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"Molecular analysis of the transcription factors MAZR and Runx complexes during T cell development"

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1 Abstract

CD4/CD8 cell fate choice of double-positive ($CD4^+CD8^+$) thymocytes during T cell development is tightly regulated by the activity of several transcription factors that act together in a transcription factor network. MAZR and Runx complexes are part of this transcription factor network and both regulate the expression of ThPOK, the master transcriptional regulator of CD4 lineage commitment. Since we previously showed that MAZR interacts with Runx complexes we hypothesized that MAZR might act in synergy with Runx complexes during T cell development. In my master thesis I show that MAZR interacts with the activation domain of Runx1 and Runx3 in a BTB domain- and zinc finger 7-dependent manner. By analyzing MAZR single and MAZR-Runx double-deficient mice we observed that MAZR and Runx complexes cooperate in the control of ThPOK repression during CD8 lineage differentiation. Furthermore, MAZR-Runx3 double-deficient mice showed enhanced derepression of CD4 in $CD8^+$ T cells compared to Runx3 single-mutant mice. This implies an unexpected role of MAZR in *Cd4* silencing, at least in the absence of Runx3. Finally, retroviral Cre-mediated conditional deletion of MAZR in peripheral $CD8^+$ T cells led to derepression of ThPOK, indicating that MAZR was continuously required to repress ThPOK in $CD8^+$ T cells. These data indicate developmental stage-specific synergistic activities between Runx complexes and MAZR in the repression of ThPOK and CD4. Moreover, these data demonstrate that MAZR is required for the establishment and maintenance of ThPOK repression during CD8 lineage differentiation. Thus, the results of my master thesis provide new insight into the transcriptional network regulating CD4/CD8-lineage choice.

2 Zusammenfassung

Die Ausreifung von sogenannten doppelt-positiven ($CD4^+CD8^+$) Thymozyten in $CD4^+$ Helfer-T-Zellen oder in $CD8^+$ zytotoxische T-Zellen ist ein wichtiger Schritt während der T-Zellentwicklung und wird durch mehrere Transkriptionsfaktoren, die gemeinsam in einem Transkriptionsfaktor-Netzwerk agieren, kontrolliert. MAZR und Mitglieder der Runx-Familie sind Teile dieses Transkriptionsfaktor-Netzwerkes und regulieren auf molekularer Ebene die Genexpression des Transkriptionsfaktors ThPOK, welcher essentiell für die Differenzierung von $CD4^+$ T-Helfer-Zellen ist. In früheren Studien konnten wir zeigen, dass MAZR und Runx-Komplexe miteinander interagieren und daraus leitete sich unsere Hypothese ab, dass MAZR möglicherweise synergistisch mit Runx-Komplexen während der T-Zellentwicklung agiert. In meiner Masterarbeit konnte ich zeigen, dass MAZR über seine BTB Domäne sowie Zinkfinger 7 mit der Aktivierungsdomäne von Runx1 und Runx3 interagiert. Weiters konnte durch die vergleichende Analyse von konditionellen MAZR Knockout-Mäusen und MAZR-Runx Doppelknockout-Mäusen gezeigt werden, dass eine gemeinsame Aktivität von MAZR und Runx-Komplexen essentiell für die Aufrechterhaltung der ThPOK Repression während der $CD8^+$ T-Zelldifferenzierung ist. Weiterführende Analysen von ausdifferenzierten peripheren zytotoxischen $CD8^+$ T-Zellen ergaben, dass MAZR kontinuierlich benötigt wird, um die Transkription von ThPOK in $CD8^+$ T-Zellen zu unterdrücken. Darüber hinaus haben wir im Vergleich zu Runx3 defizienten-Mäusen in MAZR-Runx3 Doppelknockout-Mäusen eine erhöhte Expression des Co-Rezeptormoleküls CD4 in $CD8^+$ T-Zellen beobachtet, was auf eine bisher unbekannte Rolle von MAZR, zumindest in Abwesenheit von Runx3, in der transkriptionellen Stilllegung des *Cd4* Genlocus hindeutet. Die Ergebnisse meiner Arbeit haben daher zu neuen molekularen Einblicken in die transkriptionelle Regulation der Entstehung von $CD4^+$ und $CD8^+$ T-Zellen geführt.

3 Introduction

3.1 The thymus

The ancient Greeks had already noted a large mass of tissue in the chest above the heart of young animals, after performing sacrificial rites on them. Over centuries the function of this organ remained a mystery. Until 1960 it was believed that it had become redundant during the course of evolution and just served as a graveyard for dying lymphocytes (Miller 2002). But more than fifty years ago the puzzle started to be unraveled. 1961 Jaques Miller published a paper in the *Lancet* where he came to the conclusion that the thymus must be responsible for the development of immunologically competent cells (Miller 1961). Thus, one can say the 1960s were the starting point for T cell development research and continued to be a fascinating topic with many questions still to be answered.

For a long time the thymus was an underestimated organ but today its importance for adaptive immunity is a proven fact. For proper T cell development, including several differentiation and proliferation steps, instructive signals from the thymic microenvironment are necessary to generate functional helper and cytotoxic T cells that can exert their specific functions in peripheral lymphoid organs (Anderson and Takahama 2012). The function of $CD4^+$ helper T cells is to stimulate and coordinate other immune cells and help B cells with antibody production, whereas $CD8^+$ cytotoxic T cells kill virus infected cells or tumor cells (Krogsgaard and Davis 2005).

The effect of thymic involution on peripheral T cell senescence is getting obvious during aging. Aging correlates with reduced ability of the immune system to generate antigen-specific responses to pathogens and vaccination. Therefore in older individuals one can see an increase in infections, neoplastic and autoimmune diseases, depicting the importance of the thymus throughout life (Aw et al. 2007, McElhaney et al. 2012, Palmer 2013).

3.2 T cell development

For T cell development hematopoietic stem cell (HSC)-derived lymphocyte progenitors have to exit the bone marrow and enter the thymus (Figure 1) (Schwarz and Bhandoola 2006). Only the thymic microarchitecture, including specialized

stromal and epithelial cells, provides the required environment for appropriate thymocyte differentiation. In contrast, B cells stay and mature in the bone marrow (Zuniga-Pflucker 2004). Committed lymphoid progenitors (CLPs) arriving in the thymus via the blood stream do no longer have the potential to become B cells but still retain some myeloid potential. From this stage on they are designated as double-negative (DN) thymocytes that do not express CD4 nor CD8 coreceptor molecules on their surface.

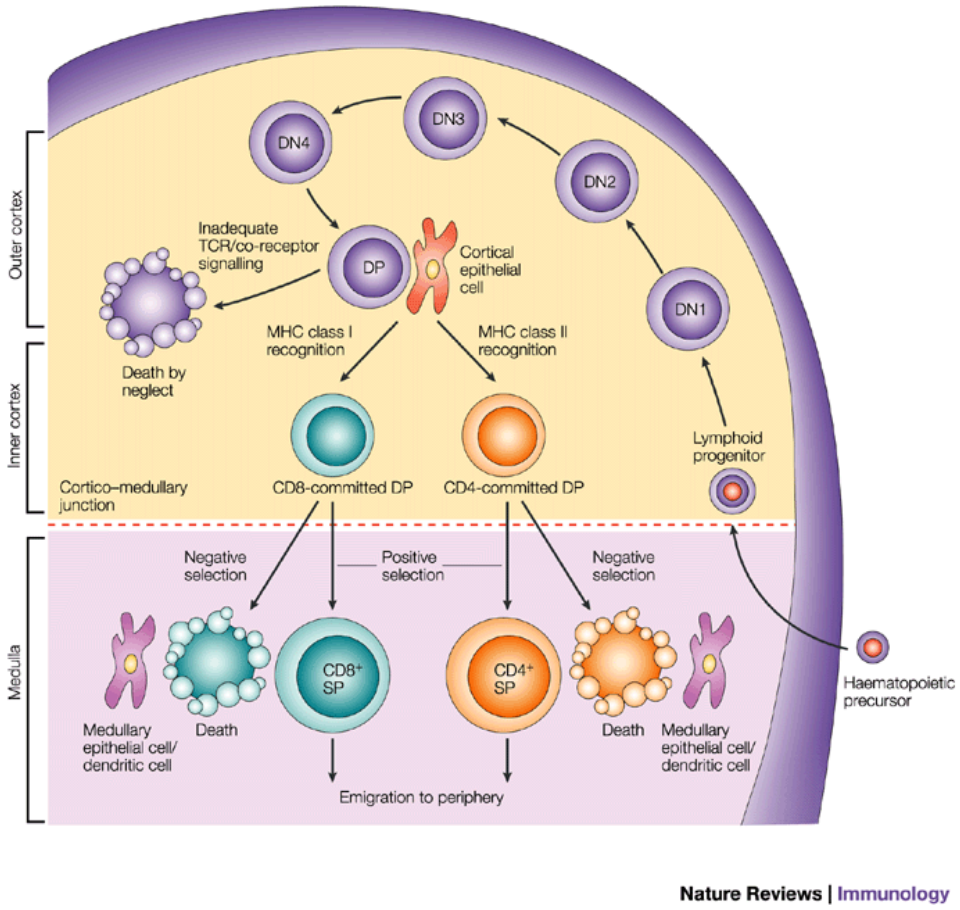

Figure 1. T cell development in the thymus
This cartoon depicts the different developmental stages of thymocytes and the selection checkpoints during T cell development in the thymus. See chapter 3.2 for details (adapted from Germain 2002).

DN thymocytes are subdivided into four sequential phenotypic subsets that are defined by the expression of CD44 and CD25 (DN1: $CD44^{+}CD25^{-}$; DN2: $CD44^{+}CD25^{+}$; DN3: $CD44^{-}CD25^{+}$; DN4: $CD44^{-}CD25^{-}$). T cell lineage commitment comes along with the loss of multipotency and is not completed before DN2 stage. At the DN3 stage rearrangements at the *Tcrb* locus, that are controlled by

recombination-activating gene (RAG)1 and RAG2 proteins, take place (Germain 2002, Carpenter and Bosselut 2010). A successful recombination of the *Tcrb* locus leads to the cell surface expression of the pre-TCR complex (formed by the TCRβ chain together with the invariant pre-TCRα chain) that is associated with a CD3ζ complex that is involved in proximal signal transduction (Mombaerts et al. 1992, Shinkai et al. 1993). DN thymocytes that have productively rearranged the TCR β-chain genes (beta selection) undergo several cell divisions to expand the pool of precursors with successfully rearranged TCR β-chains, start to express CD8 and CD4 and hence progress to the double-positive (DP) stage of thymocyte development. DP cells recombine the *Tcra* chain locus to generate the second chain of the mature αβ T cell antigen receptor (Germain 2002, Carpenter and Bosselut 2010). These αβ (DP, $CD4^+$, $CD8^+$) expressing immature cells constitute 90% of the lymphoid compartment in the thymus of young individuals (Hoffman et al. 1996).

Upon expression of a functionally mature αβTCR complex, DP thymocytes undergo a positive/negative selection process and CD4/CD8 cell fate choice. This selection process ensures that DP thymocytes with a TCR incapable of engaging self-major histocompatibility complex (MHC) molecules are eliminated and undergo 'death by neglect' within a few days. Cells with a very high avidity for self-MHC molecules undergo apoptosis in a process called negative selection, which leads to the elimination of self-reactive cells. Only cells with a TCR able to recognize self-MHC molecules with an appropriate affinity survive and maturate. During the positive selection process, DP cells develop into either $CD4^+$ or $CD8^+$ single-positive (SP) T cells. DP thymocytes that are MHC class II-restricted differentiate into $CD4^+$ T cells and MHC class I-restricted cells differentiate into $CD8^+$ T cells. The CD4 and CD8 molecules play an important role in positive/negative selection process. CD4 and CD8 bind to invariant domains of the MHC class II and class I molecules, respectively and thus influence the avidity of the TCR-self-peptide/MHC interactions (Germain 2002, Carpenter and Bosselut 2010).

3.3 The CD4/CD8-lineage choice

To understand how CD4 and CD8 cells develop in the thymus it is essential to unravel the molecular mechanism that regulates CD4/CD8-lineage choice. Based

on many experimental data, several models were proposed during the last 15-20 years to describe how DP thymocytes acquire their appropriate lineage fate (Germain 2002, Ellmeier et al. 2013). The most accepted model to date that is supported by many experimental data is the “kinetic signaling model”. This model was built on by the observation that DP thymocytes transiently terminate *Cd8* gene transcription in TCR-signaled DP thymocytes at the onset of positive selection, regardless of the MHC-restriction of the TCR. This leads to the appearance of $CD4^+CD8^{lo}$ cells that still retain the potential to become either $CD4^+$ or $CD8^+$ T cells. According to the “kinetic signaling model”, $CD4^+CD8^{lo}$ cells that express a TCR restricted to MHC class II have continuous TCR signaling, which is unaffected by the loss of surface CD8 protein due to terminated Cd8 transcription and $CD4^+CD8^{lo}$ cells develop into $CD4^+$ helper T cells. However, if $CD4^+CD8^{lo}$ cells express a TCR that is MHC class I-restricted, then the TCR signaling is interrupted due to the down-regulation of CD8. As a consequence, $CD4^+CD8^{lo}$ cells become susceptible to IL-7 and IL-15 cytokine signaling. This leads to the development of $CD4^+CD8^{lo}$ cells into the CD8 lineage during which CD4 expression is shut-off and CD8 re-expressed (“coreceptor-reversal”) (Brugnera et al. 2000, Singer 2002, Yu et al. 2003, Singer and Bosselut 2004, Singer et al. 2008). For the moment this model provides an explanation of how different instructive signals are generated to guide cells into either the CD4 or CD8 lineage. However, many unanswered questions still remain. How are these signals transmitted into the cell nucleus? How is the genetic program behind this cell fate determination switched on? It was shown that the CD4 and CD8 expression correlates to almost 100% with CD4/CD8-lineage choice in mature conventional $\alpha\beta TCR^+$ T cells (Singer 2002). Therefore it was hypothesized that transcription factors that regulate the expression of these two coreceptor genes might also play a role in the CD4/CD8-lineage choice. The *Cd4* and *Cd8* loci were intensively studied and these studies resulted in the isolation of several transcription factors regulating these genes. It became clear that these factors are part of a complex transcription factor network that regulates helper versus cytotoxic lineage decision (Egawa 2009, Naito and Taniuchi 2010). In the next chapters I will focus on three transcription factors that are part of the transcription factor network regulating CD4/CD8-lineage choice.

3.4 Transcription factors involved in CD4/CD8-lineage choice

3.4.1 Runx complexes

Runx proteins are transcriptional regulators that play an important role during hematopoiesis, bone development and neurogenesis in mice (Kundu et al. 2002, Levanon et al. 2002, Wang et al. 2013). Mutations in Runx proteins have also been identified in several human diseases, for example mutations of RUNX1/AML1 in acute myeloid leukemia patients (Osato 2004) and mutations of RUNX3 playing a role in gastric cancer (Li et al. 2002). Runx complexes are heterodimers consisting of one of the three mammalian Runt domain proteins Runx1, Runx2 or Runx3 and its dimerization partner core-binding factor beta (Cbfβ). The common feature of Runx proteins is the evolutionally conserved 128 amino acid Runt domain that is responsible for sequence-specific DNA-binding and for dimerization with Cbfβ. Cbfβ itself can not bind to the DNA but assists Runt domain proteins to bind to the DNA by an unknown mechanism. Runx transcription factor complexes can act either as transcriptional repressors or activators of tissue-specific target genes (Wheeler et al. 2000, Collins et al. 2009). The VWRPY pentapeptide sequences at the C-terminal end of Runx proteins are another evolutionally conserved domain within this family of proteins. It has been shown that by recruiting corepressors like Groucho/TLE Runx1 and Runx3 can exert their function as transcriptional repressors (Levanon et al. 1998). Interestingly, other corepressors, such as HDACs can interact with Runx proteins in a VWRPY-independent manner (Westendorf 2006, Taniuchi and Ellmeier 2011).

In the last decade Runx proteins were also shown to have important function during T cell development. It was shown that two Runx binding sites in the core *Cd4* silencer are essential to repress *Cd4* transcription in immature DN thymocytes and for the establishment of epigenetic *Cd4* silencing during the development of the cytotoxic T cell lineage. Taniuchi and colleagues showed that Runx1 is required to repress CD4 expression in DN thymocytes, while Runx3 is required for establishment of epigenetic silencing and for specification of functional $CD8^+$ T cells. Thus, Runx proteins do not seem to be functionally identical and cross-regulation should be considered too (Taniuchi et al. 2002, Taniuchi and Ellmeier 2011).

To further study the role of Runx factors in T cells, mice with T cell-specific deletions of Runx factors were generated since germline Runx-deficient mice are embryonic or neonatal lethal (Okuda et al. 1996, Wang et al. 1996, Li et al. 2002). The inactivation of both Runx1 and Runx3 in DP thymocytes leads to a dramatic loss of $CD8^+$ T cells, although a subset of $CD8^+$ T cells can still develop due to an escape from Cre-mediated deletion (Setoguchi et al. 2008). In order to circumvent the issue about the "escape", they used mice with a mutation in the VWRPY motif of Runx1 ($Runx1^{\Delta 446/\Delta 446}$) and crossed them with mice on a Runx3-deficient background. In these compound mutant mice, virtually no $CD8^+$ T cells develop, highlighting a crucial role of the Runx complexes for $CD8^+$ T cell development. To further investigate the reason for the loss of $CD8^+$ T cells in the periphery they crossed $Runx1^{\Delta 446/\Delta 446}Runx3^{f/f}$ *Cd4*-Cre mice onto a MHC class II-deficient background. The results indicate that in the absence of Runx complexes the majority of class I-restricted cells are "redirected" to differentiate into $CD4^+$ T cells (Figure 2) that are functionally helper-like T cells (Setoguchi et al. 2008). This "redirection" is also observed in the MAZR knockout (Sakaguchi et al. 2010) and in MHC class I-restricted thymocytes with ectopically expressed ThPOK (He et al. 2005, Sun et al. 2005). These data highlight the importance of a strict regulation of these three transcription factors during CD4/CD8-lineage choice.

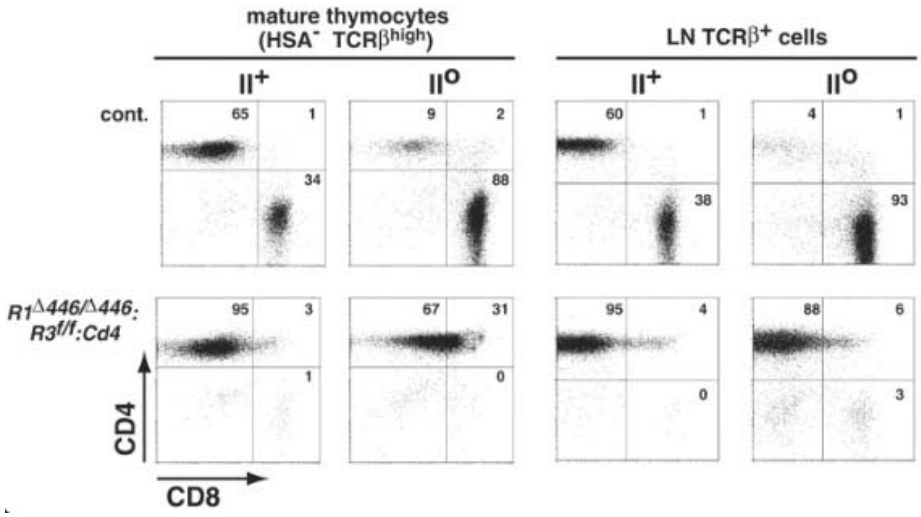

Figure 2. Redirection of MHC class I-restricted cells into $CD4^+CD8^-$ T cells in the absence of Runx1 (Δ446/Δ446) and Runx3 (f/f)
CD4 and CD8 expression in mature thymocytes and lymph node (LN) $TCR\beta^+$ T cells either in the presence (II+) or absence (II°) of I-A MHC class II molecules (adapted from Setoguchi et al. 2008).

3.4.2 ThPOK

In 1998 a mouse strain with a spontaneous autosomal recessive mutation was identified. These mice have almost no MHC class II-restricted $CD4^+$ T cells because of a specific block in thymic development. This mouse strain was termed HD for "helper T cell-deficient" (Dave et al. 1998) and was further analyzed. The *hd/hd* mice were crossed with a variety of different knockout mice and it was shown that thymocytes with MHC class II TCRs were redirected to the CD8 lineage although positive selection was still intact. This finding showed that the CD4/CD8-lineage choice is independent from positive selection (Keefe et al. 1999). Finally, in 2005 the molecular cause of the HD phenotype was identified. A single-base pair mutation in the *Zbtb7b* locus, encoding for the transcription factor ThPOK, leads to a point mutation in the DNA-binding domain of ThPOK (He et al. 2005). This transcription factor belongs to the BTB/POZ domain-containing zinc finger transcription factor family (Siggs and Beutler 2012). This protein family will be described in chapter 3.5 in more detail. Ectopic expression of ThPOK leads to a redirection of MHC class I-restricted thymocytes into the CD4 helper lineage (He et al. 2005, Sun et al. 2005). Since the gain- and loss-of-function analyses indicate that ThPOK is necessary and sufficient for helper lineage differentiation, ThPOK is considered as a "master regulator" of $CD4^+$ T cell development (He et al. 2005).

The next step in understanding the transcriptional control of the CD4/CD8-lineage choice was to examine the molecular mechanism behind the CD4 lineage-specific expression of the *Thpok* gene. Several groups were investigating the *Thpok* locus and in 2008 two groups independently showed that a region in the *Thpok* locus is essential for helper-lineage-specific expression of ThPOK. This region was named the *Thpok* silencer because of its transcriptional silencer activity (He et al. 2008, Setoguchi et al. 2008). Due to the fact that overexpression of ThPOK leads to a redirection of MHC class I-restricted cells into $CD4^+CD8^-$ cells (He et al. 2005, Sun et al. 2005), Setoguchi and colleagues analyzed ThPOK in several Runx mutant mice. Their results suggested that ThPOK repression is Runx-mediated in peripheral $CD8^+$ T cells via binding to the *Thpok* silencer. They also showed that Runx is associated with the *Thpok* silencer in ThPOK-expressing cells indicating a role of additional Runx interacting molecules in regulating *Thpok* silencer activity

(Setoguchi et al. 2008). In the last years the search for Runx-interacting factors was intensified and MAZR occurred as a promising candidate.

3.4.3 MAZR

Myc-associated zinc finger-related factor (MAZR) is another transcription factor involved in the CD4/CD8 cell fate choice. It is also known as PATZ1 and encoded by the *Patz1* gene (Fedele et al. 2000, Bilic and Ellmeier 2007). Kobayashi and colleagues initially identified MAZR as an interacting partner of Bach2. Bach2 is a B- and neuron-cell-specific transcriptional repressor but it was also shown that Bach2 is part of an activating complex at the *fgf4* promoter. They showed that MAZR and Bach2 interact via their BTB/POZ domains and suggested that MAZR works as an architectural transcription factor that induces structural changes of regulatory regions of Bach2 target genes and thereby facilitating transcriptional activation. MAZR also functions as a transcriptional activator of the *c-myc* promoter in B cells, although it has no typical activation domain (Kobayashi et al. 2000). It was shown *in vivo* and *in vitro* that MAZR interacts with RNF4. RNF4 is a transcriptional activator and by interacting with MAZR a switch from activation to repression function was observed (Fedele et al. 2000). These observations show that MAZR acts both as a positive and negative transcriptional regulator depending on the cellular context.

MAZR is also essential for spermatogenesis and could be a potential tumor suppressor in testicular germ cell tumors (Fedele et al. 2008). MAZR knockout mice spontaneously develop tumors such as Non-Hodgkin lymphomas, sarcomas and hepatocellular carcinomas (Pero et al. 2012). Moreover, MAZR appears to have an oncogenic but also an anti-oncogenic activity in carcinogenesis. Recently it was shown that MAZR interacts with p53 and thereby regulating p53-target genes (Valentino et al. 2013). One of the latest studies shows that MAZR is an important regulator of pluripotency in embryonic stem cells (Ow et al. 2014).

In the T cell lineage, MAZR plays a role in DN to DP transition during thymocyte development as a negative regulator of *Cd8* gene expression. An explanation for the molecular mechanism behind the repression of CD8 in DN thymocytes is the interaction of MAZR with the nuclear receptor corepressor (N-CoR). MAZR eventually recruits N-CoR repressor complexes that have HDAC

activity to the *Cd8* gene locus. Via histone modifications an epigenetic and transcriptional "off-state" can be established (Bilic et al. 2006). By generating *Mazr* knockout mice the role of MAZR as a negative regulator of CD8 expression during the DN to DP transition was proven (Sakaguchi et al. 2010).

In addition, the analysis of MAZR-null mice revealed that MAZR represses ThPOK expression in MHC class I-restricted thymocytes during the CD4/CD8-lineage choice. In the absence of MAZR MHC class I-restricted thymocytes redirect into $CD4^+$ helper-like cells due to ThPOK derepression (Figure 3). ChIP assays revealed that MAZR binds to the *Thpok* silencer and thereby controls ThPOK repression. These data showed the importance of MAZR in this complex transcription factor network that regulates CD4/CD8-lineage choice in DP thymocytes (Sakaguchi et al. 2010).

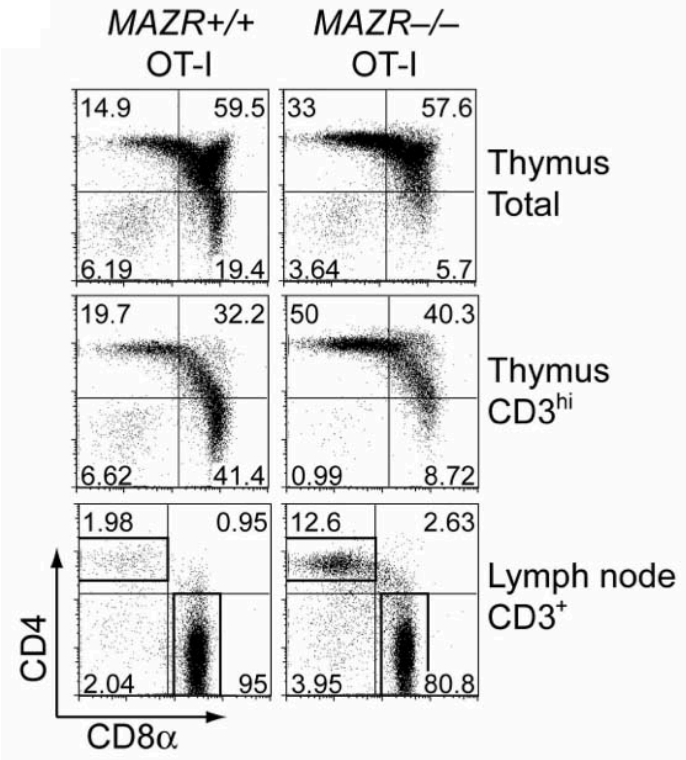

Figure 3. Redirection of MHC class I-restricted cells into $CD4^+$ helper-like T cells in the absence of MAZR

CD4 and CD8α expression on total (upper panel) and $CD3^{hi}$ (middle panel) thymocytes, and on lymph node T cells (lower panel) isolated from Mazr+/+,OT-I and Mazr−/−,OT-I littermates (adapted from Sakaguchi et al. 2010).

3.5 The BTB-ZF family of transcription factors

Members of this protein family, including MAZR and ThPOK, have important roles during T cell development and are therefore described in this chapter in more detail. Broad complex, tramtrack, bric-a-brac and zinc finger (BTB-ZF) proteins are a group of transcriptional regulators that are evolutionally conserved. They have one or more Cys_2His_2 zinc finger, necessary for DNA-binding, and a N-terminal BTB domain that mediates protein-protein interactions (Siggs and Beutler 2012). Their transcriptional regulation depends on sequence-specific binding of the zinc finger (ZF) domain to regulatory regions in target genes and the recruitment of cofactors involved in chromatin remodeling, transcriptional silencing and activation. The BTB domain, also known as poxvirus and zinc finger (POZ) domain, mediates the cofactor complex formation and directly interacts with corepressors (e.g. N-CoR) and histone modification enzymes (e.g. HDACs) (Beaulieu and Sant'Angelo 2011). BTB-ZF proteins can self-associate and form oligomers but they can also act as heterodimers with other BTB-ZF proteins (Kobayashi et al. 2000, Beaulieu and Sant'Angelo 2011). In mammals about 200 proteins were found that contain this protein-protein interaction motif at their N-terminus and approximately 60 BTB-ZF proteins are encoded by the human genome (Stogios et al. 2005, van Roy and McCrea 2005).

Sequence alignments of BTB-ZF proteins involved in thymocyte development and T cell function showed a homology at critical residues for dimerization and nuclear corepressor interaction. This implicates a common molecular mechanism of how BTB-ZF proteins regulate their target genes (Bilic and Ellmeier 2007). The number of zinc fingers at the C-terminus varies between BTB-ZF proteins (Figure 4) and they also have different DNA-binding sequences. The linker region between the C-terminus and N-terminus is not well conserved. Further they all function at different stages of T cell development (Bilic and Ellmeier 2007, Beaulieu and Sant'Angelo 2011). For example MAZR is highly expressed in DN thymocytes but decreases during further development (Bilic et al. 2006). To date nine BTB-ZF proteins, including the eight BTB-ZF proteins depicted in Figure 4, are implicated to have regulating functions in T cell development (Ellmeier and Taniuchi 2014).

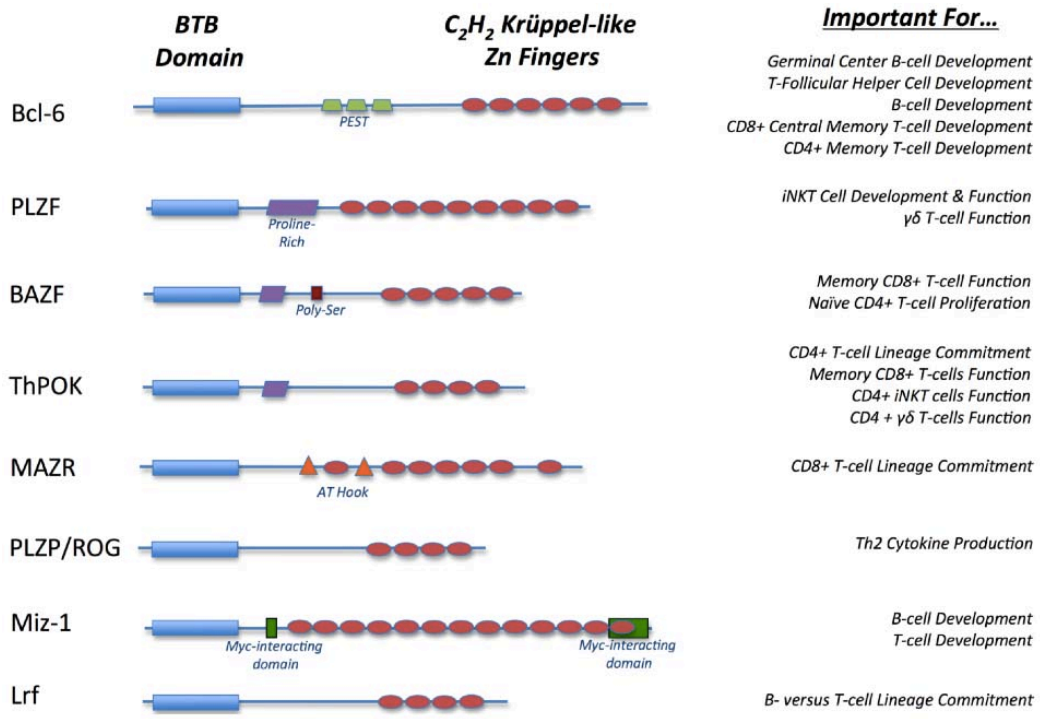

Figure 4. BTB-ZF transcription factors in T cell development
This schematic map shows the location of the BTB domain at the N-terminus and the number of Cys_2His_2 zinc finger motifs at the C-terminus of several BTB-ZF proteins with a role in T cell development and the regulation of peripheral T cell function (adapted from Beaulieu and Sant'Angelo 2011).

Many of the BTB-ZF proteins are cancer-associated proteins and are linked to tumorigenic processes. One prominent example for a BTB-ZF protein involved in cancer is promyelocytic leukemia zinc finger (PLZF) (Kelly and Daniel 2006). PLZF is located on chromosome 11 in humans and in some cases it undergoes a chromosomal translocation (t(11;17)(q23;q21)). Due to this translocation PLZF fuses to the retinoic acid receptor alpha (RARα) and leads to acute promyelocytic leukemia. The mutant PLZF-RARα fusion protein recruits transcriptional corepressors and acts as a negative inhibitor of wild-type RARα (Chen et al. 1993, Lin et al. 2001). In further consequence target genes involved in DNA repair, apoptosis and cell cycle are affected by this translocation (Grignani et al. 1998, Kelly and Daniel 2006). PLZF also plays a role as an important regulator of $CD44^{hi}$ memory phenotype T cell development and is essential for natural killer T cell development (Raberger et al. 2008, Alonzo and Sant'Angelo 2011).

4 Aim of the Thesis

We recently identified that the transcription factor MAZR, a member of the BTB/POZ domain containing family of zinc finger transcription factors, is one of the essential transcription factors that are part of the transcriptional network that controls CD4/CD8 cell fate choice of double-positive (DP) thymocytes. MAZR regulates CD4/CD8 lineage differentiation by repressing ThPOK in MHC class I-signaled DP thymocytes, presumably, via binding to the *Thpok* silencer. Since MAZR has been shown to interact with members of the Runx protein family that itself regulate ThPOK expression, we hypothesized that MAZR might act in synergy with Runx factors in the repression of ThPOK. The aim of my master thesis was to test this hypothesis by using genetic, molecular and biochemical approaches.

Specific aim 1: Characterize the interaction between MAZR and Runx complexes.

By using a molecular and biochemical approach I addressed whether MAZR and Runx1/Runx3 interact *in vitro* and determined domains on MAZR important for Runx1/Runx3 interaction. Moreover, to investigate the role of MAZR and Runx complexes in regulating ThPOK expression *in vivo*, I analyzed the phenotype of mice deficient in both MAZR and Runx3.

Specific aim 2: Mechanistic insight into the regulation of ThPOK expression by MAZR.

In this aim I addressed the role of various MAZR protein domains in the repression of ThPOK by retroviral-mediated overexpression of wild-type and mutant MAZR forms in mature $CD8^+$ T cells. Furthermore, I investigated the potential involvement of histone deacetylases (HDACs) in MAZR-mediated ThPOK repression.

5 Results

5.1.1 MAZR interacts with Runx complexes via direct protein-protein interaction

The results obtained during my master thesis work are part of a FWF-funded project conducted by Dr. Shinya Sakaguchi. For a better understanding of my experiments I start with a short summary of preliminary results obtained by Dr. Shinya Sakaguchi. He performed several co-immunoprecipitation (CoIP) assays to investigate whether MAZR and Runx complexes can interact. The results showed that MAZR interacts with Runx1d and Runx3d but not with the dimerization partners Cbfβ1 and Cbfβ2 (Figure 5). Based on these results I performed additional experiments.

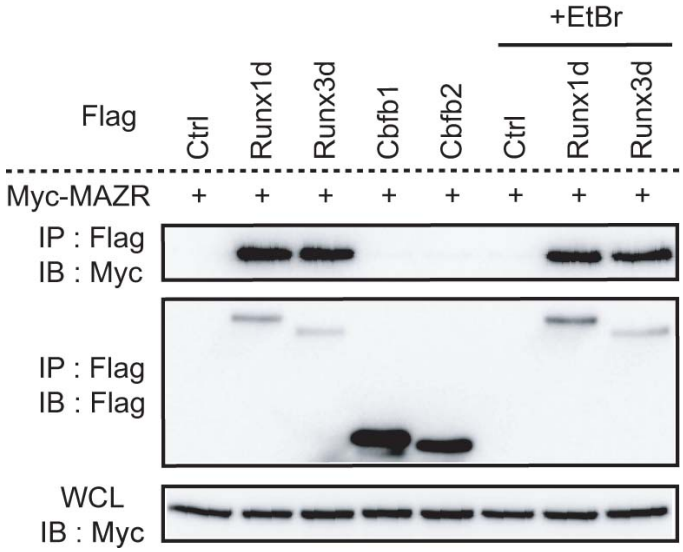

Figure 5. MAZR interacts with Runx1 and Runx3 in a DNA-independent manner
Immunoblots of lysates of HEK293T cells overexpressing Myc-tagged MAZR and either Flag-tagged Runx1d (lane 2 and 7), Runx3d (lane 3 and 8), Cbfβ1 (lane 4), Cbfβ2 (lane 5) or empty control vector (lane 1 and 6) immunoprecipitated (IP) with anti-Flag antibody and blotted (IB) with anti-Myc (upper panel) or with anti-Flag (middle panel) antibodies. The immunoblot in the lower panel shows whole cell lysate (WCL) blotted with anti-Myc antibody. In some samples (lanes 6-8) ethidium bromide (EtBr) was added to the CoIP reaction.

The interaction between MAZR and Runx complexes was also analyzed in the presence of ethidium bromide (Figure 5). Ethidium bromide intercalates with bases in the double helix of the DNA and thereby disrupting protein interactions mediated

by DNA (Nguyen and Goodrich 2006). This experimental setup showed that MAZR and Runx complexes directly interact via protein-protein interaction.

5.1.2 MAZR interacts with Runx1 and Runx3 via its 7th zinc finger domain

Once the interaction between MAZR and Runx complexes was shown, we addressed the question about the domains of MAZR, Runx1 and Runx3 necessary for these interactions. Preliminary results showed that the activation domain of Runx1 is important for the interaction with MAZR (data not shown). As a next step I generated Myc-tagged MAZR mutant constructs to narrow down the region for possible interaction between MAZR and Runx complexes (Figure 6A). The constructs were transfected into HEK293T cells together with the constructs expressing either Flag-tagged Runx1 or Runx3 and CoIPs were performed. Figure 6B shows that the deletion of the 7th zinc finger domain of MAZR protein almost completely abrogates the MAZR/Runx1 and MAZR/Runx3 interactions while other constructs containing the 7th zinc finger domain of MAZR can still interact with Runx1 and Runx3 (Figure 6C). These results demonstrate that the 7th zinc finger of MAZR is necessary for the interaction with Runx complexes.

5.1.3 Two conserved amino acids in the BTB domain of MAZR are dispensable for MAZR/Runx interactions

BTB-ZF proteins often interact with corepressors such as N-CoR and SMRT via their BTB domain, and thereby regulating the target gene expression negatively (Huynh and Bardwell 1998). In a previous study our group showed that MAZR can also interact with N-CoR, and that 2 amino acids in its BTB domain are crucial for the interaction (Bilic et al. 2006). To test if these 2 amino acids are also essential for binding Runx complexes I generated a plasmid containing the *Mazr* gene with these 2 point mutations in the BTB domain. Figure 6C shows that the mutant form of MAZR can still interact with Runx1 and Runx3, indicating that these 2 amino acids are dispensable for the MAZR/Runx1 and MAZR/Runx3 interactions.

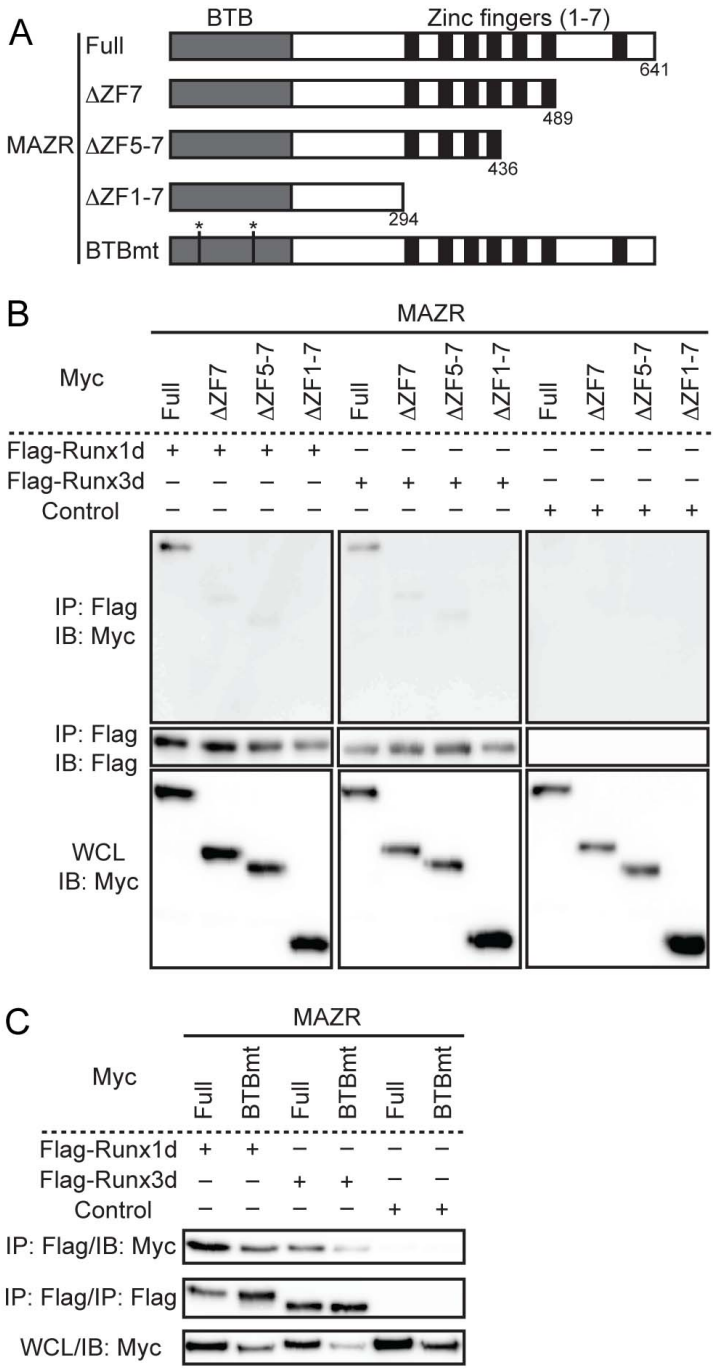

Figure 6. Mapping of interaction domains between MAZR and Runx complexes

(A) Schematic map of various MAZR constructs used for transfections.

(B) Immunoblots of lysates of HEK293T cells overexpressing Flag-tagged Runx1d (lanes 1-4), Flag-tagged Runx3d (lanes 5-8) or empty control vector (lanes 9-12) and various Myc-tagged variants of MAZR (shown in 6A) immunoprecipitated (IP) with anti-Flag antibody and blotted (IB) with anti-Myc (upper panel) or with anti-Flag (middle panel) antibodies. The immunoblot in the lower panel shows whole cell lysate (WCL) blotted with anti-Myc antibody.

(C) Immunoblots of lysates of HEK293T cells overexpressing Flag-tagged Runx1d (lanes 1-2), Flag-tagged Runx3d (lanes 3-4) or empty control vector (lanes 5-6) and either Myc-tagged MAZR or BTBmt-MAZR (shown in 6A) immunoprecipitated (IP) with anti-Flag antibody and blotted (IB) with anti-Myc (upper panel) or with anti-Flag (middle panel) antibodies. The immunoblot in the lower panel shows whole cell lysate (WCL) blotted with anti-Myc antibody.

5.1.4 The BTB domain of MAZR can also interact with Runx1 and Runx3

In addition to a series of mutant MAZR proteins described above, I generated a construct expressing only the BTB domain of MAZR protein, and tested its interaction with Runx1 and Runx3 proteins (Figure 7A and 7B). Surprisingly, the BTB domain alone is sufficient for the interaction between MAZR and Runx proteins, which is somewhat puzzling considering the results indicating the importance of the 7th zinc finger domain for their interactions. The structure of the BTB domain is highly conserved among the BTB-ZF proteins (Stogios et al. 2005), and we speculated that the ability to interact with Runx proteins might be a "general" feature of the BTB domain when it is expressed alone. To test this hypothesis, I generated plasmids expressing the BTB domain of other BTB-ZF proteins (ThPOK and PLZF), and tested for their interactions with Runx3 protein. As shown in Figure 7C, the BTB domain of both ThPOK and PLZF proteins failed to interact with Runx3, indicating that the ability to interact with Runx proteins is an unique feature of the MAZR BTB domain.

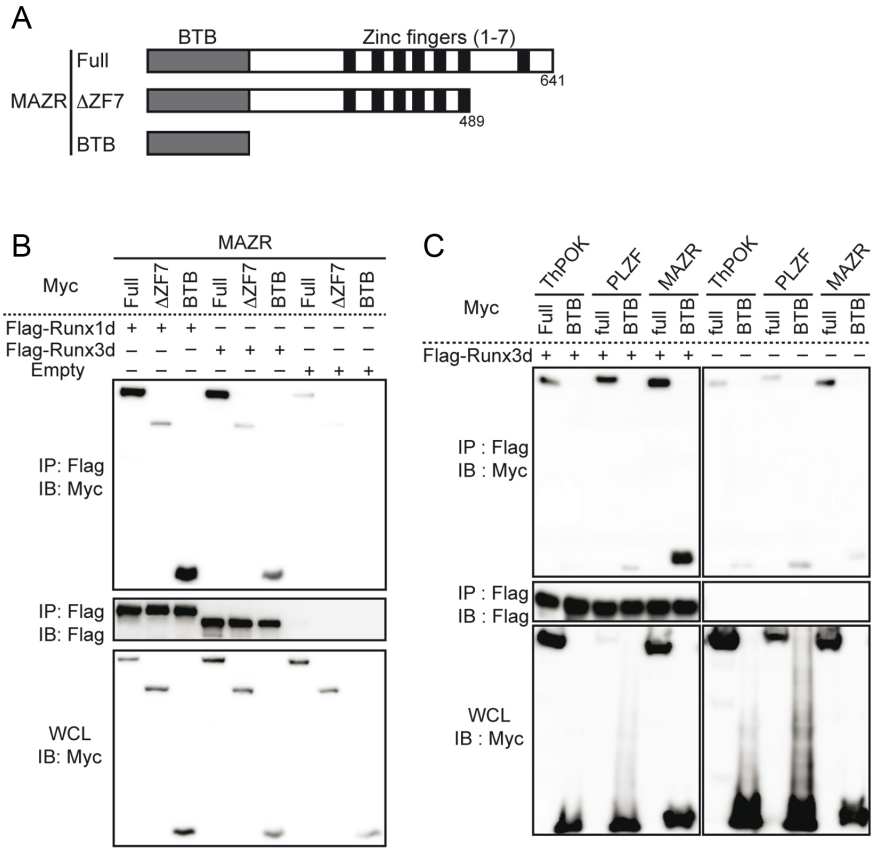

Figure 7. BTB-ZF proteins and the BTB domain of MAZR interact with Runx complexes

(A) Schematic map of various MAZR constructs used for transfections.
(B) Immunoblots of lysates of HEK293T cells overexpressing Flag-tagged Runx1d (lanes 1-3), Flag-tagged Runx3d (lanes 4-6) or empty control vector (lanes 7-9) and various Myc-tagged variants of MAZR (shown in 7A) immunoprecipitated (IP) with anti-Flag antibody and blotted (IB) with anti-Myc (upper panel) or with anti-Flag (middle panel) antibodies. The immunblot in the lower panel shows whole cell lysate (WCL) blotted with anti-Myc antibody.
(C) Immunoblots of lysates of HEK293T cells overexpressing Flag-tagged Runx3d (lanes 1-6) or empty control vector (lanes 7-12) and Myc-tagged ThPOK, PLZF, MAZR and the only BTB domain variants of these BTB-ZF proteins. Immunoprecipitation (IP) was performed with anti-Flag antibody and blotted (IB) with anti-Myc (upper panel) or with anti-Flag (middle panel) antibodies. The immunoblot in the lower panel shows whole cell lysate (WCL) blotted with anti-Myc antibody.

5.1.5 Synergistic activities of MAZR and Runx3 in *Thpok* and *Cd4* gene repression

After showing *in vitro* that MAZR and Runx3 interact with each other I took genetic approaches to investigate if these transcription factors synergize during cytotoxic lineage differentiation. Dr. Shinya Sakaguchi crossed recently generated *Mazr*$^{f/f}$ mice (Abramova et al. 2013) with *Cd4*-Cre transgenic mice (where Cre recombinase is expressed from the DP thymocyte stage on) (Lee et al. 2001), and furthermore introduced a ThPOK-GFP knock-in allele as a “surrogate” marker for ThPOK expression (Setoguchi et al. 2008). Finally, he crossed *Mazr*$^{f/f}$ mice with *Runx3*$^{mt/mt}$ mice expressing a mutant Runx3 protein lacking the VWRPY repressor motif at the C-terminus (Yarmus et al. 2006), and thus generated *Mazr*$^{f/f}$*Runx3*$^{mt/mt}$*Thpok*$^{GFP/+}$*Cd4*-Cre mice.

Consistent with Dr. Sakaguchi`s previous publication *Mazr*$^{f/f}$*Thpok*$^{GFP/+}$*Cd4*-Cre mice displayed elevated CD4 to CD8 ratios in peripheral T cells and ThPOK derepression in CD8$^+$ T cells (Figure 8) (Sakaguchi et al. 2010). *Runx3*$^{mt/mt}$*Thpok*$^{GFP/+}$ mice showed CD4 derepression in CD8$^+$ T cells as previously described in *Runx3*$^{mt/mt}$ mice (Yarmus et al. 2006). Notably, in *Mazr*$^{f/f}$*Runx3*$^{mt/mt}$*Thpok*$^{GFP/+}$*Cd4*-Cre mice ThPOK derepression in CD8$^+$ T cells was enhanced compared to wild-type, *Mazr*$^{f/f}$*Thpok*$^{GFP/+}$*Cd4*-Cre and *Runx3*$^{mt/mt}$*Thpok*$^{GFP/+}$ CD8$^+$ T cells (Figure 8).
Moreover, the *Mazr*$^{f/f}$*Runx3*$^{mt/mt}$*Thpok*$^{GFP/+}$*Cd4*-Cre CD8$^+$ T cells displayed enhanced CD4 derepression compared to *Runx3*$^{mt/mt}$*Thpok*$^{GFP/+}$ T cells, indicating that MAZR is involved in *Cd4* silencing, at least in the absence of Runx3.

These data together indicate a synergistic activity of MAZR and Runx3 in repression of *Thpok* and *Cd4* genes during CD8$^+$ T cell development.

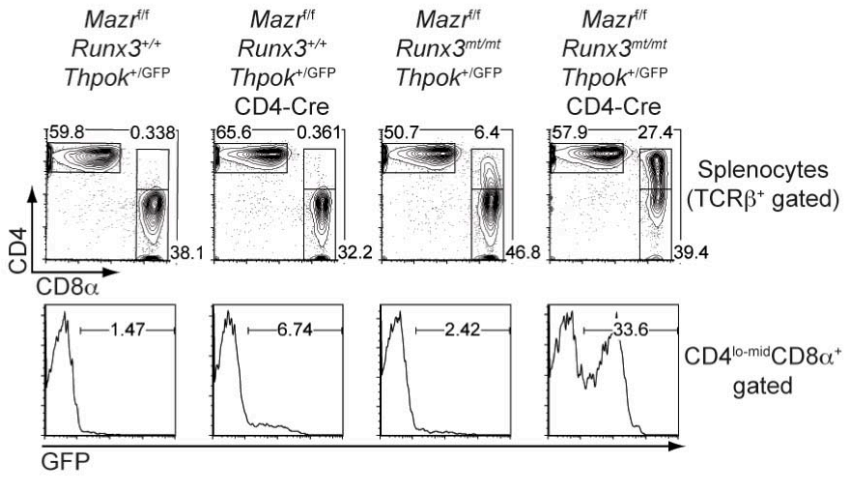

Figure 8. Synergistic activities of MAZR and Runx3 in the repression of *Thpok* and *Cd4* genes
Flow cytometric analysis showing CD4, CD8α and GFP expression on splenocytes isolated from mice with the indicated genotype. Flow cytometric analysis of CD4 and CD8α expression was performed on TCRβ$^+$ splenocytes (upper panel). Histograms showing GFP expression (lower panel) in CD4$^{lo\text{-}mid}$CD8α$^+$ T cells. Numbers in histograms show the percentages of the GFP-positive population.

5.1.6 Zinc fingers 1-4 and the BTB domain are necessary to repress ThPOK

It was not known so far whether ThPOK derepression in MAZR-deficient CD8$^+$ T cells can be rescued by re-expressing ectopic MAZR. To address this question I retrovirally overexpressed wild-type and truncated versions of MAZR in *Mazr$^{f/f}$Thpok$^{GFP/+}$Lck*-Cre CD8$^+$ T cells (Figure 9A), and monitored GFP expression which correlates with ThPOK expression (Figure 9B). The results demonstrated that the deletion of MAZR leads to ThPOK derepression in a fraction of peripheral CD8$^+$ T cells. The immunoblot analysis in Figure 9C shows that all versions of MAZR protein were overexpressed. Overexpression of wild-type MAZR protein as well as MAZR protein lacking the 5th to 7th zinc finger domains could revert the phenotype of ThPOK derepression in MAZR-deficient CD8$^+$ T cells, whereas the mutant versions of MAZR lacking all the zinc fingers and harboring the 2 point mutations in the BTB domain failed to rescue the phenotype. These results suggest that ThPOK derepression in peripheral MAZR-deficient CD8$^+$ T cells is not fixed during cytotoxic lineage differentiation, and that it can be readily reverted upon MAZR overexpression. Moreover, my data suggest that MAZR-mediated ThPOK repression might take place via its interaction with the corepressor N-CoR, whereas MAZR/Runx interactions (which take place via the 7th zinc finger of MAZR) are dispensable for this process.

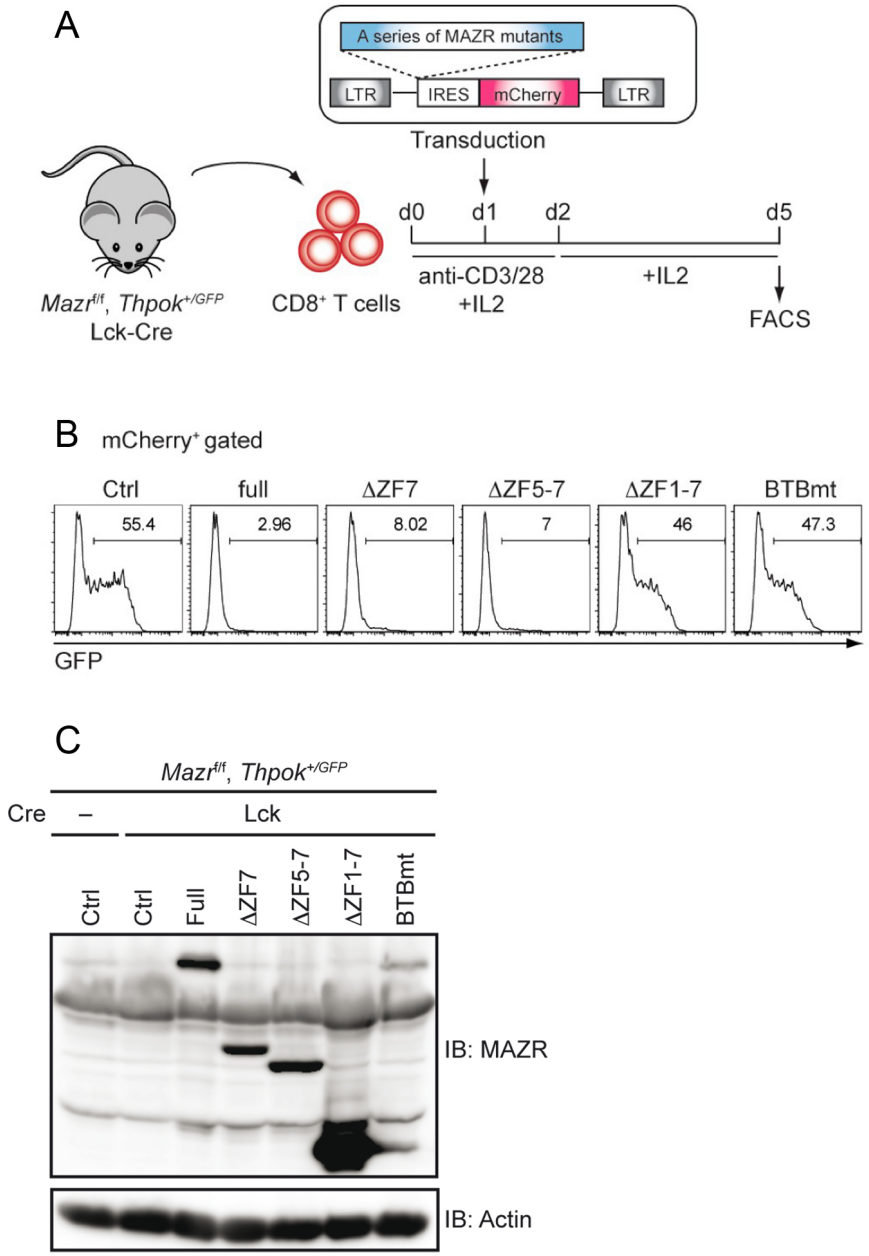

Figure 9. Zinc fingers 1-4 and the BTB domain are necessary to repress ThPOK

(A) Map of the retroviral constructs and experimental strategy. Transduced CD8$^+$ T cells were identified by gating on the mCherry-positive population.

(B) *Mazr*$^{f/f}$*Thpok*$^{GFP/+}$ and *Mazr*$^{f/f}$*Thpok*$^{GFP/+}$*Lck*-Cre CD8$^+$ T cells were transduced with retroviral vectors containing wild-type or various deletion mutants of MAZR (shown in 6A), or with an empty control vector. Histograms show GFP expression in mCherry-positive CD8$^+$ T cells 5 days after transduction.

(C) Immunoblot of lysates of CD8$^+$ T cells from *Mazr*$^{f/f}$*Thpok*$^{GFP/+}$ and *Mazr*$^{f/f}$*Thpok*$^{GFP/+}$*Lck*-Cre mice that were transduced with a retroviral vector containing wild-type or various deletion mutants of MAZR, or with an empty control vector. Cell lysates were blotted (IB) with anti-MAZR antibody (upper panel) or anti-Actin antibody (lower panel).

5.1.7 HDAC inhibitor has no effect on MAZR-mediated ThPOK repression

Next we wanted to elucidate the mechanism of how MAZR represses ThPOK. My results described above suggested a possible involvement of MAZR/N-CoR interaction in ThPOK repression. N-CoR is a component of complexes containing proteins involved in epigenetic modifications such as histone deacetylases (HDACs) (Horlein et al. 1995, Jepsen et al. 2000). Our group recently reported that in the combined absence of HDAC1 and HDAC2 $CD8^+$ T cells display derepressed expression of ThPOK, indicating a potential involvement of HDAC1 and HDAC2 in *Thpok* gene regulation in peripheral $CD8^+$ T cells (Boucheron et al. 2014). I therefore tested whether inhibition of HDAC function leads to impairment of MAZR-mediated ThPOK repression in $CD8^+$ T cells. I cultured MAZR-overexpressed *Mazr*$^{f/f}$*Thpok*$^{+/GFP}$*Lck*-Cre $CD8^+$ T cells in the presence of Trichostatin A (TSA), a pan-HDAC inhibitor, and analyzed ThPOK expression (Figure 10). I could not detect any effect of the inhibitor on MAZR-mediated ThPOK repression compared to cells treated with DMSO as a control. These data suggest that MAZR represses ThPOK independently of HDACs.

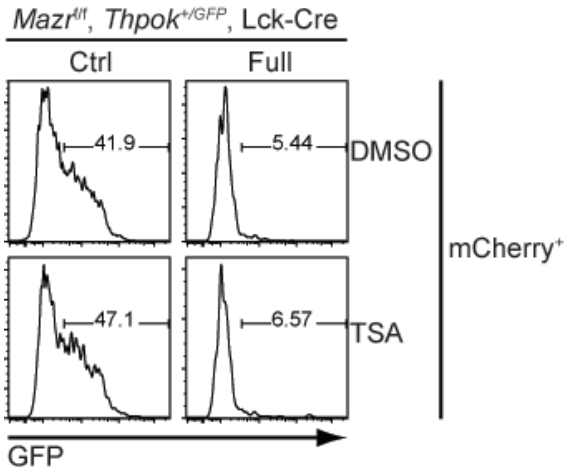

Figure 10. HDAC inhibitor has no effect on MAZR-mediated ThPOK repression
Mazr$^{f/f}$*Thpok*$^{+/GFP}$*Lck*-Cre $CD8^+$ T cells were either transduced with a retroviral vector containing wild-type MAZR (right panels) or with an empty control vector (left panels). Histograms show GFP expression in mCherry-positive $CD8^+$ T cells treated with Trichostatin A (TSA) (lower panels) and DMSO (upper panels) as a control.

6 Discussion

Eukaryotic gene expression depends on the coordination of multiple proteins. By establishing transcription factor networks the higher regulatory demand of multicellular organisms can be accomplished. *Cis*-acting DNA-elements such as promoters, silencers and enhancers, mediate the assembly of stereo-specific protein-DNA complexes (Tjian and Maniatis 1994, Rohs et al. 2010). Upon DNA-binding transcription factors can change their protein structure. Further they can homo- or heterodimerize and thereby increasing structural complexity. To answer the question how different patterns of eukaryotic gene expression are generated a deep understanding of protein-protein and protein-DNA interactions is necessary (Remenyi et al. 2004).

Helper-versus-cytotoxic lineage choice provides one of the best biological models to study how the tightly regulated gene expression coordinates the differentiation of bipotential progenitors to functionally distinct cells (Singer and Bosselut 2004, Taniuchi et al. 2004). During the last two decades, intensive studies have been conducted to understand CD4/CD8-lineage choice at a molecular level, leading to the identification of key transcription factors as well as their cooperation activities in the transcription factor network. Among several transcription factors identified, ThPOK plays a central role for helper lineage differentiation, and the elucidation of the gene regulation mechanisms underlying helper-specific ThPOK expression is currently one of the key issues in the field (Naito and Taniuchi 2010). MAZR and Runx complexes are both transcription factors that bind to the *Thpok* silencer and thereby promoting CD8 lineage commitment (Setoguchi et al. 2008, Sakaguchi et al. 2010). During my master thesis work I aimed to investigate whether, and if yes, how MAZR and Runx cooperatively control T cell development, particularly focusing on their *Thpok* gene regulation.

We first performed CoIP assays to investigate whether MAZR and Runx complexes can interact *in vitro*. We could show that MAZR interacts with Runx1 and Runx3 but does not bind to the Cbfβ subunit. In addition, we observed the interaction of MAZR and Runx complexes in the presence of ethidium bromide, indicating the interaction is mediated through a direct protein-protein association. These data implicate that MAZR and Runx complexes might function together via direct protein-protein interactions. We also tested the interaction between MAZR

and Runx complexes *in vivo* by performing CoIP assays with thymocytes and naïve $CD8^+$ T cells, but so far we failed to confirm this interaction (data not shown). A reason for that could be that the amount of endogenous proteins is too low to detect the interaction compared to an overexpression setup. To solve this problem one could try to overexpress e.g. Runx proteins in $CD8^+$ T cells via retroviral transduction (thereby increasing the amount of Runx proteins in the cells) and to co-immunoprecipitate endogenous MAZR protein, although this approach would not faithfully reflect the *in vivo* situation.

Next, we aimed to map the domains within MAZR and Runx proteins that are required for their interaction. Preliminary results from my supervisor Dr. Sakaguchi showed that the activation domain of Runx1 is essential for the interaction with MAZR. Therefore in a next step I searched for the Runx interaction domains within MAZR. After a series of deletion analysis and CoIP experiments, I identified that the 7^{th} zinc finger of MAZR is essential for the interaction with Runx molecules because the deletion of the 7^{th} zinc finger domain of MAZR almost completely abolished the interaction with Runx1 and Runx3. Many studies have reported that zinc fingers do not only play a role as a DNA-binding motif but also interact with RNA and mediate protein-protein interactions (Gamsjaeger et al. 2007). Thus, the interaction between MAZR and Runx proteins via zinc finger 7 demonstrates a typical feature of zinc fingers in protein-protein interactions. The N-terminal BTB domain is another important domain within the MAZR protein. It is known that BTB-ZF proteins interact with nuclear corepressors (e.g. N-CoR and SMRT) via this BTB domain (Huynh and Bardwell 1998), thereby negatively regulating gene expression. Previous studies have shown that MAZR interacts with N-CoR and that 2 conserved amino acids in the BTB domain are necessary for the interaction (Bilic et al. 2006). We observed that MAZR can still interact with Runx1 and Runx3 even if the 2 conserved amino acids are mutated in the BTB domain, indicating that MAZR and Runx proteins can interact in a corepressor complex-independent manner.

I also generated a construct containing only the BTB domain of MAZR. Surprisingly the BTB domain alone was sufficient for the interaction between MAZR and Runx proteins. This result appears to be inconsistent with our findings that zinc finger 7 is essential for the protein-protein interaction between MAZR and Runx proteins. One explanation would be that the BTB domain, which is highly conserved among BTB-ZF proteins, “generally” possesses an ability to interact with Runx

proteins if it is expressed alone without any additional domains such as zinc finger motifs. To test our hypothesis, I generated constructs containing only the BTB domain of two other BTB-ZF proteins, ThPOK and PLZF, and tested their interaction with Runx proteins. Our results show that full-length versions of ThPOK, PLZF and MAZR proteins interact with Runx3, however only the BTB domain of MAZR interacts with Runx3 when their BTB domains are expressed alone. This indicates a unique ability of the MAZR BTB domain to interact with Runx3. Similar observation was made during the analysis of the structure of BCL6 and PLZF proteins, which demonstrated that the BTB domain of BCL6 but not PLZF can interact with N-CoR (Ahmad et al. 2003). However, these results do not provide a molecular explanation for the inconsistency between our results showing an essential role of the 7th zinc finger of MAZR for the MAZR/Runx interaction, and the ones indicating its BTB domain alone is sufficient for the interaction.

Having confirmed that MAZR and Runx complexes interact via direct protein-protein interactions at least in an *in vitro* setting, we took genetic approaches to elucidate the synergistic function of MAZR and Runx in the regulation of CD4$^+$ and CD8$^+$ T cell development. Interestingly, MAZR-Runx double mutant mice showed enhanced ThPOK derepression in CD8$^+$ T cells compared to wild type, MAZR single knockout (KO) and Runx single KO cells. Moreover, the MAZR-Runx double mutant mice displayed enhanced CD4 derepression compared to Runx single KO cells, indicating a role of MAZR in *Cd4* silencing, at least in the absence of Runx3.

Next we investigated how MAZR mechanistically regulates ThPOK expression. We demonstrated that ectopic expression of MAZR can revert the phenotype of ThPOK derepression in MAZR-deficient activated CD8$^+$ T cells. As overexpression of MAZR in MAZR-sufficient activated CD8$^+$ T cells also leads to ThPOK repression (data not shown), this indicates that MAZR-mediated ThPOK repression operates in wild-type CD8$^+$ T cells which have been generated under “normal” physiological conditions. This idea was further confirmed by our finding that the conditional deletion of MAZR in activated CD8$^+$ T cells leads to ThPOK derepression. Together, our gain- and loss-of-function analyses of MAZR suggest that MAZR is actively repressing ThPOK expression in CD8$^+$ T cells. Taniuchi and his colleagues have recently demonstrated that during CD8$^+$ T cell development the status of the *Thpok* locus becomes epigenetically repressed, and that ThPOK repression in CD8$^+$ T cells can be maintained independently of the *Thpok* silencer

(Tanaka et al. 2013). This suggests that MAZR represses ThPOK expression without its recruitment to the *Thpok* silencer in $CD8^+$ T cells. In addition, our finding implies that MAZR might be part of a molecular machinery required for the establishment/maintenance of the epigenetic status of the *Thpok* locus in peripheral $CD8^+$ T cells. Interestingly, it has been shown that ThPOK expression in activated $CD8^+$ T cells is mediated by the activity of the proximal enhancer and distal promoter of the *Thpok* locus (Muroi et al. 2013). Therefore it might be possible that MAZR is involved in controlling the activity of those elements in direct or indirect ways.

To gain more insight into the mechanism by which MAZR represses ThPOK expression in $CD8^+$ T cells, we transduced a series of truncated MAZR proteins as well as MAZR protein with point mutations into MAZR-deficient $CD8^+$ T cells, and monitored *Thpok* gene expression. We demonstrated that a MAZR version containing the BTB domain and zinc finger (ZF) 1-4 is sufficient for ThPOK repression. Since the deletion of ZF 7 almost completely abrogates the MAZR/Runx interaction, this suggests that MAZR can repress ThPOK expression independently of its association with Runx complex. Interestingly, we also found that MAZR protein harboring the two point mutations essential for its interaction with N-CoR fails to repress ThPOK expression, indicating that MAZR-mediated recruitment of corepressor complexes to the locus is indispensable for ThPOK repression. It is well known that N-CoR corepressor complexes also contain histone-modifying enzymes such as HDACs, and that they thereby change the chromatin status of the target loci and repress gene expression (Mottis et al. 2013).

Our group recently reported a reduced expression of ThPOK in the absence of HDAC1 and HDAC2 in activated $CD4^+$ T cells. These data implicate a potential role of HDAC1 and HDAC2 in *Thpok* gene regulation in peripheral T cells (Boucheron et al. 2014). I therefore tested whether the inhibition of HDAC function leads to a loss of MAZR-mediated ThPOK repression in $CD8^+$ T cells, and failed to detect any effect of a pan-HDAC inhibitor on MAZR-mediated ThPOK repression. These data suggest that MAZR represses ThPOK independently of HDAC activities. Another possible mechanism to repress ThPOK could be the recruitment of DNA methyltransferases to the *Thpok* silencer by MAZR. Further investigations are necessary to elucidate the exact mechanism behind MAZR-mediated ThPOK repression.

Conclusion

Together, my data demonstrate synergistic activities of MAZR and Runx3 in repressing *Thpok* and *Cd4* genes during $CD8^+$ T cell development. By using molecular and biochemical approaches it was possible to gain a deeper understanding of the interaction between MAZR and Runx complexes on a molecular level. The results of this work provide further insight into understanding the transcription factor network underlying the CD4/CD8-lineage choice. Moreover from the results of my thesis further questions are arising. Does MAZR directly regulate CD4 via binding to the *Cd4* silencer? Is CD4 derepression induced by ThPOK derepression? To answer these questions, in future studies ChIP assays have to be performed and MAZR/Runx3/ThPOK triple mutant mice have to be generated and analyzed. Finally, it will also be important to study the role of MAZR and Runx complexes in the differentiation and function of cytotoxic $CD8^+$ effector T cells.

7 Material and Methods

7.1 Cell culture media

7.1.1 For HEK293T and Phoenix cells

DMEM (Sigma)
supplemented with:

10%	FBS (PAA)
100 U/ml	penicillin
100 µg/ml	streptomycin
0,3 µg/ml	L-glutamine

7.1.2 For $CD8^+$ T cells

RPMI-1640 (Sigma)
supplemented with:

10%	FBS
100 U/ml	penicillin
100 µg/ml	streptomycin
0,3 µg/ml	L-glutamine
1%	100 mM sodium pyruvate solution (PAA)
1%	100x non essential amino acids (Lonza)
0.5%	β-mercaptoethanol (GIBCO)

7.2 Cell lines

7.2.1 HEK293T cells

HEK293T human embryonic kidney cell stocks were stored in liquid nitrogen. Frozen cells were rapidly thawed at 37°C, immediately diluted in 10ml DMEM medium and centrifuged at 1200 rpm for 5 min to remove cryoprotective but toxic DMSO (Carl-Roth). The cell pellet was resuspended in DMEM medium and transferred to 10cm tissue culture dishes (Thermo Scientific). Cells were grown in 37°C incubator supplied with 5% CO_2 and split every three days depending on the density. For splitting cells were gently rinsed once with 10ml PBS (Sigma) and trypsinized with 2ml 0.25% trypsin-EDTA solution (Sigma) until the cells easily

detach. Trypsinization was stopped with 8ml fresh DMEM medium. The cell suspension was centrifuged at 1200 rpm for 5 min and then the cell pellet was resuspended in fresh DMEM medium and split on 10cm tissue culture dishes.

7.2.2 Phoenix cells

Phoenix is a retrovirus producer line based on the 293T line (Swift et al. 2001). They were used as packaging cells for virus production. The viral supernatant was then used for retroviral transductions. Phoenix cells were treated the same way as HEK293T cells regarding thawing, splitting and culturing.

7.2.3 Cell stock preparation

To prepare one cryotube (Sigma) for cell stock all cells on a confluent 10cm tissue culture dish (approx. $10x10^6$ cells) were detached by adding 2ml of 0,25% trypsin-EDTA solution and collected in a 15ml falcon (BD Biosciences). The cell suspension was centrifuged at 3200 rpm for 3 min and the cell pellet was resuspended in 1ml freezing medium (90% FBS, 10% DMSO). The cryotubes were stored at -80°C overnight and on the next day they were put in liquid nitrogen tanks for long-term storage.

7.3 Cloning

Initially all MAZR mutant genes (full, ΔZF7, ΔZF5-7, ΔZF1-7 and BTBmt) had a pcDNA3 backbone. For the retroviral transductions these genes had to be cloned into a retroviral vector containing mCherry as a reporter gene (Table 1).
In addition ThPOK full, ThPOK BTB, PLZF full and PLZF BTB had to be cloned into a pcDNA3-Myc vector to overexpress them in HEK293T cells. For cloning ThPOK BTB, PLZF full and PLZF BTB I first designed forward and reverse primers (Table 2) and then amplified the region of interest by PCR. The primers contain EcoRI restriction sites at the 5' end to easily clone the PCR-products in the vector backbone that is also cut with EcoRI.

Table 1. Plasmids

Plasmid name	Vector backbone	Insert name	Insert description
pcDNA3-Myc (Invitrogen, nowadays Life Technologies)	• **mammalian expression vector** • CMV-promoter • ampicillin-resistance • transient • Myc-tag	empty	without insert
		MAZR full	full-length
		MAZR ΔZF7	lacks ZF7
		MAZR ΔZF5-7	lacks ZF5-7
		MAZR ΔZF1-7	lacks ZF1-7
		MAZR BTBmt	2 point mutations in the BTB domain
		MAZR BTB	only BTB domain
		ThPOK full	full-length
		ThPOK BTB	only BTB domain
		PLZF full	full-length
		PLZF BTB	only BTB domain
pcDNA3-Flag (Invitrogen, nowadays Life Technologies)	• **mammalian expression vector** • CMV-promoter • ampicillin-resistance • transient • Flag-tag	empty	without insert
		Runx1d	Runx1 distal promoter
		Runx3d	Runx3 distal promoter
pMIG-IRES-mCherry (provided by B. Erman, Sabanci University Istanbul, Turkey)	• **retroviral expression vector** • IRES-mCherry casette, allowing identification of transduced cells by their mCherry expression • LTR promoter • ampicillin-resistance • stable	empty	without insert
		MAZR full	full-length
		MAZR ΔZF7	lacks ZF7
		MAZR ΔZF5-7	lacks ZF5-7
		MAZR ΔZF1-7	lacks ZF1-7
		MAZR BTBmt	2 point mutations in the BTB domain
pGEM®-T Easy Vector (Promega)	• **cloning vector** • T-overhang for easy PCR cloning • blue/white screening • ampicillin-resistance		

Table 2. Primers

Primer name	Sequence (5'-3')
ThPOK fwd	TATAGAATTCCGTTGCTGTCGGCGAGTGGGGAAGGGGCAG CGACCATGGGGAGCCCCGAGG
ThPOK BTB only rev	ATTAGAATTCTCACCCACTGCCTTGTAGAATCTC
PLZF fwd	TATAGAATTCCGTTGCTGTCGGCGAGTGGGGAAGGGGCAG CGACCATGGATCTGACAAAGATGGGGATG
PLZF BTB only rev	ATTAGAATTCTCAGATGGTCTCCAGGATCTTCAG
PLZF full rev	ATTAGAATTCTCACACATAACACAGGTAGAGG

PCR reaction mix:

5µl	10x High Fidelity PCR buffer (Thermo Scientific #K0191)
1µl	10mM dNTPs
1µl	10µM forward primer
1µl	10µM reverse primer
1,5 units	High Fidelity PCR Enzyme mix (Thermo Scientific #K0191))
1µl	DNA (conc. 1ng/µl)
	ad 50µl dH_2O

PCR program:

96°C	5 min	
59°C	30 sec	} 39 cycles
72°C	2 min 30 sec	
94°C	30 sec	
59°C	30 sec	
72°C	4 min	
4°C	forever	

Then the PCR products were cloned into a pGEM®-T Easy vector (according to the protocol provided by Promega corporation) and transformed into *E.coli* DH5α cells. By blue/white-screening clones with inserts were identified, picked, mini-prepped and sent to eurofins (www.eurofins.at) for sequencing. Clones with correct sequences were transformed and liquid LB-medium was inoculated. The bacterial overnight culture was mini-prepped (see chapter 7.3.7) and the purified plasmids were digested with EcoRI. In general the steps described in the next chapters were performed to generate functional plasmids for further experiments.

To clone ThPOK full-length into a pcDNA3-Myc vector I had to use an alternative cloning strategy to the strategy mentioned above. A pcDNA3-Myc plasmid (containing ThPOK BTB) and the pcDNA3-cKrox plasmid (WE699 – contains ThPOK full-length) were digested with the restriction enzymes XhoI and XbaI (Fermentas) and the desired fragments were ligated.

7.3.1 Restriction digest

Unique restriction sites of EcoRI are located at the ends of the genes encoding the various MAZR mutants in each plasmid. To cut out the DNA sequence encoding the respective MAZR variant plasmids were digested with the restriction enzyme EcoRI. Also the plasmid with the desired vector backbone was cut with EcoRI.

Reaction mix:

80 units	EcoRI (New England BioLabs #R0101S),
5µl	10x buffer EcoRI (New England BioLabs #B0101S)
0.5µl	BSA (New England BioLabs #B9000S)
10-40µl*	DNA template
	ad 50µl dH_2O

incubate at 37°C for 1.5 hours

(* The used DNA template amount varied because of different plasmid concentrations.)

7.3.2 Alkaline phosphatase treatment

Following the restriction digest of the vectors with EcoRI a calf intestinal alkaline phosphatase (CIAP, Promega #M182A) was used for dephosphorylation of 5' and 3' ends of DNA to prevent religation of linearized plasmid DNA. 2 units CIAP were added to the restriction digest and the mix was incubated for 15 min at 37°C. Phosphatase treatment was stopped by an incubation step of 10 min at 75°C.

7.3.3 Agarose gel electrophoresis

After the restriction digest and the dephosphorylation step the DNA inserts and vectors were analyzed on agarose gel. For 150ml of a 1% gel 1.5g agarose and 150ml 1x TAE buffer were mixed and heated until the agarose was completely dissolved. Three drops of DNA Stain Clear G (SERVA) were added to the agarose gel solution and immediately poured into a gel-casting frame. After approx. 30 min the gel had hardened and was ready for loading.
6x DNA loading dye (Thermo Scientific #R0611) was added to the 50µl restriction digest mix and a GeneRuler DNA ladder mix (Fermentas #SM0333) was used to

estimate the size of the DNA fragments. The gels were run at 200V for approx. 30 min and afterwards the separated DNA fragments were visualized with QUANTUM Multi-Imagingsystem (PEQLAB). The desired bands were cut out with a razor blade and transferred into a pre-weighed 1.5ml tube Eppendorf tube to easily estimate the weight of the gel slice.

7.3.4 Purification of DNA fragments from agarose gel (GeneJET™ Gel Extraction Kit, Fermentas)

For 100mg agarose gel 100µl binding buffer was added to the tube and incubated at 55°C for 10 min until the gel slice was completely dissolved. The dissolved gel solution was transferred to the GeneJET™ purification column and centrifuged at 10000 rpm for 1 min. 500µl of wash buffer was added to the GeneJET™ purification column and centrifuged. This washing step was performed twice and the residual wash buffer was removed by an additional centrifugation step. Finally, the GeneJET™ purification column was transferred into a clean 1.5ml tube and 20µl elution buffer were added directly to the center of the membrane. The DNA was eluted by centrifugation at 10000 rpm for 1 min and directly used for the ligation set up or stored at -20°C.

7.3.5 Ligation

Before setting up the ligation it is important to determine the amount of cut insert and vector to be used for the ligation reaction. The quantification of DNA concentration was done by using the NanoDrop spectrophotometer. As an alternative method for quantification 1µl DNA sample was run on agarose gel together with a series of DNA standards with known concentrations (e.g. different GeneRuler DNA Ladder mix dilutions). DNA was quantified in comparison with the fluorescent yield of the standards.
The DNA amount used in the ligation mix was then calculated according to a molar ratio of 4 x insert : 1 x vector.

Ligation reaction:

2µl	T4 DNA 10x ligase buffer (Promega #C126B)
3 units	T4 DNA ligase (Promega #M180A)
100ng	vector
x ng*	insert
	ad 20µl dH_2O

incubated at 16°C (3 hours or overnight)

(* insert DNA amount depends on the calculation)

7.3.6 Transformation

LB-medium Luria/Miller (Carl Roth)

25g in 1 liter deionized water

contain:

10g	trypton
5g	yeast extract
10g	NaCl

autoclaved for 1h at 125°C

before use supplemented with: 100µg/ml ampicillin

stored at 4°C

LB-agar Luria/Miller (Carl Roth)

40g in 1 liter deionized water

contain:

10g	trypton
5g	yeast extract
10g	NaCl
15g	agar-agar

autoclaved for 1h at 125°C

stored at 4°C

5x KCM buffer

0.5 M	KCl
0.15 M	$CaCl_2$
0.25 M	$MgCl_2$
	dH_2O

sterile filtration, stored at 4°C

Transformation mix:

10µl	ligation reaction
100µl	chemically competent *E.coli* DH5α cells
20µl	5x KCM buffer
80µl	dH_2O

The transformation mix was incubated on ice for 30 min followed by an incubation step at room temperature for 10 min. Then 1ml LB media without ampicillin was added and the tube was incubated for 1h at 37°C. The transformation mix was centrifuged at 5000 rpm for 3 min and the supernatant was removed. 200µl supernatant were left in the tube and the bacterial cell pellet was resuspended. By using an aseptic technique these 200µl of bacterial solution were plated on agar plates containing ampicillin for selection. On the next day clones were picked and 2.5ml liquid LB-medium were inoculated and incubated at 37°C overnight.

7.3.7 Plasmid DNA purification from bacteria cultures (GeneJET Plasmid Miniprep Kit, Thermo Scientific)

2.5 ml bacterial overnight cultures were centrifuged at 10000 rpm for 5 min at room temperature. After removing the supernatant the cell pellet was completely resuspended in 250µl Resuspension Solution. To liberate the plasmid DNA 250µl Lysis Solution was added and the tube was inverted six times until the solution became viscous. 350µl Neutralization Solution was added and the tube was inverted six times to optimize binding conditions of plasmid DNA on the silica membrane in the spin columns. To pellet cell debris and SDS the lysate was centrifuged for 5 min at 10000 rpm. Then the supernatant containing the plasmid DNA was transferred to the GeneJET™ spin column and centrifuged for 1 min.

500µl Wash Solution was added to the column and centrifuged for 1 min at 10000 rpm. To elute the plasmid DNA the spin column was transferred into a fresh 1.5ml tube and 50µl Elution Buffer were directly added on the membrane and centrifuged for 1 min at 10000 rpm.

7.4 Co-Immunoprecipitation analysis

7.4.1 Calcium phosphate transfection of HEK293T cells

$CaCl_2$-solution

1.25 M	$CaCl_2$
	dH_2O

sterile filtration, stored at 4°C

2x HEPES-buffered saline (HEBS) buffer

50 mM	HEPES
10 mM	KCl
12 mM	glucose
280 mM	NaCl
1.5 mM	Na_2HPO_4
	dH_2O

sterile filtration, stored at 4°C

Lysis buffer

20 mM	Tris-HCl (pH 8)
138 mM	NaCl
10 mM	EDTA
1%	NP-40
10%	glycerol
100 mM	NaF

before use supplemented with:

1 mM	PMSF
2 mM	Na_3VO_4
1 tablet/20ml	protease inhibitor cocktail tablet (Roche)

HEK293T cells were thawed and fresh culture medium was immediately added. To remove DMSO the cell suspension was centrifuged at 1400 rpm for 4 min. The supernatant was removed and 30ml of culture medium was added. The cell pellet was resuspended and 10ml were transferred to each 10cm cell culture plate. The plates were incubated at 37°C and 5% CO_2 until the cells were confluent and in good condition they were seeded out. Therefore the plates were washed with 5ml PBS and 2ml 0.25% trypsin-EDTA solution were added. To enforce the detachment of the cells the plates were incubated at 37°C for 2 min. To stop trypsinization 8ml of FBS-containing culture medium was added. The cell suspensions of all plates were pipetted into a 50ml Falcon tube (BD Falcon) and centrifuged at 1400 rpm for 4 min. The supernatant was removed and 30ml culture medium was added. Cells were counted with a CASY cell counter and $3.5x10^6$ cells were seeded on each 10cm plate. The cells were incubated overnight and on the next day in the morning the transfection mix for each combination was prepared in round-bottom polystyrene tubes (BD Falcon).

Transfection mix:

120µl	1.25 M $CaCl_2$
460µl	deionized water
600µl	2x HEBS buffer
20µl	plasmids

First 1.25 M $CaCl_2$, deionized water and the plasmids were pipetted into a round-bottom polystyrene tube. Each transfection mix contained Flag-Runx1d, Flag-Runx3d or empty vector and one of the Myc-tagged MAZR mutant constructs. 10µl of each plasmid were added to the transfection mix and in total 20µl (= 20µg) of plasmids were used for one transfection.

At last the 2x HEBS buffer was added carefully. By using a pasteur pipette stuck into a pipette boy air bubbles were carefully introduced in the transfection mix and 1ml 2x HEBS buffer was added dropwise with a second pipette. Afterwards the transfection mix was vortexed and slowly pipetted on the HEK293T plates that were prepared the day before. The plates were put back in the incubator and after 10 min first precipitates were visible. After 6 hours the media was sucked off, the cells were

washed once with 5ml PBS and 10ml of fresh culture medium was gently added to each plate.

48 hours later the cells were harvested on ice. The medium was removed and the cells were carefully rinsed with ice-cold PBS containing PMSF. Then 5ml ice-cold PBS containing 1mM PMSF was added, adherent cells were collected with a cell scraper (Carl-Roth) and collected in 15ml Falcon tube (BD Falcon). The cell suspensions were centrifuged at 1400 rpm for 4 min, the supernatant was sucked off and the cell pellet was resuspended in 1ml ice-cold lysis buffer supplemented with PMSF, Na_3VO_4 and protease inhibitor cocktail tablets. The lysed cell solution was transferred to a new 1.5ml tube, centrifuged at 12000 rpm at 4°C for 15 min to remove cell debris. Finally the supernatant containing the proteins was transferred to a new tube and the samples were stored at -80°C or immediately used for co-immunoprecipitation.

7.4.2 Co-Immunoprecipitation (CoIP)

4x Laemmli sample buffer

250 mM	Tris-Cl (pH 6.8)
8%	SDS
20%	β-mercaptoethanol
43,4%	glycerol
	bromphenol blue

1x Running buffer

25 mM	Tris-base
192 mM	glycine
0.1%	SDS

The frozen cell lysates were thawed and 30µl were aliquoted for the whole cell lysate (WCL) immunoblot. 300µl lysate were filled up with lysis buffer to reach a volume of 1ml and were used for CoIP assays.

The CoIP consists basically of three parts: preclear, immunoprecipitation and washing steps. For the preclear 20µl Protein A Sepharose CL 4B beads (Sigma) per sample were washed twice with 1ml lysis buffer. After each wash step the beads

were centrifuged at 8000 rpm for 1 min at 4°C to the bottom of the tube. For efficient pipetting of the beads the pipette tips were cut off. For the IP 10µl of anti-Flag beads and 10µl Protein A Sepharose CL 4B beads were mixed together for each sample and washed the same way as the preclear beads.

20µl washed Protein A Sepharose CL 4B beads were added to the lysate and the samples rotated for 1 hour at 4°C. Afterwards the beads were spun down at 8000 rpm for 1 min at 4°C. The supernatant was used for the IP step and the beads were discarded. 20µl of washed IP beads were added to the precleared lysate and rotated at 4°C overnight.

On the next day the washing steps were performed. These steps are necessary to remove unbound proteins that would otherwise falsify the results. The beads/lysate solution from the IP step was spun down at 8000 rpm for 1 min at 4°C and 500µl fresh prepared lysis buffer was added. This step was performed twice. Then two further washing steps were performed but this time the lysis buffer contained additional 150 mM NaCl to increase the stringency of the washing. This step was performed twice as well. After the last washing step all lysis buffer was removed and 40µl 1x Laemmli sample buffer was added to the beads.

At this step the WCL samples were included again and 10µl 4x Laemmli sample buffer was added. All the samples were boiled at 95°C for 5 min and spun down. The cooked lysate was either stored at -20°C or directly used for SDS-PAGE.

7.4.3 SDS polyacrylamide gel electrophoresis (SDS-PAGE)

The WCL and IP samples with the added Laemmli buffer were boiled at 95°C for 5 min to denature the proteins and the beads were spun down before loading the supernatant on the gel. The proteins were separated on a gel (10% resolving gel, 5% stacking gel) and 1x Running buffer was used. The gel was run at 200V for 50 min. To determine the size of the loaded proteins 7µl of PageRuler prestained protein ladder (Thermo Scientific #26616) was used as a marker.

<u>10% resolving gel (10ml for 1 gel)</u>

4 ml	dH_2O
3.3 ml	30% acrylamide mix (Carl-Roth)
2.5 ml	1.5 M Tris (pH 8.8)

0.1 ml	10% SDS
0.1 ml	10% APS
0.004 ml	TEMED

5% stacking gel (4ml for 1 gel)

2.7 ml	dH_2O
0.67 ml	30% acrylamide mix
0.5 ml	1 M Tris (pH 6.8)
0.04 ml	10% SDS
0.04 ml	10% APS
0.004 ml	TEMED

7.4.4 Western Blot

1x Transfer buffer (10l)

37.88g	Tris base
180.25g	glycine
	dH_2O

Stripping buffer

62.5 mM	Tris-HCl (pH 7.5)
100 mM	β-mercaptoethanol
2%	SDS
	dH_2O

1x Tris-buffered saline (TBST)

50 mM	Tris-HCl (pH 8)
100 mM	NaCl
0.1%	Tween-20
	dH_2O

After separation the proteins were electro-blotted by wet transfer on a PVDF membrane (BioRad) for 2 hours at constant 200mA in transfer buffer at 4°C.

Subsequently, the blot was blocked overnight at 4°C in TBST with 5% non-fat dried milk (Fixmilk Instant).

On the next day the membranes were incubated with the primary antibodies (Table 3), diluted in TBST with 5% non-fat dried milk, for 1 hour at room temperature and then washed five times (30s, 15 min, 5 min, 5 min, 5min) with TBST. Then the membranes were incubated with the HRP-coupled secondary antibodies (Table 4), diluted in 5% non-fat dried milk for 1 hour at room temperature and finally washed five times with TBST. Amersham ECL Plus Western Blotting Detection System (GE Healthcare) was used for development of the immunoblots and imagined with Fujifilm LAS-4000. For detection of total protein the membranes were stripped with stripping buffer for 15 min at 60°C and again incubated with first and secondary antibodies.

Table 3. Primary antibodies for Western blotting

Name	recognizes	Clone	Host	Company	Dilution
Anti-Myc Tag	Myc-Tag	4A6	mouse	Millipore	1:2500
ANTI-FLAG®M2	Flag-Tag	M2	mouse	Sigma	1:5000
PATZ1 antibody	MAZR	H-300	rabbit	Santa Cruz	1:1000
Anti-β-actin	β-actin	AC-74	mouse	Sigma	1:5000

Table 4. Secondary antibodies for Western blotting

Name	Type	Company	Product code	Dilution
Peroxidase-conjugated Affinipure Goat Anti-Mouse IgG	polyclonal	Jackson ImmunoResearch	115-035-003	1:5000
Peroxidase-conjugated Affinipure Goat Anti-Rabbit IgG	polyclonal	Jackson ImmunoResearch	111-035-003	1:5000

7.5 Mice

All mice used in the experiments were backcrossed on a C57BL/6 background and bred at the animal facility of the Institute of Immunology at the Medical University of Vienna according to the institutional guidelines. Cells were isolated from mice 6 to 8 weeks of age.

Mazr$^{f/f}$*Thpok*$^{+/GFP}$ and *Mazr*$^{f/f}$*Thpok*$^{+/GFP}$*Lck*-Cre mice

To study the role of MAZR in regulating ThPOK expression MAZR-deficient mice were used. Because Mazr-/- mice on a C57BL/6 background are embryonic lethal our group generated mice that harbor a conditional floxed *Mazr* allele (Abramova et al. 2013). To monitor *Thpok* expression ThPOK/GFP knock-in allele was introduced (Setoguchi et al. 2008). The *Mazr*$^{f/f}$*Thpok*$^{+/GFP}$ mice were crossed with *Lck*-Cre transgenic mice where Cre recombinase is expressed under control of the *Lck* promoter, enabling thymocyte-specific excision of loxP-flanked sequences (Lee et al. 2001).

Mazr$^{f/f}$*Runx3*$^{+/+}$*Thpok*$^{+/GFP}$, *Mazr*$^{f/f}$*Runx3*$^{+/+}$*Thpok*$^{+/GFP}$CD4-Cre, *Mazr*$^{f/f}$*Runx3*$^{mt/mt}$ *Thpok*$^{+/GFP}$ and *Mazr*$^{f/f}$*Runx3*$^{mt/mt}$*Thpok*$^{+/GFP}$CD4-Cre mice

To study the synergistic role of MAZR and Runx complexes in regulating *Thpok* expression double knockout mice with a ThPOK/GFP knock-in allele were generated. *Mazr*$^{f/f}$ mice were crossed with mice containing a mutant form of Runx3 (Runx3$^{mt/mt}$) that lacks the VWRPY motif necessary for the repressive function of Runx3 (Yarmus et al. 2006). These double mutant mice were crossed with CD4-Cre transgenic mice where the Cre recombinase is expressed from the double-positive thymocyte stage on (Lee et al. 2001).

7.6 $CD8^+$ T cell isolation

PBS/FBS/EDTA (P/F/E) solution

2%	FBS
1 mM	EDTA
	PBS

sterile filtration, stored at 4°C

ACK lysis buffer

9 volumes	0,83% NH_4Cl
1 volume	Tris (pH 7.65)

sterile filtration

$CD8^+$ T cells were isolated from spleens and lymphnodes of wild-type and knockout mice that were humanly sacrificed by cervical dislocation. The mice were sprayed with alcohol to avoid contamination in further steps. For the isolation 6 well plates with a cell strainer and 3ml P/F/E in each well were prepared on ice. From now on all steps were performed on ice. The spleens and lymphnodes were homogenized by pushing the plunge of a syringe against the cell strainer and the single cell suspension was pipetted into a 15ml Falcon. The cell strainer was rinsed with 1ml P/F/E to remove remaining cells and they were collected into the same Falcon tube. This suspension was centrifuged at 1600 rpm for 4 min at 4°C and then the supernatant was removed. To remove erythrocytes the cell pellet was resuspended in 1ml ACK lysis buffer and vortexed afterwards. After 2 min incubation at room temperature the lysis was stopped with 5-fold volume of P/F/E and formed precipitates were removed. The cells were pelleted by centrifugation at 1200 rpm for 5 min at 4°C and the supernatant was removed. In the meantime the antibody mix for the negative depletion was prepared. The negative depletion is necessary to remove all immune cells except $CD8^+$ T cells. The antibody mix was prepared in P/F/E and for one mouse 400µl were needed. The concentrations of the biotinylated antibodies used for negative depletion are shown in Table 5.

Table 5. Negative depletion antibodies

Name	recognizes	Clone	Company	final conc.	diluted in
Biotin Rat Anti-Mouse Ly-6G and Ly6C	granulocytes	RB6-8C5	BD Pharmingen™	4 µg/ml	P/F/E
Biotin Rat Anti-Mouse CD45R/B220	B cells	RA3-6B2	BD Pharmingen™	4 µg/ml	P/F/E
Biotin Rat Anti-Mouse TER-119/Erythroid cells	erythrocytes	-	BD Pharmingen™	1 µg/ml	P/F/E
Biotin Mouse Anti-Mouse NK-1.1	NK cells	PK136	BD Pharmingen™	1 µg/ml	P/F/E
Biotin Hamster Anti-Mouse CD11c	dendritic cells	HL3	BD Pharmingen™	1 µg/ml	P/F/E
Biotin Rat Anti-Mouse CD11b	macrophages	M1/70	BD Pharmingen™	1 µg/ml	P/F/E
Biotin Rat Anti-Mouse CD4	helper T cells	RM4-5	BD Pharmingen™	3 µg/ml	P/F/E

The antibody mix was pipetted on the cell pellet, resuspended and incubated 30-60 min on ice. Then the suspension was washed with 10-fold volume of P/F/E and centrifuged at 1600 rpm for 5 min at 4°C.

Magnetic activated cell sorting (MACS) was used to separate $CD8^+$ T cells from other immune cells. Therefore Streptavidin Particles Plus-DM (BD IMag™) (200µl/mouse) were pipetted on the cell pellet, vortexed and incubated on ice for exactly 15 min. Then 3ml of P/F/E were pipetted to the beads and this solution was poured into a 14ml polypropylene tube (BD Falcon) and put on a magnet. After 6 min the solution cleared and was then pipetted into a new polypropylene tube. In the meantime the tube was kept on ice. The remaining beads were washed of the wall with 1ml P/F/E and this tube was placed on the magnet again. After further 6 min the cleared solution was pipetted into the tube on ice and put on the magnet. 6 min later the cleared solution containing only $CD8^+$ T cells was transferred into a 15ml Falcon and left on ice until the cells were put in culture.

$CD8^+$ T cells were plated with a density of 0.25-0.5 million cells/ml/well on coated 48 well plates and cultured in RPMI-1640 medium supplemented with 20U human interleukin 2 (h-IL2)/ml. At this step the medium of transfected phoenix cells was changed to 6ml RPMI-1640 per plate.

7.7 Retroviral-mediated gene transfer into $CD8^+$ T cells

7.7.1 Calcium phosphate transfection of Phoenix cells

Phoenix cells were treated like HEK293T cells regarding thawing, splitting and culturing. One day before transfection they were seeded out with a density of $3.5x10^6$ cells per 10cm cell culture plate.

14µg of plasmids containing the MAZR constructs and 7µg of the helper Ψ (Psi) plasmid were transfected by using the calcium phosphate method. After six hours the medium was changed. On the same day 48 well plates were coated with anti-CD3 and anti-CD28 antibodies (Table 6) and incubated overnight at 4°C.

Table 6. Coating antibodies

Name	Clone	Company	final concentration	diluted in
Purified NA/LE Hamster Anti-Mouse CD3ε	145-2C11	BD Pharmingen™	2µg/ml	PBS
Purified NA/LE Hamster Anti-Mouse CD28	37.51	BD Pharmingen™	2µg/ml	PBS

7.7.2 Retroviral transduction

48 hours after transfection of the phoenix cells the virus titer in the medium is high enough to continue with retroviral transduction. The viral supernatant was filtered with a syringe, through a 0.45µm filter and mixed with polybrene (final concentration 10µg/ml) to increase the retroviral gene transfer efficiency (Davis et al. 2002).

The medium of the $CD8^+$ T cells was removed and the viral supernatant was slowly pipetted on the cells. The plate was centrifuged with 1800 rpm at 32°C for 2 hours with low acceleration and low deceleration of speed. After spin infection the viral supernatant was aspirated and 1ml of fresh RPMI-1640 supplemented with 20U hIL2/ml was added and the cells were put back into the incubator.

24 hours later the cells were split 1:2 and the medium was changed to RPMI-1640 supplemented with 100U hIL-2/ml. From now on every second day the cells were split depending on their density and on day 5 cells were analyzed by flow cytometry.

7.8 Flow cytometry

On day 5 350µl of cells were harvested into FACS tubes and centrifuged at 1600 rpm at 4°C for 4 min. The supernatant was removed and 50µl antibody mix (Table 7) was added to each tube. The cells were incubated with the antibody mix for 30 min and afterwards washed with 500µl P/F/E. The suspension was centrifuged at 1600 rpm at 4°C for 4 min and almost all of the supernatant was removed, only 50µl were left in the tube. The stained cells were kept on 4°C dark until FACS data were acquired on LSRFortessa (BD Biosciences). The FACS data were analyzed using FlowJo software (Tree Star, Inc).

Table 7. Staining scheme for FACS

Name	visualizes	Clone	Company	Dilution	diluted in
GFP knock-in allele	ThPOK	-	-	-	-
mCherry reporter	MAZR	-	-	-	-
Anti-Mouse CD4 PE-Cy7	CD4	RM4-5	ebioscience	1:400	P/F/E
Anti-Mouse CD8α APC	CD8α	53-6.7	ebioscience	1:200	P/F/E
Anti-Mouse TCRβ APC-eFluor780	TCRβ	H57-597	ebioscience	1:400	P/F/E

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9 List of Figures and Tables

10 Nomenclature

ACK	ammonium-chloride-potassium
AML1	acute myeloid leukemia 1 protein
APS	ammonium persulfate
BSA	bovine serum albumin
$CaCl_2$	calcium chloride
ChIP	chromatin immunopreciptitation
CMV	cytomegalovirus
CO_2	carbon dioxide
dH_2O	deionized water
DMEM	Dulbecco`s Modified Eagle Medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTPs	deoxynucleoside triphosphate
EDTA	ethylenediaminetetraacetic acid
FBS	fetal bovine serum
fgf4	fibroblast growth factor 4 gene locus
HCl	hydrogen chloride
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	horseradish peroxidase
IRES	internal ribosome entry site
KCl	potassium chloride
LTR	long terminal repeat
$MgCl_2$	magnesium chloride
Na_2HPO_4	disodium phosphate
Na_3VO_4	sodium orthovanadate
NaCl	sodium chloride
NaF	sodium fluoride
N-CoR	nuclear receptor corepressor
NH_4Cl	ammonium chloride
NK	natural killer
NP-40	nonyl phenoxypolyethoxylethanol
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PMSF	phenylmethanesulfonylfluoride
PVDF	polyvinyl difluoride
RNF4	RING finger protein 4
rpm	revolutions per minute
RPMI	Roswell Park Memorial Institute
SDS	sodium dodecyl sulfate
SMRT	silencing mediator for retinoid and thyroid receptors
Tcra	T cell receptor alpha gene locus
Tcrb	T cell receptor beta gene locus
TEMED	tetramethylethylenediamine

11 Curriculum Vitae

Curriculum Vitae
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