Published in final edited form as:
Nat Immunol. 2010 May ; 11(5): 442–448. doi:10.1038/ni.1860.

The zinc finger protein MAZR is part of the transcription factor network controlling CD4/CD8 cell fate decision of DP thymocytes

Shinya Sakaguchi[1], **Matthias Hombauer**[1,5], **Ivan Bilic**[1,3,5], **Yoshinori Naoe**[2,4], **Alexandra Schebesta**[1], **Ichiro Taniuchi**[2], and **Wilfried Ellmeier**[1]

[1]Division of Immunobiology, Institute of Immunology, Center for Pathophysiology, Infectiology and Immunology, Medical University of Vienna, 1090 Vienna, Austria

[2]Laboratory for Transcriptional Regulation, RIKEN Research Center for Allergy and Immunology, Yokohama, Kanagawa 230-0045, Japan

Abstract

CD4/CD8 lineage specification of thymocytes is linked with coreceptor expression. Previously, the transcription factor MAZR was identified as an important regulator of *Cd8* gene expression. Here we show that variegated CD8 expression by loss of *Cd8* enhancers is reverted in MAZR-deficient mice, confirming that MAZR negatively regulates the *Cd8* loci during the transition to the double-positive (DP) stage. Moreover, loss of MAZR led to a partial redirection of MHC class I-restricted thymocytes into $CD4^+$ helper-like T cells correlating with derepression of ThPOK, a central transcription factor for helper-lineage development. MAZR bound the ThPOK silencer, indicating direct regulation of the *ThPOK* locus by MAZR. Thus, MAZR is part of the transcription factor network regulating CD8 lineage differentiation of DP thymocytes.

INTRODUCTION

CD4 and CD8 coreceptor expression correlates with the functional phenotype of T cells. Understanding the transcriptional regulation of *Cd4* and the adjacent genes *Cd8a* and *Cd8b1* (*Cd8*) might therefore provide insight into the cell fate decision of DP thymocytes[1-3].

CD8 coreceptor expression is tightly regulated during thymocyte development by the activity of at least five different *cis*-regulatory elements[4, 5]. Combined deletion of certain *Cd8* enhancers leads to variegated expression of CD8 in DP thymocytes[6-8] and CD8 variegation correlates with an epigenetic "off" state[9]. These studies indicate a complex regulatory network of developmental stage- and subset-specific *cis*-regulatory elements and link *Cd8* enhancer function with chromatin remodeling of the *Cd8ab* gene complex[10]. The transcriptional regulator MAZR appears to be one of the factors that are involved in keeping

Correspondence should be addressed to W.E. (wilfried.ellmeier@meduniwien.ac.at).
[3]Present address: Research Institute of Molecular Pathology, 1030 Vienna, Austria
[4]Present address: Department of Mechanism of Aging, National Institute for Longevity Sciences, National Center for Geriatrics and Gerontology, Obu, Aichi 474-8522, Japan
[5]These authors contributed equally to this work.

AUTHOR CONTRIBUTIONS S.S. designed the research, performed most of the experiments, analyzed the data and wrote the manuscript; M.H. performed co-immunoprecipitations and ChIP assays; A.S. and Y.N. performed ChIP assays; I.B. contributed to the generation of *Mazr*-deficient mice and performed some of the bone marrow transplantation experiments; I.T. provided reagents and experimental mice, and contributed to the design of the research; W.E. designed the research, contributed to the generation of *Mazr*-deficient mice, analyzed the data and wrote the manuscript.

COMPETING INTERESTS STATEMENT The authors declare that they have no competing financial interest.

Europe PMC Funders Author Manuscripts

the local chromatin at the *Cd8* gene loci in a transcriptional "off" state at the double-negative (DN) stage[9]. MAZR (also known as PATZ1 or Zfp278), encoded by the *Patz1* gene (referred to here as *Mazr*), belongs to the family of BTB (bric-a-brac, tramtrack, broad complex; also known as POZ domain: poxvirus zinc finger) domain-containing zinc finger (ZF) factors[11, 12]. *Mazr* is expressed in the brain, thymocytes, and B cells[13]. A recent study also reported that MAZR is essential for spermatogenesis[14]. In DN thymocytes MAZR interacts with several *Cd8 cis*-regulatory elements. MAZR expression is down-regulated during the transition from the DN to double-positive (DP) stage, and forced expression of MAZR results in variegated expression of CD8 in a proportion of DP thymocytes. Mechanistically, the transcriptional "off" state is linked to the nuclear corepressor NCoR, which interacts with the BTB domain of MAZR. Based on these observations MAZR has been proposed as an important negative regulator of *Cd8ab* gene complex activation during the DN to DP transition of thymocyte development[9].

To further investigate the physiological function of MAZR during T cell development, $Mazr^{-/-}$ mice were generated. While deletion of MAZR is not sufficient to activate CD8 expression in DN thymocytes, it partially reverted variegated expression of *Cd8* genes in $E8_I$,$E8_{II}$ enhancer doubly-deficient DP thymocytes. Furthermore, $Mazr^{-/-}$ mice displayed elevated CD4 to CD8 ratios in mature thymocytes and in peripheral T cells. By using either MHC class I-restricted TCR transgenic mice or $Mazr^{-/-}$ bone marrow (BM) chimeric MHC class II-deficient mice, we demonstrated that the increase in CD4 lineage cells in $Mazr^{-/-}$ mice was in part due to redirected differentiation of MHC class I-restricted CD8 SP thymocytes into the CD4 helper lineage. In addition, we could show derepression of ThPOK (encoded by the *Zbtb7* gene, referred to here as *ThPOK*), an essential molecule for helper lineage differentiation[15, 16], in MHC class I-signaled DP and $CD4^+CD8^{lo}$ $Mazr^{-/-}$ thymocytes. MAZR bound the *ThPOK* locus, suggesting a direct control of ThPOK expression. Thus, our data provide genetic evidence that MAZR is part of the transcription factor network that controls CD4/CD8 cell fate decision of DP thymocytes by regulating ThPOK expression.

RESULTS

Altered CD4 to CD8 T cell ratios in $Mazr^{-/-}$ mice

Mazr-deficient ($Mazr^{-/-}$) mice were generated by gene-targeting approaches (see Supplementary Fig. 1). $Mazr^{-/-}$ mice were born at a reduced Mendelian frequency (Supplementary Fig. 2a) and were smaller in size compared to $Mazr^{+/+}$ littermates (Supplementary Fig. 2b,c). Since MAZR is expressed at high amounts in DN and DP thymocytes[9], a comprehensive analysis of the T cell lineage was performed. The smaller size of $Mazr^{-/-}$ mice correlated with an approximately 40% and 50% reduction of thymocyte and splenocyte numbers, respectively (Fig. 1a). CD4, CD8α and CD8β protein expression levels were normal in $Mazr^{-/-}$ mice (Fig. 1b,c, and data not shown). However, the CD4SP to CD8SP thymocyte ratio was increased in $Mazr^{-/-}$ compared to $Mazr^{+/+}$ mice, and was most notable in the $CD3^{hi}$ population (Fig. 1b,d). In contrast, the CD4/CD8 profile of $CD3^{lo}$ cells was similar between $Mazr^{+/+}$ and $Mazr^{-/-}$ mice (Fig. 1b). The increased CD4SP to CD8SP ratio led also to an increased $CD4^+$ to $CD8^+$ T cell ratio in peripheral lymphoid organs of $Mazr^{-/-}$ mice (Fig. 1c,d). However, the expression of several cell surface markers such as CD62L, CD44, CD25 and CD122 on peripheral $CD4^+$ and $CD8^+$ T cells was normal, with the exception of a small increase in the $CD8^+CD44^{hi}$ T cell subset in the absence of MAZR (Supplementary Fig. 3a and data not shown). Moreover, $CD25^+FoxP3^+$ regulatory T cells, CD1d-restricted invariant NKT cells, $TCR\gamma\delta^+$ T cells and CD8αα positive $TCR\gamma\delta^+$ intraepithelial lymphocytes (IEL) were present at similar frequencies in $Mazr^{+/+}$ and $Mazr^{-/-}$ mice (Supplementary Fig. 3b-e). $Mazr^{-/-}$ mice showed

normal B cell development, and all major myeloid subsets were present under homeostatic conditions (data not shown).

MAZR affects CD8 expression

MAZR is proposed to function as a negative regulator of the activation of the *Cd8ab* gene complex during the DN to DP transition of thymocyte development[9]. DN thymocytes were still present in *Mazr*$^{-/-}$ mice (Fig. 1b, lower panel), and the distribution of the DN1-4 subsets was similar between *Mazr*$^{+/+}$ and *Mazr*$^{-/-}$ mice (Supplementary Fig. 3f,g). This indicates that early stages of thymocyte development are not affected by the loss of MAZR, and that the deletion of MAZR is not sufficient to activate *Cd8a* and *Cd8b* gene expression in DN thymocytes.

Combined deletion of the *Cd8* enhancers $E8_I$ and $E8_{II}$ leads to CD8 variegation in DP thymocytes[6], which correlates with epigenetic differences at the *Cd8ab* gene complex[9]. We hypothesized that in the absence of $E8_I$ and $E8_{II}$, fewer positive factors are recruited to the *Cd8* loci in DP thymocytes, thus changing the balance between a closed and an open state of chromatin. Since MAZR is one of the negative factors that controls the *Cd8ab* gene complex, one would predict reversion of CD8 variegation in $E8_I$,$E8_{II}$ doubly-deficient mice in the absence of MAZR. To test our hypothesis, *Mazr*$^{-/-}$ mice were intercrossed with $E8_I$,$E8_{II}$ doubly-deficient mice. The numbers of viable *Mazr*$^{-/-}$ offspring from heterozygous intercrosses dropped dramatically when mice were backcrossed to a C57BL/6 background (S.S. and W.E., unpublished observation). Therefore, E13.5 *Mazr*$^{+/+}$ and *Mazr*$^{-/-}$ fetal liver (FL) cells (CD45.2$^+$) on an $E8_I$,$E8_{II}$ doubly-deficient background were transferred into irradiated CD45.1$^+$ congenic mice (CD45.1$^+$). As previously reported[9], there was a strong CD8 variegation in *Mazr*$^{+/+}$,$E8_I$,E8 thymocytes. However, CD8 variegation was dramatically reduced in *Mazr*$^{-/-}$ II ,$E8_I$,$E8_{II}$ thymocytes (Fig. 2a, upper panel), which was even more evident within the CD3^{lo} subset that represents primarily DP thymocytes (Fig. 2a, lower panel and Fig. 2b). These findings provide genetic evidence that MAZR is an essential negative regulator of CD8 expression during DN to DP transition, as proposed in our previous studies.

T cell-intrinsic defects in *Mazr*$^{-/-}$ mice

The reduced thymocyte and T cell numbers could be the consequence of the smaller size of *Mazr*$^{-/-}$ mice, or due to a T cell-intrinsic defect, or both. Furthermore, the thymic microenvironment could be altered in *Mazr*$^{-/-}$ mice, and this could lead to altered CD4/CD8 ratios. To distinguish between these possibilities, competitive bone marrow (BM) reconstitution experiments were performed. For this, BM cells from wild-type CD45.1$^+$ congenic mice were mixed in a 1:1 ratio with either *Mazr*$^{+/+}$ (CD45.2$^+$) or *Mazr*$^{-/-}$ (CD45.2$^+$) BM cells and transplanted into irradiated CD45.1$^+$ recipients. *Mazr*$^{-/-}$ BM reconstituted the T cell lineage as well as the B cell lineage as efficiently as *Mazr*$^{+/+}$ BM in a competitive environment (Fig. 3a, upper panel and Fig. 3b). This indicates that reduced T cell numbers in *Mazr*$^{-/-}$ mice is the consequence of the smaller size of *Mazr*$^{-/-}$ mice. In contrast, while the CD4 and CD8 expression profile on thymocytes and peripheral T cells derived from *Mazr*$^{+/+}$ (CD45.2$^+$) BM cells was normal, the CD4 to CD8 ratio was increased in the *Mazr*$^{-/-}$ BM-derived (CD45.2$^+$) T cell compartment (Fig. 3a, lower panel and Fig. 3c). This reveals that the altered CD4/CD8 ratio in *Mazr*$^{-/-}$ mice is a T cell-intrinsic defect.

Partial redirection of MHC class I-restricted thymocytes

To investigate the reason for an increased CD4 to CD8 T cell ratio, *Mazr*$^{-/-}$ mice were either crossed with OT-I or OT-II TCR transgenic mice, which express TCRs ($V_\beta5$, $V_\alpha2$) restricted to MHC class I or class II, respectively[17, 18]. The thymic CD4/CD8 profile in *Mazr*$^{-/-}$,OT-II mice was similar to the one in *Mazr*$^{+/+}$,OT-II cells (Fig. 4a and

Supplementary Fig. 4a). In the periphery, there was a mild increase in the percentage of $CD4^+$ T cells and a corresponding decrease in $CD8^+$ T cells in *Mazr*$^{-/-}$,OT-II mice, however, the percentage of transgenic $V_\alpha 2$-expressing T cells was similar (Fig. 4a,b and Supplementary Fig. 4b). This is in contrast to *Mazr*$^{-/-}$,OT-I TCR transgenic mice, which showed dramatic changes in the CD4/CD8 expression profile. While *Mazr*$^{+/+}$,OT-I thymocytes were predominantly selected into the cytotoxic T cell lineage, *Mazr*$^{-/-}$,OT-I thymocytes displayed a marked increase in the percentage of CD4SP cells and a corresponding reduction of the CD8SP population (Fig. 4c, upper and middle panels, and Supplementary Fig. 4c). In the periphery of *Mazr*$^{+/+}$,OT-I mice, only few $CD4^+$ T cells were present (Fig. 4c, lower left panel). In contrast, a substantial number of $CD4^+$ T cells appeared in *Mazr*$^{-/-}$,OT-I mice (Fig. 4c, lower right panel and Supplementary Fig. 4d). Moreover, these $CD4^+$ T cells predominantly expressed the $V_\alpha 2$ TCR chain, suggesting that these T cells were selected on the basis of MHC class I-restricted OT-I TCR (Fig. 4d,e).

To exclude the possibility that *Mazr*$^{-/-}$,OT-I cells were selected on MHC class II-restricted TCRs consisting of an endogenous $V_\alpha 2$ chain, *Mazr*$^{-/-}$,OT-I mice were crossed onto a *Rag2*$^{-/-}$ background. E13.5 FL cells ($CD45.2^+$) from either *Mazr*$^{+/+}$*Rag2*$^{-/-}$,OT-I or *Mazr*$^{-/-}$ *Rag2*$^{-/-}$,OT-I embryos were transferred into irradiated wild-type $CD45.1^+$ congenic mice. Similar to *Mazr*$^{-/-}$,OT-I mice, a significant population of peripheral $V_\alpha 2^+CD4^+$ T cells developed in *Mazr*$^{-/-}$*Rag2*$^{-/-}$,OT-I chimeric mice (Fig. 5a). This resulted in a marked increase in the absolute number of $CD4^+$ T cells in *Mazr*$^{-/-}$*Rag2*$^{-/-}$,OT-I chimeric mice compared *Mazr*$^{+/+}$*Rag2*$^{-/-}$,OT-I chimeric mice (Fig. 5b). Moreover, the MHC class I-restricted $CD4^+$ T cells in *Mazr*$^{-/-}$ *Rag2*$^{-/-}$,OT-I chimeric mice displayed characteristic features of helper T cells, such as expression of *ThPOK* and upregulation of CD154 (CD40L) upon activation, but produced low amounts of IFN-γ and did not express *Prf1* (encoding perforin) (Supplementary Fig.5a,b,c). Of note, most of the *Mazr*$^{-/-}$*Rag2*$^{-/-}$,OT-I $CD4^+$ T cells were $CD44^{lo}CD62L^{hi}$, suggesting that they are not innate-like T cells (such as iNKT cells) (Supplementary Fig. 5d).

Next, we investigated whether MHC class I-restricted MAZR-deficient $CD4^+$ T cells also develop in a non-TCR transgenic setting. Thus, *Mazr*$^{+/+}$ and *Mazr*$^{-/-}$ BM cells ($CD45.2^+$) were transplanted into MHC class II-deficient recipients ($CD45.1^+$) and the number of peripheral T cells and thymocytes upon reconstitution was determined. There was a significant increase in the percentage and absolute number of $CD3^+CD4^+$ T cells ($CD45.2^+$) and a corresponding decrease of $CD3^+CD8^+$ T cells in the spleen and thymus of *Mazr*$^{-/-}$ chimeric mice compared to *Mazr*$^{+/+}$ chimeras (Fig. 5c,d, and Supplementary Fig.6a,b). To exclude innate-like T cells such as invariant NKT cells, only CD1d tetramer-negative cells were analyzed. A similar increase in CD4 lineage cells was also detected on $TCR\alpha\beta^+$-gated T cells (to exclude γδT cells, data not shown). We noticed that the majority of *Mazr*$^{+/+}$ and *Mazr*$^{-/-}$ $CD4^+$ T cells were $CD44^{hi}$, most likely due to altered homeostasis in an MHC class II-deficient environment. However, there was no difference in the percentage of IFN-γ-producing $CD44^{hi}$ T cells upon PMA/ionomycin stimulation between *Mazr*$^{+/+}$ and *Mazr*$^{-/-}$ MHC class II-deficient chimeras, indicating that the increase in *Mazr*$^{-/-}$ $CD4^+$ T cells is not due to an increase of innate-like T cells (Supplementary Fig. 6c).

Together, the data observed from either MHC class I-restricted TCR transgenic mice or BM chimeric MHC class II-deficient mice indicate that a fraction of *Mazr*$^{-/-}$ MHC class I-restricted thymocytes redirects into the helper T cell lineage.

Derepression of *ThPOK* in MAZR-deficient thymocytes

Molecules such as ThPOK, Runx1, Runx3, GATA-3 and Tox are implicated in CD4/CD8 lineage commitment of DP thymocytes[15, 16, 19-24]. To investigate whether loss of MAZR induces alterations in the expression of these factors, qRT-PCR experiments on sorted

$CD69^{-}V_{\alpha}2^{lo}$DP (DP $CD69^{-}$; i.e. pre-selected DP cells), $CD69^{+}V_{\alpha}2^{int\text{-}hi}$ DP (DP $CD69^{+}$; i.e. signaled DP cells) and $CD69^{+}V_{\alpha}2^{int\text{-}hi}CD4^{+}CD8^{lo}$ ($CD4^{+}CD8^{lo}$ $CD69^{+}$) thymocyte subsets from *Mazr*$^{+/+}$*Rag2*$^{-/-}$,OT-I and *Mazr*$^{-/-}$*Rag2*$^{-/-}$,OT-I FL-transplanted mice were performed. An increased expression of *ThPOK* gene in DP $CD69^{-}$ and DP $CD69^{+}$ subsets of *Mazr*$^{-/-}$*Rag2*$^{-/-}$,OT-I chimeric mice compared to *Mazr*$^{+/+}$*Rag2*$^{-/-}$,OT-I chimeric mice was observed. In contrast, *Runx1, Runx3*, *Gata3*, and *Tox* expression was similar in *Mazr*$^{-/-}$*Rag2*$^{-/-}$,OT-I and *Mazr*$^{+/+}$*Rag2*$^{-/-}$,OT-I DP (both $CD69^{-}$ and $CD69^{+}$) subsets, while *Runx3* expression was reduced in $CD4^{+}CD8^{lo}CD69^{+}$ subsets (Fig. 6a and Supplementary Fig. 7).

To investigate in detail the expression pattern of ThPOK in MAZR-deficient thymocytes, *Mazr*$^{-/-}$,OT-I mice were crossed with a ThPOK GFP reporter mouse, in which a cDNA encoding GFP is inserted into the *ThPOK* gene locus[20]. Thus, GFP expression is driven by the endogenous *ThPOK* regulatory elements. ThPOK expression is primarily induced by MHC class II signaling, and after positive selection a large fraction of $CD4^{+}CD8^{lo}CD69^{+}$ thymocytes expresses ThPOK15, 16, 20, 21, 25. However, some ThPOK-expressing $CD4^{+}CD8^{lo}CD69^{+}$ cells retain the potential to develop into the cytotoxic cell lineage[21, 25]. During CD4 lineage differentiation, *ThPOK* mRNA expression is upregulated and all CD4SP thymocytes and $CD4^{+}$ T cells express high levels of ThPOK, while only a small population of CD8SP thymocytes and peripheral $CD8^{+}$ T cells expressed very low levels of ThPOK[21, 25]. In *Mazr*$^{+/+}$ *ThPOK*$^{+/GFP}$,OT-I mice, GFP^{+} cells were detected in DP $CD69^{+}$ and in $CD4^{+}CD8^{lo}$ $CD69^{+}$ populations (Fig. 6b and Supplementary Fig. 8a). The percentage of GFP^{+} cells increased almost to 100% within the CD4SP subset, whereas CD8SP thymocytes terminated GFP expression. Consistent with the qRT-PCR data, a substantial number of GFP^{+} cells appeared in DP $CD69^{-}$ thymocytes of *Mazr*$^{-/-}$ *ThPOK*$^{+/GFP}$,OT-I mice, indicating derepression of ThPOK (Fig. 6b and Supplementary Fig. 8a). Moreover, the percentage of GFP^{+} cells within the DP $CD69^{+}$ and $CD4^{+}CD8^{lo}CD69^{+}$ populations of *Mazr*$^{-/-}$ *ThPOK*$^{+/GFP}$,OT-I thymocytes was increased compared to *Mazr*$^{+/+}$ *ThPOK*$^{+/GFP}$,OT-I thymocytes (Fig. 6b). In CD8SP cells, endogenous *ThPOK* expression was already decreased compared to CD4SP cells (Supplementary Fig. 9a,b), even though a certain fraction of CD8SP remained GFP-high (Fig. 6b), most likely due to slow GFP protein degradation, Notably, peripheral $CD8^{+}$ T cells in *Mazr*$^{-/-}$ *ThPOK*$^{+/GFP}$,OT-I mice expressed GFP^{+}, although at lower levels compared to $CD4^{+}$ T cells (Fig. 6b), consistent with the observation that *ThPOK* expression was detected in $CD8^{+}$ T cells of *Mazr*$^{-/-}$*Rag2*$^{-/-}$,OT-I mice (Supplementary Fig.5c).

To determine whether derepression of ThPOK is also observed in a non-TCR transgenic setting, we analyzed GFP expression in *Mazr*$^{-/-}$ *ThPOK*$^{+/GFP}$ mice. GFP expression (or *ThPOK* mRNA expression) was not detected in *Mazr*$^{-/-}$ DP $CD69^{-}$ thymocytes (Fig.7a, and data not shown), suggesting that MAZR is not essential for ThPOK repression in pre-signaled DP cells. However, an increased percentage of GFP^{+} DP $CD69^{+}$ and $CD4^{+}CD8^{lo}CD69^{+}$ thymocyte subsets (i.e. $CD69^{+}TCR\beta^{int\text{-}hi}$DP and $CD69^{+}TCR\beta^{int\text{-}hi}CD4^{+}CD8^{lo}$ subsets) was observed in *Mazr*$^{-/-}$ *ThPOK*$^{+/GFP}$ mice compared to *Mazr*$^{+/+}$ *ThPOK*$^{+/GFP}$ mice, while CD8SP cells terminated GFP expression (Fig.7a and Supplementary Fig. 8b). Similar to *Mazr*$^{-/-}$ *ThPOK*$^{+/GFP}$,OT-I mice, a fraction of peripheral $CD8^{+}$ T cells in *Mazr*$^{-/-}$ *ThPOK*$^{+/GFP}$ mice re-expressed GFP (Fig. 7a), indicating that MAZR is required to maintain ThPOK repression in $CD8^{+}$ T cells. Next, we determined whether TCR interactions with either MHC class I or MHC class II induced increased percentages of GFP^{+} cells. Therefore, BM cells from *Mazr*$^{+/+}$ *ThPOK*$^{+/GFP}$ and *Mazr*$^{-/-}$ *ThPOK*$^{+/GFP}$ mice were transferred either into irradiated MHC class I- or MHC class II-deficient mice. Similar amounts of GFP were expressed in DP $CD69^{+}$ and $CD4^{+}CD8^{lo}CD69^{+}$ thymocyte subsets derived from *Mazr*$^{+/+}$ *ThPOK*$^{+/GFP}$ and *Mazr*$^{-/-}$ *ThPOK*$^{+/GFP}$ BM in MHC class I-deficient recipients. In contrast, the percentage of GFP^{+}

cells in those subsets in MHC class II-deficient recipients was much higher in the absence of MAZR (Fig. 7b and Supplementary Fig. 8c). This indicates derepression of ThPOK in *Mazr*$^{-/-}$ thymocytes in MHC class I-signaled thymocytes.

To provide mechanistic insight into how MAZR is involved in ThPOK repression, we tested whether MAZR could bind a newly identified *ThPOK* silencer, which is indispensable for the helper lineage-specific expression of ThPOK[20, 26]. Chromatin immunoprecipitation assays (ChIP) revealed that MAZR binds to the *ThPOK* silencer region (Fig 7c). This result strongly suggests a direct regulation of the *ThPOK* gene by MAZR.

DISCUSSION

In this study we provide evidence that the transcriptional regulator MAZR has important functions at two distinct stages of T cell development. MAZR is a negative regulator of the *Cd8ab* gene complex during the DN to DP transition, and it represses ThPOK expression in MHC class I-signaled thymocytes during the CD4/CD8 cell fate decision.

First, we observed that the deletion of MAZR almost completely reversed CD8 variegation in *Cd8* enhancer $E8_I$,$E8_{II}$ doubly-deficient mice. Thus, our data provide strong genetic evidence that MAZR negatively regulates the activation of the *Cd8* gene loci during the DN to DP transition. MAZR binds to *Cd8* enhancers and interacts with a the nuclear corepressor NCoR complex in thymocytes[9]. The co-repressor NCoR is a component of multiple protein complexes that also contain histone deacetylases that repress target genes[27]. Therefore, MAZR might repress *Cd8* expression via recruitment of repressor complexes containing NCoR. Based on this model, one would predict reversion of CD8 variegation in $E8_I$,$E8_{II}$ doubly-deficient mice in the absence of MAZR. This was indeed observed. Thus, our results indicate that the balance between negative and positive factors at the *Cd8ab* gene complex can determine the initiation of *Cd8a* and *Cd8b* gene expression. Even though the *Cd8* gene loci are released from MAZR-mediated repression in the absence of MAZR, positive factors required for CD8 expression might not be sufficiently available in DN thymocytes. In contrast, in the absence of $E8_I$ and $E8_{II}$, less positive factors are recruited to the *Cd8* loci in DP thymocytes, leading to CD8 variegation. However, deletion of the negative regulator MAZR in $E8_I$,$E8_{II}$ doubly-deficient DP cells shifts the balance at the *Cd8* gene loci towards an “on” state and thus facilitates CD8 expression.

A second important result of our study is the demonstration that MAZR is part of the transcription factor network that controls CD4/CD8 cell fate specification of DP thymocytes. Several transcription factors (such as ThPOK, Runx1, Runx3, GATA-3 and Tox) act in a complex regulatory network that orchestrates CD4/CD8 cell fate decision[15, 16, 19-24, 28-30]. Among those, ThPOK is considered as a “master” CD4 commitment factor, in part by shutting off lineage inappropriate genes[21, 22, 31, 32]. Therefore, the regulation of ThPOK expression is crucial for correct lineage choice. We now show that in the absence of MAZR, a substantial proportion of MHC class I-restricted thymocytes redirects into $CD4^+$ T cells with helper lineage characteristics due to derepression of ThPOK. This was demonstrated either by using transgenic mice expressing MHC class I-restricted TCR or by generating *Mazr*$^{-/-}$ BM chimeric MHC class II-deficient mice. Although only a minor subset of *Mazr*$^{+/+}$ DP 69^+ and $CD4^+CD8^{lo}CD69^+$ cells expresses low levels of *ThPOK* mRNA in response to MHC class I stimulation[15, 16, 20, 21, 25], a higher percentage of these cells derepressed ThPOK in the absence of MAZR, providing a potential molecular explanation for the redirected differentiation of *Mazr*$^{-/-}$ thymocytes. This suggests a role for MAZR in repressing ThPOK expression in MHC class I-signaled thymocytes during positive selection.

The *ThPOK* silencer is required for ThPOK repression and cytotoxic lineage development[20, 26]. Runx complexes are essential for ThPOK repression at the pre-selection DP CD69^{-} stage via binding to the *ThPOK* silencer[20, 21, 29]. We cannot formally exclude the possibility that MAZR regulates ThPOK expression indirectly, e.g by regulating the expression of Runx3 in CD4^{+}CD8^{lo} cells, which would then lead to an altered expression of ThPOK. Alternatively, MAZR might have pleiotropic effects on gene expression, e.g. it might regulate the expression of a factor involved in TCR signaling. This could lead to qualitative and/or quantitative changes in TCR signaling and as a consequence changes in ThPOK expression. However, our ChIP result showing that MAZR binds to the *ThPOK* silencer clearly suggests direct control of the *ThPOK* locus by MAZR. It is tempting to speculate that MAZR represses ThPOK expression in cooperation with Runx complexes in MHC class I-signaled cells. Runx1 and Runx3 expression was normal in DP thymocytes, and Runx complexes were still bound to the *ThPOK* silencer in the absence of MAZR (data not shown), indicating that the recruitment of Runx complexes is independent of MAZR. In preliminary experiments we detected an association of MAZR with Runx1 but not Runx3 upon overexpression in HEK293T cells (see Supplementary Fig. 10). Thus, MAZR might be recruited to the *ThPOK* silencer via interaction with Runx complexes that are bound already to the *ThPOK* silencer. Alternatively, MAZR recruitment to the *ThPOK* silencer could occur independently of Runx complexes, however the interaction between MAZR and Runx complexes might be necessary for the recruitment of additional factors to repress ThPOK. Further studies are required to investigate potential mechanism(s) of ThPOK repression mediated by MAZR and Runx interactions. Moreover, since Runx1 promotes the expression of CD8 in unsignaled DP thymocytes[19], and since MAZR regulates the activation of the *Cd8ab* gene complex, further studies should also address whether MAZR-Runx1 interactions might regulate CD8 expression during the DN to DP transition.

We also observed that a fraction of peripheral MAZR-deficient CD8^{+} T cells expressed ThPOK. This suggests that MAZR might also be part of the molecular machinery that maintains ThPOK silencing in CD8^{+} T cells. A recent study demonstrated that certain DNase I hypersensitive sites within the *ThPOK* locus preferentially appear in CD4SP thymocytes, supporting the notion that ThPOK expression is accompanied by structural changes of chromatin[26]. Based on the variegated expression of ThPOK in *Mazr*$^{-/-}$ *ThPOK*$^{+/GFP}$ CD8^{+} T cells, MAZR might regulate ThPOK expression at the epigenetic level, in a similar manner to which it regulates *Cd8* gene expression[9]. In contrast to CD8^{+} T cells, CD8SP thymocytes in *Mazr*$^{-/-}$ *ThPOK*$^{+/GFP}$ mice did not express ThPOK. This suggests different mechanisms of ThPOK silencing in CD8SP and in CD8^{+} T cells, a situation that is reminiscent of the transcriptional control of CD4 silencing, where the *Cd4* silencer is required in different ways in CD8SP versus CD8^{+} T cells[19, 33, 34]. Further analysis is required to elucidate the molecular mechanism(s) by which MAZR shuts-off ThPOK expression and/or maintains ThPOK silencing in CD8 lineage cells. Thus, in MHC class I-signaled CD4^{+}CD8^{lo} thymocytes, MAZR is involved in keeping the *ThPOK* locus repressed during CD8 lineage differentiation, and/or in peripheral CD8^{+} T cells MAZR is likely to function in establishment and/or maintenance of ThPOK silencing (see Supplementary Fig. 11a,b for a working model of MAZR function). Of note, MHC class I- and MHC class II-signaled CD4^{+}CD8^{lo} thymocytes expressed similar levels of *Mazr* mRNA (data not shown), and MAZR bound the *ThPOK* silencer in DN, DP, CD4SP, and CD8SP cells (data not shown). These data suggest that ThPOK repression by MAZR in MHC class I-signaled cells is not due to differences in MAZR expression or differential recruitment to the *ThPOK* locus.

We observed that ThPOK was derepressed in a proportion of MHC class I-signaled *Mazr*$^{-/-}$ *ThPOK*$^{+/GFP}$ thymocytes, and that *ThPOK* mRNA expression levels were broadly distributed leading to cells expressing higher and lower levels of ThPOK. It might be

possible that only those thymocytes in which derepressed ThPOK expression levels remain above a certain threshold redirect into the helper lineage, thus leading to a partial redirection phenotype. In contrast, if derepression of ThPOK is below a certain threshold level, their differentiation into the cytotoxic lineage is impaired. ThPOK expression might even be regulated by TCR strength. Indeed, in contrast to the OT-I TCR transgenic system, thymocytes expressing the MHC class I-restricted P14 transgenic TCR are not redirected by loss of MAZR, although CD8 lineage development is severely impaired (data not shown). A similar dependency on the transgenic TCR system was observed in GATA-3-deficient mice, where redirected differentiation of MHC class II-restricted thymocytes occurs on the 5CC7 TCR transgenic background, but not on AND or DO11.10 TCR transgenic background[22]. Further studies are required to clarify the potential relation between TCR signaling strength and ThPOK derepression in the absence of MAZR, and the subsequent impact for CD4/8 cell fate decision.

Taken together, our data provide genetic evidence that MAZR controls the *Cd8* gene loci. Moreover, our study reveals that loss of MAZR leads to derepression of ThPOK causing redirected differentiation of MHC class I-restricted thymocytes into helper T cells. Thus, MAZR is part of the transcription factor network that controls cell fate decision of DP thymocytes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors thank J. Kaye for information about primer sequences detecting *Tox* gene expression, M. Willheim and G. Hofbauer from the FACS facility, Medical University of Vienna, and D. Prinz from St. Anna Children hospital for cell sorting, H. Tanaka from Taniuchi lab for technical advice, E. Pfeiffer for help with irradiation, Meinrad Busslinger for help with some animal experiments, and D. Stoiber-Sakaguchi, J. Raberger and N. Boucheron for critical reading of the manuscript. PBS57-loaded and unloaded CD1d reagents were kindly provided by the NIH Tetramer facility. This study was supported by the Austrian Science Fund (FWF; research grants P16708, P19930), and by the START Program (Project Y-163) of the Austrian Ministry of Science and Research (BM:WF), and by a RIKEN RCAI International collaboration award.

REFERENCES

1. Bosselut R. CD4/CD8-lineage differentiation in the thymus: from nuclear effectors to membrane signals. Nat Rev Immunol. 2004; 4:529–540. [PubMed: 15229472]
2. He X, Kappes DJ. CD4/CD8 lineage commitment: light at the end of the tunnel? Curr Opin Immunol. 2006; 18:135–142. [PubMed: 16480861]
3. Singer A, Adoro S, Park JH. Lineage fate and intense debate: myths, models and mechanisms of CD4- versus CD8-lineage choice. Nat Rev Immunol. 2008; 8:788–801. [PubMed: 18802443]
4. Ellmeier W, Sawada S, Littman DR. The regulation of CD4 and CD8 coreceptor gene expression during T cell development. Annu Rev Immunol. 1999; 17:523–554. [PubMed: 10358767]
5. Kioussis D, Ellmeier W. Chromatin and CD4, CD8A and CD8B gene expression during thymic differentiation. Nat Rev Immunol. 2002; 2:909–919. [PubMed: 12461564]
6. Ellmeier W, Sunshine MJ, Maschek R, Littman DR. Combined deletion of CD8 locus cis-regulatory elements affects initiation but not maintenance of CD8 expression. Immunity. 2002; 16:623–634. [PubMed: 12049715]
7. Garefalaki A, et al. Variegated expression of CD8 alpha resulting from in situ deletion of regulatory sequences. Immunity. 2002; 16:635–647. [PubMed: 12049716]
8. Feik N, et al. Functional and molecular analysis of the double-positive stage-specific CD8 enhancer E8III during thymocyte development. J Immunol. 2005; 174:1513–1524. [PubMed: 15661911]

9. Bilic I, et al. Negative regulation of CD8 expression via Cd8 enhancer-mediated recruitment of the zinc finger protein MAZR. Nat Immunol. 2006; 7:392–400. [PubMed: 16491076]
10. Taniuchi I, Ellmeier W, Littman DR. The CD4/CD8 lineage choice: new insights into epigenetic regulation during T cell development. Adv Immunol. 2004; 83:55–89. [PubMed: 15135628]
11. Bilic I, Ellmeier W. The role of BTB domain-containing zinc finger proteins in T cell development and function. Immunol Lett. 2007; 108:1–9. [PubMed: 17084908]
12. Kelly KF, Daniel JM. POZ for effect--POZ-ZF transcription factors in cancer and development. Trends Cell Biol. 2006; 16:578–587. [PubMed: 16996269]
13. Kobayashi A, et al. A combinatorial code for gene expression generated by transcription factor Bach2 and MAZR (MAZ-related factor) through the BTB/POZ domain. Mol Cell Biol. 2000; 20:1733–1746. [PubMed: 10669750]
14. Fedele M, et al. PATZ1 gene has a critical role in the spermatogenesis and testicular tumours. J Pathol. 2008; 215:39–47. [PubMed: 18241078]
15. He X, et al. The zinc finger transcription factor Th-POK regulates CD4 versus CD8 T-cell lineage commitment. Nature. 2005; 433:826–833. [PubMed: 15729333]
16. Sun G, et al. The zinc finger protein cKrox directs CD4 lineage differentiation during intrathymic T cell positive selection. Nat Immunol. 2005; 6:373–381. [PubMed: 15750595]
17. Barnden MJ, Allison J, Heath WR, Carbone FR. Defective TCR expression in transgenic mice constructed using cDNA-based alpha- and beta-chain genes under the control of heterologous regulatory elements. Immunol Cell Biol. 1998; 76:34–40. [PubMed: 9553774]
18. Hogquist KA, et al. T cell receptor antagonist peptides induce positive selection. Cell. 1994; 76:17–27. [PubMed: 8287475]
19. Taniuchi I, et al. Differential requirements for Runx proteins in CD4 repression and epigenetic silencing during T lymphocyte development. Cell. 2002; 111:621–633. [PubMed: 12464175]
20. Setoguchi R, et al. Repression of the transcription factor Th-POK by Runx complexes in cytotoxic T cell development. Science. 2008; 319:822–825. [PubMed: 18258917]
21. Egawa T, Littman DR. ThPOK acts late in specification of the helper T cell lineage and suppresses Runx-mediated commitment to the cytotoxic T cell lineage. Nat Immunol. 2008; 9:1131–1139. [PubMed: 18776905]
22. Wang L, et al. Distinct functions for the transcription factors GATA-3 and ThPOK during intrathymic differentiation of CD4(+) T cells. Nat Immunol. 2008; 9:1122–1130. [PubMed: 18776904]
23. Aliahmad P, Kaye J. Development of all CD4 T lineages requires nuclear factor TOX. J Exp Med. 2008; 205:245–256. [PubMed: 18195075]
24. Egawa T, Tillman RE, Naoe Y, Taniuchi I, Littman DR. The role of the Runx transcription factors in thymocyte differentiation and in homeostasis of naive T cells. J Exp Med. 2007; 204:1945–1957. [PubMed: 17646406]
25. Muroi S, et al. Cascading suppression of transcriptional silencers by ThPOK seals helper T cell fate. Nat Immunol. 2008; 9:1113–1121. [PubMed: 18776907]
26. He X, et al. CD4-CD8 lineage commitment is regulated by a silencer element at the ThPOK transcription-factor locus. Immunity. 2008; 28:346–358. [PubMed: 18342007]
27. Jepsen K, Rosenfeld MG. Biological roles and mechanistic actions of co-repressor complexes. J Cell Sci. 2002; 115:689–698. [PubMed: 11865025]
28. Taniuchi I. Transcriptional regulation in helper versus cytotoxic-lineage decision. Curr Opin Immunol. 2009; 21:127–132. [PubMed: 19361971]
29. Collins A, Littman DR, Taniuchi I. RUNX proteins in transcription factor networks that regulate T-cell lineage choice. Nat Rev Immunol. 2009; 9:106–115. [PubMed: 19165227]
30. Egawa T. Runx and ThPOK: A balancing act to regulate thymocyte lineage commitment. J Cell Biochem. 2009
31. Wang L, et al. The zinc finger transcription factor Zbtb7b represses CD8-lineage gene expression in peripheral CD4+ T cells. Immunity. 2008; 29:876–887. [PubMed: 19062319]

32. Jenkinson SR, et al. Expression of the transcription factor cKrox in peripheral CD8 T cells reveals substantial postthymic plasticity in CD4-CD8 lineage differentiation. J Exp Med. 2007; 204:267–272. [PubMed: 17296789]

33. Zou YR, et al. Epigenetic silencing of CD4 in T cells committed to the cytotoxic lineage. Nat Genet. 2001; 29:332–336. [PubMed: 11687799]

34. Leung RK, et al. Deletion of the CD4 silencer element supports a stochastic mechanism of thymocyte lineage commitment. Nat Immunol. 2001; 2:1167–1173. [PubMed: 11694883]

Europe PMC Funders Author Manuscripts

Europe PMC Funders Author Manuscripts

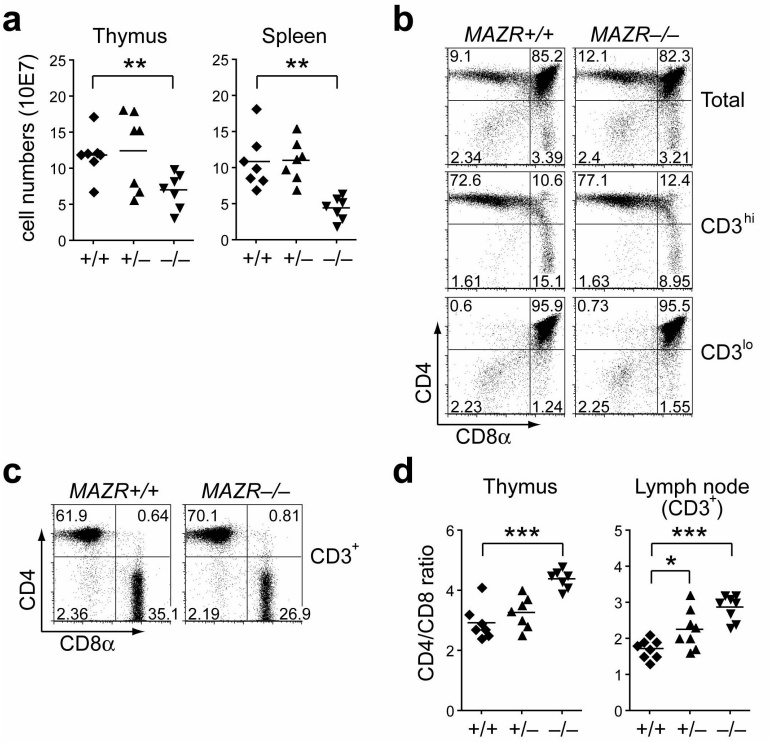

Figure 1.

Altered CD4 to CD8 T cell ratio in the absence of MAZR. (**a**) Diagrams show thymocyte and splenocyte numbers of 6-8 weeks old *Mazr*$^{+/+}$, *Mazr*$^{+/-}$ and *Mazr*$^{-/-}$ female mice. Each symbol represents one mouse, and horizontal lines indicate average values. (**b**) Flow cytometric analysis of CD4 and CD8α expression on thymocytes isolated from *Mazr*$^{+/+}$ and *Mazr*$^{-/-}$ littermates. Cells were gated on total thymocytes (upper panel), or on CD3^{hi} (middle panel) and on CD3^{lo} (bottom panel) subsets. Numbers indicate the percentage of cells in the respective quadrant. Data are representative of more than 10 independent experiments. (**c**) CD4 and CD8α expression pattern on lymph node cells isolated from *Mazr*$^{+/+}$ and *Mazr*$^{-/-}$ littermates. Cells were gated on the CD3^{+} population. Numbers in the dot plots indicate the percentage of cells in the respective quadrant. Data are representative of more than 10 independent experiments. (**d**) Diagrams show CD4 to CD8 ratio of SP thymocytes (left panel) and of CD3^{+} lymph node T cells (right panel) from *Mazr*$^{+/+}$, *Mazr*$^{+/-}$ and *Mazr*$^{-/-}$ mice. Each symbol represents one mouse, and horizontal lines indicate average values.

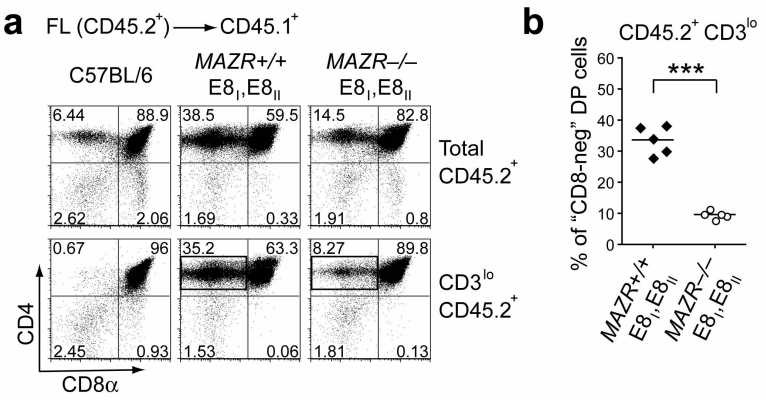

Figure 2.

Reduced variegation of CD8 in $E8_I,E8_{II}$ double-deficient mice in the absence of MAZR (**a**) CD4 and CD8α expression on $CD45.2^+$ thymocytes isolated from $Mazr^{+/+}$,$E8_I,E8_{II}$ and $Mazr^{-/-}$,E8 ,E8 + I II fetal liver (FL)-transplanted CD45.1 recipient mice (middle and right panel, respectively). Left panel shows thymic CD4 and CD8α expression profile of C57BL/6 mice. Cells were gated on total $CD45.2^+$ thymocytes (upper panels), or on $CD45.2^+$ $CD3^{lo}$ thymocytes (lower panels). Numbers indicate the percentage of cells in the respective quadrant. Data shown are representative of five mice each. (**b**) Diagram showing the percentage of $CD4^+CD8^-$ (i.e. "$CD8^-$") DP thymocytes within the $CD45.2^+CD3^{lo}$ population in $Mazr^{+/+}$,$E8_I,E8_{II}$ and $Mazr^{-/-}$,$E8_I,E8_{II}$ FL-transplanted mice. Each dot represents one mouse, and horizontal lines indicate average values. The gating areas for $CD4^+CD8^-$ cells is shown in **a** (lower panel).

Europe PMC Funders Author Manuscripts

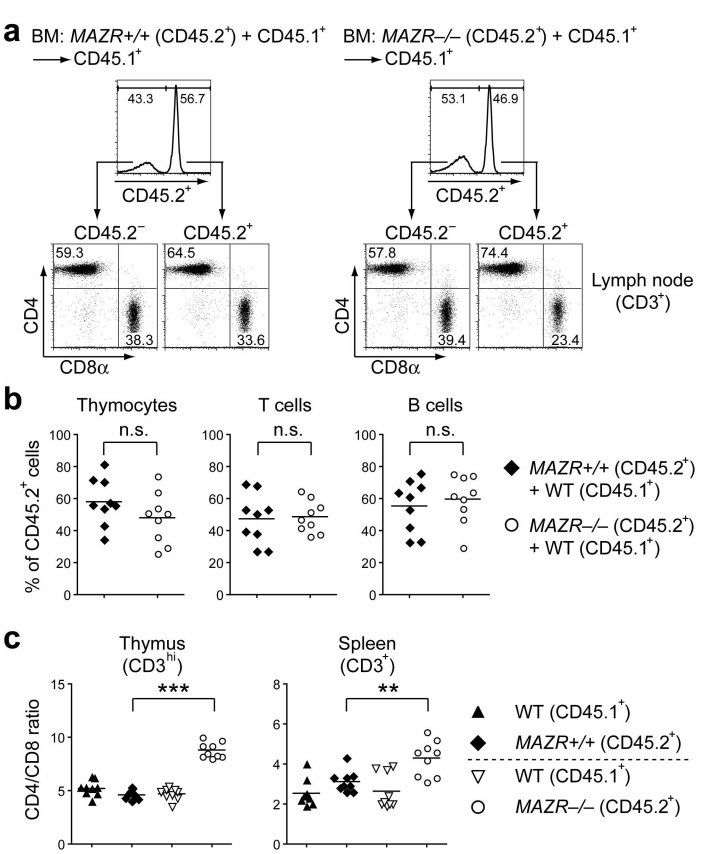

Figure 3.

T cell-intrinsic defects lead to an altered CD4 to CD8 ratio in *Mazr*$^{-/-}$ mice (**a**) *Mazr*$^{+/+}$ (left panel) or *Mazr*$^{-/-}$ (right panel) BM (CD45.2^{+}) was mixed in a 1:1 ratio with wild-type BM (CD45.1^{+}) and injected into irradiated CD45.1^{+} recipients. Histograms show CD45.2 expression on CD3^{+} lymph node cells in BM chimeric mice. Numbers indicate the percentage of CD45.2^{-} and CD45.2^{+} cells. Dot plots indicate CD4 and CD8α expression on CD45.2^{-} and CD45.2^{+} lymph node T cells. Data are representative of 9 mice each. (**b**) Panels show the percentage of CD45.2^{+} thymocytes (left panel), T cells (CD3^{+} splenocytes; middle panel), and B cells (B220^{+} splenocytes; right panel) in BM chimeric mice. (**c**) Diagrams indicate CD4 to CD8 ratio in CD3^{hi} thymocytes (left panel) and CD3^{+} splenocytes (right panel) in the CD45.1^{+} wild-type and CD45.2^{+} *Mazr*$^{+/+}$ or CD45.2^{+} *Mazr*$^{-/-}$ subsets of BM chimeric mice. In **b** and **c**, each symbol represents one mouse, and horizontal lines indicate average value.

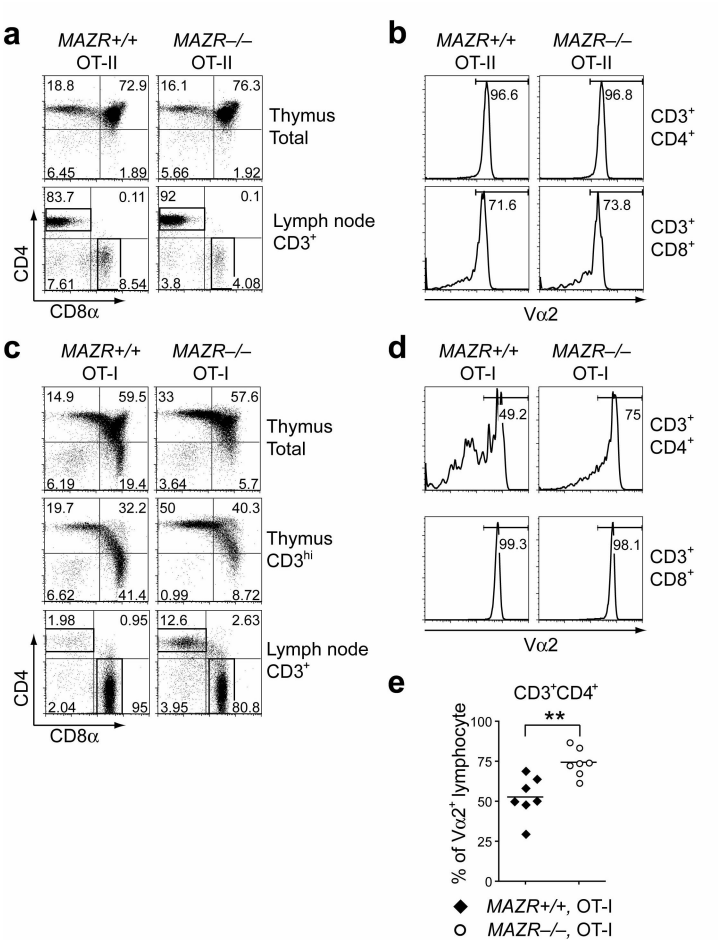

Figure 4.

T cell development in MHC class I- and class II-restricted TCR transgenic mice in the absence of MAZR (**a**) CD4 and CD8 expression pattern on thymocytes and lymph node T cells isolated from *Mazr*$^{+/+}$,OT-II and *Mazr*$^{-/-}$,OT-II littermates. Numbers indicate the percentage of cells in the respective quadrants. Data are representative of 3 independent experiments. (**b**) Histograms show $V_{\alpha}2$ expression on CD4^{+} and CD8^{+} lymph node T cells isolated from *Mazr*$^{+/+}$,OT-II and *Mazr*$^{-/-}$,OT-II littermates. Numbers show the percentage of $V_{\alpha}2^{hi}$ cells within the indicated region. Gating areas for CD4^{+} and CD8^{+} T cell populations are shown in the lower panel of **a**. Data are representative of 3 independent experiments. (**c**) 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. Numbers indicate the percentage of cells in the respective quadrant. Data are representative of 7 independent experiments. (**d**) Histograms show $V_{\alpha}2$ expression on CD4^{+} and CD8^{+} lymph node T cells isolated from *Mazr*$^{+/+}$,OT-I and *Mazr*$^{-/-}$,OT-I littermates. Numbers show the percentage of $V_{\alpha}2^{hi}$ cells within the indicated region. Gating areas for CD4^{+} and CD8^{+} T cell populations are shown in the lower panel of **c**. Data shown are representative of 7 independent mice. (**e**) Panels showing the percentage of $V_{\alpha}2^{hi}$ cells in CD4^{+} lymph node T cells isolated from *Mazr*$^{+/+}$,OT-I and *Mazr*$^{-/-}$,OT-I mice. Each symbol represents one mouse, and horizontal lines indicate average values.

Europe PMC Funders Author Manuscripts

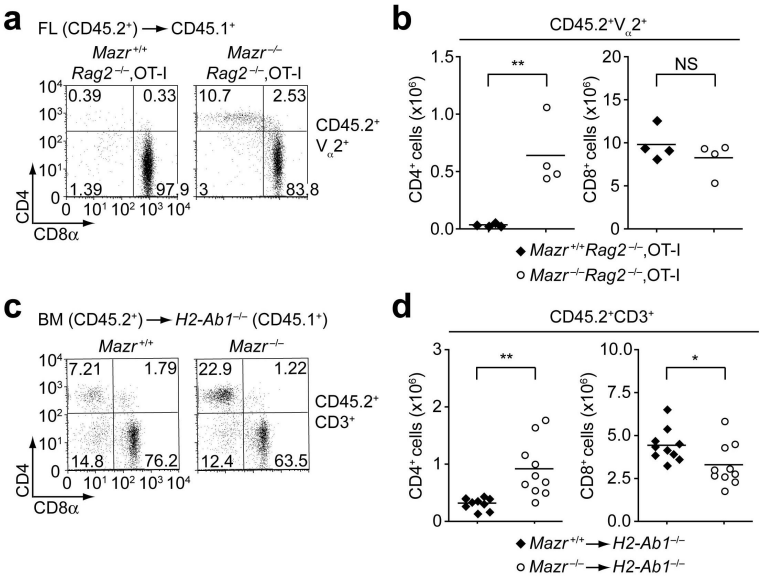

Figure 5.

Redirected differentiation of MHC class I-restricted thymocytes into helper lineage cells in the absence of MAZR. (**a**) CD4 and CD8α expression on lymph node T cells isolated from *Mazr*$^{+/+}$*Rag2*$^{-/-}$,OT-I and *Mazr*$^{-/-}$*Rag2*$^{-/-}$,OT-I fetal liver (FL)-transplanted mice. Cells were gated on the CD45.2$^+$V$_\alpha$2$^+$ population. Numbers indicate the percentage of cells in the respective quadrants. Data are representative of 7 independent experiments. (**b**) Panels show CD45.2$^+$V$_\alpha$2$^+$CD4$^+$ and CD45.2$^+$V$_\alpha$2$^+$CD8$^+$ T cell numbers isolated from the spleens of *Mazr*$^{+/+}$*Rag2*$^{-/-}$,OT-I and *Mazr*$^{-/-}$*Rag2*$^{-/-}$,OT-I FL-transplanted mice. Each symbol represents one mouse, and horizontal lines indicate average values. (**c**) T cell-depleted BM cells from *Mazr*$^{+/+}$ and *Mazr*$^{-/-}$ mice (CD45.2$^+$) were transferred into MHC class II-deficient (*H2-Ab1*$^{-/-}$, CD45.1$^+$) recipient mice. Dot plots shows CD4 and CD8α expression pattern of CD45.2$^+$ CD3$^+$ T cells in the spleen of *Mazr*$^{+/+}$ (left) and *Mazr*$^{-/-}$ (right) BM chimeric MHC class II-deficient mice. To exclude invariant NKT cells, CD1d:PBS57 tetramer-positive cells were excluded from the analysis. Data are representative of 10 independent experiments. (**d**) Diagrams show CD45.2$^+$CD3$^+$CD4$^+$ and CD45.2$^+$CD3$^+$CD8$^+$ T cell numbers isolated from the spleens of *Mazr*$^{+/+}$ and *Mazr*$^{-/-}$ BM chimeric MHC class II-deficient (*H2-Ab1*$^{-/-}$) mice. Each symbol represents one mouse, and horizontal lines indicate average values.

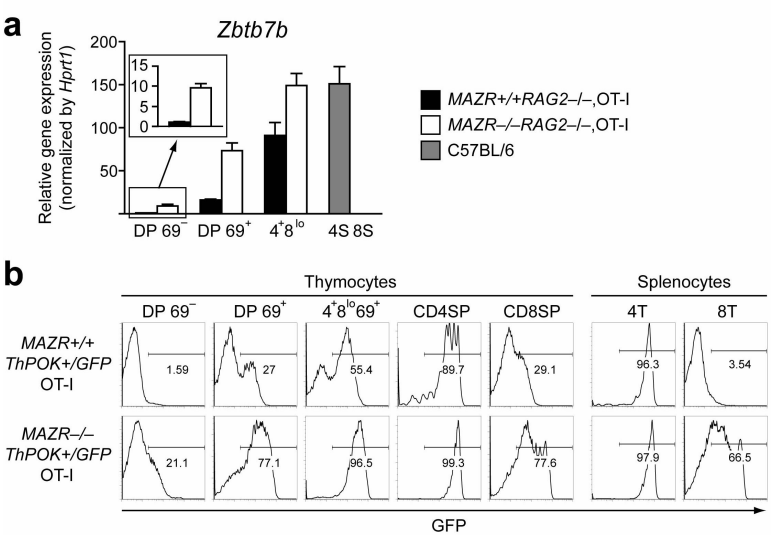

Figure 6.

Derepression of ThPOK in *Mazr*$^{-/-}$*Rag2*$^{-/-}$,OT-I thymocytes and CD8^{+} T cells (**a**) Quantitative real-time PCR showing *ThPOK* expression in DP CD69^{-} (DP 69^{-}), DP CD69^{+} (DP 69^{+}), and CD4^{+}CD8^{lo}CD69^{+} (4^{+}8^{lo}) thymocytes isolated from *Mazr*$^{+/+}$*Rag2*$^{-/-}$,OT-I (black bars) and *Mazr*$^{-/-}$*Rag2*$^{-/-}$,OT-I (white bars) FL-chimeric mice. As a reference, the expression of *ThPOK* in wild-type CD4SP (4S) and CD8SP (8S) thymocytes is shown at the right (gray bars). Values are relative to *Hprt1* expression. *ThPOK* expression in *Mazr*$^{+/+}$*Rag2*$^{-/-}$,OT-I DP CD69^{-} thymocytes is set as 1. Data are representative of 3 independent experiments (**b**) Histograms show GFP expression in DP CD69^{-} (DP 69^{-}), DP CD69^{+} (DP 69^{+}) and CD4^{+}CD8^{lo}CD69^{+} (4^{+}8^{lo}69^{+}) subsets, in CD4SP and CD8SP thymocytes, and in splenic CD4^{+} (4T) and CD8^{+} (8T) T cells. Gating areas are shown in Supplementary Fig. 8a. Upper and lower panels show thymocyte and splenocyte subsets analyzed from *Mazr*$^{+/+}$*ThPOK*$^{+/GFP}$,OT-I and from *Mazr*$^{-/-}$*ThPOK*$^{+/GFP}$,OT-I mice, respectively. Numbers show percentage of cells in the indicated region. Data are representative of 3 independent experiments.

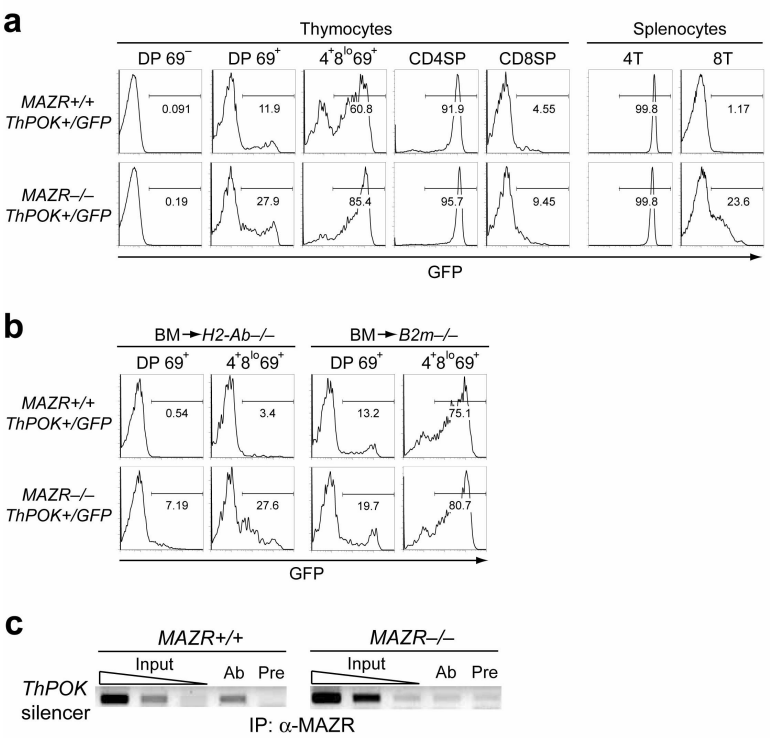

Figure 7.

Derepressed ThPOK expression in MHC class I-signaled *Mazr*$^{-/-}$ thymocytes (**a**) Histograms show GFP expression in DP CD69^{-} (DP 69^{-}), DP CD69^{+} (DP 69^{+}) and CD4^{+}CD8^{lo}CD69^{+} (4^{+}8^{lo}69^{+}) subsets, in CD4SP and CD8SP thymocytes, and in splenic CD4^{+} (4T) and CD8^{+} (8T) T cells. Gating areas are shown in Supplementary Fig. 8b. Upper and lower panels show thymocyte and splenocyte subsets analyzed from *Mazr*$^{+/+}$ *ThPOK*$^{+/GFP}$ and from *Mazr*$^{-/-}$ *ThPOK*$^{+/GFP}$, respectively. Numbers show percentage of cells in the indicated regions. Data are representative of 5 independent experiments. (**b**) T cell-depleted BM cells from *Mazr*$^{+/+}$ *ThPOK*$^{+/GFP}$ and *Mazr*$^{-/-}$ *ThPOK*$^{+/GFP}$ mice were transferred into MHC class II-deficient (*H2-Ab1*$^{-/-}$) or MHC class I-deficient (*B2m*$^{-/-}$) recipient mice. Histograms show GFP expression in DP CD69^{+} (DP 69^{+}) and CD4^{+}CD8^{lo}CD69^{+} (4^{+}8^{lo}69^{+}) thymocyte subsets of *Mazr*$^{+/+}$ *ThPOK*$^{+/GFP}$ (upper panels) and *Mazr*$^{-/-}$ *ThPOK*$^{+/GFP}$ (lower panels) BM chimeric MHC class II- (left) and MHC class I-deficient (right) mice. Gating areas are shown in Supplementary Fig. 8c. Numbers show percentage of cells in the indicated regions. Data are representative of 4 mice from 2 independent experiments. (**c**) Chromatin immunoprecipitation analysis of the *ThPOK* silencer. *Mazr*$^{+/+}$ and *Mazr*$^{-/-}$ thymocytes chromatin was immunoprecipitated with anti-MAZR serum (Ab) or pre-serum (Pre), followed by PCR with primers specific for the *ThPOK* silencer region (Silencer) or for a non-MAZR binding region from the *Cd8ab* gene complex (Ctrl). Input DNA was PCR amplified undiluted or at a dilution of 1:5 or 1:25 (wedges) to ensure PCR quantification in a non-saturated amplification range. Data are representative of three independent experiments.