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# Thymic Development of a Unique Bone Marrow–Resident Innate-like T Cell Subset with a Potent Innate Immune Function

**Ryusuke Yamamoto,*,† Yan Xu,* Satoshi Ikeda,* Kentaro Sumida,* Hiroki Tanaka,* Katsuto Hozumi,‡ Akifumi Takaori-Kondo,† and Nagahiro Minato***

**Mainstream CD8$^+$ and CD4$^+$ T cells of αβ lineage are developed in the thymus through TCR-mediated selection in the context of MHC class I and MHC class II in association with self-peptides, respectively. In addition, minor αβT cells bearing invariant TCRs, NKT cells, and mucosal-associated invariant T cells are selected via MHC-like molecules, CD1d, and MR1 complexed with nonpeptide Ags, respectively, parts of which express neither CD4 nor CD8. In this study, we indicate that bone marrow (BM), but barely other lymphoid tissues, harbors CD4/CD8 double-negative αβT cells with an apparently diverse TCR repertoire at considerable proportions in healthy adult mice. The BM-resident double-negative αβT (BMDNT) cells are developed in the thymus in a Notch and IL-7–dependent manner but independently of known restriction elements, including MHC class I, MHC class II, CD1d, and MR1. These cells are sustained in BM throughout the adult stage with "homeostatic" proliferation via IL-1β derived from normal myeloid cells dominating the BM environment. Although BMDNT cells secrete a unique set of cytokines, including IL-17, GM-CSF, IL-3, and CCL chemokines on TCR stimulation, these T cells also express a series of NK receptors and exhibit a potent NK-like cytotoxic activity. Furthermore, BMDNT cells show robustly accelerated proliferation and activation following systemic administration of TLR ligands likely through the enhanced production of IL-1β by myeloid cells in situ. Our results suggest that αβT lineage cells that are developed in the thymus by default of TCR-mediated selection are maintained and differentiated to innate-like T cells in BM and may play a role in innate immunity in the hematopoietic environment.** ***The Journal of Immunology*****, 2019, 203: 167–177.**

Mainstream T cells of αβT lineage are developed in the thymus through TCR-based selection in the context of class I and II MHC molecules expressed on thymic epithelial cells or dendritic cells, where the TCR coreceptors, CD4 and CD8, play crucial roles (1). Thus, immature CD4$^+$ CD8$^+$ double-positive thymocytes derived from the earliest CD4$^-$ CD8$^-$ double-negative (DN) progenitors are positively selected and differentiate into mature CD4$^+$ and CD8$^+$ single-positive (SP) T cells upon TCR engagement with self-peptide/MHC class II (MHC-II) and self-peptide/MHC class I (MHC-I) complexes, respectively (2). In addition to the mainstream αβT cells, minor T cell subsets with distinctive "invariant" TCR structures, NKT cells, and mucosal-associated invariant T (MAIT) cells are, respectively, selected in the context of nonpolymorphic MHC-I–related CD1d and MR1 expressed on hematopoietic cells (thymocytes, B cells) (3, 4). Involvement of TCR coreceptors in the selection of invariant T cells remains elusive; whereas NKT and MAIT cells may, respectively, exhibit CD4 and CD8 expression during their development, significant fractions of the peripheral invariant T cells express neither CD4 nor CD8 and are recognized as DN αβT cells similar to the majority of γδT lineage cells (5–7).

Notably, the minor T cell subsets often show the functional characteristics of innate immune cells during inflammation, microbial infection, and even cancer (8–10). Although this feature may be attributed partly to the unique TCR specificity for the metabolites in microbial riboflavin metabolic pathways and glycosphingolipids in invariant T cells (11, 12) reminiscent of germline coded "pattern recognition receptors" in innate immune cells (13), these T cells also express NK cell–related receptors (NKRs), such as CD161 (NK1.1), CD314 (NKG2D), and CD94, and show an immediate response during microbial infection (14, 15). Also, these minor T cell subsets of both αβ and γδT cell lineages tend to show unique tissue residence, including various epithelial tissues directly exposed to external stimuli, such as in the gut, liver, lungs, and skin, and are considered to play important roles in tissue-stress responses (7, 15, 16). It is suggested that the expression of NKRs in these T cells is induced at a late stage of their development, probably at the peripheral tissues (5).

In our current study, we found that DN αβT cells are present in unexpectedly high proportions in bone marrow (BM) but barely in other lymphoid tissues of healthy adult mice. The BM-resident DN αβT (BMDNT) cells showed a diverse TCR repertoire and thus were distinct from known invariant T cells. The development of BMDNT cells crucially depended on the thymic Notch ligand Dll4 and IL-7 but was independent of any of the hitherto known

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Abbreviations used in this article: BM, bone marrow; BMDNT, BM-resident DN αβT; CM, conditioned medium; CTV, CellTrace Violet; DN, double-negative; ILC, innate lymphoid cell; $\beta_2$m, $\beta_2$-microglobulin; MAIT, mucosal-associated invariant T; MHC-I, MHC class I; MHC-II, MHC class II; NKR, NK cell–related receptor; poly I:C, polyinosinic-polycytidylic acid; SP, single-positive; Wt, wild type.

restriction elements, MHC-II, or $\beta_2$-microglobulin ($\beta_2$m)–associated MHC-I, CD1d, or MR1. The BMDNT cells became detectable only after birth and were maintained throughout adult stages with "homeostatic" proliferation in the BM environment. BMDNT cells also exhibited functional features of innate lymphoid cells (ILCs) expressing a series of NKRs and were robustly increased and activated in situ in the BM following the systemic administration of TLR ligands. We propose that minor αβT lineage cells, by default of thymic selection with specific restriction molecules, may be settled and maintained in BM and play a role in innate immunity in the adult hematopoietic environment.

## Materials and Methods

### *Mice*

CD45.2 C57BL/6 (B6), BALB/c, and BALB/c *nu/nu* mice were purchased from SLC (Shizuoka, Japan), and CD45.1 congenic B6 mice were purchased from Charles River Laboratories Japan (Kanagawa, Japan). *Il7*$^{-/-}$ and *Il15*$^{-/-}$ mice were provided by Dr. K. Ikuta (Institute for Frontier Life and Medical Sciences, Kyoto University, Kyoto, Japan) and Dr. Y. Yoshikai (Medical Institute of Bioregulation, Kyushu University, Fukuoka, Japan), respectively. *β2m*$^{-/-}$ and *IAβ*$^{-/-}$ mice were obtained from Dr. Y. Takahama (National Cancer Institute, National Institutes of Health, Bethesda, MD). *Mr1*$^{-/-}$ mice were a kind gift from Dr. S. Gilfillan (Washington University School of Medicine, St. Louis, MO). BALB/c *Cd1d*$^{-/-}$ mice were provided by Dr. M. Taniguchi (RIKEN Research Center, Yokohama, Japan). *FoxN1-Cre:Dll4*$^{flox/flox}$ mice were previously reported (17). All the mutant mice, except for *Cd1d*$^{-/-}$ mice, were of a B6 genetic background. In all experiments, age- and sex-matched mutant and cohoused wild type (Wt) mice between 8 and 16 wk of age were used, and the numbers of mice used in experiments are indicated in the figures. All mice were maintained in specific pathogen-free conditions at the Center for Experimental Animals of Kyoto University, and all animal experiments were performed strictly according to the institutional guidelines of the Kyoto University Animal Ethics Committee.

### *Cell lines*

The leukemia cell lines YAC-1, RLmale1, EL-4, and P815 were purchased from American Type Culture Collection and maintained in complete RPMI 1640 medium supplemented with 10% heat-inactivated FCS.

### *Cell preparation*

Murine BM cells were isolated from femurs and tibiae, and peripheral blood cells were obtained by retro-orbital bleed. Lungs were finely chopped and treated with 1 mg/ml collagenase type IV (Sigma-Aldrich, St. Louis, MO) and 100 μg/ml DNase I (Worthington Biochemical, Freehold, NJ) in DMEM containing 2% FCS for 60 min at 37˚C, and the mononuclear cells were isolated by Percoll density gradient centrifugation. Human BM mononuclear cells from healthy donors were purchased from AllCells (Alameda, CA). T cells, B cells, myeloid cells, and dendritic cells were isolated from BM cells by positive magnetic-bead separation with AutoMACS (Miltenyi Biotec, Bergisch, Gladbach, Germany) using PE-conjugated anti-Thy1.2 (53-2.1; eBioscience, San Diego, CA), anti-B220 (RA3-6B2; BD Biosciences, San Jose, CA), anti-CD11b (M1/70; BD Biosciences), and anti-CD11c (N418; BioLegend, San Diego, CA) Abs, followed by anti-PE beads (Miltenyi Biotec). All separated subpopulations were analyzed by FACSCanto II (BD Biosciences), and the purity of the cells were routinely above 80–90% of the intended cell types. Separation of T cell subsets was performed using FACSAria II cell sorter (BD Biosciences).

### *Conditioned medium*

Subpopulations of BM were cultured at 1–2 × $10^6$ cells/ml for 24 h, and the culture supernatants were harvested. When indicated, the conditioned medium (CM) was preincubated with anti–IL-1β (B122; Bio X Cell, West Lebanon, NH), anti–IL-6 (MP5-20F3; BioLegend), anti–IL-12 p40 (C17.8; Bio X Cell), anti–TNF-α (MP6-XT22; BioLegend) Abs, or isotype-matched IgG before adding in the cultures.

### *Flow cytometry*

Multicolor flow cytometric analysis was performed with FACSCanto II, and the data were analyzed with FlowJo (BD Biosciences). For the staining of murine cells, the cell preparations were Fc blocked with anti-CD16/CD32 Ab and stained with the following Abs: FITC-conjugated anti-CD4 (GK1.5), anti–granzyme B (GB11), anti-TCRγδ (GL3), anti-TCR Vα3.2 (RR3-16), anti-TCR Vα8.3 (B21.14), anti-TLR9 (M9.D6), and rat IgG2a,κ (eBR2a); PE-conjugated anti-α4/β7 Integrin (DATK32), anti-CD3ε (145-2C11), anti-CD49b (DX5), anti-CD94 (18d3), anti-CD122 (5H4), anti-CD127 (A7R34), anti-CD135 (A2F10), anti–c-Kit (2B8), anti-GATA3 (TWAJ), anti-Ki67 (SolA15), anti-Ly49A (YE1/48.10.6), anti-NK1.1 (PK136), anti-NKG2D (CX5), anti-NKp46 (29A1.4), anti-PLZF (9E12), anti-RORγt (AFKJS-9), anti-TCRβ (H57-597), anti-TCR Vα2 (B20.1), anti–Sca-1 (D7), Armenian hamster IgG (HTK888), mouse IgG2a, κ (MOPC-173), rat IgG1, κ (RTK2071), rat IgG2a, κ (RTK2758), rat IgG2b, κ (RTK4530), and rat IgM, κ (RTK2118); PE/Cy7-conjugated anti-CD4 (GK1.5), anti-CD8a (53-6.7), anti-EOMES (Dan11mag), anti–T-bet (eBio4B10), anti-TCRβ (H57-597), mouse IgG1, κ (MOPC-21), and rat IgG2a, κ (RTK2758); allophycocyanin-conjugated anti-CD45.1 (A20), anti–IFN-γ (XMG1.2), and anti-TCRγδ (GL3); allophycocyanin/Cy7-conjugated anti-CD4 (GK1.5), and anti-CD8a (53-6.7); and BV421-conjugated anti-TCRβ (H57-597), and BV510-conjugated anti-CD8a (53-6.7) Abs. For the analysis of human BM cells, the following Abs were used: FITC-conjugated anti-CD4 (RPA-T4), PE-conjugated anti-NKG2D (1D11) and mouse IgG1,κ (MOPC-21), PE/Cy7-conjugated anti-TCRα/β (IP26), allophycocyanin-conjugated TCR Vα24-Jα18 (6B11), allophycocyanin/Cy7-conjugated CD8a (RPA-T8), and BV421-conjugated CD3e (OKT3) Abs. All Abs were purchased from BD Biosciences, BioLegend, eBioscience, and Tonbo Biosciences (San Diego, CA). For the detection of NKT cells, allophycocyanin-conjugated CD1d tetramers (MBL, Woburn, MA) loaded with α-galactosylceramide (Kirin Brewery, Gunma, Japan) were used according to the manufacturer's instructions. For detection of MAIT cells, PE-conjugated MR1 tetramers loaded with the ligand 5-OP-RU provided by the National Institutes of Health Tetramer Core Facility were used (11). Anti-Mouse TCR Vβ Screening Panel (BD Biosciences) and LEGENDScreen Mouse PE Kit (BioLegend) were used for TCR repertoire analysis and surface-marker screening, respectively. Intracellular staining was performed using IC Fixation Buffer (eBioscience) or the Foxp3/Transcription Factor Staining Buffer Set (eBioscience) according to the manufacturer's instructions. Propidium iodide (Sigma-Aldrich) or Ghost Dye Violet 450 (Tonbo Biosciences) was used to exclude dead cells.

### *Cytotoxicity assay*

Sorted BMDNT cells were cultured in complete RPMI 1640 medium with 10% FCS in the presence of 1 ng/ml recombinant human IL-2 (Wako Pure Chemical, Osaka, Japan) and 20 ng/ml murine IL-15 (PeproTech, Rocky Hill, NJ). The BMDNT cells were mixed with target cells labeled using the N-SPC nonradioactive cellular cytotoxicity assay kit (Techno Suzuta, Nagasaki, Japan) at the indicated ratio in 96-well round-bottom plates. Two hours later, the time-resolved fluorescence of europium signal in the supernatants was measured with a multilabel plate reader ARVO *X5* (PerkinElmer, Foster City, CA).

### *Cytokine assay*

Sorted BMDNT cells were cultured in the absence or presence of coated anti-CD3ε Ab (1 μg/well, 145-2C11; eBioscience) for 3 d, and cytokines in the supernatant were measured using a mouse cytokine array, Panel A (R&D Systems, Minneapolis, MN) with ImageQuant LAS 4000 instrument (GE Healthcare, Buckinghamshire, U.K.). For intracellular staining of cytokines, cells were stimulated with coated anti-CD3ε Ab, recombinant human IL-2 (1 ng/ml; Wako Pure Chemical), or murine IL-15 (20 ng/ml; PeproTech) for 18 h in the presence of brefeldin A (BioLegend) for the last 4 h, followed by flow cytometric analysis.

### *Proliferation assay*

Sorted BMDNT cells or Thy1.2$^+$ T cells were labeled with CellTrace Violet (CTV) (Thermo Fisher Scientific, Waltham, MA) and cocultured with 2–10-fold various tissue cells pretreated with 20 μg/ml mitomycin C (Kyowa Hakko Kirin, Tokyo, Japan) for 60 min at 37˚C in a 96-well flat-bottom plate or a 24-well transwell plate (pore size: 0.4 μm; Corning, New York, NY). When indicated, the BMDNT cells were cultured with diluted CM of sorted BM-CD11b$^+$ cells cultured in the absence or presence of 0.1 μg/ml CpG oligodeoxynucleotide (CpG, 5′-TCCATGACGTTCCTGATGCT-3′, ODN1668; Hokkaido System Science, Sapporo, Japan) for 24 h. When indicated, recombinant murine IL-1β (Wako Pure Chemical) or IL-6 (Wako Pure Chemical) was included in the cultures at 1 ng/ml. In all cases, the cultures were supplemented with 20 ng/ml recombinant murine IL-7 (Wako Pure Chemical) to support cell viability. After culturing, the CTV dilutions at the BMDNT cell gate were analyzed with FACSCanto II.

### *Real-time quantitative PCR*

RNA extractions were prepared using TRIzol reagent with DNase I treatment (Life Technologies, Carlsbad, CA), and cDNAs were synthesized

by Superscript II Reverse Transcriptase and Oligo(dT) primers (Life Technologies). Relative quantification of mRNA was performed using an Applied Biosystems StepOnePlus Real-Time PCR System with PowerUp SYBR Green Master Mix (Applied Biosystems, Foster City, CA). The relative transcript levels were normalized to those of *β-actin* or *cyclophilin*. Primer sequences were as follows: *Il1a,* 5′-TTGGTTAAATGACCTGCAACA-3′ and 5′-GAGCGCTCACGAACAGTTG-3′; *Il1b,* 5′-TGTAATGAAAGACGGCACACC-3′ and 5′-TCTTCTTTGGGTATTGCTTGG-3′; *Tnfa,* 5′-TCTTCTCATTCCTGCTTGTGG-3′ and 5′-GGTCTGGGCCATAGAACTGA-3′; *β-actin,* 5′-GGCTGTATTCCCCTCCATCG-3′ and 5′-CCAGTTGGTAACAATGCCATGT-3′; *Ifng,* 5′-ATCTGGAGGAACTGGCAAAA-3′ and 5′-TTCAAGACTTCAAAGAGTCTGAGG-3′; *Gmcsf,* 5′-GCATGTAGAGGCCATCAAAGA-3′ and 5′-CGGGTCTGCACACATGTTA-3′; *Ccl1,* 5′-CCCCTGAAGTTTATCCAGTGTTA-3′ and 5′-GCAGCTTTCTCTACCTTTGTTCA-3′; *Ccl3,* 5′-CAAGTCTTCTCAGCGCCATA-3′ and 5′-GGAATCTTCCGGCTGTAGG-3′; *Ccl4,* 5′-AGCAACACCATGAAGCTCTG-3′ and 5′-GAGGGTCAGAGCCCATTG-3′; *Ccl5,* 5′-CCTACTCCCACTCGGTCCT-3′ and 5′-GCTGATTTCTTGGGTTTGCT-3′; *Gzma,* 5′-CTCCGTGGTGGAAAGGACTC-3′ and 5′-AGAGGTGATGCCTCGCAAAA-3′; *Gzmb,* 5′-GACAACACTCTTGACGCTGG-3′ and 5′-CGATGATCTCCCCTGCCTTT-3′; and *Cyclophilin,* 5′-GACGAAGGTAGCCAGTCACAAG-3′ and 5′-AATCAGGCCTGTGGAATGTGAG-3′.

### *May–Giemsa staining*

Freshly isolated cells were cytospun onto glass slides (Matsunami Glass, Osaka, Japan) using a CytoSpin 3 (Shandon, Pittsburgh, PA) or were incubated on collagen-coated glass slides (Matsunami Glass), fixed, and stained with May-Grünwald and Giemsa stain solution (Muto Pure Chemicals, Tokyo, Japan).

### *TLR ligand administration*

B6 mice were administered i.p. in a single shot with 50 μg LPS (Sigma-Aldrich), 50 μg polyinosinic-polycytidylic acid (poly I:C) (Sigma-Aldrich), or 50 μg CpG (Hokkaido System Science), each in 200 μl PBS or with 200 μl PBS alone.

### *Statistical analyses*

Statistical analyses were performed using the unpaired two-tailed Student *t* test; the *p* value $<0.05$ was considered to indicate a significant difference. Hierarchical clustering of surface-marker expressions in T cell subsets was performed using JMP software (SAS Institute, Cary, NC).

## Results

### *Postnatal development of a unique αβT cell population in BM*

In the BM of adult B6 mice, CD3$^+$ T cells consisted of major TCRβ$^+$ (αβT) and minor TCRγδ$^+$ (γδT) cells at ~90 and 10%, respectively. α-galactosylceramide–CD1d$^+$ NKT cells comprised ~3% of the αβT cells on average (Fig. 1A, left), and MR1-5-OP-RU$^+$ MAIT cells were negligible in BM ($<0.1\%$), although significant

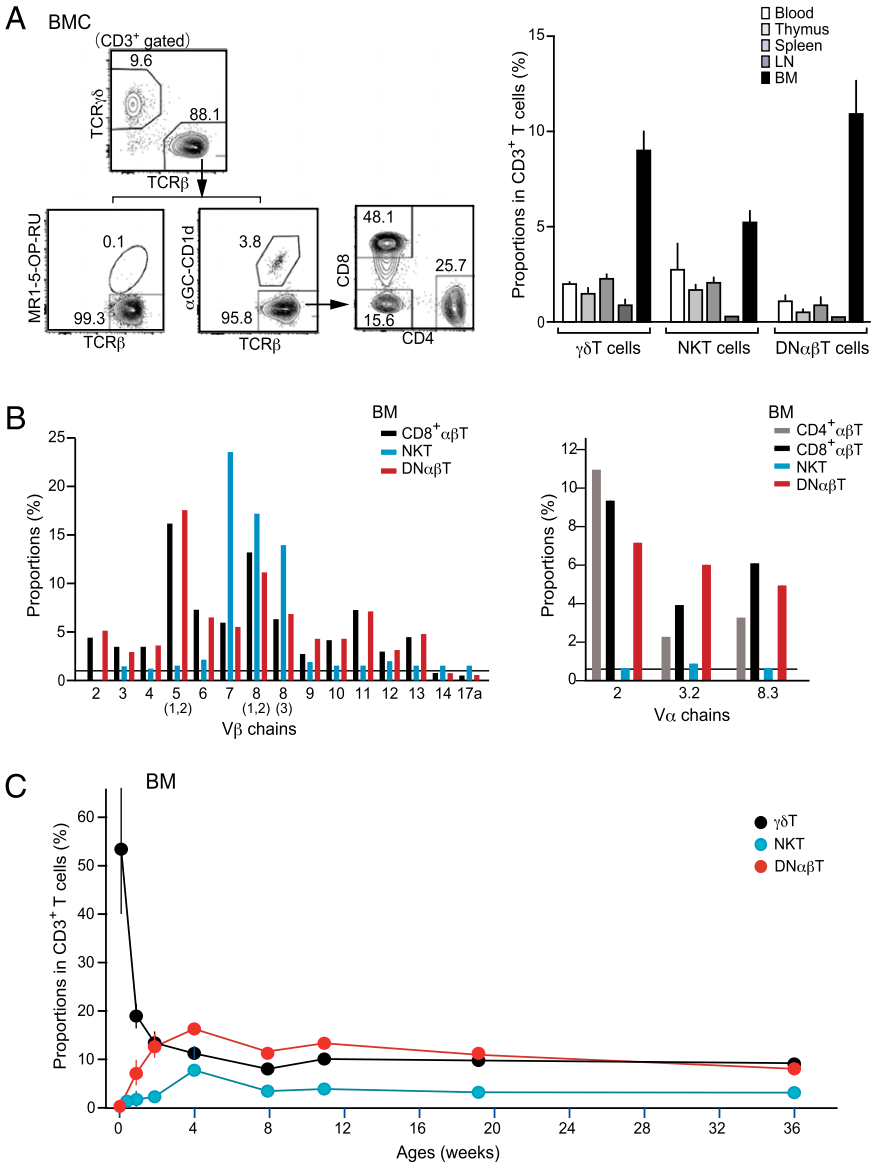


**FIGURE 1.** Postnatal generation of DN αβT cells with diverse TCR repertoire exclusively in BM. (**A**) BM cells from B6 mice were analyzed with the indicated gating strategy (left) and the proportions of minor T cell subsets, γδT cells, NKT cells, and DN αβT cells excluding invariant T cells in total CD3$^+$ cells in indicated tissues are shown (right). The means and SEs of four mice are shown. DN αβT cells were detected almost exclusively in BM among lymphoid tissues. LNs, pooled from axillary and inguinal lymph nodes. (**B**) Expression of all TCR Vβ-chains (left) and major Vα-chains (right) was analyzed in the indicated T cell subsets of BM from 8-wk-old B6 mice. Data are representative of three independent experiments, and the means of four mice are shown. DN αβT cells in BM showed diverse TCR Vα/Vβ usages comparable to SP αβT cells. (**C**) The proportions of the indicated T cell subsets were analyzed in the BM from neonatal to adult stages of B6 mice. The means and SEs of three to six mice per group pooled from three independent experiments are shown. Antithetical developmental patterns were noted after birth in γδT cells and DN αβT cells.

MAIT cells were detected in the lungs as previously reported (18) (Fig. 1A, left, Supplemental Fig. 1A). Among αβT cells in BM, excluding the invariant T cells, we found unexpectedly high proportions of DN T cells at 10–15% of all αβT cells; we also noted more $CD8^+$ T cells than $CD4^+$ T cells in BM as opposed to other lymphoid tissues (Fig. 1A, left). Such DN αβT cells were detected minimally in peripheral blood and other lymphoid tissues (<1%) (Fig. 1A, right, Supplemental Fig. 1B). The unlike NKT cells, exhibited a diverse TCR Vβ-chain expression comparable to SP αβT cells (Fig. 1B, left). These T cells also expressed selected major TCR Vα-chains largely similarly to SP αβT cells (Fig. 1B, right), suggesting that the DN αβT cells in BM show a diverse TCR repertoire. For comparison, we also surveyed the expression of a series of lymphocyte-related cell surface markers (129 molecules) among DN αβT, NKT, $CD4^+$ αβT, $CD8^+$ αβT, and γδT cells purified from BM. The hierarchical clustering analysis indicated that the DN αβT cells showed an overall expression profile closer to γδT cells rather than other αβT lineage cells (Supplemental Fig. 2). Of note, the ontogeny of DN αβT cells in BM showed a quite antithetical profile to that of γδT cells; DN αβT cells were undetectable in newborn BM but increased rapidly until 4 wk of age before reaching a steady level in adults, whereas γδT cells were maximal in newborn BM but reduced sharply until 4 wk of age (Fig. 1C). These results indicate that adult BM harbors a unique αβT cell population with no expression of CD4/CD8 coreceptors.

*Development of BMDNT cells requires the thymic Notch signal but is independent of known restriction elements for positive selection*

The DN αβT cells were absent in the BM of adult *nu/nu* mice like mainstream SP αβT cells (Fig. 2A). In agreement with the crucial role of the Notch signal in the development of the earliest thymic T cell progenitors (17, 19), the numbers of SP αβT cells in BM were reduced profoundly, if not completely, in *FoxN1-Cre:Dll4$^{fl/fl}$* mice (Fig. 2B). DN αβT cells, as well as NKT and γδT cells, were essentially undetectable in the BM of adult *FoxN1-Cre:Dll4$^{fl/fl}$* mice (Fig. 2B, Supplemental Fig. 3A). The development of DN αβT cells was unaffected in the BM of *β2m$^{-/-}$* mice, which, as anticipated, showed selective abolishment of $CD8^+$ αβT cells and NKT cells (Fig. 2C, Supplemental Fig. 3B). Similarly, *IAβ$^{-/-}$* mice showed unaffected, or even increased, numbers of DN αβT cells in the BM (Fig. 2D). Although $CD4^+$ αβT cells were negligible in the spleen of *IAβ$^{-/-}$* mice (Supplemental Fig. 3C), the decrease in BM was less dramatic (only ∼50%), similar to that in the thymus (20) (Fig. 2D). In any case, these results eliminated the possibility that BMDNT cells represented the mainstream SP αβT cells that had completely lost their TCR coreceptor expression postthymically. We also confirmed that the development of DN αβT cells in BM was unaffected in both *Cd1d$^{-/-}$* and *Mr1$^{-/-}$* mice, in which the development of NKT cells and MAIT cells, respectively, was completely abolished; it was noted that Wt B6 mice contained more total αβT cells in the BM and accordingly more DN αβT cells than Wt BALB/c mice (Fig. 2E, 2F, Supplemental

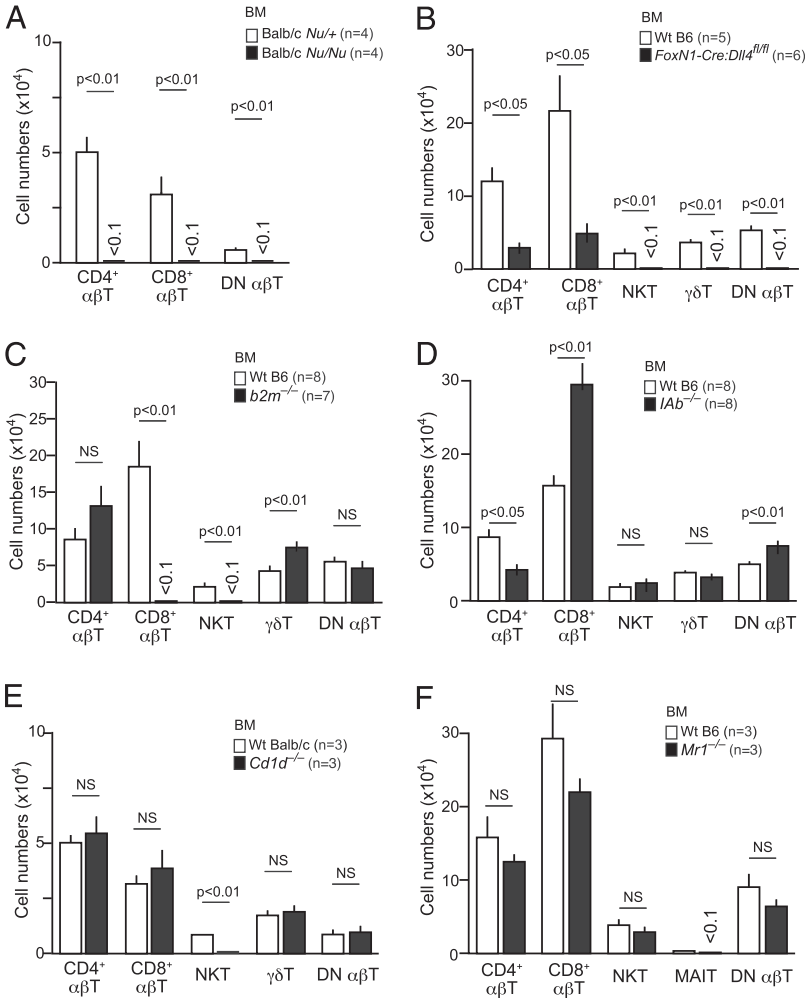


**FIGURE 2.** DN αβT cells in BM are developed in the thymus independently of known selection elements, including MHC-I, MHC-II, CD1d, and MR1. (**A**–**F**) BM cells from adult (∼8–12 wk old) BALB/c *nu/nu* mice ($n$ = 4) (A), B6 *FoxN1-Cre:Dll4$^{fl/fl}$* mice ($n$ = 6) (B), B6 *β2m$^{-/-}$* mice ($n$ = 7) (C), B6 *IAβ$^{-/-}$* mice ($n$ = 8) (D), BALB/c *Cd1d$^{-/-}$* mice ($n$ = 3) (E), and B6 *Mr1$^{-/-}$* mice ($n$ = 3) (F) were analyzed for the indicated T cell subsets in BM along with comparable numbers of age- and sex-matched Wt mice. The means and SEs in BM are indicated, and $p$ values were determined with an unpaired two-tailed Student $t$ test. The numbers of BM DN αβT cells tended to be smaller in the genetic background of BALB/c than in B6 mice. Although DN αβT cells were essentially absent in the BM of *nu/nu* and *FoxN1-Cre:Dll4$^{fl/fl}$* mice, their numbers were unaffected in the BM of *β2m$^{-/-}$*, *IAβ$^{-/-}$*, *Cd1d$^{-/-}$*, and *Mr1$^{-/-}$* mice. NS, not significant.

Fig. 3D, 3E). Altogether, these results suggest that BMDNT cells are developed in the thymus in a Notch-dependent manner but under no apparent selection via known MHC or MHC-related restriction molecules, and we termed them as BMDNT cells.

*IL-7 and IL-15 play essential and mutually nonredundant roles in the development and maintenance of BMDNT cells*

IL-7 also plays an important role in thymic T cell development (21), and all T cell subsets were essentially undetectable in the spleen of *Il7*$^{-/-}$ mice as reported, including SP αβT cells, invariant αβT cells, and γδT cells (data not shown). In BM, the decrease of mainstream CD4$^+$ and CD8$^+$αβT cells was less prominent, with residual T cells at ∼50 and 20% of Wt levels on average, respectively (Fig. 3A). However, BMDNT cells were almost completely absent in the BM of *Il7*$^{-/-}$ mice along with γδT cells (Fig. 3A). In contrast, IL-15 is dispensable for the thymic development of mainstream αβT cells (22). In BM, whereas CD4$^+$αβT cells were unaffected, CD8$^+$αβT cells were significantly reduced in agreement with the report that *Il15*$^{-/-}$ mice show impaired development or maintenance of peripheral memory phenotype CD8$^+$ T cells (22), which are dominant in BM (23) (Fig. 3A, right). Unexpectedly, however, BMDNT cells were completely absent either in the BM of *Il15*$^{-/-}$ mice, whereas γδT cells were reduced only partially (Fig. 3A, right). To investigate the possibility for the postthymic requirement of these cytokines in the development of BMDNT cells, we examined the responsiveness of the BMDNT cells to them. Although BMDNT cells freshly isolated from BM exhibited CD127 expression essentially comparable to mainstream CD4$^+$ and CD8$^+$αβT cells (Fig. 3B, left), the great majority of BMDNT cells exhibited much higher levels of CD122 expression than SP αβT cells (Fig. 3B, right). Further, the BMDNT cells showed significant cell divisions in culture in the presence of IL-15 but not in the presence of IL-7 (Fig. 3C), suggesting that IL-15 may play a role in their postthymic development and/or maintenance in BM. Phenotypically, BMDNT cells hardly expressed Sca-1, c-Kit, and Flt3, unlike ILCs without TCR expression (24), but strongly exhibited α4/β7 integrin expression (Fig. 3D). Although these T cells exhibited RORγt and weak EOMES expression, they showed an undetectable expression of GATA3, T-bet, and PLZF (Fig. 3E). These results suggest that the thymic and postthymic peripheral development of BMDNT cells crucially require IL-7 and IL-15.

*BMDNT cells express multiple NKRs with a potent NK-like cytotoxic activity and secrete a unique set of cytokines including IL-17 on TCR stimulation*

The BMDNT cells freshly isolated from BM were blastic lymphoid cells larger than mainstream αβT cells and comparable to NK cells (Fig. 4A, left) and tended to be spread when incubated on biomatrix (Fig. 4A, right); the freshly sorted cells may appear larger

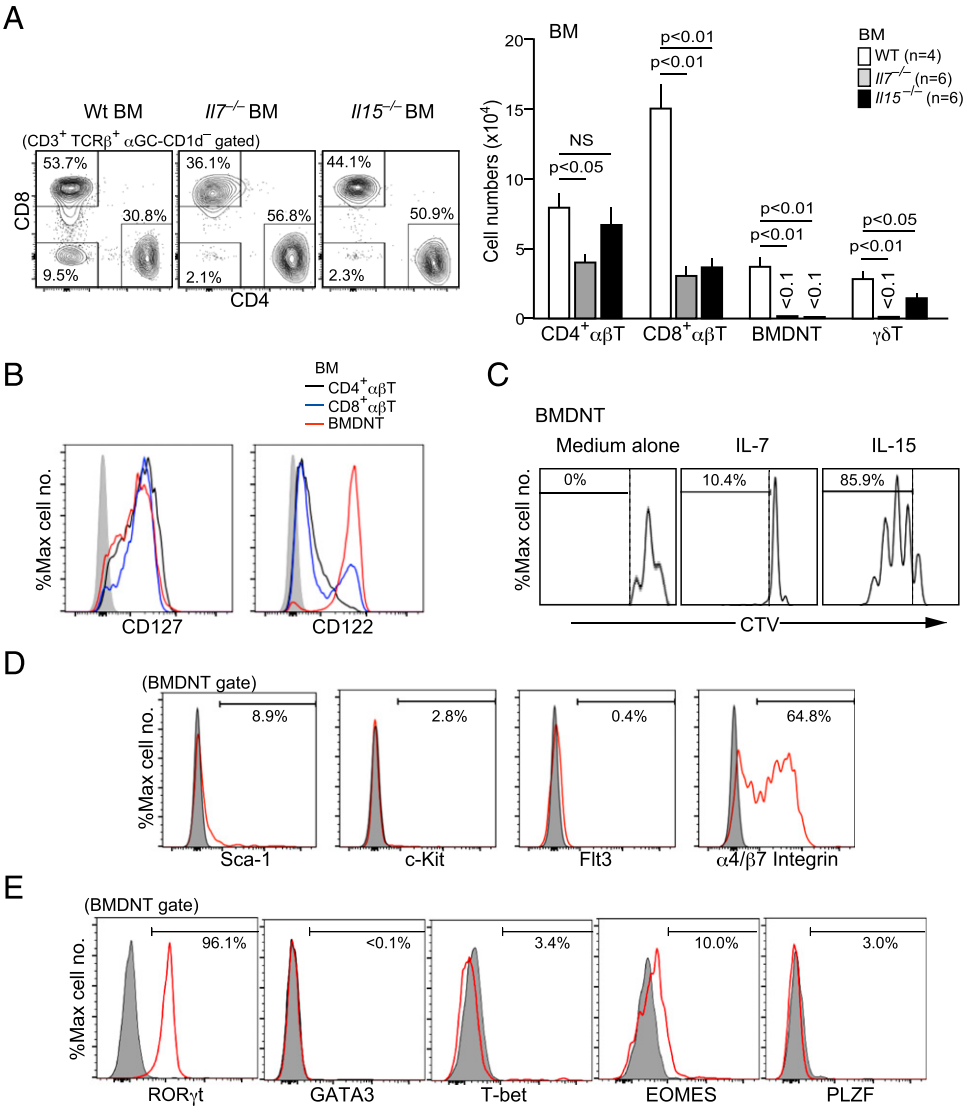


**FIGURE 3.** IL-7 and IL-15 play essential and nonredundant roles in the development and/or the maintenance of BMDNT cells. (**A**) BM cells from 8-wk-old Wt ($n$ = 4), *Il7*$^{-/-}$ ($n$ = 6), and *Il15*$^{-/-}$ ($n$ = 6) B6 mice were multicolor analyzed with the indicated Abs (left), and the numbers of the CD4$^+$ αβT cells, CD8$^+$ αβT cells, DN αβT cells, excluding invariant T cells called BMDNT cells thereafter, and γδT cells were determined (right). The $p$ values were determined with an unpaired two-tailed Student $t$ test. BMDNT cells were essentially undetectable in both *Il7*$^{-/-}$ and *Il15*$^{-/-}$ mice. NS, not significant. (**B**) The expression of CD127 and CD122 in the indicated T cell subsets are shown. Shaded areas indicate control staining with isotype-matched IgG. Similar results were obtained in three B6 mice. (**C**) Sorted BMDNT cells were labeled with CTV and cultured in the absence or presence of IL-7 (20 ng/ml) or IL-15 (20 ng/ml) for 4 d, followed by flow cytometric analysis for the CTV dilution. BMDNT cells showed significant cell divisions in the presence of IL-15 but not IL-7. Similar results were obtained in two independent experiments. (**D** and **E**) B6 BM cells were analyzed for the expression of indicated cell surface markers (D) or intracellular transcription factors (E) in the BMDNT cell gate. Shaded areas indicate control staining with isotype-matched IgG.

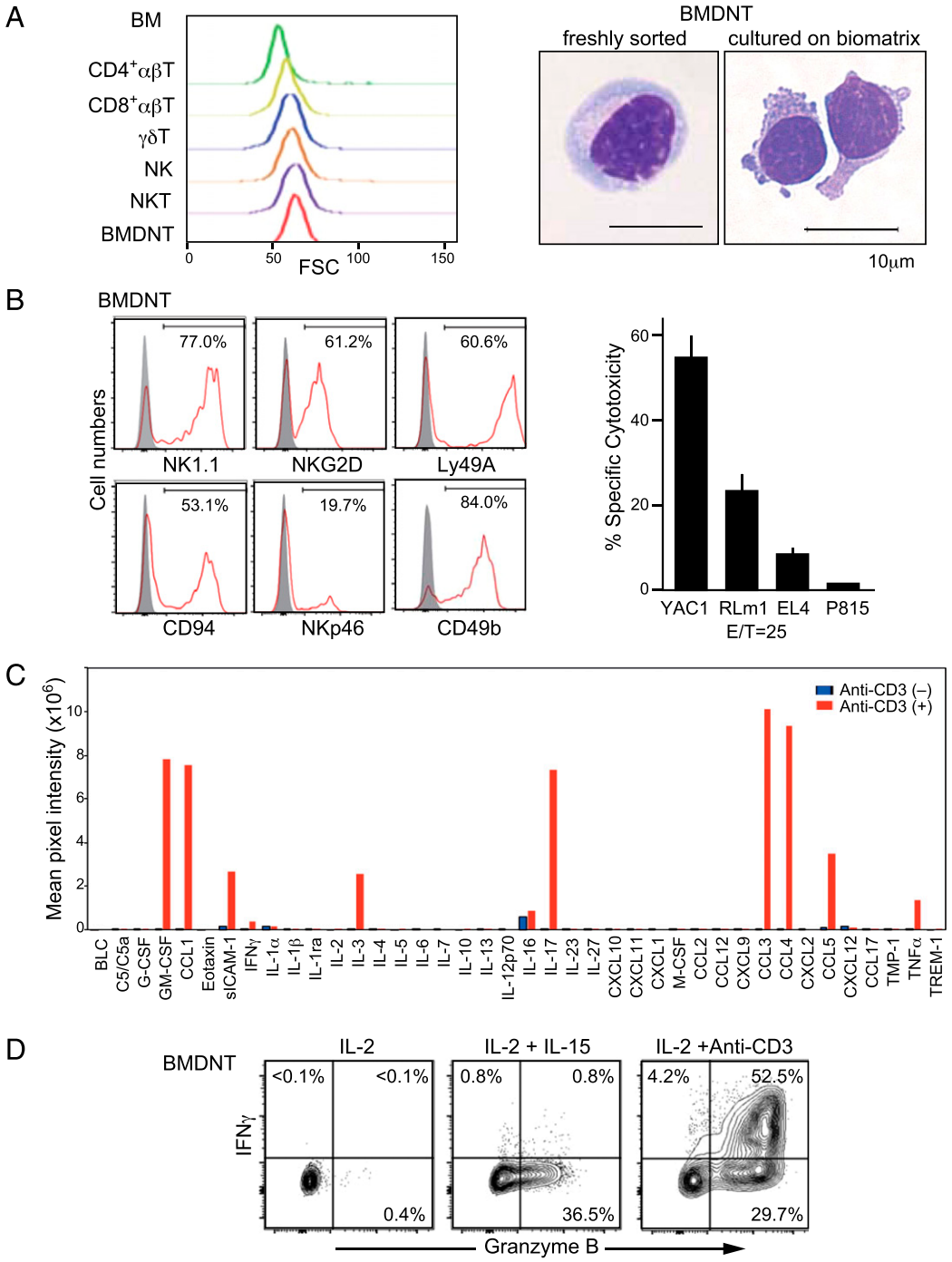


**FIGURE 4.** BMDNT cells express a series of NK receptors with a potent NK-like cytotoxicity and produce a unique set of cytokines on TCR stimulation. (**A**) Forward scatters (FSCs) of the indicated T cell subsets and NK1.1$^+$ CD3$^-$ NK cells in BM from B6 mice are shown (left). Freshly sorted BMDNT cells and those incubated overnight on collagen-coated slide glass were stained with May–Giemsa solution (right). Scale bar, 10 μm. The former appears larger because of the cytospin before staining. (**B**) The expression of indicated NKRs in the BMDNT cell gate are indicated (left). Sorted BMDNT cells were incubated with the indicated tumor cells at 25:1 ratio, and the cytotoxicity was determined (right). The means and SEs of the percentage of specific cytotoxicity in duplicate cultures are shown. BMDNT cells showed a characteristic cytotoxic activity reminiscent of NK cells. (**C**) Sorted BMDNT cells were cultured with or without solid phase anti-CD3 Ab, and 3 d later, the culture supernatants were analyzed for the secretion of a series of cytokines and chemokines. Similar results were obtained in two independent experiments. (**D**) Sorted BMDNT cells were cultured in the presence of IL-2 (1 ng/ml) alone, IL-2 + IL-15 (20 ng/ml), or IL-2 + anti-CD3 Ab (1 μg/well). Eighteen hours later, the cells were analyzed for the expression of intracellular IFN-γ and granzyme B. Whereas IL-2 alone hardly induced either factor, additional IL-15 stimulation caused significant granzyme B expression, and anti-CD3 stimulation caused a remarkable increase in IFN-γ in addition to granzyme B. Similar results were obtained in two independent experiments.

because of cytospin before staining. Also, BMDNT cells strongly expressed a series of NKRs, including NK1.1 (CD161), NKG2D (CD314), CD94, NKp46 (CD335), Ly49A, and VLA2 (CD49b), and exhibited a spontaneous cytotoxic activity against certain tumor cells reminiscent of NK cells at a relatively low E:T ratio (25) (Fig. 4B). Upon TCR stimulation with anti-CD3 Ab alone, the sorted BMDNT cells secreted a unique set of cytokines (IL-17, GM-CSF, IL-3, and to a much lesser extent, IFN-γ) and CCL-type

chemokines (CCL1, 3, 4, and 5) capable of inducing proliferation and chemoattraction of myeloid cells (26–28) (Fig. 4C). In the presence of costimulatory IL-2, anti-CD3 Ab and, to a lesser extent, IL-15, also caused a remarkable increase in the intracellular granzyme B and IFN-γ (Fig. 4D). These results suggest that BMDNT cells are bifunctional, bearing NKR-mediated cytotoxic and TCR-mediated proinflammatory activity. We also investigated the BM cells from healthy adult humans and found a small yet significant $CD3^+$ DN $TCR\alpha\beta^+$ cell population, the great majority of which strongly expressed NKG2D similarly to the murine BMDNT cells (Supplemental Fig. 4).

### *BMDNT cells are homeostatically proliferated in BM environment via IL-1β derived from normal myeloid cells*

Given the blastic morphology of freshly isolated BMDNT cells, we examined their cell cycling states in vivo. In BM, BMDNT cells showed a significantly higher proportion of $Ki67^+$ cells than SP αβT cells (Fig. 5A). When sorted $CD45.1^+$ $Thy1.2^+$ BM cells were labeled with CTV and cultured for 4 d with total $CD45.2^+$ BM cells, the BMDNT cells, but barely the SP αβT cells, showed significant cell divisions (CTV dilution in <2% of SP αβT cells and 36.5% of BMDNT cells). To examine whether the spontaneous proliferation was cell autonomous or not, we isolated the $CD45.1^+$ BMDNT cells from BM, labeled them with CTV, and cultured alone or with various tissue cells from CD45.2 B6 mice. Although BMDNT cells alone showed minimal cell divisions (11.1%), they showed markedly enhanced proliferation in the presence of total BM and spleen cells (36.5 and 34.4%, respectively) and, to a lesser extent, with thymic cells (18.8%). Among BM cells, $CD11b^+$ myeloid lineage cells and, to a greater extent, $CD11c^+$ dendritic cells induced robust cell divisions, whereas $B220^+$ B lineage and $Thy1^+$ T lineage cells did so only minimally (Fig. 5B). Also, the BMDNT cells sorted from B6 BM showed comparable proliferation in the presence of syngeneic and allogeneic cells (62.1% with B6 spleen cells and 63.4% with BALB/c spleen cells), whereas BM $CD8^+$ αβT cells exhibited robust cell divisions only in response to allogeneic cells as expected (14.2% with B6 spleen cells and 82.9% with BALB/c spleen cells). The proliferation of BMDNT cells also occurred in the presence of $CD11b^+$ cells separated with cell-impermeable filters (Fig. 5C), indicating the involvement of myeloid cell-derived soluble factors. Consistently, the CM of BM $CD11b^+$ cells induced significant BMDNT cell proliferation; however, the effect was almost completely abolished in the presence of anti–IL-1β Ab, whereas Abs for IL-6, IL-12, and TNF-α were without effects (Fig. 5D). Further, the remarkable proliferation of isolated BMDNT cells, but not SP αβT cells, was induced in the presence of IL-1β but barely in the presence of IL-6 (Fig. 5E); γδT cells were also proliferated in response to IL-1β as reported previously (29), although the extent was less than that of BMDNT cells (Fig. 5E). Although IL-15 was capable of inducing the cell divisions of isolated BMDNT cells (see Fig. 3C), the $CD11b^+$ cells from *Il15*$^{-/-}$ mice caused BMDNT cell divisions comparable to those from Wt mice (Fig. 5F), indicating that the mitogenic effect via the BM $CD11b^+$ cells was not accounted for by IL-15, which is derived from BM stroma cells (30). The results suggest that BMDNT cells are homeostatically proliferated in BM via IL-1β derived from normal myeloid cells that are most abundant in normal hematopoietic environment.

### *The proliferation of BMDNT cells is rapidly accelerated in situ following systemic administration with TLR ligands*

Although a significant proportion of BMDNT cells were in a cycling state at a steady-state, the proportion of $Ki67^+$ cells was increased rapidly and remarkably following the systemic administration of various TLR ligands, including CpG (TLR9-L) and, to lesser extents, LPS (TLR4-L) and poly I:C (TLR3-L), resulting in the selective increase in BMDNT cell proportions among the αβT cells in BM (Fig. 6A). The increase in $Ki67^+$ BMDNT cells occurred as early as 48 h after TLR ligand administration, and this was reflected by a marked increase in the actual numbers of BMDNT cells in BM by 72 h (Fig. 6B), whereas the numbers of BM SP αβT cells tended to be rather decreased (Fig. 6C). Such an increase in DN αβT cells was not observed in peripheral blood or other peripheral lymphoid tissues after CpG administration the level of DN αβT cells remained at <1%, suggesting that the proliferation of BMDNT cells took place in situ within a BM environment. Although BMDNT cells exhibited intracellular TLR9 expression largely comparable to mainstream SP T cells (Fig. 6D, left), CpG alone hardly induced the proliferation of isolated BMDNT cells in culture (Fig. 6D, right). However, BMDNT cells exhibited markedly enhanced proliferation in the presence of CM from BM $CD11b^+$ cells cocultured with CpG compared with that from $CD11b^+$ cells without CpG (Fig. 6D, right). We also found that *Il1b* and *Il1a*, but not *Tnfa*, transcripts were increased in the BM $CD11b^+$ cells 1 d after CpG injection (Fig. 6E). Further, BMDNT cells isolated from the BM of mice that had been injected with CpG 2 d before revealed a markedly increased expression of *Gzma*, *Gzmb*, *Gmcsf*, *Ifng*, and *Tnfa* compared with those from PBS-injected mice (Fig. 6F). These results suggested that the proliferation and function of BMDNT cells are rapidly enhanced by systemic TLR ligands, most likely via the augmented IL-1β production by myeloid cells in the BM microenvironment.

## Discussion

BM is an essential hematopoietic tissue for both erythromyeloid and lymphoid lineage cells in healthy adults. In addition, BM also functions as a storage site for fully differentiated Ab-producing cells as well as memory T cells and may even support the initiation of immune responses (31–33). A minor population of αβ lineage T cells exhibiting neither CD4 nor CD8 TCR coreceptor expression has been reported to be present in various tissues in healthy and pathological conditions (6, 16, 34–36). In the current study, we have identified a unique BM-resident DN αβT (BMDNT) cell population in healthy adult mice, which are apparently distinct from mainstream αβT cells and known invariant T cells.

Like mainstream SP and invariant αβT cells (21, 37), the development of BMDNT cells was dependent on the thymus and, in particular, the Notch ligand (Dll4) on the thymic epithelial cells as well as IL-7. However, genetic analysis revealed that the development of BMDNT cells was unaffected in mice deficient in MHC-I and MHC-II, showing defective development of $CD8^+$ and $CD4^+$ SP αβT cells, respectively, and thus it was strongly suggested that the BMDNT cells were developed in a manner distinct from SP αβT cells in the thymus rather than merely represented the SP αβT cells that somehow lost their coreceptor expression postthymically. Further, BMDNT cells apparently showed a diverse αβTCR repertoire, and mice deficient in CD1d and MR1 with defective development of invariant NKT and MAIT cells, respectively (3, 38), also exhibited BMDNT cell development comparable to Wt mice. These results have suggested that BMDNT cells are developed in the thymus by default of TCR-based positive selection via restriction molecules, although a possibility remains that these αβT cells are selected via hitherto unknown restriction elements. A similar default developmental pathway has been proposed for γδ17 cells of γδT lineage T cells

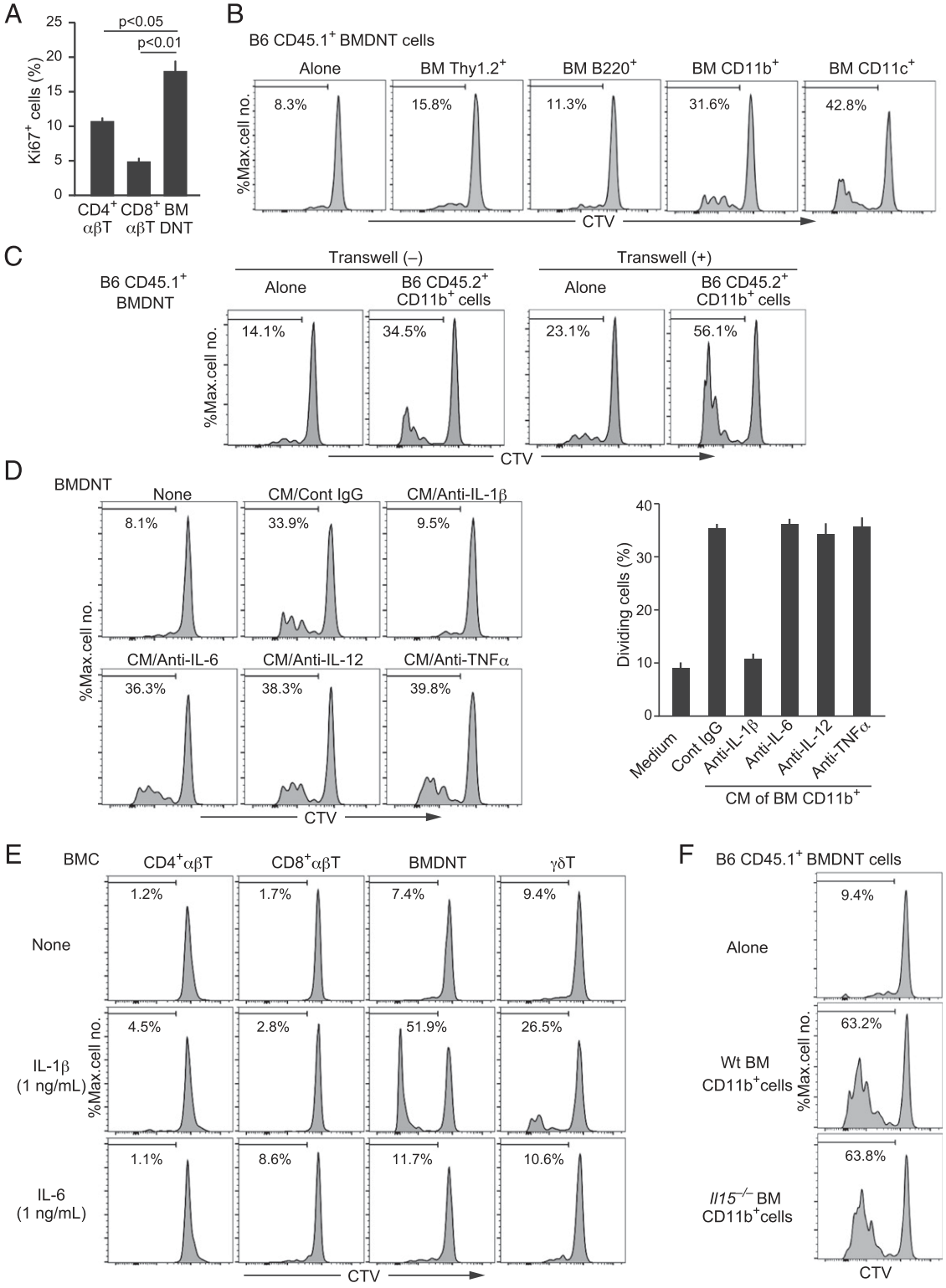


**FIGURE 5.** BMDNT cells show robust cell divisions in the presence of normal myeloid cells via IL-1β. (**A**) The mean proportions of Ki67$^+$ cells and SEs in the indicated T cell subsets of BM from B6 mice ($n = 4$). The $p$ values were determined with unpaired two-tailed Student $t$ tests. BMDNT cells showed a significantly higher proportion of Ki67$^+$ cells than SP αβT cells. Similar results were obtained in three independent experiments. (**B**) BMDNT cells sorted from CD45.1$^+$ B6 BM cells and labeled with CTV were cultured alone or with the indicated BM cell fractions from CD45.2$^+$ B6 mice for 4 d, followed by the analysis of CTV dilution. Robust cell divisions of BMDNT cells were induced in the presence of CD11b$^+$ or CD11c$^+$ cells. (**C**) Sorted CD45.1$^+$ BMDNT cells were cultured alone or with CD45.2$^+$ B6 BM CD11b$^+$ cells, directly or separately in the Boyden chamber for 4 d. Remarkable cell divisions of BMDNT cells were induced in the presence of CD11b$^+$ cells separated with cell-impermeable filters. (**D**) CTV-labeled BMDNT cells were cultured alone or with 50% CM of BM CD11b$^+$ cells in the presence of control IgG or the indicated Abs (10 μg/ml) for 4 d (left). The mean proportions of dividing cells and SEs of triplicate cultures are also indicated (right). Anti–IL-1β Ab completely abolished the cell divisions of BMDNT cells in the presence of CD11b$^+$ cell CM. (**E**) Thy1.2$^+$ T cells sorted from B6 BM cells were labeled with CTV and cultured in the absence or presence of indicated cytokines for 4 d. IL-1β, but not IL-6, caused robust cell divisions of BMDNT cells and, to a lesser extent, γδT cells. (**F**) Thy1.2$^+$ T cells sorted from CD45.1$^+$ B6 BM cells were cultured in the absence or presence of BM CD11b$^+$ cells from CD45.2$^+$ Wt or *Il15*$^{-/-}$ mice for 4 d. *Il15*$^{-/-}$ CD11b$^+$ cells induced BMDNT cell divisions comparable to Wt CD11b$^+$ cells. In (B)–(F), the experiments were done in duplicate or triplicate and repeated twice with similar results.

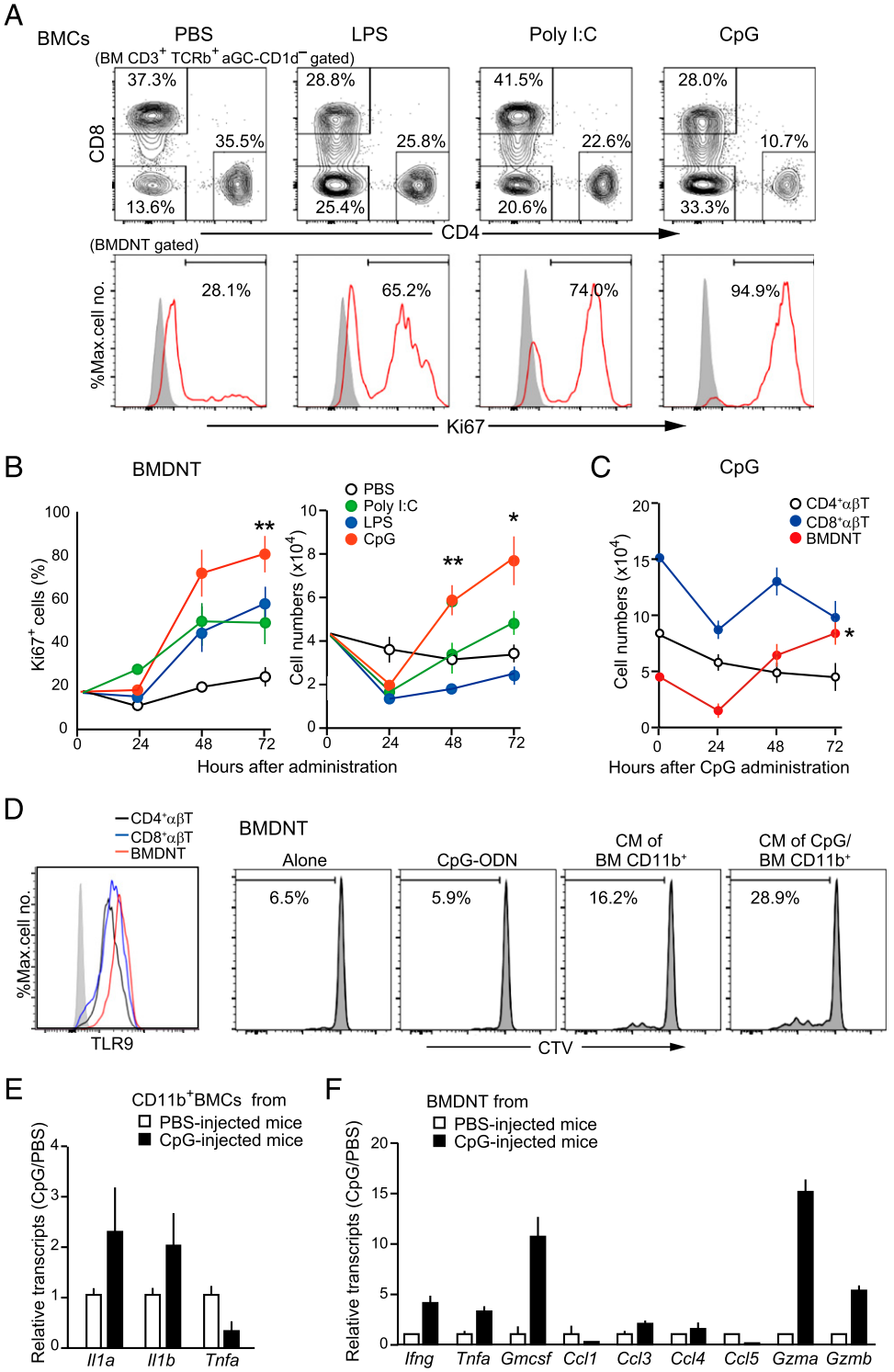


**FIGURE 6.** Systemic administration of TLR ligands causes robust proliferation and activation of BMDNT cells. (**A**–**C**) B6 mice were injected i.p. with LPS, poly I:C, CpG, or PBS as a control, and 3 d later, the BM cells were analyzed for the proportions of BMDNT cells and their Ki67 expression (A). The proportions of Ki67$^+$ cells and the actual numbers of BMDNT cells (B) and SP αβT cells (C) in BM at the indicated days after the injection are also shown. The means and SEs of six to eight mice per group pooled from two independent experiments are shown. The *p* values were determined with unpaired two-tailed Student *t* tests. $*p < 0.05$, $**p < 0.01$, versus PBS (B) or day 0 (C). CpG and, to lesser extents, LPS and poly I:C caused selective expansion of BMDNT cells. (**D**) B6 BM cells were analyzed for intracellular TLR9 expression in the indicated T cell subsets (left). Sorted BMDNT cells labeled with CTV were cultured alone or with CpG-ODN (0.1 μg/ml), 50% CM of BM CD11b$^+$ cells, or CM of BM CD11b$^+$ cells precultured with CpG (0.1 μg/ml) for 4 d. CpG alone hardly induced the BMDNT cell divisions, whereas CM of CpG-stimulated CD11b$^+$ cells caused greater cell divisions than the CM of unstimulated CD11b$^+$ cells. The experiments were done in duplicate and repeated twice with similar results. (**E**) CD11b$^+$ cells were sorted from the BM of B6 mice 1 d after the injection of PBS or CpG, and the expression of the indicated transcripts normalized to *β-actin* were compared. The means and SEs of three B6 mice per group are shown. (**F**) BMDNT cells were sorted from the BM of B6 mice 2 d after the injection of PBS or CpG, and relative transcripts of indicated genes normalized to *cyclophilin* were compared. The means and SEs of triplicate determination are shown. Eight mice per group were pooled for an experiment, and independent experiments were done three times with similar results. The BMDNT cells showed a remarkable increase in the expression of cytotoxicity-related (*Gzma*, *Gzmb*) and proinflammatory (*Gmcsf*, *Ifng*, *Tnfa*) genes following CpG administration.

(39, 40). The TCRs of BMDNT cells are functional, in that these cells secreted a unique set of cytokines on anti-CD3 Ab stimulation, including IL-17, GM-CSF, IL-3, and CCL-chemokines, all of which affect the myeloid cell proliferation and function (28, 41, 42); this was consistent with the strong expression of RORγt with negligible GATA3 and T-bet expression. With the TCR costimulation with IL-2, BMDNT cells also produced IFN-γ and abundant granzymes. These results may suggest that BMDNT cells are functionally programmed for cytotoxic and proinflammatory activity.

BMDNT cells freshly isolated from BM also expressed a series of NKRs, such as NK1.1, NKG2D, CD94, and NKp46, and exhibited a potent NK-like spontaneous cytotoxic activity against certain tumor cells. The features of ILCs may be shared among minor T cell subsets, including invariant T cells and γδT cells (9, 43), which often show unique tissue residence (44, 45). The expression of NKRs in these T cells is suggested to occur at a late stage of their development, probably at the peripheral tissues, rather than during the thymic development (5). Notably, whereas IL-15 is dispensable for mainstream SP αβT cell development (22), the BMDNT cells were essentially absent in the BM of *Il15*$^{-/-}$ mice, being reminiscent of NK cells (22). Also, BMDNT cells in BM exhibited a much higher CD122 expression than SP αβT cells and showed significant proliferation and granzyme B expression in response to IL-15, but not to IL-7, suggesting that IL-15 plays an important role in the postthymic development of BMDNT cells. The induction of granzyme B expression via IL-15 may be consistent with the role of IL-15 in the peripheral differentiation or maturation of BMDNT cells. Recently, it was reported that unique TCRαβ$^+$ NK1.1$^+$ cells characterized by predominant granzyme B expression and potent NK-like cytotoxicity, termed innate-like T cells, were increased in tumor tissues (8). These innate-like T cells are nonrecirculating, tissue-resident T cells with features distinct from mainstream or invariant T cells and are also absent in *Il15*$^{-/-}$ mice (8), although the relation to BMDNT cells remains to be seen. Curiously, the overall profile of cell surface Ags of BMDNT cells was much closer to that of γδT cells in BM, which were also decreased in *Il15*$^{-/-}$ mice, than mainstream SP αβT cells. As such, it appears that the thymus-derived BMDNT cells are functionally differentiated to ILCs in adult BM in a manner similar to NK cells and fetal-type γδT cells.

Current results have indicated that a considerable proportion of BMDNT cells are cycling in a steady-state. The homeostatic proliferation of BMDNT cells in BM was not cell autonomous but was attributable to IL-1β constitutively secreted by normal CD11b$^+$ myeloid cells, the most abundant cell population in the BM hematopoietic environment, again similarly to BM γδT cells. Because *Il15*$^{-/-}$ CD11b$^+$ myeloid cells were similarly capable of supporting the BMDNT cell proliferation, it was suggested that IL-15 most likely derived from nonhematopoietic stroma cells (30) and IL-1β secreted from myeloid cells, both playing distinct roles in supporting the development and maintenance of BMDNT cells in the BM environment. Besides homeostatic proliferation in the BM, the cell cycling rates of BMDNT cells were rapidly and robustly accelerated following systemic administration of TLR ligands, most potently CpG, and accordingly, the numbers of BMDNT cells were increased remarkably and selectively within 3 d. Such an increase was confined to BM, suggesting that the cell divisions of BMDNT cells occurred in situ within BM. It was suggested that the effects were attributed to the activation of CD11b$^+$ myeloid cells with CpG, in that the CM of CpG-treated BM CD11b$^+$ cells induced greater proliferation of BMDNT cells than that of untreated CD11b$^+$ cells, whereas CpG alone caused negligible proliferation of BMDNT cells in vitro. Because *Il1b* transcripts in BM CD11b$^+$ cells were increased after CpG administration, it is quite likely that the enhanced cell cycling rate of BMDNT cells is attributed to the increased IL-1β production by myeloid cells. The BMDNT cells from CpG-injected mice also revealed a remarkably increased expression of cytotoxic and inflammatory genes, such as *Gzma*, *Gzmb*, *Gmcsf*, *Ifng*, and *Tnfa*, suggesting that BMDNT cells play a role in innate immunity against blood-born microbes invading the BM hematopoietic environment. It is reported that DN αβT cells exist in other solid organs, such as livers and kidneys (16, 46). Although the population seems to consist of heterogenous cell types, at least a portion of the DN αβT cells in normal kidneys are suggested to develop in a classic MHC-independent manner (47, 48). Further, such kidney resident T cells show spontaneous cell cycling at a steady-state and respond immediately against tissue insults (48). It remains to be investigated whether the similar mechanisms for the development and maintenance of BMDNT cells are involved in other resident DN αβT cells in epithelial tissues.

In conclusion, we have demonstrated that a unique subset of αβT cells, BMDNT cells with innate immune function, emerge soon after birth and are sustained throughout the adult stage in BM. It has been suggested that BMDNT cells are developed in the thymus by default of a TCR-based positive selection process and are maintained with homeostatic proliferation via normal myeloid cells in the hematopoietic environment, being rapidly expanded and activated there via TLR ligands. Our results indicate the presence of similar DN αβT cells constitutively expressing high levels of NKG2D other than SP and invariant αβT cells in the adult human BM, although further analysis is needed to confirm that these T cells are equivalent to murine BMDNT cells. BMDNT cells may play a unique role in protecting hematopoietic tissue from blood-born microbes and possibly cancer cells.

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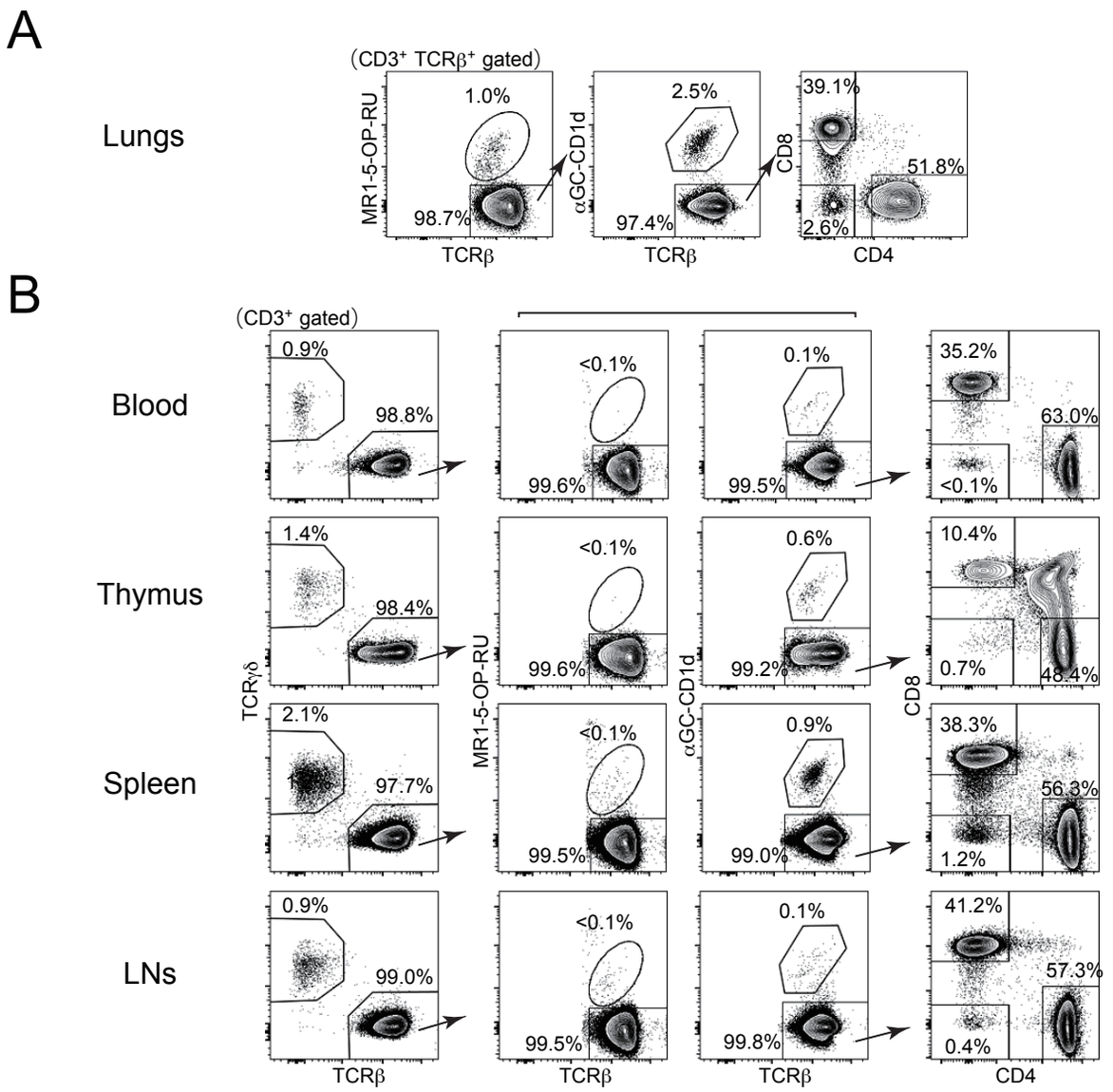
## Disclosures

The authors have no financial conflicts of interest.

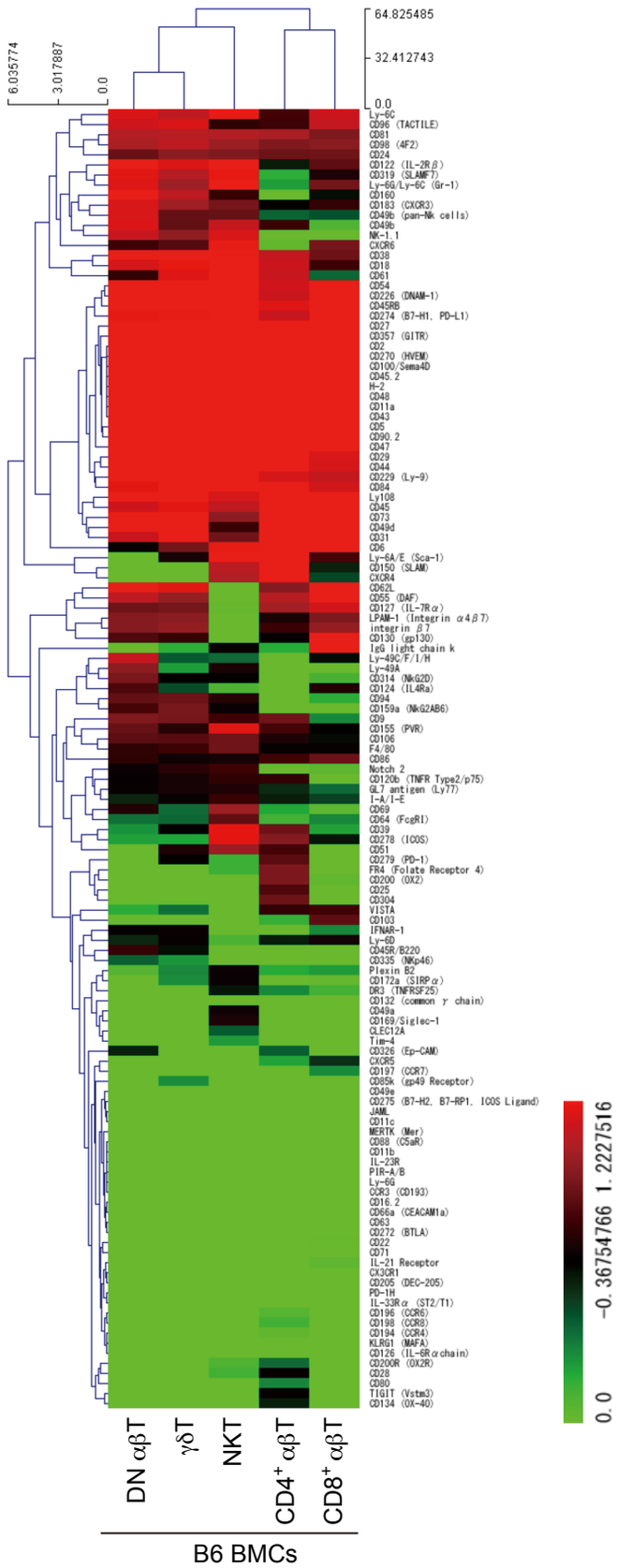
## References

1. Klein, L., M. Hinterberger, G. Wirnsberger, and B. Kyewski. 2009. Antigen presentation in the thymus for positive selection and central tolerance induction. *Nat. Rev. Immunol.* 9: 833–844.
2. Brugnera, E., A. Bhandoola, R. Cibotti, Q. Yu, T. I. Guinter, Y. Yamashita, S. O. Sharrow, and A. Singer. 2000. Coreceptor reversal in the thymus: signaled CD4+8+ thymocytes initially terminate CD8 transcription even when differentiating into CD8+ T cells. *Immunity* 13: 59–71.
3. 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.
4. Bendelac, A. 1995. Positive selection of mouse NK1+ T cells by CD1-expressing cortical thymocytes. *J. Exp. Med.* 182: 2091–2096.
5. Bendelac, A., P. B. Savage, and L. Teyton. 2007. The biology of NKT cells. *Annu. Rev. Immunol.* 25: 297–336.
6. Cowley, S. C., and K. L. Elkins. 2003. Multiple T cell subsets control *Francisella tularensis* LVS intracellular growth without stimulation through macrophage interferon gamma receptors. *J. Exp. Med.* 198: 379–389.
7. Hayday, A. C. 2009. Gammadelta T cells and the lymphoid stress-surveillance response. *Immunity* 31: 184–196.
8. Dadi, S., S. Chhangawala, B. M. Whitlock, R. A. Franklin, C. T. Luo, S. A. Oh, A. Toure, Y. Pritykin, M. Huse, C. S. Leslie, and M. O. Li. 2016. Cancer immunosurveillance by tissue-resident innate lymphoid cells and innate-like T cells. *Cell* 164: 365–377.
9. Mori, L., M. Lepore, and G. De Libero. 2016. The immunology of CD1- and MR1-restricted T cells. *Annu. Rev. Immunol.* 34: 479–510.
10. Gao, Y., and A. P. Williams. 2015. Role of innate T cells in anti-bacterial immunity. *Front. Immunol.* 6: 302.
11. Corbett, A. J., S. B. Eckle, R. W. Birkinshaw, L. Liu, O. Patel, J. Mahony, Z. Chen, R. Reantragoon, B. Meehan, H. Cao, et al. 2014. T-cell activation by

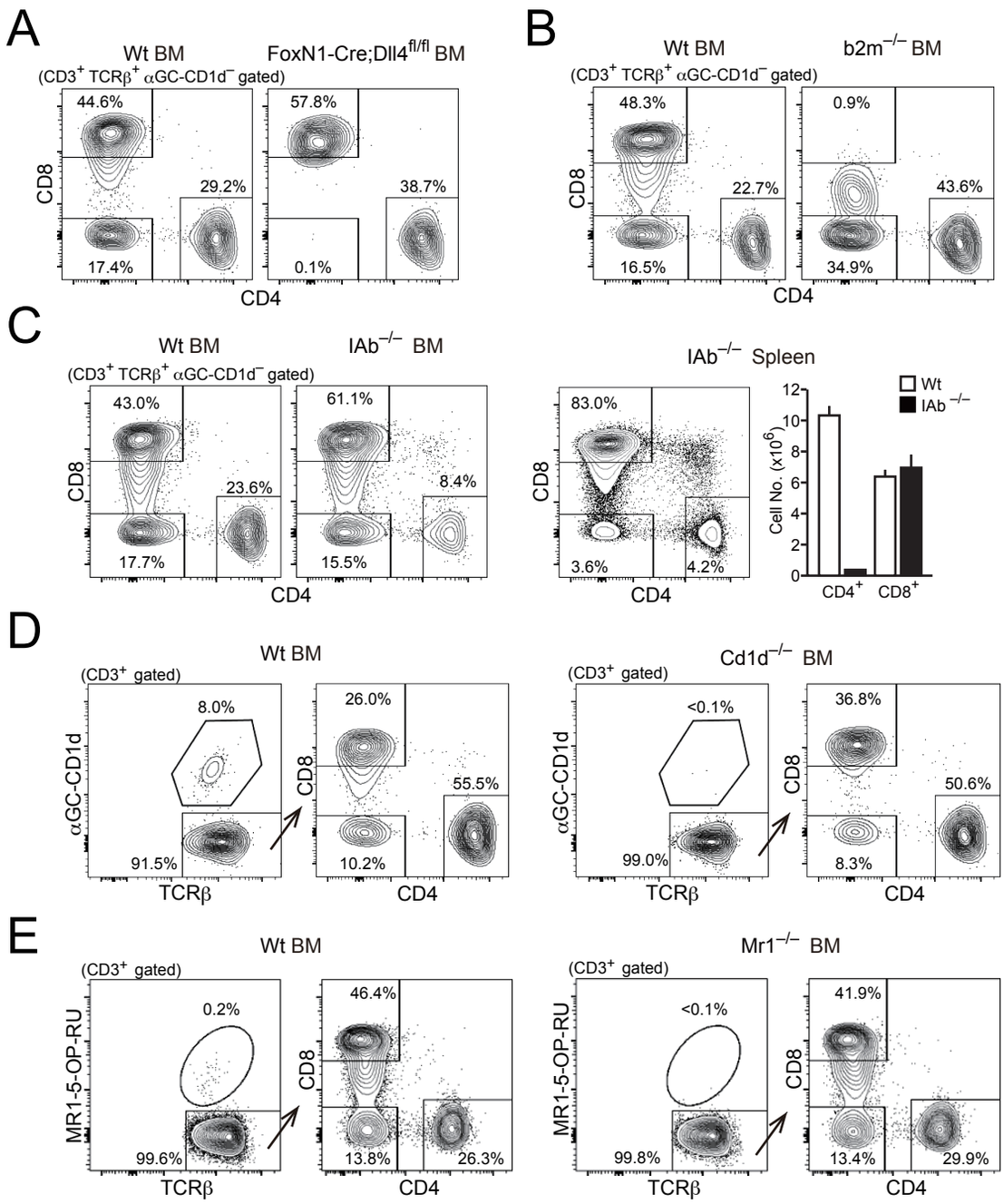
transitory neo-antigens derived from distinct microbial pathways. *Nature* 509: 361–365.
12. Kawano, T., J. Cui, Y. Koezuka, I. Toura, Y. Kaneko, K. Motoki, H. Ueno, R. Nakagawa, H. Sato, E. Kondo, et al. 1997. CD1d-restricted and TCR-mediated activation of valpha14 NKT cells by glycosylceramides. *Science* 278: 1626–1629.
13. Brubaker, S. W., K. S. Bonham, I. Zanoni, and J. C. Kagan. 2015. Innate immune pattern recognition: a cell biological perspective. *Annu. Rev. Immunol.* 33: 257–290.
14. Cowley, S. C. 2014. MAIT cells and pathogen defense. *Cell. Mol. Life Sci.* 71: 4831–4840.
15. Trottein, F., and C. Paget. 2018. Natural killer T cells and mucosal-associated invariant T cells in lung infections. *Front. Immunol.* 9: 1750.
16. D'Acquisto, F., and T. Crompton. 2011. CD3+CD4-CD8- (double negative) T cells: saviours or villains of the immune response? *Biochem. Pharmacol.* 82: 333–340.
17. Hozumi, K., C. Mailhos, N. Negishi, K. Hirano, T. Yahata, K. Ando, S. Zuklys, G. A. Holländer, D. T. Shima, and S. Habu. 2008. Delta-like 4 is indispensable in thymic environment specific for T cell development. *J. Exp. Med.* 205: 2507–2513.
18. 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.
19. Koch, U., E. Fiorini, R. Benedito, V. Besseyrias, K. Schuster-Gossler, M. Pierres, N. R. Manley, A. Duarte, H. R. Macdonald, and F. Radtke. 2008. Delta-like 4 is the essential, nonredundant ligand for Notch1 during thymic T cell lineage commitment. *J. Exp. Med.* 205: 2515–2523.
20. Cosgrove, D., D. Gray, A. Dierich, J. Kaufman, M. Lemeur, C. Benoist, and D. Mathis. 1991. Mice lacking MHC class II molecules. *Cell* 66: 1051–1066.
21. von Freeden-Jeffry, U., P. Vieira, L. A. Lucian, T. McNeil, S. E. Burdach, and R. Murray. 1995. Lymphopenia in interleukin (IL)-7 gene-deleted mice identifies IL-7 as a nonredundant cytokine. *J. Exp. Med.* 181: 1519–1526.
22. Kennedy, M. K., M. Glaccum, S. N. Brown, E. A. Butz, J. L. Viney, M. Embers, N. Matsuki, K. Charrier, L. Sedger, C. R. Willis, et al. 2000. Reversible defects in natural killer and memory CD8 T cell lineages in interleukin 15-deficient mice. *J. Exp. Med.* 191: 771–780.
23. Becker, T. C., S. M. Coley, E. J. Wherry, and R. Ahmed. 2005. Bone marrow is a preferred site for homeostatic proliferation of memory CD8 T cells. *J. Immunol.* 174: 1269–1273.
24. Vivier, E., D. Artis, M. Colonna, A. Diefenbach, J. P. Di Santo, G. Eberl, S. Koyasu, R. M. Locksley, A. N. J. McKenzie, R. E. Mebius, et al. 2018. Innate lymphoid cells: 10 Years on. *Cell* 174: 1054–1066.
25. Sugie, T., H. Kubota, M. Sato, E. Nakamura, M. Imamura, and N. Minato. 1996. NK 1+ CD4- CD8- alphabeta T cells in the peritoneal cavity: specific T cell receptor-mediated cytotoxicity and selective IFN-gamma production against B cell leukemia and myeloma cells. *J. Immunol.* 157: 3925–3935.
26. Krstic, A., S. Mojsilovic, G. Jovcic, and D. Bugarski. 2012. The potential of interleukin-17 to mediate hematopoietic response. *Immunol. Res.* 52: 34–41.
27. Broxmeyer, H. E. 1996. Is interleukin 17, an inducible cytokine that stimulates production of other cytokines, merely a redundant player in a sea of other biomolecules? *J. Exp. Med.* 183: 2411–2415.
28. Griffith, J. W., C. L. Sokol, and A. D. Luster. 2014. Chemokines and chemokine receptors: positioning cells for host defense and immunity. *Annu. Rev. Immunol.* 32: 659–702.
29. Skeen, M. J., and H. K. Ziegler. 1995. Activation of gamma delta T cells for production of IFN-gamma is mediated by bacteria via macrophage-derived cytokines IL-1 and IL-12. *J. Immunol.* 154: 5832–5841.
30. Cui, G., T. Hara, S. Simmons, K. Wagatsuma, A. Abe, H. Miyachi, S. Kitano, M. Ishii, S. Tani-ichi, and K. Ikuta. 2014. Characterization of the IL-15 niche in primary and secondary lymphoid organs in vivo. *Proc. Natl. Acad. Sci. USA* 111: 1915–1920.
31. Pabst, R. 2018. The bone marrow is not only a primary lymphoid organ: the critical role for T lymphocyte migration and housing of long-term memory plasma cells. *Eur. J. Immunol.* 48: 1096–1100.
32. Di Rosa, F. 2016. Two niches in the bone marrow: a hypothesis on life-long T cell memory. *Trends Immunol.* 37: 503–512.
33. Feuerer, M., P. Beckhove, N. Garbi, Y. Mahnke, A. Limmer, M. Hommel, G. J. Hämmerling, B. Kyewski, A. Hamann, V. Umansky, and V. Schirrmacher. 2003. Bone marrow as a priming site for T-cell responses to blood-borne antigen. *Nat. Med.* 9: 1151–1157.
34. Cowley, S. C., E. Hamilton, J. A. Frelinger, J. Su, J. Forman, and K. L. Elkins. 2005. CD4-CD8- T cells control intracellular bacterial infections both in vitro and in vivo. *J. Exp. Med.* 202: 309–319.
35. Straus, S. E., M. Sneller, M. J. Lenardo, J. M. Puck, and W. Strober. 1999. An inherited disorder of lymphocyte apoptosis: the autoimmune lymphoproliferative syndrome. *Ann. Intern. Med.* 130: 591–601.
36. 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.
37. Radtke, F., H. R. MacDonald, and F. Tacchini-Cottier. 2013. Regulation of innate and adaptive immunity by Notch. *Nat. Rev. Immunol.* 13: 427–437.
38. Smiley, S. T., M. H. Kaplan, and M. J. Grusby. 1997. Immunoglobulin E production in the absence of interleukin-4-secreting CD1-dependent cells. *Science* 275: 977–979.
39. Turchinovich, G., and A. C. Hayday. 2011. Skint-1 identifies a common molecular mechanism for the development of interferon-γ-secreting versus interleukin-17-secreting γδ T cells. *Immunity* 35: 59–68.
40. Jensen, K. D., X. Su, S. Shin, L. Li, S. Youssef, S. Yamasaki, L. Steinman, T. Saito, R. M. Locksley, M. M. Davis, et al. 2008. Thymic selection determines gammadelta T cell effector fate: antigen-naive cells make interleukin-17 and antigen-experienced cells make interferon gamma. *Immunity* 29: 90–100.
41. Schwarzenberger, P., V. La Russa, A. Miller, P. Ye, W. Huang, A. Zieske, S. Nelson, G. J. Bagby, D. Stoltz, R. L. Mynatt, et al. 1998. IL-17 stimulates granulopoiesis in mice: use of an alternate, novel gene therapy-derived method for in vivo evaluation of cytokines. *J. Immunol.* 161: 6383–6389.
42. Schwarzenberger, P., W. Huang, P. Ye, P. Oliver, M. Manuel, Z. Zhang, G. Bagby, S. Nelson, and J. K. Kolls. 2000. Requirement of endogenous stem cell factor and granulocyte-colony-stimulating factor for IL-17-mediated granulopoiesis. *J. Immunol.* 164: 4783–4789.
43. Silva-Santos, B., K. Serre, and H. Norell. 2015. γδ T cells in cancer. *Nat. Rev. Immunol.* 15: 683–691.
44. Salio, M., J. D. Silk, E. Y. Jones, and V. Cerundolo. 2014. Biology of CD1- and MR1-restricted T cells. *Annu. Rev. Immunol.* 32: 323–366.
45. Nielsen, M. M., D. A. Witherden, and W. L. Havran. 2017. γδ T cells in homeostasis and host defence of epithelial barrier tissues. *Nat. Rev. Immunol.* 17: 733–745.
46. Ascon, D. B., M. Ascon, S. Satpute, S. Lopez-Briones, L. Racusen, R. B. Colvin, M. J. Soloski, and H. Rabb. 2008. Normal mouse kidneys contain activated and CD3+CD4- CD8- double-negative T lymphocytes with a distinct TCR repertoire. *J. Leukoc. Biol.* 84: 1400–1409.
47. Martina, M. N., S. Noel, A. Saxena, S. Bandapalle, R. Majithia, C. Jie, L. J. Arend, M. E. Allaf, H. Rabb, and A. R. Hamad. 2016. Double-negative αβ T cells are early responders to AKI and are found in human kidney. *J. Am. Soc. Nephrol.* 27: 1113–1123.
48. Sadasivam, M., S. Noel, S. A. Lee, J. Gong, M. E. Allaf, P. Pierorazio, H. Rabb, and A. R. A. Hamad. 2019. Activation and proliferation of PD-1$^+$ kidney double-negative T cells is dependent on nonclassical MHC proteins and IL-2. *J. Am. Soc. Nephrol.* 30: 277–292.



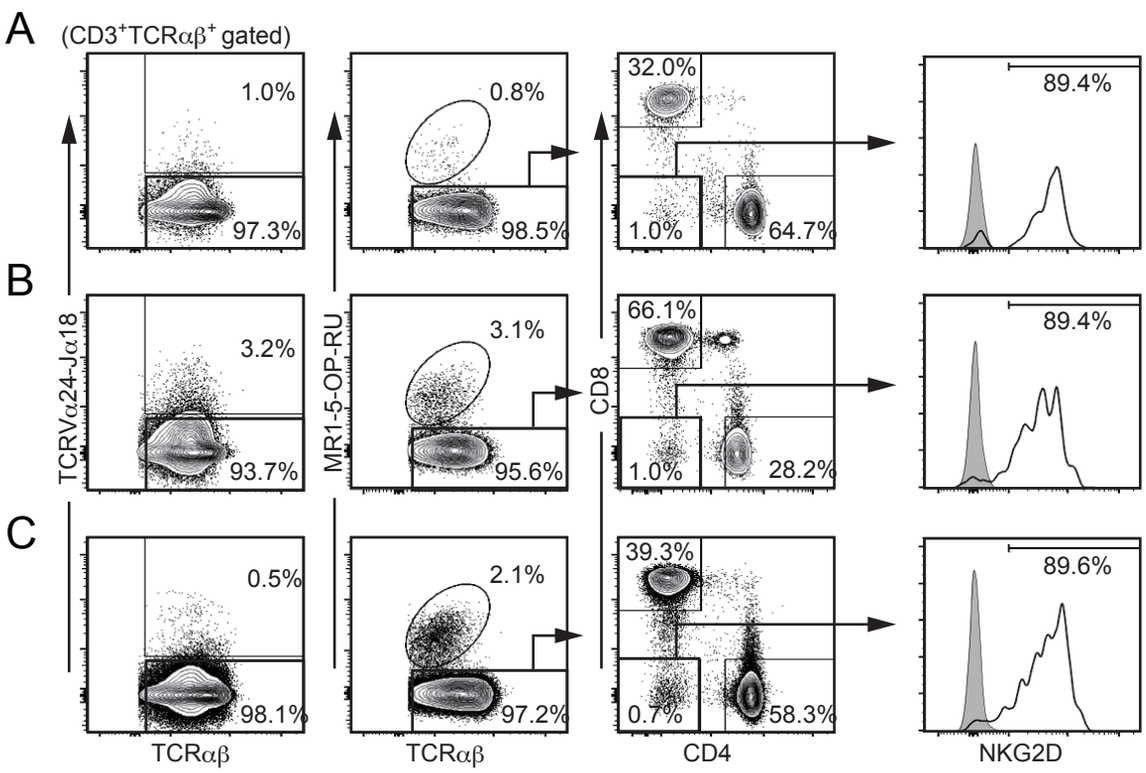

**Supplementary Fig. S1. DN αβT cells other than invariant T cells are detected minimally in the peripheral blood and other lymphoid tissues.** (**A)** MR1-5-OP-RU$^+$ MAIT cells are detectable in the lungs, but these αβT cells were rarely detected in blood and lymphoid tissues. (**B**) DN αβT cells other than invariant NKT cells were detected rarely in the blood and only minimally if any in the non-hematopoietic lymphoid tissues of adult mice.




**Supplementary Fig. S2. BM DN αβT cells show cell surface phenotype closer to BM γδT cells than SP αβT cells and NKT cells.** Expression of surface markers in DN αβT cells, γδT cells, NKT cells, and SP αβT cells of BM from adult B6 mice was analyzed using LEGENDScreen™ Mouse PE kit (Biolegend). The positive proportions of 129 markers that were expressed in more than 5% of at least one T cell subset excluding those defining each subset (for example, CD4, CD8a) were hierarchically clustered and ordered by a Manhattan distance metric using JMP software (SAS Institute Inc.).




**Supplementary Fig. S3. Development of BM DN αβT cells is defective in athymic *nu/nu* and *FoxN1-Cre:Dll4$^{fl/fl}$* mice, but is unaffected in *b2m$^{-/-}$*, *IAb$^{-/-}$*, *Cd1d$^{-/-}$*, and *Mr1$^{-/-}$* mice.** (**A-C**) The representative profiles of CD4 vs. CD8 expression in BM cells at CD3$^+$ TCRβ$^+$ αGC-CD1d$^+$ cell gate are indicated. In *IAb$^{-/-}$* mice, analysis of the spleen cells is also shown, indicating almost complete abolishment of CD4$^+$ T cells. (**D, E**) Similar analysis was done in the BM cells from *Cd1d$^{-/-}$* and *Mr1$^{-/-}$* mice. Although *Cd1d$^{-/-}$* and *Mr1$^{-/-}$* mice showed complete absence of NKT and MAIT cells, the development of DN αβT cells was unaffected at all.




**Supplementary Fig. S4. BMDNT-like cells in the BM from healthy adult humans.** BM cells from healthy adult humans (A-C) at the ages of 24 to 26 years were multi-color analyzed with indicated antibodies. The BM cells contained DN αβT cells distinct from invariant TCRVα24-Jα18$^+$ NKT cells and MR1-5-OP-RU$^+$ MAIT cells at around 1% of αβT cells, the great majority of which constitutively expressed high levels of NKG2D.