Supplemental information

Ontogenic timing, T cell receptor signal strength, and Notch signaling direct \( \gamma\delta \) T cell functional differentiation \textit{in vivo}

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Controlled Initiation of T-Cell Development Reveals a Temporal Regulation in the Generation of T-Cells

In utero test the ability of RBPJκ expression in spleen and lymph nodes of fetal-, neonatal-, or adult-induced adult mice. Cells are pre-gated as indicated.

Fetal-Induced and Neonatal-Induced T-Cell Development

Different epithelial-rich tissues in adult animals. The development of fetal-, neonatal-, or adult-induced adult mice. Cells are pre-gated as indicated.

Figure S1: Doxycycline Treatment Periods for Temporal Induction of T-Cell Development:

Fetal Induction Neonatal Induction Adult Induction Analysis

Birth 3 Weeks 6 Weeks 9 Weeks 12 Weeks

B

E16 Fetal Thymus Day 12 Neonatal Thymus Day 54 Adult Thymus

C

Fetal Thymus Adult Thymus

| Days post Dox treatment | % of γδ T-cells |
|------------------------|-----------------|
| E16                    | 78.3            |
| E17                    | 61.5            |
| E18                    | 45.9            |
| E19                    | 33.5            |
| E20                    | 21.8            |
| E21                    | 10.1            |

| Days post Dox treatment | % of γδ T-cells |
|------------------------|-----------------|
| Day 9                  | 30.0            |
| Day 11                 | 35.0            |
| Day 13                 | 40.0            |
| Day 15                 | 45.0            |
| Day 17                 | 50.0            |
| Day 19                 | 55.0            |

D

Spleen

| # of cells |
|------------|
| CD3        |

LN

| # of cells |
|------------|
| CD3        |

γδ TCR

CD45

γδ T cells in the lungs. At approximately ten days post-infection, there is an increase in γδ T cells not normally localized to lymphoid tissues. Evidence in support of the involvement of γδ T cells in the initial stages of the response to infection. These results obtained using various infectious disease models. Mouse models of virus and parasite infection. Although fewer in numbers, fetal Vγδ T cells were enriched for PLZF expression.

This staged response does not, as evidenced by the initial stages of the response to infection. These results obtained using various infectious disease models. Mouse models of virus and parasite infection. Although fewer in numbers, fetal Vγδ T cells were enriched for PLZF expression. Therefore, not only the anatomical location of T cells nor their functional activities are active. Infection is one approach that has proved informative for elucidating T-cell function. This involves the successive activation and expansion of two distinct populations of T cells in the context of a biological response. Therefore, not only the anatomical location of T cells nor their functional activities are active. Infection is one approach that has proved informative for elucidating T-cell function.
Figure S1. Temporal restriction of T-cell development in RBPJ\textsuperscript{ind} mice. Related to Figure 1. (A) Doxycycline treatment strategies for fetal, neonatal, or adult specific induction of T-cell development in RBPJ\textsuperscript{ind} mice. (B) Flow cytometry analysis of T-cell development and presence of γδ T-cells in the thymus of fetal, neonatal, or adult RBPJ\textsuperscript{ind} mice, with or without Dox. (C) Kinetics of Vγ1, Vγ4, Vγ5, Vγ7, and other (Vγ1\textsuperscript{-}Vγ4\textsuperscript{-}Vγ5\textsuperscript{-}Vγ7\textsuperscript{-}) development in the thymus of fetal or adult RBPJ\textsuperscript{ind} mice following indicated days of Dox treatment; flow cytometry analysis and pre-gated on γδ T-cells. (D) Flow cytometry analysis of presence of γδ T-cells in spleen and lymph nodes of uninduced (-Dox), fetal-, neonatal-, or adult-induced RBPJ\textsuperscript{ind} mice; pre-gated on γδ T-cells. Data are representative of three independent experiments. Data are presented as means ± standard deviation of three independent experiments. n = 3 mice per group.
**Figure S2**

A) 

Gated on IL-17⁺ γδ T-cells

B) 

Mock  | TDM

IL-17

Gated on γδ T-cells

C) 

Doxycycline Treatment Periods

Fetal Induction  | Adult Induction

Birth | 3 Weeks  | 6 Weeks  | 9 Weeks  | 12 Weeks

D) 

Lung IL-17⁺ γδ T-Cells:

| Comparison                          | Percentage Statistics | Total Number Statistics |
|-------------------------------------|-----------------------|-------------------------|
| Control vs Fetal -Dox               | *                     | ns                      |
| Control vs Fetal +Dox               | *                     | ns                      |
| Control vs Adult -Dox               | ns                    | **                      |
| Control vs Adult +Dox               | ns                    | **                      |
| Fetal -Dox vs Fetal +Dox            | ns                    | ns                      |
| Fetal -Dox vs Adult -Dox            | **                    | *                       |
| Fetal -Dox vs Adult +Dox            | **                    | *                       |
| Fetal +Dox vs Adult -Dox            | **                    | **                      |
| Fetal +Dox vs Adult +Dox            | **                    | **                      |
| Adult -Dox vs Adult +Dox            | ns                    | ns                      |
Figure S2. TDM inflammation model to elicit IL-17 production by lung γδ T-cells. Related to Figure 3. (A) Flow cytometry analysis of Vγ1+ and Vγ4+ cells among IL-17+ lung γδ T-cells of adult-induced RBPJind mice. Data are representative of three independent experiments. (B) Flow cytometry analysis of IL-17 production by γδ T-cells in lung of normal mice after 2 days of mock or TDM injection (left), with right panels showing percentages and numbers. Data are representative of two independent experiments. Data are presented as means ± standard deviation of two independent experiments. n = 4 mice per group. (C) Doxycycline treatment strategies for fetal or adult specific induction of T-cell development in RBPJind mice, followed by continued arrest or re-initiation of Notch responsiveness in peripheral γδ T-cells, followed by TDM challenge. (D) Statistical analyses between groups as described in Figure 3C. ns = not significant, *P<0.05, **P<0.01, ****P<0.0001 (two-tailed unpaired t-test for B and one-way ANOVA for D).
Figure S3

A. RBPJ$^{\text{ind}}$ (B6) x KN6$^{\text{ig}}$ (BALB/c) → KN6$^{\text{ig}}$ or RBPJ$^{\text{ind}}$KN6$^{\text{ig}}$

- $\text{H-2T}^{b/b}$
- $\text{H-2T}^{d/d}$
- $\text{H-2T}^{b/d}$ or $\text{H-2T}^{d/d}$

B. CD3 MFI

- **

C. Gated on Vγ4$^+$ cells

- CD3
- CD24
- CD73
**Figure S3.** Modulation of TCR signal strength in KN6tg mice. Related to Figure 4. (A) Generation of KN6tg and RBPJindKN6tg mice (H-2Tb/d or H-2Td/d). (B) CD3 MFI of KN6 cells in the thymus of b/d and d/d KN6tg mice. (C) Flow cytometry analysis of CD24 and CD73 expression by KN6 cells in the thymus of b/d and d/d KN6tg mice. Data are representative of three independent experiments. Data are presented as means ± standard deviation of three independent experiments. n = 3 mice per group. **P<0.01 (two-tailed unpaired t-test).**
Figure S4

A

IFNγ+ Cells

% of Total Vγ4 Cells

Total Cell #

b/d  d/d  ns  b/d  ns  d/d

0 10 20 30 40 50 60

0 10^4 2 10^4 3 10^4 4 10^4 5 10^4

IFNγ+ Cells

B

Spleen  LN  Lung

IFNγ

b/d  d/d

Gated on Vγ4+ cells
PMA + Ionomycin stimulated

C

Spleen  LN  Lung

IL-17

b/d  d/d

Gated on Vγ4+ cells
PMA + Ionomycin stimulated
Figure S4. TCR signal strength influence peripheral γδ T-cell functionality. Related to Figure 4. (A) Percentage and number of IFNγ+ thymic KN6 cells of b/d and d/d KN6tg mice. (B) Flow cytometry analysis of IFNγ production by KN6 cells in spleen, lymph nodes, lung, and IL-4 production by KN6 cells in spleen of b/d and d/d KN6tg mice stimulated with PMA and ionomycin in vitro. (C) Flow cytometry analysis of IFNγ and IL-17 production by KN6 cells in spleen, lymph nodes, lung of b/d and d/d KN6tg mice stimulated with PMA and ionomycin in vitro. Data are representative of three independent experiments. Data are presented as means ± standard deviation of three independent experiments. n = 3 mice per group. ns = not significant (two-tailed unpaired t-test).
**Figure S5. Cell cluster gene expression of b/d and d/d thymic KN6 cells.** Related to Figure 5. UMAP analysis of Cd24a and Nt5e (A), Ccr9 (B), and Klrk1, Klrd1, Klrc1, Blk, Maf, and Rorc (C) gene expression of KN6 cells in the thymus of b/d and d/d KN6\textsuperscript{tg} mice. Data are from one independent experiment. \( n = 2112 \) cells from 3 pooled mice for b/d and \( n = 2753 \) cells from 3 pooled mice for d/d.
Figure S6

A

Day 1  |  Day 2  |  Day 3  |  Day 4  |  Day 5  
CD44  |  0.168  |  0.543  |  0.699  |  7.70  
CD25  |  0.168  |  1.55  |  1.05  |  5.86  

B

Day 1  |  Day 2  |  Day 3  |  Day 4  |  Day 5  |  Day 6  
CD44  |  0.917  |  0.827  |  0.8  |  0.611  |  1.25  
CD4  |  0.108  |  0.571  |  1.23  |  0.141  |  0.325  
CD8  |  2.17  |  0.611  |  0.618  |  1.25  |  2

C

Day 1  |  Day 2  |  Day 3  |  Day 4  |  Day 5  |  Day 6  
γδTCR  |  0.0168  |  0.0181  |  0.0246  |  0.0276  |  0.0501  
CD3  |  0.911  |  0.896  |  0.554  |  2.3  |  4.01  

Gated on γδ T-cells
Figure S6. DN2/DN3, DP, and γδ T-cell developmental kinetics. Related to Figure 6. Flow cytometry analysis of kinetics of DN2/DN3 development (A), DP development (B), and γδ T-cell development (C) in the thymus of RBPJind mice following indicated days of Dox treatment. Data are representative of three independent experiments. $n = 3$ mice per group.
Figure S7

A

-Dox

Dox+5d+7d

Dox+5d-7d

CD4

CD8

Total Cell # CD4+CD8+ T-Cells

B

-Dox

Dox+5d+7d

Dox+5d-7d

γδTCR

CD3

CD24

CD73

Gated on γδ T-cells

Total Cell # CD73- γδ T-Cells

Total Cell # CD73+ γδ T-Cells
Figure S7. Notch signaling is dispensable for γδ-lineage commitment in vivo. Related to Figure 6. (A) Flow cytometry analysis of DP development with continued (Dox\(^{+5d+7d}\)) or discontinued (Dox\(^{-5d+7d}\)) Notch responsiveness in developing DN2/DN3 cells in the thymus of RBP\(^{\text{ind}}\) mice (left), with right panels showing numbers. (B) Flow cytometry analysis of γδTCR\(^+\)CD73\(^-/-\) development with continued (Dox\(^{+5d+7d}\)) or discontinued (Dox\(^{-5d+7d}\)) Notch responsiveness in developing DN2/DN3 cells in the thymus of RBP\(^{\text{ind}}\) mice (left), with right panels showing numbers. Data are representative of three independent experiments. Data are presented as means ± standard deviation of three independent experiments. \(n = 3\) mice per group. ns = not significant, **\(P<0.01\), ****\(P<0.0001\) (two-tailed unpaired \(t\)-test).
Figure S8

Panel A: Gated on Vγ4+ cells

Panel B: Gated on Vγ4+ cells
PMA + Ionomycin stimulated
Figure S8. Notch signaling regulates γδ-lineage commitment in the presence of less strong TCR signaling. Related to Figure 6. (A) Flow cytometry analysis of CD24 and CD73 expression by KN6 cells in the thymus of b/d and d/d RBPJ<sup>ind</sup>KN6<sup>tg</sup> treated with Dox for 8 days, followed by continued (Dox<sup>+8d+4d</sup>) or discontinued (Dox<sup>+8d-4d</sup>) Dox treatment for 4 days (left), with right panels showing numbers (++ = Dox<sup>+8d+4d</sup>; + = Dox<sup>+8d-4d</sup>). Data are representative of three independent experiments. Data are presented as means ± standard deviation of three independent experiments. n = 6 mice per b/d group and n = 3 mice per d/d group. (B) Flow cytometry analysis of IFNγ and IL-17 production by KN6 cells in the thymus of b/d and d/d RBPJ<sup>ind</sup>KN6<sup>tg</sup> (Dox<sup>+8d+4d</sup> or Dox<sup>+8d-4d</sup>), and stimulated with PMA and ionomycin in vitro (left), with right panels showing percentages and numbers (++ = Dox<sup>+8d+4d</sup>; + = Dox<sup>+8d-4d</sup>). Data are representative of three independent experiments. Data are presented as means ± standard deviation of three independent experiments. n = 4 mice per b/d group and n = 3 mice per d/d group. ns=not significant, **P<0.01 (two-tailed unpaired t-test).
Figure S9. Transcriptomic analysis of Notch signaling in γδ T-cell functional differentiation with strong TCR signals. Related to Figure 6. (A) UMAP analysis of cluster 1 enriched genes of thymic KN6 cells from b/d++ (Dox*8d+4d) and b/d+- (Dox*8d-4d) RBPJ^{ind}KN6^{tg} mice. (B) UMAP analysis of Hivep3 and Zbtb16 expression in cluster 1 of thymic KN6 cells from b/d++ and b/d+- RBPJ^{ind}KN6^{tg} mice (left). Ratio of cluster 1 Hivep3^{+}Zbtb16^{+} to other of thymic KN6 cells from b/d++ and b/d+- RBPJ^{ind}KN6^{tg} mice (right). Statistical significance of ratio was determined by a chi-squared test (Table S3). Data are from one independent experiment. n = 2775 cells from 5 pooled mice for b/d++ and n = 3065 cells from 5 pooled mice for b/d+-.