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Diplomarbeit

Molecular studies on the transcriptional regulation of the *Cd8ab* gene loci during T cell development

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Abstract

CD8 coreceptor expression is tightly regulated during T cell development and is coupled with the functional phenotype of cytotoxic T cells. Understanding the transcriptional control mechanisms of *Cd8* gene regulation will therefore provide new insights into cell fate decisions of DP thymocytes towards CD4/CD8 lineage differentiation.

Five developmental stage- and subset-specific *Cd8* enhancers were identified in previous studies. However, some important aspects remain to be adressed. It is still unclear where the core enhancer regions are located within the known *Cd8* enhancers. Since the *Cd8* *cis*-regulatory elements were initially mapped on large genomic fragments (4-8kb), it is essential to identify the core enhancer regions within those fragments in order to use them as molecular baits for the isolation of binding factors. Furthermore, it is not known which *cis*-regulatory elements direct the expression of CD8 in CD8$\alpha\alpha^+$ dendritic cells. Important *Cd8* *cis*-regulatory elements are expected to be evolutionary conserved, thus a cross-species comparison of the *Cd8a* and *Cd8b* genomic loci to search for evolutionary conserved regions (ECR) was performed. This approach led to the identification of 10 ECRs ranging from 200bp to 500bp. In vivo transgenic reporter expression assays and in vitro transient luciferase assays were performed to test ECR containing fragments for enhancer activity. These studies led to the identification of the $E8_I$ core enhancer and we could also identify a novel enhancer element ($E8_{VI}$) that drives expression of *Cd8* in CD8$\alpha\alpha^+$ dendritic cells.

Previous findings of our laboratory demonstrated epigenetic control of the *Cd8* gene loci. We further identified that the transcription factor MAZR acts as a negative regulator of CD8 expression. In order to learn more about the role of MAZR, we generated MAZR deficient mice and observed an altered CD4/CD8 ratio in the thymus of MAZR knock-out mice as well as the development of class I-restricted CD4 lineage cells. Furthermore we observed an increase of Th-POK expression in MAZR -/- $CD4^+CD8^{lo}$ thymocytes, indicating that MAZR might regulate the expression of Th-POK. By using ChIP experiments we showed that MAZR is binding to the Th-POK silencer region RBS-1, indicating a molecular mechanism where MAZR is recruited to the Th-POK silencer region RBS-1 and in turn contributes to the appropriate repression of Th-POK expression.

Zusammenfassung

Die Expression des CD8 Co-Rezeptormoleküls wird während der Entwicklung von T Zellen streng reguliert und ist an den funktionellen Phänotyp von zytotoxischen T Zellen gekoppelt. In vorangegangenen Experimenten konnten fünf *Cd8* spezifische Enhancer Elemente identifiziert werden. Nachdem diese *Cd8* *cis*-regulatorischen Elemente ursprünglich als große genomsiche Abschnitte (4-8kb) nachgewiesen wurden, ist es von großer Bedeutung die Kern-Enhancer Regionen zu identifizieren um diese als molekulare Köder für die Isolation von Bindungsfaktoren zu nutzen. Von wichtigen *Cd8* *cis*-regulatorische Elemente wird angenommen, dass sie evolutionär konserviert sind und daher wurde am *Cd8* Lokus ein Sequenzvergleich verschiedener Spezies durchgeführt um evolutionär konservierte Regionen (ECRs) nachzuweisen. Dieses Experiment führte zur Identifizierung von 10 ECRs in der Größe zwischen 200bp und 500bp. Um die gefundenen Fragmente auf Ihre Enhancerfunktion zu testen wurden in vivo transgene Reporter Expressions Analysen zusammen mit in vitro Luciferase Analysen durchgeführt. Mit Hilfe dieser Experimente konnten wir sowohl den $E8_I$ Kern-Enhancer identifizieren sowie ein neues Enhancer-Element ($E8_{VI}$), welches die *Cd8* Expression in CD8$\alpha\alpha^+$ dendritischen Zellen reguliert, lokalisieren.

Bisherige Experimente in unserem Labor zeigten auch, dass der *Cd8* Lokus epigenetisch reguliert wird. Weiters konnten wir den Transkriptionsfaktor MAZR als negativen Regulator des CD8 Co-Rezeptormoleküls identifizieren. Um mehr über MAZR zu lernen, generierten wir MAZR knock out Mäuse welche zu einem veränderten CD4/CD8 Verhältnis im Thmyus führten und die Ausbildung von MHC-Klasse I selektierten CD4 Zellen zeigte. Des Weiteren konnten wir einen Anstieg der Th-POK Expression in MAZR -/- $CD4^+CD8^{lo}$ Thymocyten feststellen, dass einen Hinweis darauf gibt, dass MAZR in der Regulierung der Th-POK Expression beteilig ist. Durch ChIP-Experimente konnten wir zeigen dass MAZR an die Th-POK silencer Region RBS-1 bindet. Daher schlagen wir einen molekularen Mechanismus vor, bei dem MAZR an die Th-POK silencer Region RBS-1 rekrutiert wird um die Th-POK Expression zu unterdrücken.

1 Introduction

1.1 T cell development: starting from scratch

Haematopoietic stem cell (HSC)-derived lymphocyte progenitors migrate from the bone marrow through the blood stream to the thymus, the primary site of T cell lymphopoiesis.[1] Different developmental stages of thmyocytes can be distinguished on the basis of the expression of CD4 and CD8 co-receptor molecules.[2] After common lymphoid progenitors enter the cortico-medullary junction of the thymus they become double negative (DN, $CD4^-CD8^-$) cells (Figure 1.1). This stage can be further subdivided into four stages of differentiation regarding CD44 and CD25 expression: DN1 ($CD44^+CD25^-$), DN2 ($CD44^+CD25^+$), DN3 ($CD44^-CD25^+$), and DN4 ($CD44^-CD25^-$).[3] As cells progress through the DN2 to DN4 stages, they express the pre-T-cell receptor (TCR) which is composed of the non-rearranging pre-Tα-chain and a rearranged TCRβ-chain.[4] Successful pre-TCR expression leads to substantial cell proliferation during the DN3/DN4 stage and the cells become $CD8^+$ immature single-positive (ISP) cells. After expressing CD4 co-receptor molecules the cells are termed double positive (DP, $CD4^+CD8^+$) thymocytes and the replacement of the pre-TCR α-chain with a newly rearranged TCR α-chain results in the expression of a mature $\alpha\beta$-TCR. The $\alpha\beta$-$TCR^+CD4^+CD8^+$(DP) thymocytes then interact with cortical epithelial cells that express a high density of MHC molecules associated with self-peptides.[5] Most DP thymocytes (90%) express TCRs that bind with a low avidity to MHC-self peptide complexes and the intracellular signals that are required to sustain viability are not generated, and the cells undergo apoptosis (death by neglect). A small fraction of immature T cells (5%) sustains TCRs that bind with a high avidity to MHC-self peptide complexes. These thymocytes are potentially autoreactive and therefore are eliminated by rapid apoptotic death (nega-

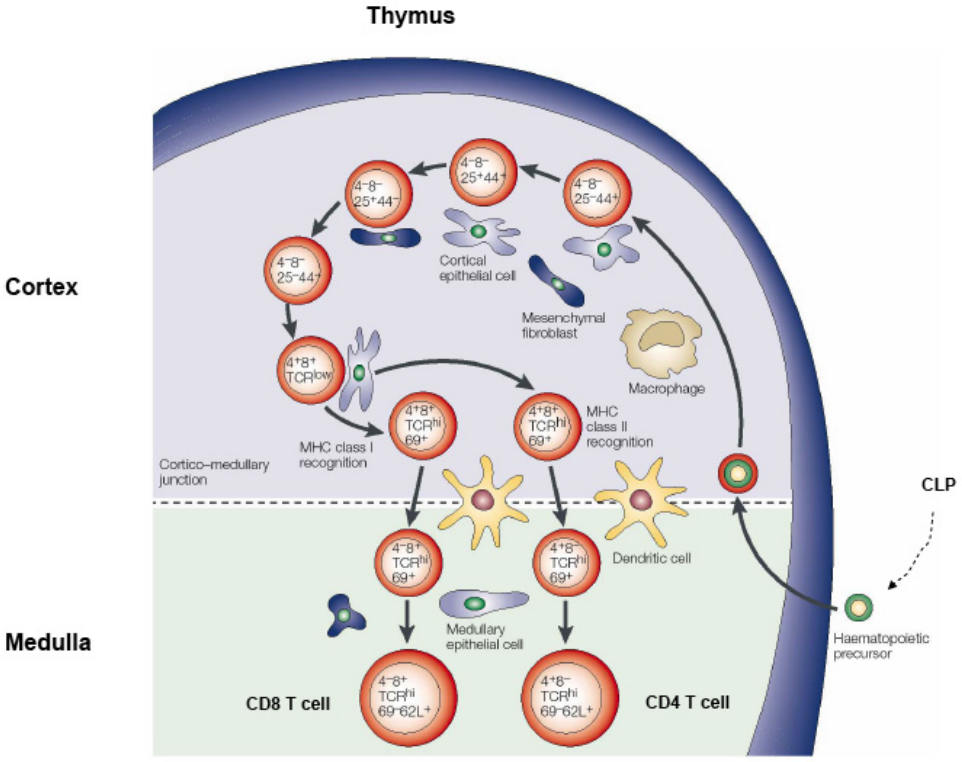

Figure 1.1: T cell development in the thymus. The thymic architecture is organized into cortex and medulla, each of which is characterized by the presence of thymocyte precursors at defined maturation stages and particular stromal cell types.[6] Thymocyte differentiation is characterized by the cell surface markers CD4, CD8, CD44, and CD25 as well as the status of the T-cell receptor.[3,7,8]

tive selection). Cells that express TCRs that recognize self-ligands and generate signals with an intermediate avidity for MHC-self peptide complexes undergo a process called positive selection.[5] Thymocytes that express TCRs that bind self-peptide–MHC-class-I complexes become $CD8^+$ cytotoxic T cells, whereas those that express TCRs that bind self-peptide–MHC-class-II ligands become $CD4^+$ helper T cells.[5] The positive selected naive $\alpha\beta$ T cells emigrate after 4 – 5 days through a "conveyor belt" mechanism, where the oldest thymocytes leave the thymus first to migrate to peripheral lymphoid sites.[9]

1.2 The murine *Cd8ab* gene complex

1.2.1 *Cis*-regulatory elements

Thymus-derived cytotoxic T cells (CTLs) express the cell surface glycoprotein CD8 that consists of CD8α and CD8β heterodimers. Nonconventional T cells such as intraepithelial lymphocytes (IELs) from the gut,[10,11] a subset of human natural killer (NK)[12] cells, and CD8$^+$ dendritic cells (DCs)[13] express only CD8$\alpha\alpha$ homodimers. Mice with a disrupted *Cd8a* gene can not express the CD8 heterodimer and therefore lack mature CTLs, indicating that CD8 is crucial for thymocyte development.[14] Removal of the cytoplasmic domain of the CD8β-chain[15] or deletion of the *Cd8b* gene[16] resulting in disruption of positive and negative selection in thymocytes. This expression pattern of the *Cd8a* and *Cd8b* genes, which are linked at a distance of 36 kb on mouse chromosome 6[17] , indicates that their expression must be both coordinately and independently regulated. Therefore, *cis*-regulatory elements that are specific for CD8α or CD8β might exist.[18]
A DNase I Hypersensitivity (DH) analysis of an 80 kb region in the murine *Cd8* loci identified four clusters (I, II, III, IV) of DH sites, whereas three of them are thymocyte specific (II, III, IV).[19] Transgenic mice carrying the identified *Cd8ab* genomic locus, expressed the transgenic CD8 in a developmental stage- and lineage-specific manner, showing that the main *cis*-regulatory elements are active in this 80kb region.[2,19] Additional transgenic reporter-expression assays led to the identification of at least five genomic fragments that are involved in the regulation of *Cd8a* and *Cd8b* gene expression.[19–29] These results revealed a complex regulatory network that involves the developmental stage-, subset-, and lineage-specific use of multiple closely linked *cis*-regulatory elements during T-cell development[2] (Figure 1.2).
The results from the transgenic reporter expression assays raised the question of why so many different, and probably partially redundant, *cis*-acting elements are required for the regulation of CD8 expression. One could argue that embedded within the endogenous *Cd8* gene loci, the enhancers which show activity in the same subsets, such as E8$_{\mathrm{I}}$ and E8$_{\mathrm{II}}$ in CD8 SP and mature CD8$^-$ T cells and E8$_{\mathrm{II}}$ and E8$_{\mathrm{III}}$ in DP thymocytes, are together required for high-level expression of CD8α and CD8β.[22,24,28] When analyzed

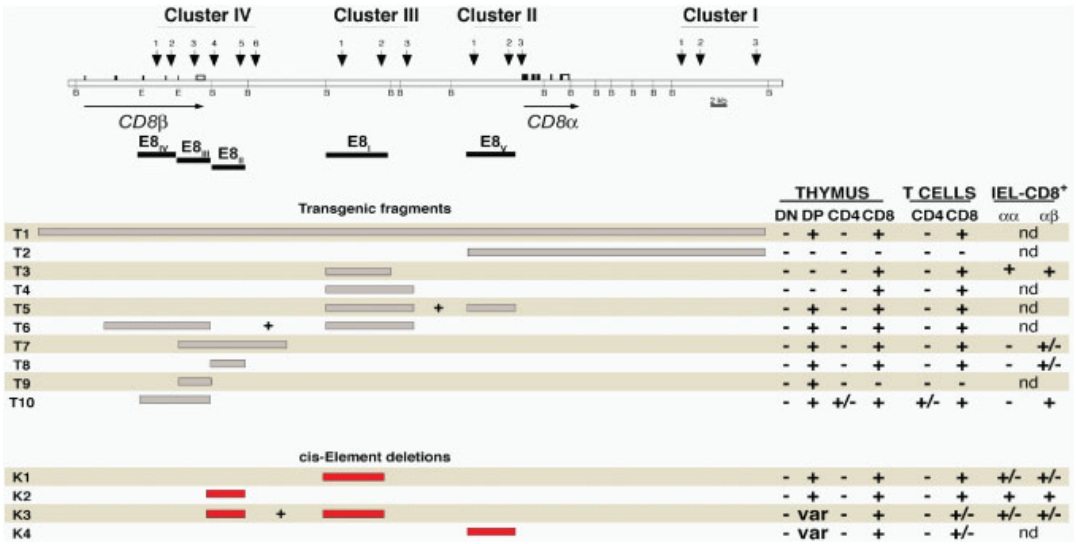

Figure 1.2: Mouse *Cd8a* and *Cd8b* genomic loci: transgenic constructs and enhancer deletions. *Upper panel* – genomic organization of the *Cd8ab* gene complex including the localization of DNase I hypersensitivity clusters (CI-CIV). Individual DH sites are indicated by vertical arrows. Horizontal arrows indicate the position and the transcriptional orientation of the *Cd8a* and *Cd8b* genes. Thick bars below the diagram represent genomic fragments used to define the *Cd8* *cis*-regulatory elements ($E8_I$-$E8_V$). All BamHI (B), but only relevant EcoRI (E) restriction sites are shown. *Middle panel* – grey bars represent the key genomic fragments used to identify Cd8 enhancers. the table on the left indicates the developmental stage-, and lineage- specific activity of individual transgenic constructs. For intraepithelial lymphocytes (IELs) only expression in the CD8$\alpha\alpha$ lineage is shown. *Lower panel* – red bars represent mouse germline deletions of CD8 genomic fragments. The effect on the CD8 expression is shown on the right. +, normal expression; +/-, reduced expression; var, variegated expression; nd, not determined.[2]

in transgenic reporter assays isolated from their genomic context, they therefore display similar activities. Another possible reason for the requirement of multiple *cis*-elements is if some of the enhancers direct specifically the expression of either CD8α or CD8β. Based on the results from transgenic reporter expression assays, one would have predicted that individual deletions of enhancers should (at least) lead to a reduction of CD8 expression in the subsets in which the enhancers show activity, and that combined deletions of enhancers with similar T cell subset activities should lead to a more dramatic

reduction of CD8 expression compared to individual deletions. Dependent on whether some of the enhancers are specific for CD8α or CD8β, one may also expect a reduction in the expression of only one of these co-receptor genes.

1.2.2 Single and combinatorial deletions of *cis*-regulatory elements

Because it is difficult to experimentally test these possibilities in transgenic reporter assays, a systematic deletional analysis of the various enhancers and *cis*-elements in the mouse germ line has been initiated (Figure 1.2). Initial studies focused on the following *cis*-regulatory regions: Enhancer $E8_I$ (overlapping with cluster III, DH sites 1 and 2 also termed CIII-1,2 which is active as a transgene in $CD8^+$ SP T cells and IELs; enhancer $E8_{II}$ (CIV-4,5) directed expression in DP and $CD8^+$ SP T cells; $E8_{III}$ (CIV-3) was active only in immature DP thymocytes; $E8_{IV}$ (CIV-1,2) displayed low expression in $CD4^+$ SP and mature T cells and directed expression in DP and CD8 lineage cells; and DH cluster II($E8_V$; CII-1,2) which by itself has no enhancer activity, but in combination with cluster III directs expression in mature $CD8^+$ T-cell lineage and in DP thymocytes.[22,24] Mice homozygous for deletion of $E8_I$ had a 3-5 fold reduction in Cd8α expression levels on IEL indicating that $E8_I$ is the major regulatory element for Cd8α expression on IEL.[22] However, $E8_I$ deficient mice showed a normal expression pattern of *Cd8a* and *Cd8b* genes in thymic-derived T cells. Mice with targeted deletions in $E8_{II}$ had normal expression of Cd8α and Cd8β in conventional T cells and IELs.[28] These results together showed that individual deletions of either $E8_I$ or $E8_{II}$ did not cause any change in the expression pattern of CD8 in the various T cell subsets. Previous experiments with transgenic mice showed that $E8_I$ and $E8_{II}$ individually direct expression of Cd8α and Cd8β in mature $CD8^+$ T cells and so it might be possible that these two enhancers can compensate for each other.[2] In addition, in $E8_{II}$-deficient mice, $E8_{III}$ or $E8_V$ (in combination with other regulatory regions) could similarly compensate to direct CD8 expression in DP thymocytes.[22,24]

In order to address the question if enhancers can compensate for each other, $E8_I$ and $E8_{II}$ double-deficient mice were generated. In contrast to individual deletions, the deletion of both enhancers had a major effect on the expression of CD8 during thymocyte

development, but not in mature CD8 lineage cells.[28] A population of $CD8^{-}CD4^{+}$ (CD8 negative DP) thymocytes were observed that could not be distinguished from DP thymocytes by surface marker expression and functional phenotype. The presence of CD8 negative DP thymocytes indicates that the *Cd8a* and *Cd8b* gene expression is variegated in DP thymocytes.[28] Strikingly, a similar population of CD8 negative DP thymocytes were identified by deletion of $E8_V$ or by altering the activity of either the Ikaros family of transcriptional regulators or the BAF (Brahma-related gene/Brahma-associated factor) chromatin remodelling complex.[29–31] These results indicate that the CD8 enhancers may also function as recruitment sites for factors involved in chromatin remodeling to activate the *Cd8a* and *Cd8a* gene loci during the DN to DP transition in T cell development.[2]

In more recent studies we have compared the epigenetic state of the Cd8α and Cd8β gene loci in sorted CD8 negative DP and DP thymocytes isolated from $E8_I$,$E8_{II}$ doubly deficient mice.[32] Chromatin immunoprecipitation (ChIP) assays and DNA methylation analysis revealed that CD8 negative DP thymocytes have epigenetic modifications at the *Cd8a* and *Cd8b* gene complex that correspond to an "off" state of chromatin. Variegation of CD8 expression in DP thymocytes could be partially reverted by intercrossing $E8_I$,$E8_{II}$ doubly deficient mice with conditional DNA methyltransferase 1 (Dnmt1)-deficient mice,[33] further indicating a partial "epigenetic block" of CD8 expression due to the absence of *cis*-regulatory elements. Taken together, these studies link *Cd8* enhancer function with the epigenetic regulation of CD8 expression. Surprisingly, expression of CD8 on those cells in which it was up-regulated (i.e. DP thymocytes and peripheral $CD8^{+}$ T cells) was only slightly reduced in the mutant mice. Thus, it is possible that other (novel) Cd8 enhancers than $E8_I$, $E8_{II}$, and $E8_V$ are required to maintain expression of CD8 once the expression has been initiated, or that epigenetic mechanism renders mature T cells independent of further function of these *cis*-regulatory elements.

1.3 Transcription factors in the epigenetic control of the *Cd8ab* gene loci

1.3.1 MAZR: A negative regulator of CD8 expression in DN T cells

MAZR (Myc-associated Zinc finger related factor) was originally isolated as an interaction partner of Bach2, a transcriptional repressor in B cells and neuronal cells.[34] The MAZR transcription factor (641aa) is encoded by the Zfp278 gene and contains a N-terminal BTB-POZ motif and seven C_2H_2 zinc fingers (Figure 1.3). The BTB (broad complex, tramtrack, bric a brac) domain or POZ (poxvirus zinc finger) domain, is a protein–protein interaction motif that was first identified in the fruit fly drosophila melanogaster and is present in about 200 genes in mammalian genomes.[35,36] Proteins with a BTB domain are involved in a number of cellular processes such as protein targeting for ubiquitination, cytoskeleton dynamics, and transcriptional regulation.[35] Among all proteins containing a BTB domain a large group of proteins have also C_2H_2 zinc finger (ZF, Krüppel-like C2H2 type) motifs that are important for DNA binding.[37] BTB-ZF proteins can either form oligomers or heteromers with other BTB-ZF proteins and have been mainly associated with transcriptional repression.[34,38,39] Until now, six BTB-ZF have been identified to play a role in T cell development and/or T cell function: MAZR,[32] Th-POK (cKrox),[40,41] PLZF,[42] Bcl6,[39] ROG (PLZP/TZFP/FAZF),[43] and BAZF (Bcl6b).[44]

MAZR is expressed in many tissues, including fetal liver, bone marrow and thymus and was first isolated in a yeast one-hybrid screen in a search for Cd8 enhancer $E8_{II}$ binding proteins.[32] We have previously shown that MAZR is highly expressed in DN thymocytes where it is interacting with N-CoR corepressor complexes, but as thymocyte development progress MAZR expression is down modulated and almost no expression of MAZR could be found in peripheral T cells. Forced expression of MAZR affect the activation of CD8 expression in a proportion of DP thymocytes and resulting in a variegated expression of CD8.[32] Previous studies have described that transcription factors together with chromatin-modifying complexes can alter the epigenetic pattern at the closely linked *Cd8a* and *Cd8b* gene loci to a transcriptional "on" state and thereby facilitating expres-

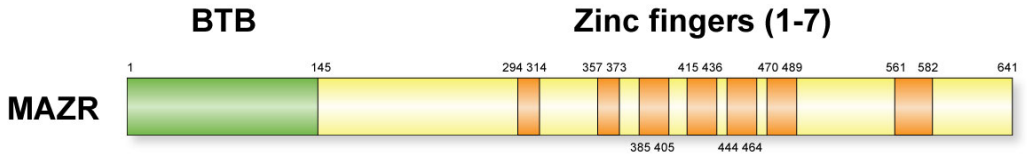

Figure 1.3: MAZR (Myc-associated Zinc finger related factor). The transcription factor MAZR with its N-terminal BTB domain (green) and seven C_2H_2 zinc fingers (orange). The alternative variant of MAZR lacks the last zinc finger.

sion.[31,45] In contrast, it might be that cells with variegated CD8 expression keeping the local chromatin at the Cd8 gene loci in a transcriptional "off " state at the DN stage. Recently our group generated MAZR deficient mice and we observed an altered CD4/CD8 ratio in the thymus as well as redirection of class I-restricted thymocytes into the $CD4^+$ lineage (Sakaguchi et al. manuscript in preparation). Furthermore, we detected a three fold increase of Th-POK expression in MAZR -/- $CD4^+CD8^{lo}$ thymocytes, indicating that MAZR might regulate Th-POK expression. Taken our results together we propose a model were MAZR is a critical negative regulator of CD8 expression[32] and is also involved in CD4/CD8 lineage commitment.

1.3.2 Th-POK: A master regulator of CD4 T cells

The BTB-ZF factor Th-POK (T-helper-inducing POZ/Kruppel like factor; also termed cKrox; encoded by the Zbtb7b gene) contains a N-terminal BTB domain and four C_2H_2 zinc finger motifs.[40] Recently, a spontaneous mouse mutant (helper-deficient (HD) mouse) has been reported in which the commitment to the $CD4^+$ helper T cell lineage was diminished without any defects in positive selection.[46,47] The mice with single recessive HD mutation showed a normal CD4 and MHC-II expression as well as a proximal intact TCR signaling.[46,47] It has been found that a single base pair mutation in the second zinc finger of Th-POK redirects all class-II restricted thymocytes to the $CD8^+$ cytotoxic lineage.[40] Strikingly, transgenic expression of Th-POK in the thymus lead to redirection of all positively selected thymocytes to differentiate into $CD4^+$ T cells, even

those with MHC class I-restricted TCRs.[40,41] When the mutated (HD) form of Th-POK or a truncated version lacking the BTB domain was overexpressed in thymocytes no effect was observed.[40,41] This indicates that the HD mutation plays a crucial role in the ZF DNA-binding domain for the proper function of Th-POK during CD4 lineage development. Therefore Th-POK is termed as master regulator of CD4-lineage choice and $CD4^+$ T-cell differentiation[48] (Figure 5.1). Th-POK influences both *Cd4* and *Cd8* gene transcription. At the *Cd4* locus, Th-POK maintains *Cd4* gene transcription by preventing factors, such as RUNX3, from silencing[49] , whereas *Cd8* gene expression is decreased by downregulating the enhancer activity of $E8_I$.[50]

1.3.3 RUNX: A negative regulator of Th-POK

RUNX (AML, acute myeloid leukaemia) proteins belong to the Runt-domain family of transcription factors that share similar structural organization as well as conserved DNA-binding sites.[51] The three mammalian RUNX genes (RUNX1, RUNX2, and RUNX3) are known to encode for transcription factors that bind DNA as part of the core-binding factor (CBF) complex. Together with the CBFβ cofactor, the CBF complex activates and represses transcription of key regulators of survival, growth and differentiation pathways.[52] In the thymus, RUNX1 is mainly expressed in DN thymocytes whereas RUNX3 expression was observed in $CD8^+$ SP thymocytes.[49] Previous studies showed that both RUNX1 and RUNX3 bind to the *Cd4* silencer element and silence *Cd4* gene transcription and therefore indicating a role of RUNX proteins in CD4/CD8 lineage choice.[49]
In thymocyte development, RUNX3 is first expressed in $CD4^+CD8^{lo}$ intermediate thymocytes and is upregulated in $CD8^+$ T cells.[53,54] During T cell differentiation, RUNX3 binds to the *Cd4* silencer element to silence *Cd4* gene transcription,[49] and at the same time RUNX3 is binding to $E8_I$ at the Cd8 gene loci and reinitiates Cd8 gene transcription.[53] Recent data showed that RUNX proteins can also bind to Zbtb7b, the gene that encode for Th-POK, and shut down Th-POK expression.[55] Thus, RUNX proteins promote $Cd8^+$ T cell differentiation in a variety of possible control mechanisms.

1.4 Aims of the master thesis

As outlined in the introduction, five developmental stage- and subset-specific-*Cd8* enhancers (E_I - E_V) have been identified and extensively studied in transgenic reporter expression assays and by deletional analysis in the mouse germ line. These results revealed important insights into epigenetic and transcriptional control mechanisms during T cell development. Nevertheless, several important aspects remain to be adressed. The core enhancer regions for most of the known Cd8 enhancers have not been described. This would be important to use the Cd8 enhancers as molecular baits to identify binding factors. In addition, it is not known which *cis*-regulatory elements direct the expression of CD8 in CD8$\alpha\alpha^+$ dendritic cells. Previous findings in our laboratory demonstrated epigenetic control of the *Cd8ab* gene loci by a complex network of transcription factors. Nevertheless, it is still poorly understood how these transcription factors are regulated during T cell development. Therefore, we revisited the *Cd8ab* gene complex to gain more insight into the complex regulated *Cd8* gene loci by using a combination of bioinformatic and molecular biology approaches.

Specific aim 1 was to identify the $E8_I$ core enhancer region and novel *Cd8* enhancer elements within the *Cd8ab* gene complex. Since the identified enhancer elements are 4-8 kb in length it is crucial to find the core enhancers in order to use them as molecular baits for the identification of binding factors. It is expected that important *Cd8 cis*-regulatory elements are evolutionary conserved. Thus we performed a cross-species comparison of the *Cd8a* and *Cd8b* genomic loci to identify evolutionary conserved regions (ECRs). Furthermore we wanted to test the identified ECRs for enhancer activity and thus we used a combination of an in vitro transient luciferase reporter assay and an in vivo transgenic reporter assay.

Specific aim 2 was to identify the role of MAZR in the regulation of CD4/CD8 lineage commitment. We have previously shown that the transcription factor MAZR is a negative regulator of CD8 expression. In ongoing studies in the laboratory we observed an altered CD4/CD8 ratio in the thymus of MAZR deficient mice. Furthermore, we detected a three fold increase of Th-POK expression in MAZR -/- $CD4^{+}CD8^{lo}$ thymocytes, indicating that MAZR might regulate Th-POK expression. In order to investigate that in more detail we performed Chromatin-Immunoprecipitation (ChIP) experiments of the Th-POK silencer region RBS-1 as well as Co-Immunoprecipitation of MAZR and RUNX3.

2 Results

2.1 *Cis*-regulatory elements in the *Cd8ab* gene locus

2.1.1 Evolutionary conserved regions (ECRs) in the *Cd8ab* gene locus

It has been shown that important cis-regulatory elements are evolutionary conserved.[56] Therefore we asked the question, if this is also the case for the *Cd8ab* gene complex and performed a bioinformatic cross-species comparison. By using the algorithm MULAN (http://mulan.dcode.org/)[57] we compared the sequences of the *Cd8a* and *Cd8b* gene loci of mouse, human, rat and dog, respectively. The evolutionary conserved regions (ECRs) were defined as regions having more than 70% homology over a stretch of at least 100bp. 10 ECRs in the range of 200-500bp that matched the selection criteria were identified (Figure 2.1). Strikingly, ECR-2, -3, -6, -7, -8 and -10 overlapped with previously identified enhancer elements whereas ECR-3 and -8 also correlates with DNase I Hypersensitivity sites (HS) at the *Cd8ab* gene complex. To our surprise we also identified ECR-1, -4, -5, and -9 as novel elements that have never been described in the literature before. In order to test the ECRs for enhancer activity we performed both in vitro transient luciferase assays of ECR-4, -5 and -9 and in vivo transgenic reporter assays of ECR-4, -5, and -8.

2.1.2 Identification of the $E8_I$ core enhancer region

Recent studies identified a 1.6kb HindIII fragment[22] that shows the same enhancer activity as the original 7.6kb enhancer $E8_I$ fragment.[21] By using the MULAN algorithm we identified ECR-8, a 200bp region that overlaps with the 1.6kb HindIII fragment and the hypersensitivity site CIII-2 of enhancer $E8_I$ (Figure 2.1). Since the enhancer elements are 4-8 kb in length it is crucial to find the core enhancers in order to use them as molecular

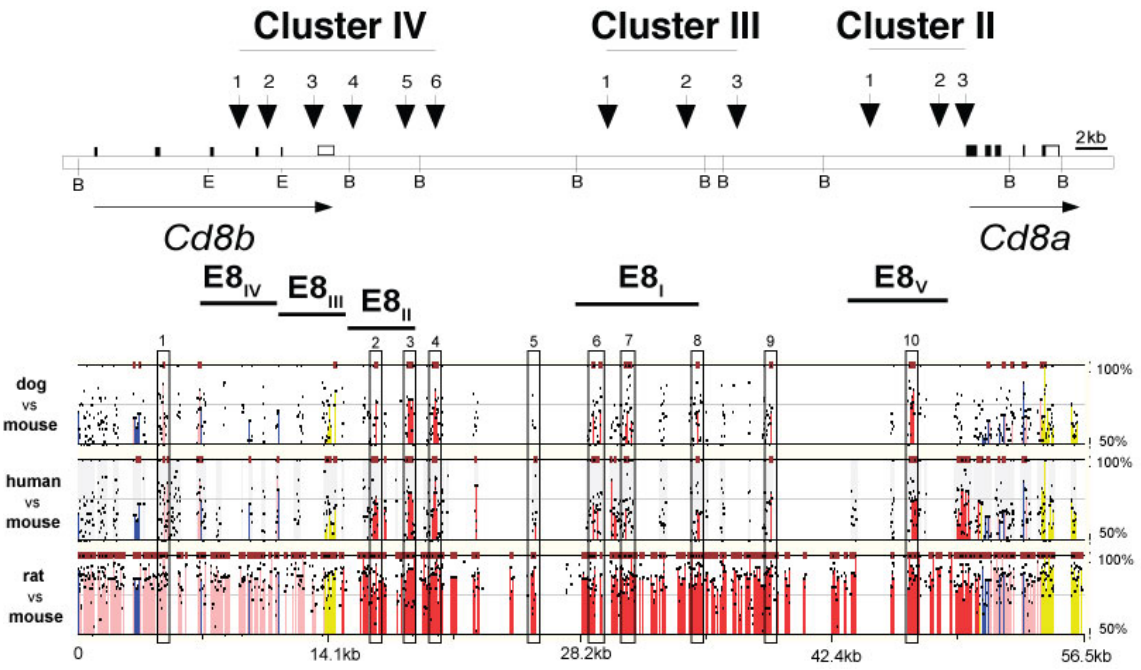

Figure 2.1: Genomic organization of the *Cd8a* and *Cd8b* genomic loci. *Upper panel* - Schematic map of the *Cd8a* and *Cd8b* gene loci. Horizontal arrows indicate the transcriptional orientation of the *Cd8a* and *Cd8b* genes. Vertical arrows indicate the localization of DNase I hypersensitivity (DH) sites that constitute clusters II, III, and IV. The horizontal thick bars below DH sites indicate genomic fragments used to define enhancers $E8_I$, $E8_{II}$, $E8_{III}$, $E8_{IV}$, and $E8_V$, respectively. All BamHI (B), but only relevant EcoRI (E) sites are shown. *Lower panel* - Evolutionary conserved non-coding regions within the *Cd8a* and *Cd8b* genomic loci. Pairwise comparison of mouse with dog, human, and rat, respectively. ECRs (1-10) are indicated by the open boxes. Intergenomic sequences are shown in red. Degree of homology within exon sequences is indicated in blue, UTRs in yellow, and intronic sequences in pink.

bait. Therefore, we cloned a small genomic fragment (539bp) containing ECR-8 into a basic hCD2 reporter construct (Figure 4.2). We used a larger fragment than the 200bp ECR-8 since there was also a high-degree of homology in the 300bp downstream of ECR-8. Transgenic mice were generated and positive founders were tested. The analysis of ECR-8 transgenic mice showed that ECR-8 directs hCD2 expression in mature $CD8^+$ SP thymocytes, peripheral $CD8^+$ T cells and in $CD8\alpha\alpha^+$ IEL (Figure 2.2). The same

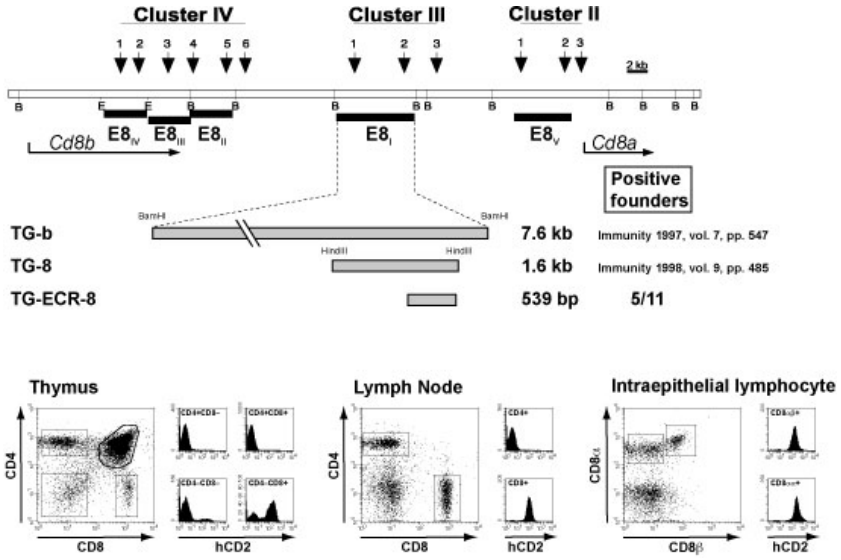

Figure 2.2: Schematic map of the genomic fragments used for in vivo transgenic reporter gene expression assays and Flow cytometric analysis of ECR-8 transgenic mice. *Upper panel* - Schematic map of the *Cd8a* and *Cd8b* gene loci and of the genomic fragments used for the generation of transgenic mice (TG-b, -8, -ECR-8). The length of the genomic transgenic inserts and the number of transgenic founders expressing hCD2 among total founders are shown at the right. *Lower panel* - Flow cytometric analysis of ECR-8 transgenic mice. Dot blots show CD4 vs. CD8 expression pattern in thymocytes (left panel) and lymph nodes (middle panel) of ECR-8 transgenic mice. Human CD2 expression is shown in the histograms next to the dot plots. Left panel shows CD8α vs. CD8β expression in IEL. Histograms show hCD2 expression in CD8$\alpha\alpha^+$ and CD8$\alpha\beta^+$ IEL.

expression pattern was observed with the original large $E8_I$ fragment, indicating that the genomic fragment that contains ECR-8 represents the $E8_I$ core enhancer.

2.1.3 Identification of the novel *Cd8-cis* regulatory element $E8_{VI}$

To test whether ECR-4 (246bp), -5 (115bp) and -9 (119bp) have enhancer activity, we performed in vitro luciferase assays. ECR-4 is located downstream of Cd8 enhancer $E8_{II}$ and overlaps with the HS site CIV-6 (Figure 2.1). In contrast, ECR-5 and ECR-9 do not overlap with known HS sites. The three evolutionary conserved regions were inserted in the basic Cd8 promoter luciferase reporter construct (Figure 4.3) and were transiently transfected either into AKR1.1 cells (DP T cell line) or 1200M cells ($CD8^+$ T cell lymphoma cell line) (Figure 2.3). Neither ECR-4 nor ECR-5 and -9 could drive the expression of the luciferase gene in vitro, indicating that these fragments have no enhancer activity at least in these particular cell lines.

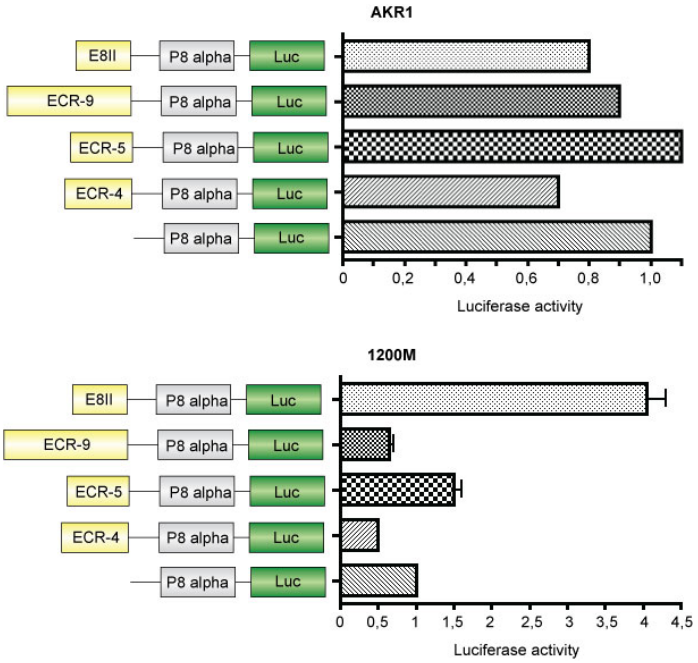

Figure 2.3: Luciferase assay of ECR-4, ECR-5 and ECR-9 in AKR1.1 and 1200M cells. *Upper panel* - Reporter constructs are shown on the left side and were transfected into AKR1.1 cells. Normalized luciferase expression is shown by bars on the right side. *Lower panel* - The reporter constructs were transfected into 1200M cells. The empty vector was used as the background control whereas the $E8_{II}$ construct was used as a positive control.Data are representative of two independent experiments.

Previous studies of the $E8_I$ core enhancer did not reveal any enhancer activity in vitro (Sakaguchi et al., unplublished), although the element was active in transgenic reporter assays in vivo. Therefore it remained possible that ECR-4 and -9 still show enhancer activity in vivo. ECR-4 and ECR-9 were inserted in the basic Cd8 promoter transgenic reporter construct (Figure 4.2) and transgenic mice were generated. For ECR-9, five human CD2 transgenic founders were obtained. Human CD2 expression could not be detected in thymocytes and peripheral T cells of ECR-9 founders (data not shown), indicating that ECR-9 is not a cis-regulatory element that contains enhancer activity (at least if tested individually). In contrast, hCD2 expression was detected in peripheral $CD8^+$ but not in $CD4^+$ T cells in 2 out of 11 ECR-4 transgenic founders (data not shown). This indicated that ECR-4 has enhancer activity specifically in $CD8^+$ T cells. To test whether ECR-4 is also active during T cell development, transgenic ECR-4 lines were analyzed in more detail. This revealed that ECR-4 was active in mature CD8 SP thymocytes and in CD8 T cells but not in immature DP cells. Furthermore we could not detect hCD2 expression in both CD8$\alpha\alpha^+$ or CD8$\alpha\beta^+$ IEL (Figure 2.4). Another population that express CD8 are $CD11c^+$ CD8$\alpha\alpha^+$ dendritic cells (DCs). Surprisingly, we observed that ECR-4 can drive hCD2 expression in this specific cell population (Figure 2.5).

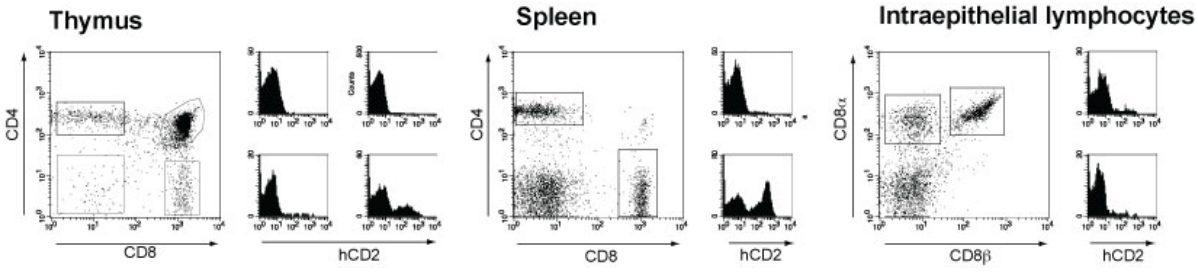

Figure 2.4: Flow cytometric analysis of ECR-4 transgenic mice. Dot blots show CD4 vs. CD8 expression in thymocytes (left panel) and splenocytes (middle panel) of ECR-4 transgenic mice. Human CD2 expression is shown in the histograms next to the dot plots. Left panel shows CD8α vs. CD8β expression in IEL. Histograms show hCD2 expression in CD8$\alpha\alpha^+$ and CD8$\alpha\beta^+$ IEL.

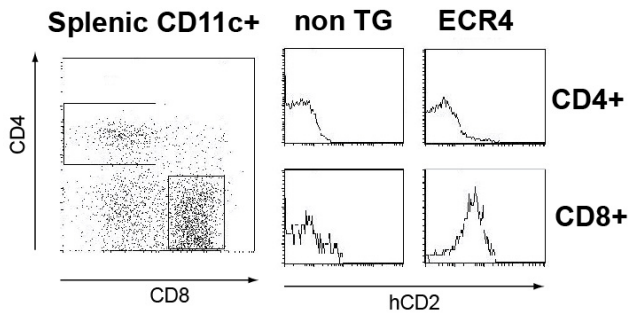

Figure 2.5: Flow cytometric analysis of splenic CD11c$^+$ DC in ECR-4 transgenic mice. Dot blots show CD4 vs. CD8 expression pattern on Cd11c-gated splenocytes. Human CD2 expression in non transgenic controls and in ECR-4 transgenic mice is shown in histograms next to the dot plot.

2.2 The role of MAZR in regulation of CD4/CD8 lineage commitment

2.2.1 MAZR binds to Th-POK silencer region RBS-1

Recently, we have shown that MAZR is a negative regulator of CD8 expression and an altered CD4/CD8 ratio in the thymus has been observed in MAZR deficient mice. Furthermore we observed that there is redirection of class I-restricted thymocytes into the $CD4^+$ lineage (Sakaguchi et al., unpublished). Real time PCR analysis of MAZR knockout mice revealed a three fold increase of Th-POK expression in MAZR -/- $CD4^+$ $Cd8^{lo}$ thymocytes, indicating that MAZR might regulate Th-POK expression.

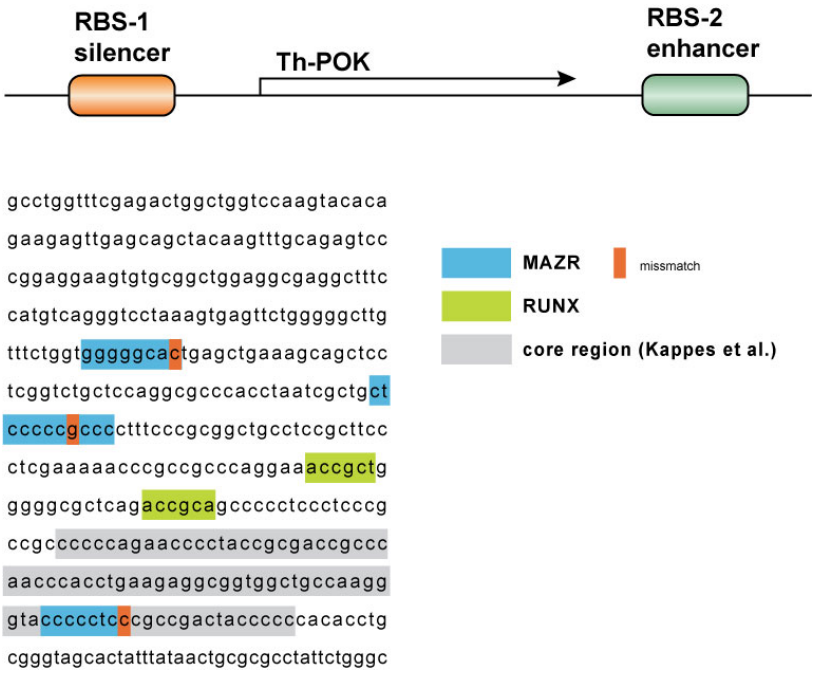

Figure 2.6: Overview of the Th-POK gene locus. *Upper panel* - Th-POK gene locus including RBS-1 silencer element (orange) and the RBS-2 enhancer element (green). Horizontal arrow indicates the transcriptional start of the Th-POK gene. *Lower panel* - Sequence of the Th-POK silencer region RBS-1. The Th-POK core region is indicated in grey.[58] Potential MAZR binding sites are indicated in blue. Red boxes signal missmatches compared to known MAZR binding sites. RUNX sites are shown in green.

The expression of Th-POK is repressed during differentiation of MHC class I-restricted thymocytes toward $CD8^+$ cytotoxic T cells by the Th-POK silencer. Therefore we analysed the Th-POK silencer region RBS-1 for potential MAZR binding sites. We detected three MAZR and two RUNX binding sites within the Th-POK silencer region RBS-1 (Figure 2.6).

To test if MAZR binds to the Th-POK silencer region RBS-1, ChIP experiments were performed. Therefore we used MAZR serum for the Immunoprecipitation followed by a PCR analysis of the RBS-1 sequence. Strikingly, we identified MAZR binding to the Th-POK silencer region RBS-1 in MAZR +/+ mice. As a negative control we used MAZR -/- mice (Figure 2.7).

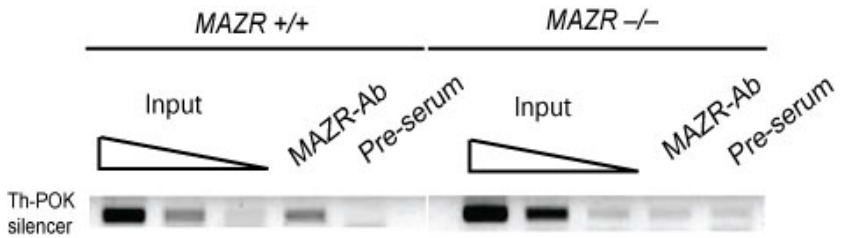

Figure 2.7: Chromatin-Immunoprecipitation of MAZR on the Th-POK silencer RBS-1. MAZR +/+ (left) and MAZR -/- (right) mice were used for the ChIP experiment. The input dilutions were as follows; undiluted, 1:5, 1:25. MAZR Preserum was used as negative control.

2.2.2 RUNX3 binds to Th-POK silencer region RBS-1 independent of MAZR

Recent data showed that RUNX proteins can also bind to Th-POK silencer region RBS-1 and shut down Th-POK expression.[55] As mentioned before RUNX complexes are composed of two subunits: the DNA binding domain and the unique non-DNA binding CBFβ protein. We performed a CBFβ2 ChIP experiment to test if RUNX3 binding to the Th-POK silencer region RBS-1 is dependent on the expression of MAZR. We could detect binding of RUNX3 to the Th-POK RBS-1 locus in wildtpye and MAZR deficient mice. This result indicates that MAZR is not necessary for proper RUNX3 binding (Figure 2.8).

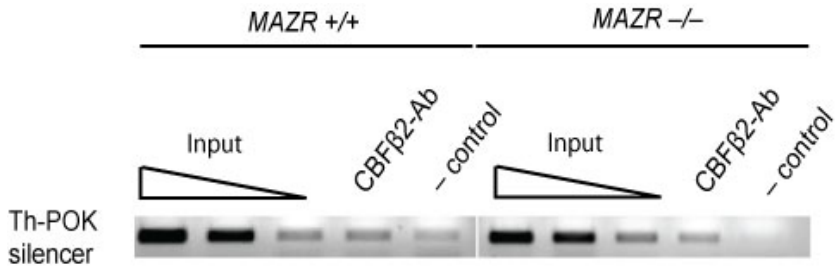

Figure 2.8: Chromatin-Immunoprecipitation of CBFβ2 on the Th-POK silencer RBS-1. MAZR +/+ (left) and MAZR -/- (right) mice were used for the ChIP experiment. The input dilutions were as follows; undiluted, 1:5, 1:25. dH_2O was used as negative control.

2.2.3 MAZR does not interact with the RUNX3

After we have identified MAZR binding next to the RUNX sites within the Th-POK silencer region RBS-1 we wanted to determine whether MAZR can directly interact with RUNX3. Therefore we overexpressed MAZR and myc-tagged RUNX3 in 293T cells and performed Co-Immunoprecipitation experiments. These experiments revealed that MAZR does not interact with RUNX3 (Figure 2.9).

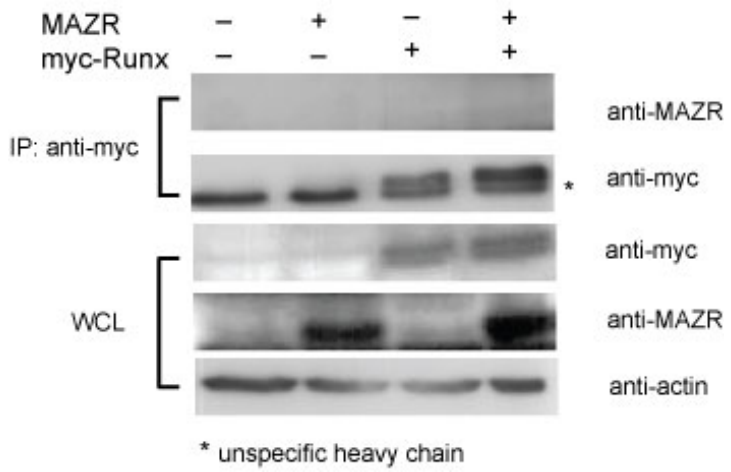

Figure 2.9: Co-Immunoprecipitation of MAZR and RUNX3. Immunoblots of Immunoprecipitated (IP) samples and whole cell lysates (WCL) are shown. We used 293T cells where either none, MAZR or myc-tagged RUNX3 or both proteins were over-expressed.Whole cell lysates were used as a control for the expression of the proteins. Actin was used as a control of protein loading.

3 Discussion

3.1 The core enhancer region of $E8_I$ and a novel *Cd8* enhancer element within the *Cd8ab* gene complex.

The expression of CD8 is coupled to the phenotype of cytotoxic T cells. Recent studies identified five developmental stage-, subset-, and lineage-specific *Cd8* enhancers that are involved in the regulation of *Cd8a* and *Cd8b* gene expression. A better understanding how the *cis*-regulatory elements are functioning and the knowledge of the factors that bind to the *cis*-regions within the enhancers will therefore provide molecular details of lineage commitment and T cell development.

In order to study the murine *Cd8ab* gene complex, we used a combination of bioinformatic and molecular biology approaches. Since the *Cd8 cis*-regulatory elements were initially mapped on large genomic fragments (4-8kb),[21,22,28] it is important as a first step to identify the core enhancer regions within those fragments in order to use them as molecular baits for the isolation of binding factors. Important *Cd8 cis*-regulatory elements are expected to be evolutionary conserved, thus we performed a cross-species comparison of the *Cd8a* and *Cd8b* genomic loci to search for evolutionary conserved regions (ECR). First, we focused on enhancer $E8_I$ that is localized initially on a 7.6 kb BamHI genomic fragment.[21] This approach led to the identification of a 539bp fragment (ECR-8) which overlapped with the hypersensitivity site CIII-2 of enhancer $E8_I$. Several studies showed that DH sites indicate the location of promoters and enhancers of genes, including those *cis*-elements that control expression of the *Cd4*, *Cd8a* and *Cd8b* loci.[2,19] Thus, indicating that the DNA sequence of the ECR-8 region has open chromatin status and is accessible for binding of transcription factors. By performing in vivo transgene

reporter assays with a reporter construct containing the 539-bp genomic fragment we identified enhancer activity in $CD8^+$ SP thymocytes, peripheral $CD8^+$ T cells and in $CD8\alpha\alpha^+$ IEL. The same expression pattern was observed with the original large $E8_I$ fragment, indicating that the genomic fragment that contains ECR-8 represents the $E8_I$ core enhancer.

In addition to the five known CD8 enhancer elements, we focused also on the question if we can identify novel enhancer elements within the *Cd8ab* gene loci. Therefore we used the cross-sequence comparison to search for novel ECRs and identified three ECRs (ECR-4, -5 and -9) that mapped outside known enhancer elements (Figure 2.1). We performed in vivo transgenic reporter assays of ECR-4 and ECR-9. By using a reporter construct containing the 246bp (ECR-4) fragment we identified enhancer activity of hCD2 in mature CD8 SP thymocytes and in CD8 T cells but not in immature DP cells (Figure 2.4). The new *cis*-regulatory element was named $E8_{VI}$ (Figure 3.1). Transgenic founders which had the ECR-9 fragment integrated into their genome did not show any hCD2 expression.

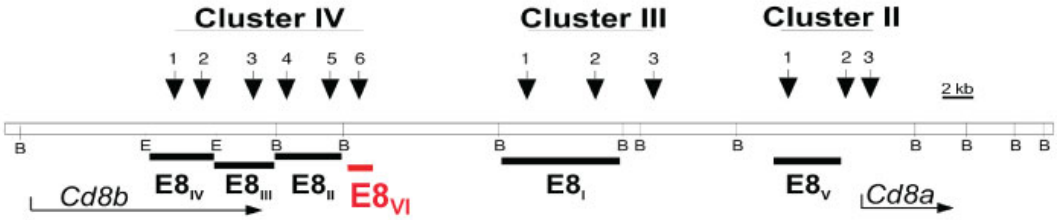

Figure 3.1: Updated map of enhancer regions located within the *Cd8a* and *Cd8b* genomic loci. Schematic map of the *Cd8a* and *Cd8b* gene loci including the novel enhancer element $E8_{VI}$ in red.

By performing in vitro luciferase assays of ECR-4, -5 and -9 we were not able to detect luciferase activity in 1200M and AKR1.1 cells (Figure 2.3). In previous studies of the $E8_I$ enhancer we also could not reveal any enhancer activity in vitro (Sakaguchi et al.,

unpublished), although the element was active in transgenic reporter assays in vivo. One reason could be that factors binding to these ECRs are not present in these cell lines and therefore they can not express the reporter gene. Another reason might be that *cis*-elements are only active when they are present in defined chromatin structures. A developmental programming of the *cis*-elements might be necessary to obtain enhancer activity.

Another population that express CD8 are CD11c$^+$ CD8$\alpha\alpha^+$ DCs.[13] However, neither E8$_{\mathrm{I}}$ nor E8$_{\mathrm{II}}$ was able to direct the expression of a reporter gene in dendritic cells (Ellmeier et al., unpublished). A more precise analysis of the positive E8$_{\mathrm{VI}}$ transgenic founders revealed expression of hCD2 also in CD8$\alpha\alpha^+$ DCs and E8$_{\mathrm{VI}}$ is to our knowledge the first Cd8 *cis*-element that is described to be active in CD8$\alpha\alpha^+$ DCs (Figure 3.1).

Comparing E8$_{\mathrm{I}}$ and E8$_{\mathrm{VI}}$ shows that they have similar activities in cytotoxic T cells (Figure 3.2). Targeted deletion of E8$_{\mathrm{I}}$ resulted in a dramatic loss of CD8α expression in IEL, but the expression in conventional T cells was normal in the absence of E8$_{\mathrm{I}}$. Based on our preliminary results, it is conceivable that E8$_{\mathrm{VI}}$ may compensate for loss of E8$_{\mathrm{I}}$.

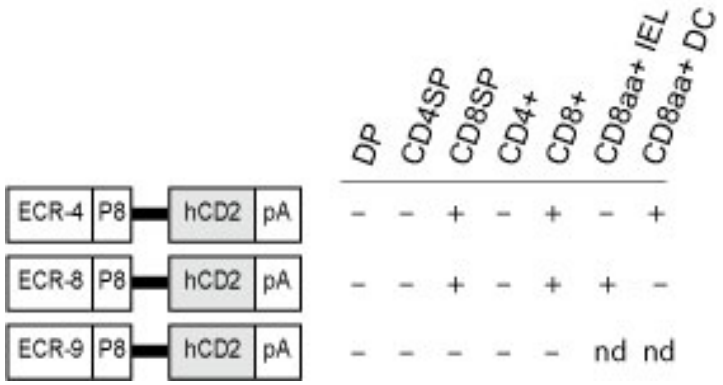

Figure 3.2: Map of transgenic ECR constructs and the summary of their activity in various T cell lineages. Left panel - Schematic map of the transgenic constructs. Right panel - Expression pattern of the hCD2 reporter gene. nd: not determined

In the future, we will further dissect ECR-4 ($E8_{VI}$) and ECR-8 ($E8_{I}$). For both ECRs, that will be achieved with additional transgenic reporter assays, since we could not identify a T cell line in which $E8_{I}$ (Ellmeier et al., unpublished) or $E8_{VI}$ displays in vitro enhancer activity . In addition we will generate ECR-4-deficient mice and the CD8 expression pattern in conventional T cells, IELs and DCs of $E8_{VI}$-knock out mice will then be analyzed in detail.

Conclusion

In this study, we have demonstrated that a bioinformatics approach is a powerful method for complementing biological assays in the identification of *cis*-regulatory elements within the genomic *Cd8ab* loci. By employing this approach we identified the core enhancer region of $E8_{I}$ which is the first step in the isolation and identification of transcription factors that regulate CD8 expression. Furthermore we identified a novel *Cd8* enhancer element $E8_{VI}$ that is active in mature CD8 SP thymocytes, in CD8 T cells as well as in $CD11c^{+}$ $CD8\alpha\alpha^{+}$ DCs. $E8_{VI}$ is to our knowledge the first *Cd8 cis*-element that is active in $CD8\alpha\alpha^{+}$ DCs.

3.2 The transcription factors MAZR, Th-POK and RUNX

Previous studies have identified several transcription factors that are involved in the regulation of coreceptor gene expression and CD4/CD8 cell fate decisions. We recently demonstrated epigenetic control of the *Cd8a* and *Cd8b* gene loci by the transcription factor MAZR.[32] MAZR was isolated using a yeast one-hybrid screen in a search for *Cd8* enhancer $E8_{II}$ binding proteins and we showed that MAZR is a negative regulator of CD8 expression during the DN to DP transition of thymocyte development.[32] To analyse the function of MAZR in more detail, we recently generated MAZR deficient mice. An altered CD4/CD8 ratio in the thymus as well as the development of class I-restricted CD4 lineage cells in MAZR -/- mice was observed (Sakaguchi et al., paper in preparation). Real time PCR analysis of MAZR knockout mice also revealed a three fold increase of Th-POK expression in MAZR -/- $CD4^+$ $Cd8^{lo}$ thymocytes, indicating that MAZR might regulate Th-POK expression. Therefore we analysed the interaction of MAZR with other transcription factors such as Th-POK and RUNX that are known to regulate CD4/CD8 cell fate decision.[59] (Figure 3.3).

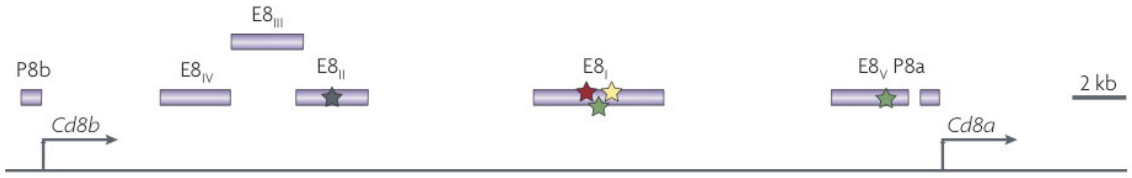

Figure 3.3: Regulation of *Cd8* gene expression. Expression of the *Cd8a* gene is controlled by the stage and lineage specific enhancer elements $E8_I$-$E8_V$. Transcription factors that bind to these regions include Ikaros[31] (green), RUNX[49] (yellow), MAZR[32](blue) and STAT5 (signal transducer and activator of transcription 5)[60] (red).[59]

Recent findings in our group indicate that the redirection in MAZR deficient mice was induced by de-repressed expression of Th-POK. One possible molecular mechanism would be that MAZR represses Th-POK expression via binding to the Th-POK silencer RBS-1. To test our hypothesis, we searched the Th-POK silencer region RBS-1 for potential MAZR binding sites and this lead to the identification of three MAZR target sequences. By performing ChIP experiments we detected binding of MAZR to the Th-POK silencer region RBS-1, and therefore providing a potential molecular mechanism.
Another transcription factor that is involved in CD4/CD8 lineage decision of DP thymocytes is RUNX. RUNX is a negative regulator of Th-POK and previous studies have shown that RUNX proteins bind to the Th-POK silencer region RBS-1. Therefore we asked the question if MAZR is necessary for proper binding of RUNX3 to the Th-POK silencer region RBS-1. ChIP experiments revealed that RUNX3 binding to the Th-POK silencer region RBS-1 is independent of MAZR. Another ChIP experiment in RUNX deficient mice showed, that the binding of MAZR to the Th-POK silencer region RBS-1 is weaker compared to wildtype mice (unpublished data). These results indicate that MAZR is recruited to the Th-POK silencer region RBS-1 by the RUNX complex and in turn contributes to the appropriate repression of Th-POK expression in cooperation with the RUNX complex. Furthermore we could not detect a direct interaction between MAZR and RUNX3 by using co-immunoprecipitation assays (data not shown). One reason might be, that the in vitro conditions differ from those in vivo. Therefore we are currently testing whether we can detect the interaction in ex vivo isolated thymocytes.

To gain more insights into the role of MAZR in CD4/CD8 lineage decision, we will performed a DNA microarray analysis to compare the gene expression profile between MAZR +/+ and −/− mice at the DP stage of thymocytes. This experiment will help us to find new genes of transcription factors that might be involved in the regulation of MAZR during cell fate decision processes.

Conclusion

We have recently demonstrated epigenetic control of the *Cd8a* and *Cd8b* gene loci by the transcription factor MAZR.[32] In order to learn more about the role of MAZR, we generated MAZR deficient mice and observed an altered CD4/CD8 ratio in the thymus of MAZR knockout mice as well as the development of class I-restricted CD4 lineage cells indicating altered lineage differentiation. Furthermore we observed an increase of Th-POK expression in MAZR -/- $CD4^{+}CD8^{lo}$ thymocytes, indicating that MAZR might regulate the expression of Th-POK. In this study, we propose a molecular mechanism where MAZR is recruited to the Th-POK silencer region RBS-1 via RUNX and in turn contributes to the appropriate repression of Th-POK expression.

4 Materials and Methods

4.1 Cell lines and culture

4.1.1 1200M cell line

1200M cells were cultivated in RPMI 1640 (Gibco) supplemented with 10% foetal calf serum (Gibco) which was heat inactivated at 56°C for 40 min, 50μM ß-mercaptoethanol, 100 U/ml Penicillin, 0,3μg/ml L-Glutamine, and 100μg/ml Streptomycin.

BM cyclin was used for treatment of mycoplasma-contaminated 1200M cell cultures. After splitting the cells 1:20 in a new cell culture flask, BM cyclin 1 stock solution was added to reach a final concentration of 5μg/ml. 1200M cells were cultivated for 3 days, the medium was removed BM cyclin 2 was added to reach an end concentration of 2μg/ml. The cells were cultivated for 4 days and the treatment was repeated twice.

4.1.2 AKR1.1 cell line

AKR1.1 cells were cultivated in RPMI 1640 (Gibco) supplemented with 10% foetal calf serum (Gibco), 100 U/ml Penicillin, 0,3μg/ml L-Glutamine, and 100μg/ml Streptomycin.

4.1.3 293T cell line

293T cells were cultivated in DMEM (Gibco) supplemented with 10% foetal calf serum (Gibco), 100 U/ml Penicillin, 0,3μg/ml L-Glutamine, and 100μg/ml Streptomycin.

4.1.4 Cell stocks

Suspension cells were centrifuged at 3200 rpm for 3 min and resuspended in 90% FCS and 10% DMSO to reach a final concentration of $10x10^6$ cells/ml. Cryotubes were incubated in -80°C over night and finally stored in liquide nitrogen.

4.2 Bacterial Techniques

4.2.1 Bacteria LB-media (Luria/Miller)

25g of LB Media or 40g of LB Agar was resolved in 1l of deionized water and autoclaved at 125°C for 1h. After autoclaving, the media was left to cool down to 55°C. For selection of bacterial strains carrying plasmids with an ampicillin resistance gene, ampicillin was added to a final concentration of 100μg/ml before inoculation. For solid media plates, the LB Agar was poured into 10cm dishes, left at room temperature until the LB Agar became solid and were finally stored at 4°C.

4.2.2 Preperation of competent E.coli cells

Competent cells were prepared by inoculating 3ml LB medium with the DH5α strain. This culture was incubated by shaking at 200 rpm at 37°C over night. Next morning 100ml of LB medium was inoculated with 1ml of the over-night culture and incubated on a shaker at 37°C until the OD_{600} reached 0,3-0,6. Then the cells were centrifuged at 5000 rpm for 5 min. The cell pellet was resuspended in 10ml sterile filtrated TBS buffer (LB containing 10% Polyethylenglycol 3350, 5% Dimethylsulfoxid, 10mM $MgCl_2$, and 10mM $MgSO_4$). Then the cells were aliquoted in eppendorf tubes, snap frozen in liquide nitrogen and stored at -80°C.

4.2.3 Transformation

Transformation was performed by mixing 10μl ligation reaction with 100μl competent E.coli cells (DH5α), 80μl dH2O and 20μl KCM-Buffer (0,5M KCl, 0,15M $CaCl_2$ and 0,25M $MgCl_2$). After an incubation of 40 min on ice and a following incubation for 10 min at RT, bacterial cells were plated out on LB-Amp (100μg/μl) plates and incubated at 37°C, 0,5% CO2 overnight.

4.3 DNA Methodes

4.3.1 Restriction digest

Restriction digest was performed using 40 Units EcoRI restriction enzym (New England Biolabs #R0101s), 10-15μl DNA templates, and 10x buffer EcoRI (Fermentas #B12) in a volume of 50μl. The following incubation step was performed at 37°C for 2 h.

4.3.2 Ligation

Ligation of the PCR products was performed by mixing 50ng pGEM-T Easy vector (Promega #TM042, Figure 4.1) with a 4 molar access of PCR products, dH2O, 5μl 1x Rapid Ligation Buffer (Promega) and 3 Units T4 DNA Ligase (Promega) in a volume of 10μl. The ligation mix was incubated for 1,5 h at RT. DNA ligation was carried out with 30ng of the luciferase (WE310)- and transgenic vector (WE-191) and a 4 molar access of inserts as estimated from the gel in a volume of 10μl. The reactions were incubated with 3 Units T4 DNA Ligase (Promega) at 16°C for 3 h.

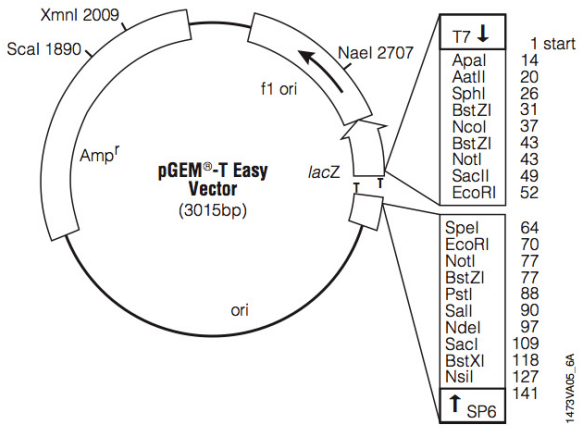

Figure 4.1: pGEM-T Easy Vector circle map

4.3.3 PCR reactions

Genomic DNA was isolated from C57BL/6 ES cells using phenol/chloroform precipitation. The evolutionary conserved regions (ECR-4, -5 and -9) were amplified with the following forward and reverse primers:

Primer	Sequence (5´to 3´)
ECR-4 forward	ATGAATTCGACAACTCAAGGCCAGCAC
ECR-4 reverse	ATGAATTCGGGGTGGGTGCTAGATGA
ECR-5 forward	ATGAATTCTTTTTCAATTCCTGTCCGTGT
ECR-5 reverse	ATGAATTCGCCTTGGAATCTTGATCCTC
ECR-9 forward	ATGAATTCTCCCACACCAGAAAAAGAAGA
ECR-9 reverse	ATGAATTCCCTAGGTTGTCCTTGAATTTACA

The underlined sequences indicating EcoRI restriction sites that were added on the 5´ end of the primer. ECR-4 has a length of 246bp and the PCR product was 497bp. ECR-5 is 115bp and after PCR amplificaton the product size was 228bp, whereas ECR-9

has a size of 119bp and after amplification it was 271bp.
The PCR reactions were performed on a PTC-200 Peltier Thermal cycler (MJ research) under the following conditions: 1x Taq Buffer, 0,1μM of each primer, 0,2mM dNTP mix, and DMSO in a volume of 25μl. For cloning purposes 2,5 U Taq DNA Polymerase (New England Biolabs #M0267L) was used. The PCR program was as follows: 96°C for 5 min, 39 cycles at 94°C for 30 sec, 60°C for 30 sec, and 72°C for 2 min; and 72°C for 7 min. The PCR products were seperated on an 1% agarose gel.

For ChIP experiments the following forward and reverse primer were used:

Primer	Sequence (5´to 3´)
RBS-1 forward	TGGTTTCGAGACTGGCTGGT
RBS-1 reverse	GACCGAGGAGCTGCTTTCAG

The PCR reactions were performed on a PTC-200 Peltier Thermal cycler (MJ research) under the following conditions: 1x Taq Buffer (5 Prime), 0,1μM of each primer, 0,2mM dNTP mix in a volume of 25μl. 2,5 U Taq DNA Polymerase (5 Prime) was used. The PCR program was as follows: 96°C for 5 min, 35 cycles at 94°C for 30 sec, 58°C for 30 sec, and 72°C for 40 sec; and 72°C for 2 min. The PCR products were seperated on an 1% agarose gel.

4.3.4 Agarose Gel Electrophoresis

DNA fragments were separated on 1% agarose gels using 1 x TAE buffer (40mM Tris-acetate, 1mM EDTA pH 8,3) as running and preparation buffer. 1-2 drops of ethidium-bromide solution (0,08%) were added to the agarose gel solution. Samples were loaded on the gel with 6x DNA loading Dye (10 mM Tris-HCl (pH 7.6), 0.03% bromophenol blue, 0.03% xylene cyanol FF, 60% glycerol, 60 mM EDTA; Fermentas #R0611). GenRuler DNA Ladder Mix (Fermentas #SM0333) was used as DNA size marker and the

gels were run at 100V. DNA bands were visualized with an UV illuminator and a Lumi-Imager (Boehringer Mannheim), respectively.

4.3.5 Gel purification (Wizard SV Gel clean-Up system, Promega)

The fragments were cut out and 10μl Membran binding solution (per 10mg gel) was added, vortexed and incubated at 55°C until the gel slice was completely dissolved. The samples were transfered to the SV Minicolumn, incubated for 1 min at RT and centrifuged at 16000 g for 1 min. 700μl Membran wash solution was added to the minicolumn and centrifuged at 16000 g for 1 min. Then 500μl Membran wash solution was added to the minicolumn and centrifuged at 16000 g for 5 min. Finally, the empty minicolumn was centrifuged at 16000 g for 1 min. DNA was eluated with 30μl dH_2O, incubated 1 min and centrifuged at 16000 g for 1 min. Samples were stored at -20°C.

4.3.6 Plasmid DNA Purification (Wizard Plus SV Miniprep DNA Purification System, Promega)

1 ml of overnight cultures was centrifuged at 10000 g for 5 min to pellet the bacteria. All centrifugation steps were done at room temperature. The cell pellet was resuspended in 250μl Cell Resuspension Solution. 250μl Cell Lysis Solution was added, the sample was mixed by inverting 4 times and incubated until the cell suspension clears. 10μl Alkaline Protease Solution was added, mixed by inverting the tube 4 times and incubated for 5 min. Next 350μl Wizard Plus SV Neutralization Solution was added and the sample was mixed 4 times. The bacterial lysate was centrifuged at 14000 g for 10 min. The lysate was transfered into the Spin Column and centrifuge at 14000 g for 1 min. 750μl Wash Solution was added and the sample was centrifuge at 14000 g for 1 min. 250μl Wash Solution was added and the sample was centrifuge at 14000 g for 1 min. To ensure removal of ethanol the column was centrifuged at 14000 g for 2 min. In the final step 100μl dH_2O was added and the Plasmid DNA was eluted by a final centrifugation step

at 14000 g for 1 min.

4.3.7 Plasmid DNA Purification (Plasmid Maxi Kit, QIAGEN)

100 ml of LB medium was inoculated with 1 ml of competent bacterial cells and grown overnight at 37°C. The bacterial cells were harvested by centrifugation at 5000 g for 15 min at 4°C. For efficient lysis the bacterial pellet was resuspended in 10 ml Buffer P1 (Resuspension buffer) and was vortexed until no cell clumps remained. Then 10 ml Buffer P2 (Lysis buffer) was added, mixed gently by inverting 4-6 times until it appeared viscous and was incubated at room temperature for 5 min. By adding 10 ml of chilled buffer P3 (Neutralization buffer) and inverting 4-6 times a fluffy white material formed, which contained genomic DNA, proteins, cell debris and SDS. The mixture was then incubated on ice for 20 min. After this precipitation step the mixture was centrifuged at 5000 g for 30 min at 4°C and the supernatant containing plasmid DNA was removed promptly. In the intervening period the QIAGEN-tip 500 was equilibrated by applying 10 ml Buffer QBT (Equilibration buffer). The supernatant was loaded onto the QIAGEN-tip using a paper towel in addition and was allowed to enter the resin by gravity flow. Then the QIAGEN-tip was washed with 30 ml Buffer QC (Wash buffer) for two times. The washing steps are sufficient for removing all contaminants in the majority of plasmid DNA preparations. Afterwards the DNA was eluted with 15 ml Buffer QF (Elution buffer), precipitated by adding 10.5 ml (0.7 volumes) isopropanol and was centrifuged at 5000 g for 1 h at 4°C. The supernatant was removed and the DNA pellet was washed with 5 ml of room-temperature 70% ethanol and was centrifuged 5000 g for 1 h. The resulting supernatant was removed again, the DNA pellet was air-dried for 10 min and redissolved in 200μl H_2O.

4.3.8 DNA purification using Phenol/Chloroform extraction and ethanol precipitation

An equal volume of Phenol-Chloroform-Isoamyethanol (23:24:1) was added to the DNA sample, mixed and centrifuged at 12000 rpm for 5 min at 4°C. The supernatant was transfered to a fresh tube. Next an equal volume of Chloroform was added, the sample was mixed and centrifuged at 12000 rpm for 5 min at 4°C. The supernatant was removed and 20μg of glycogen was added in order to visualize the small DNA pellet. 0.1 volume of 5M NaCl and 2.5 volumes of ice cold 100% Ethanol were added to precipitate DNA, mixed and centrifuged at 12000 rpm for 20 min at 4°C. The supernatant was removed, 1 ml 70% Ethanol was added and centrifuged at 12000 rpm for 10 min at 4°C. The supernatant was removed again and the DNA pellet was air dried for 10 min at room temperature. Finally the DNA was resuspended in an appropriate amount of dH_2O.

4.3.9 Generation of transgenic constructs

The generation of the basic Cd8a promoter-human CD2 (hCD2) reporter construct has been previously described.[21] ECR-4 and -5 were amplified by PCR and subcloned into the Cd8a promoter-hCD2 reporter construct as described above (Figure 4.2).

Figure 4.2: Schematic map of the transgenic reporter construct used for the generation of TG mice. The basic construct contains part of the first Cd4 intron as a splicing module between the Cd8a promoter and the human CD2 reporter gene (not shown). Inserted genomic fragments are shown in yellow, Cd8a promotor in grey, hCD2 reporter gene in blue and poly-A in grey.

4.3.10 Generation of transgenic mice

F2 eggs of (B6/D2) mice were injected with the transgenic constructs according to standard procedures . Transgenic founders were identified by Southern blotting of tail DNA with a probe from the CD4 splicing module of the transgenic reporter construct. Transgenic animals were backcrossed to C57BL/6 to generate transgenic lines. All animals analyzed were between 4 and 16 wk old. Transgenic mice were generated at the RCAI in Yokohama, Japan.

4.3.11 Transfection of 293T cells

Frozen 293T cells were thawed and washed with 50 ml of 293T medium. The following centrifugation step at 1400 rpm for 4 min removed DMSO which is included in the freezing medium and is toxic for the cells. The supernatant was sucked off, 40 ml of new 293T medium was added and 4x10 ml was transfered to a $10cm^2$ tissue plate (Corning) to reach a dilution of 1/4. After 2 days the cell culture medium was sucked off and the confluent cells were washed with 5 ml PBS. After this washing step, 2 ml Trypsin was added, the cells were incubated at 37°C for 5 min and a following centrifugation step at 1400 rpm for 4 min was performed. The supernatant was removed, cells were counted with a Casy counter and $3x10^6$ cells were seeded to a $10cm^2$ tissue plate. The next morning 10μg of myc-RUNX3 and/or full length MAZR was transfered into a polystyrene round bottom tube (BD falcon) and mixed with 200μl 1,25M $CaCl_2$ and 790μl dH_2O. Using a pasteur pipet, bubbles of the samples were formed and 1 ml 2xHBS buffer was added dropwise. The samples were then vortexed, incubated for 2 min on ice and transfered slowly to the $10cm^2$ tissue plates. After 6 hours the media was sucked off, the cells were washed with 5 ml PBS and 10 ml 293T medium was added. After 48 hours the supernatant was removed and the cells were washed twice with ice cold 1x PBS. A Cell Scraper (Greiner-Bio one) was used to remove adherent cells and the cells were collected in a 15 ml tube (Iwaki). The samples were then centrifuged at 14000 rpm for 4 min, the supernatant was sucked off and the cells were resuspended in 250μl Carin

Lysis Buffer (20mM Tris HCl, pH 8.0, 138mM NaCl, 10mM EDTA, 100mM NaF, 1% NP40, 10% glycerol, 2mM Na-Ortho-Vanadate and Proteaseinhibitor). Finally the cells were transfered to screwed cryo tubes, immediately frozen in liquide nitrogen and stored at -80°C.

4.4 Protein Analysis

4.4.1 SDS polyacrylamide gel Electrophoresis (SDS-page)

Protein samples were mixed with 4x Laemmli buffer (250mM Tris-base (pH 6.8), 8% SDS, 20% ß-mercaptoethanol, 43,4% glyerol), boiled at 95°C for 10 min and spinned down before loading on the gel. The denatured protein samples were separated on an 8% polyacrylamide gel using 1x running buffer (25mM Tris-base, 192mM glycine, and 0.1% sodium dodecyl sulfat) at 60 Volt for stacking gel and 120 Volt for resolving gel, respectively. 7μl PageRuler prestained protein ladder (Fermentas) was used to determine the fragment length of the protein samples.

4.4.2 Western Blot

After SDS polyacrylamide gel electrophoresis, proteins were transfered to ImmobilonP polyvinyl difluorid (PVDF) membranes (Millipore) using a wet blot transfer system. The PVDF membranes were equilibrated in methanol and sandwiched with the polyacrylamide gel between 2 layers of wattmann papers and 2 layers of sponges. For the transfer process 200 mA constant current was used for 2 hours at 4°C. After the transfer, PVDF membranes were blocked in Tris-buffered saline (TBST) (50mM Tris-HCl pH 8, 100mM NaCl, 0,1% Tween-20) with 5% non-fat dried milk at room temperature for 1 hour and at 4°C over night, respectively. Then the membranes were incubated with MAZR serum (1:7500) and anti-myc antibody (1:5000)diluted in TBST containing 3% non fat dried milk for total protein at room temperature for 1 hour, washed with TBST 6 times for 10 min, incubated with the secondary horseradish peroxidase (HRP)-coupled

anti-rabbit (1:5000) antibody, diluted in TBST containing 1% non fat dried milk at room temperature for 1 hour and finally washed with TBST 6 times for 10 min. Immunoblots were developed using enhanced chemilumiscent (ECL) reagent (Amersham) and imaged on high performance chemiluminescent films (Hyperfilm, ECL, Amersham). Blots were re-probed with antibodies recognizing total protein after stripping in Stripping buffer (62.5mM Tris-HCl pH 7.5, 100mM ß-mercaptoethanol, 2% SDS) for 15 min at 60°C.

4.4.3 ChIP (Chromatin Immunoprecipitation)

6-8 weeks old wildtype (C57BL/6) and MAZR deficient mice (males and females) were humanely killed by cervical dislocation. The thymus was isolated and homogenized in 3 ml PBS/1% FCS by using a syringe element in combination with a cell strainer. The single cell suspension was transfered into a new 50 ml falcon tube, 1ml PBS/1% FCS was added and the sample was centrifuged at 1600 rpm for 4 min at 4°C. Next the supernatant was removed and 1 ml of Ack lysis buffer (9 volumes 0,83% NH4Cl + 1 volume Tris pH 7.65) was added to lyse erythrocytes. The sample was mixed, incubated for 2 min at RT and 10 ml PBS/1%FCS was added. The sample was transfered to a new 50 ml falcon using a cell strainer and centrifuged at 1600 rpm for 3 min at 4°C. The supernatant was decanted, 10 ml PBS/1%FCS was added and thymocytes were counted with the Casey cell counter.

$1x10^7$ thymocytes were centrifuged at 1200 rpm for 5 min and washed twice in ice cold 1x PBS/1mM PMSF. For crosslinking, the cells were resuspended in 10 ml of 0,6% formaldehyde solution in PBS + Proteaseinhibitor (PI). The samples were placed immediately on 37°C for 2:30 min. The crosslinking was stopped by addition of 500μl 2,5M glycine, the tubes were inverted several times and incubated on a rotating wheel for 5 min at room temperature. Next, thymocytes were centrifuged at 3200 rpm for 5 min at 4°C. To remove formaldehyde, the cells were washed with 10 ml of ice cold PBS + PI twice. In the final wash step, the supernatant was removed and the pellets were snap frozen on dry ice for several minutes. Afterwards the cell pellets were resuspended in 500μl lysis buffer (50mM Tris ph 8.0, 10mM EDTA, 1% SDS, PI) and incubated on

ice for 20 min.

The lysates were sonicated using a Bandelin Sonoplus 70 Sonicator with the following settings: 7x10 sec intervals at 30% power. Between the sonication intervalls a 20 sec break on ice was performed. In case of foaming, the samples were spinned down, the foam was removed and dH_2O was added to a final volume of 500μl. The sonicator tip was washed with 70% EtOH and dH_2O prior to sonicating different samples. The sonicated lysates were centrifuged at 13000 rpm for 10 min at 4°C to pellet the cell debris. 400μl of sonicated supernatant was transfered into a new 15 ml falcon tube and 3,6 ml Chip buffer (0,01% SDS, 1,1% Triton X-100, 1,2mM EDTA, 16,7mM Tris pH 8.0, 167mM NaCl, PI) was added. Then the sample was split: 40μl (input sample) was transfered in a new 1,5 ml eppendorf tube and stored at -20°C. 2 x 1980μl were split into new 2 ml eppendorf tubes and correspond to the Chip sample and negative control sample. In addition the remaining 100μl of lysate was filled up to 500μl with H_2O, 20μl of 5M NaCl was added and the sample was decrosslinked o/n at 65°C while shaking.

80μl of ssDNA/Protein A (for rabbit anti-MAZR and rabbit anti-CBFb2) Agarose slurry was added to the Chip and negative control samples and rotated 45 min at 4°C to preclear the lysates from proteins that unspecifically bind to the Agarose slurry and protein A. The agarose slurry was then centrifuged at 1000 rpm for 1 min and the supernatant was transfered to a new 2 ml eppendorf tube. 10μl MAZR Preserum or dH_2O was used as negative control and 10μl MAZR-serum or 20μl (10μg) CBFb2 serum was added to the samples following an incubation overnight at 4°C with gentle rotation. The next day 60μl of ssDNA/Protein A/Agarose slurry was added to precipitate specific DNA-protein-Ab complexes and the samples were rotated gently for 1,5 - 2 hours at 4°C. A centrifugation step at 1000 rpm for 1 min at 4°C was performed to pellet down the agarose beads. The supernatant was removed and the following washing steps were performed by rotating the samples for 3-5 min at 4°C and centrifugation at 1000 rpm for 1 min: 1) 1 ml of ice-cold Low Salt wash buffer (0,1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris pH 8.0, 150mM NaCl, PI), 2) 1 ml of ice-cold High Salt wash buffer (0,1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris pH 8.0, 500mM NaCl, PI), 3) 1 ml of ice-cold LiCl immunocomplex wash buffer (0,25M LiCl, 1% NP40, 1% Deoxycholate, 1mM EDTA,

10mM Tris pH 8.0, PI), 4) 2x with 1 ml of ice-cold 1xTE (10mM Tris pH 8.0, 1mM EDTA, PI). Afterwards the DNA-histone complex was eluted from the antibodies by adding 250μl of Elution buffer (1% SDS, 0,1M NaHCO3) to the agarose slurry/protein A/histone complex. The samples were shortly vortexed and shook for 15 min at RT. The agarose beads were centrifuged at 1000 rpm for 1 min at RT and the supernatant was collected. The elution step was repeated again to collect a total of 500μl eluate and the input samples were filled up with 460μl dH_2O to a total of 500μl. 20μl of 5M NaCl was added and the samples were reverse crosslinked at 65°C o/n while shaking. The next day 10μl 0,5M EDTA, 20μl 1M TRIS-HCl pH 6.5 and 2μl of 10mg/ml Proteinase K was added to the reverse crosslinked eluate and incubated for 1 hour at 45°C. Finally, DNA was isolated by using Phenol/Chloroform precipitation as described above. All steps were performed by using filter tips.

4.4.4 Co-Immunoprecipitation

Frozen transfected cells were thawed on ice and centrifuged at 13000 rpm for 20 min at 4°C. For Western blotting 30μl of samples were mixed with 10μl Laemmli Buffer and frozen in liquide nitrogen. The rest of the lysates were filled up to 500μl with Carin-Lysisbuffer (20mM Tris HCl, pH 8.0, 138mM NaCl, 10mM EDTA, 100mM NaF, 1% NP40, 10% glycerol, 2mM Na-Ortho-Vanadate and Proteaseinhibitor). In the intervening period a 30% protein G sepharose was prepared. Following a washing step with Carin lysis buffer, 50μl 30% protein G sepharose was transfered to the IP samples and incubated for 1 hour at 4°C on a rotating wheel. After a centrifugation step at 1200 rpm for 1 min at 4°C, the supernatant was removed and 10μg of α-myc-Ab was added. Then the samples were incubated overnigt at 4°C on a rotating wheel.
The next day another 50μl 30% protein G sepharose was added to the samples and rotated for 1 hour at 4°C. After a centrifugation step at 1500 rpm for 1 min at 4°C the supernatant was sucked off and 1 ml Carin Lysis buffer was added. The samples were mixed, centrifuged at 1500 rpm for 1 min at 4°C. The washing steps were done in a total of four times. 30μl Carin Lysis buffer and 10μl Laemmli Buffer were added to the samples. Finally, the samples were boiled at 95°C for 10 min and centrifuged at 132000

rpm for 10 min at 4°. The supernatant was frozen in liquide nitrogen and either stored at -80°C or used immediately for SDS page. Western blotting was performed due to the protocol describes above.

4.5 Biological assays

4.5.1 Luciferase assay

1200M cells ($5x10^6$cells/500μl of RPMI 1640, 25μM Hepes) were electroporated (960μF, 220V; Bio-Rad Gene Pulser) in 0.4 cm cuvettes (Bio-Rad) with either 10 μg WE310 + ECR4 or an equimolar amount of plasmids WE310 + ECR-5, -9 or WE511. (Figure 4.3)

Figure 4.3: Schematic map of the luciferase reporter construct used for luciferase assays. The basic construct contains part of the first Cd4 intron as a splicing module between the Cd8a promoter and the luciferase reporter gene (not shown) . Genomic fragments (yellow) were inserted upstream of the Cd8a promoter. Cd8a promotor is indicated in grey, luciferase reporter gene in green and poly-A in grey.

In addition 2 μg of SV40 Renilla-luciferase plasmid (Promega) was cotransfected to normalize the transfection efficiency. The total amount of transfected DNA was equal for each transfection and adjusted by adding dH_2O. After electroporation, the cells were washed with RMPI 1640 medium and finally cultured in 15 ml of 1200M medium (RPMI 1640, 10% FCS, and antibiotics). After 24-36 hours the luciferase activity was measured using the Dual luciferase reporter assay system (Promega). The cells were transfered to a 15 ml falcon and centrifuged at 1300 rpm for 5 min, RT. The supernatant was removed and the cells were washed with PBS. After another centrifugation at 1300 rpm for 5 min, 100μl of passive lysis buffer (5x stock) was added to the pelleted cells and incubated for 10 min on ice. The cells were centrifuged at 5000 rpm for 20 min at 4°C and 20μl of

the supernatant was transfered to a 5ml tube (Sarstedt). The samples were measured in duplicates. 100μl luciferase reagent II was added and the firefly luciferase activity was measured using a Luminometer. For the Renilla Luciferase activity 100μl of Stop and glo solution (50x stock) was used.

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