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Cutting Edge: Intrinsic Programming of Thymic γδT Cells for Specific Peripheral Tissue Localization

Yan Jin,* Mingcan Xia,* Christina M. Saylor,* Kavitha Narayan,† Joosoo Kang,† David L. Wiest,‡ Yanming Wang,§ and Na Xiong*  

Various innate-like T cell subsets preferentially reside in specific epithelial tissues as the first line of defense. However, mechanisms regulating their tissue-specific development are poorly understood. Using the prototypical skin intraepithelial γδT cells (sIELs) as a model, we show in this study that a TCR-mediated selection plays an important role in promoting acquisition of a specific skin-homing property by fetal thymic sIEL precursors for their epidermal location, and the skin-homing potential is intrinsically programmed even before the selection. In addition, once localized in the skin, the sIEL precursors develop into sIELs without the requirement of further TCR–ligand interaction. These studies reveal that development of the tissue-specific lymphocytes is a hard-wired process that targets them to specific tissues for proper functions. The Journal of Immunology, 2010, 185: 000–000.

Besides conventional αβT cells that reside in secondary lymphoid organs (SLOs) for adaptive responses, various unconventional T cell subsets, such as γδT cells with innate properties, preferentially reside in epithelial tissues as the first line of defense. However, their tissue-specific development processes are poorly understood.

Recent studies suggest that development of the tissue-resident T cells is determined intrathymically. We found that fetal thymic precursors for skin-specific intraepithelial γδT cells (sIELs; also called dendritic epidermal T cells) express a pattern of migration molecules, such as chemokine receptor CCR10 that plays a role in their homing to the epidermis (1, 2). Similarly, subsets of adult thymic αβ and γδ T cells were found to display homing properties associated with their intestinal location (3, 4).

A TCR-mediated thymic selection is likely involved in the acquisition of unique homing properties by the specific T cell subsets (1, 4). Fetal thymic Vγ3+ sIEL precursors deficient of TCR signaling molecule Itk could not acquire the skin-homing property properly (5). In addition, Vγ3+ sIEL precursors remain at an immature stage and could not develop into sIELs in a strain of FVB mice (Tac) that bear mutated Skint1, a selecting molecule for the Vγ3+ sIEL precursors (6). However, how the different thymic T cell subsets acquire unique homing properties is unknown. We show in this study that the development of tissue-specific T cells is an intrinsically programmed process.

Materials and Methods

Mice

KN6 and G8 γδ TCR transgenic (Tg) mice were described (7, 8). BALB/c, C57BL/6 (B6), TCRbΔβ−/−, and homozygous knockout mice deficient for β2-microglobulin (β2m−/−) were purchased from The Jackson Laboratory (Bar Harbor, ME); FVB(NCI) from the National Cancer Institute (Bethesda, MD), and FVB(Tac) from Taconic Farms (Hudson, NY). KN6 or G8 mice on a B6, BALB/c, or β2m−/− background were obtained by proper crossing. One CCR10-knockout/enhanced GFP (EGFP)-knockin allele (CCR10+/EGFP) was also introduced into KN6 or G8 mice of the different backgrounds or mice bearing Skint1FVB(Tac) alleles as a reporter for CCR10 expression (2). All animal experiments were approved by The Pennsylvania State University Institutional Animal Care and Use Committee.

Cell isolation, Abs, and flow cytometry (FACS)

Isolation of epidermal cells, thymocytes, and splenocytes was performed as described (1). Anti-CD3, -CD122, -CD62L, and -γδ TCR Abs were from BioLegend (San Diego, CA); anti-Vγ2, -Vγ3, and αβT from BD Biosciences (San Jose, CA); and anti-CCR9 from R&D Systems (Minneapolis, MN). 17D1 Ab was described (9).

Reconstituted fetal thymic organ culture

The experiment was performed as described (10). Rag1−/−γC−/− or 2-deoxyguanosine–treated E15 fetal thymic lobes were reconstituted with donor cells of different origins and cultured in vitro.

Chromatin immunoprecipitation

The experiment was performed similarly as described (11). Formaldehyde-fixed cells were sonicated to generate fragmented chromatin which was immunoprecipitated with anti-dimethylated Lys4 of histone 3 (H3K4m2) or -trimethylated Lys4 of histone 3 (H3K9m3) Abs (Abcam, Cambridge, MA).

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The online version of this article contains supplemental material.

Abbreviations used in this paper: A, adult; EGFP, enhanced GFP; F, fetal; FTOC, fetal thymic organ culture; H3K4m2, dimethylated Lys4 of histone 3; H3K9m3, trimethylated Lys4 of histone 3; β2m−/−, homozygous knockout mice deficient for β2-microglobulin; ND, nondetectable; sIEL, skin intraepithelial γδ T cell; SLO, secondary lymphoid organ; Tg, transgenic.

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DNA purified from the immunoprecipitated samples was analyzed by real-time PCR with primer sets PT5’-CCACCGTGAGGAGGATG-3’/PR5’-AGCCGTGAACCCAGAGAAAAGC-3’ for the CCR10 promoter region and CF5’-GGGGTGGGAATGTCTTACACCGT-3’/CR5’-GGCCATTGCCAGCCAGCCC-3’ for the CCR10 coding region.

Adoptive transfer of fetal thymic γδT cells into recipient mice

The experiment was performed as described (5). Positively selected CD122+ fetal thymic Tg Vy3+ of B6 background or wild-type Vy3+ γδT cells were injected i.p. into 2- to 3-d-old newborn mice. Two months after the transfer, recipients were analyzed.

In situ staining of epidermal sheets and fluorescent microscopy

The experiment was performed as previously described (5). Ear epidermal sheets were fixed and stained with fluorescently labeled anti-Vγ3 or -Vγ2 Abs and, if for TUNEL staining, an in situ cell death detection kit (TMR red) (Roche Applied Science, Indianapolis, IN). The stained sheets were analyzed by fluorescent microscopy.

Results and Discussion

Impaired selection promotes acquisition of different homing properties by fetal thymic Vy3+ T cells

To dissect how selection is involved in the acquisition of the skin-homing property by fetal thymic Vy3+ T cells, we analyzed their differentiation in mice bearing the mutant Skint1 gene (6). In contrast to those of wild-type Skint1B6 background, Vy3+ fetal thymocytes of Skint1FVB(Tac) background could not develop into the mature CD122+CCR10+ stage that is associated with a unique homing molecule pattern (CCR10+CCR9−CCR7−CD62Llowα4β7−) and the ability to home to the skin (Fig. 1A, Supplemental Fig. 1) (2). Instead, many Vy3+ fetal thymocytes of Skint1FVB(Tac) background differentiated into a CD122+CCR10+ population with a homing molecule expression pattern similar to that of Vy3− γδT cells (CCR9−CCR7−CD62Lhighα4β7+). Consistent with their altered homing property, Vy3− cells were found in tissues other than the skin, such as the uterus, in FVB(Tac) mice (Supplemental Fig. 2).

The CD122−CCR10+ or CD122−CCR10− Vy3+ fetal thymocytes of Skint1FVB(Tac) background differentiated into the CD122−CCR10+ stage when reconstituted and cultured in fetal thymic lobes of B6 mice bearing a wild-type Skint1 (Fig. 1B), confirming that a unique selection is required for acquisition of the skin-homing property by the Vy3+ cells.

A strong γδTCR signal promotes fetal thymic transgenic Vy2+ T cells to acquire the Vy3-like skin-homing property

To assess how the TCR signaling is involved in acquisition of the skin-homing property, we tested whether γδT cell subsets that normally do not localize to the skin could acquire the unique skin-homing property if provided different TCR signals. For this purpose, we used two well-studied Vy2+ γδTCR Tg mice, G8 and KN6 (7, 8). Both G8 and KN6 γδTCRs recognize ligands T10/22, two nonclassic MHC molecules encoded in the H-2 region that are expressed highly in B6 (H-2b), low in BALB/c (H-2d), but not in β2m−/− mice (12). Because Vy2+ T cells are normally generated in adult thymi and preferentially localize into SLOs, these Tg mice also allow us to compare how fetal and adult thymic TγδT cells are selected to acquire specific homing properties.

Only on the ligand-high B6 background did the fetal thymic G8 or KN6 γδT cells undergo a developmental process same as the Vy3+ sIEL precursors to reach the mature CD122+CCR10+ stage (CD24−CD62LlowCCR7−) (Fig. 2A, Supplemental Fig. 3), demonstrating that a strong γδTCR signal promotes positive selection-associated acquisition of the skin-homing property in fetal thymic γδT cells, irrespective of γδTCR composition. The Tg γδT cells of ligand-
low BALB/c background showed signs of partial selection, such as the moderate CD122 upregulation and CD62L down-regulation, but could not reach the CD122+CCR10+ stage.

Unlike their fetal counterparts, adult thymic G8 or KN6 γδT cells of B6 background were negatively selected (Fig. 2B) (8). Although the adult thymic Tg γδT cells of BALB/c background were not negatively selected, they preferentially expressed homing molecules characteristic of the SLO localization, such as CCR7 and CD62L, but no or low CCR10 (Fig. 2C). Therefore, T cells bearing identical γδTCRs, if generated at different ontogenic stages and/or selected in different thymic environments, acquire different homing properties.

The skin-homing potential is intrinsically programmed in fetal thymic γδT cells before the γδTCR-mediated selection

To dissect mechanisms underlying the selective acquisition of skin-homing property by the fetal thymic γδT cells, we reconstituted B6 fetal thymic lobes with purified unselected adult or fetal thymic KN6 Vγ2+ T cells for fetal thymic organ cultures (FTOCs). Although the fetal donor cells differentiated efficiently into the CD122+CCR10+ stage, few cells could be recovered from lobes reconstituted with the adult donors (Fig. 3A). These results are consistent with the notion that adult thymic γδT cells are negatively selected in the ligand-high environment and suggest that intrinsic properties of fetal and adult thymic γδT cells play an important role in their selection consequences. To dissect this further, we reconstituted B6 fetal thymic lobes with earlier-stage γδTCRαβTCRγδTCRαβTCRγδTCR adult or fetal thymic progenitors of KN6 mice. In this scheme, KN6 γδT cells were efficiently generated of both adult and fetal donors (Fig. 3B). However, KN6 γδT cells of the adult donors still preferentially expressed the molecules associated with the SLO localization (CD62Llow/CCR10low/CCR10low/), whereas those of the fetal donors displayed the unique skin-homing property (CD122+CCR10+) (Fig. 3C), indicating that the skin-homing potential is intrinsically programmed in the fetal thymic progenitors even before the TCR-mediated selection.

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β2m−/− background, which do not express CCR10 at all (Fig. 2A). Levels of H3K4m2, a modification correlating with gene activation, and H3K9m3, a modification implicated in gene silencing, were determined by a chromatin immuno-precipitation assay (11, 13). Compared to their adult counterparts, the fetal thymic Tg γδT cells had higher levels of H3K4m2 and lower levels of H3K9m3 at both CCR10 promoter and coding regions (Fig. 3D, 3E), all suggesting that the fetal cells have a more active CCR10 locus. These results indicate that before its expression, CCR10 locus is already in an OPEN and transcription permissive configuration in the fetal, but not adult, thymic γδT cells, suggesting that it is programmed for different expression potentials.

Positively selected fetal thymic γδT cells develop into sIELs without requirement of peripheral TCR-ligand interaction

Different from Vγ3+ cells, the positive selection and skin-homing properties of the fetal thymic Tg Vγ2+ T cells were not associated with the efficient development of Tg Vγ2+ sIELs (Fig. 4A). There were nearly no Tg sIELs in adult G8(B6) mice, whereas β2m−/−G8 mice had many. KN6(B6) mice also had fewer sIELs than Kn6(BALB/c) mice. The development of Tg sIELs in G8 versus KN6 mice of same backgrounds was also different. For example, there were more Tg sIELs in β2m−/−G8 mice than in β2m−/−KN6 mice.

Several factors might contribute to these complex phenotypes. First, although a strong γδ TCR–ligand signal promotes acquisition of the skin-homing property in fetal thymic γδT cells, peripheral TCR–ligand interaction might also affect their development into sIELs. A ligand for G8 and KN6 γδTCRs is expressed in the skin of B6, but not BALB/c, mice (12). Complicating the issue, G8 γδTCR has a higher affinity for the ligand than KN6 γδTCR (14), and they could possibly have additionally different ligands, which altogether would result in the phenotypic difference between G8 and KN6 mice. Furthermore, adult thymic Tg γδT cells might contribute to sIELs. In fact, γδT cells of adult SLOs could migrate into the skin in absence of normal sIELs (15). Therefore, correlation between positive selection of the fetal thymic Tg γδT cells and their development into sIELs could not be directly assessed in the Tg mice.

We therefore transferred positively selected fetal thymic G8 or KN6 γδT cells of B6 background into ligand-negative TCRδ−/−β2m−/−B6 or ligand-high TCRδ+/−β2m−/−B6 mice, which lack endogenous γδT cells. The transferred G8 γδT cells developed into sIELs only in the ligand-negative recipients (Fig. 4B). The transferred KN6 γδT cells also developed efficiently into sIELs in the ligand-negative recipients, although some KN6 sIELs developed in the ligand-high recipients (Supplemental Fig. 4D). These results demonstrate that: 1) the positively selected fetal thymic Tg γδT cells could develop into sIELs without requirement of peripheral TCR–ligand interaction; and 2) continuous peripheral TCR–ligand interaction impairs the sIEL development, likely due to activation-induced apoptosis. Consistent with this, there were higher percentages of apoptotic sIELs in KN6 mice of B6 background than in β2m−/−KN6 or wild-type mice (Fig. 4C, Supplemental Fig. 5). The extents of impairment of the G8 or KN6 sIEL development in the ligand-high recipients are also consistent with this conclusion in that a higher G8TCR–ligand affinity is associated with the reduced KN6 sIELs. Unselected fetal thymic Tg γδT cells could not develop into sIELs in any recipients (Supplemental Fig. 4B), confirming that the positive selection is a prerequisite for their development into sIELs. Together, these results demonstrate that the positive selection of fetal thymic γδT cells is necessary and sufficient for their development into sIELs. Further supporting this, transfected positively selected fetal thymic Vγ3+ γδT cells of Skint1-sufficient FVB(NCI) mice developed into sIELs in Skint1-deficient FVB (Tac) recipients (Fig. 4D).

In conclusion, proper localization of various T cell subsets in specific tissues is important in the local protection but mechanisms regulating the process are unclear. We identify in this study an intrinsically programmed process in the fetal thymus that determines selective acquisition of the skin-homing property by the sIEL precursors. This finding could provide a guide in understanding development of other tissue-resident T cells.

Our findings on the differential selection processes of the fetal versus adult thymic Tg γδT cells also help in understanding roles of TCR signals in the γδT cell development, a poorly understood question. Early suggestion on the requirement of TCR signals for positive selection of the adult thymic Tg γδT cells (16) was later attributed to negative selection or genetic background (17). However, a recent study found that KN6 γδT cells divert to αβT cell lineage on a β2m−/− background (18), suggesting requirement of γδ TCR signals for the γδT cell development. In light of our findings, it is likely that different γδT cell subsets have differential requirements of TCR signals for their development.

Acknowledgments

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Disclosures

The authors have no financial conflicts of interest.

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2. Jin, Y., M. Xia, A. Sun, C. M. Saylor, and N. Xiong. 2010. CCR10 is important for the development of skin-specific γδ T cells by guest on July 25, 2017 http://www.jimmunol.org/ Downloaded from
**SUPPLEMENTAL FIGURES**

**Supplementary Figure 1.** Comparison of expression patterns of migration molecules by E16-17 fetal thymic CD122⁺CCR10(EGFP)⁺Vγ3⁺ T cells of CCR10⁺EGFP mice of mutant Skint1<sup>FVB(Tac)</sup> background (CCR10⁺EGFP<sup>Skint1<sup>FVB(Tac)</sup></sup>) vs. CD122⁺CCR10(EGFP)⁺Vγ3⁺ or CD122⁺CCR10(EGFP)⁺Vγ3⁺γδT cells of CCR10⁺EGFP mice of the wild type Skint1<sup>B6</sup> background (CCR10⁺EGFP<sup>Skint1<sup>B6</sup></sup>) by flow cytometry. The gray areas are of staining with respective iso-type controls. More than 3 mice were analyzed for each staining. The CCR10⁺EGFP mice on the Skint1<sup>FVB(Tac)</sup> background were obtained by crossing CCR10⁺EGFP mice of B6 background with FVB(Tac) mice, progeny of which were intercrossed. The mutant Skint1<sup>FVB(Tac)</sup> allele was distinguished from the wild-type Skint1<sup>B6</sup> allele by a genomic PCR with primers Skint1-F1: GGACCTTGCCTAAAATCAAT and Skint-R1: CCCCAGAACCCTGAAGGTC, followed by restriction enzyme digestion with MboII, which gave rise to different band patterns for Skint1<sup>B6</sup> (150/50/230bp) and Skint1<sup>FVB(Tac)</sup> allele (150/280bp), due to a mutation in one MboII site of the latter.

**Supplementary Figure 2.** Detection of Vγ3⁺γδT cells in the uterus of the Skint1<sup>FVB(Tac)</sup> mice. FACS analysis of lymphocytes isolated from the uterus of adult B6 and FVB(Tac) mice for Vγ3 expression. To isolate lymphocytes from uterus, uterus were minced and digested with collagenase for 1-2 hours with gentle shaking to dissociate the cells. Mononucleocytes were enriched from the dissociated cell preparations using Percoll gradients (40%/80%) and stained with anti-Cδ and Vγ3 antibodies, followed by staining with the 17D1 antibody that recognizes anti-Cδ antibody-bound Vγ4/Vδ1⁺ TCR (Roark,
CL, et al. J. Leuk. Biol. 75: 68. 2004). The histograms were on gated γδTCR+ T cells.
Three mice were analyzed in each group.

**Supplementary Figure 3.** Effects of different levels of ligand expression on the positive selection and homing molecule expression of fetal thymic KN6 and G8 transgenic γδT cells. E16-17 fetal KN6 or G8 transgenic (Tg) Vγ2+ thymocytes of three different ligand-expressing backgrounds, as indicated, were FACS analyzed for CD122, CD24, CD62L, CCR7 and CCR10(EGFP) expression. All histograms were gated on transgenic Vγ2+ cells. Gray areas showed isotype controls. N>3.

**Supplementary Figure 4.** Reconstitution of sIELs in ligand-negative vs. ligand-high recipients by fetal thymic transgenic γδT cells of different ligand-expressing backgrounds. A. Differential reconstitution of sIELs in ligand-negative vs. ligand-high recipients by fetal thymic KN6Tg γδT cells of B6 background. Two-three day-old TCRδ−/−β2M−/− or TCRδ−/−β2M+/− mice of B6 background were adoptively transferred with the indicated fetal thymic donor cells. Two months after the transfer, ear epidermal sheets of the recipients were analyzed by immunofluorescent microscopy for the transgenic Vγ2+ sIELs. N≥3. B. Unselected fetal thymic G8 transgenic γδT cells could not reconstitute sIELs in recipients. The 2-3 day old TCRδ−/−β2M−/− or TCRδ−/−β2M+/+ mice of B6 background were transferred with fetal thymic transgenic γδT cells of G8Tgβ2M−/− mice of the same B6 background. Two months after the transfer, ear epidermal sheets of the recipients were analyzed by immunofluorescent microscopy for the transgenic Vγ2+ sIELs. N≥3.
Supplementary Figure 5. In situ TUNEL analyses of ear epidermal sheets to detect apoptotic sIELs. Ear epidermal sheets of the KN6/B6, KN6/β2M−/− and wild-type (WT) mice were stained with the TMR red TUNEL staining mixture and FITC conjugated anti-Vγ2 (or Vγ3 for the WT) antibody and analyzed by confocal fluorescent microscopy. Apoptotic sIELs were double positive for green and red, indicated by arrowheads. Insets in the top panel show an apoptotic and a non-apoptotic sIEL at the high magnification. The numbers in each panel are percentages of apoptotic sIELs, calculated based on ratios of numbers of apoptotic vs. total sIELs from multiple analyses of at least four mice in each strain. Note that there were many other apoptotic skin cells besides the apoptotic sIELs due to high turnover of epidermal cells.
Supplementary Figure 1, Jin, et al

- CCR10^{+/EGFP}Skint^{B6}
- CCR10^{+/EGFP}Skint^{FVB(Tac)}

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Supplementary Figure 4, Jin, et al

A
Donor: KN6TgCCR10\textsuperscript{+/+} to TCR\textsuperscript{δ\neg\neg}\beta2M\textsuperscript{−/−}
Recipient: TCR\textsuperscript{δ\neg\neg}\beta2M\textsuperscript{−/−}

B
Donor: G8Tg\beta2M\textsuperscript{−/−} to TCR\textsuperscript{δ\neg\neg}\beta2M\textsuperscript{+/+}
Recipient: TCR\textsuperscript{δ\neg\neg}\beta2M\textsuperscript{−/−}
Supplementary Figure 5, Jin, et al