The Journal of Immunology

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J Immunol 2020; 205:133-142; Prepublished online 20 May 2020;
doi: 10.4049/jimmunol.2000156
http://www.jimmunol.org/content/205/1/133

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

MHC-Independent Thymic Selection of CD4 and CD8 Coreceptor Negative αβ T Cells

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It is becoming increasingly clear that unconventional T cell subsets, such as NKT, γδ T, mucosal-associated invariant T, and CD8αα T cells, each play distinct roles in the immune response. Subsets of these cell types can lack both CD4 and CD8 coreceptor expression. Beyond these known subsets, we identify $CD4^{-}CD8^{-}TCR\alpha\beta^{+}$, double-negative (DN) T cells, in mouse secondary lymphoid organs. DN T cells are a unique unconventional thymic-derived T cell subset. In contrast to $CD5^{high}$ DN thymocytes that preferentially yield $TCR\alpha\beta^{+}$ CD8αα intestinal lymphocytes, we find that mature $CD5^{low}$ DN thymocytes are precursors to peripheral DN T cells. Using reporter mouse strains, we show that DN T cells transit through the immature $CD4^{+}CD8^{+}$ (double-positive) thymocyte stage. Moreover, we provide evidence that DN T cells can differentiate in MHC-deficient mice. Our study demonstrates that MHC-independent thymic selection can yield DN T cells that are distinct from NKT, γδ T, mucosal-associated invariant T, and CD8αα T cells. ***The Journal of Immunology*****, 2020, 205: 133–142.**

T cells play a central role in the immune response. Their specificity is conferred by their TCR repertoire, which is selected following a series of complex molecular and cellular events (1–4). Specifically, hematopoietic progenitor cells migrate from the fetal liver or bone marrow to the thymus, where they differentiate into mature T cells (5, 6). For conventional T cells, progression through a series of maturation stages is primarily identified based on the differential expression of the CD4 and CD8 coreceptors and the TCR (7). The earliest T cell progenitors do not express a TCR or the CD4 or CD8 coreceptors and are called triple-negative (TN) thymocytes. TN thymocytes that successfully generate a functional TCRβ-chain proliferate, progress to the $CD4^{+}CD8^{+}$ (double-positive [DP]) thymocyte stage, and, subsequently, rearrange the TCRα-chain. DP thymocytes that express an αβ TCR are subjected to positive and negative selection dependent on TCR interactions with self-peptide/MHC complexes, giving rise to conventional $CD4^{+}$ or $CD8^{+}$ $TCR_{\alpha\beta}$ T cells that exit the thymus to populate peripheral tissues (7).

In addition to conventional T cells, a number of unconventional T cell subsets such as γδ T, NKT, and mucosal-associated invariant T (MAIT) cells as well as $TCR_{\alpha\beta}{}^{+}$ $CD8\alpha\alpha^{+}$ intraepithelial lymphocytes (IELs) are also found in peripheral tissues (8–10). The thymic differentiation process of these unconventional T cell subsets has been at least partly resolved. γδ T cells deviate from the conventional T cell differentiation pathway at the TN thymocyte stage, and their maturation into functional T cells is thought to result from the induction of a strong TCR signal (11–13). In contrast to γδ T cells, the thymic differentiation pathway of NKT cells, MAIT cells, and $TCR_{\alpha\beta}{}^{+}$ $CD8\alpha\alpha^{+}$ IELs proceeds through the DP thymocyte stage (14–16). However, their thymic selection differs from conventional T cells, which are selected on classical MHC molecules, in that NKT and MAIT cell differentiation strictly depend on nonclassical MHC class I (MHC I) molecules, whereas $TCR_{\alpha\beta}{}^{+}$ $CD8\alpha\alpha^{+}$ IELs can be selected on both nonclassical and classical MHC molecules (15, 17–24). Because of the nonpolymorphic nature of nonclassical MHC I molecules, the TCR repertoire of both NKT and MAIT cells is more restricted. Therefore, the differentiation of unconventional T cell subsets proceeds via unique thymic selection processes (10).

TCR signal strength influences T cell positive selection, which is reflected by the level of expression of both CD5 and Nur77. Accordingly, positive selection of conventional T cells leads to a relatively low expression of CD5 and Nur77 when compared with that of unconventional T cells, which are subjected to stronger TCR signals associated with agonist selection (10, 23–29). Using TCR transgenic mice, we have previously identified a subset of unconventional T cells that lack CD4 and CD8 coreceptor expression

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Received for publication February 10, 2020. Accepted for publication April 23, 2020.

This work was supported by Canadian Institutes of Health Research (CIHR) (PJT 159603) and Cancer Research Society (21006, 23189) grants to S.L. and a CIHR (MOP-142254) grant to H.J.M. R.C. was supported by a Ph.D. scholarship from the Fonds de Recherche du Québec–Santé (FRQ-S). F.L.-V. is supported by Ph.D. scholarships from the FRQ-S, the Cole Foundation, and the Fondation de l'Hôpital Maisonneuve-Rosemont. M.-È.L. is supported by FRQ-S and L'Oréal-UNESCO for Women in Science postdoctoral fellowships. E.E.H. was recipient of a CIHR Ph.D. scholarship. H.J.M. is a Junior 1 Scholar of the FRQ-S and a CIHR New Investigator (MSH-141967). S.L. is a Research Scholars Emeritus awardee of the FRQ-S.

R.C. designed and conducted most of the experiments, prepared the figures, and drafted the manuscript. F.L.-V. performed the in vivo thymocyte transfer experiments as well as some phenotypic characterization of double-negative T cells and prepared the figures. E.E.H. performed some of the TCR Vβ repertoire experiments and drafted and revised the manuscript. M.-È.L. and G.C.-R. contributed to the analysis of transgenic reporter mice. G.C.-R. also performed some phenotypic characterization of double-negative T cells. H.J.M. contributed to the experimental design and revision of the manuscript. S.L. supervised the study and revised the manuscript.

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The online version of this article contains supplemental material.

Abbreviations used in this article: B6, C57BL/6; B2m, β2-microglobulin; BV, Brilliant Violet; DN, double-negative; DP, double-positive; E, embryonic day; IEL, intraepithelial lymphocyte; MAIT, mucosal-associated invariant T; MHC I, MHC class I; MHC II, MHC class II; TN, triple-negative.

and that express low levels of CD5 (30). We now demonstrate that this subset is also present in non-TCR transgenic mice and that it is distinct from γδ T cells, NKT cells, MAIT cells, and $TCR_{\alpha\beta}^+$ CD8αα$^+$ IELs. These dual coreceptor negative $TCR_{\alpha\beta}^+$ T cells (double-negative [DN] T cells) differentiate from a $CD5^{low}$ thymic precursor. DN T cells transit through a DP thymocyte stage and are present in MHC-deficient mice. Altogether, our data suggest that $TCR_{\alpha\beta}^+$ DN T cells can differentiate in the thymus in an MHC-independent manner.

Materials and Methods

Mice

C57BL/6 (B6) (no. 000664), $CD1d^{-/-}$ (no. 008881), B6.SJL (no. 002014), CD4-Cre (no. 022071), dLck-Cre (no. 012837), ROSA-fl-STOP-fl-YFP (RosaYFP, no. 007903), $Rag1^{-/-}$ (no. 002216), $\beta2m^{-/-}$ (no. 002087), MHC class II (MHC II)$^{-/-}$ (no. 003584), and $Nur77^{GFP}$ (no. 016617) mice were originally purchased from Jackson Laboratories. The $B6.Rag2^{GFP}$ (31) and $K^bD^{b-/-}$ (32) mice were generous gifts from M. Rafei and C. Shatz, respectively. The E8I-Cre (no. 008766; Jackson Laboratories) and E8III-Cre (33) strains were generously provided by N. Labrecque and A. Singer. B6.SJL, $B6.Rag2^{GFP}$, CD4-Cre, dLck-Cre, E8I-Cre, E8III-Cre, RosaYFP, $K^bD^{b-/-}$, MHC $II^{-/-}$, and $Nur77^{GFP}$ were crossed onto the $CD1d^{-/-}$ background. The $\beta2m^{-/-}$MHC $II^{-/-}$ were generated by intercrossing $\beta2m^{-/-}$ and MHC $II^{-/-}$ mice. For timed pregnancies, the presence of a vaginal plug was defined as day 0.5. Data for both female and male mice are combined for all experiments because no phenotypic differences were observed. All of the mouse strains were maintained at the Maisonneuve-Rosemont Hospital animal facility (Montreal, Canada). The Maisonneuve-Rosemont Hospital ethics committee, overseen by the Canadian Council for Animal Protection, approved the experimental procedures.

Flow cytometry

Thymi and spleen were pressed through a 70-μm cell strainer (Thermo Fisher Scientific). Spleen cell suspensions were treated with NH_4Cl to lyse RBCs. IELs were isolated from 1-cm pieces of the small intestine that were agitated for 20 min at 37°C in RPMI 1640 medium supplemented with 3% FBS, 5 mM EDTA, and 0.145 mg/ml DL-Dithiotreitol (Sigma-Aldrich), and subsequently shaken in RPMI 1640 containing 2 mM EDTA followed by a 30% Percoll (GE Healthcare) gradient. For all tissues, live cells were counted using trypan blue exclusion, and single-cell suspensions were stained extracellularly with combinations of the following Abs, listed as "target (clone; dyes)." CD3 (17A2; Brilliant Violet [BV]711), CD4 (RM5-5; BV786), CD4 (GK1.5; PE-Cy7), CD5 (53-7.3; FITC, PerCP), CD8α (53-6.7; PerCP, Allophycocyanin-Cy7, BV510), CD19 (6D5; FITC, BV605), CD25 (PC61; Pacific Blue), CD45.1 (A20.1; FITC), CD45.2 (104; Pacific Blue), TCRβ (H57-597; FITC, PE, Allophycocyanin), TCRγδ (GL3; PerCP-Cy5.5) were all purchased from BioLegend. The CD4 (GK1.5; eFluor 450) and CD8β (eBioH35-17.2; PE-Cy7) Abs, as well as the viability dyes (eFluor 780 and eFluor 506), were purchased from eBioscience. The PE- and BV421-coupled PBS-57 mCD1d-tetramers as well as the PE-conjugated MR1 tetramer was provided by the National Institutes of Health tetramer core facility. All Abs were titrated for optimal use. Fluorescence minus one stainings were performed to determine background noise. Cells were incubated with the tetramer reagents at 4°C for 15 min before adding the Ab mixtures. Cells were incubated with Abs at 4°C for an additional 30 min and washed twice prior to data acquisition on flow cytometers. Staining for Ki-67 (B56; FITC; from Becton Dickinson) and for PLZF (9E12; PE; BioLegend) was performed using the eBioscience intracellular staining kit. The TCR Vβ repertoire was analyzed by flow cytometry with an anti-mouse TCR Vβ screening panel (BD Bioscience) according to the manufacturer's instructions. Unless otherwise stated, DN T cells are defined as $TCR_\beta^+CD4^-CD8\alpha^-$, $CD4^+$ T cells are defined as $TCR_\beta^+CD4^+CD8\alpha^-$, and $CD8^+$ T cells are defined as $TCR_\beta^+CD4^-CD8\beta^+$. In some experiments, CD19 or a viability dye were also used as additional negative markers before gating on TCR_β^+ cells. In CD1d-sufficient mice, type 1 NKT were excluded from $CD4^+$ T, $CD8^+$ T, and DN T cells by the use of PBS-57 CD1d-tetramer. Data were collected using Becton Dickinson instruments, namely an LSRFortessa X20, an LSR II, or a FacsCanto and analyzed using Flow-Jo software (FlowJo LLC).

Thymic graft under the kidney capsule

Embryonic thymi from embryonic day (E)15.5 or E16.5 $CD1d^{-/-}$ (CD45.1 or CD45.2) mice were harvested in cold sterile PBS. Six- to eight-week-old $CD1d^{-/-}$ (reciprocally CD45.2 or CD45.1) mice were anesthetized by isoflurane inhalation. The left flanks of the mice were shaved, and an incision was made to expose the left kidney. One embryonic thymus was grafted per mouse. Anafen (Merial) was injected at 5 mg/kg per mouse immediately after surgery and every day for three additional days.

In vivo transfer of $CD5^{low}$ and $CD5^{high}$ DN thymocytes

Thymocytes from $B6.SJL.CD1d^{-/-}$ mice were stained with Abs against TCRβ, CD4, CD8α, and CD5. Thymic DN T cells ($TCR\beta^+CD4^-CD8\alpha^-$) were sorted based on their cell surface levels of CD5. Between 25,000 and 350,000 cells were injected in the tail vein of $Rag1^{-/-}$ mice. Six weeks after injection, the $Rag1^{-/-}$ recipients were sacrificed, and organs were collected for flow cytometry analysis.

In vitro DN T cell activation

Total spleen cells from $B6.CD1d^{-/-}$ mice were incubated in vitro for 18 h in the presence of plate bound anti-CD3 (1 μg/ml, 145-2C11; BioLegend) and anti-CD28 (10 μg/ml, 37.51; BioLegend) Abs. Cells were stained and analyzed by flow cytometry. Cells incubated in the absence of Abs were used as unstimulated controls.

Statistics

Significance was tested using IBM SPSS software by one-way ANOVA with Games–Howell post hoc test for comparisons between more than two groups. Paired or unpaired *t* tests or Mann–Whitney tests were used for comparisons between two groups, where appropriate.

Results

DN T cells are distinct from other T cell subsets

Many unconventional T cell subsets lack CD4 and/or CD8 coreceptors (8, 24, 34, 35). We thus aimed to determine the proportion of DN T cells that were distinct from other defined unconventional T cells, namely NKT cells, γδ T cells, IELs, and MAIT cells. First, although type I NKT cells, which express an invariant Vα-chain (Vα14), are readily identified using an α-GalCer/CD1d tetramer (36, 37) or the PBS57 analogue of α-GalCer (38), there are currently no means to identify all type II polyclonal NKT cells (39, 40). Moreover, CD49b and NK1.1, which are commonly used to identify NKT cells, are neither expressed by all NKT cells nor are they exclusive to NKT cells (41–43). Nevertheless, all NKT cells express a TCR restricted to CD1d molecules, such that both type I and type II NKT cells are absent in $CD1d^{-/-}$ mice (17, 18). Therefore, we performed our studies in $CD1d^{-/-}$ mice, in which all NKT cell subsets are absent (Fig. 1A) (17, 18). Importantly, apart from NKT cells, the absence of CD1d did not affect the proportion and absolute numbers of other T cell subsets, including conventional $CD4^+$ and $CD8^+$ T cells, as well as unconventional γδ T cells, CD8αα$^+$ IELs, and MAIT cells (Fig. 1A, 1B, Supplemental Fig. 1). To quantify the proportion and absolute numbers of $TCR_{\alpha\beta}^+$ DN T cells in the spleen, in addition to excluding all NKT cells, we also exclude γδ T cells by specifically selecting for $TCR\beta^+$ cells and exclude $TCR_{\alpha\beta}^+$ CD8αα$^+$ IELs based on CD8α expression (Fig. 1A middle panel and Supplemental Fig. 1). MAIT cells were not excluded but composed a negligible proportion of the $CD4^-CD8^-TCR_{\alpha\beta}^+$ cells (Fig. 1A). This is not surprising considering that MAIT cells are most abundant in nonlymphoid tissues (14). After genetic exclusion of NKT cells in $CD1d^{-/-}$ mice and selective exclusion of γδ T cells and $TCR_{\alpha\beta}^+$ CD8αα$^+$ IELs by flow cytometry, we find that mature $TCR_{\alpha\beta}^+$ DN T cells are present in comparable numbers to type 1 NKT cells, composing $<1\%$ of total splenocytes (Fig. 1B). We also find that DN T cells differ from other unconventional T cell subsets such as some γδ T cells, NKT, and MAIT cells, as they lack expression of the PLZF transcription factor (Fig. 1C) (15, 44–49). Together, these data show that $TCR_{\alpha\beta}^+$ DN T cells in the spleen are a distinct unconventional T cell subset.

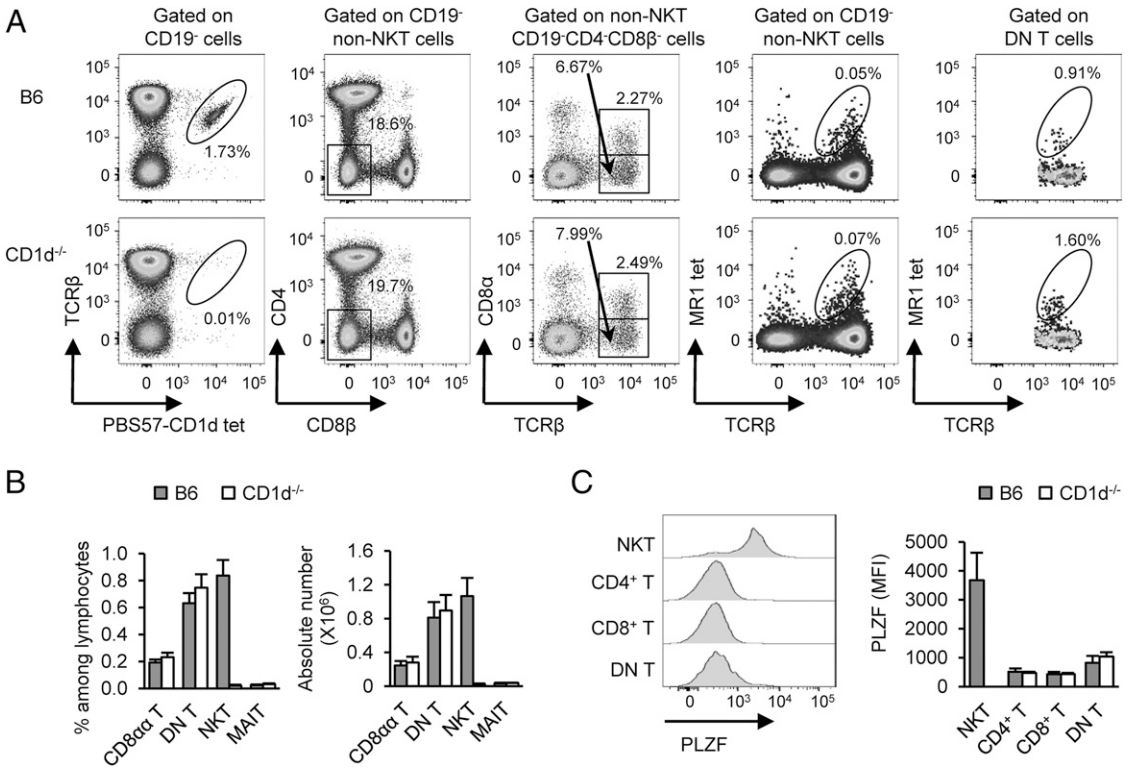

FIGURE 1. Mature DN T cells are distinct from other T cell subsets. (**A**) The spleen was harvested from 8- to 12-wk-old B6 and CD1d$^{-/-}$ mice and analyzed by flow cytometry to determine the proportion and absolute number of T cell subsets. The complete gating strategy for the various cell subsets is detailed in Supplemental Fig. 1. Specifically, type 1 NKT cells, MAIT cells, CD8αα$^+$ T cells, and DN T cells were identified as PBS-57 CD1d-tetramer$^+$, MR1-tetramer$^+$, CD4$^-$CD8β$^-$CD8α$^+$, and CD4$^-$CD8β$^-$CD8α$^-$ cells, respectively. (**B**) A compilation of the percentages and absolute numbers are plotted ($n \geq 4$ mice per group). Mean ± SD are shown. (**C**) T cell subsets from the spleens of B6 and CD1d$^{-/-}$ mice were analyzed by flow cytometry for the expression of PLZF ($n = 3$ per genotype).

Mature DN T cells are of thymic origin

T cells typically complete their maturation in the thymus before exiting to the periphery. We thus examined embryonic thymi from CD1d$^{-/-}$ mice for the presence of mature TCR$_{\alpha\beta}^+$ DN thymocytes. Thymi from 14.5- to 19.5-d-old CD1d$^{-/-}$ embryos harbored mature TCR$_{\alpha\beta}^+$ DN thymocytes that develop in the thymus with similar kinetics to both conventional CD4$^+$ and CD8$^+$ single positive thymocytes (Fig. 2A). Embryos lack peripheral T cells such that the presence of mature TCR$_{\alpha\beta}^+$ DN thymocytes cannot be attributed to recirculation of mature T cells back to the thymus. As mature TCR$_{\alpha\beta}^+$ DN thymocytes are present in fetal thymi, we next grafted thymic lobes from CD1d$^{-/-}$ embryos under the kidney capsule of adult CD1d$^{-/-}$ mice expressing a different CD45 allele to track thymus-derived cells in the peripheral organs of the host. A sizeable number of mature DN T cells derived from the grafted thymus were detected in the host spleens at 2, 3, and 4 wk postgraft (Fig. 2B). Together, these experiments demonstrate that mature TCR$_{\alpha\beta}^+$ DN thymocytes can be found in the thymus and suggest that they may be precursors to the mature DN T cells found in the spleen.

Thymic CD5^{low} and CD5^{high} DN T cells exhibit distinct properties

We next sought to test whether mature TCR$_{\alpha\beta}^+$ DN thymocytes were indeed precursors to mature TCR$_{\alpha\beta}^+$ DN T cells in the spleen. In the thymus, the mature TCR$_{\alpha\beta}^+$ DN thymocyte pool includes precursors to TCR$_{\alpha\beta}^+$ CD8αα$^+$ IELs (23, 27–29, 50). Several studies suggest that the CD5^{high} fraction of the TCR$_{\alpha\beta}^+$ DN thymocyte subset are precursors to TCR$_{\alpha\beta}^+$ CD8αα$^+$ IELs (23, 27–29, 51). Interestingly, we find that mature DN T cells in the spleen express rather low levels of CD5 relative to conventional T cells (Fig. 3A). Therefore, we segregated the TCR$_{\alpha\beta}^+$ DN thymocytes based on CD5 expression (Fig. 3B, top). Specifically, we analyzed both CD5^{high} and CD5^{low} TCR$_{\alpha\beta}^+$ DN thymocytes from B6.CD1d$^{-/-}$.Rag2^{GFP} reporter mice, where GFP expression coincides with time since TCR rearrangement (52). Both CD5^{high} and CD5^{low} TCR$_{\alpha\beta}^+$ DN thymocyte subsets expressed GFP, suggesting that they had recently undergone TCR rearrangement, yet the proportion of GFP$^+$ cells was lower in the CD5^{low} relative to the CD5^{high} TCR$_{\alpha\beta}^+$ DN thymocytes (Fig. 3B, 3C). Of note, GFP expression is rapidly lost upon cell cycling. Indeed, NKT cells are GFP$^-$ even prior to their exit from the thymus [McCaughtry et al. (53) and data not shown], likely due to both proliferation post–TCR rearrangement and their long-term residency in the thymus (54, 55). To test whether cell proliferation may explain the loss of GFP expression in CD5^{low} TCR$_{\alpha\beta}^+$ DN thymocytes, we quantified Ki-67 expression, a protein expressed during cell proliferation (56). We found that a higher proportion of thymic CD5^{low} TCR$_{\alpha\beta}^+$ DN thymocytes were Ki-67$^+$ in comparison with the CD5^{high} subset (Fig. 3B). Interestingly, the proportion of GFP$^+$ CD5^{low} TCR$_{\alpha\beta}^+$ DN thymocytes was highest in younger mice and declined with age (Fig. 3C), suggesting that the thymic residency time of CD5^{low} TCR$_{\alpha\beta}^+$ DN thymocytes may be greater than that of the CD5^{high} subset. Altogether, these results suggest that CD5 expression can discriminate two TCR$_{\alpha\beta}^+$ DN thymocyte subsets, where CD5^{low} TCR$_{\alpha\beta}^+$ DN thymocytes are more actively cycling.

Thymic CD5^{low} DN T cells are precursors to peripheral DN T cells

Although CD5^{high} TCR$_{\alpha\beta}^+$ DN thymocytes are precursors to TCR$_{\alpha\beta}^+$ CD8αα$^+$ IELs, the ability of CD5^{low} TCR$_{\alpha\beta}^+$ DN thymocytes

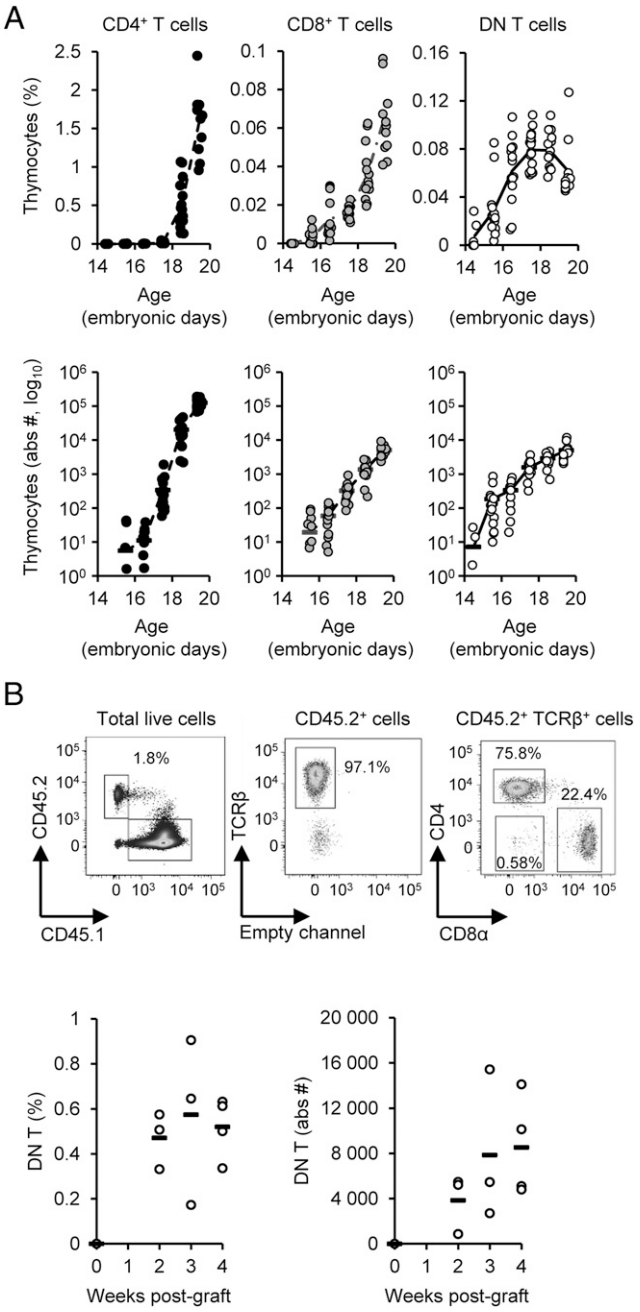

FIGURE 2. Peripheral mature DN T cells are of thymic origin. (**A**) T cell populations were quantified in CD1d$^{-/-}$ embryonic thymi from E14.5 to E19.5 d. The proportion and absolute number of thymocyte subsets are shown among total thymocytes. Each dot represents data from one embryo (n = the number of mice for each embryonic day; 14.5: n = 6; 15.5: n = 16; 16.5: n = 14; 17.5: n = 18; 18.5: n = 15; 19.5: n = 12; for at least 2 litters per time point). (**B**) Thymi from E15.5 or E16.5 CD1d$^{-/-}$ embryos (CD45.2$^+$) were grafted under the kidney capsule of CD1d$^{-/-}$ mice (CD45.1$^+$), where CD45 allotypic markers allow for cell tracking. DN T cells derived from the grafted thymus were quantified in the recipient spleen 2 (n = 3), 3 (n = 3) or 4 (n = 4) wk postgraft. Top, Representative profile of graft-derived cells (CD45.2$^+$) 14 d postgraft. Bottom, The proportion of DN T cells among graft-derived cells and the total number of graft-derived DN T cells are shown. Each dot represents data from one mouse.

to generate T cells in the periphery is not known. To test the lineage fate of these different precursors, we sorted TCR$_{\alpha\beta}^+$ DN thymocytes from CD1d$^{-/-}$ mice based on CD5^{low} and CD5^{high} expression and transferred them into Rag1$^{-/-}$ mice, where the donor and recipient mice expressed different CD45 alleles (Supplemental Fig. 2). As expected, CD5^{high} TCR$_{\alpha\beta}^+$ DN thymocytes homed to both the spleen and intestine (Fig. 4A) (23, 28, 50). In contrast, CD5^{low} TCR$_{\alpha\beta}^+$ DN thymocytes preferentially homed to the spleen (Fig. 4A). Consistent with previous reports, most CD5^{high} TCR$_{\alpha\beta}^+$ DN thymocytes acquired a CD8$^+$ T cell phenotype in the spleen and differentiated into TCR$_{\alpha\beta}^+$ CD8αα$^+$ IELs in the intestine (Fig. 4B, 4C) (9, 29). Conversely, the CD5^{low} TCR$_{\alpha\beta}^+$ DN thymocytes primarily maintained a CD4$^-$CD8$^-$ TCR$_{\alpha\beta}^+$ phenotype in the spleen (Fig. 4B, 4D). These in vivo transfer studies confirm that CD5^{high} TCR$_{\alpha\beta}^+$ DN thymocytes preferentially yield TCR$_{\alpha\beta}^+$ CD8αα$^+$ IELs and demonstrate that CD5^{low} TCR$_{\alpha\beta}^+$ DN thymocytes are precursors to mature DN T cells in the spleen.

Thymic differentiation of DN T cells proceeds through the DP stage

We next sought to define the differentiation steps that precede the generation of mature DN T cells. As such, we took advantage of transgenic reporter mice that allow for cell-fate tracking. Specifically, we used three Cre transgenic mouse models, namely CD4-Cre (57), dLck-Cre (58), and E8I-Cre (59), where the Cre enzyme is first expressed in DP, late DP, and CD4$^-$CD8$^+$ thymocytes, respectively. These Cre-transgenic mice were crossed to RosaYFP mice (60). In this model, activation of the promoter induces Cre expression leading to permanent expression of YFP in the cell and all its daughter cells. For all reporter systems, thymocytes from both CD1d-sufficient and -deficient mice were studied, and no notable differences were observed other a lack of NKT cells in all CD1d$^{-/-}$ reporter mice (Fig. 5). As expected, we found that all DP thymocytes are YFP$^+$ in CD4-Cre reporter mice, a subset of DP thymocytes expressed YFP in dLck-Cre reporter mice, and all DP thymocytes were YFP$^-$ in E8I-Cre reporter mice (Fig. 5A–C). Moreover, as γδ T cells do not transition through the DP thymocyte stage (61), γδ T cells were mostly negative for YFP expression in the three reporter strains (Fig. 5A–C). When analyzing TCR$_{\alpha\beta}^+$ DN thymocytes, we found that almost all mature TCR$_{\alpha\beta}^+$ DN thymocytes were positive for YFP expression in the CD4-Cre reporter mice (Fig. 5A), demonstrating that mature TCR$_{\alpha\beta}^+$ DN thymocytes transit through the DP thymocyte stage. Similar results were obtained using the E8III-Cre mice, which also drives Cre expression in early DP thymocytes (data not shown). However, in dLck-Cre reporter mice, ~20% of the mature TCR$_{\alpha\beta}^+$ DN thymocytes were positive for YFP expression (Fig. 5B). Because only a fraction of the TCR$_{\alpha\beta}^+$ DN thymocytes expressed YFP in the dLck-Cre reporter mice, we further segregated these cells based on CD5 expression. A similar proportion of both CD5^{high} and CD5^{low} TCR$_{\alpha\beta}^+$ DN thymocytes were YFP$^+$ (Fig. 5D). This suggests that both TCR$_{\alpha\beta}^+$ DN thymocyte subsets primarily do not transit through the late DP stage and thus differentiate earlier than most conventional CD4$^+$ and CD8$^+$ single positive thymocytes. Finally, in E8I-Cre reporter mice, almost none of the mature TCR$_{\alpha\beta}^+$ DN thymocytes were positive for YFP expression (Fig. 5C), showing that mature CD8$^+$ single-positive thymocytes are not the main precursors to mature TCR$_{\alpha\beta}^+$ DN thymocytes. Altogether, these findings demonstrate that, as for NKT and MAIT cells, mature TCR$_{\alpha\beta}^+$ DN thymocytes transit through the DP thymocyte stage.

Low CD5 and Nur77 levels on mature TCR$_{\alpha\beta}^+$ DN thymocytes reflect low TCR signal strength for their differentiation

CD5 levels can serve as surrogate markers for TCR signaling during the thymic selection process (10, 26), suggesting that CD5^{low} TCR$_{\alpha\beta}^+$ DN thymocytes are selected by weak TCR signals. In

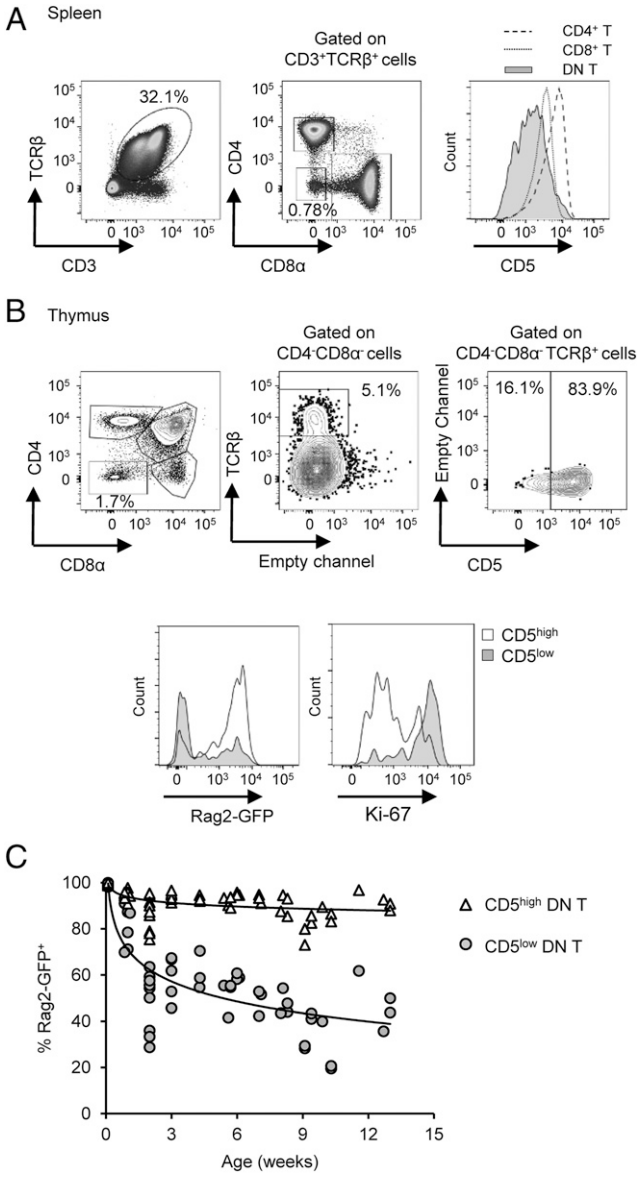

FIGURE 3. $CD5^{low}$ and $CD5^{high}$ $TCR_{\alpha\beta}{}^{+}$ DN thymocytes exhibit different properties. (**A**) Left and middle, Gating for T cell subsets in the spleen. Right, Representative overlay of CD5 expression in $CD4^{+}$ (dashed), $CD8^{+}$ (dotted), and DN T (filled histogram) cells from the spleen of a $CD1d^{-/-}$ mouse (n = 7). (**B**) Top, Gating strategy for separating $CD5^{high}$ and $CD5^{low}$ $TCR_{\alpha\beta}{}^{+}$ DN thymocytes. Bottom, Representative GFP expression in both $CD5^{high}$ (line) and $CD5^{low}$ (shaded) $TCR_{\alpha\beta}{}^{+}$ DN thymocytes from B6.$Rag2^{GFP}$.$CD1d^{-/-}$ mice (left) and Ki-67 expression (right) from $CD1d^{-/-}$ mice ($Rag2^{GFP}$: n = 14; Ki-67: n = 2). (**C**) GFP expression in $CD5^{high}$ (triangles) and $CD5^{low}$ (circles) $TCR_{\alpha\beta}{}^{+}$ DN thymocytes from 1- to 13-wk-old B6.$Rag2^{GFP}$.$CD1d^{-/-}$ mice. Each dot represents data from one mouse for age in weeks as follows: 0: n = 20; 1: n = 7; 2: n = 9; 3: n = 5; 4: n = 3; 5: n = 1; 6: n = 6; 7: n = 3; 8: n = 4; 9: n = 4; 10: n = 3; 12: n = 1; 13: n = 3. Exponential regression curves of GFP^{+} cells among each thymocyte subset as a function of age are shown.

addition to CD5 levels, Nur77 represents a robust marker for the relative TCR signal strength that occurs during thymocyte differentiation (25). Therefore, we quantified Nur77 expression in thymocyte subsets from $Nur77^{GFP}$.$CD1d^{-/-}$ reporter mice (Fig. 6). As expected, among the single positive thymocytes, the expression levels of CD5 and $Nur77^{GFP}$ are highest in thymic $CD4^{+}CD25^{+}$ regulatory T cells, followed by conventional $CD4^{+}$ and $CD8^{+}$ single-positive thymocytes (Fig. 6), in line with their reported relative affinity for self-peptide MHC (10). $CD5^{high}$ $TCR_{\alpha\beta}{}^{+}$ DN thymocytes, which are selected on very high affinity ligands, expressed the highest level of $Nur77^{GFP}$ (Fig. 6) (10). In contrast, $CD5^{low}$ $TCR_{\alpha\beta}{}^{+}$ DN thymocytes expressed very low levels of $Nur77^{GFP}$, well below the levels observed for $CD4^{+}$ and $CD8^{+}$ thymocytes that have undergone positive selection (Fig. 6). These results suggest that mature DN T cells are selected by weak TCR–Ag interactions.

DN T cells differentiate in the absence of MHC expression

We have shown that DN T cells can differentiate in the thymus, transit through a DP thymocyte stage, and receive weak TCR signals relative to other T cell subsets. During thymocyte positive selection, both CD4 and CD8 coreceptor expression at the DP thymocyte stage participates in stabilizing TCR to self-peptide/MHC interactions. As mature $TCR_{\alpha\beta}{}^{+}$ DN thymocyte transit through the DP thymocyte stage, we next sought to define their MHC restriction. We assessed the proportion and number of $CD5^{low}$ $TCR_{\alpha\beta}{}^{+}$ DN thymocytes of five different mouse strains, each lacking different combinations of MHC molecules and all precluding NKT cell differentiation. Specifically, we quantified thymocyte subsets in MHC-sufficient mice (B6.$CD1d^{-/-}$), classical MHC I–deficient ($K^{b}D^{b-/-}$.$CD1d^{-/-}$), both classical and nonclassical MHC I–deficient (β2-microglobulin $[B2m]^{-/-}$), MHC II-deficient (MHC $II^{-/-}CD1d^{-/-}$), and total MHC-deficient ($B2m^{-/-}$MHC $II^{-/-}$) mice. Of note, B2m is an essential component both of classical and of most nonclassical MHC I molecules, including CD1d and MR1; therefore, these mice lack NKT and MAIT cells (data not shown) (62–64). As anticipated, mice lacking MHC I or II molecules contained a paucity of CD8 and CD4 single-positive thymocytes, respectively (Fig. 7). The $CD5^{high}$ $TCR_{\alpha\beta}{}^{+}$ DN thymocytes, which are precursors to $TCR_{\alpha\beta}{}^{+}$ $CD8\alpha\alpha^{+}$ IELs, are largely dependent on MHC I expression (Fig. 7), as previously reported (23, 27, 65). Notably, the percentage and absolute number of $CD5^{low}$ $TCR_{\alpha\beta}{}^{+}$ DN thymocytes, precursors to peripheral DN T cells, was similar in all strains (Fig. 7). Taken together, we provide evidence that unconventional mature DN T cells can develop in the absence of MHC.

Polyclonal DN T cells can be maintained in periphery in the absence of MHC

As T cells are typically selected on MHC molecules, we wanted to determine if the absence of MHC influenced the pool of peripheral DN T cells. We thus quantified the proportion and absolute number of DN T cells in the spleen of the five different mouse strains, each lacking different combinations of MHC molecules, as detailed above. Although we observed some variations, DN T cells were present in the spleen of all five strains. Specifically, the proportion and absolute numbers of DN T cells in mice lacking either MHC I ($K^{b}D^{b-/-}$.$CD1d^{-/-}$ and $B2m^{-/-}$) or MHC II (MHC $II^{-/-}CD1d^{-/-}$), were decreased or increased relative to $CD1d^{-/-}$ mice, respectively (Fig. 8A). This suggests that MHC may influence peripheral survival and/or expansion of DN T cells. In contrast, we found that both the proportion and absolute number of DN T cells are similar in $CD1d^{-/-}$ and $B2m^{-/-}$MHC $II^{-/-}$ mice (Fig. 8A). Therefore, DN T cell that differentiate in the absence of MHC (i.e., $B2m^{-/-}$MHC $II^{-/-}$ mice) are able to survive in an MHC-deficient setting.

MHC restriction in the thymus can influence the TCR repertoire (66). Therefore, we examined the TCR Vβ repertoire of DN T cells from $CD1d^{-/-}$, $B2m^{-/-}$, and MHC $II^{-/-}CD1d^{-/-}$ mice. In contrast to unconventional T cells for which the TCR repertoire is typically rather narrow (8), DN T cells from these three mouse strains all expressed a polyclonal TCR Vβ repertoire (Fig. 8B).

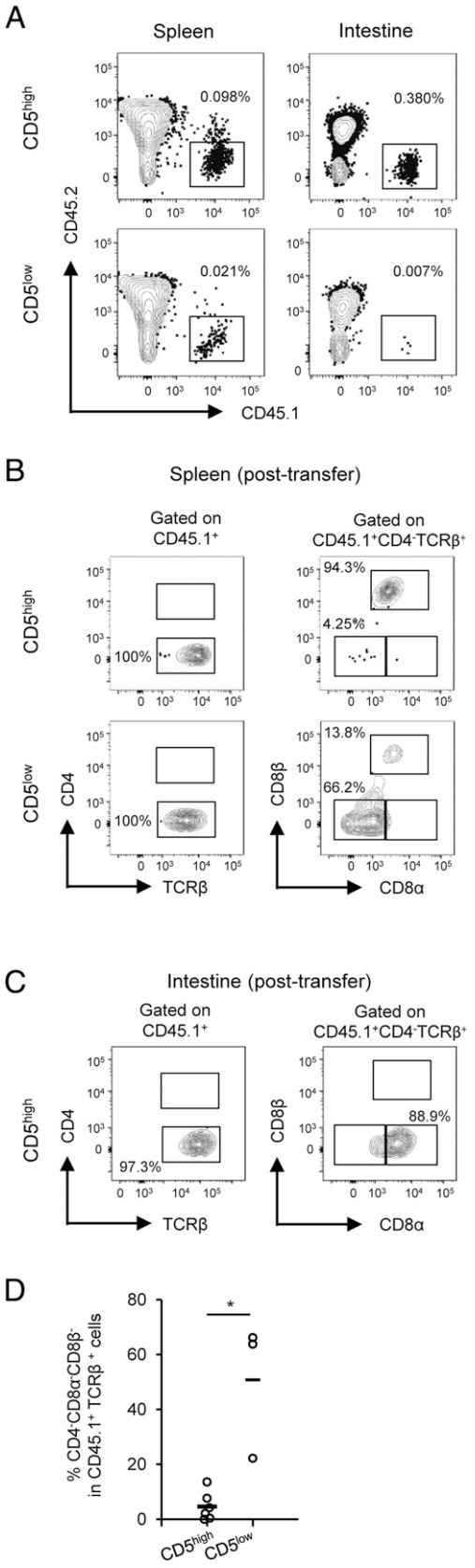

FIGURE 4. Thymic precursors to DN T cells express low levels of CD5. (**A**) CD5^{high} and CD5^{low} TCR$_{\alpha\beta}^{+}$ DN thymocytes from B6.SJL.CD1d$^{-/-}$ (CD45.1) mice were injected in Rag1$^{-/-}$ (CD45.2) mice and tracked 6 wk later in the spleen and intestine. (**B**) Phenotype of the transferred cells in the spleen of mice described in (A). (**C**) Phenotype of the transferred CD5^{high} TCR$_{\alpha\beta}^{+}$ DN thymocytes in the intestine of mice described in (A). (**D**) Compilation of the proportion of splenic DN T cells that originate from either CD5^{high} or CD5^{low} TCR$_{\alpha\beta}^{+}$ DN thymocytes, upon transfer. CD5^{high}: $n = 6$; CD5^{low}: $n = 3$. $*p < 0.05$ according to the paired Student t test.

However, differences in the proportion of select Vβs were observed among the different strains of mice, suggesting that MHC may influence the DN T cell TCR repertoire. Of note, the TCR Vβ repertoire remains polyclonal in B2m$^{-/-}$MHC II$^{-/-}$ mice that lack MHC I and MHC II expression (Supplemental Fig. 3). Altogether, these results suggest that although the presence or absence of MHC influences the TCR repertoire, DN T cells selected in the thymus in the absence of MHC express a polyclonal TCR repertoire and can survive in the periphery.

TCR–MHC interactions are typically needed for T cell differentiation and survival. However, our data show that at least some DN T cells can differentiate and survive in the periphery in the absence of MHC. We thus wondered whether DN T cells could respond to TCR stimulation. In vitro stimulation with anti-CD3 increased the proportion of CD69^{+} DN T cells, albeit to a lesser extent than for conventional T cells (Fig. 8C). It thus appears that the TCR signal is functional in these cells.

Discussion

Each T cell subset plays a unique role in the immune response. In addition to conventional CD4^{+} and CD8^{+} T cells, many unconventional T cell subsets have been described, including NKT cells, MAIT cells, and γδ T cells, among others (8). These unconventional T cells differ from conventional CD4^{+} and CD8^{+} T cells, in that they are typically selected on nonclassical MHC molecules and express a limited TCR repertoire. They also express a memory-like signature in secondary lymphoid organs and, as for memory T cells, respond more quickly upon antigenic challenge (8). Another characteristic of unconventional T cells that distinguishes them from conventional T cells is that subsets of these cells lack both CD4 and CD8 coreceptor expression. T cells expressing a CD4^{-}CD8^{-}CD3^{+} phenotype include NKT cells, MAIT cells, and γδ T cells, in addition to DN T cells defined in this study. There are no unique markers to define DN T cells, posing a considerable challenge for characterizing the phenotype and function of these cells. To address this challenge we exploit CD1d$^{-/-}$ mice to completely exclude all NKT cells (17, 18), and, by flow cytometry, we also carefully exclude γδ T and CD8αα T cells. This approach has allowed us to specifically study DN T cells and demonstrate that they are a distinct T cell subset with a polyclonal TCR repertoire. Using thymic grafts, we unequivocally demonstrate that DN T cells can originate in the thymus. In complementary cell-fate mapping experiments, we also show that DN T cells transit through an early DP thymocyte stage. This contrasts with γδ T cells, which directly differentiate from immature DN thymocytes. The thymic selection of DN T cells is distinct from NKT and MAIT cells, as DN T cells differentiate in B2m$^{-/-}$ mice, whereas both NKT and MAIT cells require B2m for selection on CD1d and MR1, respectively (62, 63). We also demonstrate that DN T cells and TCR$_{\alpha\beta}^{+}$ CD8αα^{+} IELs arise from distinct TCR$_{\alpha\beta}^{+}$ DN thymic precursors, based on the relative expression of CD5. Indeed, CD5^{low} and CD5^{high} TCR$_{\alpha\beta}^{+}$ DN thymocytes preferentially yield mature DN T and TCR$_{\alpha\beta}^{+}$ CD8αα^{+} IELs, respectively. Altogether, we find that thymic differentiation of DN T cells can proceed in an MHC-independent setting.

It could be argued that DN T cells in the spleen are derived from peripheral CD4^{+} or CD8^{+} T cells that have lost coreceptor

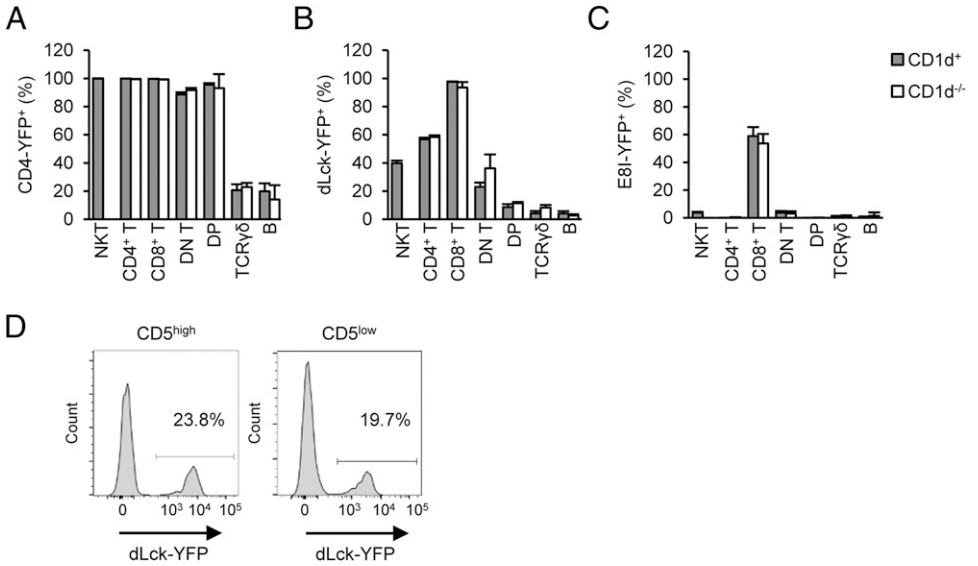

FIGURE 5. Thymic differentiation of DN T cells proceeds through the DP stage. Quantification of YFP^+ cells among the indicated thymocyte subsets from (**A**) CD4-Cre.RosaYFP, ($CD1d^+$: $n = 4$, $CD1d^{-/-}$: $n = 5$) (**B**) dLck-Cre.RosaYFP, ($CD1d^+$: $n = 4$, $CD1d^{-/-}$: $n = 6$), and (**C**) E8I-Cre.RosaYFP ($CD1d^+$: $n = 11$, $CD1d^{-/-}$: $n = 6$) reporter mice on both $CD1d^+$ (gray) or $CD1d^{-/-}$ (white) genetic backgrounds. The data show the mean ± SD of YFP^+ cells. Littermate controls were used in these experiments. (**D**) Representative flow cytometry of YFP expression in $CD5^{high}$ and $CD5^{low}$ $TCR_{\alpha\beta}{}^+$ DN thymocytes from the dLck-Cre.RosaYFP mice ($n = 3$).

expression. We provide different lines of evidence that peripheral DN T cells do not arise from conventional T cells that have lost coreceptor expression. First, <5% of thymic DN T cells are YFP^+ in E8I reporter mice, suggesting that DN T cells do not arise from $CD8^+$ T cells. Second, we find that transfer of $CD4^-CD8^-CD5^{low}$ $TCR_{\alpha\beta}{}^+$ thymocytes effectively yield mature DN T cells, but not conventional T cells, upon in vivo transfer, demonstrating that DN T cells and conventional T cells differentiate from distinct thymic pathways. Third, DN T cells can differentiate in the absence of MHC. Still, in some inflammatory contexts, conventional T cells may lose coreceptor expression and provide additional heterogeneity in the pool of $CD4^-CD8^-CD3^+$ cells (67–71). Experimental settings with adoptive transfers of allogeneically marked $CD4^+$ or $CD8^+$ T cells into $CD1d^{-/-}$ mice subjected to various inflammatory or infectious insults could help resolve this issue.

One of the most striking features of the thymic selection process for DN T cells is the observation that they can differentiate in the absence of MHC. This finding is somewhat reminiscent of the Quad-ko mice, which are genetically deficient for CD4, CD8, B2m, and MHC II and where T cells can be found in secondary lymphoid organs (72). Because of the genetic deletion of CD4 and CD8, all of these T cells also lack coreceptor expression. In the absence of coreceptors and MHC, the $CD4^-CD8^-$ $TCR_{\alpha\beta}{}^+$ T cells from the Quad-ko mice were shown to recognize antigenic structures in an Ab-like manner (72–74). In $CD1d^{-/-}$ mice, the mode of Ag recognition for DN T cells remains to be defined, but it is tempting to speculate that some DN T cell may recognize non-MHC antigenic structures, similar to what has been observed in Quad-ko mice (72–74).

We had previously shown that TCR transgenic DN T cells could prevent autoimmune diabetes (30). Others have also reported that $CD4^-CD8^-CD3^+$ T cells exhibit broad immunoregulatory functions (75–78). In contrast, there are some reports of pathogenic activity and of antitumor activity mediated by $CD4^-CD8^-CD3^+$ T cells (70, 79–82). However, as $CD4^-CD8^-CD3^+$ T cells represent a heterogeneous cell population, which includes NKT cells, MAIT cells, γδ T cells, and $TCR_{\alpha\beta}{}^+$ $CD8\alpha\alpha^+$ IELs, in addition to $TCR_{\alpha\beta}{}^+$ DN T cells, the functional and phenotypic data from these studies is not directly comparable to the phenotype and function of $TCR_{\alpha\beta}{}^+$ DN T cells described in this study (75, 78). In future work, it will be important to determine if $TCR_{\alpha\beta}{}^+$ DN T cells confer Ag-specific or broad immunoregulatory activity.

In summary, we revealed key properties of DN T cells that identify them as a unique T cell subset, distinct from both conventional $CD4^+$ and $CD8^+$ T cells as well as from other unconventional T cell subsets. To our knowledge, this study points to the

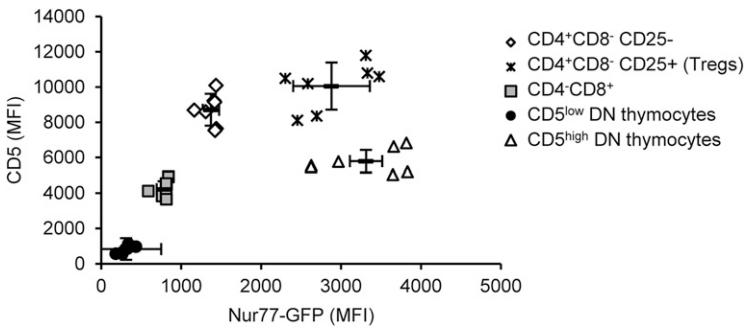

FIGURE 6. Relative expression of CD5 and $Nur77^{GFP}$ as surrogate markers of TCR signal strength. CD5 and $Nur77^{GFP}$ expression profiles on the indicated thymocyte subsets from $Nur77^{GFP}.CD1d^{-/-}$ mice. Each symbol represents data from an individual mouse ($n = 7$). Cross indicates the mean ± SD.

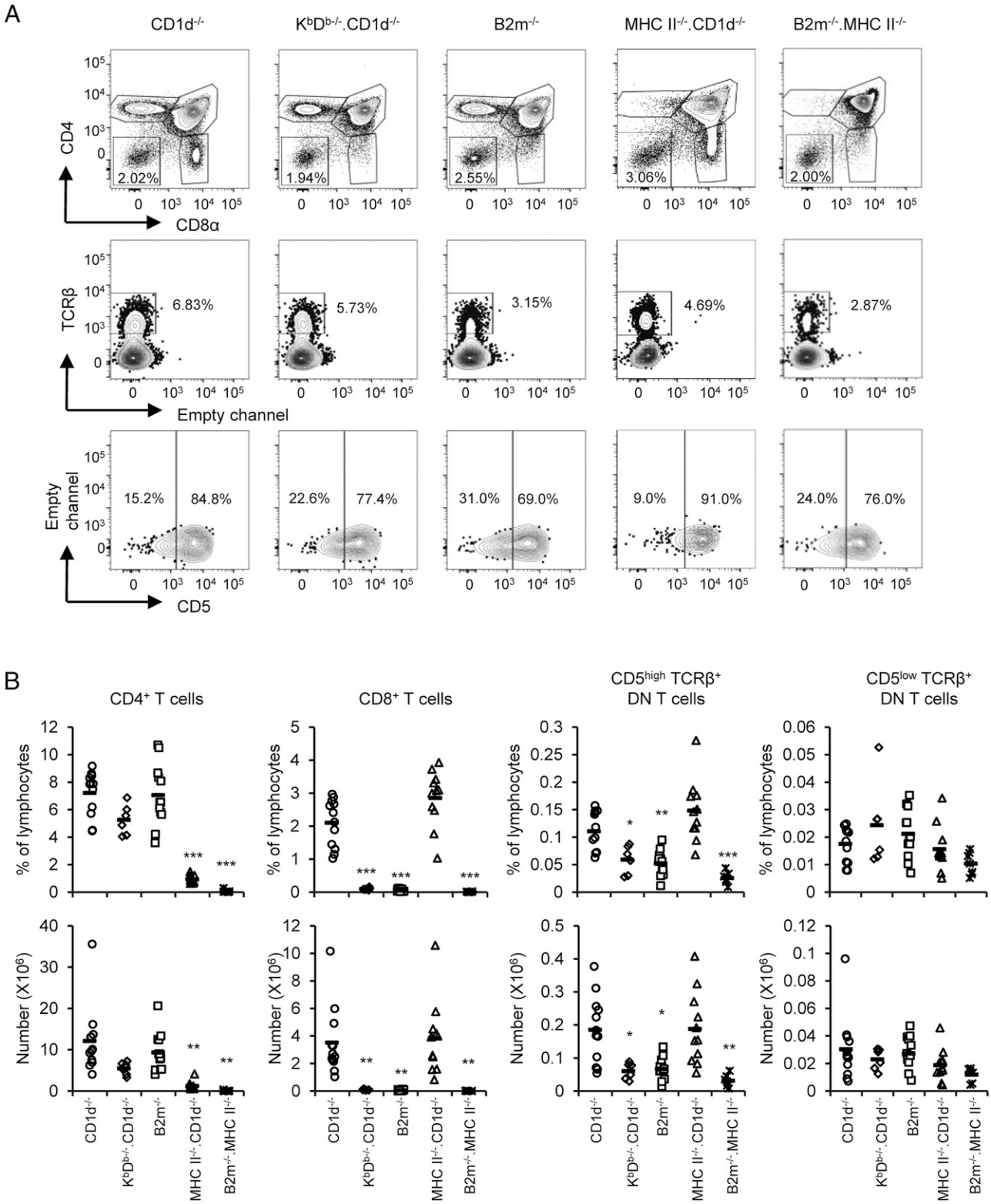

FIGURE 7. DN T cells differentiate in the absence of MHC expression. (**A**) Representative flow cytometry profiles of CD4 and CD8α expression (top), TCRβ expression on $CD4^-CD8\alpha^-$ thymocytes (middle), and CD5 expression on $TCR\beta^+CD4^-CD8\alpha^-$ thymocytes (bottom) for the indicated mouse strains. (**B**) Proportion and absolute number of $CD4^+$ ($TCR\beta^+CD4^+CD8\alpha^-$), $CD8^+$ ($TCR\beta^+CD4^-CD8\alpha^+$), $CD5^{high}TCR_{\alpha\beta}{}^+$ DN ($CD5^{high}TCR\beta^+CD4^-CD8\alpha^-$), and $CD5^{low}TCR_{\alpha\beta}{}^+$ DN ($CD5^{low}TCR\beta^+CD4^-CD8\alpha^-$) thymocytes for the indicated mouse strains ($CD1d^{-/-}$: $n = 12$; $K^bD^{b-/-}.CD1d^{-/-}$: $n = 6$; $B2m^{-/-}$: $n = 12$; MHC $II^{-/-}CD1d^{-/-}$: $n = 11$; $B2m^{-/-}$MHC $II^{-/-}$: $n = 7$). $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ according to one-way ANOVA with Games–Howell post hoc.

first T cell subset that can differentiate in the thymus in the absence of MHC. Based on the TCR signal strength model required for differentiation, our data position DN T cells at the opposite end of the spectrum to agonist selection. As we have now described that thymic $CD5^{low}$ $TCR_{\alpha\beta}{}^+$ DN T cells are precursor to $TCR_{\alpha\beta}{}^+$ DN T cells in the spleen, this will facilitate the investigation of

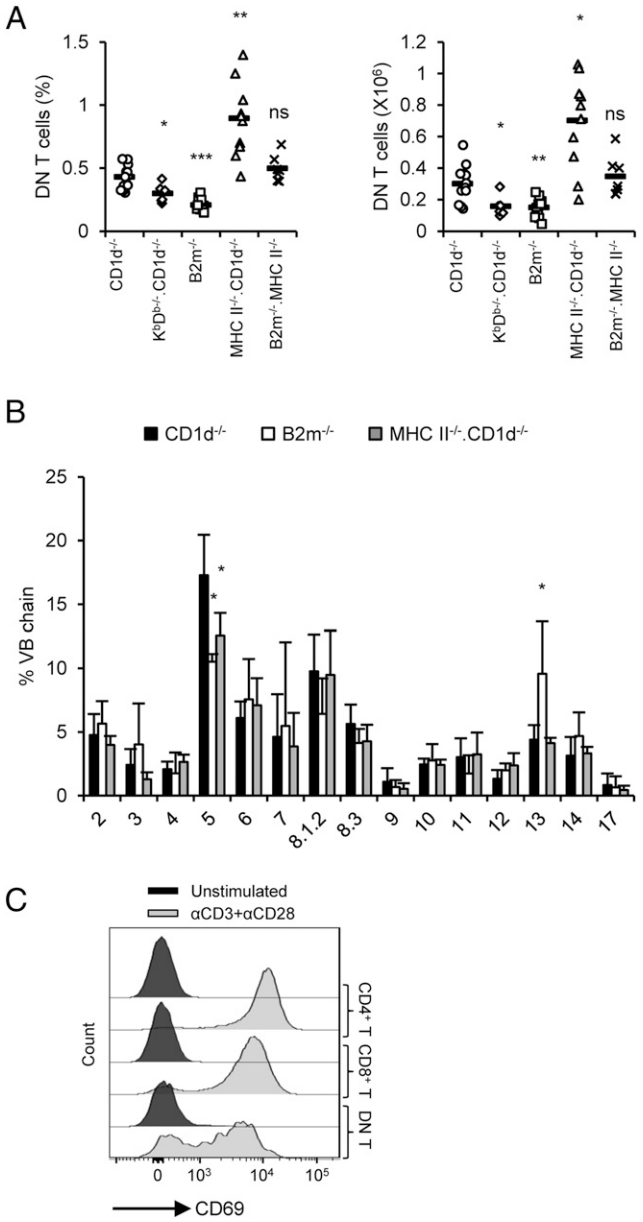

FIGURE 8. DN T cell characterization in the periphery. (**A**) Proportion and absolute number of $TCR_{\alpha\beta}{}^{+}$ DN ($CD5^{low}TCR\beta^{+}CD4^{-}CD8\alpha^{-}$) spleen cells for the indicated mouse strains ($CD1d^{-/-}$: $n = 12$; $K^bD^{b-/-}.CD1d^{-/-}$: $n = 6$; $B2m^{-/-}$: $n = 12$; MHC $II^{-/-}CD1d^{-/-}$: $n = 11$; $B2m^{-/-}$MHC $II^{-/-}$: $n = 7$). (**B**) TCR Vβ repertoire on DN T cells from the spleens of the indicated mouse strains ($CD1d^{-/-}$: $n = 6$; $B2m^{-/-}$: $n = 7$; MHC $II^{-/-}CD1d^{-/-}$: $n = 3$). Data are graphed as mean + SD. (**C**) CD69 expression on $CD4^{+}$, $CD8^{+}$, and DN T cells from the spleen of a $CD1d^{-/-}$ mouse following stimulation with plate-bound anti-CD3 and anti-CD28 (gray histograms). Profiles in black are from unstimulated cells. Histograms are representative of three experiments. $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$ according to one-way ANOVA with Games–Howell post hoc (A) or two-way ANOVA with Bonferroni post hoc (B).

this rare cell type and will help define its biological function in various contexts.

Acknowledgments

We are grateful to Drs. Nathalie Labrecque, Al Singer, Carla Shatz, and Rafei Moutih for providing E8I-Cre, E8III-Cre, $K^bD^{b-/-}$, and B6.$Rag2^{GFP}$ mice, respectively. We also thank Martine Dupuis for cell sorting experiments, Ianula Mihaela Banu and Catherine Mauger-Labelle, and all of the animal house staff at the Maisonneuve-Rosemont research center for management of the mouse colonies.

Disclosures

The authors have no financial conflicts of interest.

References

1. Labrecque, N., T. Baldwin, and S. Lesage. 2011. Molecular and genetic parameters defining T-cell clonal selection. *Immunol. Cell Biol.* 89: 16–26.
2. Kurd, N., and E. A. Robey. 2016. T-cell selection in the thymus: a spatial and temporal perspective. *Immunol. Rev.* 271: 114–126.
3. Bhandoola, A., H. von Boehmer, H. T. Petrie, and J. C. Zúñiga-Pflücker. 2007. Commitment and developmental potential of extrathymic and intrathymic T cell precursors: plenty to choose from. *Immunity* 26: 678–689.
4. Klein, L., B. Kyewski, P. M. Allen, and K. A. Hogquist. 2014. Positive and negative selection of the T cell repertoire: what thymocytes see (and don't see). *Nat. Rev. Immunol.* 14: 377–391.
5. Bhandoola, A., and A. Sambandam. 2006. From stem cell to T cell: one route or many? [Published erratum appears in 2006 *Nat. Rev. Immunol.* 6: 252.] *Nat. Rev. Immunol.* 6: 117–126.
6. Love, P. E., and A. Bhandoola. 2011. Signal integration and crosstalk during thymocyte migration and emigration. *Nat. Rev. Immunol.* 11: 469–477.
7. Starr, T. K., S. C. Jameson, and K. A. Hogquist. 2003. Positive and negative selection of T cells. *Annu. Rev. Immunol.* 21: 139–176.
8. Godfrey, D. I., A. P. Uldrich, J. McCluskey, J. Rossjohn, and D. B. Moody. 2015. The burgeoning family of unconventional T cells. [Published erratum appears in 2016 *Nat. Immunol.* 17: 214; Published erratum appears in 2016 *Nat. Immunol.* 17: 469.] *Nat. Immunol.* 16: 1114–1123.
9. Cheroutre, H., F. Lambolez, and D. Mucida. 2011. The light and dark sides of intestinal intraepithelial lymphocytes. *Nat. Rev. Immunol.* 11: 445–456.
10. Stritesky, G. L., S. C. Jameson, and K. A. Hogquist. 2012. Selection of self-reactive T cells in the thymus. *Annu. Rev. Immunol.* 30: 95–114.
11. Coffey, F., S. Y. Lee, T. B. Buus, J. P. Lauritsen, G. W. Wong, M. L. Joachims, L. F. Thompson, J. C. Zúñiga-Pflücker, D. J. Kappes, and D. L. Wiest. 2014. The TCR ligand-inducible expression of CD73 marks γδ lineage commitment and a metastable intermediate in effector specification. *J. Exp. Med.* 211: 329–343.
12. Haks, M. C., J. M. Lefebvre, J. P. Lauritsen, M. Carleton, M. Rhodes, T. Miyazaki, D. J. Kappes, and D. L. Wiest. 2005. Attenuation of gammadeltaTCR signaling efficiently diverts thymocytes to the alphabeta lineage. *Immunity* 22: 595–606.
13. Hayes, S. M., L. Li, and P. E. Love. 2005. TCR signal strength influences alphabeta/gammadelta lineage fate. *Immunity* 22: 583–593.
14. Le Bourhis, L., Y. K. Mburu, and O. Lantz. 2013. MAIT cells, surveyors of a new class of antigen: development and functions. *Curr. Opin. Immunol.* 25: 174–180.
15. Koay, H. F., N. A. Gherardin, A. Enders, L. Loh, L. K. Mackay, C. F. Almeida, B. E. Russ, C. A. Nold-Petry, M. F. Nold, S. Bedoui, et al. 2016. A three-stage intrathymic development pathway for the mucosal-associated invariant T cell lineage. *Nat. Immunol.* 17: 1300–1311.
16. Egawa, T., G. Eberl, I. Taniuchi, K. Benlagha, F. Geissmann, L. Hennighausen, A. Bendelac, and D. R. Littman. 2005. Genetic evidence supporting selection of the Valpha14i NKT cell lineage from double-positive thymocyte precursors. *Immunity* 22: 705–716.
17. Bendelac, A., O. Lantz, M. E. Quimby, J. W. Yewdell, J. R. Bennink, and R. R. Brutkiewicz. 1995. CD1 recognition by mouse NK1+ T lymphocytes. *Science* 268: 863–865.
18. Bendelac, A., P. B. Savage, and L. Teyton. 2007. The biology of NKT cells. *Annu. Rev. Immunol.* 25: 297–336.
19. Exley, M., J. Garcia, S. P. Balk, and S. Porcelli. 1997. Requirements for CD1d recognition by human invariant Valpha24+ CD4-CD8- T cells. *J. Exp. Med.* 186: 109–120.
20. Bendelac, A. 1995. Positive selection of mouse NK1+ T cells by CD1-expressing cortical thymocytes. *J. Exp. Med.* 182: 2091–2096.
21. Le Bourhis, L., L. Guerri, M. Dusseaux, E. Martin, C. Soudais, and O. Lantz. 2011. Mucosal-associated invariant T cells: unconventional development and function. *Trends Immunol.* 32: 212–218.
22. Franciszkiewicz, K., M. Salou, F. Legoux, Q. Zhou, Y. Cui, S. Bessoles, and O. Lantz. 2016. MHC class I-related molecule, MR1, and mucosal-associated invariant T cells. *Immunol. Rev.* 272: 120–138.
23. Ruscher, R., R. L. Kummer, Y. J. Lee, S. C. Jameson, and K. A. Hogquist. 2017. CD8αα intraepithelial lymphocytes arise from two main thymic precursors. *Nat. Immunol.* 18: 771–779.
24. McDonald, B. D., B. Jabri, and A. Bendelac. 2018. Diverse developmental pathways of intestinal intraepithelial lymphocytes. *Nat. Rev. Immunol.* 18: 514–525.
25. Moran, A. E., K. L. Holzapfel, Y. Xing, N. R. Cunningham, J. S. Maltzman, J. Punt, and K. A. Hogquist. 2011. T cell receptor signal strength in Treg and iNKT cell development demonstrated by a novel fluorescent reporter mouse. *J. Exp. Med.* 208: 1279–1289.
26. Azzam, H. S., A. Grinberg, K. Lui, H. Shen, E. W. Shores, and P. E. Love. 1998. CD5 expression is developmentally regulated by T cell receptor (TCR) signals and TCR avidity. *J. Exp. Med.* 188: 2301–2311.
27. McDonald, B. D., J. J. Bunker, I. E. Ishizuka, B. Jabri, and A. Bendelac. 2014. Elevated T cell receptor signaling identifies a thymic precursor to the TCRαβ(+) CD4(-)CD8β(-) intraepithelial lymphocyte lineage. *Immunity* 41: 219–229.
28. Golec, D. P., R. E. Hoeppli, L. M. Henao Caviedes, J. McCann, M. K. Levings, and T. A. Baldwin. 2017. Thymic progenitors of $TCR\alpha\beta^{+}$ CD8αα intestinal intraepithelial lymphocytes require RasGRP1 for development. *J. Exp. Med.* 214: 2421–2435.

29. Gangadharan, D., F. Lambolez, A. Attinger, Y. Wang-Zhu, B. A. Sullivan, and H. Cheroutre. 2006. Identification of pre- and postselection TCRalphabeta+ intraepithelial lymphocyte precursors in the thymus. *Immunity* 25: 631–641.
30. Dugas, V., C. Beauchamp, G. Chabot-Roy, E. E. Hillhouse, and S. Lesage. 2010. Implication of the CD47 pathway in autoimmune diabetes. *J. Autoimmun.* 35: 23–32.
31. Yu, W., H. Nagaoka, M. Jankovic, Z. Misulovin, H. Suh, A. Rolink, F. Melchers, E. Meffre, and M. C. Nussenzweig. 1999. Continued RAG expression in late stages of B cell development and no apparent re-induction after immunization. *Nature* 400: 682–687.
32. Vugmeyster, Y., R. Glas, B. Pérarnau, F. A. Lemonnier, H. Eisen, and H. Ploegh. 1998. Major histocompatibility complex (MHC) class I KbDb $^{-/-}$ deficient mice possess functional CD8+ T cells and natural killer cells. *Proc. Natl. Acad. Sci. USA* 95: 12492–12497.
33. Park, J. H., S. Adoro, T. Guinter, B. Erman, A. S. Alag, M. Catalfamo, M. Y. Kimura, Y. Cui, P. J. Lucas, R. E. Gress, et al. 2010. Signaling by intrathymic cytokines, not T cell antigen receptors, specifies CD8 lineage choice and promotes the differentiation of cytotoxic-lineage T cells. *Nat. Immunol.* 11: 257–264.
34. Lew, A. M., D. M. Pardoll, W. L. Maloy, B. J. Fowlkes, A. Kruisbeek, S. F. Cheng, R. N. Germain, J. A. Bluestone, R. H. Schwartz, and J. E. Coligan. 1986. Characterization of T cell receptor gamma chain expression in a subset of murine thymocytes. *Science* 234: 1401–1405.
35. Bendelac, A., M. N. Rivera, S. H. Park, and J. H. Roark. 1997. Mouse CD1-specific NK1 T cells: development, specificity, and function. *Annu. Rev. Immunol.* 15: 535–562.
36. Matsuda, J. L., O. V. Naidenko, L. Gapin, T. Nakayama, M. Taniguchi, C. R. Wang, Y. Koezuka, and M. Kronenberg. 2000. Tracking the response of natural killer T cells to a glycolipid antigen using CD1d tetramers. *J. Exp. Med.* 192: 741–754.
37. MacDonald, H. R. 2000. CD1d-glycolipid tetramers: a new tool to monitor natural killer T cells in health and disease. *J. Exp. Med.* 192: F15–F20.
38. Liu, Y., R. D. Goff, D. Zhou, J. Mattner, B. A. Sullivan, A. Khurana, C. Cantu, III, E. V. Ravkov, C. C. Ibegbu, J. D. Altman, et al. 2006. A modified alpha-galactosyl ceramide for staining and stimulating natural killer T cells. *J. Immunol. Methods* 312: 34–39.
39. Arrenberg, P., I. Maricic, and V. Kumar. 2011. Sulfatide-mediated activation of type II natural killer T cells prevents hepatic ischemic reperfusion injury in mice. *Gastroenterology* 140: 646–655.
40. Arrenberg, P., R. Halder, Y. Dai, I. Maricic, and V. Kumar. 2010. Oligoclonality and innate-like features in the TCR repertoire of type II NKT cells reactive to a beta-linked self-glycolipid. *Proc. Natl. Acad. Sci. USA* 107: 10984–10989.
41. Godfrey, D. I., H. R. MacDonald, M. Kronenberg, M. J. Smyth, and L. Van Kaer. 2004. NKT cells: what's in a name? *Nat. Rev. Immunol.* 4: 231–237.
42. Godfrey, D. I., K. J. Hammond, L. D. Poulton, M. J. Smyth, and A. G. Baxter. 2000. NKT cells: facts, functions and fallacies. *Immunol. Today* 21: 573–583.
43. Pellicci, D. G., K. J. Hammond, J. Coquet, K. Kyparissoudis, A. G. Brooks, K. Kedzierska, R. Keating, S. Turner, S. Berzins, M. J. Smyth, and D. I. Godfrey. 2005. DX5/CD49b-positive T cells are not synonymous with CD1d-dependent NKT cells. *J. Immunol.* 175: 4416–4425.
44. Rahimpour, A., H. F. Koay, A. Enders, R. Clanchy, S. B. Eckle, B. Meehan, Z. Chen, B. Whittle, L. Liu, D. P. Fairlie, et al. 2015. Identification of phenotypically and functionally heterogeneous mouse mucosal-associated invariant T cells using MR1 tetramers. *J. Exp. Med.* 212: 1095–1108.
45. Cui, Y., K. Franciszkiewicz, Y. K. Mburu, S. Mondot, L. Le Bourhis, V. Premel, E. Martin, A. Kachaner, L. Duban, M. A. Ingersoll, et al. 2015. Mucosal-associated invariant T cell-rich congenic mouse strain allows functional evaluation. *J. Clin. Invest.* 125: 4171–4185.
46. Lu, Y., X. Cao, X. Zhang, and D. Kovalovsky. 2015. PLZF controls the development of fetal-derived IL-17+Vγ6+ γδ T cells. *J. Immunol.* 195: 4273–4281.
47. Kreslavsky, T., A. K. Savage, R. Hobbs, F. Gounari, R. Bronson, P. Pereira, P. P. Pandolfi, A. Bendelac, and H. von Boehmer. 2009. TCR-inducible PLZF transcription factor required for innate phenotype of a subset of gammadelta T cells with restricted TCR diversity. *Proc. Natl. Acad. Sci. USA* 106: 12453–12458.
48. Savage, A. K., M. G. Constantinides, J. Han, D. Picard, E. Martin, B. Li, O. Lantz, and A. Bendelac. 2008. The transcription factor PLZF directs the effector program of the NKT cell lineage. *Immunity* 29: 391–403.
49. Kovalovsky, D., O. U. Uche, S. Eladad, R. M. Hobbs, W. Yi, E. Alonzo, K. Chua, M. Eidson, H. J. Kim, J. S. Im, et al. 2008. The BTB-zinc finger transcriptional regulator PLZF controls the development of invariant natural killer T cell effector functions. *Nat. Immunol.* 9: 1055–1064.
50. Pobezinsky, L. A., G. S. Angelov, X. Tai, S. Jeurling, F. Van Laethem, L. Feigenbaum, J. H. Park, and A. Singer. 2012. Clonal deletion and the fate of autoreactive thymocytes that survive negative selection. *Nat. Immunol.* 13: 569–578.
51. Li, K. P., A. Fähnrich, E. Roy, C. M. Cuda, H. L. Grimes, H. R. Perlman, K. Kalies, and D. A. Hildeman. 2017. Temporal expression of bim limits the development of agonist-selected thymocytes and skews their TCRβ repertoire. *J. Immunol.* 198: 257–269.
52. Boursalian, T. E., J. Golob, D. M. Soper, C. J. Cooper, and P. J. Fink. 2004. Continued maturation of thymic emigrants in the periphery. *Nat. Immunol.* 5: 418–425.
53. McCaughtry, T. M., M. S. Wilken, and K. A. Hogquist. 2007. Thymic emigration revisited. *J. Exp. Med.* 204: 2513–2520.
54. Benlagha, K., T. Kyin, A. Beavis, L. Teyton, and A. Bendelac. 2002. A thymic precursor to the NK T cell lineage. *Science* 296: 553–555.
55. Berzins, S. P., F. W. McNab, C. M. Jones, M. J. Smyth, and D. I. Godfrey. 2006. Long-term retention of mature NK1.1+ NKT cells in the thymus. *J. Immunol.* 176: 4059–4065.
56. Whitfield, M. L., L. K. George, G. D. Grant, and C. M. Perou. 2006. Common markers of proliferation. *Nat. Rev. Cancer* 6: 99–106.
57. Lee, P. P., D. R. Fitzpatrick, C. Beard, H. K. Jessup, S. Lehar, K. W. Makar, M. Pérez-Melgosa, M. T. Sweetser, M. S. Schlissel, S. Nguyen, et al. 2001. A critical role for Dnmt1 and DNA methylation in T cell development, function, and survival. *Immunity* 15: 763–774.
58. Wang, Q., J. Strong, and N. Killeen. 2001. Homeostatic competition among T cells revealed by conditional inactivation of the mouse Cd4 gene. *J. Exp. Med.* 194: 1721–1730.
59. Maekawa, Y., Y. Minato, C. Ishifune, T. Kurihara, A. Kitamura, H. Kojima, H. Yagita, M. Sakata-Yanagimoto, T. Saito, I. Taniuchi, et al. 2008. Notch2 integrates signaling by the transcription factors RBP-J and CREB1 to promote T cell cytotoxicity. *Nat. Immunol.* 9: 1140–1147.
60. Madisen, L., T. A. Zwingman, S. M. Sunkin, S. W. Oh, H. A. Zariwala, H. Gu, L. L. Ng, R. D. Palmiter, M. J. Hawrylycz, A. R. Jones, et al. 2010. A robust and high-throughput Cre reporting and characterization system for the whole mouse brain. *Nat. Neurosci.* 13: 133–140.
61. Shah, D. K., and J. C. Zúñiga-Pflücker. 2014. An overview of the intrathymic intricacies of T cell development. *J. Immunol.* 192: 4017–4023.
62. Miley, M. J., S. M. Truscott, Y. Y. Yu, S. Gilfillan, D. H. Fremont, T. H. Hansen, and L. Lybarger. 2003. Biochemical features of the MHC-related protein 1 consistent with an immunological function. *J. Immunol.* 170: 6090–6098.
63. Treiner, E., L. Duban, S. Bahram, M. Radosavljevic, V. Wanner, F. Tilloy, P. Affaticati, S. Gilfillan, and O. Lantz. 2003. Selection of evolutionarily conserved mucosal-associated invariant T cells by MR1. [Published erratum appears in 2003 *Nature* 423: 1018.] *Nature* 422: 164–169.
64. Bevan, M. J. 2010. The earliest knockouts. *J. Immunol.* 184: 4585–4586.
65. Mayans, S., D. Stepniak, S. Palida, A. Larange, J. Dreux, B. Arlian, R. Shinnakasu, M. Kronenberg, H. Cheroutre, and F. Lambolez. 2014. αβT cell receptors expressed by CD4(-)CD8αβ(-) intraepithelial T cells drive their fate into a unique lineage with unusual MHC reactivities. *Immunity* 41: 207–218.
66. La Gruta, N. L., S. Gras, S. R. Daley, P. G. Thomas, and J. Rossjohn. 2018. Understanding the drivers of MHC restriction of T cell receptors. *Nat. Rev. Immunol.* 18: 467–478.
67. Zhang, Z. X., K. Young, and L. Zhang. 2001. CD3+CD4-CD8- alphabeta-TCR+ T cell as immune regulatory cell. *J. Mol. Med. (Berl.)* 79: 419–427.
68. Zhang, D., W. Yang, N. Degauque, Y. Tian, A. Mikita, and X. X. Zheng. 2007. New differentiation pathway for double-negative regulatory T cells that regulates the magnitude of immune responses. *Blood* 109: 4071–4079.
69. Zhang, D., W. Zhang, T. W. Ng, Y. Wang, Q. Liu, V. Gorantla, F. Lakkis, and X. X. Zheng. 2011. Adoptive cell therapy using antigen-specific CD4$^-$CD8$^-$T regulatory cells to prevent autoimmune diabetes and promote islet allograft survival in NOD mice. *Diabetologia* 54: 2082–2092.
70. Crispín, J. C., M. Oukka, G. Bayliss, R. A. Cohen, C. A. Van Beek, I. E. Stillman, V. C. Kyttaris, Y. T. Juang, and G. C. Tsokos. 2008. Expanded double negative T cells in patients with systemic lupus erythematosus produce IL-17 and infiltrate the kidneys. *J. Immunol.* 181: 8761–8766.
71. Cohen, P. L., and R. A. Eisenberg. 1991. Lpr and gld: single gene models of systemic autoimmunity and lymphoproliferative disease. *Annu. Rev. Immunol.* 9: 243–269.
72. Van Laethem, F., S. D. Sarafova, J. H. Park, X. Tai, L. Pobezinsky, T. I. Guinter, S. Adoro, A. Adams, S. O. Sharrow, L. Feigenbaum, and A. Singer. 2007. Deletion of CD4 and CD8 coreceptors permits generation of alphabetaT cells that recognize antigens independently of the MHC. *Immunity* 27: 735–750.
73. Tikhonova, A. N., F. Van Laethem, K. Hanada, J. Lu, L. A. Pobezinsky, C. Hong, T. I. Guinter, S. K. Jeurling, G. Bernhardt, J. H. Park, et al. 2012. αβ T cell receptors that do not undergo major histocompatibility complex-specific thymic selection possess antibody-like recognition specificities. *Immunity* 36: 79–91.
74. Van Laethem, F., A. N. Tikhonova, and A. Singer. 2012. MHC restriction is imposed on a diverse T cell receptor repertoire by CD4 and CD8 co-receptors during thymic selection. *Trends Immunol.* 33: 437–441.
75. Hillhouse, E. E., and S. Lesage. 2013. A comprehensive review of the phenotype and function of antigen-specific immunoregulatory double negative T cells. *J. Autoimmun.* 40: 58–65.
76. Hillhouse, E. E., J. S. Delisle, and S. Lesage. 2013. Immunoregulatory CD4(-) CD8(-) T cells as a potential therapeutic tool for transplantation, autoimmunity, and cancer. *Front. Immunol.* 4: 6.
77. Juvet, S. C., and L. Zhang. 2012. Double negative regulatory T cells in transplantation and autoimmunity: recent progress and future directions. *J. Mol. Cell Biol.* 4: 48–58.
78. Martina, M. N., S. Noel, A. Saxena, H. Rabb, and A. R. Hamad. 2015. Double negative (DN) αβ T cells: misperception and overdue recognition. *Immunol. Cell Biol.* 93: 305–310.
79. Rodríguez-Rodríguez, N., S. A. Apostolidis, L. Fitzgerald, B. S. Meehan, A. J. Corbett, J. M. Martín-Villa, J. McCluskey, G. C. Tsokos, and J. C. Crispín. 2016. Pro-inflammatory self-reactive T cells are found within murine TCR-αβ(+) CD4(-) CD8(-) PD-1(+) cells. *Eur. J. Immunol.* 46: 1383–1391.
80. Crispín, J. C., and G. C. Tsokos. 2009. Human TCR-alpha beta+ CD4- CD8- T cells can derive from CD8+ T cells and display an inflammatory effector phenotype. *J. Immunol.* 183: 4675–4681.
81. Lee, J. B., H. Kang, L. Fang, C. D'Souza, O. Adeyi, and L. Zhang. 2019. Developing allogeneic double-negative T cells as a novel off-the-shelf adoptive cellular therapy for cancer. *Clin. Cancer Res.* 25: 2241–2253.
82. Lee, J., M. D. Minden, W. C. Chen, E. Streck, B. Chen, H. Kang, A. Arruda, D. Ly, S. D. Der, S. Kang, et al. 2018. Allogeneic human double negative T cells as a novel immunotherapy for acute myeloid leukemia and its underlying mechanisms. *Clin. Cancer Res.* 24: 370–382.