Brief Definitive Report

Defined αβ T Cell Receptors with Distinct Ligand Specificities Do Not Require Those Ligands to Signal Double Negative Thymocyte Differentiation

Batu Erman, Terry I. Guinter, and Alfred Singer

Experimental Immunology Branch, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892

Abstract

During T cell development in the thymus, pre–T cell receptor (TCR) complexes signal CD4$^-$ CD8$^-$ (double negative [DN]) thymocytes to differentiate into CD4$^+$ CD8$^+$ (double positive [DP]) thymocytes, and they generate such signals without apparent ligand engagements. Although ligand-independent signaling is unusual and might be unique to the pre-TCR, it is possible that other TCR complexes such as αβ TCR or αγ TCR might also be able to signal the DN to DP transition in the absence of ligand engagement if they were expressed on DN thymocytes. Although αγ TCR complexes efficiently signal DN thymocyte differentiation, it is not yet certain if αβ TCR complexes are also capable of signaling DN thymocyte differentiation, nor is it certain if such signaling is dependent upon ligand engagement. This study has addressed these questions by expressing defined αβ TCR transgenes in recombination activating gene $2^{-/-}$ pre-T$\alpha^{-/-}$ double deficient mice. In such double deficient mice, the only antigen receptors that can be expressed are those encoded by the αβ TCR transgenes. In this way, this study definitively demonstrates that αβ TCR can in fact signal the DN to DP transition. In addition, this study demonstrates that transgenic αβ TCRs signal the DN to DP transition even in the absence of their specific MHC–peptide ligands.

Key words: DN to DP transition • αβ TCR transgene • ligand-independent signaling • pre-TCR/αγ TCR

Introduction

Lymphocytes respond to their environment by integrating signals generated by interaction of plasma membrane receptors with extracellular ligands. Mature T lymphocytes use the multicomponent TCR to respond to their ligands that are MHC–peptide complexes. In developing αβ lineage T cells, rearrangement and expression of TCRβ genes initiate at the CD4$^-$ CD8$^-$ (double negative [DN]) stage of thymocyte differentiation. DN thymocytes differentiate into CD4$^+$ CD8$^+$ (double positive [DP]) cells if they are signaled by pre-TCR complexes that consist of newly generated TCRβ proteins associated with nonrearranging pre-Tα chains and CD3 components (1). However, it is not known how pre-TCR signals are generated. Because pre-TCR complexes do not require an extracellular domain to transduce signals in DN cells, their ability to transduce signals in DN thymocytes might be ligand independent (2, 3). In fact, unlike the αβ TCR, the pre-TCR has no known ligands.

Whether ligand-independent signaling by the pre-TCR is a property unique to this receptor or a general property of DN thymocytes is a matter of debate. The ability of the pre-TCR to localize in lipid rafts in the absence of ligand engagement has argued for the uniqueness of this receptor (4). The palmitoylation of a juxtamembrane cysteine residue uniquely present on pre-Tα chains was initially thought to be necessary for pre-TCR raft associations, but this residue has recently been shown to be dispensable for pre-TCR signaling (5–8). Alternatively, there are data that support the perspective that ligand-independent signaling is a general property of antigen receptors on DN thymocytes. Haks et al. (7) have shown that retrovirally induced TCRα chains successfully substituted for pre-Tα in signaling DN thymocytes to differentiate into DP cells, and they did so whether or not MHC ligands were present. These observations demonstrated that engagement of MHC ligands was not required for TCRα-dependent signaling in pre-T$\alpha^{-/-}$ DN thymocytes, but the receptor complexes doing the sig-

Address correspondence to Alfred Singer, Experimental Immunology Branch, National Cancer Institute, National Institutes of Health, Building 10, Room 4B36, Bethesda, MD 20892. Phone: (301) 496-5461; Fax: (301) 496-0887; email: SingerA@nih.gov

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naling were not necessarily αβ TCR, as they might have been αγ TCR complexes. Indeed, TCRα expression in RAG^+ DN thymocytes promotes formation of novel αγ TCR complexes that are very efficient at signaling DN thymocytes to differentiate into DP thymocytes (9). Precisely the same caveat also limits conclusions that can be drawn from other experiments in which transgenic (Tg) αβ TCRs were expressed in RAG^+ pre-$T\alpha^{-/-}$ DN thymocytes, as the possibility was not excluded that alternative αγ TCR complexes, composed of Tg TCRα and endogenously encoded TCRγ proteins, were in fact the receptor complexes that signaled the DN to DP transition in these αβ TCR Tg mice (10). In experiments in which bone marrow progenitors from MHC I–specific αβ TCR Tg mice differentiated into DP thymocytes in MHC I–deficient recipients, it was possible that the DN to DP transition was signaled by pre-TCR (endogenous pre-Tα paired with Tg TCRβ) rather than Tg αβ TCR complexes (11–13). Thus, whether αβ TCRs are able to signal the DN to DP transition has not yet been definitively demonstrated, nor has their dependence or independence on ligand engagements been determined.

This study has used two different αβ TCR transgenes with defined ligand specificities. Unlike endogenously encoded αβ TCRs that are first expressed at the DP stage, these transgene-encoded αβ TCRs are first expressed in DN thymocytes as a result of the transcriptional control elements each transgene used (14, 15). To unequivocally determine the ability of these two different αβ TCR complexes to signal the DN to DP transition, they were expressed in RAG-$2^{-/-}$ pre-$T\alpha^{-/-}$ double deficient mice that are genetically incapable of expressing any endogenously encoded TCR subunit (TCRα, β, γ, δ, pre-Tα) so that the only antigen receptors expressed were the αβ TCRs encoded by the αβ TCR transgenes. Thus, this study definitively documents that αβ TCR complexes are capable of signaling the DN to DP transition. In addition, even though αβ TCRs require specific ligand engagements to transduce signals in DP thymocytes and mature T cells, this study further indicates that these same αβ TCRs do not require those ligands to transduce signals in DN thymocytes.

Materials and Methods

Mice. C57BL/6 (B6) mice were purchased from The Jackson Laboratory. The TCRα transgene containing the 2B4 TCRα cDNA under the control of a human CD2 (hCD2) enhancer/promoter was described previously (9). $TCR\beta^{-/-}$, RAG-$2^{-/-}$, pre-$T\alpha^{-/-}$, $A\beta^{-/-}$ ($CD45.1^+$ $CD45.2^-$), $\beta 2m^{-/-}$ ($CD45.1^+$ $CD45.2^-$), AND αβ TCR Tg, and HY αβ TCR Tg mice were bred in our colony and were previously described (14–20). Each αβ TCR transgene was introduced into a RAG-2 and pre-Tα gene knockout background by breeding and screened for the presence of the transgene and the absence of RAG-2 and pre-Tα genes by PCR. Experimental mice were confirmed to be RAG-2 and pre-Tα knockout by PCR on tail DNA. DNA samples that did not amplify a genomic band using the oligos pta1 (TAA CCA GTG AGC CCA AAG GGT CTG CCT GTC TAC) and pta2 (CCC ACA CAC ACA CAC ACA CGG AAC CTA TTC) in a 35-cycle PCR reaction at 67°C were considered to be pre-Tα knockout. The same DNA samples were confirmed to be RAG-2 knockout by the amplification of a 1,100-bp targeted genomic band and not an 851-bp WT genomic band in a PCR reaction at 55°C using the oligos rag1 (GAT AAA AGA CCT ATT CAC AAT C) and rag2 (TTT CAA TCG TGT TGT CCC C). The same DNA samples were confirmed to be positive for the AND or HY transgene in a 35-cycle PCR reaction at 59°C using the oligos and1 (GAC TTG GAG ATT GCC AAC CCA TAT CTA AGT) and and2 (TGA GCC GAA GGT GTA GTC GGA GTT TGC ATT), or hy1 (GCA TGG GCT GAG GCT GAT CCA TTA) and hy2 (TGA GAG CTG TCT CCT ACT ATC GAT). All Tg mice used in this study were heterozygous for the transgene. All mice used in this study were cared for in accordance with National Institutes of Health (NIH) guidelines.

Antibodies, Flow Cytometry, and Analysis of Donor-derived Cell Populations. Thymocytes from donor mice were surface stained with FITC-conjugated anti-HY TCRα (T3.70), anti-TCR Vα11 (RR8-1) or anti-IA^b (25-9-17), Cy5-conjugated anti-CD8α (CT-CD8α; Caltag), and PE-conjugated anti-CD4 (GK1.5). Single cell suspensions of thymocytes from $A\beta^{-/-}$ ($CD45.1^+$) recipient mice that had been intrathymically injected with AND αβ TCR Tg pre-$T\alpha^{-/-}$ RAG-$2^{-/-}$ donor thymocytes were assessed by four color flow cytometry using anti-CD45.1 biotin (A20; BD Biosciences) plus streptavidin Texas red, anti-CD45.2 FITC (104; BD Biosciences), anti-CD8 CY5 (CT-CD8α; Caltag), and anti-CD4 PE (GK1.5; Becton Dickinson). Staining with antibodies to both CD45 alleles allowed us to unambiguously identify donor-derived cells as $CD45.1^-$ $CD45.2^+$ in every experiment. Single cell suspensions of thymocytes from $A\beta^{-/-}$ (CD45.1) or $\beta 2m^{-/-}$ (CD45.1) recipient mice that had been injected with AND αβ TCR Tg RAG-$2^{-/-}$ pre-$T\alpha^{-/-}$ or HY αβ TCR Tg RAG-$2^{-/-}$ pre-$T\alpha^{-/-}$ (CD45.2) donor bone marrow were assessed in a similar fashion. Cell fluorescence was typically measured on 1.25×10^5 cells using a FACS Vantage™ SE (Becton Dickinson) and analyzed with software designed by the Division of Computer Research and Technology at the NIH. Dead cells were excluded from analysis of surface staining by electronic gating on forward scatter light and propidium iodide staining.

Cell Purification, Intrathymic Injections, and Bone Marrow Chimeras. DN thymocytes were purified using MACS beads conjugated with anti-CD8α (50-3-6.7) and anti-CD4 (GK1.5) according to the manufacturer's instructions (Miltenyi Biotec). Purified thymocyte populations from AND αβ TCR Tg pre-$T\alpha^{-/-}$ RAG-$2^{-/-}$ mice (CD45.2) were injected into the thymi of unirradiated $A\beta^{-/-}$ (CD45.1) mice as described previously (21). 10^6 DN IA^{b-} cells were resuspended in a volume of 10 μl PBS with 1% B6 mouse serum and injected intrathymically. Analysis of recipient mice was performed 3–4 d after injection. Radiation bone marrow chimeras were prepared as described previously (22). Recipient mice were lethally irradiated with 950 rad and reconstituted with 10^7 T cell–depleted bone marrow cells injected into the tail vein. Analysis of chimeras was performed 4–6 wk after reconstitution.

Results and Discussion

DN thymocytes have the capacity to express different types of TCR complexes, even in the presence of TCR transgenes. We recently demonstrated that early expression of TCRα in DN thymocytes leads to the formation of novel αγ TCR complexes that bypass TCRβ selection and

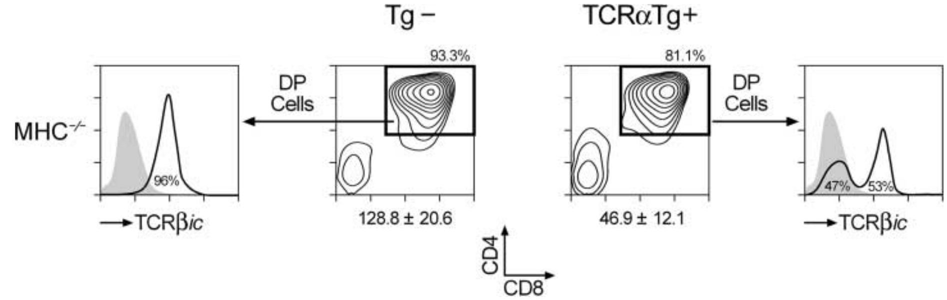

Figure 1. αγ TCRs can signal independently of MHC ligands. A transgene encoding a TCRα cDNA under the control of human CD2 promoter/enhancer elements was introduced into MHC$^{-/-}$ mice. Thymocytes from transgene$^+$ or transgene$^-$ MHC$^{-/-}$ mice were stained for CD4, CD8 surface expression, and intracellular TCRβ (TCRβ*ic*). CD4 and CD8 expression are shown as two parameter contour plots, whereas intracellular TCRβ expression is shown as a histogram. Numbers under the contour plots indicate the total number of thymocytes (± SEM) in each strain (calculated from at least three mice for each group), whereas numbers above the contour plots indicate the percentage of DP thymocytes. Intracellular TCRβ staining of DP thymocytes (solid lines) is compared with that of TCRβ$^-$ thymocytes from TCRβ$^{-/-}$ mice as a negative control (shaded areas). The percentages of TCRβ$^-$ and TCRβ$^+$ DP thymocytes are indicated.

efficiently signal the differentiation of DN into DP thymocytes (9). The ligand specificities of novel αγ TCR complexes are entirely unknown (9). To determine if engagement by MHC ligands were required for the biological activity of αγ TCR complexes, we introduced a TCRα transgene into MHC$^{-/-}$ mice. The telltale sign of signaling in DN thymocytes by αγ TCR complexes in TCRα Tg mice is the generation of TCRβ$^-$ DP thymocytes because αγ TCR signals bypass the β-selection checkpoint and promote the differentiation of TCRβ$^-$ DN thymocytes into DP cells. Therefore, we examined if TCRα Tg MHC$^{-/-}$ mice contained any TCRβ$^-$ DP thymocytes (Fig. 1). DP thymocytes in non-Tg MHC$^{-/-}$ mice were all TCRβ$^+$ by intracellular staining, indicating that they were all generated by pre-TCR signals. In contrast, TCRα Tg MHC$^{-/-}$ mice contained nearly 50% of DP thymocytes that were TCRβ$^-$ by intracellular staining and so had been signaled by αγ TCR. In these mice, the 50% of DP thymocytes that were TCRβ$^+$ were presumably generated by pre-TCR signals, although some TCRβ$^+$ cells might have also been induced by αγ TCR signals. Thus, this experiment indicates that αγ TCRs can signal the DN to DP transition in the absence of MHC ligands. Importantly, the demonstration that αγ TCRs can efficiently signal the generation of DP thymocytes in the absence of MHC ligands raises the possibility that αγ TCR complexes might have actually signaled the DN to DP transition in experiments that attributed the generation of DP thymocytes to αβ TCR signals (7, 11–13). Notably, it is impossible to exclude such a possibility in RAG$^+$ αβ TCR Tg mice because all DP thymocytes in such mice would be forced to express the TCRβ transgene, even if they were generated in response to αγ TCR signals.

To exclude αγ TCR complexes and to ensure that Tg αβ TCR were the only TCRs that DN thymocytes could express, we bred αβ TCR transgenes into RAG-2$^{-/-}$ pre-Tα$^{-/-}$ double deficient mice that were incapable of expressing any endogenously encoded TCR or pre-TCR complexes. We used two αβ TCR transgenes that encode clonotypic receptors with defined ligand specificities in

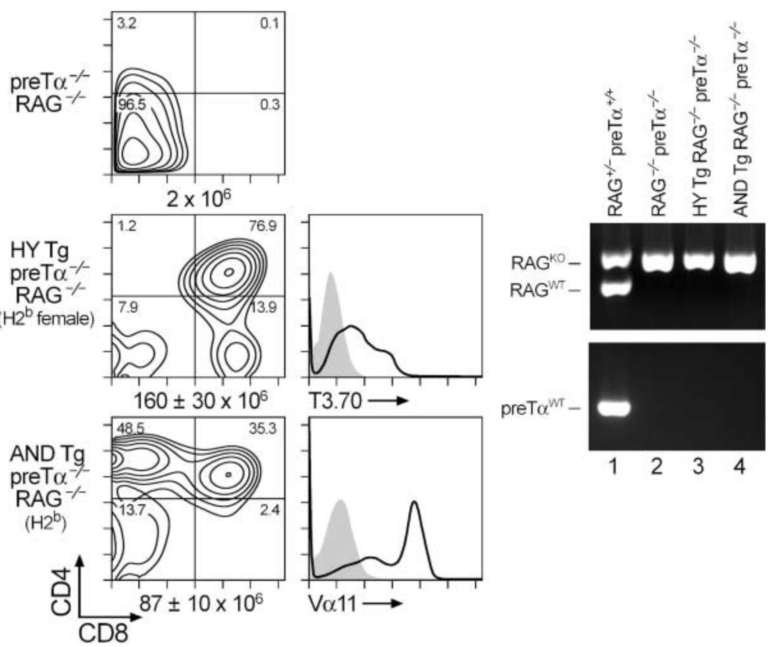

Figure 2. Expression of αβ TCR transgenes is sufficient to signal thymocyte differentiation. The HY and AND αβ TCR transgenes were introduced into RAG-2$^{-/-}$ pre-Tα$^{-/-}$ mice. CD4 and CD8 expression on thymocytes is shown as contour plots. Numbers under the contour plots indicate the number of thymocytes (± SEM) for each strain (n = 3 mice for each group). Thymocytes were also stained with anti-TCR antibodies: T3.70 for HY transgenes and Vα11 for AND transgenes (solid lines) or negative control antibodies (shaded areas). Tg mice were confirmed to be RAG-2$^{-/-}$ pre-Tα$^{-/-}$ by PCR analysis of their tail DNA. Tail DNA from experimental HY Tg RAG-2$^{-/-}$ pre-Tα$^{-/-}$ and AND Tg RAG-2$^{-/-}$ pre-Tα$^{-/-}$ mice was purified and PCR amplified using oligonucleotides that amplify the WT and knockout alleles of the RAG-2 gene or the WT allele of the pre-Tα gene. Unlike control DNA from mice heterozygous for RAG-2 and WT for pre-Tα (lane 1), DNA from RAG-2$^{-/-}$ pre-Tα$^{-/-}$ (lane 2), HY Tg RAG-2$^{-/-}$ pre-Tα$^{-/-}$ (lane 3), and AND Tg RAG-2$^{-/-}$ pre-Tα$^{-/-}$ (lane 4) mice amplified only the knockout band for RAG-2 (top) and failed to amplify the WT band for pre-Tα (bottom).

H2^b mice: the HY αβ TCR that is specific for D^b plus peptide, and the AND αβ TCR that is specific for IA^b plus peptide (Fig. 2). Expression of both Tg αβ TCRs initiates at the DN stage as a result of the transcriptional control elements used to drive transgene expression (23). We confirmed that αβ TCR Tg RAG-2$^{-/-}$ pre-Tα$^{-/-}$ mice were indeed deficient for both RAG-2 and pre-Tα molecules by performing PCR on their tail DNA (Fig. 2). Introduction of either the HY or AND αβ TCR transgene overcame the developmental block that existed at the DN stage and generated both DP and single positive (SP) thymocytes. In accordance with their ligand specificities, the HY αβ TCR generated only CD8 SP thymocytes, and the AND αβ TCR transgene generated only CD4 SP thymocytes (Fig. 2). More important for the purposes of this study, both αβ TCR transgenes signaled RAG-2$^{-/-}$ pre-Tα$^{-/-}$ DN thymocytes to differentiate into DP thymocytes (Fig. 2).

To determine if ligand engagement was required for these Tg αβ TCRs to signal DN thymocyte differentiation into DP cells, we attempted to generate αβ TCR Tg RAG-2$^{-/-}$ pre-Tα$^{-/-}$ mice that were additionally deficient in the specific MHC ligands engaged by each Tg TCR. Unfortunately, β2 microglobulin and RAG-2 gene loci are both located on mouse chromosome 2, whereas MHC and pre-Tα gene loci are both located on mouse chromosome 17. Consequently, it was not possible to generate either HY Tg RAG-2$^{-/-}$ pre-Tα$^{-/-}$ β2m$^{-/-}$ or AND Tg RAG-2$^{-/-}$ pre-Tα$^{-/-}$ MHC II$^{-/-}$ mice by simple breeding. Rather, screening for a relatively infrequent crossover recombination event was required. However, we failed in our attempts to identify any recombination event in Tg offspring.

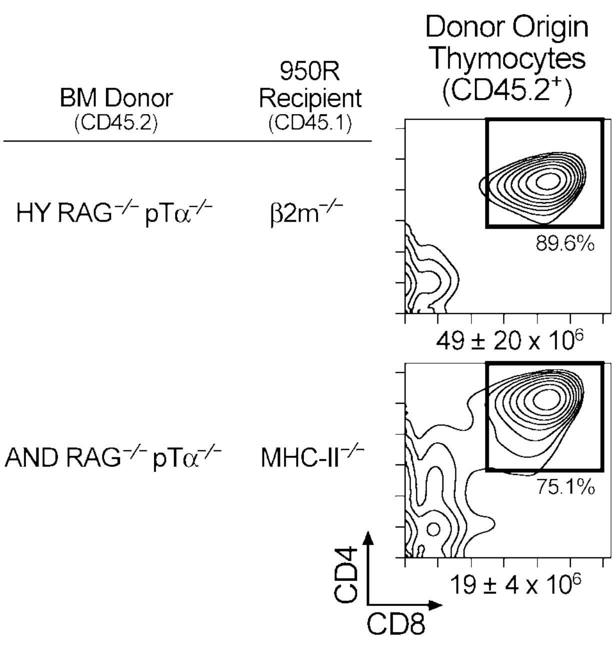

Figure 3. Thymocyte precursors from αβ TCR Tg RAG-2$^{-/-}$ pre-Tα$^{-/-}$ bone marrow donors develop into DP thymocytes in recipient mice lacking MHC ligands. Donor (CD45.2$^+$ CD45.1$^-$) HY αβ TCR Tg RAG-2$^{-/-}$ pre-Tα$^{-/-}$ bone marrow was transferred into lethally irradiated host (CD45.1$^+$ CD45.2$^-$) MHC I–deficient (β2m$^{-/-}$) mice, whereas donor (CD45.2$^+$ CD45.1$^-$) AND αβ TCR Tg RAG-2$^{-/-}$ pre-Tα$^{-/-}$ bone marrow was transferred into lethally irradiated host (CD45.1$^+$ CD45.2$^-$) MHC II–deficient (Aβ$^{-/-}$) mice. 1 mo after bone marrow reconstitution, recipient thymi were harvested and assessed for CD4 and CD8 surface expression by four color flow cytometry in which donor thymocytes were identified as CD45.1$^-$ CD45.2$^+$ cells. Numbers under the contour plots indicate the number of donor-derived thymocytes (± SEM) for each strain (n = 4 mice for each group).

We then constructed radiation bone marrow chimeras as an alternative way of assessing a potential requirement for MHC ligand engagements in αβ TCR signaling in DN thymocytes (Fig. 3). In these experiments, CD45.2$^+$ donor bone marrow from MHC I–specific HY Tg RAG-2$^{-/-}$ pre-Tα$^{-/-}$ mice were injected into 950R irradiated β2m$^{-/-}$ (MHC I–deficient) CD45.1$^+$ host mice (Fig. 3, top), and CD45.2$^+$ donor bone marrow from MHC II–specific AND Tg RAG-2$^{-/-}$ pre-Tα$^{-/-}$ mice were injected into 950R irradiated Aβ$^{-/-}$ (MHC II–deficient) CD45.1$^+$ host mice (Fig. 3, bottom). In both cases, TCR Tg donor bone marrow gave rise to DN and DP thymocytes, but not to SP thymocytes (Fig. 3). That is, in a β2m$^{-/-}$ host thymus, HY Tg TCRs were able to signal DN thymocytes to differentiate into DP thymocytes, but were unable to signal DP thymocytes to differentiate into mature CD8$^+$ T cells. And, similarly, in an MHC II$^{-/-}$ host thymus, AND Tg TCRs were able to signal DN thymocytes to differentiate into DP thymocytes, but were unable to signal DP thymocytes to differentiate into mature CD4$^+$ T cells. These results indicated that ligand engagements were required for signaling by αβ TCRs in DP thymocytes, but apparently were not required for signaling by the same αβ TCRs in DN thymocytes.

Although the relevant MHC ligands were absent from host thymic elements in these radiation bone marrow chimeras, the relevant MHC ligands (i.e., MHC I for the HY transgene and MHC II for the AND transgene) were nevertheless expressed on donor bone marrow–derived elements. Although unlikely, it was conceivable that αβ TCR signaling in DN thymocytes had been initiated by engagement of MHC ligands on donor-derived bone marrow elements in these radiation bone marrow chimeras. Consequently, we performed an intrathymic transfer experiment in which we assessed the differentiation of donor (CD45.2$^+$) DN thymocytes from AND Tg RAG-2$^{-/-}$ pre-Tα$^{-/-}$ mice (that were devoid of surface MHC II expression) in host (CD45.1$^+$) thymi of MHC II–deficient (Aβ$^{-/-}$) mice. Thus, in these experiments, AND TCR Tg RAG-2$^{-/-}$ pre-Tα$^{-/-}$ DN thymocytes were differentiating in host thymi in which both donor and host elements were devoid of MHC II expression. 4 d after intrathymic injection, the transferred thymocytes were assessed for CD4 and CD8 expression (Fig. 4). It can be seen that transferred DN thymocytes from AND Tg RAG-2$^{-/-}$ pre-Tα$^{-/-}$ mice differentiated into DP thymocytes in an MHC II–deficient host thymus, indicating that the AND TCR had signaled the DN to DP transition despite the absence of any MHC

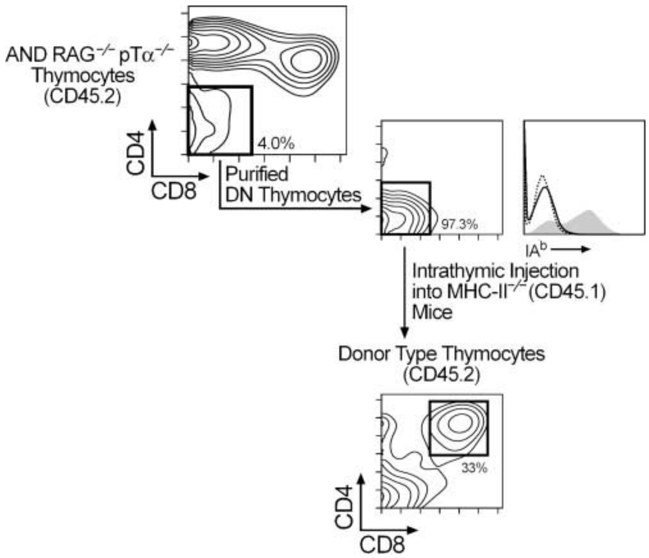

Figure 4. DN thymocytes from AND αβ TCR Tg RAG-$2^{-/-}$ pre-T$\alpha^{-/-}$ develop into DP thymocytes in the absence of MHC II ligands. AND αβ TCR Tg RAG-$2^{-/-}$ pre-T$\alpha^{-/-}$ donor ($CD45.2^+$ $CD45.1^-$) DN thymocytes were intrathymically injected into $CD45.1^+$ MHC II–deficient ($A\beta^{-/-}$) mice. 3 d after intrathymic injection, recipient thymi were harvested and assessed for CD4 and CD8 surface expression by four color flow cytometry in which donor thymocytes were identified as $CD45.1^-$ $CD45.2^+$ cells. An aliquot of donor thymocytes was stained for CD4 and CD8 surface expression (contour plot) to demonstrate that the donor inoculum was DN, and was stained for IA^b (solid line of histogram plot) to demonstrate that donor inoculum was MHC II^-. A positive control for IA^b staining (shaded area) and a negative control for IA^b staining (dotted line) are shown.

II expression. We conclude that αβ TCRs with known MHC ligand specificities do not require those ligands to signal DN thymocytes to differentiate into DP thymocytes.

This study demonstrates that Tg αβ TCRs with defined MHC I or II ligand specificities can signal DN thymocytes to differentiate into DP thymocytes, and that they can do so in the absence of their specific MHC ligands. Unlike previous experiments with αβ TCR transgenes, this study was performed in RAG-$2^{-/-}$ pre-T$\alpha^{-/-}$ double deficient mice to exclude αγ TCR complexes and to ensure that Tg αβ TCRs were the only antigen receptors that DN thymocytes could possibly express. MHC–peptide complexes are the defined ligands for αβ TCRs. In this study, we constructed chimeric animals in which αβ TCR Tg bone marrow from RAG-$2^{-/-}$ pre-T$\alpha^{-/-}$ mice was transferred into lethally irradiated MHC-deficient host mice. To be even more rigorous in minimizing the potential exposure of αβ TCR Tg DN thymocytes to MHC ligands, we also intrathymically injected AND Tg DN thymocytes that were MHC II^- into the thymus of MHC $II^{-/-}$ mice. In these ways, developing thymocytes expressing Tg αβ TCR complexes differentiated in host thymi that did not express their relevant MHC ligands. Indeed, in these experiments, αβ TCR Tg thymocytes did not differentiate beyond the DP stage because of the absence of their relevant MHC ligand. Even though the Tg AND and HY αβ TCRs failed to signal DP thymocytes, they did signal DN thymocytes to differentiate into DP thymocytes, indicating that the ligand requirements for signaling by the same αβ TCRs were different in DN and DP thymocytes. Thus, this study supports the perspective that ligand-independent signaling is a general property of antigen receptors on DN thymocytes (5, 7).

Because we found that αβ TCR signaling in DN thymocytes was ligand independent, it might be argued that Tg αβ TCRs did not actually signal the further differentiation of DN into DP thymocytes, but simply prolonged their survival so that DN thymocytes could "spontaneously" differentiate into DP thymocytes. Indeed, maneuvers that prolong the survival of DN thymocytes do result in the inefficient differentiation of DN into DP cells (24). Importantly, spontaneous differentiation of unsignaled DN thymocytes does not involve a proliferative burst and therefore results in the generation of very few ($<10^6$) DP thymocytes (24). In contrast, our current experiments found that Tg αβ TCRs promoted the generation of ~20–50 × 10^6 DP thymocytes in the absence of their specific MHC ligands, indicating that αβ TCRs induced a proliferative burst even in the absence of MHC ligand expression. Thus, our current findings are most consistent with the perspective that Tg αβ TCRs actively signal the DN to DP transition independently of ligand engagement.

A number of possible explanations for ligand-independent signaling in DN thymocytes have been proposed. One possibility is that DN thymocyte membranes might be so enriched in lipid rafts that signaling by all antigen receptor complexes occurs without ligand engagement (4). A second possibility is that the DN thymocyte membrane permits αβ TCRs and the pre-TCRs to spontaneously aggregate in the absence of ligand, in a manner analogous to developing pre–B lymphocytes in which spontaneous pre–B cell receptor aggregation results in ligand-independent signaling (25–28). A third possibility is that DN thymocytes, because they are developmentally immature, have an imbalance between intracellular kinase activity and intracellular phosphatase activity, resulting in constitutive kinase activity that allows TCR signaling even without ligand engagement (29). We would like to propose an additional possibility, namely that a component of the TCR signal transduction machinery may function to inhibit ligand-independent signaling and that this inhibitory component is specifically absent from TCR complexes on DN thymocytes. According to this model, the pre-TCR and Tg αβ TCR can signal independently of ligand in DN thymocytes because both receptor complexes lack a ligand-restricting component of the cellular signaling machinery. Whatever the molecular basis for ligand-independent signaling turns out to be, this study supports the perspective that ligand-independent signaling by antigen receptors is a general property of DN thymocytes.

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