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

On the Regulation of T Cell-dependent Immune Responses

Kun-Hui Lu

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presented by

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On the Regulation of T Cell-dependent Immune Responses

Referees: Prof. Dr. Hans-Reimer Rodewald
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Contents

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‚But by the grace of God I am what I am: and his grace which was bestowed upon me was not in vain; but I laboured more abundantly than they all: yet not I, but the grace of God which was with me.‘

1 Corinthians 15:10

Summary

The immune system responds to an enormous variety of pathogens as well as to malignant cells. Since healthy tissues may also be damaged during immune responses, multiple mechanisms have evolved to shape and limit immune responses, in order to protect the integrity of the respective tissue. Understanding the basic mechanisms of immune regulation will help to successfully reprogram immune reactivity in immune-mediated diseases. T cell responses can be regulated by multiple factors including intrinsic and extrinsic modulators.

In the first part of this thesis we aimed to understand how the Nck adaptor proteins, as intrinsic modulators, control T cell effector function. Nck adaptor proteins stabilize proximal signaling complexes of T cell receptors (TCR) in the cytoplasm, thereby enhancing TCR signaling. We found that T cell-specific deletion of Nck proteins ($Nck.T^{-/-}$) lead to impaired germinal center formation, which is the central event for productive T cell-dependent antibody production. The number of follicular helper T (Tfh) cells, which are essential for germinal center formation, was decreased in the spleen of $Nck.T^{-/-}$ mice in comparison to wild type controls. The production of cytokines, such as IL-4, IL-10, and IL-21, by Tfh cells was reduced. The dysfunction of Tfh cells was associated with decreased Akt phosphorylation and intensified apoptosis of Tfh cells. Consequently, T cell-dependent antibody responses were reduced in regard to quantity as well as quality by affinity maturation. Furthermore, using experimental autoimmune encephalomyelitis as an autoimmune model lower disease scores, delayed dynamics and faster recovery were observed in $Nck.T^{-/-}$ mice in comparison to wild type animals. Together, our findings show an essential role for the Nck adapter proteins in the generation of potent effector T cells. Thus, defects in Nck protein function may have a so far un-recognized role in human diseases with defective T cell responses.

The second part focused on the role of the secreted protein Dickkopf-3 (DKK3) in modulating T cell responses against transplanted tumors. Mesenchymal stem cells (MSCs) are known to limit T cell responses *in vivo*. We found that DKK3 is produced by MSCs and contributed to MSC-mediated immune-suppression. Wild type MSCs inhibited anti-tumor responses whereas DKK3 deficient MSCs did not affect the rejection process. Impaired chemokine production by $DKK3^{-/-}$ MSCs could be related to enhanced infiltration of $CD8^{+}$ T cells within the $DKK3^{-/-}$ MSC-inoculated tumors. In addition, loss of DKK3 in MSCs resulted in increased expression of MHC class II antigens that may render MSCs more immunogenic rather than immune-suppressive. The higher expression of MHC II in $DKK3^{-/-}$ MSCs was associated with decreased mTOR activity and thereby enhanced autophagy. We hypothesize that DKK3 may act

as a positive modulator of the non-canonical Wnt/PCP pathway. Presently, DKK3 is being suggested as a potential anti-tumoral agent in human cancers based on reports that DKK3 is a tumor-suppressor. Our studies showing an immune-suppressive effect of DKK3 in the tumor mass may counteract these optimistic expectations and call for further detailed studies on the role of DKK3 in tumor development and in the respective immune responses before starting clinical trials.

Together, our studies contribute to a better understanding of mechanisms, which are involved in the control of T cell responses, and open new perspectives for further investigations.

Zusammenfassung

Das Immunsystem schützt uns gegen alle möglichen Krankheitserreger sowie gegen maligne Zellen. Da während einer Immunantwort auch gesundes Gewebe zerstört werden kann, haben sich Mechanismen entwickelt, um Immunantworten zu begrenzen und um somit die Integrität des entsprechenden Gewebes zu schützen. Ein besseres Verständnis der grundlegenden Mechanismen der Immunregulation ist notwendig, um immunologische Reaktivität bei entsprechenden Krankheiten therapeutisch neu zu programmieren. T-Zellantworten können durch verschiedene Faktoren reguliert werden, die intrinsische und extrinsische Modulatoren einschließen.

Der erste Teil dieser Arbeit hatte das Ziel, zu verstehen, wie die Nck Adapterproteine, als T-Zell-intrinsische Modulatoren, T-Zell-Effektor-Funktion kontrollieren können. Nck Adapterproteine stabilisieren den proximalen Signalkomplex des T-Zell-Rezeptors (TCR) im Zytoplasma und verstärken dadurch das TCR-Signal. Wir haben gefunden, dass T-Zell-spezifische Deletion von Nck Proteinen (Nck.$T^{-/-}$) zu einer Beeinträchtigung der Keimzentrum-Bildung führt, die das zentrale Ereignis bei einer produktiven T-Zell-abhängigen Antikörper-Bildung ist. Die Anzahl follikulärer Helfer T-Zellen (Tfh), die für die Keimzentrum-Bildung wichtig sind, war in der Milz von Nck.$T^{-/-}$ Tieren im Vergleich zu Wild-Typ Kontrollen verringert. Die Produktion der Zytokine IL-4, IL-10 und IL-21 durch Tfh Zellen war reduziert. Diese Dysfunktion von Tfh Zellen war mit einer verminderten Akt Phosphorylierung und einer verstärkten Apoptose dieser Zellen verbunden. Folglich waren die Menge und die Qualität T-Zell-abhängiger Antikörper reduziert. Weiterhin haben wir in einem Autoimmunmodel, der Experimentellen Autoimmunen Enzephalomyelitis, verringerte Krankheitswerte, einen verzögerten Verlauf und eine schnellere Erholung bei Nck.$T^{-/-}$ Mäusen im Vergleich zu Wild-typ Tieren beobachtet. Unsere Befunde weisen auf eine wichtige Rolle der Nck Adapterproteine in der Generierung von potenten Effektor-T-Zellen hin. Möglicherweise haben die Nck Proteine eine bisher noch nicht erkannte Bedeutung bei Erkrankungen mit defekter T-Zell-Reaktivität.

Der zweite Teil der Arbeit konzentriert sich auf die Rolle des sezernierten Proteins Dickkopf 3 (DKK3) in der Modulation von T-Zellantworten gegen transplantierte Tumoren. Es ist bekannt, dass mesenchymale Stammzellen (MSC) T-Zellantworten *in vivo* begrenzen können. Wir haben gefunden, dass DKK3 von MSC produziert wird und zu ihrer immunsuppressiven Wirkung beiträgt. Wild-Typ MSC inhibierten die Immunantwort gegen Tumore, während DKK3$^{-/-}$ MSC die Tumorabstoßung nicht beeinflussten. Eine verminderte Produktion von Chemokinen durch DKK3$^{-/-}$ MSC konnte mit einer verstärkten Infiltration von $CD8^+$ T-Zellen in die DKK3$^{-/-}$ MSC-

inokulierten Tumoren korreliert werden. Zusätzlich zeigten DKK3$^{-/-}$ MSC eine erhöhte MHC-Klasse II Expression, die zu einer besseren Antigenpräsentation und damit zu einer effizienteren T-Zellantwort führen könnte. Die höhere Expression von MHC II Antigenen war mit einer verringerten mTOR Aktivität und dadurch mit einer verbesserten Autophagozytose in DKK3$^{-/-}$ MSC verbunden. Wir nehmen an, dass DKK3 positiv auf den nicht-kanonischen Wnt Signalweg wirkt. Derzeit wird DKK3 als ein mögliches Krebsmittel diskutiert, da DKK3 als Tumor-Suppressor beschrieben wurde. Unsere Ergebnisse zeigen eine immune-suppressive Wirkung von DKK3 in Tumoren und unterstützen daher diese optimistischen Einschätzungen nicht. Vielmehr sind detaillierte Studien zur Wirkung von DKK3 in der Tumorentwicklung und bei den entsprechenden Immunantworten gegen diese Tumoren notwendig, bevor klinische Studien begonnen werden.

Unsere Studien tragen zu einem besseren Verständnis der Regulation von T-Zellantworten bei und eröffnen neue Perspektiven für weitere Untersuchungen.

Abbreviations

AICD	Activation-induced Cell Death
AIRE	Autoimmune Regulator
APC	Antigen Presenting Cell
Atg	Autophagy-related Gene
BCR	B Cell Receptor
Breg	Regulatory B Cell
BSA	Bovine Serum Albumin
CD	Cluster of Differentiation
CFA	Complete Freund's Adjuvant
CMA	Chaperon-mediated Autophagy
CNS	Central Nervous System
CREB	Cyclic AMP-responsive Element-binding Protein
CVID	Common Variable Immunodeficiency
DAMP	Damage-associated Molecular Pattern
DKK	Dickkopf
DN	Double Negative
EAE	Experimental Autoimmune Encephalomyelitis
EGFP	Enhanced Green Fluorescence Protein
ELISA	Enzyme-linked Immunosorbent Assay
ERK	Extracellular Signal-regulated Kinase
FACS	Fluorescence-activated Cell Sorting
FBS	Fetal Bovine Serum
GALT	Gut-associated Lymphoid Tissue
GC	Germinal Center
HRP	Horseradish Peroxidase
HSC	Heat Shock Cognate Protein
i.p.	Intraperitoneal
ICOS	Inducible T Cell Co-stimulator
iDC	Immature Dendritic Cell

IDO	Indoleamine-dioxygenase
IFA	Incomplete Freund's Ajuvant
IFNγ	Interferon γ
Ig	Immunoglobulin
IL-x	Interleukine-x
ILC	Innate Lymphoid Cell
iTreg	Inducible Regulatory T Cell
JNK	c-Jun-NH2-Kinase
KLH	Keyhole Limpet Hemocyanin
KO	Knock-out
Krm	Kremen
KYN	Kynurenine
LAMP	Lysosome-associated Membrane Protein
LSEC	Liver Sinusoidal Endothelium Cell
MDSC	Myeloid-derived Suppressor Cell
MFI	Mean Fluorescence Intensity
MHC	Major Histocompatibility Complex
MIIC	MHC Class II-containing Compartment
MMP	Metalloproteinase
MOG	Myelin Oligodendrocyte Glycoprotein
mOVA	Membrane-bound Ovalbumin
MS	Multiple Sclerosis
MSC	Mesenchymal Stem Cell
mTOR	Mammalian Target of Rapamycin
mTORC1	mTOR Complex 1
NK	Nature Killer
NKT	Nature Killer T
NLR	Nod-like Receptor
NO	Nitric Oxygen
nTreg	Natural Regulatory T Cell
PAK	p21-Activated Kinase

PAMP	Pathogen-associated Molecular Pattern
PD	Programmed Death
PE	Phosphatidylethanolamine
PGE2	Prostaglandin E2
PID	Primary Immunodeficiency Disease
PMA	Phorbol 12-Myristate 13-Acetate
PRR	Pattern Recognition Receptor
REIC	Reduced Expression in Immortalized Cells
ROR	Receptor Tyrosine Kinase-like Orphan Receptor
RORγt	Retinoid-Acid Receptor-related Orphan Receptor γ t
ROS	Radical Oxygen Species
s.c.	Subcutaneous
SARS	Severe Acute Respiratory Syndrome
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SH	Src Homology
TAM	Tumor-associated Macrophage
TAN	Tumor-associated Neutrophil
TCR	T Cell Receptor
Tfh	Follicular Helper T Cell
TGFβ	Tumor Growth Factor β
Th	Helper T Cell
TLR	Toll-like Receptor
TNFα	Tumor Necrosis Factor α
TNP	Trinitrophenol
Tr1	T Regulatory Type 1
TRAIL	TNF Receptor Apoptosis-inducing Ligand
Treg	Regulatory T Cell
VEGF	Vascular Endothelial Growth Factor
WASP	Wiskott-Aldrich Syndrome Protein
WT	Wild Type

Introduction

1. Introduction

1.0.0.0. Immune System

To reach homeostasis, evolution built up several systems within an organism, such as the neural system, the endocrine system, and the immune system. These systems react to external stress or internal dysregualtion and help the organism to overcome such challenges. Reciprocal regulations are found between different systems. For example, the central nervous system (CNS) can regulate the immune system through hormones and direct neural signaling. On the other hand, the immune system affects neurological functions through various cytokines [1].

The immune system is responsible for both external and internal threats. It fights against invading pathogens from outside and also monitors malignant cells inside of the body. Based on response dynamics, immunological memory formation, and antigen recognition, the immune system can be divided in two rough categories: the innate immunity and the adaptive immunity [2]. Innate immunity serves as cardinals to sense dangers and alert the whole immune system. Antigen presenting cells (APCs) from innate cells such as dendritic cells [3] and macrophages [4] will present antigens to T cells in the adaptive compartment. In addition, APCs direct T cell homing to inflammatory sites through chemokines and produce cytokines like IL-12 and TNFα to help T cell activation [3]. Reciprocally, T cell-derived cytokines affect the differentiation and activities of APCs [3]. The boundary can somehow be blurred by cells like innate lymphoid cells (ILCs) [5-7] and nature killer T (NKT) cells [8], which possess characteristics from both sides and by which innate and adaptive immunity may also be bridged and collaborate.

1.1.0.0. Innate Immunity

Once the immune system is triggered by danger signals [9], an immune response is initiated (Fig. 1.1). Danger signals may be derived from external invading pathogens (pathogen-associated molecular patterns, PAMPs) or internal damaged cells (damage-associated molecular patterns, DAMPs) [9, 10]. PAMPs, like sugar chains, DNA, or RNA will be recognized by soluble factors or pattern recognition receptors (PRRs) [10, 11] of innate immune cells. Toll-like receptors (TLRs) [12] are for example the most well-defined PRRs. PRRs of innate cells can also detect DAMPs derived from cytosolic or nulear components of necrotic cells [13]. Innate immunity serves the first wave of recognition, pathogen elimination, and clearance of undesirable wastes or debris. In addition, it initiates the adaptive immunity afterwards through antigen presentation, chemotaxis, and cytokine secretion.

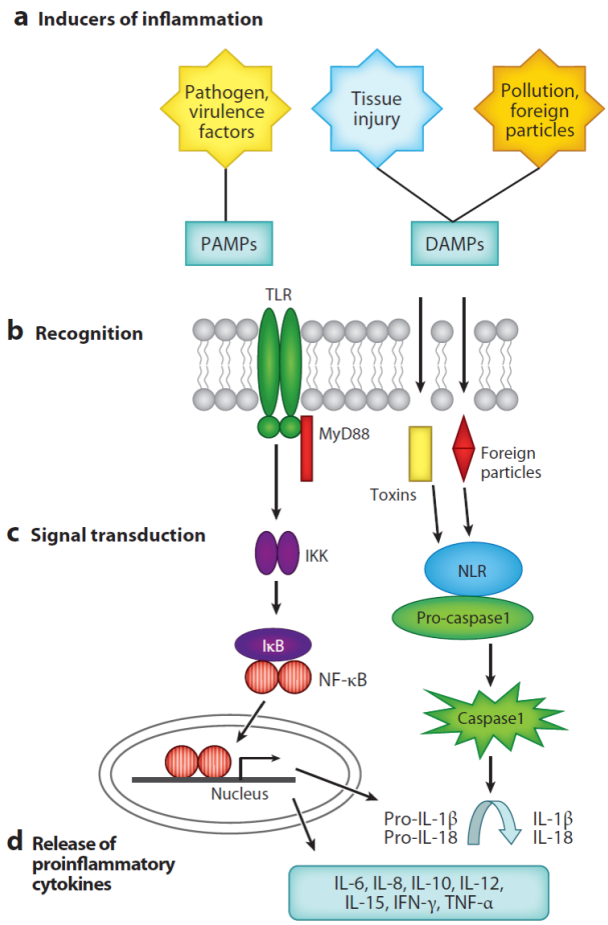


Figure 1.1 Initiaton of innate immunity by the pattern recognition receptors (PRRs). Molecules from invading pathogens, damaged tissues, or toxic particles can be recognized by pattern recognition receptors (PRRs) either on the cell surface or inside of the cell. The signals relayed then may induce the production of pro-inflammatory cytokines and chemokines as well as phagocytic and antigen presentating activities by the stimulated innate immune cells. The cytokines and chemokines mediate the differentiation and migration of both innate and adaptive immune cells while the antigen presentation bridges the innate and adaptive compartments together and promote further immune responses. The figure is derived from *Ashley, et al., Annu. Rev. Ecol. Evol. Syst. 2012, 43:385–406*

Soluble factors, such as complement proteins [14], natural antibodies [15], or Type-I interferons [16], are essential for innate responses. For example, complement proteins can form attacking complexes directly on the surface of bacteria and destroy targets by damaging the cell wall. The complexes are also involved in building up pro-inflammatory signals for further response cascades [14]. While the complement proteins work directly on the target pathogens, the interferons function through indirect routes. Type-I interferons trigger the intracellular anti-viral machinary and therefore help cells setting up barriers to defend themselves [16].

Cellular compartments including both hematopoietic immune cells and tissue cells contribute to innate immunity. Other than immune cells, various tissue cell types, including epithelium cells, fibroblasts, and mesenchymal stem cells (MSCs), can present antigens [17, 18], produce cytokines [19] and regulate the homing of T cells [18].

1.2.0.0. Adaptive Immunity

Specific antigen recognition and memory formation are the most critical properties which distinguish adaptive immunity from innate immunity. Through mechanisms, like clonal expansion, somatic hypermutation, and epitope editing, the adaptive immune system can select and refine its specificity and efficiency. Though delayed at the first moment when encountering new threats, adaptive immunity serves as a more powerful and specific defending system for the organism. The major humoral components for adaptive immunity are antigen-specific antibodies, which are produced by B cells. The cellular parts are composed by T cells and B cells.

1.2.1.0. T Cells

The 'T' for T cells is derived from the first letter of thymus, in which the T cells mature. T cells express T cell receptors (TCRs) on their surface to recognize specific peptides [20] presented by major histocompatibility complex (MHCs) on antigen-presenting cells (APCs). By different combinations of peptide chains expressed to form heterodimeric TCR, T cells can be identified as αβT or γδT cells [21]. Based on other surface markers, αβT cells may be further divided into $CD4^+$, $CD8^+$, and NKT cells [22, 23]. In general, $CD4^+$ T cells are "helpers" which guide the differentiation and functions of other immune cells. Specific regulatory populations, the regulatory T cells (Treg) [24, 25], serve as immunological brakes to cease immune responses. On the other hand, most $CD8^+$ T cells are canonical effectors which carry out the cytotoxic responses to kill target cells. However, like in the case of $CD4^+$ T cells, some $CD8^+$ T cells are suppressors for immune responses and possess regulatory capacity [25]. NKT cells are T cells recognizing antigens loaded on CD1d rather than MHCs on APCs [8]. They are not only capable to kill the target cells but also have been described to help B cells or become regulatory during immune responses [26, 27]. Through out the life span of one T cell, combinations of signals and quality of one single signal can control the fate of T cells. Among all the diverse signals including cytokines and co-stimulatory molecules, TCR signaling is the central event. The signaling strength as well as signaling duration of TCR make indispensable contribution to survival, development, differentiation, effector functions, and memory formation of T cells [28-34]. TCR signaling can be tuned by both external and internal factors. The affinity of antigens, the conformation of MHC molecules, the interaction duration and the timing during immune responses are all external factors contributing to TCR signaling. On the other hand, the co-receptors, cytoplasmic adaptor proteins, and other signaling molecules are internal factors to modulate the TCR signaling.

1.2.1.1. TCR Signaling Strength in T Cell Lineage and Fate

T cell precursors develope from hematopoietic stem cells in the bone marrow and then migrate to the thymus for further maturation [21]. In the thymus, T cells have to survive positive and negative selection to become mature and emigrate to the periphery [30]. The survival in the two selection processes depends on signaling strength of TCR. T cells bearing TCRs with extremely low affinity and therefore transducing very weak signals can not initiate sufficient survival signals to pass positive selection. On the other hand, the T cells which survive positive selection but express TCRs with extremely high affinity and produce very strong TCR signaling are eliminated by apoptosis in negative selection [30, 35] (Fig. 1.2).

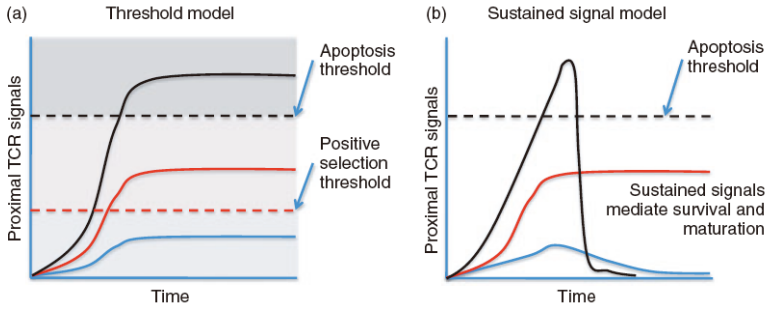


Figure 1.2 TCR signaling strength determines fate of T cells in thymic selection. While extremely strong TCR signals lead to apoptosis in negative selection, insufficient TCR signaling causes cell death or ignorance in positive selection. Only T cells with TCR signals in optimal range can survive and mature. Model (a) is relatively simple and considers only whether the TCR signal strength reaches thresholds defined in positive or negative selection. Once the threshold is reached, the fates of T cells are decided. To be noted, if the apoptosis fails to eliminate doomed cells, the escaped cells will be kept alive. Model (b) takes the duration of signals into consideration. Only when the optimal signals are sustained the T cells can survive. Overall, strength and duration of TCR signaling are essential for thymic selection. The chart is derived from *Moran and Hogquist, Immunology 2012, 135, 261–267*

Moreover, the signaling strength of TCR can determine not only the survival but also the fate between αβ/γδ T cell lineages in this stage (Fig. 1.3). In early stage of development (the $CD4^-CD8^-$ double negative stage "DN"), T cells express γδ TCR isoforms. Lower signaling strength from γδ TCR favours T cells to express αβ isoforms instead. However, stronger signaling promote T cells to maintain themselves in the γδ lineage [28, 36, 37]. Overall, TCR signaling is the central event to decide the developmental fate of T cells in the thymus.

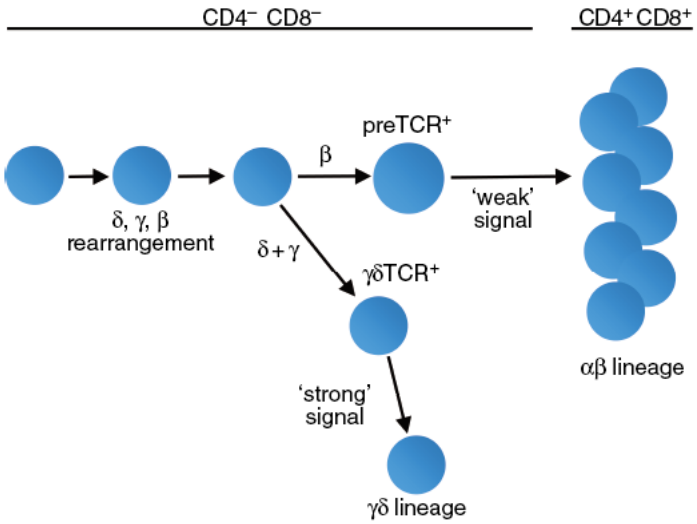


Figure 1.3 TCR signaling strength favors αβ/γδ T cell fates. γδ TCR is expressed earlier than the TCRβ chain during the development. Strong signals from γδ TCR will maintain the development of γδ T cells. Relatively weak γδ TCR signals favors the commitment of αβ lineage. The figure is derived from *Hayes and Love, Immunological Reviews 2006, Vol. 209: 170–175.*

Signaling strength of TCR also decides whether T cells can be activated and go on further differentiation as well as effector function. Inappropriate TCR signaling, either too high or too low, will lead to cell death or unresponsiveness. Only when appropriate TCR signaling occurs T cells can be activated [38, 39]. After initial T cell activation, the signaling strength of TCR is coordinated with other signals, such as cytokines, to drive T cells into diverse differentiation [29]. During differentiation T cells acquire specialized functions, cytokine profiles, and chemotaxis properties along with expression of specific master transcriptional factors. For example, $CD4^+$ T cells can differentiate into lineages such as Th1, Th2, Th17, Tfh (follicular helper T), or Treg. Newly defined populations like Th9 or $Th_{GM\text{-}CSF}$ keep emerging on the list [40]. Previous studies have shown that stronger TCR signaling promotes Th1 [41] or Tfh [42, 43] (also Th2 when the TCR signal is extremely high [44]) differentiation while lower strength favours Th2 [45] or Th17 [46] (Fig. 1.4). On the molecular basis, TCR signaling strength decides the phosphorylation of signaling molecules which can essentially contribute to the production of respective cytokines [47-49].

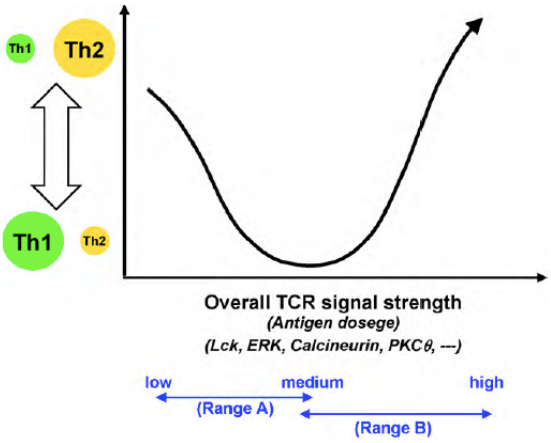


Figure 1.4 TCR signaling strength favors Th1/Th2 differentiation. Antigen dosages, TCR affinity, co-receptors, co-stimulatory molecules, and intracellular signaling complexes contribute to spectrum of TCR signaling strength. Extremely high or low strength favors Th2 differentiation while the medium range confers Th1-promoting capacity. The figure is derived from *Nakayama and Yamashita, Seminars in Immunology 2010, 22:303–309*

1.2.1.2. Nck Adaptor Proteins for Tuning of TCR Signaling Strength

Nck proteins are adaptor proteins, which exhibit no enzymatic activities but possess protein-interacting domains to bring enzymes and substrates together [50]. Nck1 and Nck2 are highly homologous, widely expressed adaptor proteins, which contain three Src homology (SH)3 domains and a single SH2 domain [51] (Fig. 1.5). In many cell types, the Nck proteins link phosphotyrosine signals to actin cytoskeleton reorganization through the Wiskott-Aldrich syndrome protein (WASP) and the p21 activated kinase (PAK) [51] (Fig. 1.5). Nck adaptor proteins are also key players to enhance and tune TCR signaling strength [52, 53]. The involvement of Nck in TCR signalling [38] has been suggested on the basis of its binding to CD3ε [54, 55] and SLP-76 [56] (Fig. 1.5). Using a conditional knock-out approach, it was demonstrated that *in vivo* Nck deletion induced a severe impairment in thymic selection of low avidity T cells [52]. In peripheral lymphoid organs, Nck deletion resulted in profound T cell lymphopenia and hypo-reactivity to TCR-mediated stimulation. Nck-deficient T cells expressing TCRs with low avidity for self-antigens were strongly reduced, while T cell proliferation was defective upon weak antigenic stimulation. Downstream TCR signaling events such as phosphorylation of ERK and the calcium influx are found to be diminished [53]. Thus, Nck adaptors reduce the threshold of TCR responsiveness in both developing and mature T cells.

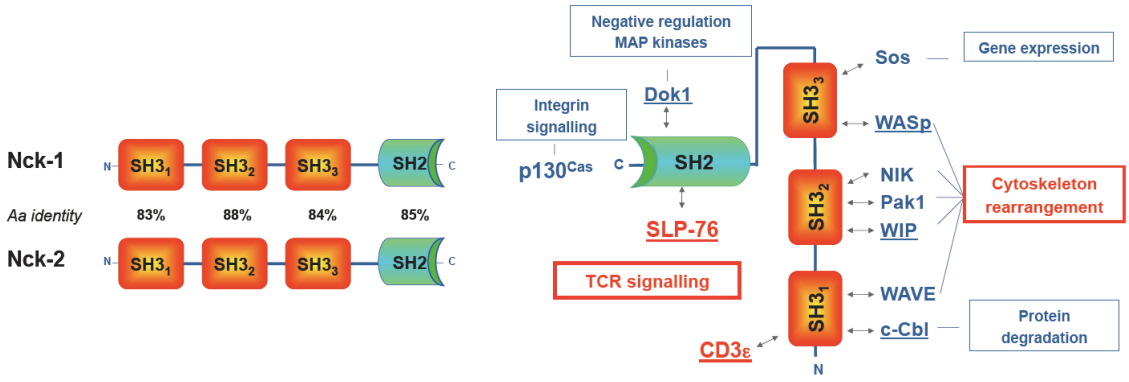


Figure 1.5 Nck protein structure and interacting partners. Nck proteins are composed with 3 SH3 domains and 1 C-terminal SH2 domain. Two highly conserved homologues are found in the murine system. Various interacting partners are shown.

1.2.2.0. B Cells

B cells are named after an avian-specific lymphoid organ, 'bursa', where they were first classified. B cells originate from HSCs and then mature in the bone marrow [57, 58]. They express B cell receptors (BCRs) on their surface to recognize antigens directly [58]. B cells can be classified into B1 [15] and B2 cells, with low expression of CD45R(B220) for B1 and high expression for B2 cells. B1 cells can be further defined by CD5-positive B1a and CD5-negative B1b cells [15]. They are mainly resident cells

within body cavities such as the peritoneal cavity [59]. By contrast, B2 cells can circulate or reside in spleen, lymph nodes or bone marrow. After activation by signaling of BCRs, B cells home to secondary lymphoid organs to proliferate, differentiate, and form germinal centers (GCs) [60, 61]. Most B cells, especially B2 cells respond in adaptive immunity to produce antibodies. B1 cells can take part in innate immunity by producing natural antibodies [15] to recognize invading pathogens and also autoantigens [62] at the very beginning of immune responses. To improve the affinity and quality of antibodies, B cells may undergo antibody class-switching and somatic hypermutation. These processes will lead to so called affinity maturation. Affinity maturation occurs within the GCs [61]. To initiate affinity maturation, B cells require help from T cells with cell-cell contact [43, 63]. In addition, cytokines such as IL-4, IL-10, and IL-21 produced by T cells are essential for the survival and affinity maturation of B cells [43, 63]. Similar to T cells, BCR signaling strength is also critical for the differentiation and effector functions of B cells [58]. Nck adaptor proteins, which are expressed also in B cells and enhance BCR down-stream signaling, were found to be important for efficient antibody production [64].

1.2.3.0 Follicular Helper T Cells (Tfh)

Within the germinal centers, B cells require help from T cells [63, 65-67], mainly from follicular helper T (Tfh) cells [43, 63, 68] (Fig. 1.6), to proliferate, differentiate, and produce high affinity antibodies through class-switching and somatic hypermutation. The name 'follicular' is derived from B cell follicles in secondary lymphoid organs. Tfh cells can be defined by the expression of CD4, CXCR5, and other markers, like GL-7, ICOS, and PD-1 [43, 69, 70] (Fig. 1.6). Specific cytokines such as IL-4, IL-10, IL-21 can be produced by Tfh. Bcl-6 is reported to be the master transcriptional factor for Tfh [71-73] which may be supported by low expression of other transcriptional factors [74]. Several models are proposed for the origin of Tfh cells [43]. Some findings suggest that the Tfh cells are only the B cell follicle-resident counterparts of other T helper populations [43].

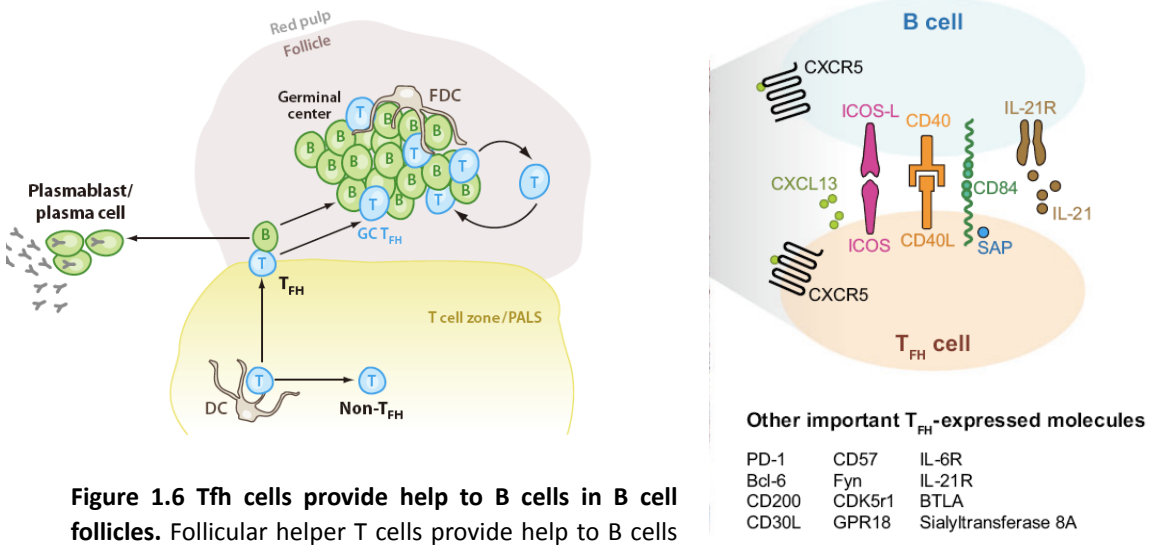


Figure 1.6 Tfh cells provide help to B cells in B cell follicles. Follicular helper T cells provide help to B cells within the B follicles through cell-cell contact and soluble factors. The figure is derived from *King, et. al., Annu. Rev. Immunol. 2008, 26:741–66 and Crotty, Annu. Rev. Immunol. 2011, 29:621–63.*

1.3.0.0. Regulation of Immune Responses: Immune Tolerance

During lymphocytes development, TCRs or BCRs are generated by recombination of a limited repertoire of gene segments in somatic immune cells. The recombination assembles randomly selected V(variable), D(diversity), and J(joining) segments by RAG (recombination activating genes) recombinases [75] to form immune receptors. This process, named V(D)J recombination [76], is conserved among vertebrates through the evolution [77]. The randomized recombination, together with individual variations in one single gene locus, mutations, and also secondary recombination [78], created tremendous variety of receptors for antigen-specific recognition [76]. However, the randomly created specificities inevitably generate autoreactive receptors [79, 80] which recognize self antigens.

Once immune responses against foreign antigens are initiated, there must be ceased in order to repair the damaged tissues and restore homeostasis. If the immune responses cannot be regulated, as observed in dengue virus infection [81] and sepsis [82], catastrophic cytokine storm and multiple organ failure may be induced [9]. For example, during the 1918 Spanish flu [83, 84] or SARS [85] outbreak in 2003, massive death was not only caused by the viral virulence but also by excessive immune responses. Many victims were immune competent adults or youth and were killed by overwhelming cytokine storm and respiratory failure caused by lung-infiltrating immune cells [83, 85]. Furthermore, immune responses in different areas show qualitative and quantitative differences. Destructive immune responses have to be prevented in tissues with a limited capacity of regeneration, such as the central nervous system [86], eyes [86], liver [87], testis [88], placenta [89], and hair follicles [90]. The concept of so-called immune privilege [91-95] was initially based on the observation that skin allografts were rejected when placed on the skin of a recipient but were accepted in the anterial chamber of the eye [89]. It has been shown that immune privilege can be established by both active and passive processes [91, 92].

During pregnancy, paternal antigens from the fetus can also be taken as foreign antigens. Dysregulated immunological tolerance during pregnancy can lead to immune responses against fetus and subsequent abortion [96-98].

Several mechanisms exist to tolerize immune responses against food antigens to avoid food allergy [99]. If these mechanisms fail, allergic diarrhea, malnutrition, and even lethal allergic responses could be induced [100]. Desensitization and tolerance induction [101] are important clinical application of immunue tolerance [102] to treat the food allergy.

To avoid undesirable immune responses, either autoreactive or excessive, the immune system is equipped with mechanisms defined as immune tolerance [103]. Tolerance represents a ‘physiological state in which the immune system does not

react destructively against the organism that harbours it', which is quoted from the definition by Ronald H. Schwartz in 1993 [104]. It can be achieved according to two principles. One is to delete, allegedly to 'purge', autoreactive cells from the immune repertoire. The most well known mechanism to carry out this part is clonal deletion. The other principle is to compromise, allegedly to 'tune', autoreactivity or excessive immune responses by receptor editing, clonal anergy, or by regulation [105, 106]. Immune tolerance can be further categorized into central or peripheral tolerance as discussed below.

1.3.1.0. Central Tolerance

Central tolerance is used as term to summarize processes that induce autoreactive lymphocytes tolerance in primary lymphoid organs such as the thymus and the bone marrow [80, 107, 108]. Taking T cells as examples, thymic epithelial cells (TECs) express self-antigens in a randomized manner [107] by promiscuous gene expression [109], which is regulated by AIRE [110]. Most autoreactive T cells are eliminated or become hyporesponsive. Only cells surviving the negative selection can mature and emigrate from the thymus to the periphery. In addition, natural regulatory T cells (nTregs) develop in the thymus and are exported to the periphery to control immune responses [106].

nTregs are phenotypically identified as $CD4^+CD25^+Foxp3^+$ T cells [25]. Foxp3 is the master transcriptional factor of the nTreg lineage [111] although it can be transiently expressed by newly activated effector T cells [112, 113]. Scurfy mice, in which the Foxp3 gene is deleted, develop sever autoimmune diseases [114-116] while in humans the corresponding dysfunction of the human Foxp3 gene leads to the IPEX syndrome (immunodysregulation, polyendocrinopathy, enteropathy, X−linked syndrome) [117-119]. Strong TCR signaling strength favours the development of nTreg [120, 121] (Fig. 1.7). The expression of Foxp3 [122] as well as cytokines like IL-2 [123] and TGFβ [124] are essential to support the development of nTreg. Enhanced NFκB activities also favour the expression of Foxp3 and therefore the development of nTreg [125]. As strong TCR signaling risks clonal deletion of the developing T cells by negative selection, a two step model is proposed to link the requirements of strong TCR and pro-survival activities of cytokines like IL-2 [126, 127]. Thus, strong TCR signaling up-regulates the expression of CD25, the high-affinity receptor of IL-2, and therefore increases the pro-survival signals to counteract with the driving force of negative selection [126].

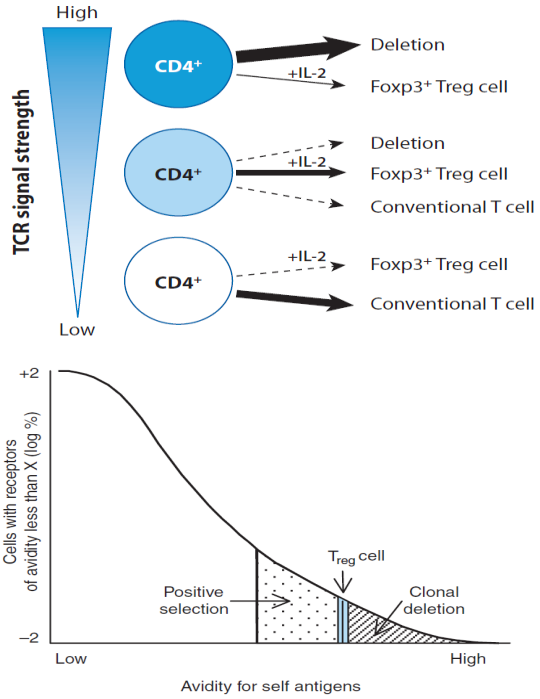


Figure 1.7 Optimal TCR signaling strength drives developing T cells toward the fate of regulatory T cells. The weight of arrows stands for the probability of different developmental fates. Basically, strong TCR signaling risks clonal deletion of the developing T cells and low TCR signaling favours the development of conventional T cells. Foxp3$^+$ nTregs require optimal range of TCR signaling, which is relatively narrow, and the support from IL-2. Note that nTreg still can be raised from other suboptimal signaling strength but only with less probability. The model of TCR signaling strength may explain the low composition ratio (10-20%) of Tregs to the total T cells. The upper and lower figures are derived from *Josefowicz, et al., Annu. Rev. Immunol. 2012, 30:531–64* and *Schwartz, Nat. Rev. Immunol. 2005, 6(4):327-330* respectively.

1.3.2.0. Peripheral Tolerance

Despite of central tolerance induction, autoreactive lymphocytes are still found in the periphery and have to be controlled by mechanisms of peripheral tolerance. Here, the respective cell populations [25, 128, 129] and soluble factors will be discussed.

1.3.2.1. Tolerogenic Cells: Adaptive Immunity Compartment

Among the adaptive immune cells, regulatory T cells (Tregs) [130, 131], regulatory B cells (Breg) [132, 133], and NKT cells [134, 135] are responsible for regulating immune responses. Taking Tregs as example, they can be classified by their origins into the already mentioned nTregs [131] and the inducible regulatory T cells (iTreg) [130].

nTreg cells can produce Immunoregulatory cytokines such as IL-10 [136] and TGFβ [137] to suppress immune responses [25, 121]. High expression of CD25 [121, 131], the high affinity receptor of IL-2, enables nTreg to deprive the pro-survival IL-2 in the environment and to compete out effector T cells from survival and productive immune responses [138]. nTreg can also indirectly regulate activities of other T cells by inducing tolerogenic dendritic cells via downregulating co-stimulatory molecules on dendritic cells [25, 139].

iTregs include various types of regulatory T cells induced in the periphery under diverse conditions [130]. For example, IL-10 can induce T regulatory type 1 (Tr1) cells [140, 141]. $CD103^+$ dendritic cells in the gut-associated lymphoid tissues (GALT) can induce regulatory T cells by presenting antigens along with production of TGFβ and rentinoid acid [142-144]. Immature dendritic cells [145, 146] and mTOR-mediated metabolic conditioning [147, 148] also drive distinct iTreg populations in different milieus. iTreg can be induced to restrain ongoing immune responses in infections, autoimmune diseases, or tumor/transplant rejection [25, 130]. iTreg cells may also be induced in the peripheral tissues such as in mucosal system [149]. By long-term and low-dose exposure to common antigens such as food antigens or commensal flora, iTreg can be induced and conduct peripheral tolerance in specific sites [150].

1.3.2.2. Tolerogenic Cells: Innate Immunity Compartment

Immature dendritic cells (iDCs) express only low levels of co-stimulatory molecules, such as CD80 and CD86, and are barely capable to produce cytokines such as IL-6, IL-12, or TNFα. Instead, they produce soluble immunosuppressive factors, such as IL-10, TGFβ and indoleamine 2,3-dioxygenase [108, 146, 151-153]. These properties favour the differentiation of iTreg. In addition, dendritic cells can induce clonal deletion of autoreactive T cells.

Macrophages can differentiate into classically activated proinflammatory M1 or alternatively activated immunosuppressive M2 macropages depending on the environmental conditions [154, 155]. M2 macrophages can be identified by high expression of the mannose receptor CD206 [156]. They are associated with wound healing and are capable to suppress immune responses by producing polyamines [154, 157] and high levels of IL-10 but reduced levels of the proinflammatory IL-12 [154]. Tryptophan metabolites produced by macrophages are also reported to suppress T cells [158]. Inside of solid tumors, tumor associated macrophages (TAMs), in particular M2 macrophages, are often recruited and correlated with tumor growth and poor prognosis [128, 155].

Furthermore, myeloid-derived suppressor cells (MDSCs), covering a broad spectrum of immature myeloid cells, can produce radical oxygen species (ROS) or nitric oxygen (NO) to inhibit T cell activities directly. Moreover, they can recruit nTregs and induce iTregs inside of the inflammatory sites or tumors [25, 128].

1.3.2.3. Tolerogenic Cells: Peripheral Tissues

Other than the professional immune cells, peripheral tissue cells, such as hepatocytes [159-162], hepatic stellate cells [163-165], and liver sinusoidal endothelium cells (LSECs) [166-168] in the liver are also capable to induce peripheral T cell tolerance (Fig. 1.8). In the case of hepatocytes, they can tolerate $CD8^+$ T cell

responses by down-regulating the expression of TCR on the surface of autoreactive $CD8^+$ T cells [159]. They also provide insufficient co-stimulatory signals thereby causing activation induced cell death (AICD) [169-171]. Moreover, they can express the ligand of programmed death 1 receptor (PD-1L) to impair the cytotoxic activities of $CD8^+$ T cells [172]. Hepatic stellate cells also express PD-1L [163] as well as TNF receptor apoptosis-inducing ligand (TRAIL) [173] to induce the apoptosis of T cells. In addition, they promote the IL-2-dependent Treg expansion [164], by which along with induction of T cell apoptosis the hepatic stellate cells may help pancreatic islet allografts to survive [129, 165]. Besides hepatocytes and hepatic stellate cells, LSECs are also potent inducers of peripheral tolerance in the liver by inducing $CD4^+Foxp3^-$ Treg [168], tolerizing $CD8^+$ T cells with PD-1 signaling [174], and leading to FasL-Fas mediated apoptosis of T cells [167, 175]. The capacities of hepatic cells to induce tolerance are important since the liver is a major site for the body to encounter foreign antigens, including food antigens. In humans, about 1.5 liter of blood passes through the liver every minute and the whole volume of blood circulates through the liver for about 360 times per day [104].

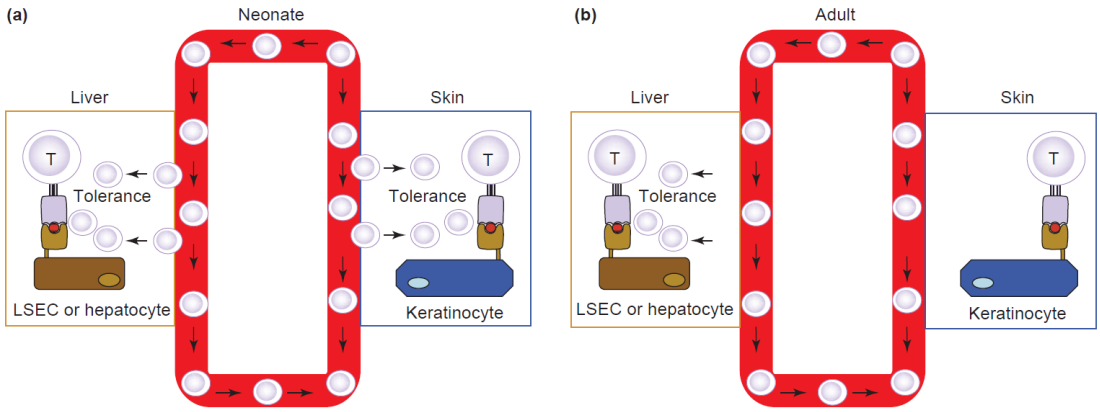


Figure 1.8 Peripheral tolerance induced by tissue cells of neonates or adults in homeostasis. Hepatic cells including hepatocytes and LSECs are capable to induce peripheral tolerance of T cells in both neonates and adults since T cells are capabel to reach them throughout the life time. However, keratinocytes are only tolerogenic in neonates because the accessibility of skin for circulating T cells is limited. The figure is derived from *Arnold, et al., TRENDS in Immunology 2005, 26(8):406-411*

The accessibility of naive lymphocytes to tissues is limited in adults [176] (Fig. 1.8). However, in neonates, naive T cells are able to travel into tissues and encounter antigens, for example in the skin [177] (Fig. 1.8). Our group showed that mice, which expressed an antigen on keratinocytes in hair follicles, were tolerant to skin and tumor transplants expressing this antigen [177]. Tolerance was induced during the neonatal life, when the skin is accessible to naive T cells, and was maintained by long-lived $CD8^+$ regulatory T cells [178, 179]. These $CD8^+$ regulatory T cell prevented activation of respective antigen-specific naïve $CD8^+$ T cells which were newly exported from the thymus during adulthood.

1.3.2.4. Tolerogenic Cells: Mesenchymal Stem Cells (MSCs)

Mesenchymal stem cells (MSCs) are tissue-resident stem cells which exhibit multipotency giving rise to various cell lineages [180, 181] (Fig. 1.9).

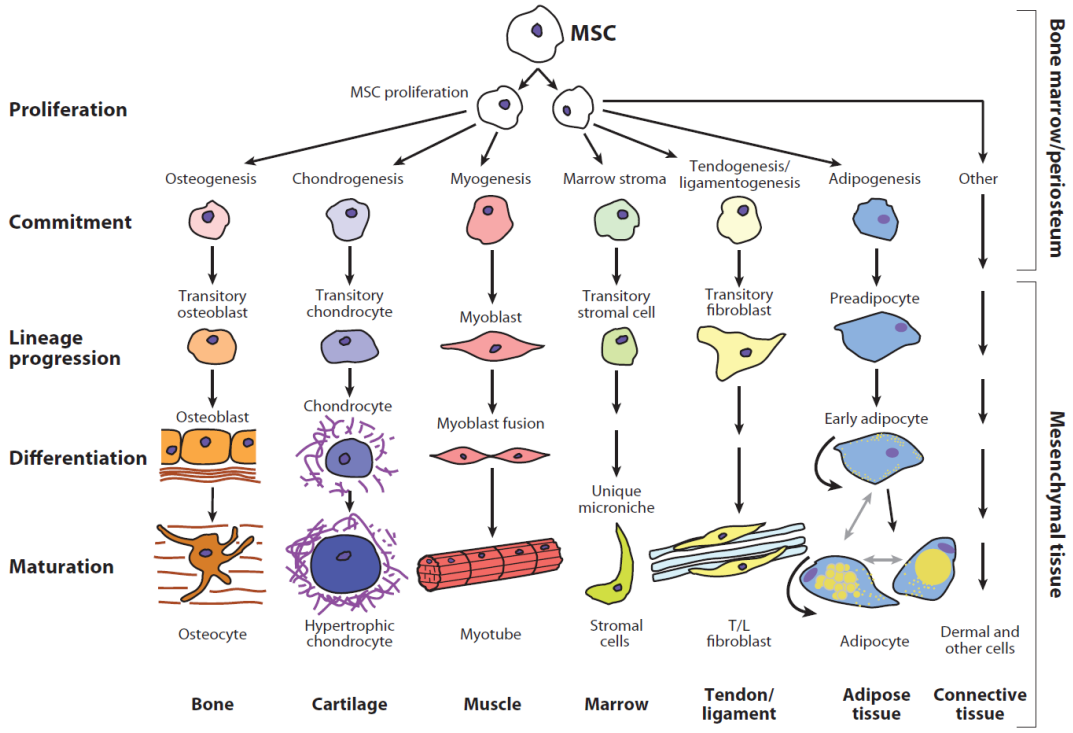


Figure 1.9 MSCs can give rse to multiple lineages of tissue cells. The figure is derived from *Singer and Caplan, Annu. Rev. Pathol. Mech. Dis. 2011, 6:457–78*

MSCs are derived from adult tissues and taken as promising substitution for embryonic stem cells for regenerative medicine. However, despite of their well-known roles in tissue repairing and renewing, accumulating data suggest that MSCs are involved in limiting undesirable immune responses, such as hypersensitivity, autoimmune disease, or alloreactivity against transplants [180, 182, 183]. It has been reported that MSCs can suppress human graft-versus-host disease (GvHD) [184, 185] and experimental autoimmune encephalomyelitis (EAE) [186, 187], which is the murine model for human multiple sclerosis (MS). Also in the contact-dependent hypersensitivity disease, systemically infused MSCs can sufficiently ameliorate local immune responses [188]. A long list of clinical trials has been launched to test the possible applications of MSCs to regulate immunological diseases [180, 183, 189] (Table 1.1).

Indication	MSC type	Phase*	Sponsor and location	Clinical trial number‡
Graft-versus-host disease	Allogeneic BM-MSCs	Expanded access	Osiris Therapeutics, USA	NCT00826046
	Allogeneic BM-MSCs	I/II	UMC Utrecht, The Netherlands	NCT00827398
	Allogeneic BM-MSCs	II	University Hospital of Liege, Belgium	NCT00603330
	Allogeneic umbilical cord MSCs	I/II	Hadassah Medical Organization, Israel	NCT00749164
	Allogeneic adipose tissue MSCs	I/II	Fundacion Progesio y Salud, Spain	NCT01222039
Crohn's disease	Allogeneic BM-MSCs	I/II	Leiden UMC, The Netherlands	NCT01144962
	Autologous adipose tissue MSCs	I/II	Universidad de Navarra, Spain	NCT01157650
	Allogeneic BM-MSCs	III	Osiris Therapeutics, USA	NCT00482092
	Allogeneic adipose tissue MSCs	I	Anterogen, South Korea	NCT01440699
Ulcerative colitis	Allogeneic umbilical cord MSCs	I/II	Qingdao University, China	NCT01221428
Multiple sclerosis	Autologous BM-MSCs	I	Cleveland Clinic Foundation, USA	NCT00813969
	Allogeneic umbilical cord MSCs	I/II	Shenzhen Beike Bio-Technology, China	NCT01364246
Systemic lupus erythematosus	Allogeneic BM-MSCs	I/II	Nanjing Medical University, China	NCT00698191

Table. 1.1. Examples for clinical trials of MSC-mediated treatments on inflammatory diseases. The table is derived from *Le Blanc and Mougiakakos, Nat. Rev. Immunol. 2012, 12:383-396*

Besides these promising clinical applications for the immunoregulatory capacities, MSCs have been reported to support the tumor growth [190] by promoting the formation of tumor stroma [191], inducing angiogenesis[192, 193], and maintaining cancer stem cells [194] (Fig. 1.10). MSCs may also promote the metastasis of tumors by producing chemokines, such as CCL5 in the case of breast cancer [195]. MSCs help tumors to escape anti-tumor immunity by inhibiting T cells proliferation [196, 197] or by inducing regulatory T cells [198]. TGFβ is one of the central mediators for the protumoral vicious cycle (Fig 1.10).

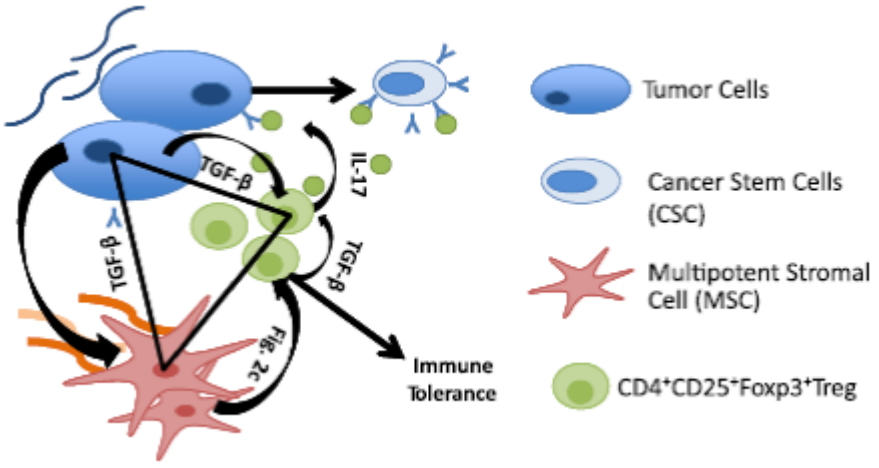


Figure 1.10 MSCs are part of the Vicious Triangle to inhibit anti-tumor immunity and promote tumor growth. TGFβ production by MSCs and tumor cells promotes tumor growth by enhancing the formation of tumor stem cells (CSCs) and induction of regulatory T cells (Treg). The figure is derived from *Ilmer, et al., FASEB J. 2014, 28:1-14*

MSCs are sensitive to immunological stimuli. They can express pattern recognition receptors, such as Toll-like receptors (TLRs) and NOD-like receptors (NLRs) [183]. In addition, they also express complement receptor C3aR and C5aR [183]. They are sensitive to cytokine stimulations by IL-1β and IFNγ [183]. The respective receptors enable MSCs to respond to inflammation and initiate their immunoregulatory functions (Fig. 1.11) by production of immunosupressive TGFβ [183] (Fig. 1.10), prostaglandin E2 (PGE2) and kynurenine (KYN). These soluble factors can repolarize M1 proinflammatory macrophages into immunosupressive M2 macrophages [183] and also can directly modulate T cell function [199, 200].

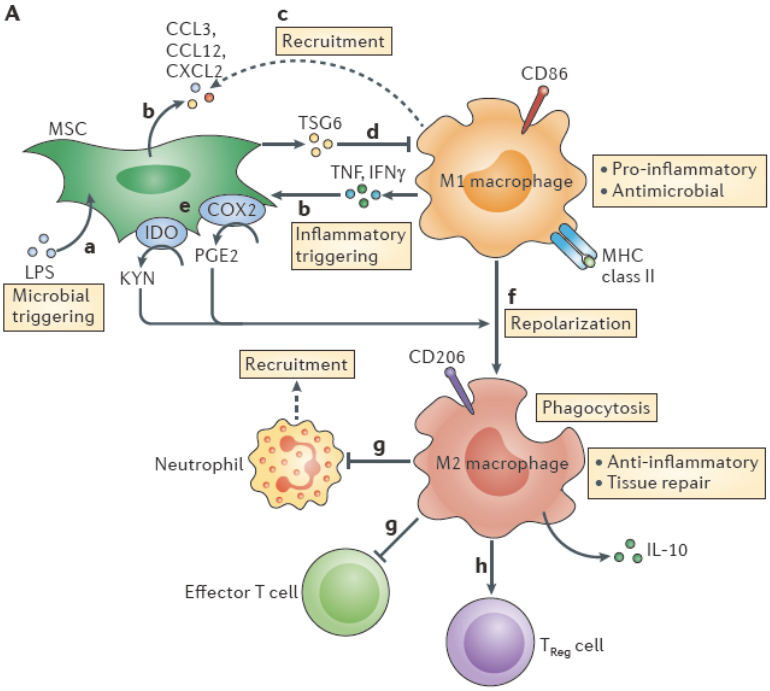


Figure 4.2 MSC regulates differentiation of macrophages. The figure is derived from *Katarina Le Blanc and Dimitrios Mougiakakos, Nat. Rev. Immunol. 2012, 12:383-396*

In addition to these soluble factors, cell-cell contact-mediated mechanisms are also contributing to MSC-mediated immune regulation [18, 196, 201]. MSCs are capable to express MHC molecules and act as antigen presenting cells to T cells [17, 18, 182, 202-204]. MHC class I expression is up-regulated in IFNγ-stimulated MSCs [205]. By contrast, expression of MHC class II molecules is often negligible [206-208] and only found in a narrow window of IFNγ stimulation [202]. Low expression of MHC class II decreases the allogenicity and consequential allogeneic rejection of MSCs during transplantation [206-208].

MSCs may induce regulatory $CD4^+$ or $CD8^+$ T cells by direct cell-cell contact [209]. They inhibit $CD8^+$ T cells in mixed lymphocytes responses and in rejection of allogenic tumors [210]. MSCs can also induce unresponsiveness of T cells by affecting the maturation of antigen presenting cells [211]. MSCs favour the differentiation of T cells from Th17 phenotypes to Tregs [183]. MSCs may inhibit the *de novo* Th17 differentiation [212, 213] directly or reprogram the existing Th17 cells [212].

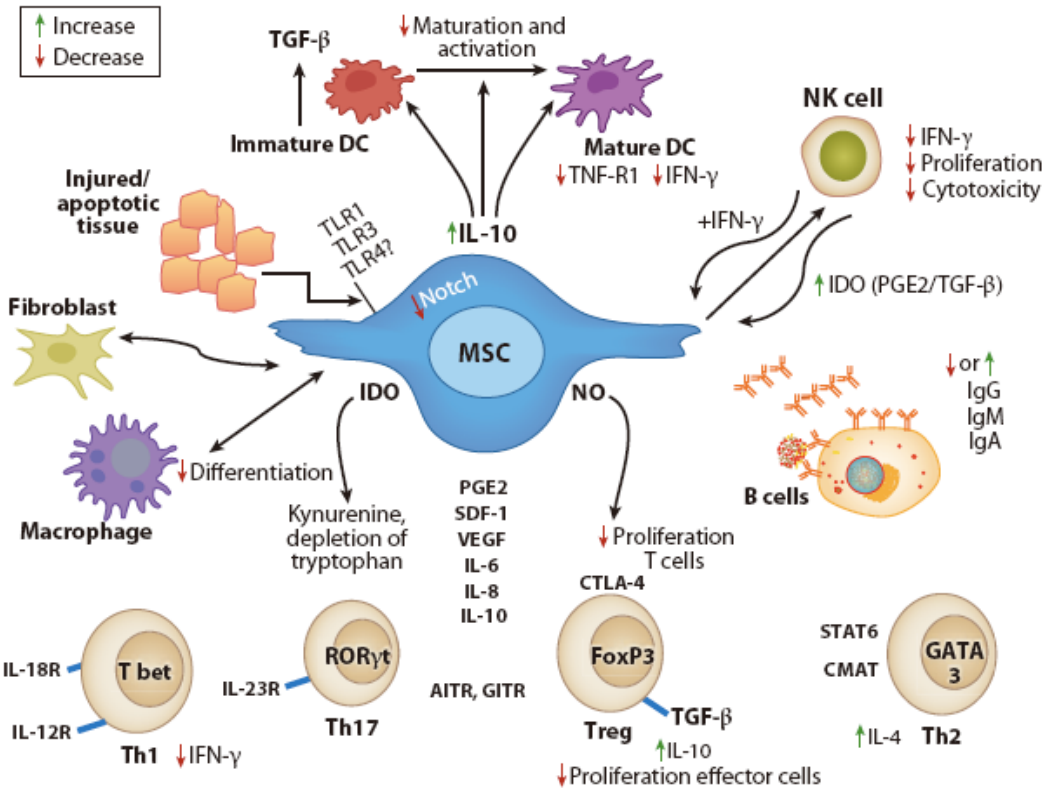


Figure 1.11 Mechanisms of MSC-mediated immune regulations. The figure is derived from *Singer and Caplan, Annu. Rev. Pathol. Mech. Dis. 2011, 6:457–78*

Taken together, cells from both innate and adaptive immune compartments as well as non-hematopoietic cells can contribute to immunological tolerance.

1.4.0.0. Dickkopf-3 (DKK3) and Immunoregulation

Dickkopf-3 (DKK3) belongs to the Dickkopf protein family (DKKs) [214-216], which contains only 4 evolutionary conserved members (DKK1-4) and 1 distantly related member, Soggy (Dickkopf-like 1, DKKL-1) [214, 215] (Fig. 1.12). DKK1/2/4 possess around 46% to 50% of homologous sequences between each other while DKK3 shares only 37% to 40% with all three others [215]. Besides less homology in sequence [214], DKK3 is also functionally distinct (Fig. 1.13).

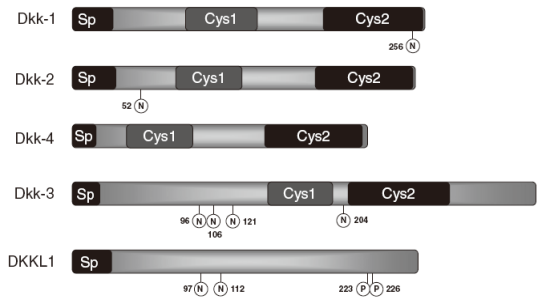


Figure 1.12 Structures of DKKs. The figure is derived from *Veeck and Dahl, Biochimica et Biophysica Acta 2012, 1825:18–28.*

DKK1, DKK2, and DKK4 are found to be antagonists of the canonical Wnt/β-catenin signaling pathway (Fig. 1.14) by competing with Wnt ligands for the Lrp5/6 co-receptors [214, 217]. However, DKK3 has been reported to have no binding capacity for the Lrp-5/6 co-receptor [214]. Only in certain cell lines, over-expressed DKK3 has been reported to reduce the cytoplasmic level of β-catenin and therefore may affect the canonical Wnt/β-catenin pathway differently from other DKKs [218] (Fig. 1.13). Moreover, intrinsically expressed DKK3 has been shown to promote, instead of inhibit, the Wnt3a-mediated canonical Wnt/β-catenin pathway by binding onto co-receptors Kremen (Krm)1 and Krm2 [219]. Intriguingly, this has not been found for extrinsic DKK3 in conditioned medium [219]. Additionally, DKK3 may regulate Wnt signaling pathway through the non-canonical Wnt/planar cell polarity (Wnt/PCP) pathway [214, 215, 220] because over-expression of DKK3 induces downstream c-Jun-NH2-Kinase (JNK) activity (Fig. 1.13). So far, no clear picture has emerged whether and how DKK3 influences Wnt signaling.

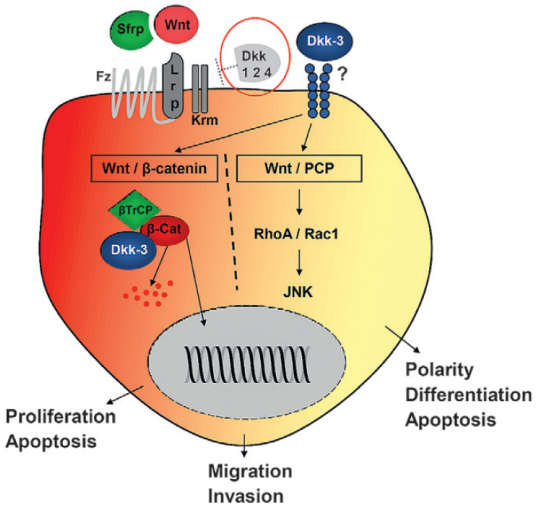


Figure 1.13 DKKs regulate the Wnt signaling pathway. DKK1/2/4 can compete with Wnt ligands for co-receptors Lrp5/6 or Kremen and inhibit Wnt signaling through the canonical Wnt/β-catenin pathway. However, DKK3 cannot bind as other DKK members and fails to inhibit Wnt signaling in various situations. Only in certain cell lines DKK3 can reduce cytoplasmic β-catenin and affect the canonical Wnt/β-catenin pathway. It is also controversial whether DKK3 can inhibit non-canonical Wnt/PCP pathway. The figure is from *Veeck and Dahl, Biochimica et Biophysica Acta 2012, 1825:18–28.*

The distribution of Wnt ligands and temporal/spatial differences of Wnt signaling activities decide developmental events, such as antero–posterior axial patterning, limb development, and eye formation [214]. Dysregulated Wnt signaling may disrupt the developmental plans [221, 222]. Aberant Wnt signaling is reported in various cancers [215]. In addition, Wnt signaling is also essential in immunological responses [223]. Since DKK family members regulate Wnt signaling, it is not surprising that DKK proteins are also involved in developmental processes [214, 222]. Indeed, the name *dickkopf* of the DKK family is based on abnormal head formation in embryos in the absence of DKK1 during development [216].

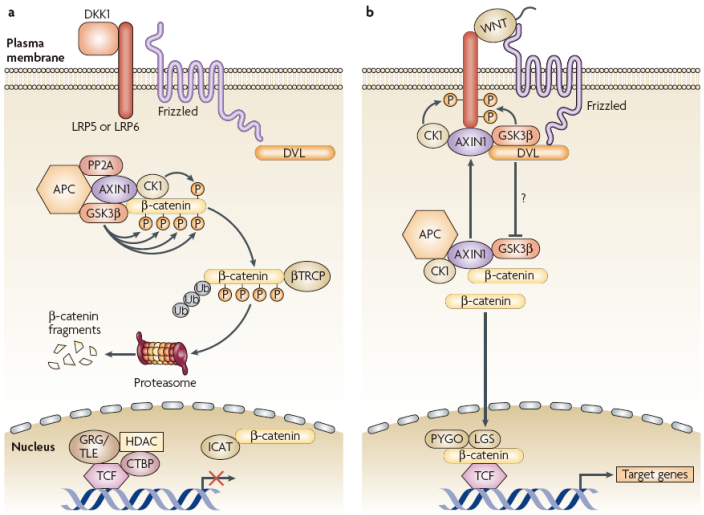


Figure 1.14 Canonical Wnt signaling pathway. DKKs can bind onto co-receptor LPR5/6 and Kremen, inducing internalization of co-receptors and impair the Wnt signaling. However, when Wnt ligands can compete out DKKs for the binding of co-receptors and the receptor, Frizzled, cytoplasmic β-catenin will be translocated into the nucleus and initiate downstream events. The figure is from *Frank Staal, et al, Nat. Rev. Immunol.2008,12:581-593*

DKK3 has been described as a tumor suppressor in various types of tumors [215, 224]. Down-regulation of DKK3 has been found in solid tumors of lung, colon, kidney, prostate, and breast as well as in hematopoietic malignancies. As a result, DKK3 has been identified as REIC (Reduced Expression in Immortalized Cells) when it was discovered in transcriptome screening among primary tumors [225]. Hypermethylation of the DKK3 promoter silences its expression in tumors [215]. Demethylation drugs can reverse the suppression of DKK3 expression in various tumors and may be relevant to the beneficial outcomes observed for these demethylation drugs in tumor patients [215, 226].

Since DKK3 is a tumor suppressor, efforts to up-regulate DKK3 expression in cancer patients have been considered for novel treatment strategies [215]. However, the observation that patients with deletion at the DKK3 locus had lower lymph node metastasis and better prognosis in head and neck squamous cell carcinomas point towards a tumor-promoting function of DKK3 [227]. Thus, systemic or on-site up-regulation of DKK3 may abort anti-tumor responses.

Indeed, our group has shown that DKK3 is essential for $CD8^+$ T cell tolerance [228]. Overall, the exact roles of DKK3 in tumor development and in the respective immune responses call for further investigation.

1.5.0.0. Autophagy

Proteasome and lysosome are two major machineries to destroy and recycle cellular components [229]. While the proteasomes are responsible for short-lived proteins labeled by ubiquitylation [230] or sumoylation [231], lysosomes deal with damaged organelles and long-lived proteins [229]. Autophagy is defined as the process by which the cells deliver damaged organelles or cellular proteins into the lysosomes for degradation [229]. The degraded components can therefore be reused as anabolic elements for other purposes. From single-cell yeasts to human, autophagy is conserved through evolution as a crucial mechanism to maintain the homeostasis of biological blocks and energy inside of cells. With autophagy cells can struggle to survive during starvation. Additional signals such as endoplasmic reticulum (ER) stress, oxidative stress, and immune activation can also stimulate autophagy [232].

Autophagy can be categorized into three major pathways: macroautophagy, microautophagy, and chaperon-mediated autophagy (CMA) [229] (Fig. 1.15). Macro- and microautophagy can pack their cargos in both selective and non-selective ways [233, 234], while CMA performs selective processes [233]. CMA deals with mainly cytosolic unfolded proteins containing degenerated sequences of signal peptides [229]. The signal peptides can be recognized by the cytosolic HSC-70-centered chaperone system and then docked onto the lysosomal membrane [235]. On the lysosomal membrane, the lysosome-associated membrane protein (LAMP)-2A is multimerized to form translocating channels [236, 237] and mediate the trafficking of CMA cargos. Lysosomal HSC-70 chaperones and the C-terminal cytosolic domain of LAMP-2A are also essential for the trafficking [238]. Microautophagy is initiated by inward protrusions of the lysosomal membranes [229]. Soluble proteins can be included inside of the pocket and delivered into the lysosome when the protrusions finally round up and are isolated from the membrane. By contrast, macroautophagy requires a cup-shaped isolated double-membrane system, of which the origin is controversial, to pack the target cargo in the cytoplasm independently and then to fuse with the destined lysosome [229, 232, 239]. Protein products of the yeast autophagy-related genes (ATG) [232, 240] and their corresponding homologues in higher eukaryotes are required to initiate and process macroautophagy [229, 232, 239]. The isolation of the double-membrane system is initiated by Atg6 with signaling inputs from PI3K signaling [233, 239, 241, 242] and is inhibited by active mTOR signaling [233, 239, 241-243]. The isolated double-membranes start as cup-shaped omegasomes [232] and elongate to surround cargos. Two protein systems are involved in further formation of autophagosomes. The core of the first system is the Atg8 protein (named LC3 in human). Atg8 proteins are cleaved by Atg4 to expose the C-terminal glycine residues (G_{120}). Cleaved Atg8 proteins are activated by E1-like activating Atg7 and then ligated onto the phosphatidylethanolamine (PE) residues on both outer and inner elongating membranes by E2-like conjugating Atg3. The other protein system process Atg12 proteins. Atg12 proteins are conjugated with Atg5

through the help from E1-like Atg7 and E2-like Atg10. The conjugated Atg12-Atg5 forms complexes with Atg16L afterwards and stays onto the outer elongating membrane. It is believed that Atg12-Atg5-Atg16L complexes act as E3-like ligase to facilitate the ligation of Atg8 onto PE residues. All accessory proteins except for Atg8 proteins attached onto the inner membrane leave the autophagosomes upon completion. The completed autophagosomes then fuse with lysosomes to form autolysosomes and the cargos are degraded [229, 232]. Both macroautophagy and chaperone-mediated autophagy are observed in higher eukaryotes and related to immune responses, while microautophagy is only documented in yeasts and other lower eukaryotes [229].

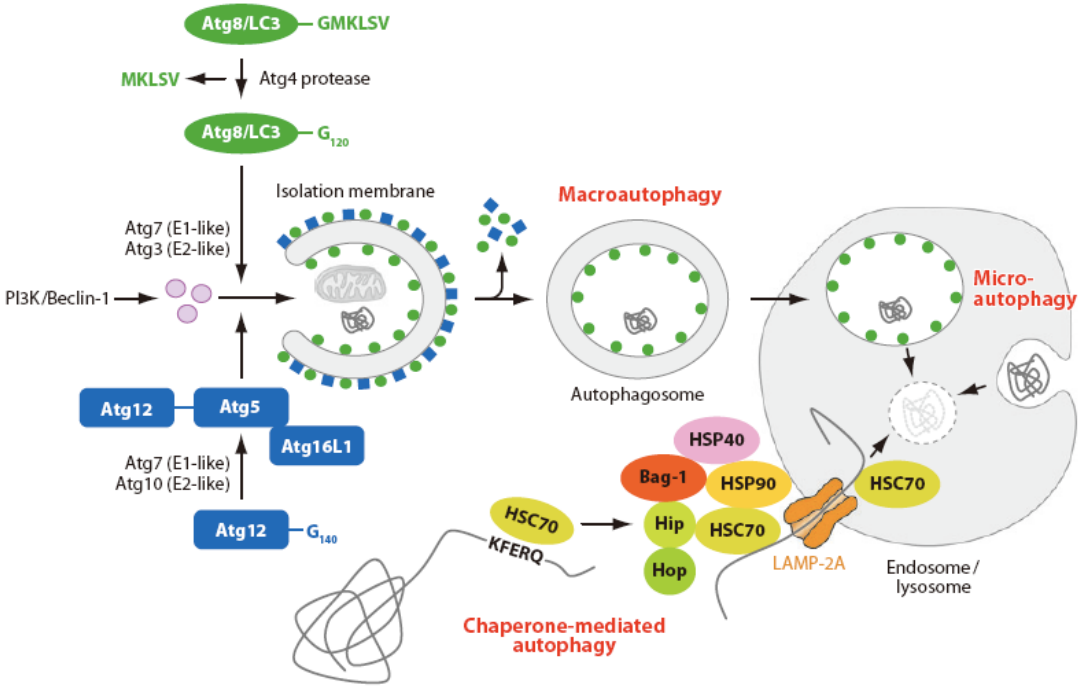


Figure 1.15 Summary of autophagy. There are three types of autophagy, including macroautophagy, microautophagy, and chaperon-mediated autophagy. The figure is derived from *Münz, Annu. Rev. Immunol. 2009, 27:423–49*

mTOR signaling suppresses autophagy (Fig. 1.16). Amino acids, glucose, metabolites, growth factors, hormones, cytokines, and Wnt signaling can modulate mTOR activities [243]. Therefore, mTOR is a central node for cells to respond to environmental stress. For example, during nutritional deprivation, low concentration of amino acids and glucose in the environment or an increased AMP:ATP ratio inactivate mTOR activities (Fig. 1.16). Inactivated mTOR stops biosynthesis and cell cycle, however, trigger autophagic processes instead to reserve metabolic blocks for cell survival [242, 243]. Wnt signaling can activate mTOR by inhibiting the mTOR inhibitor GSK3β (Fig. 1.16), providing another control of mTOR activities [244] and possible regulation of autophagy.

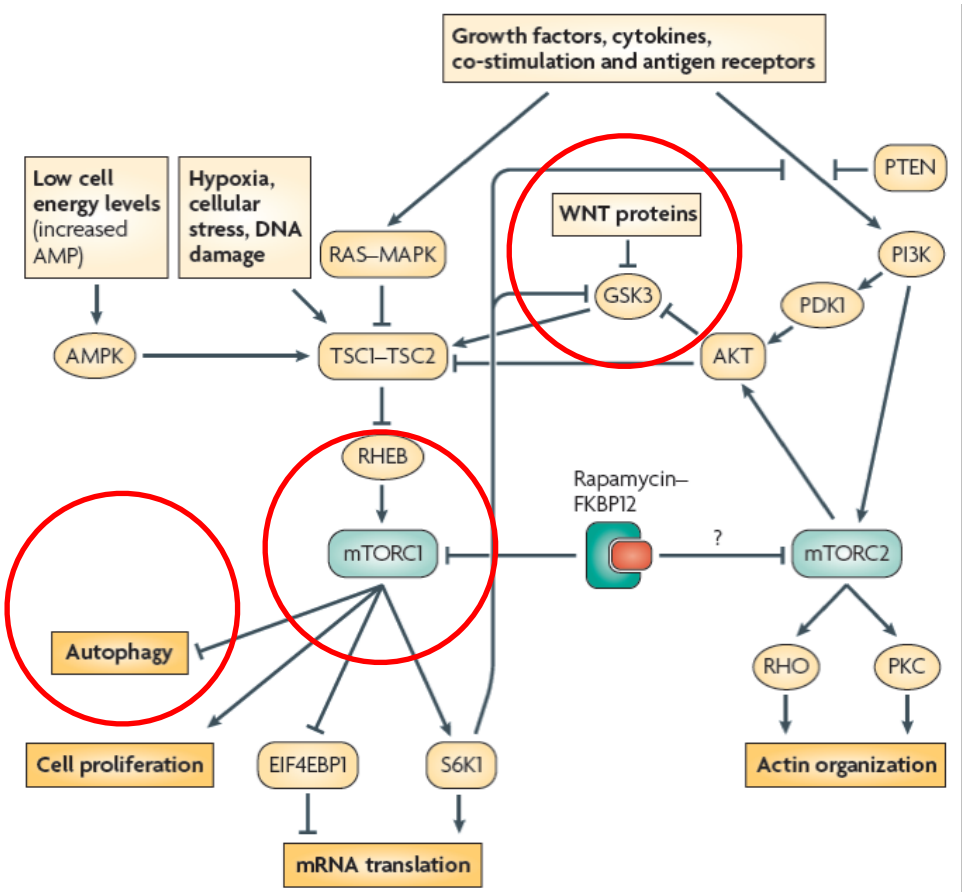


Figure 1.16 Summary of the regulation of mTOR signaling. Wnt signaling can activate mTOR activities by inhibiting mTOR inhibitor GSK3β. mTOR activities suppress autophagy. Therefore, Wnt signaling can be a negative regulator of autophagic activities. The figure is derived from *Thomson, et al., Nat. Rev. Immunol. 2009, 9:324-337*

Autophagy has been correlated with broad ranges of diseases, including various cancers [233, 241] and neurodegenerative diseases like Alzheimer's disease [233, 241]. It is also implicated in aging and longevity [233]. In immune responses, autophagy contributes to eliminate intracellular pathogens [233, 245]. It is also involved in both MHC class I [246] and MHC class II [247, 248] presentation, providing help to enhance both innate and adaptive immunity [229]. Both macro- and chaperone-mediated autophagy (CMA) contribute to the input of antigens into the MHC class II-containing compartments (MIICs). Autophagosome from macroautophagy can fuse with MIICs while CMA directly import unfolded protein into MIICs [229]. On the other hand, immunological signaling by cytokines, co-stimulatory molecules, and TLRs can regulate autophagy [229]. The reciprocal regulation between autophagy and the immune system raises the issue how a basic cellular metabolic event can be translated into immune responses. Autophagy may also be the link between nutritional status and efficiency of immunity.

1.6.0.0. Aim of the study

Attenuating T cell responses is a key therapeutic goal in transplant rejection and autoimmune diseases, while an enhanced T cell reactivity is a pre-requisite for rejection of solid tumors. Understanding the molecular mechanisms regulating T cell reactivity is, therefore, required for a successful reprogramming of immune responses in such medical conditions. T cell responses can be regulated by multiple factors including intrinsic and extrinsic modulators. The aim of this study is to investigate the role of Nck adaptor proteins, as T cell intrinsic factors, and of Dkk3, as an extrinsic modulator, in the control of T cell responses.

In the first part of the thesis, we will assess the contribution of the Nck adaptor proteins to *in vivo* T cell responses to foreign antigens. It will be investigated whether loss of Nck function could cause any alterations in CD4 helper T cell activity, using antibody production by B cells as read-out system. In addition, EAE will be employed to study the role of the Nck adaptors in a T cell-mediated autoimmune disease.

The second part of the thesis will focus on the question whether and how DKK3 can contribute to the immune-suppressive activity of MSCs in a system of T cell-mediated tumor rejection.

Materials and Methods

2. Materials and Methods

Mice

Nck.T$^{-/-}$ mice were bred as described before [249, 250] (Fig. 2.1). In short, Nck1 single knock-out mice were crossed with Nck2$^{flx/flx}$ mice, in which exon1 of Nck2 was flanked by loxP sites. The offsprings were further crossed with Lck-Cre deleter mice in which the Cre recombinase was under the control of T cell specific *Lck* promoter. This T cell-specific Nck knock-out mice were then maintained by sibling mating. Absence of Nck in T cells was confirmed on protein level (Fig. 2.2) and regularly controlled by PCR. Lck-Cre deleter mice were used as controls and noted as Nck.T$^{+/+}$.

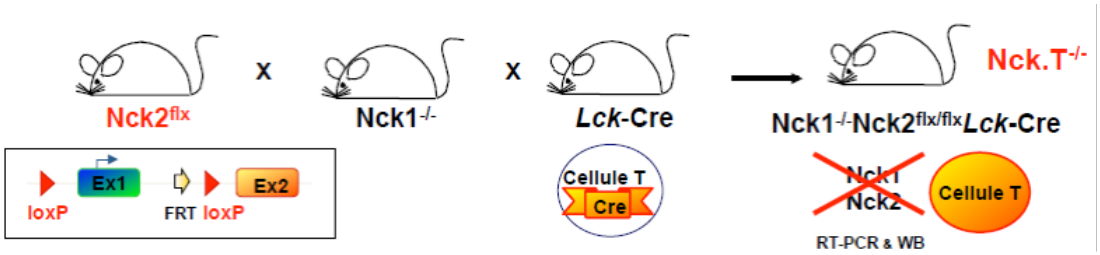


Figure 2.1 Summary of Nck.T$^{-/-}$ murine model. The first exon of Nck2 was flanked by loxP sites (Nck2^{flx}). The Nck2flx mice were then crossed with Nck1 knock-out (Nck1$^{-/-}$) mice and Lck-Cre mice, which expressed the deleter gene *cre* under the control of T cell-specific promotor *Lck*. Descendant Nck.T$^{-/-}$ mice were monitored by checking the expression of Nck1 and Nck2 genes in the mice through regular PCR. The figure is derived from the illustration of Dr. Anna Tafuri.

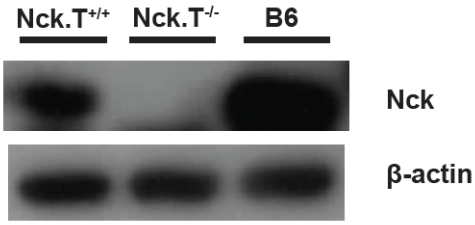


Figure 2.2 Ansence of Nck expression in T cells of Nck.T$^{-/-}$ murine model. The expression of Nck proteins in splenic T cells was specifically abrogated in Nck.T$^{-/-}$ mice.

C57BL/6 (B6) or RAGE-EGFP$^{+/+}$ x B6 (EGFP$^{+/-}$) mice were used as control in different experiments. DKK3 knock-out (DKK3$^{-/-}$) mice were mice with systemic deletion of DKK3 expression [251]. RAGE-EGFP$^{+/+}$ mice were crossed with DKK3$^{-/-}$ mice to generate EGFP-expressing mice with DKK3 systemic deletion (EGFP$^{+/-}$xDKK3$^{-/-}$). Rag2$^{-/-}$ mice were with systemic deletion of Rag2 gene as described before [75]. Mice were bred and housed in central animal laboratory in German Cancer research Center.

Immunization and Sampling

For subcutaneous primary immunization, TNP_{23}- or TNP_{26}-BSA (#T-5050, Biosearch Technology) was diluted in 100µl DPBS and mixed with 100µl complete Freund's adjuvant. Spleen and blood were collected 10 days after primary immunization or 7 days from secondary immunization.

Mesenchymal stem cells (MSCs) isolation

Isolation of MSCs was based on the protocol described before [252, 253] and the manufacturer's guides from STEMCELL Technologies by which the MesenCult™ selection medium system was produced. In short, the femur and tibia bones were isolated from two mice scarified by CO_2. Furs and muscles were removed by scalpels. Openings were trimmed at two ends of the bones to allow the bone marrows being washed out by isolation buffer (1x PBS with 2%FBS and 1mM EDTA). Washed out bone marrow was suspended in isolating buffer and kept on ice. Bones were then cracked down then by scalpels into small fragments without scratching and damaging the bone membranes. Fragments of bones were soaked in digesting medium (1x PBS with 20% FBS and 0.25% collagenase type I) in room temperature for 5 minutes. Soaked bone fragments were further cracked to fine pieces. The digesting buffer was then filled up to 10ml and the containing tubes were shook in 37°C for 45 minutes. Treated bone pieces were suspended by directly adding isolation buffer to final volume of 30ml and then filtered through 70µm cell strainers. The cell strainers were washed by additional 10ml isolation buffer. Cells from bone and bone marrow were centrifuged and the respective cell pellets were suspended in MesenCult™ selection medium. Cells were cultured in the selection medium for at least 3 weeks. After checking for expression of MSCs surface markers, the selection medium was gradually replaced by complete DMEM. When the differentiating capacities of these cells were confirmed, the MSCs were cultured only in complete DMEM for further experiments.

Mesenchymal stem cells (MSCs) differentiation assays

The differentiating medium for adipogenesis was prepared by MesenCult™ selection medium containing 5µg/ml insulin (#I6634, Sigma), 50µM indomethacin (#I7378, Sigma), 1µM dexamaethasone (#D4902, Sigma), and 0.5µM IBMX (#I7018, Sigma). The medium for osteogenesis was MesenCult™ selection medium containing 20mM β-glycerol phosphate (#G9891, Sigma), 1nM dexamaethasone (#D4902, Sigma), and 0.5µM ascorbate 2-phosphate (#A8960, Sigma). 2x105 MSCs were seeded in 6-wells microplates one day before inducing the differentiation. On the day of differentiation, culture medium was replaced with differentiating medium. The differentiating medium was changed every 3 days for 3 weeks. 3 weeks later, the medium was

removed and the cells were washed with 1x PBS twice and then fixed with 10% formalin in room temperature for 20 minutes. After the fixation, cells were washed with 1x PBS and processed for staining. For staining of adipocytes, 0.5% Oil Red O (#O0625, Sigma) was dissolved in methanol. For staining of osteogenesis, 2% Alizarin Red S (#S5533, Sigma) was dissolved in distilled water in pH 4.1. Fixed cells were stained by indicated solution in room temperature for 20 minutes and then washed twice with 1x PBS before filled up with 2mL 1x PBS and observed by optical microscope in 40x magnificence.

Culture and Re-stimulation of Splenic Cells

Single cells from spleens were suspended and cultured in complete RPMI 1640 medium. For re-stimulation, anti-CD3 antibodies (LEAF™ purified, clone 145-2C11, #100314 BioLegend) were diluted in 500µl PBS in 5µg/ml and coated on 24-wells microplates in 37°C for 24 hours. Coating solution was removed right before adding 500µl single cells suspensions. 48 hours later cells were harvested for further analysis. For the intracellular staining of cytokines, 4 hours before the harvest, 500µl fresh culture medium containing PMA and ionomycin along with monensin (GolgiStop™, #554724 BD) was added into the culture.

Culture and IFNγ stimulation of MSCs

Established MSCs were cultured in complete DMEM containing 10% FBS, 2mM L-Glutamine, and 1% HEPES. RMA-mOVA cells were cultured in complete RPMI 1640 containing 10% FBS, 2mM L-Glutamine, and 1% HEPES. For IFNγ stimulation, mouse recombinant IFNγ (#300-02, PeproTech) was prepared into the concentration of 20ng/ml in complete DMEM. One day before the stimulation, $1x10^6$ WT or $DKK3^{-/-}$ MSCs were seeded in 10cm petri dishes (or $1x10^5$ cells in 6-wells microplates). On the day of stimulation, culture medium was replaced with stimulating medium and the cells were cultured for further 48 hours. Before every experiment, the MSCs were checked by their surface markers for quality control. RMA-mOVA cells were controlled by flow cytometry for the surface expression of membrane-bound OVA protein and MHC class I.

Flow Cytometry

To stain surface markers, harvested cells were washed twice with staining buffer (3% FBS and 0.1% sodium azide in DPBS) and stained with antibody combinations diluted in staining buffer in the dark on ice for 30 minutes. Stained cells were washed twice before analysis on the BD FACSCanto™ II flow cytometry. For intracellular staining of cytokines or transcriptional factors, surface markers were stained first. The fixation, permeabilization, and cytokine staining were carried out with the buffer sets

(#560409, BD and 88-8824-00, eBioscience) and manufacturer's instructions. The same buffer set was applied for the staining of intracellular DKK3. For staining of transcriptional factors, an alternative buffer set from eBioscience (#00-5523-00) or BD™ (#560409) was used instead. For staining of apoptotic cells, apoptosis detection kit (#559793, BD) was used. For staining of phosphorylated Akt, BD™ Phosflow Perm Buffer III (#558050, BD) was used and the manufacturer's instructions were followed.

ELISA

For the quantification and affinity assay of serum antibodies, TNP_{26}-BSA, TNP_{16}-KLH, or TNP_5-KLH (#T-5050, #T-5060 Biosearch Technology) molecules were diluted in coating buffer (50mM sodium carbonate, pH9.5) and then coated on the 96-wells ELISA plates in 4°C overnight. For TNP_{26}-BSA the coating concentration was 1µg/ml and for TNP_{16}-KLH or TNP_5-KLH was 10µg/ml. Coating buffer was discarded and the wells were washed 3 times by wash buffer (1x TBS with 0.1% Tween-20). Gelatin-containing blocking buffer was used to block the wells in 4°C overnight. Samples, standards, or HRP-conjugated secondary antibodies recognizing various antibody isotypes were diluted in wash buffer and then incubated in room temperature for 1 hour. Serial dilutions were made for samples and standards. Secondary antibodies were diluted in 1:1000. Signals were developed by 100µl OPD solution (400µg/ml in 0.1M K_2PO_4, pH6.0) containing 0.03% peroxide and then stopped by adding 50µl 1M sulfuric acid solutions. The optical density at wavelength of 495 nM (O.D.495) was measured by automatic reader (Victor™ 1420 Multilabel Counter, Perkin Elmer). Antibody concentrations were calculated by O.D.495 values gained from TNP_{26}-BSA binding and standard curves. Affinity units were calculated by dividing the O.D.495 values gained from TNP_{16}-KLH binding with corresponding antibody concentrations. Reduction ratio was calculated by dividing the O.D.495 values gained from TNP_5-KLH binding with values from TNP_{16}-KLH binding in the same serum dilution [26]. For quantification of cytokine concentrations in the serum, Ready-SET-Go!® kits for mouse IL-4 or IL-10 (#88-7044, #88-7104, eBioscience) were used. The protocols were according to the manufacturer's instructions.

Western blotting

MSCs were lysed directly on the 10cm petri dishes after being washed by 1x PBS for two times. Lysis buffer (#78501, Thermo Scientific) contained protease and phosphatase inhibitors cocktail (#11873580001 and #04906837001, Roche) was prepared according to the manufacturer's instruction. Cell debris was removed by 10 minutes of 13000r.p.m. centrifugation. Supernatants containing proteins of interest were analyzed by Western blotting [254]. Samples were denatured by cooking with DTT for 10 minutes in 96°C. Western blotting was carried out by mini gel system from BioRad for the SDS-PAGE. Acrylamide gels were prepared for 10% and 15% of

acrylamide concentration. Proteins were transferred by Trans-Blot® SD Semi-Dry Transfer Cell and detected by specific primary antibodies and HRP-conjugated antibodies. The results were visualized by HRP substrates (#WBKLS0100, Merck Millipore) and optical films according to the manufacturer's guides.

Antibodies

For analysis by flow cytometry: anti-mouse CD3ε PerCP-eFluor®710 (clone 17A2, #46-0032-82 eBioscience); anti-mouse CD4 Pacific Blue™ (clone RM4-5, #100531 BioLegend); anti-mouse CD8α APC-Cy7 (clone 53-6.7, #100714 Biolegend); anti-mouse CD11b PE-Cy7 (clone M1/70, #101216 Biolegend); anti-mouse CD11c AlexaFluor®647 (clone N418, #117312 Biolegend); anti-mouse CD29 biotinylated (clone HMβ1-1, #102203 Biolegend); anti-mouse/human CD44 PE-Cy7(clone IM7, #103030 Biolegend); anti-mouse CD69 FITC (clone H1.2F3, #553236 BD); anti-mouse CD105 biotinylated (clone MJ7/18, #120404 Biolegend); anti-mouse CD185(CXCR5) PerCp-eFluor®710 (clone SPRCL5, #46-7185 eBioscience); anti-mouse CD206(MMR) Brilliant Violet 421™ (clone C068C2, #141717, Biolegend); anti-human/mouse/rat CD278 Alexa Fluor®647 (clone C398.4A, #313516 BioLegend); anti-mouse CD279 PE-Cy7 (clone 29F.1A12, #135215 BioLegend); anti-human/mouse GL-7 PE (clone GL-7, #12-5902 eBioscience); anti-mouse NK1.1 PE (clone PK136, #108708 Biolegend); anti-mouse F4/80 AlexaFluor® 647 (clone BM8, #123122 Biolegend); anti-mouse IL-4 Alexa Fluor®488 (clone 11B11, #504111 BioLegend); anti-mouse IL-10 Alexa Fluor®647 (clone JES5-16E3, #505014 BioLegend); anti-mouse IL-21 PE (clone mhalx21, #12-7213 eBioscience); anti-pAkt(pS473) PE (clone M89-61, #560378 BD); anti-human/mouse Bcl-6 purified (clone 603406, #MAB5046 R&D Systems); anti-human/mouse GATA-3 eFluor®660 (clone TWAJ, #50-9966-41 eBioscience); Annexin V PE (#51-65875X BD); 7-AAD (#51-68981E BD).

For ELISA: goat-anti-mouse IgM, IgG1, IgG2a, IgG2b HRP-conjugated (#1021-05, #1070-05, #1080-05, #1090-05 Southern Biotech); AffiniPure goat-anti-mouse IgG +IgM(H+L) (#115-005-068 Jackson ImmunoResearch); purified mouse IgM (#550963 BD), IgG1 (#553485 BD), IgG2a (home-made), IgG2b (home-made).

For Western blotting: Autophagy Antibody Sampler Kit (#4445 Cell Signaling Technology®), mTOR Pathway Antibody Sampler Kit (#9964 Cell Signaling Technology®), mTOR Regulation Antibody Sampler Kit (#9864 Cell Signaling Technology®), Wnt Signaling Antibody Sampler Kit (#2915 Cell Signaling Technology®), Wnt/β-catenin Activated Targets Antibody Sampler Kit (#8655 Cell Signaling Technology®), LC3A(D50G8) XP®Rabbit mAb (#4599 Cell Signaling Technology®), LC3B(D11) XP® Rabbit mAb (#3868 Cell Signaling Technology®), MCP-1 Antibody (Mouse Specific) (#2029 Cell Signaling Technology®), CCL-3 antibody (#GTX29927 GeneTex), RANTES Anitibody (Rodent Specific) (#2989 Cell Signaling

Technology®), CXCL-8 antibody (#GTX62979 GeneTex), CXCL-10 antibody (#ab8098 abcam®), CXCL-12 (#NBP1-45849Novus Biologicals), anti-β-actin antibody (#A5441 Sigma), Anti-mouse IgG HRP-linked Antibody (#7076 Cell Signaling Technology®), Anti-rabbit IgG HRP-linked Antibody (#7074 Cell Signaling Technology®).

Histology

Mice were immunized subcutaneously with TNP_{23}-BSA mixed by complete Freund's adjuvant. The spleens were recovered after 10 days. Frozen sections were made for H&E staining and further light microscopy imaging.

Experimental Autoimmune Encephalomyelitis (EAE)[255]

Mice were immunized by 20μg MOG_{35-55} peptides mixed with complete Freund's adjuvant (CFA) subcutaneously in the tail bases. 1μg pertussis toxin (#P2980 Sigma-Aldrich) in 500μL DPBS was injected simultaneously into the peritoneal cavity (i.p.). Additional pertussis toxin was injected i.p. after 2 days. Disease scores were recorded as described before [256]. Mice were sacrificed on day 17 after EAE induction. Infiltrated lymphocytes were extracted from brains and spinal cords by scissors and forceps. Isolated tissues were cut into small pieces and then digested by complete RPMI culture medium containing collagenase IV in 37°C for 10 minutes. Softened tissues were pressed through metal sieves and the extract suspension was separated by Percoll (GE Healthcare Life Sciences) gradients. For analyzing Th17 cells, isolated cells were re-stimulated with PMA plus ionomycin along with monensin, which was added 4 hours before harvesting. FACS analysis was performed after 6 hours of re-stimulation or right after the separation.

Tumor growth and isolation

$4x10^5$ cells of RMA-mOVA mixed with equal numbers of WT or $DKK3^{-/-}$ MSCs were washed three times with 1xPBS and then suspended in 200μl 1xPBS. Mice were shaved at their right flanks. Subcutaneous injection was performed to inoculate the cells. Tumor size was measured in mm and calculated by the following equation: $\boldsymbol{0.5 \times (width)^2 \times (length)}$ for approximate volume [257]. The tumor growth was then followed for about 3 weeks. For analysis of tumor-infiltrating cells, survival and stemness of MSCs, tumors were isolated after 14-17 days of inoculation. Tumor-bearing mice were sacrificed by CO_2 on the day of tumor isolation. Tumor nodules were taken out by scissors and forceps. Isolated nodules were cut into smaller pieces and then digested by digesting buffer in room temperature for 3 hours. Digested tissues were filtered through 40μm cell strainer with one round of additional wash by complete RPMI 1640 medium. Collected cells were then ready for FACS staining.

Statistics

For the first part of study, unpaired t-test was applied throughout the studies except for the analysis on antibody affinity maturation, for which the 2-ways ANOVA was applied. For the second part, unpaired t-test was applied throughout the studies except for the analysis on tumor growth, for which 2-ways ANOVA was applied.

Results

3. Results

3.1 Nck Adaptor Proteins Modulate Differentiation and Effector Functions of T Cells

3.1.1 Nck deletion results in impaired germinal center formation and reduction of follicular helper T cells.

To study the role of Nck adaptors in helper T cell function, Nck.$T^{-/-}$ and Nck.$T^{+/+}$ mice were immunized with keyhole limped hemocyanin (KLH) in Complete Freund's Adjuvant (CFA) and germinal center (GC) formation was investigated in the respective spleens. The GC architecture was disrupted in Nck.$T^{-/-}$ mice (Fig.3.1 A). In addition, the cellularity of GC B cells [258] with the phenotype [259, 260] $B220^{+}GL7^{+}CD95^{dim}$ was decreased in Nck.$T^{-/-}$ mice in comparison to Nck.$T^{+/+}$ mice (Fig.3.1 B). Based on these initial findings we performed a detailed analysis of the splenic Tfh cell compartment in both types of mice after immunization with the T cell-dependent antigen TNP_{26}-BSA. Splenic $CD4^{+}CXCR5^{+}$ Tfh cells [261] (Fig.3.1 C) as well as GC-associated Tfh cells (GC-Tfh) [69] expressing the germinal center marker GL-7 [262, 263] (Fig.3.1 D) were reduced in Nck.$T^{-/-}$ mice. Furthermore, Tfh cells which expressed the activation marker CD69 or the co-stimulatory proteins ICOS and PD-1 [258] were also reduced (Fig.3.1 D). Similar alterations were observed after secondary immunization (Fig. 3.2 A-B). Taken together, loss of Nck function resulted in impaired GC formation and in a reduction of GC-associated B and Tfh cells.

3.1.2 Nck adaptors are required for efficient cytokine production by $CD4^{+}$ helper T cells.

To determine the capacity of $CD4^{+}$ helper T cells deficient for Nck adaptors to produce cytokines, we immunized Nck.$T^{-/-}$ and Nck.$T^{+/+}$ mice with TNP_{26}-BSA. Splenic T cells were isolated 10 days later and re-stimulated with α-CD3 antibodies for 48 hours. Protein levels of IL-4 and IL-10, both of which can be produced by Tfh cells and support germinal center formation [261, 264], were strongly reduced in the supernatant of T cells isolated from Nck.$T^{-/-}$ mice (Fig.3.3 A). When single Tfh cells were further assessed by intracellular staining, IL-10 production was significantly reduced in Tfh cells from Nck.$T^{-/-}$ mice while IL-4 or IL-21 [261] production was slightly reduced without reaching statistical significance (Fig.3.3 B). However, strong reduction of all three cytokines was observed in PD-1^{+} Tfh cells (Fig.3.3 B). Thus, Nck adapters are required for efficient cytokine production by Tfh cells.

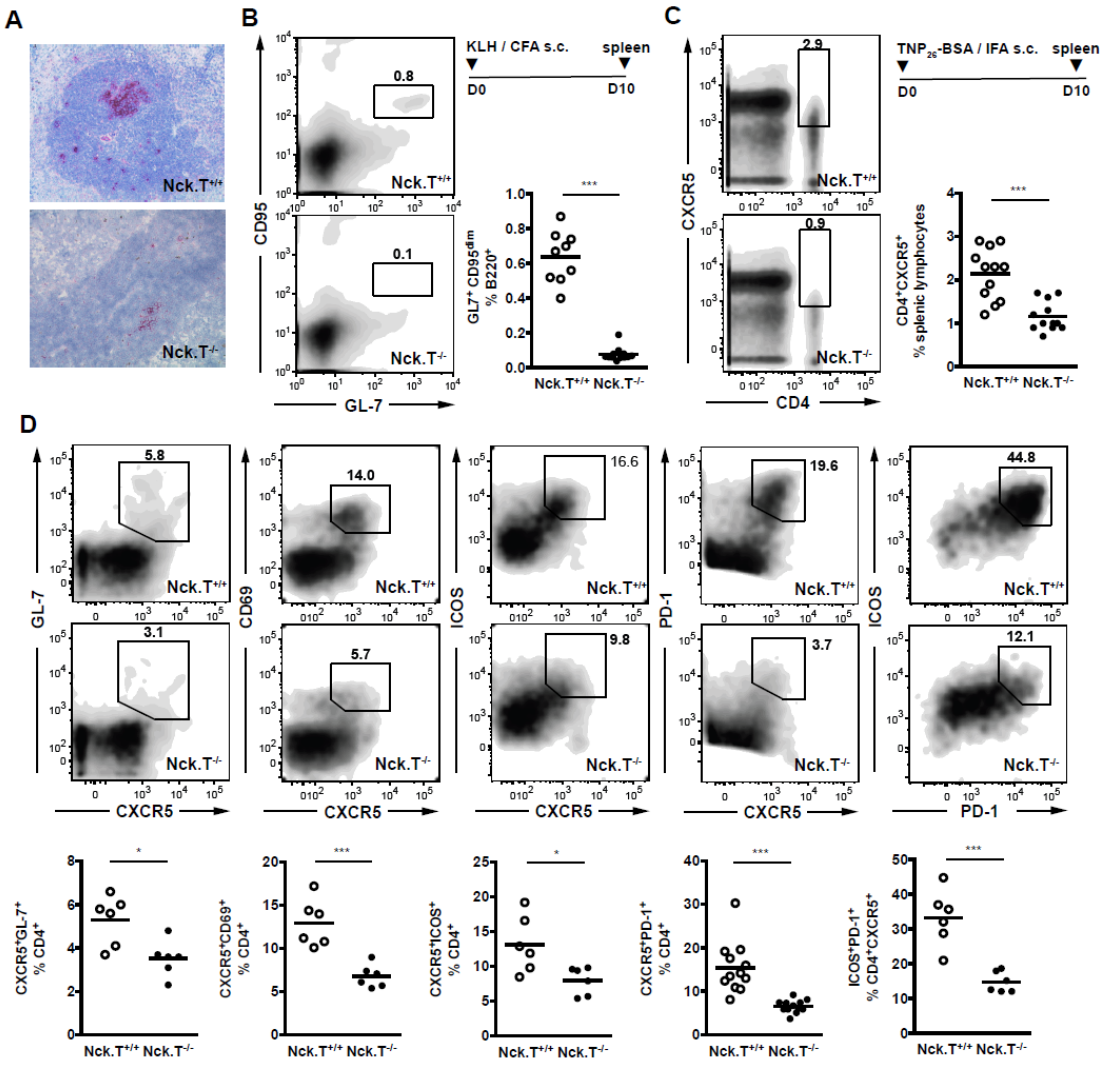


Figure 3.1 Loss of Nck in T cells leads to impaired germinal center (GC) formation and reduction of GC-associated B cells and follicular helper T (Tfh) cells. (A) In contrast to Nck.T$^{+/+}$ mice, Nck.T$^{-/-}$ mice showed impaired GC formation 9-10 days after subcutaneous immunization of 100µg KLH/CFA. Three independent experiments were carried out and representative H&E staining on frozen sections were presented. 3-4 mice were used in each experiment. (B) Nck.T$^{-/-}$ (●, N=12) and Nck.T$^{+/+}$ (○, N=9) mice were immunized with 100µg KLH/CFA and splenic cells were isolated on day 10 after immunization. GC associated B220^{+}GL-7^{+}CD95^{dim} B cells were significantly decreased in Nck.T$^{-/-}$ mice ($p<0.0001$). (C) Nck.T$^{-/-}$ (●, N=6) and Nck.T$^{+/+}$ (○, N=6) mice were immunized with 100µg TNP_{26}-BSA/IFA i.p.. 10 days later the splenic T cells were analyzed. CD4^{+}CXCR5^{+} Tfh cells were significantly reduced in Nck.T$^{-/-}$ mice in comparison with Nck.T$^{+/+}$ ($p<0.0001$). (D) Further analysis of these Nck.T$^{-/-}$ mice showed also significant reduction of the following Tfh cell populations: CD69^{+} cells ($p=0.0006$), GL-7^{+} (GC-associated) cells ($p=0.0111$), ICOS^{+} cells ($p=0.0188$), PD-1^{+} cells ($p<0.0001$), and ICOS^{+}PD-1^{+} cells ($p=0.0004$). Unpaired t test was carried out for analysis.

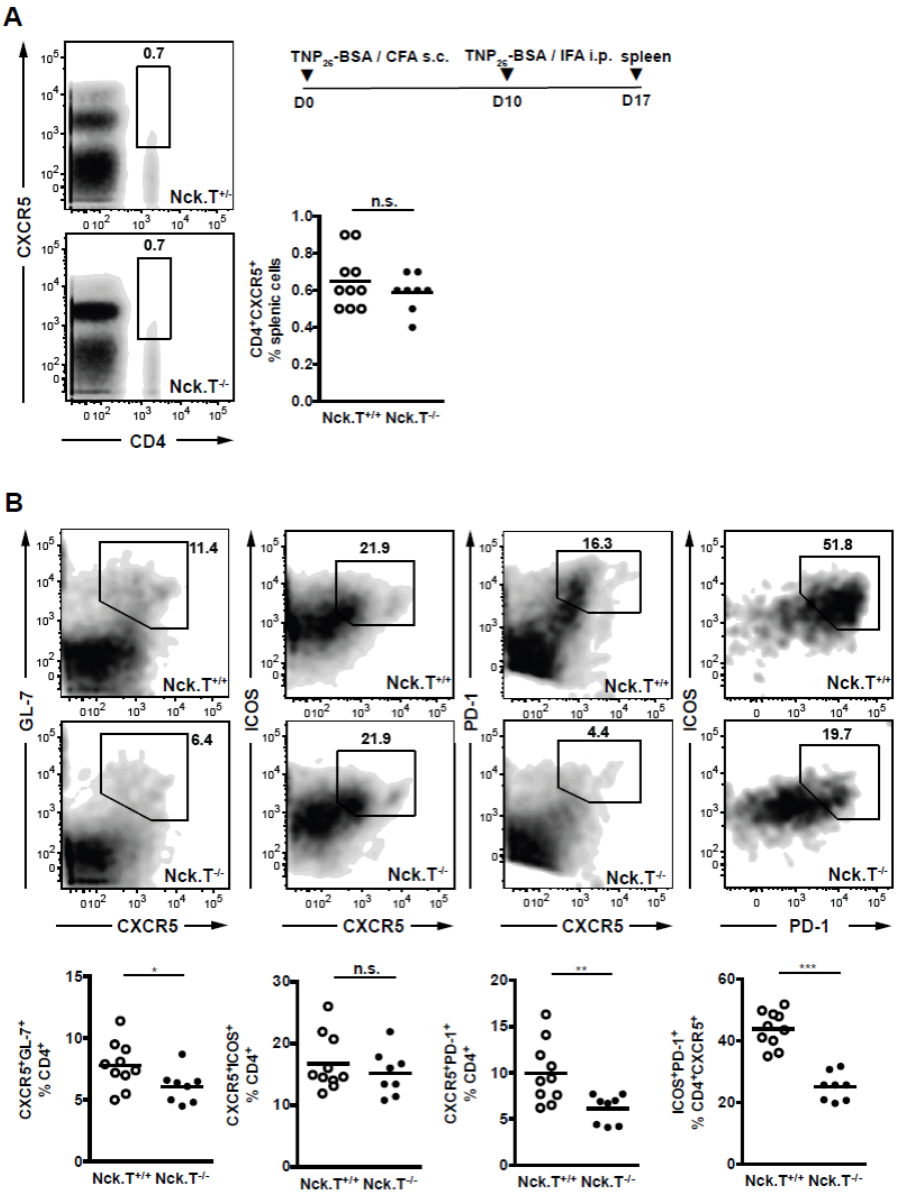


Figure 3.2 Altered compositions of splenic Tfh cells in Nck.T-/- mice during secondary immune responses. Nck.T$^{-/-}$ (●, N=8) and Nck.T$^{+/+}$ (○, N=10) mice were immunized with 100μg TNP_{26}-BSA/CFA s.c. and then boosted with TNP_{26}-BSA/IFA 10 days later. Splenic cells were analyzed on day 7 after the secondary immunization. (A) The percentage of CD4^{+}CXCR5^{+} follicular helper T (Tfh) cells did not change significantly between Nck.T$^{-/-}$ and Nck.T$^{+/+}$ mice (p=0.3288). (B) However, the GL-7^{+} (GC-Tfh), PD-1^{+}, or ICOS^{+}PD-1^{+} Tfh cells were reduced significantly in Nck.T$^{-/-}$ mice (p=0.0437, p=0.0083, p<0.0001), while the ICOS^{+} Tfh remained comparable (p=0.4366). Unpaired t test were used for the analysis.

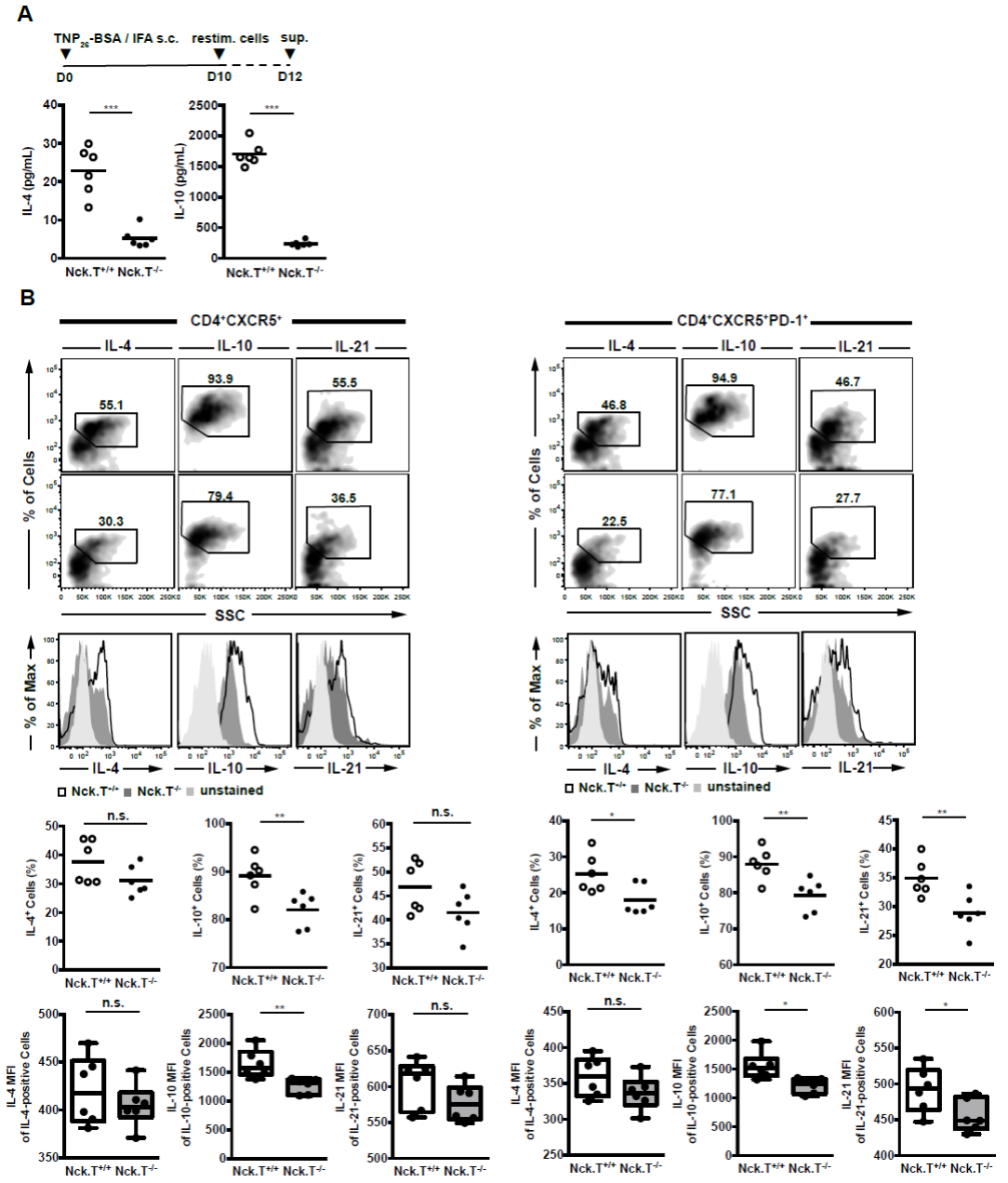


Figure 3.3 Loss of Nck reduces the production of Th2/Tfh cytokines. Nck.T$^{-/-}$ (●, N=6) and Nck.T$^{+/+}$ (○, N=6) mice were immunized with 100µg TNP26-BSA/IFA. Splenic T cells were isolated 10 days later and re-stimulated with 5µg/mL plate-bound α-CD3 antibodies for 48 hours. (A) Cytokines in supernatant were analyzed by ELISA. IL-4 and IL-10 were significantly reduced in the supernatant of T cells isolated from Nck.T$^{-/-}$ (both p<0.0001). (B) For intracellular staining of cytokines, 50ng/ml PMA and 1µg/mL Ionomycin along with monensin were added to the culture medium 4 hours before cell harvesting and antibody staining. IL-10 producing CD4^{+}CXCR5^{+} Tfh cells were significantly decreased in Nck.T$^{-/-}$ mice (p=0.0096), while IL-4 and IL-21 producers were decreased by average but without reaching statistical significance (p=0.1096, p=0.0922). PD-1^{+} Tfh cells producing IL-4, IL-10, or IL-21 were all significantly reduced in Nck.T$^{-/-}$ mice (p=0.0248, p=0.0066, p=0.0086). When specifically investigating the cytokine producing populations in PD-1^{+} Tfh cells from Nck.T$^{-/-}$ mice, the mean fluorescence intensities (MFIs) of IL-10 and IL-21 were both significantly reduced (p=0.0107, p=0.0453). The MFI of IL-4 reduced but reached no significance (p=0.1602). When analyzing the total Tfh cells in the same way, similar reduction was found for three respective cytokines but only reached significance in the case of IL-10 (p=0.0084). Unpaired t test was applied.

3.1.3 Nck is not essential for the expression of Th2/Tfh transcriptional factors.

To investigate whether the observed decrease in Tfh cellularity and the reduction in cytokine production might be attributed to impaired T cell differentiation we determined the expression of the transcription factors GATA-3 [265] and Bcl-6 [258, 261, 266]. Nck.T$^{-/-}$ and Nck.T$^{+/+}$ mice were immunized with TNP$_{26}$-BSA and splenic T cells were isolated 10 days later. Expression of both transcription factors was assessed in total Tfh and GC-Tfh populations after re-stimulation with α-CD3 antibodies in vitro. Neither the percentage of expressing cells nor the expression intensity was found to be different in the studied cell populations of both types of mice for Bcl-6 (Fig.3.4 A) and GATA-3 (Fig.3.4 B). Therefore, Nck adaptors do not influence expression of transcription factors promoting Th2/Tfh cell differentiation.

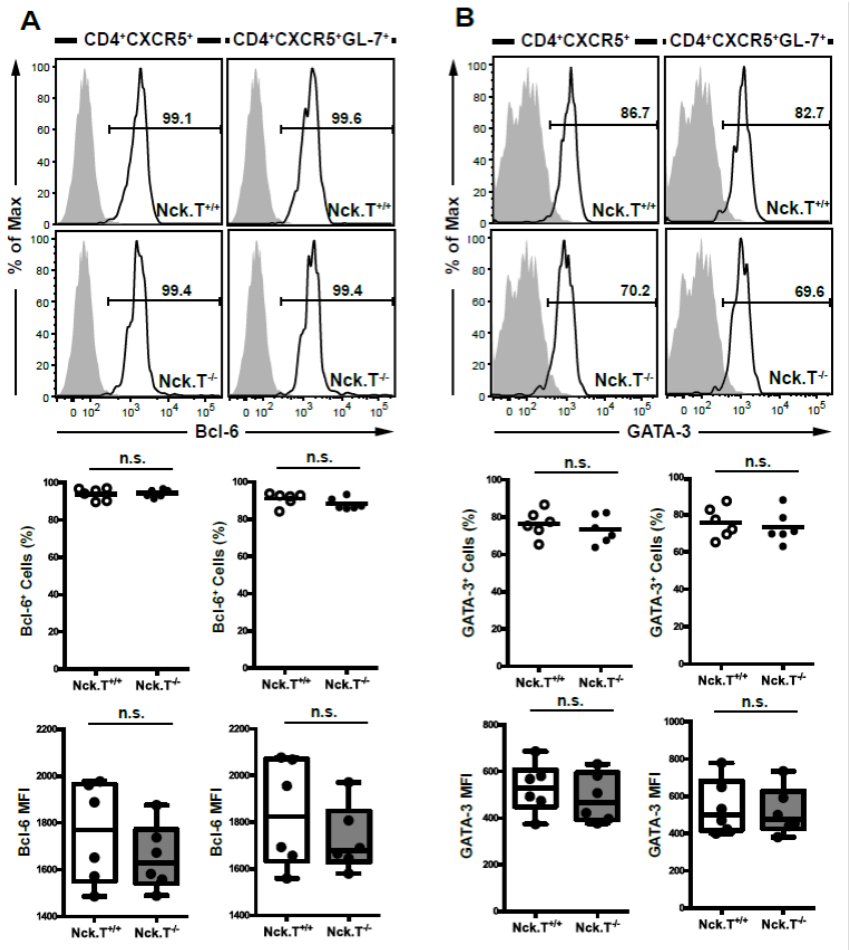


Figure 3.4 Nck is not essential for the expression of Th2/Tfh transcriptional factors. (A) Nck.T$^{-/-}$ (●, N=6) and Nck.T$^{+/+}$ (○, N=6) mice were immunized with 100µg TNP$_{26}$-BSA/IFA i.p. and splenic cells were isolated 10 days later. Expression of Bcl-6 and GATA-3 was assessed by flow cytometry after re-stimulation with 5µg/ml plate-bound α-CD3 antibodies for 48 hours. Similar percentages of Bcl-6 and GATA-3 positive populations were detected among Tfh cells (p=0.8113, p=0.4717) and (B) GL7^{+} Tfh (GC-Tfh) cells (p=0.1891, p=0.6304). Mean fluorescence intensities were comparable in Tfh or GC-Tfh cells (p=0.3432, p=0.3411, p=0.4970, p=0.7529). Unpaired t test was applied.

3.1.4 Nck deficient T cells are more susceptible to apoptosis.

Although Nck deficient Tfh cells express the respective transcription factors for successful differentiation, it is possible that their complete differentiation or survival is impaired. Therefore, we investigated apoptotic cell death of $CD4^+$ T cells isolated from TNP_{26}-BSA immunized $Nck.T^{-/-}$ and $Nck.T^{+/+}$ mice after re-stimulation with anti-CD3 antibodies in vitro. Cell staining with 7-AAD and fluorochrome-conjugated Annexin V did not show a significant difference in apoptosis of total $CD4^+$ T cells isolated from both types of mice. (Fig.3.5 A). However, more than 60% of Nck deficient $GL\text{-}7^+$ GC-T cells entered the late phase of apoptosis while wild-type T cells mostly remained alive or entered only the early phase of apoptosis within the tested time frame (Fig.3.5 A). Since Nck adaptors influence Akt signaling [267] and since Akt signaling takes part in anti-apoptotic pathways [268, 269] we studied Akt phosphorylation (pAkt) in the activated Nck-deficient and -sufficient $CD4^+$ T cells. pAkt levels were significantly decreased in total $CD4^+$ T cells and in Tfh cells from $Nck.T^{-/-}$ mice (Fig.3.5 B). Taking together, loss of Nck leads to enhanced apoptosis of Tfh cells, which is associated with decreased anti-apoptotic signaling by Akt phosphorylation.

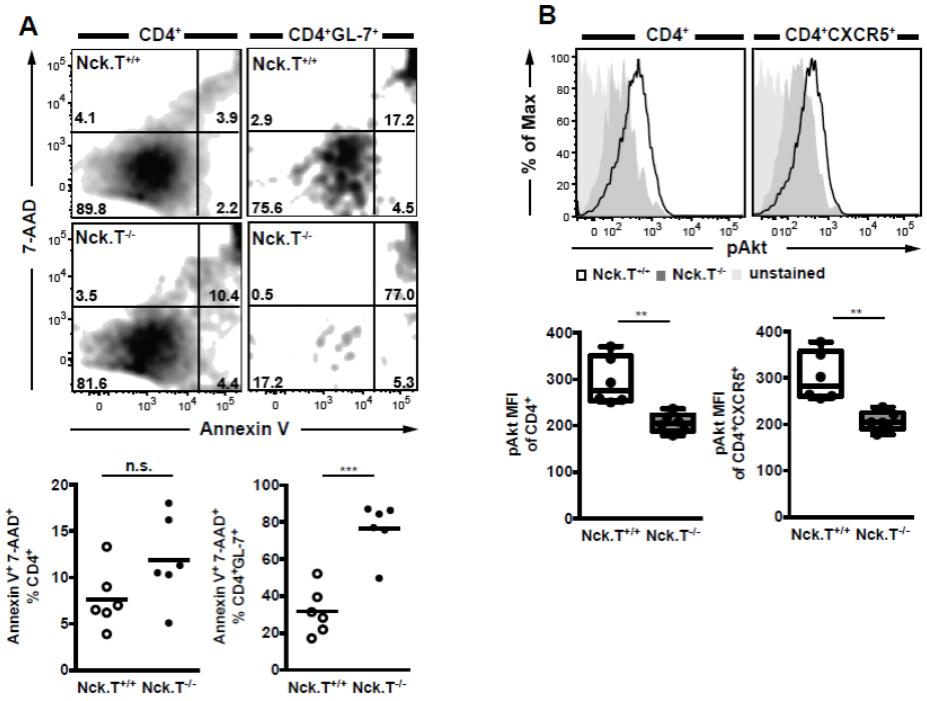
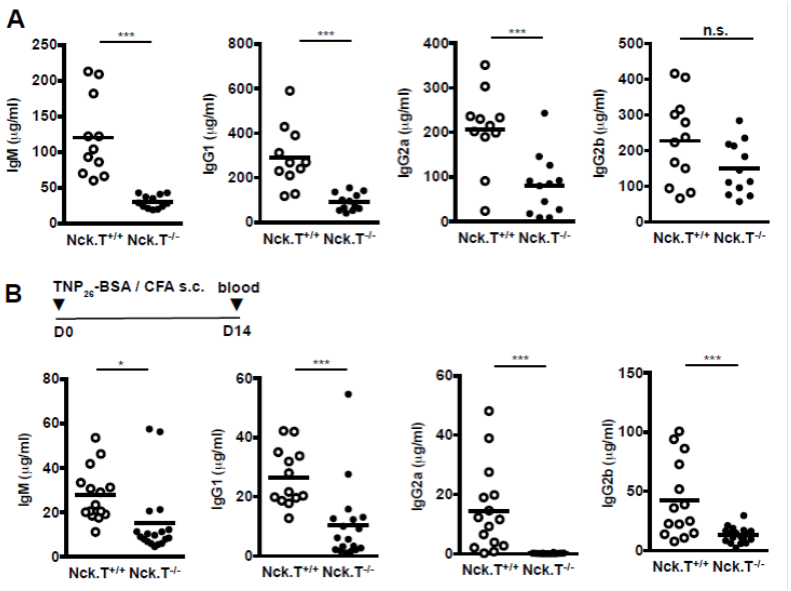


Figure 3.5 Nck deficient T cells are more susceptible to apoptosis. Ten days after immunization of 100µg TNP26-BSA/IFA i.p. splenic cells were isolated from $Nck.T^{-/-}$ (●, N=6) or $Nck.T^{+/+}$ (○, N=6) mice and re-stimulated with 5µg/ml plate-bound α-CD3 antibody. (A) Re-stimulated cells were stained with 7-AAD and PE-conjugated Annexin V. Among total $CD4^+$ T cells, cells in late stage apoptosis (Annexin $V^+7\text{-}AAD^+$) were increased but without reaching significance (p=0.0942). Among $GL\text{-}7^+CD4^+$ T cells, cells in late stage of apoptosis increased significantly in $Nck.T^{-/-}$ mice (p=0.0001). (B) Phosphorylated Akt (pAkt) was detected by flow cytometry. pAkt levels (MFI) dropped significantly in total $CD4^+$ T cells (p=0.0026) as well as in total $CD4^+CXCR5^+$ Tfh cells (p=0.0024) from $Nck.T^{-/-}$ mice. Unpaired t test was carried out.

3.1.5 Nck.T$^{-/-}$ mice show reduced antibody production and insufficient affinity maturation.

Finally, we extended our studies on the role of Nck adaptors in helper T cell function by measuring the production and quality of antibodies in Nck.T$^{-/-}$ and Nck.T$^{+/+}$ mice. The basal levels of natural IgM, IgG1, IgG2a, and IgG2b were significantly decreased in the serum of Nck.T$^{-/-}$ mice (Fig.3.6 A). This phenomenon was consistent with reported T cell-dependency of natural antibody production [270]. Similarly, primary antibody responses against TNP_{26}-BSA were also impaired in all four measured isotypes in Nck.T$^{-/-}$ mice (Fig.3.6 B). Moreover, when Nck.T$^{-/-}$ mice experienced secondary and tertiary immunization, the production of IgG2a remained significantly lower while IgG1 reduced in average without reaching significance (Fig.3.6 C). Thus, Nck ablation in T cells leads to a reduction in antibody production of both primary and secondary responses.

We now asked whether Nck expression in T cells has also an impact on the quality of the produced antibodies as measured by affinity maturation. As an assay for affinity maturation we firstly determined hapten binding per mass unit of antibodies in the serum to the 2 lower hapten conjugates on one carrier, TNP_5-KLH and TNP_{16}-KLH, after secondary immunization with TNP_{26}-BSA. We then calculated the ratio of the obtained signal strength of TNP_5-binding to TNP_{16}-binding[26]. All isotypes except for IgG1 showed significantly lower strength ratio in sera from Nck.T$^{-/-}$ mice compared to sera from Nck.T$^{+/+}$ mice (Fig. 3.6D). This indicated that Nck.T$^{-/-}$ antibody populations contained less high-affinity antibodies than controls, because they could not bind as strong as control antibodies when the number of haptens on the carrier was decreased. Hence, we propose that affinity maturation is impaired in Nck.T$^{-/-}$ mice.



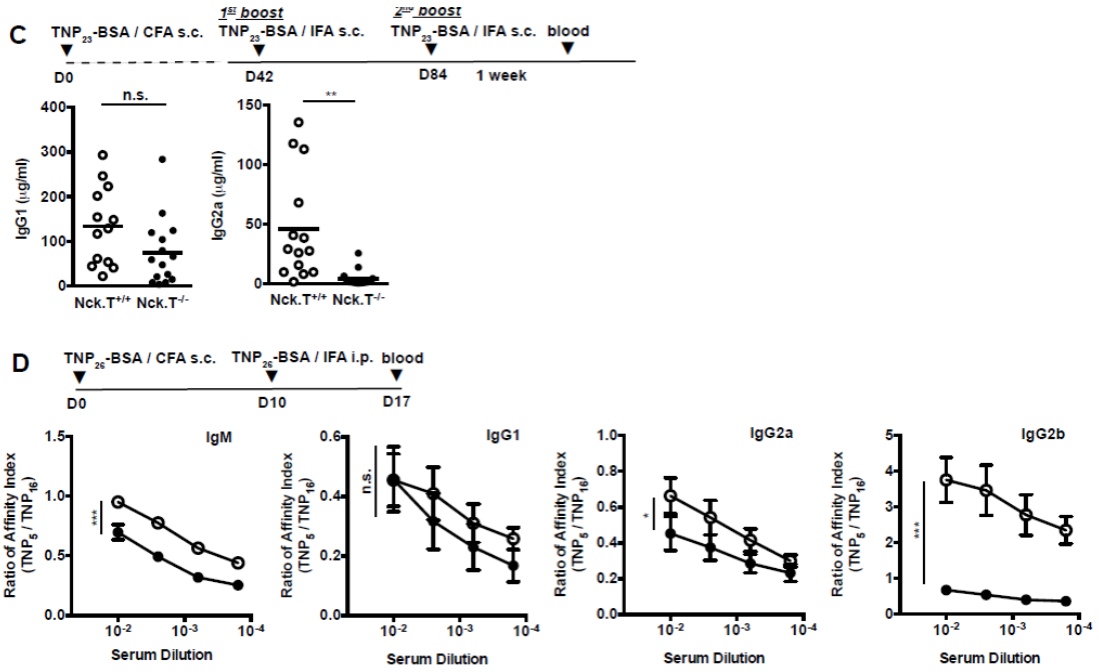


Figure 3.6 Nck.T$^{-/-}$ mice show reduced antibody production and insufficient affinity maturation. (A) Basal immunoglobulin levels in the serum of 6 to 8 weeks-old Nck.T$^{-/-}$ (●, N=12) and Nck.T$^{+/+}$ (○, N=11) mice were assessed by ELISA. The serum levels of IgM ($p<0.0001$), IgG1 ($p<0.0001$), and IgG2a ($p=0.001$) were significantly reduced in Nck.T$^{-/-}$ mice, when compared to the Nck.T$^{+/+}$ mice. By contrast, the levels of IgG2b were comparable in both types of mice ($p=0.0696$). (B) Nck.T$^{-/-}$ (●, N=18) and Nck.T$^{+/+}$ (○, N=13) mice were immunized with 200μg TNP_{26}-BSA/CFA s.c. in 4 different sites of the belly. Antigen specific antibodies were analyzed in the serum on day 7, 14, and 21. Significantly reduced levels of IgM ($p=0.0163$), IgG1 ($p=0.0007$), IgG2a ($p=0.0002$), and IgG2b ($p=0.0008$) were observed on day 14. (C) Nck.T$^{-/-}$ (●, N=15) and Nck.T$^{+/+}$ (○, N=13) mice were immunized with 100mg TNP_{23}-BSA/CFA s.c. on day 0 and boosted twice with 100mg TNP_{23}-BSA/IFA s.c. 42 and 84 days after the primary stimulation. Serum was collected on day 7 after the secondary boosting. The levels of TNP-specific IgG1 and IgG2a were assessed by ELISA. The levels of IgG1 were comparable between Nck.T$^{-/-}$ and Nck.T$^{+/+}$ mice ($p=0.0708$) , whereas the levels of IgG2a were significantly decreased ($p=0.0015$) in the absence of Nck. (D) Nck.T$^{-/-}$ (●, N=8) and Nck.T$^{+/+}$ (○, N=10) mice were immunized with 100μg TNP_{26}-BSA/CFA s.c. in the tail base and boosted with TNP_{26}-BSA/IFA i.p. 10 days later. Sera were collected on day 7 after the secondary immunization. The levels of antigen-specific IgM, IgG1, IgG2a and IgG2b were assessed by ELISA. 10μg of TNP_5-KLH or TNP_{16}-KLH were coated on ELISA plates. Sera were 4-fold diluted from 1/100 serially. Binding signal (O.D. values) of every serum dilution was acquired by ELISA. Affinity Index (AI) was calculated by dividing the O.D. values with corresponding serum concentrations of antibody isotypes. AIs from TNP_5-KLH detection were then divided by corresponding AIs from TNP_{16}-KLH detection. All isotypes showed significantly stronger decrease in ratio of AIs ($p<0.0001$, $p=0.0104$, $p<0.0001$) except for IgG1($p=0.2525$). Unpaired t test was applied for analyzing (A) and (B). 2-ways ANOVA was carried out for analyzing (C) and (D).

3.1.6 EAE disease is ameliorated in Nck.T$^{-/-}$ mice.

To verify whether the aberration of immunity after loss of Nck is only limited to T cell-dependent humoral responses, we extended our studies to experimental autoimmune encephalomyelitis(EAE) [255] as an autoimmune model.

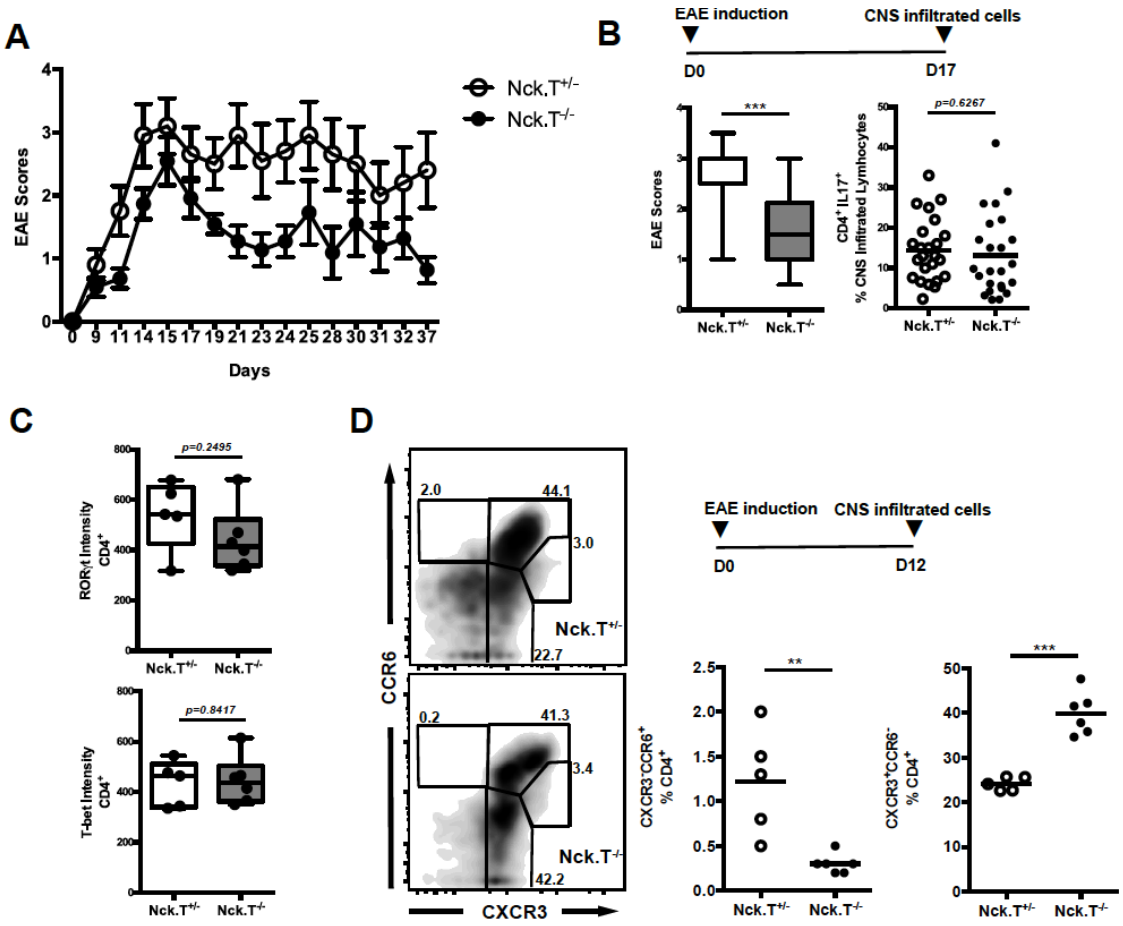


Figure 3.7 Attenuated EAE disease and T cells with altered chemotaxis profiles were observed in Nck.T$^{-/-}$ mice. (A) EAE was induced on Nck.T$^{-/-}$ (●, N=11) or Nck.T$^{+/+}$ (○, N=10) mice. Disease scores were recorded since day 9 after the induction. Scoring criteria were according to descriptions in published protocol [256]. Nck.T$^{-/-}$ mice were observed for significantly attenuated and delayed disease. The remission of disease also progressed faster in Nck.T$^{-/-}$ mice ($p<0.0001$). 2-way ANOVA was carried out. (B) On day 14 after induction of EAE, Nck.T$^{-/-}$ (●, N=24) or Nck.T$^{+/+}$ (○, N=24) mice were sacrificed and the CNS infiltrated cells were isolated. Isolated cells were stimulated by 500ng/ml PMA and 1µg/ml Ionomycin for 4 hours before intracellular staining. On day 14, the disease was close to the peak but significantly ameliorated in Nck.T$^{-/-}$ mice ($p<0.0001$). However, CD4$^+$IL-17$^+$ Th17 cells were comparable ($p=0.6267$). Unpaired t test was applied. (C) For transcriptional factors RORγ-t and T-bet, comparable expression levels were observed among CD4$^+$ T cells ($p=0.2495$ and $p=0.8417$). (D) CNS infiltrated cells were isolated from Nck.T$^{-/-}$ (●, N=7) or Nck.T$^{+/+}$ (○, N=6) mice on day 10 after EAE induction. CCR6-positive CD4$^+$ T cells were significantly reduced in Nck.T$^{-/-}$ mice ($p=0.0061$). Unpaired t test was used.

The EAE was induced by immunization with the murine MOG_{35-55} peptides in CFA. Attenuated EAE disease was observed in Nck.$T^{-/-}$ mice (Fig. 3.7A). The disease scores were lower, the dynamics were delayed, and the recovery from disease was faster. Although Th17 cells were characterized as the signature population for EAE induction [271, 272], intriguingly, the size of Th17 population was not changed significantly in the CNS samples from Nck.$T^{-/-}$ mice (Fig. 3.7B), indicating that the differentiation of Th17 might not cause the attenuated disease. This assumption was supported by the observation that no significant change in the expression of the transcriptional factors RORγt or T-bet could be detected (Fig. 3.7C). As a chemotaxis marker for the 'first wave' of Th17 cells migrating into the CNS, CCR6(CD196) expression [273] on $CD4^+$ T cells was assessed. Numbers of CCR6-positive CD4 T cells were significantly decreased in Nck.$T^{-/-}$ mice (Fig. 3.7D), indicating that less disease initiating Th17 cells were able to find their ways into CNS. On the other hand, an increase in Th1 chemokine receptor CXCR3(CD183) expression [274] was found among CNS-infiltrating $CD4^+$ T cells in Nck.$T^{-/-}$ mice (Fig. 3.7D), which further supported the idea for an altered chemotaxis behavior of Nck.$T^{-/-}$ T cells.

By summary, we found that Nck adaptor proteins are essential for successful TCR signaling in responses to foreign antigens leading to fully competent effector $CD4^+$ T cells.

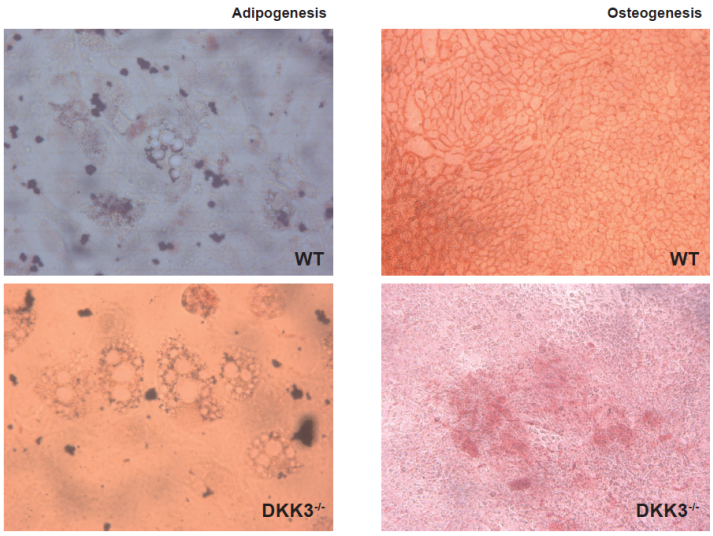
3.2 Dickkopf-3 contributes to the immune-suppressive actions of mesenchymal stem cells.

There have been accumulating data demonstrating the immunosuppressive capacity of mesenchymal stem cells (MSCs) [180, 183] mediated by cell-cell contact as well as soluble factors, such as transforming growth factor beta (TGFβ) and indoleamine-dioxygenase (IDO) [180, 183, 275]. However, these factors did not count for the entire suppressive activity of MSCs. Therefore, we asked whether the newly identified immunoregulatory Dickkopf-3 (DKK3) protein [228] may also play a role in MSCs-mediated immunosuppression. Indeed, there was already preliminary evidence in our group that MSCs express DKK3 (Dr. Amel Tounsi, unpublished data).

3.2.1 Mesenchymal stem cells express Dickkopf-3

Bone marrow-derived MSCs were isolated from the femur and tibia of wild-type (WT) B6 mice or the DKK3$^{-/-}$ mice according to the protocols described in Materials and Methods. Both WT and DKK3$^{-/-}$ MSCs were capable to differentiate into adipocytes and osteocytes [276, 277] (Fig. 3.8A). In addition, the expression of MSCs markers [276, 277] such as CD29, CD34, CD44, and CD105 were maintained in both MSCs while neither of them expressed myeloid or lymphoid markers (Fig. 3.8B). To be noted, during the FACS analysis, MSCs exhibited high background autofluorescence. This phenomenon counted for the dim signals of myeloid and lymphoid markers in WT MSCs. (Fig. 3.8B) Thus, the isolated WT and DKK3$^{-/-}$ MSCs were both potent MSCs by phenotype and function. In the following studies we kept monitoring the quality of MSCs by staining for cell surface markers.

A



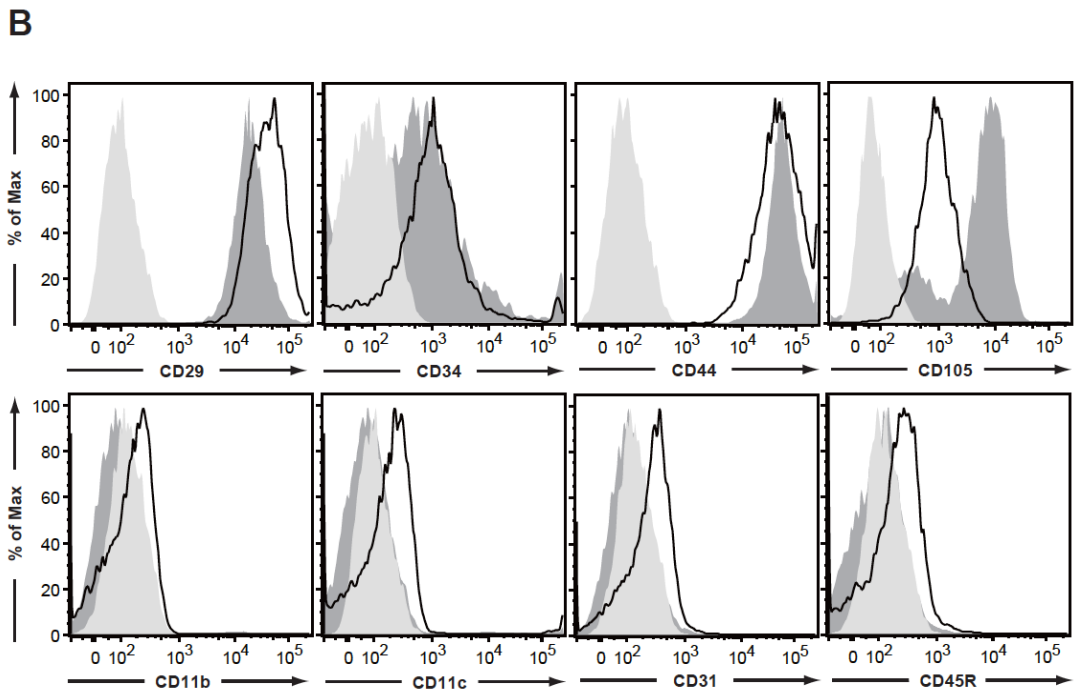


Figure 3.8 DKK3$^{-/-}$ MSCs and WT MSCs were potent MSCs. The induction of adipogenesis and osteogenesis was done as described before. In short, MSCs were cultured in conditioned MesenCult™ selection medium for 3 weeks. MSCs were stained by Oil Red O for adipogenesis and Alizarin Red S for osteogenesis. (A) Both WT and DKK3$^{-/-}$ MSCs were able to differentiate. Images were taken under 100x magnificence by light microscope. Representative images from both types of MSCs are shown. (B) MSCs were cultured in complete DMEM when they were stabilized after 4 weeks of selection by MesenCult® selection medium. Surface staining was performed for FACS analysis. Both WT and DKK3$^{-/-}$ MSCs expressed MSC markers CD29, CD34, CD44, and CD105 while both were negative for myeloid, endothelium, or lymphoid markers CD11b, CD11c, CD31, or CD45R. The light grey shadow represents for the staining control, while the solid line and dark shadow indicate WT and DKK3$^{-/-}$ MSCs respectively.

By using the MSCs isolated in our study, we could confirm that MSCs express high levels of DKK3 (Fig. 3.9).

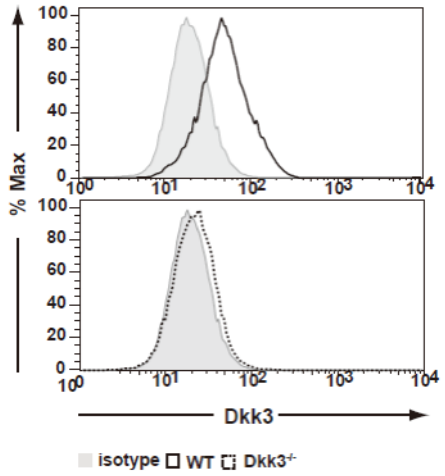


Figure 3.9 MSCs expressed DKK3 proteins. Bone-derived MSCs were isolated from the femur and tibia of wild-type (WT) B6 mice or the DKK3$^{-/-}$ mice according to the protocols and principles described before. Intracellular staining for flow cytometry was performed to detect the DKK3 exprssion within the MSCs. The DKK3 was expressed within WT MSCs while it was not detectable in DKK3$^{-/-}$ MSCs. The grey shadow represents the isotype control staining. The solid line and dashed lines stand for WT and DKK3$^{-/-}$ MSCs respectively.

3.2.2 DKK3$^{-/-}$ MSCs do not have immunosuppressive capacity *in vivo*.

We next asked whether loss of DKK3 would compromise the immunosuppressive capacity of MSCs. We chose growth of RMA-mOVA tumor cells as our model for two reasons. First, tumor rejection in this model was highly dependent on CD8^{+} T cells and it was shown before that DKK3 could abrogate CD8^{+} T cells responses [228]. Second, MSCs can support tumor growth by reducing CD8^{+} T cells and promoting regulatory T cells [198]. Hence, loss of DKK3 may disable MSCs to inhibit CD8^{+} T cell responses. By inoculating mice with MSCs together with tumor cells expressing the membrane-bound ovalbumin (mOVA) antigen, we observed tumor growth in contrast to tumor rejection by mice which had not received any MSCs or DKK3-defective MSCs (Fig. 3.10A). To facilitate the identification of tumor-infiltrating host cells we confirmed these results in C57BL/6 (B6) mice carrying EGFP (EGFP$^{+/-}$) as a transgene [278] (Fig. 3.10B). Moreover, the capacity of WT MSCs to sustain tumor growth in DKK3$^{-/-}$ mice (Fig. 3.10C) indicated that it was the MSC-derived but not environmental DKK3 which mediated the immunosuppressive function of MSCs in tumors. Tumor growth was similar when tumor cells with DKK3-sufficient or -deficient MSCs were injected into the Rag2$^{-/-}$ mice lacking any lymphocytes (Fig. 3.10D). Taken together, DKK3 does not influence tumor growth directly but was essential for MSCs to promote tumor growth by modulating lymphocyte reactivity.

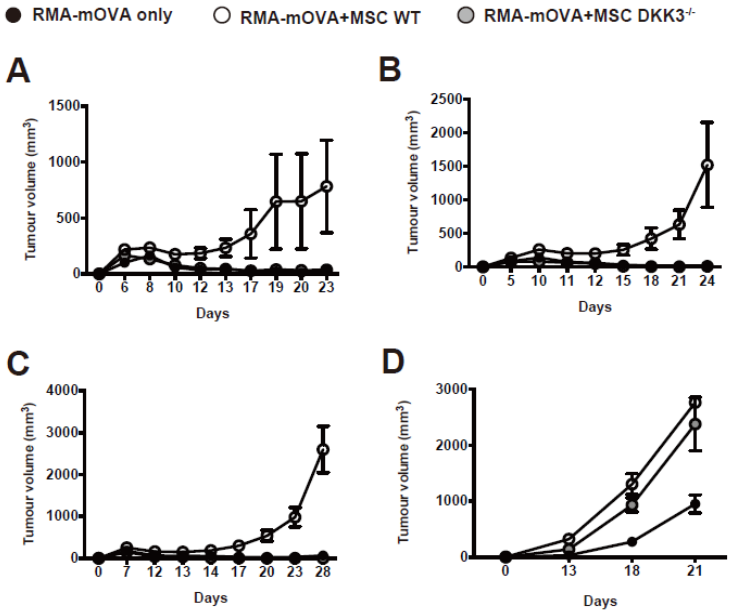


Figure 3.10 MSC-derived DKK3 enabled MSCs to promote tumor growth by modulating lymphocyte activity. 4x10^{5} RMA-mOVA cells were inoculated subcutaneously alone or together with equal numbers of WT or DKK3$^{-/-}$ MSCs. The tumor volume was observed on indicted days. In (A) B6, (B) EGFP $^{+/-}$ B6, or (C) DKK3$^{-/-}$ mice, DKK3$^{-/-}$ MSCs failed to support the tumor growth. In (D) Rag2$^{-/-}$ mice, which lacked of lymphocytes, the tumors were not rejected even with DKK3$^{-/-}$ MSCs. For every experimental group 6 mice were used.

3.2.3 Loss of DKK3 does not affect viability and stemness of MSCs in tumors.

Since it was possible that the decreased immune-suppressive capacity by DKK3$^{-/-}$ MSCs was simply due to poorer survival or loss of MSC characteristics, we investigated whether there was any difference on viability or MSC phenotypes between WT and DKK3$^{-/-}$ MSCs within the tumors.

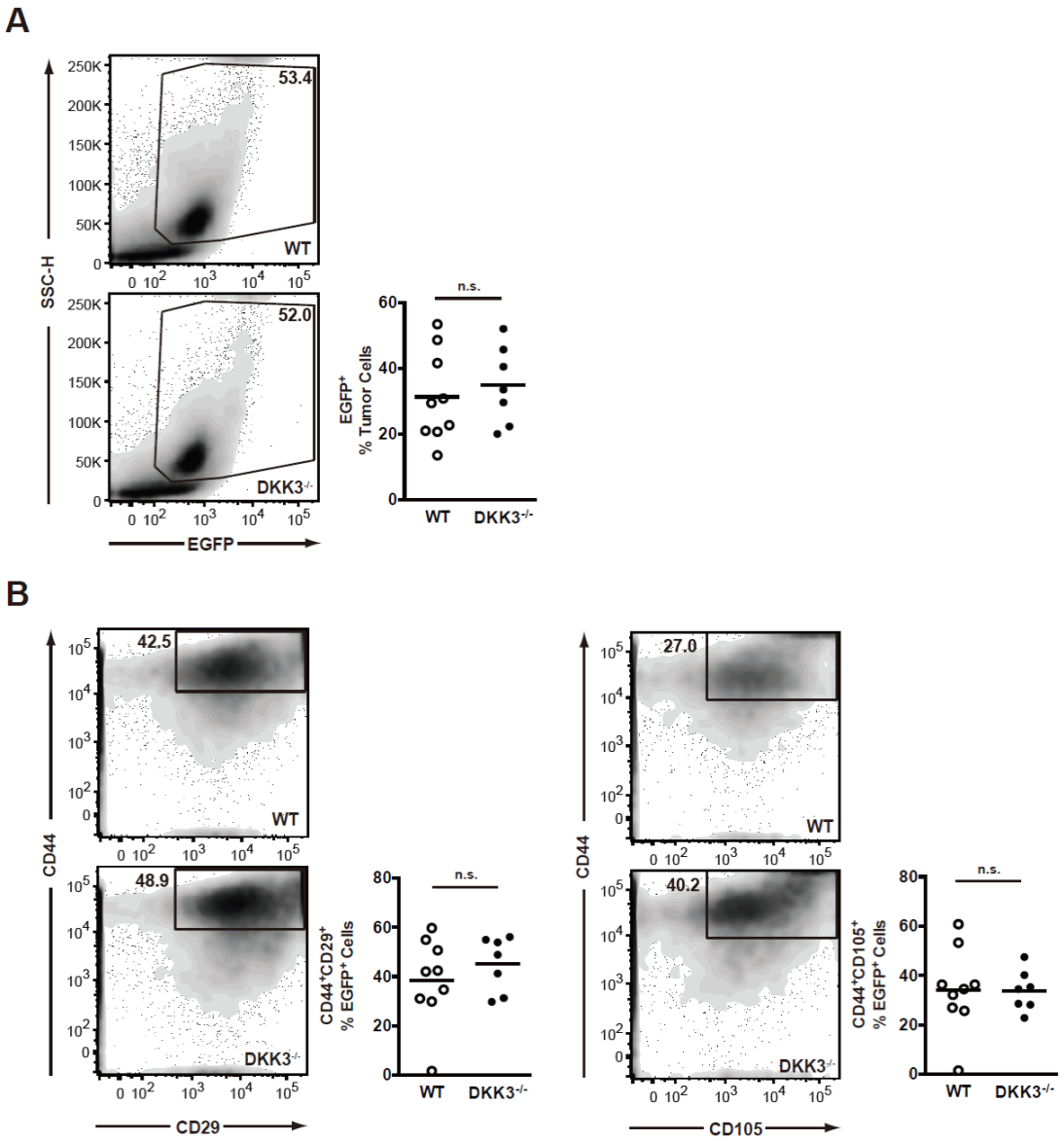


Figure 3.11 Loss of DKK3 did not compromise the survival and stemness of MSCs. MSCs derived from EGFP$^{+/-}$ B6 or EGFP$^{+/-}$xDKK3$^{-/-}$ mice were subcutaneously inoculated together with RMA-mOVA cells into B6 mice. On day 14 the tumors were isolated and homogenized for single cell suspension. The EGFP^{+} cells were identified as inoculated MSCs or their descendant cells. (A) The intratumoral EGFP $^{+}$MSCs were comparable in percentage between WT and DKK3$^{-/-}$ MSCs. When further analyzing the EGFP^{+}MSCs surface markers (B) no difference was found in CD29^{+}CD44^{+} or CD44^{+}CD105^{+} cells.

Both the percentage of MSCs within the tumors (Fig. 3.11A) and the MSC markers (Fig. 3.11B) were not different between WT and DKK3$^{-/-}$ MSCs. This indicated that loss of DKK3 did not affect the survival or stemness of MSCs. Thus, the DKK3$^{-/-}$ MSCs were persistent within tumors but failed to carry out any immunosuppressive function.

3.2.4 Increased numbers of CD8^{+} T cells infiltrate into DKK3$^{-/-}$ MSC-inoculated tumors.

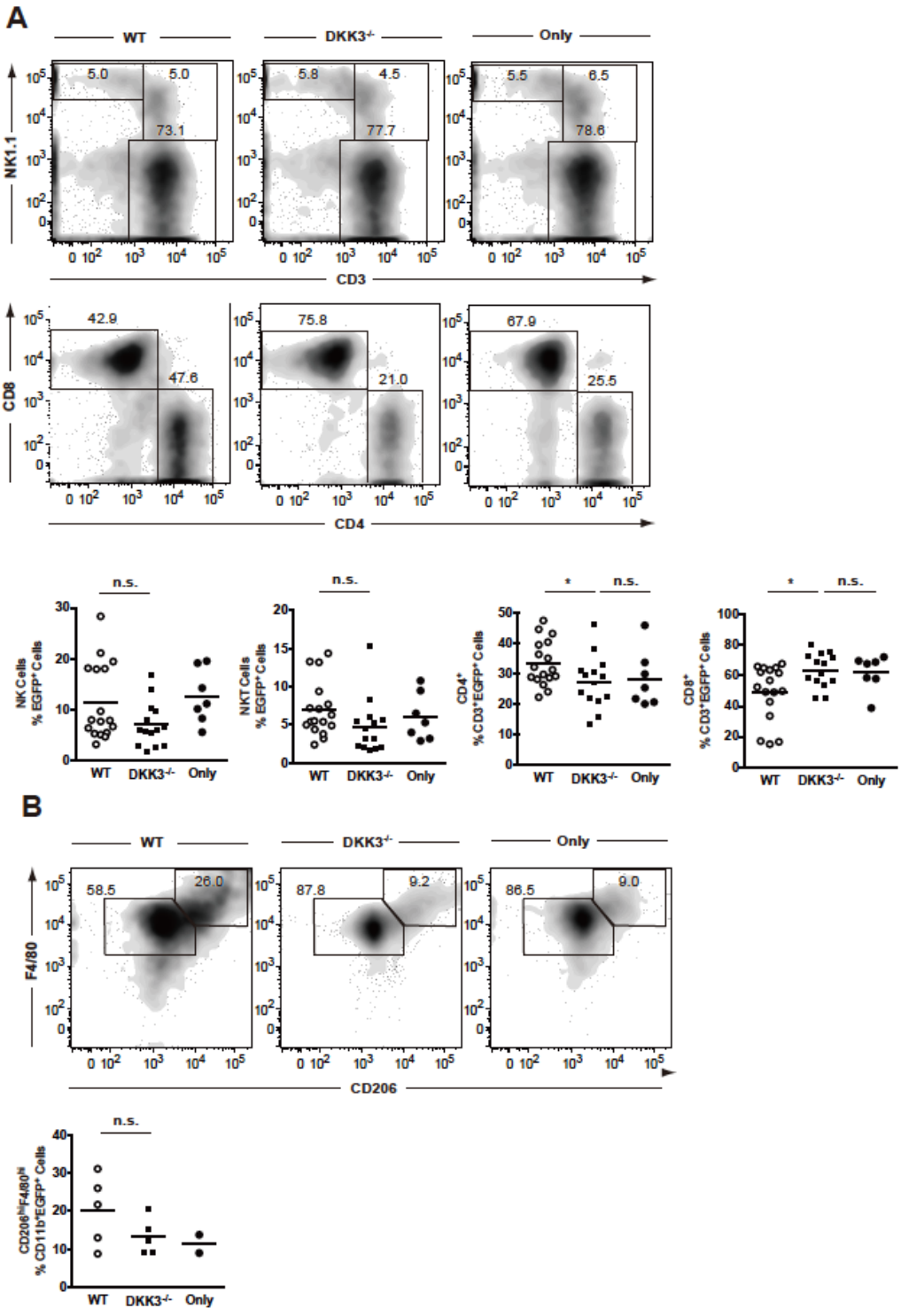


Figure 3.12 DKK3$^{-/-}$ MSCs failed to reduce the infiltration of CD8^{+} T cells. MSCs derived from B6 or DKK3$^{-/-}$ mice were subcutaneously inoculated together with RMA-mOVA cells into EGFP$^{+/-}$ B6 mice. 14 days later the tumors were isolate and analyzed by FACS. The EGFP^{+} cells were identified as infiltrated cells. If not specifically mentioned, the comparisons were done between WT and DKK3$^{-/-}$ MSC-inoculated tumors. (A) When looking into the compositions, the NK1.1^{+} NK cells or NK1.1^{+}CD3^{+} NKT cells were reduced in DKK3$^{-/-}$ MSC-inoculated tumors but without reaching significance. Among NK1.1^{-} CD3^{+} T cells, CD8^{+} cells were decreased while CD4^{+} cells were increased in DKK3$^{-/-}$ MSC-inoculated tumors. (B) In the case of CD11b^{+}F4/80^{hi}CD206^{hi} M2 type macrophages, less recruitment was found in DKK3$^{-/-}$ MSC-inoculated tumors though without reaching statistical significance. Unpaired t test was carried out for analysis.

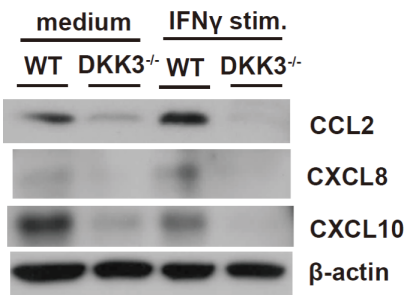
Next, we analyzed tumor-infiltrating immune cells. The percentage of infiltrating CD8^{+} T cells was significantly increased in DKK3$^{-/-}$ MSC-inoculated tumors in comparison to WT MSC-inoculated tumors (Fig. 3.12A) and reached the level of CD8^{+} T cells in tumors without MSC inoculation (Fig. 3.12A). This indicated that MSCs could reduce CD8^{+} T cells infiltration into tumors and DKK3 was essential for it. By contrast, the percentage of infiltrating CD4^{+} T cells was significantly decreased in the DKK3$^{-/-}$ MSC-inoculated tumors while the percentages of NK or NKT cells were decreased but without reaching significance (Fig. 3.12A). We also observed reduced numbers of M2 macrophages in the DKK3$^{-/-}$ MSC-inoculated tumors in comparison with the WT MSC-inoculated tumors (Fig. 3.12B) but without reaching significance. The infiltration of M2 macrophages called for further verification.

Overall, WT MSCs could reduce the infiltration of CD8^{+} T cells and slightly but not significantly enhance the recruitment of M2 macrophages. These could contribute to suppress the anti-tumor immunity and fitted to our observations of sustained tumor growth in WT MSC-inoculated tumors. DKK3 was essential for these MSC-mediated immunoregulation.

3.2.5 DKK3$^{-/-}$ MSCs produce less chemokines, such as CCL2, CXCL8, and CXCL10.

Since the pattern of tumor-infiltrating cells changed depending on the types of MSCs inoculated, we asked for the underlying mechanisms. MSCs are capable to secret various chemokines to regulate migration of immune cells [183]. Therefore we measured the expression profiles of chemokines in both WT and DKK3$^{-/-}$ MSCs.

Figure 3.13 DKK3$^{-/-}$ MSCs showed differed chemokine profiles. WT and DKK3$^{-/-}$ MSCs were lysed by lysis buffer in resting status or after stimulated with 20ng/ml IFNγ in complete DMEM. Western blotting was performed. Diminished expression of CCL2, CXCL8, and CXCL10 were found in DKK3$^{-/-}$ MSCs, no matter in resting status or after IFNγ-stimulation. Representative results wre shown for 5 independent experiments.



In both resting and IFNγ-stimulated status, DKK3$^{-/-}$ MSCs expressed merely no CCL2, CXCL8, or CXCL10. However, WT MSCs were competent to produce such chemokines (Fig. 3.13). Our results showing aberrant chemotaxis capacity of MSCs deficient for DKK3 might explain the observed abnormalities in macrophage infiltration. CCL2 was reported to be a chemoattractant to leukocytes, such as monocytes and M2 macrphages [279].

3.2.6 DKK3$^{-/-}$ MSCs fail to elicit MHC class I expression upon IFNγ-stimulation, while constantly expressing higher, but still minimal, level of MHC class II.

Besides the production of chemokines for recruitment, cell-cell contact dependent mechanisms between MSCs and immune cells might regulate immune activities of the recruited immune cells. Expression of MHC class I was shown as a mechanism for IFNγ-activated MSCs to prevent NK cell-mediated cytolysis [205]. On the other hand, MSCs can express MHC class II and present antigens under certain conditions, such as stimulation by low concentration of IFNγ [202, 203]. Therefore, we tested MHC class I and class II expression in both DKK3-sufficient and -deficient MSCs.

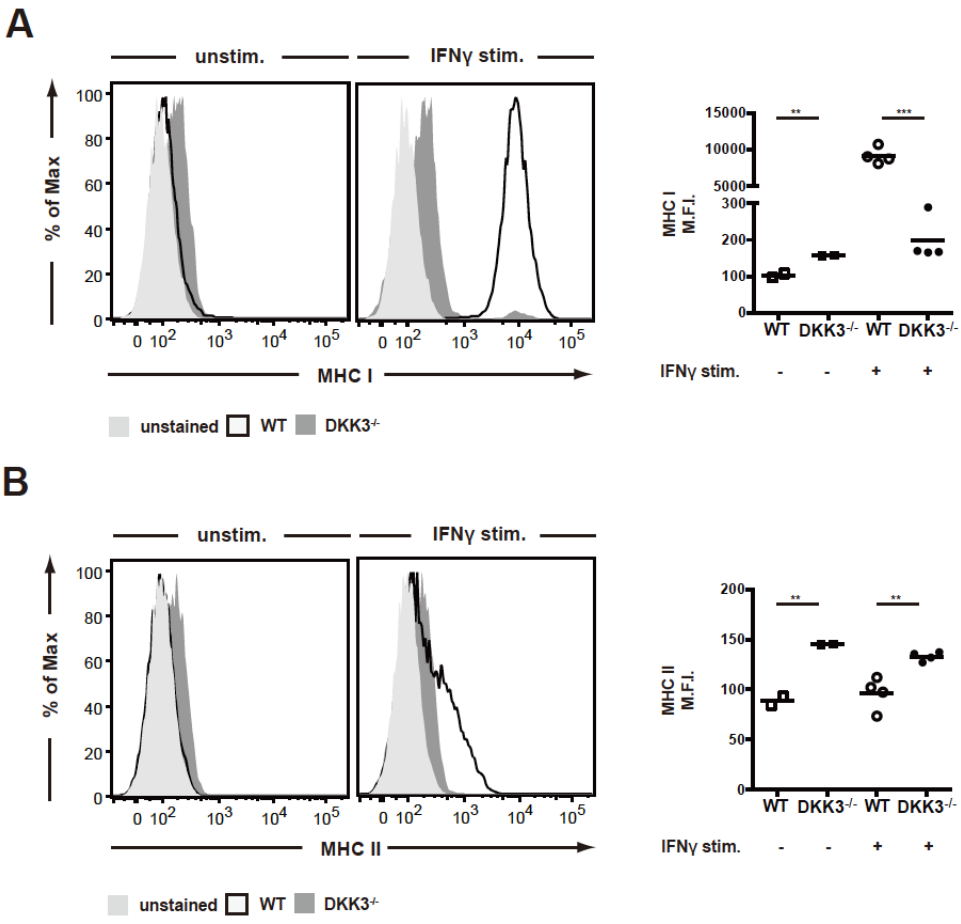


Figure 3.14 DKK3$^{-/-}$ MSCs failed to elicit MHC class I expression after IFNγ-stimulation, while expressing higher MHC class II molecules. MSCs were stimulated with 20ng/ml IFNγ for 48hrs. The surface expression of MHC class I or II was analyzed by FACS. (A) In the resting status, the expression of MHC class I by either WT or DKK3$^{-/-}$ MSCs was low. After IFNγ-stimulation, only WT MSCs dramatically elicited the expression of MHC class I while DKK3$^{-/-}$ MSCs were impaired. (B) In the case of MHC class II, IFNγ-stimulation did not induce up-regulation in either WT or DKK3$^{-/-}$ MSCs. However, in both resting and IFNγ-stimulated status, DKK3$^{-/-}$ MSCs expressed higher levels of MHC class II. Unpaired student t test was applied for analysis.

DKK3$^{-/-}$ MSCs were strongly impaired in their expression of MHC class I after IFNγ-stimulation (Fig. 3.14A). However, they expressed higher levels of MHC class II in either resting or IFNγ-stimulated status (Fig. 3.14B). These results suggest that the DKK3$^{-/-}$ MSCs may be less capable to suppress NK cell activities and may be more capable to present antigens to CD4^{+} T cells.

3.2.7 Intensified autophagy is found in DKK3$^{-/-}$ MSCs

Next we asked what might be the mechanisms beneath the altered surface expression of MHC molecules by DKK3$^{-/-}$ MSCs. As discussed earlier, autophagy could contribute to the antigen influx into MIIC and therefore could enhance the MHC class II expression. Since we have found constitutively higher expression of MHC class II in DKK3$^{-/-}$ MSCs, we hypothesized that autophagy might be increased in these cells.

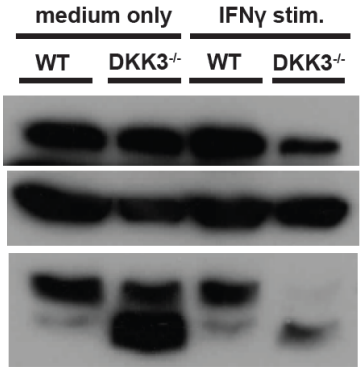


Figure 3.15 DKK3$^{-/-}$ MSCs showed intensified autophagic activities. WT or DKK3$^{-/-}$ MSCs were cultured with or without 20ng/ml IFNγ for 48hrs. MSCs were then washed and lysed by lysis buffer. Proteins were analyzed by Western blotting. In the lysates from DKK3$^{-/-}$ MSCs, either unstimulated or stimulated with IFNγ, stronger LC3B-II signals were detected. Beclin-1 was reduced only in DKK3$^{-/-}$ MSCs after IFNγ stimulation.

Beclin-1, which initiates the formation of autophagosomes, remained unchanged in untreated DKK3$^{-/-}$ MSCs and was decreased after IFNγ-stimulation (Fig. 3.15). However, we identified enhanced autophagic activities by observing intensified expression of LC3B-II (Fig. 3.15). LC3 proteins are cleaved by proteases like Atg4 from LC3-I to become LC3-II. Cleaved LC3 proteins are then incorporated into the autophagosome. Hence, intensified signals of cleaved LC3 proteins indicate active autophagy. Therefore higher cleavage and consumption rates of LC3 in DKK3-deficient MSCs point to an inhibition of autophagy by DKK3.

Thus, DKK3$^{-/-}$ MSCs exhibited stronger autophagic activities, which were compatible with better expression of MHC class II in DKK3$^{-/-}$ MSCs.

3.2.8 Diminished mTOR signaling in DKK3$^{-/-}$ MSCs

Since mTOR was reported to inhibit autophagy [243, 280, 281], we asked whether DKK3 could modulate mTOR signaling. In DKK3$^{-/-}$ MSCs, decreased phosphorylation of mTOR proteins at both serine 2448(S2448) and serine 2481(S2481) sites indicated diminished mTOR activation (Fig. 3.16). Moreover, the phosphorylation of Raptor, a member of the mTORC1, was also reduced, further supporting the observation of weakened mTOR activities (Fig. 3.16). These results are compatible with intensified autophagy and MHC class II expression we found in DKK3$^{-/-}$ MSCs.

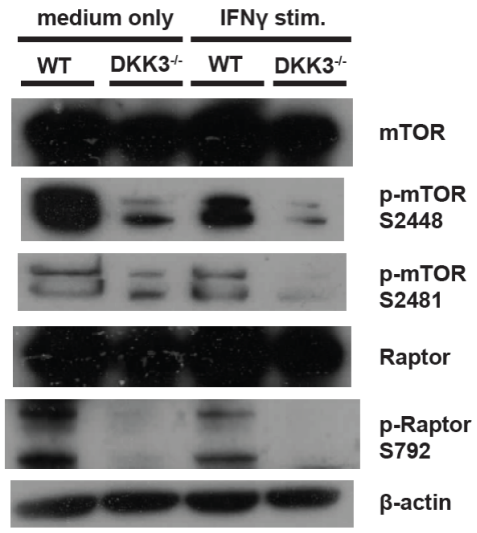


Figure 3.16 Loss of DKK3 caused diminished mTOR activities in MSCs. WT or DKK3$^{-/-}$ MSCs were cultured with or without 20ng/ml IFNγ stimulation for 48hrs. Cells were washed and lysed by lysis buffer for Western blotting. The signals of phosphorylated mTOR, p-mTOR S2448 and p-mTOR S2481, were diminished in DKK3$^{-/-}$ MSCs with or without IFNγ stimulation. Phsphorylated Raptor, p-Raptor S792, was also decreased in DKK3$^{-/-}$ MSCs in both conditions. The expression of total mTOR or Raptor was not significantly changed. Representative images from five repetitive experiments were shown here.

Discussions

4. Discussions

The immune system defends organisms against harmful environmental insults. Thereby, it is essential to have a tight balance between the strength of the immune response to eliminate the respective pathogen and the protection of the respective organ against excessive inflammation and subsequent tissue damage. A detailed knowledge of the processes contributing to this balance is required for the development of novel therapeutic strategies for the treatment of diseases, in which the immune system is involved.

In this study we investigated the role of proteins, which contribute to regulate T cell responses: the T cell intrinsic Nck adapter proteins and the extrinsic modulator DKK3.

4.1 Nck Adaptor Proteins Modulate Differentiation and Effector Functions of T Cells

4.1.1 T cell-dependent antibody responses were reduced in Nck.T$^{-/-}$ mice

Here we provide evidence that Nck proteins are essential for survival and effector functions of Tfh cells. Loss of Nck proteins lead to impaired GC formation along with reduced cellularity of splenic Tfh cells and GC-Tfh cells. Production of key Th2 and Tfh cytokines, such as IL-4, IL-10, and IL-21 [261, 282], was also diminished. These signs of dysfunction were associated with decreased levels of Akt phosphorylation and intensified apoptosis of Tfh cells. Consequently, T cell-dependent antibody responses were reduced in regard to quantity as well as quality by affinity maturation.

In our Nck.T$^{-/-}$ model Nck1 is deleted among all somatic cells, including B cells. Although Nck2 remains intact and the two Nck homologues are highly conserved in sequence and most likely functional redundant [283, 284], one may still argue that the loss of Nck1 in B cells may contribute to the observed dysfunctions in antibody production. Deletion of Nck1 in all somatic cells or both Nck homologues specifically in B cells has been studied in detail [267] and the patterns of antibody production were distinct from our results in the Nck.T$^{-/-}$ system. For example, somatic deletion of Nck1 or B cell-specific deletion of Nck proteins lead to increased basal levels of IgG1 and IgG2a in the serum. By contrast, both isotypes are diminished in our Nck.T$^{-/-}$ mice. Therefore, the defective antibody responses we observed can be attributed to the role of Nck proteins in T helper cell functions.

During humoral immune responses GCs are formed in secondary lymphoid organs and provide microenvironments for differentiation and activation of B cells [285]. GCs

were normally generated in mice with Nck-deficient B cells [267]. In contrast, we found impaired GC formation in Nck.T$^{-/-}$ mice. Follicular helper T (Tfh) cells, which are characterized by high expression of CXCR5 and ICOS, contribute to GC formation [261, 285]. Un-physiological excess of GC-Tfh associated with increased cytokine secretion could lead to increased antibody production with possible autoimmune pathology [286]. The function of GC-Tfh has been reported to be regulated by the strength of TCR binding to peptide-MHC complexes [287]. Our findings show that also T cell-intrinsic modulation of TCR signal strength by Nck proteins can regulate the function of GC-Tfh and thereby the formation of GC.

Reduced numbers of GC-Tfh cells and enhanced apoptosis of these cells suggest that defects in Tfh cell survival limit productive T cell-dependent antibody responses in Nck.T$^{-/-}$ mice. In addition, Nck proteins may also be involved in regulation of T helper cell differentiation. Expression of transcription factors promoting Th2/Tfh cell differentiation, such as Bcl-6 and GATA-3, was comparable in *in vitro* stimulated total Tfh and GC-Tfh populations of wild-type and Nck.T$^{-/-}$ mice. However, Tfh cells from Nck.T$^{-/-}$ mice produced less amounts of IL-10, IL-4 and IL-21 per cell than the respective cells from wild-type mice (Fig. 3.3B). This difference was most pronounced when PD-1^{+} Tfh cells were compared. Thus, these cytokine-producing cells may be able to differentiate but fail to be fully equipped to secrete normal levels of cytokines. This observation could be explained by decreased Akt activities and reported impairment of ERK phosphorylation and Ca^{2+} flux in Nck.T$^{-/-}$ T cells [249]. Akt activity is reported to support the production of different cytokines by T cells, including IL-4, IL-10, and IL-21 [288-290]. However, although ERK signaling promotes IL-10 production [291], it also negatively regulates the production of IL-4 or IL-21 in certain situations [292-294]. Therefore, the reduction of IL-10 after loss of Nck may be explained by synergistic effects of decreased Akt and ERK signaling. However, the observed changes of IL-4 and IL-21 in Nck.T$^{-/-}$ T cells may be the results of opposite activities by decreased Akt activities and decreased ERK signaling. This may help to explain why the reduction of IL-4 and IL-21 is not universally significant among all Tfh but specifically pronounced in PD-1^{+} Tfh. The detailed balance between decreased Akt and ERK signaling after loss of Nck proteins requires further investigation.

4.1.2 EAE disease was attenuated in Nck.T$^{-/-}$ mice

To investigate whether loss of Nck in T cells is perturbing T cell function in general, we extended our studies to the autoimmune model EAE. This demyelinating disease is associated with multiple CNS lesions, mostly pronounced in the brain stem and the spinal cord. The inflammatory process is characterized by the disruption of the blood-brain barrier and the infiltration of various cell types.

Attenuated EAE disease was observed in Nck.T$^{-/-}$ mice (Fig. 3.7A) with lower disease scores, delayed dynamics and faster recovery. The size of disease initiating Th17 cells

was not changed in CNS samples from Nck.T$^{-/-}$ in comparison to wild type mice, suggesting normal T cell differentiation. This assumption was supported by similar expression levels of the transcription factors RORγt or T-bet in CD4 T cells of both types of mice. However, CCR6^{+}CD4^{+} T cells were significantly decreased in Nck.T$^{-/-}$ mice (Fig. 3.7C). The alteration in the expression of this receptor mediating chemotaxis suggests an impairment of Th17 cell migration within the CNS. This finding requires further investigation, as Nck proteins have so far not been associated with migration. In contrary, normal migration of Nck-deficient B cells was observed in secondary lymphoid organs [267].

Together, our findings and previously published data point to an important role of Nck proteins in fine tuning TCR signaling strength during all T cell stages, including development (Fig. 4.1), maintenance, activation and effector function. Nck adaptor proteins contribute to shaping the pre-immune T cell repertoire during thymic selection, contribute to the size and sensitivity of the peripheral T cell repertoire and, as shown here, regulate helper T cell functions during T cell-dependent antibody responses in GC. Thus, defects in Nck protein function may have a so far un-recognized role in human diseases with defective T-dependent antibody responses.

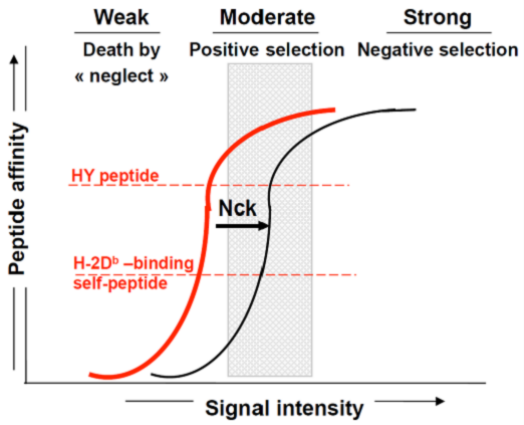


Figure 4.1 Model of Nck adaptor tuning TCR signaling strength in thymic selection. Nck adaptor proteins enhance the signaling strength of TCR on developing thymocytes to appropriate range. Tuning of TCR signaling by Nck proteins are also essential for peripheral maintenance, responsiveness, and effector functions of mature T cells. The figure is based on the fiindings of *Roy, et al., J. Immunol. 2010, 185(12): 7518-26.*

4.2 Dickkopf-3 can limit T cell-mediated anti-tumor responses.

In this part of the studies we showed that DKK3 is produced by MSCs and can limit T cell mediated anti-tumor responses. Impaired chemokine production by DKK3 deficient MSCs could be related to enhanced infiltration of $CD8^+$ T cells within the $DKK3^{-/-}$ MSC-inoculated tumors. In addition, loss of DKK3 in MSCs resulted in increased expression of MHC class II antigens, which was associated with intensified autophagy and reduced mTOR activity.

4.2.1 Dickkopf 3 is essential for the immune-suppressive capacity of mesenchymal stem cells.

The RMA-mOVA transplantation tumor is rejected by C57BL/6 mice after a short growth period but is growing in Rag2-deficient mice lacking lymphocytes. Therefore, it is a suitable model to study possible immune-modulatory capacities of MSCs. WT MSCs inhibited anti-tumor responses whereas DKK3-deficient MSCs did not affect the rejection process. This functional difference between WT and $DKK3^{-/-}$ MSC was not due to different *in vivo* survival of the two types of cells. Therefore, we conclude that DKK3 can contribute to the reported immune-suppressive capacity of MSCs, which can affect both innate and adaptive immune responses [180, 183] by cell-cell contact or soluble factors. To clarify mechanisms resulting in the tolerogenic capacity of Dkk3 we analyzed the composition of tumor-infiltrating cells using eGFP-transgenic recipient mice. WT MSCs could reduce $CD8^+$ T cells infiltration into tumors, whereas the percentage of infiltrating $CD8^+$ T cells was significantly increased in $DKK3^{-/-}$ MSC-inoculated tumors and reached the level of $CD8^+$ T cells in tumors without MSC inoculation (Fig. 3.12A).

We also observed reduced numbers of M2 macrophages in the $DKK3^{-/-}$ MSC-inoculated tumors in comparison with the WT MSC-inoculated tumors but without reaching significance. MSCs are known to modulate the recruitment and differentiation of macrophages. TNFα and IFNγ produced by pro-inflammatory M1 macrophages can trigger MSCs to produce prostaglandin E2 (PGE2) and kynurenine (KYN), by which the recruited M1 macrophages can be repolarized to an immunosuppressive M2 type [183]. For example, in a sepsis model, MSCs were transferred and M2 macrophages were polarized upon MSCs transfer and the IL-10 produced by M2 macrophages decreased further infiltration of pro-inflammatory neutrophils and tissue damage [295]. As macrophages belong to the main target cells of MSCs to exert their immune-regulatory function, further investigations are required to clarify the role of DKK3 produced by MSCs in macrophage recruitment to inflammatory sites.

4.2.2 Dickkopf-3 contributes to the control of chemokine production by MSCs

We next investigated whether the observed differences in inflammatory cell recruitment in the WT and DKK3$^{-/-}$ MSC-inoculated tumors could be related to differential chemokine production by the two types of MSCs. Impaired production of CCL2, CXCL8, and CXCL10 by DKK3$^{-/-}$ MSCs was found (Fig. 3.13).

CCL2 is associated with the recruitment of macrophages and monocytes in tumors [296, 297]. In various human cancers, such as breast [298], ovarian [299], and non-small cell lung cancer [300] as well as glioblastoma [301], expression of CCL2 is positively related to the infiltration of tumor-associated macrophages (TAM), which are mainly M2 type and suppress the anti-tumor immunity. Higher expression of CCL2 in the epithelial regions of various tumors [302-304] indicates the primary sites for monocyte recruitment (Fig. 4.3). In murine melanoma, higher expression of CCL2 is also associated with enhanced recruitment of monocyte and macrophages [305]. Impaired CCL2-mediated chemotaxis of monocytes and macrophages by siRNAs silencing CCR2 [306] or antibody neutralizing CCL2 [307] lead to delayed tumor progression and impaired metastasis. All these findings indicate the CCL2 production in tumors is important for immunosuppression and tumor growth. MSCs have been reported to recruit monocytes by CCL2 [279]. Therefore, the reduction of CCL2 production by DKK3$^{-/-}$ MSCs may be causal for reduced recruitment of monocytes and macrophages to the tumor.

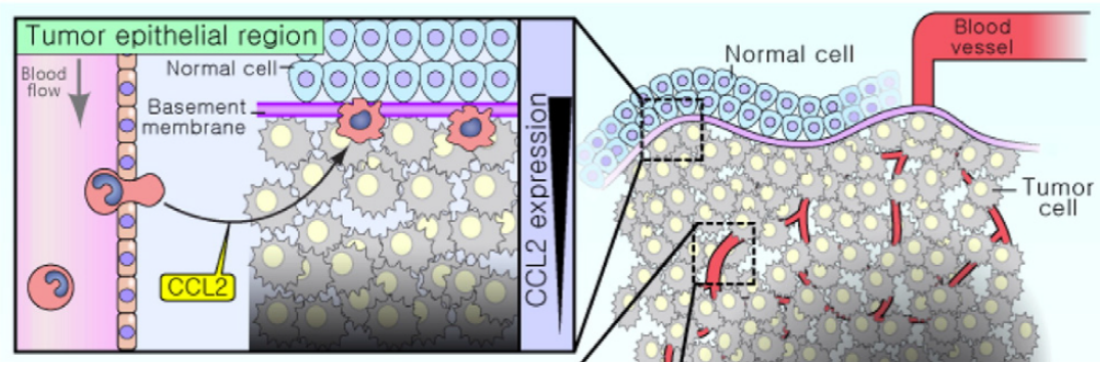


Figure 4.3 CCL2 mediates the recruitment of monocytes into the tumor in the epithelial region of tumor. The figure is derived from *Lee, et al., Biochimica et Biophysica Acta 2013, 1835:170–179.*

CXCL8 is essential for the recruitment of neutrophils [297, 308, 309]. Although in early studies both CXCL8 and neutrophils were characterized as pro-inflammatory elements in the immune system, emerging evidences have shown that neutrophils can produce anti-inflammatory IL-10 [310] and can differentiate into immunosuppressive 'N2' neutrophils according to environmental cues, like TGFβ [311, 312]. Tumor-associated neutrophils (TANs) are composed of both anti-tumoral N1 and pro-tumoral N2 neutrophils [313]. The anti-tumoral effect of N1 neutrophils is dependent on the release of tumorcidal molecules, such as ROS [314-316] and

HOC1 [317] as well as on the support of $CD8^+$ T cell activity [311, 313, 318]. By contrast, pro-tumoral N2 neutrophils can suppress the cytotoxicity and cytokine production of $CD8^+$ T cells [311] and can support tumor survival by production of matrix metalloproteinase-9 (MMP-9) [319, 320]. MMP-9 produced by TANs can support angiogenesis and neovascularization [321-323]. The roles of N2 neutrophils can be analogous to the function of M2 macrophages [320], which are found to be reduced in rejected $DKK3^{-/-}$ MSC-inoculated tumors (Fig. 3.12). Therefore, impaired production of CXCL8 by $DKK3^{-/-}$ MSCs may possibly lead to reduced immunosuppressive N2 neutrophils in the tumor mass and thereby may fail to restrain the tumor rejection. This hypothesis need to be investigated by distinguishing $CD11b^+Ly6G^+Ly6C^{neg.\rightarrow int.}$ N2 neutrophils from $CD11b^+Ly6G^+$ N1 cells in tumor mass.

The impaired CXCL8 production can also be a sign of reduced PGE2 by $DKK3^{-/-}$ MSCs or by other stromal cells. PGE2 can promote the production of CXCL8 by Caco2 colonic epithelial cells through receptor EP4-CREB (cyclic AMP-responsive element-binding protein) signaling [324]. Hypoxia can also synergize with PGE2 to promote the production of CXCL8 [325]. Since hypoxia can be found in internal regions of growing tumors, MSCs may encounter the hypoxic conditions along with PGE2 from the environment or in an autocrine fashion and may be stimulated to produce CXCL8. Since PGE2 can be an important immunosuppressive mediator for MSCs, it will be interesting to determine whether reduced CXCL8 in $DKK3^{-/-}$ MSCs is associated with reduced PGE2 in these cells or in the tumor mass compromising immune-suppression.

Furthermore, MSCs have been reported to be involved in promoting angiogenesis of tumors by producing VEGF [192]. In addition, CXCL8 is reported to promote angiogenesis [326, 327]. Hence, loss of CXCL8 production in $DKK3^{-/-}$ MSCs may limit tumor growth by depriving the nutritional supply. The question whether an increased angiogenesis or the recruitment of immunosuppressive cells weights more for the DKK3-competent MSC-mediated tumor growth requires further investigation.

CXCL10 is known to recruit Th1 T cells, $CD8^+$ T cells as well as NK cells into inflammatory sites [308, 328-330] and is considered to promote immune responses. Since significantly higher numbers of $CD8^+$ T cells infiltrated $DKK3^{-/-}$ MSC-inoculated tumors and since these tumors were rejected, higher expression of CXCL10 in $DKK3^{-/-}$ MSCs was expected. However, $DKK3^{-/-}$ MSCs produced far less CXCL10 in comparison to WT MSCs. We assume that other mechanisms might mask the recruitment of those anti-tumoral immune cells, because the expression of CXCL10 in DKK3-competent MSCs did not cause the rejection of tumors. The co-localization of MSCs and target cells may be essential for short-distanced immunoregulatory molecules like PGE2 and KYN to be effective. PGE2 produced by MSCs can limit the proliferation of T cells by down-regulating the production of IL-2 [331] and decreasing the

expression of IL-2 receptors [332] of T cells [199, 333]. For $CD4^+$ T cells, PGE2 favours Th2 [334, 335] and Th17 [336] differentiation rather than tumor-reactive Th1 cells [199]. Moreover, PGE2 can induce the differentiation of regulatory T cells[337-339]. The cytotoxic activity of $CD8^+$ T cells can be abolished by PGE2 in the activating [340] as well as in the effector stage [341]. KYN [200, 342] can inhibit Th1 responses by supporting Th2 instead [200, 342, 343]. It is also capable to induce the differentiaiotn of $CD4^+Foxp3^+$ iTreg [344]. Therefore, the expression of CXCL10 may serve as a 'trap' of MSCs to recruit T cells closely and then unarm their anti-tumoral capacities.

4.2.3 Dickkopf 3 may regulate MHC class II expression by activation of mTOR and suppression of autophagy

Besides the impaired chemokine production by $DKK3^{-/-}$ MSCs, we also observed altered expression of MHC antigens in these cells. It remains controversial whether or not the expression levels of MHC molecules contribute to the immunosuppressive activity of MSCs [345]. It has been reported that autophagy could contribute to the antigen influx into MIIC compartments and therefore could enhance the MHC class II expression [247]. Thus, the reduced mTOR signaling and the enhanced autophagy which we observed in $DKK3^{-/-}$ MSCs may explain the constitutively higher expression of MHC class II antigens in these cells. Consequently, one may argue that the $DKK3^{-/-}$ MSCs could better present antigen to $CD4^+$ T cells in comparison to DKK3-competent MSCs and therefore support the anti-tumor response. It requires further investigations to clarify, whether or not $DKK3^{-/-}$ MSCs can indeed induce destructive $CD4^+$ T cell responses.

As discussed earlier, it is unclear whether or not DKK3 is an antagonist of the canonical Wnt/β-catenin signaling pathway. Wnt signaling has been reported to activate mTOR signaling by inhibiting GSK3β [244]. Hence, Wnt signaling should suppress the autophagy. Indeed, three recent reports confirmed that the canonical Wnt/β-catenin pathway could suppress autophagy by Wnt1 or Wnt5a induced signaling [346-348]. Furthermore, silencing or depletion of β-catenin enhanced autophagy [347]. On the other hand, autophagy could modulate Wnt signaling by degrading β-catenin [347] or Dishevelled proteins [349, 350], indicating reciprocal regulation. If DKK3 could antagonize the canonical Wnt/β-catenin signaling pathway, mTOR activity should be intensified and autophagy should be diminished in $DKK3^{-/-}$ MSCs. However, our findings (Fig. 3.15-16) do not support this hypothesis. Instead, we assume that DKK3 affects autophagy by modulating JNK signaling. Previous findings demonstrated that DKK3 over-expression in tumor cells induced JNK phosphorylation [220] and that JNK signaling activates mTOR signaling [351, 352]. Therefore, we like to propose that DKK3 may activate mTOR signaling and suppress autophagy via promoting JNK signaling (Fig. 4.3).

This assumption would be in line with the abrogated chemokine production in DKK3$^{-/-}$ MSCs. JNK signaling can promote the production of CCL2 and CXCL8 in various cell types by different stimuli [353-355]. Inhibition of JNK signaling reduces the production of both chemokines [354, 356, 357]. Moreover, Wnt5a, the Wnt ligand protein that activates the non-canonical Wnt/PCP pathway via JNK signaling [358-362], can induce the production of both CCL2 and CXCL8 [358, 363] (Fig. 4.5). Interfering with Wnt5a signaling by sFRP5 inhibits the production of CCL2 [364]. Hence, positive modulation of the non-canonical Wnt/PCP pathway by DKK3 via JNK signaling, would explain the observed, impaired production of CCL2 and CXCL8 in DKK3$^{-/-}$ MSCs.

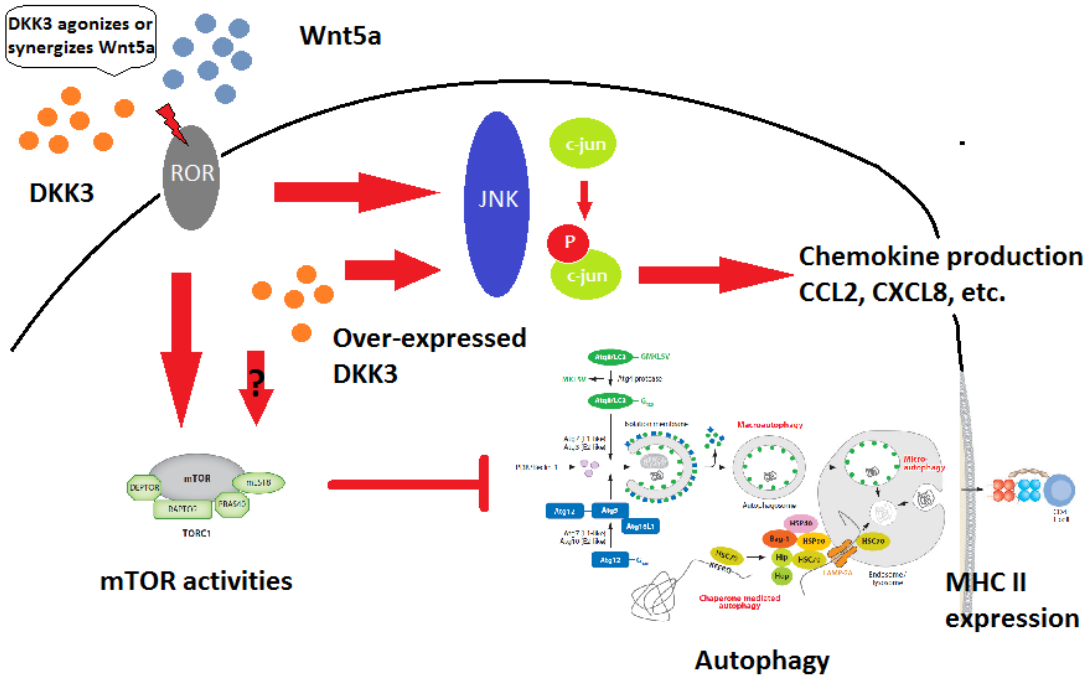


Figure 4.5 Summary of the DKK3 working model in MSC-mediated immunosuppression and tumor growth. In MSCs, DKK3 may synergize or agonize with the non-canonical Wnt signaling to activate mTOR and then to inhibit autophagy and MHC II expression. Also, DKK3 may synergize or agonize with the non-canonical Wnt signaling to enhance JNK activities and to induce the expression of chemokines such as CCL2 and CXCL8. These DKK3-mediated activities may support MSCs to suppress anti-tumor immunity and therefore support the tumor growth. The figure is modified from *Münz, Annu. Rev. Immunol. 27:423–49 and Powell, et al., Annu. Rev. Immunol. 30:39–68.*

4.3 Outlook

Our studies contribute to a better understanding of mechanisms, which are involved in the control of T cell responses, and open new perspectives for further investigations.

Previous studies and our work provided evidence that the Nck adaptor proteins are essential for thymic selection, peripheral maintenance and effector function [52, 53]. Our finding that secondary T cell-dependent antibody responses are impaired in Nck.T$^{-/-}$ mice suggests defects in memory T cell formation in the absence of Nck. Mutations on SLP-76, which is the central adaptor in proximal TCR signaling [31, 365] and can be a binding partner of Nck adaptor proteins, have been shown to dampen the TCR signaling and favour the memory formation in T cells [366]. Deficiency of SLP-76 in CD4^{+} T cells led to poorer persistence of formed CD4^{+} memory T cells and impaired production of cytokines in memory responses [367]. Intriguingly, the persistence of CD8^{+} memory T cells seems to be less dependent on SLP-76 [368]. Therefore, it will be interesting to further clarify the contribution of TCR signaling strength and of different signaling components, such as Nck adaptor proteins, to memory T cell formation and to evaluate possible consequences for vaccination.

Based on our findings, Nck proteins may serve as possible clinical index for human primary immunodeficiency diseases (PIDs) [369]. Among common variable immunodeficiency (CVID) [370] patients, around 90% cases are caused by unknown factors. About 10 to 20% of patients demonstrate familial inheritent patterns of diseases, indicating an involvement of the genetic information. The basic criteria for CVID include reduced serum concentrations of IgG, impaired specific antibody responses, decreased T cell responses, and recurrent infections [371]. These phenotypes of CVID in antibody and T cell responses may recall our findings in the Nck.T$^{-/-}$ murine model. Impaired germinal center formation is also reported in the lymphadenopathy of patients with CVID [372]. Hence, it may worth to study whether Nck genes may be linked to the pathogenesis of the immunodeficiencies.

Based on our studies, DKK3 promotes tumor growth through MSCs. However, DKK3 is found to be commonly reduced in tumor tissues of developing tumors [215], which indicates adversary roles of DKK3 for tumor growth. Nowadays, increasing efforts are carried on to develop DKK3 as a potential anti-tumoral agent in various types of human cancer [373-375]. Our studies revealed immunosuppressive effects of DKK3 in the tumor mass. These findings may counteract the optimmistic expectation for the antitumoral capacity of DKK3. Hence, we propose further detailed studies on the role of Dkk3 in tumor development and immune responses against these tumors before starting clinical trials.

Furthermore, MSCs have been applied in murine autoimmune models, such as EAE [376] to evaluate the therapeutic potential for corresponding human autoimmune diseases. In some studies MSC-treated mice show ameleorated disease outcome [377] whereas such a reduction in EAE was not observed in others [378], suggesting that the heterogenicity of the used MSCs population and thereby possibly its capacity to produce DKK3 may be critical for successful treatment. Moreover, autopahgy was found to inhibit the immunosuppressive function of MSCs in EAE [379]. This is corresponding to our findings that the enhanced autophagy in DKK3$^{-/-}$ MSCs is correlated with the loss of the immunosuppressive capacity. Hence, it is possible that DKK3 can play an important role in the MSC-based therapy of autoimmune diseases.

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