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MHC-restricted T cell receptor signaling is required for αβ TCR replacement of the pre T cell receptor

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A developmental block is imposed on $CD25^+CD44^-$ thymocytes at the β-selection checkpoint in the absence of the pre T cell receptor (preTCR) α-chain, pTα. Early surface expression of a transgenic αβ TCR has been shown to partially circumvent this block, such that thymocytes progress to the $CD4^+CD8^+$ double-positive stage. We wanted to analyze whether a restricting MHC element is required for αβ TCR-expressing double-negative (DN) thymocytes to overcome the developmental block in pTα-deficient animals. We used the HY-I knock-in model that endows thymocytes with αβ TCR expression in the DN compartment but has the advantage of physiological expression levels, in contrast to conventional TCR transgenes. On a pTα-deficient background, this HY-I TCR transgene 'rescued' $CD25^+CD44^-$ thymocytes from apoptosis and enabled progression to later differentiation stages. On a non-selecting MHC background, however, pTα-deficient HY-I mice presented a pronounced reduction in numbers of splenocytes and thymocytes when compared to animals of selecting MHC genotype, showing that MHC restriction is necessary to drive HY-TCR-mediated rescue of pTα-deficient thymocytes.

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Introduction

For antigen recognition, most T lymphocytes clonally express the heterodimeric αβ T cell receptor (TCR). To generate a diverse T cell repertoire, the genes encoding the variable regions of the TCR are assembled by somatic rearrangement of genomic gene segments during development of the T cells in the thymus. This process is called V(D)J recombination and is crucially dependent on the presence of the proteins RAG1 and RAG2 [1, 2]. Under normal circumstances, the genes for the TCRβ chain are rearranged during the $CD4^-CD8^-$ (double-negative, DN) stage, followed by rearrangement of the genes for the TCRα chain in $CD4^+CD8^+$ (double-positive, DP)-stage thymocytes. Recent findings, however, show that some TCRα chain recombination takes also place during the early DN stage [3]. The DN stage can be further subdivided according to expression of CD25 and CD44. Recombination of TCRβ is initiated in cells of $CD25^+CD44^+$ (DNII) phenotype and peaks at the $CD25^+CD44^-$ (DNIII) stage [4]. Only upon surface expression of a TCRβ chain can thymocytes proceed to the $CD25^-CD44^-$ (DNIV) stage and beyond [3]. Transition through this checkpoint is termed β-selection and requires pairing of the newly generated TCRβ chain with a surrogate TCRα chain, the pre T cell receptor (preTCR)

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Abbreviations: DN: double-negative · **DP:** double-positive · **HYtg:** HY-TCR transgenic · **preTCR:** pre T cell receptor · **pTα:** preTCR α-chain

α-chain, pTα [5]. The resulting heterodimer comprises the surface-expressed preTCR, which is required to signal for termination of recombination at the TCRβ locus, progression to the DP stage, and initiation of recombination at the TCRα locus. Genetic deficiencies of either TCRβ or pTα, the preTCR core components, leads to a block in T cell development at DNIII [3, 6]. A ligand for the preTCR has not been identified and experimental data concerning the necessity of a preTCR ligand remain inconclusive. It appears, however, that the extracellular domain is not required for signaling of the preTCR [7–9]. This led to the hypothesis that a constitutive signal emanates from the preTCR. It was suggested that this constitutive signal required the significant cytoplasmic tail featured by pTα and absent in the TCRα chain [5]. However, experiments using a mutant pTα chain lacking the cytoplasmic portion showed only a slight decrease in thymic cellularity (2–3-fold) compared to mice with WT pTα [8, 10, 11]. Unfortunately, the differences in transgenic pTα expression in these transgenic models meant that the observed transition to the DP stage was either the result of pTα overexpression or of the insignificance of the cytoplasmic tail [12]. Nevertheless, the seemingly normal T cell development in mice expressing a mutant pTα lacking a cytoplasmic tail demonstrates that the cytoplasmic tail is not an absolute requirement for the function of the pTα molecule. This conclusion is supported by the observation that anti-CD3ε antibody treatment [10], a CD4 transgene [13] and an Lck transgene [10] can alleviate the block induced by pTα deficiency. Also the αβ TCR itself, when transgenically expressed at the early DN stage, can partially replace the preTCR [14]. Thus, transgenic expression of the αβ TCR has become a model for understanding the signaling requirements at the β-selection checkpoint. Initially, it was shown that transgenic expression of a full HY-TCR in DN thymocytes allows a fraction of these cells to proceed beyond the block imposed by pTα deficiency [14]. Later, a similar outcome was observed when the MHC class II-restricted AND TCR was used [15]. However, it still remains controversial to what extent the mature αβ TCR can act in place of the preTCR. In some cases, it could replace the preTCR at the β-selection point [14], while in others its expression imposed a developmental block [12, 16]. In addition to its usefulness as a model for understanding the physiological properties of β-selection by the preTCR, it is also currently unclear how, in animals that do not carry a TCR transgene, thymocytes that have acquired αβ TCR expression at the DNII or DNIII stage proceed through the β-selection checkpoint. It seems conceivable that they could either rely on preTCR- or on αβ TCR-mediated signals.

Because preTCR-mediated β-selection appears to depend on constitutive signaling through this surrogate receptor, it has been a matter of debate whether β-selection by αβ TCR additionally depends on the TCR recognizing a ligand. It seems plausible that either mere expression of the αβ TCR at DNII/III or its expression and interaction with its restricting MHC element could be required to imitate the preTCR signal. Two studies indicated that the restricting MHC elements were not required [15, 17], but due to the nature of the used model systems – viral TCR expression, bone marrow chimeras, and adoptive transfer – the authors could not rigorously exclude a contribution of MHC-mediated signals in the experimental outcome.

We therefore wanted to readdress this question in a system well suited to provide new insights. After breeding the HY-I system [18], which expresses the HY-TCR, onto a pTα-deficient background, we wanted to assess the influence of the restricting MHC class I molecule H-2D^b in comparison to the non-restricting MHC class I molecule H-2D^d. However, since the H-2 locus and pTα are located on the same chromosome, we had to screen for a crossover combining H-2^d and the mutated pTα allele, the latter originally made in H-2^b. After identification of such a crossover and further breeding, we observed that in pTα-deficient HY-I mice on a non-selecting H-2^d background the block at the β-selection point was significantly more severe than in HY-I mice on a selecting H-2^b background. Thus, and in contrast to previously published data [15, 17], in preTCR-deficient animals, a differentiation and survival signal emanating from a prematurely expressed HY-TCR appears to be most efficient in the presence of its positively selecting MHC molecules, facilitating optimal transition from DNIII to DNIV.

Results

Premature expression of the HY-TCR in the HY-I model

We have previously reported the generation of the first TCRα insertion model [18] allowing the study of thymocyte development with a transgenic TCRα chain under control of its normal genomic regulatory elements. This strain was generated by introduction of recombined HY-TCR VαJα elements into the appropriate location in the TCRα locus, including deletion of intervening Vα, TCRδ and Jα elements. The genomic location of the introduced VαJα exon is indistinguishable from that of VαJα exons generated by V(D)J recombination (Fig. 1A). Breeding of this HYα$^{I\Delta}$ allele with a conventional transgene encoding the TCRβ chain (HYβ) [19] results in surface expression of the HY-TCR, which recognizes a Y chromosome-encoded peptide in the context of H-2D^b [19, 20]. Although this HY-I model

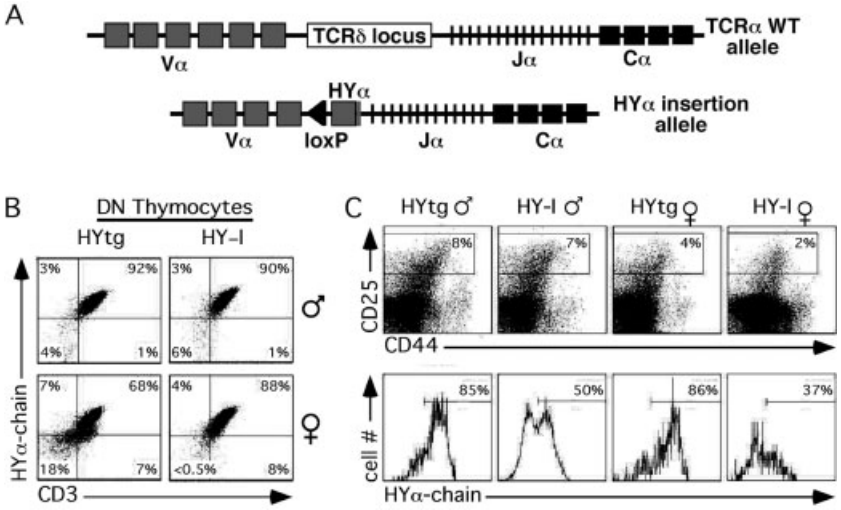

Figure 1. Premature HY-TCR expression in HYtg and HY-I mice. (A) Scheme of the HYα insertion locus in comparison to the WT locus. The HYα insertion locus mimics the product of a Vα6/δ3-to-Jα57 RAG-mediated recombination event. The intervening TCRδ locus and some TCRα elements were deleted *via* Cre-loxP. The VαJα of the HY-TCR α-chain was introduced with its Vα9.1 promoter. (B) HY-TCR expression on DN cells. Thymocytes of male and female HYtg and HY-I mice were stained with antibodies for CD4, CD8, CD3 and the HYα chain and analyzed by flow cytometry. CD3 vs. HYα analysis of live DN cells is shown. (C) Analysis of DN stages and HYα chain expression. Thymocytes of male and female HYtg and HY-I mice were stained for CD4/CD8, CD25, CD44, and T3.70 and analyzed. The dot plots (upper row) show cells not expressing CD4 and CD8. The percentage of cells lying in the selected gate is indicated. The histograms (lower row) show HYα chain expression on $CD4^{-}CD8^{-}CD25^{high}$ cells according to the gate shown in the upper row. The percentage of cells lying in the indicated gate is given within the plots.

expresses the TCRα chain under physiological transcriptional control, we observed early expression of the full αβ TCR in the DN compartment [18] (Fig. 1B). Previous reports have shown that also the transgenic TCR of conventional HY-TCR transgenic (HYtg) mice was first expressed at this stage, in contrast to WT thymocytes that at that time in development only express TCRβ. To further investigate the premature expression of the TCRα chain in HY-I mice, we characterized the onset of TCRα chain expression in more detail. Within the DN compartment, we were interested in finding expression of the HY-TCR on a high percentage of thymocytes, as defined by surface expression of CD3 and HYα chain (Fig. 1B). Using thymocyte expression of CD25 and CD44, we then further dissected HYα chain expression within the cells of the DN compartment in the two strains. We observed that also in the HY-I model, in which α-chain expression is controlled by the endogenous transcription control elements, a significant proportion of $CD25^{high}$ thymocytes express the full HY-TCR (Fig. 1C). In contrast, β-chain rearrangement at the $CD25^{high}$ stage in WT mice precedes α-chain rearrangement and thus full TCR expression, which takes place at the DP stage. It seemed, however, that the proportion of HYα-expressing cells in the knock-in HY-I mice was lower than in conventional HYtg mice (Fig. 1C). We also observed that male mice of the knock-in model express the HY-α chain on a larger proportion of $CD25^{high}$ thymocytes compared to females (Fig. 1C), which may be the result of the presence of the cognate ligand.

Rescue of T cell development by HYtg and HY-I transgenes in pTα deficiency

In WT mice, successful rearrangement of the β-chain locus at DNIII is followed by a down-regulation of CD25 expression and induction of coreceptor expression. Cells with out-of-frame rearrangement products of both TCRβ alleles will remain in the DNIII compartment and die eventually of apoptosis. Thus, in WT mice a pronounced DNIII compartment is observed, in contrast to TCRβ- and αβ TCR-transgenic mice, in which cells move quickly from DNIII to DNIV (Fig. 2 and data not shown) [14]. pTα deficiency, on the other hand, results in an almost complete developmental block at the DNIII-to-DNIV transition and is characterized by an increased proportion of DN thymocytes expressing CD25 (Fig. 2D) [6]. The subsequent developmental stages show a pronounced reduction in cell number, such that there is an acute reduction of DP and single-positive (SP) thymocytes and peripheral $CD4^{+}$ and $CD8^{+}$ T cells (Fig. 2C) [6]. Transgenic expression of the HY-TCR within DN cells was previously shown to lead to efficient development of thymocytes in the absence of a preTCR, replenishing the cellularity among the subsequent developmental stages. We asked whether the prema-

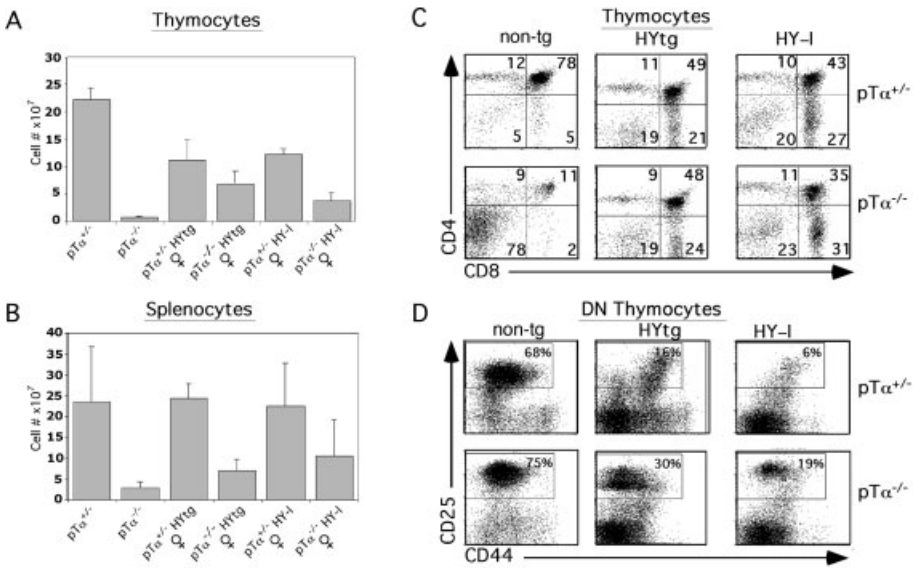

Figure 2. Rescue of T cell development in pTα-deficient mice by HYtg and HY-I transgenes. (A, B) Absolute cell numbers and the respective standard deviations for thymi (A) and spleens (B) of the indicated mouse strains (n >5) are shown. (C, D) The thymocytes were stained for CD4 and CD8 (C) or CD4/CD8, CD25, and CD44 (D) and analyzed by FACS. Shown are either live lymphocytes (C) or $CD4^-CD8^-$ live lymphocytes (D). Compartmental percentages are given in the respective gates.

turely expressed TCRα chain in the HY-I model was able to rescue T cell development in the absence of pTα, as observed for the HYtg model. This was of interest, since HY-I mice demonstrated a decreased proportion of HY-TCR expressing cells at the DNII/III stage when compared to HYtg mice, suggesting a later onset of transgenic α-chain expression and a possible decrease in efficiency of HY-TCR-mediated thymocyte rescue. We therefore crossed the HY-I and HYtg models onto the pTα-deficient background and analyzed thymocytes and splenocytes by flow cytometry.

As observed before, the conventional transgenic model mediated efficient rescue through the β-selection block into the DP compartment, leading to the presence of DP, SP, and peripheral T cells (Fig. 2). The presence of the HY transgene increased cellularity in the thymus by 13-fold, and by 3-fold in the spleen (Fig. 2A, B). Also upon analysis of the HY-I model on the pTα-deficient background, we could detect increased numbers of cells in all thymocyte compartments following the β-selection block (Fig. 2C) and the spleen (Fig. 2B). Thymocyte numbers were increased sevenfold in HY-I pTα-deficient mice as compared to mice that were pTα deficient only (Fig. 2A). Likewise, T cell counts in the spleen increased by fourfold (Fig. 2B). Thus, both the HY-I and the HYtg transgenes alleviate the β-selection block in pTα-deficient animals, with the efficiency of the HY-I knock-in to rescue thymic development of pTα-deficient deficient mice appearing to be somewhat limited compared to the conventional HY transgene. The increased proportion of $CD25^{high}$ thymocytes in the DN compartment of TCR-transgenic pTα-deficient mice reaffirms, however, the position of pTα as a principle factor in mediating the DN-to-DP transition of thymocytes (Fig. 2D). Even in the presence of a prematurely expressed HY-TCR transgene, development is more efficient in the presence of a functional pTα locus, indicating that both the preTCR and the prematurely expressed αβ TCR contribute to the enhanced β-selec- observed in TCR-transgenic mouse strains (Fig. 2D).

Rescue of thymic development in TCR-transgenic pTα-deficient animals depends on MHC restriction

It is a matter of debate whether or not recognition of the restricting MHC molecules by the TCR is required to signal further development on a pTα-deficient background. To investigate whether the HY-TCR could still ameliorate the developmental block at the β-selection point in the absence of its restricting MHC element, we bred the $pT\alpha^{-/-}$ HY-I model onto the $H\text{-}2^d$ background, which does not support positive selection of the HY-TCR [21]. Because the MHC and the pTα gene are located approximately 11 megabases apart on the same chromosome (www.ensembl.org) [22], mice had to be screened for a recombinant harboring the pTα-deficient allele on the same chromosome as $H\text{-}2^d$. To this end, pTα-deficient mice were crossed with BALB/c mice, such that the resulting F1 generation was heterozygous for pTα deficiency and $H\text{-}2^{d/b}$. F1 animals were intercrossed and offspring were screened to obtain $pT\alpha^{-/-}$ $H\text{-}2^{d/b}$ or

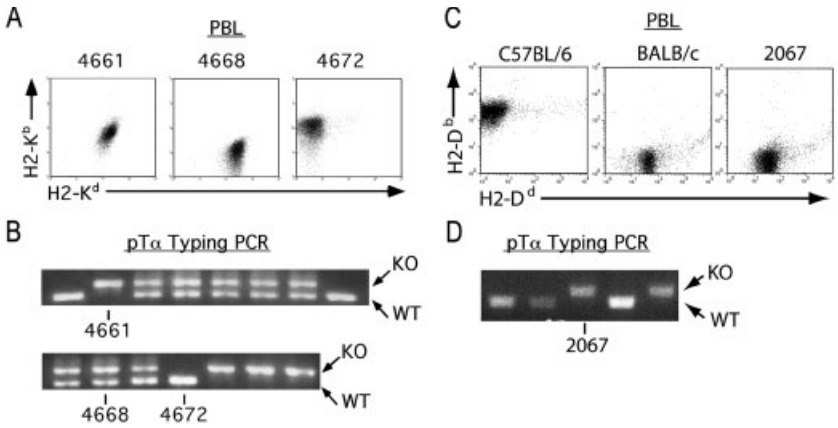

Figure 3. A crossover brings H-2^d and pTα deficiency into cis. F2 animals of an intercross of pTα-deficient HY-I (H-2^b) and BALB/c (H-2^d) mice were typed for H-2K^b and H-2K^d by FACS analysis of peripheral blood lymphocytes (A) and for the presence of pTα WT and deficient alleles by PCR (B). A crossover was identified by either the H-2K$^{d/b}$ pTα$^{-/-}$ or the H-2K$^{d/d}$ pTα$^{+/-}$ genotypes as shown for the mice with the numbers 4661 and 4668, respectively. An H-2K$^{b/b}$ mouse (4672) is shown as negative control of the H-2K^d staining. After breeding to homozygosity, the crossover was also confirmed for the H-2D genes by additional FACS analysis, as exemplified by mouse 2067 (C, D).

pTα$^{+/-}$ H-2$^{d/d}$ animals, which would carry the desired crossover event. Mice bearing a recombinant chromosome were found (Fig. 3A, B) with a frequency of 4% (3 out of 83 screened animals). Their pTα-deficient, H-2^d chromosome was bred to homozygosity (Fig. 3C, D); the HYα$^{I\Delta}$ and HYβtg alleles were retained during breeding. To analyze the requirement for the restricting MHC molecules on β-selection by an αβ TCR in the absence of pTα, we compared thymic development in pTα$^{-/-}$ H-2$^{d/d}$ HY-I and pTα$^{-/-}$ H-2$^{d/b}$ HY-I animals. We found that thymic cellularity was reduced fourfold in the absence of the restricting MHC molecules (Fig. 4A). This reduction was mostly due to a greatly impaired transition from DN to DP thymocytes as exemplified by a highly significant reduction in the number of DP thymocytes in HY-TCR-bearing mice on an H-2D^d compared to an H-2D^b background (Fig. 4B, D). This reduction in DP and total thymic cell numbers translated into a profound reduction in splenic cellularity (Fig. 4C) and a higher percentage of non-T cells in the LN (Fig. 4D). Although such a profound phenotype with respect to whole thymocyte numbers, generation of DP thymocytes, and even peripheral T cell numbers was observed, the absence of the restricting MHC element did not result in a significant increase in the percentage of CD25-expressing DN cells, usually another indicator of less effective DNIII-to-DNIV transition (Fig. 2D and 4E, F). In summary, αβ TCR-mediated alleviation of the developmental block seen in pTα-deficient animals appears to be more efficient in the presence of the MHC molecules to which the αβ TCR is restricted.

Discussion

Until now it was an open question whether or not the αβ TCR, when replacing the preTCR, requires binding to its appropriate MHC ligand. Aside from its relevance to the understanding of β-selection by signaling through the preTCR, this question has recently acquired additional significance after the identification of a population of thymocytes that rearrange and express both TCR genes, TCRα and TCRβ, already at the early DNII and DNIII stages [3]. These early αβ TCR expressers could potentially use the αβ TCR or the preTCR for achieving β-selection. In addition, as discussed below, it is still not understood what the differences are between the preTCR and the αβ TCR in respect to signaling β-selection. An MHC requirement for the αβ TCR but not the preTCR at this stage would significantly change the way experimental data dealing with this topic has to be interpreted. With respect to the data available, it has been thought that both TCR complexes signal β-selection cell-autonomously, but if the αβ TCR in fact required self-MHC at this stage it would rely on the availability of this ligand. This availability may differ for each developing cell, which in turn would affect the quantitative outcome of β-selection as measured in numbers of cells found in stages before and after β-selection.

The data provided in this manuscript show clearly that for efficiently overcoming a block in development imposed by the absence of a functional preTCR, the αβ TCR requires the presence of its restricting MHC element. We performed our analysis in a set-up generated through breeding together the respective alleles, in contrast to the data published so far which had revealed no such requirement.

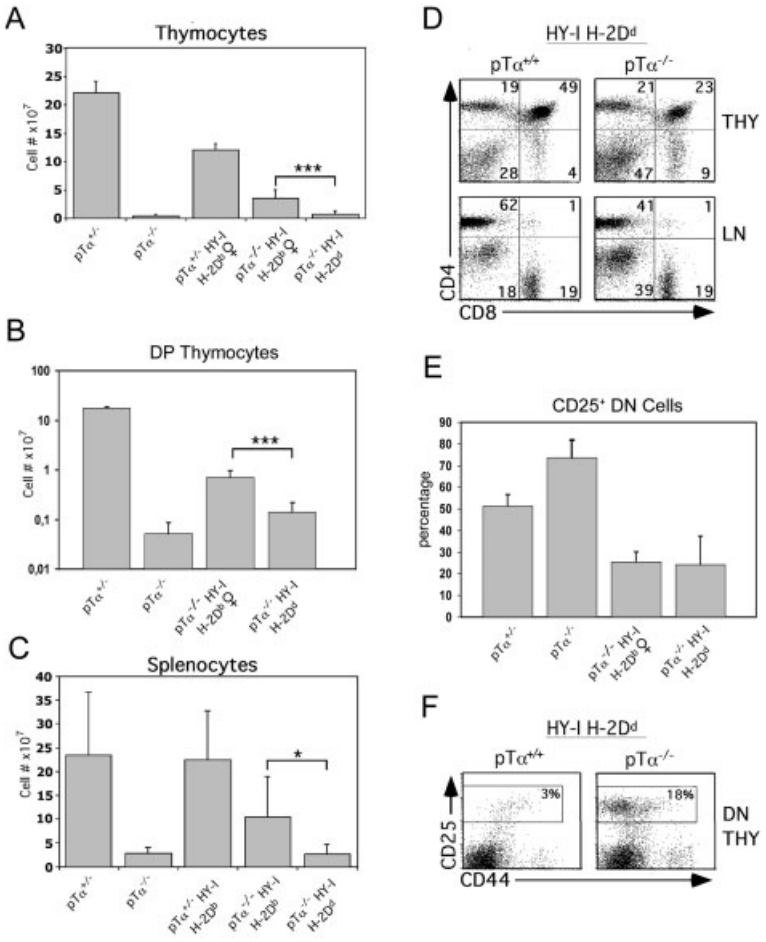

Figure 4. Rescue of T cell development by a TCR transgene in pTα deficiency depends on the restricting MHC element. (A–C) Absolute cell numbers of (A) total thymocytes, (B) DP thymocytes and (C) splenocytes are shown for the indicated strains and respective MHC restriction (n = 5). Statistical significance was calculated using Student's t-test. (D) CD4 and CD8 stainings were performed on thymocytes and LN-derived lymphocytes in the presence or absence of the pTα for the HY-I H-2D^d strain. Percentages of thymocytes and T cells are given in the representative quadrants. (E) Proportion of CD25$^+$ thymocytes in the DN compartment of the HY-I H-2D^d and HY-I H-2D^b strains are compared in the absence of pTα. (F) An additional CD44 CD25 staining was carried out to illustrate the DN compartment of the indicated strain in the presence or absence of pTα. Percentages of CD25$^+$ thymocytes are shown in the representative gates. THY: thymus; LN: lymph nodes; DN: CD4$^-$CD8$^-$. * $p < 0.5$, *** $p < 0.01$ in Student's t-test.

One group had addressed the question of the relevance of TCR-MHC interaction for β-selection through αβ TCR by retroviral gene transfer of a pigeon cytochrome C-specific TCRα chain into pTα-deficient fetal thymocytes [17]. Upon organ culture in MHC class I/II-deficient or -proficient thymic lobes, significant progression to the DP stage was observed in both environments. However, since the TCRβ chain was not co-transduced, the specificity of any expressed TCR was unknown. It is conceivable that some TCR recognized non-conventional MHC molecules or MHC class I molecules on the surface of the transduced thymocytes, thus resulting in a selecting signal that allowed developmental progression. Another group [15] assessed ligand dependence of the αβ TCR in mediating DNIII-to-DNIV transition by use of bone marrow chimeras. pTα$^{-/-}$ HYtg or pTα$^{-/-}$ AND bone marrow was transferred into MHC class I- and class II-deficient hosts, respectively. Because these experiments could not exclude MHC expression by the donor bone marrow-derived cells, the authors also conducted an experiment using intrathymic injection of DN cells from one of their TCR-transgenic models (the MHC class II-restricted AND TCR) into MHC class II-deficient thymi and could once again not detect any evidence for an influence of MHC on development αβ TCR-expressing DN cells into DP cells.

Because the aforementioned experiments involved the use of conventional TCR-transgenic models or retroviral gene transfer – systems that are non-physiological in terms of expression levels of the transgenic TCR and also involved artificial and compli-

cated experimental set-ups – we reassessed the question of the involvement of restricting MHC in the contribution to the signal that allows a transgenic αβ TCR to replace the preTCR at the β-selection checkpoint. To exclude an artificial contribution to our observations from non-physiological TCR gene expression – a phenomenon that has been observed in the analysis of conventional pTα-transgenic mouse strains [12] – we wanted to use the HY-I knock-in model. To simplify the analysis and to keep the cells in an intact *in vivo* environment, we decided to breed the pTα deficiency into a different MHC class I background. This allowed us to investigate the different behavior of HY-TCR-expressing pTα-deficient DN cells in the presence or absence of the restricting MHC element. First, we confirmed that the premature expression of the αβ TCR in the HY-I insertion model also conferred alleviation of the β-selection block in pTα-deficient animals (Fig. 2). Subsequently, we excluded that αγ TCR dimers with the capacity to signal transition to the DP stage [23] contributed significantly to β-selection, by showing that the block at DNIII remained in pTα-deficient mice carrying solely the HYα chain (data not shown). We then crossed the HY-I system onto pTα deficiency in the non-selecting H-2D$^{d/d}$ background (Fig. 3). The comparison of thymic development in HY-I pTα$^{-/-}$ mice in the presence and absence of the restricting MHC element revealed a significant contribution of restricting MHC-derived signaling at the β-selection checkpoint. Cellularities of the DP compartment, the whole thymus and spleen were lower in the H-2D^{d} background compared to the H-2D^{b} background (Fig. 4). It seems unlikely that this is a result of the changed genetic background other than the MHC loci as we had shown thymic cellularity before to be similar in both backgrounds in the presence of a WT pTα allele [18]. It is interesting to note that, despite the observed differences in efficacy of β-selec-, thymic development is not completely disrupted in the absence of selecting ligand. This cannot be attributed to the formation of αγ TCR dimers because, as mentioned above, we could not detect any rescue of T cell development by breeding of the TCRα insertion transgene alone onto pTα deficiency. Some development of T cell precursors to the DP stage and further in the absence of pTα and the selecting MHC may be facilitated by recombination of the α-chain genes at the DN stage [3], thus generating specificities that could possibly be of a type that facilitates β-selection. Some remaining selection may also be the result of γδ TCR-mediated β-selection, as shown before [14].

In conclusion, our results contradict the data of Erman *et al.* [15] in that we do observe an influence of MHC on the ability of αβ TCR to signal transition through the β-selection block in pTα deficiency. The reason for this observation can lie (a) in the use of different TCR transgenes – Erman *et al.* used the HY-TCR system in all but their most rigorous experiments; (b) in the expression level of the transgenic TCR – supported by the fact that expression levels and timing of pTα transgenes strongly influence the observed effect on T cell development [12]; or (c) in the possible presence of MHC class II-expressing cells after intrathymic transfer of MHC class II-negative precursors of dendritic cells or other APC – indicated by the fact that a certain subpopulation of DN thymocytes can differentiate into MHC class II-expressing dendritic cells [24].

Our data therefore support a model of preTCR-mediated β-selection that involves a constitutive signal of at least medium strength. Thus, signal strength does not only determine the outcome of positive and negative selection of DP cells; it appears also to be an important feature of β-selection. It has been suggested that strong and weak signals at the β-selection checkpoint favor development towards the γδ and αβ T cell lineages, respectively [25, 26]. Our data is in agreement with this conclusion in that a certain minimal signal strength is required for facilitating β-selection through the αβ (HY-) TCR. The observation, that HY-TCR-transgenic males in the presence of restricting MHC develop a larger thymic population of DN HY-TCR-expressing cells, probably resembling γδ T cells [27], would support the theory of distinct TCR signaling thresholds for the two T cell types, modulated by other additional signals like the Notch system. Nevertheless, it remains to be shown whether or not the preTCR generates a signal distinct from the αβ TCR or whether the only difference between TCR signaling and preTCR signaling at the β-selection point lies in the signal strength. Because we find a dependence of β-selection by αβ TCR on the presence of the restricting MHC, our data support the signal strength hypothesis. Nevertheless, the HY-TCR in the presence of H-2D^{b} does not permit β-selection with the same efficiency as the preTCR. This may be explained by either a failure of delivering a signal of the right strength in most cells or indeed by a specific signal that only the preTCR delivers. It is possible that this specific signal can be replaced by other mechanisms, but with a lower probability to occur for each individual cell. Another level of complexity that we have now added to the picture is the fact that the αβ TCR but not the preTCR obviously needs to encounter a ligand for signaling β-selection. Thus, cells that depend on αβ TCR for continuing the differentiation process need to encounter a factor outside the respective cell. This factor, self-MHC, is not evenly distributed in the thymus and not always available to the same degree to every individual developing thymocyte, a fact that may already account for the different efficiencies of αβ TCR *versus* preTCR in mediating the same biological process, *e.g.* β-selection. Thus, our finding of MHC restriction during β-selection

by αβ TCR changes significantly our view of understanding the differences between the preTCR and the αβ TCR. In addition, our data now build the basis for further experiments that could unravel the different options regarding the mode of action of αβ TCR and preTCR at the β-selection point.

Materials and methods

Mouse maintenance and genotyping

Mice were kept in barrier and SPF animal facilities according to institutional guidelines. DNA for typing was prepared from tail biopsies. The presence of the different alleles was tested by PCR using the following primer pairs: for HYβ tg 5′-cac atg gag gct gca gtc ac-3′ and 5′-gtt tct gca ctg tta tca cc-3′ (307 bp), for $HY\alpha^{I\Delta}$ 5′-cag gca cca cag acc atc ctt gc-3′ and 5′-cca gac agt tcc tac tac acc acg ttc-3′ (947 bp), for pTα 5′-tca cac tgc tgg tag atg gaa gg-3′ and 5′-ggc tca aga gat aac ctg aac cat g-3′ and 5′-gtt tgc tcg aca ttg ggt gg-3′ (KO 625 bp and WT 532 bp).

Flow cytometry

Single-cell suspensions were stained with FITC-, PE-, Cychrome, APC-, and biotin-conjugated antibodies as described [28]. The following antibodies supplied by BD Biosciences were used: anti-CD44-FITC, anti-CD25-PE, anti-TCRβ-PE and anti-CD4-Cychrome. Streptavidin (SA)-Cy7-PE (Caltag) and SA-Cychrome (BD Biosciences) were used to reveal biotin-coupled antibodies. The following antibodies were prepared in our laboratory: anti-HYα-biotin (T3.70), anti-CD4-APC (GK1.5-4) and anti-CD8-APC (53–6.7).

Dead cells were excluded from analysis by either propidium iodide (PI; 0.2 μg/mL; Molecular Probes) or TOPRO-3 (10 nM; Molecular Probes) staining. Data were acquired on a FACSCalibur (BD Biosciences) and analyzed with CellQuest software (BD Biosciences).

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References

1 **Mombaerts, I. J., Johnson, R. S., Herrup, K., Tonegawa, S and Papaionnou, V. E.,** RAG-1 deficient mice have no mature B and T lymphocytes. *Cell* 1992. **68:** 869–877.

2 **Shinkai, Y., Rathbun, G., Lam, K. P., Oltz, E. M., Stewart, V., Mendelsohn, M., Charron, J. *et al.*,** RAG-2-deficient mice lack mature lymphocytes owing to inability to initiate V(D)J rearrangement. *Cell* 1992. **68:** 855–867.

3 **Aifantis, I., Bassing, C. H., Garbe, A. I., Sawai, K., Alt, F. W. and von Boehmer, H.,** The E delta enhancer controls the generation of $CD4^-$ $CD8^-$ alphabetaTCR-expressing T cells that can give rise to different lineages of alphabeta T cells. *J. Exp. Med.* 2006. **203:** 1543–1550.

4 **Kisielow, P. and von Boehmer, H.,** Development and selection of T cells: Facts and puzzles. *Adv. Immunol.* 1995. **58:** 87–209.

5 **Saint-Ruf, C., Ungewiss, K., Groettrup, M., Bruno, L., Fehling, H. J. and von Boehmer, H.,** Analysis and expression of a cloned pre-T cell receptor gene. *Science* 1994. **266:** 1208–1212.

6 **Fehling, H. J., Krotkova, A., Saint-Ruf, C. and von Boehmer, H.,** Crucial role of the pre-T-cell receptor alpha gene in development of alpha beta but not gamma delta T cells. *Nature* 1995. **375:** 795–798.

7 **Irving, B. A., Alt, F. W. and Killeen, N.,** Thymocyte development in the absence of pre-T cell receptor extracellular immunoglobulin domains. *Science* 1998. **280:** 905–908.

8 **Gibbons, D., Douglas, N. C., Barber, D. F., Liu, Q., Sullo, R., Geng, L., Fehling, H. J. *et al.*,** The biological activity of natural and mutant pTalpha alleles. *J. Exp. Med.* 2001. **194:** 695–703.

9 **Xu, Y., Davidson, L., Alt, F. W. and Baltimore, D.,** Function of the pre-T-cell receptor alpha chain in T-cell development and allelic exclusion at the T-cell receptor beta locus. *Proc. Natl. Acad. Sci. USA* 1996. **93:** 2169–2173.

10 **Fehling, H. J., Iritani, B. M., Krotkova, A., Forbush, K. A., Laplace, C., Perlmutter, R. M. and von Boehmer, H.,** Restoration of thymopoiesis in pT $alpha^{-/-}$ mice by anti-CD3epsilon antibody treatment or with transgenes encoding activated Lck or tailless pT alpha. *Immunity* 1997. **6:** 703–714.

11 **Aifantis, I., Borowski, C., Gounari, F., Lacorazza, H. D., Nikolich-Zugich, J. and von Boehmer, H.,** A critical role for the cytoplasmic tail of pTalpha in T lymphocyte development. *Nat. Immunol.* 2002. **3:** 483–488.

12 **Lacorazza, H. D., Tucek-Szabo, C., Vasovic, L. V., Remus, K. and Nikolich-Zugich, J.,** Premature TCR alpha beta expression and signaling in early thymocytes impair thymocyte expansion and partially block their development. *J. Immunol.* 2001. **166:** 3184–3193.

13 **Norment, A. M., Forbush, K. A., Nguyen, N., Malissen, M. and Perlmutter, R. M.,** Replacement of pre-T cell receptor signaling functions by the CD4 coreceptor. *J. Exp. Med.* 1997. **185:** 121–130.

14 **Buer, J., Aifantis, I., DiSanto, J. P., Fehling, H. J. and von Boehmer, H.,** Role of different T cell receptors in the development of pre-T cells. *J. Exp. Med.* 1997. **185:** 1541–1547.

15 **Erman, B., Guinter, T. I. and Singer, A.,** Defined alphabeta T cell receptors with distinct ligand specificities do not require those ligands to signal double negative thymocyte differentiation. *J. Exp. Med.* 2004. **199:** 1719–1724.

16 **Terrence, K., Pavlovich, C. P., Matechak, E. O. and Fowlkes, B. J.,** Premature expression of T cell receptor (TCR)αβ suppresses TCRγδ gene rearrangement but permits development of γδ lineage T cells. *J. Exp. Med.* 2000. **192:** 537–548.

17 **Haks, M. C., Belkowski, S. M., Ciofani, M., Rhodes, M., Lefebvre, J. M., Trop, S., Hugo, P. *et al.*,** Low activation threshold as a mechanism for ligand-independent signaling in pre-T cells. *J. Immunol.* 2003. **170:** 2853–2861.

18 **Buch, T., Rieux-Laucat, F., Forster, I. and Rajewsky, K.,** Failure of HY-specific thymocytes to escape negative selection by receptor editing. *Immunity* 2002. **16:** 707–718.

19 **Uematsu, Y., Ryser, S., Dembic, Z., Borgulya, P., Krimpenfort, P., Berns, A., von Boehmer, H. and Steinmetz, M.,** In transgenic mice the introduced functional T cell receptor beta gene prevents expression of endogenous beta genes. *Cell* 1988. **52:** 831–841.

20 **Markiewitz, M. A., Girao, C., Opferman, J. T., Sun, J., Hu, Q., Agulnik, A. A., Bishop, C. E. *et al.*,** Long-term T cell memory requires the surface expression of self-peptide/major histocompatibility complex molecules. *Proc. Natl. Acad. Sci. USA* 1998. **95:** 3065–3070.

21 **Kisielow, P., Teh, H. S., Bluthmann, H. and von Boehmer, H.,** Positive selection of antigen-specific T cells in thymus by restricting MHC molecules. *Nature* 1988. **335:** 730–733.

22 **Fehling, H. J., Laplace, C., Mattei, M. G., Saint-Ruf, C. and von Boehmer, H.,** Genomic structure and chromosomal location of the mouse pre-T-cell receptor alpha gene. *Immunogenetics* 1995. **42:** 275–281.

23 **Erman, B., Feigenbaum, L., Coligan, J. E. and Singer, A.,** Early TCRalpha expression generates TCRalphagamma complexes that signal the DN-to-DP transition and impair development. *Nat. Immunol.* 2002. **3:** 564–569.

24 **Shimizu, C., Kawamoto, H., Yamashita, M., Kimura, M., Kondou, E., Kaneko, Y., Okada, S. *et al.*,** Progression of T cell lineage restriction in the earliest subpopulation of murine adult thymus visualized by the expression of *lck* proximal promoter activity. *Int. Immunol.* 2001. **13:** 105–117.

25 **Hayes, S. M., Li, L. and Love, P. E.,** TCR signal strength influences alphabeta/gammadelta lineage fate. *Immunity* 2005. **22:** 583–593.

26 **Haks, M. C., Lefebvre, J. M., Lauritsen, J. P., Carleton, M., Rhodes, M., Miyazaki, T., Kappes, D. J. and Wiest, D. L.,** Attenuation of gamma-deltaTCR signaling efficiently diverts thymocytes to the alphabeta lineage. *Immunity* 2005. **22:** 595–606.

27 **Bruno, L., Fehling, H. J. and von Boehmer, H.,** The alpha beta T cell receptor can replace the gamma delta receptor in the development of gamma delta lineage cells. *Immunity* 1996. **5:** 343–352.

28 **Förster, I. and Rajewsky, K.,** Expansion and functional activity of Ly-1$^+$ B cells upon transfer of peritoneal cells into allotype-congenic newborn mice. *Eur. J. Immunol.* 1987. **17:** 521–528.

