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Unusual selection and peripheral homeostasis for immunoregulatory CD4$^-$ CD8$^-$ T cells

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Summary

Immunoregulatory CD4$^-$ CD8$^-$ (double-negative; DN) T cells exhibit a unique antigen-specific mode of suppression, yet the ontogeny of DN T cells remains enigmatic. We have recently shown that 3A9 T-cell receptor (TCR) transgenic mice bear a high proportion of immunoregulatory 3A9 DN T cells, facilitating their study. The 3A9 TCR is positively selected on the H2^k MHC haplotype, is negatively selected in mice bearing the cognate antigen, namely hen egg lysozyme, and there is absence of positive selection on the H2^b MHC haplotype. Herein, we take advantage of this well-defined 3A9 TCR transgenic model to assess the thymic differentiation of DN T cells and its impact on determining the proportion of these cells in secondary lymphoid organs. We find that the proportion of DN T cells in the thymus is not dictated by the nature of the MHC-selecting haplotype. By defining DN T-cell differentiation in 3A9 TCR transgenic CD47-deficient mice as well as in mice bearing the NOD.*H2^k* genetic background, we further demonstrate that the proportion of 3A9 DN T cells in the spleen is independent of the MHC selecting haplotype. Together, our findings suggest that immunoregulatory DN T cells are subject to rules distinct from those imposed upon CD4 T cells.

Keywords: CD47; double-negative T cells; non-obese diabetic; T-cell receptor transgenic; thymus.

Introduction

Immunoregulatory CD4$^-$ CD8$^-$ (double-negative; DN) T cells compose approximately 1–3% of total T cells in non-transgenic mice and in humans.[1–3] This rare cell type has been shown to regulate immune responses in a unique antigen-specific manner.[4] Indeed, the antigen-specific property of DN T cells is conferred, at least in part, by their ability to acquire peptide–MHC complexes on antigen-presenting cells,[1–3] a process known as trogocytosis.[5] *In vivo*, DN T cells exhibit immunoregulatory properties in multiple settings, including allografts and xenografts, graft-versus-host disease as well as autoimmune diabetes.[1–3] Yet, the ontogeny of immunoregulatory DN T cells remains enigmatic.

Many hypotheses have been put forth in an attempt to explain the developmental origin of DN T cells. The introduction of T-cell receptor (TCR) $\alpha\beta$ transgenes was shown to favour the differentiation of CD4$^-$ CD8$^-$ TCR-$\alpha\beta$ T cells.[6–9] Some reports suggested that DN T cells from TCR-$\alpha\beta$ transgenic mice represent aberrant differentiation of $\gamma\delta$ T cells arising as a consequence of forced TCR-$\alpha\beta$ expression in the immature stages of thymic differentiation.[7,10,11] Studies investigating $\alpha\beta$ versus $\gamma\delta$ T-cell lineage commitment also support a potential lineage relationship between TCR-$\alpha\beta$ DN T cells and $\gamma\delta$ T cells.[11–15] Alternatively, a large proportion of CD4$^-$ CD8$^-$ TCR-$\alpha\beta^+$ B220$^+$ cells are also found in lpr and gld mice, as well as in humans, as a result of genetic mutations in either CD95 or CD95 ligand.[16–20] In these lymphoproliferative settings, DN T cells were

Abbreviations: DN, CD4$^-$ CD8$^-$ double negative; HEL, hen egg lysozyme; iHEL, HEL expressed under the rat insulin promoter; TCR, T-cell receptor

proposed to accumulate as a result of either a defect in lymphocyte apoptosis or a down-regulation of either CD4 or CD8 co-receptor (reviewed in ref. 2). Recent work supports the view that, at least in lpr mice, DN T cells accumulate as a result of defective apoptosis.[21–24] Finally, there are also data that support a possible extrathymic differentiation pathway explaining the origin of DN T cells.[25,26]

Most recently, to address the role of the CD4 and CD8 co-receptor and their respective MHC ligands in the selection of thymocytes, Van Laethem *et al.*[27] generated CD4, CD8, MHC I and MHC II quadruple-deficient mice (referred to as quad-deficient mice). Surprisingly, $CD4^-$ $CD8^-$ T cells were selected in the MHC-deficient thymus on native antigen structures.[27,28] Together, these findings suggest that mature DN T cells may arise through a unique thymic differentiation pathway.

Importantly, it should be emphasized that the investigation of thymic differentiation of immunoregulatory DN T cells poses many challenges. For one, in non-transgenic mice, not only are immunoregulatory DN T cells scarce,[29] but they must be successfully distinguished from the immature $CD4^-$ $CD8^-$ TCR^{low} thymic T-cell pool. In addition, to specifically identify immunoregulatory DN T cells, we must exclude other mature $CD4^-$ $CD8^-$ T-cell populations, including $CD4^-$ $CD8^-$ TCR$\gamma\delta$ T cells as well as both type I and type II $CD4^-$ $CD8^-$ NKT cells.[30–32] Finally, to address questions of TCR-specificity for positive selection and peripheral homeostasis, a TCR of defined specificity must be used.

As such, we herein exploit mice carrying the 3A9 TCR transgene, which recognizes a peptide from hen egg lysozyme (HEL) in the context of $I\text{-}A^k$.[33,34] We have recently shown that the 3A9 TCR, as for other TCR transgenes,[4,9,10,15,29,35] is non-permissive to NKT cell differentiation and leads to an increased proportion of immunoregulatory DN T cells.[29,36] Our previous findings also demonstrate that a low proportion of 3A9 DN T cells associates with an increased susceptibility to develop autoimmune diabetes in the 3A9 TCR:iHEL (where HEL is expressed under the rat insulin promoter) transgenic BALB.K CD47-deficient mice as well as in mice carrying the 3A9 TCR:iHEL transgenes on the $NOD.H2^k$ genetic background.[29,37] It is therefore of interest to determine the factors that participate in defining the proportion of 3A9 DN T cells. Importantly, the findings presented herein demonstrate that the size of the DN T-cell compartment is not defined by the same factors as that of CD4 T cells.

Materials and methods

Mice

3A9 TCR[34] and iHEL (HEL under the rat-insulin promoter a.k.a ILK-3)[38] transgenic mice on B10.Br background and 3A9 TCR $NOD.H2^k$ have been previously described.[37] Non-TCR transgenic mice, B10.Br (#000465), C57BL/6 (#000664) and Rag1-deficient on C57BL/6 (#002216) and NOD (#003729) backgrounds, were purchased from The Jackson Laboratory (Bar Harbor, ME). 3A9 TCR on B10.Br background, hereafter referred to as 3A9 $TCR.B10.H2^k$ mice, were bred to C57BL/6 mice to generate 3A9 $TCR.B10.H2^{k,b}$ mice, which were then intercrossed to produce mice carrying the 3A9 $TCR.B10.H2^b$ genotype. The Rag1 deficiency on C57BL/6 background was similarly introgressed onto the 3A9 TCR $B10.H2^k$ genotype to generate three lines of mice each carrying the 3A9 $TCR.Rag1.H2^k$, the 3A9 $TCR.Rag1.H2^b$ or the 3A9 $TCR:iHEL.Rag1.H2^k$ genotype. The $NOD.Rag1^{-/-}$ mice were interbred with 3A9 TCR $NOD.H2^k$ mice to generate 3A9 TCR $NOD.Rag1.H2^k$ mice. CD47-deficient mice, provided by Dr Oldenborg (Umeå University, Sweden) had been backcrossed 18 generations on BALB/c background. These BALB/c $CD47^{-/-}$ mice were backcrossed for at least six generations to 3A9 $TCR.B10.H2^k$ mice to yield 3A9 $TCR.B10.H2^k$ CD47-deficient mice. All mice were used between 6 and 12 weeks of age. Maisonneuve-Rosemont Hospital ethics committee, overseen by the Canadian Council for Animal Protection, approved all experimental procedures.

Flow cytometry

Six- to 12-week-old non-diabetic mice (Diascreen-negative) were analysed. Single cell suspensions of thymi and spleens were prepared by mechanical disruption through a 70-μm sterile cell strainer (BD BioSciences, Mississauga, ON, Canada). NH_4Cl was used for erythrocyte lysis of single-cell suspensions from spleens. Cell counts were performed by trypan blue exclusion using a haemocytometer. One million cells were stained with fluorochrome-conjugated antibodies to CD4, CD8, TCR-β, TCR-δ, B220, CD44 and CD25, as specified in each figure legend. Anti-clonotypic 1G12 antibody recognizing the expression of the 3A9 TCR[39] was used as a culture supernatant and detected using allophycocyanin - labelled X56 from BD Biosciences. Anti-CD25 and anti-CD44 antibodies were purchased from Caltag (Carlsbad, CA). All other antibodies were purchased from Biolegend (San Diego, CA). Data were collected on a FACSCalibur (BD Biosciences), unless otherwise specified, and all data were analysed with FlowJo software (Treestar, Ashland, OR).

Statistical analyses

Data were tested for significance using a non-parametric Mann–Whitney test with a minimal threshold of 0·05 as specified in the figure legends.

Results

The 3A9 TCR allows the exploration of defects in antigen-specific T-cell-mediated immune tolerance

processes.[37,40–44] Using this model, we recently demonstrated that a low proportion of 3A9 DN T cells is observed in mice most susceptible to autoimmune diabetes.[29] To determine the factors that define the proportion of 3A9 DN T cells, we further take advantage of the 3A9 TCR transgenic mice on the B10.Br (C57BL/10 mice congenic for the H2^k MHC locus) genetic background,[37] hereafter denoted as 3A9 TCR.B10.H2^k.

The proportion of 3A9 DN T cells in the spleen may be dictated by thymic selection events. To determine the ontogeny of 3A9 DN T cells, we assessed T-cell differentiation during embryonic development of 3A9 TCR B10.H2^k mice. Due to its restriction to MHC class II molecules, the 3A9 TCR transgene effectively skews the thymic differentiation process in favour of CD4 T cells in 3A9 TCR B10.H2^k mice.[37] As expected, we found efficient differentiation of mature 3A9 CD4 T cells, where almost all mature CD4 T cells expressed the 3A9 TCR, detected with the 1G12 clonotypic antibody (Fig. 1). We also observed a high proportion of 3A9 DN T cells in the fetal thymus (Fig. 1). Importantly, these DN T cells lack CD24 expression (Fig. 1), demonstrating that they exhibit a mature phenotype. CD24$^-$ mature 3A9 DN T cells were present during fetal development as early as day 15, 1 day before the appearance of CD4$^+$ CD8$^+$ thymocytes and preceding the appearance of CD4$^+$ CD8^{low} T cells by at least 2 days (Fig. 1). Taken together, these results suggest that the 3A9 TCR transgene is expressed very early in the thymic differentiation process, generating a high number of 3A9 DN T cells in the fetal thymus. Hence, mature 3A9 DN T cells do not arise in the thymus as a consequence of cells recirculating from the periphery.

This early onset of 3A9 TCR expression is expected to skew the thymic differentiation process and alter early thymic differentiation events as well as prevent $\gamma\delta$ T-cell development.[7,10,11,45] The differentiation of CD4$^-$ CD8$^-$ TCR$^-$ thymocytes in non-transgenic mice can be delineated in four steps by the regulated expression of CD44 and CD25; namely DN1 CD44$^+$ CD25$^-$, DN2 CD44$^+$ CD25$^+$, DN3 CD44$^-$ CD25$^+$, DN4 CD44$^-$ CD25$^-$.[46,47] Remarkably, these early thymic differentiation steps were unperturbed in CD4$^-$ CD8$^-$ 1G12$^-$ thymocytes from 3A9 TCR B10.H2^k mice, when compared with that of non-transgenic B10.Br mice (Fig. 2a). Moreover, 3A9 TCR transgenic mice presented with a sizeable proportion of mature CD4$^-$ CD8$^-$ $\gamma\delta$ T cells (Fig. 2b). These results demonstrate that the insertion of the 3A9 TCR transgene does not significantly alter early thymic selection events.

Next, we aimed to evaluate the impact of thymic selection processes in defining the proportion of 3A9 DN T cells. To that effect, we further exploited the 3A9 TCR transgenic model, wherein the H2^k MHC allele is known to provide efficient positive selection of 3A9 CD4 T cells[37] (Fig. 3a). In addition, the H2^b MHC haplotype is poorly permissive to positive selection of 3A9 TCR transgenic CD4 T cells in 3A9 TCR B10.H2^b mice (Fig. 3a).[38] Finally, the presence of the HEL antigen in the thymus of 3A9 TCR:iHEL B10.H2^k mice leads to efficient thymic negative selection of 3A9 CD4 T cells.[37,42] Hence, together, the 3A9 TCR B10.H2^k, the 3A9 TCR B10.H2^b and the 3A9 TCR:iHEL B10.H2^k mice, respectively, represent models of efficient positive selection, weak positive selection and typical negative selection towards a self-antigen. Importantly, introgression of the Rag1 deficiency, which prevents the expression of endogenous TCR chains, does not affect the efficiency of both positive and negative selection in 3A9 TCR Rag1.H2^k and 3A9 TCR:iHEL Rag1.H2^k mice, respectively (Fig. 3b). Yet, it completely precludes the differentiation of 3A9 CD4 T cells in 3A9 TCR Rag1.H2^b mice (Fig. 3b), suggesting that the weak positive selection observed in 3A9 TCR B10.H2^b mice results from selection of a cell bearing dual TCRs, namely the 3A9 transgene as well as an endogenous TCR (Fig. 3b). Together, these 3A9 TCR transgenic models on both Rag1-sufficient and Rag1-deficient backgrounds provide useful tools to study the impact of thymic selection processes.

Notably, we observed a similar proportion and absolute number of 3A9 DN T cells in the thymus of all strains, namely 3A9 TCR B10.H2^k, 3A9 TCR B10.H2^b and 3A9 TCR:iHEL B10.H2^k mice (Fig. 3a), as well as in 3A9 TCR Rag1.H2^k and 3A9 TCR:iHEL Rag1.H2^k mice (Fig. 3b). We also noted that 3A9 TCR Rag1.H2^b, a genetic background that effectively precludes 3A9 CD4 T-cell differentiation, was permissive to the production of 3A9 DN T cells (Fig. 3b). This rather invariable proportion and number of 3A9 DN T cells in the different strains of mice is in line with the observation that the early expression of the TCR transgene promotes the production of thymic 3A9 DN T cells before the CD4$^+$ CD8$^+$ thymocyte stage (Fig. 1), hence preceding positive and negative selection events. Evaluation of the 3A9 DN T-cell proportion in the thymus is therefore unlikely to reflect the impact of various MHC haplotypes and negatively selecting ligands.

Consequently, to determine the impact of thymic selection processes in defining the proportion of 3A9 DN T cells, we took advantage of the fact that the proportion of T-cell subsets in secondary lymphoid organs often reflects the outcome of previous thymic selection events. As such, we next assessed the proportion and absolute number of both 3A9 CD4 and DN T cells in the spleen of 3A9 TCR B10.H2^k, 3A9 TCR B10.H2^b and 3A9 TCR:iHEL B10.H2^k mice. As expected, we found a high proportion and number of 3A9 CD4 T cells in 3A9 TCR B10.H2^k mice, relative to both 3A9 TCR B10.H2^b and 3A9 TCR:iHEL B10.H2^k mice (Fig. 4a). Similar results were observed when the Rag1 deficiency was introgressed onto the different strains, such that the 3A9 CD4 T cells were most abundant in 3A9 TCR Rag1.H2^k mice relative to both 3A9 TCR Rag1.H2^b and 3A9 TCR:iHEL Rag1.H2^k mice (Fig. 4b). Notably, as the Rag deficiency is not permissive to the expression of endogenous TCR, the proportion of

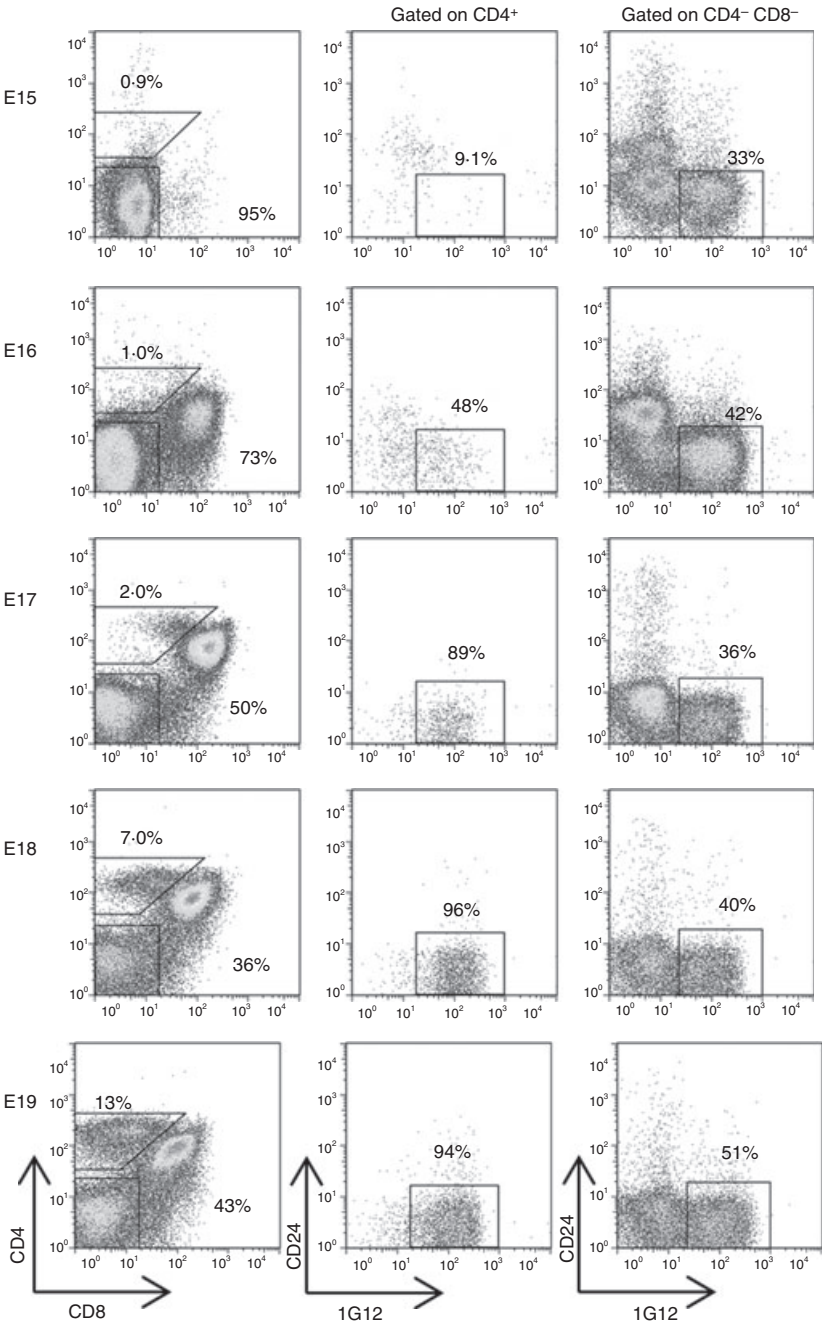

Figure 1. Embryonic thymocyte development in 3A9 T-cell receptor (TCR) B10.H2^{k} mice. Thymocytes from day 15 to day 19 embryos were stained for CD4, CD8, CD24 and 1G12. Representative thymocyte profiles of CD4 and CD8 expression (left column) and CD24 and 1G12 expression among CD4^{+} CD8^{-} (middle column) and CD4^{-} CD8^{-} (right column) are depicted. The thymus of at least four 3A9 TCR B10.H2^{k} embryos was analysed for each time-point.

3A9 CD4 T cells is lowest in the 3A9 TCR Rag1.H2^{b} mice. In the Rag-deficient background, both the proportion and absolute number of 3A9 DN T cells mirrored that of 3A9 CD4 T cells (Fig. 4b), where the highest number of 3A9 DN T cells was found in the spleen of 3A9 TCR Rag1.H2^{k} mice. Together, these findings suggest that although the proportion and number of 3A9 DN T cells in the thymus is invariable, the proportion of 3A9 DN T cells in the spleen appears to be defined by the nature of the MHC selecting haplotype and the MHC-negative selecting ligands. Therefore, although 3A9 DN T cells are generated at an early step in thymic differentiation, they may be subject to positive and negative selection before exiting the thymus and populating the periphery. Alternatively, the size of the 3A9 DN T-cell compartment in the spleen may be subject to peripheral homeostatic mechanisms.

We have previously shown that CD47 deficiency leads to a reduced proportion and absolute number of 3A9 DN T cells in the spleen.[29] Therefore, we took advantage of 3A9 TCR B10.H2^{k} mice and introgressed the CD47 deficiency to determine whether the 3A9 DN T-cell

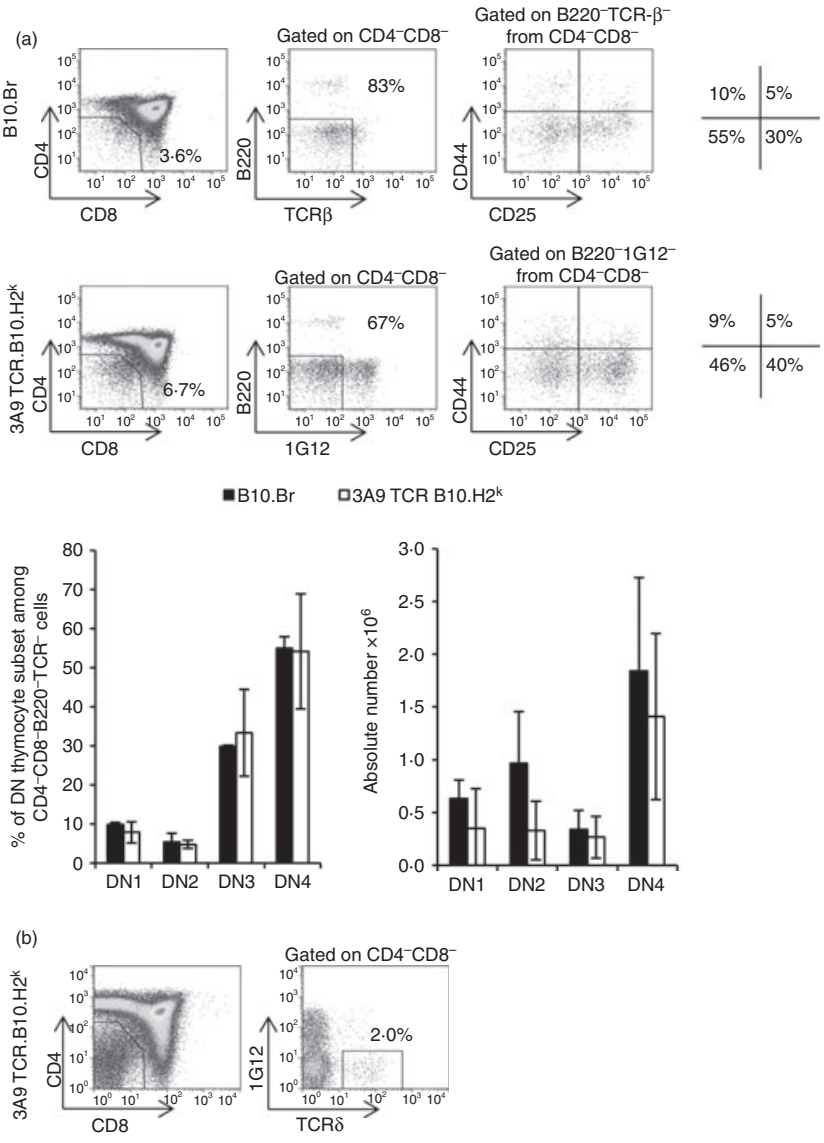

Figure 2. Early thymic differentiation events proceed normally in 3A9 T-cell receptor (TCR) B10.H2^k mice. (a) Thymocytes from B10.Br and 3A9 TCR B10.H2^k mice were stained for CD4, CD8, B220, CD44 and CD25 as well as TCR-β (B10.Br) or 1G12 (3A9 TCR B10.H2^k). The samples were acquired on the LSR II (BD Biosciences). CD4$^-$ CD8$^-$ thymocytes were gated from representative thymocyte profiles of CD4 and CD8 expression (left column). B cells and mature TCR$^+$ T cells were electronically excluded (centre column), before analysis of the CD44 and CD25 expression profiles (right column). Percentages are shown on the far right. A compilation of the data showing the percentage (left) and absolute number (right) of thymic double-negative (DN) subsets is depicted in two separate graphs. (n = 3). No significant differences were observed between the two groups for all thymic DN subsets; $P > 0{\cdot}05$. (B) TCR-δ expression is shown (right panel) on CD4$^-$ CD8$^-$ thymocytes from 3A9 TCR B10.H2^k mice (n = 3).

proportion in the spleen was defined as a result of either thymic or peripheral regulation. Note that, as the CD47-deficient mice exhibit a severe reduction in total thymocyte cellularity (ref. 44 and data not shown), the absolute number of 3A9 CD4 T cells and 3A9 DN T cells in the thymus must here be interpreted with caution, such that the comparison of cellular proportion between the two strains is most representative. Expectedly, as for the H2^b MHC haplotype and the iHEL negatively selecting ligand (Fig. 3a), the absence of CD47 did not influence the proportion of 3A9 DN T cells in the thymus (Fig. 5a). Indeed, 3A9 DN T cells were present in similar proportions in the thymus of both 3A9 TCR B10.H2^k and 3A9 TCR B10.H2^k.CD47$^{-/-}$ mice (Fig. 5a). Moreover, as we have previously reported for 3A9 TCR BALB.K CD47-deficient mice,[29] we observed a reduced proportion of 3A9 DN T cells in the spleen of 3A9 TCR B10.H2^k CD47-deficient mice relative to the CD47-sufficient counterpart (Fig. 5b). However, in stark contrast to CD47-sufficient mice, the reduced proportion of 3A9 DN T cells in the spleen did not parallel that of 3A9 CD4 T cells in CD47-deficient mice. Indeed, positive selection of 3A9 CD4 T cells proceeds normally in the thymus of CD47-deficient mice and the proportion of 3A9 CD4 T cells in the spleen is comparable between 3A9 TCR B10.H2^k and 3A9 TCR B10.H2^k.CD47$^{-/-}$ mice (Fig. 5). These results suggest that the proportion of 3A9 DN T cells in the spleen is not defined by the efficiency of thymic positive and negative selection processes. Rather, it appears that CD47 deficiency specifically influences the proportion of 3A9 DN T cells in the spleen.

Other than CD47, we have also previously demonstrated that the proportion and absolute number of 3A9 DN T cells is much reduced in 3A9 TCR.NOD.H2^k mice

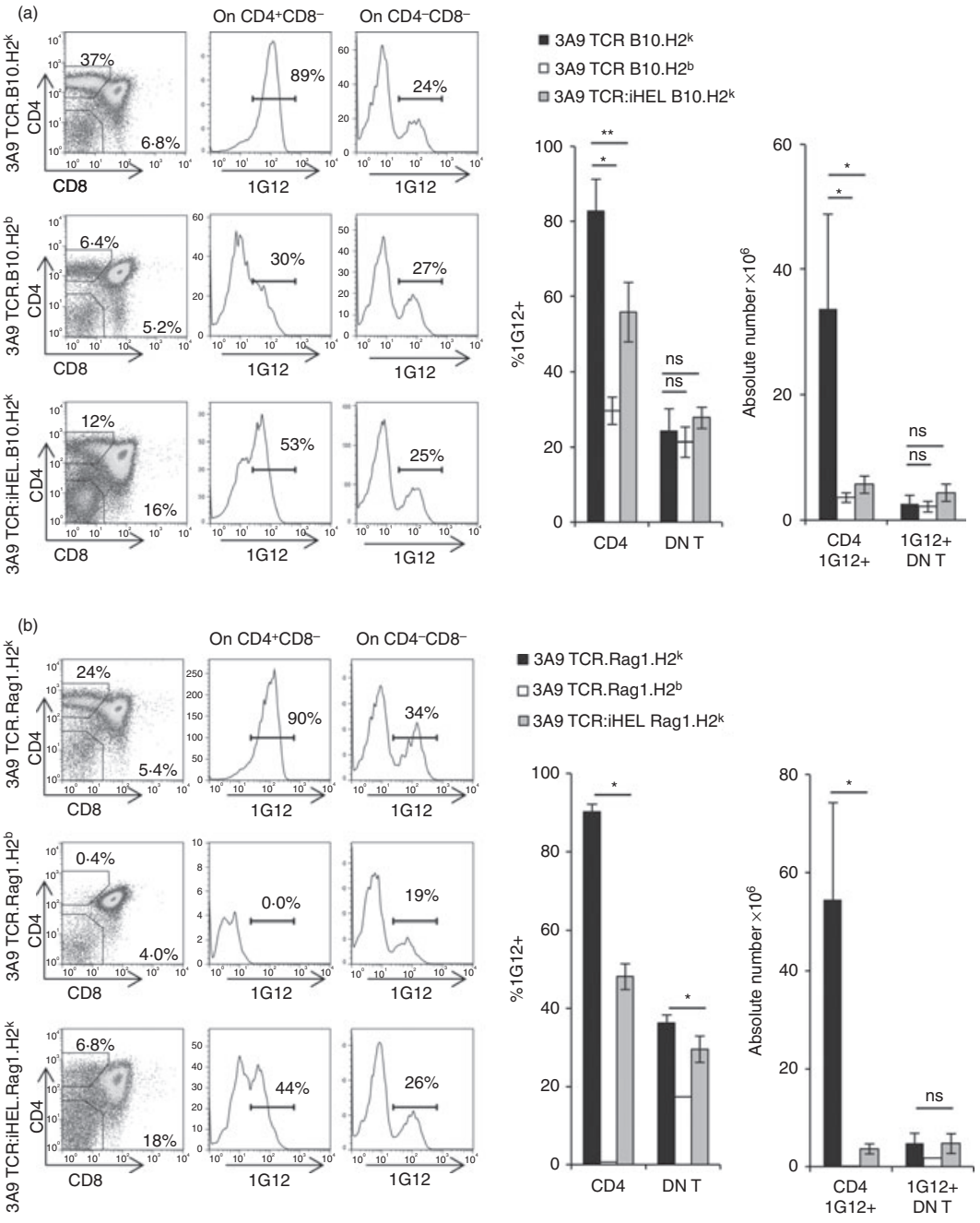

Figure 3. Proportion and absolute number of 3A9 CD4 and 3A9 double-negative (DN) T cells in the thymus. Thymocytes from (a) 3A9 T-cell receptor (TCR) B10.H2^k, 3A9 TCR B10.H2^b and 3A9 TCR:iHEL B10.H2^k mice and from (b) 3A9 TCR.Rag1.H2^k, 3A9 TCR.Rag1.H2^b and 3A9 TCR:iHEL.Rag1.H2^k mice were stained for CD4, CD8, and 1G12. Representative profiles of CD4 and CD8 expression (left column), 1G12 expression among CD4$^+$ CD8$^-$ (middle column) and among CD4$^-$ CD8$^-$ thymocytes (right column) are depicted for each mouse strain. Compilations of the average percentage of 1G12$^+$ cells among CD4$^+$ CD8$^-$ and CD4$^-$ CD8$^-$ cells and the absolute number of total CD4 1G12$^+$ and 1G12$^+$ DN T cells in each group of mice is depicted in the histograms. At least four mice per group were analysed, except 3A9 TCR.Rag1.H2^b which shows representative data of two mice. *$P < 0{\cdot}05$, **$P < 0{\cdot}01$.

relative to 3A9 TCR.B10.H2^k mice.[29] We therefore further attempted to determine the impact of the NOD genetic background in defining the proportion and absolute number of 3A9 DN T cells. To exclude the possible role of endogenous TCRs, we introgressed the Rag1 deficiency on the 3A9 TCR.NOD.H2^k background. As for the 3A9 TCR B10.H2^k.CD47$^{-/-}$ mice, although the proportion and number of 3A9 CD4 T cells in the thymus and spleen of 3A9 TCR NOD.Rag1.H2^k mice is suggestive of efficient positive selection, the proportion and absolute number of

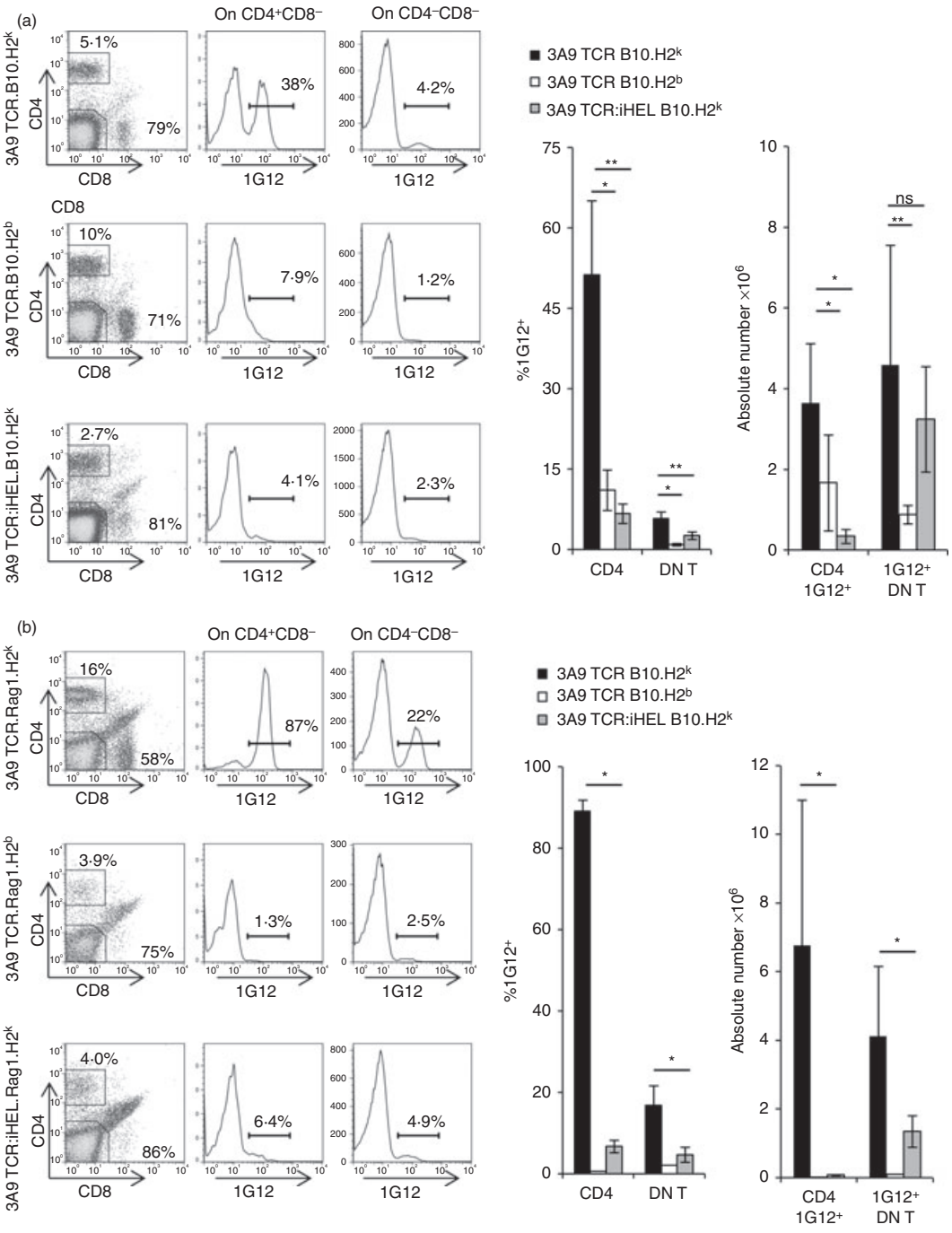

Figure 4. Proportion and absolute number of 3A9 CD4 and 3A9 double-negative (DN) T cells in the spleen. Spleen cells from (a) 3A9 T-cell receptor (TCR) B10.H2^k, 3A9 TCR B10.H2^b and 3A9 TCR:iHEL B10.H2^k mice and from (b) 3A9 TCR.Rag1.H2^k, 3A9 TCR.Rag1.H2^b and 3A9 TCR:iHEL.Rag1.H2^k mice were stained for CD4, CD8 and 1G12. Representative profiles of CD4 and CD8 expression (left column) and 1G12 expression among CD4$^+$ CD8$^-$ (middle column) and CD4$^-$ CD8$^-$ (right column) are depicted for each mouse strain. Compilations of the average percentage of 1G12$^+$ cells among CD4$^+$ CD8$^-$ and CD4$^-$ CD8$^-$ cells and the absolute number of total CD4.1G12$^+$ and 1G12$^+$ DN T cells in each group of mouse is depicted in the histograms. At least four mice per group were analysed, except 3A9 TCR.Rag1.H2^b, which shows representative data of two mice. *$P < 0{\cdot}05$, **$P < 0{\cdot}01$.

3A9 DN T cells is reduced in the spleen of 3A9 TCR NOD.Rag1.H2^k mice relative to 3A9 TCR B10.H2^k mice (Fig. 6b). In contrast to the CD47-deficient mice, the proportion of 3A9 DN T cells was also reduced in the thymus of 3A9 TCR NOD.Rag1.H2^k mice relative to 3A9 TCR B10.H2^k mice (Fig. 6a). Taken together, these results

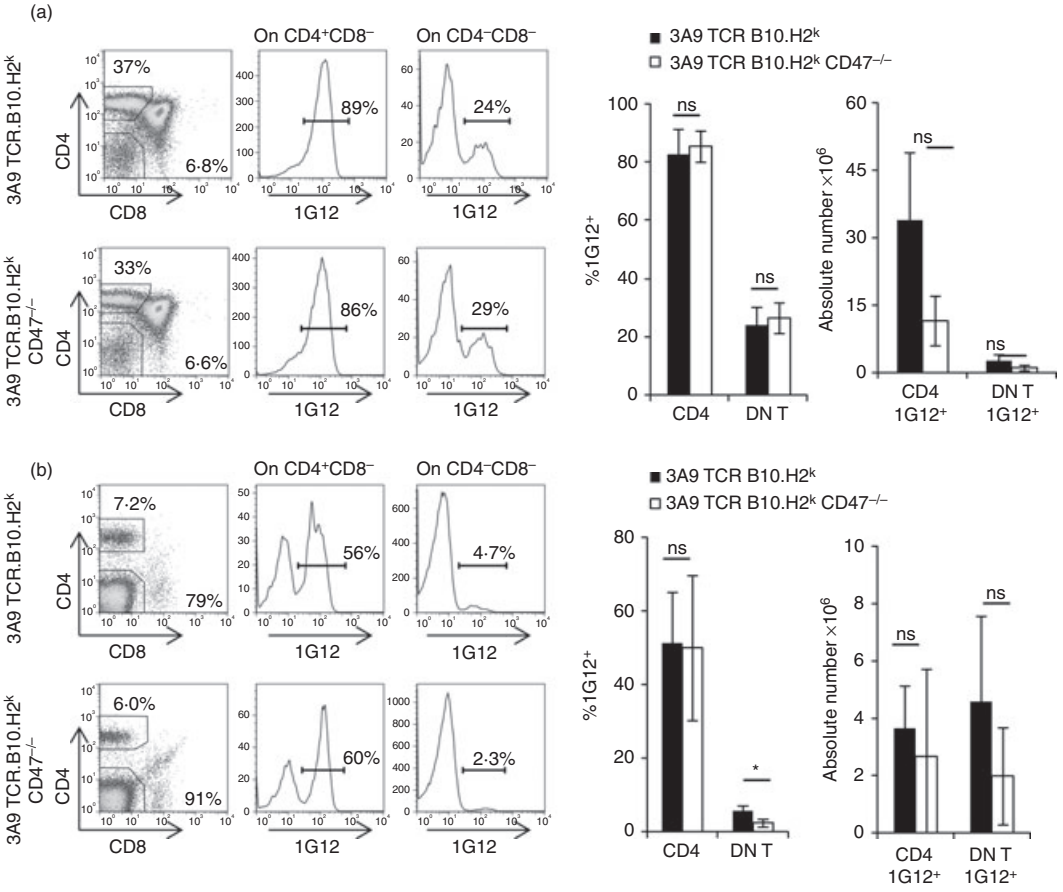

Figure 5. CD47 defines the proportion of peripheral 3A9 double-negative (DN) T cells. Cell suspension of thymocytes and spleens from 3A9 T-cell receptor (TCR) B10.H2^k and 3A9 TCR B10.H2^k CD47$^{-/-}$ mice were stained for CD4, CD8, and 1G12. Representative thymocyte (a) and spleen cell (b) profiles of CD4 and CD8 expression (left column) and 1G12 expression among CD4$^+$ CD8$^-$ (middle column) and CD4$^-$ CD8$^-$ (right column) are depicted for each mouse strain, namely 3A9 TCR B10.H2^k (n = 5), 3A9 TCR B10.H2^k CD47$^{-/-}$ (n = 3). Compilations of the average percentage of 1G12$^+$ cells among CD4$^+$ CD8$^-$ and CD4$^-$ CD8$^-$ cells and the absolute number of total CD4.1G12$^+$ and 1G12$^+$ DN T cells in each group of mouse is depicted in the histogram. *P < 0·05.

are in line with the postulate that the proportion of 3A9 DN T cells does not parallel that of 3A9 CD4 T cells, suggesting that the proportions of 3A9 DN T cells and 3A9 CD4 T cells are defined by distinct mechanisms. In addition, we found that both central and peripheral mechanisms may contribute to defining the proportion of 3A9 DN T cells.

Discussion

In the present work, we take advantage of the 3A9 TCR transgenic model to investigate the thymic selection events leading to the production of 3A9 DN T cells. We find that although the 3A9 TCR transgene does not appear to perturb early thymic differentiation events, the production of 3A9 DN T cells precedes that of CD4$^+$ CD8$^+$ thymocytes. In line with the early appearance of 3A9 DN T cells, the proportion of 3A9 DN T cells in the thymus is not influenced by the presence of either positive or negative selecting ligands. This directly contrasts with the differentiation of CD4 thymocytes. Moreover, we find that the size of the 3A9 DN T-cell niche in the spleen is not always dictated by thymic selection processes. Rather, we conclude that both central and peripheral homeostatic mechanisms define the proportion of 3A9 DN T cells.

Differentiation of immunoregulatory DN T cells has also been proposed to occur extrathymically.[26] However, these data should be interpreted with caution considering that the conclusions were drawn from thymectomized mice and that the secondary cervical thymus could well have provided a sufficient environment to allow differentiation of DN T cells.[48,49] Nonetheless, although we show that 3A9 DN T cells are present in the thymus and can be observed in embryonic thymi, our data do not exclude an extrathymic differentiation pathway for immunoregulatory DN T cells. Indeed, it remains possible that 3A9 DN T cells found in the spleen do not develop from the thymic 3A9 DN T-cell population, but rather arise as a

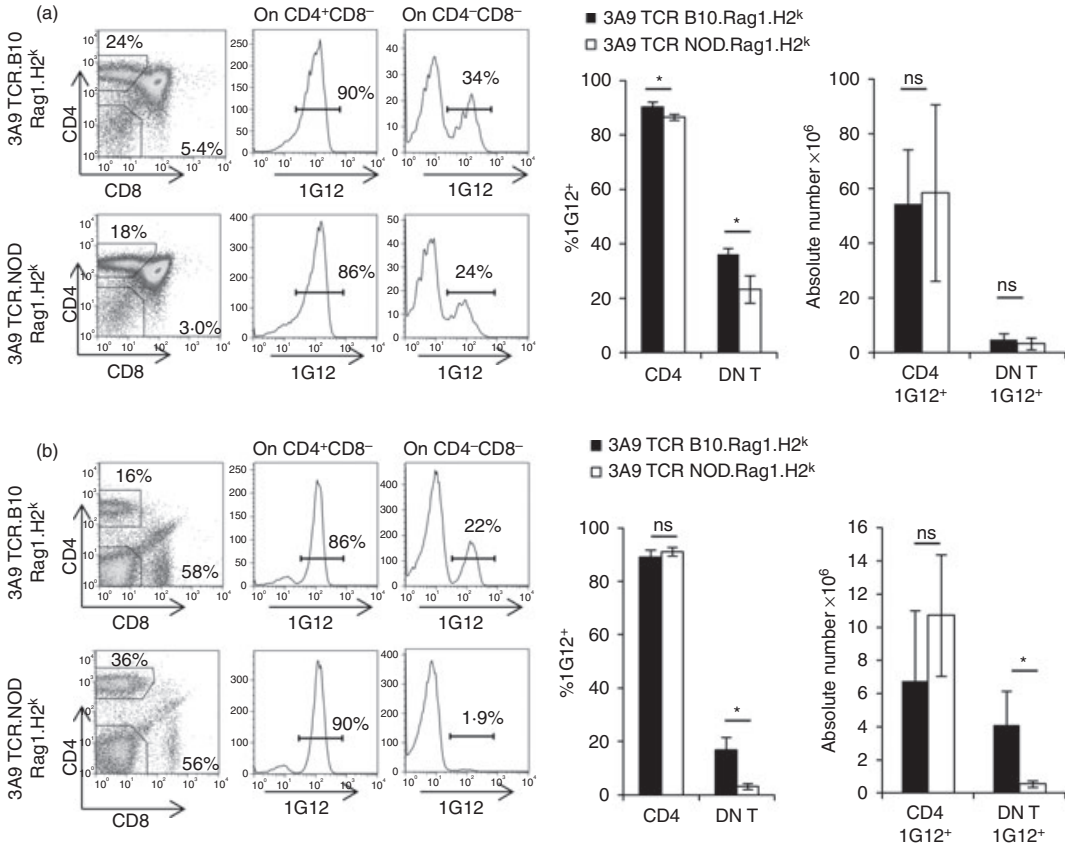

Figure 6. Influence of the non-obese diabetic (NOD) genetic background in defining 3A9 double-negative (DN) T-cell proportion and absolute number. Cell suspension of thymocytes and spleens from 3A9 T-cell receptor (TCR) B10.Rag1.H2^k and 3A9 TCR NOD.Rag1.H2^k mice were stained for CD4, CD8 and 1G12. Representative thymocyte (a) and spleen cell (b) profiles of CD4 and CD8 expression (left column) and 1G12 expression among CD4$^+$ CD8$^-$ (middle column) and CD4$^-$ CD8$^-$ (right column) are depicted for each mouse strain, namely 3A9 TCR B10.H2^k (n = 4) and 3A9 TCR NOD.Rag1.H2^k mice (n = 3). Compilations of the average percentage of 1G12$^+$ cells among CD4$^+$ CD8$^-$ and CD4$^-$ CD8$^-$ cells and the absolute number of total CD4 1G12$^+$ and 1G12$^+$ DN T cells in each group of mouse is depicted in the histogram. *$P < 0{\cdot}05$.

consequence of CD4 or CD8 down-regulation on 3A9 T cells in the periphery. Yet, it should be noted that if either 3A9 CD4 T cells or 3A9 CD8 T cells lose the expression of their co-receptor to acquire the 3A9 DN T-cell phenotype, this does not arise as a consequence of recognition of cognate antigen. Indeed, 3A9 TCR: iHEL.B10.H2^k mice exhibit fewer 3A9 DNT cells than the 3A9 TCR.B10.H2^k counterpart. Future experiments evaluating thymic export of 3A9 DN T cells will help to clarify these issues.

T-cell-receptor transgenes impose the expression of TCR-$\alpha\beta$ on early differentiating CD4$^-$ CD8$^-$ thymocytes. This non-physiological timing in the expression of the TCR has been shown to alter various thymocyte differentiation steps, namely the differentiation of CD4$^-$ CD8$^-$ TCR$^{-/low}$ cells from the CD44$^+$ CD25$^-$ phenotype to the CD44$^-$ CD25$^-$ phenotype.[45] In addition, the early expression of TCR-$\alpha\beta$ at the CD4$^-$ CD8$^-$ TCR$^{-/low}$ thymocyte differentiation steps has been proposed to preclude the expression of the TCR-$\gamma\delta$.[7] However, in the 3A9 TCR model, we find that these early thymic differentiation steps proceed normally and that $\gamma\delta$ T-cell differentiation is not impeded. Nonetheless, in the 3A9 TCR transgenic model, as for other TCR transgenic models, the proportion of immunoregulatory 3A9 DN T cells is increased to non-physiological levels.[29] Common features of these immunoregulatory DN T cells include a naive phenotype, anergy and immunoregulatory properties.[1] Interestingly, most of these characteristics are also shared with DN T cells obtained from humans and non-transgenic mice, suggesting that TCR transgenic mice may well serve as a relevant model to dissect some of the properties of these rare cells carrying a unique antigen-specific immunoregulatory potential.[1] These results clearly highlight the unique and subtle differences of TCR transgenic models, where comparison of each of these models may yield important clues in the ontogeny, phenotype and function of immunoregulatory DN T cells.

The most characterized TCR model for immunoregulatory DN T cells is unarguably the 2C TCR.[1–3] In this transgenic model, it has been proposed that agonist selection

favoured the differentiation of 2C DN T cells.[8,35] In stark contrast with the 2C TCR transgenic model, we provide strong evidence to support that agonist selection does not promote 3A9 DN T-cell differentiation. First, 3A9 DN T cells are found in higher numbers in the spleen of 3A9 TCR B10.H2^k mice, which lack the presence of the HEL cognate antigen. Second, in comparison to 3A9 TCR B10.H2^k mice, we observe fewer 3A9 DN T cells, as opposed to an increase in number, in the spleen of TCR: iHEL B10.H2^k mice, in which the cognate antigen is present. Third, the thymic selection of 3A9 DN T cells also does not result from self-antigen recognition by a self-TCR composed of an endogenous TCR-α chain as a result of potentially incomplete allelic exclusion, as 3A9 DN T cells are found in similar numbers in the thymus of 3A9 TCR B10.H2^k and 3A9 TCR.Rag1.H2^k mice and are even increased in proportion in the spleen of 3A9 TCR.Rag1.H2^k mice. Together, these findings argue against a role for self-antigen or agonist selection in the differentiation of 3A9 DN T cells.

The reason for the discrepancy between the 2C TCR and the 3A9 TCR transgenes in the generation of immunoregulatory DN T cells is unclear. The timing of the TCR expression for these two transgenes during thymocyte development or the specific affinity to their respective self-ligands may account for the divergence in contribution of self-ligand towards the effective production of DN T cells. Alternatively, the differential contribution of agonist selection for DN T cells in these two models may lie in the fact that the 2C TCR is positively selected on MHC class I, whereas the 3A9 TCR is restricted to MHC class II. Interestingly, the production of TCR transgenic DN T cells in the MHC class I-restricted OT-1 and H-Y models also appears to be favoured in the presence of self-antigen, whereas the MHC class II-restricted DO11.10, 4E3 and 1H3.1 models effectively generate transgenic DN T cells irrespective of the presence of cognate antigen.[9,15,50,51] Moreover, the phenotype and function of DN T cells generated in MHC class II-restricted TCR transgenic mice are very similar,[1,4,9,29,35,36,50,51] but there are notable differences when comparing DN T cells from TCR transgenic mice restricted to either MHC class I or MHC class II.[1,36] These observations strongly suggest that the differentiation of MHC class I-restricted and MHC class II-restricted DN T cells proceeds through different means. Interestingly, thymic differentiation of non-transgenic DN T cells was recently reported to occur as a result of agonist selection leading to the production of CD8$\alpha\alpha$ T cells.[52] Hence, we would like to propose that these non-transgenic DN T cells are MHC class I-restricted. Clearly, additional studies in non-transgenic mice are needed to explore the thymic selection process of both MHC class I-restricted and MHC class II-restricted DN T cells, as well as their respective biological role.

In conclusion, the 3A9 TCR transgenic system has enabled us to examine the impact of thymic positive and negative selection on 3A9 DN T cells. Our results support a model in which MHC class II-restricted DN T cells do not arise as a result of agonist selection on self-ligands. This observation may point to distinct roles for both MHC class I-restricted and MHC class II-restricted DN T cells in non-transgenic settings. Our results also suggest that the proportion of DN T cells in secondary lymphoid organs is dictated by unique parameters, including the CD47 pathway. Together, these findings lay the ground for future work towards understanding the regulation of a rare cell type with a prominent antigen-specific immunotherapeutic potential.

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Disclosure

The authors have no conflicts of interest to disclose.

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