SKIN TRANSPLANTATION IN A MOUSE MODEL OF NAÏVE T-CELL DEFICIENCY

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To my Father, who showed me how to be a GOOD person, which I hope I became.

I seem to have been only like a boy playing on the seashore, (…) finding a smoother pebble or a prettier shell than ordinary, whilst the great ocean of truth lay all undiscovered before me.

– Isaac Newton –

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Abbreviations

Ab	Antibody
ABM	Antigen-specific CD4+ T cells towards the I-A^{bm12}
ACK	Ammonium-Chloride-Potassium
ADCC	Antibody-dependent cell-mediated cytotoxicity
Ag	Antigen
AICD	Activation-induced cell death
APC	Allophycocyanin
APC	Antigen-presenting cell
Bcl-2	B cell lymphoma 2
BL/6 or B6	C57BL/6 mouse
BrdU	5-bromo-2'-deoxyuridine
Ca^{2+}	Calcium ions
CC	Coiled-coil
CCR7	C-C chemokine receptor type 7
CD	Cluster of differentiation
cCD4	Conventional CD4+ T cells
CD40L	CD40 ligand
CD62L	CD62 L-selectin
CICD	Cytokine-induced cell death
Cor1-/-	Coronin 1-deficient mouse
CRAC	Calcium release activated channel
CTL	Cytotoxic T lymphocytes
CTLA-4	Cytotoxic T-lymphocyte antigen 4
CTV	Cell trace violet
DC	Dendritic cells
DMSO	Dimethyl sulfoxide
DN	Double negative
DNA	Deoxyribonucleic acid
DP	Double positive

EAE	Experimental autoimmune encephalomyelitis
EBV	Epstein-Barr Virus
EC	Endothelial cell
EDTA	Ethylenediaminetetraacetic acid
ER	Endoplasmic reticulum
EtOH	Ethanol
FACS	Fluorescence-activated cell sorting
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanat
Foxp3	Forkhead box protein 3
GM-CSF	Granulocyte macrophage colony-stimulating factor
IFN	Interferon
ICAM	Intercellular adhesion molecule
Ig	Immunoglobulin
IL	Interleukin
IP3	Inositol-1,4,5-triphosphate
IP3R	Inositol-1,4,5-triphosphate receptor
kDa	Kilodalton
Lamp-1	Lysosomal associated membrane protein 1 (CD107a)
LCMV	Lymphocytic Choriomeningitis Virus
LEAF	Low Endotoxin, Azide-Free
LFA-1	Lymphocyte-function-associated antigen 1
LN	Lymph node
mAB	Monoclonal antibody
Mac-1	Macrophage-1 antigen (αMβ2 integrin)
MHC	Major histocompatibility complex
MLR	Mixed lymphocyte reaction
mTECs	Medullary thymic epithelial cells
Na-Py	Sodium Pyruvate
NEAA	Non-essential Amino Acid Solution
NFAT	Nuclear factor of activated T cells

NK	Natural Killer cells
OD	Optical density
PBS	Phosphate buffer saline
PD-1	Programmed cell death protein 1
PE	Phycoerithrin
PE/Cy7	Phycoerithrin/Cyanine 7
PerCp	Peridinin Chlorophyll protein
PerCp/Cy5.5	Peridinin Chlorophyll/Cyanine 5.5
PLCγ1	Phospholipase Cγ1
PMA/I	Phorbol-12-myristate-13-acetate and Ionomycin stimulation
Rag2	Recombinase-2 gene
RPMI	Roswell Park Memorial Institute
RTE	Recent thymic emigrants
SCID	Severe combined immunodeficiency
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate - polyacrylamide gel electrophoresis
SF-IMDM	Serum-Free Iscove's Modified Dulbecco's Medium
SNP	Single-nucleotide polymorphism
SP	Single positive
SPF	Specific pathogen free
STIM1	Stromal interaction molecule 1
TACO	Tryptophan-Aspartate containing coat protein
TCR	T-cell receptor
TGF-β	Transforming growth factor beta
T_H	T helper cell
TNF	Tumor necrosis factor
TNFR	Tumor necrosis factor receptor
Treg	Regulatory T cell
TSAs	Tissue specific antigens
UD	Unique domain

WD	Tryptophan/aspartate
Wt	Wild type
VCAM-1	Vascular cell adhesion molecule 1
VLA-4	Very late antigen 4 (α4-integrin)
VSV	Vesicular stomatitis virus

1 Summary

Coronin 1 is one of seven mammalian coronin family members that is preferentially expressed in hematopoietic cells and brain tissue (Ferrari et al. 1999; Jayachandran et al. 2014). It was initially discovered in macrophages where it is involved in *Mycobacterium tuberculosis* pathogenesis and survival (Ferrari et al. 1999). Interestingly, analysis of coronin 1-deficient mice showed a drastic reduction in the numbers of T cells in the periphery (Föger et al. 2006; Shiow et al. 2008; Haraldsson et al. 2008; Mueller et al. 2008). However, segregation and development of T cells in the thymus showed no significant changes when compared with wild type mice (Mueller et al. 2008). Recent data demonstrate that despite the paucity in naïve T cells, coronin 1-deficient mice are resistant to infections but fail to induce autoimmune diseases (Tchang et al. 2013; Haraldsson et al. 2008; Shiow et al. 2008; Siegmund et al. 2011). The physiological relevance of coronin 1 deficiency in allorejection is yet to be discovered, however, the importance of T cells in this process suggests a critical role for coronin 1. When organs are transplanted between members of the same species, an immune response to such a graft develops alloreactivity. It consists of the same mechanism as an immune response for defense against pathogens but it is mediated against differences in major histocompatibility complexes (MHC) class I and II molecules between the host and the donor (Goldsby et al. 2002).

The goal of my project was to dissect the ability of coronin 1-deficient T cells to recognize foreign MHC complexes and to reject the allograft. I focused on finding the mechanisms that allow coronin 1-deficent mice to keep the graft while at the same time drive the immunity against infection. To this end, I combined skin transplantation experiments with in-depth *in vitro* analysis.

Interestingly, we found prolonged survival of Balb/c skin transplanted onto coronin 1-deficient BL/6 recipients. Further investigations showed tolerance induction in a minor mismatch setting where BL/6 skin presenting minor histocompatibility antigens from

Balb/c were transplanted onto coronin 1-deficient mice. This could be the outcome of reduced frequency and/or impaired activation of T cells in the absence of coronin 1. However, despite increasing coronin 1-deficient CD4+ T cells numbers, we could not induce rejection in Rag2-/- mice transplanted with bm12Rag2-/- skin. Although the retained lower counts of CD4+ T cells could indicate a defect in proliferation or antigen recognition after skin transplantation, further *in vitro* experiments suggested that coronin 1-deficient T cells were able to recognize antigen through the T cell receptor (TCR) and respond, could become activated and could proliferate. Furthermore, cytokines and cell markers analysis suggest that coronin 1-deficient T cells were under the stress of high activation ($IFN\gamma^{hi}$, $CD5^{hi}$, IL-$7R^{lo}$) and intense proliferation (Ki-67^{hi}) that could cause exhaustion (PD-1^{hi}) and consequently lead to their death (FasL). As the same markers were not changed in the thymus of coronin 1-deficient mice and the mice could produce recent thymic emigrants this can provide insight into the time point at which coronin 1 is essential for T cells' survival. In-depth analysis of T cells *in vitro* and *in vivo* confirmed the different impact of coronin 1-deficiency in CD8+ and CD4+ T cells, as the prolonged graft survival resembled the depletion of CD4+ T cells in skin transplantation model (Goldsby et al. 2002). In a CD4+ T cell-dependent model, where bm12Rag2-/- skin was transplanted onto coronin 1-deficient mice, we observed acceptance of the graft. What is more, the bm1 (CD8-dependent) skin was rejected in coronin 1-deficient recipients upon transplantation. This absence or delay in rejection in coronin 1-deficient mice caused by induction of tolerance may be due to elevated rate of regulatory T cells (Tregs), the suppressive abilities of conventional CD4+ T cells or combination of these. Finding increased frequencies of Tregs in the coronin 1-deficient mice, compared to wild type mice, supported this statement. In-depth analysis of regulatory T cells in coronin 1-deficient mice showed no difference in suppressive abilities compared with wild type cells. Moreover, we demonstrated that the cells did not generate or proliferate better in coronin 1-deficient mice. Nevertheless, their superior survival over conventional coronin 1-deficient CD4+ T cells *in vitro* could explain their higher frequencies and could be linked to enhanced expression of PD-1. This, in turn, promotes apoptosis in antigen-specific T cells while increasing survival of Tregs (Francisco, Sage, and Sharpe 2010). Overall, the data suggests a critical role of coronin 1 in T cell-mediated allograft rejection. However, its precise function and mechanistic details are subject for further study.

2 Introduction

2.1 Coronin protein family

Coronins constitute an evolutionary conserved family of proteins widely expressed from yeast to human (Eckert, Hammesfahr, and Kollmar 2011; Jean Pieters, Müller, and Jayachandran 2013). Lower eukaryotes, such as yeast *Saccharomyces cerevisiae* and *Dictyostelium discoideum*, have only single coronin gene, called Crn and Coronin A (Clemen, Rybakin, and Eichinger 2008), respectively, while mammalian cells can co-express several coronin molecules. Seven coronin isoforms have been identified in mammalians so far (Jean Pieters, Müller, and Jayachandran 2013; Okumura et al. 1998). Interestingly, gene expression suggests that most of the mammalian coronins are expressed in immune cells and the nervous system (Jean Pieters, Müller, and Jayachandran 2013).

Coronin	Alternative names	Expression	Chromosomal localization		Protein length	
			Human	Mice	Human	Mice
Coronin 1	CORO1A, p57, TACO, CLIPINA, CRN 4, CLABP	Immune cells, neurons	16p11.2	7F3	461	461
Coronin 2	CORO1B, Coroninse, p66, CRN1	Immune cells, neurons	11q13.2	19A	489	484
Coronin 3	CORO1C, HCRNN4, CRN2	Ubiquitous	12q24.1	5F	474	474
Coronin 4	CORO2A, IR10, CLIPINB, WDR2, CRN5	Brain	9q22.3	4B1	525	524
Coronin 5	CORO2B, CLIPINC, CRN6	Brain	15q23	9B	480	480
Coronin 6	CLIPONE	Ubiquitous	17q11.2	11B5	472	471
Coronin 7	POD1, p70	Immune cells, brain, fibroblasts	16p13.3	16A1	925	922

Table 1. ***Mammalian coronin isoforms.*** *Table presents alternative names, expression, localization in the chromosome and protein length of different mammalian coronin isoforms [adapted from* (Jean Pieters, Müller, and Jayachandran 2013)*].*

The coronin protein structure is characterized by an amino-terminal tryptophan/aspartate (WD) repeat-containing domain followed by a unique region of variable length connected

to a coiled-coil (CC) carboxy-terminal domain, which is essential for oligomerization (de Hostos 1999; John Gatfield et al. 2005). The mammalian coronin family can be divided into two groups based on their structure (Fig. 1). Coronin 1 to coronin 6, coronin of *D. discoideum* and *S. cerevisiae* share the classical coronin structure described above (Kammerer et al. 2005). Coronin 7 presents a tandem repetition of the core WD-repeats region and lacks the coiled-coil domain (Rybakin et al. 2004; Yuan et al. 2014). Biophysical analysis of the coronin 1 C-terminal domain demonstrated that this protein forms a trimeric structure (Kammerer et al. 2005; Appleton, Wu, and Wiesmann 2006). Although coronin proteins lack a transmembrane domain, many of them co-localize with membranes possibly through their interaction with cholesterol and the cytoskeleton (John Gatfield et al. 2005; J. Gatfield and Pieters 2000).

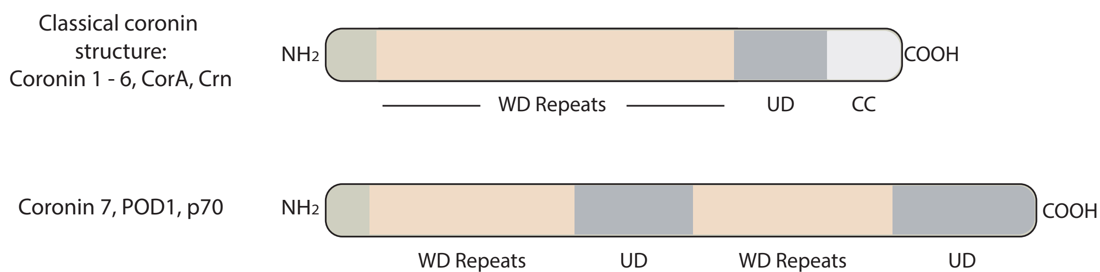


Figure 1. *Structure of coronins.* *Classical structure of coronin proteins is shared by coronin 1 to coronin 6, CorA from D. discoideum and Crn of S. cerevisiae. Coronin 7, POD1 and p70 consist of tandem repeats of WD fragment and lack the coiled coil region. WD Repeats – tryptophan/aspartate repeats; UD – unique domain; CC – coiled coil domain [adapted from* **(Jean Pieters, Müller, and Jayachandran 2013)***].*

Coronin A, the first identified member of the coronin protein family in *D. discoideum* (Eckert, Hammesfahr, and Kollmar 2011; Jean Pieters, Müller, and Jayachandran 2013), was shown to co-precipitate with actin-myosin complexes (de Hostos et al. 1991). Deletion of this protein in *D. discoideum* manifests defects in motility, phagocytosis and chemotaxis of a slime mold (de Hostos et al. 1993; Gerisch et al. 1995). In addition, recently coronin A has been identified to be involved in cAMP signaling, sensing and development (Vinet et al. 2014). In mammals coronin 1, 2, 4 and 7 have been associated with F-actin (John Gatfield et al. 2005; W. Huang et al. 2011; Cai, Makhov, and Bear 2007). Moreover, the interaction between coronin and actin also occurs in invertebrates and yeasts (John Gatfield et al. 2005; W. Huang et al. 2011; de Hostos et al. 1993; Galkin

et al. 2008). Together, it would suggest that coronins have evolved from a family of actin binding proteins in lower eukaryotes to regulators of physiological processes in mammals. Recent studies have shown an involvement of coronins in the development of several diseases including T cells immunodeficiency, development of invasive cancer and the resistance to autoimmunity (Haraldsson et al. 2008, 3; Shiow et al. 2009; Moshous et al. 2013).

2.1.1 Discovery, general features and function of Coronin 1

Mammalian coronin 1 is the best-studied coronin, both in terms of structure and function. This protein is highly expressed in cells of hematopoietic origin, such as leukocytes (Ferrari et al. 1999; Jean Pieters 2008a) as well as in certain neural cell types (Jayachandran et al. 2014). Coronin 1 was initially discovered in macrophages where it prevents the intracellular degradation of *Mycobacterium tuberculosis* (Ferrari et al. 1999) which was linked to the ability of coronin 1 to activate the Ca^{2+}/calcineurin pathway (Jayachandran et al. 2007). *M. tuberculosis* is the causing pathogen of tuberculosis disease, and is able to evade the host's immune response through manipulating the phagosomes in which it resides. Macrophages, normally, represent the first barrier of the innate immune system by killing pathogens upon entry into the host (Jean Pieters 2008b). After internalization by macrophages, *M. tuberculosis* prevents maturation of phagosomes and its fusion with the lysosome (Armstrong and Hart 1975; D. G. Russell 2001). In doing so, *M. tuberculosis* induces favorable environment for its survival (Hasan et al. 1997). Interestingly, this ability is only found in live bacilli (Jean Pieters 2008b). To understand how mycobacteria prevent lysosomal delivery mechanistic level phagosomes containing live bacteria were isolated and a 51kDa protein was identified as being involved. The protein was termed Tryptophan-Aspartate containing coat protein (TACO) (Hasan et al. 1997; Ferrari et al. 1999). After discovery of several homologues proteins, which were all related to the coronin family, also present in *D. discoideum* and *S. cerevisiae*, TACO is now referred to as a coronin 1. Several studies have implicated that coronin 1 is essential for intracellular survival of not only mycobacteria but also *Mycobacterium leprea* and *Helicobacter pyroli* in macrophages (Ferrari et al. 1999; Seto, Tsujimura, and Koide

2012; Montoya and Modlin 2010; Zheng and Jones 2003). The mechanism that enables coronin 1 to modulate mycobacterial survival is connected with calcium signaling following their entry into macrophages. Mycobacterial uptake is associated with transient increase of intracellular calcium levels, which activates the calcium-dependent phosphatase calcineurin (Jayachandran et al. 2007). It was shown that a depletion of intracellular calcium ions (by chelating agents) phenocopies coronin 1 deficiency leading to clearing of mycobacteria. Simultaneously, an increase of intracellular calcium ions in coronin 1-deficient macrophages results in survival of mycobacteria (Jayachandran et al. 2007). Therefore, mycobacterial survival within macrophages is tightly linked to the coronin 1-dependent activation of the calcineurin pathway.

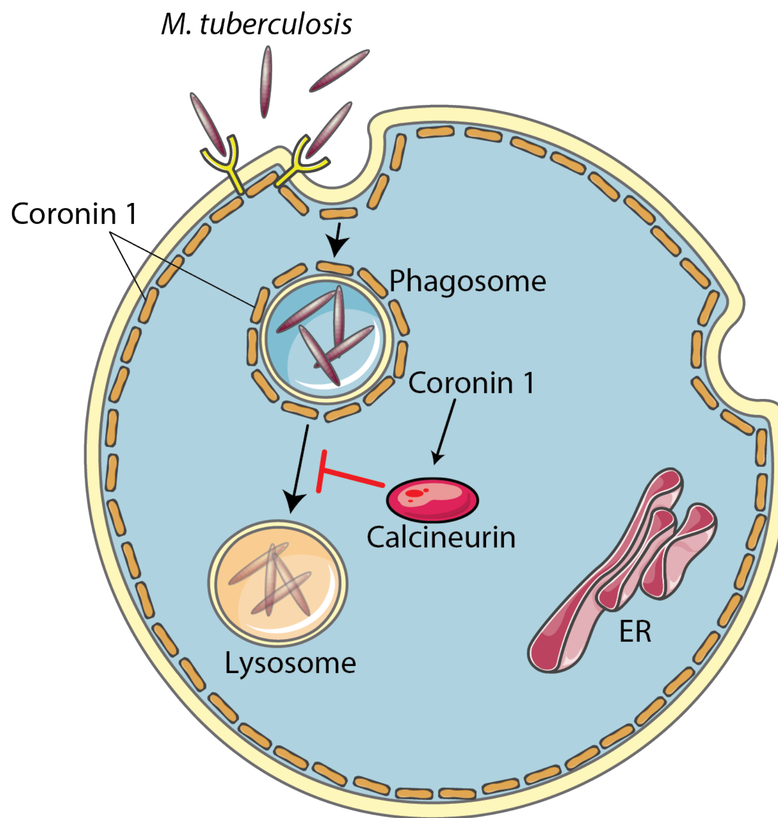


Figure 2. ***Model of mycobacterial entry into macrophage and coronin 1 function.*** *Upon mycobacterial entry into macrophage coronin 1 is recruited to the phagosomes containing M. tuberculosis. That leads to the activation of the calcium-dependent phosphatase calcineurin. Calcineurin blocks phagosome-lysosome fusion therefore allowing M. tuberculosis to survive. ER – endoplasmic reticulum [adapted from* (Jean Pieters, Müller, and Jayachandran 2013)*].*

2.1.2 Coronin 1 and T lymphocytes

To understand the role of coronin 1 *in vivo*, a mouse line in which coronin 1 was deleted by homologous recombination was established. The analysis of these mice revealed that coronin 1 is essential for T cell homeostasis (Föger et al. 2006; Shiow et al. 2008; Haraldsson et al. 2008; Mueller et al. 2008) as it is involved in the generation of the second messenger inositol-1,4,5-triphosphate (IP3) after TCR activation (Jayachandran et al. 2007; Föger et al. 2006; Mueller et al. 2008). Thus, in the absence of coronin 1, intracellular Ca^{2+} release is impaired leading to defective activation of Ca^{2+}-dependent phosphatase calcineurin (Winslow et al. 2006; Oh-hora and Rao 2008). As a result, the Ca^{2+}-dependent phosphatase calcineurin fails to dephosphorylate the transcription factor NFAT and as a result there is defective expression of pro-survival genes. Thus coronin 1 plays a crucial role in naïve T cells survival. That would explain the deletion of naïve T cells from the periphery in mice lacking coronin 1, which is the most striking phenotype of this mouse line. As an alternative explanation, defective F-actin dynamics in the absence of coronin 1 has been suggested to result in T cell death (Föger et al. 2006). However, more recent work has failed to find a correlation between increased phalloidin staining and naïve T cell survival, which indicates that changes in the levels of F-actin might not necessarily correlate with survival defects in T cells (Jean Pieters, Müller, and Jayachandran 2013; Shiow et al. 2008; Mueller, Liu, and Pieters 2011; Haraldsson et al. 2008). Furthermore, coronin 1 mutation in human is also associated with severe peripheral T cell depletion (Moshous et al. 2013; Shiow et al. 2009; Shiow et al. 2008; Stray-Pedersen et al. 2014; Yee et al. 2016). Coronin 1-deficient mice have mainly depleted naïve T cells while the effector and memory T cells are less affected both in numbers and functionality (Föger et al. 2006; Mueller et al. 2008). That is why, the main fraction of residual T cells in these mice are central memory T cells characterized by high expression of CD44 and low expression of CD62L (Mueller et al. 2008; Mueller, Liu, and Pieters 2011; Mugnier et al. 2008). Findings that coronin 1 modulates downstream of the TCR signal transduction cascade is consistent with studies showing that T cell homeostasis and survival are tightly linked to the capacity of these cells to receive a tonic TCR stimulus. Additionally, coronin 1-deficient naïve T cells express more caspase 3 and have higher levels of cleaved caspase 3 product, and lower levels of the anti-apoptotic

molecule B cell lymphoma 2 (Bcl-2) (Mueller, Liu, and Pieters 2011). Thereby, coronin 1 deficiency imitates the absence of TCR or MHC molecules in the periphery suggesting an essential role for this protein in tonic TCR stimulation (Surh and Sprent 2008; Polic et al. 2001; Labrecque et al. 2001). What is more, thymic selection and cellularity is normal in the absence of coronin 1 (Mueller et al. 2008). Also, coronin 1-deficient mice show normal antigen processing and presentation by dendritic cells (Westritschnig et al. 2013), and the neutrophil population is fully functional (Moshous et al. 2013; Siegmund et al. 2013; Combaluzier and Pieters 2009). Other immune cell types, such as B cells, macrophages, mast cells, and NK cells, that also express coronin 1, appear not to be affected by the deletion of coronin 1 (Combaluzier et al. 2009; Westritschnig et al. 2013). Interestingly, when coronin 1-deficient mice were infected with Lymphocytic Choriomeningitis Virus (LCMV), the CD8+ T cell response was relatively normal compared to wild type controls, and led to the clearance of the virus (Tchang et al. 2013). Simultaneously, after infection with Vesicular Stomatitis Virus (VSV) where the CD4+ T cells play crucial role, their response was impaired and mice were not protected from the virus (Tchang et al. 2013). These findings indicate that coronin 1 deficiency has a different impact on CD4+ and CD8+ T cell. Furthermore, these mice age normally and are not more susceptible to tumor development or infections despite their depletion in T cells (unpublished observations). Despite a relatively normal immune response against bacterial and viral antigens, mice deficient in coronin 1 are not susceptible to experimental autoimmune encephalomyelitis (EAE) (Siegmund et al. 2011) and protected against systemic lupus erythematosus (Haraldsson et al. 2008). Recent studies, using T cells-specific coronin 1 knockout mouse model, confirmed an intrinsic role of coronin 1 in T cell function, as the phenotype observed in this study resembled whole-body coronin 1 knockout mice (Siegmund et al. 2016). Although coronin 1 is expressed in all immune cells, its deficiency leads to specific kind of severe combined immunodeficiency (SCID), which is characterized by naïve T cells depletion and the ability to resist to specific infection while at the same time resistance to autoimmune diseases.

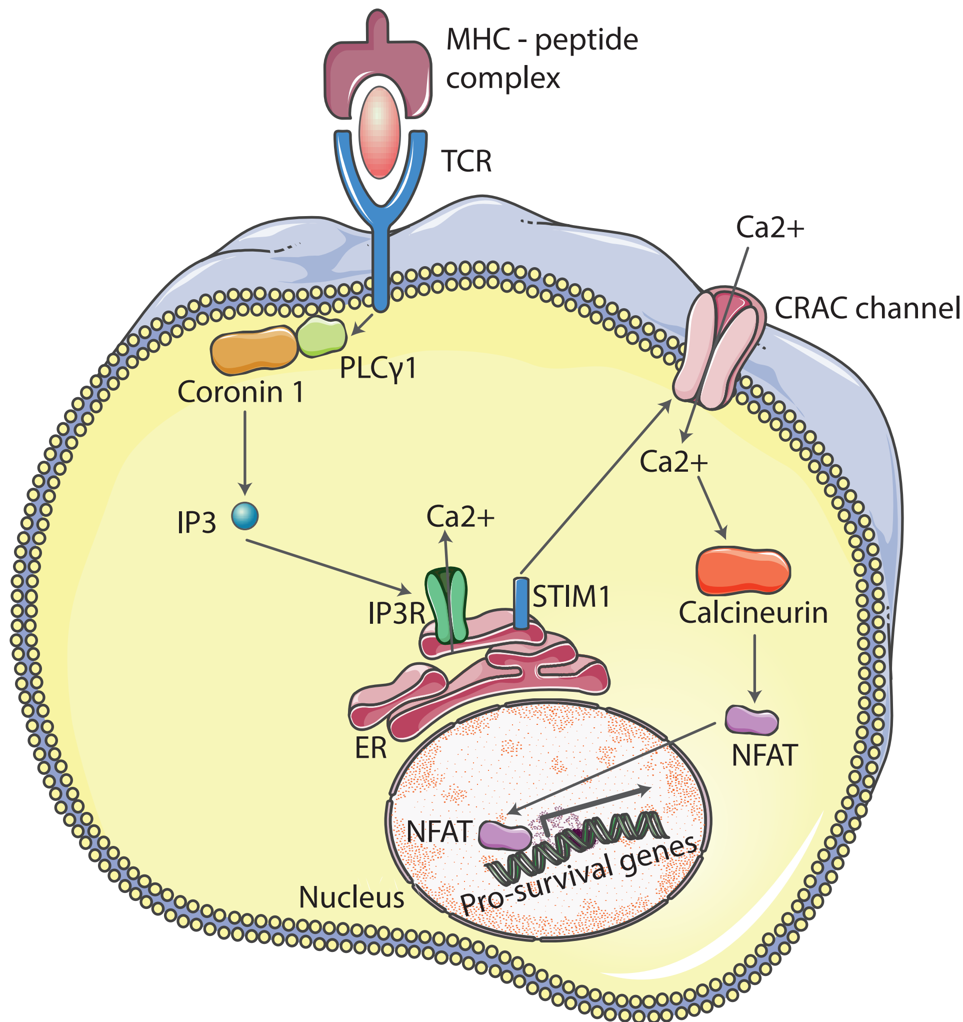


Figure 3. ***The impaired survival of coronin 1-deficient T cells in the periphery.*** *The deletion of coronin 1 in mice causes defective calcium mobilization downstream of T cell receptor (TCR) upon stimulation with peptide-MHC complexes. This impaired calcium signaling blocks activation of calcineurin, which in the end results in disturbed expression of pro-survival genes. CRAC – calcium release activated channel; ER – endoplasmic reticulum; IP3 – inositol-1,4,5-triphosphate; IP3R – IP3 receptor; NFAT – nuclear factor of activated T cells; PLCγ1 – phospholipase Cγ1; STIM1 – stromal interaction molecule 1 [adapted from* (Jean Pieters, Müller, and Jayachandran 2013)*]*.

2.1.3 Coronin 1 mutations effect in humans

Similarly to murine immune system, coronin 1 plays an important role in humans. So far, all described coronin 1-deficient patients manifested with severe T cell lymphocytopenia,

while B cells and NK cells were present (Shiow et al. 2009; Shiow et al. 2008; Moshous et al. 2013; Stray-Pedersen et al. 2014; Yee et al. 2016). Patients having mutation or deletion in coronin 1 gene, which resulted in total abrogation of the protein, were diagnosed with recurrent respiratory infections at early age (within first two years of age). Additionally, they suffered from Epstein-Barr Virus (EBV)-induced B-cell lymphomas and impairment in the development of cognitive function (such as social deficits, increased aggression and learning abilities) (Shiow et al. 2008; Shiow et al. 2009; Moshous et al. 2013; Jayachandran et al. 2014). Another two siblings, diagnosed with T-B+NK+ cells SCID, described by Stray-Pedersen et al., showed late disease onset at the age of 7 years old. They possessed compound heterozygous variants and mutation in coronin 1 gene. Both suffered from multiple viral infections and the older patient died of EBV-induced lymphoma. Interestingly, despite of recurrent epidermal-dermal lesions (from tuberculosis leprea, epidermodysplasia verruciformis and molluscum) both patients did not suffer from pain (Stray-Pedersen et al. 2014). Strikingly, all of the male patients diagnosed with coronin 1 deficiency survived longer than female patients (Shiow et al. 2008; Shiow et al. 2009; Moshous et al. 2013). There are two more young adult patients described with coronin 1 mutation, which resulted in deletion of coiled-coil (CC) domain that impairs oligomerization of coronin 1. These patients were diagnosed with CD4+ T cell lymphopenia resulting in recurrent viral infection as well as disseminated varicella and pneumonia, however, they continue to survive into young adulthood without SCID phenotype and lymphoproliferative disease (Yee et al. 2016).

2.2 Principles of immune activation

Our body is constantly exposed to environmental pathogenic attacks, such as viruses and bacteria. Fortunately, we developed an immune system that serves as a safeguard shielding us from most of the dangers. The immune system provides innate and adaptive immune responses.

Innate immunity consists of molecular and cellular mechanisms that are ready for immediate defense against infectious agents (viruses, bacteria, fungi or parasites). Innate response is considered as the less specific defense, however, it provides the first line of protection for the host. The main physical barriers of the innate immune system are the epithelial layers of the skin and the mucosal and glandular tissue surfaces, which block pathogen entry to the body. Additionally, soluble substances possessing antimicrobial activity and acidic pH are produced at these surfaces. The next barrier that is faced by pathogens is the cellular response of the innate immune system. This response is rapid, beginning within minutes after invasion. Cellular responses are triggered by cell surface and intracellular receptors that recognize conserved molecular patterns of pathogens (Akira, Uematsu, and Takeuchi 2006).

Despite the many barriers of innate immune system, some pathogens are able to survive innate defenses and invade the organism. In this situation the adaptive immune responses are ready to take part in the defense. The adaptive immune system counters infection with a specific response to the attacking pathogen. This specific response is mounted within five to six days after infection. It is characterized by its high specificity to the recognized antigen and provides immunological memory. The recognition of billions of single structures is possible thanks to the capacity of the adaptive immunity to generate a remarkable diversity in recognized molecules. What is more important, the adaptive immune system has the capacity to discriminate self from non-self (Kindt, Osborne, and Goldsby 2006b; Billingham, Brent, and Medawar 2010; Burnet, F., M. and Fenner 1941; Burnet 1976). Adaptive immunity responses are conducted by B and T lymphocytes. Major task of B cells is to produce specific antibodies, whereas T cells play a role in recognizing, neutralizing and eliminating the invaders. T lymphocytes can be divided into two main populations: CD4+ and CD8+ T cells. CD4+ T lymphocytes provide help to B

cells by CD40L:CD40 interaction and production of cytokines. Furthermore, CD4+ T cells facilitate CD8+ T cell expansion and activation of macrophages through cytokine production, such as IL-2, IFNγ and TNF. CD8+ T lymphocytes, termed cytotoxic T cells, kill infected cells, via cell-cell interaction and by secretion of cytokines for example, IFNγ (S. Huang et al. 1993; Suzuki et al. 1988). Peptides derived from infectious agents and intracellular antigens are recognized by T cell receptors (TCR) only when bound to major histocompatibility complexes (MHC). There are two types of MHC molecules: MHC class I, expressed on almost all nucleated cells, and MHC class II molecules expressed only on professional antigen presenting cells (APC) (Kindt, Osborne, and Goldsby 2006a). According to the convention, only cells presenting peptide via MHC class II molecules are called professional APC, cells presenting peptide on class I molecules are referred to as target cells. Three types of cells belong to APC: dendritic cells (DC), macrophages and B lymphocytes (Banchereau et al. 2000; Guermonprez et al. 2002). Antigen presenting cells are able to induce T cell responses following infection or organ transplantation. Dendritic cells constitutively express high levels of MHC class I and II molecules as well as co-stimulatory proteins of B7 family. Consequently, they are more potent in antigen presentation than macrophages and B cells, which have to be activated prior to antigen presentation. Additionally, DC can migrate to the site of inflammation in the periphery (Pulendran, Palucka, and Banchereau 2001). There are studies showing that also non-hematopoietic cells (like endothelial cells) can act as APC. Endothelial cells (EC) can be induced to express MHC class II upon inflammation and present antigens to T cells for short period of time (Marelli-Berg et al. 2001). Endothelial cells act as APC in the context of an allograft transplantation where they display antigens to T cells. Presentation by endothelial cells can be carried out through the direct presentation where they expose allo-MHC-peptide complexes, or via indirect pathway, which involves cross-presentation of antigens (Marelli-Berg et al. 2001; Epperson and Pober 1994; Bagai et al. 2005; Rose 1998).

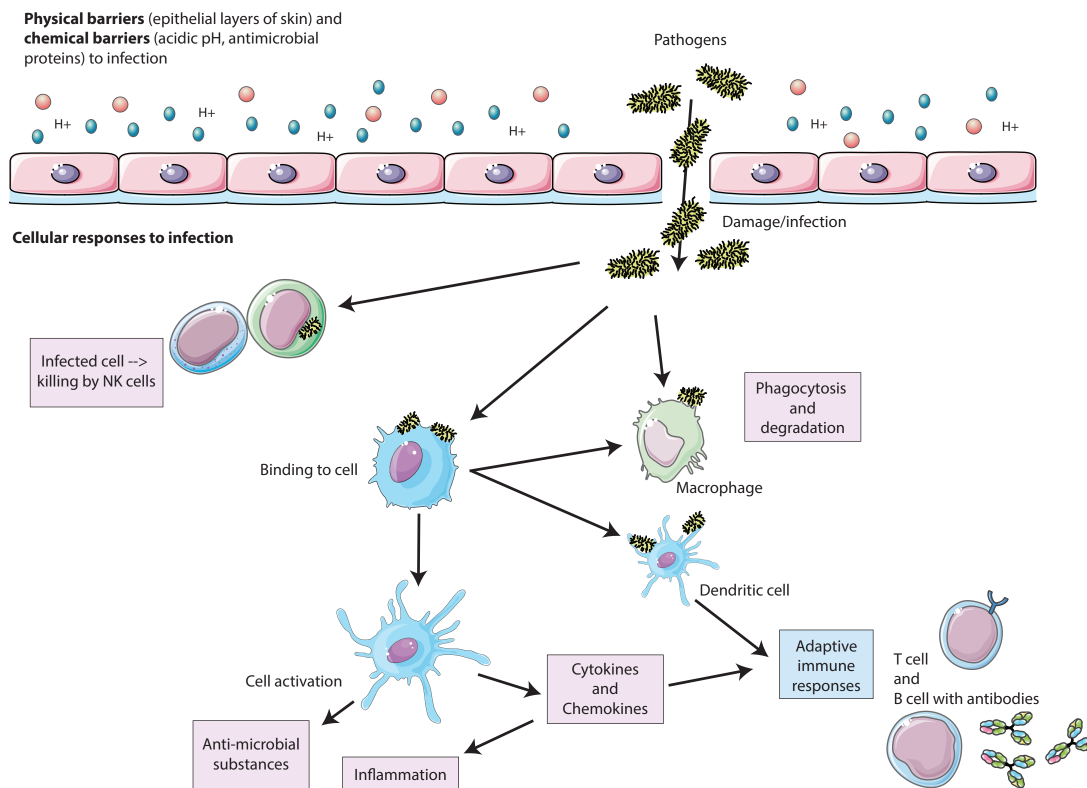


Figure 4. ***Innate and adaptive immune responses.*** *Physical and chemical barriers, that prevent infection, are key elements of innate immunity. Pathogen entry results in multiple cellular responses with cell surface and intracellular receptors recognizing molecular patterns on pathogens. After recognition of the pathogen, some cells are activated to phagocytose and degrade the intruder, other cells produce variety of antimicrobial substances that kill the pathogen. Secretion of cytokines and chemokines recruits leukocytes to the side of infection leading to the inflammation and stimulating adaptive immune responses. Additionally, natural killer (NK) cells of the innate immune system recognize and kill cells infected with viruses. Dendritic cells present pathogen to lymphocytes activating adaptive immune system [adapted from (Kindt, Osborne, and Goldsby 2006a)].*

2.2.1 T cell development and activation

T cells are the cellular mediators of the adaptive immune system. They develop in the thymus in a strictly regulated process which task is to produce maximal degree of diversity and to remove self-reactive T cells. Immature T lymphocyte precursors when traveling from the bone marrow to the thymus express no mature T cell features and no antigen receptor. T cell development in the thymus can be divided into two groups of

events: early thymocyte development and selection processes. Early thymocyte development starts with uncommitted double negative (DN) CD4-CD8- cells and ends with T-cell receptor expressing double positive (DP) CD4+CD8+ cells. During this stage hematopoietic precursors are committed to the T cell lineage, TCR rearrangements are initiated and the selection and expansion of cells with successfully rearranged one of TCR genes conducted (Chiei et al. 1987; Hayday et al. 1985; Raulet et al. 1985). The second phase of selection events is mainly dependent on T-cell receptor interactions. This stage brings CD4+CD8+ cells to the mature CD4+ or CD8+ single positive (SP) T cells. During this second phase of development immature T cells need to undergo positive selection, that select for T cells which possess TCR responding to self-MHC. Afterwards, T cells are subjected to negative selection, in which cells with TCR reacting strongly to self-peptide-MHC complexes are eliminated. In the end thymocytes are committed to effector cell lineages: CD4+ helper or CD8+ cytotoxic T cell populations. When leaving the thymus, mature T cells possess diverse specific receptors, which are tolerant to self and restricted to self-MHC (Carpenter and Bosselut 2010; Germain 2002; Singer 2010; Starr, Jameson, and Hogquist 2003; Teh et al. 1988).

After development in the thymus, naïve T cells migrate to the periphery where their number is strictly regulated by T cells homeostasis. This process aims at keeping the naïve T cells in a non-proliferative state while maintaining diversity such that their numbers remain constant (Goldrath and Bevan 1999; Takada and Jameson 2009). Several mechanisms have been proposed to be responsible for the survival of naïve T cells. One is based on the T cell receptor (TCR) that is triggered by the self-peptide presented in a complex with major histocompatibility complex (MHC) class I and class II molecules (Kirberg, Berns, and Boehmer 1997); another is based on IL-7 signaling (Takada and Jameson 2009). Naïve T lymphocytes circulate in the blood and home with the help of CD62L and CCR7 to secondary lymphoid organs which include spleen and lymph nodes (Sallusto et al. 1999). Without previous activation, they are not able to enter peripheral non-lymphoid tissue. To become activated and develop into effector cells, T cells need to be triggered through antigen presentation (signal 1) and co-stimulatory signals (signal 2), both presented by antigen presenting cells (Salomon and Bluestone 2001) (Fig. 5). These interactions are based on cell-cell contact. Antigen presenting cells can present peptides to

T cells only in the context of the MHC molecules. This phenomenon is called MHC-restriction, where CD4+ T cells recognize antigenic peptide bound to MHC class II and CD8+ T cells recognize antigenic peptide bound to MHC class I molecules (Zinkernagel and Doherty 1974a; Zinkernagel and Doherty 1974b). Following interaction of the TCR with antigen presenting cells, T cells up-regulate the expression of CD28 and CD40L. One or more of these surface molecules is engaged with their specific ligands on APC, including CD40, CD80 and CD86. This interaction provides co-stimulation of T cells. After this process, activated T cells are recruited to the sites of inflammation. Adhesion molecule ligands expressed on activated T cells (L-selectin, LFA-1, VLA-4, Mac-1) enable contact with adhesion molecules of the activated endothelium (P-selectin, E-selectin, VCAM-1, ICAM-1) resulting in entry of T cells to the periphery (Campbell et al. 1998; R. M. Rao et al. 2007).

2.2.2 T cell tolerance (central and peripheral tolerance)

Each mature T cell expresses a unique antigen receptor that has been assembled in the thymus during T-cell development through random gene rearrangement (Tonegawa et al. 1977; von Boehmer et al. 1988). Therefore, T cell development must be a highly controlled process where potentially self-reactive T cells are eliminated. This major self-tolerance mechanism, which plays a key role, is referred to as central tolerance (Kappler, Roehm, and Marrack 1987; Hogquist, Baldwin, and Jameson 2005). Developing thymocytes proliferate and differentiate following determined pathways to become functionally distinct populations of T cells. During positive and negative selection they are submitted to the test of recognizing self-peptides and self-MHC molecules. These self-antigens consist of peptides either ubiquitously expressed or those restricted to specific tissues, named tissue specific antigens (TSAs). Medullary thymic epithelial cells (mTECs) and bone marrow-derived dendritic cells, which capture the TSAs from mTECs and present them to the thymocytes, express TSAs (Kyewski and Derbinski 2004; Gallegos and Bevan 2004; Klein et al. 2009). However, it is possible that potentially self-reactive T cells can be released to the periphery after they escape negative selection. Consequently, self-reactive T cells can become activated and induce autoimmunity.

Therefore, peripheral tolerance was developed in the periphery to control self-reactivity (Zehn and Bevan 2006). One mechanism of peripheral tolerance is an interaction of a T cell with an immature DC, which leads to T cell anergy rather than activation. Dendritic cells not activated by inflammation or infection lack expression of co-stimulatory molecules. Although immature DCs can deliver signal 1, they are not able to deliver signal 2 causing T cell tolerance (Fig. 5). As has been already described by Jenkins in 1987, these two signals are essential for full T cell activation (Jenkins et al. 1987). Moreover, dendritic cells can express negative co-stimulatory signals, which inhibit TCR signaling. The molecules PD-1 and CTLA-4 expressed on the DCs are able to maintain peripheral tolerance of T cells. CTLA-4 is a member of the CD28 family and binds to CD80 and CD86 antagonizing T cell activating signals. Similarly, PD-1 inhibits TCR-mediated T cell activation, and has been recently described to also regulate the differentiation of CD4+ CD25+ Foxp3+ regulatory T cells (Tregs) (Probst et al. 2005; Sakaguchi et al. 2006). Hence dendritic cells have been shown to induce tolerance by activation of Tregs (Sakaguchi 2004). Regulatory T cells play a crucial role in the prevention of autoimmunity (Sakaguchi et al. 2006), inhibition of anti-tumoral immunity (Beyer and Schultze 2006) and, additionally, maintenance of maternal tolerance to the fetus (Aluvihare, Kallikourdis, and Betz 2004). Natural regulatory T cells develop in the thymus where they are positively selected and enriched in auto specific cells (Bensinger et al. 2001). Dendritic cells specifically activate these regulatory cells through the presentation of autoantigens. Additionally, Tregs need IL-2 to proliferate. Regulatory T cells down-regulate inflammation via secretion of IL-10 and TGF-β, which have inhibitory effects on APCs, or by direct interaction with T cells (Asseman et al. 1999; Fahlén et al. 2005). Peripheral tolerance, through Tregs, is responsible for the induction of tolerance towards foreign antigens. Therefore, following transplantation, when the induction of specific allogeneic tolerance is a main aim to prevent transplant rejection, Tregs are of the great importance. The importance of regulatory T cells is also reflected in the scurfy mouse model, where mutation within the Foxp3 gene causes the lack of functional regulatory T cells. Scurfy mice develop a lymphoproliferative disease and multiorgan inflammation caused by unrestrained activity of autoreactive CD4+ T cells. This X-linked mutation results in a death of homozygous males by 22 to 26 days of age (Brunkow et al. 2001; Clark et al. 1999; Godfrey et al. 1991).

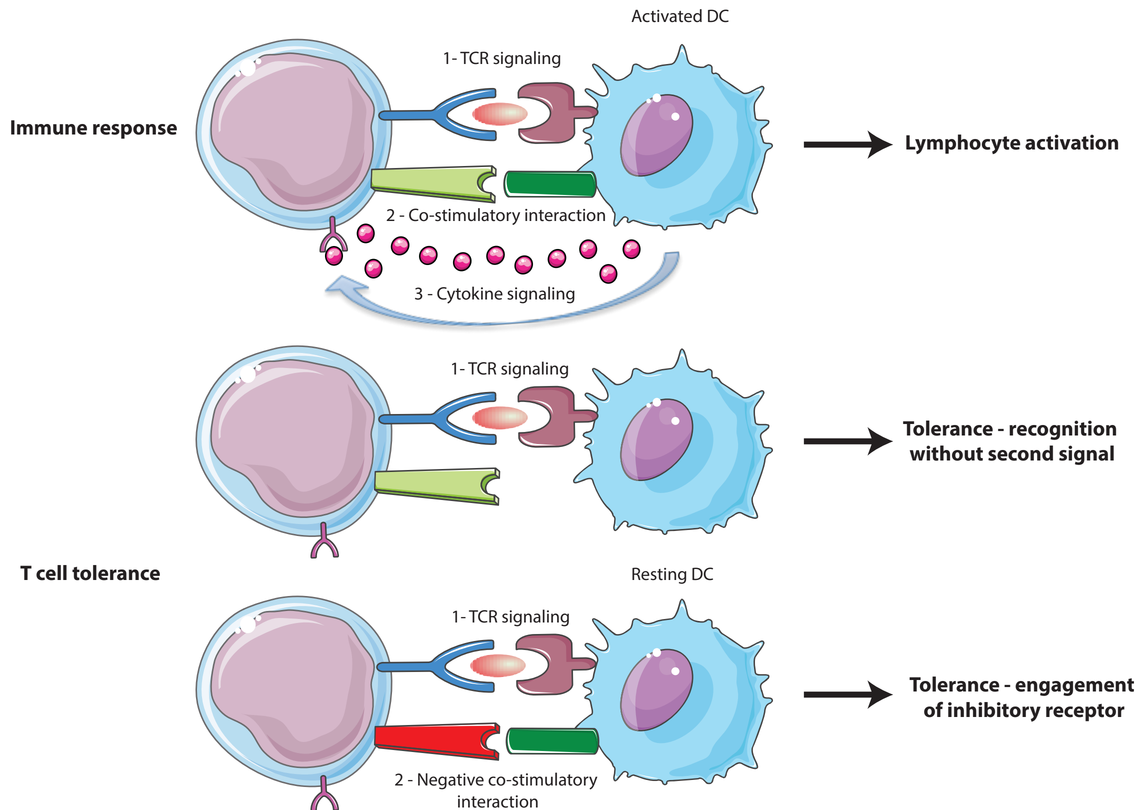


Figure 5. Immune activation and tolerance induction by dendritic cells. *Three signals are required for the activation of naïve T cells: TCR signaling, co-stimulatory binding and cytokine secretion. Signal 1 is provided by TCR/peptide-MHC complex interaction together with CD4+/CD8+ co-receptors and adhesion molecules. Co-stimulatory molecules, including CD28 family of proteins, supply co-stimulation in signal 2. Signal 1 and signal 2 initiate signal transduction that results in activation of transcription factors and secretion of cytokines providing signal 3 [adapted from (Owen, Punt, and Stranford 2013)].*

2.3 Transplantations

The elaborate mechanisms maintaining self-tolerance that are described above, are also responsible for rejection of any transplanted tissue or cells whenever the donor is not genetically identical to the recipient. Transplantation is an act of transferring cells, tissues or organs from one individual to another. We can distinguish several types of transplantation: autograft, the self-tissue transfer from one body site to another; isograft, where tissue is grafted between genetically identical individuals (inbred strains of mice or identical twins); allograft, tissue transfer between genetically different members of the same species (from one mouse strain to another); xenograft, is when the tissue is transferred between individuals from different species. Usually, autografts and isografts are accepted due to genetic identity of the donor and recipient. Allograft tissue, genetically different from the recipient, expresses unique antigens, thus is recognize as foreign by the immune system and therefore rejected. Xenograft, being the greatest challenge for the immune system, due to its genetic difference, displays vigorous graft rejection response (Alonso Arias, López-Vázquez, and López-Larrea 2012; Chinen and Buckley 2010).

Transplantation of organs or cells saves or prolongs many lives each year. Bone marrow transplantation is an extremely effective treatment of malignant blood cell diseases and kidney transplantation is the most commonly performed therapy nowadays. The subsequent most commonly transplanted organ is liver, heart comes next, followed by lung and pancreas. In addition, corneal tissue graft is also frequent. However, despite advances in surgical technics and improved immunosuppressive drugs, successful long-term survival of transplants is still restricted by late graft failure due to the immune responses (Alonso Arias, López-Vázquez, and López-Larrea 2012; Sayegh and Carpenter 2004). The frequency of transplantations for a given organ depends primarily on: organ availability, alternative treatment, the level of procedure difficulty and successful survival rate of grafted tissue. Skin transplantation has been a subject of a great interest for surgeons for many years. Its vitality, easiness in dissecting a large portion of skin from a donor and its adaptation in a recipient made the skin transplantation a valuable tool for plastic surgeries. However, already in 1870 doctors described many limitations in using this technique (Steele 1870). As we know now, this limitation comes from recipient's

immune system and alloreactivity. Therefore, most skin transplantations are conducted with autologous tissue. Rarely, after severe burns allogeneic skin grafts are used as a natural dressing, which in time is replaced with autologous skin. Allogeneic skin graft still gives high risk of rejection and infection nowadays (Scheuher 2016).

2.3.1 Alloreactivity

Transplantations are mainly performed between members of the same species thus referred to as allotransplants. The immune response to allotransplantation is called alloreactivity. The most intense graft rejection happens after transplantation between individuals with differences in ABO blood group. If the recipient carries antibodies to any of donor antigens, the grafted tissue will induce rapid antibody-mediated lysis of foreign donor cells. The next important match is the MHC compatibility between a donor and a recipient. But even when the MHC antigens are identical, the transplant can be rejected due to differences in the minor histocompatibility antigens (Sayegh and Carpenter 2004).

When T cells mature in the thymus and undergo positive and negative selection, they are selected to recognize self-MHC bound with non-self peptides. There is no process preparing them to bind to foreign MHC molecules that are not expressed in the body. The direct recognition of alloreactive T cells with non-self MHC molecule presenting foreign peptide is not MHC-restricted. Allogeneic T cells recognize the foreign MHC complex in the process of the recognition of major histocompatibility antigens. Minor histocompatibility antigens, unlike MHC, are recognized only when presented in the context of self-MHC. Rejection caused by minor histocompatibility variability is less robust but can lead to graft rejection (Alonso Arias, López-Vázquez, and López-Larrea 2012; Chinen and Buckley 2010). There are two models describing allogeneic T cell recognition of peptide-allogeneic-MHC complexes. One explains that T cell activation derives from the interaction between TCR and the allogeneic-MHC molecules (Bevan 1984), whereas the other claims that the activation is due to the recognition of foreign peptide (Matzinger and Bevan 1977) (Fig. 6).

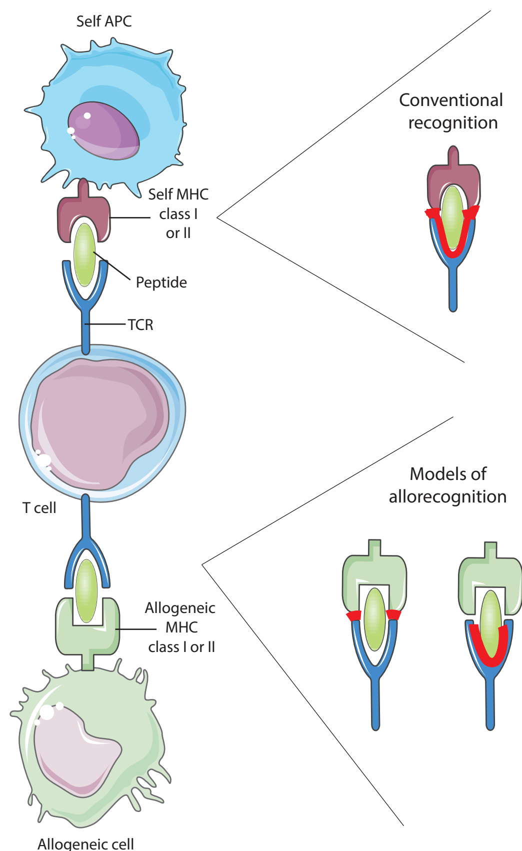


Figure 6. ***Conventional recognition and models of allorecognition of allogeneic-MHC complexes by T cells.*** *T cell receptor recognizes peptide-self-MHC (conventional recognition, upper panel) as well as peptide-allogeneic-MHC (allorecognition, lower panel) complexes. While in conventional recognition TCR makes contact with a peptide and a self-MHC, during allorecognition some models suggest that the TCR makes contact with a peptide, other models claim that TCR makes contact with allogeneic-MHC molecule. However, in many situations, the interaction of allospecific T cell with peptide-allogeneic-MHC complex seems to be identical to conventional recognition. Red line indicates interaction between TCR and peptide/MHC [adapted from (Felix and Allen 2007)].*

2.3.2 Major histocompatibility complexes

MHC molecules are highly polymorphic, which means that more than one allele is present at the same locus. There are hundreds of variants of MHC molecules and although they show a huge degree of diversity all of them are relatively common in the population. Thanks to so many polymorphic amino acids localized in the binding groove, each MHC variant can present a different range of peptides. Polymorphism in the α-helical proteins

influences allorecognition, while polymorphism in the peptide-binding site affects the type of bound peptide and conformation of the whole complex (peptide-allogeneic MHC). It means that the type of peptide that a particular individual recognizes depends on their MHC proteins and varies from person to person (Alonso Arias, López-Vázquez, and López-Larrea 2012).

The physiological function of MHC proteins is to present peptides to T lymphocytes. Thus, they are responsible for the organisms to be able to distinguish between self and foreign cells, consequently being responsible for the acceptance or rejection of transplant. There are two types of MHC complexes: MHC class I, which presents cytoplasmic peptides to CD8+ T cells, and MHC class II, binding proteins derived from extracellular molecules and presenting them to CD4+ T cells. MHC class I molecule consist of a cytoplasmic domain and a transmembrane region linked to an α-chain, which is composed of three domains α_1, α_2 and α_3, and a small extracellular domain known as β_2-microglobulin (Simonsen 1985). The α_3 domain has a relatively conserved structure that interacts with CD8 molecules of T cells. The α_2 and α_3 form a groove for binding peptides presented by the molecule. The MHC class I groove can only recognize peptides of 8-10 amino acids (Pamer and Cresswell 1998). Only MHC molecule loaded with a peptide is expressed on the cell surface as the interaction between α-chain and β_2-microglobulin is stabilized with the binding of the antigen to the groove MHC class I molecules are expressed on the surface of all nucleated cells and are considered as the identification card for T cell control (Alonso Arias, López-Vázquez, and López-Larrea 2012; Cunningham 1977). The MHC class II molecules are constructed from two equal transmembrane molecules, α and β-chain. The α_1 and β_1 amino terminal segments interact with one another forming a binding groove, which is the most polymorphic site of the MHC class II molecule. The binding groove of MHC class II can bind peptides up to 30 amino acids. These peptides usually come from extracellular antigens that have entered the cell by endocytosis. The binding site for CD4 molecules is located in the β_2 segment. Cytoplasmic tails follow transmembrane regions of both α and β-chains. Additionally, there is one more polypeptide which binds to recently synthesized MHC class II molecules, knows as the invariant chain. The invariant chain is involved in the formation and prevents binding of intracellular peptides to class II molecule. Also, invariant chain

facilitates transport of MHC class II proteins from ER to Golgi apparatus, which is followed by a fusion with late endosome containing extracellular degraded proteins, consequently is involved in loading proteins onto MHC class II (Cresswell 1994; Claesson et al. 1983; Tulp et al. 1994; Pieters 2001). MHC class II molecules are mainly expressed on a professional antigen presenting cells (APCs), which were described before in this Chapter (Alonso Arias, López-Vázquez, and López-Larrea 2012; Felix and Allen 2007).

The wide range of MHC variety is useful for the recognition and fighting pathogens, however, at the same time it is a great barrier to the transplantation. The fact that every individual carries different MHC molecules means that grafted tissues express foreign set of antigens that are recognized by the recipient and lead to rejection process. Naturally, when carrying out transplantation, the aim is to match MHC molecules between recipient and donor as similar as possible, consequently reducing the risk of rejection and increasing survival of the graft.

2.3.3 Allograft rejection

T lymphocytes play a major role in the process of rejection. These are the cells that recognize foreign tissue by distinguishing self from non-self MHC molecules in a process called allorecognition. Allorecognition is an immune response that the recipient develops against a transplant. The process consists of the same mechanism as the immune response for defense against pathogens. Without immunosuppressive drugs, alloreactivity leads to rejection of the graft. This is characterized by infiltration of host lymphocytes, mainly CD4+ and CD8+ T cells. There are two pathways in which the foreign antigens can be presented to the host T cells: directly – the antigen presenting cells originate from the donor graft; and indirectly – the antigen presenting cells come from the recipient (Fig. 7). The direct presentation involves both CD8+ and CD4+ T cells that recognize intact donor MHC class I and II molecules, respectively, on donor antigen presenting cells (Hernandez-Fuentes, Baker, and Lechler 1999; Larsen, Austyn, and Morris 1990). The allelic difference between graft and host MHC molecules is referred to as a major

histocompatibility antigen mismatch. Over time, more and more host antigen presenting cells enter the graft, pick up donor antigens and present it to host naïve T cells making the indirect presentation pathway more predominant (Felix and Allen 2007). Both, the direct and indirect pathway, lead to rejection of the graft through activation and proliferation of allospecific T cells and direct killing of donor cells (Benichou et al. 2011).

The rate of allograft rejection differs according to the transferred tissue. Skin grafts are rejected faster than other tissues. Rejection occurs after a skin from inbred mouse is transplanted to another mouse from different strain. Firstly, the skin is revascularized within 3 to 7 days, and afterwards it is infiltrated with inflammatory cells. Then, after 7 to 10 days the vascularization starts to decrease with sign of necrosis by day 10. Twelve to 14 days after transplantation the skin is completely rejected (Benichou et al. 2011; Goldsby et al. 2002).

The graft rejection process can be divided into two stages: sensitization and effector phase, and is caused by humoral and cell-mediated immune responses to alloantigens expressed on cells of the graft. The first stage of graft rejection – sensitization phase, begins shortly after transplantation and lasts until alloreactive lymphocytes of the recipient proliferate as a result of allorecognition. Upon tissue transplantation, polymorphonuclear cells, macrophages, dendritic cells, cytokines and acute-phase proteins of recipient infiltrate the graft. Donor dendritic cells carrying donor antigens migrate from the transplanted tissue to the secondary lymphoid organs of the recipient. By presenting donor antigen and allogeneic MHC molecules they elicit an adaptive immune response. The adaptive immune response is initiated by recipient T cells, which recognize foreign peptide-foreign MHC complexes. Following allorecognition, CD4+ and CD8+ T cells become activated and proliferate thereby secreting proinflammatory cytokines. Both major and minor histocompatibility antigens are recognized, however the response to minor histocompatibility antigens is weaker (Fig. 7B). Host CD4+ T helper (T_H) cells become activated after receiving two signals (interaction of TCR-MHC complex and co-stimulation) from APCs. Donor APCs, after traveling into recipient draining lymph nodes, as well as recipient APCs, which can migrate into transplant and endocytose foreign antigens, can provide the interaction with host T cells. Langerhans cells of the skin and endothelial cells lining blood vessels can present alloantigens in addition to dendritic cells

(Epperson and Pober 1994; Marelli-Berg et al. 2001; Bagai et al. 2005). Naïve T cells can only be activated within the secondary lymphoid organs and are not able to reject the graft in the absence of those organs (Lakkis et al. 2000). Only after differentiating into effector cells, T cells leave the secondary lymphoid organs and infiltrate the graft, where they mediate rejection by killing donor cells (Benichou et al. 2011). This event starts the second effector phase of graft rejection, which consists of immune destruction of the graft. The effector phase consist of cell-mediated reactions, antibody-mediated complement lysis and destruction by antibody-dependent cell-mediated cytotoxicity (ADCC). Cell mediated reactions involve an influx of immune cells, mainly T cells (mainly CD4+), APCs and macrophages, into the graft. Additionally, host CD8+ T cells can mediate CTL-mediated killing (cytotoxic T lymphocytes) (Chávez-Galán et al. 2009). Cytokines secreted by T helper cells play a central role in the mechanism of graft rejection. IL-2 and IFNγ (produced by T_H1 cells) promote T cell proliferation and synthesis of IgG by B cells, leading to complement activation. Furthermore, cytokines produced by T_H2 and T_H17, have also been shown to be involved in graft rejection (Benichou et al. 2011). B cell activation cytokines, IL-4, -5 and -13, as well as IL-17 have all been related to allograft rejection. Antibody-mediated rejection, dependent on maintenance of alloreactive B cells by T lymphocytes, also contributes to the rejection of transplanted tissue (Benichou et al. 2011) (Fig. 7A).

The role of CD4+ and CD8+ T cells in allograft rejection of skin grafts was outlined in studies using transplanted mice injected with monoclonal antibodies. The antibodies depleted one or two subpopulations of T cells and then the graft survival was accessed. Removal of CD8+ T cells alone had no effect on the graft survival. However, deletion of CD4+ T cells prolonged survival of the graft. Further, removing both of T cell populations, CD4+ and CD8+, resulted in long-term survival of the graft, thus confirming the collaboration between them (Fig. 8) (Auchincloss et al. 1993). Interestingly, inhibition of dendritic cells facilitate acceptance of the graft in mice (Cobbold et al. 1986)

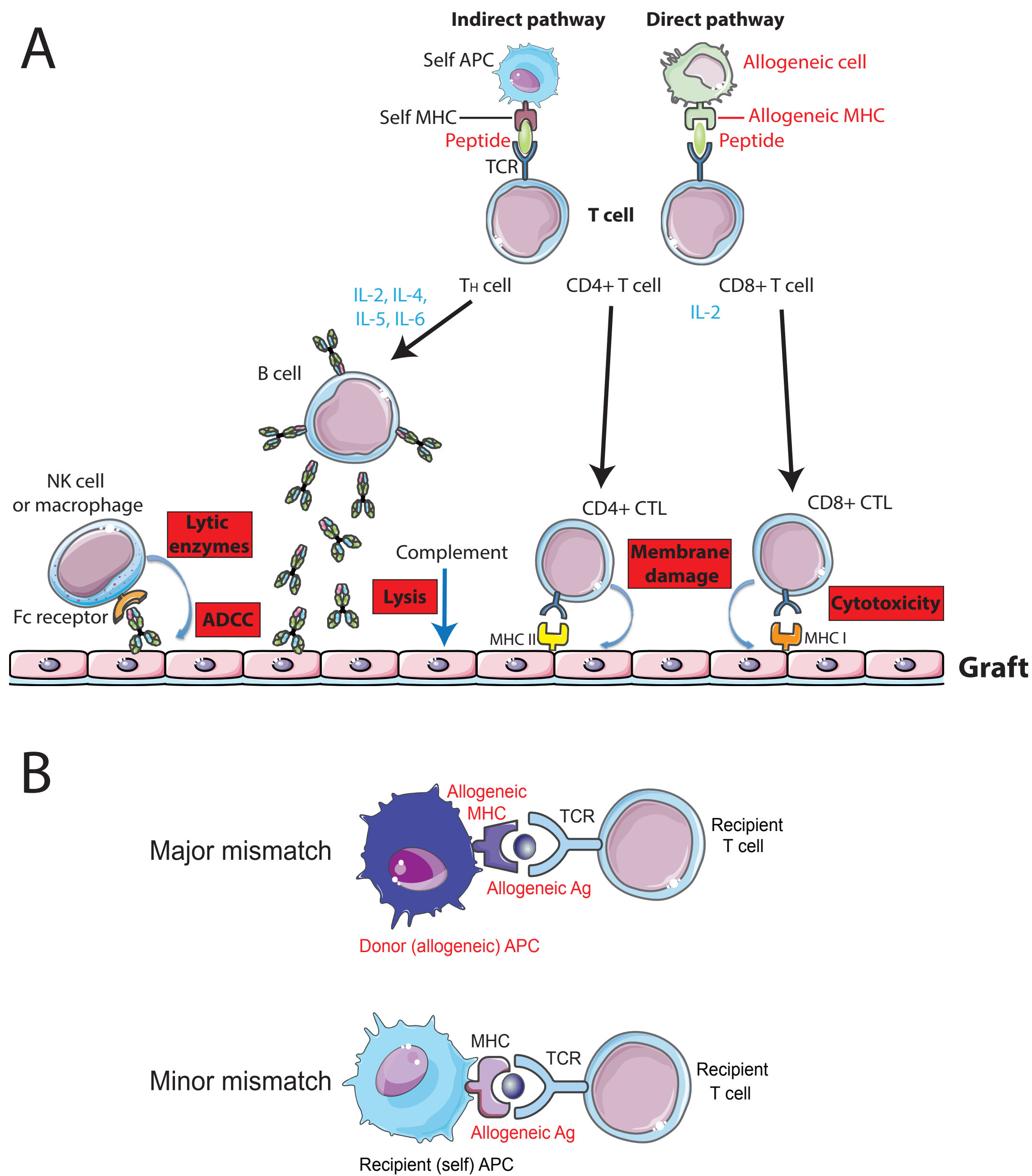


Figure 7. ***Panel A. Different mechanisms of graft rejection process.*** *The activation, proliferation and generation of various effector cells during graft rejection are stimulated by either direct or indirect recognition of alloantigens by T cells. B cells, stimulated by T_H cells producing cytokines (IL-2, IL-4, IL-5, IL-6; marked with blue), secrete antibodies, which stimulate NK cells and macrophages to attack cells of the graft. Activated CD4+ and CD8+ T cells recognize MHC complexes on the surface of the graft causing membrane damage of grafted cells and cytotoxicity. Complement activation leads to lysis of foreign cells [adapted from (Goldsby et al. 2002)].* ***Panel B. Major and minor mismatch model.*** *We refer to major mismatch when both MHC molecule and presented antigen are recognized as foreign (allogeneic). In this model donor and recipient come from different mouse strains. In minor mismatch only antigen is allogeneic and is presented by self-MHC thus only antigen is recognize as foreign by recipient T cell.*

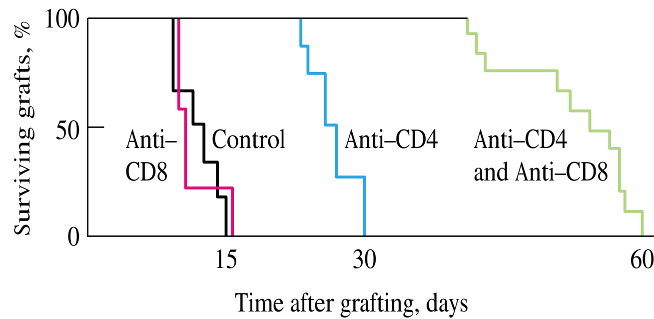


Figure 8. *CD4+ and CD8+ T cell role in graft survival.* *Mice grafted with tail skin were treated with depleting monoclonal antibodies for CD4 and CD8, and observed daily for graft rejection. The depletion of CD8+ T cells did not delay rejection of skin grafts. Mice depleted of CD4+ T cells showed prolonged survival of transplants. Additionally, mice injected simultaneously with anti-CD4 and anti-CD8 antibodies kept the graft even longer than those injected only with anti-CD4 (Goldsby et al. 2002).*

2.3.3.1 Hyperacute rejection

Hyperacute rejection is caused by preexisting host antibodies specific for antigens of the graft and results in rejection within the first 48 hours after transplantation. It is more common for highly vascularized graft, such as kidney and heart. During this process preexisting antibodies bind to endothelial cells of the graft activating the complement system, which leads to infiltration of neutrophils. This causes destruction of the endothelium, haemorrage, and thrombosis, which in the end results in necrosis of the grafted organ (Alonso Arias, López-Vázquez, and López-Larrea 2012).

2.3.3.2 Acute rejection

This type or rejection is a cell-mediated rejection, which can begin about 7 to 10 days after transplantation but may also occur within 3 months. It is manifested as an infiltration of macrophages and lymphocytes (both CD4+ and CD8+ T cells with activated or memory phenotype) at the site of tissue destruction (Alonso Arias, López-Vázquez, and López-Larrea 2012).

2.3.3.3 Chronic rejection

The mechanisms of chronic rejection include both humoral and cell-mediated responses and can develop months or years after transplantation. Although, nowadays, survival of the graft within the first year after transplantation is increased due to the availability of immunosuppressive drugs, long-term survival is still a challenge. Immunosuppressive drugs usually do not prevent chronic rejection and therefore in many cases transplants show slow deterioration of function (Alonso Arias, López-Vázquez, and López-Larrea 2012).

2.3.4 Therapy of allograft rejection

Allogeneic transplantations always require immunosuppression. There are several described methods to prevent allograft rejection. Most of these are nonspecific involving generalized suppression of responses to all antigens, not only those from the allograft. Nonspecific immunosuppressive drugs expose recipients to infection, which in fact, is the most common cause of transplant-related death. Recipients undergoing long-term immunosuppressive therapy are at risk of hypertension and metabolic bone disease. Selection of proper immunosuppressive drugs and eventually ceasing the therapy is the biggest challenge for doctors nowadays. One way, to obtain more specific suppression, is the use of immunosuppressive drugs that inhibit T cell signaling, for example Calcineurin inhibitors (Crabtree and Olson 2002; A. Rao, Luo, and Hogan 1997). Moreover development of drugs like cyclosporine A, FK506 (tacrolimus) and rapamycin, was a great breakthrough in a field of transplantation. These drugs are able to block activation and proliferation of resting T cells, and also prevent transcription of genes encoding T-cell activation molecules (such as IL-2). The most specific immunosuppression is achieved with the use of monoclonal antibodies and soluble ligands that bind specific molecules on the T cell surface. This allows depletion of the recipient's particular T cell population. Another way is the blockade of co-stimulatory signals between antigen presenting and T cells (for example CD28/CTLA:CD80/CD86 (Salomon and Bluestone 2001; Lin et al. 1993) and CD40/CD40L (Guo et al. 2001)). Moreover, transfer of

regulatory T lymphocytes (CD4+CD25+Foxp3+ T cells) in mice has been described to prevent acute and chronic rejection (Joffre et al. 2004; Joffre et al. 2008).

2.3.5 Tolerance induction in transplantation – the role of CD4+CD25+Foxp3+ T cells

Regulatory T cells are a powerful mechanism to induce antigen-specific self-tolerance. A number of studies imply that these cells play a role in transplantation tolerance (Gorantla et al. 2010; Waldmann and Cobbold 2004; Joffre et al. 2004; Joffre et al. 2008). The survival of grafts in the absence of immunosuppressive drugs is accompanied by increased number of Tregs in the periphery and the grafted tissue. Tregs are believed to inhibit rejection by direct contact with alloreactive cells and secretion of TGF-β, IL-10 and IL-35. The population of these cells is small which causes difficulties in their isolation and analysis, and limits Tregs use in inducing transplant tolerance. However, currently, different ways to induce specific T cell tolerance are described, including combination of CD4+CD25+Foxp3+ T cells and induction of chimerism. This prevents acute and chronic allograft rejection (Salisbury, Game, and Lechler 2014; Joffre et al. 2004; Joffre et al. 2008; Thebault et al. 2007). Although regulatory T cells are a subject of intense studies, the mechanism of their suppression and regulation is not yet fully understood.

2.4 Survival and homeostasis of naïve T cells

T cell development and homeostasis is an exhausting process for the body. Around 95% to 98% of thymocytes die by apoptosis due to defective TCR development (Starr, Jameson, and Hogquist 2003; Fesus 1991). Apoptosis also regulates immune cell homeostasis by restoring T cell numbers to their appropriate levels after their intense proliferation provoked by infection. T cell homeostasis is a process of maintaining mature naïve T cells in the periphery at constant level and requires signaling by TCR and appropriate cytokines. The maintenance of appropriate numbers of lymphocytes is an important mechanism to maintain an effective immune system (Takada and Jameson 2009). Apoptosis, also called programmed cell death, is a process in which a cell induces its own death. Apoptotic cells dismantle their content without disturbing its membrane. Apoptotic cells are cleared by phagocytes without releasing any inflammatory materials, therefore without triggering a damaging inflammatory response. Apoptosis is characterized by a decrease in cell volume, modification of the cytoskeleton, condensation of chromatin and degradation of DNA into fragments (P. Waring, Kos, and Müllbacher 1991). There are several ways in which lymphocytes can be triggered to undergo apoptosis, all resulting in the activation of caspases. In the periphery, most of TCR-mediated apoptosis is induced by membrane-associated death receptors (including Fas, also known as CD95), which are called extrinsic pathways. Fas and Fas ligand (FasL) are co-expressed on the T cell surface upon persistent stimulation of TCR, causing Fas/FasL mediated cell death. This type of apoptosis induction is known as activation-induced cell death (AICD). AICD is a major homeostatic mechanism, which reduces the number of activated T cells, after antigen is cleared, and removes autoreactive T cell (McConkey, Orrenius, and Jondal 1990). Similarly, another member of the same receptor family, tumor necrosis factor (TNF) and its receptor (TNFR) can induce apoptosis via activation of caspase proteases. B-cell lymphoma 2 (Bcl-2) family of proteins consists of both anti-apoptotic and pro-apoptotic proteins. Proteins of Bcl-2 family respond to the cell's stress and regulate cytochrome c release by their incorporation into mitochondrial membranes. One of the members of this family, Bcl-2, contributes to long survival of lymphocytes. Therefore, Bcl-2 expression levels play an important role in regulating the life span of hematopoietic cells, for example, by eliminating unneeded activated lymphocytes.

Consequently, activated lymphocytes express lower levels of Bcl-2 and are more susceptible to apoptosis than naïve and memory cells, which express high levels of this protein. However, if the TCR activation continues, the expression of Bcl-2 is sustained thereby blocking the apoptotic pathway (Chávez-Galán et al. 2009; Bouillet et al. 2002).

The next important player in maintaining homeostasis of T cells is programmed cell death protein 1 (PD-1), which is inducibly expressed on peripheral CD4+ and CD8+ T cells. Its expression is provoked by TCR signaling and remains at a high level upon persistent antigen stimulation. Non-functional and exhausted T cells, for example during chronic infection, express high levels of PD-1. This molecule transmits inhibitory signals when stimulated together with TCR. These inhibitory signals block T cell proliferation, cytokine production and cytolytic function, therefore impairs T cell survival. PD-1 inhibits T cell function and survival directly via blocking activation signals (through CD28) or IL-2. However, the intensity of inhibition depends on the strength of the TCR signal. Lower levels of TCR signaling lead to a greater inhibition of T cells function. PD-1 signaling protects against self-reactive T cells, thus prevents autoimmunity suggesting that PD-1 has a role in maintenance of tolerance. Furthermore, the PD-1 pathway promotes Treg development, maintenance, and function. Tregs induced by this pathway can assist in maintaining homeostasis, regulating the level of T cell activation. PD-1 ligands (PD-L1 and PD-L2) are found on tolerogeneic dendritic cells where they can control induction of T cell activation or tolerance (Francisco, Sage, and Sharpe 2010).

Recent thymic emigrants, which arrive in the periphery, so-called naïve T cells, are antigen inexperienced cells with the greatest potential to proliferate and differentiate. The maintenance of naïve T cells is necessary for lifelong immunocompetence. Survival of naïve CD8+ T cells requires signaling by IL-7 and engagement of TCR. This is a complex process, which demands precise intermittent stimulation with IL-7 and a proper strength of TCR induction to keep the cells alive. Continuous IL-7 signaling as well as insufficient TCR triggering induces CD8+ T cells to proliferate and produce IFNγ. Consequently, this leads to IFNγ-triggered cell death refer to as cytokine-induced cell death (CICD) (Kimura et al. 2013). The mechanisms involved in the survival of naïve CD4+ T cells in the periphery are less clear. However, most of the studies describe CD4+ T cell homeostatic proliferation to be dependent on interaction between TCR and low affinity self-peptide-

MHC complexes. Engagement of TCR with self-MHC class II is required for long-term survival of CD4+ T cells, but the interaction should not be specific. The selective engagement of TCRs by peptide-MHC molecules promotes proliferation of CD4+ T cells, which is associated with activation. There are some studies describing this process to be additionally dependent on cytokines, such as IL-2 or IL-7 (Morris and Allen 2012; Clarke and Rudensky 2000; Martin et al. 2006; van der Geest et al. 2015) (Fig. 9).

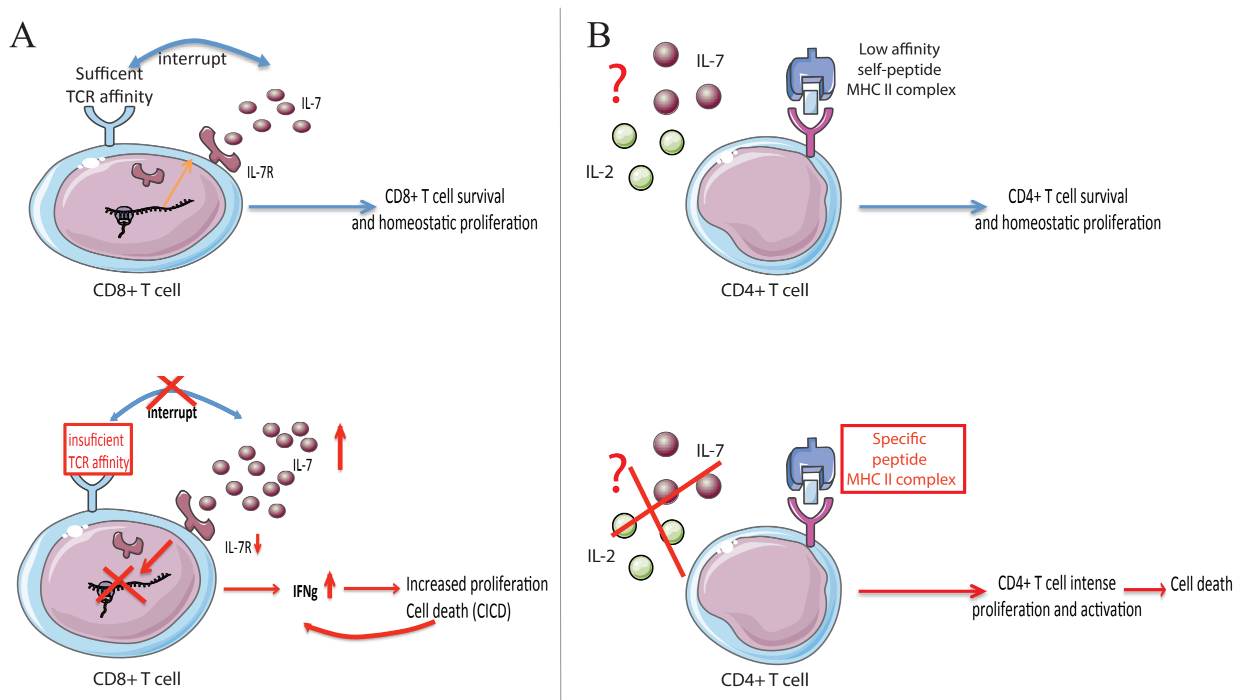


Figure 9. ***Homeostatic maintenance of naïve T cells.*** *A. Scheme of proposed mechanisms involved in survival of CD8+ T cells. Low-affinity engagement of TCR by self-antigens and IL-7 signaling in the periphery are necessary for the homeostasis of CD8+ T cells. At the same time continuous strong IL-7 signaling and inappropriate TCR triggering induces CD8+ T cells proliferation and activation. This in turns inhibits IL-7R expression and leads to increased production of IFNγ, which causes apoptosis by cytokine induces cell death (CICD) [based on (Kimura et al. 2013)]. B. Survival of naïve CD4+ T cells is a controversial topic. Most of the studies describe it to be dependent on low affinity self-peptide-MHC complexes interaction with TCRs [based on (Morris and Allen 2012)].*

3 Aim of the study

Tissue transfer from a donor to a recipient triggers potent reaction by the recipient's immune system. Mechanisms that are responsible for maintenance of self-tolerance participate in rejection of grafted tissue by a process called allorecognition. Allorecognition consists of the same mechanism as an immune response for defense against pathogens but it is mediated against differences in MHC class I and class II molecules between the host and the donor. T cells are the main players in allograft rejection (Alonso Arias, López-Vázquez, and López-Larrea 2012). Coronin 1-deficient mice show a drastic reduction in the numbers of T cells in the periphery (Föger et al. 2006; Mueller et al. 2008). Interestingly, coronin 1 mutation in human is associated with severe peripheral T cell depletion (Moshous et al. 2013). Recently, coronin 1-deficient mice have been found to possess relatively normal T cell antigen-induced responses upon infection while at the same time resisting the development of autoimmune diseases (Siegmund et al. 2011; Haraldsson et al. 2008; Tchang et al. 2013). Preliminary observations of experiments in our laboratory showed prolonged survival of transplanted skin grafts in coronin 1-deficient recipients (not published). This observation suggests a critical role of coronin 1 in T cell-mediated allograft rejection. However, the physiological relevance for coronin 1 deficiency in allograft rejection is yet to be discovered.

Based on the data described above, we hypothesized that coronin 1 plays an essential role in the activation of alloreactive CD4+ and CD8+ T cells during graft rejection. The goal of my thesis was to investigate the ability of coronin 1-deficient T cells to recognize foreign MHC complexes and to reject the allograft. We approached that by designing an appropriate experimental setup, in which we tested skin graft rejection in wild type and coronin 1-deficient mice. Furthermore, we analyzed the outcome of reduced frequency of coronin 1-deficient T cells on the graft rejection. We studied the ability of coronin 1-deficient T cells to become activated and proliferate. We also

explored the induction of tolerance after graft transplantation in coronin 1-deficient mice as a potential reason for the delay in graft rejection.

4 Results

4.1 Skin transplantation models: major and minor mismatch

T lymphocytes play a major role in allorecognition and lead to rejection of transplanted tissue. Dendritic cells are the cells presenting foreign antigens to alloreactive T cells (Guermonprez et al. 2002). As mentioned above coronin 1-deficient mice show decreased numbers of T cells (Moshous et al. 2013) and normal antigen processing and presentation by dendritic cells (Westritschnig et al. 2013). When coronin 1-deficient mice were infected with LCMV the CD8+ T cell response was relatively normal compared to wild type controls and led to the clearance of the virus (Tchang et al. 2013). On the other hand, after infection with VSV CD4+ T cells response was impaired and mice were not protected from the virus (Tchang et al. 2013). Moreover, mice deficient in coronin 1 are not susceptible to EAE (Siegmund et al. 2011) and protected against systemic lupus erythematosus (Haraldsson et al. 2008). These data demonstrate that despite the paucity in naïve T cells, coronin 1-deficient mice are resistant to a variety of infections but not susceptible to autoimmune diseases. Since allorecognition is an immune response that the recipient develops against a transplant, the process consists of the same mechanism as the immune response for defense against pathogens (Benichou et al. 2011). Interestingly, preliminary results of experiments in our laboratory showed prolonged survival of transplants in coronin 1-deficient recipients. Although this could be the outcome of reduced frequency and/or impaired activation of T cells in the absence of coronin 1 we would have expected similar defects in immune response against pathogens. To address the question regarding responses to allografts in coronin 1-deficient mice, two types of experiments were set up in our lab. These experiments included major and minor mismatch skin grafts. In the major mismatch model the donor and the recipient come from MHC-mismatched mouse strains and differ in both presented antigen and MHC molecules. When donor and recipient possess identical MHC molecules but

differ regarding the presented antigen, this is referred to as a minor mismatch (Fig.7B).

4.1.1 Rejection of major mismatched grafts in coronin 1-deficient recipients

Initially, to address the question how coronin 1-deficient mice behave in allorecognition, tissue transplantation was performed using mice from two different strains. Balb/c mice were used as donors and BL/6 wild type or coronin 1-deficient (on BL/6 background) mice were used as recipients. Donor skin was harvested from the tail of Balb/c mice and the transplantation procedure followed the protocol described before (Schmaler, Broggi, and Rossi 2014, and in this thesis Chapter 6.4). Following transplantation, all mice were scored for a duration of at least 100 days and tested for composition of immune cells at different time points. Transplantation of fully mismatched skin onto BL/6 mice resulted in rejection within 10-12 days, whereas grafts transplanted onto coronin 1-deficient mice survived until day 33 (Fig. 10). The results indicated an impaired response to foreign tissue in mice lacking coronin 1.

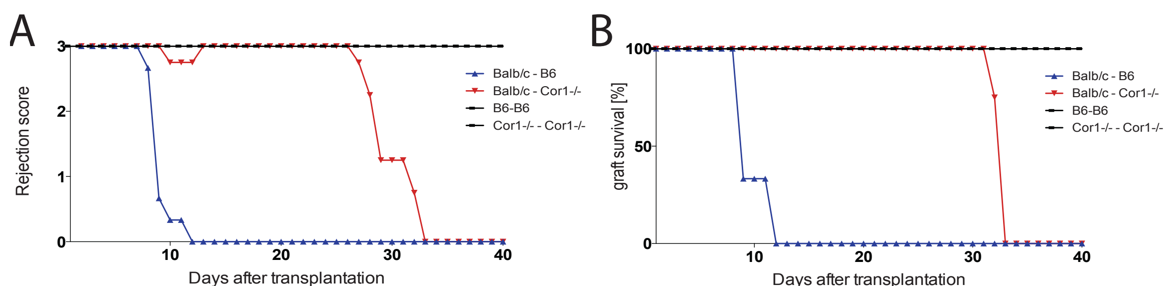


Figure 10. ***Rejection score (A) and skin graft survival (B) in BL/6 wild type and coronin 1-deficient recipients of Balb/c tail skin grafts.*** *The surgery was done following the transplantation protocol described in Materials and Methods (Chapter 6.4). The survival of the graft was verified daily for at least 100 days. Each point represents mean of two mice for control groups (BL/6 onto BL/6 and Cor1-/- onto Cor1-/-) and four mice for experimental groups (Balb/c onto BL/6 and Balb/c onto Cor1-/-1). The experiment was repeated four times, each time 2 to 6 mice were used per group and similar results were observed for each repeat. B6 – BL/6 wild type; Cor1-/- - coronin 1-deficient. Presented data were obtained by Rajesh Jayachandran.*

Flow cytometry analysis of immune cell composition (B cells, NK cells and neutrophils) in transplanted mice did not show many significant differences between wild type and coronin 1-deficient mice (Fig. 11 and 12). T cell numbers in lymph nodes of coronin 1-deficient mice with skin transplants were decreased and maintained at levels comparable to naïve coronin 1-deficient mice (Fig. 11B and D). The retained counts of T cells (between naïve and transplanted coronin 1-deficient mice) could indicate defects in proliferation due to impaired antigen recognition or an impaired process of antigen recognition in coronin 1-deficient T cells. Although numbers of both CD4+ and CD8+ T cells in the lymph nodes of coronin 1-deficient transplanted mice were lower than in BL/6 recipients, they increased in coronin 1-deficient and wild type mice (Fig. 12B and D). This could indicate an activation and proliferation of coronin 1-deficient T cells after transplantation but at a low level. Both CD4+ and CD8+ T cells numbers did not change in spleens of coronin 1-deficient and wild type mice after transplantation. B cells numbers in naïve controls BL/6 wild type and mice lacking coronin 1 were similar. However, counts of B cells in lymph nodes of coronin 1-deficient recipients were lower than in wild type. Typically, after organ transplantation and stimulation by T cells, B cells undergo uncontrolled mobilization and activation which drives inflammation and results in graft loss (Coelho et al. 2013; Thibault-Espitia et al. 2012; Zarkhin, Chalasani, and Sarwal 2010). Lower counts of B cells in coronin 1-deficient lymph nodes could be explained by lower activation of B cells by decreased or impaired T cell numbers in recipients lacking coronin 1 (Fig. 11E and F) since B cells responses to protein antigens require help from T cells (Kwun et al. 2012). Moreover, although the overall numbers of B cells were reduced in coronin 1-deficient mice after transplantation, the rate of increase of these cells was comparable to wild type recipients. These results imply that the T cells were able to activate B cells but the activation was not as efficient as in the wild type recipients. That could be explained by decreased activation and numbers of T cells themselves.

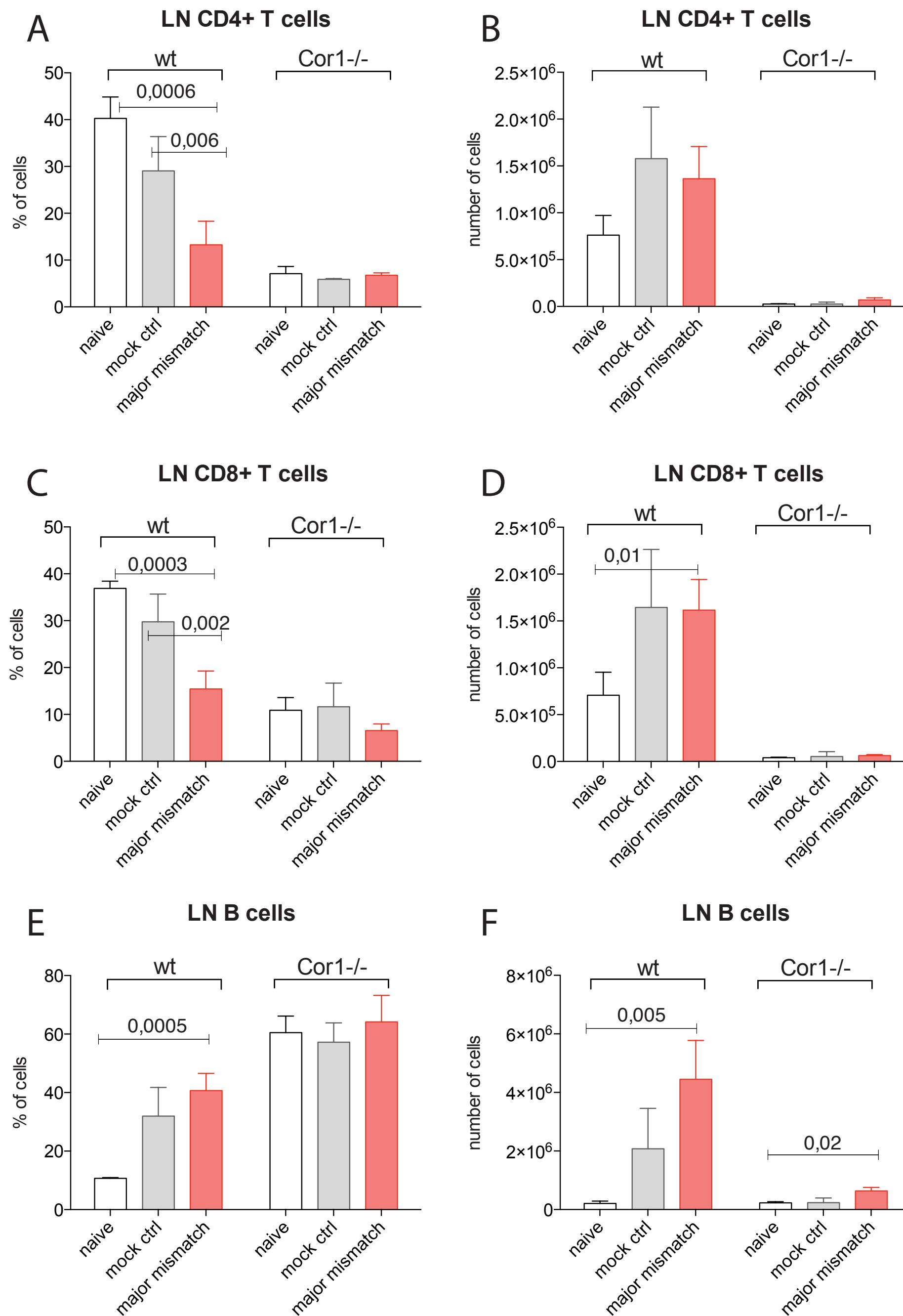


Figure 11. ***Immune cells in lymph nodes of BL/6 wild type and coronin 1-deficient recipients of Balb/c skin grafts.*** *The mice were transplanted following the transplantation protocol (Chapter 6.4) and analyzed at the day of rejection: day 12 for wild type and day 33 for Cor1-/- recipients. B cells and T cells were analyzed from lymph nodes (2x axillary, 2x brachial, 2x inguinal) with appropriate antibodies. Results were acquired using FACS. Each bar graph represents results for 3 (naïve and mock controls) or 6 individual mice (major mismatch). A and B shows CD4+ T cells in lymph nodes both in % (A) and numbers (B). C and D represents CD8+ T cells in % (C) as well as in numbers (D). Percentages of B cells in lymph nodes are shown in graph E, numbers in F. B6 – BL/6 wild type; Cor1-/- - coronin 1-deficient.*

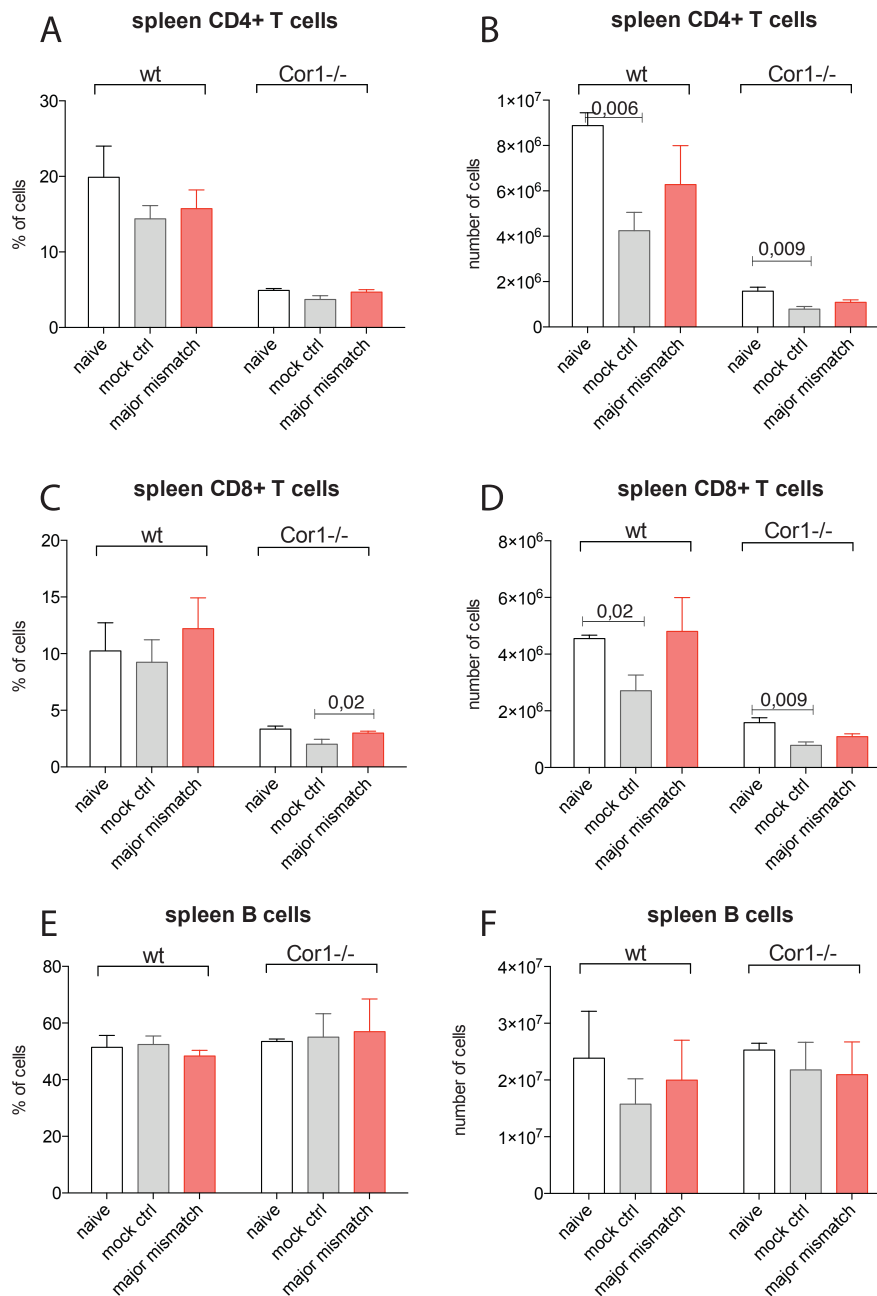


Figure 12. ***Immune cells in spleen of BL/6 wild type and coronin 1-deficient mice transplanted with Balb/c tail skin (major mismatch) or mock controls.*** *Skin transplantations were performed following the transplantation protocol (Chapter 6.4). The mice were analyzed at the day of rejection: day 12 for wild type and day 33 for Cor1-/- recipients. Cells from spleens were analyzed with antibodies specific for T and B cells. The results were acquired using FACS. Each bar graph represents results for 3 (naïve and mock controls) or 6 individual mice (major mismatch). A and B shows CD4+ T cells in spleen both in % (A) and numbers (B). C and D represents CD8+ T cells in % (C) as well as in numbers (D). Percentages of splenic B cells are shown in graph E, numbers in F. B6 – BL/6 wild type; Cor1-/- - coronin 1-deficient.*

From previous studies we know that coronin 1-deficient mice can respond to viral and bacterial infection (Tchang et al. 2013) thus the above results suggest that coronin 1-deficient mice have a specific problem with allorecognition by T cells or their efficient activation after transplantation.

4.1.2 Survival of minor mismatched grafts in wild type and coronin 1-deficient mice

In order to further investigate impaired allorecognition of T cells in coronin 1-deficient mice, we performed minor mismatch surgeries. To this end we used mouse strain 6.25% Balb/c in BL/6 generated in the lab (described in Materials and Methods). First, the strain was characterized for the expression of MHC complexes. We stained 6.25% Balb/c in BL/6, Balb/c and BL/6 mice for Balb/c specific MHC complexes: I-Ad (MHC class II), H2-Dd and H2-Kd (MHC class I) and BL/6 specific MHC complexes: I-Ab (MHC class II), H2-Db and H2-Kb (MHC class I) (Supplementary Fig. 1). The results showed that 6.25% Balb/c in BL/6 mice expressed all three BL/6 MHC proteins and none from Balb/c. This suggests that the response induced after transplantation was caused by differences in presented antigens but not by allorecognition of foreign MHC proteins. Additionally, 6.25% Balb/c in BL/6 mice were analyzed by the presence of single-nucleotide polymorphism (SNP) typical for either BL/6 or Balb/c mouse strain (data not shown). According to the SNP analysis the strain showed 96.9% of BL/6 recipient genome.

As can be seen in Fig. 13A and B minor mismatched skin was rejected after 70 days in BL/6 mice. In coronin 1-deficient mice transplants were not rejected (data shown until day 100). Analysis of NK cells and neutrophils in transplanted mice did not show any significant differences between wild type and coronin 1-deficient mice. The T cell numbers in coronin 1-deficient mice with skin transplants were decreased and showed a slight (not significant) increase upon transplantation, which did not induce graft rejection (Fig. 13C – F). Similarly to major mismatch surgeries, B cells numbers in coronin 1-deficient recipients were lower than in the wild type.

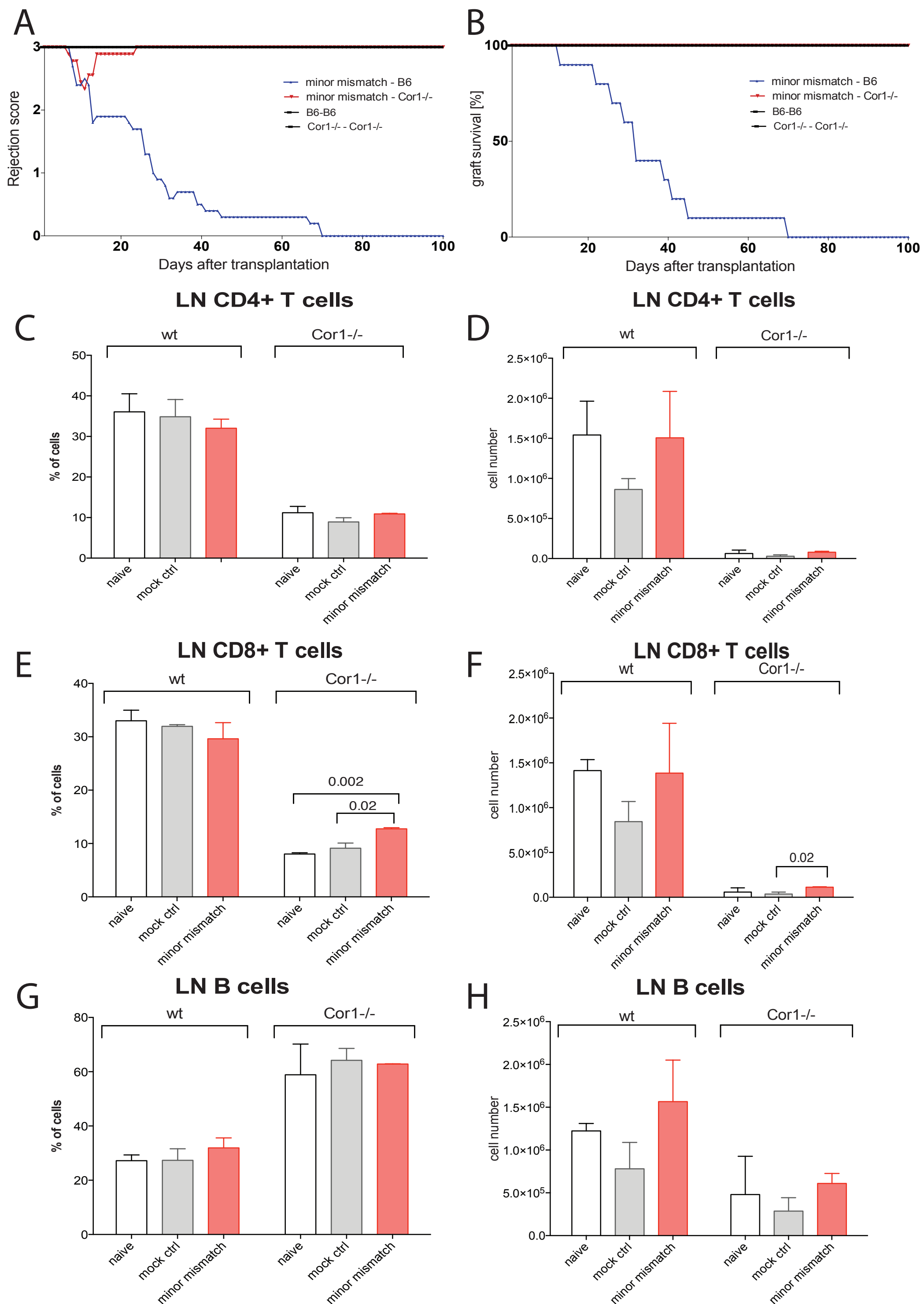


Figure 13. ***A – B. Rejection score (A) and skin graft survival (B) in BL/6 wild type or coronin 1-deficient recipients of 6.25% Balb/c in BL/6 tail skin grafts (minor mismatch).*** *The surgery was done following the transplantation protocol (Chapter 6.4). The survival of grafts was verified daily for at least 150 days. Each point represents mean of three mice for control transplantations and five mice for minor mismatched surgeries. The experiment was repeated four times, each time 3 mice were used in controls and 5 mice were used in minor mismatched group. C – H. Composition of immune cells in*

lymph nodes of transplanted or naïve mice 87 days after surgery. The mice were transplanted with minor mismatch skin grafts following the transplantation protocol (Chapter 6.4). Axillary, brachial and inguinal lymph nodes were harvested 87 days after surgery, processed and analyzed for immune cell composition by FACS staining with anti-CD3, CD4, CD8 and CD19 antibodies. C and D shows % and numbers of CD4+ T cells, respectively. CD8+ T cells percentage (E), CD8+ T cell numbers (F), B cells percentage (G) and B cell numbers (H) are represented on the bar graphs. Graphs represent mean ± standard deviation (SD) of 3 mice for naïve and mock controls, and mean ±SD of 5 mice of minor mismatch groups. B6 – BL/6 wild type; Cor1-/- - coronin 1-deficient. Data presented in Fig. 13A and B were obtained by Rajesh Jayachandran.

In summary, coronin 1-deficient mice showed delayed rejection of major mismatch skin grafts compared with the wild type recipients. The coronin 1-deficient animals kept the graft approximately 3 times longer than BL/6 wild type. At the same time, coronin 1-deficient mice were not able to elicit rejection in minor mismatch setup. Additionally, cell analysis in the recipient mice showed relatively normal numbers of different immune cell types (neutrophils, NK cells and B cells) in blood, spleen and lymph nodes of coronin 1-deficient compared with BL/6 wild type recipients (Table 2). Only the rates and numbers of T cells (CD4+ T cells as well as CD8+ T cells) were reduced in coronin 1-deficient mice when compared with wild type. Therefore the delayed rejection could be the consequence of the reduced numbers of T cells that need more time to reach the appropriate cell levels to be able to reject the graft.

Therefore, we asked three major questions:

I. Is the delayed rejection due to the lower number of T cells in the coronin 1-deficient mice?
II. Are coronin 1-deficient T cells impaired in their function or allorecognition?
III. Is the prolonged acceptance of the graft due to the induction of tolerance in coronin 1-deficient recipients?

4.2 CD4+ T cell-mediated allograft rejection (MHC class II mismatched transplantations)

Interestingly, the delayed rejection in the major mismatched experiment resembled literature data in which CD4+ T cells were depleted with anti-CD4 antibody in skin transplantation model (Cobbold et al. 1986; Auchincloss et al. 1993) (Fig. 8). Based on this observation and previously described results, where the CD4+ T cells were impaired during infection (Tchang et al. 2013), we decided to perform CD4+ T cell-dependent transplantation using bm12Rag2-/- mice as donors.

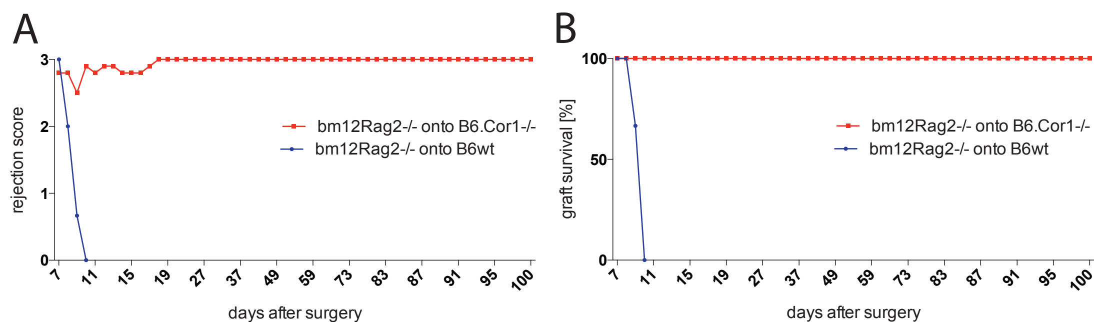


Figure 14. *Rejection score (A) and graft survival (B) in BL/6 wild type and coronin 1-deficient recipients of bm12Rag2-/- tail skin.* *BL/6 wt and coronin 1-deficient mice were transplanted according to the transplantation protocol and the graft survival was observed for at least 100 days. BL/6 wild type recipient group consist of 3 mice and Cor1-/- group of 6 mice. The experiment was repeated six times; each time at least 3 mice were used per group. Figure shows representative plot from one experiment. Coronin 1-deficient mice tolerate the bm12Rag2-/- skin grafts until day 100 while BL/6 wild type recipients rejected grafts within 10 days. B6 – BL/6 wild type; Cor1-/- - coronin 1-deficient.*

In the CD4-dependent transplantation model we were using B6.C.H-2-bm12Rag2-/- (bm12Rag2-/-) mice as donors. Bm12 mice are BL/6 mice that have three-points mutation localized in the I-A subregion of the murine MHC class II (Lee, Hansen, and Cullen 1982). As a result, the CD4+ T cells of BL/6 mice will recognize MHC class II molecules of bm12 mice as foreign and thus induce a strong allogeneic activation of these cells (Schmaler, Broggi, and Rossi 2014). After transplantation of bm12Rag2-/- skin onto BL/6 wild type and coronin 1-deficient mice, wild type recipients rejected grafts within 10 days (Schmaler, Broggi, and Rossi 2014) while coronin 1-deficient mice showed no rejection (data shown until day 100, Fig. 14). The analysis of CD4+ T cells in transplanted mice did not show any significant differences in proliferation

or activation of these cells compared with major mismatch model (Fig. 15). Interestingly, we observed tolerance of bm12Rag2-/- skin graft in coronin 1-deficient mice, which could be caused by the defect in CD4+ T cells or induction of tolerance.

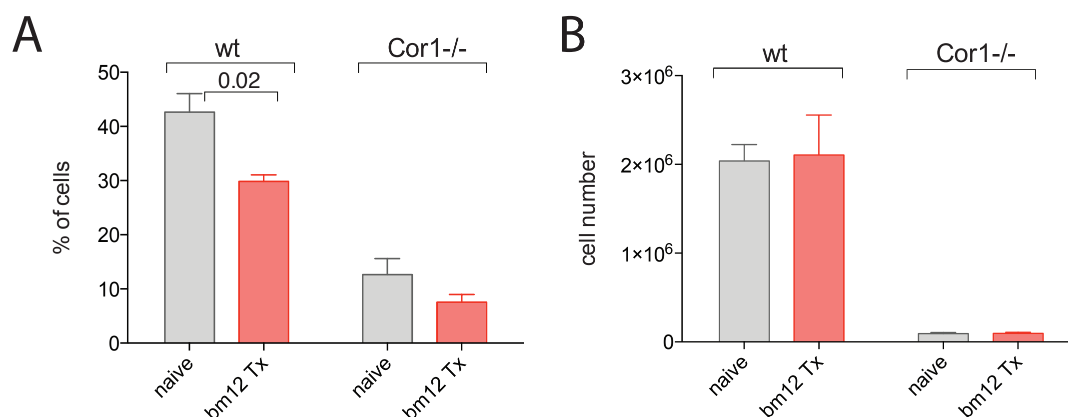


Figure 15. ***CD4+ T cells in lymph nodes of BL/6 wild type and coronin 1-deficient mice with and without bm12Rag2-/- skin grafts.*** *The mice were processed following the transplantation protocol (Chapter 6.4). BL/6 wt mice were sacrificed at day 13 after the surgery (graft rejection), Cor1-/- recipients were analyzed at day 50 after the surgery. Lymph nodes (2x axillary, 2x brachial, 2x inguinal) were harvested at the day of sacrifice and FACS stained with T cells specific antibodies (antiCD3 and anti-CD4). The group of naïve mice consisted of 3 mice; the transplanted group consisted of 4 mice. The experiment was performed several times, each time at least three mice were used per group. Figure shows representative plot from one experiment. A. % of CD4+ lymphocytes for naïve or transplanted BL/6 wt and Cor1-/- mice with bm12Rag2-/- tail skin. B. Number of CD4+ T cells for naïve or transplanted BL/6 wt and Cor1-/- mice with bm12Rag2-/- tail skin. B6 – BL/6 wild type; Cor1-/- - coronin 1-deficient; bm12 – bm12Rag2-/-; Tx – transplanted mice.*

We then went on to investigate the ability of CD8+ T cells of coronin 1-deficient mice to induce rejection. For MHC class I mismatched transplantations we used B6.C-H2-K-bm1 (bm1) mice as a skin donors. The bm1 mice differ from the parental BL/6 gene by seven nucleotides, which resolve in substitution of three amino acids in a subregion of class I H-2Kb (Schulze et al. 1983). Survival of bm1 skin graft on the BL/6 wild type or coronin 1-deficient mice is shown in Fig. 16. As can be seen bm1 skin transplanted onto BL/6 wild type mice was rejected within 13 days. Coronin 1-deficient recipients started to reject the graft after 36 days post transplantation. At day 50 two out of four mice rejected the transplanted skin completely. At the end of the experiment (day 90) only one mouse kept the graft but showed signs of undergoing rejection (score 2). This result confirmed the previous findings that CD8+ T cells of

coronin 1-deficient mice are able to mount immune response (Tchang et al. 2013). The delayed rejection could be due to a lower number of these cells which need more time to proliferate and become activated sufficiently in order to reject a graft. Therefore, the result confirmed our hypothesis that the delayed rejection of major mismatch skin grafts was due to impairment in coronin 1-deficient CD4+ T cells. Thus, from now on, this thesis will focus mainly on CD4+ T cell responses using MHC class II mismatched transplantation model.

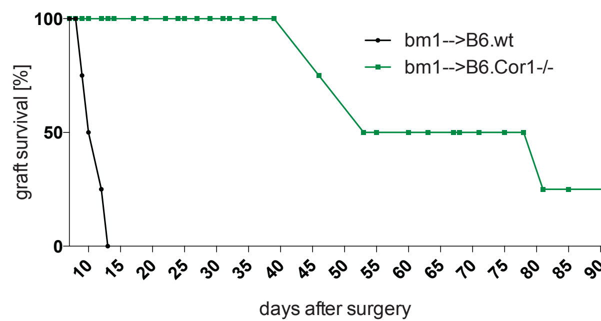


Figure 16. ***Graft survival after CD8+ T cells-dependent (bm1) skin transplantations.*** *The mice were transplanted following the transplantation protocol (Chapter 6.4), graft score was observed for at least 90 days. Each transplanted group consisted of minimum three mice. Bm1 skin transplanted onto BL/6 wild type mice was rejected within 13 days, coronin 1-deficient recipients started to reject the graft after 36 days post transplantation; at day 50 two out of four mice rejected the transplanted skin completely; at the end of the experiment only one mouse kept the graft (with score 2). B6 – BL/6 wild type; Cor1-/- - coronin 1-deficient.*

4.3 Analysis of differences in T cell numbers between wild type and coronin 1-deficient mice and their influence on graft rejection

To investigate whether decreased numbers of coronin 1-deficient T cells are the cause of delayed rejection, bm12Rag2-/- skin was transplanted onto Rag2-/- mice. The absence of T cells in Rag2-/- mice allows skin graft tolerance. The acceptance of the graft can be overcome by adoptive transfer of 20 000 BL/6 wild type CD4+ T cells (Schmaler, Broggi, and Rossi 2014). Therefore, three weeks after surgeries, we adoptively transferred CD4+ T cells purified from either BL/6 wild type or coronin 1-deficient spleens. Transplanted mice injected with 20 000 of BL/6 wild type CD4+ T cells rejected the grafts within 12 days (Fig. 17). Coronin 1-deficient CD4+ T cells recipients received 20 000, 50 000, 100 000 and 1 000 000 cells by adoptive transfer and none of the group rejected the graft (Fig. 17). The data suggests that increasing coronin 1-deficient CD4+ T cells numbers does not lead to rejection in this model. Consequently decreased numbers of T cells in coronin 1-deficient mice alone were not responsible for delayed rejection. Additionally, to confirm the above statement we performed analysis of survival of wild type and coronin 1-deficient CD4+ T cells in Rag2-/- mice. We noticed defects in survival of coronin 1-deficient cells when compared with wild type. This issue is discussed later in Section 4.4.5 (*Concentration dependence in survival of coronin 1-deficient T cells in vivo*) of this Chapter.

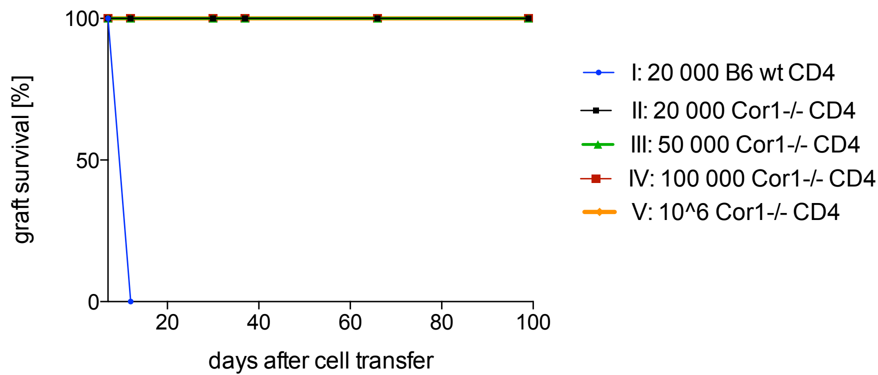


Figure 17. ***Survival of bm12Rag2-/- skin grafts in Rag2-/- recipients after transfer of BL/6 wild type and coronin 1-deficient CD4+ T lymphocytes.*** *The mice were transplanted following the transplantation protocol (Chapter 6.4); CD4+ T cells were adoptively transferred into tail vein 30 days after surgery. Rejection was scored for 100 days. Group I shows survival of bm12Rag2-/- skin grafts in Rag2-/- mice after transfer of 20 000 negatively sorted BL/6 wild type CD4+ T cells. All mice in group I rejected skin within 12 days after cell transfer. Group II to V showed survival of bm12Rag2-/- grafts in Rag2-/- recipients after transfer of: 20 000 (group II), 50 000 (group III), 100 000 (group IV) and 1 000 000 (group V) coronin 1-deficient CD4+ T cells. Mice from group II to V kept graft until day 100 after cell transfer (end point of the experiment). Each group consisted of 5 mice. B6 – BL/6 wild type; Cor1-/- - coronin 1-deficient.*

4.4 Study of T cell function in coronin 1-deficient compared to wild type mice

4.4.1 Mixed lymphocyte reaction experiment in the study of proliferation of T cells

In order to analyze proliferation and activation of T cells after allorecognition we performed mixed lymphocyte reaction experiments (MLR). This *in vitro* system allowed us to use the same number of wild type and coronin 1-deficient T cells thereby to compensate for the decreased numbers of these cells in the periphery of coronin 1-deficient mice. Mixed lymphocyte reaction experiment mimics rejection *in vitro*, where MHC mismatched cells are used as stimulators and responders. This experiment is an assay between two allogeneic populations, which was originally used to analyze leukocytes, mixed from two unrelated individuals in culture, to identify their compatibility as a donor and a recipient. Reaction of cultured lymphocytes was measured by blast formation, DNA synthesis and their proliferation quantified using incorporation of radioactive nucleosides (3H-thymidine or 5-bromo-2'-deoxyuridine, BrdU) (Bach and Hirschhorn 1964; Bain, B, Vas, M., and Lowenstein, L. 1963).

In our MLR experiments Balb/c splenocytes, depleted of erythrocytes and CD3+ cells, and then treated with mitomycin c to prevent proliferation, were used as stimulators. Total wild type or coronin 1-deficient T cells isolated from spleens were added to the Balb/c cultures and incubated for 52 hours. Afterwards thymidine was added and the cultures were further incubated for 20 hours. The thymidine incorporation into DNA reflects proliferation of cells. As can be seen in Fig. 18A, there was a significant difference in proliferation of total T cells from wild type and coronin 1-deficient mice. The coronin 1-deficient T cell proliferation rate was decreased, however it showed a similar trend as in for wild type with cells the highest proliferation at 400 000 stimulators. When the CD4+ (Fig. 18B) and CD8+ (Fig. 18C) T cells were purified and plated separately there was also a variety in the proliferation

rate between wild type and coronin 1-deficient cells – the coronin 1-deficient cells had reduced cell growth for both types of T cells. Additionally, the proliferation of CD4+ T cells was much lower that CD8+ T cells, which is consistent with the literature (Foulds et al. 2002). We concluded that the proliferation of coronin 1-deficient cells is impaired compared with the wild type. However, with this type of experiment we could not measure survival of T cells and thus we could not be sure if the decreased rate of proliferation in coronin 1-deficient T cells is due to lower activation or lower survival rate of these cells compared to wild type. Therefore we decided to introduce immune staining instead of thymidine incorporation to investigate the status of the T cells within the MLR assay further.

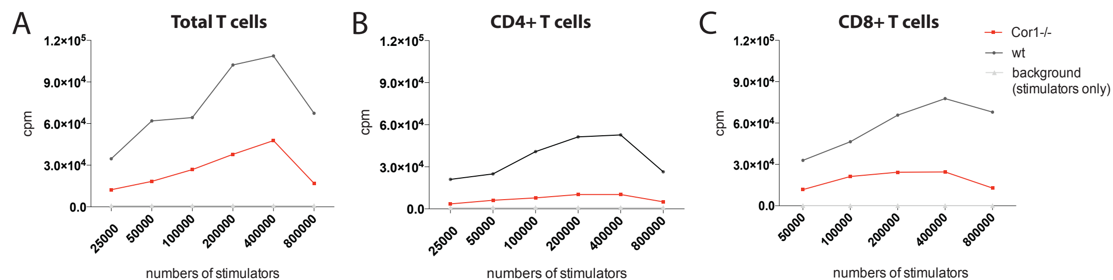


Figure 18. ***Mixed lymphocyte reaction (MLR) experiments with major mismatch antigens (Balb/c) for total (A), CD4+ (B) and CD8+ (C) T cells from BL/6 wild type or coronin 1-deficient mice.*** *The cells were purified from mice spleen following the MLR protocol described in Materials and Methods (Chapter 6.20), plated onto 96-well plate and cultured for 72h. Balb/c splenocytes treated with mitomycin C were used as stimulators. Thymidine incorporation was measured as an indicator of proliferation of stimulated cells. Each point is a mean of three replicates; each experiment was repeated three times. All the points showing Cor1-/- proliferation were statistically different from wt. B6 – BL/6 wild type; Cor1-/- - coronin 1-deficient.*

4.4.2 Proliferation and survival of wild type and coronin 1-deficient T cells upon allorecognition

The use of flow cytometry analysis provided important information about activation, survival and proliferation rate of T cells. The Balb/c splenocytes depleted of erythrocytes and CD3+ T cells were treated with mitomycin c and used as stimulators. Responder BL/6 wild type and coronin 1-deficient T cells were purified from mouse

spleens, labeled with Cell Trace Violet (CTV) and plated together with Balb/c stimulators. Antibody staining allowed to distinguish the CD4+ and CD8+ populations of T cells. The MLR experiments were performed with different numbers of stimulators and incubation times. Fig. 19 shows results of experiment performed with 100 000 total T cells as responders and analyzed for CD4+ T cells. Interestingly coronin 1-deficient CD4+ T cells seemed to start to proliferate earlier than wild type cells as can be seen, for example, in Fig. 21A and F. However, after 72h wild type CD4+ T cells started intense proliferation and general numbers of proliferated wild type cells were higher than for coronin 1-deficient T cells. Interestingly for the highest number of stimulators, coronin 1-deficient CD4+ T cells proliferated better than the wild type cells. We also detected similar differences in proliferation for CD8+ T cells (Supplementary Fig. 2). Coronin 1-deficient CD8+ T cells started to proliferate sooner (between 48 – 72h), however, generally wild type CD8+ T cells proliferated better than coronin 1-deficient cells for all the conditions. These data suggest that coronin 1-deficient T cells are able to initiate proliferation earlier than wild type cells upon activation. The proliferation of coronin 1-deficient T cells was noticeable after 48h in culture while wild type cells need at least 48 hours to initiate activation in MLR experiment (Elves 1969). Interestingly, when we compared rates of proliferation (percentage of proliferated cells within live cell population) between wild type and coronin 1-deficient T cells, they were similar for both CD4+ and CD8+ T cells. This suggests that the same percentage of surviving coronin 1-deficient T cells as in the wild type sample proliferated hence the proliferation itself is not decreased in coronin 1-deficient cells. These results further support our hypothesis that coronin 1-deficient T cell have impaired survival compared to wild type cells. The mechanism underlying a similar or even enhanced proliferation of coronin 1-deficient T cells upon an increase in stimulators (Fig. 19F) is unclear and subject to further investigations (Section 4.4.4 *Analysis of concentration dependence in survival of coronin 1-deficient T cells in vitro*).

Moreover, to test the specific activation of CD4+ T cells in a MHC class II mismatched model, we performed MLR experiments using bm12 splenocytes as stimulators. The activation of coronin 1-deficient CD4+ T cells was decreased

compared to wild type cells and similar to the background activation level. Additionally, coronin 1-deficient CD8+ T cells did not proliferate. These results suggest that in this experiment coronin 1-deficient CD4+ T cells could not be activated alone or their activation was suppressed, possibly indicating the impairment in coronin 1-deficient CD4+ T cells function to activate CD8+ T cells.

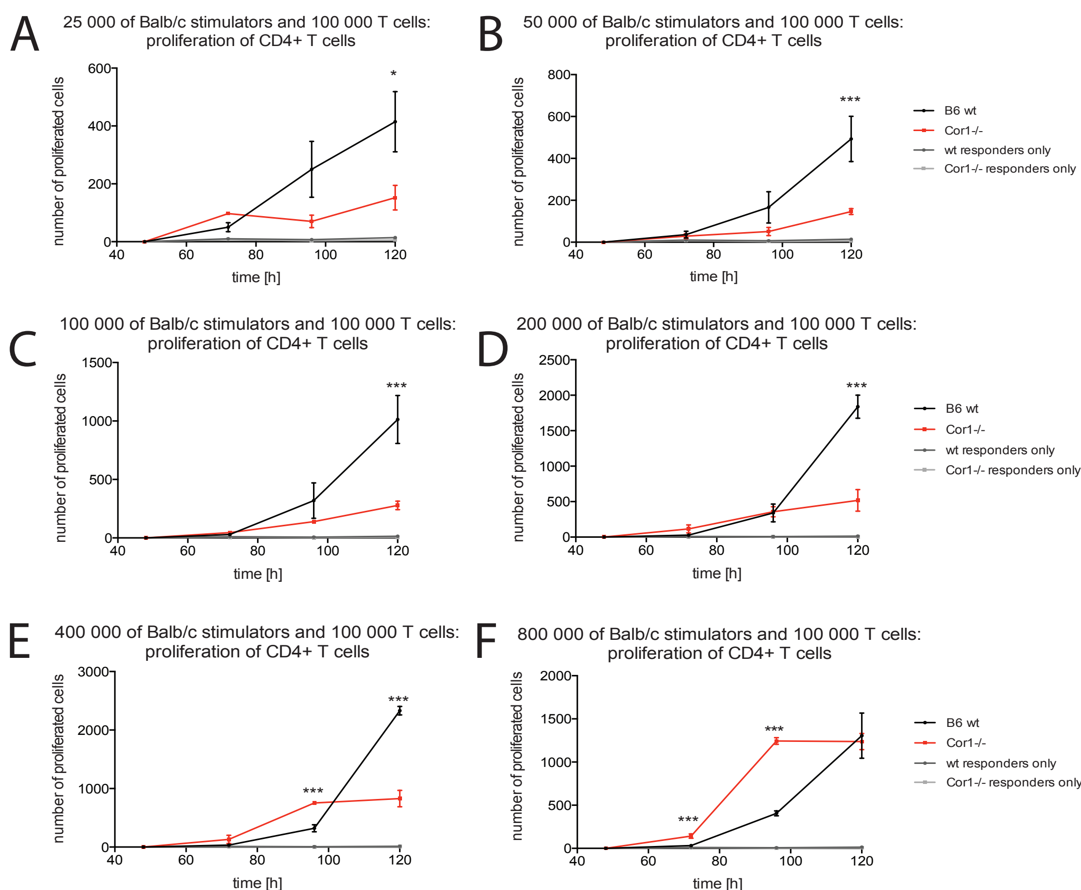


Figure 19. ***CD4+ T cells proliferation analysis in a mixed lymphocyte reaction (MLR) for total T cells from BL/6 wild type and coronin 1-deficient mice as responders and different concentrations of Balb/c splenocytes as stimulators.*** *Total T cells were purified as described in Materials and Methods Chapter 6.20. Stimulators Balb/c splenocytes were depleted of CD3 positive cells and erythrocytes (with anti-CD3 and anti-TER119 antibody, respectively). The cells were treated with mitomycin c to eliminate background proliferation of the stimulators. Then both cells were plated together in numbers indicated on each graph, in 96-well plates and cultured for 48, 72, 96 or 120h. The cells were analyzed by FACS using the same sample volume, time and speed of acquisition. A – F. Analysis of CD4+ T cells proliferation in a culture of 100 000 T cells with different numbers of Balb/c stimulators. An asterisk always shows the significance in the difference between BL/6 wt and Cor1-/- sample. Each time point shows a mean of 3 replicates. B6 – BL/6 wild type; Cor1-/- - coronin 1-deficient.*

4.4.3 Survival of wild type and coronin 1-deficient T cells

Furthermore, we decided to analyze survival of coronin 1-deficient T cells in *in vitro* assays. We established a series of negatively purified CD4+ and CD8+ T cells cultures with or without stimulation in 96-well plates. Fig. 20 shows a short-term experiment (5 – 300 min) in which the cells were cultured either in an absence of stimuli or stimulated with dendritic cells, CD3/CD28 antibodies or PMA/I (phorbol 12-myristate 13-acetate/ionomycin). As we can see coronin 1-deficient T cells, both CD4+ and CD8+ T cells, had decreased survival with the biggest difference when stimulated with PMA/I and the smallest in the co-culture with dendritic cells. These results suggest that the coronin 1-deficient cells died more in response to a strong stimulation (Fig. 20C, G) and/or needed a survival signal from other cells (Fig. 20D, H). The difference in T cell survival between coronin 1-deficient and wild type cells could be explained by our previous observation that coronin 1-deficient T cells respond to the stimuli faster than wild type cells (Section 4.4.2. *Proliferation and survival of wild type and coronin 1-deficient T cells upon allorecognition*).

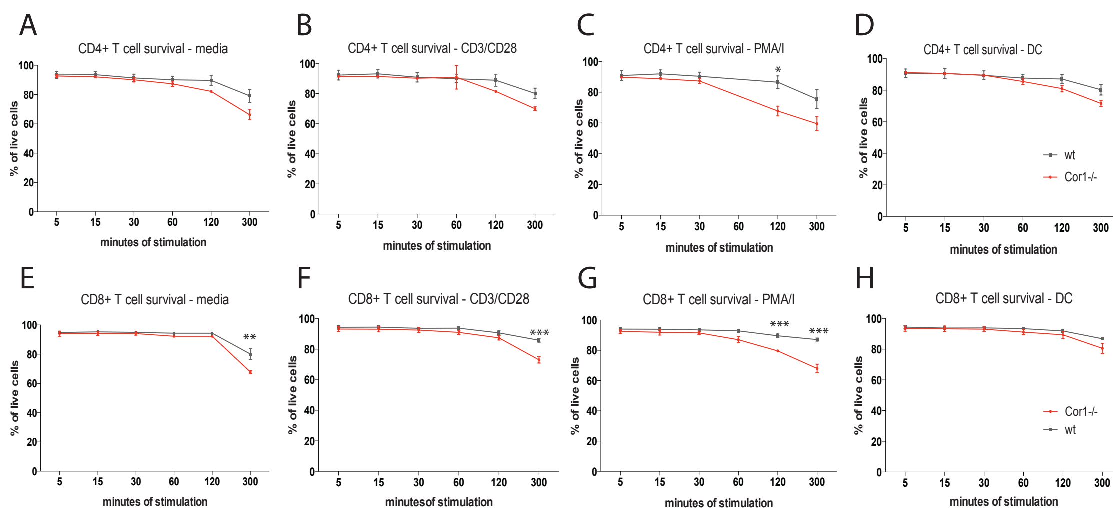


Figure 20. *Survival of CD4+ (A-D) and CD8+ (E-H) T cells in vitro without (A and E) or with stimulation with anti-CD3 and anti-CD28 (B and F), PMA and ionomycin (C and G), or dendritic cells (D and H).* *The CD4+ and CD8+ T cells were negatively sorted following the protocol described in Chapter 6.7, plated onto 96-well plate and cultured for 5, 15, 30, 60, 120 and 300 min at 37°C. Then the cells were stained following the FACS staining protocol (Chapter 6.6) and analyzed by FACS. Each point shows a mean of three replicates. Both experiments were repeated at least three times. Graphs show difference in survival between cell purified from BL/6 wt and coronin 1-deficient mice. B6 – BL/6 wild type; Cor1-/- - coronin 1-deficient.*

4.4.4 Analysis of concentration dependence in survival of coronin 1-deficient T cells *in vitro*

It drew our attention that defect of coronin 1-deficient T cells to proliferate is overcome by increasing the numbers of stimulators. The highest concentration of stimulators (800 000) in major mismatched MLR experiment induced proliferation of coronin 1-deficient CD4+ T cells at similar level to the wild type (Fig. 19). Therefore, we decided to double the number of plated T cells. Interestingly, 200 000 of coronin 1-deficient CD4+ T cells proliferated at a similar level as wild type cells (Fig. 21). Still, some differences could be seen (for 100 000, 200 000 and 400 000 stimulators) but much lower than for 100 000 responder CD4+ T cells. This indicates that the coronin 1-deficient T cells were sensitive to the cell density and behaved better when plated in higher numbers. Higher numbers of responders (Supplementary Fig. 3) improved coronin 1-deficient CD8+ T cells proliferation, yet did not rescue it to the wild type levels.

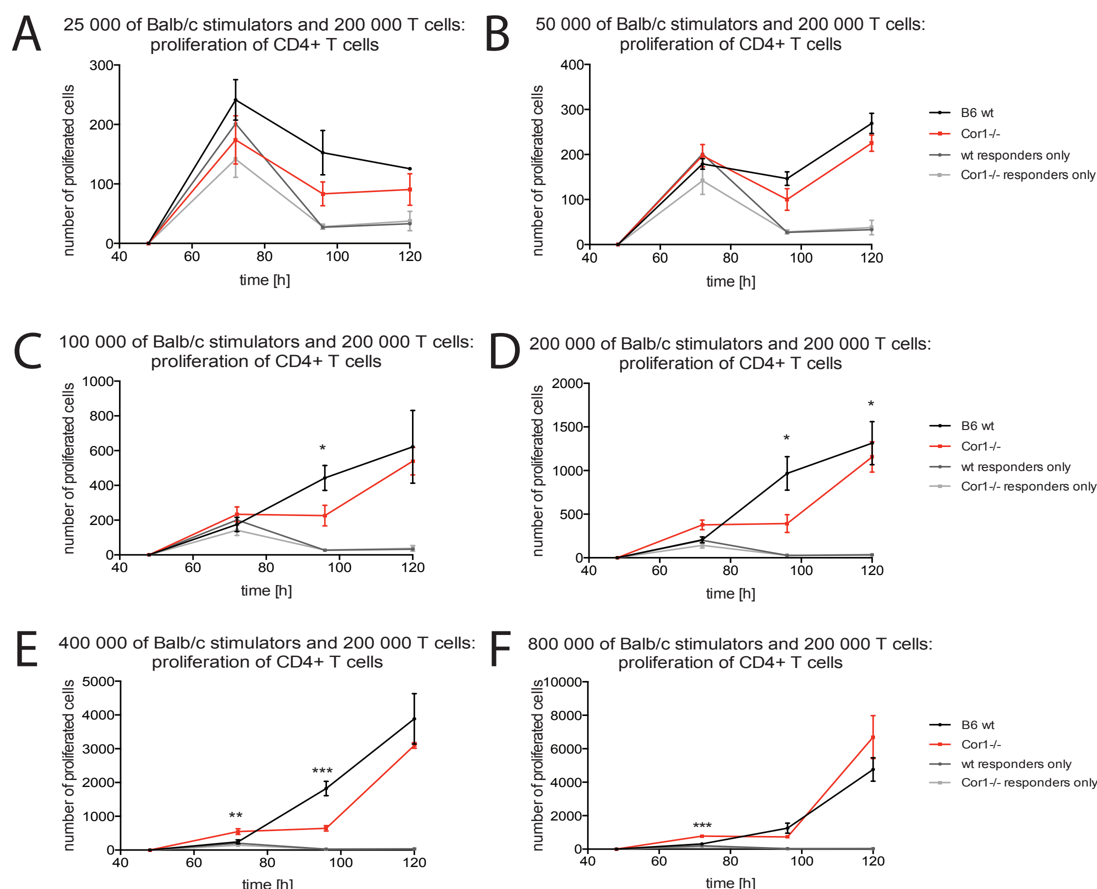


Figure 21. ***CD4+ T cells proliferation analysis in a mixed lymphocyte reaction (MLR) for total T cells from BL/6 wild type and coronin 1-deficient mice as responders and different concentrations of Balb/c splenocytes as stimulators.*** *Total T cells were purified as described in Materials and Methods Chapter 6.20. Stimulators Balb/c splenocytes were depleted of CD3 positive cells and erythrocytes (with anti-CD3 and anti-TER119 antibody, respectively). The cells were treated with mitomycin c to eliminate background proliferation of the stimulators. Then both cells were plated together in numbers indicated on each graph, in 96-well plates and cultured for 48, 72, 96 or 120h. The cells were analyzed by FACS using the same sample volume, time and speed of acquisition. A – F. Analysis of CD4+ T cells proliferation in a culture of 200 000 T cells with different numbers of Balb/c stimulators. An asterisk always shows the significance in the difference between BL/6 wt and Cor1-/- sample. Each time point shows a mean of 3 replicates. B6 – BL/6 wild type; Cor1-/- - coronin 1-deficient.*

4.4.5 Concentration dependence of the survival of coronin 1-deficient T cells *in vivo*

In order to further characterize coronin 1-deficient T cell survival, we decided to analyze the survival rate of coronin 1-deficient CD4+ T cells *in vivo*. As mentioned

above coronin 1-deficient T cells survival and proliferation seemed to be dependent on the density of plated cells therefore, we adoptively transferred 20 000, 100 000 and 1 000 000 of wild type or coronin 1-deficient CD4+ T cells into Rag2-/- mice (Fig. 22). At day 30 after the cell transfer, mice were sacrificed and analyzed for numbers of surviving CD4+ T cells.

When 20 000 of coronin 1-deficient cells were injected we could not recover many cells from Rag2-/- mice while 20 000 of wild type cells survived and additionally proliferated in the immunodeficient environment (Fig. 22A and B). However we could find coronin 1-deficient CD4+ T cells after transfer of 100 000 cells but the numbers were significantly lower than for the wild type (22 993 vs 254 987 cells, Fig 22C and D). Transfer of 1 000 000 of cells gave relatively better survival of coronin 1-deficient CD4+ T cells when compared with the wild type (Fig. 22E and F). These three experiments were done separately and it is surprising that the general number of surviving cells was the lowest for 1 000 000 wild type CD4+ T cells injected, where the difference between wild type and coronin 1-deficient cells was also the lowest. On the contrary, survival of wild type CD4+ T cells was similar for the adoptive transfer of 20 000 and 100 000. Observation that number of surviving T cells, upon adoptive transfer into lymphopenic environment, is independent of the number of injected T cell population was described before in a study on homeostatic proliferation and a pool size of T cells in lymphopenic environment *in vivo* (Rocha, Dautigny, and Pereira 1989). The T cells, both CD4+ and CD8+, after transfer to the lymphopenic host always expand to a certain number. The accumulation of their number is not dependent on the number of injected cells or their potential to proliferate; however, higher number of injected cells enables to reach the plateau faster. Moreover, they do not require exogenous antigen stimulation to proliferate. There must be a homeostatic mechanism controlling the number of T cells in the periphery that maintain the T cell pool size (Rocha, Dautigny, and Pereira 1989). However, survival of coronin 1-deficient CD4+ T cells differed between those experiments significantly. Taken together, these results indicate that coronin 1-deficient CD4+ T cells had impaired proliferation and/or survival upon transfer into Rag2-/- mice, but which is dependent on their density.

To analyze the activation and proliferation level of coronin 1-deficient and wild type cells, we stained for the expression of three markers: Ki-67, CD62L and CD44 (Fig. 23G-I). Ki-67 is a marker related to the proliferation (Scholzen and Gerdes 2000) and it was significantly higher on coronin 1-deficient cells when compered with wild type CD4+ T cells indicating that they were undergoing more intense proliferation than the wild type. CD62L and CD44 are markers of the activation of cells. Additionally, high expression of CD62L and CD44 indicates homeostatic proliferation of cells, which keeps the cell number at the constant level (Sprent and Surh 2011; Goldrath, Bogatzki, and Bevan 2000). The expression of CD62L and CD44 markers was high on the wild type cells. At the same time CD62L was low on the coronin 1-deficient CD4+ T cells, which signified their activation. The data suggested that the proliferation of coronin 1-deficient CD4+ T cells could not be impaired, however the cells were more activated and were undergoing more intense proliferation than wild type cells. However, increased activation of coronin 1-deficient T cells in general can be explained by the difference in subpopulation compared to wild type T cells. As reported before, coronin 1-deficient mice are depleted mainly of naïve T cells while memory/effector T cells are still present (Föger et al. 2006; Mueller et al. 2008).

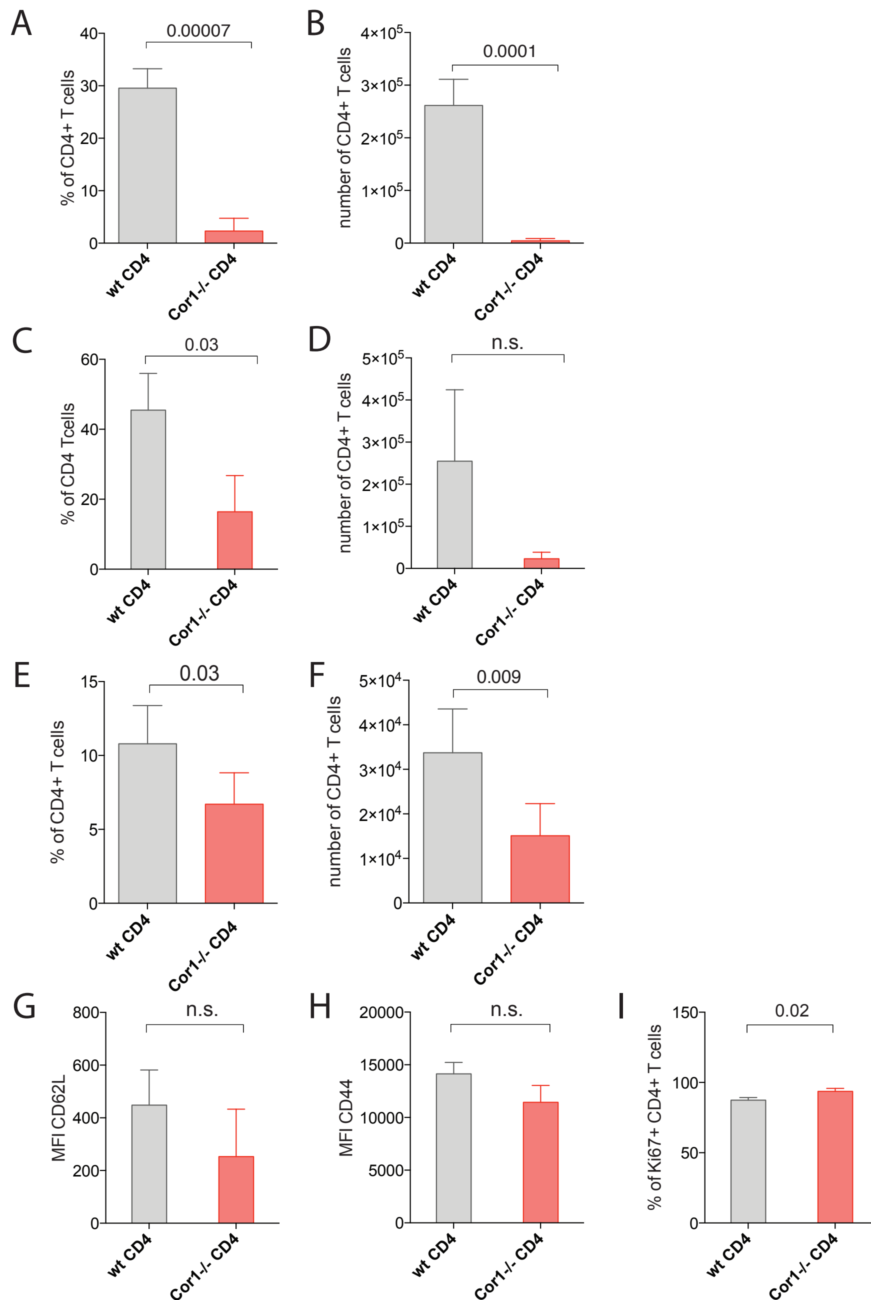


Figure 22. ***Survival of CD4+ T cells from BL/6 wild type and coronin 1-deficient mice after adoptive transfer into tail vein of Rag2-/- mice.*** *A and B. Transfer of 20 000 CD4+ T cells. C and D. Transfer of 100 000 CD4+ T cells. E and F. Transfer of 10^6 CD4+ T cells. G and H. Surface expression of CD62L and CD44 after adoptive transfer of 100 000 CD4+ T cells. I. Expression of Ki67 after transfer of 100 000 CD4+ T cells. CD4+ T cells were negatively purified from spleens of BL/6 wt or Cor1-/- mice and adoptively transferred into Rag2-/- mice. The mice were sacrifice 30 days after cell injection. Immune cells from lymph nodes (2x axillary, 2x brachial, 2x inguinal) were harvested, stained using anti-CD3 and anti-CD4 antibodies and analyzed with FACS. Each bar graph is a mean of 3 to 5 mice. B6 – BL/6 wild type; Cor1-/- - coronin 1-deficient.*

Consistent with the result obtained in MLR assays, we noticed that survival of coronin 1-deficient CD4+ T cells was dependent on the density of these cells. Higher numbers of transferred cells resulted in better survival of coronin 1-deficient CD4+ T cells compared with the wild type. Furthermore, high expression level of Ki-67 in coronin 1-deficient CD4+ T cells and similar proliferation rate between wild type and coronin 1-deficient cells in MLR suggested that the proliferation of coronin 1-deficient CD4+ T cells was not impaired. However the survival of cells seemed to be decreased in coronin 1-deficient T cells when compared with wild type. High expression of CD44 and low expression of CD62L of coronin 1-deficient CD4+ T cells indicate that these cells were activated and did not maintain homeostatic proliferation after transfer into Rag2-/- mice, in contrary to wild type cells.

4.4.6 Characterization of cell proliferation in co-culture of coronin 1-deficient with wild type T cells

In order to investigate whether differences in cytokine production or surface protein expression between wild type and coronin 1-deficient T cells have an influence on the proliferation rate of these cells we co-cultured wild type and coronin 1-deficient T cells with different stimuli (major mismatched stimulators, dendritic cells, anti-CD3 and anti-CD28 or PMA/I, Fig. 23). Fig. 23A illustrates the proliferation of total T cells (either wild type or coronin 1-deficient) in separate cultures (lines) and in a co-culture of these cells together (using half of the cell number, bars). The cells were plated with Balb/c splenocytes depleted of CD3+ cells and erythrocytes as stimulators. The proliferation rate was measured as an incorporation of ^{3}H-thymidine into the DNA. As described previously in this thesis, coronin 1-deficient T cells had decreased proliferation rate comparable with wild type cells. However, the proliferation of wild type and coronin 1-deficient T cells increased together with elevated numbers of stimulators. Although, co-culture of these cells showed better proliferation than coronin 1-deficient T cells alone, it did not a show similar rise in proliferation. These results suggest suppression of proliferation in co-culture of coronin 1-deficient and wild type T cells. To define a possible source of suppression

we co-cultured negatively sorted CD8+ T cells isolated from wild type and coronin 1-deficient mice (Fig. 23B). Since in co-culture we used half of the coronin 1-deficient and wild type cell numbers the total proliferation rate for co-culture is decreased. However, growth of separate types of cells for coronin 1-deficient (orange line) and wild type (grey line) cells, in co-culture were similar to the proliferation in separate cultures. Therefore, we conclude that coronin 1-deficient CD8+ T cells did not interfere the proliferation of wild type cells. These results suggest that the suppression of T cells growth came from CD4+ T cells.

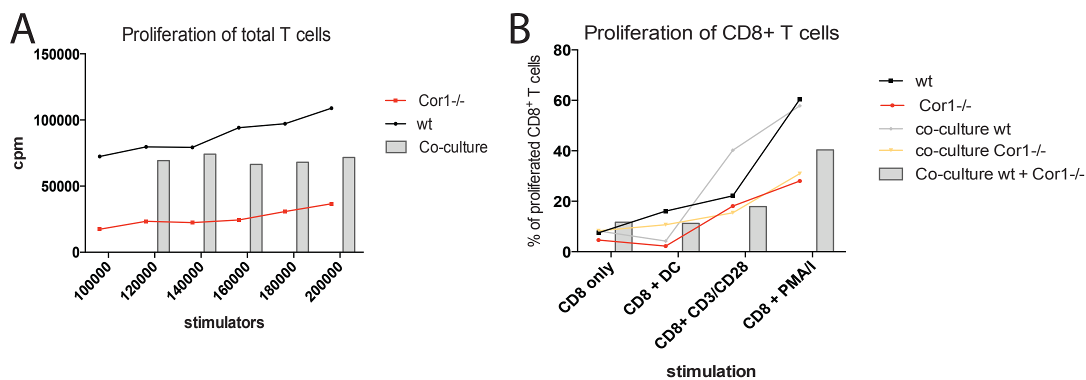


Figure 23. ***Co-culture of BL/6 wild type and coronin 1-deficient T cells after stimulation with either MHC mismatched splenocytes (A) or autologous dendritic cells, anti-CD3 and anti-CD28, and PMA/I (B).*** *A. Co-culture of total T cells (gray bar) and separate culture for BL/6 wt (black line) and Cor1-/- (red line) in the present of Balb/c splenocytes as a stimulators. The cells were obtained and plated as described before (MLR experiments). The proliferation was measured by incorporation of thymidine. B. Co-culture of CD8+ T cells (grey bar) and separate culture of BL/6 wt (black line) and Cor1-/- (red line) cells. Grey line represents proliferation of CD8+ T cells from BL/6 wt in the co-culture, orange line – proliferation of Cor1-/- in the co-culture. The cells were processed and plated following the protocol from proliferation and cytokine production assay (Chapter 6.18). For the separate culture 300 000 cells/well were used (from either BL/wt or Cor1-/- mice). For the co-culture 150 000 cells were used from both BL/6 wt and Cor1-/- mice. The cells were analyzed using FACS. Each time point shows a mean of three individual mice used per sample. wt – BL/6 wild type; Cor1-/- - coronin 1-deficient.*

4.4.7 Cytokine production by coronin 1-deficient and wild type T cells

Cytokines, important players in modulating immune responses, are secreted by different types of cells, immune as well as non-immune cells, and are able to

stimulate or inhibit other cell responses. Since the development of mature immunocompetent lymphoid cells from precursors, their antigen-driven responses and suppression are highly dependent on cytokines, to characterize the functionality of coronin 1-deficient T cells in response to different stimuli we analyzed secretion of three cytokines by these cells compared with the wild type. We chose to stain for interleukin-2 (IL-2), interferon-γ (IFNγ) and tumor necrosis factor-α (TNFα). IL-2 is a pleiotropic cytokine that drives T cell growth and development, induces differentiation of regulatory T cells and mediates activation-induced cell death. Therefore the appropriate level of IL-2 is important for proper T cell homeostasis and survival (Malek and Castro 2010; Liao, Lin, and Leonard 2011). IFNγ is another pleiotropic cytokine that modulates an adaptive immune response, for example, by promoting differentiation of cytotoxic T cells and enhancing antigen-presentation abilities causing increase in expression of MHC complexes (Cannon 2000). Recently, IFNγ has been reported to be involved, together with IL-7R, in CD8+ T cells maintenance (Kimura et al. 2013). IFNγ, together with TNFα, is considered a proinfammatory cytokine. IFNγ enhances activity of TNFα, which stimulates leukocyte proliferation, cytotoxicity and secretion of other cytokines (Dinarello 2000). These three cytokines are involved in T maintenance, therefore their abnormal secretion can influence the T cell function and survival.

To investigate secretion levels of IL-2, IFNγ and TNFα of coronin 1-deficient T cells compared with the wild type, we performed a series of *in vitro* experiments. Negatively purified CD4+ and CD8+ T cells were cultured either in the absence of stimuli or stimulated for up to 300 min with dendritic cells, CD3/CD28 antibodies or PMA/I (Supplementary Fig. 4, 5 and Table 2). Interestingly, as reported before (Dinarello 2000), the level of IFNγ produced by coronin 1-deficient CD8+ T cells was higher than for wild type; this could mean increased activation of these cells (Kimura et al. 2013). Enhanced levels of INFγ upon stimulation by IL-7 signaling leads to activation-induced cell death, which may explain impaired survival of coronin 1-deficient CD8+ T cells (Kimura et al. 2013). Also, after 300 min of stimulation with PMA/I, IFNγ was increased in coronin 1-deficient CD4+ T cells when compared with wild type. Hence indicating that coronin 1-deficient CD4+ T cells, upon activation,

would preferably differentiated into T_H1 helper cell. However, there were no significant changes in the expression of IL-2, which is also associated with T_H1 maturation (Mosmann et al. 1986). Interestingly, CD4+ T cells are reported to enhance their proliferation and survival rate when exposed to IFNγ (Reed, Branigan, and Bamezai 2008), which is the opposite of what we observed in the MLR experiments. As mentioned above, IL-2 secretion was not changed in coronin 1-deficient CD4+ T cells in the culture with or without stimulation. The same observation was true for coronin 1-deficient CD8+ T cells. TNFα secretion was decreased in coronin 1-deficient CD4+ T cells after 120 min of activation with PMA/I, which indicates activation of these cells (Hill et al. 2000). To further characterize activation and exhaustion of T cells we analyzed expression of several cell surface markers. The results are discussed below.

<table>
<tr><th rowspan="2">T cell type</th><th rowspan="2">Stimuli</th><th colspan="3">Cytokine stained</th><th rowspan="2">LAMP-1</th></tr>
<tr><th>IFNγ</th><th>TNFα</th><th>IL-2</th></tr>
<tr><td rowspan="4">CD4+</td><td>No (medium)</td><td rowspan="3">No significant differences</td><td rowspan="3">No significant differences</td><td rowspan="4">No significant differences</td><td>No significant differences</td></tr>
<tr><td>DC</td><td>Significant increase</td></tr>
<tr><td>CD3/CD28</td><td rowspan="2">No significant differences</td></tr>
<tr><td>PMA/I</td><td>Increase after 300 min in Cor1-/- cells</td><td>Decrease after 120h in Cor1-/- cells</td></tr>
<tr><td rowspan="4">CD8+</td><td>No (medium)</td><td rowspan="2">No difference between BL/6 wt and Cor1-/-</td><td rowspan="4">No significant differences</td><td rowspan="4">No significant differences</td><td rowspan="4">No significant differences</td></tr>
<tr><td>DC</td></tr>
<tr><td>CD3/CD28</td><td rowspan="2">Significant increase in Cor1-/- cells compared with wt</td></tr>
<tr><td>PMA/I</td></tr>
</table>

Table 2. ***Summary comparison of cytokine (IFN-gamma, TNF-alpha, IL-2) and LAMP-1 production in BL/6 wild type and Cor1-/- mice after stimulation with dendritic cells (DC), CD3/CD28 or PMA/I over time.*** *Dendritic cells were obtained from bone marrow of BL/6 wt mice after one week of culture in the presence of GM-CSF (as described in Materials and Methods). Then the cells were plated in 96-well plate at the concentration of 30 000 cells/well one day before T cells culture started. CD4+ or CD8+ T cells were negatively purified following the protocol (Chapter 6.7), plated in 96-well plate at the concentration of 200 000 cells/well, in the absence or presence of the stimuli. The cells were analyzed using FACS. The table shows the summary of results attached in the supplement (Supplementary Fig. 4 and 5).*

In addition to cytokine staining, we analyzed surface expression of lysosomal associated membrane protein 1 (Lamp-1). Lamp-1 (CD107a) is a transmembrane protein residing within lysosomes that upon stimulation can be expressed at the cell surface. For example, cell surface expression of Lamp-1 occurs due to the lysosomal fusion with the cell membrane, and it is found on cytotoxic T cells or metastatic cancer cells where it acts as a ligand for selectins (Kima, Burleigh, and Andrews 2000; Laferte and Dennis 1989). We found a significant increase in surface expression of Lamp-1 in coronin 1-deficient CD4+ T cells only when stimulated with dendritic cells (Supplementary Fig. 4 and 5, Table 2). The expression was enhanced between 30 and 120 min with noticeable decrease at 300 min, possibly as a result of cell-to-cell interaction in this cultured condition.

4.4.8 Expression of specific markers by coronin 1-deficient T cells

Furthermore, we analyzed specific markers in coronin 1-deficient and wild type T cells (Table 3 and Supplementary Fig. 6, 7 and 8).

Ki-67 a proliferation marker

As mentioned before in this thesis, we characterized growth of cells by staining for expression of proliferation marker Ki-67. Since this protein can be detected in active phases of cell cycle (G_1, S, G_2 and mitosis) and is absence in resting cells (phase G_0), it is strictly associated with proliferation and can be used to determine growth of cells (Scholzen and Gerdes 2000). Expression of this marker was enhanced in spleen and lymph nodes in both CD4+ and CD8+ T cells of coronin 1-deficient mice when compared with the wild type. This indicates that the coronin 1-deficient T cells underwent more intense proliferation than the wild type in the absence of external stimuli. Which implies that the coronin 1-deficient T cells are activated even in naïve mice, which could cause their exhaustion. Interestingly, Ki-67 expression was decreased in coronin 1-deficient thymocytes compared with wild type cells. Reduced expression of Ki-67 and increased apoptosis in the thymus is associated with thymic

involution (Kanavaros et al. 2001). However, the thymus in coronin 1-deficient mice is normal compared with the wild type (Mueller et al. 2008).

PD-1 and Bcl-2 expression in coronin 1-deficient and wild type T cells

Bcl-2 is an anti-apoptotic marker regulating normal life span of the cell. High expression of this protein occurs in long-lived cells, thus activated lymphocytes, when not continuously stimulated, express lower levels of Bcl-2. Memory lymphocytes have more of this marker on the surface (Chávez-Galán et al. 2009; Bouillet et al. 2002). Bcl-2 expression was significantly increased in coronin 1-deficient CD8+ T cells of spleen and lymph nodes. CD4+ T cells Bcl-2 levels were not affected by the deletion of coronin 1, as shown previously (Mueller, Liu, and Pieters 2011). Being consistent with this result, PD-1 levels were enhanced in spleen and lymph nodes in both CD4+ and CD8+ T cells of coronin 1-deficient mice compared with the wild type. PD-1, an immune exhaustion marker, and its high expression is induced by persistent TCR signaling. During chronic infection, continuously activated exhausted T cells overexpress PD-1 at the cell surface. This inhibits T cell function and survival, and has been reported to promote Treg development (Francisco, Sage, and Sharpe 2010). Bcl-2 and PD-1 are highly expressed on the surface of continuously stimulated and activated T cell, we noticed increased expression of both markers on coronin 1-deficient CD8+ T cells when compared with wild type. PD-1 level was also increased on coronin 1-deficient CD4+ T cells.

Expression of CD5 molecule

CD5 molecule associates with T and B cell receptor complexes modulating their signals and plays an essential role in T cell development. This molecule is a positive as well as a negative regulator of antigen receptor signaling. Therefore, alteration of CD5 expression levels in T cells affects TCR responses, for example, its high expression effects in the development of antigen-specific unresponsiveness in T cells (Soldevila, Raman, and Lozano 2011). Moreover, CD5 has a pro-survival role, is associated with strong activation of T cells (Osman, Ley, and Crumpton 1992) and high level of this protein is found on Tregs (Ordoñez-Rueda et al. 2009). CD5

expression levels were increased in spleen and lymph nodes in both CD4+ and CD8+ T cells of coronin 1-deficient mice compared with wild type T cells.

Surface levels of IL-7 receptor

IL-7 signaling plays a crucial role in T cell homeostasis. Increased continuous IL-7 level and insufficient TCR signaling result in induced CD8+ T cells proliferation and activation. This in turn triggers enhanced secretion of INFγ and suppresses IL-7 receptor expression on cell surface leading to cell death (Kimura et al. 2013). Moreover, IL-7 signaling participates in survival of CD4+ T cells, however, the mechanisms of that are still under an intensive studies (Fig. 8) (Hataye et al. 2006). Expression of IL-7 receptor was decreased in CD4+ and CD8+ T cells of coronin 1-deficient spleen and lymph nodes when compared with the wild type. These results, in agreement with previously described staining, shows increase in activation of coronin 1-deficient T cells. Furthermore, decrease in IL-7R surface expression together with enhanced levels of INFγ (described in Section 4.4.7 *Cytokine production by coronin 1-deficient and wild type T cells)* suggests a cytokine-induced cell death (CICD) as a possible apoptotic pathway of CD8+ T cells of coronin 1-deficient mice.

Expression of FasL

Fas ligand (FasL) is expressed on activated T cells and it induces cell death via apoptosis. This cell death induced by engagement of Fas/FasL pathway plays an important role in modulation of immune homeostasis through stimulation of activation-induced cell death (AICD) which limits expansion of T cells after elimination of pathogen and inactivate autoreactive peripheral T cells (Paul Waring and Müllbacher 1999; Schneider et al. 1997; Ashwell, Longo, and Bridges 1987). We found increased expression of FasL on coronin 1-deficient CD4+ T cells in spleen, lymph nodes and thymus compared to wild type cells. Surface level of FasL was also increased on coronin 1-deficient CD8+ T cells when compared to wild type cells but the increase was not significant.

Marker	T cell type	Organ		
		spleen	**LNs**	**thymus**
Ki67	**CD4+**	Significant increase in Cor1-/-	Significant increase in Cor1-/-	Decrease in Cor1-/-
	CD8+	Significant increase in Cor1-/-	Significant increase in Cor1-/-	Significant decrease in Cor1-/-
PD-1	**CD4+**	Significant increase in Cor1-/-	Significant increase in Cor1-/-	No significant difference
	CD8+	Significant increase in Cor1-/-	No significant difference	No significant difference
IL-7R	**CD4+**	Significant decrease in Cor1-/-	Significant decrease in Cor1-/-	Significant decrease in Cor1-/-
	CD8+	Decrease in Cor1-/-	Decrease in Cor1-/-	Significant decrease in Cor1-/-
CD5	**CD4+**	Significant increase in Cor1-/ -	No significant difference	No significant difference
	CD8+	Significant increase in Cor1-/-	Significant increase in Cor1-/-	No significant difference
Bcl-2	**CD4+**	No significant difference	No significant difference	No significant difference
	CD8+	Significant increase in Cor1-/-	Significant increase in Cor1-/-	No significant difference
FasL	**CD4+**	Significant increase in Cor1-/-	Significant increase in Cor1-/-	Significant increase in Cor1-/-
	CD8+	No significant changes	No significant changes	No significant changes

Table 3. ***Summary of cell markers expression.*** *The cells from spleen, lymph nodes and thymus were harvested and processed following the protocol (Chapter 6.5). Then they were FACS stained for Ki67, PD-1, IL-7R, CD5 and Bcl2 as described in the protocol for surface and intracellular FACS staining in the Materials and Methods Section 6.6. The results are attached in the supplement (Supplementary Fig.6, 7 and 8). Cor1-/- - coronin 1-deficient mice.*

Taken together, coronin 1-deficient T cells expressed increased levels of PD-1, Bcl-2, FasL and CD5 when compared with wild type cells. Enhanced surface expression of these markers is correlated with intense stimulation, high activation and exhaustion of T cells. Additionally, expression of IL-7R on coronin 1-deficient T cells was decreased compared to wild type cells, which indicates continuous stimulation of T cell or insufficient TCR signaling and is correlated with increased secretion of IFNγ (data described above). Interestingly, activated T cells usually undergo intense

proliferation and coronin 1-deficient T cells showed increased levels of Ki-67 compared to wild type cells.

At the same time, expression of most of those markers was not changed in the thymus of coronin 1-deficient mice compared with the wild type (no significant differences in PD-1, CD5 and Bcl-2 levels). IL-7R showed significant decrease in the thymus of coronin 1-deficient mice compared with the wild type, which could indicate defects in the thymocytes development and impaired balance between naïve and activated/memory T cells in the periphery (Nasi et al. 2006; Shitara et al. 2013). Therefore, we decided to perform an in-depth analysis of T cell numbers in wild type and coronin 1-deficient mice.

4.4.9 Numbers of T cell subsets in coronin 1-deficient mice

The peripheral T cell population is maintained at a stable number thanks to two mechanisms: peripheral T cell homeostasis and thymic output of newly generated T cells. Mature T cells leaving the thymus are referred to as recent thymic emigrants (RTE). They are a distinct population as they are not as functionally mature as peripheral naïve T cells (in terms of proliferation and cytokine production) (Boursalian et al. 2004). Using two different markers (CD24 and Qa-2 (Paiva et al. 2013; Boursalian et al. 2004)) we were able to obtain similar results, which showed that, although coronin 1-deficient mice have reduced numbers of recent thymic emigrants compared with the wild type, we could find them in the periphery of adult mice (Fig. 24E, F, M and N). All of the other T cell type numbers were decreased in the periphery (Fig. 24, as reported before (Föger et al. 2006; Mueller et al. 2008)). Generally decreased numbers of peripheral T cells explained elevated percentage of RTE in coronin 1-deficient mice. In order to eliminate T cell arrest in the thymus of coronin 1-deficient mice we analyzed thymus for the percentage and number of cells (Fig. 25). Rates and numbers of double positive (CD4+CD8+), double negative, CD4+ and CD8+ thymocytes were comparable between wild type and coronin 1-deficient mice. These results suggest that the production and egress of T cells in the

thymus of coronin 1-deficient mice are functional. However, it drew our attention that coronin 1-depleted mice showed elevated rate of CD25+ cells within the CD4+ T cell population (Fig. 24G and O). This observation is further discussed in the next Section of this Chapter.

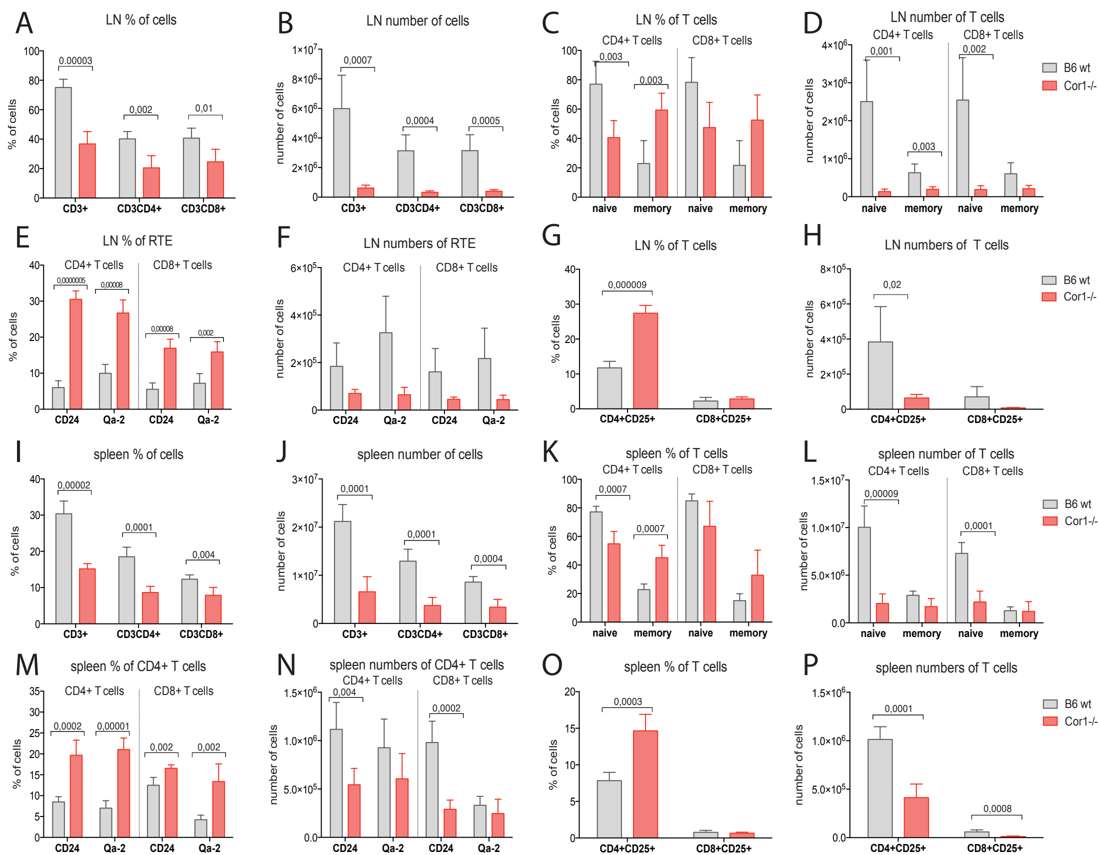


Figure 24. ***Different T cells subsets in BL/6 wild type compared with coronin 1-deficient mice.*** *A and B show T cells percentage and numbers in lymph nodes, respectively. C and D – naïve, memory CD4+ and CD8+ T cell rates and numbers in lymph nodes. E and F, recent thymic emigrants (RTE) quantities in lymph nodes. G and H represent activated CD4+ and CD8+ T cells (CD25+) in lymph nodes. I and J show T cells percentage and numbers in spleen, respectively. K and L – naïve, memory CD4+ and CD8+ T cell rates and numbers in spleens. M and N, recent thymic emigrants (RTE) quantities in spleens. O and P represent activated CD4+ and CD8+ T cells (CD25+) in spleens. The organs were harvested and processed following the protocol (Chapter 6.5). The cells were stained using surface and intracellular staining protocol from Materials and Methods. Afterwards, the cells were analyzed with the FACS. The figure shows representative results from one experiment where five mice were used per group. B6 wt – BL/6 wild type; Cor1-/- - coronin 1-deficient.*

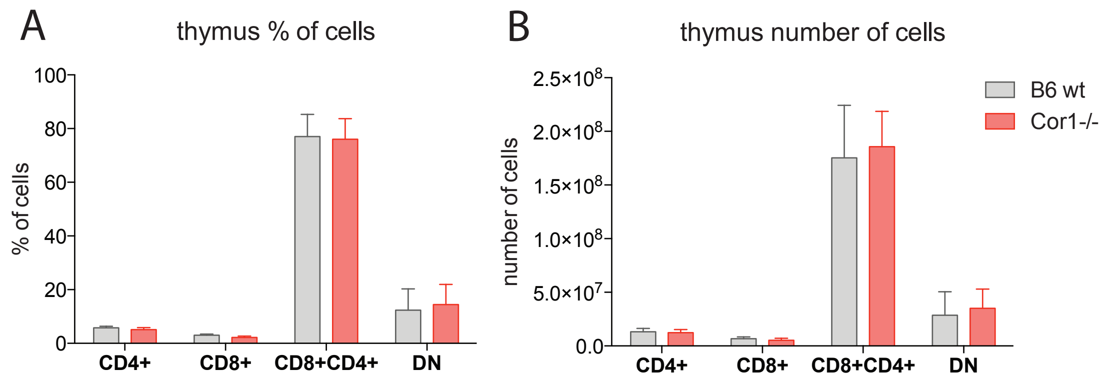


Figure 25. ***Percentage (A) and numbers (B) of different T cells subsets in the thymus of BL/6 wt and Cor1-/- mice***. *Thymi were harvested and processed following the protocol described in Chapter 6.5. The cells were stained for surface antigens. The figures shows representative results from one experiment where five mice were used per group. B6 wt– BL/6 wild type; Cor1-/- - coronin 1-deficient.*

4.5 Analysis of tolerance induction after skin transplantation in coronin 1-deficient recipients

4.5.1 Regulatory T cells in coronin 1-deficient mice

To address the question whether prolonged graft survival in coronin 1-deficient recipients is due to tolerance induction, first we characterized frequencies and numbers of regulatory T cells (CD4+CD25+Foxp3+ T cells, called Tregs) in naïve and transplanted mice. Tregs provide an additional mechanism, beside anergy and apoptosis, to maintain tolerance. They are found not only in the secondary lymphoid organs, but also in sites of infection where they can down-regulate immune process (Sakaguchi 2004). Thus, after transplantations Tregs are present in recipient lymphoid tissue and at the graft site. In resting conditions Tregs represent about 5 – 10% of total CD4+ T cells in the periphery (X. C. Li and Turka 2010). The analysis showed increased frequencies of regulatory T cells in lymph nodes and spleens of knockout animals compared with wild type mice (Fig. 26A). However, the number of Tregs remained decreased in coronin 1-deficient mice when compared with the wild type. This suggests that Tregs were also affected by coronin 1-deficiency but at a lower level than other T cells, thereby influencing the balance between regulatory and conventional CD4+ T cells. Additionally, analysis of transplanted animals showed enhanced rates of regulatory T cells in coronin 1-deficient recipients when compared with the wild type (Fig. 26B).

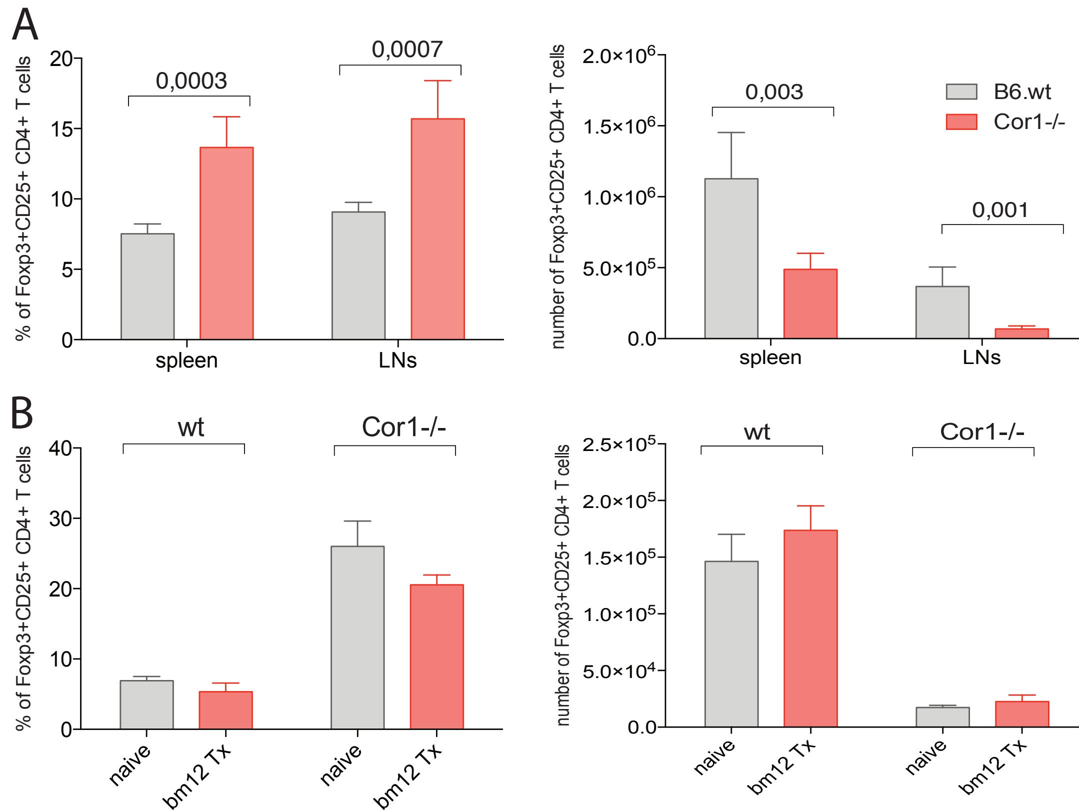


Figure 26. ***Frequencies and numbers of regulatory T cells (Tregs) in naïve (A) and mice transplanted with bm12Rag2-/- skin (B).*** *A. Percentage and number of Tregs in spleens and lymph nodes of naïve BL/6 wild type and Cor1-/- mice. Organs were harvested and processed followed the protocol described in Chapter 6.5. Then the cells were stained with CD3, CD4, CD25 and Foxp3 antibodies as described in intracellular FACS staining protocol. The experiment shows a representative graph from one out of three separate experiment were 5 mice per group were used for staining. B. Percentage and number of Tregs in lymph nodes of BL/6 wild type and Cor1-/- after transplantation with bm12Rag2-/- skin graphs compared with naïve mice. The mice were transplanted according to the transplantation protocol described in Chapter 6.4. The wild type mice were sacrificed at the day of the rejection (day 12 after transplantation), coronin 1-deficient mice were sacrificed at day 100 after the surgery. Lymph nodes were processed and stained as described before. The graph shows results pooled from two separate experiments where at least 3 mice were used per experimental group. B6 wt – BL/6 wild type; Cor1-/- - coronin 1-deficient.*

4.5.2 Tolerance maintenance after skin graft transplantation

To further determine if increased frequencies of Tregs and induction of tolerance are the main reason for the prolonged graft survival in coronin 1-deficient recipients, we challenged them with transfer of wild type CD4+ T cells. To this end we performed

bm12Rag2-/- skin transplantation onto coronin 1-deficient mice. Additionally, we transplanted Rag2-/- mice with bm12Rag2-/- skin graft to use as a control. Then, 48 days after surgeries we injected wild type CD4+ T cells into coronin 1-deficient and Rag2-/- recipients. Rag2-/- mice, when transplanted with skin grafts, did not show any rejection. After adoptive transfer of 20 000 wild type CD4+ T cells, Rag2-/- recipients rejected within 29 days (Fig. 27). Similarly, coronin 1-deficient recipients did not reject bm12Rag2-/- skin grafts. However, after injection of wild type CD4+ T cells, we observed several rejections within coronin 1-deficient mice. However, all rejections were delayed when compared with Rag2-/- controls. Interestingly, we injected different numbers of wild type CD4+ T cells into coronin 1-deficient recipients (20 000, 100 000, 10^6 and $5x10^6$) and the rate of rejection varied between different groups. In the group where 20 000 wild type CD4+ T cells were adoptively transferred, two out of four mice rejected skin transplants. Similar rejection was observed after injection of $5x10^6$ cells (2 out of 4 rejected). Mice injected with 100 000 cells showed rejection in two out of five mice. Interestingly, none of graft recipients receiving 10^6 of wild type CD4+ T cells rejected (five mice in the group). When we repeated this experiment we obtained even longer delay in rejection (after transfer with 20 000, 100 000 and 10^6 of wild type CD4+ T cells) when compared with Rag2-/- controls (50 vs. 14 days, data not shown). Notwithstanding, the delayed rejection in mice injected with wild type CD4+ T cells suggests suppression of these cells through interference in their function or their death. The variability in graft tolerance within the same group could be caused by different survival of transferred wild type CD4+ T cells and the adequate rate of Tregs:cCD4 that is required to maintain tolerance. This rate varies according to the level of allospecificity of recipient regulatory T cells (Graca et al. 2002).

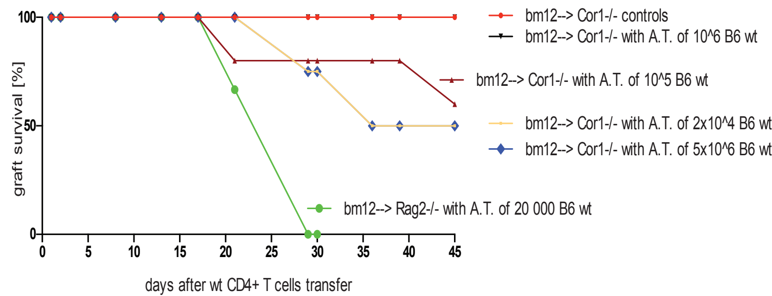


Figure 27. *Adoptive transfer of BL/6 wt CD4+ T cells into Cor1-/- mice transplanted with bm12Rag2-/- skin.* *The coronin 1-deficient (Cor1-/-), Rag2-/- and BL/6 wild type (B6 wt) mice were transplanted with bm12Rag2-/- skin according to the protocol. The BL/6 wt recipients rejected grafts after two weeks; Rag2-/- and Cor1-/- mice kept the grafts and were intravenously injected with BL/6 wt CD4+ T cells 48 days after transplantation. Rag2-/- recipients were used as a control for the transfer and vitality of injected cells. Graph shows transfer of 20 000, 100 000, 1 00 000 and 5 000 000 wt CD4+ T cells. Graphs represent survival of skin grafts after adoptive transfer of cells up to 100 days after transplantation. Each group represents at least 4 mice.*

4.5.3 Induction of graft rejection in coronin 1-deficient recipients

The transgenic ABM mice have antigen-specific CD4+ T cells towards the $I\text{-}A^{bm12}$, which can be tracked using the expression of the Vα2.1/Vβ8.1 TcR chains (Bäckström et al. 1998). Therefore, we decided to use bm12-specific (ABM) T cells to induce specific rejection in coronin 1-deficient mice after transplantation with bm12Rag2-/- skin. In order to do so, we performed bm12Rag2-/- skin transplantations onto Rag2-/- or coronin 1-deficient mice. Afterwards, at day 28 after surgeries the mice were injected with different numbers of ABM cells (20 000, 100 000 and 10^6, Fig. 28). Rag2-/- recipients rejected skin grafts within 12 days after adoptive transfer (both groups receiving 20 000 and 100 000 wild type CD4+ T cells). In the experimental groups we observed tolerance in most of the coronin 1-deficient recipients while several animals showed rejection. Consistent with previous experiments (Section 4.5.2. *Tolerance maintenance after skin graft transplantation*) the rejection rate did not depend on the number of transferred cells. One out of four mice rejected in both groups receiving 100 000 and 10^6 of ABM cells, while two out of four mice rejected after injection of 20 000 ABM cells. It seemed that there was a slight difference between tolerance and rejection in coronin 1-deficient mice, however

the reasons are unknown. One possibility is that these differences in graft tolerance of coronin 1-deficient mice depend on the number of recipient's suppressive cells, which can vary from mouse to mouse due to their impaired survival in the absence of coronin 1. Indeed, there are studies describing that the balance between effectors and regulatory T lymphocytes controls the outcome of allografts (Benghiat et al. 2005; Graca et al. 2004; Tang and Lee 2012; Graca et al. 2002).

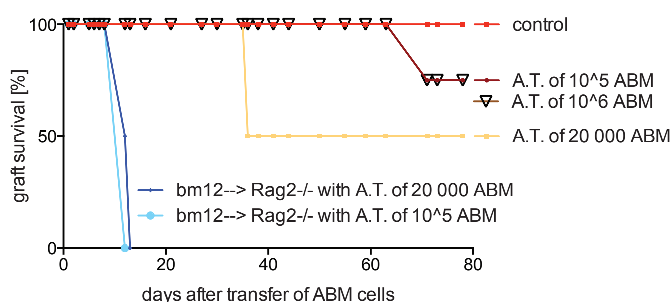


Figure 28. ***Adoptive transfer of ABM cells into Cor1-/- mice transplanted with bm12Rag2-/- skin.*** *The Cor1-/-, Rag2-/- and BL/6 wild type mice were transplanted with bm12Rag2-/- skin as described in Materials and Methods. The BL/6 wt recipients rejected grafts within two weeks; Rag2-/- and Cor1-/- mice kept the grafts and were intravenously injected with 20 000, 100 000 and 1 00 000 of ABM cells 28 days after transplantation. Rag2-/- recipients were used as a control for the transfer and vitality of injected cells. Each group consisted of at least 3 mice. Cor1-/- - coronin 1-deficient; bm12 – bm12Rag2-/- mice.*

4.5.4 Induction of rejection in immunodeficient recipients

To minimize the influence of other immune cells (such as B cells, NK cells, neutrophils and dendritic cells) and the effect of reduced CD4+ T cell numbers in coronin 1-deficient mice we decided to use Rag2-/- mice as graft recipients. Due to the fact that Rag2-/- mice are lacking T cells (Shinkai et al. 1992) we could manipulate numbers of injected T cells from coronin 1-deficient and wild type mice. Additionally, we tried to induce rejection by adoptive transfer of ABM cells. Firstly, the Rag2-/- mice were transplanted with bm12Rag2-/- skin grafts, 30 days after they were adoptively transferred with 20 000 coronin 1-deficient CD4+ T cells. Afterwards, at day 30 after CD4+ T cell transfer, the mice were additionally injected with 100 000 ABM cells. The results show that only one out of five mice rejected the

skin transplant when challenged with ABM cells (Fig. 29). The single rejection could be explained by poor survival of coronin 1-deficient CD4+ T cells after injection of such a low number (20 000) of cells in this mouse, since the other four mice kept the graft and therefore the results suggest that coronin 1-deficient CD4+ T cells can maintain tolerance of bm12Rag2-/- skin.

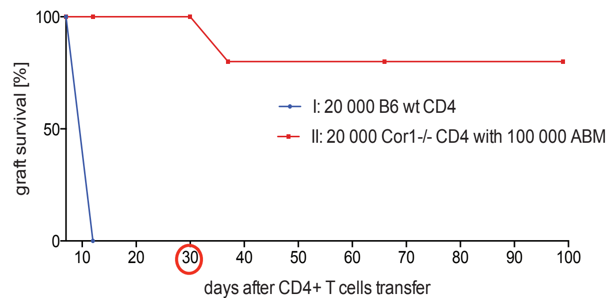


Figure 29. *Transfer of BL/6 wild type and coronin 1-deficient CD4+ T cells into Rag2-/- mice transplanted with bm12Rag2-/- skin and additional challenge with ABM cells.* *Rags2-/- mice were transplanted with bm12Rag2-/- following the transplantation protocol (Chapter 6.4). 30 days later recipients were injected with either BL/6 wt or Cor1-/- CD4+ T cells. Recipients of the wild type CD4+ T cells rejected skin grafts 12 days after injection. Rag2-/- recipients receiving Cor1-/- CD4+ T cells were additionally adoptively transferred with 100 000 ABM cells 30 days after the first injection. Only one out of five mice rejected the skin graft in this group. Each experimental group consisted of 5 mice. B6 wt – BL/6 wild type; Cor1-/- - coronin 1-deficient.*

4.5.5 Analysis of graft rejection in CD25-depleted skin transplantation model

When the transplanted tissue is accepted Tregs numbers are increased in the periphery and in the graft (Salisbury, Game, and Lechler 2014; Joffre et al. 2008). Removal of CD25+ cells subset, which contains Tregs, results in prevention of spontaneous transplant tolerance in mice (W. Li et al. 2006). Depleting rat anti-mouse CD25 mAb (PC61) is widely used to characterize Tregs *in vivo* (Setiady, Coccia, and Park 2010; Siegmund et al. 2011). PC61 antibody removes 70% of CD4+CD25+Foxp3+ T cells from the peripheral blood after single dose. The mechanism of depletion by PC61 is based on phagocytosis of antibody-bound cells. While the antigen-binding fragment of the PC61 antibody binds to the CD25+ cells the Fc fragment is recognized by

FcγRIII receptor expressed on phagocytes, such as macrophages. The binding of Fc fragment to its receptor mediates antibody-dependent phagocytosis thereby depleting CD25+ cells (Setiady, Coccia, and Park 2010). To further determine if Tregs are the main reason for the prolonged graft survival in coronin 1-deficient recipients, we performed bm12Rag2-/- skin transplantation onto coronin 1-deficient mice with either depleted or not CD25+ cells. Depleting anti-mouse CD25 mAb was given to the recipients 5 and 3 days pretransplantation and additionally 3 days after surgeries at a dose 0.5 mg per mouse. The level of Tregs was controlled in the blood by staining the day before surgery, and additionally 7 days after surgery (Supplementary Fig. 9). Moreover, coronin 1-deficient mice kept low numbers of Tregs until day 28. Treg numbers in coronin 1-deficient recipients recovered by day 65 after surgery. As expected, wild type recipients rejected skin grafts after 12-14 days (controls and CD25-depleted mice, Fig. 30). Normally, skin grafts in CD25+ T cells depleted mice are rejected earlier compared to non-depleted mice (Mai et al. 2014). Interestingly, in coronin 1-deficient mice depleted of CD25+ cells the graft survived longer than in controls. While eventually mice were able to reject skin grafts the rejection occurred at the time Tregs were replenished (started rejecting at day 58, rejected by day 78 after surgery). The delay in rejection could be explained by decreased number of conventional CD4+ (cCD4+) T cells that needed more time to proliferate and lead to the rejection, or the suppressive function of coronin 1-deficient cCD4+ T cells.

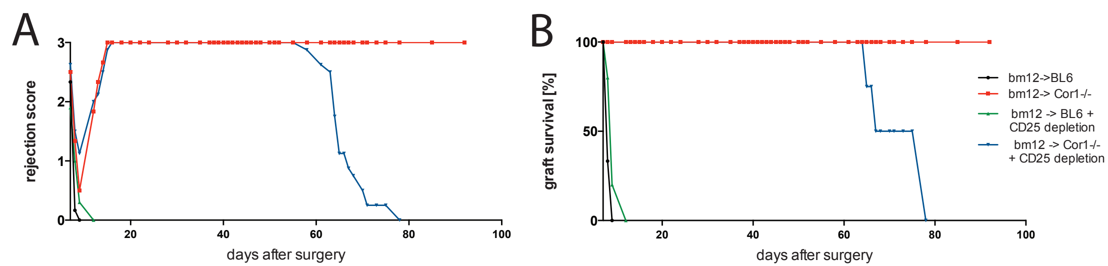


Figure 30. ***Rejection score (A) and graft survival (B) after transplantation of bm12 Rag2-/- skin grafts onto BL/6 wild type and Cor1-/- mice depleted or not of CD25+ cells.*** *The mice were injected with 0.5mg of PC61 antibody. The injection was performed 5 and 3 days before and 3 days after transplantation to remove CD25+ cells. The transplantation was done following the transplantation protocol (Chapter 6.4) and the mice were observed up to 100 days after surgery. At least 3 mice were used in each experimental group. BL6 – BL/6 wild type; Cor1-/- - coronin 1-deficient.*

To dissect this hypothesis and find the source of increased Treg frequency in coronin 1-deficient mice, we performed two sets of experiments: first, to test the function and proliferation of Tregs; second, to investigate the suppressive ability of coronin 1-deficient cCD4+ T cells.

4.5.6 *In vitro* induction of regulatory T cells from wild type and coronin 1-deficient cCD4+ T cells

Regulatory T cells, produced by the thymus, are referred to as naturally occurring Tregs. They are functionally unique T cell population, proliferate poorly in the response to antigens, unless the IL-2 is present, and have a potential to suppress the activation of other T cells in an antigen non-specific manner. There exists another population of regulatory T cells, called induced Tregs, which develops in the periphery from naïve T cells upon TCR stimulation with signaling from appropriate cytokines (TGF-β and IL-2) (Chen et al. 2003; X. C. Li and Turka 2010). Regulatory T cells are generated *in vitro* from CD4+CD25- T cells through co-stimulation with TCR in a presence of TGF-β and IL-2 (Fantini et al. 2007; Chen et al. 2003). To test induction of wild type and coronin 1-deficient Tregs *in vitro*, we stimulated conventional (CD4+CD25-) wild type or coronin 1-deficient CD4+ T cells with anti-CD3 and anti-CD28 in the presence of IL-2 and TGF-β (Fantini et al. 2007). Firstly, the CD4+CD25- cells were isolated from spleens of wild type and coronin 1-deficient mice by FACS sort. The cells were cultured for 2, 3, 4 and 5 days in the presence of stimulation or not. Then the surviving cells were analyzed for the percentage of CD4+CD25+Foxp3+ in coronin 1-deficient and wild type samples. The percentage of surviving Tregs in coronin 1-deficient samples after 3 days in culture was higher than in the wild type. However the numbers of coronin 1-deficient Tregs were significantly decreased (Fig. 31). At day 5 of culture almost all coronin 1-deficient Tregs were dead, in addition, the total number of cells in culture was low when compared to the wild type sample. Wild type Treg percentage and numbers were the highest at day 5 in culture which is consistent with the literature (Fantini et al. 2007). These results suggest that coronin 1-deficient Tregs survived better than conventional CD4+ T cells

(until day 3), however not as good as the wild type cells. The conversion of coronin 1-deficient cCD4+ into Tregs occurred earlier than in the wild type, and the cells survived for a shorter period of time.

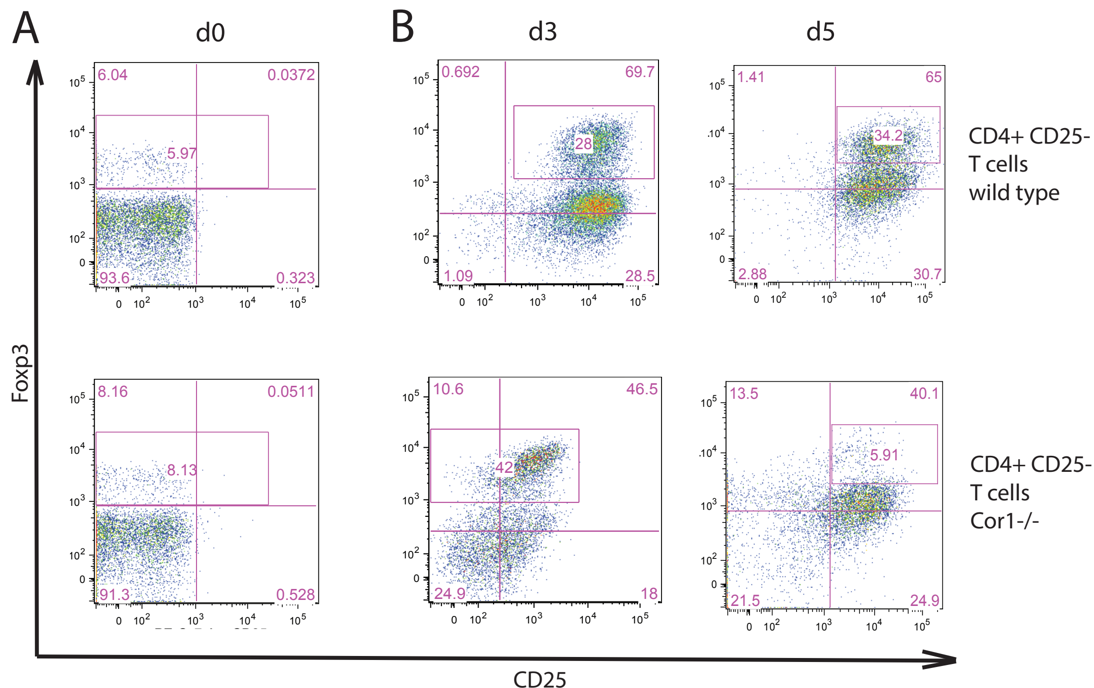


Figure 31. *In vitro generation of Tregs from CD25- CD4+ T cells.* *The experiment followed the procedure described before elsewhere (Fantini et al. 2007) and in Materials and Methods of this thesis. CD4+ T cells from wild type and coronin 1-deficient mice were negatively purified and depleted of CD25+ cells using proper antibodies and magnetic beads. The cell purity was tested before plating (A). The cells were cultured in presence of IL-2 and TGFβ and analyzed with the FACS at day 1, 2, 3, 4 and 5 (B). The figure shows representative results from one out of three repetitions. Cor1-/- - coronin 1-deficient.*

4.5.7 *In vivo* regulatory T cells numbers after skin transplantation

Separately, Treg frequencies were analyzed after skin transplantation *in vivo*. To this end we transplanted coronin 1-deficient and wild type mice with bm12Rag2-/- skin. Following transplantation, mice were analyzed for frequencies and numbers of immune cells at different time points (Fig. 32). Fig. 32A shows the rejection score for wild type and coronin 1-deficient transplanted mice and, as previously shown in this thesis, we observed fast rejection in BL/6 wild type mice and no rejection in coronin

1-deficient recipients. Flow cytometry analysis of immune cell composition showed that enhanced rate of Treg cells was present in coronin 1-deficient recipients compared with wild type mice. Numbers of Tregs in coronin 1-deficient were lower than in wild type, however they increased in a similar manner. Elevation of regulatory T cells percentages in coronin 1-deficient mice can be partially explained by low changes in numbers of other CD4+ and CD8+ T cells. Additionally, proliferation of B cells in lymph nodes of coronin 1-deficient recipients was similar to that in the wild type mock transplantations (BL/6→BL/6, Fig. 32I). This is consistent with the observation that the activation of B cells in coronin 1-deficient mice was not sufficient to cause rejection alone. Interestingly, the level of Tregs was similar after transplantation of bm12Rag2-/- onto coronin 1-deficient mice and mock surgeries (Cor1-/-→Cor1-/-, Fig. 32B and C), which indicates that these cells were not generated specifically in response to alloantigen.

In order to further study the increase of Tregs in coronin 1-deficient and wild type mice after transplantation, we performed a time course experiment with shorter time point for analysis. This time we examined only mock surgeries, as we found their Tregs rates similar to MHC class II mismatched transplantations. The results showed that Tregs percentage increase was higher in coronin 1-deficient than in the wild type (slope of 1.186 and 0.584 for coronin 1-deficient and wild type cells, respectively, Fig. 33A), the numbers however are not changing a lot (Fig. 33E). The increase in the Tregs numbers was greater for wild type recipients than coronin 1-deficient (slope of 26255 and 4104 for coronin 1-deficient and wild type cells, respectively). The rise of CD4+ and CD8+ cells was decreased in the coronin 1-deficient recipients (in both numbers as well as percentage, Fig. 33B, C, F and G). Interestingly, B cells growth (%) was similar between wild type and coronin 1-deficient mice, indicating that these cells are not impaired in coronin 1-deficient recipients upon mock transplantation (Fig. 33D and H).

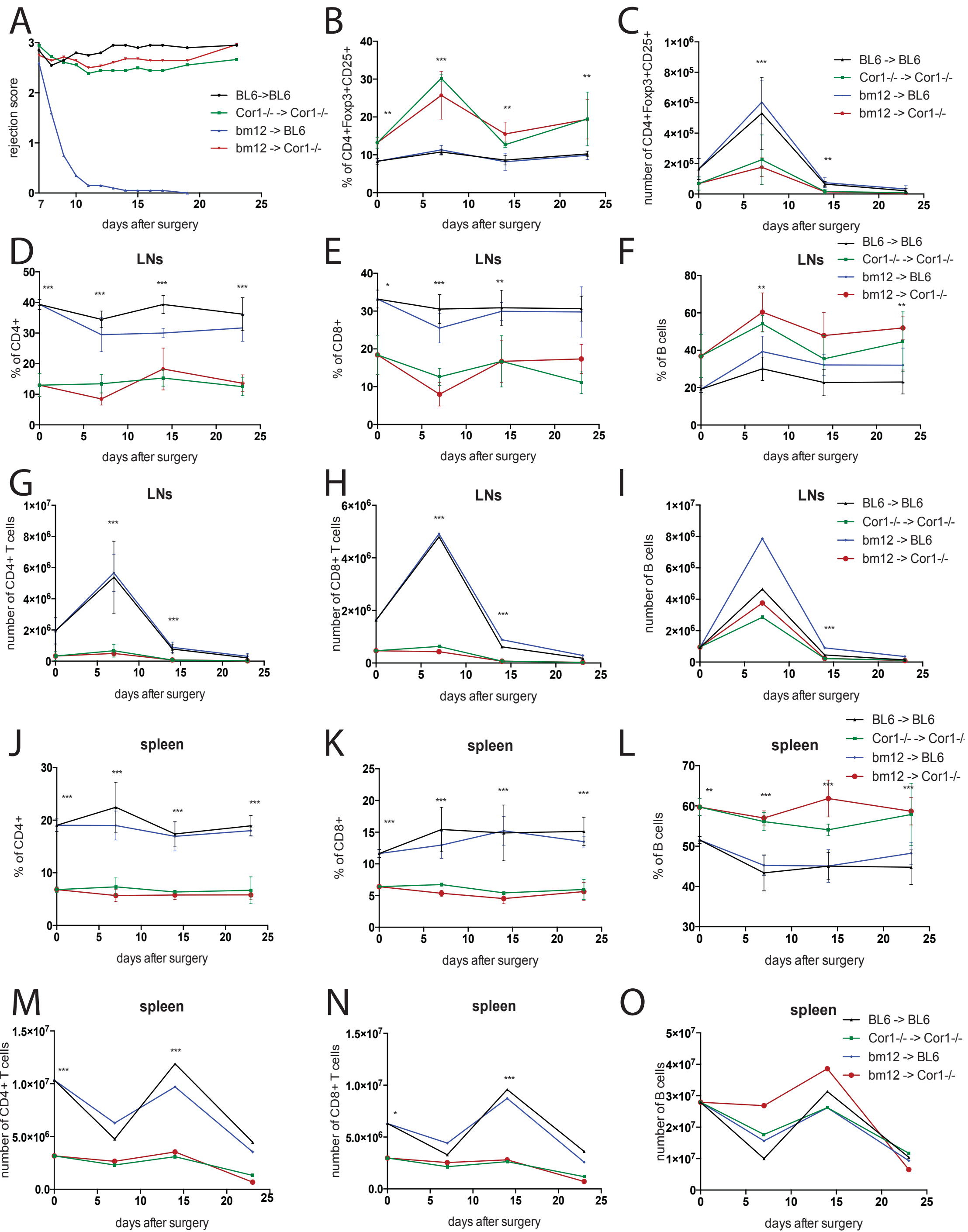


Figure 32. ***Regulatory T cells generation after skin transplantation in bm12Rag2-/- recipients.*** *Rejection score (A) and variability of cells (B – O) in spleen and lymph nodes of BL/6 and Cor1-/- mice after transplantation with bm12Rag2-/- skin and mock surgeries. BL/6 wild type and coronin 1-deficient mice were transplanted with bm12 Rag2-/- skin following the transplantation protocol (Chapter 6.4). Each group consisted of 10 mice for mock surgery and 15 mice for the mismatch transplantations. 7, 14 and 23 days later groups of 3 to 4 (mock surgery) and 5 (bm12Rag2-/-) mice*

were sacrifice and the quantities of Tregs (CD4+Foxp3+CD25+ T cells), CD4+ and CD8+ T cells, and B cells were characterized with antibody staining and flow cytometry analysis. B and C show percentage and number of Tregs in the lymph nodes, respectively. D, E and F cell rate of CD4+, CD8+ and B cells in lymph node. G, H and I number of CD4+, CD8+ and B cells in lymph nodes. J, K and L show percentage of CD4+, CD8+ and B cells in spleen. M, N and O - number of CD4+, CD8+ and B cells in spleen. Asterix always signify statistical difference between coronin 1-deficient and wild type transplant recipients. BL6 – BL/6 wild type; Cor1-/- - coronin 1-deficient; bm12 – bm12Rag2-/- mice.

Taken together the greater increase of Tregs percentages in coronin 1-deficient mice can be explained by changes in other cell types – in wild type recipients we can see an increase in both CD4+ and CD8+ T cells while these cell numbers are decreased in coronin 1-deficient mice. This further confirmed our previous finding, with *in vitro* Tregs induction, that coronin 1-deficient regulatory T cells were not induced at a higher level than the wild type in the periphery. Although, Treg numbers were lower in coronin 1-deficient recipients, their enhanced levels in comparison with conventional T cells could be responsible for the suppression of T cells in coronin 1-deficient recipients after allotransplantions.

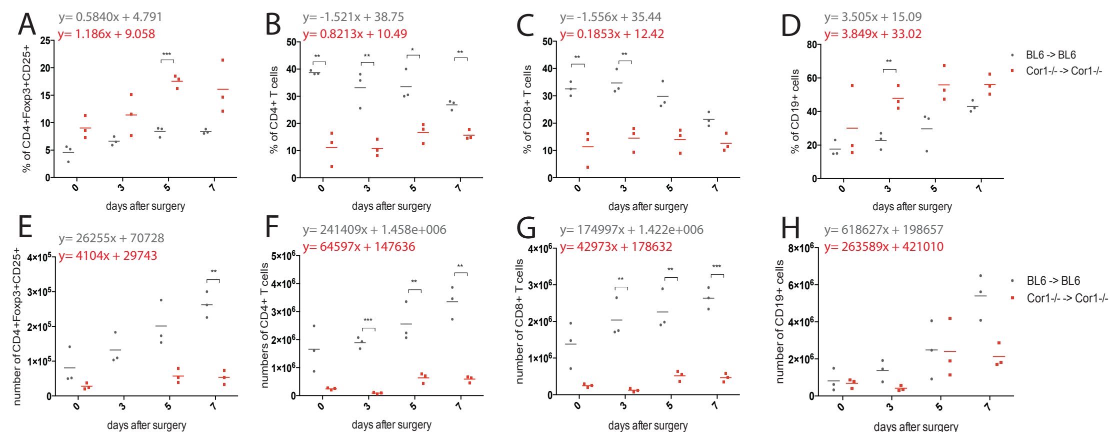


Figure 33. ***Mock skin transplantations (BL/6 wild type onto BL/6 wild type and Cor1-/- onto Cor1-/-) and cell quantities 3, 5 and 7 days after surgeries.*** *A – D shows percentage of regulatory T cells (CD4+Foxp3+CD25+), CD4+, CD8+ and B (CD19+) cells, respectively. E – H. Numbers of Tregs, CD4+, CD8+ and B cells after skin transplantation. Each graph shows linear regression lines for coronin 1-deficient and wild type cells. Mice were transplanted following the transplantation protocol (Chapter 6.4). 3 mice were sacrificed at each time point from both BL/6 wild type and coronin 1-deficient recipient group. Lymph nodes were harvested and the cells were analyzed using appropriate antibody, both following the protocols from Materials and Methods (Chapter 6.5 and 6.6). BL6 – BL/6 wild type; Cor1-/- - coronin 1-deficient.*

4.5.8 Analysis of the suppression capacity of coronin 1-deficient and wild type regulatory T cells *in vitro*

The coronin 1-deficient Treg cells suppressive abilities have been studied before and described not to differ from the wild type regulatory T cells (Siegmund et al. 2011). However, we decided to confirm this result to exclude their more efficient function as a reason of prolonged graft survival. To investigate that we performed *in vitro* suppression assay with wild type T cells as responders and either wild type or coronin 1-deficient Tregs as repressors. The cells were plated together in a different ratio, as described in Supplementary Fig. 10, and stimulated with Balb/c splenocytes (depleted of CD3+ cells) for 72h. The proliferation rate was measured by 3H-thymidine incorporation. The assay showed no difference in suppressive abilities between coronin 1-deficient and wild type Treg (Supplementary Fig. 10A). Therefore we concluded that coronin 1-deficient Tregs were not better in their suppression of wild type CD4+ CD25- T cell proliferation excluding this as a reason for prolonged graft tolerance in coronin 1-deficient recipients.

4.5.9 Proliferation of CD4+ T cells in the absence of CD4+CD25+ T cells

We then could confirm that *in vitro*, coronin 1-deficient CD25+ T cells were not responsible for the decreased proliferation of coronin 1-deficient T cells. To this end we used MLR experiments in which we cultured T cells either CD25-depleted or not (responder cells) with splenocytes from Balb/c mice (stimulators). The data shown in Supplementary Fig. 10B and C indicates that there was no significant difference in proliferation between total T cells and T cells depleted of CD25+ cells. These results further supported our hypothesis that coronin 1-deficient Tregs are not the reason for reduced proliferation of coronin 1-deficient T cells *in vitro*.

4.5.10 Analysis of suppressive abilities of conventional coronin 1-deficient CD4+ T cells

To analyze whether the suppressive activity could be conferred by coronin 1-deficient conventional CD4+ T cells, we decided to use cCD4+ T cells (CD4+CD25- T cells) as repressors in several assays *in vitro* as well as *in vivo*.

In vitro co-culture of coronin 1-deficient and wild type cCD4+ T cells

First, we carried out a co-culture of coronin 1-deficient with wild type cCD4+ T cells *in vitro* (Workman et al. 2011). Wild type Ly5.1 CD4+ and coronin 1-deficient Ly5.2 CD4+ T cells were negatively sorted with magnetic beads. Then, the cells were stained with anti-CD4 and anti-CD25 antibodies and FACS sorted for CD4+CD25- T cells. Afterwards, both isolated populations were plated either together or separately in 96-well plates. The cultures were stimulated or not with anti-CD3 and anti-CD28 antibodies. After 72 hours of culture the cells were analyzed for proliferation. When cultured without stimulation, coronin 1-deficient cCD4+ T cells were not able to suppress proliferation of wild type cCD4+ T cells. On the contrary, there was an enhancement in numbers of recovered wild type cCD4+ T cells when co-cultured with coronin 1-deficient cells upon stimulation with anti-CD3 and anti-CD28 antibody (Fig. 34A and B). Furthermore, we noticed improved survival of coronin 1-deficient cCD4+ when co-cultured with wild type cells without stimulation. Therefore we were not able to confirm the suppressive abilities of coronin 1-deficient cCD4+ T cells *in vitro*.

In vivo competition assay

To further characterize the potential suppressive abilities of coronin 1-deficient cCD4+ T cells we performed *in vivo* suppression assay where instead of regulatory T cells we used coronin 1-deficient cCD4+ T cells as repressors. In this experiment two types of cells, responders (in our case wild type Ly5.1 CD4+CD25- T cells) and repressors (coronin 1-deficient Ly5.2 CD4+CD25- T cells) were adoptively transferred together into immunodeficient Rag2-/- mice. Reduction of cell proliferation defined the suppressed population (Song et al. 2012). Both cell

populations were purified from murine spleens and enriched for CD4+ T cells with negative magnetic beads separation as described in Material and Methods (Chapter 6.7). Subsequently, the cells were stained with anti-CD4 and anti-CD25 antibodies and sorted for CD4+CD25- cells. We co-transferred 1 000 000 CD4+CD25- T cells purified from the wild type and 1 000 000 CD4+CD25- T from coronin 1-deficient mice into Rag2-/- recipients. The mice were sacrificed two weeks after injection and flow cytometry analysis of spleen and lymph nodes cell composition were performed. In contrast to the results obtained *in vitro* a significant decrease in recovered number of wild type cells was observed after co-transfer with coronin 1-deficient cCD4+ T cells, when compared with transfer of wild type cells alone (Fig. 34C). This reduced proliferation of wild type cells in the present of coronin 1-deficient CD4+CD25- T cells suggests a suppressive ability of coronin 1-deficient cells.

Graft survival in a transfer model with cCD4+ T cells

Regulatory T cells adoptively transferred into immunodeficient mouse with an allograft are able to prevent rejection mediated by conventional CD4+ T cells (Nagahama, Nishimura, and Sakaguchi 2007). Therefore, to test the hypothesis whether coronin 1-deficient CD4+CD25- T cells are able to suppress rejection of an allograft, we decided to perform *in vivo* suppression assays in transplanted Rag2-/- mice using coronin 1-deficient CD4+CD25- T cells instead of Tregs. To this end, Rag2-/- mice were transplanted with bm12Rag2-/- skin grafts. Subsequently, 30 days after transplantations, the mice were injected with CD4+CD25- T cells isolated from wild type and coronin 1-deficient mice (Fig. 34D). Mice receiving 20 000 of wild type CD4+CD25- T cells rejected grafts within 20 days. The group adoptively transferred with 20 000 of wild type and 20 000 of coronin 1-deficient CD4+CD25- T cells started to show signs of rejection earlier than wild type controls and at the end of experiment 2 out of 3 mice rejected skin completely. Interestingly, mice co-transferred with 20 000 of wild type and 100 000 of coronin 1-deficient CD4+CD25- T cells started to reject rapidly (day 12), and all mice rejected within 19 days after surgeries. What is more, group transferred with coronin 1-deficient 100 000 CD4+CD25- T cells also showed signs of rejection. At the end of the experiment one mouse rejected the graft completely, one was about to reject and one started the

process of rejection (showing some redness on grafted tissue). This shows that CD4+CD25- T cells from coronin 1-deficient mice were not able to suppress wild type cell but, in contrary, accelerated the rejection of bm12 grafts. Thus, from these experiments, we could not conclude that suppressive abilities of conventional CD4+ T cells contribute to the delayed rejection in coronin 1-deficient recipients.

Together these data show that while *in vitro* coronin 1-deficient cCD4+ T cells were not able to suppress wild type cell proliferation, *in vivo* the numbers of wild type cCD4+ T cells after co-transfer with coronin 1-deficient cells were decreased, suggesting suppressive activity. However, in a skin transplantation setting, coronin 1-deficient conventional CD4+ T cells did not alter the rejection course. Additionally, coronin 1-deficient CD4+ T cells depleted of regulatory T cells were able to induce rejection of bm12 grafts in Rag2-/- recipients.

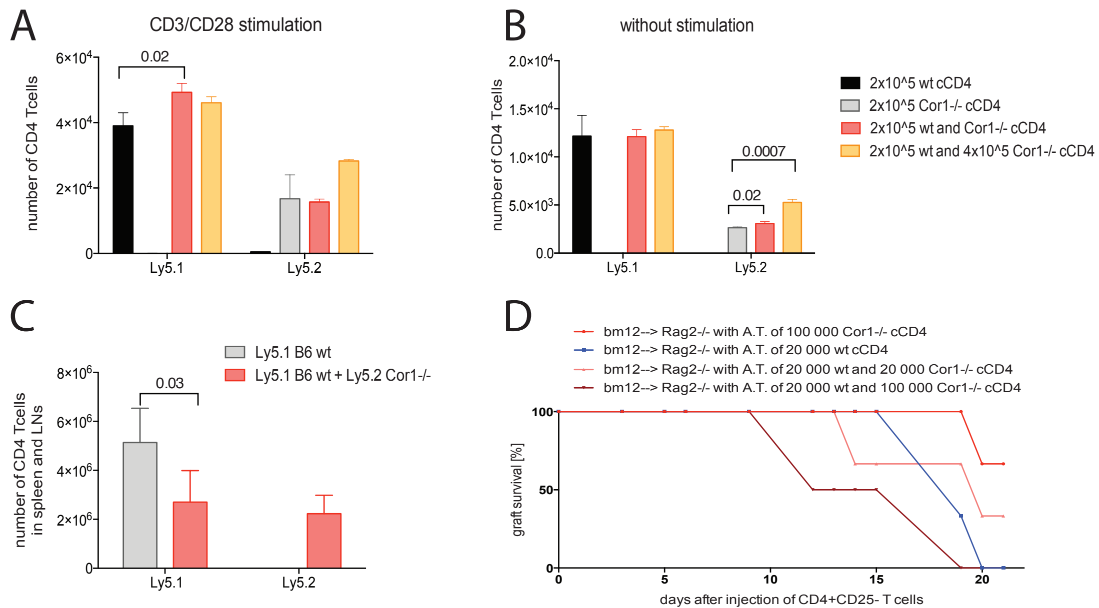


Figure 34. ***In vitro (A and B) and in vivo (C and D) competition assay of conventional CD4+ T cells (CD4+CD25-) from BL/6 wt and Cor1-/- mice.*** *A. and B. Co-culture of cCD4+ T cells from BL/6 wt (Ly5.1) and Cor1-/- (Ly5.2) mice in the absence (B) or presence (A) of CD3/CD28 antibody stimulation. The cells were sorted following the negative selection of CD4+ T cells and FACS sort for CD25- cells. They were plated in 96-well plate and incubated for 72h. Afterwards, the cells were stained and acquired with the FACS. The experiment was repeated 3 times. Figure shows representative results. C. In vivo competition assay where 1 000 000 of BL/6 wild (Ly5.1) type and Cor1-/- (Ly5.2) cells were injected together into tail vein of Rag2-/- mice. The cells were obtained from spleen after negative CD4+ T cells sort with the kit and additional FACS sort of CD4+CD25- cells. The BL/6 wt cells were either adoptively transferred alone or together with Cor1-/- cells. Mice (5 per group) were sacrifice two weeks later and the cells were quantified by FACS staining. The plot shows number of cells obtained from spleen and 6 lymph nodes of Rag2-/- mice (axillary, brachial and inguinal). The experiment was repeated 3 times. The plot shows representative result from one experiment. D. Suppression of BL/6 wt cCD4+ T cells (CD4+CD25-) by Cor1-/- cCD4+ T cells. Rag2-/- mice were transplanted with bm12Rag2-/- skin grafts following the transplantation protocol (Chapter 6.4). 28 days after surgery the mice were injected with 20 000 of BL/6 wt (Ly5.1), 100 000 Cor1-/- (Ly5.2) cCD4 T cells and co-transferred with 20 000 BL/6 wt and 20 000 Cor1-/- cCD4+ T cells or 20 000 BL/6 wt and 100 000 Cor1-/- cCD4+ T cells. The mice were observed for graft survival and sacrificed 21 days after cell transfer. Numbers of cCD4+ T cells were quantified by FACS staining of spleen and lymph nodes (axillary, brachial and inguinal) cells. Three mice were used per group. B6 wt– BL/6 wild type; Cor1-/- - coronin 1-deficient.*

5 Discussion

After recognition of an allogeneic skin graft by a recipient's immune system an organism induces several mechanisms by which the graft can be destroyed. The allograft can be rejected via nonspecifically activated inflammatory cells infiltrating the tissue, which leads to the vascular occlusion or by cytolityc soluble factors secreted by those activated inflammatory cells. The more specific mechanism responsible for graft destruction consist of antigen-specific responses conducted by antibodies and T cells. Antigen-specific T cells appear to be the major effector cells mediating skin graft rejection. T helper (T_H) cells activated by antigen recognition secrete cytokines that, when combined with antigen presentation, induce cytotoxic T (T_C) cells to proliferate and differentiate into effector cells that are able to reject grafted tissue. T cells are most efficient in rapid graft rejection when they combine both the helper and cytotoxic function (Rosenberg and Singer 1992; Boisgérault et al. 2009). Considering the severely reduced numbers of peripheral T cells in coronin 1-deficient mice, we would expect them to be highly susceptible to various infections but impaired in graft rejection (Föger et al. 2006; Mueller et al. 2008; Haraldsson et al. 2008; Shiow et al. 2008). Interestingly, while coronin 1-deficient mice are able of mounting an response against infection (Tchang et al. 2013) these mice fail to normally reject allografts.

It is known that both CD4+ and CD8+ T cells contribute to allograft rejection. The detailed interaction of T cells subpopulations during rejection has been investigated in several studies (Cobbold et al. 1986; Rosenberg et al. 1987; Auchincloss et al. 1993; Rosenberg and Singer 1992; Auchincloss et al. 1993; Boisgérault et al. 2009). All of these studies showed that CD4+ T cells are required in the rapid rejection of major mismatched skin grafts. Addtionally, CD4+ T cells are capable of elicitading acute rejection of a major mismatched transplants in the absence of CD8+ T cells (Boisgérault et al. 2009). Interestingly, when a minor mismatched skin is transplanted

onto recipients depleted of CD4+ T cells grafts are tolerated even longer (Auchincloss et al. 1993; Youssef et al. 2004, 4). As described in this thesis, when major mismatched skin was transplanted onto coronin 1-deficient mice, the graft rejection was delayed when compared with wild type recipients. Minor mismatched transplantations showed tolerance in coronin 1-recipients. Therefore we hypothesized that coronin 1-deficient CD4+ T cells are impaired in their function upon allograft stimulation.

To find out whether coronin 1-deficient CD4+ T cells are the main impaired population of T cells we performed surgeries with MHC class II (bm12) or MHC class I (bm1) allogeneic skin. Indeed, our results showed that coronin 1-deficient CD8+ T cells were able to induce rejection of bm1 grafts, however, with a delay. The delay could be due to the decreased numbers of coronin 1-deficient CD8+ T cells. Coronin 1-deficient mice, on the contrary, did not reject bm12 skin grafts. These results suggest that not only the CD4+ T cells were impaired in rejection but also there had to be a mechanism that induced tolerance in coronin 1-deficient recipients. Following these findings, in subsequent experiments, we focused on CD4+ T cell dependent rejection.

In our subsequent studies we considered three hypothesis that could possible explain the delayed rejection in coronin 1-deficient mice: it could be the result of decreased numbers of T cells in coronin 1-deficient recipients, there was the possibility that T cells posses an impaired capacity to recognize alloantigens, or coronin 1-deficient mice could be very efficient in induction and maintenance of tolerance.

5.1 Is the delayed rejection due to the lower number of T cells in the coronin 1- deficient mice?

We concluded that delayed rejection of major mismatched grafts could not be the outcome of decreased numbers of coronin 1-deficient T cells only. This was shown by the fact that adoptive transfer of highest numbers of coronin 1-deficient CD4+ T cells used in the Rag2-/- recipients did not lead to rejection. Even despite the impaired

survival of coronin 1-deficient CD4+ T cells after injection into Rag2-/- mice as we transferred high number of these cells (1 000 000 of Cor1-/- vs 20 000 of wt cells). Thus, the reduced T cell numbers per se do not explain the absence of graft rejection in coronin 1-deficient mice.

5.2 Are coronin 1-deficient T cells impaired in their functionality?

Although the retained lower counts of CD4+ T cells after major mismatched skin transplantation could indicate a defect in proliferation or alloantigen recognition, further in vitro experiments suggest that coronin 1-deficient T cells were able to recognize antigen through their TCR's and respond, could become activated and could proliferate. Furthermore, cytokines and cell marker analysis suggest that coronin 1-deficient T cells were under the stress of high activation and intense proliferation that could cause exhaustion, which may contribute to their in vivo depletion. The same markers were not changed in the thymus of coronin 1-deficient mice and the mice could produce recent thymic emigrants. Since TCR-mediated signaling is crucial for thymocyte development this suggest that the inability to reject allografts by coronin 1-deficient T cells is not due to an impaired capacity to respond towards TCR ligands.

Activation of coronin 1-deficient T cells

From previous studies we know that coronin 1-deficient mice can respond to various viral and bacterial infections thus our preliminary results suggest that coronin 1-deficient mice might have a problem specifically with allorecognition by T cells or their efficient activation after transplantation (Tchang et al. 2013). Alloantigens can be recognized by T cells either as an intact MHC molecule presented by donor APC (direct presentation), or after processing of those that produces allopeptides presented by recipients MHC complexes expressed by recipient APC (indirect presentation) (Guermonprez et al. 2002). We tested both of this recognition using major and minor mismatched skin donors. Interestingly, as described before, coronin 1-deficient mice were able to reject major mismatched (although with significant delay) but fully

tolerated minor mismatched grafts. Moreover, in vitro, when stimulated with major mismatched splenocytes, coronin 1-deficient T cells became activated and proliferated. Therefore the allorecognition by coronin 1-deficient T cells could not be impaired and could not be the reason of delayed rejection in coronin 1-deficient mice.

Survival and proliferation of coronin 1-deficient T cells

Although in vivo, the survival of coronin 1-deficient T cells is decreased compared with the wild type, interestingly, both the proliferation rate and cell survival of coronin 1-deficient CD4+ T cells in mixed lymphocyte reaction (MLR) experiments turned out to be dependent on the starting number of cultured cells. When we plated 100 000 of T cells, the number of proliferated CD4+ T cells was significantly lower in the coronin 1-deficient sample as compared to the wild type sample. However, plating 200 000 of T cells resulted in similar proliferation of CD4+ T cells in the wild type and coronin 1-deficient sample. Since the cell density is very important for the in vitro activation and expansion of T cells (Ma et al. 2010; Pilling et al. 2000), these results indicate that coronin 1-deficient T cells are even more sensitive to cell density than wild type T cells. In vitro activated T cells secrete abundant cytokines and growth factors, which improves their proliferation and survival (Ma et al. 2010). One possible explanation therefore is that coronin 1-deficient T cells are impaired in secretion of such a factor and therefore require a higher density for their proper survival. Consistent with these in vitro observations, the survival of coronin 1-deficient CD4+ T cells in vivo, after adoptive transfer into Rag2-/- mice, was also found to be dependent on the numbers of injected cells. Transfer of small numbers of mature T cells into immunodeficient mice, such as Rag2-/-, results in rapid T cell proliferation in the periphery (Clarke and Rudensky 2000). While is known that increasing numbers of wild type T cells injected into lymphopenic mice does not increase the number of surviving cells, it improved survival for the coronin 1-deficient T cells. Normally, adoptively transferred T cells have their capacity to proliferate and expand to a certain population size. The number of injected cells determines the time needed for the T cells to reach their growth plateau (Rocha, Dautigny, and Pereira 1989). In our study, population of coronin 1-deficient CD4+ T cells that were adoptively transferred into Rag2-/- mice could never reach the size of wild type sample, however

the number of these cells that we could recover depended on the number of injected cells. Together these results suggest that both in vitro and in vivo coronin-1 deficient T cells survive better upon being seeded at higher density.

Cell markers expression and cytokines secretion

A characterization of specific markers and cytokines expressed by wild type and coronin 1-deficient T cells suggested that coronin 1-deficient T cells showed a stimulated (IFNγ^{hi} and IL-7R^{lo}) and strongly activated (CD5^{hi}) phenotype undergoing intense proliferation (Ki67^{hi}) and being exhausted (PD-1^{hi}). Interestingly the co-culture of coronin 1-deficient and wild type T cells reduced the growth of wild type cells. As the CD8+ T cells from wild type and coronin 1-deficient mice co-cultured together did not influence each other's proliferation this suggests a suppressive function of coronin 1-deficient CD4+ T cells.

Analysis of coronin 1-deficient and wild type T cells showed that coronin 1-deficient T cells, both CD4+ and CD8+ T cells, produced increased levels of IFNγ, when compared to wild type cells. Enhanced levels of IFNγ together with intense or defective TCR signaling might lead to the activation of coronin 1-deficient T cells and cause the downregulation of the IL-7R receptor (which we observed in our studies) as well as increase proliferation of those cells (indicated by upregulation of Ki-67). All of these factors, mainly endogenous IFNγ, promote activation-induced cell death (AICD) (Thebault et al. 2007; Sobek et al. 2002). Interestingly, AICD is linked with antigenic engagement of TCR, which, in our studies, was correlated with enhanced expression of CD5 in coronin 1-deficient compared with wild type cells. However, higher expression of CD5 in coronin 1-deficient T cells can be related with their stage of maturation (coronin 1-deficent T cells are CD44^{hi} and CD62L^{lo}), as CD5^{hi} expression is typical in mature T cells (Foulds et al. 2002).

High activation of coronin 1-deficient T cells can lead to their exhaustion, which is related to enhanced expression of PD-1 by coronin 1-deficient T cells when compared to wild type cells (Francisco, Sage, and Sharpe 2010). Increased expression of PD-1 can facilitate apoptosis of T cells and induction of tolerance. PD-1 ligands are expressed on APCs and peripheral tissues. Binding of PD-1 to its ligands eliminates

self-reactive T cells via promoting AICD thereby protecting organism against autoimmunity and self-destruction (Francisco, Sage, and Sharpe 2010).

What is more, AICD leads to the cell death through Fas/FasL pathway. FasL was another marker which increased expression was found on the coronin 1-deficient T cells. Expression of FasL has been reported to be dependent on nuclear factor of activated T cells (NFAT) which function is disrupted in coronin 1-deficient mice (Winslow et al. 2006; Oh-hora and Rao 2008; J. H. Russell 1995; Zhang, Xu, and Liu 2004).

Taken together, coronin 1-deficient T cells express high levels of cell death markers (Ki-67, PD-1, CD5 and FasL) while downregulating IL-7R (which is also a sign of AICD (Kimura et al. 2013; Mai et al. 2014)), which could suggest that they undergo activation-induced cell death resulting in decreased numbers in coronin 1-deficient mice. These results are supported by the findings that coronin 1-deficient T cells express more caspase 3, which is involved in apoptosis (Mueller, Liu, and Pieters 2011; Tewari et al. 1995; Nicholson et al. 1995). On the other hand, coronin 1-deficient T cells could have impaired TCR self-signals causing their malfunction, which results in a stage where they are about to die and express multiple markers involved in cell death and survival.

Interestingly, several markers involved in the apoptosis of activated T cells promote survival of regulatory T cells. Expression of PD-1 and its ligation with PD-L1 or PD-L2 promotes survival of Tregs (Francisco, Sage, and Sharpe 2010). PD-1 ligands are expressed on tolerogenic dendritic cells, controlling the decision between T cell activation or tolerance by induction of regulatory T cells and control of their development, maintenance and function (Francisco, Sage, and Sharpe 2010). Moreover blockade of IL-7R and enhanced IFNγ secretion has been described to be important for the survival of Tregs (Thebault et al. 2007; Mai et al. 2014). Therefore, this could explain increased frequencies of regulatory T cells in coronin 1-deficient mice compared with wild type. After skin transplantation coronin 1-deficient T cells travel to the transplantation tissue, which expressed PD-1 ligands. Highly expressed PD-1 on coronin 1-deficient T cells could bind to its ligand causing apoptosis of

conventional T cells and promoting survival and proliferation of coronin 1 regulatory T cells thereby inducing graft tolerance.

5.3 Is the prolonged acceptance of the graft due to induction of tolerance in coronin 1-deficient recipients?

In-depth analysis of T cells subtypes in the periphery and thymus of coronin 1-deficient mice showed decreased numbers of T cells in the periphery, with RTE present also at lower numbers, enhanced rates of CD4+CD25+ T cells and normal composition of thymus. Normal development of T cells in the thymus and reduced numbers of RTE suggest their immediate death after egress or during maturation into naïve then activated/memory T cells. Both of these T cell subsets might have impaired survival themselves.

Further analysis of regulatory T cells in coronin 1-deficient mice showed no difference in suppressive abilities compared with wild type cells. Moreover, we demonstrated that the cells did not generate or proliferate better in coronin 1-deficient mice. Nevertheless, their superior survival over conventional CD4+ T cells in vitro could explain their higher frequencies in coronin 1-deficient mice and could be linked to the enhanced expression of PD-1. This, in turn, promotes apoptosis in antigen-specific T cells while increasing the survival of Tregs (Francisco, Sage, and Sharpe 2010). Therefore, the absence or delay in rejection in coronin 1-deficient recipients caused by induction of tolerance may be due to elevated rate of Tregs, the suppressive abilities of conventional CD4+ (cCD4+) T cells or combination of these. Indeed, there are studies describing that the balance between effectors and regulatory T lymphocytes controls the outcome of allografts (Benghiat et al. 2005; Graca et al. 2004; Tang and Lee 2012). Interestingly, when we tried to interfere the ratio between Tregs and cCD4+ T cells in coronin 1-deficient mice by adoptive transfer of either wild type CD4+ T cells or ABM cells, we did not observe rapid rejection in recipient mice. Although, some of the injected mice rejected skin grafts, the rejection was delayed when compared with controls and additionally, we observed acceptance of

the graft in most cases. Therefore we suspected that cCD4+ T cells in coronin 1-deficient mice might have suppressive abilities and support tolerance maintenance after transplantation. Moreover, preliminary results suggest increased levels of cAMP in coronin 1-deficient CD4+ T cells (unpublished). This secondary messenger, cAMP, is also increased in regulatory T cells and is described to play a role in suppression by these cells (Bopp et al. 2007).

Furthermore, CD25-depleted coronin 1-deficient recipients rejected skin grafts with the delay compared with the control, which could indicate suppression within coronin 1-deficient cCD4+ T cell subset as well. Although, there are studies showing that depletion of CD25+ cells result in rapid rejection (W. Li et al. 2006; Mai et al. 2014), there are others describing that under certain conditions the influence of CD25-depletion on suppression of graft rejection may be limited (Graca et al. 2002, 4; Benghiat et al. 2005). The same paper discusses the suppressive abilities of CD4+CD25- cells in graft rejection suggesting that higher numbers of cCD4+ T cells are able to suppress the rejection of skin grafts as well as CD4+CD25+ cells (Benghiat et al. 2005).

To find out whether coronin 1-deficient cCD4+ T cells are able to suppress the rejection we performed several in vitro and in vivo assays. While in vivo after adoptive transfer into Rag2-/- mice, coronin 1-deficient cCD4+ T cells caused the decreased survival of wild type cells, the in vitro co-culture did not show any influence of coronin 1-deficient cCD4+ T cells on the proliferation of wild type cells. Additionally, when we co-transferred coronin 1-deficient and wild type cCD4+ T cells into Rag2-/- mice, previously transplanted with bm12 skin grafts, we observed rapid rejection of grafts. At the same time, control mice, transferred only with coronin 1-deficient cCD4+ T cells, also rejected bm12 grafts. This suggests that not only coronin 1-deficient cCD4+ T cells are not able to suppress the rejection by wild type cells, but also they are able to induce rejection by themselves in the absence of regulatory T cells. However, the results obtained in these studies were contradictory and difficult to interpret.

5.4 Conclusions

The exact role of coronin 1 protein in promoting T cells homeostasis as well as the role of the coronin 1-dependent T cells in allorecognition and graft rejection remains largely unknown. This project provides novel insight into the role of coronin 1 in the induction of prolonged graft survival. Despite advances in immunosuppressive therapies, current clinical skin transplantation is still limited to autotransplantation. Thus, there is a need for new methods to induce immune tolerance to allografts. The knowledge of the role of coronin 1 in the regulation of tolerance might deliver new therapeutic approaches in the prevention of allograft rejection in humans. As stated earlier, one way to prevent allograft rejection is the use of immunosuppressants. However, currently used immunosuppressive drugs, such as cyclosporine A and FK506, target a pathway (Ca^2+/calcineurin) that operates in every cell type, and hence these drugs are associated with severe toxicity and risks towards opportunistic infections (Crabtree and Olson 2002; A. Rao, Luo, and Hogan 1997). Thanks to the restricted expression of coronin 1, deletion or suppression of this protein might not lead to toxicity.

In this thesis we demonstrated that, coronin 1-deficient mice showed delayed rejection of major mismatched skin grafts compared with wild type recipients. There are several explanations for the observed prolonged graft tolerance. First, it could be that the decreased numbers of T cells in coronin 1-deficient mice are not able to induce rejection of skin graft. However, since adoptive transfer of large numbers of coronin 1-deficient T cells, failed to induce rejection, this is less likely to be the main reason for observed tolerance. Second, there is the possibility that T cells posses an impaired capacity to recognize alloantigens and therefore fail to induce rejection. The proliferation, activation and survival of coronin 1-deficient T cells were investigated in this thesis and were shown to depend on the density of coronin 1-deficient T cells. However, these also appeared to be implausible to be the main reason for the delayed graft rejection since we observed tolerance in minor mismatched and an MHC class II-dependent model. Moreover the delayed graft rejection and tolerance in some models could be explained by impaired homing of dendritic cells into secondary lymphoid organs. That would prevent antigen presentation to T cells and their

efficient activation (Rosenberg and Singer 1992). Since the interaction of dendritic cells with T cells has been studied before we excluded this hypothesis in our studies (Westritschnig et al. 2013). Lastly, we hypothesized that the main reason of the prolonged graft survival in coronin 1-deficient mice was the tolerance induction. As we found increased frequencies of regulatory T cells in coronin 1-deficient mice this could contribute to suppression of graft rejection. Additionally, this was shown by the fact that adoptively transferred coronin 1-deficient CD4+ T cells depleted of CD25+ cells were able to induce graft rejection. Furthermore, preliminary results showed that coronin 1-deficient mice, when depleted of CD25+ cells, rejected skin graft, although with significant delay due to the impact of decreased T cells in general

Taken together, our results demonstrated that the delay of graft rejection in coronin 1-deficient mice may be a result of several factors combined together: the tolerance induction by increased Tregs numbers, the reduced numbers of T cells in general as well as a consequence of impaired T cells survival and proliferation.

6 Materials and Methods

6.1 Buffers and media

- **Ammonium-Chloride-Potassium (ACK) buffer**: 155mM NH_4Cl, 10mM $KHCO_2$, 1mM EDTA, pH 7.4
- **Complete media for dendritic cells**: RPMI 1640 (Sigma-Aldrich, Ref. No R8758) media containing 10% FCS (PAA clone, The Cell Culture Company, Ref. No A15-101) heat inactivated, 2mM L-glutamine, 50µM 2-mercaptoethanol (Sigma-Aldrich, Ref. No M7522) and 10ng/ml GM-CSF (Recombinant Mouse GM-CSF CF, Biolegend, Ref. No 576306)
- **Complete media for T cells**: RPMI 1640 (Sigma-Aldrich, Ref. No R8758) supplemented with 10% FSC (PAA clone, The Cell Culture Company, Ref. No A15-101), 500µg/ml Pen-Strep (Gibco, Ref. No 15140-122), 1% NEAA (Non-essential Amino Acid Solution, Sigma-Aldrich, Ref. No M7145), 10µM β-mercaptoethanol (Sigma-Aldrich, Ref. No M7522), 1µM Na-Py (Sodium Pyruvate, Sigma-Aldrich, Ref. No S8636)
- **Culture media for hybridoma cells**: SF-IMDM, 2% FCS low IgG, Pen-Strep
- **FACS buffer**: PBS, 2%FCS, 5mM EDTA
- **FACS Lysing Solution** (BD Biosciences, Ref. No 349202)
- **Foxp3 Permeabiliation/Permeabilization buffer** (FOXP3 Fix/Perm Buffer, BioLegend, Ref. No 421401)
- **Foxp3 Permeabiliation buffer** (FOXP3 Perm Buffer, BioLegend, Ref. No 421402)
- **PBS**: 10mM NaCl, 50mM HNa_2PO_4 $12H_20$, pH 8
- **RPMI 1640 without phenol red** (Life Technologies, Ref. No 11835-063)

6.2 Antibody list

Name	Antigen	Specificity	Clone	Isotype	Company	Ref. No	Dilution/ concentration
CD3 LEAF	CD3ε chain	mouse	145-2C11	Armenian Hamster IgG, κ	BioLegend	100314	10µg/ml
CD3-biotin						100304	50µg/ml
CD3-PE						100308	1:200
CD3-PE/Cy7						100320	1:100
CD3-Pacific Blue						100334	1:50
CD3-Alexa Fluor 700						100216	1:100
CD4-Brillinat Violet 510	CD4 (L3T4)	mouse	RM 4-5	Rat IgG2a, κ	BioLegend	100559	1:50
CD4-Pacific Blue						100531	1:50
CD4-PE/Cy7						100528	1:100
CD4-PE						100512	1:100
CD4-PerCP						100538	1:100
CD4-APC						100516	1:100
CD8-APC	CD8a (Ly-2)	mouse	53-6.7	Rat IgG2a, κ	BioLegend	100712	1:100
CD8-Pacific Blue						100725	1:50
CD8-Brilliant Violet 510						100751	1:50
CD8-Alexa Fluor 700						100730	1:100
CD8-PE						100708	1:100
CD11c-Alexa Fluor 647	CD11c	mouse	N418	Armenian Hamster IgG1, λ2	BioLegend	117312	1:100
CD19-APC	CD19	mouse	6D5	Rat IgG2a, κ	BioLegend	115512	1:100
CD19-PE/Cy7	CD19	mouse	6D5	Rat IgG2a, κ	BioLegend	115520	1:100
CD24-Brilliant Violet 421	CD24	mouse	M1/69	Rat IgG2b, κ	BioLegend	101826	1:100
CD25-biotin	CD25 (IL-2Rα chain p55)	mouse	PC61	Rat IgG1, λ	BioLegend	102004	50µg/ml
CD25-PE/Cy7						102016	1:100
CD28 LEAF	LEAF purified anti-CD28	mouse	37.51	IgG	BioLegend	102112	2µg/ml
CD44-APC	CD44 (Pgp-1, Ly-24)	mouse/ human	IM7	Rat IgG2b, κ	BioLegend	103012	1:100
CD44-Brilliand Violet 421						103039	1:50

CD44-PE/Cy						103030	1:100
CD45.1-Brilliand Violet 421	CD45.1 (Ly5.1)	mouse	A20	Mouse IgG2a, κ	BioLegend	110732	1:100
CD45.1-Alexa Fluor 700						110724	1:200
CD45.2-Brilliand Violet 510	CD45.2 (Ly5.2)	mouse	104	Mouse, IgG2a, κ	BioLegend	109837	1:100
CD62L-PerCP	CD62L (L-selectin)	mouse	MEL14	Rat IgG2a, κ	BioLegend	104430	1:200
CD62L-PerCp/Cy5.5						104432	1:200
CD69-PE/Cy7	CD69 (Very Early Activation Antigen)	mouse	H1.2F3	Armenian Hamster IgG1, λ3	BioLegend	104512	1:100
CD107a-Brilliand Violet 421	LAMP-1	mouse	1D4B	Rat, IgG2a, κ	BioLegend	121617	1:50
CD127-PE	IL-7Ra	mouse	A7R34	Rat, IgG2a, κ	BioLegend	135010	1:100
CD178-PE	FasL	mouse	MFL3	Armenian Hamster, IgG	BioLegend	106606	1:100
CD279-PE/Cy7	PD-1	mouse	RMP1-30	Rat, IgG2b, κ	BioLegend	109110	1:100
Gr1-PerCP/Cy5.5	Ly-6G and Ly-6C	mouse	RB6-6C6	Rat IgG2b, κ	BioLegend	108428	1:100
H-2D^b-APC	B6 MHC class I	mouse	KH95	Mouse, IgG2b, κ	BioLegend	111513	1:25
H-2D^d-PE	Balb/c MHC I	mouse	34-2-12	Mouse, IgG2a, κ	BioLegend	110608	1:50
H-2K^b-PerCp/Cy5.5	B6 MHC class I	mouse	AF6-88.5	Rat, IgG2a, κ	BioLegend	116515	1:25
H-2K^d-Pacific Blue	Balb/c MHC I	mouse	SF1-1.1	Mouse, IgG2a, κ	BioLegend	116615	1:25
I-A^b-PE/Cy7	B6 MHC class II	mouse	AF6 120.1	Mouse, IgG2a, κ	BioLegend	116420	1:50
I-A^d-FITC	Balb/c MHC II	mouse	39-10-8	Mouse, IgG3, κ	BioLegend	115005	1:50
IL-2-Alexa Fluor 647	IL-2	mouse	JES6-5H4	Rat, IgG2b, κ	BioLegend	503814	1:100
IFN-γ-PE	IFN-γ	mouse	XMG1.2	Rat, IgG1, κ	BioLegend	505808	1:100
NK1.1-Alexa Fluor 647	NK1.1	mouse	PK136	Mouse IgG2a, κ	BioLegend	108720	1:100
TNF-α-PerCP/Cy5.5	TNF-α	mouse	MP6-XT22	Rat, IgG1, κ	BioLegend	506322	1:100
TER-119-Alexa Fluor 488	TER-119/ erythrocytes	Mouse	TER-119	Rat, IgG2b, κ	BioLegend	116215	1:100

TER119-biotin	TER-119/ erythrocytes	mouse	TER-119	Rat, IgG2b, κ	BioLegend	116204	50µg/ml
Qa-2-Alexa Fluor 647	H2-Qa2	mouse	695H1-9-9	Mouse, IgG2a, κ	BioLegend	121708	1:100
TCR Vα2-APC	Vα2 T cell receptor	mouse	B20.1	Rat IgG2a, λ	BioLegend	127810	1:100
TCR Vα2-PE	Vα2 T cell receptor	mouse	B20.1	Rat IgG2a, λ	BioLegend	127808	1:100
TCR Vβ5.1/2-APC	Vβ5.1/2 T cell receptor	mouse	MR9-4	Mouse, IgG1, κ	BioLegend	139506	1:100
TCR Vβ8.1/2	TCR Vβ8.1/2 T cell receptor	mouse	KJ16-133.18	Rat IgG2a, κ	BioLegend	118408	1:100
Live/dead APC/Cy7	reaction with free cellular amines	-	-	-	Life technologies	L10119	1µl of 20µl dilution/stain

Table 4. ***Antibody list.***

6.3 Mouse lines

C57BL/6 mice and the previously generated transgenic coronin-1 mice (Jayachandran et al. 2007) were bred in-house and were kept under specific pathogen free (SPF) conditions. Bm12Rag2-/- (I-A^{bm12}), ABMRAG2-/- (TCR transgenic for I-A^{bm12}) and Rag2-/- mice were a gift from Dr. Simona Rossi Girard and were farther bred in-house under SPF conditions (Lee, Hansen, and Cullen 1982).

Ly5.1 mice were obtained from SwIMMR (*Swiss* Immunological Mutant *Mouse Repository*) and farther bred in-house. Bm1 (H-2^{bm1}) were a gift from Prof. Antonius Rolink (Schulze et al. 1983). Balb/c mice were bought from Janvier Labs. 6.5% Balb/c in the C57BL/6 (Cor1 wt or Cor1-/-) were bred in-house by crossing Balb/c mice with C57BL/6 by a two times backcrossing of a F1 (BL/6xBalb/C) mouse to the BL/6 background. The mice were tested for SNP (Taconic tests) and according to 1449 SNP analysis data sample had 96.90% of C57BL/6 genome and 3% of Balb/c SNPs. All animals' experiments were conducted according to the regulations of the Cantonal Veterinary Office (Basel, Switzerland). 6-12 old mice were used for experiments, if not indicated otherwise.

Experimental set up skin transplantation

Major mismatch model: Balb/C skin is transplanted onto BL/6 wild type or coronin 1-deficient (Cor1-/-) recipients.

Minor mismatch model: Skin from a BL/6 mouse, which has been generated by two-time backcrossing of a F1 (BL/6xBalb/C) mouse to the BL/6 background (6.25% Balb/c in BL/6), is transplanted onto BL/6 wild type and Cor1-/- recipients.

B6.C.H-2-bm12 model: this mice express a mutated MHC-II molecule (I-Ab) which has been shown to induce graft rejection by bm12-specific CD4+ T cells if transplanted onto wild type BL/6 mice (McKenzie et al. 1979).

Rag2-/- model: Rag2-/- mice are lacking the recombinase-2 gene needed for the generation of B and T cells (Shinkai et al. 1992); transplanted with B6.C.H-2-bm12Rag2-/- (bm12Rag2-/-) skin, the mice tolerate skin, however after transfer of BL/6 wild type CD4+ T cells they induce graft rejection and become major mismatch model (Schmaler, Broggi, and Rossi 2014).

6.4 Transplantation procedure and graft scoring

Skin transplantation procedure followed the protocol by Dr. Simona Rossi Girard described in paper: Transplantation of tail skin to study allogeneic CD4+ T cell responses in mice (Schmaler M, Broggi MA, Rossi SW.) In general: the donor mouse was sacrificed using CO_2. The tail was cleaned with tissue soaped with Betadine (Mundipharma, swissmedic No 34282021) and the skin was ripped off using scalpel and tweezers. The skin was placed in the Petri dish and cleaned with sterile cold PBS. The tail skin was cut into 1cm pieces. Corners of each piece were cut off to make the skin graft more round. Skin graft prepared this way was ready for transplantation. Recipient mouse was injected with Temgesic (50µg/kg of mouse diluted in sterile PBS, ESSEZ Chemie AG, Swissmedic No 41931035) at least 30min before surgery. Then the mouse was anesthetized with Isoflurane (Attane, Swissmedic No 56761002)). Sleeping mouse was placed on the surgery (with a heating pad) table with a continuous access to the Isoflurane containing anesthetic tube. The back of the

mouse was shaved, the hair was removed and the skin was cleaned with Betadine. A piece of skin was cut off with a round-ended scissors. The previously prepared donor skin graft was placed in the cut without overlapping the edges of the skin. The skin graft was glued using tissue adhesive Histo-Acryl (BBRAUN, Ref. No 1050052) glue. Then the graft was bandaged with Pflaster (Hansaplast Finger Strips, Swissmedic No 02321607) covered with Vaseline (Vifor SA, Swissmedic No 01667290) and Leukotype (2cmx10m,). The mouse was marked with the ear punch. Each mouse was kept under red heating lamp until it woke up after surgery. For one week after surgery, transplanted mice were given water with Defalgan syrup (3mg/ml in H_20, UPSA, Swissmedic No 01340235). The bandage was removed 7 days after surgery and the graft score was assess from that point every day for first month, then every second day and later twice a week.

6.4.1 Rejection score

The graft health and survival was scored with the mark scaling from 0 to 3, where 0 means the rejection of skin graft (no remaining graft on recipient mouse) and 3 means healthy and accepted skin graft (with the black stripe for B6 donor) with growing hair.

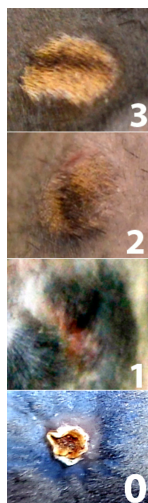


Figure 35. ***Scoring for the health and rejection of skin grafts after the transplantation.*** *Examples of scores given to BL/6 wild type or coronin 1-deficient mice transplanted with bm12Rag2-/- skin grafts. The mice were transplanted following the protocol then observed for the signs of rejection at least for 100 days. Score 3 – healthy skin graft with black stipe and growing hair. Score 2 – graft shrank significantly and shows red dot (wound). Score 1 – whole grafted skin is cover with wound (the rejection process ongoing). Score 0 – the graft is rejected and only the scrub is left that soon fells out leaving a scar.*

6.5 Organ harvesting and processing for analysis

The mice were euthanized with CO_2. Spleen, thymus and LNs were harvested in an ice cold PBS, smashed through a grid of stainless steel. Cell debris was removed by

quick spin (500rpm, 10s, 4^0C). The supernatant was transferred to new tubes. Afterward, cells were centrifuged for 10min, 350g at 4^0C. The supernatant was removed. Spleen cells (pallet) were treated with Ammonium-Chloride-Potassium (ACK) buffer (155mM NH_4Cl, 10mM $KHCO_2$, 1mM EDTA, pH 7.4), for 1min at room temperature, to remove red blood cells. Then, 10ml of PBS was added and cells were centrifuged for 10min, 350g at 4^0C. Cell pallets from spleen, thymus and LN were resuspended in PBS. Cells were counted using Neubauer chamber and dead cells were excluded using trypan blue. Cells prepared that way were taken for a FACS staining analysis.

6.6 FACS staining of cells

Cells were resuspended at the concentration of 20x10^6 cells/ml and 100µl/well was distributed into 96-well plate. The cells were washed by centrifugation for 5min, 350g at 4^0C. The cells were stained in 50µl/well FACS buffer (PBS, 2%FCS, 5mM EDTA) containing proper antibodies for 30min at 4^0C in the dark.

- For surface markers staining: the cells were washed with 150µl/well of FACS buffer and resuspended in 200ul of FACS buffer. The fluorescence was measured using BD LSR Fortessa or CantoII FACS Analyzer.
- For intracellular staining: the cells were washed with 150µl/well of FACS buffer and incubated in 100µl/well Foxp3 Fixation/Permeabilization buffer (FOXP3 Fix/Perm Buffer, BioLegend, Ref. No 421401) for 20min, room temperature in the dark. Afterwards, the cells were washed twice: with 150µl/well FACS buffer and 150µl/well Foxp3 Permeabiliation buffer (FOXP3 Perm Buffer, BioLegend, Ref. No 421402). The cells were incubated in Foxp Permeabilization buffer for 15min, room temp. in the dark. Then, the cells were centrifuged (5min, 350g at 4^0C), incubated in 50µl/well Foxp3 Permeabilzation buffer containing Foxp3-APC or appropriate isotype control antibody for 30min at room temp. in the dark. After incubation cells were washed twice with the same buffer and resuspended in 200ul of FACS buffer. The fluorescence was measured with BD LSR Fortessa or CantoII FACS Analyzer.

6.7 Cell enrichment procedure (total T cells, CD4+, CD8+ T cells)

Spleens from either B6 wt or Cor1-/- mice were harvested in PBS supplemented with 2% FCS and 2mM EDTA, and then smashed through a grid of stainless steel. Cell debris was removed by quick spin (500rpm, 10s). The cell numbers were counted and cells were sorted for total T cells, CD4+ or CD8+ T cells using negative selection T cell, CD4+ or CD8+ T Cell Enrichment Kit, respectively (EasySep, StemCell technologies, Ref. No 19751, 19752, 19753). Cells were sorted following the protocol from proper selection kit. Cells were counted using Neubauer chamber and dead cells were excluded using trypan blue.

6.8 Cell sorting by FACS sorter for CD25- and CD25+CD4+ T cells

After CD4+ enrichment with StemCell kit the cells were additionally sorted using FACS sorter. Enriched CD4+ T cells were resuspended at the concentration of 20x10^6 cells/ml in PBS supplemented with 2% FCS and 2mM EDTA and stained with saturating amount of antibodies (1/200 CD3-PE, 1/50 CD4-Brilliant Violet 510, 1/100 CD25-PE-Cy7, LIVE/DEAD® Fixable Near-IR Dead Cell Stain). The cells were incubated for 30min at 4^0C in the dark. After incubation, stained cells were washed with PBS supplemented with 2% FCS and 2mM EDTA, centrifuged 5min at 350g, resuspended at the concentration of 8x10^6 cells/ml and filtered through 30 µm Pre-Separation Filters (Miltenyi Biotec, Ref. No 130-041-407). Then the cells were sorted with Aria III BD FACS Sorter for CD4+CD25- cells. After sorting the cells were counted, washed and resuspended in plain RPMI 1640 without phenol red (Life Technologies, Ref. No 11835-063) for adoptive transfer of cells or in RPMI 1640 (Sigma-Aldrich, Ref. No R8758) supplemented with 10% FSC (PAA clone, The Cell Culture Company, Ref. No A15-101), 500µg/ml Pen-Strep (Gibco, Ref. No 15140-122), 1% NEAA (Non-essential Amino Acid Solution, Sigma-Aldrich, Ref. No M7145), 10µM β-mercaptoethanol (Sigma-Aldrich, Ref. No M7522), 1µM Na-Py (Sodium Pyruvate, Sigma-Aldrich, Ref. No S8636) for *in vitro* culture of cells.

6.9 Adoptive transfer of cells into mouse recipients

For the adoptive transfer of cells, the cells were enriched with the StemCell kit (for CD4+ T cells transfer) and sorted with FACS sorter (for CD4+CD25- T cells transfer) as described before. The cells were resuspended in plain RPMI 1640 without phenol red (Life Technologies, Ref. No 11835-063) and transferred to Insulin Syringe with sterile interior (BD Micro Fine, Ref. No 324824). 200ul of cell suspension was injected into tail veil per mouse.

6.10 Blood test

Blood test was done to phenotype some lines of mice (OT-I, OT-II, 6.25% Balb/c in BL6 and ABM) and to confirm the depletion of CD25+ cells by the injection of antibody. For taking blood, the mouse was placed in the mouse restrainer, tail vein was punched with a Needle Violet 24G (BD Microlance 3, Ref. No 304100) and about 10 drops of blood was collected into FACS tube containing 3ml of FACS buffer (PBS, 2%FCS, 5mM EDTA). The samples were kept on ice all the time. The cells were spin down at 450g, 4^0C for 5min. The supernatant was aspirated using the sucking pomp. Afterwards, the pellet was resuspended in 50ul FACS buffer containing proper antibodies. The samples were vortexed shortly and incubated for 20min at 4^0C in the dark. Then, the samples were washed by addition of 3ml of FACS buffer and centrifugation (450g, 4^0C for 5min). The supernatant was aspirated with the pomp and red blood cells were lysed with 1-2ml of BD FACS Lysing Solution (BD Biosciences, Ref. No 349202) and incubation at room temperature for about 60s. 3ml of FACS buffer was added to the samples and they were centrifuged (450g, 4^0C for 5min). The supernatant was pour off and pellet was resuspened in 250μl of FACS buffer. The samples were acquired using FACS Canto II or LSR Fortessa BD Analyzer.

6.11 CD25-depletion

CD25+ cells were depleted from mice by intraperitoneal (i.p.) injection with 0.5mg of anti-CD25 (PC61) antibody per mouse and per injection at day 5, 3 before and day 3

after transplantation. The antibody was made in-house from hybridoma cells culture as described in point Antibody production and purification. Mice were bled to establish level of CD25+ cells at day 6, 1 before and day 7 after surgery. Additionally Cor1-/- recipients (that did not reject the grafts) were bled at day 15, 28 and 65 after transplantation. Blood was obtained as described in point Blood test. The samples were stained for surface markers: CD3, CD4, CD25, LIVE/DEAD® Fixable Near-IR Dead Cell Stain and intracellular transcription factor Foxp3 as described in point FACS staining of cells.

6.12 CD25 (PC61) monoclonal antibody production from hybridoma cells

The hybridoma cells were thawed out in 37^0C water bath. The cells were transferred into 15ml falcon tube containing 10ml of pre-wormed culture media (SF-IMDM, 2% FCS low IgG, Pen-Strep). Then, the cells were spun down at 300g, 3min, room temperature. The pellet was resuspended gently with 1ml of culture media and then transferred into T-75cm^2 flask with 15ml of culture media. The cells were placed in humidified 37°C, 5% CO_2 incubator. When the cells reached 70% confluence, they were split into 15cm gridded cell culture treated petri dishes with 50ml of culture media. Then the step was repeated: when the cells reached 70% confluence each petri dish was split into 10 new petri dishes. The total culture volume was 1l. The cells were allowed to grow for two weeks (until the media turned yellow). After that the cells were harvested into Oakridge styled tubes and centrifuged at 4000rpm for 30min, 4^0C. The supernatant was transferred into sterile bottles and filtered.

6.13 CD25 (PC61) antibody purification from culture supernatant

The column used for purification: self-packed column (Bio-Rad poly-Prep Chromatography column, Ref. No 731-1550) containing 3.5ml of GammaBind Plus Sepharose (GE Healthcare Life Sciences, Ref. No 170886-01).

The column was equilibrated with 15ml PBS. The culture supernatant was loaded by gravity flow. Then the column was washed with 50ml of PBS. The antibody was

eluted with 5ml of 100mM Glycine pH3.0 and collected as 500µl fractions in tubes containing 100µl 1M Tris-HCl, pH 8.0. After elution the column was washed with 15ml of 100mM Glycine pH 2.3 and 40ml of PBS. From each step the eluent was kept and 20µl of each sample was evaluated by SDS-PAGE gel electrophoresis. The samples were mixed with SDS loading buffer and boiled for 7min. 10µl of sample was run in 10% Acrylamide gel. The gel was stained with Coomassie blue. The fractions containing the highest concentration of antibody were pooled and dialyzed against PBS for 1h, 3h and overnight in 1l of buffer at 4^{0}C. The protein solution was collected, sterile filtered and the concentration was measured by OD280. The antibody was stored at 4^{0}C.

The specificity of produced antibody was analyzed by FACS staining using splenocytes from B6 wild type mouse as a sample, goat anti-rat-PE (1:200) as secondary antibody and commercial antibodies: anti-CD25-PE/Cy7 (1:100, clone PC61, Biolegend, Ref. No 102016) and anti-CD25-PE (1:100, clone 3C7, Biolegend, Ref. No 101904) as controls.

6.14 Dendritic cells differentiation and culture

Femurs and tibiae were harvested from 6-8 weeks old mice. The bones were washed once with 70% EtOH and twice with sterile PBS. Both ends of bones were cut and the bone marrow was flush out with 21-gauage needle (BD Microlance) and 20ml syringe (BBraun, Ref. No 4616200V) into 50ml falcon tube. The bone marrow was resuspended with the needle and centrifuged for 5min, 350g, room temperature. The red blood cells were lysed with ACK buffer (described before), incubated 1min at room temperature, neutralized with 10ml of PBS and centrifuged for 5min, 350g, room temp. Then the cells were washed twice with RPMI 1640 media (Sigma-Aldrich, Ref. No R8758) and counted using Neubauer chamber (dead cells were excluded using trypan blue). The cells were spin down (5min, 350g, RT) and resuspended at the concentration of $1x10^{6}$ cells/ml in RPMI media containing 10% FCS PAA clone (heat inactivated), 2mM L-glutamine, 50µM 2-mercaptoethanol and 10ng/ml GM-CSF (Recombinant Mouse GM-CSF CF, Biolegend, Ref. No 576306) freshly added to the media. 8ml of cell suspension was plated per $10cm^{2}$ bacteria (non

cell culture treated) petri dish. The cells were cultured in humidified 37°C, 5% CO_2 incubator. 4ml of the media was replaced with a fresh one (freshly adding 10ng/ml og GM-CSF) at day 2, 4 and 6 of culture. At day 7 the cells were used farther for the co-culture proliferation experiment.

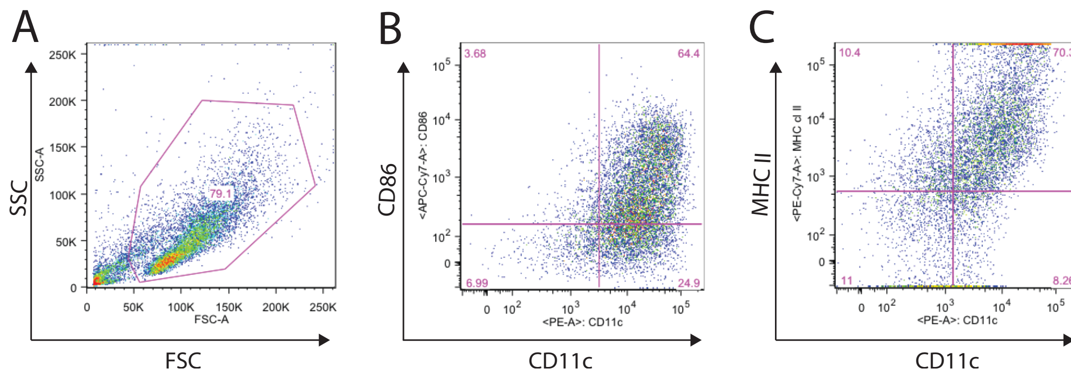


Figure 36. ***Test of dendritic cell culture obtained from bone marrow of BL/6 wild type mice after one-week culture in the presence of GM-CSF (10ng/ml).*** *The cells were use as stimulators in proliferation, survival and cytokine secretion experiments. A shows side and forward scatter of dendritic cells after one week of culture. B – expression of dendritic cell markers (CD11c and CD86), C – expression of MHC calls II.*

6.15 Plate coating and stimulation with antibodies

For stimulation with CD3 and CD28 antibodies the plate was either coated or soluble antibodies were added to the media. The method of stimulation is indicated in every experiment. 96-well plate was coated with 10μg/ml of LEAF purified anti-mouse CD3 antibody (Biolegend, Ref. No 1003014) in 100μl/well of PBS at 37^0C for two hours. After incubation the plate was centrifuged (350g, 5min, 4^0C), the supernatant was removed and then it was washed once with 100μl of PBS. LEAF purified anti-mouse CD28 antibody (Biolegend, Ref. No 102112) was added directly to the culture media at the concentration of 2μg/ml. Soluble antibodies were added directly to the culture media at the concentration of 1ug/ml for anti-CD3 and 2ug/ml for anti-CD28.

6.16 Cell Trace Violet (CTV) labeling

Purified total, CD4+ or CD8+ T cells were resuspended at the concentration of 10^7 cells/ml in RPMI medium. CTV was resuspend in DMSO at the concentration of

5mM and 1µl of the CTV was added to every 1ml of cell suspension. The cells were incubated for 20min at 37^0C. After that cells were washed with culture T cells medium to quench any unbound die (incubated 5min with the medium at room temperature and centrifuged at 350g for 5min, 4^0C). The cells were resuspend in culture T cell medium and counted.

6.17 Proliferation assay

Generated dendritic cells were collected and centrifuged (350g, 10min, RT). The cells were counted using Neubauer chamber (dead cells were excluded using trypan blue). Dendritic cells were resuspended at the concentration of $3x10^5$ cells/ml in RPMI complete media for dendritic cells. 100µl per well of cell suspension was plated in 96-well plate. The cells were incubated over night in humidified 37^oC, 5% CO_2 incubator. The next day, before addition of T cells, the plate was centrifuged at 350g, 5min, 4^0C and the supernatant was removed.

Spleens were harvested following the protocol described in section 6.5. *Organ harvesting and processing for analysis*. Total, CD4+ or CD8+ T cells were enriched using Cell Enrichment Kit, (EasySep, StemCell technologies) as described before (section 6.7. *Cell enrichment procedure*). The cells were labeled with CTV (Section 6.16) and resuspended at the concentration of $2x10^6$ cells/ml in complete RPMI media for T cells and 100µl of cell suspension was plated per well in 96-well plate.

The T cells were co-cultured with dendritic cells (generation protocol described in Dendritic cells differentiation and culture), or stimulated with CD3 and CD28 antibody (Plate coating and stimulation with antibodies) or PMA (phorbol 12-myristate 13-acetate, 20ng/ml, Sigma, Ref. No P8139) and Ionomycin (500ng/ml, Calbiochem, Ref. No 407951) for 24-96h in humidified 37^oC, 5% CO_2 incubator. After incubation cells were centrifuged (350g, 5min, 40C) and processed following FACS staining protocol.

6.18 *In vitro* cytokine analysis

For *in vitro* cytokine staining, the cells were processed as described in Proliferation assay (Chapter 6.17). Brefeldin A (10µg/ml, Calbiochem, Ref. No 203729) and anti-LAMP1-Brilliand Violet 421 (1:50, CD107a, Biolegend, Ref. No 121617) antibody was added to the cell culture and the cells were incubated for: 5, 15, 30, 60 and 120min at 37^0C. Afterwards, the cells were stained following intracellular FACS staining protocol (from Chapter 6.6) for CD3, CD4 or CD8, L/D marker, INF-g, TNF-a and IL-2 (LAMP1 was already labeled with fluorophore).

6.19 *In vitro* generation of regulatory T cells from CD4+CD25- T cells

The experiment was done following the protocol from Natire Protocols paper (*In vitro* generation of CD4+CD25+ regulatory cells from murine naïve T cells) and the procedure was as described below. 6 to 8 week old mice from B6 wt and Cor1-/- mice were sacrifice and the spleen was harvested from each mouse following the procedure described in point 5 (Organ harvesting). The red cells were lysed using ACK buffer for 1min at room temperature. Then PBS was added to the cell suspension and the cells were centrifuged for 10min at 400g, 4^0C. The pellet was resuspended and cells were counted as described before. The cells were enriched for CD4+ T cells using StemCell Enrichment Kit (negative selection described before). As a recommended medium RPMI supplemented with 10% FCS was used. Afterwards the CD25+ cells were depleted using magnetic sorting with anti-CD25-biotin (50µg/ml cells Biolegend, Ref. No 102004) labeled antibodies and streptavidin-magnetic beads. The samples were incubated with beads for 15min at 4^0C and 2.5min with beads at room temperature. The negative fraction was kept from each sample and the cells were counted. $4x10^5$ cell/well was plated in 96-well plate coated with anti-CD3 antibody (described before) with addition of anti-CD28 (2µg/ml), TGF-β1 (5ng/ml, R&D Systems, Ref. No 7666-MB) and IL-2 (20ng/ml, recombinant mouse IL-2 CF, Biolegend, Ref. No 575406) in X-Vivo15 serum-free medium (Lonza, Ref. No BE04-744Q) in the absence of antibiotics. The cells were cultured in the humidified 37^oC, 5% CO_2 incubator for 2 to 5 days. At the day of analysis the cells were stained as

described in intracellular FACS staining of cells (point 6) for L/D marker, CD3, CD8, CD8, CD25 and Foxp3.

6.20 Mixed lymphocyte reaction (MLR)

Stimulators preparation: spleen from Balb/c, bm12 or 6.25% Balb/c in BL/6 mouse was harvested as described before. Cells were counted and resuspended in PBS 2% FCS and 2mM EDTA at concentration $20x10^6$ cells/ml. Splenocytes were depleted of CD3+ cells and erythrocytes by adding anti-CD3-biotin and anti-TER119-biotin antibodies (50µg/ml), incubating 10min at room temperature. Then streptavidin-coated beads were added and the protocol from StemCell Enrichment Kit (EasySep, StemCell technologies) was followed. Obtained cells were treated with mitomycin C (50µg/ml, Sigma- Aldrich, Ref. No M4287-2MG) for 1h at 37^0C. The cells were washed twice with plain RPMI 1640 medium (R8758) and resuspended at the concentration of $8x10^6$ cells/ml in complete RPMI media for T cells. 200µl of cells suspension was plated per well in 96-well plate and then serial dilution were done in order to get 800 000, 400 000, 200 000, 100 000, 50 000 and 25 000 cells/well.

Responders preparation: spleens from either B6 wt or Cor1-/- mice were harvested as described before in PBS supplemented with 2% FCS and 2mM EDTA. The cell numbers were counted and cells were sorted for CD4+, CD8+ or total T cells using negative selection T Cell Enrichment Kit (EasySep, StemCell technologies). Afterwards, enriched CD4+ T cells were labeled with CTV as described above (Section 6.16). Washed cells were resuspended at the concentration of 1x 10^6 or 2x10^6 cells/ml in complete RPMI media for T cells. Responder T cells were plated at the concentration of 1x10^5 or $2x10^5$ cells/well. The cells were incubated in the humidified incubator at 37^0C with 5% of CO_2 for 48 - 120h.

After incubation cells were stained and analyzed with the FACS analyzer by acquiring every sample for 1.5min at the same speed.

For thymidine incorporation analysis: 52h after plating the cells, thymidine (1υCi/well) was added and the cells were further incubated for 20 hours. The incorporation of radioactive label was stopped by freezing and thawing the sample at -

20^0C and the DNA incorporated label was assessed by harvesting the lysates on a GF/C filters and counting with a scintillation counter (TopCount, Packard).

6.21 *In vitro* suppression assays

In vitro suppression assay followed the MLR protocol with the exceptions:

- B6 Ly5.2 cells were used as stimulators
- Anti-CD3 (10µg/ml) antibody was added to the culture
- The cells were enriched with StemCell enrichment kit for CD4+ cells and sorted with FACS sorter for CD4+CD25- cells (both methods described before)
- B6 wt Ly5.1 CD4+CD25- were use as responders and Cor1-/- Ly5.2 CD4+CD25- cells were use as suppressors. Suppressor Cor1-/- CD4+ CD25- T cells were added to responder cells at two concentrations: $2x10^5$ and $4x10^5$ cells/well.

6.22 *In vivo* suppression assay

Spleens from either B6 wt Ly5.1 or Cor1-/- Ly5.2 mice were harvested in PBS supplemented with 2% FCS and 2mM EDTA and proceed as described before. The cells were sorted for CD4+ T cells using negative selection CD4+ T StemCell Enrichment Kit as described before. Cells were counted and sorted with FACS sorted for CD4+CD25- cells (point 8). After sorting the cells were counted, washed and resuspended at the concentration of 10 $x10^6$ cells per ml in plain RPMI 1640 without phenol red. For the co-transfer of B6 wt (Ly5.1) CD4+CD25- with Cor1-/- (Ly5.2) CD4+CD25- cells were diluted 1:1. 200ul of cell suspension was injected into tail veil of Rag2-/- mice. All mice were sacrificed two weeks after injection and spleen and lymph nodes (axillary, brachial, inguinal) were analyzed by flow cytometry staining.

6.23 Statistical analysis

The significance in a difference between samples was assessed by multiple t test analysis. P value lower than 0.05 was marked as significantly different with *; p value lower than 0.01 as ** and p value lower than 0.001 as ***.

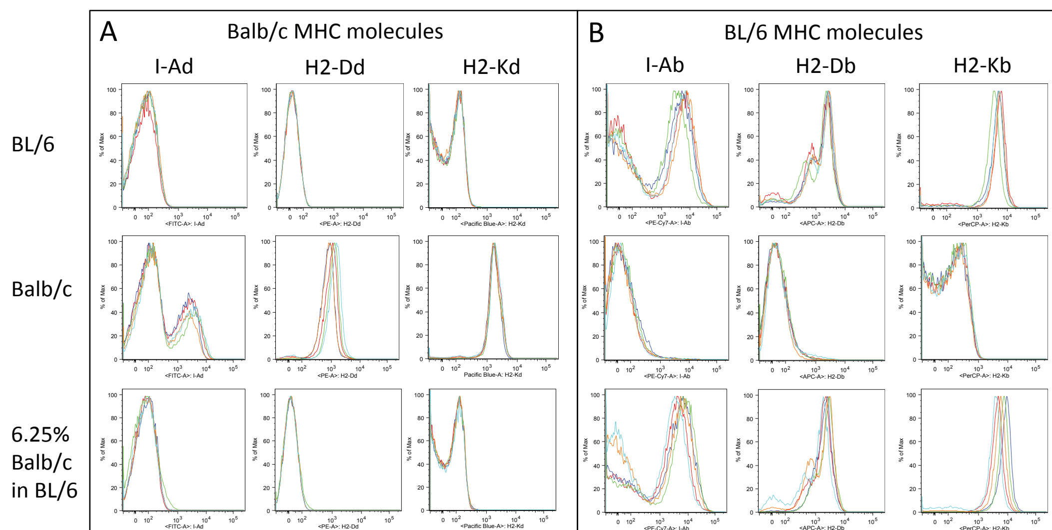
6.24 Software used

To analyze data and write the thesis several softwares were used:

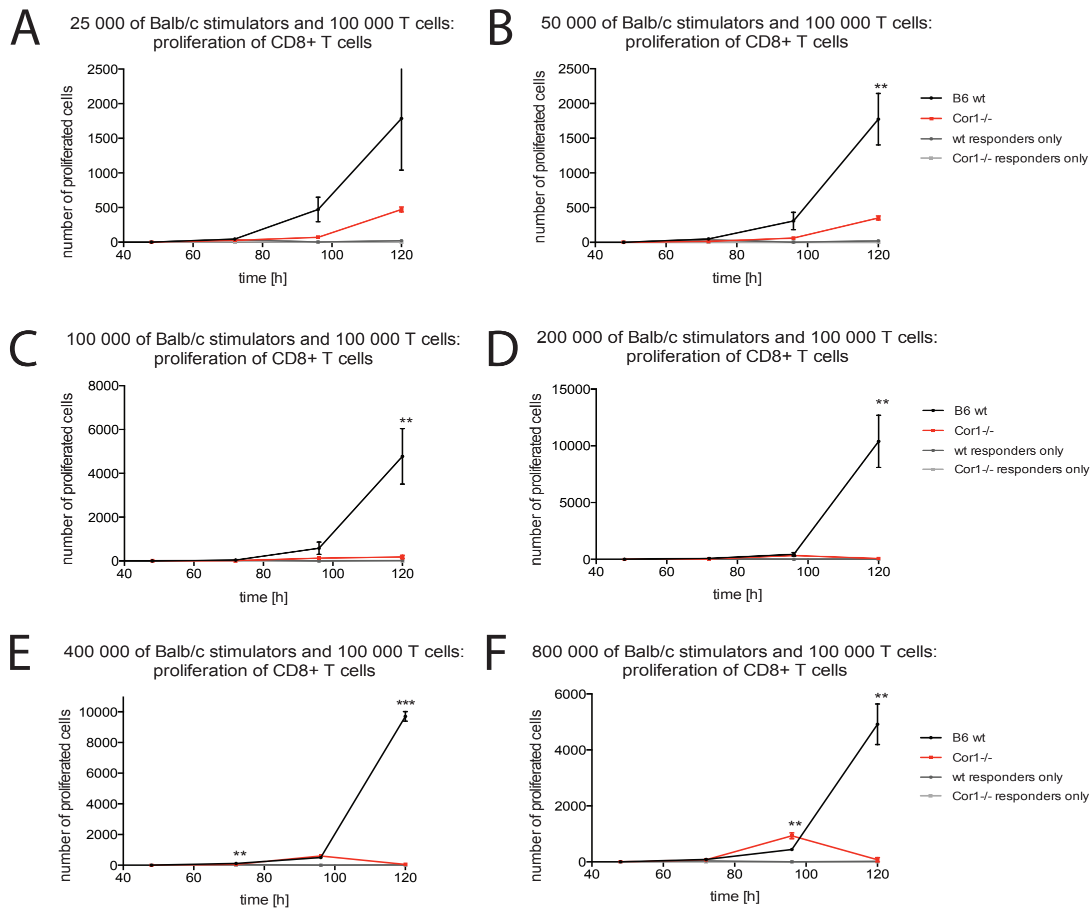
- BD FACSDiva for flow cytometry acquisition and analysis
- FlowJo for cell staining analysis acquired by FACS analyzer
- GraphPad Prism for graph preparation and statistical calculations
- Microsoft Office for writing
- Adobe (Photoshop, Illustrator) – figures preparation
- Zotero - bibliography

7 Appendix

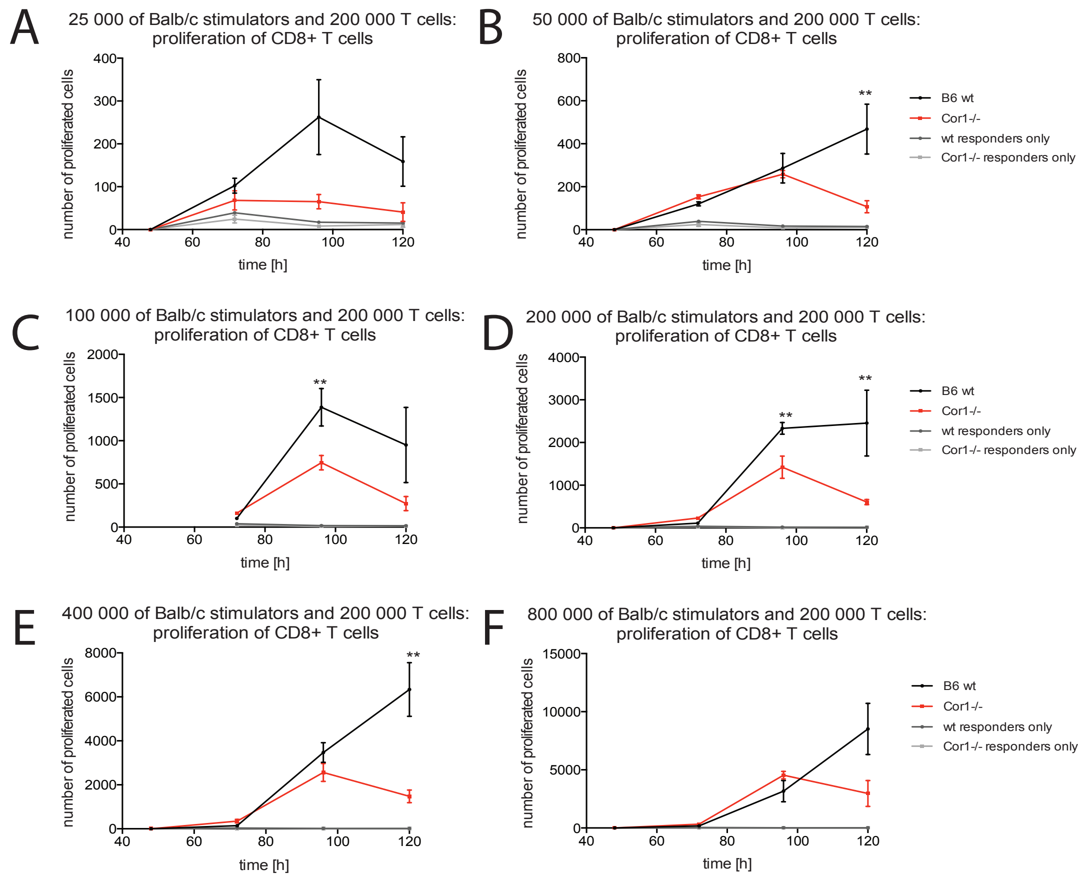
7.1 Supplementary Figures



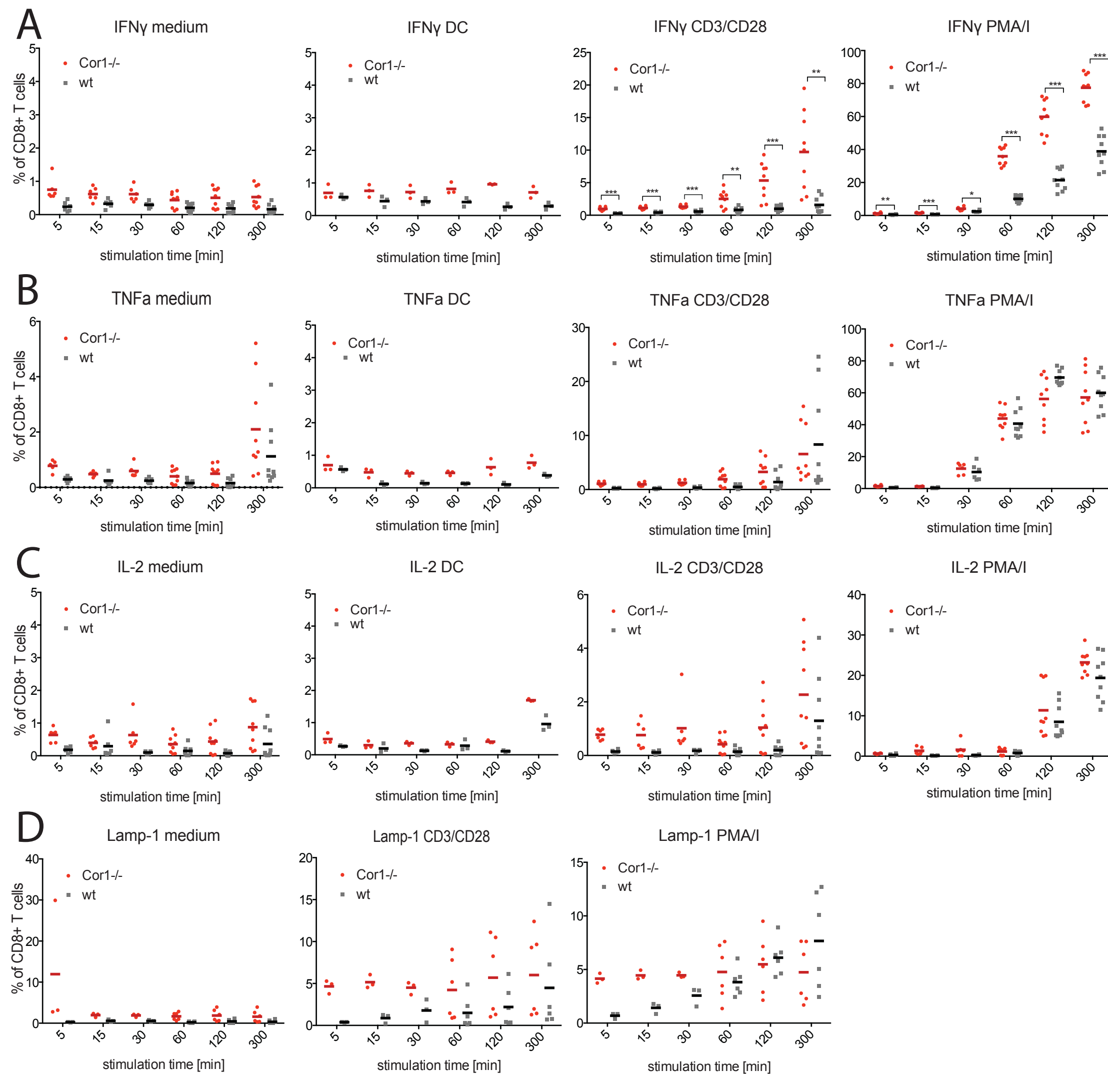
Supplementary Fig. 1. ***Major histocompatibility complexes (MHC) staining for 6.25% Balb/c in BL/6 mice.*** *Blood cells were processed following the blood staining protocol, stained with anti- I-Ab, H2-Db, H2-Kb, I-Ad, H2-Dd and H2-Kd antibodies and analyzed with FACS. Five mice were used per group; the experiment was repeated two times. A. An antibody staining for Balb/c specific MHC complexes: I-Ad (MHC class II), H2-Dd and H2-Kd (MHC class I). B. An antibody staining for BL/6 specific MHC complexes: I-Ab (MHC class II), H2-Db and H2-Kb (MHC class I). Each plot shows blood staining of five mice from Balb/c, BL/6 and 6.25% Balb/c in BL/6 for all antibodies. MHC molecules expressed by BL/6 are positive in BL/6 and 6.25% Balb/c in BL/6 mice strains when Balb/c specific MHC molecules are expressed only in Balb/c mice strains and negative in 6.25% Balb/c in BL/6 mice.*



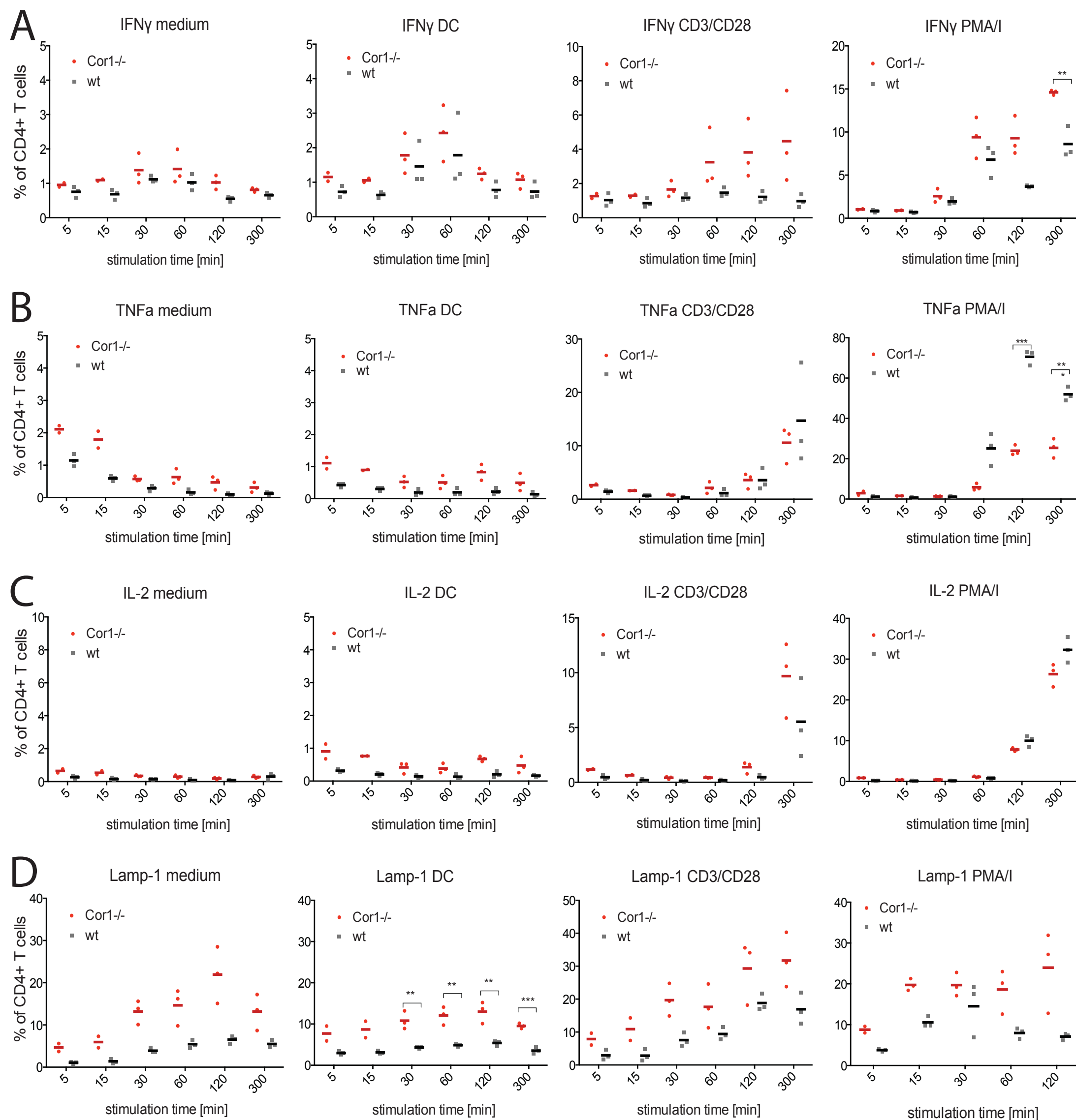
Supplementary Fig. 2. ***Analysis of CD8+ T cells in a mixed lymphocyte reaction (MLR) for total T cells from BL/6 wild type and coronin 1-deficient mice as responders and Balb/c splenocytes as stimulators (plated in different concentrations).*** *Total T cells were purified as described in Materials and Methods (chapter 4.20). Stimulators – Balb/c splenocytes were depleted of CD3 positive cells and erythrocytes (with anti-CD3 and anti-TER119 antibody, respectively). The cells were treated with mitomycin c to eliminate background proliferation of stimulators. Then both cells were plated together, in numbers indicated on each graph, in 96-well plate and cultured for 48, 72, 96 and 120h. The cells were analyzed by FACS using the same sample volume, time and speed of acquisition. A – F. Analysis of CD8+ T cells proliferation in a culture of 100 000 T cells with different numbers of Balb/c stimulators. An asterisk always shows the significance in the difference between BL/6 wt and Cor1-/- sample. Each time point shows a mean of 3 replicates. B6 – BL/6 wild type; Cor1-/- - coronin 1-deficient.*



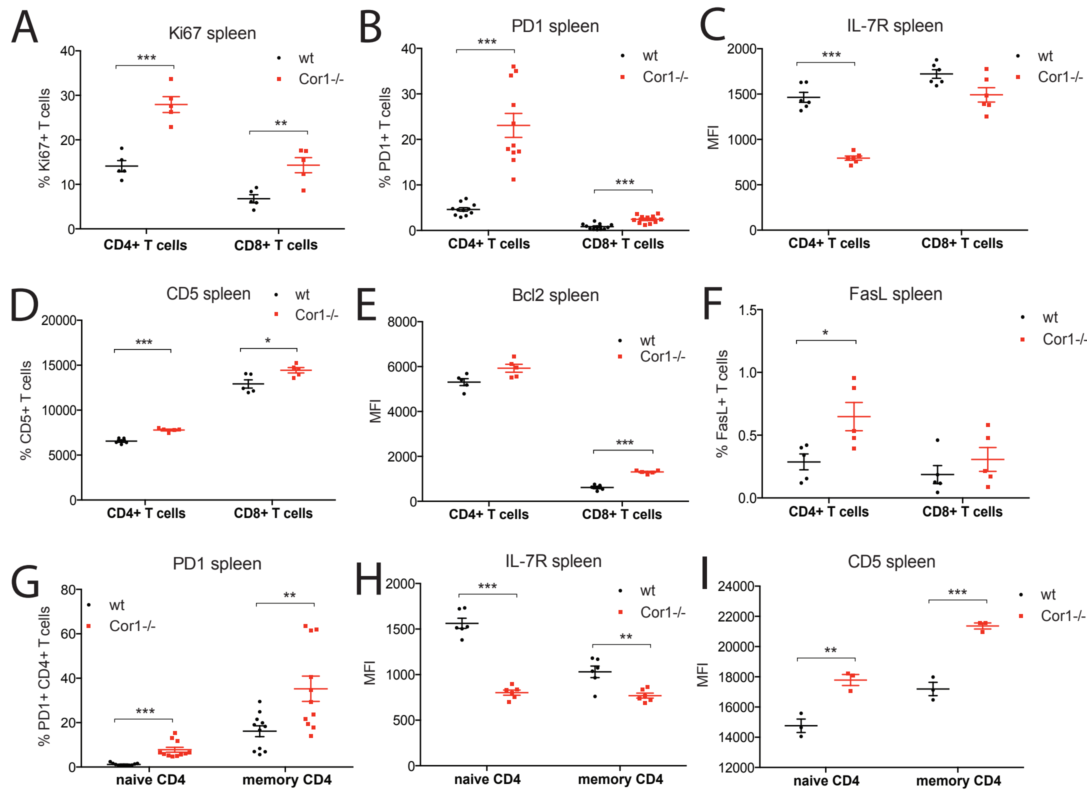
Supplementary Fig. 3. ***Analysis of CD8+ T cells in a mixed lymphocyte reaction (MLR) for total T cells from BL/6 wild type and coronin 1-deficient mice as responders and Balb/c splenocytes as stimulators (plated in different concentrations).*** *Total T cells were purified as described in Materials and Methods (chapter 4.20). Stimulators – Balb/c splenocytes were depleted of CD3 positive cells and erythrocytes (with anti-CD3 and anti-TER119 antibody, respectively). The cells were treated with mitomycin c to eliminate background proliferation of stimulators. Then both cells were plated together, in numbers indicated on each graph, in 96-well plate and cultured for 48, 72, 96 and 120h. The cells were analyzed by FACS using the same sample volume, time and speed of acquisition. A – F. Analysis of CD8+ T cells proliferation an a culture of 200 000 T cells with different numbers of Balb/c stimulators. An asterisk always shows the significance in the difference between BL/6 wt and Cor1-/- sample. Each time point shows a mean of 3 replicates. B6 – BL/6 wild type; Cor1-/- - coronin 1-deficient.*



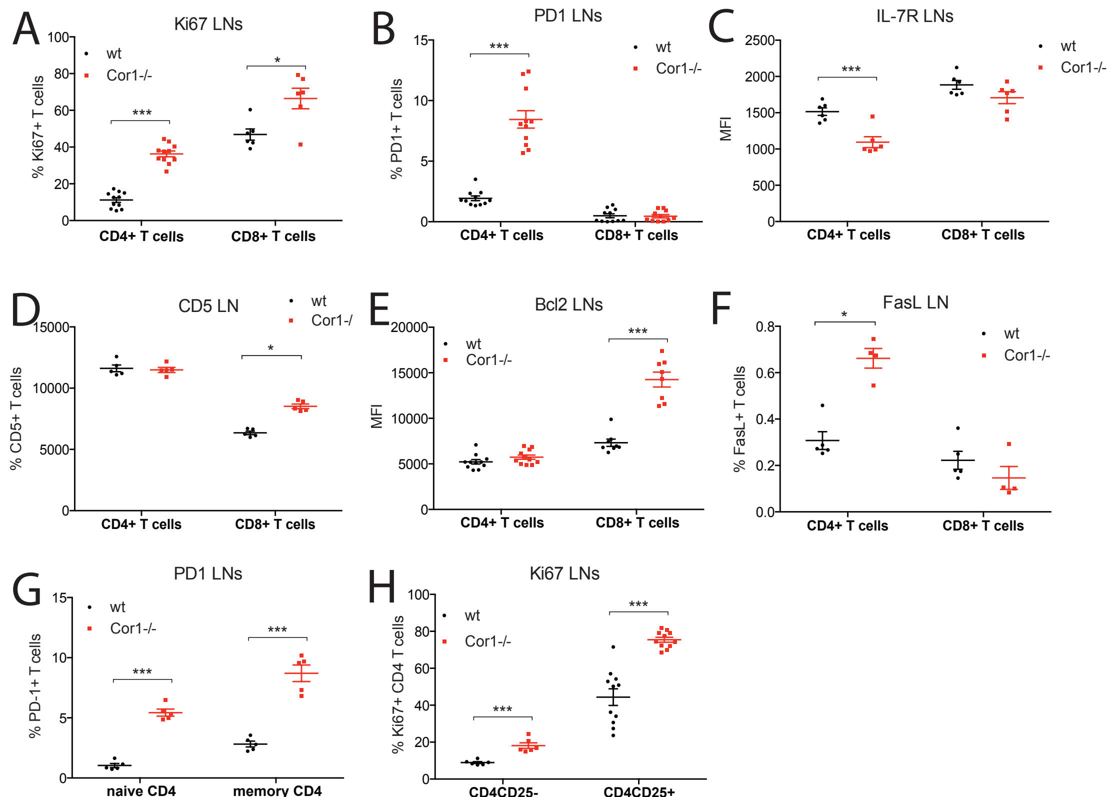
Supplementary Fig. 4. ***CD8+ T cells from BL/6 wild type and coronin 1-deficient mice analyzed for cytokine secretion in a culture without (medium) and with stimulation with dendritic cells (DC), anti-CD3 and anti-CD28 (CD3/CD28), and PMA and ionomycin (PMA/I).*** *A. Production of IFN-gamma (IFNγ). B. Secretion of TNF-alpha. C. IL-2 secretion and expression of Lamp-1 (D). Dendritic cells were obtained from bone marrow of BL/6 wt mice after one week of culture in the presence of GM-CSF (as described in Materials and Methods). Then the cells were plated in 96-well plate at the concentration of 30 000 cells/well one day before CD8+ T cells culture started. CD8+ T cells were purified following the protocol. The cells were plated in 96-well plate at the concentration of 200 000 cells/well and cultured for 5, 15, 30, 60, 120 and 300min. The cells were analyzed by FACS staining. Each time point shows a mean of 3 separate experiments where three individual mice were used per sample.*



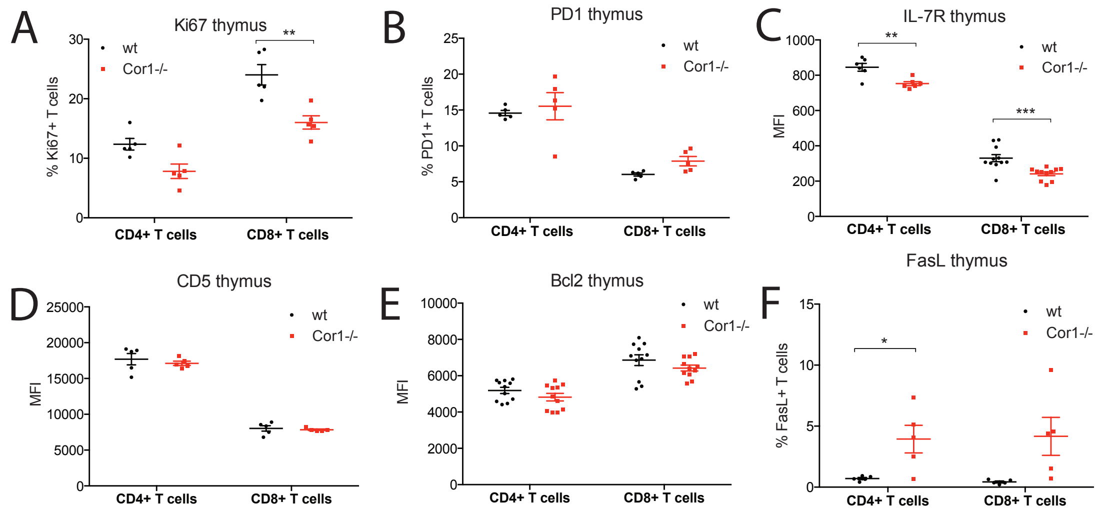
Supplementary Fig. 5. ***CD4+ T cells from BL/6 wild type and coronin 1-deficient mice analyzed for cytokine secretion in a culture without (medium) and with stimulation with dendritic cells (DC), anti-CD3 and anti-CD28 (CD3/CD28), and PMA and ionomycin (PMA/I).*** *A. Production of IFN-gamma (IFNγ) B. Secretion of TNF-alpha. C. IL-2 secretion and expression of Lamp-1 (D). Dendritic cells were obtained from bone marrow of BL/6 wt mice after one week of culture in the presence of GM-CSF (as described in Materials and Methods). Then the cells were plated in 96-well plate at the concentration of 30 000 cells/well one day before CD4+ T cells culture started. CD4+ T cells were purified following the protocol. The cells were plated in 96-well plate at the concentration of 200 000 cells/well and cultured for 5, 15, 30, 60, 120 and 300min. The cells were analyzed using FACS. Each time point shows a mean of three individual mice used per sample.*



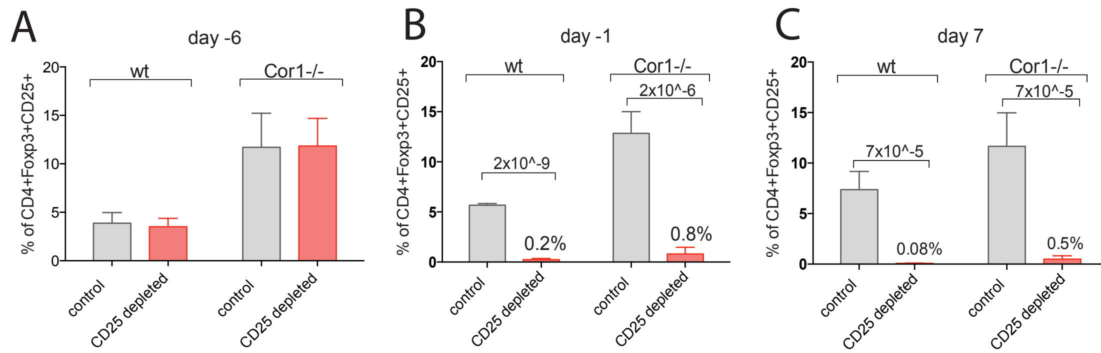
Supplementary Fig. 6. ***Expression of different cell markers in BL/6 wild type compared to coronin 1-deficient T cells from spleens.*** *A. Intracellular expression of Ki67 (proliferation marker) in BL/6 wt and Cor1-/- CD4+ and CD8+ T cells. B. PD1 (immune exhaustion marker) expression. C. Surface expression of IL-7 receptor. D. Expression of CD5 (marker of strong activation of T cells). E. Expression of Bcl-2 (pro-survival marker). F. Surface levels of FasL on wild type compared to coronin 1-deficient T cells. G - I. Expression of PD1, IL-7R and CD5 in different subsets of T cells. The cells from spleen were harvested and processed following the protocol described in Materials and Methods (Chapter 4.5). Then they were FACS stained as described in the protocol for surface or intracellular FACS staining in the Material and Methods (Chapter 4.6). Each graph shows a mean from at least one to three separate experiments where up to 5 individual mice were used per staining. Wt – BL/6 wild type; Cor1-/- - coronin 1-deficient.*



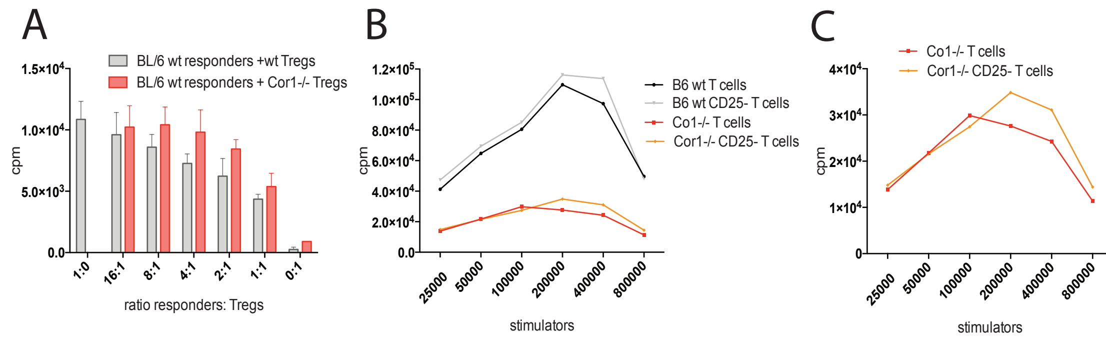
Supplementary Fig. 7. ***Expression of different cell markers in BL/6 wild type compared to coronin 1-deficient T cells from lymph nodes.*** *A. Intracellular expression of Ki67 (proliferation marker) in BL/6 wt and Cor1-/- CD4+ and CD8+ T cells. B. PD1 (immune exhaustion marker) expression. C. Surface expression of IL-7 receptor. D. Expression of CD5 (marker of strong activation of T cells). E. Expression of Bcl-2 (pro-survival marker). F. Surface levels of FasL on wild type compared to coronin 1-deficient T cells. G and H. Expression of PD1and Ki67 in different subsets of T cells. The cells from lymph nodes (axillary, brachial and inguinal) were harvested and processed following the protocol described in Materials and Methods (Chapter 4.5). Then they were FACS stained as described in the protocol for surface or intracellular FACS staining in the Material and Methods (Chapter 4.6). Each graph shows a mean from at least one to three separate experiments where up to 5 individual mice were used per staining. Wt – BL/6 wild type; Cor1-/- - coronin 1-deficient.*



Supplementary Fig. 8. ***Expression of different cell markers in BL/6 wild type compared to coronin 1-deficient T cells from thymus.*** *A. Intracellular expression of Ki67 (proliferation marker) in BL/6 wt and Cor1-/- CD4+ and CD8+ T cells. B. PD1 (immune exhaustion marker) expression. C. Surface expression of IL-7 receptor. D. Expression of CD5 (marker of strong activation of T cells). E. Expression of Bcl-2 (pro-survival marker). F. Surface levels of FasL on wild type compared to coronin 1-deficient T cells. G and H. Expression of PD1and Ki67 in different subsets of T cells. The cells from thymi were harvested and processed following the protocol described in Materials and Methods (Chapter 4.5). Then they were FACS stained as described in the protocol for surface or intracellular FACS staining in the Material and Methods (Chapter 4.6). Each graph shows a mean from at least one to three separate experiments where up to 5 individual mice were used per staining. Wt – BL/6 wild type; Cor1-/- - coronin 1-deficient.*



Supplementary Fig. 9. ***CD4+Foxp3+CD25+ (regulatory T) cells in blood of BL/6 wild type and Cor1-/- recipients of bm12Rag2-/- skin treated or not with PC61 antibody (CD25 depletion).*** *The mice were injected with 0.5mg of PC61 antibody 5 and 3 days before and 3 days after skin transplantation. They were bleed 6 (A) and 1 (B) day before and 7(C) days after surgery and the CD4+Foxp3+CD25+ T cells rates were define by antibody staining and flow cytometry analysis (following the protocol for Blood Staining).*



Supplementary Fig. 10. ***Regulatory T cells studies. A. Suppression assay comparing Tregs from BL/6 wt and Cor1-/- mice.*** *Tregs and responders were obtained from spleens after T cells negative sort with the STEM Cell Kit and additional FACS sorting for CD4+ CD25+ (Tregs) and CD4+CD25- (responders) cells. As stimulators BL6/ wt splenocytes depleted of CD4+ and CD8+ cells and treated with mitonycin c were used. The cells were plated in 96-well plate in different dilutions indicated on the graph. The cells were cultured for 72h and the proliferation was estimated by incorporation of thymidine. B. MLR experiment with total T cells depleted or not of CD25+ cells from BL/6 wt and Cor1-/- spleens. The experiment was done following the Mixed Lymphocyte Reaction protocol (Chapter 4.20) and the proliferation was measured by incorporation of thymidine. There was no statistically significant difference in proliferation between B6 wt T cells depleted and not of CD25+ cells as well as between Cor1-/- T cells depleted and not of CD25+ cells. However Cor1-/- samples (both depleted of CD25+ and not) show statistically significant difference in proliferation compared to B6 wt samples (depleted of CD25+ or not) for all conditions. C. MLR of Cor1-/- T cells depleted or not of CD25+ cells. Experiments were repeated three times and the figure shows representative results from one experiment. B6 wt– BL/6 wild type; Cor1-/- - coronin 1-deficient.*

7.2 Contribution

In part, this work was done under the supervision of Beatrice Bolinger, to whom I am immensely grateful for sharing the project with me.

Experiments described in Figures 18, 23A and Supplementary Fig. 10 were performed by Beatrice Bolinger.

Experiments described in section 4.3 (Figure 17) and 4.5.4 (Figure 29) were performed with the help of Simona Rossi Girard and Mathias Schmaler.

Repetitions of results presented in Figures 10 and 13A and B were performed by me, Beatrice Bolinger, Rajesh Jayachandran and Helene Rossez.

7.3 Bibliography

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7.4 Curriculum Vitae

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Email: a.gumienny@unibas.ch
Date of birth: 23rd September 1987
Nationality: Polish

Education

Jan 2018 – present
Technical Expert
Single Cell Facility, D-BSSE, ETH Zurich

Feb – Aug 2017
Technical Associate
FACS Core Facility, Biozentrum, University of Basel, Basel, Switzerland

2012 - 2016
Ph.D. in Biochemistry (Immunology)
Biozentrum, University of Basel, Basel, Switzerland
Topic: Skin transplantation in a mouse model of naïve T cell deficiency.
Group of Prof. Jean Pieters (due to completion October 2016)

2010 – 2011
M.Sc. in Biochemistry with specialization in Molecular and Cellular Biology (studies in English)
Université d'Orléans, Orléans, France (4.5/5.0)
Topic: Adhesion of B cells to endothelial cells.
Group of Prof. Claudine Kieda

2006 – 2011
M.Sc. in Biotechnology
Jagiellonian University, Cracow, Poland (5.0/5.0)
Topic: Influence of ADAM10 inhibition on adhesion of plasma cells to endothelial cells.
Group of Prof. Joanna Cichy

2003 – 2006
High School with splecialization in chemistry and biology
Powstancow Slaskich High School No. 1, Rybnik, Poland

Research experience

2011 – 2012
Research technician;
Instituto Gulbenkian de Ciência, Lisbon, Portugal

	Group of Prof. Jocelyne Demengeot Project: Usage of Vβ RSS at TCRβ locus.
July – Sep 2010	**Amgen Scholar;** *Addenbrooke's Hospital, University of Cambridge, Cambridge, UK* Group of Dr. Robert Busch Project: Monitoring MHC protein levels during biosynthetic labelling of dendritic cells.
Jan – June 2010	**Erasmus Scholar;** *Centre National de la Recherche Scientifique (CNRS), Université d'Orléans, France* Group of Prof. Claudine Kieda Project: Adhesion between B cells and high endothelial cells.
June – Aug 2009	**Scholar;** *Immunology and Transplant Biology Laboratory, Regenerative Medicine Institute (REMEDI), The National Centre for Biomedical Engineering Science (NCBES), National University of Ireland, Galway* Group of Prof. Matthew Griffin Project: Effect of Toll-like Receptor Activation on Interleukin-7 Production by Renal Fibroblasts.
Sep 2008 – Jan 2009	**Volunteer;** *Department of Physical Biochemistry's Laboratory, Jagiellonian University* Group of Dr. Sylwia Kędracka-Krok Project: Purification of proteins for two-dimensional gel electrophoresis.

Publications

Allele-Independent Turnover of Human Leukocyte Antigen (HLA) Class Ia Molecules; Prevosto C, Usmani M, McDonald S, **Gumienny A**, Key T, Goodman R, Gaston JSH, Deery M, Busch R, PLOS ONE, August 2016

Awards, honors and competitive fellowships

July – Oct 2016	Burckhardt-Bürgin Foundation grant for Early Career Researchers' Development (awarded scholarships to talented students and grant funding for scientific and academic research)

July – Sep 2010	Highly competitive scholarship for Cambridge University Undergraduate Programme founded by Amgen Foundation (awarded to 25 students selected from Universities worldwide).
Jan – June 2010	Erasmus Program Scholarship for studies and research training at CNRS, Université d'Orléans, France
June – Aug 2009	Undergraduate Research Experience and Knowledge Award, Summer Undergraduate Fellowships in a Biomedical Research Network (SURF BioNET UREKA) funded by Science Foundation Ireland, NUIG, Ireland (highly competitive scholarship awarded to 15 students from Universities worldwide).

Teaching experience

2012 - 2015	Teaching the practical part of Biochemistry block course, *Biozentrum, University of Basel*
Oct 2013 – June 2014	Supervisor of a bachelor thesis [Understanding T cell proliferation in a coronin 1-/- model], *Biozentrum, University of Basel*

Trainings and certificates

Nov 2011	Federation of European Laboratory Animal Science Associations (FELASA) Theoretical and Practical Course on Animal Handling and Experimentation Category B

Conferences awards

Jan 2015	2nd Poster Prize, Biozentrum Symposium
Feb 2014	2nd Poster Prize, Biozentrum Symposium
June 2013	1st Poster Prize, Biozentrum PhD Symposium

Languages

English	Full professional proficiency
German	A1 (basic)
Polish	Native

Voluntary work:

2007 - 2011	Student's Association of Biotechnology (Jagiellonian University, Faculty of Biochemistry, Biophysics and Biotechnology);
2010 - 2011	Students' Self-Government at Jagiellonian University;
2010 - 2011	National Biotechnology Student Academic Association;

Any knowledge that doesn't lead to new questions quickly dies out: it fails to maintain the temperature required for sustaining life.

– Wisława Szymborksa –