The role of coronin 1
in T cell signalling
and development

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Doktor Boktor out.

2. <u>Index</u>

3. Summary

The immune system of a mammalian organism consists of two major players, the innate and adaptive immune system. While the innate system is used as a first line of defence based on fixed recognition patterns, the adaptive immune system has the capabilities to develop highly specific ways to recognize different pathogens and malignant cells. The main players of the adaptive immune system are B and T lymphocytes. While the major responsibility of a B cell is the production of antibodies, T cells act in a helping (CD4$^+$ helper cells), regulatory (CD4$^+$ regulatory) or killing (CD8$^+$ cytotoxic) manner. The recognition of antigens by T cells is mediated by the T Cell Receptor (TCR), which needs to be modulated and rearranged during T cell development in the thymus. The passage of cells, then called thymocytes, through the thymus is a highly specified and controlled process in which around 95% of thymocytes die. Their death is linked to signals mediated by the TCR, as a thymocyte egressing from the thymus needs to (i) be able to detect and react to foreign antigens while (ii) not reacting upon encountering self-antigens as this could result in auto-immunity. However, thymocytes which have a functional TCR and are either CD4 or CD8 single-positive, have not fully matured yet. They need to modulate various surface expression markers including CD62L, CD69 or CD24, to ensure that they will also be able to egress from the thymus and perform their function in peripheral secondary lymphoid organs such as the spleen and lymph nodes. These modulations take place during the late stages of thymocyte development and also during a transition phase after thymic egress. Those recent thymic emigrant (RTE) cells are a functionally and phenotypically distinct T cell subset, which shows reduced activation but an increased capacity to populate the T cell niche in the organism. As the development from thymocytes to mature naive T cells (MNT) is a complex process, there are various studies published on defective thymocyte or T cell maturation. Interestingly, most of the studies show survival deficits in other cell types as well. Coronin 1 appears to be responsible for survival only in the peripheral T cell population, but not in other cells of the immune system. This WD-repeat domain protein has originally been identified as being important for the intracellular survival of mycobacteria in macrophages. Subsequent studies using gene modified mice lacking coronin 1 have revealed no major roles in other immune cells with the exception of MNT survival. Coronin 1, however, is not only expressed in immune cells, it has a weak expression in excitatory neurons, where it has been shown to be needed for the production of the second messenger cyclic AMP (cAMP). While the cAMP pathway has not been linked to peripheral T cell survival, it has been shown to be involved in thymocyte apoptosis and to be needed for the full activation of a T cell by TCR stimuli. In addition, coronin 1 increases cAMP

production in neurons, coronin 1-deficient cells T cells however show increased levels of cAMP. This increase is not only seen in peripheral T cells but also in CD4 and CD8 single-positive thymocytes. Interestingly, the bulk of increased cAMP stems from conventional CD4+ cells in the coronin 1-deficient animals. However, closer examination revealed that membranes from coronin 1-deficient T cells still show reduced production of cAMP, similar to the phenotype seen in neurons, whereas the increased cAMP production is due to the lack of phosphodiesterases present in T cells lacking coronin 1.

This study confirms previous findings that coronin 1 is dispensable for the development of thymocytes using a defined set of surface markers known to be involved in the transition from late stage thymocytes to RTE. Additionally, there no defect was found in the survival of coronin 1-deficient RTE cells *in vivo*. As T cells are known to behave differently under T cell depleted conditions, newborn animals were used as early development is the only physiological healthy state in which animals are considered to be lymphodepleted. Interestingly, the data suggests a coronin 1-dependent switch after two weeks of age, with T cells numbers steadily increasing in wild-type mice situation but not in coronin 1-deficient animals. To assess if the peripheral lack of T cells was due to the inability of these cells to transduce survival signals, thymocytes in the last stage before egress were sorted and cultured *in vitro*. Similar to wild-type cells, the coronin 1-deficient thymocytes were capable of surviving on signals mediated via cell-cell contact with antigen-presenting cells such as dendritic cells (DCs) or by cytokines such as interleukin-7 (IL-7).

Taken together, this study shows a spatial and phenotypical elucidation of T cell development upon the deletion of coronin 1 over the first month of mouse life. Additionally it shows that coronin 1 is only needed for the survival of MNT cells but not of cells in earlier stages, as the earlier stages are (i) not decreased in numbers *in vivo* and (ii) are capable of survival by known pro-survival mechanisms.

4. <u>Thesis statement</u>

The work described here has been performed in the laboratory of Prof: Jean Pieters at the Biozentrum of the University of Basel. All work has been performed by Mathias Lang, except for the RNAseq data shown for PDE isoforms in conventional CD4+ T cells, which is kindly provided by Rajesh Jayachandran (Fig 12 D)

The work was supervised by the thesis committee consisting of

- Prof. Jean Pieters (Biozentrum Basel)
- Prof. Sebastian Hiller (Biozentrum Basel)
- Prof. Michel Steinmetz (Paul Scherrer Institut, Villingen)
- Prof. Daniela Finke (Department Biomedizin, Basel)

Some results are used in the following manuscripts:

Data on cAMP production in T cells:

A coronin 1-dependent cAMP/PKA/pCREB signaling axis in T cells is essential for graft rejection and dispensable for anti-microbial responses; Jayachandran *et al,* in preparation

Data on T cell development in newborn and adult animals and *in vitro* survival of late stage thymocytes:

A coronin 1-dependent decision switch in juvenile mice determines population of the peripheral naïve T cell compartment; Lang & Pieters, in preparation

5. Introduction

5.1. Overview of T cell development

The human body is continuously exposed to microorganisms. Not all of these microorganisms are detrimental to the health of the host organism; some even share a symbiotic relationship. However, the human body needs a mechanism to defend against infectious microorganisms. This role is taken over by the immune system, which by itself rests on two major pillars. The first line of defence is the innate immune system, consisting of cells which recognize signals upon an infection. Detection of signals is mediated by pattern recognition receptors (reviewed in (1)), which are further subdivided into pathogen-associated molecular pattern receptors (PAMP) or damage-associated molecular pattern receptors (DAMP). As the name suggests, the former as associated with an exogenous pathogen, such as bacteria or fungi whereas the second one is a response to endogenous signals such as cell damage. Upon recognition of a non-host molecule, the innate immune system mounts a response, initiated by specific receptors for defined sets of pathogen-associated molecular patterns such as parts of the bacterial cell wall (e.g. lipopolysaccharides) or the remains of nucleic acids from a virus called toll-like receptors (reviewed in (2)). The second component of the immune system is the adaptive immune system.

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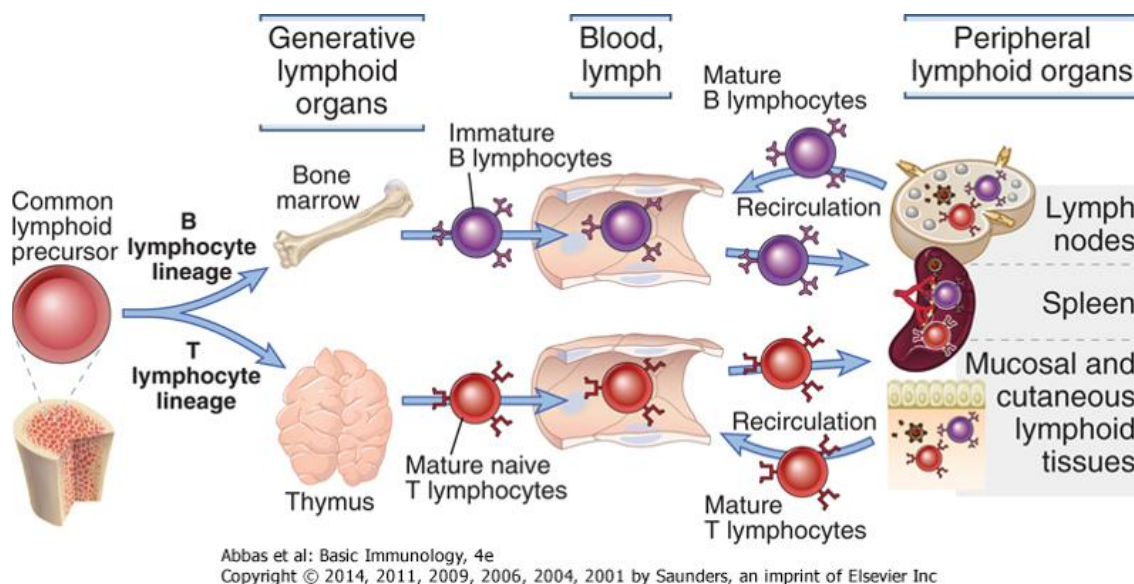
Fig 1: Life cycle of the two major players in the adaptive immune system, namely B and T cells. Both cell types originate in the bone marrow but their organ of differentiation differs. While B cells remain in the bone marrow, T cells migrate to the thymus for further maturation steps until both cell types then circulate through blood and secondary lymphoid organs.

As the name suggests, the main difference is the ability to adapt to different pathogens. The adaptive immune system consists mainly of B and T lymphocytes. Both cell lineages originate in the bone marrow as a common lymphoid progenitor cell, with B cells undergoing maturation steps in the bone marrow and the final maturation step in secondary lymphoid organs such as spleen or lymph nodes. T cell progenitors on the other hand, migrate from the bone marrow to the thymus, where they undergo a defined set of maturation steps (Fig 1). The mature thymocytes egress from the thymus to migrate to secondary lymphoid organs where they undergo further maturation steps into different T cell subsets.

5.1.1. *Thymocyte maturation*

T cell progenitors originate in the bone marrow as common lymphoid progenitor cells and migrate to the thymus. There they undergo four distinct maturation stages (Fig 2), named after the expression of CD4 and CD8 surface molecules (4, 5). During these four stages (double negative, double positive and CD4 or CD8 single positive stage) the cells rearrange their T cell receptor (TCR) and complete two selection processes (6, 7). The two selection processes, negative and positive selection, are needed to ensure that the mature T cells egressing from the thymus are (i) capable of mounting an immune response based on TCRα stimulus and (ii) do not mount immune responses against self-antigens, which could result in autoimmunity (8, 9). The maturation and survival of thymocytes is linked to their stimulation via TCR, depending on the

Fig 2: Development of thymocytes. Precursor cells enter the thymus and undergo a defined cascade of maturation steps from double negative cells to cells expressing either CD4 or CD8 molecules on the surface. Around 95% of thymocytes do not survive their maturation and either die by neglect or are actively killed (from (3)).

signal strength mediated the cells survive, are killed or die by neglect (10). As can be seen in Fig 4, semi-mature (SM) cells are single-positive for CD4 or CD8, but are still susceptible to apoptosis, mainly mediated by TNFα. Additionally, cells in the SM stage are behaving differently upon TCR stimulus: while mature thymocytes and peripheral T cells will respond with proliferation, the cells in the SM stage will die upon TCR engagement (11).

5.1.1.1. Rearrangement of the TCR

During the generation of T cells, the organism cannot anticipate which antigen the T cell will need to recognize. In fact, it's likely that the immune system might not even have encountered the antigen yet. To ensure that any antigen possible is covered by the immune system, the TCR locus is subjected to rearrangement procedures during the thymocyte double positive stage. Both α and β chains of the TCR consist of a Variable (V) and a Constant (C) region. While α chains consist of VJ arrangements, the β chain has a full set of VDJ segments. Fig 3 shows the genetic concept behind the different rearrangements. α and β gene segments are localized on two different gene loci. The gene undergoes somatic recombination (this mechanism has been elucidated by S. Tonegawa (12) and results in a DNA strand with VJ and C segments for the α chain; the β chain is made up from a strand with VDJ and C segments, resulting in roughly 2.5×10^7 possible combinations. The recombination depends highly on a specific pair on enzymes encoded by the recombination-activating genes (RAG), resulting in RAG1 and RAG2 (13). These two proteins are highly expressed during the TCR rearrangement and deletion of either leads to mice developing no T or B cells (as B cells depend on the same rearrangement mechanism for survival).

As this is a random process, the possibility exists to generate a T cell which would detect an endogenous self-molecule and therefore lead to an autoimmune disease. To avoid autoimmunity, cells undergo a negative selection step during the transition of double positive to single positive thymocytes and then migrate to the corticomedullary junction for exit. On this journey, thymocytes pass by special thymic endothelial cells, which present host-antigens (i.e. antigens which belong to the host and are presented on any cell in the body). If a thymocyte is triggered during this process, its migration is slowed down and the cell actively goes into apoptosis. This mechanism is mainly based on the pro-apoptotic BH3-only protein Bim, with animals lacking the expression of this protein showing severe autoimmune phenotypes (reviewed in (10)).
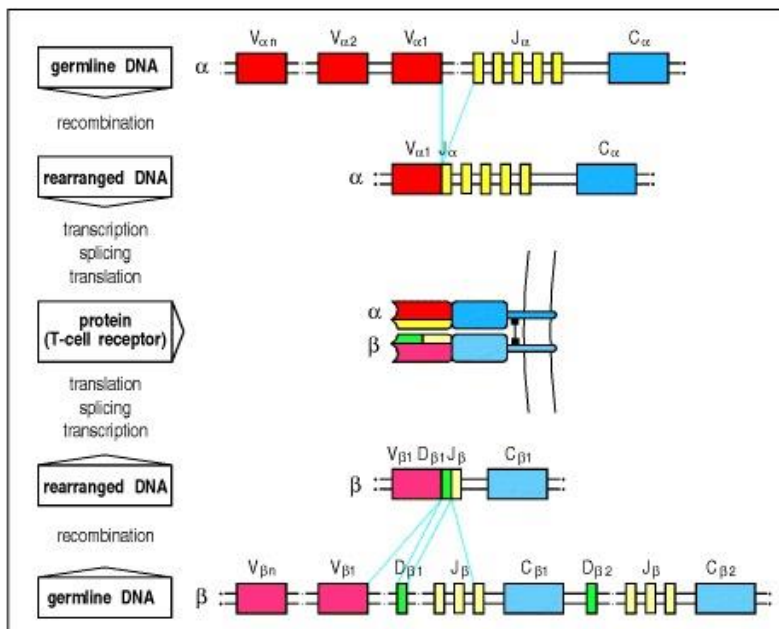
Fig 3: Schematic of the T cell receptor rearrangement. TCR α and β gene segments are joined by somatic recombination during thymocyte development. This results in every T cell having a different constellation of V, D and J segments and therefore leads to a wide possible coverage to detect antigens (from (14)).

5.1.1.2. *Single positive thymocytes*

During the last maturation stages from SM into the final mature 2 (M2) subset of thymocytes, the cells up- and down-regulate specific surface markers and transcription factors. It has been shown that thymocytes that have passed the double positive stage are still not responding fully to peripheral signals via the TCR. While mature thymocytes and peripheral T cells respond to a stimulus via TCR by proliferation, SM thymocytes go into apoptosis (11). It therefore became clear that thymocytes have not completed their maturation once they have passed the double positive stage. Thereafter they upregulate certain molecules (e.g. Qa2, MHC-I, CD62L or S1P1) and downregulate others (e.g. CD69, CD24, CCR9) (4, 15–17) to achieve a further mature state.

However, identification and isolation of this early T cell subset remained a challenge (described in further detail in 5.1.2.1), but one elegant solution was provided by Nussenzweig in 1999 and adapted by Fink *et al.* (18, 19). By taking advantage of the specific expression pattern of the RAG genes, they generated mice which produce GFP under the control of RAG2 regulatory elements. This leads to the expression of GFP before positive selection and the degradation of the GFP molecule can be used as a molecular clock to assess the age of a cell (19). With the help of this model system, Hogquist *et al.* could validate a list of several surface markers, which are differentially expressed during thymocyte maturation (Fig 4 and Table 1). The validation of the specific surface markers for, not only RTE, but also very late stages of thymocytes now allows for a better temporal resolution on thymocyte and early T cell development.
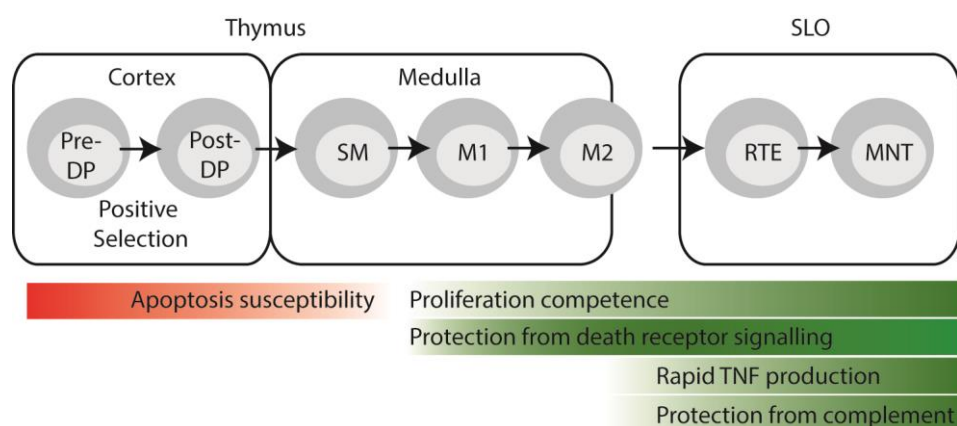
Fig 4 and Table 1: Development from mature thymocytes to mature naive T cells (adapted from Hogquist et al, 2015). As cells progress through positive and negative selection, they migrate from the cortex into the medulla. During this journey, they acquire proliferation competence, protection from death receptor signalling and, once egressed from the thymus, cytokine production and protection from complement. These are tightly regulated processes in which the cells need to up- or downregulate a specific set of proteins including surface markers or transcription factors. DP Double-Positive, SM Semi-Mature, RTE Recent Thymic Emigrants, MNT Mature Naïve T cells.

	Pre-DP	Post-DP	SM	M1	M2	RTE	MNT
TCR-β	-/+	+	+	+	+	+	+
CD24	++++	++++	++++	+++	++	+	-
CD62L	-	-	-	-	+	+	+
CD69	-	+	+	+	-	-	-
S1P1	-	-	-	-	+	+	+
Qa2	-	-	-	-	+	++	+++
CCR7	-	-	-/+	+	+	+	+
CCR9	+	+	-/+	-	-	-	-
CD45RB	-	-	-	-	-/+	+	++
IL-7Rα	-	-	-	-/+	+	+	++
CD55/DAF	-	-	-	-	-/+	+	++
Coronin 1	-/+	-/+	+	+	+	+	+
KLF-2	-	-	-	-/+	+	+	+
Bcl-2	-	+	+	+	+	+	+

5.1.1.3.   Important proteins in single-positive thymocyte maturation and thymic egress

During thymocyte maturation, the cell needs to be protected from death receptor signalling via TNFα. This has been shown to be mainly mediated by the transcription machinery surrounding the NFκB protein complex (reviewed in (20)). In the stages of M1 or M2, this axis is mediated by the protein kinase TAK1 (21). Therefore, mice having a CD4-CRE knock-out of TAK1 kinase lack the maturation of thymocytes into the M1 or M2 stages. The single positive thymocytes which are present in the animals lacking TAK1 are therefore susceptible to apoptosis induced by TNFα. Additionally, upon activation of NFκB in thymocytes, the cells start to express an anti-apoptotic molecule called c-FLIP. Mice which lack this specific protein show a blockage at the step of SM to M1 stage during thymocyte development (22).

Beside the protection from death signals, mature thymocytes also acquire surface markers needed for finding their niche once they egress into the bloodstream. The main driving factor for this is the transcription factor Krüppel-Like Factor 2 (KLF2), which is expressed at the late M1 stage (23). Once this factor is activated, it subsequently induces the expression of two surface markers needed for thymocyte egress, CD62L and S1P1 (24). While CD62L (or L-Selectin) belongs to the family of selectins, the sphingosine-1-phosphate receptor 1 (S1P1) is a G-protein coupled receptor. S1P1 is used in lymphoid cells for the thymic egress (25), but also has been postulated to be involved in the suppression of the innate immune system. Outside of the immune system it is involved in vasculogenesis, which is also the reason why S1P1 knock-out animals die during development. CD62L is needed by T cells to home into secondary lymphoid organs (SLO). The lack of either of these proteins leads to the accumulation of single positive thymocytes in the cortical region of the thymus. While CD62L and S1P1 are upregulated during thymocyte maturation, other proteins are downregulated. It has been shown that cells upregulate CD69 during thymocyte selection processes, but its expression acts inhibitory on the expression of S1P1 (26, 27). Interestingly, animals deficient for CD69 show normal peripheral T lymphocyte numbers (28). Additionally, the chemokine receptor CCR7 is needed for normal egress of thymocytes (29), as it mediates the migration of cells from cortex to medulla (16). It has been postulated that the egress of thymocytes is a chemokine gradient dependent progress, where cells migrate within 4 days of becoming single positive from cortex to medulla and then to the corticomedullary junction site (30). While the start of this migration progress is CCR7 dependent, later stages depend on the GPCR receptor S1P1, which has a high concentration of its ligand in the bloodstream (31, 32).

Another important protein for thymocyte and T cell development is Ubc13. This E2 ubiquitin-conjugating enzyme is expressed in immune cells and knock-out mice show defect in B-cell receptor, TLR/IL1-R and CD40 activated signalling cascades. A knock-out of the Ubc13 protein using a Lck_CRE system has revealed a role of Ubc13 in thymocyte development and peripheral T cell survival (33). NFκB signalling is only partially affected, interestingly also phosphorylation of TAK1 was abolished. Despite this, thymocyte numbers are comparable to wild-type. However, thymocytes show defective proliferation upon CD3/CD28 or PMA/Ionophore stimulation. Similar to coronin 1-deficient animals (discussed in following chapters), the animals have a severe defect in the survival of peripheral T cells.

As thymocyte development is a complex cascade of different signals, these are only examples and a more complete list of different proteins involved in thymocyte maturation and egress has been recently reviewed by Hogquist *et al* (34).

5.1.2. *Identification and functional capacity of recent thymic emigrants*

5.1.2.1. *Means of identification and isolation of recent thymic emigrants*

The murine thymus is an organ whose function was originally discovered by Jacques Miller around 1960. Over the years a lot of research has been done to investigate the capabilities of the thymus to produce T cells, including studies on the timeframe for an average T cell to mature from bone marrow to thymus. The timeframe has been set around 3 weeks in mice from bone marrow to thymic egress, with roughly $1x10^6$ cells/day egressing (35, 36). Only mid 80s it became clear that there is post-thymic maturation and that an egressed thymocyte is functionally and phenotypically different from a MNT cell (31, 37) and the cells were coined recent thymic emigrants (RTE). This first identification was done by injection of the fluorescent dye FITC directly into the thymus and subsequent analysis of labelled cells by flow cytometry. This is a fairly stressful procedure for the mice as it requires surgery. Additionally, it also labels any cell which recirculates from the periphery into the thymus and thus not exclusively RTEs. Another method to identify RTEs has been established for human samples and then introduced into the murine system. It depends on the TCR rearrangement procedure (see Fig 3). The somatic recombination used during this important step of thymocyte maturation results in non-replicative DNA segments (Fig 5), so called T cell receptor excision circles (TREC). These episomal DNA

circles are then diluted with every cell division As RTEs have just left the thymus and have not undergone many cell divisions, they contain a higher number of excision circles and TREC molecules can be measured by qPCR. One culprit in this identification process is the fact that cells cannot be used for further studies, as the detection method depends on the isolation of genomic DNA.
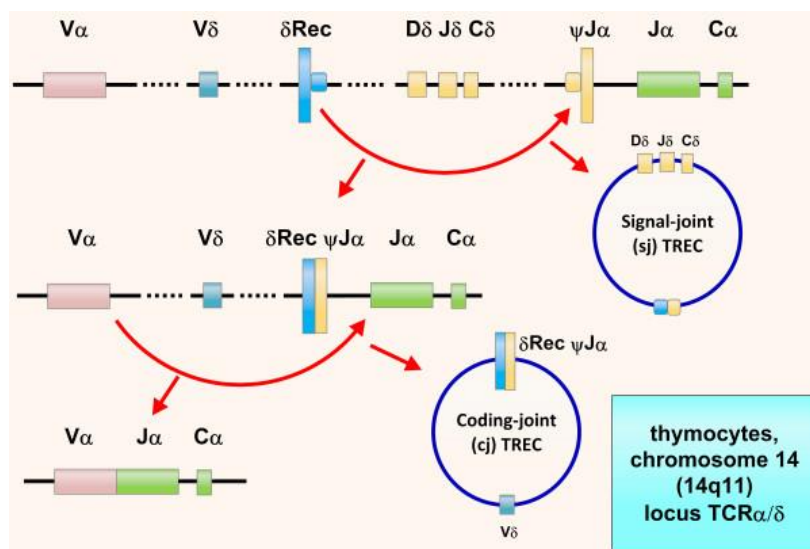
Fig 5: The generation of T Cell Receptor Excision Circles (TREC). The somatic recombination of TCR gene loci to produce different DNA segments for TCR α and β chains, results in signal-joint (sj) and coding-joint (cj) TRECs, which remain as non-replicated episomal DNA fragments and are diluted over time by cell division (from (14)).

More recently another system used for identification of different thymocyte stages has been developed. The RAG2p-GFP mice, originally used to track the RAG expression in B cells (18), have been adapted to be used to study RTEs ((38) and mentioned in section 0). This allows for phenotypical and, more importantly, functional assessment of RTE cells as cells can be easily sorted by FACS.

5.1.2.2. *Function of recent thymic emigrants*

In terms of population dynamic, RTEs are inferior at populating the T cell niche compared to MNT when used in competitive adoptive transfer experiments (i.e. injection of MNT and RTEs), but better than MNTs when injected separately (39). RTEs have higher expression of CD24, which could explain their better potential as this surface markers has been shown to be key for homoeostatic proliferation (40). Additionally, in a physiological healthy organism, RTEs and MNTs should not encounter the possibility of competing for repopulation of the T cell niche. After thymic egress, RTEs downregulate certain surface markers, including CD24 and upregulate others such as Qa2 or CD45RB. Additionally, cells upregulate proteins that are involved in protecting cells from attacks by the complement system. This has been shown using mice with a

CD4-CRE driven knock-out of the NFκB Activating Protein (NKAP). While these mice have a normal development of thymocytes and thymocyte egress, the resulting RTEs do not mature into MNTs (41). This maturation defect has been shown to be due to the defective expression of the complement decay-accelerating factor CD55, which prevents the formation of the membrane attack complex (42). Functionally RTEs have been shown to express lower levels of IL-7 receptor (43) and higher expression of TCR-CD3 complexes. RTEs also produce less interleukin-2 (IL-2) when adoptively transferred (44). The IL-2 production is comparable to the production by MNTs one week after the transfer, indicating that the injected cells undergo maturation steps in the periphery. Additionally studies have shown RTEs to be enriched for IL-4 producers for at least three days after they have egressed from the thymus (45), on the other hand, RTEs isolated and stimulated *in vitro* without the addition of cytokines produce less IL-4, IL-2 and interferon-γ (IFN-γ) (38, 46). In the same assays, the RTE cells also show reduced proliferation compared to mature counterparts. Overall, RTEs are a defined subset which is needed for the population of the T cell niche, but also for continuing replenishment.

5.1.2.3.   *Maturation of recent thymic emigrants to mature naïve T cells*

What exactly drives the maturation of RTE to MNT cells and how their survival is exactly regulated is currently not fully understood. Thus far the only proven factor to be needed is the requirement of cells to enter secondary lymphoid organs, as restraining the cells in the bloodstream by blocking VLA4 (very late antigen 4) and CD62L leads to no further maturation of RTEs (47). The second known requirement is an intact compartment of dendritic cells (DC), as it is mandatory for the maturation of RTEs to MNTs (48). In terms of survival, the interaction between TCR and major histocompatibility complex is a major factor for MNT cells (49), but is discussed for RTEs (50). The second major player in peripheral T cell survival, IL-7 (51), has recently been linked to an increase in the pro-survival molecule Bcl-2 in RTEs (43), suggesting a possible RTE survival mechanism. The exact survival dependencies of RTEs and how they differ from the survival needs of a MNT cell are therefore not yet fully elucidated and remain a topic of discussion.

5.2. The development of the immune system in mice

In the murine embryo, the bulk of haematopoiesis happens in the fetal liver. While there are hematopoietic stem cells (HSC) present in a rodent fetus, they do not undergo steps for lineage differentiation until embryonic day 10 (E10) (52, 53) and, at least in terms of B cell lineage, no mature lymphocytes are present until E18 (54). The thymus, as the main precursor organ for mature T lymphocytes, is formed around E10 in mice, with T cell precursor cells populating the developing thymus from E11 on (52). As thymocyte development takes around 12 days in the thymus, this would result in mature T lymphocytes egressing into the bloodstream of newborn animals within 3 days of birth (see Fig 6 for scheme). At E17, the primary hematopoietic tissue switches from the fetal liver to the bone marrow and by the time of birth (after 21 days of gestation), the liver has no hematopoietic properties anymore and all hematopoietic cells originate from the bone marrow. As this is a continuous process, it results in a chimeric hematopoietic system in newborn and young mice, with some cells having originated from pre-natal (hepatic) and others from post-natal (bone marrow) HSCs (55, 56). In terms of egress capacity, as soon as T cells originate from the thymus, the egress rates are comparable between newborn and adult animals (35).
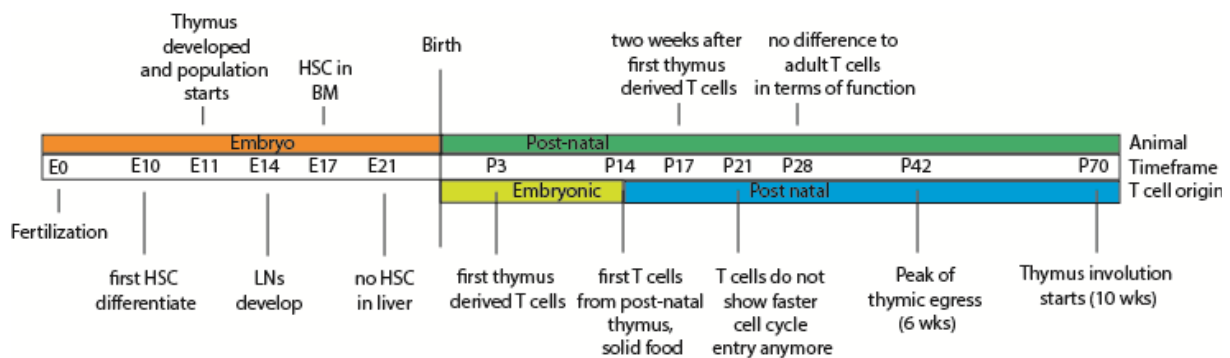
Fig 6: Schematic of T cell development during embryonic and post-natal stages based on literature reviewed in (57, 58). In the embryo, the fetal liver is the main hematopoietic organ, until this role is transferred to the bone marrow by E17. The thymus as the main T cell producing organ is developed by E11 and populated by progenitor cells. Due to the roughly two week timeframe for thymocyte maturation, recent thymic emigrants in the first two weeks after birth are of fetal liver origin. By P14, animals start to eat solid food and show the first T cells which have originated via the adult-like BM → Thymus axis.

5.2.1. *Differences in newborn and adult T cells*

Until the mid-90s, newborn rodents were considered immunologically immature, with their T cells not being able to mount immune responses in the same manner as cells obtained from adult animals do (reviewed in (57)). However, this idea stems from experiments analysing the production of IL-2 and for the proliferation potential of T cells. This concept was questioned with the increased knowledge on different T cell populations, namely the T helper type 1 (Th1) and type 2 cells (Th2). It became clear that the lack of IL-2 production from neonatal T cells was due to a bias for the Th2 lineage (59). Additionally, stimulation of neonatal T cells via the TCR results, in contrast to adult cells, not in proliferation but in apoptosis (60). Also, the neonatal T cells show reduced production of IL-2 and IFN-γ, both markers for Th1 lineage, and increased production of IL-4, marker for Th2 lineage (61). Taken together, this indicated that the idea of immature immune responses of neonatal T cells was actually more an experimental problem and that not enough was known about the system to assess functional maturity. Indeed in 1996, three papers showed that T cells obtained from neonatal animals (up to 7 days after birth) are capable of mounting adult immune responses, but only when triggered with "correct" stimulations. It became clear that neonatal mice can mount mature cytotoxic T lymphocyte responses, shown by injection of mature male dendritic cells into female neonates (62). Additionally, when considering the bias to Th2 responses, neonates injected with antigen under Th1 promoting conditions responded with a Th1 polarized adult like response (63). The third paper proving adult like immune responses in neonates used reduced doses of virus. While an adult level dose would result in Th2 cell response and no protection from the disease, neonates responded with adult-like Th1 and Th2 responses resulting in a protective cytotoxic T lymphocyte response to dosage adapted for their smaller size (64).

Thus, while neonatal T cells can mount an adult immune response, it does not happen very often. One emerging hypothesis is that, as newborn animals encounter an immense amount of new and unseen antigens, the developing organism would be in a continuous inflammatory stage. This would not only deplete resources which the organism needs to develop itself, but also could damage the developing organs (reviewed in (57)). While there are still questions to be answered, it

became clear that the developing immune system is under tight regulation to ensure growth and development of the animal while keeping it safe from potentially life-threating infections.

5.2.1.1.   *T cell population kinetics in newborn animals*

Many studies have analysed the function of neonatal T cells and their differences, but studies on developmental kinetics are rare (reviewed in (65)). It has been shown that the T cell niche is filled in waves, with the cells from the first two weeks originating from the fetal HSCs and being functionally different from the second wave (66). This study has been done by grafting thymi of newborn animals in adult mice, therefore additionally altering the environment that the egressed T cells encounter. Other studies used a more physiological approach by assessing *in vivo* data on splenocytes after injection with antigen presenting cells (62). This study reports around 1.5x10^6 peripheral T cells on day 1 in uninjected conditions, with the number rising to 20x10^6 within the next two days. The authors also report a slowing down of this massive increase in peripheral T cell numbers during the first three days to roughly doubling their numbers every week. As expected, all cells present in a newborn animal would be considered RTEs, with the number of RTEs declining around 2 weeks (19). In terms of the function of these neonatal cells, most knowledge has been gained using *in vitro* assays and assessing their cytokine production, apoptosis or proliferation (61, 66–69). However, one recent study has used more modern immunological methods combining flow cytometry and TCR transgenic mice (70). These authors found that stimulation of neonates and adult animals in a transgenic background, where the TCR can be triggered with a specific peptide, cells of neonates and adults proliferate equally. In the same study, when wild-type mice are injected with CD3 antibodies or the TCR stimulus is mimicked by PMA/ionomycin stimulation, the proliferation is higher in adult animals (reviewed in (57)). This goes along the lines of newborn animals needing a powerful stimulus to mount adult like responses. Additionally, the same study has shown proliferation rates for T cells, with neonatal T cells showing faster cell cycle progression. This difference vanishes with age, with T cells obtained from animals above 3 weeks of age having the same entry into cell cycle as adults (71). The most recent study on the differences of neonatal RTEs and their adult counterparts comes from Opiela *et al.* (72). There, authors have used the RAG2p-GFP system to compare neonatal 7 days old RTEs to RTEs from adult animals and show differences in the surface expression of

known RTE markers such as Qa2 and CD24 but also CD28. Additionally, they could show that when looking only at the RTE population, neonatal cells produce more IL-2 and Th1/2 effector cytokines. The most striking difference for survival is the different response of neonatal RTEs to IL-7. While an adult cell takes around 6 hours to downregulate the IL-7 receptor after stimulation, this occurs in neonatal RTEs after only 30 minutes. This goes in line with an increased level of the downstream target pSTAT5. It has been shown that IL-7 is a limiting factor in the repletion of T cells (73) and that this cytokine is mainly produced by stromal cells (74). Given the fact that adult RTEs do not respond with proliferation upon IL-7 production (75) and that neonates have higher levels of IL-7 (76), this would suggest a role for IL-7 in the homoeostatic proliferation during the lymphodepleted stages of animal development. This is further strengthened by data from human samples, showing an increase in IL-7 upon lymphodepletion (77) and that cord blood derived CD4 cells proliferate upon stimulation with IL-7 but not cells obtained from peripheral blood samples (78). Taken together, RTEs from newborn and adult animals are functionally different, but RTEs from newborn animals can be pushed to show adult-like features. It is however unclear if the differences are due to the RTEs of newborns being from hepatic origin or because of differences in the thymic microenvironment during development.
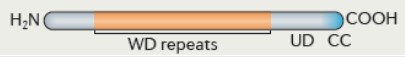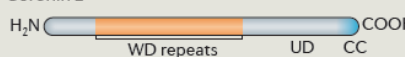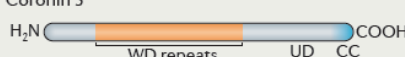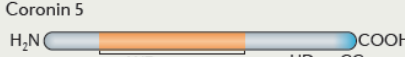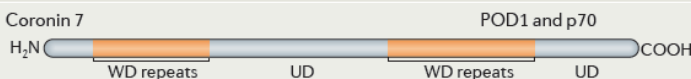
5.3. Coronin Protein Family

5.3.1. Discovery of Coronins

Coronin A was originally identified in the slime mold *Dictyostelium discoideum* as a molecule co-eluting with an actin/myosin complex (79). Taken together with the report that coronin deletion resulted in impaired particle uptake, chemotaxis and cytokinesis it was concluded that coronin is an actin-binding and modulating molecule (80).

With the onset of gene sequencing, it became clear that this protein is not restricted to *D. discoideum*. It has now been shown that the coronins constitute a protein family occurring throughout the eukaryotic kingdom, with 723 coronin molecules so far identified in 358 species (82). In mammals, the protein family consists of seven members, which are characterised by a WD-containing repeat domain followed by a unique domain (UD) (see Fig 7). In terms of structure, the WD-repeat domains and their flanking with β-sheets form a seven bladed β propeller (83, 84). The UD domain is of variable length and serves as a linker for the C-terminal coiled-coil domain (84). However, there is also coronin 7, a tandem version of coronin, lacking the coiled coil domain, but having two WD and UD fused together. In terms of expression

Coronin*	Alternative names	Chromosomal location		Protein length (amino acids)	
		Humans	Mice	Humans	Mice
Coronin 1	CORO1A, p57, TACO, CLIPINA, CRN 4 and CLABP	16p11.2	7F3	461	461
Coronin 2	CORO1B, Coroninse, p66, and CRN1	11q13.2	19A	489	484
Coronin 3	CORO1C, HCRNN4 and CRN2	12q24.1	5F	474	474
Coronin 4	CORO2A, IR10, CLIPINB, WDR2 and CRN5	9q22.3	4B1	525	524
Coronin 5	CORO2B, CLIPINC, and CRN6	15q23	9B	480	480
Coronin 6	CLIPINE	17q11.2	11B5	472	471
Coronin 7	POD1 and p70	16p13.3	16A1	925	922

CC, coiled-coil domain; UD, unique domain; WD repeats, tryptophan–aspartate repeats. *The nomenclature and phylogeny for coronin proteins has been subject to many changes; novel strategies to classify the different coronin proteins continue to result in the generation of new phylogenies, which are accompanied by novel names[1,7]. The original nomenclature for mammalian coronin 1–coronin 7 (REFS 5, 8) has been adopted in this table to avoid confusion that might arise from any new classification approaches.

Fig 7: Classification and schematic of the coronin protein family (from (81))

patterns, coronin 2, 3 and 4 appear to be ubiquitously expressed while the other members are specifically expressed in different tissues (our unpublished data and (85)).

5.3.2. *The coronin 1 protein*

5.3.2.1. *Structure and function*

Coronin 1, localising to the plasma membrane in a cholesterol dependent manner (86), has originally been identified in our laboratory as a factor actively recruited by mycobacteria (87, 88). Coronin 1 recruitment to the phagosome leads to an increased survival of phagocytosed mycobacteria in macrophages. This recruitment depends on the trimerization of coronin 1 (89) and phosphorylation by CDK5 (90). Once mycobacteria are internalized, phagosome-lysosome fusion is blocked, as long as coronin 1 is present on the membrane of the phagosome. This is only observed with living mycobacteria and has been shown to be dependent on calcineurin (88, 91). With coronin 1 being expressed mainly in cells of the immune system and excitatory neurons, most studies focused on the role of coronin 1 in cells of the immune system. For the majority of immune cells, coronin 1 is dispensable for their function or survival. No differences could be seen in the dendritic cell compartment (92) or neutrophil function (93). The same holds true for natural killer cells (94). B cells have no survival deficit and are capable of generating immune responses *in vivo* regardless of coronin 1 expression status. The only difference can be seen in IgM mediated Ca^{2+} response, which is reduced upon the deletion of coronin 1 (95). This is also seen in T cells where the lack of coronin 1 has been shown to result in a diminished Ca^{2+} mobilization and inositol-1,4,5-trisphosphate generation upon TCR triggering (96), that has been linked to the severe T cell lymphocytopenia observed in coronin 1-deficient animals (96–100). The reduced Ca^{2+} results in an impaired activation of the $Ca2^+$ dependent phosphatase calcineurin, which is itself responsible for the dephosphorylation of the Nucleated Factor of Activated T cells (NFAT) (see Fig 8). This transcription factor has been shown to be not only involved in the activation of T cells but also in their regulation and survival (reviewed in (101)).
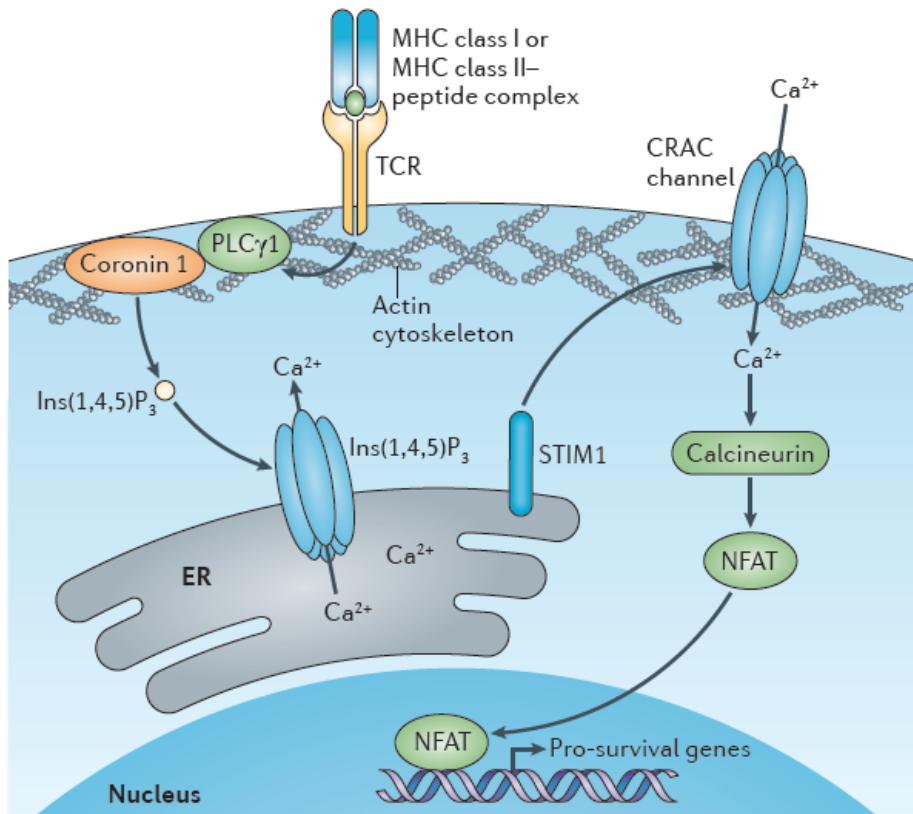
Fig 8: Coronin 1 dependent T cell survival. The lack of coronin 1 leads to reduced levels of Ins(1,4,5)P$_3$ and subsequent reduced levels of intracellular Ca^{2+}. This results, via the Ca^{2+} dependent phosphatase Calcineurin, to a reduced activation of NFAT and therefore reduced transcription of pro-survival genes (from (81)).

As peripheral T cells depend on tonic TCR:MHC stimulus for survival (102, 103), it has been suggested that the impaired survival of coronin 1 deficient T cells is due to their limited response to TCR mediated stimuli. It needs to be noted, however, that coronin 1-deficient animals with a transgenic TCR show similar response in terms of proliferation to TCR stimulus as wild-type animals (97) and our unpublished observations). This could indicate that a strong enough trigger would still be able to yield a response and that the TCR signalling is not completely defective but dampened. Additionally, while coronin 1 has been postulated as an actin-modulating protein, the migration and homeostasis of T cells has been shown to be independent of any coronin 1-dependent actin modulation (104) and intracellular Ca^{2+} stores are not affected by actin modulation (105).

5.3.2.2.   Coronin 1 in thymocyte development and thymic egress

During thymocyte development, the cells rearrange their TCR and only if signalling via TCR is functioning, the cells survive (10). If coronin 1-deficient T cells show dampened TCR responses, one would expect coronin 1 deletion to influence thymocyte survival as well. While the expression of coronin 1 is present in all immune cells, its expression pattern is not uniform. In fact, thymocytes show a differential activation of the coronin 1 promoter activity and protein expression during their maturation steps (this study and (106)). While a DN thymocyte does express coronin 1, the expression increases with maturation and only SP thymocytes express similar levels of coronin 1 compared to peripheral T cells. Interestingly, while peripheral T cells show reduced Ca^{2+} mobilization upon TCR stimulus, this phenotype is not seen in DP thymocytes (our unpublished observations), further indicating that the influence of coronin 1 on TCR signalling is only present after TCR rearrangements are completed. Therefore, coronin 1 would only be fully expressed by the time thymocytes go through negative selection and are sieved out for any self-recognition. This process itself is either not-impaired in coronin 1-deficient animals, maybe due to strong enough signals coming through the TCR, or the peripheral survival deficit results in too few T cells to actually develop an autoimmune disease, as coronin 1-deficient animals show no induction of EAE (107) or lupus (98).

In terms of thymocyte populations, the literature for coronin 1-deficient animals is diverse. Coronin 1 has been linked to a thymic egress phenotype in one specific study, characterising a mouse harbouring a point mutation of coronin 1 (*Ptcd*). Expression of the mutant coronin 1 protein leads to an increased inhibition of the Arp2/3 actin regulator complex. In terms of thymus population, the study shows equal numbers of DP cells but an increase in SP CD4 and CD8 cells (99). Additionally, the *Ptcd* mutants show impaired thymocyte migration to a S1P1 or CCL21 gradient, which has also been shown in coronin 1-deficient thymocytes (104). Both receptors (S1P1 and CCR7) have been shown to be expressed in normal levels in coronin 1-deficient thymocytes ((97, 99) and this study). While CCL21 is a shared ligand for CCR7, it has been shown that it is not important for thymocyte migration (29), whereas S1P1 is needed for correct thymocyte maturation and egress (25). The observed *in vitro* migratory phenotype could explain the accumulation of single-positive thymocytes in the *Ptcd* mutant. However, the same study also characterises a coronin 1-deficient animal from an ENU mutagenesis screen, which showed no accumulation of single positive thymocytes, but, similar to published literature from gene edited animals, a reduction in peripheral T lymphocytes. While studies have suggested an

involvement of F-actin for coronin 1-dependent T cell survival, the *Ptcd* mutant actually shows a stronger phalloidin staining in comparison to the wild-type, failing to show a simple correlation. Additionally no difference in apoptosis between wild-type and *Ptcd* mutant animals could be found. However, studies have reported an increase in cell death in *in vitro* studied thymocytes of coronin 1-deficient animals (both from ENU mutagenesis as well as gene edited animals (99, 104). In contrast to Shiow *et al.* describing an accumulation, others have shown a reduction in single positive thymocytes, but only CD8 positive ones with no differences in any other thymocyte population (97). However, the original publication for the coronin 1 knock-out mouse also shows a slight reduction of single positive CD4 thymocytes when the data is analysed for the late stage markers CD69 and CD62L, displaying roughly half the percentage for late stage thymocytes (97). Additionally, it has been reported that intrathymic injection of FITC leads to less peripheral FITC$^+$ cells in coronin 1-deficient animals. As they do not report an accumulation of FITC$^+$ cells in the thymus, this would indicate normal thymic egress but followed by death upon cells entering the bloodstream. The last study showing coronin 1 involvement in thymocyte development reports again a different picture. In this case, the reduction of late stage thymocytes is not due to an egress phenotype but due to an accumulation of double positive cells (108). The same study also describes a differential expression of certain late stage surface markers for thymocytes such as CD24, CD69 and TCR, which can partially be explained by the reduction of overall SP cells. It is however striking that this specific knock-out mouse is the only one showing an accumulation of cells during thymocyte maturation. One intriguing observation is the single-positive CD8 cells displaying increased levels of pJNK1/2 and reduced levels of p-IκB upon stimulation with CD3/CD28. As the NFκB pathway is involved in thymocyte maturation and survival, the increase in double-positive thymocytes could be due to a defect in thymocyte maturation.

Taken together, the data on thymic egress in coronin 1-deficient or coronin 1-mutated animals is contradictory. What is however clear from the several studies with different mouse models but also in human patients is the importance of coronin 1 in peripheral T cell survival.

5.4. GPCR signalling and its downstream messenger cAMP

5.4.1.   *Seven transmembrane or G protein-coupled receptors*

5.4.1.1.   *General mechanism and structure*

The receptor family consists of seven transmembrane domains, with the C-terminal end linked to heterotrimeric G-protein complex. This complex consists of three different subunits, namely Gα, Gβ and Gγ. While Gβ and γ are similar for all G-protein coupled receptors, the Gα subunit comes in different forms (110, 111). Depending on the different Gα isoform, the second messenger is either cAMP (for Gα-s or Gα-i subunits) or Ca^{2+} and subsequent activation of Phospholipase C (Gα-q or Gα-12/13). Once a ligand binds to the extracellular domain of a GPCR, the activated receptor acts as a guanine nucleotide exchange factor and swaps the GDP bound to the Gαβγ complex to a GTP. This leads to the disassociation of the Gα subunit from the βγ complex and the Gα subunit is free to provide downstream signalling (Fig 9). In case of
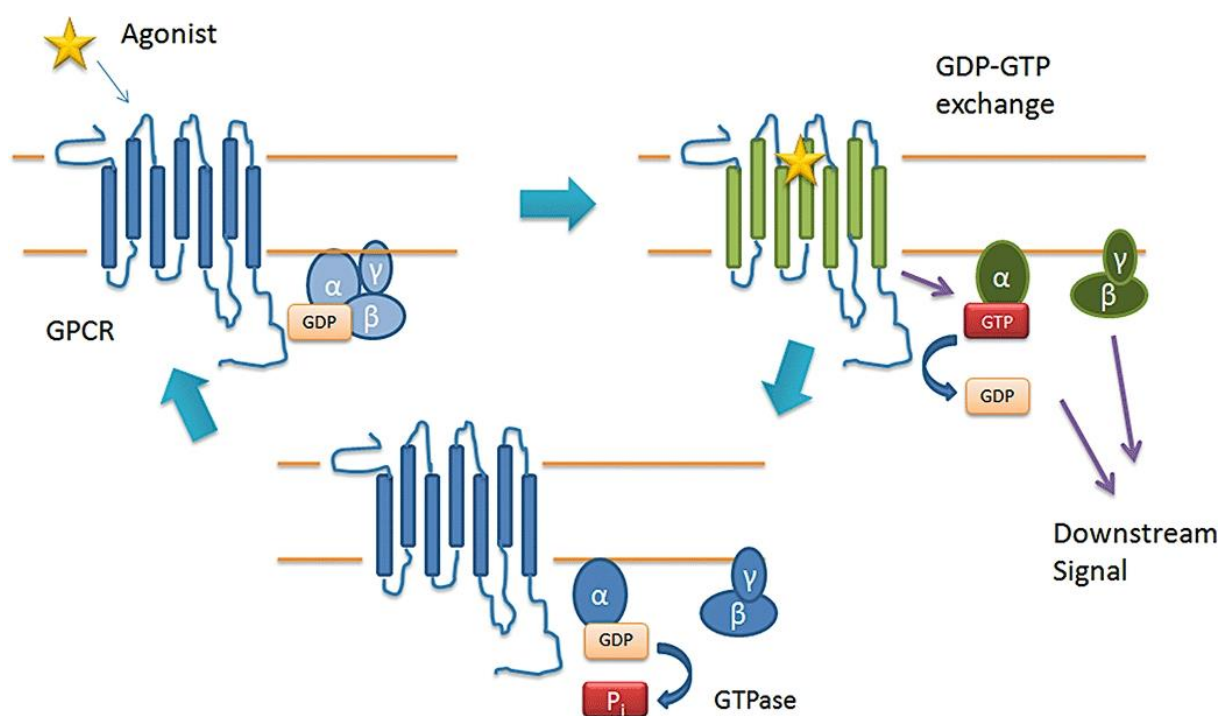
Fig 9: Simplified schematic of G-protein coupled receptor activation. Ligand-bound GPCR acts as a guanine nucleotide exchange factor and therefore exchange GDP to GTP at the Gαβγ complex. The now GTP-bound Gα subunit disassociates from the βγ G-proteins and provides downstream signalling until GTP is hydrolysed back to GDP and the Gα protein can associate again with the βγ complex (from (109))

the Gα-s (stimulatory) subunit, the downstream signal is the activation of the adenylate cyclase (AC) enzyme and subsequent increase in cAMP production. Gα-i (inhibitory) subunit leads to either the inhibition of the adenylate cyclase or the activation of phosphodiesterases, resulting in a reduced production or increased degradation of cAMP respectively (112).

5.4.1.2.   *The structural homology of coronin 1 to G-protein subunit β*

In unstimulated conditions, the Gα-s subunit is linked to the βγ complex via salt bridges. Interestingly, the Gβ subunit is a WD-repeat domain containing protein (113) with structural similarities to coronin 1 (83). Recently, coronin 1 has been linked to cAMP production in neurons, where it is needed to ensure sufficient cAMP production for the correct formation of long term memory (114). In addition to this, coronin 1 was associated with Gα-s in a stimulus-dependent manner. Therefore, with in coronin 1-deficient neurons, the production of cAMP is reduced, where fibroblasts with exogenous expression of coronin 1 show higher levels of cAMP production. As the association is only seen with the stimulatory subunit, but not with the inhibitory, the coronin 1 – Gα-s association was more closely studied. Indeed, shown by surface plasma resonance, the association of coronin 1 with the Gα-s subunit depends on the same salt bridges as the Gα-s and Gβ interaction (Pieters J, Steinmetz M and BosedasGupta S, unpublished observations). This would indicate the existence of a coronin 1-dependent shuttling mechanism of the GTP bound Gα-s subunit and a role of coronin 1 in the production of cAMP.

5.4.2.   *Generation and downstream signalling of cAMP in T cells and thymocytes*

cAMP was the first second messenger identified which exerts an intracellular function upon the stimulation by an extracellular ligand (115). As the GPCR-cAMP signalling cascade is ubiquitously used in all cell types, it is also present in T cells. There it is responsible for the downstream signalling of different ligand stimulations such as prostaglandins (116), histamines (117) and β-adrenergic agonists (118). As in other cell types, the cAMP is produced by ACs, which exist in 10 different isoforms (119), In case of T cells, AC 3, 6 and 9 are expressed at low levels whereas AC 7 is expressed in higher levels (120). Indeed, AC 7 has an impact on T cell mediated antibody responses and the deletion of the protein leads to an impairment in the

generation of memory T cells (120). While the other three isoforms are expressed at lower levels, AC 3 and 6 are localized to cholesterol rich membrane sections (121, 122), possibly close to the localization of coronin 1 (86). To reduce the levels of intracellular cAMP, cells express specific enzymes which degrade cAMP to AMP (123), the family of phosphodiesterases (PDE). While over 100 different enzymatic variants have been identified and classified into eleven families (124), the main PDEs in T cells are PDE 1-5, 7 and 8. It has been shown that of the seven PDEs expressed, PDE 4 is the major cAMP degradation enzyme in T cells (125, 126). In T cells, the main downstream effector of cAMP is the protein kinase A (PKA). This serine/threonine kinase consists of two catalytic subunits linked to one regulatory unit. As soon as two cAMP molecules bind, the catalytic subunits are released and phosphorylate downstream targets (127). While there are different PKA subunits, namely four regulatory and three catalytic ones, the major form present in T cells is the type I PKA isoform (around 80%), the remaining part is type II PKA (128). The deletion of type II PKA has no major phenotype, while in contrast mice lacking type I do not survive embryonic development (129, 130). An inducible anti-sense system for type I PKA has however revealed a role in tumour development, with animals developing lymphomas with age (131).

5.4.2.1. The effect of cAMP on T cell function

T cells can be activated by different signals with the physiological most relevant one being the presentation of an antigen, which is subsequently detected by the TCR. The antigen binding leads to the release of Ca^{2+} from intracellular stores (see Fig 8 for scheme) but also to the production of cAMP, possibly due to an increase in cyclooxygenase 2 (COX2) and subsequent prostaglandin production (132). The engagement of CD3/TCR depends on the protein kinase Lck but does not lead to a full activation of the T cell. It needs co-stimulatory molecules, mainly either CD28 or IL-2 (133). As depicted in Fig 10, an increase in cAMP due to extracellular stimulation by e.g. prostaglandins, leads to activation of PKA type I linked to A kinase anchoring proteins (AKAP) (134). After the subsequent recruitment of phosphoproteins associated with glycosphingolipid–enriched membrane domains (Cbp/PAG), the tyrosine protein kinase Csk is kept close to its Src family member Lck. As Lck is needed for full activation of a T cell, its phosphorylation at Tyr505 by Csk keeps it in an inactive state (135). It has been proposed that the recruitment of co-stimulatory molecules such as CD28, leads to a recruitment of PDE4 and β-arrestins to the plasma membrane, resulting in a decrease of cAMP at the plasma membrane (reviewed in (136).
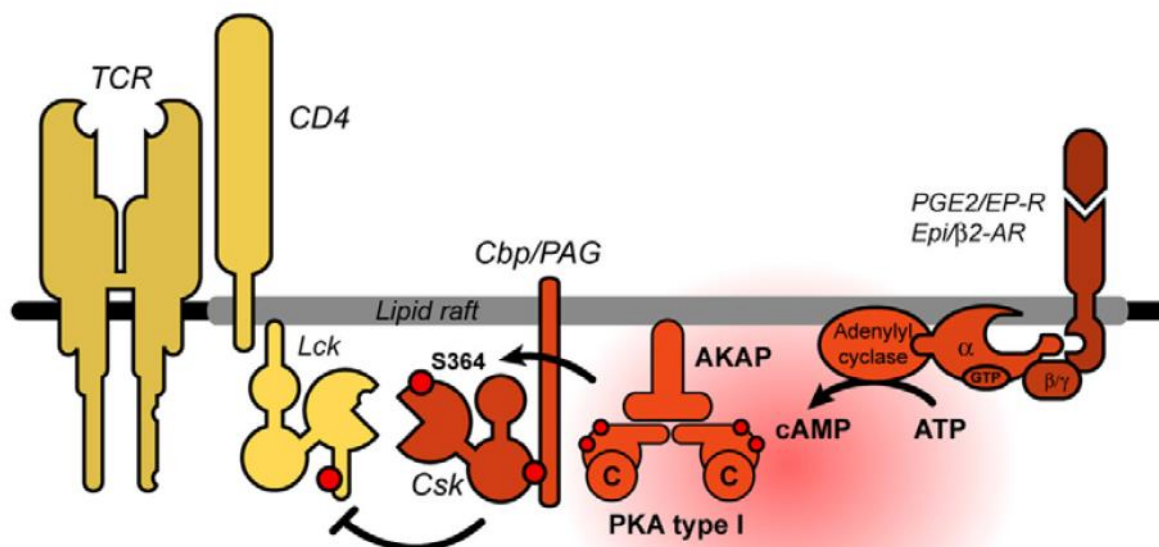
Fig 10: Model of TCR and cAMP interaction. The increase of cAMP upon stimulation with prostaglandin 2 leads to an inhibitory function on the Lck kinase, which is needed for TCR activation (from (136)). TCR T cell receptor, Lck lymphocyte-specific protein tyrosine kinase, Csk C-terminal SRC kinase, Cbp/PAG glycosphingolipid-enriched membrane domain, AKAP A kinase anchoring proteins, PKA protein kinase A, PGE2 prostaglandin E2.

Reduced cAMP levels would lower the inhibition of Lck by Csk allowing full activation of the T cell receptor and its downstream signalling mechanisms such as NFAT activation and cytokine release. Overall, cAMP plays an important regulatory and modulatory role in T cells and is the most potent and acute inhibitor of T cell activation. Additionally, as TCR activation itself already leads to cAMP production, the co-stimulatory molecules need to be tightly regulated to ensure T cell activation at the right time and strength. As already mentioned, the main degradation of cAMP is due to PDEs and indeed the blockage of PDEs to dampen TCR signals in pathological setting is being elucidated (137).

5.4.2.2.   The effect of prostaglandin on thymocytes and T cells

Prostaglandins are produced from an arachidonic acid precursor by a process dependent on the expression of COX1 and COX2. While COX1 is constitutively expressed and is considered to be used for the "household" production of prostaglandins, COX2 is inducible and has been linked to pro-inflammatory processes and also cancer development. The cyclooxygenases produce prostaglandin G2 and the intermediate form prostaglandin H2. This form is then used by specific synthases to produce different prostaglandins (e.g. PGE2, PGI2 …). PGE2 is synthesized by the PGE2 synthase which exists as one cytosolic and two membrane-bound forms, with only the PGE2 synthase I form being inducible. In terms of an effect on the immune system, PGE2 has been shown to possess pro- but also anti-inflammatory activities (reviewed in (138, 139). Surface receptor expression for any of the four PGE2 receptors in thymocytes is unclear, but all four prostaglandin E2 receptors (EP 1-4) are expressed in hematopoietic stem cells (140). In T cells the two receptors that result in an increase of cAMP (EP2 and EP4) are expressed (141) whereas the other two (EP1 and EP3) are not expressed (142).

cAMP is an important player in the development and maturation of thymocytes and is differentially regulated (143). Additionally the molecule has been shown to be involved in pro-survival but also pro-apoptotic processes (144). As the majority of thymocytes die during their developmental process, studies have linked cAMP signalling to thymocyte development and survival, which has revealed that the artificial increase of cAMP *in vitro* leads to a developmental block in fetal thymic organ culture (145). It is however unclear if this blockage would occur *in vivo* as well, as the treatment of mice with prostaglandin E2 (PGE2) leads to increased induction of apoptosis in thymocytes (146). While prostanoids are expressed and found in thymocytes (147), it is unclear whether PGE2 is physiologically important for thymocyte maturation as no thymus oriented studies have been performed in genetically modified animals. It has however been shown that cAMP-mediated apoptosis can be abrogated by blocking TNFα, the main effector for DP thymocyte apoptosis (148). Additionally, cAMP has not only been shown to be involved in thymocyte maturation but also in the adhesion of thymocyte in a stage-specific manner, with late stage thymocytes being less dependent on cAMP (149).

In mature T cells, PGE2 has been shown to have a suppressive effect on T cell activation. While this has been reported over 50 years ago, only later studies have found a major link between PGE2, cAMP and the blockage of IL-2 dependent gene expression (150, 151). These observations have been further strengthened by the revelation that an increase in cAMP leads to

the dampening of Ca2$^+$ dependent responses, such as the ones triggered by CD3/TCR engagement (152). Additionally it has been proposed that increased cAMP can influence the activity of ion channels, resulting in increased potassium concentration and subsequent impairment in G protein-mediated signalling (153). Overall, the suppressive effects of prostaglandin have been linked to the inhibition of NFκB (154) and calcineurin-dependent pathways (155). Another effect of PGE2 on T cells is lineage dependent., as PGE2 treatment inhibits Th1 effector cytokines but not Th2 cytokines (156). PGE2 also is involved in the production of regulatory T cells, which are capable of providing a tolerogenic environment (157, 158).

The pro-inflammatory effects of PGE2 are less studied, but interestingly it seems that while PGE2 can have suppressive effects, it acts different when used in micro molar quantities. Using memory T cells, Napolitani *et al* have shown that PGE2 treatment leads to an enrichment of IL-17 producing cells and inhibits the production of IFN-γ (159). While not a direct effect of PGE2 on T cells, DCs cultured in the presence of PGE2 can influence T cells to differentiate into the Th1 helper subtype (160). This is due to the induction of expression of surface markers such as CD70, which facilitates T cell/T cell interaction and therefore could act in a pro-inflammatory manner (161).

6. <u>Aims of the Study</u>

This study describes the importance of coronin 1 in the production of the second messenger cAMP, which is known to be a major player in all cell types. As coronin 1 has been shown to be important for the production of cAMP in neurons, the relation between coronin 1 and cAMP in cells of T cell lineage was further studied. During these experiments, it became clear that there is a need for pin-pointing the exact time point at which T cell survival becomes dependent on the expression of coronin 1. To this end the effect of coronin 1 deletion on different thymocytes and T cell subsets in adult and newborn animals was analysed and put in the developmental context of T cell maturation.

Additionally, the appendix of this study focuses on the potential of coronin 1 to interact with other proteins and thereby forming a supra-molecular activation complex needed for correct and efficient signal transmission within the cell. This has been studied in cells stimulated with isoproterenol and subsequently analysed by biochemical methods such as size-exclusion chromatography and co-immunoprecipitation. Additionally, as the localization of a protein is of importance for correct function, the coronin 1 protein has been mutated to study which domains are of importance for its proper localization to the plasma membrane. As both of these approaches have had their own difficulties, these projects were put on hiatus and the data presented here is considered to be preliminary.

7. <u>Material and Methods</u>

7.1. Materials

7.1.1. *Detergents, GPCR agonists and Inhibitors*

Name	Producer	Cat #
Ro 20-1724	Tocris	415
Clonidine	Tocris	690
Cilostamide	Tocris	915
Immepip	Tocris	932
Zardaverine	Tocris	1046
Rolipram	Tocris	1349
Milrinone	Tocris	1504
Iloprost	Tocris	2038
Prostaglandin E2	Tocris	2296
IBMX	Tocris	2845
TCS 2510	Tocris	4069
NP-40	Fluka	74385
Protease/Phosphatase Inhibitors 100x	Pierce	78440
Thesit	Sigma	88315
Roche Complete Tablets	Roche	11836153001
ATP	Sigma	A2383
BSA	EquiTech Bio	BAC62
Carbamyl-beta- methycholine chloride (Bethanechol chloride)	Sigma	C5259
DTT	Sigma	D0632
Digitonin	Sigma	D141
Na-b-D-maltoside	Sigma	D4641
EGTA	Sigma	E3889
EDTA	Sigma	E6758
Forskolin	Sigma	F6886
HEPES	Sigma	H3375
Dopamine	Sigma	H8502
Isoproterenol	Sigma	I6504
Melatonin	Sigma	M5250
N-octyl-glucopyranoside	Sigma	O8001
Sodium Orthovanadate	Sigma	S6508
Sodium Fluoride	Sigma	S7290
Tris	Sigma	T1503

7.1.2. *Cytokines*

Name	Producer	Cat #
GM-CSF	Biolegend	576306
IL-4	Biolegend	574306
IL-2	Biolegend	575406
IL-7	Biolegend	577806

7.1.3. *Antibody List*

Name	Antigen	Specificity	Clone	Isotype	Company	Ref. No
CD3 LEAF	CD3ε chain	Mouse	145-2C11	Armenian Hamster IgG	BioLegend	100314
CD3-biotin						100304
CD3-PE						100308
CD3-PE/Cy7						100320
CD3-Pacific Blue						100334
CD3-Alexa Fluor 700						100216
CD3-Alexa Fluor 488						100321
CD4-Brillinat Violet 510	CD4 (L3T4)	Mouse	RM4-5	Rat IgG2a	BioLegend	100559
CD4-Pacific Blue						100531
CD4-PE/Cy7						100528
CD4-PE						100512
CD4-PerCP						100538
CD4-APC						100516
CD8-APC	CD8a (Ly-2)	mouse	53-6.7	Rat IgG2a	BioLegend	100712
CD8-Pacific Blue						100725

CD8-Brilliant Violet 510						100751
CD8-Alexa Fluor 700						100730
CD8-PE						100708
CD19-APC	CD19	mouse	6D5	Rat IgG2a	BioLegend	115512
CD19-PE/Cy7	CD19	mouse	6D5	Rat IgG2a	BioLegend	115520
CD24-Brilliant Violet 421	CD24	mouse	M1/69	Rat IgG2b	BioLegend	101826
CD25-biotin	CD25 (IL-2Rα chain p55)	mouse	PC61	Rat IgG1	BioLegend	102004
CD25-PE/Cy7						102016
CD62L-PerCP	CD62L (L-selectin)	mouse	MEL14	Rat IgG2a	BioLegend	104430
CD62L-PerCp/Cy5.5						104432
CD69-PE/Cy7	CD69 (Very Early Activation Antigen)	mouse	H1.2F3	Armenian Hamster IgG1	BioLegend	104512
CD127-PE	IL-7Ra	mouse	A7R34	Rat, IgG2a	BioLegend	135010
Gr1-PerCP/Cy5.5	Ly-6G and Ly-6C	mouse	RB6-6C6	Rat IgG2b	BioLegend	108428
TER119-biotin	TER-119/erythrocytes	mouse	TER-119	Rat, IgG2b	BioLegend	116204
Qa-2-Alexa Fluor 647	H2-Qa2	mouse	695H1-9-9	Mouse, IgG2a	BioLegend	121708
Live/dead APC/Cy7	reaction with free cellular amines	-	-	-	Life technologies	L10119
S1P1	Peptide T4-H28	Mouse	713412	Rat IgG2a	R&D	MAB7089
CCR7-PE	CCR7	Mouse	4B12	Rat IgG2a	Biolegend	120105
Anti-rat IgG2a-biotin	Mixed rat IgGs	Mouse	MRG2a-83	Mouse IgG	Biolegend	497504

Rat IgG (blocking)	-	-	-	-	Sigma	I4131
Streptavidin-PE					Biolegend	405204
Streptavidin-APC					Biolegend	405207

7.1.4. *Buffer compositions*

Equilibration Buffer for Size Exclusion Column: 20 mM HEPES pH 7.5, 20 mM KCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 0.2% N-octyl-glucopyranoside

Lysis Buffer for Size Exclusion experiments: Equilibration buffer supplemented with 0.2% Digitonin, 0.3% Thesit and 0.3% NP-40

HBSS^{++}: Hanks balanced salt solution supplemented with 1.5 mM $MgCl_2$ and 1 mM $CaCl_2$

Co-immunoprecipitation Buffer: 25 mM HEPES pH 7.5, 20 mM KCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 0.2% Digitonin, 0.2% Na-b-D-maltoside, 0.3% Thesis, 0.3% NP-40

Homogenization Buffer: 10 mM Triethanolamine (Fluka #90279), 10 mM Acetic Acid (Sigma #A6283), 1 mM EDTA (Sigma E6758), 0.25 M Sucrose (Sigma #S0389) supplemented with Roche Complete Protease Inhibitor Tablets and 50 μM NaF and 5 μM Na_3VO_4 (based on (86, 162))

Ammonium-Chloride-Potassium (ACK) buffer: 155 mM NH_4Cl, 10 mM $KHCO_2$, 1 mM EDTA, pH 7.4

Complete media for dendritic cells: RPMI 1640 (Sigma-Aldrich, # R8758) media containing 10% FCS (PAA clone, The Cell Culture Company, #A15-101) heat inactivated (30' 55°C), 2 mM L-glutamine, 10 μM β-mercaptoethanol (Sigma-Aldrich, #M7522) and 10 ng/ml GM-CSF (Recombinant Mouse GM-CSF CF, Biolegend, #576306)

Complete media for thymocytes: RPMI 1640 (Sigma-Aldrich, #R8758) supplemented with 10% FSC (PAA clone, The Cell Culture Company, #A15-101), 500 μg/ml Pen-Strep (Gibco, #15140-122), 1% NEAA (Non-essential Amino Acid Solution, Sigma-Aldrich, #M7145), 50 μM β-mercaptoethanol (Sigma-Aldrich, #M7522), 1 μM Na-Pyruvate (Sodium Pyruvate, Sigma-Aldrich, #S8636)

FACS buffer: PBS, 2% FCS, 5 mM EDTA

PBS: 10 mM NaCl, 50 mM $HNa_2PO_4^+$-$12H_2O$, pH 8

D-PBS: PBS supplemented with 0.9 mM $CaCl_2$ and 0.5 mM $MgCl_2$

Resuspension buffer: 75 mM Tris pH 7.4, 12,5 mM $MgCl_2$, 1 mM EDTA supplemented with protease/phosphatase inhibitors (ThermoFisher HALT cocktail #78420)

Adenylate Cyclase Buffer: 25 mM Tris pH 7.4, 5 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT, 0.1 mg/ml BSA, 5 µM ATP

Lämmli Buffer: 10% SDS, 20% glycerol, 500 mM DTT, 300 mM Tris/Hcl pH 6.8, 0.015% bromphenol blue

RPMI 1640 without phenol red (Life Technologies, #11835)

Transfer buffer for western blot: 50 mM Tris, 32 mM Glycerin, 0.04% SDS plus 10% MeOH

7.2. Methods

7.2.1. *Animals and cells*

All animals were bred according to cantonal ethic and husbandry standards at the animal facility of the Biozentrum Basel. Coronin 1-deficient animals have been described earlier (96, 163). Ly5.1 mice were obtained from the Swiss Immunological Mutant Mouse Repository and bred in-house. If not otherwise indicated, mice were used between 6-8 weeks of age and adult mice were age and sex-matched.

Animals were sacrificed by high percentage CO_2 and organs removed. Single cell suspensions were prepared by mashing organs through a polyamide mesh (150 μm, SEFAR NITEX #03-150-38) with a plunger.

Mel Juso cells expressing coronin 1 have been described (88), Jurkat cells were obtained from ATCC (Clone E61, ATCC TIB 152) and CRISPR-Cas9 knock-outs generated and validated by Saumya Mazumder.

7.2.2. *Genotyping*

Toes of animals were cut and digested o.n. in digestion buffer (100 mM Tris-HCl pH 8.5, 5 mM EDTA, 0.2% SDS, 200 mM NaCl supplemented with 100 μg/ml Proteinase K).

GFP-negative Coronin 1-deficient animals:

PCR (1' 95°C, 30" 55°C, 40" 72°C with 35 cycles) with following primers:

Coro1aLoxSA1: 5'-GAGACAGGACTCTCTTTG-3',

Coro1aLoxLA1: 5'-GTCCTCAGTAGCTGACTG-3',

Coro1aLoxMA1: 5'-TAGCAGAAAACCCCAAGC-3'

(WT MA1/LA1: 140 bp, floxed MA1/LA1: 250 bp, KO SA1/LA1 400 bp)

GFP-positive Coronin 1-deficient animals

PCR (1' 95°C, 45" 56°C, 50" 72°C with 35 cycles) with following primers:

WTCor1: 5'-CTGTTGTAGGGGCTGATGGT-3'

WTCor2: 5'-CACTGGCCTCACAGATCAGA-3'

KOCor1: 5'-CTTCATGTGGTCGGGGTAG-3'

(WT 392 bp, het 329 bp and 478 bp, KO 478 bp)

7.2.3. *Size exclusion chromatography*

Mel Juso cells expressing Coronin 1 were cultured on 15 cm petri dishes in DMEM media supplemented with 10% FCS. 2 hours before assay, cells were washed 3x with HBSS^{++} and incubated in 10 ml HBSS^{++} at 37°C 5% CO_2. Cells were stimulated with 10 µM Isoproterenol in HBSS^{++} for 10' 37°C, briefly washed with ice cold HBSS^{++} and fixed in 1% PFA for 10' on RT. Cells were subsequently lysed in size exclusion lysis buffer, filtered by 0.2 µm membranes and loaded onto an Superose 6 column. Column was equilibrated on the day of the experiment with equilibration buffer and calibrated by running a protein standard (Biorad #1611901). Column was washed for at least one column volume (~40 ml) between runs and the general protocol consisted of 0.5 ml/min with a maximum pressure of 1.5 mPa with a fraction size of 1 or 0.5 ml. Fractions were precipitated by addition of 100% TCA (Sigma T6399) (1/5th of total volume, i.e. final concentration of 20% TCA) for 30' on ice and sedimented at 15' 21.000g 4°C. Pellets were washed with acetone twice, air dried at 95°C and resuspended in PBS for 30' on ice. After resuspension, 5x SDS Laemmli Buffer was added, samples boiled for 10' 95°C and loaded onto 10% SDS-PAGE Gels (80V 20' then 110V).

7.2.4. *Western blotting*

Protein levels were determined by BCA in 96-well flat bottom plates with gamma-globulin (1 µg/ml) used as a standard. Protein transfer was performed with a semi-dry transfer system on

nitrocellulose membranes (Biorad Transblot, 20V 45'). Transfer efficiency was assessed by ponceau staining and membranes blocked by incubation for 1 hour on RT in 5% low-fat dry milk in PBS supplemented with 0.2% Tween. All blotting was performed in PBS-T. Antibodies for western blots were diluted in PBS-T with 0.02% sodium azide and washing steps between and after antibody addition were performed for 5' 3x. Anti-Actin used 1:10000 (Millipore, MAB1501), anti-STAT6 and pSTAT6 1:1000 (from CST PhosphoStat antibody sampler kit #9914), anti-coronin 1 is made in-house (rabbit #321, 14th bleed) used 1:5000. Secondary HRP-conjugated antibodies are all from southern biotech and were used 1:5000 and incubated for 1 hr on RT. Visualization of immunoblots was performed with chemo luminescent pico substrate (Thermo Scientific #34080) either on films (for size exclusion and co-IP experiments; Fuji Super RX-N #47410 19289) or with a digital imager (blots for IL-4 stimulation; Vilber Louromat Fusion FX with Fusion Capture Advanced FX7 software).

7.2.5. *Immunofluorescence*

IF staining for HEK were performed on 10-well slides (Thermo Fisher #ER208BCE24) with 5000 cells seeded the day before analysis. For Jurkat cells, cells slides were coated with 20 μl of 1 mg/ml Poly-L-Lysine for 1 hr 37°C, washed and 20.000 cells seeded for at least 1 hour before experiment. Cells were fixed using methanol (4' at -20°C), briefly dried and rehydrated in D-PBS. For phalloidin staining, fixation was performed with 3% PFA for 15' RT and cells permeabilized by 0.1% Triton-X. Blocking was performed using D-PBS supplemented with 5% FCS. Staining was performed for 20' RT in the dark using anti-coronin 1 (in-house, same as for western blots) 1:500 in PBS-T with 2% FCS. Subsequently slides were washed 3x with D-PBS and secondary antibody conjugated to fluorochrome was used 1:200 for 20' RT. Slides were washed again, briefly dried and fixed using VectaShield mounting media (Vectorlabs #H1000). Coverslips were fixed using commercially available household nail polish.

7.2.6. *Generation of deltaN10 Cor1 constructs*

Coronin 1 was isolated from pCB6 vector by restriction enzyme digest for Sac1 and BamH1. Resulting fragment was amplified using specific primers for the C-terminal HA Tag and for deletion of the first 30 base pairs.

Fwd primer: CGCCACGTGTTTGGACAGCCA

Rev primer: CGGGAATTCCTATGCGTAGTCTGGTA

Resulting amplicon was subsequently cloned back into the pCB6 vector using Gibson Assembly provided by NEB using the following primers:

Fwd primer: gtctatataagcagagctcATGCGCCACGTGTTTGGA

Rev primer: gatccagggatgccacccgggatccTAGAGTCGACCTGCAGG

Resulting constructs were transfected into competent bacteria, bacterial colonies picked; plasmids isolated using miniprep kit from Macherey Nagel and sent to sequencing (MicroSynth) for validation. Tranfection of plasmids was performed by Calcium-Phosphate transfection of HEK cells.

7.2.7. *Determination of cAMP levels*

Stimulation of cells for cAMP levels was performed in phenol-red free RPMI media supplemented with 2 mM L-Glutamine. Jurkat cells were washed 2x and starved as 1×10^6 cells/ml for 2 hours at 37°C before experiments. No starvation was performed for primary T cells or thymocytes. For cAMP analysis, cells were spun down (5' 300g) and resuspended in the concentration needed to achieve 15.000 (Jurkat cells and coronin 1-deficient primary T cells) or 30.000 (wild-type primary T cells and wild-type/coronin 1-deficient thymocytes) cells per well measured. Measurements were performed using the HTRF cAMP assay kit (#62AM5PEB Cisbio Bioassays) using the provided protocol with stimulation and lysis done in 1.5 ml eppendorf tubes, 10 µl of lysed cells distributed into 384-well plates (Greiner BioOne #781075) and 10 µl of cryptate/lysis buffer solution added. Plates were incubated for at least 1 hour but not more than 4 hours in the dark before measuring on a Tecan F500 machine. Analysis of data is performed by building a ratio of D2 and CC signal, subtracting the background ratio and fitting values onto a sigmoidal standard curve.

7.2.8. *Membrane isolation*

Jurkat cells were spun down and washed with Homogenization Buffer and resuspended in HB. Cells were homogenized using a 27G needle and homogenization was assessed under a microscope with trypan blue. Homogenate was then spun down for 10' at 1000g 4°C and post-nuclei supernatant transferred to fresh ultracentrifuge 1.5 ml tube (Beckmann #357448) followed by another centrifugation for 30' at 100.000g. Resulting membrane pellet was carefully resuspended with a 21G needle in resuspension buffer. Protein concentrations were assessed by BCA and equal μg of membranes were spun down for 15' at 21.000g. Resulting pellet was resuspended in adenylate cyclase buffer and 2 μg of membrane was assessed for cAMP production.

7.2.9. *T cell isolation*

Spleens from either wild-type or coronin 1-deficient animals 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 or CD4+ using negative selection T cell or CD4+ T Cell Enrichment Kit, respectively (EasySep, StemCell technologies, Ref. No 19751 and 19752). Cells were sorted following the protocol from corresponding selection kit. Cells were counted using Neubauer chamber and dead cells were excluded using trypan blue.

7.2.10. *FACS staining*

S1P1 antibody from R&D Systems (MAB7086), Life/Dead Marker from ThermoFisher (#L10120).All other antibodies from Biolegend coupled to different fluorochromes. FACS acquisition was done on a BD Fortessa with Diva software (v10) and analysed using FlowJo v10 (TreeStar Software). Anti-CD3 (145-2C11), CD4 (RM4-5), CD8 (53.6-7), CD24 (M1/69), CD45.1 Ly5.1 (A20), CD62L (MEL-14), CD127 IL7Rα (A7R34), Qa2 (695H1-9-9), Ter-119 (Ter119). In general cells were counted with a Neubauer Chamber in PBS and 1.5×10^6 cells stained in 96-well U-bottom plates. Antibody solution was prepared in PBS supplemented with

2% FCS and EDTA. Cells were incubated for 30' on ice, all antibodies used 1:100. Life/Dead Marker used 1:1000. For S1P1 receptor staining, the AB was used 1:10 and no EDTA was added to the staining buffers. Following 30' on ice, cells were incubated with biotinylated 1:100 anti-ratIgG2a (MRG2a-83) for 30' on ice. After blocking with 1:100 rat-IgG (Sigma I8015) for 10', the remaining antibody cocktail was added plus Streptavidin coupled to either APC or PE. After staining cells were centrifuged (300g, 5' 4°C), resuspended in 200 µl buffer, transferred to polystyrene tubes and measured within 2 hours.

7.2.11. *FACS sort*

For sorting of thymocyte populations, single cell suspensions were prepared and stained for CD3, CD4, CD8 and Life/Dead and sorted (see Supplemental Figure 5). Sorting of different T cell populations was performed by isolating CD4+ splenic T cells using the EasySep total T cell isolation kit from StemCell (#19851). Resulting T cells were then stained for CD4, CD8 and CD25 and processed for sorting (see Supplemental Figure 4).

For sorting of late stage thymocytes, CD8 predepletion was prepared based on negative selection protocols from StemCell Technology. Thymocytes were prepared as described, counted and resuspended as 100x10^6/ml in PBS/2% FCS. Cell suspension kept in either 4 ml polystyrene tubes or 13 ml round bottom tubes as recommended by StemCell. Cells were then incubated with biotinylated antibodies for CD8 and Ter119 (12.5 µg/ml)and rat sera (50 µl/ml, from StemCell negative isolation kits #19852). After 10' on RT 75 µl/ml EasySep Streptavidin RapidSpheres 50001 (#19860) were added for 3' RT. Solution was then filled up to 2.5 or 5 ml depending on tube size and put on magnet for 3' RT. Supernatant was then poured off into fresh tubes, spun down, counted and stained for CD3, CD4, CD8, CD69, CD62L and Life/Dead (see Supplemental Figure 6).

All FACS sorts were performed on a BD Aria III at the Biozentrum FACS core facility (Janine Zankl) and purity was always >98%. After sort, cells were spun down for 10' 4°C 350*g* and resuspended either directly in TriReagent, in corresponding media in a concentration of $0.75x10^6$ cells/ml (for late stage thymocyte survival) or in RPMI supplemented with 2 mM L-Glutamine (for thymocyte stimulation assays).

7.2.12. *Quantitative RT-PCR*

Cells were resuspended in 400 μl TriReagent and stored on -80°C. Samples were processed through RNA isolation based on manufacturer's instructions (Zymo Research Direct-Zol RNA Isolation Kit #R2073) and then 250 ng transcribed into cDNA (ThermoFisher #4368814). For each reaction 10 ng of cDNA was used, reaction were performed in triplicates on MicroAmp 96-well reaction plates (ThermoFisher #N8010560). Amplicon detection was based on SYBR Green reaction mix for thymic egress markers (ThermoFisher #4367659) and on TaqMan technology for PDE4QA5 gene expression measurement (Mm01147143_m1) using Hprt1 as reference gene (Mm01545399_m1). PCR was performed on an Applied Biosystem machine (StepOne Plus) using StepOne v2.2 software. Analysis of results was done via ΔΔCt method. Primers were synthesized by MicroSynth (Balgach, Switzerland).

Primer Sequences:

NKAP

Forward: 5'-GCGTATCCCAAGAAGAGGTG-3',

Reverse: 5'-GAAGTCGAACAGCCTCCATT-3'

Foxp1

Forward: 5'-CAGCCACGAAAGAAACAGAAG -3',

Reverse 5'-GGTCCTGGTCACCTGATTATA -3'

KLF2

Forward 5'-ATGGCGCTCAGCGAGCCTAT-3',

Reverse: 5'-AGCAGCTCTGTTCCCAGGCT-3'

GAPDH

Forward: 5'-GAGCCAAACGGGTCATCATC-3',

Reverse: 5'-GAGGGGCCATCCACAGTCTT-3'

7.2.13. *TREC analysis*

Genomic DNA was isolated from single cell suspensions by digestion of 10x10^6/cells in 10 mM Tris-HCl pH 7.4 supplemented with 100 μg/ml Proteinase K for 1 hour on 56°C and 1200 rpm (based on (164)). Digests were boiled to 95°C for 10' and 5 μl per sample assessed. Primer for C57BL/6 mouse sjTREC analysis were based on (165) and DNA input was normalized by using a probe for Tfrc (Thermofisher #4458366). Data is presented as ΔΔCt values.

7.2.14. *Cell Culture and survival assays*

For preparation of dendritic cells, bone marrow of Ly5.1 mice was flushed out from femur and tibia with a 24G syringe. Bone marrow was then frozen in 10% DMSO/90% FCS (PAA LowEndotoxin FCS #A15102 Lot# A10209-2936) and used for several rounds of dendritic cell preparations. For dendritic cell preparation 6x10^6 cells were seeded into a 10cm petri dish (Falcon #351029) in 8 ml RPMI1640 with 10% FCS, 1 mM Sodium Pyruvate (Sigma, #S8636), Non-essential amino acids (Sigma, #M7145), 100u/ml Penicillin 100 μg/ml Streptomycin (Sigma #4333), 2 mM L-Glutamine (Gibco #25030) and 10 μM β-Mercaptoethanol (Sigma, M7522). Media was supplemented with 10 ng/ml GM-CSF (Biolegend, #576302). Cells were kept for 7 days, with 2 ml of media and fresh cytokines added every second day. For dendritic cell supernatant, cells were harvested on day 6, washed once in thymocyte media and cultured in thymocyte media without cytokines for 24 hours before the experiment. For co-culture experiments, dendritic cells were assessed on the experimental day by microscopy for general morphology and 100.000 cells preseeded into 96-well U-bottom plates (Falcon #351177) or transwell plates (Corning HTS Transwell 3 μm #3385).

For thymocyte survival assays, cells were adjusted to 0.75x10^6 cells and 100 μl added per well. Media formulation was RPMI1640, 10% FCS (PAA FCS #A15101 Lot# A10109-1886), 1 mM Sodium Pyruvate, NEAA, PenStrep, 2 mM L-Glutamine and 50 μM β-Mercaptoethanol (same as above). In general, media was replenished after 2 days of culture by careful removal of 75 μl media and re-adding the same amount. Culture volume was 200 μl in normal cultures, for transwell cultures 250 μl were used to ensure proper diffusion between compartments. For dendritic cell supernatant experiments, the supernatant from the day of seeding was filtered (0.2 μm) and 100 μl of supernatant was added to each well. The supernatant was stored on 4°C for

media replenishment on day 2. Cytokines (interleukin 2 #575402 and interleukin-7 #577802, both Biolegend) were added as 20 ng/ml. On the day of survival assessment, cells were sedimented (10' 4°C 350g) and stained for L/D marker and Ly5.1.

For bulk thymocyte survival assays, single cell suspensions from thymi were prepared and 100 μl were seeded in 96-well flat bottom plates to have 100.000 thymocytes per well. Media alone (100 μl) or supplemented with 20 ng/ml of cytokines (IL-2 or IL-7) was added in 2x concentration. Viability measurements were performed as described for late stage thymocyte survival assays.

7.2.15. *Statistics and software*

General calculations were performed in Microsoft Excel 2007. Graphs and statistical tests were performed in GraphPad Prism Software 5.00. FACS analysis was done with FlowJo V10 (TreeStar software).

8. <u>Results</u>

8.1. *Involvement of coronin 1 in cAMP signalling in cells of the T cell lineage*

8.1.1. *Coronin 1-dependent cAMP modulation in primary T cells and T cell lines*

8.1.1.1. *Coronin 1 deletion increases cAMP levels in T cells*

Coronin 1 has been shown to be important for the generation of cAMP in neurons. Additionally, fibroblasts transfected to express exogenous coronin 1, have an increased ability to generate cAMP (114). To study if the impairment in peripheral T cell survival of coronin 1-deficient animals is linked to differences in cAMP levels, total T cells from wild-type and coronin 1-deficient animals were used. T cells were isolated by making single cell suspensions of spleen and subsequently negatively selected using the EasySep T cell isolation kit. The cells were then either used unstimulated or stimulated with the adenylate cyclase activator Forskolin and their cAMP content measured by a homogenous time-resolved fluorescence (HTRF) assay. As can be seen in Fig 11 A, cells with a coronin 1-deficient background showed around four times higher levels of cAMP in their unstimulated state. Once the cells were stimulated, the cAMP content increases. In the wild-type situation this resulted in an increase of around 10 fold, whereas the coronin 1-deficient T cells only increase their cAMP content by ~6 fold. Nevertheless, wild-type cells generated less cAMP in total compared to their coronin 1-deficient counterparts.

The cAMP level in cells is modulated by two mechanisms, one is the production via ACs, and the other one is the degradation of cAMP to 5-AMP by phosphodiesterases (PDE). To assess the involvement of PDEs in the observed cAMP modulation upon coronin 1 deletion, T cells were pre-incubated with the PDE 4 inhibitor Rolipram for 30' before stimulation. Panel B of Fig 11 shows the fold induction of cAMP production in the presence of Rolipram after stimulation with Forskolin. While wild-type cells show an increase of cAMP production when the cAMP degrading enzyme PDE is blocked, coronin 1-deficient T cells do not display this increase in cAMP content. In summary, the deletion of coronin 1 leads to increased production of cAMP in primary murine T cells and the data suggests an involvement of the cAMP degradation pathway.
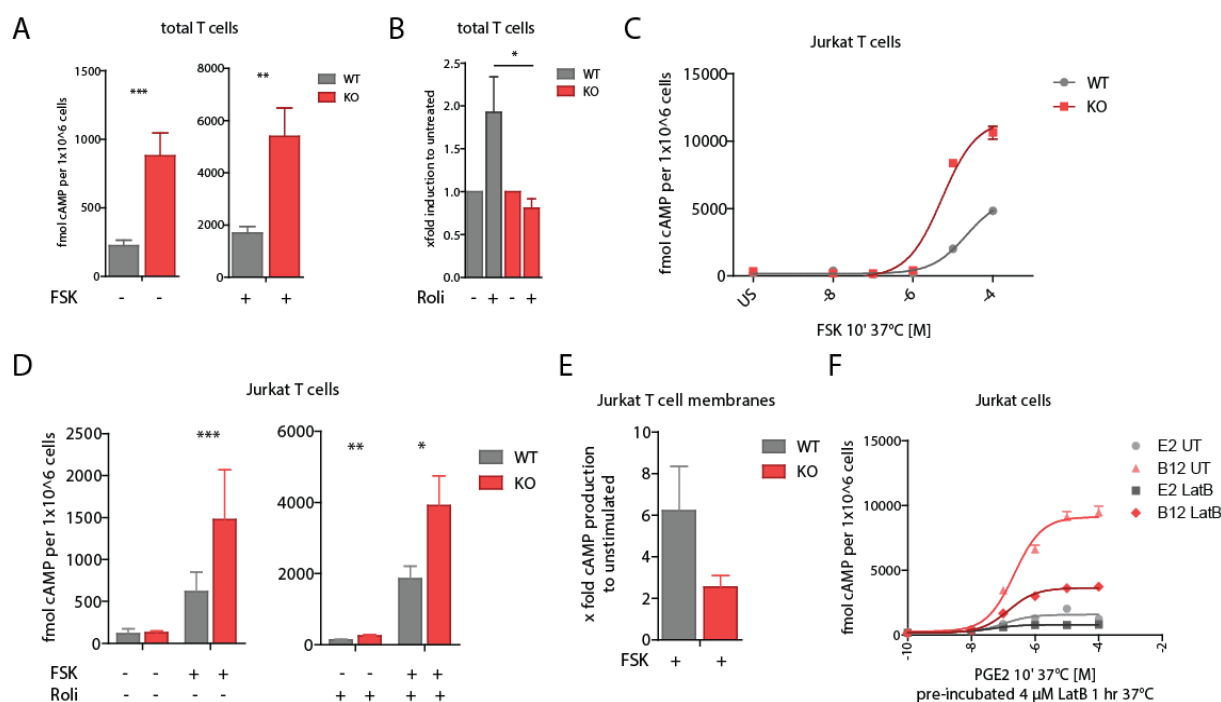
Fig 11: cAMP measurements in primary mouse T cells and the human Jurkat T cell line. (A) steady state levels of total T cells or stimulated with 100 μM FSK **(B)** T cells stimulated with 100 μM FSK with or without pre-incubation with 100 μM Rolipram for 30' at 37°C. Bars are depicted as x-fold to the unstimulated control values. **(C)** Wild-type or coronin 1-deficient Jurkat T cells stimulated with different concentrations of FSK. **(D)** Jurkat T cells stimulated with 10 μM FSK with or without Rolipram pre-incubation. **(E)** Membranes of Jurkat T cells stimulated with FSK. All stimulation steps were done for 10' at 37°C. **(F)** Jurkat T cells pre-incubated with 4 μM Latrunculin B for 1 hour on 37°C and assessed for cAMP production upon stimulation with different concentrations of PGE2 * p=<0.05, ** p<0.005, *** p<0.0005. Unpaired two-tailed students *t*-test. Graphs show pooled data from at least 3 (A-D) or 2 (E) independent experiments for A-D. cAMP measurements in cells with depolymerized actin only done once.

For further biochemical analysis of a cAMP in T cells deficient for coronin 1, a CRISPR/Cas9 knock-out of coronin 1 was established in the human Jurkat T cell line. Interestingly, the generation of this cell lines was not as straight forward as expected with many clones dying during the selection procedure (observations from Saumya Mazumder) and not all clones showing a cAMP phenotype similar to primary murine T cells (Supplemental Figure 1). Nevertheless, a cell line deficient for coronin 1 was successfully generated. Stimulating the coronin 1-deficient Jurkat cells with Forskolin resolves a similar increase in the generation of cAMP, as seen in the primary cells (compare Fig 11 C with A), indicating that the Jurkat cell line undergoes similar or the same modifications of the cAMP pathway upon a lack of coronin 1 expression.

8.1.1.2. *Increased levels of cAMP in T cells are due to decreased cAMP degradation not due to increased production*

The blockage of PDEs in wild-type of coronin 1-deficient Jurkat cells leads to an increase in general cAMP content, with both genotypes producing roughly double the amount of cAMP. This is different in comparison with primary T cells, where the blockage of PDEs does not lead to an increase in cAMP production. Also not present is the increased steady state levels of cAMP when cells are assessed without any stimulation. As coronin 1 has been postulated to be involved in the arrangement of the actin cytoskeleton, cells were also assessed under actin depolymerising conditions. Therefore, the actin cytoskeleton was depolymerized by the addition of 4 µM Latrunculin B for 1 hour. Both, wild-type and coronin 1-deficient Jurkat T cells showed reduced levels of cAMP production after treatment with Latrunculin B. However, the coronin 1-deficient cell line still produced more cAMP than its wild-type counterpart (Fig 11 F), which suggests no involvement of the actin cytoskeleton in the increased cAMP production of coronin 1-deficient T cells. To assess if the involvement of PDE could explain the increase of cAMP in coronin 1-deficient T cells, the production of cAMP in membranes was studied. For this, Jurkat cells were starved, homogenized and separated into cytosol and membrane fractions by high-speed centrifugation. Resulting membranes were assessed for their cAMP production upon FSK stimulation. Similar to what is known from a coronin 1-deficient background in neurons; the membranes of coronin 1-deficient T cells produce less cAMP than wild-type membranes (Fig 11 E).
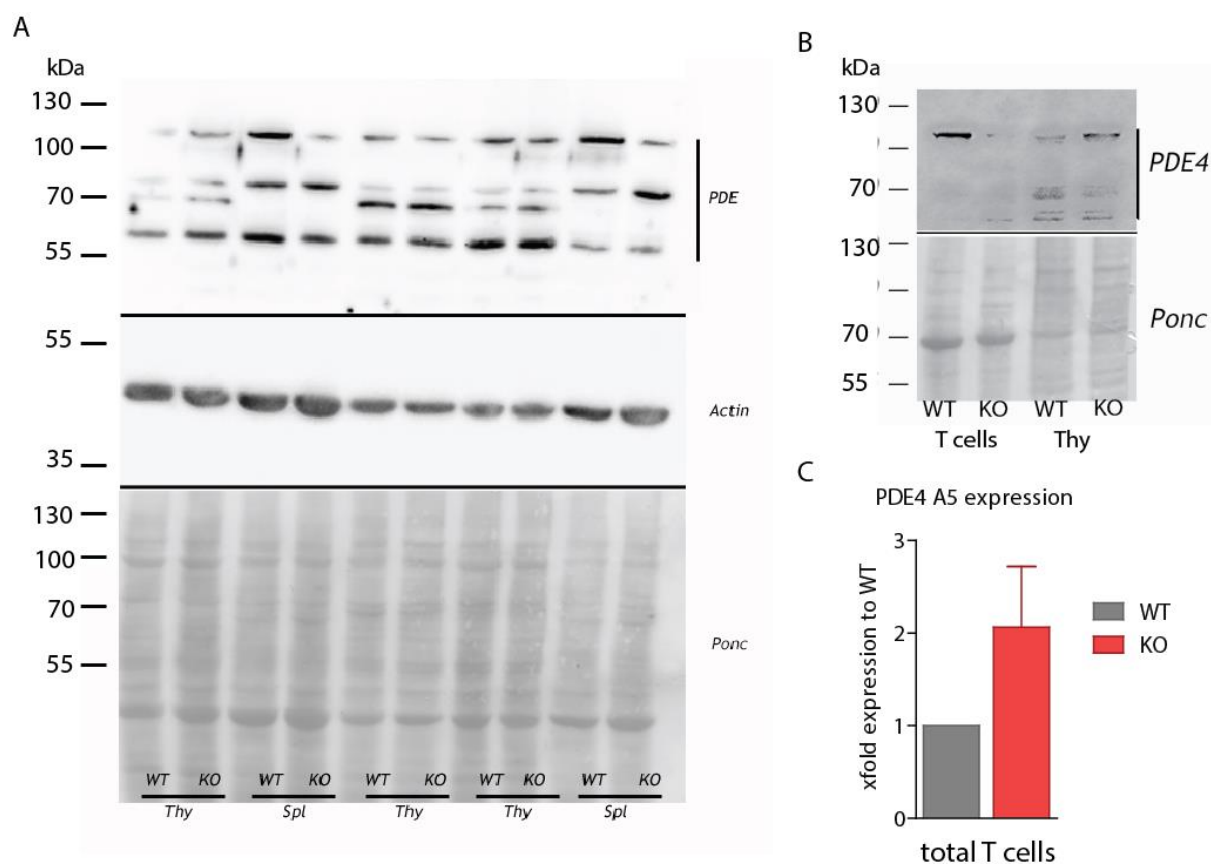
RNA seq data for PDE isoforms in conventional wild-type versus coronin 1-deficient primary murine T cells

ENSID	MGI symbol	MGI Description	log2 FoldChange
ENSMUSG00000021684	Pde8b	phosphodiesterase 8B	-0.66
ENSMUSG00000041119	Pde9a	phosphodiesterase 9A	-0.56
ENSMUSG00000023868	Pde10a	phosphodiesterase 10A	-0.95
ENSMUSG00000004347	Pde1c	phosphodiesterase 1C	-1.44
ENSMUSG00000041741	Pde3a	phosphodiesterase 3A, cGMP inhibited	-1.08
ENSMUSG00000032177	Pde4a	phosphodiesterase 4A, cAMP specific	-2.24
ENSMUSG00000053965	Pde5a	phosphodiesterase 5A, cGMP-specific	-2.11
ENSMUSG00000019990	Pde7b	phosphodiesterase 7B	-2.78

Fig 12: PDE4 protein and gene expression in T cells and thymocytes. Immunoblots for PDE4 expression in (A) Thymocytes or splenocytes (B) thymocytes or negatively isolated T cells. (C) pooled data from quantitative RT-PCR for PDE4A5 gene expression from 3 individual wild-type or coronin 1-deficient negatively isolated T cells. Threshold for altered gene expression status was set at 2 fold change. (D) data from RNAseq (3 biological replicates) for CD4+CD25⁻ wild-type or coronin 1-deficient T cells showing the different PDE isoforms detected.

Normal cAMP production at membranes suggested an impairment in the degradation of cAMPas the reason for the observed high levels of cAMP. To analyse this, T cells were assessed for their PDE4 expression status. Either total splenocytes and thymocytes or isolated T cells and thymocytes were lysed and assessed by western blot (Fig 12). In T cells, the most abundant and important isoform is PDE4A5, with a weight of 114 kD. However, the antibody detects various isoforms of PDE4. As can be seen in Fig 12 A, the band presumably corresponding to PDE4A5 showed a decrease in protein expression in splenocytes lacking coronin 1 expression. This is not observed in the samples obtained from thymi of the two genotypes. One need to note though that the samples loaded in the blot are from total thymus lysate, which consists to 80% of double-positive thymocytes. With T cells isolated from splenocytes, the difference is more noticeable, only showing one band present in the blot, presumably representing PDE4A5, although it cannot be excluded to be the isoform 6 of PDE4A. To assess if T cells deregulate PDE4 expression on a transcriptional basis, total T cells were harvested and mRNA isolated. The subsequent quantitative RT-PCR for PDE4A5 showed a slight increase in expression in coronin 1-deficient T cells (Fig 12 C). Additionally, conventional CD4$^+$CD25$^-$ T cells were assessed by RNAseq (by Rajesh Jayachandran). Fig 12 D shows these data for all PDE isoform detected in the assay. This dataset shows the same slight increase of PDE4 in T cells, but also for other PDE isoforms. It is possible that the PDE isoforms are slightly up regulated in coronin 1-deficient T cells to account for the reduction in PDE4 protein levels. Also, while using different PDE inhibitors in wild-type cells would result in different levels of cAMP, the blockage of PDEs in coronin 1-deficient cells would lead to the same increase upon stimulation with PGE2 (Fig 13).

In summary, the data suggests an enhancing role for coronin 1 in the production of cAMP in T cells as in neurons, with an impaired production upon coronin 1 deletion. The increase in cAMP levels observed in the coronin 1-deficient T cells is based on a lack of degradation of the cAMP by PDEs, since PDE4 protein levels are reduced. The fact that gene expression of PDEs does not seem to be affected upon coronin 1 deletion but protein levels are reduced, suggests a post-translational modulation of PDE4 expression in coronin 1-deficient T cells. It remains unclear when exactly the cells start to modulate their cAMP levels when coronin 1 is lacking, but based on the data obtained by peripheral T cells, only those cells which do modulate PDE4 expression are capable of surviving in the periphery.
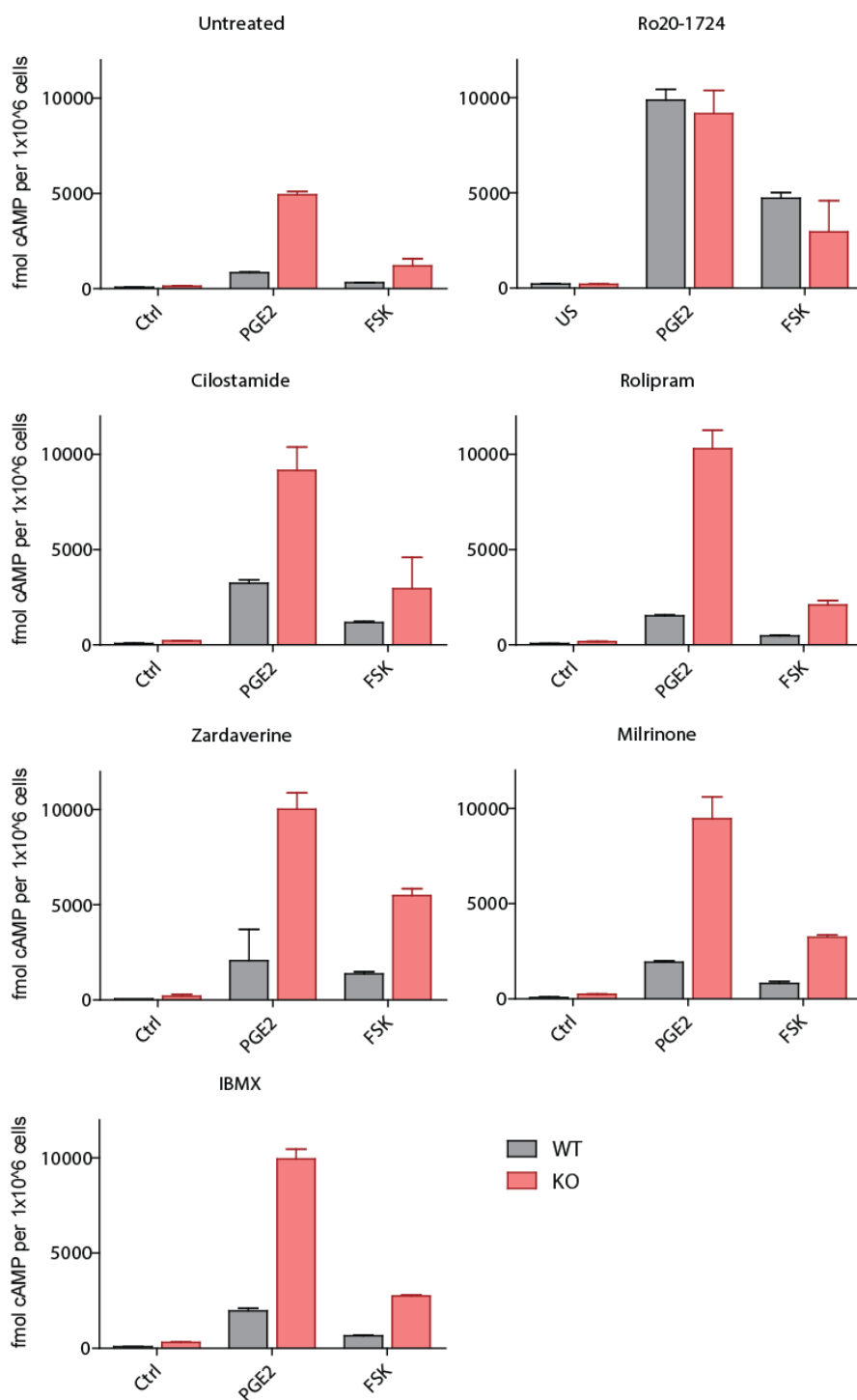
Fig 13: Blockage of PDEs in Jurkat cells. Wild-type or coronin 1-deficient Jurkat T cells were incubated with 50 μM of Ro 20-1724, Cilostamide, Rolipram, Zardaverine or Milrinone or 100 μM IBMX for 30' 37°C and stimulated with 10 μM PGE2 or Forskolin for 15' on RT. Data from one representative experiment shown.

8.1.1.3. *Coronin 1-dependent cAMP modulation is only present in stimuli linked to an increase of cAMP*

Coronin 1 in neurons has been shown to be associated with the stimulatory subunit of the $G\alpha$ protein. $G\alpha$-s is needed to induce the generation of cAMP from ATP by ACs. To assess a potential role of coronin 1 in the activation of ACs via the $G\alpha$-s pathway in T cells, Jurkat and primary T cells were stimulated with prostaglandin pathway stimulators. PGE2 activates all prostanoid receptors of the EP subtype and leads to an increase in cAMP in T cells and significantly more cAMP is generated in coronin 1-deficient cells (Fig 14). TCS2510 is a derivate, which only stimulates the EP4 receptor, linked to $G\alpha$-s. This results in the generation of cAMP in Jurkat cells with an increased response in the absence of coronin 1. Primary T cells fail to respond which could be due to the lack of surface expression of the corresponding receptor. Interestingly, the stimulation of Jurkat and primary T cells with Iloprost leads to the production of cAMP in coronin 1-deficient T cells, but not in wild-type cells (Fig 14). The small molecule Iloprost has its highest affinity with the receptor subtypes EP1 and EP3 (based on Ki values provided by manufacturer). Neither of these should lead to the production of cAMP as EP1 is associated with the q-subunit, involved in Ca^{2+} signalling and EP3 with the inhibitory subunit, blocking ACs. Iloprost has been shown to bind $G\alpha$-s mediated receptors in higher
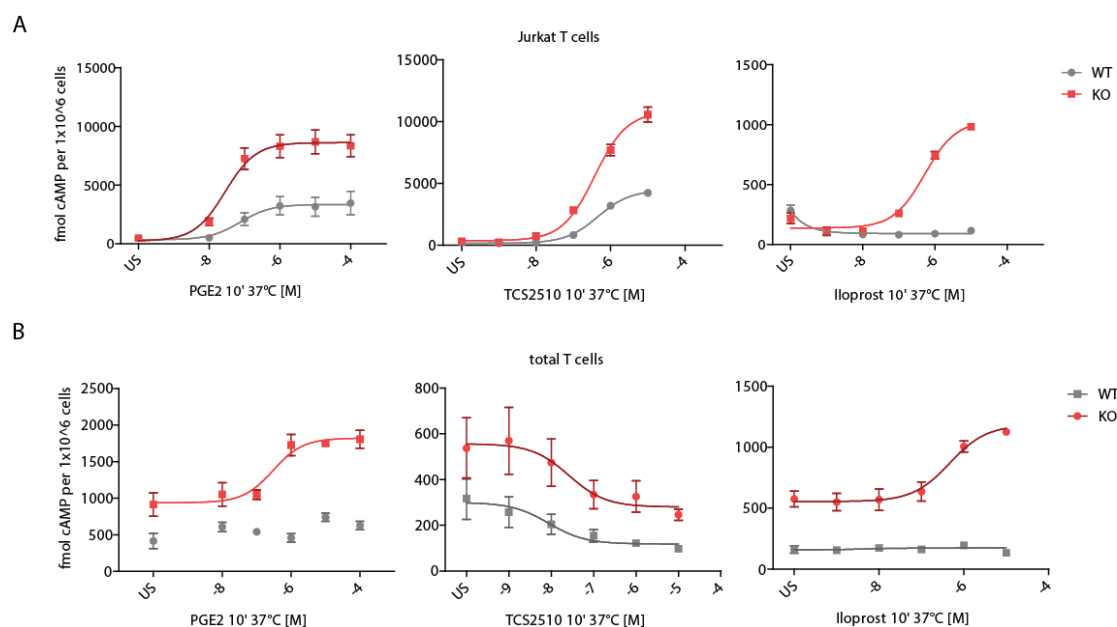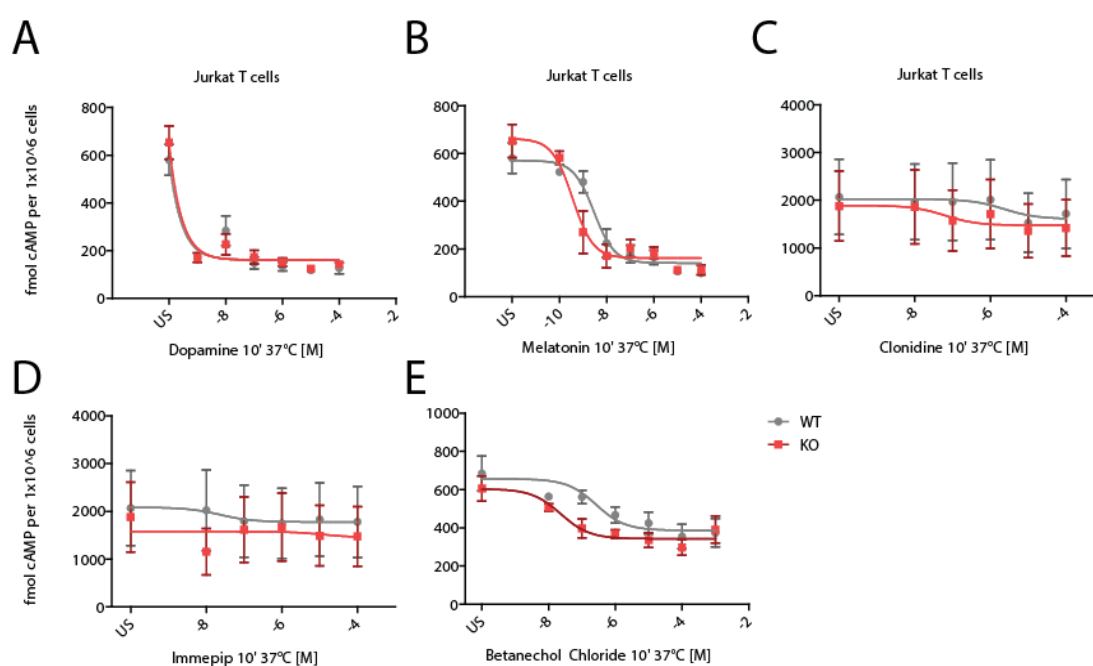

Fig 14: primary T cells or Jurkat T cells stimulated with different concentrations of indicated agonists. All stimulations were done for 10' at 37°C. Error Bars indicate SD. N=3 for primary T cell stimulation and PGE2 stimulation of Jurkat cells. TCS2510 and Iloprost in Jurkat cells n=1.

concentrations, but with wild-type cells showing zero stimulation, this effect does not explain the production of cAMP in the coronin 1-deficient cells. As the concept of G-protein coupled receptor signalling is widespread, the cells were also assessed for other receptor subtypes linked to the inhibitory or q-subunit of Gα. As can be seen in Fig 15, none of these agonists resulted in differential modulation of cAMP between cells with a wild-type or coronin 1-deficient background. In summary, the increased generation of cAMP in T cells can also be seen with stimuli dependent on Gα-s, however, there is no evidence that coronin 1 interferes with signalling via the other two Gα subunits.

Fig 15: Jurkat T cells stimulated with different concentrations of indicated agonists, All stimulations were done for 10' at 37°C. Error Bars indicate SD from 2 independent experiments.

8.1.1.4. *Majority of increased cAMP levels in coronin 1-deficient T cells comes from conventional CD4+CD25⁻ T cells*

T cells can be differentiated via surface marker expression and function into specific T cell subsets. While some of the T cells act in a cytotoxic manner, others support other cell types in their roles by modulating the immune response. To assess whether all T cells show the difference in their potential to generate cAMP, the T cells were separated into different populations. Stimulation of total T cells with PGE2 results in an increase in cAMP content in wild-type and in coronin 1-deficient cells (Fig 16 A). In T cells separated for the surface markers CD4 and CD8 and stimulated with a gradient of PGE2, CD4$^+$ coronin 1-deficient cells respond with a significant accumulation of cAMP upon stimulus, while wild-type CD4$^+$ T cells only produce low amounts. CD8$^+$ cells respond in a similar manner, however the amount of cAMP produced is lower, only resulting in about a tenth of the cAMP produced by CD4$^+$ cells (Fig 16 B). It is known that the levels of cAMP differ between different T cell subtypes (166), with regulatory T cells having elevated levels of cAMP in comparison to conventional CD4$^+$ cells and CD8$^+$ cells. To assess if the difference in cAMP is subset-specific or equal among the different populations (see Supplemental Figure 6 for details), T cells were isolated via negative selection and subsequently sorted for their CD8 and CD4/CD25 status. This allowed for the differentiation into regulatory (CD4$^+$CD25$^+$), conventional (CD4$^-$CD25$^-$) and CD8$^+$ T cells. In contrast to wild-type situations, the bulk of cAMP present in coronin 1-deficient T cells is not from the regulatory T cells, but from the conventional CD4$^+$ cells (Fig 16 C). With conventional CD4$^+$ cells producing the majority of the increased cAMP seen in coronin 1-deficient T cells, one could speculate that the increased cAMP content dampens the necessary survival signals depending on TCR:MHC interaction.
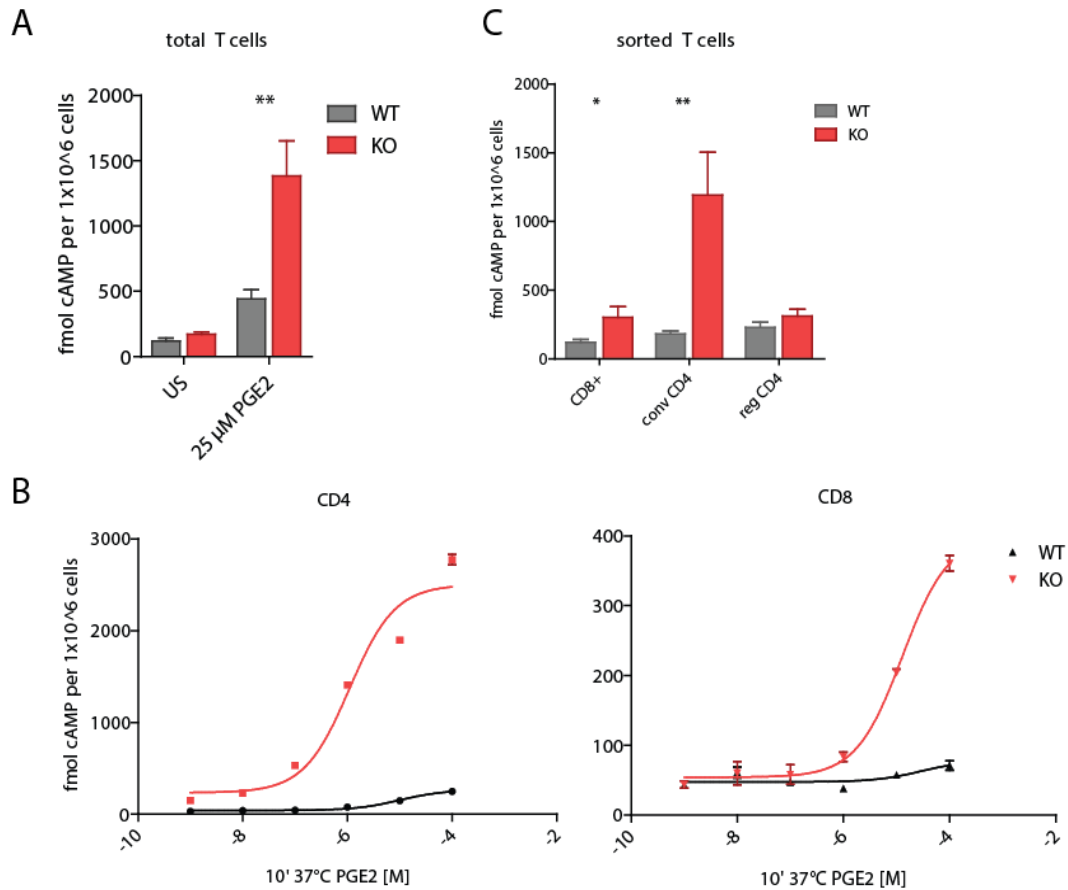
Fig 16: total or sorted T cells stimulated with PGE2. (A) T cells were negatively selected and stimulated with 25 μM PGE2. (B) CD4 or CD8 cells were negatively selected and stimulated with different concentrations of PGE2. All stimulations were performed for 10' at 37°C. (C) total T cells were isolated from spleen and subsequently sorted by flow cytometry into different populations. Steady levels were assessed for the resulting T cell populations. * p<0.05, **p<0.005. Unpaired two-tailed students *t*-test. Error Bars indicate SD from 3 independent experiments for (A) and (C) and two independent experiments for (B).

8.2. Dependency of thymocytes on coronin 1 expression

8.2.1. Coronin 1-dependent cAMP production in thymocytes

8.2.1.1. Single positive thymocytes lacking coronin 1 expression show increased cAMP production

The thymus is the main organ for T cell maturation. It is populated during early life and involutes with age. Coronin 1-deficient animals, while showing a massive peripheral T cell lymphocytopenia, do not show a thymic phenotype in terms of thymic subpopulations (see section 8.4.1). The knock-out animals used in this study represent a constitutive knock-out and the coronin 1 promoter increases in activity during thymocyte development. To assess if thymocytes show a similar cAMP phenotype observed in peripheral T cells, the thymi of wild-type and coronin 1-deficient animals were sorted for their expression status of CD4 and CD8 (see Supplemental Figure 5 for details). In terms of steady state cAMP levels, single positive thymocytes have lower levels than peripheral T cells (compare Fig 17 A with Fig 11 A). There is also no significant difference in the steady state cAMP levels of wild-type and coronin 1-deficient thymocytes, regardless of their CD4/CD8 expression status. Interestingly, the increased generation of cAMP is observed in the single positive thymocytes, with CD4$^+$ thymocytes having around 2 fold increased generation of cAMP upon stimulation with FSK. While CD8$^+$ thymocytes do show a statistical significant difference, it is not as striking as in CD4$^+$ cells. When stimulated with PGE2, double negative cells produce high levels of cAMP regardless of coronin 1 expression. The production of cAMP upon PGE2 stimulation is different in single positive thymocytes. While CD4$^+$ thymocytes produce low amounts of cAMP upon PGE2 stimulation, the CD8$^+$ cells show increased levels of cAMP upon stimulation with PGE2 (Fig 17 C). This is in stark contrast to peripheral T cells, where stimulation with PGE2 leads to a massive increase in cAMP in CD4$^+$ cells but only a slight increase in CD8$^+$ cells, which is possibly explained by differential surface expression of PGE2 receptors. Interestingly, coronin 1-dependent elevation of cAMP levels is observed in the thymocyte subpopulations (single-positive) showing the highest coronin 1 expression levels. Taken together, the data shows a correlation between the expression pattern of coronin 1 and the differential production of cAMP upon stimulus in wild-type and coronin 1-deficient thymocytes.
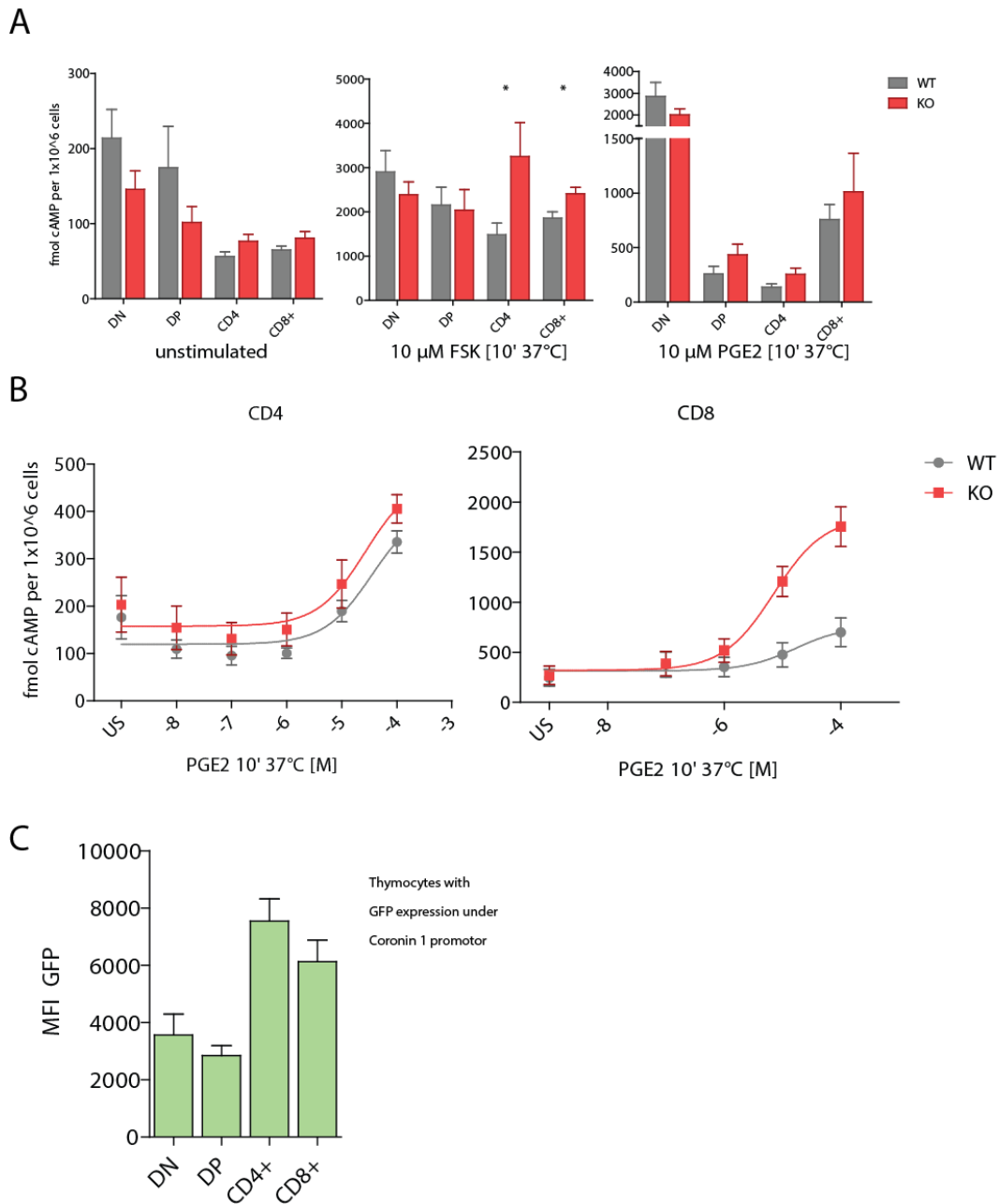
Fig 17: Assessment of cAMP production in sorted thymocytes stimulated with PGE2. (A) thymocytes sorted for expression status of CD4/CD8 and subsequently assessed for cAMP at steady state or stimulated with 10 μM FSK or PGE2. (B) cAMP production in thymocytes sorted for CD4 or CD8 status upon stimulation with different PGE2 concentrations. (C) Representative MFI measurements in thymocytes expressing GFP under the coronin 1 promoter. All stimulations done for 10' at 37°C. * $p<0.05$. Unpaired two-tailed students *t*-test. Error Bars indicate SD from pooled data of 3 (A) or 2 (B) independent experiments.

8.2.2. *Survival of coronin 1-deficient thymocytes*

8.2.2.1. *Changes in survival of thymocytes upon modulation of cAMP is comparable regardless of coronin 1 expression status*

Thymocytes from coronin 1-deficient animals show increased cAMP production, but no physiological survival phenotype. To study if thymocyte survival is dependent on cAMP signalling or if the stimulatory environment of the thymus overcomes any impairment on survival, the cells were studied in vitro. Additionally, the cAMP content was modulated by either (i) blocking cAMP degradation with Rolipram, (ii) increasing cAMP production by either direct (FSK) or indirect (PGE2) adenylate cyclase activation or (iii) by competing with endogenous cAMP (8-Br-cAMP). For this, thymi were prepared as single cell suspensions, cultured in the presence of cAMP modulating agents and their viability assessed by flow cytometry. As expected, thymocytes do not survive in culture for extended periods of time, with only around 60% of wild-type thymocytes viable after day 1. This is further reduced to around 25% on day 2. However, the coronin 1-deficient thymocytes, while not statistically significant, appear to die faster than the wild-type cells. Modulation of cAMP by inhibiting degradation by PDE4 or increasing cAMP actively by stimulation leads to the death of thymocytes, regardless of their coronin 1 expression status (Fig 18). In contrast to the other agents, 8-Br-cAMP does not modulate the cAMP content itself, it competes with endogenous cAMP for the downstream signalling via the PKA/CREB pathway. The competition did not interfere with thymocyte viability at all, showing a similar survival pattern as the untreated control samples. In summary, modulation of cAMP by either inhibiting degradation or by inducing the production leads to the death of total thymocytes which leaves any interpretation on the effect of coronin 1 deletion to be difficult.
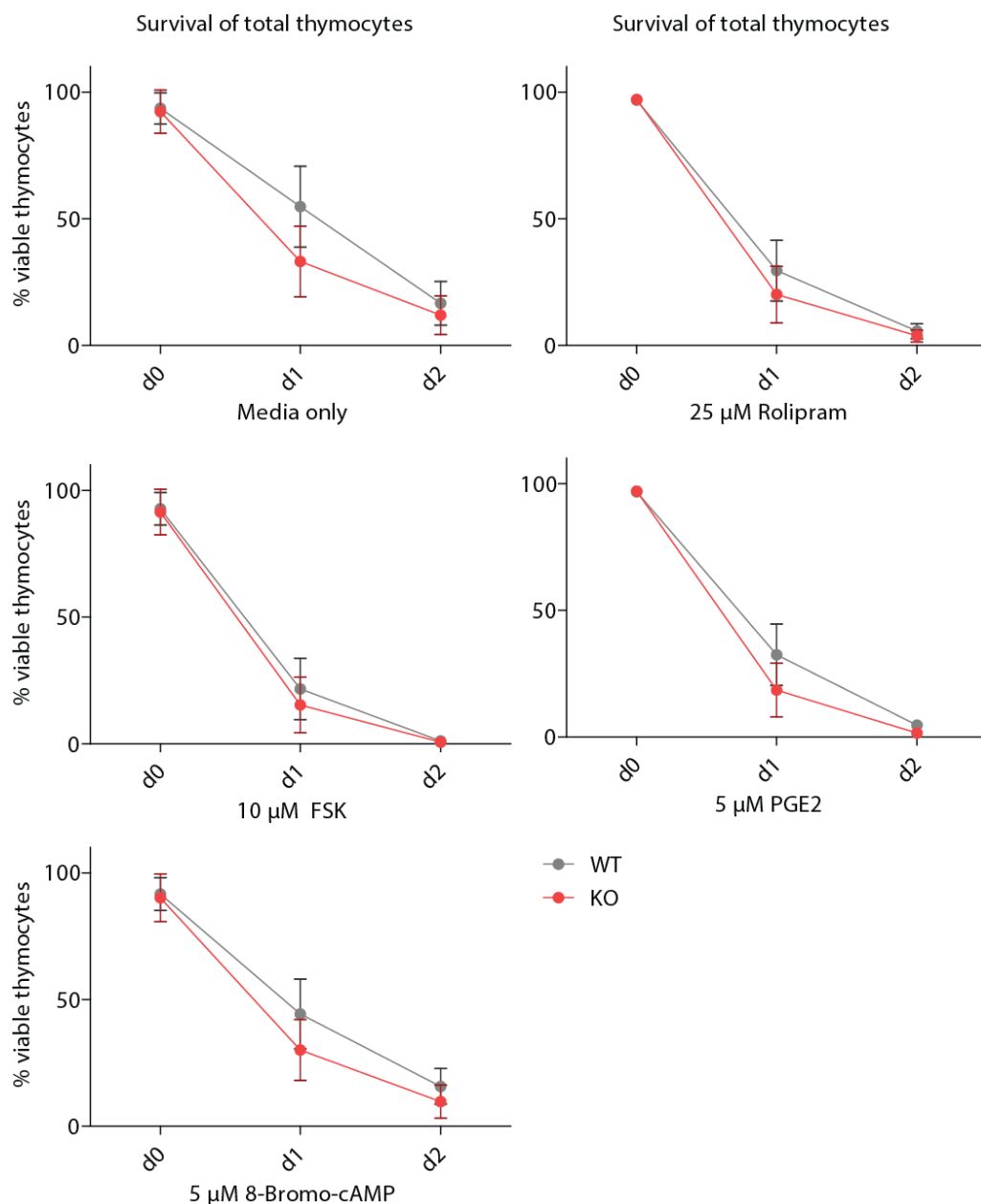
Fig 18: Survival of total thymocytes *in vitro*. Single cell suspensions from thymi of wild-type or coronin 1-deficient animals were cultured with indicated conditions and time. Viability was assessed by flow cytometry. Graphs show pooled data from at least three independent experiments.

Additionally, cells were incubated with cytokines known to be involved during thymocyte development (IL-7) but also peripheral T cell survival (IL-2 and IL-7) and their viability assessed. As seen in Fig 19, one hour incubation does not lead to thymocyte death, after around 6 hours of incubation time; the cells slowly start to show reduced viability. While thymocytes obtained from coronin 1-deficient animals seem to have a slightly lower overall viability, the trend is the same and any difference is not significant. In terms of cytokine treatment, the cells also respond

equally, with thymocytes from coronin 1-deficient and wild-type animals showing a viability of around 25% after 2 days.

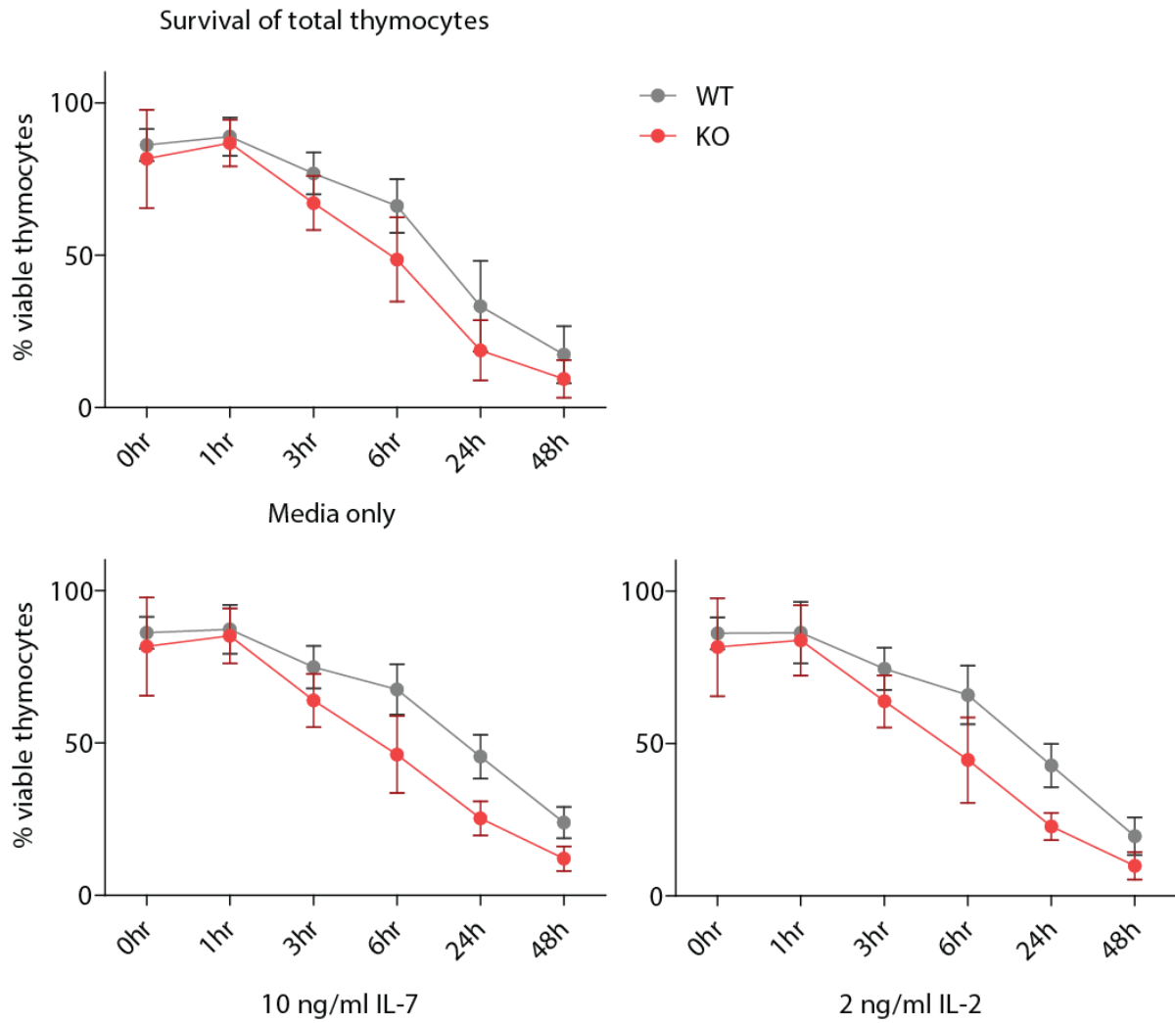**Fig 19: short term survival of total thymocytes. Single cell suspensions from thymi of wild-type or coronin 1-deficient animals were cultured with indicated conditions and time. Cell viability was assessed by flow cytometry. Graphs show pooled data from at least three independent experiments.**

8.3. Survival of coronin 1-deficient thymocytes

8.3.1. *Coronin 1-deficiency has no impact on cytokine induced late stage thymocyte survival*

As the bulk of thymocytes are double-positive, showing lower expression of coronin 1 than peripheral T cells and later stages of thymocytes, only thymocytes about to exit the thymus were used for the follow up studies. To achieve this, single cell suspension of thymocytes were first depleted with an antibody for CD8$^+$ and magnetic beads. This results in only double-negative and CD4 single positive cells remaining. The resulting cells were then stained for surface markers known to be modulated during the last stages of thymocyte maturation. By selecting only cells which have downregulated CD69 and also have acquired the expression of CD62L, the resulting cells are (i) proliferation competent and (ii) protected from death receptor signalling (see Supplementary Figure 6 for scheme). This represents a physiologically unimpaired population, in terms of survival, of cells as close to peripheral T cells as possible in a coronin 1-deficient background. Similar to the experiment performed in total thymocytes, the cells were then cultured in media alone or in the presence of the pro-survival cytokines IL-2 and/or IL-7. When provided with IL-2 and IL-7 both wild-type and coronin 1-deficient thymocytes survive equally (Fig 20 A). The IL-7 receptor is known to be upregulated during thymocyte maturation. Interestingly, the assessment of its surface expression for CD62L$^+$CD69$^-$ thymocytes was equal in wild-type and coronin 1-deficient thymocytes. However, the late thymocyte population which is the only one found to be reduced in coronin 1-deficient animals, also shows higher expression of the IL-7 receptor (Fig 20 B). Culturing the thymocytes in a single-cytokine system reveals that the rescue is not due to IL-2 (Fig 20 C) but mainly due to the presence of IL-7 (Fig 20 D). This is in line with recent thymic emigrants depending on IL-7 for their survival and IL-2 needed for later stages (167). Therefore, the severe T cell lymphocytopenia seen in coronin-1 deficient animals is not due to their inability to convey the survival signals provided by IL-7.
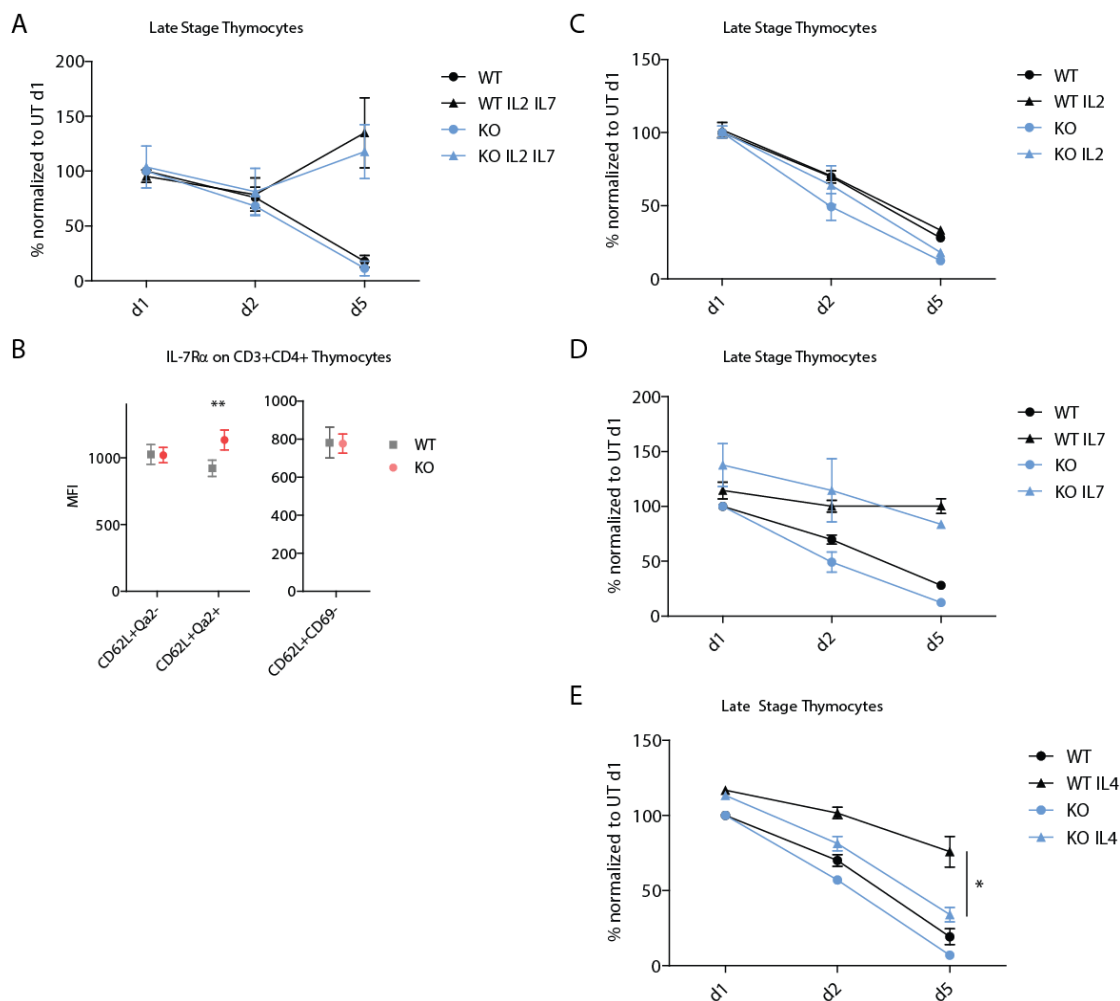
Fig 20: (A, C, D and E) Thymocytes cultured in media alone or in the presence of 20 ng/ml of indicated cytokines. Data from at least 3 individual experiments. (B) surface expression of IL-7a on Late Stage Thymocytes and thymocytes gated for CD62L/Qa2 expression status. Data from 6 animals in 2 independent experiments. Unpaired students *t*-test, two-tailed, * p<0.01 ** p<0.001.

8.3.2. *Wild-type but not coronin 1-deficient late stage thymocytes can be rescued by addition of IL-4*

Interestingly, one cytokine, IL-4, that is not needed for T cell survival but for subsequent steps in T cell maturation showed different survival responses in coronin 1-deficient thymocytes compared to wild-type cells. IL-4 is known to be important for the generation of a specific subset of T cells, namely Th2. While wild-type thymocytes survive better in the presence of IL-4, the cells lacking coronin 1 do not respond to this survival signal and die (Fig 20).
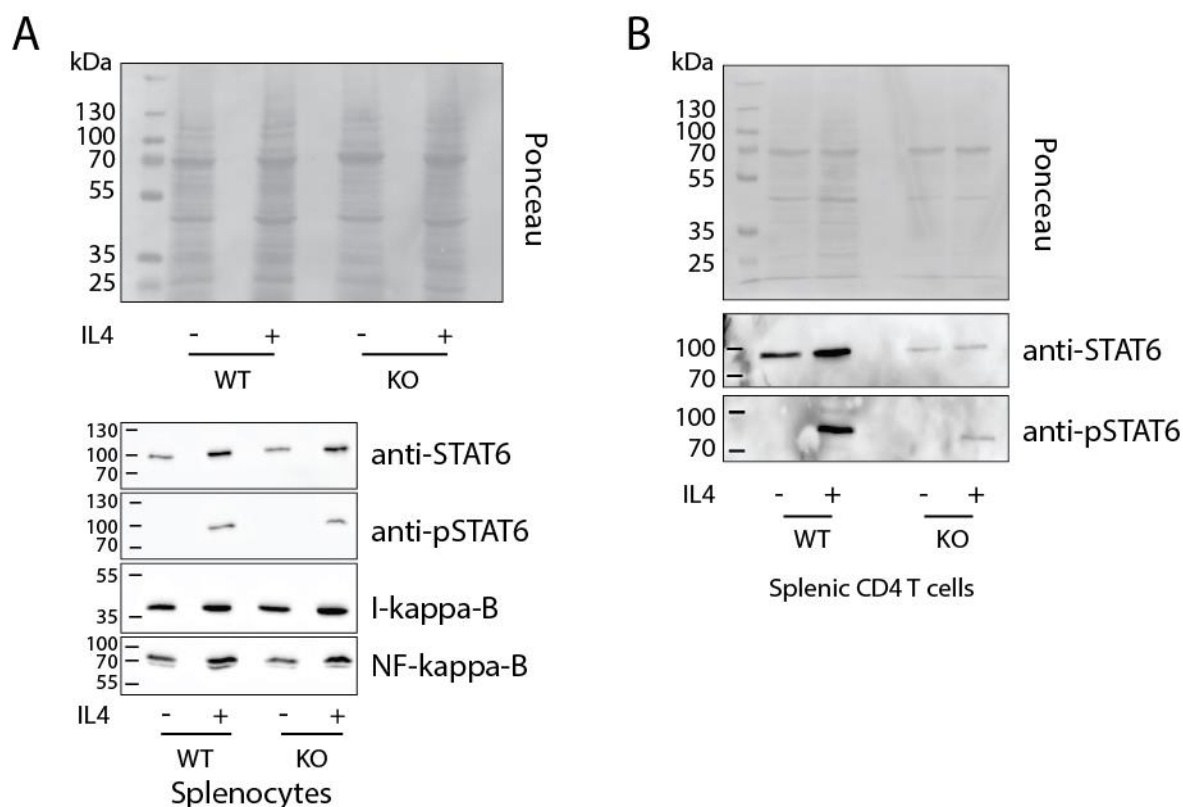
Fig 21: IL-4 induced phosphorylation of STAT6. Splenocytes (A) or isolated splenic CD4⁺ cells (B) of wild-type or coronin 1-deficient animals were stimulated with 20 ng/ml IL-4 for 24 hours and assessed by immunoblot. Each experiment is only performed once.

To assess if the defect in IL-4 induced survival of thymocytes would be due to the inability of coronin 1-deficient cells to phosphorylate the downstream target STAT6, either splenocytes (Fig 21 A) or splenic CD4⁺ cells (Fig 21 B) were cultured in the presence of 20 ng/ml IL-4 and assessed for downstream signals. Both cell types show phosphorylation of STAT6 upon treatment with 20 ng/ml IL-4 for 24 hours. The assessment of splenocytes for proteins involved in NFκB signalling displayed no difference between genotypes, which could also be due to the mixed population of cells masking any T cell specific phenotype. However, this is only an indication that coronin 1-deficient cells can be triggered by IL-4 and the lack of survival of sorted coronin 1-deficient thymocytes upon IL-4 stimulus would require further studies.

8.3.3. *Survival signals mediated by dendritic cells rescue both wild-type and coronin 1-deficient late stage thymocytes*

As the thymocytes have not shown a difference in the survival when cultured with IL-7, sorted thymocytes were also cultured in medium or in the presence of bone marrow-derived dendritic cells, which are known to offer stimuli needed for peripheral T cell survival (168). As shown in Fig 22 B, both wild-type and coronin 1-deficient late stage thymocytes do not survive in culture when not receiving any additional survival signals. In co-culture with immature dendritic cells however, both wild-type as well as coronin 1-deficient thymocytes survive over the period of five days (Fig 22 B). To assess if this survival is due to factors secreted by DCs, thymocytes were either cultured in filtered supernatant of DCs or in a transwell co-culture system. Under both conditions, the thymocytes failed to survive, suggesting a requirement for direct cell-cell contact (compare Fig 22 B to C) for late stage thymocyte survival.
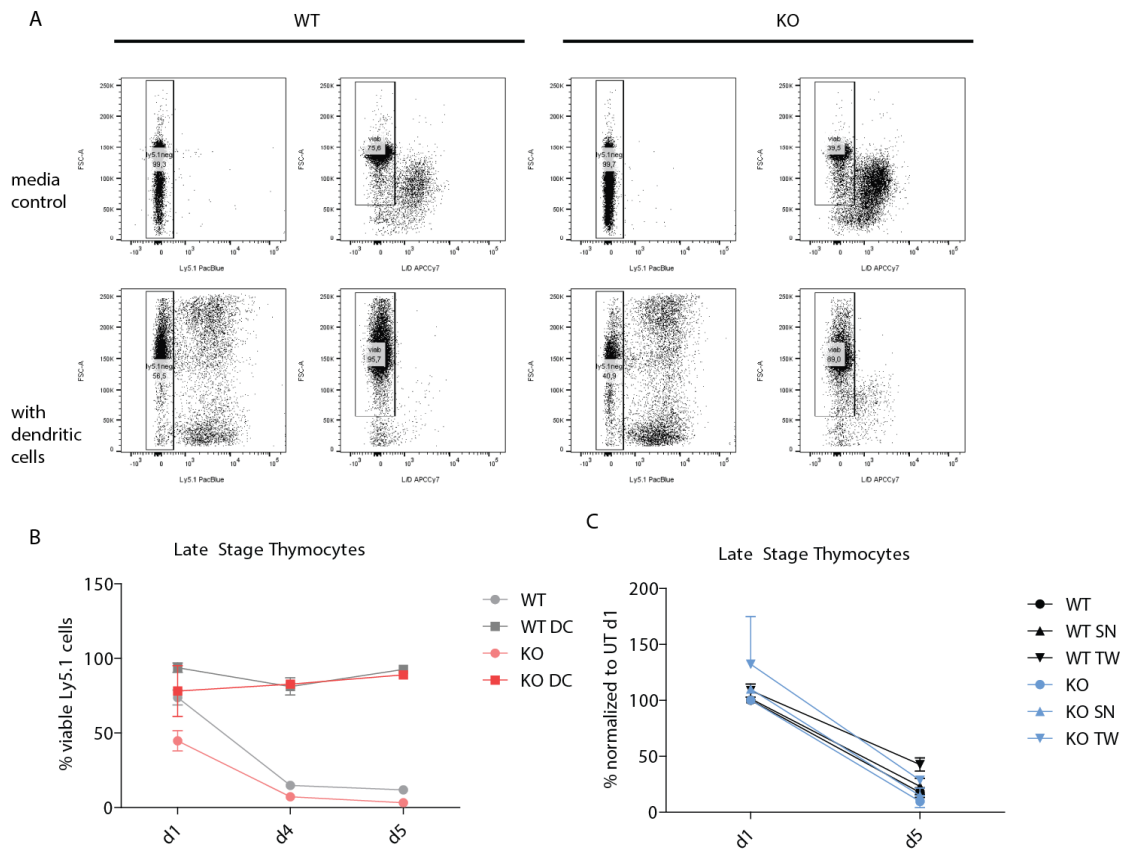
Fig 22: In vitro survival of thymocytes sorted for CD3$^+$CD4$^+$CD62L$^+$CD69$^-$. (A) Gating strategy for viability measurements. Top panels show wild-type (left side) and coronin 1-deficient (right side) samples without (top) or with dendritic cells (bottom). (B) Thymocytes cultured in media alone or in the presence of 100.000 immature dendritic cells for up to 5 days. (C) Thymocytes cultured in media alone, with 0.2 μm filtered 24 hr supernatant of immature dendritic cells (SN) or in a 3.0 μm transwell system with 100.000 immature dendritic cells. For panel C cell counts were normalised to the untreated media control of day 1. Concentration of thymocytes is 75.000 cells/well in all experiments. Media was replenished after 2 days in culture; data represents mean from at least 3 independent experiments. Error Bars indicate SD.

8.4. Thymocyte development in wild-type and coronin 1-deficient animals

8.4.1. *Major Thymocyte populations and TCR maturation are comparable in wild-type and coronin 1-deficient animals*

Coronin 1-deficient animals have been reported to show a severe lack of peripheral T cells but whether or not coronin 1 is important for the development and maturation of thymocytes is unclear (96, 97). Additionally, previous experiments have shown the thymocytes produced by coronin 1-deficient animals to have equal survival capabilities when cultured with known pro-survival stimuli. To assess which population of T cell lineage would be the first showing a phenotype upon coronin 1 deletion, thymi of wild-type or coronin 1-deficient animals were isolated and single cell suspensions were prepared. Subsequently, the cells were stained for CD3, CD4 and CD8 and viable cells were assessed by flow cytometry. No difference in the percentages of double negative, double positive or single positive cells could be found in the thymus of wild-type versus coronin 1-deficient animals (Fig 23 A).

As TCR rearrangements occur during the double positive stages of thymocyte development, the TCR usage was analysed by flow cytometry. Total T cells were isolated via negative selection from the thymus of wild-type or coronin 1-deficient animals. The resulting cells were then stained for CD3, CD4 and CD8 and additionally with antibodies detecting specific mouse Vβ T cells receptor isoforms. For analysis, $CD3^+CD4^+$ cells were identified by flow cytometry followed by the analysis of the presence of specific Vβ T cell receptor isoforms. No difference was observed in $CD3^+CD4^+$ thymocytes concerning their TCR repertoire (Fig 23 B). Together with the normal percentages of thymic populations, this indicates that coronin 1 is dispensable for thymocyte maturation up to the single positive stage.
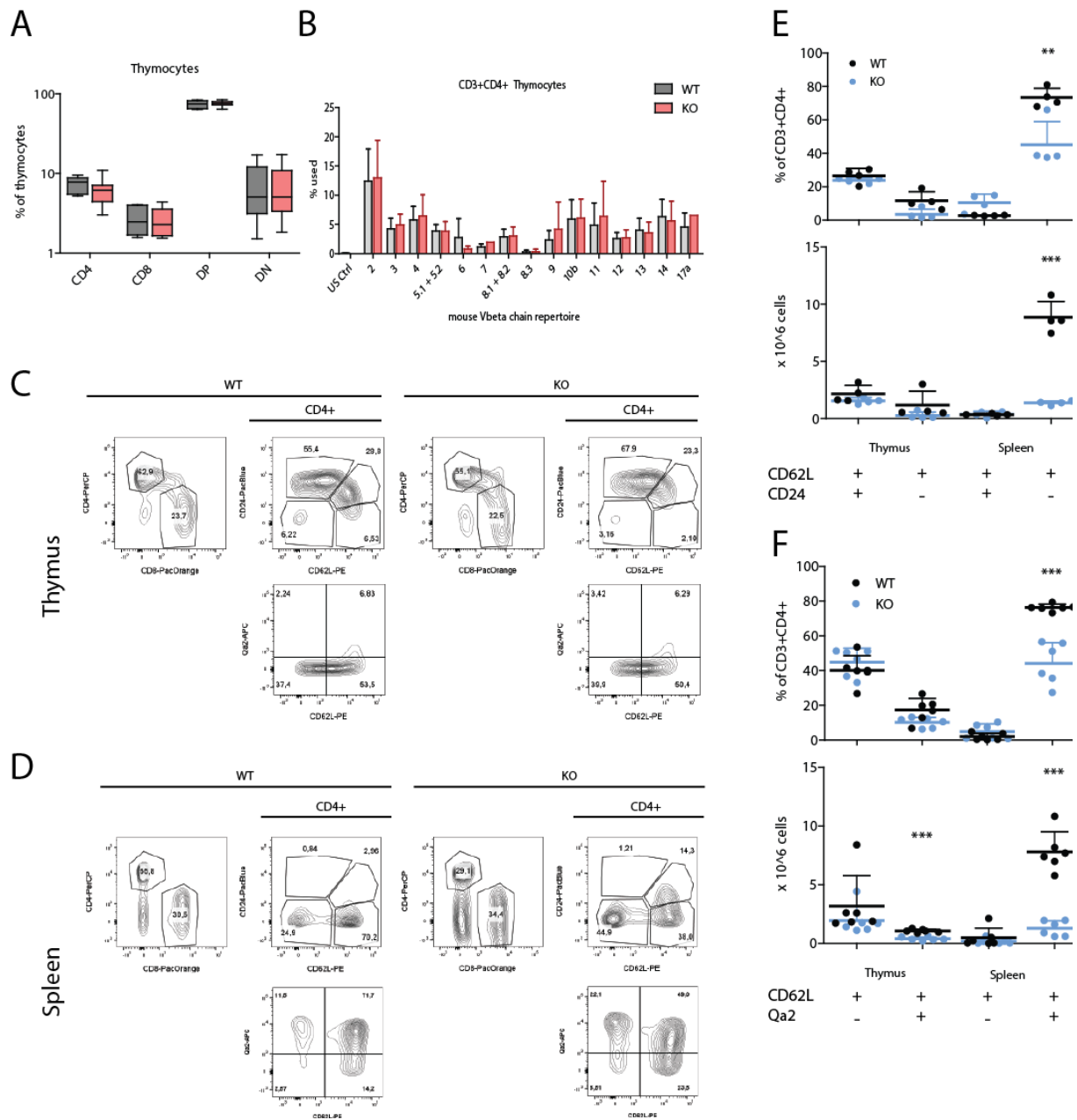
Fig 23: Analysis of thymocytes populations, TCR usage and late stage thymocyte surface markers (CD62L, CD24, Qa2) CD3+CD4+cells from wild-type and coronin 1-deficient mice. Thymi were mashed into single cell suspensions and stained for corresponding markers. (A) Analysis into thymic populations, double negative DN, double positive DP, single positive CD4 or CD8 (>3 independent experiments) (B) Usage of mouse Vβ chains for assessment of TCR repertoire (SD of 2 independent experiments) (C) Representative gating strategy for CD3+CD4+ thymocytes and (D) splenocytes, numbers indicate percentage of CD3+CD4+ cells. (E+F) Percentages and total numbers for indicated populations in thymi or spleen of wild-type and coronin 1-deficient animals of 6-8 weeks of age. (n=4 and 6 for CD62L/Qa2 and CD62L/CD24 expression respectively from at least 3 independent experiments. Unpaired students *t*-test, two-tailed, * p<0.01, ** p<0.001, *** p<0.0001. Error Bars indicate SD

8.4.2. *Equal numbers of ready to egress thymocytes are found in wild-type and coronin 1-deficient animals*

Following maturation, thymocytes exist in the thymus as late stage thymocytes before they egress out of the thymus as RTEs. To assess a possible role for coronin 1 at later developmental stages, the levels of cell surface markers that define this cell population, in particular CD62L, Qa2 and CD24 (34) were assessed. Thymocytes exiting the thymus upregulate CD62L and Qa2 while downregulating the expression of CD24. This allows the differentiation into thymocytes preparing to egress (CD62L$^+$CD24mid/CD62L$^+$Qa2$^-$) and thymocytes ready to egress (CD62L$^-$CD24$^-$/CD62L$^+$Qa2$^+$) (Fig 23 C for gating strategy). In peripheral organs such as the spleen, the same gates can be used to identify cells which have either just egressed (CD62L$^+$CD24mid/CD62L$^+$Qa2$^-$) or have already established themselves in the peripheral T cell pool (CD62L$^+$CD24$^-$/CD62L$^+$Qa2$^+$) (Fig 23 D). Analysis of surface expression for CD62L/CD24 in CD4$^+$ thymocytes revealed no difference in total numbers of thymocytes ready to egress and also not in the numbers of T cells which recently egressed (Fig 23 E). The same analysis performed for the surface expression of CD62L and Qa2, which both need to be upregulated for thymic egress, reveals again no difference in total numbers of recently egressed T cells. While there is a small but significant difference in CD62L/Qa2 double positive thymocyte numbers, this loses its significance when displayed as percentage of cells (Fig 23 F). The most striking and consistent difference is, as expected from previous observations (96, 99, 100, 108), visible in the mature stages of naïve T cells, where coronin 1-deficient animals show a clear reduction in numbers and percentages (Fig 23 E+F). Together this data suggest that coronin 1 is dispensable for thymocyte survival and development.

8.4.3. *Expression of proteins needed for thymocyte and recent thymic emigrant maturation*

Thymocytes, which are about to egress, upregulate not only CD62L but also downregulate expression of CD69. Therefore cells positive for CD62L have downregulated CD69 and are considered late stage thymocytes. As coronin 1-deficient animals show no thymic egress phenotype (i.e. accumulation of single positive thymocytes, see Fig 23A, E and F), the gene expression of proteins known to be important for thymic egress, but also for pro-survival signals

and homing/migration signals in thymocytes and RTEs were assessed (Fig 24A). Therefore, thymocytes were sorted for the M2 stage (i.e. CD62L$^+$CD69$^-$, see Supplementary Figure 6), as described for the survival studies. Subsequently mRNA was isolated and the gene expression assessed by qPCR. The krüppel-like factor 2 (KLF2), which is upstream of CD62L, was found to be expressed equally in wild-type and coronin 1-deficient thymocytes sorted for CD62L$^+$Cd69$^-$ expression. Secondly, another important transcription factor, not only for thymic egress but also for the generation of naïve mature T cells, is the Forkhead Box Protein 1 (Foxp1). Mice deficient for this factor develop an activated phenotype, shown by increased CD44 positive cells in the thymus, resulting in increased apoptosis. However, this is not the case in coronin 1-deficient animals as the thymocytes have no increased CD44 expression (data no shown) and also the gene expression of Foxp1 is unchanged. Furthermore the expression of the transcriptional repressor NFκB activation protein (NKAP) was analysed. NKAP is mandatory for the maturation of T cells, as lack of this repressor leads to functionally immature RTEs. In line with the fact that no striking reduction in late stage thymocyte numbers can be seen, NKAP expression is not significantly modulated upon the lack of coronin 1 protein. To assess the expression of CCR7, the chemokine receptor needed for successful migration from the medulla to the cortical region of the thymus, single cell suspension from thymi were measure by flow cytometry. In line with previous data (97), no differences in surface expression could be found between wild-type and coronin 1-deficient thymocytes (Fig 24).
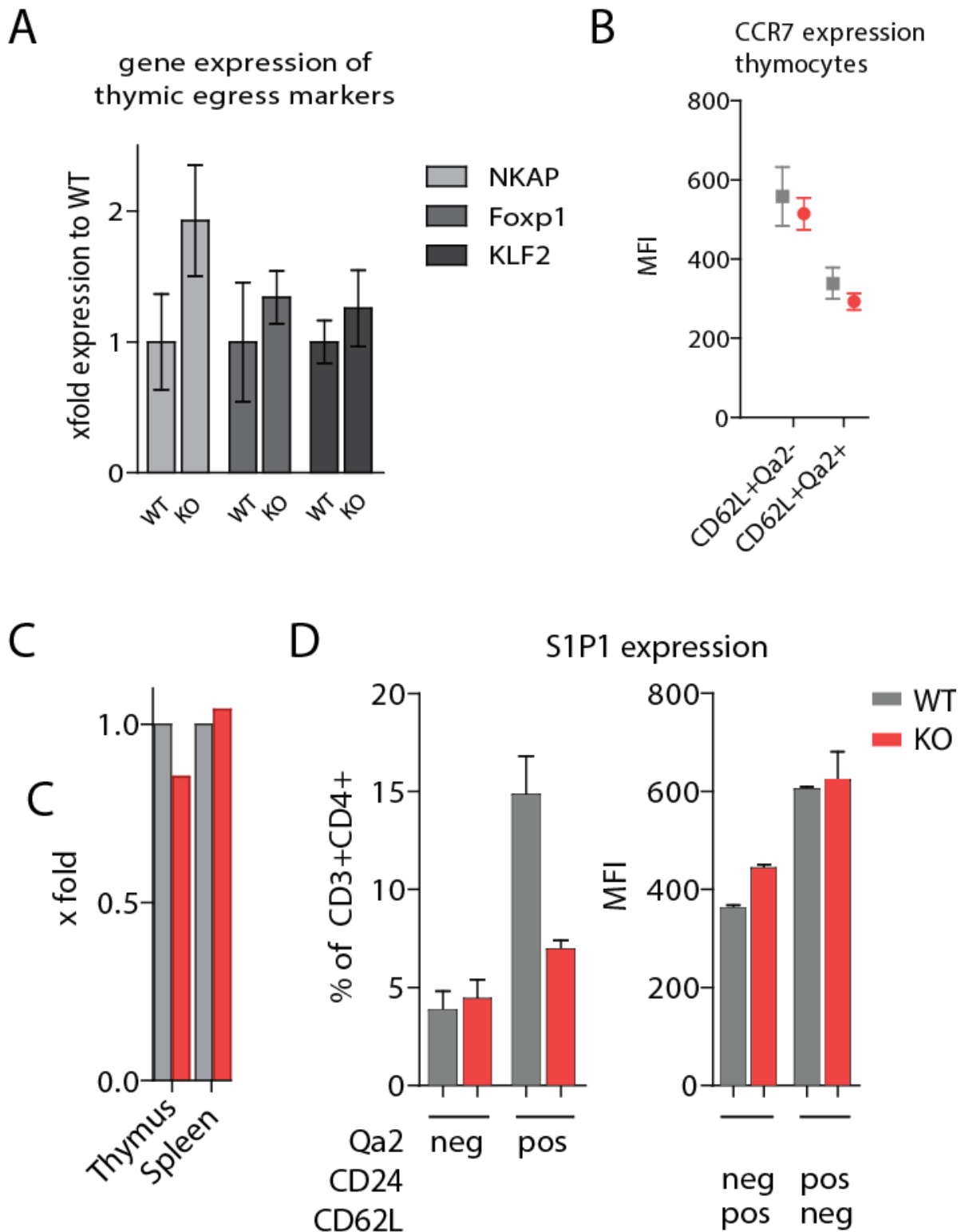
Fig 24: Analysis of markers important for thymocyte egress and maturation in animals from wild-type or coronin 1-deficient background. (A) quantitative RT-PCR for NKAP, Foxp1, KLF2 and GAPDH in thymocytes sorted for CD62L and CD69 expression. Shown is the xfold change in gene expression in KO compared to wild-type cells. Data is normalized to GAPDH expression and acquired in triplicates from RNA samples obtained in three independent experiments. >2 fold change considered significant (B)

To further analyse a requirement for coronin 1 in TCR rearrangement that could possibly result in differences in T cell population in the two genotypes, T Cell Receptor Excision Circles (TREC) were analysed. These TRECs originate during TCR rearrangement in the thymus, were small circular episomal DNA circles are produced, and persist in T cells, where they are diluted with every division and can therefore be used as a proxy to assess the age of a peripheral T cell. Therefore TRECs can be used to assess successful thymocyte maturation and egress(165). To measure the levels of TREC molecules, genomic DNA of thymocytes and splenocytes was isolated and a qPCR performed. The levels of TREC were comparable in wild type and coronin 1-deficient animals consistent with the similar T cell receptor repertoire in wild type and coronin 1-deficient mice (Fig 24 C). This also holds true for the TREC levels in splenocytes of both genotypes, further indicating a normal RTE compartment in coronin 1-deficient animals.

For the main migration marker in thymocytes egress, the receptor for S1P, literature (99) has suggested no difference in surface expression, but a functional deficit in migration. The surface expression of this receptor was therefore assessed by flow cytometry in whole thymocytes. While our data does not show an increase in any late stage thymocyte population, suggesting an *in vivo* migration defect, the surface expression of S1P1 differs between wild-type and coronin 1-deficient thymocytes gates for CD62L and Qa2 (Fig 24 C). While wild-type animals possess double the percentage of CD62L, Qa2, S1P1 triple positive cells in comparison to coronin 1-deficient animals, the expression of S1P1 on cells gates for CD62L and CD24 expression is equal. If this would hint at sequential events of surface molecule expression during the last steps of thymocyte egress remains elusive.

Taken together, the late stage thymocytes of coronin 1-deficient animals show no migratory *in vivo* defect and are capable of modulating surface markers and transcription markers needed for thymic egress and maturation.

8.5. Establishment of the peripheral T cell pool in neonatal wild-type and coronin 1-deficient mice

8.5.1. *The coronin 1-dependent survival of peripheral T cells is age dependent*

As the T cells of coronin 1-deficient animals might have accumulated unknown compensatory mechanism to cope with the deletion, newborn animals were studied to see if their RTE and MNT population would reflect the data for adult animals. Therefore the T cell population during the first three weeks of age in wild-type and coronin 1-deficient littermates was assessed. During the first two weeks after birth, in wild type and coronin 1-deficient mice peripheral $CD3^+CD4^+$ T cell numbers were found to raise to similar numbers ($2x10^6$, see Fig 25 A). While in wild type mice T cell numbers steadily increased to $\sim12x10^6$, absence of coronin 1 T cell numbers resulted in low numbers around $\sim2x10^6$. In contrast to this, thymocyte populations were found to be equal (Fig 25 A). Additionally no difference in total cellularity of spleen and thymus of the animals could be found (Fig 25 B).

The majority of T cells in the periphery of newborn animals are RTEs, therefore a subset analysis of peripheral T cells in newborn animals was performed, using the same staining as for the adults. Similar to what was observed in total peripheral T cells, only later T cell stages ($CD62L^+Qa2^+$) showed differences visible after d19, with RTE ($CD62L^+Qa2^-$) being comparable. Wild-type animals have a higher percentage of later stages of T cells in comparison to coronin 1-deficient animals (Fig 25 C). It is possible that the difference only visible after two weeks is due to a lack of expression of coronin 1 during this timeframe. To account for this possibility, we used splenocytes and thymocytes from mice which express GFP under the coronin 1 promoter. Fig 26 A shows a representative histogram from $CD3^+$ splenocytes and total thymocytes of these mice, showing equal activity of the coronin 1 promoter during development.
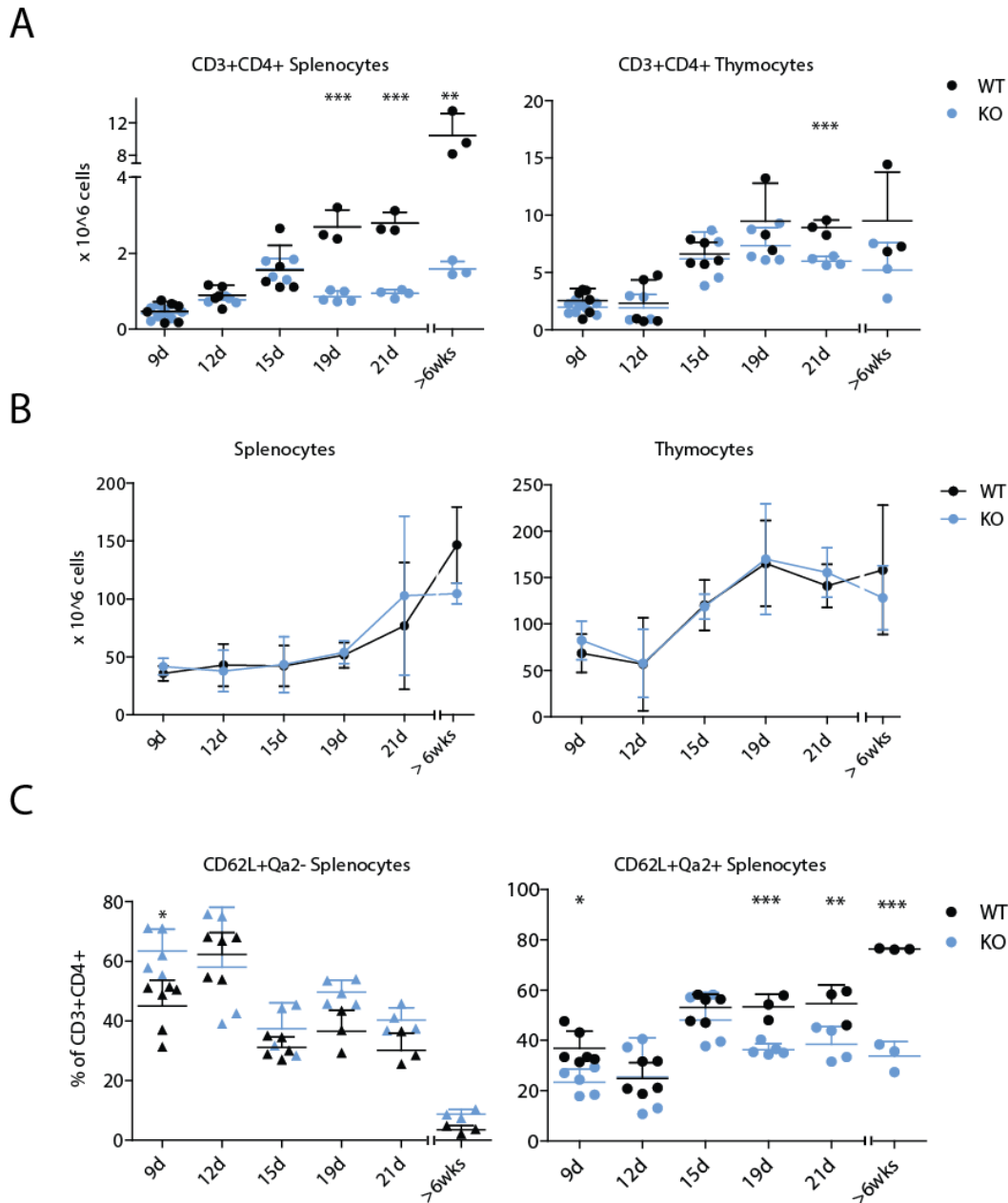
Fig 25: Presence and subset analysis of peripheral T cells and thymocytes in animals a different ages. (A) total numbers of CD3⁺CD4⁺ splenocytes (left) or thymocytes (right) (B) total cellularity for spleen and thymus (C) subset analysis of CD62L/Qa2 expression. Unpaired two-tailed students *t*-test. * p < 0.01, ** p < 0.001 *** p < 0.0001. Each dot represents one animal, data obtained from wild-type and coronin 1-deficient littermates.

As shown in Fig 26B, the assessment of TREC in splenocytes of wild-type and coronin 1-deficient newborn animals (d12) showed no difference, further indicating that the peripheral T cell pool in both genotypes is comparable (Fig 26 C).

Similar to what has been observed in the thymocytes of adult animals, the expression pattern of S1P1 is different in coronin 1-deficient cells. While the assessment of S1P1 expression in cells

gated for CD62L and CD24 status is comparable between genotypes, only about half of the cells are triple-positive for CD62L, Qa2 and S1P1. Additionally, in contrast to the adult thymocytes, the cells from animals of d10 show high expression of S1P1 on Qa2 negative cells, which is not visible in their adult counterparts.

Taken together, this suggests that survival of recent thymic emigrants is independent of coronin 1. Additionally, coronin 1-deficient thymocytes are capable of successful *in vivo* thymus egress comparable to the wild-type situation. Therefore the data points the exact time at which cells of T cell lineage become dependent on the expression of coronin 1 at the stage of MNTs, with the thymic and RTE compartment being unimpaired.
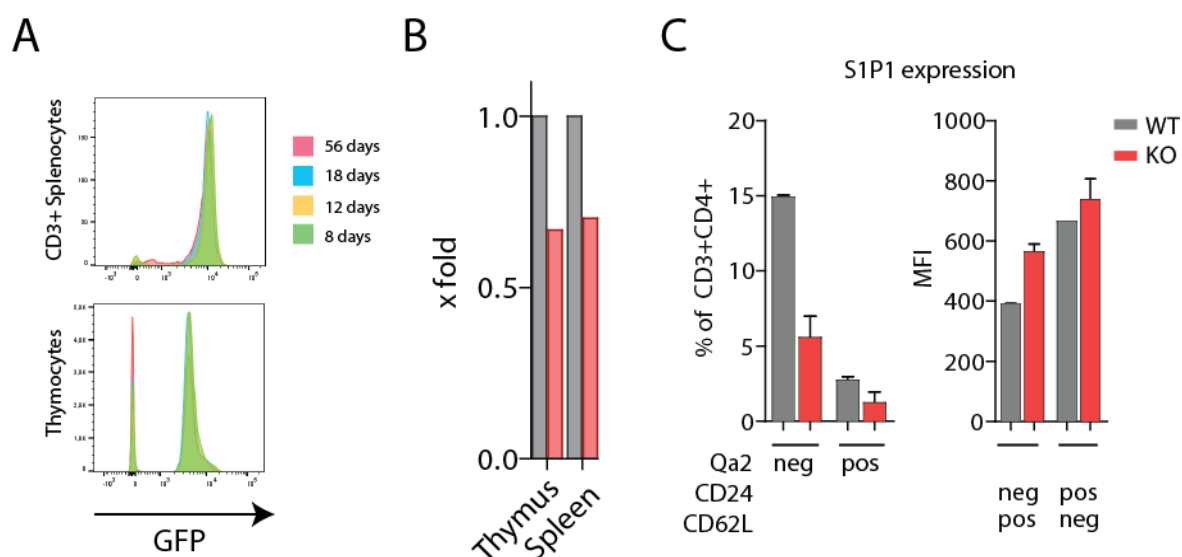

Fig 26: (A) representative histogram for CD3⁺ splenocytes (left) or thymocytes (right) from animals of different ages expressing GFP under the coronin 1 promoter. (B) Analysis of mTREC in splenocytes and thymocytes of d12 old littermates (n=2). (C) surface expression of S1P1 on thymocytes (n=3). Error Bars indicate SD.

9. <u>Discussion</u>

9.1. Cyclic AMP in coronin 1-deficient cells of T cell lineage

9.1.1. *Cyclic AMP levels are increased in coronin 1-deficient T cells*

Neurons expressing coronin 1, and fibroblast, with exogenous expression of coronin 1, show increased levels of cAMP compared to coronin 1-deficient neurons or non-transfected fibroblasts (114). The strongest expression of coronin 1 is visible in cells of the immune system, but no survival deficit is seen in any other immune cells except for peripheral T cells. Interestingly, when T cells are assessed for their cAMP levels, they show an increase in cAMP levels upon the deletion of coronin 1. This phenotype is already present in single positive thymocytes, so it is possible that T cells need finely regulated cAMP levels to achieve maturity, but this regulation is dependent on coronin 1.

Coronin 1 is expressed in all cells of the hematopoietic system and in excitatory neurons (106, 114). The expression pattern is however not equal among hematopoietic cells (http://haemosphere.org/expression/show?geneId=ENSMUSG00000030707). In T cells, coronin 1 levels increase with maturation, namely at stage of single positive thymocytes. Additionally, T cells are the only lymphocyte population with survival impairment upon coronin 1-deletion. Coronin 1, when expressed in fibroblasts, increases the levels of cAMP. In neurons, when deleted, the cAMP levels are reduced, which leads to impairment in the formation of long term memory. As cAMP is an important secondary messenger (169) and changes in cAMP have been linked to thymocyte and T cell survival and function (146, 170), the T cell survival phenotype upon coronin 1 deletion could be linked to cAMP. Thorough analysis of the cAMP pathway in coronin 1-deficient T cells has revealed two important facts (this study and Jayachandran *et al.* in prep). First, coronin 1 has a similar mode of action in neurons and T cells, with the lack of coronin 1 resulting in reduced production of cAMP. Second, the increase in overall cAMP levels is due to modulation of downstream signalling in the cAMP pathway, namely the modulation of the degradation enzyme PDE4. The reduction of PDE4 is likely to be a post-transcriptional modulation, as gene expression analysis of PDE4A5 shows no differential regulation. It is however possible that the band seen is a different isoform of PDE4A, as this enzyme family has a variety of isoforms, closely homologous to each other. One possibility would be PDE4 Isoform 6, which has recently been upgrade from a predicted isoform to a proven

isoform based on the NCBI database. Additionally, no PDE4 related proteins have been found to be modulated in an RNAseq screen in T cells sorted for conventional and regulatory CD4$^+$ cells (data from Rajesh Jayachandran).

Interestingly, while coronin 1 has been shown to be a direct interactor of Gα-s, the G-protein responsible for the activation of the AC, increased cAMP in coronin 1-deficient animals is also seen by direct activation of the AC by Forskolin. Assuming that the difference in cAMP is only due to reduction in degrading enzymes, it is unclear why the lack of coronin 1 would lead to reduced levels of cAMP upon direct activation of ACs in isolated membranes. While the interaction of coronin 1 and Gα-s has been shown to be activation dependent, it stands to reason that coronin 1 does affect AC activity by a so far unknown mechanism. As the combination of Gα-s and Forskolin has been shown to have a synergistic effect on the AC activity (171) one possibility could be just a stochastic mechanism. In a wild-type situation, coronin 1, G-proteins and AC are localized in the plasma membrane, with coronin 1 interacting with free Gα-s. Potentially, coronin 1 leaves ACs in more activated state, increasing the cAMP production.

9.1.2. *Increased cAMP stems from conventional coronin 1-deficient CD4$^+$ T-cells*

Coronin 1-deficiency leads to a distortion in T cell populations, with less naïve T cells and, percentage-wise, more cells of a regulatory subtype. Recent experiments in our laboratory have revealed that coronin 1-deficient T cells have the ability to provide a tolerogenic environment. This results in the lack of host versus graft disease, with the animals not rejecting tissue grafts (manuscript by Jayachandran et al. and thesis from Aleksandra Gumienny 2016). In a wild-type situation, tolerogenic environments are provided by regulatory T cells, but in a coronin 1-deficient situation this is not exclusively mediated by regulatory T cells but rather by conventional T cells. It is known, that regulatory T cells have higher basal levels of cAMP (166). Sorting peripheral T cells into CD8$^+$ cells or conventional and regulatory CD4$^+$ cells revealed the surprising fact that the increased cAMP levels are not due to increased regulatory T cell numbers. It is mainly due to the conventional T cells, which show around 5 fold higher levels of cAMP than their wild-type counterparts. Stimulation by PGE2 of CD4$^+$ and CD8$^+$ splenic T cells shows an increased production of cAMP in both populations. However, the production of cAMP is higher in CD4$^+$ cells, which could be due to differential expression of PGE2 surface expression in these cells. It is unclear why the coronin 1-deficiency would result in T cells down regulating

PDEs to increase their cAMP content, presumably as a compensatory mechanism. However, it has been shown that increased levels of cAMP dampen the TCR response, with increased levels of PDE4 being recruited to membranes to assure efficient TCR activation (172, 173). This indicates that cAMP needs to be fine-tuned for the survival and maturation of T cells, which could be the role of coronin 1. One could speculate that with the lack of coronin 1, the cells end up compensating to achieve sufficient levels of cAMP. This subsequently results in (i) providing a cAMP dependent tolerogenic environment and (ii) having survival impairment due to dampening of TCR signals. The dampened TCR signal leads to the normal tonic TCR stimulus, provided by the peripheral lymphoid organs, not being strong enough for survival.

9.2. T cell subsets in coronin 1-deficient animals and their survival capabilities
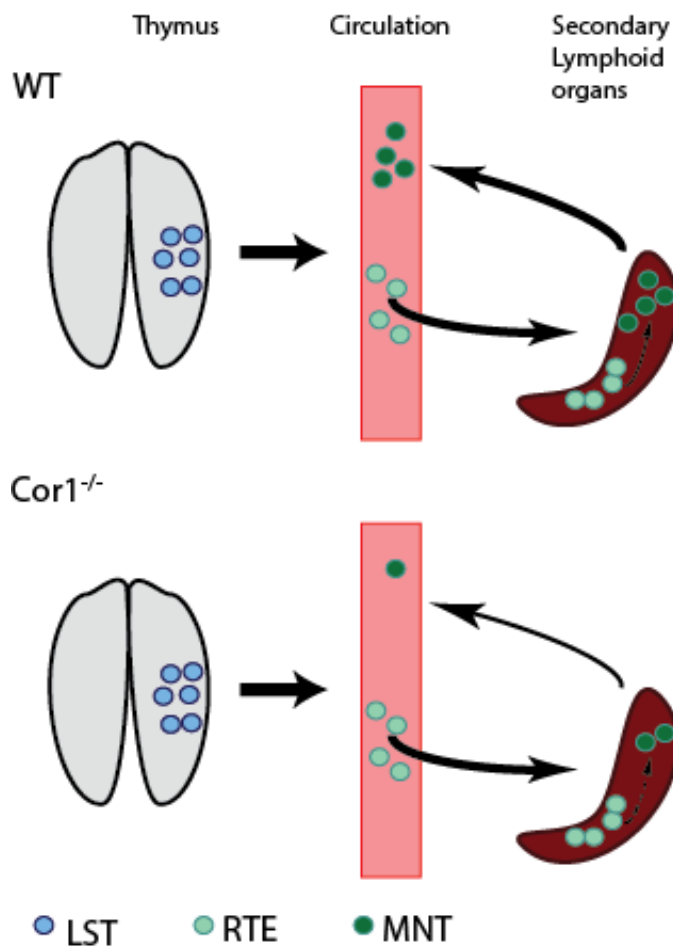
9.2.1. *Coronin 1-deficient late stage thymocytes are capable to survive on peripheral survival signals*

Total thymocytes of coronin 1-deficient animals were shown to be less viable when cultured without any stimulation, however *in vivo* the thymus consists to 80% of double positive cells, which could skew the analysis. While cells in the thymus depend on TCR signalling for their maturation steps, and mature naïve T cells depend on tonic TCR stimuli for survival, the survival and maturation of RTEs does not depend on TCR signals (50). However, it has been shown that RTEs produce higher levels of the pro-survival protein Bcl-2 upon IL-7 signalling (174, 175). As coronin 1-deficient T cell in the periphery are highly compromised, a system with the last non-compromised cell subset was used, namely the latest stage of thymocytes (M2). These cells are proliferation competent and resistant to death receptor signalling of the thymic microenvironment. Interestingly, these late stage thymocytes show comparable survival capabilities to their wild-type counterpart. In line with literature, the cells survive similar to T cells in a co-culture system and the survival is dependent on cell-cell contact (168). While the cells used in this assay would physiologically not depend on the survival signals presented by antigen-presenting cells, it stands to reason that a mature naïve T cell originating from one of these cells would be capable of surviving via TCR:MHC signalling if the signal is strong enough. As it has been shown that the downstream TCR signalling of coronin 1-deficient T cells is dampened (96), possibly also due to increased cAMP levels in these cells, the tonic stimuli provided in secondary lymphoid organs might not be enough for the cells to survive. On the other hand, T cells with a transgenic TCR, specific for parts of the ovalbumin peptide, proliferate upon stimulus regardless of coronin 1 expression status (97), although they show the same reduction in Ca^{2+} signalling as coronin 1-deficient T cells with wild-type TCR (our unpublished observations). An explanation for this discrepancy is so-far elusive; the only potential explanation would be different signals being responsible for homeostasis and proliferation of T cells.

Next to tonic TCR:MHC interaction, the second important factor for peripheral T cell survival and maturation are cytokines, with the two most important ones for survival being IL-2 and IL-7 (167, 176). While IL-2 is important for more mature stages of T cells and to uphold self-tolerance (177), IL-7 is not only needed for the survival of peripheral T cell but also for the maturation of thymocytes (178). Thymocytes, when cultured in high concentrations of IL-2 and IL-7 survive equally regardless of coronin 1 expression. Not only do the cells survive, they also proliferate equally. Given that the same cells will not survive on IL-2 but on IL-7, the late stage thymocytes of wild-type and coronin 1-deficient animals respond equally to cytokine mediated survival. This would further indicate that these cells are per-se capable of understanding survival signals provided by different environments. Interestingly, when the late stage thymocytes are treated with a cytokine, which is not necessarily needed for survival but for maturation, the response differs. While thymocytes expressing coronin 1 can survive when cultured in the presence of IL-4, the thymocytes lacking the expression of coronin 1 do not. This would be in line with total T cells of coronin 1-deficient background showing increased production of IFN-γ, which is being produced by T-helper type 1 cells. In contrast to this, IL-4 is produced in a positive feedback loop by T-helper type 2 cells. However, the lack of survival of peripheral T cells in a coronin 1-deficient animal cannot be due to the lack of response to IL-4, as the IL-4 knock-out animals show no defect in peripheral T cell survival (179). It is therefore unclear what the physiological role would be in terms of IL-4 and coronin 1 and the potential involvement of coronin 1 in the IL-4 pathway remains to be addressed.

9.2.2. *Identification and Measurement of distinct T cell subsets*

T cells from coronin 1-deficient mice show a massive reduction in numbers, regardless of specific subtype. However, this difference is pronounced the most in the compartment of mature naive T cells, with more mature T cells still showing a subtler difference. Additionally, several labs have published contradicting data on the percentages of thymocyte subtypes. Data on thymocytes range from no difference in any thymocyte subsets (96, 100), no reduction in $CD4^+$ cells but in $CD8^+$ single positive cells (97), an accumulation of double positive cells with subsequent reduction in both single positive cell subtypes (108) to accumulation of late stage thymocytes resembling a classic thymic egress phenotype (99). When assessed for surface markers known to be important in the late stages of thymocytes and being modulated upon the maturation of recent thymic emigrants to mature naive T cells, no difference was observed in terms of numbers and percentages up to the stage of recent thymic emigrants. In contrast to mature naive T cells, which depend on the tonic MHC:TCR interaction for survival, recent thymic emigrants have been shown to only depend on this interaction for shaping the TCR repertoire but not for maturation or survival (50). It is, as of now, unclear which exact signals drive RTE maturation, but the cells do need to home into secondary lymphoid organs for their maturation (47). As coronin 1-deficient animals do have mature T cells which have undergone post-thymic maturation steps and additionally show no reduction in KLF2 gene expression or CD62L surface expression, the cells should theoretically be able to efficiently

Fig 27: Schematic on T cell homeostasis in mice. Wild-type and coronin 1-deficient thymocytes egress into the bloodstream and then migrate into secondary peripheral lymphoid organs. The recent thymic emigrants further mature into mature naïve T cells. At this stage, coronin 1-deficient T cells have a survival deficit and only a small set of peripheral T cells manage to survive.

enter secondary lymphoid organs. However, there have been studies showing a deficit in migration against certain ligands in coronin 1-deficient cells (99, 104). In summary, coronin 1-deficient animals (i) have normal thymocyte and recent thymic emigrant levels, (ii) have a severe reduction in the stage of mature naive T cells and (iii) show normal expression of migration markers, but do show deficiencies in migration. This could indicate that the thymocyte egress and the circulation of recent thymic emigrants is normal, which is also consistent with normal levels of TREC in the periphery of coronin 1-deficient animals, but that these cells have a problem with maturation and therefore die off as soon as they depend on survival signals for mature naive T cells (Fig 27). This would be in line with literature showing a defect in Ca^{2+} signalling upon stimulation of the T cell receptor in coronin 1-deficient T cells (96).

9.2.3. *T cell development in newborn and adult mice*

In a normal functioning organism, the majority of T cells come out of the thymus and proliferation in the periphery is scarce. In other conditions, such as lymphocytopenia, T cells are known to proliferate as the normal thymic output cannot produce enough cells in a short period of time. This proliferation is coined homeostatic proliferation and is dependent on the expression of CD24 (40). However, there is one physiological occurrence when organisms are functioning normally but are considered lymphodepleted. During mouse development, the T cell niche is gradually filled by RTEs with the peak of thymic egress at around 6 weeks of age (19). Interestingly, the survival of coronin 1-deficient T cells in the periphery is age dependent. Early ages show equal numbers of peripheral CD4$^+$ T cells, but after around 2 weeks of age, the wild-type T cells increase in numbers, whereas the coronin 1-deficient cells plateau. This could be due to the fact that recent thymic emigrants have a survival span or roughly 2 weeks, after this the cells mature further. This would also fit with subset analysis of the cells, showing a decrease in cells which are low on Qa2 but high on CD62L (being RTEs) and with comparable TREC measurements. However, it is known that T cells of newborn animals have functional differences in comparison with cells obtained from an adult animal (58). Especially RTEs of newborns have been shown to respond differently to various pro-survival signals (72). It is therefore unclear if the equal number of peripheral T cells is due to all cells being recent thymic emigrants and therefore not depending on coronin 1 for their survival, or if the only reason is that newborn animals offer a more stimulating environment for cells to survive than adult animals.

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11. Appendix

11.1. Biochemical elucidation and localization of coronin 1

11.1.1. *Possible formation of a supra-molecular complex including coronin 1*

Previous preliminary data from our lab (by Somdeb BoseDasGupta) has shown the possibility of coronin 1 being part of supra-molecular complex, dependent on stimulation of cells by isoproterenol, a ligand for the β-adrenergic receptor. For the analysis, the melanoma cell line MelJuso was transformed to stably express coronin 1. Coronin 1 expressing cells were then grown to confluence and starved for 2 hours in HBSS containing $Mg2^+$ and $Ca2^+$. The cells were subsequently stimulated by 10 μM isoproterenol for 10' at 37°C followed by fixation with 1% paraformaldehyde. Cells were then lysed in a buffer containing 0.2% digitonin, to extract coronin 1 from the membranes and fractionated into either 1 ml (Fig 28 A) or 500 μl (Fig 28 B) fractions by a Superose 6 column using a HEPES based buffer (20 mM pH 7.5) containing 20 mM KCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$ and 0.2% N-octyl-glucopyranoside. Resulting fractions were precipitated by TCA and loaded onto 10% SDS Page gels and analysed by immunoblotting. While coronin 1 should have a size of around 51 kD, with the possibility to form a trimer structure around 150 kD (84), the blots show coronin 1 signal also in higher molecular weight fractions. In Panel A Isoproterenol stimulated fractions showed a shift into higher molecular fractions, but this also holds true for the actin signal. In Panel B, this shift is less visible with coronin 1 present in high molecular fractions, but only stimulated samples showing coronin 1 in the highest fractions. Additionally, the gels show two different elution profiles, with Panel B zoomed in on the high molecular weights. It needs to be noted, that the loading of the gels is not equal and any change in protein levels could therefore be due to unequal protein levels present.
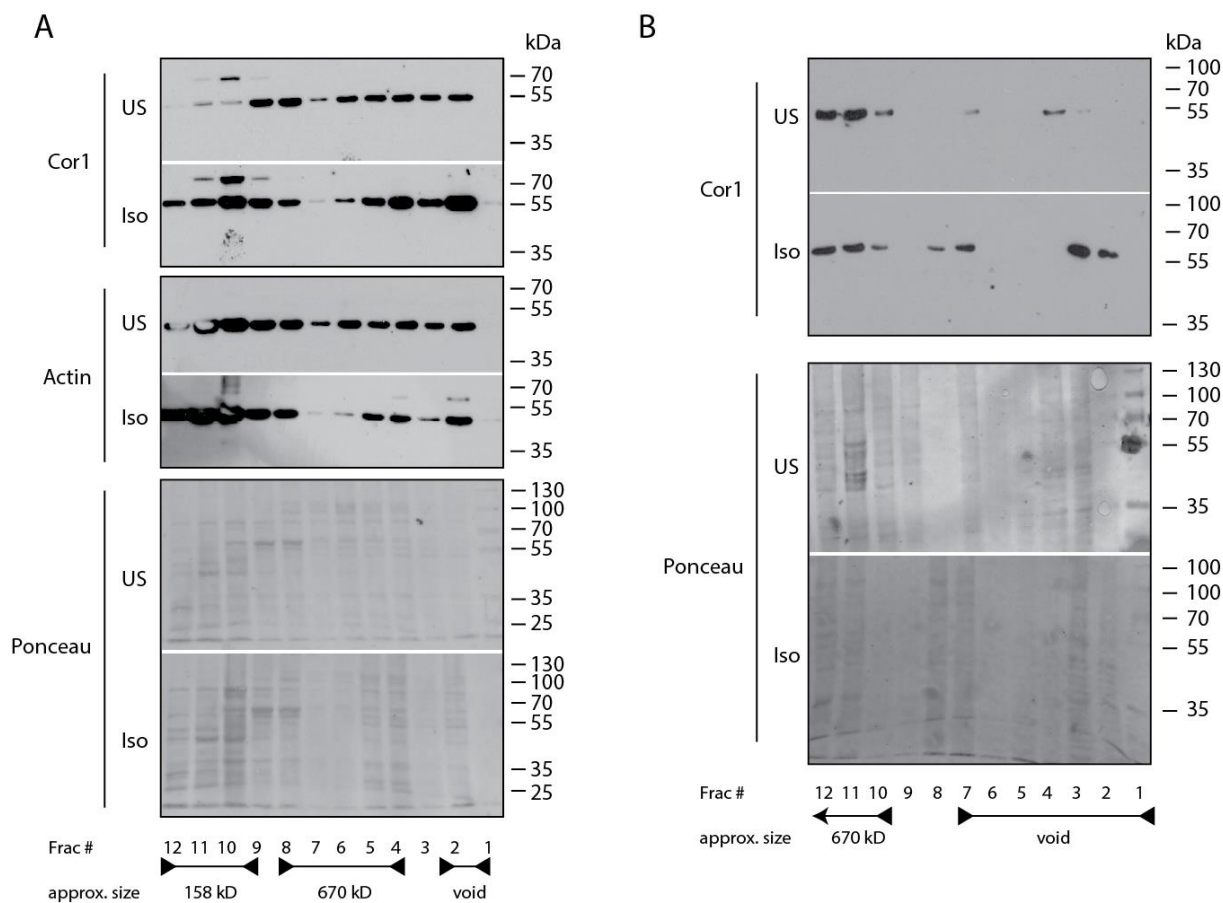
Fig 28: Fractionation of MelJuso cells. Cells were stimulated with 10 µM Isoproterenol for 10' on 37°C, lysed and fractionated by a S6 size exclusion column. Resulting fractions were loaded on SDS PAGE and analysed by immunoblotting for the presence of coronin 1 in higher molecular fractions. (A) and (B) are two representative experiments, showing the low reproducibility between experiments.

11.1.2. *Elucidation of coronin 1 interaction partners by co-immunoprecipitation*

Coronin 1 has been shown to be involved in the production of cAMP due to its association with Gα-s. In neurons, this has been linked to the formation of long-term memories and coronin 1-deficiency results in insufficient levels of cAMP produced (114). Therefore, the neuroblastoma cell line N1E115 was used, as neurons show a low expression pattern of coronin 1. To analyse interactions, N1E115 cells were starved for 2 hours in HBSS supplemented with Mg2+ and Ca2+ and stimulated with 10 μM isoproterenol. The cells were then lysed in a detergent based buffer containing 0.1% digitonin without any fixation procedure. Cell lysates were then incubated with an antibody against mouse coronin 1 for 2 hours at 4°C and antibody-protein complexes extracted by incubation with magnetic Protein G dynabeads for 1 hr at 4°C. After washing, beads were solubilised in Laemmli Buffer and boiled for 10' at 95°C. The resulting solution was then loaded on a 10% SDS PAGE Gel and immunoblotted for coronin 1 (Fig 29). To assess correct stimulation and precipitation, the samples were also probed for Gα-S. While coronin 1 is present in input and equally in the immunoprecipitated samples, it is not present in the flow-through. Gα-s is found in input and flow-through, indicating not all available Gα-s is bound by coronin 1. For the co-immunoprecipitation, only the samples stimulated with isoproterenol show Gα-s binding with coronin 1, with no signal present in unstimulated samples. Taken together, it shows that the co-immunoprecipitation for coronin 1 shows a consistent association with Gα-s and the identification of any other pulled-down proteins by mass spectrometry would be the next step.

Fig 29: Stimulus dependent association of coronin 1 with Gα-S. Neuroblastoma N1E115 cells were stimulated for 10' 37°C with 10 μM Isoproterenol and lysates immunoprecipitated with anti-Coronin 1. Bound complexes were isolated with magnetic Protein G dynabeads and resulting lysate loaded onto SDS PAGE and assessed by immunoblot. Shown is a representative blot of 3.

11.1.3. *Dependency of coronin 1 membrane association on N-terminal regions*

Coronin 1 has been shown to be mainly localized to the membrane and kept there by association with cholesterol (86). Therefore, buffers used to assess coronin 1 expression in a cell contain digitonin to sequester cholesterol and extract the membrane associated coronin 1. As Gatfield *et al.* have shown that the C-terminal regions of coronin 1 are not needed for its localization at the cell membrane but for the trimerization (84). To assess if the N-terminal part of coronin 1 would be important for its localization, cells expressing a mutated version of coronin 1, lacking the first ten amino acids, were analysed. This mutation results in all the amino acids up to the beginning of the first β propeller being deleted and therefore should leave the overall tertiary structure of coronin 1 intact (see Fig 30 A for scheme). C-terminal HA-tagged versions of wild-type coronin 1 (Cor1-HA) or coronin 1 lacking the first ten amino acids (N10-Cor1-HA) were expressed in HEK cells and assessed by immunofluorescence and western blotting. As expected wild-type coronin 1 was localized at the membrane. The deletion of the first 10 amino acids resulted in a loss of membrane association, showing instead a diffuse cytoplasm localized pattern (Fig 30 B). However, less cells show expression of the N10 version in comparison to the wild-type version. This could explain the lack of coronin 1 visible in cells lysed with RIPA buffer and assessed by immunoblotting (Fig 30 C and D).
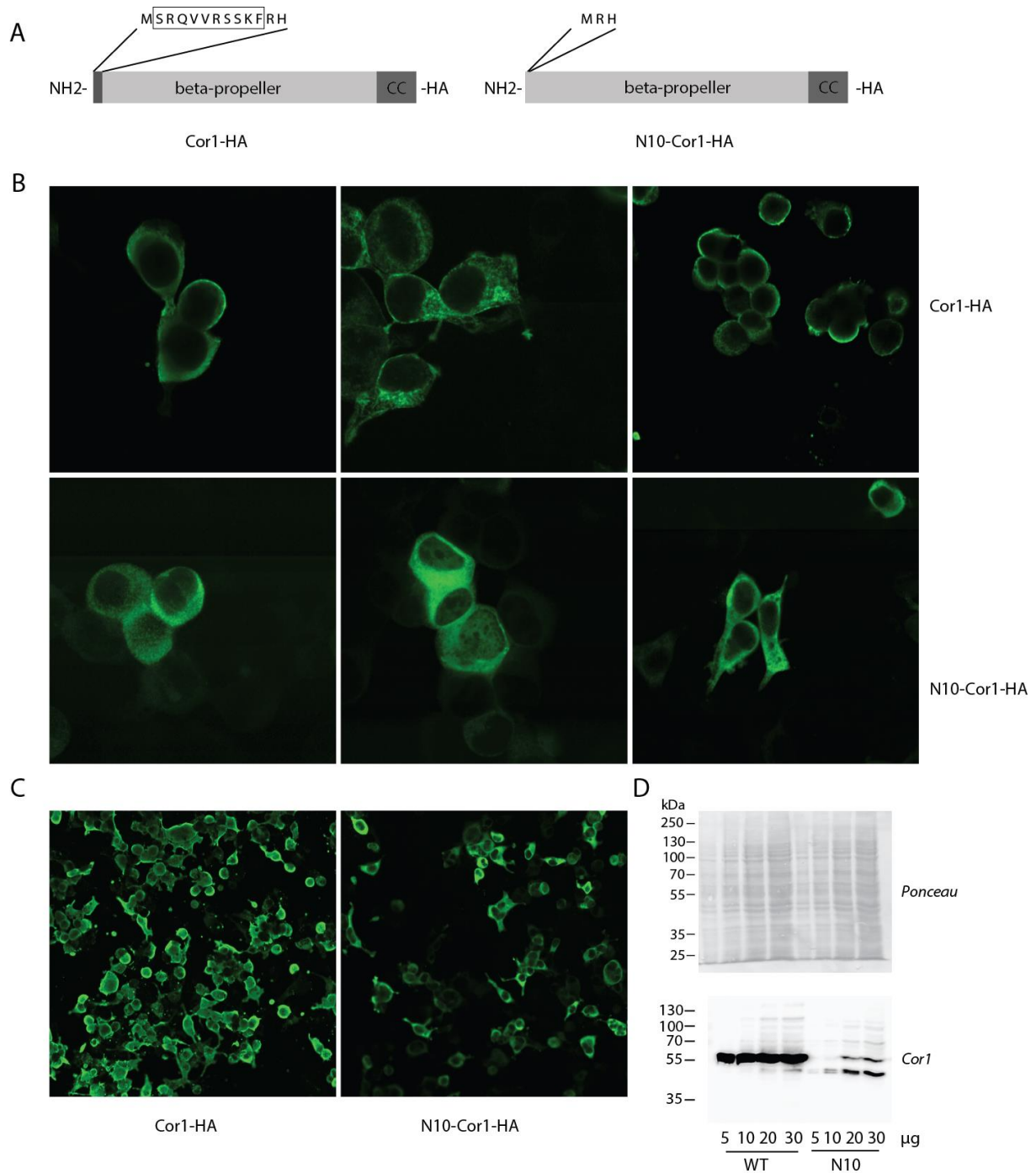
Fig 30: Localization of coronin 1 in transfected HEK cells. (A) Scheme of protein modification. Representative immunofluorescence of HEK cells transfected with either wild-type coronin 1 or a mutated version with a deletion of the first 10 amino acids in 63x magnification (A) or 20x (B). (C) Representative immunoblot for coronin 1 in HEK cells expressing wild-type or N10 mutant.

11.1. Discussion on stimulation dependent complex formation and coronin 1 localization

As this part of the project has been followed up for the first one and a half years, but has never yielded any interpretable and usable results, the discussion is more focussed on providing possible experiments to follow up than discussing the data itself.

11.1.1. *Complex isolation by size exclusion column and co-immunoprecipitation*

In line with preliminary data obtained by previous labmembers, the fractionation of cells expressing coronin 1 with a size exclusion column showed a shift of fractions containing coronin 1 upon stimulation. However, the method was not very reproducible, with sometimes displaying a shift, but not other times and often with different sizes. Additionally, the step of TCA precipitation was often only partially successful with samples sometimes having to be solubilised in 8M urea. In combination these slight changes from experiment to experiment introduced a high variation. Additionally, using transfected cells leads to an abundant expression of coronin 1, possibly resulted in false positive results. In general, loading of whole cell lysate on size exclusion columns, while covering a wide range of possible protein formations, is a method prone to variations. Using a cell line with endogenous expression of coronin 1 and an antibody based co-immunoprecipitation approach gave more reproducible results to build upon. As a first approach, the beads obtained by co-immunoprecipitation could be used to load a SDS PAGE gels and subsequently assess the gel for any additional bands not matching either the size of coronin 1, Gα-S or antibody heavy/light chains. This has not been seen by a ponceau stain, but a more sensitive approach such a silver stain could show additional bands. Therefore, beads obtained by co-IP could be used for mass spec analysis using an on-bead digestion procedure. The mass spectrometry analysis should then show coronin 1 and Gα-s as positive control with potentially additional proteins coming up.

11.1.2. *Localization of coronin 1*

The data obtained with the deltaN10 mutant of coronin 1 suggests that the membrane association is dependent on these first amino acids. A mutated version of coronin 1 with a single point mutation at amino acid 26 has been shown to have an actin phenotype, with the immunofluorescence pictures showing similar diffused pattern in the cytoplasm (180) with functional deficiencies. This amino acid 26 is already part of the first β-sheet, which could indicate that a malformation of coronin 1 is the reason for these phenotypes. While there is no evidence that deletion of the first 10 amino acids leads to proper formation of the seven-bladed propeller, further study would be warranted to assess a potential interference of deltaN10 coronin 1 with the actin cytoskeleton.

The first issue to be solved with the mutant would be however to establish an equal expression pattern, to make sure any differences are not due to differential transfection of the plasmid. This can then be followed up by further assessment of the actin cytoskeleton, preferably to be done in a cell which would usually have an endogenous coronin 1 expression, such as a coronin 1 knock-out of an immune cell or a cell of neural origin. Any influence of differential transfection of cells could then be circumvented by establishing a stable transfection. First experiments would need to cover any influence of the mutated coronin 1 protein on the actin cytoskeleton, possibly by assessing other proteins localization and F-actin polymerization and organization. Additionally further point mutations can be made to isolate the amino acid necessary for coronin 1 membrane association. As coronin 1 has been shown to be involved in cAMP signalling in neurons, signalling defects could be assessed by measuring intracellular cAMP levels. This would allow for studying the dependency of coronin 1 localization on its function.

11.2. Abbreviations

8-Br-cAMP	8-bromo-cyclic Adenosine monophosphate
AC	Adenylate Cyclase
AKAP	A-kinase anchoring protein
AMP	Adenosine monophosphate
Arp2/3	Actin-related protein 2/3
Bcl-2	B cell lymphoma 2
BH3	Bcl-2 homology domain 3
Bim	Bcl-2 interacting mediator of cell death
$CaCl_2$	Calcium chloride
cAMP	Cyclic Adenosine monophosphate
CCL21	CC-chemokine ligand 21
CCR7	CC chemokine receptor 7
CCR9	CC chemokine receptor 9
CD24	Cluster of differentiation 24, heat-stable antigen (HAS)
CD40	Cluster of differentiation 40
CD45RB	Protein Tyrosine Phosphatase, receptor type, c (PTPRC)
CD55/DAF	Decay accelarating factor
CD62L	Cluster of differentiation 62L, L-selectin
CDK5	Cyclin dependent kinase 5
c-FLIP	FLICE-like inhibitory protein
COX1/2	Cyclooxygenase 1/2
CRE	Cre recombinase
CREB	cAMP response element binding protein
CRISPR/Cas9	Clustered regularly interspaced short palindromic repeats
Csk	C-src tyrosine kinase
CTL	Cytotoxic T Lymphocyte
DAMP	Damage associated molecular pattern
DC	Dendritic cells
DN	Double negative
DNA	Desocyribonucleic acid
DP	Double positive

EAE	Experimental autoimmune encephalomyelitis
FACS	Fluorescence activated cell sorting
FITC	Fluorescein isothiocyanate
FSK	Forskolin
GDP	Guanosine Diphosphate
GFP	Green fluorescent protein
GM-CSF	Granulocyte-macrophage colony stimulating factor
GPCR	G protein-coupled receptor
GTP	Guanosine triphosphate
HSC	Hematopoietic stem cells
HTRF	Homologous time resolved fluorescence
IFN	Interferon
IgM	Immunoglobulin M
IκB	Inhibitor of κb
IL	Interleukin
JNK	Junk kinase
KCl	Potassium Chloride
KLF2	Krüppel like factor 2
Lck	Lymphocyte-specific protein tyrosine kinase
LN	Lymph node
LST	Late stage thymocyte
M1	Mature 1 stage of thymocytes
M2	Mature 2 stage of thymocytes
MgCl$_2$	Magnesium Chloride
MHC	Major histocompatibility complex
MNT	Mature naïve T cells
NFAT	Nucleated factor of activated T cells
NFκB	Nuclear factor kappa-light-chain-enhancer of B cells
NKAP	NFκB activating protein
PAMP	Pathogen-associated molecular pattern
PDE	Phosphodiesterases
PGE2	Prostaglandin E2
PKA	Protein kinase A

PMA	Phorbol 12-myristate 13-acetate
Qa2	Qa lymphocyte antigen 2
qPCR	Quantitative polymerase chain reaction
RAG	Recombinating-activating gene
RPMI	Roswell Park Memorial Institute Medium
RTE	Recent thymic emigrants
S1P1	Sphingosine-1-phsophate-1 receptor
SLO	Secondary lymphoid organs
SM	Semi-Mature thymocytes
SP	Single Positive
STAT	Signal Transducer and Activator of Transcription
TAK1	Transforming growth factor beta-activated kinase 1
TCR	T cell receptor
Th1/2	T helper cells type 1, type 2
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TREC	T cell receptor excision circles
Ubc13	E2 Ubiquitin-conjugating protein 13
UD	Unique Domain
VLA4	Very late antigen 4
WD	Tryptophan-aspartic acid repeat domain

11.3.   List of Figures

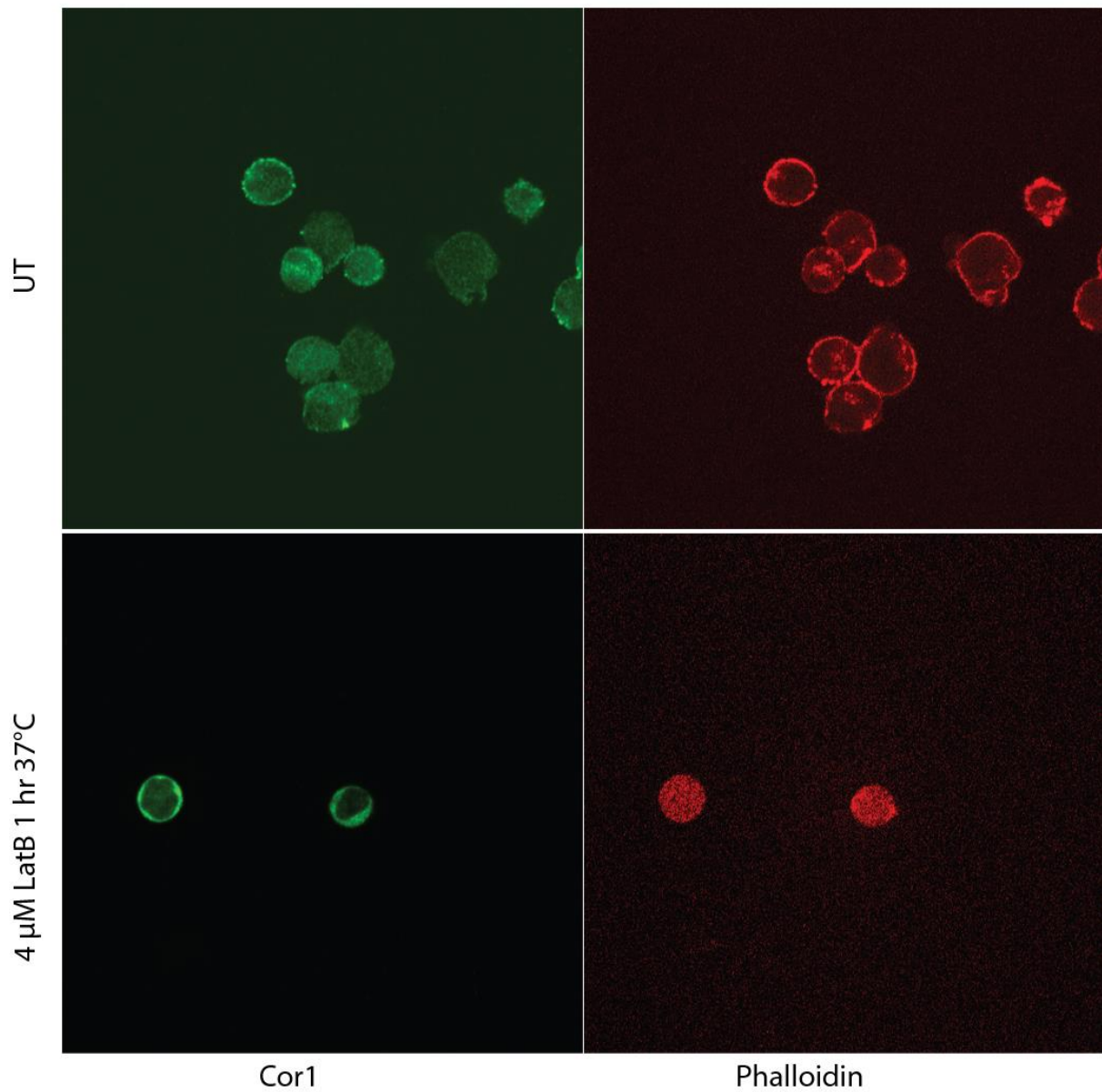## 11.4. *List of supplemental figures*

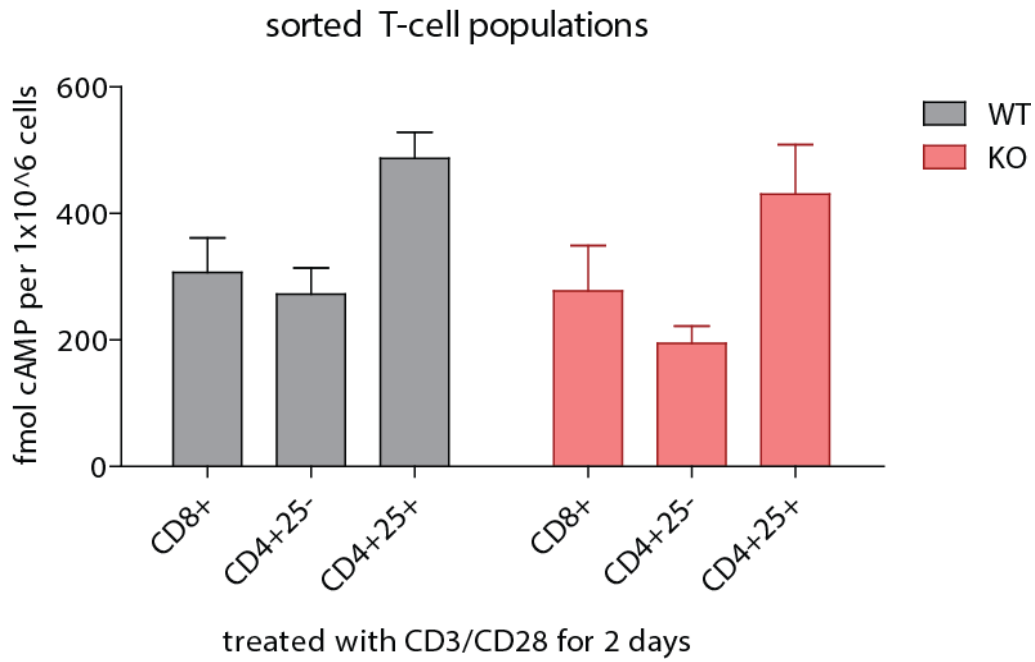## 11.5.   Supplemental Figures

Supplemental Fig 1: cAMP measurements of CRISPR/Cas9 generated coronin 1-deficient clones for the human T cell line Jurkat and SupT1. cAMP levels were assessed as for Jurkat cells used in Fig 11)

A

Supplemental Fig 2: Human Jurkat T cells were incubated with 4 μM Latrunculin B for 1 hr at 37°C, fixed with 1% PFA, permeabilized by 0.1% Triton X for 5' RT. Cells were stained for Cor1 (1:500) and actin visualized by phalloidin (1:40). Upon treatment with Latrunculin B, the localization of coronin 1 does not change, but actin staining turns from a cortical ring to a diffuse pattern.

Supplemental Fig 3: T cells obtained for Fig 16 C were seeded into 96-well plates at 100.000 cells/well and cultured for 2 days in the presence of CD3/CD28. Subsequently cells were assessed for their cAMP content, viability was only assessed by visual inspection of cells with trypan blue (n=2).

A

B
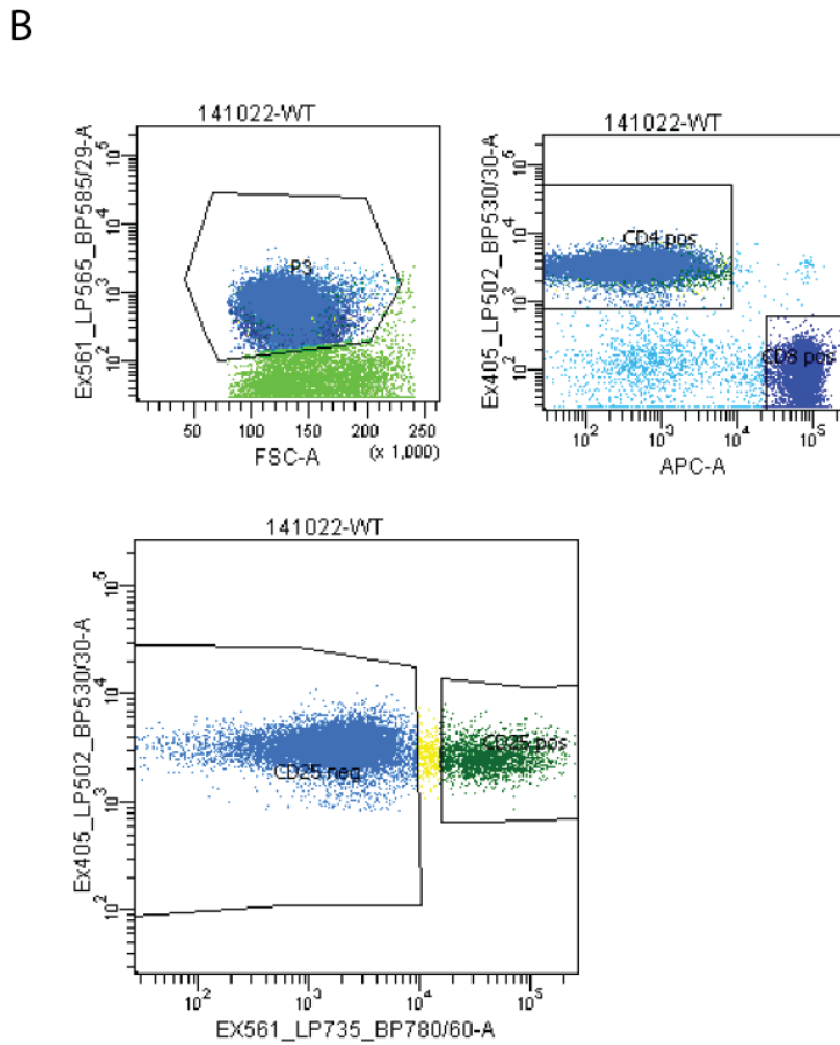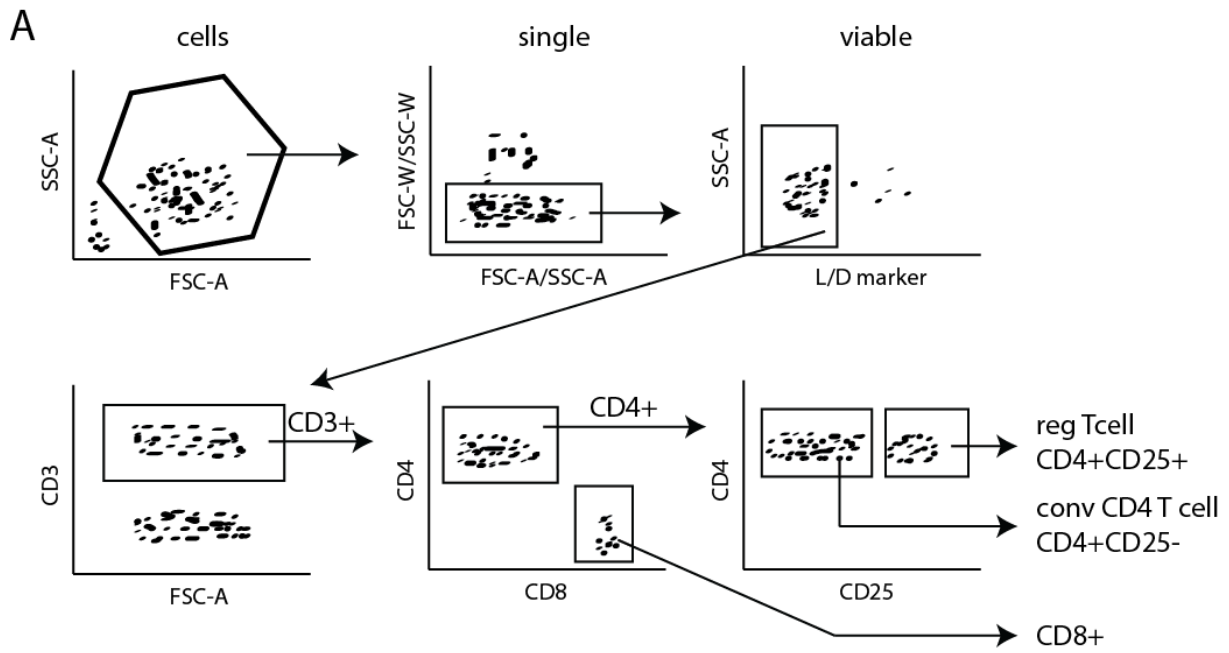
Supplemental Fig 4: Sorting protocol for conventional and regulatory CD4+ as well as CD8+ cells.
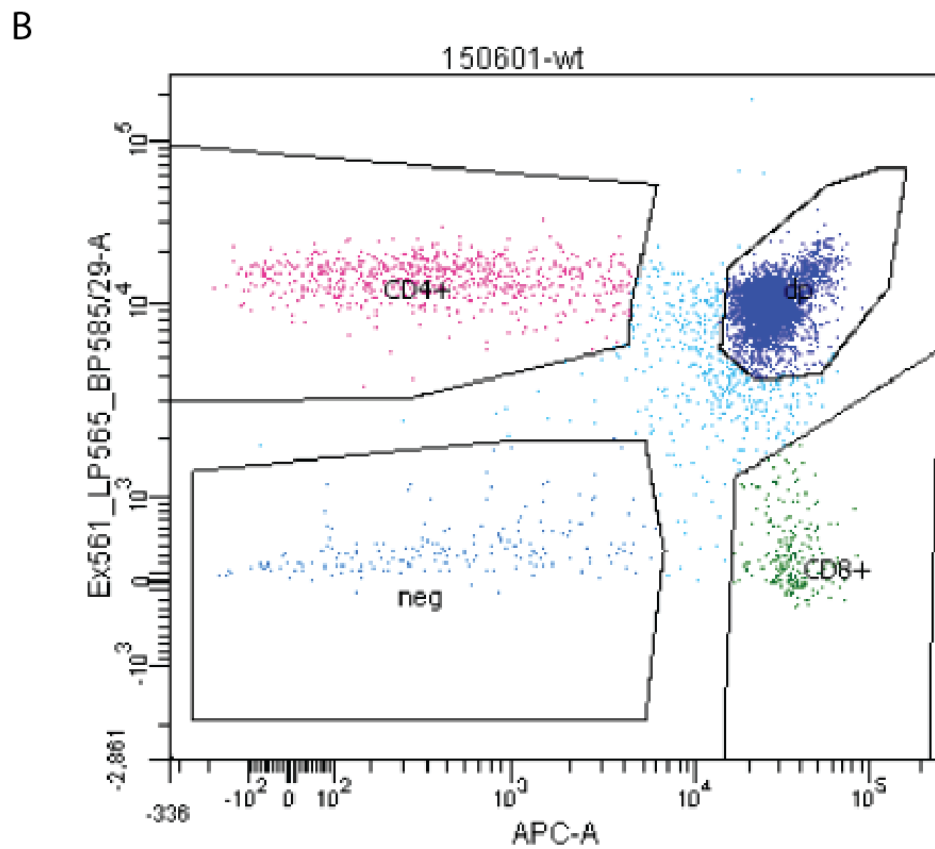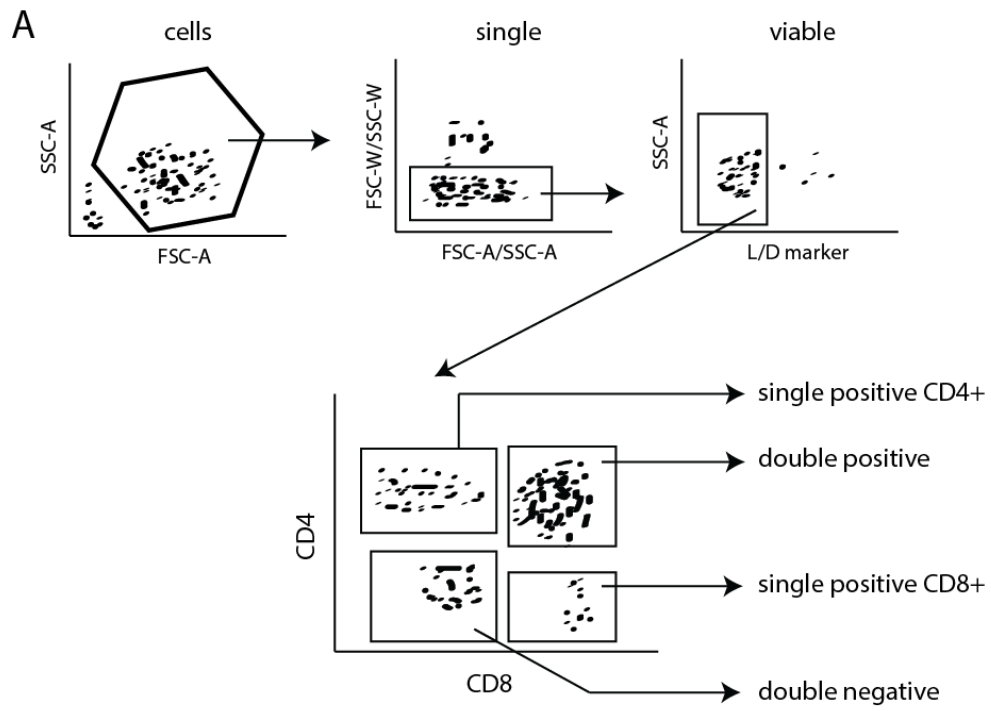
Supplemental Fig 5: Sorting protocol for sort of thymocytes into major subpopulations (DN, DP, CD4 and CD8)
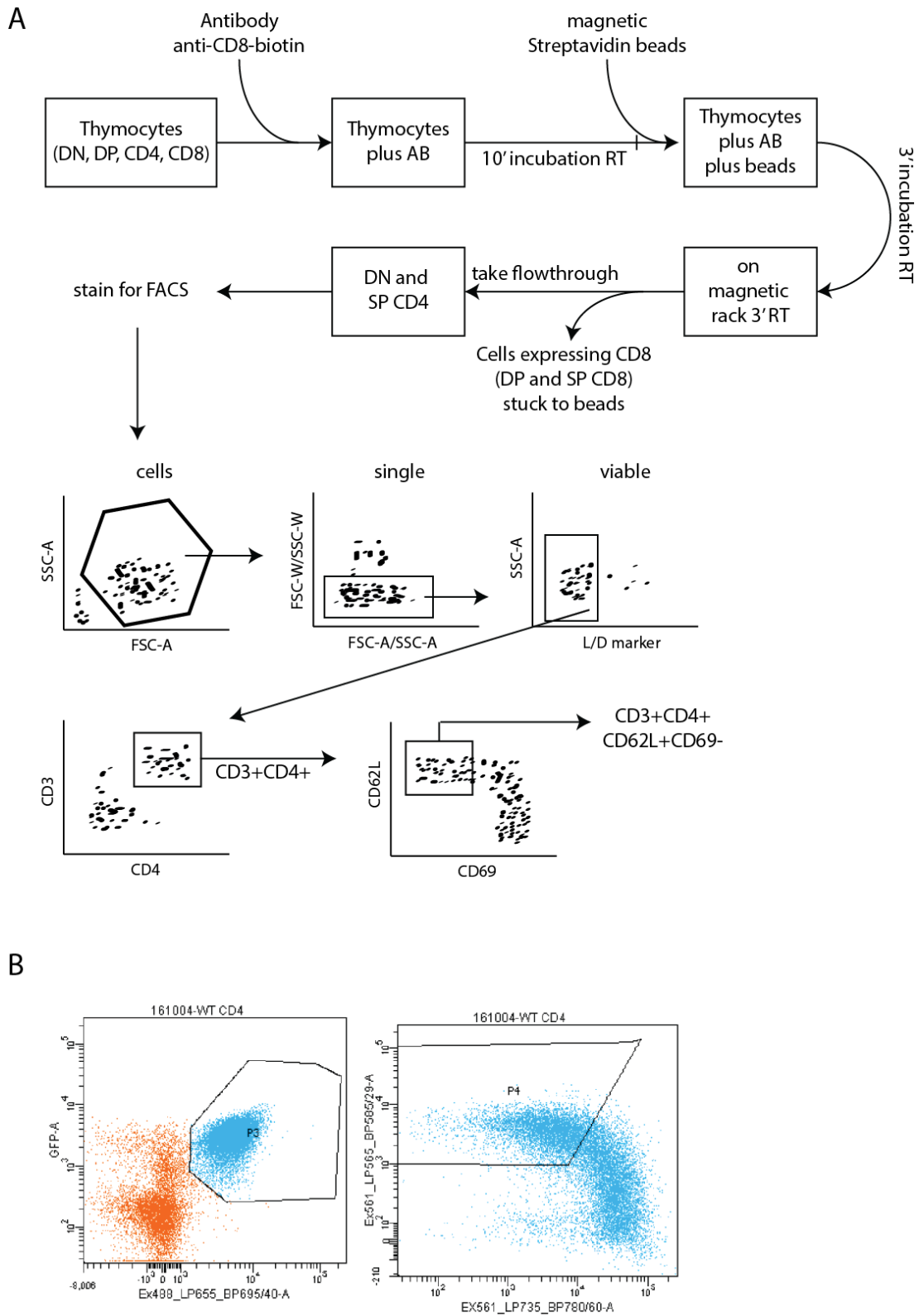
Supplemental Fig 6: Sorting protocol for obtaining late stage thymocytes.

11.6.   Curriculum Vitae

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Education:

- 2013- 2017: PhD in Biological Medical Research from the University of Basel
  - Thesis topic: The role of coronin 1 in T cell signalling and development
- 2010-2013: Master Studies: "Molecular Cell and Developmental Biology" at the University of Innsbruck, Austria
  - Thesis topic: The role of the Bcl-2 pro-survival family member A1/Bfl1 in tumorigenesis
- Nov. 2011-Sept 2012: research year at the Walter and Eliza Hall Institute for Medical Research in Melbourne, Australia
- 2006-2010: Bachelor in Biology at the University of Innsbruck, Austria
  - Thesis 1: The Immunoblot Techniques in Protein Analysis
    Thesis 2: Chemical fixation methods for ultrastructural research in yeast
- 2005: graduation from Bundesgymnasium Dornbirn, Austria

Internships:

- 2011: 8 weeks at the Division of Developmental Immunology at the Biozentrum Innsbruck (now CCB)
- 2011: 8 weeks at the Division for Cell Biology at the Biozentrum Innsbruck (now CCB)
- 2010: 6 weeks at the SanMaru Serpentarium in Taquaral, Brazil
- Summers 2009, 2008 and 2006: Internships at the Laboratory for Immunological and Molecular Cancer Research for 17 weeks in total during the three summers

Research:

- Lang MJ, Brennan MS, O'Reilly LA, Ottina E, Czabotar PE, Whitlock E, Fairlie WD, Tai L, Strasser A, Herold MJ. Characterization of a novel A1 antibody. *Cell Death Dis.* 2014 Dec 4;5:e1553
- Herold MJ, Rohrbeck L, Lang MJ, Grumont R, Gerondakis S, Tai L, Bouillet P, Kaufmann T, Strasser A. Foxo-mediated Bim transcription is dispensable for the apoptosis of hematopoietic cells that is mediated by this BH3-only protein.