of CFSE-labelled Tg4 cells was observed in draining lymph nodes when MBP-peptide loaded DCs were co-injected with the transferred T cells into Ker-MBP mice (Figure 3.4B). Additionally, the contact sensitizer treatment induced the migration of skin CD11c-positive cells into the draining lymph nodes (Figure 3.4C). Nevertheless, the MBP peptide was not presented in the draining lymph nodes of Ker-MBP mice as indicated by the lack of Tg4 T cell proliferation (Figure 3.4A). Thus, this mouse model allows the study of the consequences of antigen presentation by keratinocytes in the skin in the absence of antigen presentation by professional APC in the draining lymph nodes.

Oxazolone treatment resulted in the accumulation of $CD4^+$ T cells in the skin of Tg4 x Ker-MBP mice but not in the skin of Tg4 mice (Figure 3.4D). Thus, contact sensitizer-induced skin inflammation promotes the expansion of MBP-specific T cells in a manner that depends on antigen presentation by keratinocytes.

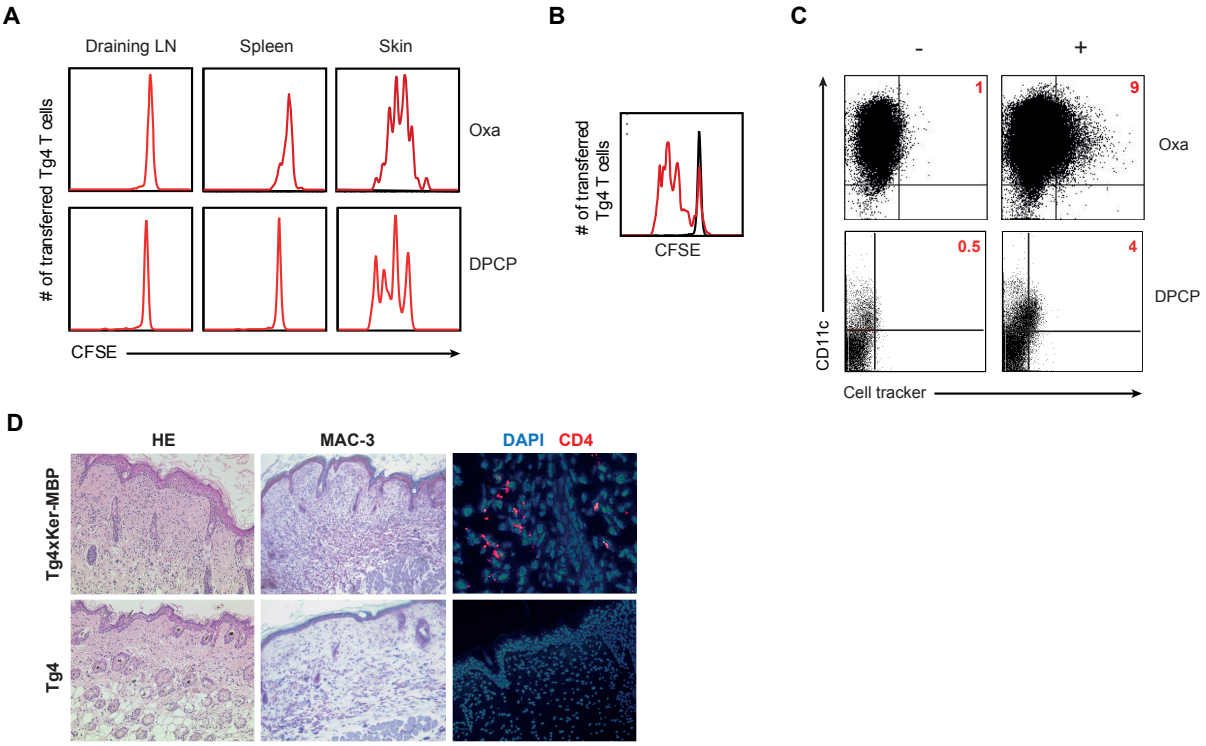

Figure 3.4 MHC class II – MBP complex presentation in Ker-MBP mice is functional and skin restricted. (A) 5x107 CFSE labelled spleenocytes from Tg4 transgenic mice were transferred i.v. into Ker-MBP mice 24h before administration of either oxazolone (3% w/v in acetone) or DPCP (2% w/v in acetone) to the shaved abdomen. 5 days after skin treatment CFSE profiles of Tg4 CD4+ T cells isolated from draining lymph nodes (LN), spleen and skin were analyzed. One representative experiment out of 3 is shown.(B) 5x107 CFSE labelled Tg4 spleenocytes were co-transferred i.v. together with MBP1-10-peptide loaded dendritic cells into Ker-MBP mice (red). As a control, CFSE labelled Tg4 spleenocytes alone were transferred (black). 5 days later, CFSE dilution profiles of Tg4 T cells from lymph nodes were analyzed. One representative experiment out of 3 is depicted. Shaved abdominal skin of Ker-MBP mice was challenged with 100 µl of either 3% w/v oxazolone or 3% w/v DPCP in acetone. As a control untreated animals were used. 400 µl CellTracker (1/20 in ethanol) was applied onto the skin of these mice. After 24h, skin draining LN were analyzed for CellTracker positive dendritic cells. One representative experiment out of 3 is shown. (D) Histological examination of the skin of Tg4xKer-MBP and Tg4 mice 8 days after irritation with oxazolone (3% w/v). Infiltration of distinct leukocytes populations into the skin was analyzed using hematoxylin/eosin, anti-MAC-3 or anti-CD4 stainings. One representative out of 3 independent experiments is shown.

Despite the expansion of Tg4 T cells in oxazolone treated Tg4 x Ker-MBP mice, these mice showed no symptoms of EAE (Figure 3.5A). To investigate whether the Tg4 T cells that are activated by keratinocytes in the inflamed skin have any encephalitogenic capacity, Tg4 x Ker-MBP and Tg4 mice were injected 2 days after oxazolone treatment with pertussis toxin, which is commonly used to elicit EAE, possibly because it breaks the blood brain barrier (Racke, Hu et al. 2005). Tg4 x Ker-MBP but not Tg4 mice developed severe but reversible EAE (Figure 3.5B,C). Pertussis toxin treatment alone did not lead to any EAE symptoms (Figure 3.5D).

It is of importance to note that EAE developed without additional immunization with MBP peptide. These findings show that the transgenic MBP peptide expressed by keratinocytes in the inflamed skin could activate MBP-specific $CD4^+$ T cells, which then can cause autoimmunity in a distal organ.

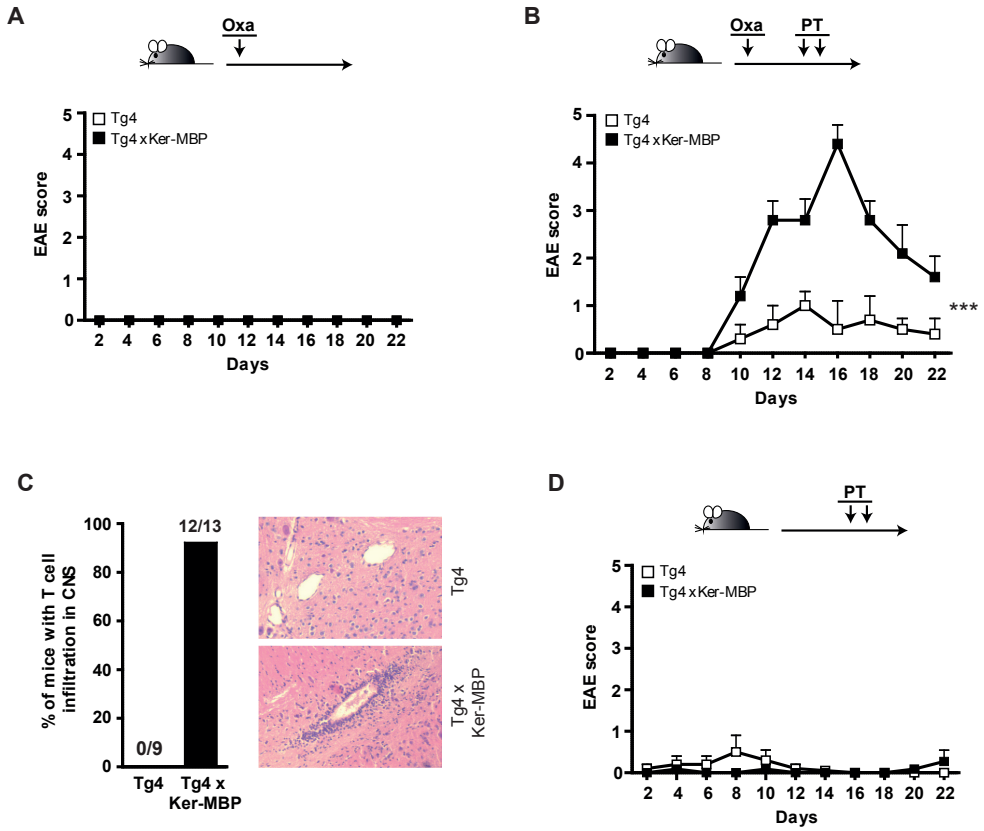

Figure 3.5 Acute skin inflammation in Tg4xKer-MBP mice activates MBP-specific $CD4^+$ T cell which cause EAE upon pertussis toxin treatment. (A) EAE scores of Tg4xKer-MBP and Tg4 mice were monitored after skin treatment with oxazolone (3% w/v in acteone). Mean clinical score ± SEM is plotted over time. One representative experiment out of 3 is depicted (n=8, n.s., repeated measures ANOVA). (B) Tg4xKer-MBP and Tg4 mice were treated like in (A) followed by 2 injections of pertussis toxin i.p. (200 ng) and clinical EAE score was monitored. Mean clinical score ± SEM is plotted over time (pooled data from two individual experiments, n=12, ***=p<0.0001, repeated measures ANOVA). (C) Brain infiltration of Tg4xKer-MBP and Tg4 mice treated as in (B). HE staining of brain sections was performed 8 days after skin irritation and infiltration was analyzed (n=13 & n=9, ***p<0.001, Fischer's exact test). (D) Tg4xKer-MBP and Tg4 mice received 2 injections of pertussis toxin i.p. (200 ng) and EAE symptoms were monitored. Mean clinical score ± SEM is plotted over time. One representative experiment out of 3 is shown (n=12, n.s. repeated measures ANOVA).

3.1.3 Chronic skin inflammation can limit the severity of EAE.

Next, we asked whether treatment of Ker-MBP mice with contact sensitizers would have a functional impact on active immunization with MBP. Untreated Ker-MBP mice containing a polyclonal T cell repertoire developed comparable symptoms of EAE after immunization with the MBP peptide in CFA as non-transgenic littermates (Figure 3.6A). A single oxazolone (Figure 3.6A) or DPCP (Figure 3.6 B) treatment 8 days before sensitization with the MBP peptide in CFA did not alter EAE levels in Ker-MBP mice significantly compared to wild type mice.

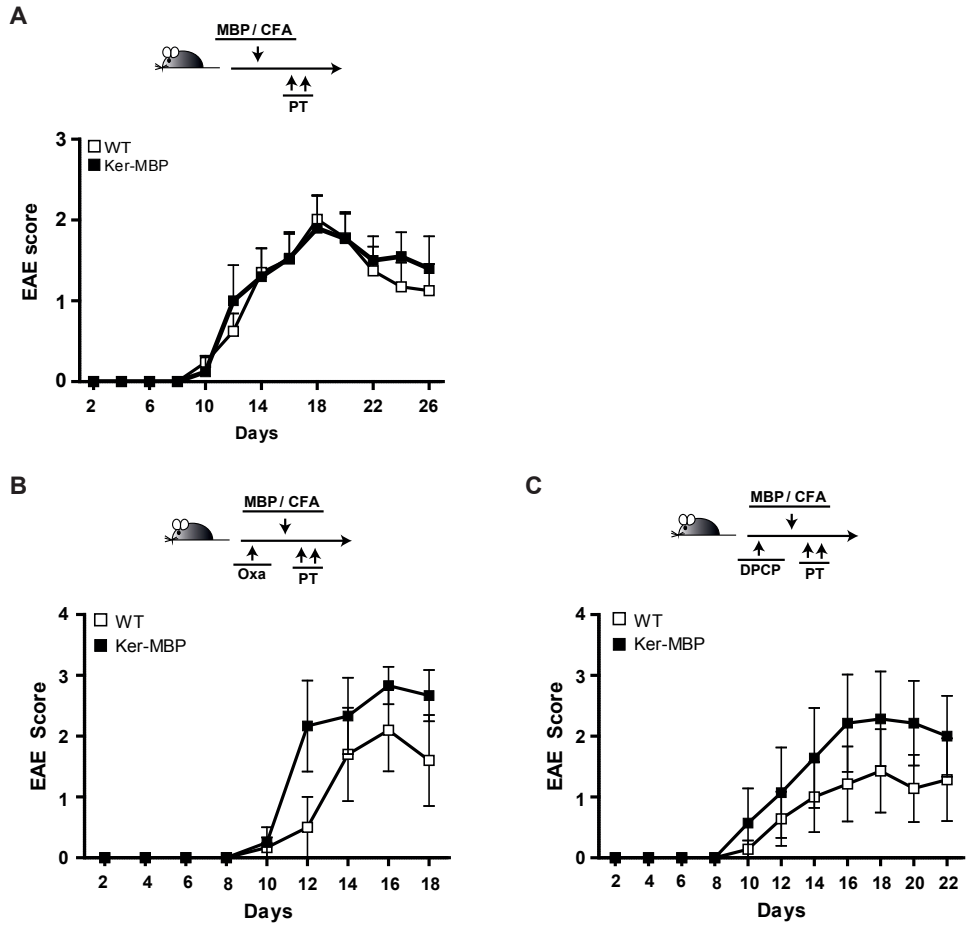

Figure 3.6 Acute skin inflammation does not limit EAE severity in Ker-MBP mice. (A) Ker-MBP and wt mice were injected s.c. with MBP1-10/CFA followed by two applications of pertussis toxin (200 ng). Subsequently, EAE symptoms were monitored. Mean clinical score ± SEM is plotted over time. Depicted is one representative experiment out of 3 (n=12, n.s. two measure ANOVA). (A) Skin of Ker-MBP and wt mice was pre-treated with 3% (w/v) oxazolone in acetone. One week later, EAE was induced by s.c. injection of MBP1-10/CFA and two applications of pertussis toxin and clinical symptoms were monitored. One representative experiment out of 2 is shown. Mean clinical score ± SEM is plotted over time (n=8, n.s., repeated measures ANOVA). (B) Skin of Ker-MBP and wt mice was pre-treated with 3% (w/v) DPCP in acetone. One week later, EAE was induced as in (A). Mean clinical score ± SEM is plotted over time. (pooled data from two individual experiments, n=8, n.s., repeated measures ANOVA).

As repeated exposure of T cells to self antigens in tissues has been reported to attenuate the severity of auto-immune responses (Lara-Corrales and Pope 2010, Rosenblum, Gratz et al. 2011), four repetitive skin treatments with oxazolone were performed before the mice were challenged with the MBP peptide in CFA. Again, no alterations in EAE symptoms were detected comparing Ker-MBP mice and littermate controls (Figure 3.7A). However, reduced severity of EAE symptoms was observed in Ker-MBP mice when DPCP was used in 4 skin pre-treatments (Figure 3.7B).

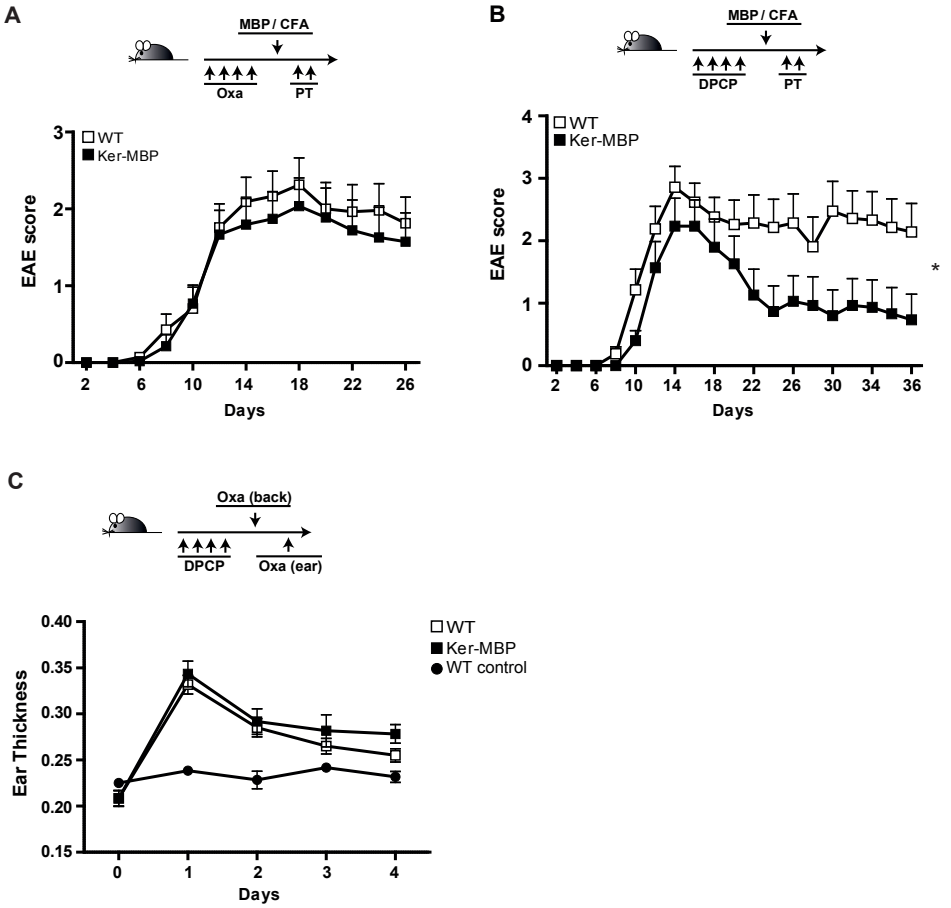

Figure 3.7 DPCP induced chronic skin inflammation limits EAE severity in Ker-MBP mice. (A) Shaved abdominal skin of individual Ker-MBP and wt mice was pre-treated 4 times with oxazolone (0.3% w/v in acetone, each one weak apart). One week later, EAE was induced like in (A) and EAE symptoms were monitored. Mean clinical score ± SEM is plotted over time (data pooled from three individual experiments, n=24, n.s., repeated measures ANOVA). (B) Skin of Ker-MBP and wt mice was 4 times pre-treated with DPCP in acetone (20µl of a 2% solution, followed by 100µl of a 0.1%, 0.25% or 0.5% (w/v) solution, each one week apart.) before induction of EAE like in (A). Clinical EAE symptoms were monitored. Mean clinical score ± SEM is plotted over time (data pooled from three individual experiments, n=18, *p < 0.05, repeated measures ANOVA). (D) Ker-MBP and wt mice were pre-treated like in (C). One week after the last pre-treatment, 3% Oxazolone (v/w) was applied to back skin of Ker-MBP and wt mice. One additional week later, the right ear of the mice was challenged by 1% oxazolone and ear thickness was monitored for the following 4 days. As a control, ears of wt mice were challenged without DPCP pre-treatment and without oxa application on the back skin. Ear thickness ± SEM is plotted over time (data pooled from 2 individual experiments, n=10, n.s., repeated measures ANOVA).

This reduction of the anti-MBP T cell response in Ker-MBP mice was not based on general DPCP-induced immune suppression in the skin because this skin pre-treatment did not affect a delayed-type hypersensitivity response to oxazolone (Figure 3.7C). Thus, DPCP-mediated chronic inflammation in the skin of Ker-MBP mice results in continuous cell surface presence of the MBP peptide on keratinocytes, which in turn down-modulates systemic responses by the antigen-specific T cells.

3.1.4 Reduced reactivity of $CD4^+$ T cells that recognize self-antigen in the chronically inflamed skin depends on the presence of Dickkopf-3

To clarify the mechanisms that limit the MBP-induced autoimmunity shown in Figure 3.7B, it was investigated whether the reduced severity of EAE symptoms in pre-treated Ker-MBP mice was associated with a dominant form of immune regulation. MBP activated spleenocytes were transferred to 4 times DPCP pre-treated wild type and Ker-MBP mice and clinical EAE scores were monitored after pertussis toxin treatment. EAE symptoms were significantly less severe in Ker-MBP mice in comparison to non-transgenic littermates (Figure 3.8A). This finding suggests the involvement of a regulatory mechanism that down-modulates MBP-specific T cell reactivity in DPCP pre-treated Ker-MBP mice. To test whether $CD4^+CD25^+FoxP3^+$ regulatory T cells contribute to the observed regulation, the percentage of these cells among $CD4^+$ T cells was determined in the lymph nodes and spleens of four times DPCP treated wild type and Ker-MBP mice. In addition, the expression of forkhead box P3 (FOXP3) was analysed in the treated skin of these mice. We could not detect a significant difference in any of these analyses comparing the 2 types of mice (Figure 3.8B, C). Thus, the observed regulation is not associated with significant changes in the $CD4^+CD25^+FOXP3^+$ regulatory T cell population.

Next, we analysed the expression levels of several immune mediators in the four times DPCP treated skin of wild type and Ker-MBP mice. No differences could be detected in the expression of interleukin-10 (IL-10), transforming growth factor β (TGFβ), programmed cell death 1 ligand (PD1-L) and cytotoxic T lymphocyte associated antigen 4 (CTLA4) in the skin of both types of mice (Figure 3.8D).

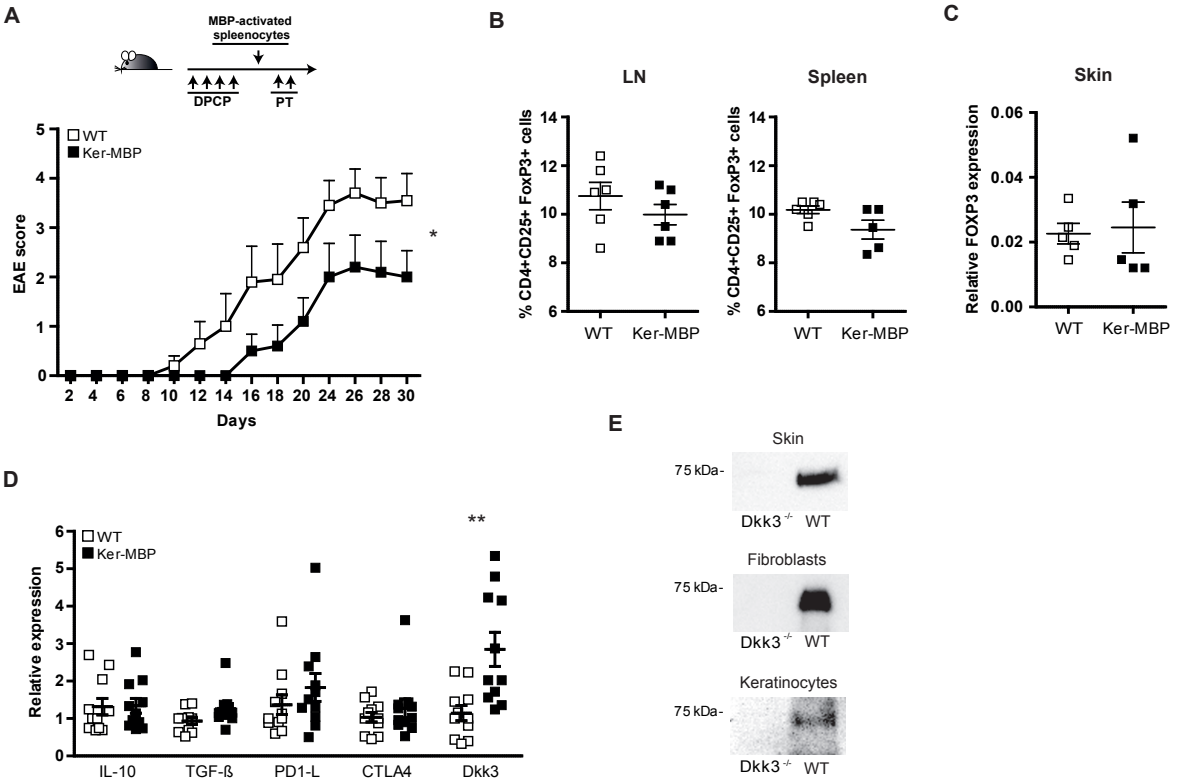

Figure 3.8 DPCP pre-treatment of Ker-MBP mice leads to a dominant form of tolerance. (A) 10 days after MBP1-10/CFA immunization of B10.PL mice, spleenocytes were taken out and re-stimulated in vitro for 3 days in the presence of MBP1-10 peptide and IL-12. Subsequently, these activated spleeenocytes were transferred i.p. to Ker-MBP or wt mice 4 times pre-treated with DPCP (20µl of a 2% solution, followed by 100µl of a 0.1%, 0.25% or 0.5% solution, each one week apart) and clinical EAE score was monitored. Mean clinical score ± SEM is plotted over time. Depicted is one out of two representative experiments (n=10, *p<0.05, repeated measures ANOVA). (B) Percentages of $CD25^{+}FOXP3^{+}$ positive $CD4^{+}$ T cells in spleen and lymph nodes of Ker-MBP and wt mice 4 times pre-treated with DPCP (20µl of a 2% solution, followed by 100µl of a 0.1%, 0.25% or 0.5% solution, each one week apart) (n=6, n.s.). (C) Quantitative real time PCR was performed on RNA isolated from skin of Ker-MBP and wt mice pre-treated as in (B). Samples were taken one week after the last DPCP application. Expression levels of *Foxp3* are expressed relative to the control gene Actb (n=6, n.s.). (D) Quantitative real time PCR was performed on RNA isolated from skin of Ker-MBP and wt mice pre-treated as in (B). Expression levels of the indicated genes are expressed relative to the control gene *Actb* (n=12, **p=0.0027). (E) Immuno-precipitation and western blotting of skin lysate or supernatants of primary kerationcyte or fibroblast cultures of wt and $dkk3^{-/-}$ mice. DKK3 protein was detected using the anti-Dkk3 mab clone 4.22. Depicted is one representative experiment out of 3.

Our lab has previously reported that the secreted protein Dkk3 can limit CD8 T cell reactivity *in vitro* and *in vivo* (Papatriantafyllou, Moldenhauer et al. 2012). Dkk3 is present in hair follicles (Ohyama, Terunuma et al. 2006) and therefore at the site of the MBP-peptide/MHC II transgene expression. So, we asked whether Dkk3 may affect $CD4^{+}$ T cell reactivity in the pre-treated mice. We confirmed expression of DKK3 in the epidermal skin and showed that Dkk3 is produced by fibroblasts and keratinocytes in vitro using tissue lysates and supernatants of primary cell cultures for immuno-precipitation and Western blotting (Figure

3.8 E). Importantly, DKK3 expression was significantly up-regulated in the four times DPCP treated skin of Ker-MBP mice as compared with the skin of DPCP-treated wild type mice (Figure 3.8D).

In order to analyse the impact of Dkk3 on the observed effect, Ker-MBP-transgenic mice deficient for Dkk3 were established and four times treated with DPCP as described above. In contrast to Dkk3-competent mice (Figure 3.7B), DPCP pre-treatment did not result in a reduction of EAE symptoms in Ker-MBP.*dkk3*$^{-/-}$ mice after MBP peptide priming in comparison to non-transgenic *dkk3*$^{-/-}$ littermates (Figure 3.9A). Absence of EAE down-modulation was not due to altered MHC class II expression. Similar amounts of the endogenous MHC class II α-chain were induced by the four DPCP pre-treatments of wild type and Ker-MBP DKK3-sufficient and deficient mice (Figure 3.9C), allowing normal cell surface expression of the transgenic MHC class II β-chain construct in Ker-MBP mice. In a different set of experiments, we blocked Dkk3 function in Dkk3-competent Ker-MBP mice and non-transgenic littermates by administering neutralizing Dkk3-specific antibodies at the beginning of the DPCP treatment (Figure 3.9B). These results show that upon neutralization of Dkk3 there was no reduction in MBP-triggered EAE as a result of pre-treatment of Ker-MBP mice with DPCP.

Finally, we investigated whether the observed changes in EAE severity were linked with the effector function of the respective MBP-specific CD4$^+$ T cells. Wild-type and Ker-MBP mice were immunized with the MBP peptide in CFA and seven days later splenic T cells were re-stimulated *in vitro* with MBP peptide. The percentages of MBP-specific CD4$^+$ T cells that produced interleukin-2 (IL-2) and tumour necrosis factor (TNF) after 6 hours of *in vitro* restimulation were comparable for the two experimental groups (Figure 3.9D). However, the percentages of IL-2 and TNF producing MBP-specific T cells were significantly reduced among *in vitro* restimulated CD4$^+$ T cells that had been isolated from Ker-MBP mice following four times treatment with DPCP. By contrast, the effector function of MBP-specific CD4$^+$ T cells from DPCP-treated Ker-MBP.*dkk3*$^{-/-}$ mice (Figure 3.9D) was not compromised *in vitro*. Thus, chronic skin inflammation, as modelled by repeated DPCP treatment and the associated priming of MBP-specific CD4$^+$ T cells by keratinocytes in the skin can reduce systemic CD4$^+$ T cell reactivity in an antigen-dependent manner through a mechanism that involves DKK3 expression.

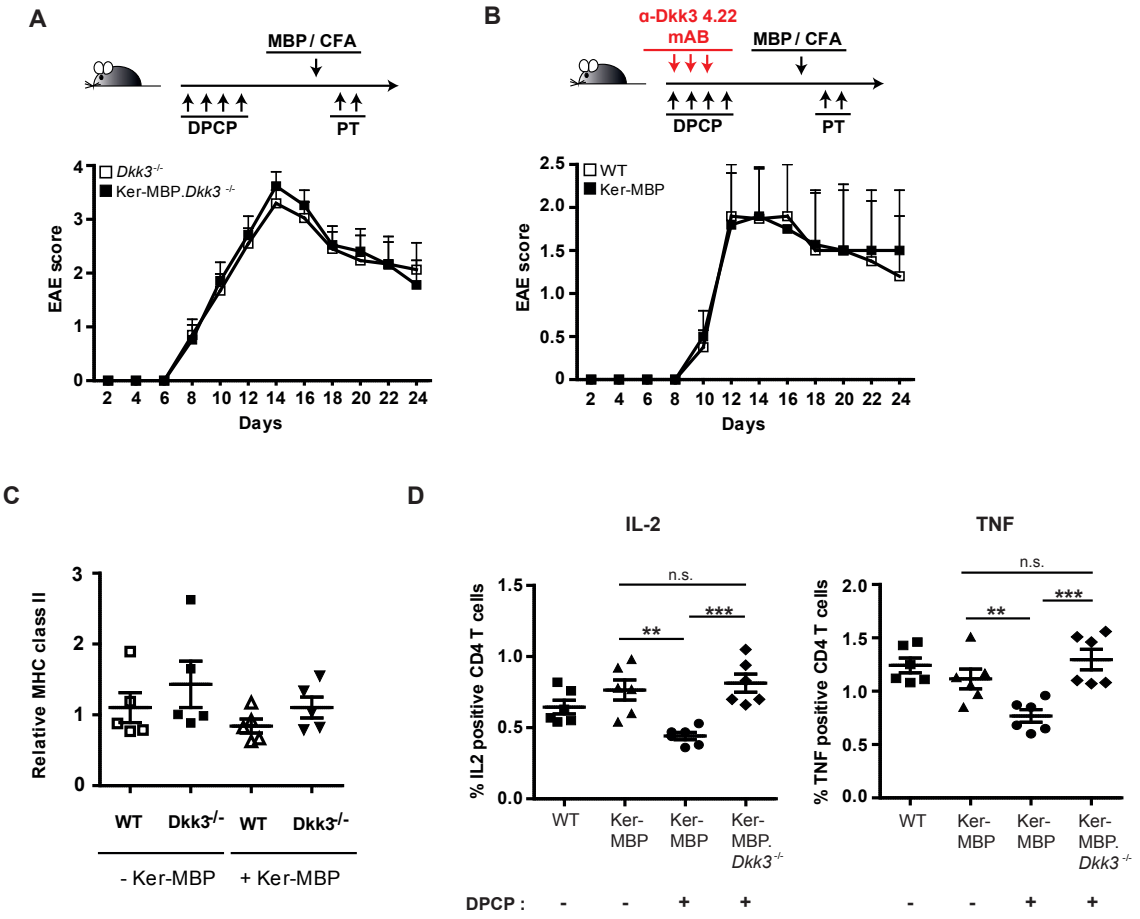

Figure 3.9 Reduction of MBP specific CD4$^+$ T cell response in Ker-MBP mice depends on Dickkopf-3. (A) Ker-MBP.dkk3$^{-/-}$ and dkk3$^{-/-}$ mice were 4 times pre-treated with DPCP in acetone (20µl of a 2% solution, followed by 100µl of a 0.1%, 0.25% or 0.5% solution, each one week apart) before induction of EAE by MBP1-10/CFA. Mean clinical score ± SEM is plotted over time (data pooled from 2 individual experiments, n=16, n.s., repeated measures ANOVA). (B) Ker-MBP and wt mice were pre-treated as in (A). In both groups, 500µg of anti-Dkk3 mab clone 4.22 was applied i.p. along with the first three DPCP applications as indicated. One week after the 4th DPCP application, mice were challenged with MBP1-10 peptide in CFA and EAE was monitored. Mean clinical score ± SEM is plotted over time (data pooled from 2 individual experiments, n=16, n.s., repeated measures ANOVA). (C) Skin of wt, *dkk3*$^{-/-}$, Ker-MBP and Ker-MBP.*dkk3*$^{-/-}$ mice was pretreated like in (A). One week after the last DPCP application, quantitative real time PCR was performed on RNA isolated from skin of the respective mice. Expression levels of H2-Aa are expressed relative to the control gene Actb. (data pooled from 2 individual experiments, n=6, n.s.) (D) Ker-MBP and Ker-MBP.*dkk3*$^{-/-}$ mice were DPCP pre-treated as in (A). As controls, untreated Ker-MBP and wt mice were used. One week after the last pre-treatment, animals were immunized s.c. with MBP1-10/CFA. 7 days later spleenocytes were in vitro re-stimulated with MBP1-10 peptide and analyzed by flow cytometry. Depicted are the percentages of IL-2 and TNFA producing CD4+ T cells (data pooled from three individual experiments, n=6, **P < 0.01; ***P < 0.001)

In summary, the results of this chapter reveal that self-antigen presentation by keratinocytes in the inflamed skin can stimulate auto-reactive T cells, which are capable to initiate an autoimmune response in the brain. In contrast, repeated antigen presentation by keratinocytes led to an antigen-specific reduction of auto-reactivity in the brain. Finally, the limitation of CD4 T cell reactivity was shown to be dependent on the presence of Dkk3.

3.2 Dkk3 expression in the steady state

Previous work in our lab as well as the results of the prevenient chapter identified Dkk3 as an immune-modulatory molecule mainly expressed by tissue cells. Therefore, the expression pattern of Dkk3 is of particular interest. So far published results, displaying prominent Dkk3 expression in several mouse tissues, could be confirmed in our lab via PCR and ELISA. Among these, so called immune-privileged organs like brain, eye, spinal cord, uterus, placenta and embryo displayed highest Dkk3 expression levels. Interestingly, no Dkk3 expression could be detected in several hematopoietic cells that were analyzed including T cells, B cells and myeloid cells.

Even though, there is already considerable insight into Dkk3 expression in the mouse, in most of the cases the particular cell types expressing Dkk3 are not yet identified. Additionally, the influence of inflammation and its mediators on Dkk3 expression within tissues remains almost unexplored.

3.2.1 Generation of a BAC-transgenic Dkk3 reporter mouse

In order to develop a tool for the detection of Dkk3 expression, a BAC-transgenic reporter mouse, expressing luciferase and mCherry under regulatory elements of the Dkk3 gene was generated (Figure 3.10A). This tool should enable visualization of Dkk3 expression in- and ex-vivo via bioluminescence imaging (luciferase) and in histology by fluorescence microscopy (mCherry). To do so, a previously established Luciferase-2A-mCherry cassette (Miloud, Henrich et al. 2007) was inserted into the start codon (ATG) of *dkk3* in a respective bacterial artificial chromosome harbouring the whole gene and up-stream regulatory sequences. After removal of the vector, the construct was injected into C57B6/J F2 Oocytes, which were transplanted to super-ovulated C57B6 females. Transgenic founders were tested for in vivo luciferase activity (Figure 3.10B). The founder showing highest bioluminescence signal (line 46) was selected for further breeding. All following experiments were conducted using littermates of this founder line.

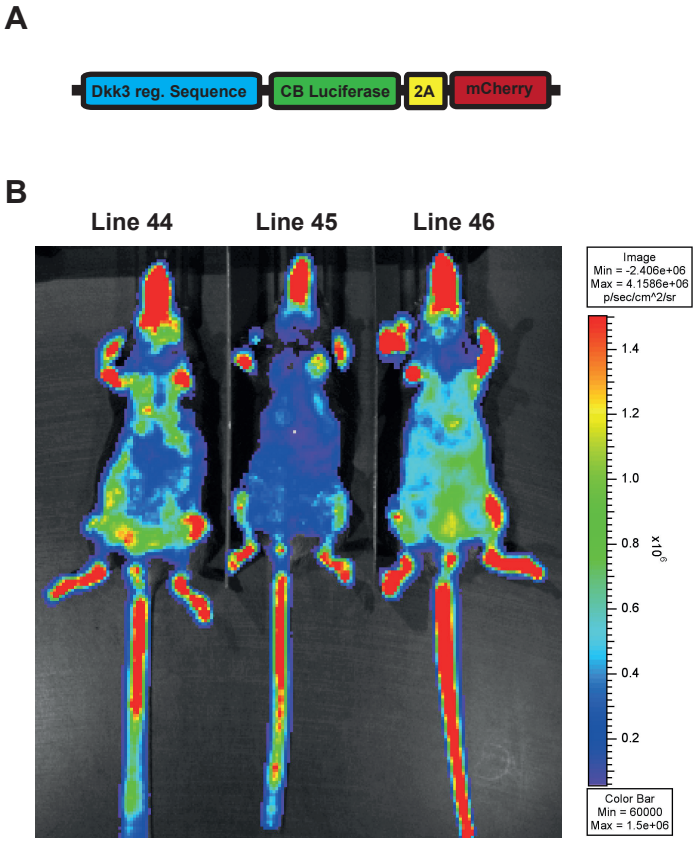

Figure 3.10 Establishment of the Dkk3-LCh transgenic Dkk3 reporter mouse line. (A) Shematic illustration of the Dkk3 reporter construct. (B) In vivo Bioluminescence imaging of Dkk3-LCh founder. Mice were injected i.p. with 150mg/kg D-luciferin, anesthesized with isoflurane and imaged for 5min. Colours display intensity of the emmited light (see scale).

Initially, identification of Dkk3 expressing sites was accomplished by ex vivo bioluminescence imaging of organs from healthy, adult Dkk3-LCh reporter mice. According to published data, the luciferase reporter signal could be detected in gal bladder, atrium of the heart, lung, eye, brain, skin, uterus, ovary, stomach and intestine. Additionally, we confirmed results generated in our lab previously, indicating Dkk3 expression in spinal cord. Surprisingly, we detected a prominent luciferase signal in esophagous/trachea and cartilage (Figure 3.11). Thus, reporter expression correlated with previously described Dkk3 expression and identified new potential sites of Dkk3 production.

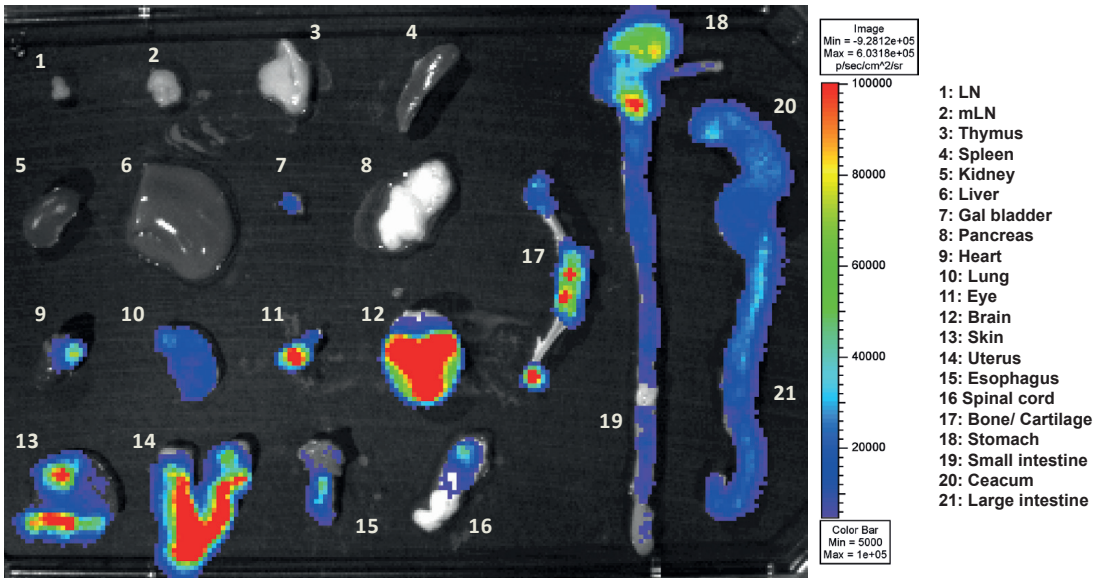

Figure 3.11 Dkk3 expression pattern in adult mouse organs. Ex vivo bioluminescence imaging of organs of healthy, 12 weeks old Dkk3-LCh mice. 5 min after i.p. injection of 150mg/kg D-luciferin, mice were sacrificed and organs were taken out. After 5min of incubation in a 1mg/ml D-luciferin solution in PBS at 37°C, organs were imaged for 5min. Colours display intensity of the emmited light (see scale). One representtive experiment out of 5 is shown.

In order to verify the correlation of Luciferase and Dkk3 expression, their mRNA levels were compared in several Dkk3-LCh tissues. As shown in Figure 3.12, expression of luciferase and Dkk3 predominantly correlated in the tested organs. For example, high expression of both genes was found in eye, brain and spinal cord while almost no signal was detected in thymus, spleen and lymph node. Thus, luciferase expression reflects Dkk3 expression in Dkk3-LCh mice.

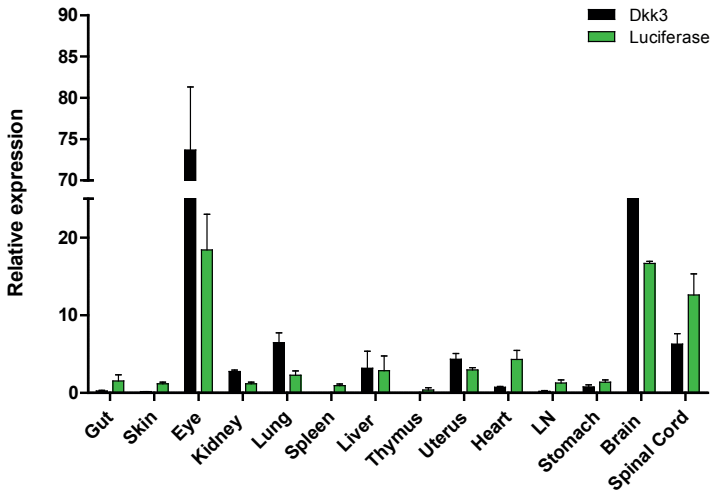

Figure 3.12 Relative expression levels of dkk3 and luciferase are comparable in Dkk3-LCh mice. QRT-PCR on total RNA isolated from the respective Dkk3-LCh organs. Dkk3 and luciferase expression levels are displayed relative to the housekeeping gene *Actb*.

As a next step, expression of the second reporter molecule present in Dkk3-LCh mice, the fluorochrome mCherry, was analysed. Therefore, whole embryo slices of p14 Dkk3-Lch embryos were generated and mCherry expression was detected by fluorescence microscopy. As a control, transgene negative embryos obtained from the same mother were used. It is of importance to mention, that mCherry fluorescence signal by itself was not detectable and had to be potentiated by fluorochrome-conjugated antibody staining. Results revealed a specific signal for mCherry mainly in muscles and cartilage like structures as well as the eye (Figure 3.13).

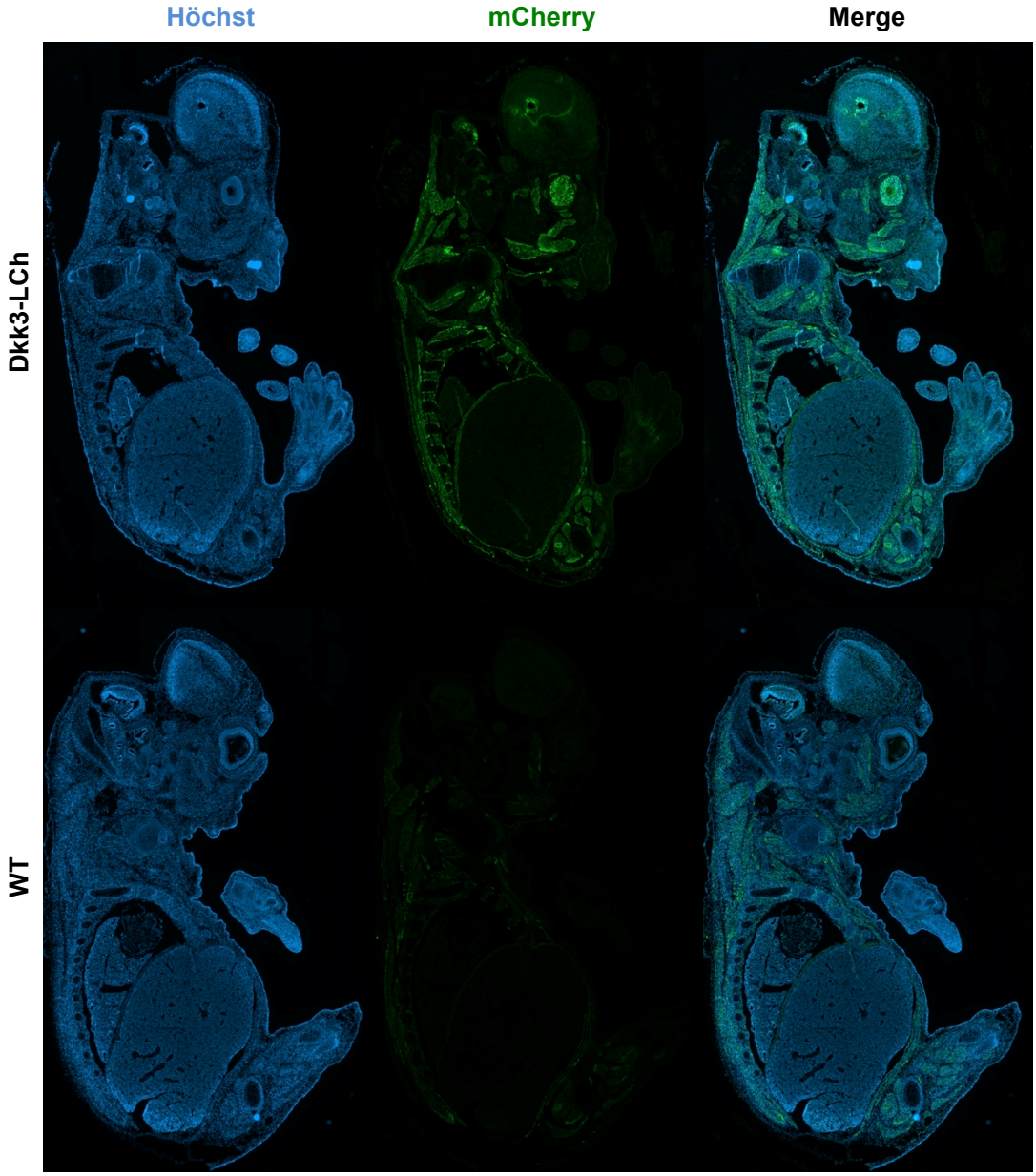

Figure 3.13 MCherry displays dkk3 expression in Dkk3-LCh embryos. Detection of mCherry expression in E14 Dkk3-LCh embryos by flourochrome conjugated immunohistochemistry. 14 days after vaginal plug detection Dkk3-LCh positive and negative (wt) embryos from the same mother were harvested, cryo sections were prepared and fluorochrome conjugated anti-mCherry staining (green) was performed. Nuclei were counter stained with Hoechst dye (blue). One representative experiment out of 3 is shown.

As expected, the most considerable mCherry expression was found in elongated cells spanning the entire thickness of the retina, referred to as Müller glial cells, in the eye of the embryos (Figure 3.14). According to the literature, these cells are strong Dkk3 expressers (Nakamura, Hunter et al. 2007). Hence, Dkk3-LCh mice provide the opportunity to reliably indicate Dkk3 expression via its two reporter molecules.

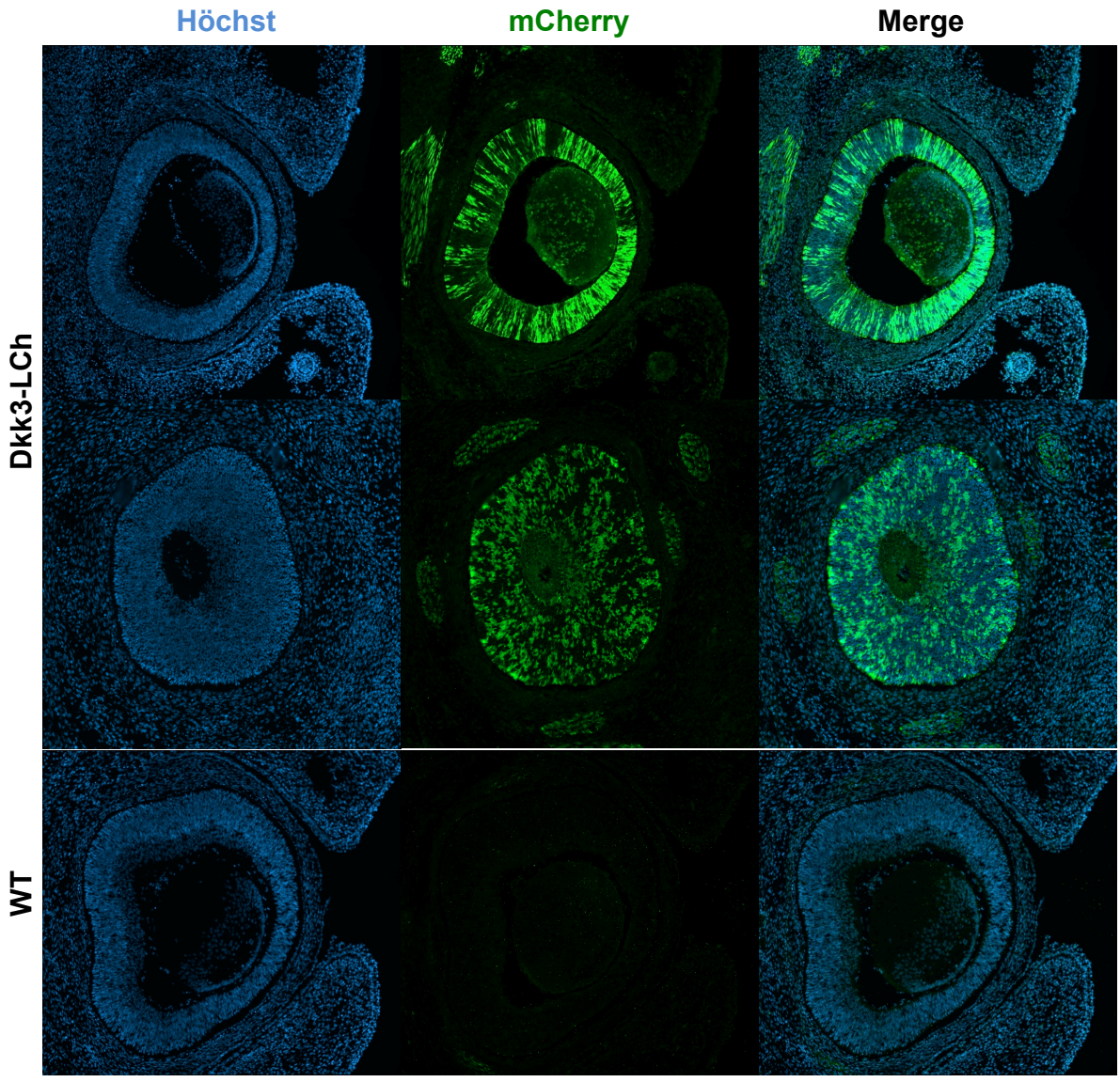

Figure 3.14 MCherry is strongly expressed in müller glia cells in the eye of Dkk3-LCh embryos. Detection of mCherry expression in the eye of E14 Dkk3-LCh embryos by flourochrome conjugated immunohistochemistry. 14days after vp detection Dkk3-LCh positive and negative (wt) embryos from the same mother were harvested, cryo sections were prepared and fluorochrome conjugated anti-mCherry staining (green) was performed. Nuclei were counter stained with Hoechst dye (blue). One representative experiment out of 3 is shown.

Furthermore, comparison of luciferase expression in organs of adult and neonatal Dkk3-LCh mice revealed a similar but not completely identical expression pattern. While luciferase activity in the adult thymus and kidney was completely absent, prominent expression in these organs could be observed in the neonatal phase (Figure 3.15A). Additionally, ex vivo bioluminescence analysis revealed that, in general, the strength of luciferase expression was drastically increased in virtually all of the neonatal organs compared to the adult phase

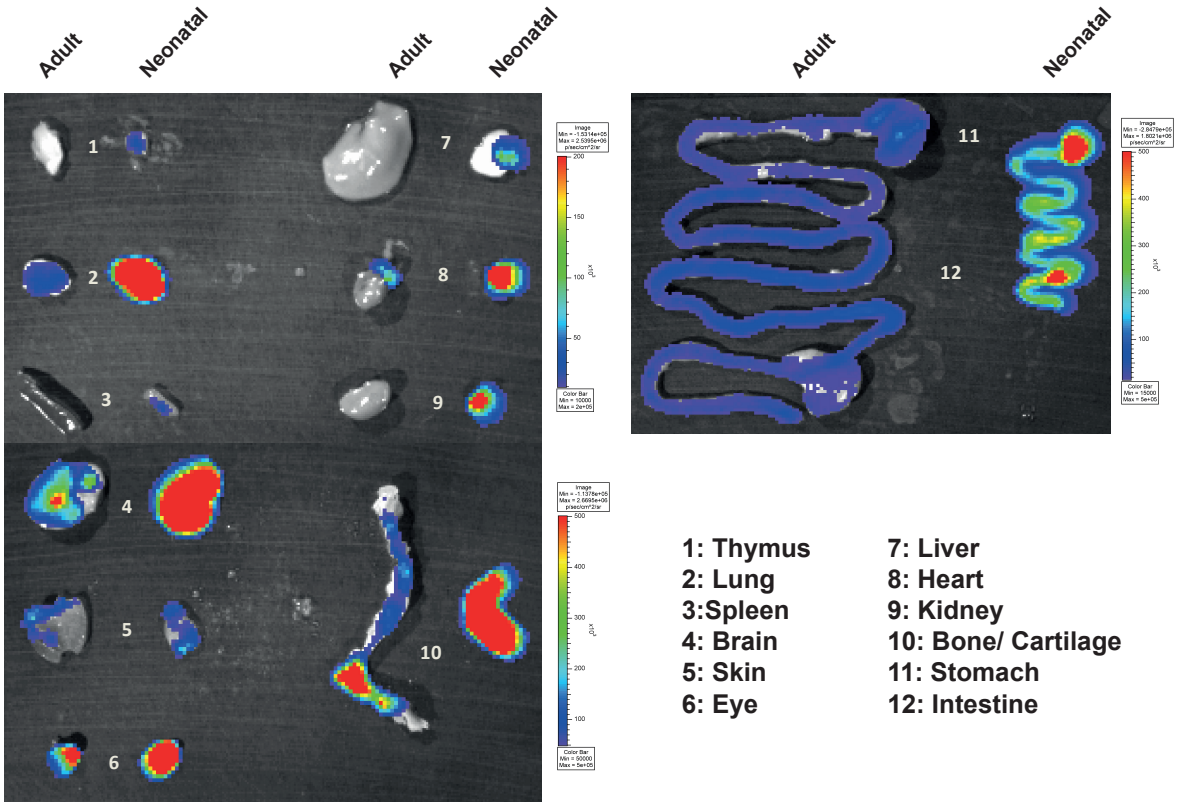

Figure 3.15 Altered Luciferase expression in Neonatal Dkk3-Lch organs compared to Adult controls. Ex vivo bioluminescence imaging of organs of healthy, one day (neonatal) and 12 weeks old (adult) Dkk3-LCh mice. 5 min after i.p. injection of 150mg/kg D-luciferin, mice were sacrifized and organs were taken out. After 5min of incubation in a 1mg/ml D-luciferin solution in PBS at 37°C organs were imaged for 5min. Colours display intensity of the emmited light (see scale). One representtive experiment out of 5 is shown.

Since the kidney exhibited the most prominent disparity in luciferase expression comparing the neonatal and the adult phase, a more detailed analysis of Dkk3 expression in this organ was accomplished. Quantitative RT-PCR displayed considerable renal Dkk3 expression in the first 2 - 3 weeks after birth followed by a drastic decrease (Figure 3.16A). According to literature, this correlates with the phase of postnatal kidney development (Little and McMahon 2012).

Moreover, we wondered whether the increased luciferase expression in neonatal organs is reflected by increased systemic Dkk3 protein level. Therefore, Dkk3 serum concentrations were analysed by ELISA. Indeed, after a peak of Dkk3 serum concentration at about 4µg/ml in the first two weeks after birth the level abruptly decreases and reaches a plateau phase of about 1µg/ml for the following weeks (Figure 3.16B).

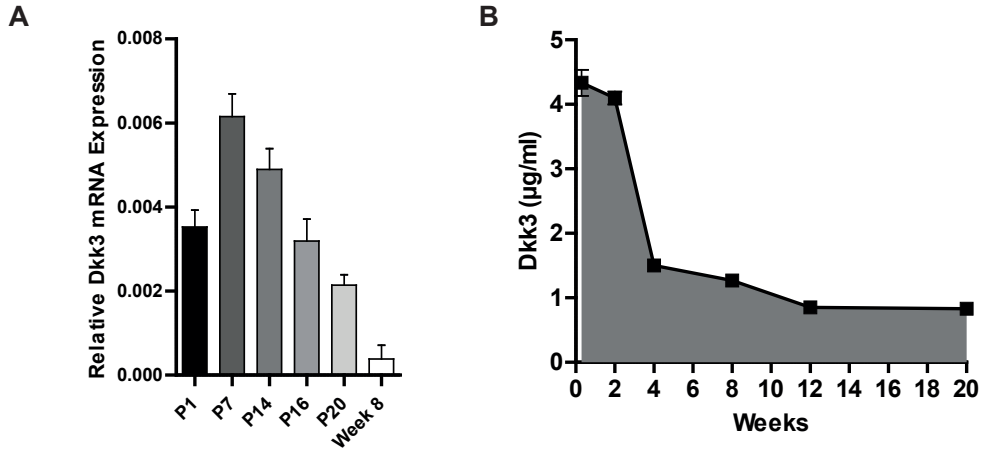

Figure 3.16 Dkk3 expression post-natally decreases with time. (A) Dkk3 mRNA expression detected by quantitative RT-PCR on total RNA from Kidneys in different developmental stages. Values are displayed relative to the expression of the housekeeping gene *Actb* (B) Dkk3 serum levels of mice with indicated age detected by ELISA.

In this chapter, we reported the successful generation of a Dkk3 reporter mouse, which can serve as an appropriate tool for the detection of Dkk3 expression via its two reporter molecules, luciferase and mCherry. By using this tool, we identified Dkk3 expression in neonatal kidney and thymus, which was absent in adult healthy mice. Additionally, we revealed that systemic Dkk3 levels in healthy mice decreases with age.

3.3 Dkk3 expression under inflammatory conditions

In the previous chapter we analysed Dkk3 expression in the steady state. However, since we are interested in the influence of Dkk3 on immune responses it is of importance to investigate if and how its expression changes under inflammatory conditions. Therefore, we assessed the impact of cytokines on Dkk3 expression in vitro and in vivo.

3.3.1 Impact of cytokines on Dkk3 expression

Previously, we identified primary dermal fibroblasts to express Dkk3 in vitro on an intermediate level. Therefore these cells were used in initial expression studies in order to identify positive as well as negative impact on Dkk3 expression by several cytokines.

As a first step, a screening to identify the impact of different cytokines on Dkk3 expression was accomplished. Primary dermal fibroblast were stimulated for 48h with increasing concentrations of recombinant Dkk3, IL-7, TNFα, IFNγ, IL-13, IL-4, TGF-β, IL-10, IL-1β, IL-12 and retionic acid. Subsequently, Dkk3 mRNA levels were analysed by qRT-PCR. As a control untreated fibroblast were used.

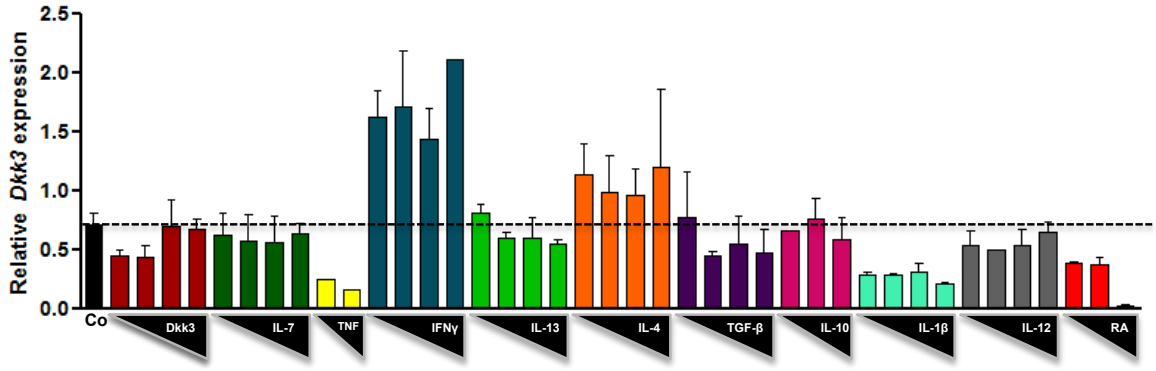

Figure 3.17 Influence of cytokines on *Dkk3* expression in primary dermal fibroblasts. $2x10^5$ primary dermal fibroblasts were stimulated with the indicated recombinant cytokines for 48h. Quantitavie RT-PCR on reverse transcribed RNA of stimulated fibroblast and controls was performed to detect Dkk3 expression levels. Values (mean and SEM) are displayed relative to the expression level of *Actb*.

As shown in Figure 3.17 different concentrations of Dkk3, IL-7, IL-13, TGF-β, IL-10 and IL-12 had no significant influence on Dkk3 expression. While TNFα, IL-1β and retinoic acid considerably decreased Dkk3 production, IL-4 slightly and IFNγ strongly enhanced it. Based on these initial results, we decided to further investigate the influence of IFNγ on Dkk3 expression in detail.

3.3.2 IFNγ enhances Dkk3 expression in primary dermal fibroblast

To evaluate the results of the cytokine screening we stimulated fibroblast for different time periods with different concentrations of IFNγ and analysed Dkk3 gene expression again by qRT PCR and additionally Dkk3 protein secretion by ELISA of the supernatant. And indeed, IFNγ stimulation increased Dkk3 gene expression as well as Dkk3 protein levels in the cell culture supernatant (Figure 3.18 A,B).

As a control IFNγ-receptor 1 deficient fibroblast were used. As expected, in these cells IFNγ failed to induce Dkk3 expression (Figure 3.18 C). Thus, enhancement of dkk3 production by IFNγ in primary dermal fibroblast is IFNγ-receptor 1 dependent.

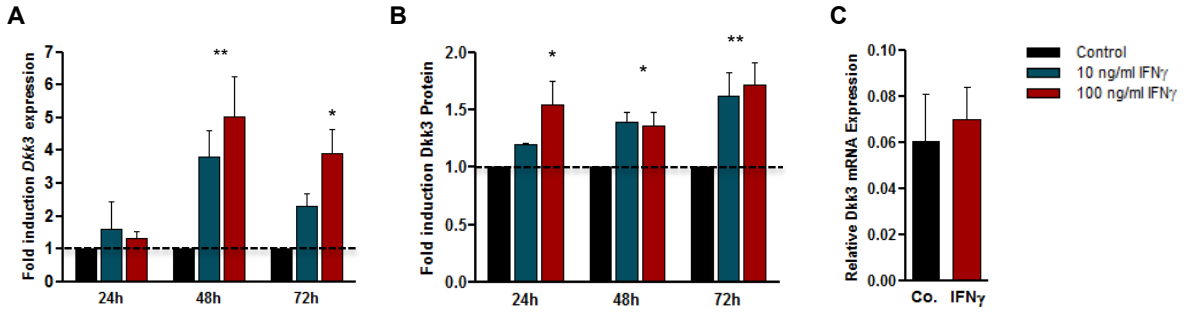

Figure 3.18 IFNγ enhances Dkk3 expression in fibroblast. (A+B) $2x10^5$ primary dermal fibroblasts were stimulated with 10 ng/ml or 100 ng/ml recombinant IFNγ for the indicated time periods. (A) Quantitavie RT-PCR on reverse transcribed RNA of stimulated fibroblast and controls was performed to detect Dkk3 expression levels. Values are displayed relative to the expression level of Actb. (B) Dkk3 protein levels in the supernatant of stimulated cells and controls was detected by ELISA. (C) $2x10^5$ IFNγ-receptor 1 deficent primary dermal fibroblasts were stimulated with 100ng/ml recombinat IFNγ for 48h. Quantitavie RT-PCR on reverse transcribed RNA of stimulated fibroblast and controls was performed to detect Dkk3 expression levels. Values are displayed relative to the expression level of *Actb*. Shown are mean and SEM for each group.

In order to extend these in vitro findings to a more general mode of action, we aimed to analyse the influence of IFNγ on Dkk3 production in vivo. To do so, we induced IFNγ release in vivo by administration of α-galactosylceramide (αGal-Cer) and measured Dkk3 serum levels before and 24 after application. αGal-Cer is a sponge derived glycolipid which induces the release of substantial amounts of IFNγ as well as several other cytokines by NKT cells. To analyse the pure impact of IFNγ on Dkk3 production, IFNγ-receptor1 deficient mice were used as a control.

Injection of 50µg of αGal-Cer led to high serum levels of IFNγ in wt and IFNg-R1$^{-/-}$ mice (Figure 3.19A). Simultaneously, Dkk3 serum levels of wt mice significantly increased 24h after αGal-Cer administration (Figure 3.19B). Such changes could not be observed in IFNgR1 deficient mice. Thus, IFNγ is also capable to induce Dkk3 expression in vivo.

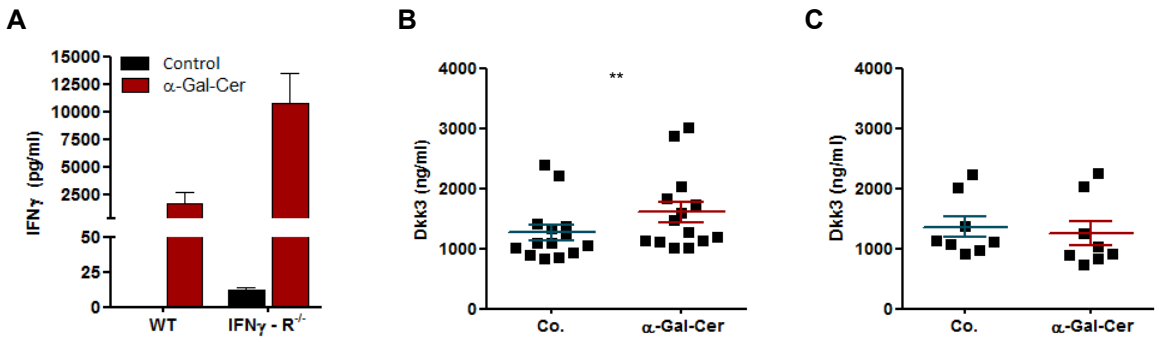

Figure 3.19 Interferon-γ dependent increase of Dkk3 protein in the serum of α-Galactosylcermide injected mice. (A) IFNγ levels in the serum of wt and IFNγ-receptor 1 deficient mice before and 24h after injection of 50µg α-galactosylcermide, detected by ELISA. (B+C) Dkk3 concentration in the serum of (B) wt (Paired students t-etst **p=0.0065) and (C) IFNγ-receptor 1 deficient mice before and 24h after injection of 50 µg α-galactosylcermide, detected by ELISA. Mean and SEM are displaeyd for each group.

Next, we addressed the question, whether the capacity of IFNγ to induce Dkk3 expression in vivo can also be observed in a pathophysiological situation.

Data generated in our lab showed, that deletion of Dkk3 results in a significant delay of experimental autoimmune encephalomyelitis remission associated with increased numbers of IFNγ producing CD4^{+} and CD8^{+} T cells in the brain (Figure 3.20) (Papatriantafyllou, 2008). It has been shown that, the presence of IFN is beneficial in the recovery phase of EAE. For example, IFNγ and IFNγ-receptor deficient mice exhibit a more severe and prolonged onset of disease (Ferber, Brocke et al. 1996, Willenborg, Fordham et al. 1996). Accordingly, we hypothesized that, during EAE, IFNγ may induce Dkk3 expression in brain and spinal cord, which in turn may contribute to the recovery of clinical symptoms.

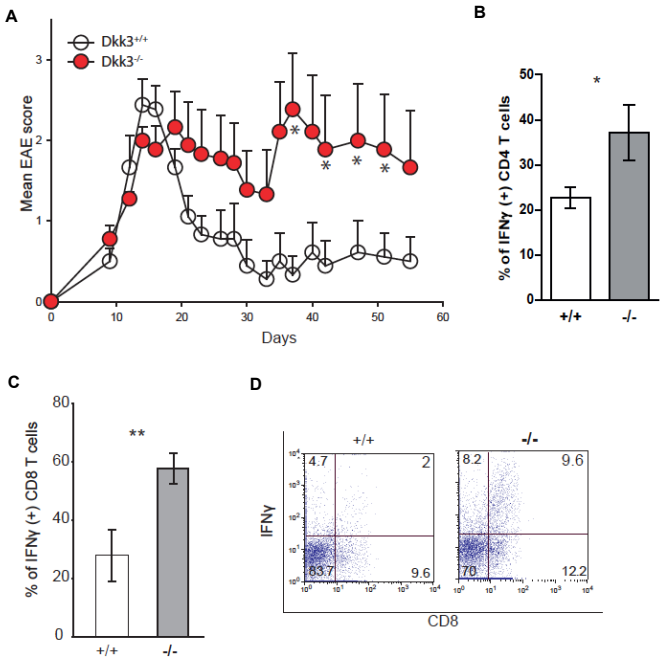

Figure 3.20 Increased numbers of IFNγ producing T cells in EAE brains of Dkk3 deficient mice. (A) EAE was induced in wt and *dkk3*$^{-/-}$ mice by s.c. immunization with MOG peptide in CFA and EAE symptoms were monitored for the following 56 days. Mean clinical score ± SEM is plotted over time. (B+C) Percentage of IFNγ positive (B) CD4+ and (C) CD8+ T cells isolated from brains of *dkk3*$^{-/-}$ and wt mice in the recovery phase of EAE. (D) Representative Dot Plot of (C). Data obtained from Papatriantafyllou, 2008.

Therefore, Dkk3 levels in brain and spinal cord of diseased mice with a minimal EAE score of 3 and healthy controls were compared. This analysis revealed a two times higher Dkk3 protein level in brain and spinal cord of EAE bearing mice. However, this induction was not detectable in IFNγ-receptor deficient mice (Figure 3.21). Thus, IFNγ up-regulates Dkk3 expression in the brain and spinal cord during EAE.

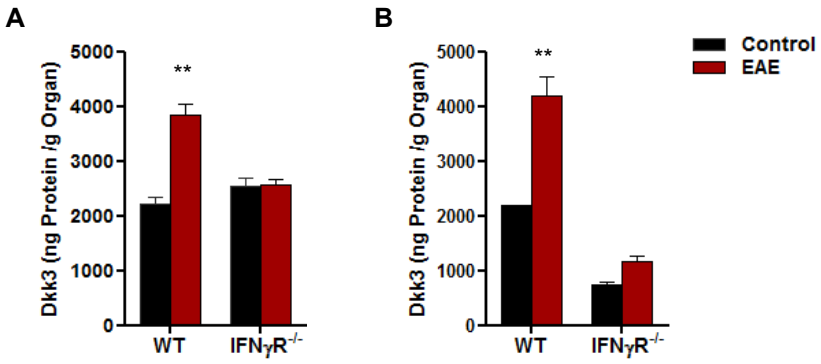

Figure 3.21 IFNγ dependent increase of Dkk3 in brain and spinal cord of EAE bearing mice. (A+B) EAE was induced in wt and IFNγ-R deficient mice by s.c. immunization with 2µg MOG peptide in CFA. Brain (A) and spinal cord (B) of mice reaching a clinical EAE score of 4 were harvested and Dkk3 protein amount per gramm organ weight was determined by ELISA. Displayed are mean and SEM.

Additionally, we analyzed dorsal route ganglia for Dkk3 expression and the potential to up-regulate Dkk3 production upon IFNγ stimulation, since these are highly affected target cells in EAE (Simmons, Buzbee et al. 1988). We found that primary dorsal route ganglia (DRGs) in culture express Dkk3 on a low level (Figure 3.22A). However, IFNγ stimulation increased mRNA expression and protein levels in the respective supernatants up to 8 times (Figure 3.22B, C). This IFNγ induced up-regulation could not be detected in *IFNγR1*$^{-/-}$ DRGs .

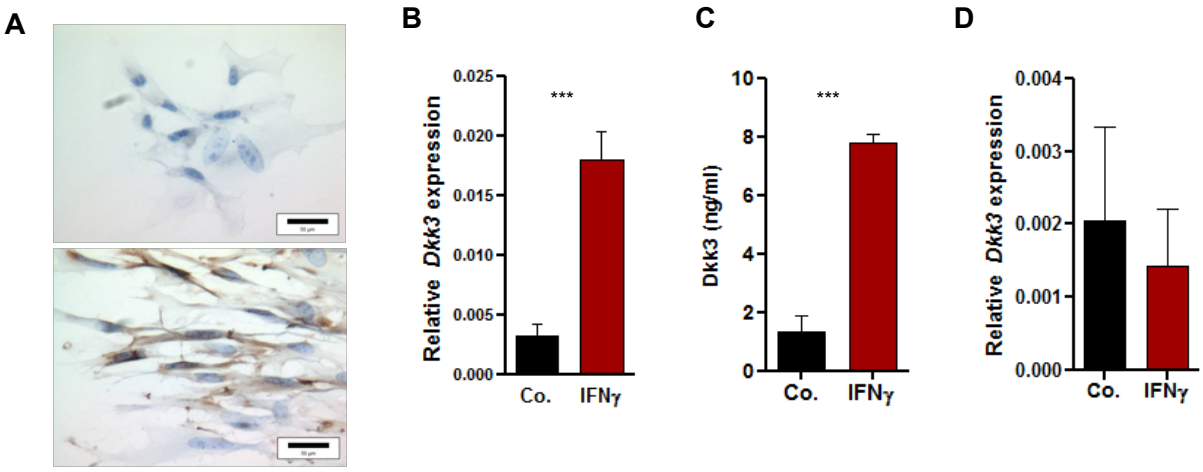

Figure 3.22 IFNγ induces Dkk3 expression in dorsal route ganglia. (A) Immnohistochemistry detecting Dkk3 expression in primary dkk3$^{-/-}$ (upper panel) and wt (lower panel) dorsal route ganglia using the Dkk3-4.22 antibody. (B+C) Primary wt and (D) IFNγ-rceptor 1 deficient dorsal route ganglia were incubated for 48h with 100ng/ml recombinat IFNγ. (B+D) Quantitavie RT-PCR on reverse transcribed RNA of stimulated DRGs and controls was performed to detect Dkk3 expression levels. Values are displayed relative to the expression level of Actb. (C) Dkk3 concentration in the supernatant of stimulated DRGs an controls, detected by ELISA. Mean and SEM are indicated for each group.

In summary, IFNγ is capable to induce Dkk3 expression in vitro and in vivo. We observed that Dkk3 expression in cultured primary dermal fibroblasts can be further increased by stimualtion with IFNγ. In vivo, systemic IFNγ induced by α-galactosylceramide increased Dkk3 serum levels. Additionally, in the pathogenic situation of experimental autoimmune encephalomyelitis Dkk3 levels in the brain and spinal cord were found to be elevated in an IFNγ dependent manner. In line with that, Dkk3 production in isolated dorsal route ganglia can be induced by IFNγ.

3.4 The role of Dkk3 in kidney fibrosis

So far, we revealed that Dkk3 is up-regulated during inflammatory responses in skin (Chapter 3.1) and brain (Chapter 3.3) and may contribute to regulation of pathogenic T cell responses. As mentioned earlier, an improved understanding of the cellular and molecular mechanisms of renal fibrosis is paramount and essential, not only for gaining novel insights into the pathogenesis of the process, but also for developing rational strategies to treat patients with fibrotic kidney disorders. Therefore, we addressed the question, whether Dkk3 exerts any impact on the outcome of the important pathological process of renal fibrosis. This project was conducted in a collaboration with the department of Molecular Pathology of Prof. Dr. H.-J. Gröne.

3.4.1 Dkk3 facilitates development of interstitial kidney fibrosis

In the following study, two well established models of kidney fibrosis in mice were applied, differing in the cause of tissue injury. (1) The model of unilateral ureteral obstruction (UUO). In this system ureteral obstruction and the resulting pressure within the kidney leads to tubulointerstitial damage and fibrosis. (2) The adenine feeding model in which the development of adenine crystals in kidney and the resulting shear forces lead to renal damage and fibrosis. All operations, adenine feeding and complete histological analysis were accomplished by Giussepina Federico in the Department of Molecular Pathology.

As a first step, the level of fibrosis in wt and Dkk3 deficient mice was compared in both models. UUO induction revealed that, Dkk3 deficient mice developed a significantly less severe renal fibrosis, compared to wild type littermate controls. This was manifested by a lower fibrosis score as shown in Figure 3.23, by less tubular damage and decreased levels of αSMA (PhD Thesis, G. Federico). Notably, while differences between the two groups were strongly pronounced 21 days after UUO induction, they were hardly detectable 7 days after operation.

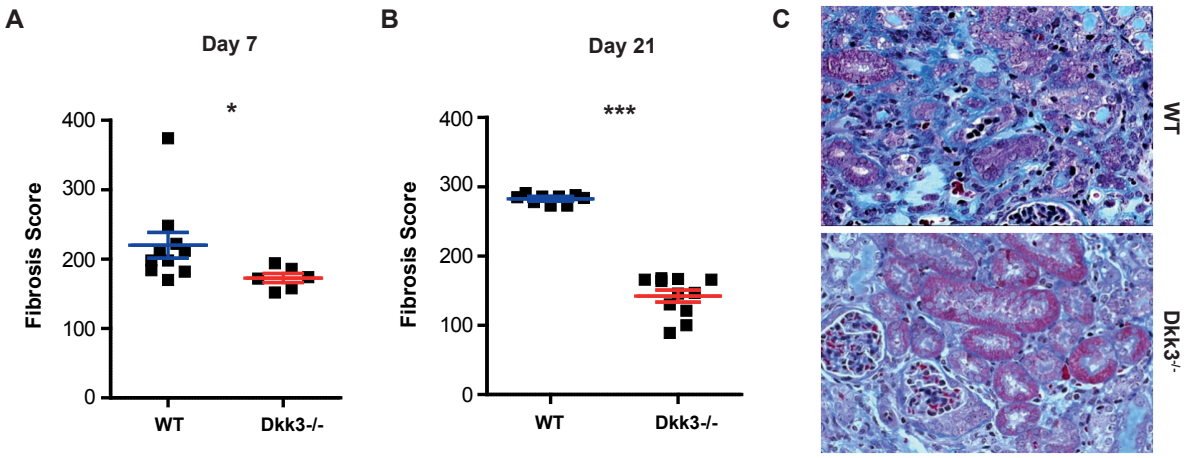

Figure 3.23 UUO induced kidney fibrosis is drastically decreased in Dkk3 deficeint mice. Fibrosis score (A) 7 days and (B) 21 days after UUO induction in *dkk3*$^{-/-}$ and wt mice kidneys. Fibrosis score was evaluated by the intensity of trichrome staining of respective kidney slices. One dot represents fibrosis score of one mouse. Mean and SEM are displayed by horizontal bars. (C) Representative trichrome stainings of kidneys from (B).

Similar results were obtained in the adenine-feeding model of kidney fibrosis. 28 days after fibrosis induction, Dkk3 deficient mice exhibited a significantly less pronounced degree of fibrosis. This was not the case 7 days after operation. Fibrosis outcome was monitored by all above mentioned parameters and is exemplary displayed by fibrosis score (Figure 3.24).

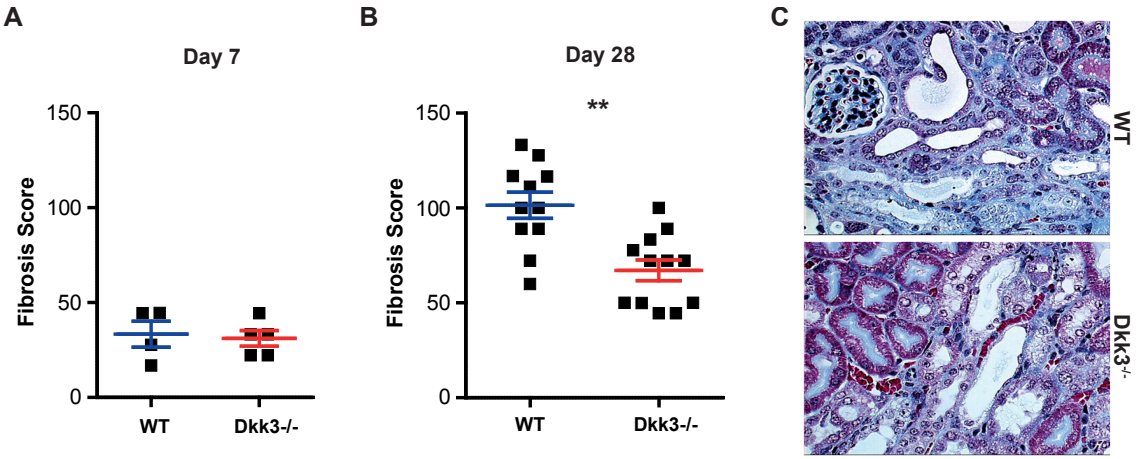

Figure 3.24 Adenine feeding induced a less severe degree of kideny fibrosis in Dkk3 deficent mice. Fibrosis score (A) 7 days and (B) 21 days after starting of adenine feeding in dkk3$^{-/-}$ and wt mice kidneys. Fibrosis score was evaluated by the intensity of trichrome staining of respective kidney slices. One dot represents fibrosis score of one mouse. Mean and SEM are displayed by horizontal bars. (C) Representative trichrome stainings of kidneys from (B).

3.4.2 Renal Dkk3 expression is induced during fibrosis development

As shown earlier (Figure 3.11), Dkk3 is hardly expressed in healthy, adult kidneys. Therefore, we wondered if systemic Dkk3 influences pathology or if Dkk3 may be induced upon injury and inflammation, mediating its properties by this means.

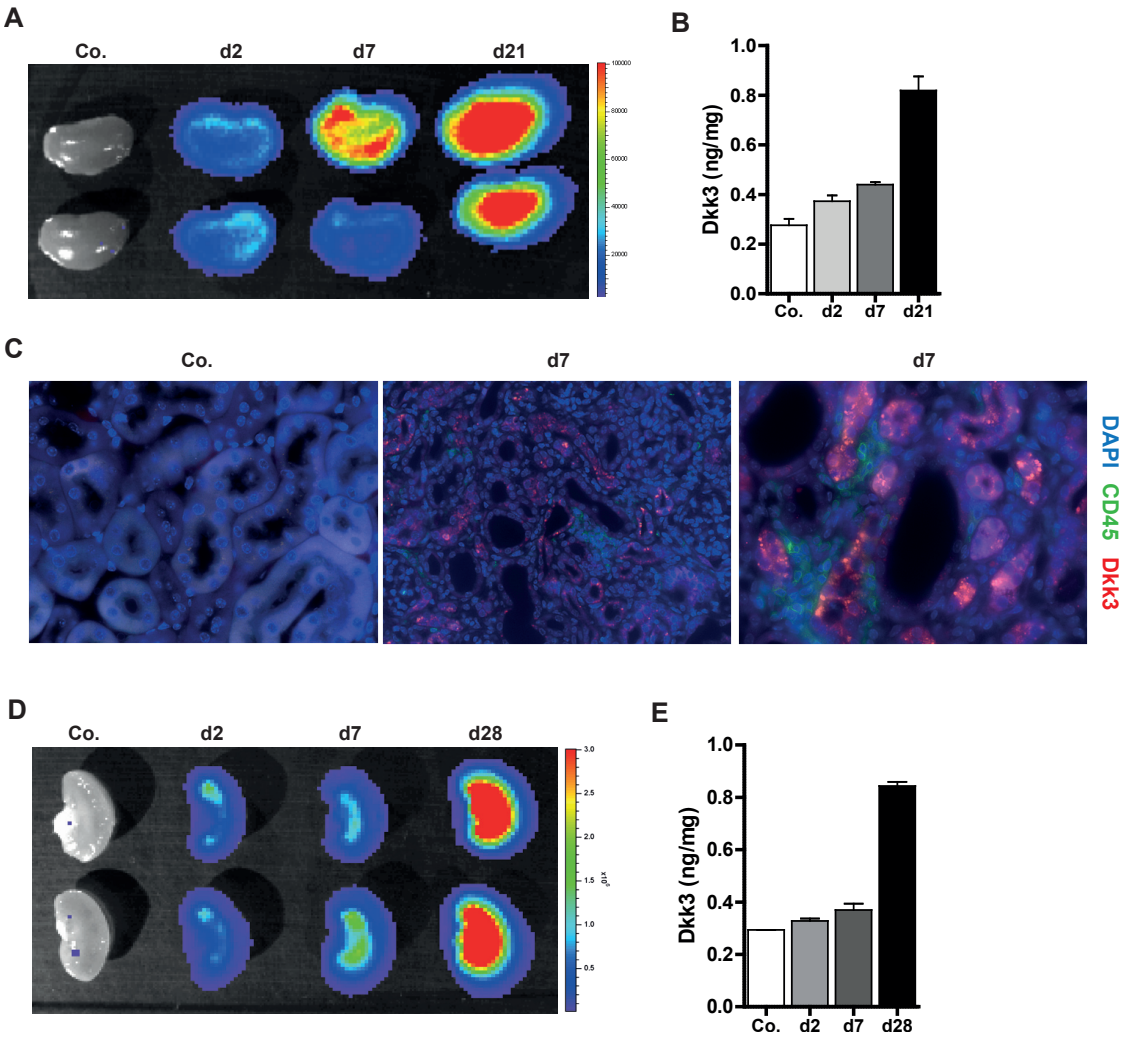

Figure 3.25 Renal Dkk3 production is induced in tubular epithelial cells during fibrosis development. (A+D) *Ex vivo* bioluminescence imaging of fibrotic kidneys of Dkk3-LCh mice (A) 2, 7, and 21 days after UUO induction or (D) 2, 7 and 28 days after starting of adenine feeding. As a control kidneys from untreated Dkk3-LCh mice were used. 5 min after i.p. injection of 150mg/kg D-luciferin, mice were sacrifized and organs were taken out. After 5min of incubation in a 1mg/ml D-luciferin solution in PBS at 37°C organs were imaged for 5min. Colours display intensity of the emmited light (see scale). One representtive experiment out of 3 is shown. (B+E) Amount of Dkk3 protein relative to organ weight in lysates of fibrotic kidneys (B) 2, 7 and 21 days after UUO induction or (E) 2, 7 and 28 days after starting of denine feeding. As a control, kidneys from untreated mice were used. (C) Detection of mCherry expression in fibrotic kidneys of Dkk3-LCh mice 7 days after UUO induction. As a control kidneys of untreated Dkk3-LCh mice were used. Cryo sections from isolated kidneys were prepared and fluorochrome conjugated anti-mCherry staining (red) and anti-CD45 staining (green) was performed. Nuclei were counter stained with DAPI (blue). Shape and localization of mCherry positive cells identified them as tubular epitheliel cells. One representative experiment out of 3 is shown.

In order to answer this question, we triggered UUO in Dkk3 reporter mice (Dkk3-LCh) and monitored luciferase expression in damaged kidneys *ex vivo*. Indeed, after 2 and 7 days a slight increase and after 21 days a strong induction of luciferase expression was detected (Figure 3.25A). This result was confirmed by the detection of Dkk3 protein levels in whole kidney lysates after UUO by ELISA. Dkk3 protein content of kidneys slightly increased 2 and 7 days after operation and reached a 4 times higher level after 21 days (Figure 3.25B). To identify the particular cell type responsible for stress induced Dkk3 production. UUO was induced in Dkk3-LCh mice and kidneys were histologically examined to identify mCherry-expressing cells. Untreated Dkk3-LCh mice were used as a control. In order to exclude hematopoietic cells as source of mCherry expression, co-staining with CD45 was accomplished. As shown in Figure 3.25C , mCherry expression could be detected in epithelial cells but not in hematopoietic cells. Detailed morphological analysis of mCherry positive cells uncovered that renal tubular cells were the main source of mCherry. Additionally, the same degree of Dkk3 induction in the kidney could be detected using the adenine-feeding model (Figure 3.25D,E).

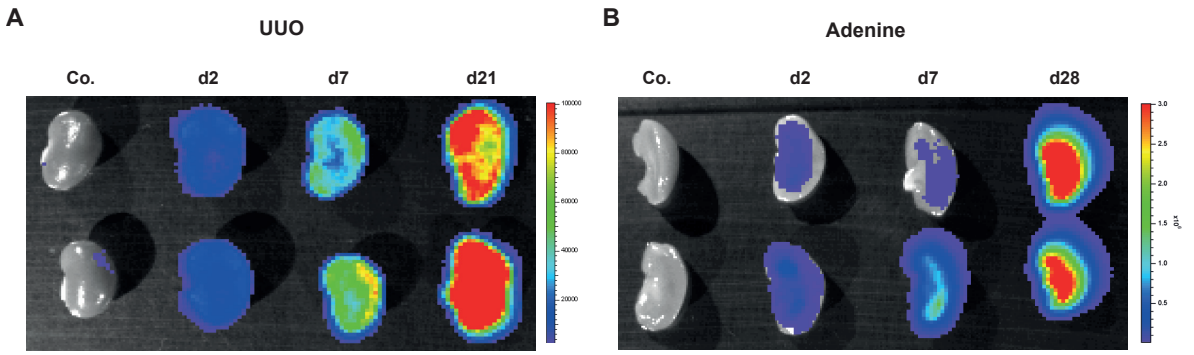

Figure 3.26 Dkk3 induction in fibrotic kidneys is IFNg independent. (A) Ex vivo bioluminescence imaging of fibrotic kidneys of Dkk3-LCh x IFNγR1$^{-/-}$ mice (A) 2, 7, and 21 days after UUO induction or (B) 2, 7 and 28 days after staring adenine feeding. As a control kidneys from untreated Dkk3-LCh mice were used. 5 min after i.p. injection of 150mg/kg D-luciferin, mice were sacrifized and organs were taken out. After 5min of incubation in a 1mg/ml D-luciferin solution organs were imaged for 5min. Colours display intensity of the emmited light (see scale). One representtive experiment out of 3 is shown.

In Chapter 2 it was shown that IFNγ can induce Dkk3 expression in vitro and in vivo. In order to clarify whether the induction of Dkk3 expression in the kidney during development of fibrosis was IFNγ dependent we crossed Dkk3 reporter mice (Dkk3-LCh) with IFN-receptor 1 deficient mice and performed fibrosis experiments. In these animals a similar degree of Dkk3 induction compared to wild type reporter mice was observed independent of the fibrosis

model (Figure 3.26). Thus, renal Dkk3 production is induced in tubular epothelial cells during fibrosis development in an IFNγ independent manner.

3.4.3 Increased inflammation in fibrotic dkk3$^{-/-}$ kidneys

Since the phenotype of *dkk3*$^{-/-}$ mice was more pronounced in the UUO model we decided to use this system for further, detailed analysis of the underlying mechanisms. Previously, we described Dkk3 as modulator of T cell responses, therefore we wanted to examine the impact of the immune system in general and T cells in particular on the present phenotype. We first analyzed cytokine expression by qRT-PCR on RNA isolated from total fibrotic kidneys of *dkk3*$^{-/-}$ and wt mice 21 days after UUO induction. This analysis revealed that the expression of TGF-β, a key molecule in fibrosis development, was not altered in kidneys of Dkk3 deficient mice compared to wt control (Figure 3.27A). However, the expression of the pro-inflammatory cytokines IFNγ and IL-1β was significantly increased in Dkk3 deficient kidneys. While elevated IFNγ levels were already detected after 7 days, IL-1β expression differed only after 21 days (Figure 3.27). Surprisingly, less severe fibrosis was accompanied by a stronger inflammation after UUO in Dkk3 deficient mice. Therefore, we decided to further analyze the impact of Dkk3 on the immune response, contributing to fibrosis development.

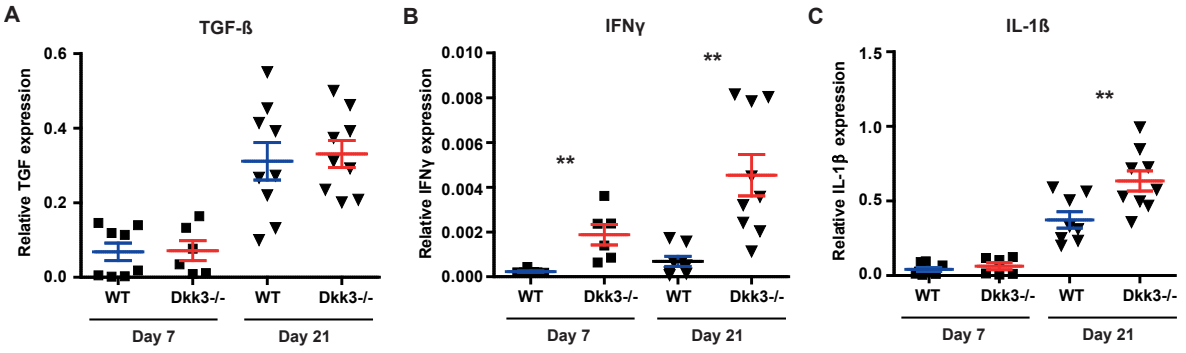

Figure 3.27 Increased expression of proinflammatory cytokines in fibrotic kidneys of Dkk3 deficient mice. (A-C) QRT-PCR on total RNA from fibrotic kidneys of Dkk3 deficient and wt mice, isolated 7 and 21 days after UUO induction. (A) TGF-β, (B) IFNγ and (C) IL-1β expression levels are displayed relative to the housekeeping gene Actb. One dot represents the cytokine level in one kidney of one mouse. Mean and SEM are displayed by horizontal bars.

The development of kidney fibrosis in the UUO model was reported to be, at least in part, T cell dependent (Tapmeier, Fearn et al. 2010). In order to confirm these results and to justify further T cell analysis, we induced UUO fibrosis in Rag2 deficient mice, which lack T cells. As expected, Rag2 deficient mice developed a much milder fibrosis phenotype compared to wt controls, located in the range of Dkk3 deficient mice 21 days after UUO induction (Figure 3.28). Thus, in our experiments, the development of severe kidney fibrosis in the UUO mouse model is T cell dependent.

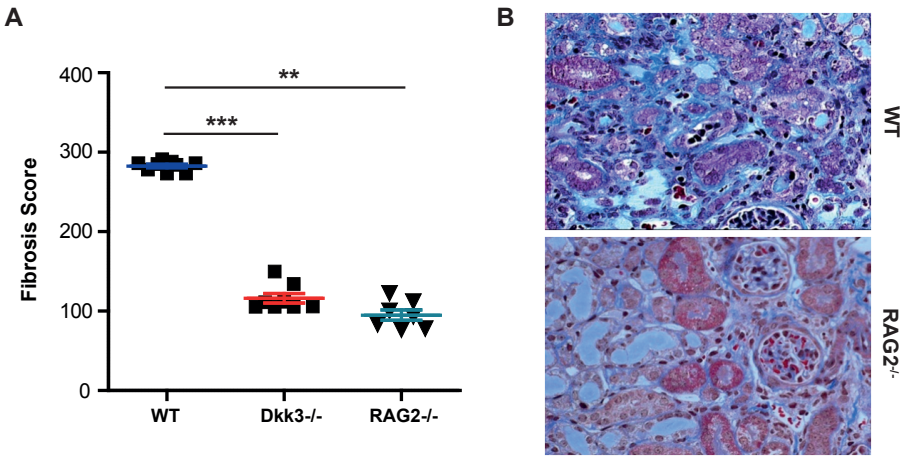

Figure 3.28 Fibrosis development in the UUO mouse model is T cell dependent. (A) Fibrosis score in kidneys of RAG2 deficient, Dkk3 deficient and wt mice 21 days after UUO induction. Fibrosis score was evaluated by the intensity of trichrome staining of respective kidney slices. One dot represents fibrosis score in the obstructed kidney of one mouse. Mean and SEM are displayed by horizontal bars. (B) Representative trichrome stainings of RAG2 deficeint and wt kidneys from (A).

Based on these results, we analyzed the degree of T cell infiltration in kidneys of *dkk3*$^{-/-}$ and wt mice via CD3 staining. Surprisingly, we found considerably increased numbers of infiltrated T cells in the kidneys of Dkk3 deficient mice compared to wt controls. While 7 days after UUO induction a moderate increase was detected, 21 days after operation the amount of T cells in Dkk3 deficient mice was about 6 times higher compared to wt controls (Figure 3.29). Increased T cell infiltration in *dkk3*$^{-/-}$ kidneys correlated with elevated cytokines levels (Figure 3.27).

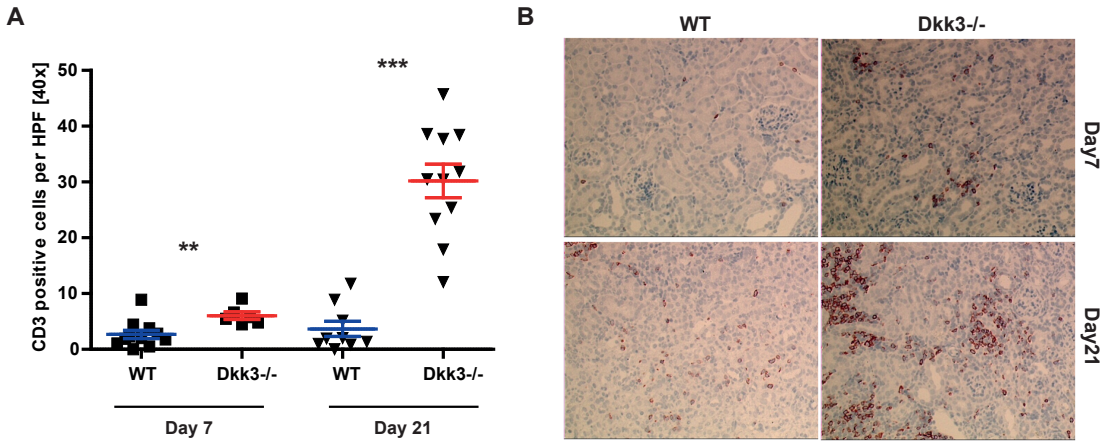

Figure 3.29 Increased T cell infiltration in fibrotic kidneys of dkk3$^{-/-}$ mice. (A) Number of CD3 positive cells per High Power Field (HPF) 7 days and (B) 21 days after UUO induction in *dkk3*-/- and wt kidneys. Data were obtained by immunohistochemistry using an anti-CD3 staining. One dot represents the number of CD3^{+} cells per HPF in the obstructed kidney of one mouse. Mean and SEM are displayed by horizontal bars (B) Representative CD3 stainings of kidneys from (A).

3.4.4 Dkk3 supports Th2 differentiation during the development of renal fibrosis

So far, severity of fibrosis was thought to correlate with the strength of the accompanying inflammation. However, in the previous experiments, we observed that despite a strongly increased inflammation and T cell infiltration, renal fibrosis outcome was significantly decreased in Dkk3 deficient mice compared to wt controls. Therefore we hypothesized that the kind of inflammation and the type of infiltrated T cells might differ in *dkk3*-/- mice. In line with that, T cell polarization towards a Th2 phenotype and the presence of the respective cytokines (IL-4/IL1-3/IL-5) was shown to drive fibrosis development (Liu, Kou et al. 2012), while a Th1 phenotype and the cytokine IFNγ has been reported to be beneficial (Oldroyd, Thomas et al. 1999).

In order to get a first insight into Th1/Th2/Treg distribution within the kidneys of *dkk3*-/- and wt mice, we analyzed mRNA expression of transcription factors described as master regulators of the respective T cell linages. As displayed in Figure 3.30, mRNA expression of GATA3 the master regulator of the Th2 linage was decreased in Dkk3 deficient mice 7 (Figure 3.30A) and 21 days (Figure 3.30B) after UUO induction. In contrast, mRNA levels of Tbet, the indispensable transcription factor for Th1 differentiation, were elevated (Figure 3.30). Additionally, we observed a slight increase in Foxp3 mRNA in *dkk3*-/- kidneys.

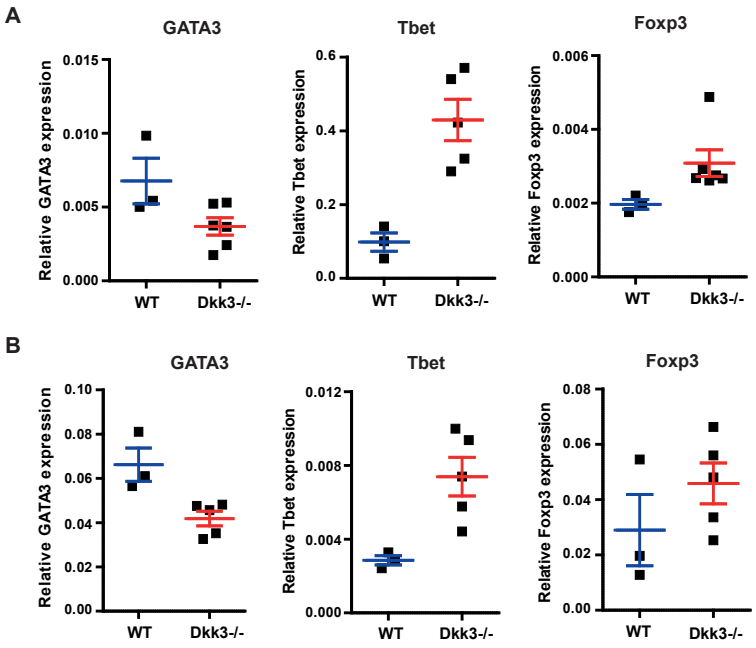

Figure 3.30 Altered expression of master regulateros of CD4 T cell linage differentiation in fibrotic *dkk3*$^{-/-}$ kidneys. (A+B) QRT-PCR on total RNA from fibrotic kidneys of Dkk3 deficient and wt mice, isolated (A) 7 and (B) 21 days after UUO induction. Expression levels of Gata3, Tbx21 (Tbet) and Foxp3 are diplayed relative to the housekeeping gene *Actb*. One dot represents the expression level in the obstructed kidney of one mouse. Mean and SEM are displayed by horizontal bars.

These results suggested an adjustment in T cell polarization from Th2 towards a Th1 phenotype in Dkk3 deficient mice. In order to verify these results on a single cell level, lymphocytes from kidneys of *dkk3*$^{-/-}$ and wt mice were isolated 21 days after UUO induction and their phenotype was analyzed. Besides transcription factor expression, cytokine production of T cells was assessed by flow cytometry.

According to histological results showing increased T cell infiltration (Figure 3.29), we found elevated numbers of infiltrating, lymphocytes in Dkk3 deficient kidneys (Figure 3.31A). Furthermore, analysis of cytokine production after in vitro re-stimulation revealed a significantly higher number of IFNγ and TNF producing CD4 (Figure 3.31B) and CD8 positive (Figure 3.31C) T cells in Dkk3 deficient kidneys compared to wt controls.

In addition, intracellular transcription factor staining of isolated cells approved qRT-PCR data by showing decreased numbers of GATA3 positive cells and a lower GATA3 mean fluorescence intensity (MFI) (Figure 3.31D). However, we failed to detect a higher proportion of Tbet positive cells in *dkk3*$^{-/-}$ kidneys. This was in contrast to qRT-PCR results (Figure 3.31E).

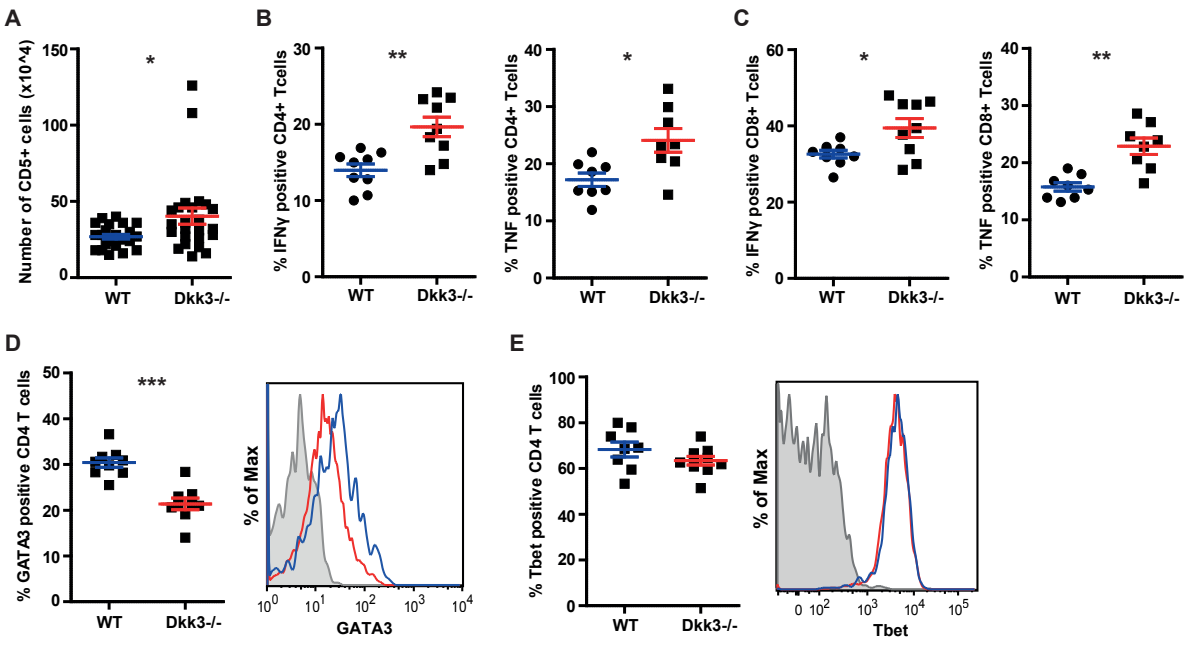

Figure 3.31 Dkk3 promotes CD4 T cell polarization towards a Th2 phenotype in fibrotic kidneys. (A-E) CD5 positive cells were isolated from fibrotic wt and *dkk3*$^{-/-}$ kidneys 21 days after UUO induction by MACS beads. (A) Absolut numbers of isolated CD5^{+} cell. (B+C) Isolated CD5^{+} cells were restimulated in vitro with PMA/Ionomycin in the presence of monesin and IFNγ an TNF production of (B) CD4^{+} and (C) CD8^{+} T cells was analyzed by intracellular staining and subsequent flow cytometry. Displayed is the percentage of IFNγ and TNF positive CD4^{+} or CD8^{+} cells. (D+E) Isolated CD5^{+} cells from fibrotic kidneys were stained intracellularly for (D) GATA3 and (E) Tbet. Displayed is the percentage of (D, left panel) GATA3 and (E, left panel) Tbet positive CD4+ cells as well as the MFI of (D, right panel) GATA3 and (E, right panel) Tbet (blue: wt, red: *dkk3*$^{-/-}$) in CD4 T cells. One dot represents pooled cells from two mice. Mean and SEM are displayed by horizontal bars.

Next, we asked whether the observed T cell phenotype was locally restricted to fibrotic kidneys or was a systemic phenomenon. Therefore, we performed similar experiments with cells from spleen 21 days after fibrosis induction. In contrast to kidneys, total numbers of lymphocytes as well as levels of TNF and IFNγ producing $CD4^+$ and IFNg producing $CD8^+$ T cells were comparable in *dkk3*$^{-/-}$ and wt spleens. Only levels of TNF producing $CD8^+$ T cells and GATA3 positive CD4 T cell were slightly altered.

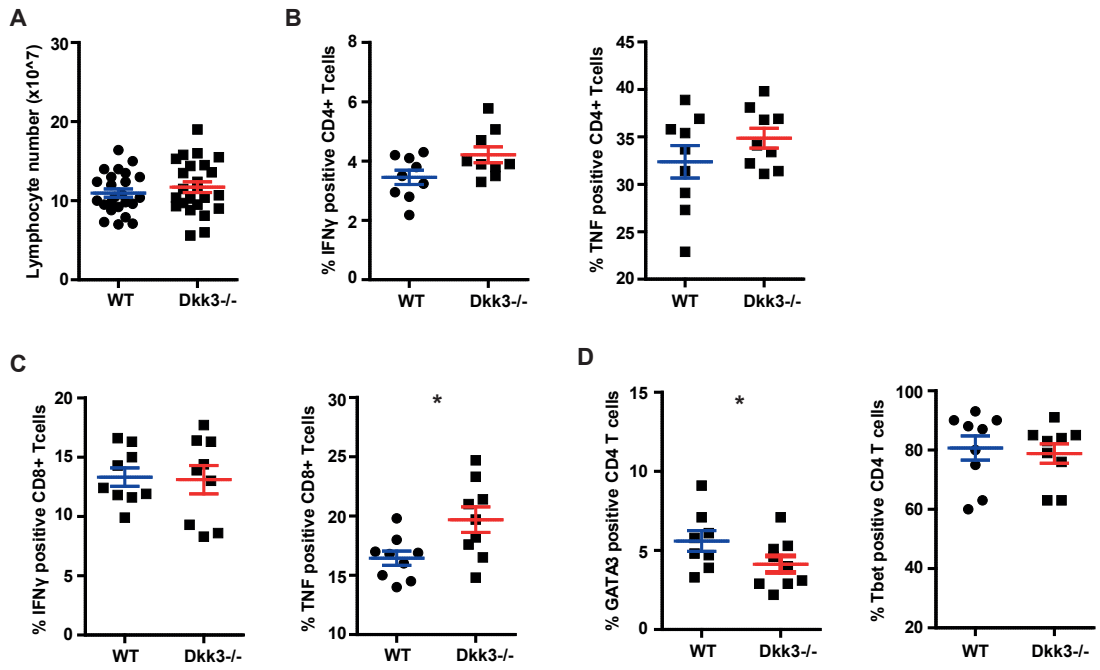

Figure 3.32 No alteration of T cell polarization in spleens of Dkk3 deficient mice bearing kidney fibrois. (A-D) Single cell suspension of spleens from *dkk3*$^{-/-}$ and wt mice 21 dys after UUO induction were generated. (A) Absolut numbers of spleenocytes. (B+C) Spleenocytes were restimulated in vitro with PMA/Ionomycin in the presence of monesin and IFNγ an TNF production of (B) $CD4^+$ and (C) $CD8^+$ T cells was analyzed by intracellular staining and subsequent flow cytometry. Displayed is the percentage of IFNγ and TNF positive (B) $CD4^+$ or (C) $CD8^+$ cells. Spleenocytes were stained intracellularly for GATA3 and Tbet. Displayed is the percentage of (D, left panel) GATA3 and (D, right panel) Tbet positive $CD4^+$ cells. One dot represents pooled cells from two mice. Mean and SEM are displayed by horizontal bars.

In addition, we compared the levels of regulatory T cells in fibrotic kidneys of *dkk3*$^{-/-}$ and wt mice as well as the respective spleens. Histological analysis of kidneys revealed an increased number of Foxp3 positive cells in Dkk3 deficient mice compared to wt controls 21 days after UUO induction. Flow cytometric analysis of cells isolated from fibrotic kidneys showed similar results. Increased proportions of $CD4^+CD25^+Foxp3^+$ cells were detected in *dkk3*$^{-/-}$ kidneys 21 days after surgery (Figure 3.33B). Again, this alteration was absent in the respective spleens (Figure 3.33C).

Thus, flow cytometric analysis of isolated T cells not only revealed stronger T cell infiltration into kidneys of Dkk3 deficient mice during fibrosis development but also uncovered an altered $CD4^+$ T cell phenotype possibly being responsible for the milder outcome of fibrosis in these mice. Since this was only observed in fibrotic kidneys but not in the respective spleen, this seemed to be a rather local than systemic effect of Dkk3.

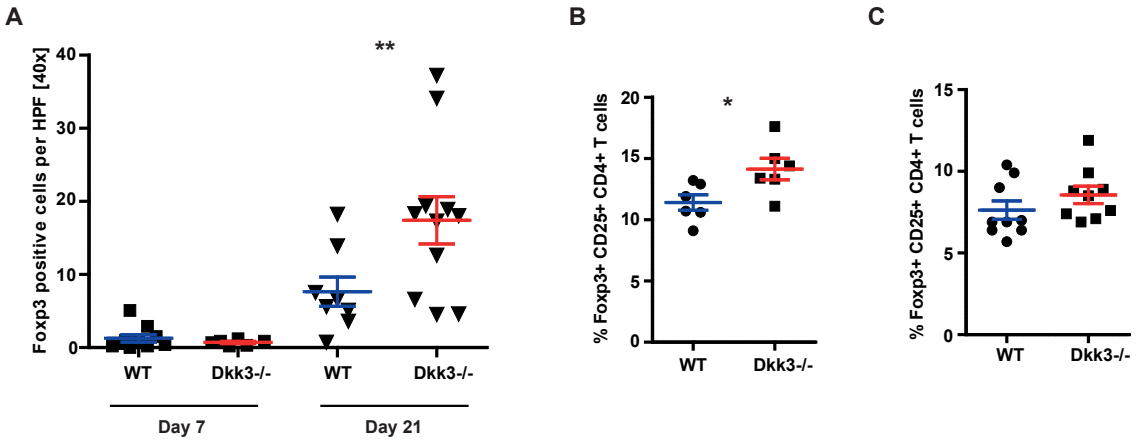

Figure 3.33 Increased numbers of Foxp3+ regulatory T cells in fibrotic kidneys of dkk3 deficient mice. (A) Number of Foxp3 positive cells per HPF in fibrotic kidneys of *dkk3*$^{-/-}$ and wt mice 7 and 21 days after UUO induction. Data were obtained by immunohistochemistry using an anti-Foxp3 staining. (B+C) Percentages of $Foxp3^+$ $CD25^+$ among $CD4^+$ T cells. (B) CD5 positive cells were isolated from fibrotic wt and *dkk3*$^{-/-}$ kidneys 21 days after UUO induction by MACS beads. After staining for the respective markers, cells were analyzed by flow cytometry. One dot represents one mouse. Mean and SEM are displayed by horizontal bars

Finally, the mechanism by which Dkk3 modulates $CD4^+$ T cell polarization in the fibrosis model was analysed. Dkk3 was shown to influences Wnt signalling in several cell lines by several mechanisms (Nakamura, Hunter et al. 2007). Additionally, particular Wnt target genes have been reported to play a pivotal role in $CD4^+$ T cell differentiation (Yu, Sharma et al. 2009) Therefore, we analysed expression levels of several Wnt target genes in $CD4^+$ T cells isolated from fibrotic *dkk3*$^{-/-}$ and wt kidneys 7 days after UUO induction. QRT-PCR analysis revealed that expression of all analysed Wnt target genes *tcf7, lef1, fzd1, c-jun, nlk* and *gata3* was decreased in T cells from *dkk3* deficient fibrotic kidneys. Therefore, Dkk3 might positively influences the Wnt pathway in CD4 T cells and thus modulate their linage commitment.

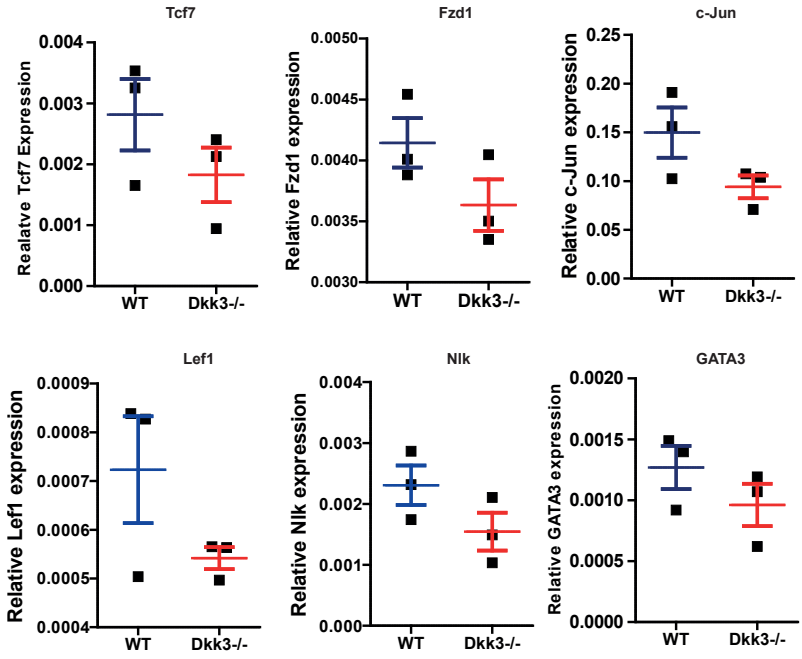

Figure 3.34 Expression of Wnt target genes is slightly decreased in CD4 T cell from fibrotic kidneys of dkk3$^{-/-}$ mice. QRT-PCR on total RNA from CD4^{+} T cells isolated from fibrotic kidneys of Dkk3 deficient and wt mice 7 days after UUO induction. Expression levels of the respective genes are diplayed relative to the housekeeping gene *Actb*. One dot represents expression in cells pooled from two mice. Mean and SEM are displayed by horizontal bars.

In summary, we found that Dkk3 promotes the development of renal indicated by considerably less signs of fibrosis in Dkk3 deficient mice in two independent mouse models. Furthermore, we observed that Dkk3 expression is induced in tubular epithelial cells in the course of fibrosis development. Less fibrosis in Dkk3 deficient mice was accompanied with elevated levels of pro-inflammatory cytokines and increased T cell infiltration in the respective kidneys. Additionally, we observed an altered polarization of infiltrating CD4^{+} T cells towards a Th1/Treg phenotype in dkk3$^{-/-}$ kidneys, which comes along with decreased expression of Wnt target genes in these cells.

4 Discussion

4.1 Self-antigen presentation by keratinocytes modulates auto-reactive T cell responses

The skin provides a first line of defence against physical insults, toxins and pathogens. Therefore, immune surveillance of this barrier has to be finely balanced to ensure its integrity: injuries have to be promptly repaired to prevent overwhelming infections and immune responses have to be limited to avoid chronic inflammation and autoimmune disease.

Here, we asked how T cells respond to cognate auto-antigens that are expressed and presented exclusively by keratinocytes of the inflamed skin, and how direct antigen presentation by keratinocytes influences the outcome of an immune response. Our data suggest that:

(i) Keratinocytes can present self-antigen to stimulate potentially destructive autoreactive $CD4^+$ T cells under inflammatory conditions.

(ii) Repeated antigen stimulation in the inflamed skin leads to down-modulation of T cell responses

(iii) This down- modulation depends on the expression of Dkk3 protein, which limits $CD4^+$ T cell reactivity.

Transgenic mice that inducibly expressed a nominal antigen, such as Ovalbumin (OVA), exclusively in keratinocytes under keratin 5 regulatory sequences have been previously reported (Rosenblum, Gratz et al. 2011). In these mice OVA-derived peptides are presented by professional APCs in the skin draining lymph nodes leading to the proliferation of OVA-specific, DO11 $CD4^+$ T cells. However, it is difficult to investigate the contribution of antigen presentation by keratinocytes to the resulting T cell response *in vivo* in such a model. In our Ker-MBP mouse model, a MBP peptide that is covalently linked to the MHC class II A^u β-chain is exclusively and constitutively expressed in keratinocytes of hair follicles. Our data indicate that in naïve Ker-MBP mice, MBP-specific $CD4^+$ T cells are not aware of their cognate antigen. This suggests that professional APCs do not take up and present the MBP-peptide under steady state conditions. Upon skin inflammation MBP might be presented

either by keratinocytes or by professional APC. If professional APCs would take up the MBP peptide in the skin one would expect MBP-specific T cell proliferation in the draining lymph nodes after induction of migration of $CD11c^+$ cells from the skin to the draining lymph nodes by contact sensitizers. However, proliferation of MBP-specific T cells was only observed in the skin and was absent from the draining lymph nodes. These observations support the view that in the Ker-MBP mouse model the MBP peptide represents a truly sessile antigen in the skin that can be presented by keratinocytes only upon inflammation.

The cutaneous inflammation that was induced by a single treatment with the contact sensitizer oxazolone was more potent in Tg4 x Ker-MBP mice than in Tg4 mice. However, severe skin disease, such as scaling and alopecia was not observed in the double transgenic mice. This is in contrast to what was observed following induction of OVA in the above mentioned Ker-OVA mouse model, in which OVA-specific DO11 T cells caused pronounced inflammatory dermatitis that spontaneously resolved after 20-30 days (Rosenblum, Gratz et al. 2011). The different results obtained in the two models might reflect the fact that OVA can be presented to DO11 T cells by professional APCs in the Ker-OVA mouse model, whereas MBP is presented to Tg4 T cells exclusively by keratinocytes in our system. Activated Tg4 T cells mediated their destructive potential in oxazolone treated Tg4 x Ker-MBP mice only after injection of pertussis toxin, which led to the occurrence of EAE symptoms. The lack of severe skin disease upon oxazolone treatment might suggest that keratinocytes cannot properly activate T cell responses. However, using EAE as read out system we found that under skin-inflammatory conditions keratinocytes can present antigen to activate potentially destructive autoreactive $CD4^+$ T cells. It seems that T cell responses in the skin may be more strongly regulated than in the CNS and/or target cells of the autoimmune attack are more resistant in the skin than in the CNS. It has been proposed that microbial infections might initiate autoimmune diseases by activation of cross-reactive T cells which recognize shared microbial and self determinants (Cusick, Libbey et al. 2012). However, accessibility of the target organ is essential for tissue destruction. Activation of MBP-specific T cells during skin inflammation did not cause an autoimmune disease in the CNS. Our data rather suggest that perturbations of tissue integrity by a toxin produced by an invading pathogen, such as the ones caused by pertussis toxin in the blood-brain barrier and the CNS, may be an additional essential factor to initiate autoimmune diseases by cross-reactive T cells that are activated in a distal site.

There is increasing evidence that repeated exposure of T cells to self-antigens in tissues attenuates the severity of autoimmune responses (Lara-Corrales and Pope 2010, Rosenblum, Gratz et al. 2011). In Ker-MBP mice, the MBP determinant is presented on the cell surface of keratinocytes only during inflammation, upon co-expression of the endogenous MHC II α-chain. DPCP is used clinically for the treatment of alopecia areata because of its proposed immune-modulatory capacity (Hoffmann, Wenzel et al. 1994). In our experiments, repeated applications of DPCP that enabled continuous availability of the MBP peptide/MHC class II complex on keratinocytes, limited the severity of EAE that was induced by immunization with MBP peptide and CFA. Such a down-modulation of the MBP-specific T cell response was observed neither after a single application of DPCP or oxazolone nor following four treatments with oxazolone. Skin inflammation, however, caused by both contact sensitizers was sufficient to induce MBP-specific T cell proliferation in the skin, even though functional consequences of this T cell activation were different. These results indicate a different mode of action in inducing T cell responses by the two types of contact sensitizers. Even though this has been analysed (Webb, Tzimas et al. 1998) the molecular basis is still unknown and requires further investigation.

Our results pointed out that regulatory mechanisms are responsible for down-modulation of MBP-induced EAE mediated by pre-treatment with DPCP. However, the numbers of $CD4^+CD25^+FoxP3^+$ regulatory T cells were not altered. In order to identifying molecular mechanisms responsible for the observed inhibition we failed to detect differences in expression levels of several immune mediators, such as IL-10, TGFβ, PD-1L and CTLA-4 in the skin of Ker-MBP compared with wild type mice. In line with that it was shown that epicutaneous immunization with the auto-antigenic MBP peptide inhibited the induction of EAE by a challenge with MBP peptide and CFA (Bynoe, Evans et al. 2003). This protection was based on $CD4^+$ T cell regulation, but was independent of $CD4^+CD25^+$ regulatory T cells and was not mediated by IL-4, IL-10 or TGFβ. The MBP peptide was shown to be presented by professional APCs in the draining lymph nodes. An involvement of MBP peptide presentation by keratinocytes was not investigated in that study. Interestingly, repeated tolerogenic epicutaneous applications of MBP peptide have similar consequences as continuous MBP peptide presentation by keratinocytes under inflammatory conditions.

In view of the notion that the skin expresses self-antigens that are also present in other tissues (Kawakami, Eliyahu et al. 1994, Kormos, Belso et al. 2011) and that the skin is

frequently the target of infections and inflammations, our data may in part explain why individuals who have experienced infections have a lower incidence of autoimmune diseases (Bach 2002).

Our data revealed that, Dkk3 was significantly up-regulated in the skin of DPCP-treated Ker-MBP mice in comparison to DPCP-treated wild type mice indicating that both, DPCP-induced inflammation and T cell activation by MBP-presenting keratinocytes were necessary for the increase in Dkk3 expression. Our lab recently reported that Dkk3 can limit $CD8^+$ T cell mediated tumor and skin graft rejection (Papatriantafyllou, Moldenhauer et al. 2012). Additionally, Dkk3 was reported to be mainly expressed at sites that have been traditionally referred to as immune privileged including the hair follicle (Barrantes Idel, Montero-Pedrazuela et al. 2006, Ohyama, Terunuma et al. 2006, Hermann, Pirkebner et al. 2007). Therefore, one could speculate that Dkk3 may contribute to the immunosuppressive milieu of such tissues. Here we showed that depletion of Dkk3 reversed the reduction of T cell-mediated EAE symptoms in DPCP-treated Ker-MBP mice. Furthermore, expression of Dkk3 in the skin was associated with decreased cytokine production by MBP-specific $CD4^+$ T cells. Neutralizing Dkk3-specific antibodies could block the down-modulation of $CD4^+$ T cell reactivity when administered at the beginning of the DPCP treatment. This observation suggests that Dkk3 is involved in the induction of the observed immune regulation by influencing the induction of T cell function in the skin.

4.2 Dkk3 expression can be visualized in a novel reporter mouse

Dickkopf-3 is a secreted glycoprotein that is prominently expressed during vertebrate development. However, it was also reported to be significantly expressed in adult organs. Our lab has previously described an immune modulatory capacity of Dkk3 (Papatriantafyllou, Moldenhauer et al. 2012). Additionally, results of this thesis (Chapter 3.1), showing its contribution to keratinocyte-mediated limitation of $CD4^+$ T cell reactivity, support this finding. Therefore, the expression pattern of Dkk3 is of particular interest. Even though there is already a considerable amount of available data dealing with Dkk3 expression. We aimed to create a tool that enables simple and reliable detection of Dkk3 expression.

Here, we reported the successful generation of a Dkk3 reporter mouse (Dkk3-LCh), a tool for the identification of Dkk3 expressing tissues and cells. In this transgenic system, the two reporter molecules CB99 Luciferase and mCherry were expressed under the regulatory sequences of the Dkk3 gene. In order to have the possibility to identify sites of Dkk3 expression via *in vivo* and *ex vivo* bioluminescence analysis, CB99 luciferase was chosen as a reporter molecule. The fluorochrome mCherry was introduced to identify particular Dkk3 expressing cell types via fluorescence-microscopy. In our construct, both genes were connected by the viral 2A sequence, which assured stoichiometric expression of both molecules (Miloud, Henrich et al. 2007).

Ex vivo analysis of luciferase expression, in organs of healthy, adult Dkk3-LCh mice, revealed prominent luciferase expression in eye, brain, spinal cord, heart, lung, skin, uterus, stomach, gut, gal bladder, esophagus and cartilage. In contrast, luciferase expression was absent in the lymphoid organs, namely lymph nodes, spleen and thymus, as well as in kidney, liver and pancreas. These findings predominantly confirmed so far published result (Krupnik, Sharp et al. 1999) and were in line with data on Dkk3 expression, previously obtained in our lab via qRT-PCR and ELISA (Papatriantafyllou, 2008). Interestingly, Dkk3 expression in cartilage was not reported in the literature or detected in our lab before. This finding could be of particular interest, as Dkk3, due to its immune modulatory functions (Papatriantafyllou, Moldenhauer et al. 2012), might influence rheumatoid arthritis, which is a devastating autoimmune disorder in joints (McInnes and Schett 2011).

As expected we failed to detect luciferase expression in lymphoid organs. So far, published studies suggested a general absence of Dkk3 expression in hematopoietic cells of wt mice, except for a rare subset of long-term memory $CD8^+$ T cells (Kaech, Hemby et al. 2002) and, as published very recently, in a subset of γδ T cells. This study revealed Dkk3 expression in

CD27 negative γδ T cells in contrast to CD27+ γδ T cells or common αβ T cells (Schmolka, Serre et al. 2013) However, the frequency of cells of these particular subtypes is probably too low to generate a prominent luciferase signal. Furthermore, the luciferase expression pattern in the heart illustrates the capacity of the Dkk3-Lch mouse to specifically indicate sites of dkk3 expression. It was shown that, in the heart, Dkk3 is exclusively expressed by specific cells in the atrium but not in the ventricle (Krupnik, Sharp et al. 1999). In Dkk3-LCh mice, luciferase could precisely reproduce this specific expression pattern. Finally, qRT-PCR analysis comparing Dkk3 and luciferase mRNA expression, revealed similar relative expression levels of both genes in respective organs. In summary, luciferase expression in Dkk3-LCh mice seems to reflect natural Dkk3 expression.

Expression analysis of the second reporter molecule in Dkk3-LCh mice, mCherry, was accomplished by fluorescence microscopy. Most considerable mCherry expression in E14.5 Dkk3-LCh embryos was found in elongated cells spanning the entire thickness of the retina, referred to as Müller glial cells. According to the literature, these cells are strong Dkk3 expressers (Nakamura, Hunter et al. 2007). It is of importance to mention, that the mCherry fluorescence signal by itself was not detectable and had to be potentiated by fluorochrome-conjugated antibody staining. This was probably due to the destruction of the protein confirmation by fixation with paraformaldehyde. It seems that, by this method, only highly mCherry positive cells were detectable, as several other organs that have been reported to be Dkk3 positive in the embryo were not stained (Monaghan, Kioschis et al. 1999). Thus, in Dkk3-LCh mice, mCherry seems to be able to indicate dkk3-expressing cells even though detection might be limited to cells which strongly express the flourochrome.

Just recently, the generation of a similar Dkk3 reporter mouse was published (Muranishi and Furukawa 2012). According to our Dkk3-LCh mouse, the reporter gene eGFP was introduced by a BAC-transgene. Displayed reporter expression in this mouse highly correlated with that in our Dkk3-LCh mice. All together, the Dkk3-LCh mouse provides the opportunity to reliably indicate Dkk3 expression via its two reporter molecules.

By using this tool, we found that Dkk3 expression in the neonate is much higher than in adulthood. In virtually all organs of neonatal Dkk3-LCh mice, reporter expression was strongly increased compared to adult controls. Measurement of systemic Dkk3 in the serum confirmed this finding. 2-3 weeks after birth, Dkk3 levels of about 4µg/ml in the serum dropped to an intermediate concentration of about 1µg/ml. This level was maintained over

the lasting lifetime. The most prominent differences in Dkk3 expression between neonate and adulthood were observed in thymus and kidney. While it was almost completely absent in adult mice, prominent expression was detected in the neonatal kidney. mRNA expression analysis in kidneys revealed that Dkk3 expression is maintained over the first 3 weeks after birth before it is silenced. These results raise several questions: (i) What is the reason for this timely restricted expression and (ii) how is this decrease of Dkk3 expression mechanistically regulated.

The early expression of Dkk3 suggests a role in developmental processes, as it was postulated before, due to its expression pattern in the embryo (Monaghan, Kioschis et al. 1999). This hypothesis is supported by studies considering a Wnt-pathway modulating activity of Dkk3, as Wnt-signaling is essential for embryonic development (van Amerongen and Nusse 2009). However, in contrast to its family member Dkk1 no functional evidence of a pivotal role of Dkk3 in developmental processes exists so far. While homozygous deletion of Dkk1 leads to embryonic lethality (Mukhopadhyay, Shtrom et al. 2001), Dkk3 knock out mice don't show any developmental defects *per se* (Barrantes Idel, Montero-Pedrazuela et al. 2006). This might be explained by redundancy in this essential system.

4.3 Dkk3 expression can be regulated by inflammatory mediators

Our interest was focused on the role of tissue derived Dkk3 in inflammatory responses. Therefore, we investigated if and how its expression is influenced by cytokines and how it changes under inflammatory conditions. Our data revealed that *in vitro*, TNFα, IL-1ß and retinoic acid reduced Dkk3 expression, whereas IL-4 slightly, and IFNγ strongly up-regulated its expression in primary dermal fibroblasts. In contrast to our results, TNFα was shown to induce Dkk3 expression in vitro in human endothelial colony forming cells capacity (ECFC) (Untergasser, Steurer et al. 2008) and human mesenchymal stem cells (Lee, Yoon et al. 2012). This discrepancy may be explained by the different species and cell types that were analysed, since other function of Dkk3, like for example, the capacity to act as tumour suppressor, was shown to be dependent on the tumour cell line that was studied. While Dkk3 was reported to block proliferation and to induce apoptosis in several tumour cells like human prostate cancer cells (Abarzua, Sakaguchi et al. 2005), it supported proliferation in cells of an human lung adenocarcinoma (Jung, Kang et al. 2010) .

Our results showing that IFNγ is capable to up-regulate Dkk3 expression, reflects the so far identified properties of this cytokine. It was reported to induce the expression of numerous inflammatory mediators in hematopoietic as well as tissue cells, acting as a kind of master regulator of inflammation (Schroder, Hertzog et al. 2004). Even though its main targets are pro-inflammatory molecules (Boehm, Klamp et al. 1997), also anti-inflammatory mediators like indoleamine-2,3 dioxygenase (IDO) (Malone, Dolan et al. 1994) and inducible NO synthase (iNOS) (Burke, Updegraff et al. 2013) were shown to be IFNγ inducible. Thus, Dkk3 may belong to the second category.

The fact that several different cytokines exert an impact on the expression of Dkk3, indicates that the course and outcome of Dkk3 production during inflammation is determined by the concerted action of several cytokines. Our data displayed that cytokines produced early in inflammation like IL-1 and TNFα repressed Dkk3 expression, while later cytokines like IFNγ and IL-4 induce its production. One could speculate that during the induction phase of an immune response the expression of the anti-inflammatory molecule Dkk3 is counterproductive and therefore repressed. However, after resolution of the primary insult, inflammation needs to be kept in check to prevent tissue damage and therefore, Dkk3 is induced.

Further investigations on the capacity of IFNγ to induce dkk3 expression were performed *in vivo.* α-galactosylceramide induced IFNγ was able to elevate systemic Dkk3 levels in mice, suggesting that the initially observed effects were not only an in vitro artifact of cultured dermal fibroblasts but represents a more general mechanism in vivo. However, the source of Dkk3 in this approach remains unclear.

In order to study Dkk3 induction by IFNγ in a pathophysiological situation we choose experimental autoimmune encephalomyelitis as disease model. There, as in other autoimmune models, IFNγ is known to play a divergent, role. While being pro-inflammatory and disease-promoting in the early phase, it was shown to exert an anti-inflammatory, beneficial function in the later phases after culmination of inflammation (Zhang 2007). IFNγ mediates its disease-providing properties by a variety of mechanisms like induction of antigen presentation, promotion of Th1 differentiation as well as up-regulation of adhesion molecules and a wide range of pro-inflammatory mediators (Boehm, Klamp et al. 1997). How the anti-inflammatory part is mediated is so far not clear. As mechanisms, inhibition of T cell expansion, induction of Foxp3 positive regulatory cells and the up-regulation of anti-inflammatory mediators like IDO or iNOS were proposed (Refaeli, Van Parijs et al. 2002, Willenborg, Staykova et al. 2007, Zhang 2007). Additionally, the circumstances that are responsible for the switch in IFNγ function remain to be elucidated in detail.

Our experiments revealed IFNγ-dependent up-regulation of Dkk3 in brain and spinal cord during EAE. Additionally, we found that IFNγ induces Dkk3 expression in cultured dorsal route ganglia, one of the main target cells in EAE. As we analyzed whole brain and spinal cord lysates, the respective cell types which are responsible for elevated Dkk3 levels in vivo could not be identified. Constitutive Dkk3 expression in the brain was localized in neurons of hippocampus and cortex (Papatriantafyllou, 2008). Therefore, these cells might also represent the source of additional Dkk3 during EAE. However, our *in vitro* experiments with DRGs suggest that up-regulation of Dkk3 expression *in vivo* also happens in these cells, at least in spinal cord.

Data previously generated in our lab show that deletion of Dkk3 results in a significant delay of EAE remission associated with increased numbers of IFNγ producing $CD4^+$ and $CD8^+$ T cells in the brain (Papatriantafyllou, 2008).

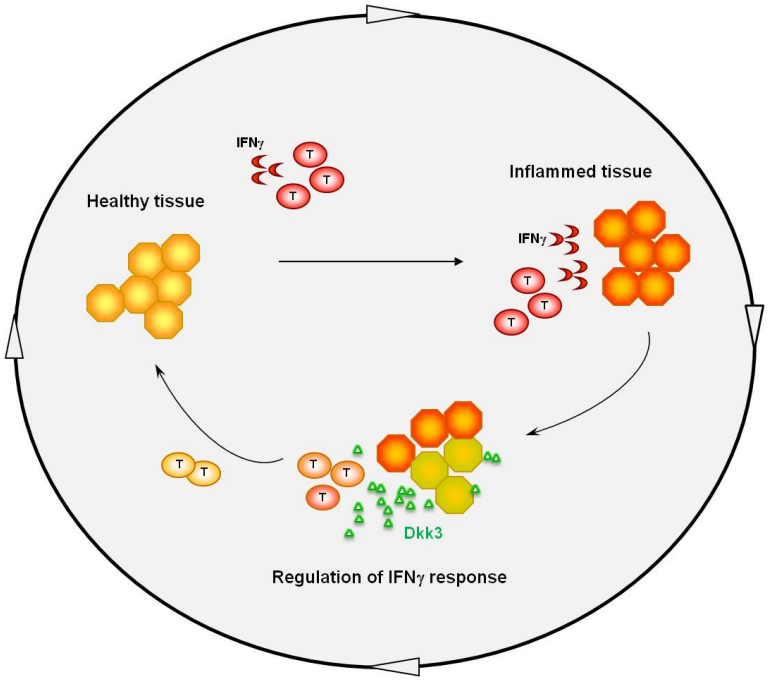

Figure 4.1 Dkk3 mediated negative feedback loop of IFNγ during EAE. T cell derived IFNγ induceds Dkk3 expression in brain and spinal cord. This Dkk3 in turn, decreases the numbers of IFNγ producing T cells.

All our findings suggest that Dkk3 is a mediator of the beneficial function of IFNγ during EAE, contributing to diseases resolution by in turn regulating IFNγ production of T cells in a negative feedback loop (Figure 4.1). However, while we could clearly show the direct induction of Dkk3 by IFNγ, the mechanism by which Dkk3 regulates IFNγ production and/or IFNγ activity remains to be elucidated.

Surprisingly, Dkk3 expression was increased in the inflamed brain although high amounts of Dkk3 are already present in the healthy brain. This finding raises the question, why this additional amount of Dkk3 is needed and whether only part of Dkk3 is in its active form. Dkk3 contains a potential dibasic cleavage site for furin type proteases and was shown to be a substrate of the MMP2 and 9 *in vitro* (Prudova, auf dem Keller et al. 2010). MMP2 and 9 are mainly produced by neutrophils, macrophages and T cells at inflammatory sites (Corbel, Boichot et al. 2000). Therefore one could speculate that Dkk3 might only be functional in inflammation, when post-translational processed, possibly by matrix metalloproteinases.

In summary, we revealed that Dkk3 is a tissue-derived molecule that can be induced by IFNγ and potentially contributes to its immune-modulatory function. Thus, our findings provide further insight into the crosstalk between immune and tissue cells during inflammation and propose a new mechanism by which tissues preserve their integrity.

4.4 Dkk3 facilitates renal fibrosis

Chronic kidney diseases, which ultimately lead to renal fibrosis and organ failure, have become a major public health problem on global scale. An improved understanding of the cellular and molecular mechanisms of renal fibrosis is essential, not only for gaining novel insights into the pathogenesis of the process, but also for developing rational strategies to treat patients with fibrotic kidney disorders. A hallmark of the development of renal fibrosis is a chronic, non-resolving inflammation.

Dkk3 is up-regulated during inflammatory responses in skin (Chapter 3.1) and brain (Chapter 3.3) and may contribute to regulation of pathogenic T cell responses.

Therefore, we addressed the question, whether Dkk3 is also induced in the course of renal fibrosis and if it exerts any impact on the outcome of this important pathological process. Our data point out that:

(i) Dkk3 expression in kidney is up-regulated in tubular epithelial cells in the course of renal fibrosis
(ii) Dkk3 facilitates the development of renal fibrosis
(iii) Dkk3 alters the immune response in damaged kidneys by modulating the phenotype of infiltrating T cells.

Dkk3 deficient mice developed significantly less renal fibrosis than wt mice. These effects were observed in two different mouse models of renal fibrosis, differing in their way of damage induction. This indicates a more general mechanism that is not restricted to only one model and not due to the way of fibrosis induction.

The function of Dkk3 in these models was contradictory to that of Dkk1, another member of the Dkk family. Dkk1 was shown to dampen renal fibrosis by inhibition of pericyte activation and transition to myofibroblasts via blocking of Wnt dependent LRP-6 signaling (Ren, Johnson et al. 2013). This suggests a completely different mode of action and an opposed function of both molecules in kidney diseases.

By using the Dkk3 reporter mouse (Dkk3-LCh) we found that, while almost absent in adult healthy kidneys *per se*, Dkk3 expression is strongly up-regulated in tubular epithelial cells during the course of fibrosis. Accordingly, the induction of mRNA expression of all Dkks during UUO was reported (He, Dai et al. 2009). Thus, Dkk3 is expressed during kidney

development (as reported in Chapter 3.2) and can be induced in pathological situation. Similar mechanisms seem to apply during development and repair. Numerous other molecules in the kidney, like for example Midkine, a retinoic acid responsive gene coding for a heparin-binding growth factor, show a similar expression course. Midkine is expressed during nephrogenesis where it influences kidney development (Vilar, Lalou et al. 2002) and is induced in tubular epithelial cells in pathological situations like diabetic nephropathy where it promotes disease development (Kosugi, Yuzawa et al. 2006).

Tubular epithelial cells are known to actively participate in the development of renal fibrosis. Beside numerous other functions, they are known to influence immune responses in the kidney. They modulate innate and adaptive immunity by a variety of mechanisms including secretion of cytokines and chemokines upon stimulation through immune cells or danger signals (Nguan and Du 2009, Hato, El-Achkar et al. 2013). In contrast to the up-regulation of Dkk3 expression during EAE, Dkk3 induction in tubular epithelial cells was not IFNγ dependent. Thus, IFNγ is not the only potent inducer of Dkk3 expression. Signals responsible for Dkk3 induction upon stress in the kidney need to be identified in future. As already discussed in the context of EAE, it is not clear yet whether only Dkk3 induced in the kidney or high amounts in serum or both influence fibrosis development. This question could be answered by the use of a conditional Dkk3 knock out mouse, in which the Dkk3 gene is only disrupted in kidney.

So far, fibrosis outcome in the kidney was correlated with the strength of inflammation within the organ. Surprisingly, although Dkk3 deficiency led to a better fibrosis outcome we observed increased inflammation, indicated by higher cytokine levels and a strongly enhanced T cell infiltration in kidneys of these mice. This is, at least to our knowledge, one of the first examples combining stronger inflammation with improved outcome of renal fibrosis. However, not only increased T cell infiltration but also an altered phenotype of kidney infiltrating $CD4^+$ T cells was observed in Dkk3 deficient mice. Increased numbers of IFNγ and TNFα producing $CD4^+$ T cells, which are considered to be Th1 cells, and decreased levels of GATA3 positive, Th2 cells, were detected. In addition, proportions of $Foxp3^+$ $CD25^+$ $CD4^+$ T cells were increased. Thus, our data suggest a Th1/Treg repressing and Th2 promoting role of Dkk3.

A shift from a Th2 to a Th1/Treg CD4 T cell phenotype might explain the improved fibrosis outcome in *dkk3*$^{-/-}$ mice. It was shown that Th2 cells and their cytokines IL-4, IL-5 and IL-13 are pro-fibrotic (Braga, Correa-Costa et al. 2012, Liu, Kou et al. 2012) while Th1 cells and particularly IFNγ were shown to be anti-fibrotic (Oldroyd, Thomas et al. 1999).

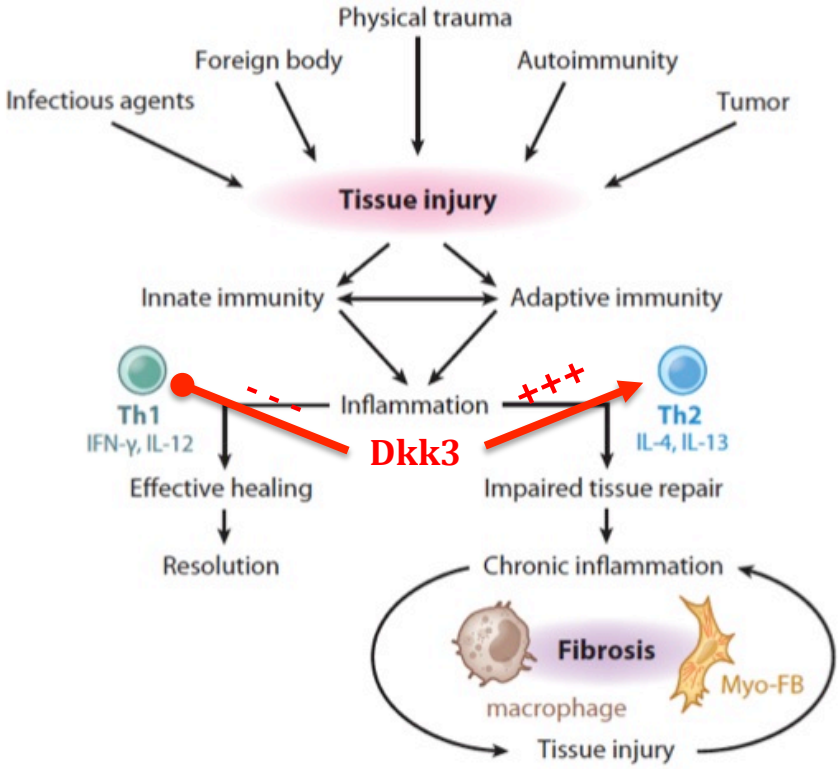

Figure 4.2 Dkk3 promotes development of kidney fibrosis by fvaoring Th2 and represing Th1/Treg responses. Dkk3 promotes the Th2 and represses the Th1 response in the development of kidney fibrosis. Modified sheme from (Wick, Grundtman et al. 2013).

Elevated lymphocyte numbers as well as the altered CD4 T cell phenotype in *dkk3*$^{-/-}$ fibrotic kidneys were almost absent in respective spleens, indicating rather a local then a systemic effect of Dkk3. Small differences in spleens were possibly due to lymphocyte re-circulation from kidneys.

Interestingly, T cell phenotype of Dkk3 deficient mice in kidney inflammation and EAE (Papatriantafyllou, 2008) are very similar. In both cases, stronger infiltration into the inflamed organ and increased numbers of IFNγ producing CD4^{+} T cells and Tregs were observed in *dkk3*$^{-/-}$ mice. In parallel, differences could only be detected locally but not systemically. These similar T cell phenotypes led to completely different results. While Dkk3

deficiency led to more severe damage and stronger symptoms in EAE, it was beneficial in the kidney and protected from fibrosis. This suggests a similar mode of action of Dkk3 under inflammatory conditions in the brain and kidney. As EAE is a Th1 mediated diseases and the development of kidney fibrosis is Th2 driven, these results further strengthen the hypothesis that Dkk3 promotes Th2- and represses Th1 differentiation.

T cell differentiation was shown to be influenced by Wnt target genes. Expression analysis of such genes in $CD4^+$ T cells isolated from *dkk3*$^{-/-}$ fibrotic kidneys revealed a slight decreased expression level, compared to wt controls. This reflects the T cell phenotype in Dkk3 deficient kidneys, as Wnt signaling, particularly TCF1 and Lef, was shown to be Th2 promoting (Yu, Sharma et al. 2009) but Th1 (Ma, Wang et al. 2012) and Treg repressing (van Loosdregt, Fleskens et al. 2013). These results raise the possibility that Dkk3 promotes Th2 differentiation and represses Th1/Treg differentiation by positively influencing Wnt signaling. This is in contrast to the majority of reports in literature, considering a suppressive function of Dkk3 on Wnt signaling (Caricasole, Ferraro et al. 2003, Hoang, Kubo et al. 2004), in parallel to its family member Dkk1 (Glinka, Wu et al. 1998). However, there are also studies postulating a Wnt promoting role for Dkk3 (Nakamura and Hackam 2010). Dkk3 seems to have a divergent function in Wnt signaling depending on the cell type it is acting on, like it is shown for its family member, Dkk2 (Mao and Niehrs 2003).

A recent study identified Dkk3 expression in the CD27 negative, IL-17 producing subset of γδ T cells. It was shown that in Dkk3 deficient mice, this subset has an increased capacity to produce IL-17 (Schmolka, Serre et al. 2013). As the downstream effector molecules of Wnt signalling, TCF1 and Lef, were reported to inhibit the differentiation of IL-17 producing cells γδ T cells (Malhotra, Narayan et al. 2013) the authors suggested a Wnt-promoting role for Dkk3 in γδ T cell differentiation (Schmolka, Serre et al. 2013). These results support the hypothesis that Dkk3 positively interferes with Wnt signaling in T cells and therefore modulates their linage commitment.

Considering that depletion of the Dkk3 considerably improves fibrosis outcome, one may regard Dkk3 as an interesting target for therapy. According to our data, Dkk3 depletion leads to a shift in T cell polarization and therefore to an improved fibrosis outcome. The strategy to redirect T cell differentiation was already tested in the context of autoimmune disease (Mouzaki, Deraos et al. 2005). Such an approach might constitute an alternative to standard therapy of chronic inflammatory diseases, which is usually accomplished by dampening the

whole immune system with steroids. Unfavorable side effects of such a steroid therapy would be omitted. For example increased susceptibility of infections caused by steroid application (Stanbury and Graham 1998) would not apply due to even increased levels of Th1 and cytotoxic T cells upon Dkk3 depletion.

However, one has to take in consideration that Dkk3 was reported to be a tumor suppressor (Hsieh, Hsieh et al. 2004) and its depletion was shown to increase autoimmunity in the mouse model of multiple sclerosis (Papatriantafyllou, 2008).

4.5 Dkk3 contributes to tissue mediated immune regulation

This study aimed to analyse the contribution of Dkk3 to tissue mediated immune regulation and provided the following results:

We observed up-regulation of Dkk3 expression in different types of inflammatory processes in different organs: Contact sensitizer induced inflammation in the skin, experimental autoimmune encephalomyelitis in the brain and fibrosis in the kidney. Thus, Dkk3 up-regulation in the course of inflammation seems to be a general mechanism operating in the crosstalk between immune and tissue cells.

In line with previous work of our lab (Papatriantafyllou, Moldenhauer et al. 2012) (Papatriantafyllou, 2008), we observed an immune modulatory capacity of Dkk3 in our experimental systems. In skin, it contributed to limit CD4 T cell reactivity. In brain, it was shown to be important for regulation of T cell activity in the recovery phase of EAE (Papatriantafyllou, 2008) and in kidney Dkk3 facilitated development of fibrosis by modulation of the T cell response. In these systems, Dkk3 deficient mice revealed a similar phenotype characterized by increased inflammation and T cell polarization towards a Th1 phenotype. Thus, it appears that Dkk3 is involved in limiting T cell reactivity and in modulating T cell differentiation. Dkk3 seems to favour Th2 responses, while at the same time repressing Th1/Treg development. Moreover, Dkk3 to acts rather locally at the site of inflammation than in a systemic manner. This suggests that the high levels of systemic Dkk3 may be not active in this regard.

Further experiments have to identify the functional part or form of Dkk3. In addition, it has to be clarified whether Dkk3 indeed interferes with Wnt signalling in T cells. The precise mechanism of such a Dkk3 - target cell interaction remains to be elucidated. Whether it binds to surface receptors or is taken up by target cells in order to act intracellularly is of importance in this regard. Moreover, it would be interesting to analyse the influence of other Dkk family members or Wnt pathway modulating molecules on inflammatory responses. This would not only give further insight into the role of Wnt signalling in inflammation but would also provide an opportunity to identify other tissue derived immune modulators. Finally, whether the observed Dkk3 function is a general mechanism that can be applied to inflammatory diseases in other organs has to be resolved in further investigations.

In conclusion, this study revealed that Dkk3 is a tissue-derived molecule that contributes to regulation of T cell responses.

5 References

Abarzua, F., Y. Kashiwakura, M. Takaoka, M. Watanabe, K. Ochiai, M. Sakaguchi, T. Iwawaki, R. Tanimoto, Y. Nasu, N. H. Huh and H. Kumon (2008). "An N-terminal 78 amino acid truncation of REIC/Dkk-3 effectively induces apoptosis." Biochem Biophys Res Commun **375**(4): 614-618.

Abarzua, F., M. Sakaguchi, M. Takaishi, Y. Nasu, K. Kurose, S. Ebara, M. Miyazaki, M. Namba, H. Kumon and N. H. Huh (2005). "Adenovirus-mediated overexpression of REIC/Dkk-3 selectively induces apoptosis in human prostate cancer cells through activation of c-Jun-NH2-kinase." Cancer Res **65**(21): 9617-9622.

Abarzua, F., M. Sakaguchi, R. Tanimoto, H. Sonegawa, D. W. Li, K. Edamura, T. Kobayashi, M. Watanabe, Y. Kashiwakura, H. Kaku, T. Saika, K. Nakamura, Y. Nasu, H. Kumon and N. H. Huh (2007). "Heat shock proteins play a crucial role in tumor-specific apoptosis by REIC/Dkk-3." Int J Mol Med **20**(1): 37-43.

Albanesi, C., A. Cavani and G. Girolomoni (1998). "Interferon-gamma-stimulated human keratinocytes express the genes necessary for the production of peptide-loaded MHC class II molecules." J Invest Dermatol **110**(2): 138-142.

Alferink, J., S. Aigner, R. Reibke, G. J. Hammerling and B. Arnold (1999). "Peripheral T-cell tolerance: the contribution of permissive T-cell migration into parenchymal tissues of the neonate." Immunol Rev **169**: 255-261.

Alferink, J., A. Tafuri, D. Vestweber, R. Hallmann, G. J. Hammerling and B. Arnold (1998). "Control of neonatal tolerance to tissue antigens by peripheral T cell trafficking." Science **282**(5392): 1338-1341.

Allakhverdi, Z., M. R. Comeau, H. K. Jessup, B. R. Yoon, A. Brewer, S. Chartier, N. Paquette, S. F. Ziegler, M. Sarfati and G. Delespesse (2007). "Thymic stromal lymphopoietin is released by human epithelial cells in response to microbes, trauma, or inflammation and potently activates mast cells." J Exp Med **204**(2): 253-258.

Anderson, G., J. J. Owen, N. C. Moore and E. J. Jenkinson (1994). "Thymic epithelial cells provide unique signals for positive selection of CD4+CD8+ thymocytes in vitro." J Exp Med **179**(6): 2027-2031.

Andl, T., S. T. Reddy, T. Gaddapara and S. E. Millar (2002). "WNT signals are required for the initiation of hair follicle development." Dev Cell **2**(5): 643-653.

Arai, T., K. Abe, H. Matsuoka, M. Yoshida, M. Mori, S. Goya, H. Kida, K. Nishino, T. Osaki, I. Tachibana, Y. Kaneda and S. Hayashi (2000). "Introduction of the interleukin-10 gene into mice inhibited bleomycin-induced lung injury in vivo." Am J Physiol Lung Cell Mol Physiol **278**(5): L914-922.

Aranami, T. and T. Yamamura (2008). "Th17 Cells and autoimmune encephalomyelitis (EAE/MS)." Allergol Int **57**(2): 115-120.

Aravind, L. and E. V. Koonin (1998). "A colipase fold in the carboxy-terminal domain of the Wnt antagonists--the Dickkopfs." Curr Biol **8**(14): R477-478.

Ardern-Jones, M. R., A. P. Black, E. A. Bateman and G. S. Ogg (2007). "Bacterial superantigen facilitates epithelial presentation of allergen to T helper 2 cells." Proc Natl Acad Sci U S A **104**(13): 5557-5562.

Arnold, B., G. Schonrich and G. J. Hammerling (1993). "Multiple levels of peripheral tolerance." Immunol Today **14**(1): 12-14.

Arrighi, J. F., C. Soulas, C. Hauser, S. Saeland, B. Chapuis, R. H. Zubler and V. Kindler (2003). "TNF-alpha induces the generation of Langerin/(CD207)+ immature Langerhans-type dendritic cells from both CD14-CD1a and CD14+CD1a- precursors derived from CD34+ cord blood cells." Eur J Immunol **33**(7): 2053-2063.

Bach, J. F. (2002). "The effect of infections on susceptibility to autoimmune and allergic diseases." N Engl J Med **347**(12): 911-920.

Baker, B. S., J. M. Ovigne, A. V. Powles, S. Corcoran and L. Fry (2003). "Normal keratinocytes express Toll-like receptors (TLRs) 1, 2 and 5: modulation of TLR expression in chronic plaque psoriasis." Br J Dermatol **148**(4): 670-679.

Bal, V., A. McIndoe, G. Denton, D. Hudson, G. Lombardi, J. Lamb and R. Lechler (1990). "Antigen presentation by keratinocytes induces tolerance in human T cells." Eur J Immunol **20**(9): 1893-1897.

Balabanov, R., K. Strand, R. Goswami, E. McMahon, W. Begolka, S. D. Miller and B. Popko (2007). "Interferon-gamma-oligodendrocyte interactions in the regulation of experimental autoimmune encephalomyelitis." J Neurosci **27**(8): 2013-2024.

Banerjee, G., A. Damodaran, N. Devi, K. Dharmalingam and G. Raman (2004). "Role of keratinocytes in antigen presentation and polarization of human T lymphocytes." Scand J Immunol **59**(4): 385-394.

Barcala Tabarrozzi, A. E., C. N. Castro, R. A. Dewey, M. C. Sogayar, L. Labriola and M. J. Perone (2013). "Cell-based interventions to halt autoimmunity in type 1 diabetes mellitus." Clin Exp Immunol **171**(2): 135-146.

Barnes, J. L. and Y. Gorin (2011). "Myofibroblast differentiation during fibrosis: role of NAD(P)H oxidases." Kidney Int **79**(9): 944-956.

Barrantes Idel, B., A. Montero-Pedrazuela, A. Guadano-Ferraz, M. J. Obregon, R. Martinez de Mena, V. Gailus-Durner, H. Fuchs, T. J. Franz, S. Kalaydjiev, M. Klempt, S. Holter, B. Rathkolb, C. Reinhard, G. Morreale de Escobar, J. Bernal, D. H. Busch, W. Wurst, E. Wolf, H. Schulz, S. Shtrom, E. Greiner, M. Hrabe de Angelis, H. Westphal and C. Niehrs (2006). "Generation and characterization of dickkopf3 mutant mice." Mol Cell Biol **26**(6): 2317-2326.

Basham, T. Y., B. J. Nickoloff, T. C. Merigan and V. B. Morhenn (1985). "Recombinant gamma interferon differentially regulates class II antigen expression and biosynthesis on cultured normal human keratinocytes." J Interferon Res **5**(1): 23-32.

Becher, B., B. G. Durell and R. J. Noelle (2002). "Experimental autoimmune encephalitis and inflammation in the absence of interleukin-12." J Clin Invest **110**(4): 493-497.

Bechtel, W., S. McGoohan, E. M. Zeisberg, G. A. Muller, H. Kalbacher, D. J. Salant, C. A. Muller, R. Kalluri and M. Zeisberg (2010). "Methylation determines fibroblast activation and fibrogenesis in the kidney." Nat Med **16**(5): 544-550.

Belkaid, Y. and S. Naik (2013). "Compartmentalized and systemic control of tissue immunity by commensals." Nat Immunol **14**(7): 646-653.

Bielesz, B., Y. Sirin, H. Si, T. Niranjan, A. Gruenwald, S. Ahn, H. Kato, J. Pullman, M. Gessler, V. H. Haase and K. Susztak (2010). "Epithelial Notch signaling regulates interstitial fibrosis development in the kidneys of mice and humans." J Clin Invest **120**(11): 4040-4054.

Black, A. P., M. R. Ardern-Jones, V. Kasprowicz, P. Bowness, L. Jones, A. S. Bailey and G. S. Ogg (2007). "Human keratinocyte induction of rapid effector function in antigen-specific memory CD4+ and CD8+ T cells." Eur J Immunol **37**(6): 1485-1493.

Boehm, U., T. Klamp, M. Groot and J. C. Howard (1997). "Cellular responses to interferon-gamma." Annu Rev Immunol **15**: 749-795.

Boitard, C. (2012). "Pancreatic islet autoimmunity." Presse Med **41**(12 P 2): e636-650.

Boor, P. and J. Floege (2011). "Chronic kidney disease growth factors in renal fibrosis." Clin Exp Pharmacol Physiol **38**(7): 441-450.

Boor, P., T. Ostendorf and J. Floege (2010). "Renal fibrosis: novel insights into mechanisms and therapeutic targets." Nat Rev Nephrol **6**(11): 643-656.

Bottcher, J. P., P. A. Knolle and D. Stabenow (2011). "Mechanisms balancing tolerance and immunity in the liver." Dig Dis **29**(4): 384-390.

Bottinger, E. P. (2007). "TGF-beta in renal injury and disease." Semin Nephrol **27**(3): 309-320.

Braga, T. T., M. Correa-Costa, Y. F. Guise, A. Castoldi, C. D. de Oliveira, M. I. Hyane, M. A. Cenedeze, S. A. Teixeira, M. N. Muscara, K. R. Perez, I. M. Cuccovia, A. Pacheco-Silva, G. M. Goncalves and N. O. Camara (2012). "MyD88 signaling pathway is involved in renal fibrosis by favoring a TH2 immune response and activating alternative M2 macrophages." Mol Med **18**: 1231-1239.

Brocker, T., M. Riedinger and K. Karjalainen (1997). "Targeted expression of major histocompatibility complex (MHC) class II molecules demonstrates that dendritic cells can induce negative but not positive selection of thymocytes in vivo." J Exp Med **185**(3): 541-550.

Burke, S. J., B. L. Updegraff, R. M. Bellich, M. R. Goff, D. Lu, S. C. Minkin, Jr., M. D. Karlstad and J. J. Collier (2013). "Regulation of iNOS gene transcription by IL-1beta and IFN-gamma requires a coactivator exchange mechanism." Mol Endocrinol **27**(10): 1724-1742.

Burkly, L. C., S. Degermann, J. Longley, J. Hagman, R. L. Brinster, D. Lo and R. A. Flavell (1993). "Clonal deletion of V beta 5+ T cells by transgenic I-E restricted to thymic medullary epithelium." J Immunol **151**(8): 3954-3960.

Bynoe, M. S., J. T. Evans, C. Viret and C. A. Janeway, Jr. (2003). "Epicutaneous immunization with autoantigenic peptides induces T suppressor cells that prevent experimental allergic encephalomyelitis." Immunity **19**(3): 317-328.

Byun, T., M. Karimi, J. L. Marsh, T. Milovanovic, F. Lin and R. F. Holcombe (2005). "Expression of secreted Wnt antagonists in gastrointestinal tissues: potential role in stem cell homeostasis." J Clin Pathol **58**(5): 515-519.

Cadigan, K. M. and Y. I. Liu (2006). "Wnt signaling: complexity at the surface." J Cell Sci **119**(Pt 3): 395-402.

Caricasole, A., T. Ferraro, L. Iacovelli, E. Barletta, A. Caruso, D. Melchiorri, G. C. Terstappen and F. Nicoletti (2003). "Functional characterization of WNT7A signaling in PC12 cells: interaction with A FZD5 x LRP6 receptor complex and modulation by Dickkopf proteins." J Biol Chem **278**(39): 37024-37031.

Carroll, T. J., J. S. Park, S. Hayashi, A. Majumdar and A. P. McMahon (2005). "Wnt9b plays a central role in the regulation of mesenchymal to epithelial transitions underlying organogenesis of the mammalian urogenital system." Dev Cell **9**(2): 283-292.

Cauza, K., A. Grassauer, G. Hinterhuber, R. Horvat, K. Rappersberger, K. Wolff and D. Foedinger (2002). "FcgammaRIII expression on cultured human keratinocytes and upregulation by interferon-gamma." J Invest Dermatol **119**(5): 1074-1079.

Chappert, P. and R. H. Schwartz (2010). "Induction of T cell anergy: integration of environmental cues and infectious tolerance." Curr Opin Immunol **22**(5): 552-559.

Cheever, A. W., M. E. Williams, T. A. Wynn, F. D. Finkelman, R. A. Seder, T. M. Cox, S. Hieny, P. Caspar and A. Sher (1994). "Anti-IL-4 treatment of Schistosoma mansoni-infected mice inhibits development of T cells and non-B, non-T cells expressing Th2 cytokines while decreasing egg-induced hepatic fibrosis." J Immunol **153**(2): 753-759.

Chen, J., M. Watanabe, P. Huang, M. Sakaguchi, K. Ochiai, Y. Nasu, M. Ouchida, N. H. Huh, K. Shimizu, Y. Kashiwakura, H. Kaku and H. Kumon (2009). "REIC/Dkk-3 stable transfection reduces the malignant phenotype of mouse prostate cancer RM9 cells." Int J Mol Med **24**(6): 789-794.

Cheng, S. and D. H. Lovett (2003). "Gelatinase A (MMP-2) is necessary and sufficient for renal tubular cell epithelial-mesenchymal transformation." Am J Pathol **162**(6): 1937-1949.

Chung, A. C. and H. Y. Lan (2011). "Chemokines in renal injury." J Am Soc Nephrol **22**(5): 802-809.

Clevers, H. (2006). "Wnt/beta-catenin signaling in development and disease." Cell **127**(3): 469-480.

Cochrane, A. L., M. M. Kett, C. S. Samuel, N. V. Campanale, W. P. Anderson, D. A. Hume, M. H. Little, J. F. Bertram and S. D. Ricardo (2005). "Renal structural and functional repair in a mouse model of reversal of ureteral obstruction." J Am Soc Nephrol **16**(12): 3623-3630.

Codarri, L., G. Gyulveszi, V. Tosevski, L. Hesske, A. Fontana, L. Magnenat, T. Suter and B. Becher (2011). "RORgammat drives production of the cytokine GM-CSF in helper T cells, which is essential for the effector phase of autoimmune neuroinflammation." Nat Immunol **12**(6): 560-567.

Corbel, M., E. Boichot and V. Lagente (2000). "Role of gelatinases MMP-2 and MMP-9 in tissue remodeling following acute lung injury." Braz J Med Biol Res **33**(7): 749-754.

Coresh, J., E. Selvin, L. A. Stevens, J. Manzi, J. W. Kusek, P. Eggers, F. Van Lente and A. S. Levey (2007). "Prevalence of chronic kidney disease in the United States." JAMA **298**(17): 2038-2047.

Corrigan, C. J., W. Wang, Q. Meng, C. Fang, G. Eid, M. R. Caballero, Z. Lv, Y. An, Y. H. Wang, Y. J. Liu, A. B. Kay, T. H. Lee and S. Ying (2011). "Allergen-induced expression of IL-25 and IL-25 receptor in atopic asthmatic airways and late-phase cutaneous responses." J Allergy Clin Immunol **128**(1): 116-124.

Crotty, S. (2011). "Follicular helper CD4 T cells (TFH)." Annu Rev Immunol **29**: 621-663.

Csato, M., B. Bozoky, J. Hunyadi and A. Dobozy (1986). "Candida albicans phagocytosis by separated human epidermal cells." Arch Dermatol Res **279**(2): 136-139.

Cumberbatch, M., R. J. Dearman, C. Antonopoulos, R. W. Groves and I. Kimber (2001). "Interleukin (IL)-18 induces Langerhans cell migration by a tumour necrosis factor-alpha- and IL-1beta-dependent mechanism." Immunology **102**(3): 323-330.

Cumberbatch, M. and I. Kimber (1995). "Tumour necrosis factor-alpha is required for accumulation of dendritic cells in draining lymph nodes and for optimal contact sensitization." Immunology **84**(1): 31-35.

Cusick, M. F., J. E. Libbey and R. S. Fujinami (2012). "Molecular mimicry as a mechanism of autoimmune disease." Clin Rev Allergy Immunol **42**(1): 102-111.

Das, D. S., N. Wadhwa, N. Kunj, K. Sarda, B. S. Pradhan and S. S. Majumdar (2013). "Dickkopf homolog 3 (DKK3) plays a crucial role upstream of WNT/beta-CATENIN signaling for Sertoli cell mediated regulation of spermatogenesis." PLoS One **8**(5): e63603.

De Ferrari, G. V. and R. T. Moon (2006). "The ups and downs of Wnt signaling in prevalent neurological disorders." Oncogene **25**(57): 7545-7553.

de Koning, H. D., D. Rodijk-Olthuis, I. M. van Vlijmen-Willems, L. A. Joosten, M. G. Netea, J. Schalkwijk and P. L. Zeeuwen (2010). "A comprehensive analysis of pattern recognition receptors in normal and inflamed human epidermis: upregulation of dectin-1 in psoriasis." J Invest Dermatol **130**(11): 2611-2620.

Delves, P. J. and I. M. Roitt (2000). "The immune system. First of two parts." N Engl J Med **343**(1): 37-49.

Derbinski, J., A. Schulte, B. Kyewski and L. Klein (2001). "Promiscuous gene expression in medullary thymic epithelial cells mirrors the peripheral self." Nat Immunol **2**(11): 1032-1039.

Doucet, C., D. Brouty-Boye, C. Pottin-Clemenceau, G. W. Canonica, C. Jasmin and B. Azzarone (1998). "Interleukin (IL) 4 and IL-13 act on human lung fibroblasts. Implication in asthma." J Clin Invest **101**(10): 2129-2139.

Duffield, J. S. (2003). "The inflammatory macrophage: a story of Jekyll and Hyde." Clin Sci (Lond) **104**(1): 27-38.

Duffield, J. S. (2010). "Macrophages and immunologic inflammation of the kidney." Semin Nephrol **30**(3): 234-254.

Duffield, J. S., C. F. Ware, B. Ryffel and J. Savill (2001). "Suppression by apoptotic cells defines tumor necrosis factor-mediated induction of glomerular mesangial cell apoptosis by activated macrophages." Am J Pathol **159**(4): 1397-1404.

Ebner, S., V. A. Nguyen, M. Forstner, Y. H. Wang, D. Wolfram, Y. J. Liu and N. Romani (2007). "Thymic stromal lymphopoietin converts human epidermal Langerhans cells into antigen-presenting cells that induce proallergic T cells." J Allergy Clin Immunol **119**(4): 982-990.

Eddy, A. A. (1995). "Interstitial macrophages as mediators of renal fibrosis." Exp Nephrol **3**(2): 76-79.

Etheridge, S. L., G. J. Spencer, D. J. Heath and P. G. Genever (2004). "Expression profiling and functional analysis of wnt signaling mechanisms in mesenchymal stem cells." Stem Cells **22**(5): 849-860.

Fadok, V. A., D. L. Bratton, A. Konowal, P. W. Freed, J. Y. Westcott and P. M. Henson (1998). "Macrophages that have ingested apoptotic cells in vitro inhibit proinflammatory cytokine production through autocrine/paracrine mechanisms involving TGF-beta, PGE2, and PAF." J Clin Invest **101**(4): 890-898.

Fan, L., B. W. Busser, T. Q. Lifsted, M. Oukka, D. Lo and T. M. Laufer (2003). "Antigen presentation by keratinocytes directs autoimmune skin disease." Proc Natl Acad Sci U S A **100**(6): 3386-3391.

Fedders, H., R. Augustin and T. C. Bosch (2004). "A Dickkopf- 3-related gene is expressed in differentiating nematocytes in the basal metazoan Hydra." Dev Genes Evol **214**(2): 72-80.

Ferber, I. A., S. Brocke, C. Taylor-Edwards, W. Ridgway, C. Dinisco, L. Steinman, D. Dalton and C. G. Fathman (1996). "Mice with a disrupted IFN-gamma gene are susceptible to the induction of experimental autoimmune encephalomyelitis (EAE)." J Immunol **156**(1): 5-7.

Fertin, C., J. F. Nicolas, P. Gillery, B. Kalis, J. Banchereau and F. X. Maquart (1991). "Interleukin-4 stimulates collagen synthesis by normal and scleroderma fibroblasts in dermal equivalents." Cell Mol Biol **37**(8): 823-829.

Fine, L. G. and J. T. Norman (2008). "Chronic hypoxia as a mechanism of progression of chronic kidney diseases: from hypothesis to novel therapeutics." Kidney Int **74**(7): 867-872.

Flacher, V., M. Bouschbacher, E. Verronese, C. Massacrier, V. Sisirak, O. Berthier-Vergnes, B. de Saint-Vis, C. Caux, C. Dezutter-Dambuyant, S. Lebecque and J. Valladeau (2006). "Human Langerhans cells express a specific TLR profile and differentially respond to viruses and Gram-positive bacteria." J Immunol **177**(11): 7959-7967.

Fort, M. M., J. Cheung, D. Yen, J. Li, S. M. Zurawski, S. Lo, S. Menon, T. Clifford, B. Hunte, R. Lesley, T. Muchamuel, S. D. Hurst, G. Zurawski, M. W. Leach, D. M. Gorman and D. M. Rennick (2001). "IL-25 induces IL-4, IL-5, and IL-13 and Th2-associated pathologies in vivo." Immunity **15**(6): 985-995.

Furlan, R., C. Cuomo and G. Martino (2009). "Animal models of multiple sclerosis." Methods Mol Biol **549**: 157-173.

Gaspari, A. A., M. K. Jenkins and S. I. Katz (1988). "Class II MHC-bearing keratinocytes induce antigen-specific unresponsiveness in hapten-specific Th1 clones." J Immunol **141**(7): 2216-2220.

Gaspari, A. A. and S. I. Katz (1988). "Induction and functional characterization of class II MHC (Ia) antigens on murine keratinocytes." J Immunol **140**(9): 2956-2963.

Germain, R. N. (2002). "T-cell development and the CD4-CD8 lineage decision." Nat Rev Immunol **2**(5): 309-322.

Glinka, A., W. Wu, H. Delius, A. P. Monaghan, C. Blumenstock and C. Niehrs (1998). "Dickkopf-1 is a member of a new family of secreted proteins and functions in head induction." Nature **391**(6665): 357-362.

Goerdt, S. and C. E. Orfanos (1999). "Other functions, other genes: alternative activation of antigen-presenting cells." Immunity **10**(2): 137-142.

Gold, R., C. Linington and H. Lassmann (2006). "Understanding pathogenesis and therapy of multiple sclerosis via animal models: 70 years of merits and culprits in experimental autoimmune encephalomyelitis research." Brain **129**(Pt 8): 1953-1971.

Goodman, R. E., F. Nestle, Y. M. Naidu, J. M. Green, C. B. Thompson, B. J. Nickoloff and L. A. Turka (1994). "Keratinocyte-derived T cell costimulation induces preferential production of IL-2 and IL-4 but not IFN-gamma." J Immunol **152**(11): 5189-5198.

Grande, M. T. and J. M. Lopez-Novoa (2009). "Fibroblast activation and myofibroblast generation in obstructive nephropathy." Nat Rev Nephrol **5**(6): 319-328.

Grice, E. A., H. H. Kong, S. Conlan, C. B. Deming, J. Davis, A. C. Young, G. G. Bouffard, R. W. Blakesley, P. R. Murray, E. D. Green, M. L. Turner and J. A. Segre (2009). "Topographical and temporal diversity of the human skin microbiome." Science **324**(5931): 1190-1192.

Guder, C., S. Pinho, T. G. Nacak, H. A. Schmidt, B. Hobmayer, C. Niehrs and T. W. Holstein (2006). "An ancient Wnt-Dickkopf antagonism in Hydra." Development **133**(5): 901-911.

Gunaratnam, L. and J. V. Bonventre (2009). "HIF in kidney disease and development." J Am Soc Nephrol **20**(9): 1877-1887.

Gurujeyalakshmi, G. and S. N. Giri (1995). "Molecular mechanisms of antifibrotic effect of interferon gamma in bleomycin-mouse model of lung fibrosis: downregulation of TGF-beta and procollagen I and III gene expression." Exp Lung Res **21**(5): 791-808.

Gutowska-Owsiak, D. and G. S. Ogg (2012). "The epidermis as an adjuvant." J Invest Dermatol **132**(3 Pt 2): 940-948.

Haak, S., G. Gyulveszi and B. Becher (2009). "Th17 cells in autoimmune disease: changing the verdict." Immunotherapy **1**(2): 199-203.

Haque, R., F. Lei, X. Xiong, Y. Bian, B. Zhao, Y. Wu and J. Song (2012). "Programming of regulatory T cells from pluripotent stem cells and prevention of autoimmunity." J Immunol **189**(3): 1228-1236.

Harder, J. and G. Nunez (2009). "Functional expression of the intracellular pattern recognition receptor NOD1 in human keratinocytes." J Invest Dermatol **129**(5): 1299-1302.

Hartung, H. P. and P. Rieckmann (1997). "Pathogenesis of immune-mediated demyelination in the CNS." J Neural Transm Suppl **50**: 173-181.

Hassan, G. A., H. A. Sliem, A. T. Ellethy and S. Salama Mel (2012). "Role of immune system modulation in prevention of type 1 diabetes mellitus." Indian J Endocrinol Metab **16**(6): 904-909.

Hato, T., T. M. El-Achkar and P. C. Dagher (2013). "Sisters in arms: myeloid and tubular epithelial cells shape renal innate immunity." Am J Physiol Renal Physiol **304**(10): F1243-1251.

He, W., C. Dai, Y. Li, G. Zeng, S. P. Monga and Y. Liu (2009). "Wnt/beta-catenin signaling promotes renal interstitial fibrosis." J Am Soc Nephrol **20**(4): 765-776.

He, W., Y. S. Kang, C. Dai and Y. Liu (2011). "Blockade of Wnt/beta-catenin signaling by paricalcitol ameliorates proteinuria and kidney injury." J Am Soc Nephrol **22**(1): 90-103.

Hermann, M., D. Pirkebner, A. Draxl, P. Berger, G. Untergasser, R. Margreiter and P. Hengster (2007). "Dickkopf-3 is expressed in a subset of adult human pancreatic beta cells." Histochem Cell Biol **127**(5): 513-521.

Herzog, E. L. and R. Bucala (2010). "Fibrocytes in health and disease." Exp Hematol **38**(7): 548-556.

Hewitson, T. D. (2009). "Renal tubulointerstitial fibrosis: common but never simple." Am J Physiol Renal Physiol **296**(6): F1239-1244.

Higgins, D. F., K. Kimura, W. M. Bernhardt, N. Shrimanker, Y. Akai, B. Hohenstein, Y. Saito, R. S. Johnson, M. Kretzler, C. D. Cohen, K. U. Eckardt, M. Iwano and V. H. Haase (2007). "Hypoxia promotes fibrogenesis in vivo via HIF-1 stimulation of epithelial-to-mesenchymal transition." J Clin Invest **117**(12): 3810-3820.

Hoang, B. H., T. Kubo, J. H. Healey, R. Yang, S. S. Nathan, E. A. Kolb, B. Mazza, P. A. Meyers and R. Gorlick (2004). "Dickkopf 3 inhibits invasion and motility of Saos-2 osteosarcoma cells by modulating the Wnt-beta-catenin pathway." Cancer Res **64**(8): 2734-2739.

Hoffmann, R., E. Wenzel, A. Huth, P. van der Steen, M. Schaufele, H. P. Henninger and R. Happle (1994). "Cytokine mRNA levels in Alopecia areata before and after treatment with the contact allergen diphenylcyclopropenone." J Invest Dermatol **103**(4): 530-533.

Hou, S. and P. C. Doherty (1993). "Partitioning of responder CD8+ T cells in lymph node and lung of mice with Sendai virus pneumonia by LECAM-1 and CD45RB phenotype." J Immunol **150**(12): 5494-5500.

Hsieh, S. Y., P. S. Hsieh, C. T. Chiu and W. Y. Chen (2004). "Dickkopf-3/REIC functions as a suppressor gene of tumor growth." Oncogene **23**(57): 9183-9189.

Huesmann, M., B. Scott, P. Kisielow and H. von Boehmer (1991). "Kinetics and efficacy of positive selection in the thymus of normal and T cell receptor transgenic mice." Cell **66**(3): 533-540.

Humphreys, B. D., S. L. Lin, A. Kobayashi, T. E. Hudson, B. T. Nowlin, J. V. Bonventre, M. T. Valerius, A. P. McMahon and J. S. Duffield (2010). "Fate tracing reveals the pericyte and not epithelial origin of myofibroblasts in kidney fibrosis." Am J Pathol **176**(1): 85-97.

Iglesias, D. M., P. A. Hueber, L. Chu, R. Campbell, A. M. Patenaude, A. J. Dziarmaga, J. Quinlan, O. Mohamed, D. Dufort and P. R. Goodyer (2007). "Canonical WNT signaling during kidney development." Am J Physiol Renal Physiol **293**(2): F494-500.

Iredale, J. P., R. C. Benyon, J. Pickering, M. McCullen, M. Northrop, S. Pawley, C. Hovell and M. J. Arthur (1998). "Mechanisms of spontaneous resolution of rat liver fibrosis. Hepatic stellate cell apoptosis and reduced hepatic expression of metalloproteinase inhibitors." J Clin Invest **102**(3): 538-549.

Ito, T., Y. H. Wang, O. Duramad, T. Hori, G. J. Delespesse, N. Watanabe, F. X. Qin, Z. Yao, W. Cao and Y. J. Liu (2005). "TSLP-activated dendritic cells induce an inflammatory T helper type 2 cell response through OX40 ligand." J Exp Med **202**(9): 1213-1223.

Jamora, C. and E. Fuchs (2002). "Intercellular adhesion, signalling and the cytoskeleton." Nat Cell Biol **4**(4): E101-108.

Jiang, M., K. Liu, J. Luo and Z. Dong (2010). "Autophagy is a renoprotective mechanism during in vitro hypoxia and in vivo ischemia-reperfusion injury." Am J Pathol **176**(3): 1181-1192.

Jiang, T., L. Huang, S. Wang and S. Zhang (2010). "Clinical significance of serum Dkk-3 in patients with gynecological cancer." J Obstet Gynaecol Res **36**(4): 769-773.

Juhler, M., D. I. Barry, H. Offner, G. Konat, L. Klinken and O. B. Paulson (1984). "Blood-brain and blood-spinal cord barrier permeability during the course of experimental allergic encephalomyelitis in the rat." Brain Res **302**(2): 347-355.

Jung, I. L., H. J. Kang, K. C. Kim and I. G. Kim (2010). "Knockdown of the Dickkopf 3 gene induces apoptosis in a lung adenocarcinoma." Int J Mol Med **26**(1): 33-38.

Kaech, S. M., S. Hemby, E. Kersh and R. Ahmed (2002). "Molecular and functional profiling of memory CD8 T cell differentiation." Cell **111**(6): 837-851.

Kaissling, B. and M. Le Hir (2008). "The renal cortical interstitium: morphological and functional aspects." Histochem Cell Biol **130**(2): 247-262.

Kalali, B. N., G. Kollisch, J. Mages, T. Muller, S. Bauer, H. Wagner, J. Ring, R. Lang, M. Mempel and M. Ollert (2008). "Double-stranded RNA induces an antiviral defense status in epidermal keratinocytes through TLR3-, PKR-, and MDA5/RIG-I-mediated differential signaling." J Immunol **181**(4): 2694-2704.

Kalluri, R. and R. A. Weinberg (2009). "The basics of epithelial-mesenchymal transition." J Clin Invest **119**(6): 1420-1428.

Kappler, J. W., N. Roehm and P. Marrack (1987). "T cell tolerance by clonal elimination in the thymus." Cell **49**(2): 273-280.

Katase, N., M. Gunduz, L. Beder, E. Gunduz, M. Lefeuvre, O. F. Hatipoglu, S. S. Borkosky, R. Tamamura, S. Tominaga, N. Yamanaka, K. Shimizu, N. Nagai and H. Nagatsuka (2008). "Deletion at Dickkopf (dkk)-3 locus (11p15.2) is related with lower lymph node metastasis and better prognosis in head and neck squamous cell carcinomas." Oncol Res **17**(6): 273-282.

Kawakami, Y., S. Eliyahu, C. H. Delgado, P. F. Robbins, L. Rivoltini, S. L. Topalian, T. Miki and S. A. Rosenberg (1994). "Cloning of the gene coding for a shared human melanoma antigen recognized by autologous T cells infiltrating into tumor." Proc Natl Acad Sci U S A **91**(9): 3515-3519.

Kawano, Y. and R. Kypta (2003). "Secreted antagonists of the Wnt signalling pathway." J Cell Sci **116**(Pt 13): 2627-2634.

Kawasaki, K., M. Watanabe, M. Sakaguchi, Y. Ogasawara, K. Ochiai, Y. Nasu, H. Doihara, Y. Kashiwakura, N. H. Huh, H. Kumon and H. Date (2009). "REIC/Dkk-3 overexpression downregulates P-glycoprotein in multidrug-resistant MCF7/ADR cells and induces apoptosis in breast cancer." Cancer Gene Ther **16**(1): 65-72.

Kelly, K. J., J. L. Burford and J. H. Dominguez (2009). "Postischemic inflammatory syndrome: a critical mechanism of progression in diabetic nephropathy." Am J Physiol Renal Physiol **297**(4): F923-931.

Kimpton, W. G., E. A. Washington and R. N. Cahill (1995). "Virgin alpha beta and gamma delta T cells recirculate extensively through peripheral tissues and skin during normal development of the fetal immune system." Int Immunol **7**(10): 1567-1577.

Kinoshita, H., T. Takai, T. A. Le, S. Kamijo, X. L. Wang, H. Ushio, M. Hara, J. Kawasaki, A. T. Vu, T. Ogawa, H. Gunawan, S. Ikeda, K. Okumura and H. Ogawa (2009). "Cytokine milieu modulates release of thymic stromal lymphopoietin from human keratinocytes stimulated with double-stranded RNA." J Allergy Clin Immunol **123**(1): 179-186.

Kipari, T. and J. Hughes (2002). "Macrophage-mediated renal cell death." Kidney Int **61**(2): 760-761.

Kisich, K. O., M. D. Howell, M. Boguniewicz, H. R. Heizer, N. U. Watson and D. Y. Leung (2007). "The constitutive capacity of human keratinocytes to kill Staphylococcus aureus is dependent on beta-defensin 3." J Invest Dermatol **127**(10): 2368-2380.

Kisielow, P. (1988). "The true function of the thymus." Immunol Today **9**(12): 367.

Kivisakk, P., J. Imitola, S. Rasmussen, W. Elyaman, B. Zhu, R. M. Ransohoff and S. J. Khoury (2009). "Localizing central nervous system immune surveillance: meningeal antigen-presenting cells activate T cells during experimental autoimmune encephalomyelitis." Ann Neurol **65**(4): 457-469.

Kobayashi, M., R. Yoshiki, J. Sakabe, K. Kabashima, M. Nakamura and Y. Tokura (2009). "Expression of toll-like receptor 2, NOD2 and dectin-1 and stimulatory effects of their ligands and histamine in normal human keratinocytes." Br J Dermatol **160**(2): 297-304.

Kock, A., T. Schwarz, R. Kirnbauer, A. Urbanski, P. Perry, J. C. Ansel and T. A. Luger (1990). "Human keratinocytes are a source for tumor necrosis factor alpha: evidence for synthesis and release upon stimulation with endotoxin or ultraviolet light." J Exp Med **172**(6): 1609-1614.

Koesters, R., B. Kaissling, M. Lehir, N. Picard, F. Theilig, R. Gebhardt, A. B. Glick, B. Hahnel, H. Hosser, H. J. Grone and W. Kriz (2010). "Tubular overexpression of transforming growth factor-beta1 induces autophagy and fibrosis but not mesenchymal transition of renal epithelial cells." Am J Pathol **177**(2): 632-643.

Kohka, H., T. Yoshino, H. Iwagaki, I. Sakuma, T. Tanimoto, Y. Matsuo, M. Kurimoto, K. Orita, T. Akagi and N. Tanaka (1998). "Interleukin-18/interferon-gamma-inducing factor, a novel cytokine, up-regulates ICAM-1 (CD54) expression in KG-1 cells." J Leukoc Biol **64**(4): 519-527.

Kohn, A. D. and R. T. Moon (2005). "Wnt and calcium signaling: beta-catenin-independent pathways." Cell Calcium **38**(3-4): 439-446.

Kohn, M. J., K. J. Kaneko and M. L. DePamphilis (2005). "DkkL1 (Soggy), a Dickkopf family member, localizes to the acrosome during mammalian spermatogenesis." Mol Reprod Dev **71**(4): 516-522.

Kollisch, G., B. N. Kalali, V. Voelcker, R. Wallich, H. Behrendt, J. Ring, S. Bauer, T. Jakob, M. Mempel and M. Ollert (2005). "Various members of the Toll-like receptor family contribute to the innate immune response of human epidermal keratinocytes." Immunology **114**(4): 531-541.

Kolodsick, J. E., G. B. Toews, C. Jakubzick, C. Hogaboam, T. A. Moore, A. McKenzie, C. A. Wilke, C. J. Chrisman and B. B. Moore (2004). "Protection from fluorescein isothiocyanate-induced fibrosis in IL-13-deficient, but not IL-4-deficient, mice results from impaired collagen synthesis by fibroblasts." J Immunol **172**(7): 4068-4076.

Komatsu, N. and H. Takayanagi (2012). "Autoimmune arthritis: the interface between the immune system and joints." Adv Immunol **115**: 45-71.

Koppen, A., R. Ait-Aissa, J. Koster, I. Ora, J. Bras, P. G. van Sluis, H. Caron, R. Versteeg and L. J. Valentijn (2008). "Dickkopf-3 expression is a marker for neuroblastic tumor maturation and is down-regulated by MYCN." Int J Cancer **122**(7): 1455-1464.

Kormos, B., N. Belso, A. Bebes, G. Szabad, S. Bacsa, M. Szell, L. Kemeny and Z. Bata-Csorgo (2011). "In vitro dedifferentiation of melanocytes from adult epidermis." PLoS One **6**(2): e17197.

Kosugi, T., Y. Yuzawa, W. Sato, H. Kawai, S. Matsuo, Y. Takei, T. Muramatsu and K. Kadomatsu (2006). "Growth factor midkine is involved in the pathogenesis of diabetic nephropathy." Am J Pathol **168**(1): 9-19.

Krupnik, V. E., J. D. Sharp, C. Jiang, K. Robison, T. W. Chickering, L. Amaravadi, D. E. Brown, D. Guyot, G. Mays, K. Leiby, B. Chang, T. Duong, A. D. Goodearl, D. P. Gearing, S. Y. Sokol and S. A. McCarthy (1999). "Functional and structural diversity of the human Dickkopf gene family." Gene **238**(2): 301-313.

Kumar, R. K., C. Herbert, M. Yang, A. M. Koskinen, A. N. McKenzie and P. S. Foster (2002). "Role of interleukin-13 in eosinophil accumulation and airway remodelling in a mouse model of chronic asthma." Clin Exp Allergy **32**(7): 1104-1111.

Kuroiwa, T., R. Schlimgen, G. G. Illei, I. B. McInnes and D. T. Boumpas (2000). "Distinct T cell/renal tubular epithelial cell interactions define differential chemokine production: implications for tubulointerstitial injury in chronic glomerulonephritides." J Immunol **164**(6): 3323-3329.

Kurschus, F. C., T. Oelert, B. Liliensiek, P. Buchmann, D. C. Wraith, G. J. Hammerling and B. Arnold (2006). "Experimental autoimmune encephalomyelitis in mice expressing the autoantigen MBP 1-10 covalently bound to the MHC class II molecule I-Au." Int Immunol **18**(1): 151-162.

Kurts, C., U. Panzer, H. J. Anders and A. J. Rees (2013). "The immune system and kidney disease: basic concepts and clinical implications." Nat Rev Immunol **13**(10): 738-753.

Lam, A. P. and C. J. Gottardi (2011). "beta-catenin signaling: a novel mediator of fibrosis and potential therapeutic target." Curr Opin Rheumatol **23**(6): 562-567.

Lange-Sperandio, B., F. Cachat, B. A. Thornhill and R. L. Chevalier (2002). "Selectins mediate macrophage infiltration in obstructive nephropathy in newborn mice." Kidney Int **61**(2): 516-524.

Lara-Corrales, I. and E. Pope (2010). "Autoimmune blistering diseases in children." Semin Cutan Med Surg **29**(2): 85-91.

Le Moine, A., V. Flamand, F. X. Demoor, J. C. Noel, M. Surquin, R. Kiss, M. A. Nahori, M. Pretolani, M. Goldman and D. Abramowicz (1999). "Critical roles for IL-4, IL-5, and eosinophils in chronic skin allograft rejection." J Clin Invest **103**(12): 1659-1667.

Lebre, M. C., J. C. Antons, P. Kalinski, J. H. Schuitemaker, T. M. van Capel, M. L. Kapsenberg and E. C. De Jong (2003). "Double-stranded RNA-exposed human keratinocytes promote Th1 responses by inducing a Type-1 polarized phenotype in dendritic cells: role of keratinocyte-derived tumor necrosis factor alpha, type I interferons, and interleukin-18." J Invest Dermatol **120**(6): 990-997.

Lebre, M. C., A. M. van der Aar, L. van Baarsen, T. M. van Capel, J. H. Schuitemaker, M. L. Kapsenberg and E. C. de Jong (2007). "Human keratinocytes express functional Toll-like receptor 3, 4, 5, and 9." J Invest Dermatol **127**(2): 331-341.

Lee, E. J., M. Jo, S. B. Rho, K. Park, Y. N. Yoo, J. Park, M. Chae, W. Zhang and J. H. Lee (2009). "Dkk3, downregulated in cervical cancer, functions as a negative regulator of beta-catenin." Int J Cancer **124**(2): 287-297.

Lee, H. M., D. M. Shin, D. K. Choi, Z. W. Lee, K. H. Kim, J. M. Yuk, C. D. Kim, J. H. Lee and E. K. Jo (2009). "Innate immune responses to Mycobacterium ulcerans via toll-like receptors and dectin-1 in human keratinocytes." Cell Microbiol **11**(4): 678-692.

Lee, R. H., N. Yoon, J. C. Reneau and D. J. Prockop (2012). "Preactivation of human MSCs with TNF-alpha enhances tumor-suppressive activity." Cell Stem Cell **11**(6): 825-835.

Li, Q., K. Shen, Y. Zhao, C. Ma, J. Liu and J. Ma (2013). "MiR-92b inhibitor promoted glioma cell apoptosis via targeting DKK3 and blocking the Wnt/beta-catenin signaling pathway." J Transl Med **11**: 302.

Lichti, U., J. Anders and S. H. Yuspa (2008). "Isolation and short-term culture of primary keratinocytes, hair follicle populations and dermal cells from newborn mice and keratinocytes from adult mice for in vitro analysis and for grafting to immunodeficient mice." Nat Protoc **3**(5): 799-810.

Lin, S. L., T. Kisseleva, D. A. Brenner and J. S. Duffield (2008). "Pericytes and perivascular fibroblasts are the primary source of collagen-producing cells in obstructive fibrosis of the kidney." Am J Pathol **173**(6): 1617-1627.

Little, M. C., R. A. Metcalfe, J. W. Haycock, J. Healy, D. J. Gawkrodger and S. Mac Neil (1998). "The participation of proliferative keratinocytes in the preimmune response to sensitizing agents." Br J Dermatol **138**(1): 45-56.

Little, M. H. and A. P. McMahon (2012). "Mammalian kidney development: principles, progress, and projections." Cold Spring Harb Perspect Biol **4**(5).

Liu, G. Y., P. J. Fairchild, R. M. Smith, J. R. Prowle, D. Kioussis and D. C. Wraith (1995). "Low avidity recognition of self-antigen by T cells permits escape from central tolerance." Immunity **3**(4): 407-415.

Liu, L., P. Kou, Q. Zeng, G. Pei, Y. Li, H. Liang, G. Xu and S. Chen (2012). "CD4+ T Lymphocytes, especially Th2 cells, contribute to the progress of renal fibrosis." Am J Nephrol **36**(4): 386-396.

Liu, Y. (2011). "Cellular and molecular mechanisms of renal fibrosis." Nat Rev Nephrol **7**(12): 684-696.

Lodygin, D., A. Epanchintsev, A. Menssen, J. Diebold and H. Hermeking (2005). "Functional epigenomics identifies genes frequently silenced in prostate cancer." Cancer Res **65**(10): 4218-4227.

Louis, H., J. L. Van Laethem, W. Wu, E. Quertinmont, C. Degraef, K. Van den Berg, A. Demols, M. Goldman, O. Le Moine, A. Geerts and J. Deviere (1998). "Interleukin-10 controls neutrophilic infiltration, hepatocyte proliferation, and liver fibrosis induced by carbon tetrachloride in mice." Hepatology **28**(6): 1607-1615.

Lundqvist, E. N. and O. Back (1990). "Interleukin-1 decreases the number of Ia+ epidermal dendritic cells but increases their expression of Ia antigen." Acta Derm Venereol **70**(5): 391-394.

Luo, D. D., A. Phillips and D. Fraser (2010). "Bone morphogenetic protein-7 inhibits proximal tubular epithelial cell Smad3 signaling via increased SnoN expression." Am J Pathol **176**(3): 1139-1147.

Lupher, M. L., Jr. and W. M. Gallatin (2006). "Regulation of fibrosis by the immune system." Adv Immunol **89**: 245-288.

Lustig, B. and J. Behrens (2003). "The Wnt signaling pathway and its role in tumor development." J Cancer Res Clin Oncol **129**(4): 199-221.

Lutz, J., Y. Yao, E. Song, B. Antus, P. Hamar, S. Liu and U. Heemann (2005). "Inhibition of matrix metalloproteinases during chronic allograft nephropathy in rats." Transplantation **79**(6): 655-661.

Ma, J., R. Wang, X. Fang and Z. Sun (2012). "beta-catenin/TCF-1 pathway in T cell development and differentiation." J Neuroimmune Pharmacol **7**(4): 750-762.

Mackay, C. R. (1993). "Homing of naive, memory and effector lymphocytes." Curr Opin Immunol **5**(3): 423-427.

Magombedze, G., P. B. Reddy, S. Eda and V. V. Ganusov (2013). "Cellular and population plasticity of helper CD4(+) T cell responses." Front Physiol **4**: 206.

Malhotra, N., K. Narayan, O. H. Cho, K. E. Sylvia, C. Yin, H. Melichar, M. Rashighi, V. Lefebvre, J. E. Harris, L. J. Berg, J. Kang and C. Immunological Genome Project (2013). "A network of high-mobility group box transcription factors programs innate interleukin-17 production." Immunity **38**(4): 681-693.

Malone, D. G., P. W. Dolan, R. R. Brown, M. V. Kalayoglu, R. A. Arend, G. I. Byrne and Y. Ozaki (1994). "Interferon gamma induced production of indoleamine 2,3 dioxygenase in cultured human synovial cells." J Rheumatol **21**(6): 1011-1019.

Mao, B. and C. Niehrs (2003). "Kremen2 modulates Dickkopf2 activity during Wnt/LRP6 signaling." Gene **302**(1-2): 179-183.

Mao, B., W. Wu, G. Davidson, J. Marhold, M. Li, B. M. Mechler, H. Delius, D. Hoppe, P. Stannek, C. Walter, A. Glinka and C. Niehrs (2002). "Kremen proteins are Dickkopf receptors that regulate Wnt/beta-catenin signalling." Nature **417**(6889): 664-667.

Mariani, T. J., S. Sandefur, J. D. Roby and R. A. Pierce (1998). "Collagenase-3 induction in rat lung fibroblasts requires the combined effects of tumor necrosis factor-alpha and 12-lipoxygenase metabolites: a model of macrophage-induced, fibroblast-driven extracellular matrix remodeling during inflammatory lung injury." Mol Biol Cell **9**(6): 1411-1424.

Matzinger, P. (2007). Friendly and dangerous signals: is the tissue in control? *Nat Immunol*. **8:** 11-13.

McInnes, I. B. and G. Schett (2011). "The pathogenesis of rheumatoid arthritis." N Engl J Med **365**(23): 2205-2219.

Medinger, M., A. Tzankov, J. Kern, A. Pircher, M. Hermann, H. W. Ott, G. Gastl, G. Untergasser and E. Gunsilius (2010). "Increased Dkk3 protein expression in platelets and megakaryocytes of patients with myeloproliferative neoplasms." Thromb Haemost **105**(1).

Mempel, M., V. Voelcker, G. Kollisch, C. Plank, R. Rad, M. Gerhard, C. Schnopp, P. Fraunberger, A. K. Walli, J. Ring, D. Abeck and M. Ollert (2003). "Toll-like receptor expression in human keratinocytes: nuclear factor kappaB controlled gene activation by Staphylococcus aureus is toll-like receptor 2 but not toll-like receptor 4 or platelet activating factor receptor dependent." J Invest Dermatol **121**(6): 1389-1396.

Meran, S. and R. Steadman (2011). "Fibroblasts and myofibroblasts in renal fibrosis." Int J Exp Pathol **92**(3): 158-167.

Miller, L. S., O. E. Sorensen, P. T. Liu, H. R. Jalian, D. Eshtiaghpour, B. E. Behmanesh, W. Chung, T. D. Starner, J. Kim, P. A. Sieling, T. Ganz and R. L. Modlin (2005). "TGF-alpha regulates TLR expression and function on epidermal keratinocytes." J Immunol **174**(10): 6137-6143.

Miloud, T., C. Henrich and G. J. Hammerling (2007). "Quantitative comparison of click beetle and firefly luciferases for in vivo bioluminescence imaging." J Biomed Opt **12**(5): 054018.

Mimura, I. and M. Nangaku (2010). "The suffocating kidney: tubulointerstitial hypoxia in end-stage renal disease." Nat Rev Nephrol **6**(11): 667-678.

Mizobuchi, Y., K. Matsuzaki, K. Kuwayama, K. Kitazato, H. Mure, T. Kageji and S. Nagahiro (2008). "REIC/Dkk-3 induces cell death in human malignant glioma." Neuro Oncol **10**(3): 244-253.

Monaghan, A. P., P. Kioschis, W. Wu, A. Zuniga, D. Bock, A. Poustka, H. Delius and C. Niehrs (1999). "Dickkopf genes are co-ordinately expressed in mesodermal lineages." Mech Dev **87**(1-2): 45-56.

Moon, R. T., A. D. Kohn, G. V. De Ferrari and A. Kaykas (2004). "WNT and beta-catenin signalling: diseases and therapies." Nat Rev Genet **5**(9): 691-701.

Moore, K. W., R. de Waal Malefyt, R. L. Coffman and A. O'Garra (2001). "Interleukin-10 and the interleukin-10 receptor." Annu Rev Immunol **19**: 683-765.

Mouzaki, A., S. Deraos and K. Chatzantoni (2005). "Advances in the treatment of autoimmune diseases; cellular activity, type-1/type-2 cytokine secretion patterns and their modulation by therapeutic peptides." Curr Med Chem **12**(13): 1537-1550.

Mukhopadhyay, M., S. Shtrom, C. Rodriguez-Esteban, L. Chen, T. Tsukui, L. Gomer, D. W. Dorward, A. Glinka, A. Grinberg, S. P. Huang, C. Niehrs, J. C. Izpisua Belmonte and H. Westphal (2001). "Dickkopf1 is required for embryonic head induction and limb morphogenesis in the mouse." Dev Cell **1**(3): 423-434.

Muranishi, Y. and T. Furukawa (2012). "BAC-Dkk3-EGFP transgenic mouse: an in vivo analytical tool for Dkk3 expression." J Biomed Biotechnol **2012**: 973140.

Mutis, T., M. De Bueger, A. Bakker and T. H. Ottenhoff (1993). "HLA class II+ human keratinocytes present Mycobacterium leprae antigens to CD4+ Th1-like cells." Scand J Immunol **37**(1): 43-51.

Na, S. Y., A. Hermann, M. Sanchez-Ruiz, A. Storch, M. Deckert and T. Hunig (2012). "Oligodendrocytes enforce immune tolerance of the uninfected brain by purging the peripheral repertoire of autoreactive CD8+ T cells." Immunity **37**(1): 134-146.

Naik, S. M., G. Cannon, G. J. Burbach, S. R. Singh, R. A. Swerlick, J. N. Wilcox, J. C. Ansel and S. W. Caughman (1999). "Human keratinocytes constitutively express interleukin-18 and secrete biologically active interleukin-18 after treatment with pro-inflammatory mediators and dinitrochlorobenzene." J Invest Dermatol **113**(5): 766-772.

Nakamura, R. E. and A. S. Hackam (2010). "Analysis of Dickkopf3 interactions with Wnt signaling receptors." Growth Factors **28**(4): 232-242.

Nakamura, R. E., D. D. Hunter, H. Yi, W. J. Brunken and A. S. Hackam (2007). "Identification of two novel activities of the Wnt signaling regulator Dickkopf 3 and characterization of its expression in the mouse retina." BMC Cell Biol **8**: 52.

Nakayama, K. (1997). "Furin: a mammalian subtilisin/Kex2p-like endoprotease involved in processing of a wide variety of precursor proteins." Biochem J **327 (Pt 3)**: 625-635.

Nathan, C. and A. Ding (2010). "Nonresolving inflammation." Cell **140**(6): 871-882.

Negre-Salvayre, A., C. Coatrieux, C. Ingueneau and R. Salvayre (2008). "Advanced lipid peroxidation end products in oxidative damage to proteins. Potential role in diseases and therapeutic prospects for the inhibitors." Br J Pharmacol **153**(1): 6-20.

Nengwen, K., F. Li, X. Li, S. Juan, L. Jianjun, Z. Chuntao and L. Youping (2009). "B7-1 induces immunosuppression when expressed in cultured neonatal mice keratinocytes." Immunobiology **214**(8): 653-663.

Nguan, C. Y. and C. Du (2009). "Renal tubular epithelial cells as immunoregulatory cells in renal allograft rejection." Transplant Rev (Orlando) **23**(3): 129-138.

Nickoloff, B. J. and L. A. Turka (1994). "Immunological functions of non-professional antigen-presenting cells: new insights from studies of T-cell interactions with keratinocytes." Immunol Today **15**(10): 464-469.

Niebuhr, M., K. Baumert and T. Werfel (2010). "TLR-2-mediated cytokine and chemokine secretion in human keratinocytes." Exp Dermatol **19**(10): 873-877.

Niedermeier, M., B. Reich, M. Rodriguez Gomez, A. Denzel, K. Schmidbauer, N. Gobel, Y. Talke, F. Schweda and M. Mack (2009). "CD4+ T cells control the differentiation of Gr1+ monocytes into fibrocytes." Proc Natl Acad Sci U S A **106**(42): 17892-17897.

Niehrs, C. (2006). "Function and biological roles of the Dickkopf family of Wnt modulators." Oncogene **25**(57): 7469-7481.

Nikolic-Paterson, D. J. (2010). "CD4+ T cells: a potential player in renal fibrosis." Kidney Int **78**(4): 333-335.

Nishida, M. and K. Hamaoka (2008). "Macrophage phenotype and renal fibrosis in obstructive nephropathy." Nephron Exp Nephrol **110**(1): e31-36.

Ohyama, M., A. Terunuma, C. L. Tock, M. F. Radonovich, C. A. Pise-Masison, S. B. Hopping, J. N. Brady, M. C. Udey and J. C. Vogel (2006). "Characterization and isolation of stem cell-enriched human hair follicle bulge cells." J Clin Invest **116**(1): 249-260.

Oldroyd, S. D., G. L. Thomas, G. Gabbiani and A. M. El Nahas (1999). "Interferon-gamma inhibits experimental renal fibrosis." Kidney Int **56**(6): 2116-2127.

Ong, C., C. Wong, C. R. Roberts, H. S. Teh and F. R. Jirik (1998). "Anti-IL-4 treatment prevents dermal collagen deposition in the tight-skin mouse model of scleroderma." Eur J Immunol **28**(9): 2619-2629.

Ostendorf, T., F. Eitner and J. Floege (2012). "The PDGF family in renal fibrosis." Pediatr Nephrol **27**(7): 1041-1050.

Otten, H. G., B. Bor, C. Ververs, L. F. Verdonck, M. De Boer and G. C. De Gast (1996). "Alloantigen-specific T-cell anergy induced by human keratinocytes is abrogated upon loss of cell-cell contact." Immunology **88**(2): 214-219.

Pantelyushin, S., S. Haak, B. Ingold, P. Kulig, F. L. Heppner, A. A. Navarini and B. Becher (2012). "Rorgammat+ innate lymphocytes and gammadelta T cells initiate psoriasiform plaque formation in mice." J Clin Invest **122**(6): 2252-2256.

Papatriantafyllou, M., G. Moldenhauer, J. Ludwig, A. Tafuri, N. Garbi, G. Hollmann, G. Kublbeck, A. Klevenz, S. Schmitt, G. Pougialis, C. Niehrs, H. J. Grone, G. J. Hammerling, B. Arnold and T. Oelert (2012). "Dickkopf-3, an immune modulator in peripheral CD8 T-cell tolerance." Proc Natl Acad Sci U S A **109**(5): 1631-1636.

Pastore, S., E. Fanales-Belasio, C. Albanesi, L. M. Chinni, A. Giannetti and G. Girolomoni (1997). "Granulocyte macrophage colony-stimulating factor is overproduced by keratinocytes in atopic dermatitis. Implications for sustained dendritic cell activation in the skin." J Clin Invest **99**(12): 3009-3017.

Pellegrini, G., M. De Luca, G. Orecchia, F. Balzac, O. Cremona, P. Savoia, R. Cancedda and P. C. Marchisio (1992). "Expression, topography, and function of integrin receptors are severely altered in keratinocytes from involved and uninvolved psoriatic skin." J Clin Invest **89**(6): 1783-1795.

Phanish, M. K., S. K. Winn and M. E. Dockrell (2010). "Connective tissue growth factor-(CTGF, CCN2)--a marker, mediator and therapeutic target for renal fibrosis." Nephron Exp Nephrol **114**(3): e83-92.

Pierson, E., S. B. Simmons, L. Castelli and J. M. Goverman (2012). "Mechanisms regulating regional localization of inflammation during CNS autoimmunity." Immunol Rev **248**(1): 205-215.

Pivarcsi, A., L. Bodai, B. Rethi, A. Kenderessy-Szabo, A. Koreck, M. Szell, Z. Beer, Z. Bata-Csorgoo, M. Magocsi, E. Rajnavolgyi, A. Dobozy and L. Kemeny (2003). "Expression and function of Toll-like receptors 2 and 4 in human keratinocytes." Int Immunol **15**(6): 721-730.

Plitz, T., P. Saint-Mezard, M. Satho, S. Herren, C. Waltzinger, M. de Carvalho Bittencourt, M. H. Kosco-Vilbois and Y. Chvatchko (2003). "IL-18 binding protein protects against contact hypersensitivity." J Immunol **171**(3): 1164-1171.

Pollard, S. L. and P. W. Holland (2000). "Evidence for 14 homeobox gene clusters in human genome ancestry." Curr Biol **10**(17): 1059-1062.

Poulsen, L. K. and L. Hummelshoj (2007). "Triggers of IgE class switching and allergy development." Ann Med **39**(6): 440-456.

Prens, E. P., M. Kant, G. van Dijk, L. I. van der Wel, S. Mourits and L. van der Fits (2008). "IFN-alpha enhances poly-IC responses in human keratinocytes by inducing expression of cytosolic innate RNA receptors: relevance for psoriasis." J Invest Dermatol **128**(4): 932-938.

Prudova, A., U. auf dem Keller, G. S. Butler and C. M. Overall (2010). "Multiplex N-terminome analysis of MMP-2 and MMP-9 substrate degradomes by iTRAQ-TAILS quantitative proteomics." Mol Cell Proteomics **9**(5): 894-911.

Pulkkinen, K., S. Murugan and S. Vainio (2008). "Wnt signaling in kidney development and disease." Organogenesis **4**(2): 55-59.

Racke, M. K., W. Hu and A. E. Lovett-Racke (2005). "PTX cruiser: driving autoimmunity via TLR4." Trends Immunol **26**(6): 289-291.

Refaeli, Y., L. Van Parijs, S. I. Alexander and A. K. Abbas (2002). "Interferon gamma is required for activation-induced death of T lymphocytes." J Exp Med **196**(7): 999-1005.

Ren, S., B. G. Johnson, Y. Kida, C. Ip, K. C. Davidson, S. L. Lin, A. Kobayashi, R. A. Lang, A. K. Hadjantonakis, R. T. Moon and J. S. Duffield (2013). "LRP-6 is a coreceptor for multiple fibrogenic signaling pathways in pericytes and myofibroblasts that are inhibited by DKK-1." Proc Natl Acad Sci U S A **110**(4): 1440-1445.

Reya, T. and H. Clevers (2005). "Wnt signalling in stem cells and cancer." Nature **434**(7035): 843-850.

Roman-Gomez, J., A. Jimenez-Velasco, X. Agirre, J. A. Castillejo, G. Navarro, M. Barrios, E. J. Andreu, F. Prosper, A. Heiniger and A. Torres (2004). "Transcriptional silencing of the Dickkopfs-3 (Dkk-3) gene by CpG hypermethylation in acute lymphoblastic leukaemia." Br J Cancer **91**(4): 707-713.

Rosenblum, M. D., I. K. Gratz, J. S. Paw, K. Lee, A. Marshak-Rothstein and A. K. Abbas (2011). "Response to self antigen imprints regulatory memory in tissues." Nature **480**(7378): 538-542.

Saadi-Kheddouci, S., D. Berrebi, B. Romagnolo, F. Cluzeaud, M. Peuchmaur, A. Kahn, A. Vandewalle and C. Perret (2001). "Early development of polycystic kidney disease in transgenic mice expressing an activated mutant of the beta-catenin gene." Oncogene **20**(42): 5972-5981.

Sakaguchi, M., K. Kataoka, F. Abarzua, R. Tanimoto, M. Watanabe, H. Murata, S. S. Than, K. Kurose, Y. Kashiwakura, K. Ochiai, Y. Nasu, H. Kumon and N. H. Huh (2009). "Overexpression of REIC/Dkk-3 in normal fibroblasts suppresses tumor growth via induction of interleukin-7." J Biol Chem **284**(21): 14236-14244.

Sakaguchi, S. (2004). "Naturally arising CD4+ regulatory t cells for immunologic self-tolerance and negative control of immune responses." Annu Rev Immunol **22**: 531-562.

Schmitz, J., A. Owyang, E. Oldham, Y. Song, E. Murphy, T. K. McClanahan, G. Zurawski, M. Moshrefi, J. Qin, X. Li, D. M. Gorman, J. F. Bazan and R. A. Kastelein (2005). "IL-33, an interleukin-1-like cytokine that signals via the IL-1 receptor-related protein ST2 and induces T helper type 2-associated cytokines." Immunity **23**(5): 479-490.

Schmolka, N., K. Serre, A. R. Grosso, M. Rei, D. J. Pennington, A. Q. Gomes and B. Silva-Santos (2013). "Epigenetic and transcriptional signatures of stable versus plastic differentiation of proinflammatory gammadelta T cell subsets." Nat Immunol **14**(10): 1093-1100.

Schnaper, H. W., S. Jandeska, C. E. Runyan, S. C. Hubchak, R. K. Basu, J. F. Curley, R. D. Smith and T. Hayashida (2009). "TGF-beta signal transduction in chronic kidney disease." Front Biosci (Landmark Ed) **14**: 2448-2465.

Schrimpf, C. and J. S. Duffield (2011). "Mechanisms of fibrosis: the role of the pericyte." Curr Opin Nephrol Hypertens **20**(3): 297-305.

Schroder, K., P. J. Hertzog, T. Ravasi and D. A. Hume (2004). "Interferon-gamma: an overview of signals, mechanisms and functions." J Leukoc Biol **75**(2): 163-189.

Semenov, M. V., K. Tamai, B. K. Brott, M. Kuhl, S. Sokol and X. He (2001). "Head inducer Dickkopf-1 is a ligand for Wnt coreceptor LRP6." Curr Biol **11**(12): 951-961.

Sempowski, G. D., M. P. Beckmann, S. Derdak and R. P. Phipps (1994). "Subsets of murine lung fibroblasts express membrane-bound and soluble IL-4 receptors. Role of IL-4 in enhancing fibroblast proliferation and collagen synthesis." J Immunol **152**(7): 3606-3614.

Sercan, O., D. Stoycheva, G. J. Hammerling, B. Arnold and T. Schuler (2010). "IFN-gamma receptor signaling regulates memory CD8+ T cell differentiation." J Immunol **184**(6): 2855-2862.

Shao, D. D., R. Suresh, V. Vakil, R. H. Gomer and D. Pilling (2008). "Pivotal Advance: Th-1 cytokines inhibit, and Th-2 cytokines promote fibrocyte differentiation." J Leukoc Biol **83**(6): 1323-1333.

Shi, X. Y., F. F. Hou, H. X. Niu, G. B. Wang, D. Xie, Z. J. Guo, Z. M. Zhou, F. Yang, J. W. Tian and X. Zhang (2008). "Advanced oxidation protein products promote inflammation in diabetic kidney through activation of renal nicotinamide adenine dinucleotide phosphate oxidase." Endocrinology **149**(4): 1829-1839.

Simmons, R. D., T. M. Buzbee and D. S. Linthicum (1988). "An immunohistological study of autoimmune encephalomyelitis and neuritis in the rabbit. Observations in the dorsal root ganglion using the freeze-dried paraffin-embedded tissue technique." J Neurol Sci **83**(2-3): 293-304.

Slavin, A., L. Kelly-Modis, M. Labadia, K. Ryan and M. L. Brown (2010). "Pathogenic mechanisms and experimental models of multiple sclerosis." Autoimmunity **43**(7): 504-513.

Song, E., N. Ouyang, M. Horbelt, B. Antus, M. Wang and M. S. Exton (2000). "Influence of alternatively and classically activated macrophages on fibrogenic activities of human fibroblasts." Cell Immunol **204**(1): 19-28.

Soumelis, V., P. A. Reche, H. Kanzler, W. Yuan, G. Edward, B. Homey, M. Gilliet, S. Ho, S. Antonenko, A. Lauerma, K. Smith, D. Gorman, S. Zurawski, J. Abrams, S. Menon, T. McClanahan, R. de Waal-Malefyt Rd, F. Bazan, R. A. Kastelein and Y. J. Liu (2002). "Human epithelial cells trigger dendritic cell mediated allergic inflammation by producing TSLP." Nat Immunol **3**(7): 673-680.

Stanbury, R. M. and E. M. Graham (1998). "Systemic corticosteroid therapy--side effects and their management." Br J Ophthalmol **82**(6): 704-708.

Strange, P., L. Skov and O. Baadsgaard (1994). "Interferon gamma-treated keratinocytes activate T cells in the presence of superantigens: involvement of major histocompatibility complex class II molecules." J Invest Dermatol **102**(2): 150-154.

Strid, J., R. E. Tigelaar and A. C. Hayday (2009). "Skin immune surveillance by T cells--a new order?" Semin Immunol **21**(3): 110-120.

Strutz, F. and G. A. Muller (2006). "Renal fibrosis and the origin of the renal fibroblast." Nephrol Dial Transplant **21**(12): 3368-3370.

Strutz, F., M. Zeisberg, B. Hemmerlein, B. Sattler, K. Hummel, V. Becker and G. A. Muller (2000). "Basic fibroblast growth factor expression is increased in human renal fibrogenesis and may mediate autocrine fibroblast proliferation." Kidney Int **57**(4): 1521-1538.

Surendran, K., S. P. McCaul and T. C. Simon (2002). "A role for Wnt-4 in renal fibrosis." Am J Physiol Renal Physiol **282**(3): F431-441.

Suwa, T., M. Chen, C. L. Hawks and P. J. Hornsby (2003). "Zonal expression of dickkopf-3 and components of the Wnt signalling pathways in the human adrenal cortex." J Endocrinol **178**(1): 149-158.

Szolnoky, G., Z. Bata-Csorgo, A. S. Kenderessy, M. Kiss, A. Pivarcsi, Z. Novak, K. Nagy Newman, G. Michel, T. Ruzicka, L. Marodi, A. Dobozy and L. Kemeny (2001). "A mannose-binding receptor is expressed on human keratinocytes and mediates killing of Candida albicans." J Invest Dermatol **117**(2): 205-213.

Tan, J. K. and H. C. O'Neill (2005). "Maturation requirements for dendritic cells in T cell stimulation leading to tolerance versus immunity." J Leukoc Biol **78**(2): 319-324.

Tapmeier, T. T., A. Fearn, K. Brown, P. Chowdhury, S. H. Sacks, N. S. Sheerin and W. Wong (2010). "Pivotal role of CD4+ T cells in renal fibrosis following ureteric obstruction." Kidney Int **78**(4): 351-362.

Terada, Y., H. Tanaka, T. Okado, H. Shimamura, S. Inoshita, M. Kuwahara and S. Sasaki (2003). "Expression and function of the developmental gene Wnt-4 during experimental acute renal failure in rats." J Am Soc Nephrol **14**(5): 1223-1233.

Thiery, J. P., H. Acloque, R. Y. Huang and M. A. Nieto (2009). "Epithelial-mesenchymal transitions in development and disease." Cell **139**(5): 871-890.

Thompson, K., J. Maltby, J. Fallowfield, M. McAulay, H. Millward-Sadler and N. Sheron (1998). "Interleukin-10 expression and function in experimental murine liver inflammation and fibrosis." Hepatology **28**(6): 1597-1606.

Tietz, W., Y. Allemand, E. Borges, D. von Laer, R. Hallmann, D. Vestweber and A. Hamann (1998). "CD4+ T cells migrate into inflamed skin only if they express ligands for E- and P-selectin." J Immunol **161**(2): 963-970.

Tigalonowa, M., J. R. Bjerke, J. K. Livden and R. Matre (1990). "The distribution of Fc gamma RI, Fc gamma RII and Fc gamma R III on Langerhans' cells and keratinocytes in normal skin." Acta Derm Venereol **70**(5): 385-390.

Tsuji, T., M. Miyazaki, M. Sakaguchi, Y. Inoue and M. Namba (2000). "A REIC gene shows down-regulation in human immortalized cells and human tumor-derived cell lines." Biochem Biophys Res Commun **268**(1): 20-24.

Untergasser, G., M. Steurer, M. Zimmermann, M. Hermann, J. Kern, A. Amberger, G. Gastl and E. Gunsilius (2008). "The Dickkopf-homolog 3 is expressed in tumor endothelial cells and supports capillary formation." Int J Cancer **122**(7): 1539-1547.

van Amerongen, R. and R. Nusse (2009). "Towards an integrated view of Wnt signaling in development." Development **136**(19): 3205-3214.

van Loosdregt, J., V. Fleskens, M. M. Tiemessen, M. Mokry, R. van Boxtel, J. Meerding, C. E. Pals, D. Kurek, M. R. Baert, E. M. Delemarre, A. Grone, M. J. Koerkamp, A. J. Sijts, E. E. Nieuwenhuis, M. M. Maurice, J. H. van Es, D. Ten Berge, F. C. Holstege, F. J. Staal, D. M. Zaiss, B. J. Prakken and P. J. Coffer (2013). "Canonical Wnt signaling negatively modulates regulatory T cell function." Immunity **39**(2): 298-310.

van Meerwijk, J. P., S. Marguerat, R. K. Lees, R. N. Germain, B. J. Fowlkes and H. R. MacDonald (1997). "Quantitative impact of thymic clonal deletion on the T cell repertoire." J Exp Med **185**(3): 377-383.

Veeck, J. and E. Dahl (2012). "Targeting the Wnt pathway in cancer: The emerging role of Dickkopf-3." Biochimica et Biophysica Acta (BBA) - Reviews on Cancer **1825**(1): 18-28.

Venkatachalam, M. A., K. A. Griffin, R. Lan, H. Geng, P. Saikumar and A. K. Bidani (2010). "Acute kidney injury: a springboard for progression in chronic kidney disease." Am J Physiol Renal Physiol **298**(5): F1078-1094.

Vernon, M. A., K. J. Mylonas and J. Hughes (2010). "Macrophages and renal fibrosis." Semin Nephrol **30**(3): 302-317.

Vilar, J., C. Lalou, J. P. Duong Van Huyen, S. Charrin, S. Hardouin, D. Raulais, C. Merlet-Benichou and M. Lelievre-Pegorier (2002). "Midkine is involved in kidney development and in its regulation by retinoids." J Am Soc Nephrol **13**(3): 668-676.

Wada, T., N. Sakai, Y. Sakai, K. Matsushima, S. Kaneko and K. Furuichi (2011). "Involvement of bone-marrow-derived cells in kidney fibrosis." Clin Exp Nephrol **15**(1): 8-13.

Wang, X., M. Fujita, R. Prado, A. Tousson, H. C. Hsu, A. Schottelius, D. R. Kelly, P. A. Yang, Q. Wu, J. Chen, H. Xu, C. A. Elmets, J. D. Mountz and C. K. Edwards, 3rd (2010). "Visualizing CD4 T-cell migration into inflamed skin and its inhibition by CCR4/CCR10 blockades using in vivo imaging model." Br J Dermatol **162**(3): 487-496.

Wangoo, A., C. Laban, H. T. Cook, B. Glenville and R. J. Shaw (1997). "Interleukin-10- and corticosteroid-induced reduction in type I procollagen in a human ex vivo scar culture." Int J Exp Pathol **78**(1): 33-41.

Webb, E. F., M. N. Tzimas, S. J. Newsholme and D. E. Griswold (1998). "Intralesional cytokines in chronic oxazolone-induced contact sensitivity suggest roles for tumor necrosis factor alpha and interleukin-4." J Invest Dermatol **111**(1): 86-92.

Wick, G., C. Grundtman, C. Mayerl, T. F. Wimpissinger, J. Feichtinger, B. Zelger, R. Sgonc and D. Wolfram (2013). "The immunology of fibrosis." Annu Rev Immunol **31**: 107-135.

Willenborg, D. O., S. Fordham, C. C. Bernard, W. B. Cowden and I. A. Ramshaw (1996). "IFN-gamma plays a critical down-regulatory role in the induction and effector phase of myelin oligodendrocyte glycoprotein-induced autoimmune encephalomyelitis." J Immunol **157**(8): 3223-3227.

Willenborg, D. O., M. Staykova, S. Fordham, N. O'Brien and D. Linares (2007). "The contribution of nitric oxide and interferon gamma to the regulation of the neuro-inflammation in experimental autoimmune encephalomyelitis." J Neuroimmunol **191**(1-2): 16-25.

Willer, C. J. and G. C. Ebers (2000). "Susceptibility to multiple sclerosis: interplay between genes and environment." Curr Opin Neurol **13**(3): 241-247.

Wynn, T. A. (2008). "Cellular and molecular mechanisms of fibrosis." J Pathol **214**(2): 199-210.

Xiang, T., L. Li, X. Yin, L. Zhong, W. Peng, Z. Qiu, G. Ren and Q. Tao (2013). "Epigenetic silencing of the WNT antagonist Dickkopf 3 disrupts normal Wnt/beta-catenin signalling and apoptosis regulation in breast cancer cells." J Cell Mol Med **17**(10): 1236-1246.

Yang, J., C. Dai and Y. Liu (2003). "Hepatocyte growth factor suppresses renal interstitial myofibroblast activation and intercepts Smad signal transduction." Am J Pathol **163**(2): 621-632.

Yang, J. and Y. Liu (2001). "Dissection of key events in tubular epithelial to myofibroblast transition and its implications in renal interstitial fibrosis." Am J Pathol **159**(4): 1465-1475.

Yao, Y., J. Zhang, D. Q. Tan, X. Y. Chen, D. F. Ye, J. P. Peng, J. T. Li, Y. Q. Zheng, L. Fang, Y. K. Li and M. X. Fan (2011). "Interferon-gamma improves renal interstitial fibrosis and decreases intrarenal vascular resistance of hydronephrosis in an animal model." Urology **77**(3): 761 e768-713.

Yu, Q., A. Sharma, S. Y. Oh, H. G. Moon, M. Z. Hossain, T. M. Salay, K. E. Leeds, H. Du, B. Wu, M. L. Waterman, Z. Zhu and J. M. Sen (2009). "T cell factor 1 initiates the T helper type 2 fate by inducing the transcription factor GATA-3 and repressing interferon-gamma." Nat Immunol **10**(9): 992-999.

Zenzmaier, C., J. Marksteiner, A. Kiefer, P. Berger and C. Humpel (2009). "Dkk-3 is elevated in CSF and plasma of Alzheimer's disease patients." J Neurochem **110**(2): 653-661.

Zhang, G., H. Kim, X. Cai, J. M. Lopez-Guisa, P. Carmeliet and A. A. Eddy (2003). "Urokinase receptor modulates cellular and angiogenic responses in obstructive nephropathy." J Am Soc Nephrol **14**(5): 1234-1253.

Zhang, J. (2007). "Yin and yang interplay of IFN-gamma in inflammation and autoimmune disease." J Clin Invest **117**(4): 871-873.

Zhou, L. L., F. F. Hou, G. B. Wang, F. Yang, D. Xie, Y. P. Wang and J. W. Tian (2009). "Accumulation of advanced oxidation protein products induces podocyte apoptosis and deletion through NADPH-dependent mechanisms." Kidney Int **76**(11): 1148-1160.

Zhu, J. and W. E. Paul (2010). "Peripheral CD4+ T-cell differentiation regulated by networks of cytokines and transcription factors." Immunol Rev **238**(1): 247-262.

Zinkernagel, R. M. and P. C. Doherty (1974). "Restriction of in vitro T cell-mediated cytotoxicity in lymphocytic choriomeningitis within a syngeneic or semiallogeneic system." Nature **248**(450): 701-702.

Cohen B., Weinshenker B. and Cerruto L. (2013) „Neurology, Multiple Sclerosis Edition, Chapter 13 - Genetics of Multiple Sclerosis: Implications for MS Susceptibility, Prognosis, and Treatment Response“. Living Medical eTextbook:http://lmt.projectsinknowledge.com/Activity/index.cfm?showfile=b&jn=2023&sj=2023.13&sc=2023.13.4.

Papatriantafyllou, M. (2008) “Molecular Mechanisms of Peripheral T cell Tolerance: Identification of Dickkopf 3 as a Novel Immune Modulator”. Inaugural-Dissertation : http://archiv.ub.uni-heidelberg.de/volltextserver/frontdoor.php?source_opus=8825.

6 Abbreviations

AICD	Activation induced cell death
AIRE	Autoimmune regulator
APC	Antigen presenting cell
BAC	Bacterial artificial chromosome
BSA	Bovine serum albumin
CCL	Chemokine C-C motif
CD	Cluster of differentiation
CFA	Complete freund´s adjuvants
CKD	Chronic kidney disease
CNS	Central nervous system
CSF	Cerebrospinal fluid
cTEC	Cortical thymic epithelial cell
CTGF	Connective tissue growth factor
CTLA-4	Cytotoxic T-lymphocyte antigen 4
CXCL	Chemokine C-X-C motif
DC	Dendrtitc cell
Dkk	Dickkof
DN	Double negative
DP	Double positive
DPCP	Diphenylcyclopropenone
DRG	Dorsal route ganglia
EAE	Experimental autoimmune encephalomyelitis
ECFC	Endothelial colony-forming cells
FGF	Fibroblast growth factor
Foxp3	Forkhead-Box Protein P3
Gata3	GATA binding protein 3
GM-CSF	Granulocyte macrophage stimulating factor
HIF1	Hypoxia-inducible factor 1
HPF	High power field
HSC	Hematopoietic stem cell
ICAM-1	Intracellular adhesion molecule - 1
IDO	Indoleamine-2,3 dioxygenase

IFNγ	Interferon-γ
IFNγ-R1	Interferon-γ receptor 1
IL	Interleukin
iNOS	Inducible NO synthase
LN	Lymph node
MBP	Myelin basic protein
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MMP	Matrix metalloproteinas
MOG	Myelin oligodendrocyte glycoprotein
mTEC	Medullary thymic epithelial cell
PD-1	Programmed cell death 1
PD-1L	Programmed cell death 1 ligand
PDGF	Platlete derived growth factor
PDGF	Platelet derived growth factor
PT	Pertussis toxin
RA	Retionic acid
RAG	Recombination activating gene
RoRγT	RAR-related orphan receptor-γ
ROS	Reactive oxygen species
SP	Single positive
Tbet	T box expressed in T cells
TCR	T cell receptor
TGF	Transforming growth factor
TNF	Tumour necrosis factor
TRA	Tissue restricted antigen
Treg	Regulatory T cell
TSLP	Thymic stromal lymphopoietin
UUO	Unilateral ureteral obstruction
wt	Wild-type
αGal-Cer	α-Galactosylceramide

7 Acknowledgements

It would have not been possible to accomplish this thesis without the invaluable help and great support of many people:

First and foremost I want to thank the head of the Department of Molecular Immunology and my supervisor Prof. Dr. Bernd Arnold. I am very grateful, not only for the possibility to accomplish this thesis in his group and to work on a highly interesting project, but also for his excellent guidance and remarkable support. It was an honour to work with you not only because you are a great scientist but also a great person.

My gratitude goes to to Prof. Dr. Günter Hämmerling, the former head of the Department of Molecular Immunology, for providing his enormous knowledge and experience whenever it was needed. Thank you for always critically accompanying the experiments and being the "first reviewer".

Furthermore, I would like to thank Dr. Thilo Oelert for his great scientific support, for providing me with his great ideas and for sharing his vast knowledge on immunology and science with me.

I want to thank Maria Papatriantafyllou for providing me with her excellent pre-work on Dkk3.

I´m indebted to Prof. Dr. H.J. Gröne and his co-workers Dr. Stefan Porubsky and Giuseppina Federico. Without their efforts, the fibrosis project would not exist. It was a great pleasure to work with you in such a close and trustful collaboration.

I´m thankfull to Prof. Dr. Thomas Tüting and his co-workers Dr. Tobias Bald and Dr. Evelyn Gaffal for their contribution to the MBP project.

A special thanks goes to Sandra Prokosch, who constantly assisted my work in an excellent way. It was always great fun to perform experiments together.

I want to thank Günter Küblbeck not only for his theoretical and technical support on everything concerning molecular biology but also for great discussions, especially about football. I thank Alexandra Klevenz for her enormous help with any kind of *in vitro* assay as well as for her constant advice. Moreover I´m thankful to Sabine Schmitt and Georg Pougialis for their assistance in all kinds of ELISA and EAE experiments.

It was a great pleasure to work with the whole stuff of the department of molecular immunology (D050). Thank you for the unique, warm and cooperative atmosphere.

Zum Schluss möchte ich meiner Familie für ihre anhaltende und bedingungslose Unterstützung danken. Ohne den Rückhalt meiner Eltern wäre es nicht möglich gewesen den Weg bis zu diesem Punkt zu gehen. Der größte Dank gilt meiner Freundin Tina und meinen wundervollen Kindern Emily und Karl. Liebe Tina, du warst unseren Kindern immer eine tolle und liebevolle Mutter, du hast mir während der ganzen Zeit den Rücken frei gehalten und hast mir so diese Arbeit erst ermöglicht. Dafür bin ich dir unendlich dankbar!