Enforcement of $\gamma\delta$ -lineage commitment by the pre-T-cell receptor in precursors with weak $\gamma\delta$ -TCR signals

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Developing thymocytes bifurcate from a bipotent precursor into $\alpha\beta$ - or $\gamma\delta$ -lineage T cells. Considering this common origin and the fact that the T-cell receptor (TCR) β -, γ -, and δ -chains simultaneously rearrange at the double negative (DN) stage of development, the possibility exists that a given DN cell can express and transmit signals through both the pre-TCR and $\gamma\delta$ -TCR. Here, we tested this scenario by defining the differentiation outcomes and criteria for lineage choice when both TCR- β and $\gamma\delta$ -TCR are simultaneously expressed in *Rag2*^{-/-} DN cells via retroviral transduction. Our results showed that *Rag2*^{-/-} DN cells expressing both TCRs developed along the $\gamma\delta$ -lineage, down-regulated CD24 expression, and up-regulated CD73 expression, showed a $\gamma\delta$ -biased gene-expression profile, and produced IFN- γ in response to stimulation. However, in the absence of Inhibitor of DNA-binding 3 expression and strong $\gamma\delta$ -TCR ligand, $\gamma\delta$ -expressing cells showed a lower propensity to differentiate along the $\gamma\delta$ -lineage. Importantly, differentiation along the $\gamma\delta$ -lineage was restored by pre-TCR coexpression, which induced greater down-regulation of CD24, higher levels of CD73, *Nr4a2*, and *Rgs1*, and recovery of functional competence to produce IFN- γ . These results confirm a requirement for a strong $\gamma\delta$ -TCR ligand engagement to promote maturation along the $\gamma\delta$ T-cell lineage, whereas additional signals from the pre-TCR can serve to enforce a $\gamma\delta$ -lineage choice in the case of weaker $\gamma\delta$ -TCR signals. Taken together, these findings further cement the view that the cumulative signal strength sensed by developing DN cells serves to dictate its lineage choice.

T-cell development | β -selection | Notch | $\gamma\delta$ T-lineage

T cells can differentiate along distinct $\alpha\beta$ - or $\gamma\delta$ -cell lineages, but bifurcate from a common bipotent precursor (1, 2). In mice, the earliest subset of T cells contains CD4⁻ CD8⁻ or double-negative (DN) thymocytes, and this can further be divided into four subgroups (DN1–4) based on the expression of CD25 and CD44 (3, 4). Single-cell progenitor analyses have identified the DN3 stage as the point of T-lineage commitment, and also the final stage at which a DN cell specifies its lineage fate as $\alpha\beta$ or $\gamma\delta$ (1, 5). The $\alpha\beta$ - or $\gamma\delta$ -lineage choice decision is governed by several factors. Two competing models have been proposed for this process: the stochastic and instructional models (2). Although evidence exists to support either model, a version of the instructional model posits that the strength of signal transduced by the T-cell receptor (TCR) expressed by the DN3 cell dictates its lineage specification (6, 7).

The apparent connection between lineage choice and the TCR expressed by the cell can be severed by manipulations of TCR signal strength. We previously noted that stimulating stronger signals via expression of the ERK/MAPK-induced Inhibitor of DNA-binding 3 (Id3) appears to promote the $\gamma\delta$ -lineage fate in developing DN3 cells in the absence of TCR expression (8), suggesting a critical role for Id3 in mediating $\alpha\beta$ - versus $\gamma\delta$ -lineage decisions at this developmental checkpoint. Nevertheless, absence of Id3 also appears to favor the emergence

of innate-like V γ 1.1/V δ 6.3 $\gamma\delta$ -TCR-bearing T cells from the thymus over other $\gamma\delta$ -TCR subsets (9, 10).

Several studies have shown that ligand engagement highly influences the $\alpha\beta$ - versus $\gamma\delta$ -lineage decision because of its effects on $\gamma\delta$ -TCR signal strength (6, 7, 9, 11, 12). $\gamma\delta$ -TCR-expressing DN3 cells develop along the $\alpha\beta$ -lineage and become CD4⁺ CD8⁺ (double-positive, DP) cells in the absence of ligand engagement (7), whereas provision of the ligand, or the use of antibodies to mimic ligand engagement (11), allows these cells to adopt the $\gamma\delta$ -lineage fate, remain DN, and down-regulate expression of CD24. Additional signals, such as those mediated by Notch, can also influence $\alpha\beta$ - versus $\gamma\delta$ -lineage fate outcomes (1, 13–16). We showed that $\gamma\delta$ -TCR-bearing thymocytes adopting the $\gamma\delta$ -lineage do not require concurrent signals from Notch to mature past the DN3 stage, whereas their pre-TCR-expressing counterparts are completely dependent upon Notch signaling to facilitate their pre-TCR-dependent differentiation to the DP stage (1, 17).

Considering the common origin of $\alpha\beta$ - and $\gamma\delta$ -lineage cells, it is possible for a bipotent DN3 cell to simultaneously express and transmit signals through a functional pre-TCR and a functional $\gamma\delta$ -TCR, especially considering that TCR- β -, γ -, and δ -genes complete their rearrangements at the DN3 stage. Additionally, $\gamma\delta$ -T cells have been shown to contain TCR- β rearrangements (18) and $\alpha\beta$ -lineage cells show evidence of both TCR- γ and δ -rearrangements (19–21). In a previous study looking to address the consequences of simultaneously expressing a TCR- β and $\gamma\delta$ -TCR in vivo using transgenic (Tg) mice, the numbers of $\alpha\beta$ -

Significance

Expression of a productively rearranged T-cell receptor (TCR)- β chain induces a program of $\alpha\beta$ T-lineage differentiation, whereas thymocytes that productively rearranged TCR- γ and TCR- δ typically give rise to $\gamma\delta$ -lineage T cells. However, given that all three TCR gene loci simultaneously undergo gene rearrangements, the possibility exists that a developing thymocyte may express a $\gamma\delta$ -TCR together with a TCR- β or pre-TCR complex, and it is not clear to what this outcome would give rise in terms of T-lineage differentiation. Our findings point to a striking conclusion, in that rather than transmitting signals that exclusively promote $\alpha\beta$ -lineage commitment/differentiation, the pre-TCR can function in concert with the $\gamma\delta$ -TCR to promote $\gamma\delta$ commitment/differentiation, a result that supports a signal strength model of $\alpha\beta$ / $\gamma\delta$ -lineage choice.

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and $\gamma\delta$ -lineage cells in TCR- $\beta/\gamma\delta$ -expressing cells were both high, and comparable to TCR- β - and $\gamma\delta$ -TCR-Tg mice, respectively (22). In this case, however, the TCR chains were expressed earlier than physiological for T-cell development, and premature expression of $\alpha\beta$ -TCR transgene can lead to aberrant developmental progression (23, 24).

Here, we attempt to definitively answer the question of lineage choice by simultaneously expressing TCR- β and $\gamma\delta$ -TCR in $Rag2^{-/-}$ DN3 cells via retroviral transduction followed by in vitro coculture, including limiting dilution and clonal analyses. We now find that $Rag2^{-/-}$ DN3 cells expressing both pre-TCR and $\gamma\delta$ -TCR mature along the $\gamma\delta$ -lineage into functionally competent cells that produce IFN- γ in response to stimulation. However, in the absence of Id3 expression and strong $\gamma\delta$ -TCR ligand, $\gamma\delta$ -expressing cells show a lower propensity to differentiate along the $\gamma\delta$ -lineage, but when expressing both pre- and $\gamma\delta$ -TCRs, these cells showed increased $\gamma\delta$ -lineage differentiation and recover functional competence to produce IFN- γ , indicating that the pre-TCR can serve to enforce to a $\gamma\delta$ -lineage choice in the case of weaker $\gamma\delta$ -TCR signals. Taken together, these findings further cement the view that the cumulative signal strength sensed by developing DN cells dictates its lineage choice.

Results

DN3 Cells Simultaneously Expressing Productive TCR- β and $\gamma\delta$ -TCR Chains Develop and Mature Along the $\gamma\delta$ -Lineage. Previously, we found that TCR- β - and $\gamma\delta$ -TCR-expressing DN3 cells have a high propensity to develop along their respective lineages (1). Furthermore, the pre-TCR and $\gamma\delta$ -TCR produced quantitatively different signals, which was responsible for directing lineage-fate outcomes (7, 8). Considering these results, we sought to determine the lineage choice of a DN3 cell that simultaneously expresses a pre-TCR (TCR- β) and a $\gamma\delta$ -TCR (KN6-TCR) (1). Specifically, we wanted to address whether strong signals transmitted from a $\gamma\delta$ -TCR would be sufficient to override developmental signals emitted from a pre-TCR. To test this possibility, we ectopically expressed TCR- β , $\gamma\delta$ -TCR, or both in $Rag2^{-/-}$ DN3 cells and assessed the role of each TCR in determining lineage fate in the presence of Notch signals by culturing them on OP9-DL cells (25). As reported previously (1), pre-TCR- (TCR- β -transduced)

expressing $Rag2$ -deficient DN3 cells proliferated robustly, developed along the $\alpha\beta$ -lineage to the DP stage, and maintained high expression of CD24 (Fig. 1). In contrast, $\gamma\delta$ -TCR-expressing DN3 cells showed lower cellular expansion, developed along the $\gamma\delta$ -lineage, and matured into DN CD24^{lo} $\gamma\delta$ cells, with only a very small proportion of cells differentiating along the $\alpha\beta$ -lineage to become DP cells (Fig. 1D). Of note, $Rag2^{-/-}$ DN3 cells that did not bear any TCRs (MigR1/MIY-transduced), did not proliferate, and remained DN and CD24^{hi}, indicating their inability to progress along either lineage (Fig. 1A–C).

Strikingly, DN3 cells simultaneously expressing both TCR- β and $\gamma\delta$ -TCR followed a developmental path much like $\gamma\delta$ -TCR-only cells, in that they progressed to become DN CD24^{lo} $\gamma\delta$ cells, with only a very small proportion of cells differentiating to become DP cells (Fig. 1A and B). Interestingly, TCR- β expression with $\gamma\delta$ -TCR slightly increased the level of CD24 down-regulation compared with $\gamma\delta$ -TCR alone (Fig. 1B). Of note, TCR- $\beta/\gamma\delta$ -expressing DN3/OP9-DL cocultures showed higher cellularity compared with their $\gamma\delta$ -TCR-only-expressing counterparts, but still much lower than that of TCR- β -only/OP9-DL cocultures (Fig. 1C). Additionally, to rule out any effects as a result of a potential limiting availability of shared CD3 complex subunits, we found that TCR- $\beta/\gamma\delta$ -expressing cells had similar levels of $\gamma\delta$ -TCR cell-surface expression and TCR- β expression as $\gamma\delta$ -TCR-expressing cells and TCR- β -expressing cells, respectively, suggesting that expression of both pre-TCR and $\gamma\delta$ -TCR does not result in lower $\gamma\delta$ -TCR or pre-TCR expression (Fig. S1A–C).

We previously showed a differential requirement for Notch signaling between DN3 cells expressing the pre-TCR and committing to the $\alpha\beta$ -lineage, and those expressing a $\gamma\delta$ -TCR and differentiating along the $\gamma\delta$ -lineage (1). Considering this finding, we addressed the role of Notch signals in the differentiation of DN3 cells simultaneously expressing both TCR- β and $\gamma\delta$ -TCR by culturing them without Notch signals on OP9-Ctrl cells. As expected, $Rag2$ -deficient cells that did not express a TCR, or those expressing a pre-TCR, were unable to develop (17). These cells remained DN and apoptosed in the absence of the trophic signals provided by Notch signaling (26), as indicated by the complete loss of cellularity in these cultures (Fig. 1A and C). Conversely, both $\gamma\delta$ -TCR- and TCR- $\beta/\gamma\delta$ -expressing $Rag2^{-/-}$ DN3 cells were able to differentiate along the $\gamma\delta$ -lineage, remained DN, and matured to become CD24^{lo}, albeit with reduced cell numbers compared with the same cells cultured with Notch signals (Fig. 1C), in agreement with previous findings (1).

Fig. 1. $\gamma\delta$ -TCR-expressing DN3 cells predominantly differentiate along the $\gamma\delta$ -lineage, irrespective of TCR- β coexpression and the availability of Notch signals. (A and B) Developmental progression of in vitro-derived $Rag2^{-/-}$ DN3 cells retrovirally transduced to express TCR- β , $\gamma\delta$ -TCR, neither, or both, and cultured for 6 d with OP9-DL or OP9-Ctrl cells. Flow cytometric analysis of cell surface expression for CD4 and CD8 (A), and CD24 along with forward size scatter (FSC) (B) are shown for GFP⁺ YFP⁺-gated cells; C shows the corresponding fold-expansion in cellularity. Fold-expansion was obtained from the total cellularity divided by the number of cells used at the start of the culture (input). (D) Bar graph showing percentage of DP from the indicated cocultures in relation to TCR- β /YFP-transduced cells set at 100. Data are derived from at least three independent experiments. GFP(G), MigR1-transduced; YFP(Y), MIY-transduced.

$\gamma\delta$ -TCR and Id3 Signal to Induce Maturation Along the $\gamma\delta$ -Lineage. Id3 is an important molecular effector of the strong signals that dictate $\gamma\delta$ -fate choice and maturation, but it is dispensable for adoption of the $\alpha\beta$ -fate (8). Considering this finding, we sought to determine whether the loss of Id3 in TCR- $\beta/\gamma\delta$ -expressing DN3 cells would affect lineage choice. As reported previously (8), differentiation of pre-TCR-expressing DN3 cells was not impaired by Id3-deficiency, and these cells were able to proliferate and develop to the DP stage (Fig. S2A). Notably, the maturation potential of $\gamma\delta$ -TCR-only DN3 cells into IFN- γ -producing $\gamma\delta$ -T cells was severely affected in the absence of Id3, but a significant rescue in IFN- γ production was seen in TCR- $\beta/\gamma\delta$ -expressing cells (Fig. 2). Furthermore, in the absence of Notch signals, $\gamma\delta$ -TCR-expressing $Rag2^{-/-}$ DN3 cells showed no functionality in terms of IFN- γ production. However, and as seen in the presence of Notch signals, TCR- $\beta/\gamma\delta$ -expressing $Rag2^{-/-}$ DN3 cells showed a rescued in their ability to produce IFN- γ (Fig. 2).

The CD24^{hi} DN phenotype is shared between preselection DN3 thymocytes and cells that have committed but not yet matured along the IFN- γ -producing $\gamma\delta$ T-cell lineage (1, 8). Considering this ambiguity associated with using the CD24 marker, we sought to verify the identity of CD24^{hi} and CD24^{lo} DN cells present in the cocultures (Fig. 1B) by determining their gene-expression profiles. Interestingly, $\gamma\delta$ -TCR- and TCR- $\beta/\gamma\delta$ -expressing $Rag2^{-/-}$ DN3 cells expressed similar or greater transcript levels of $\gamma\delta$ -biased genes *Crem*, *Nurr1*, and *Rgs1* compared with their $Rag2^{-/-}$

Fig. 2. Loss of Id3 inhibits the maturation of $\gamma\delta$ -TCR-expressing T cells as IFN- γ -producers. Sorted CD45⁺ GFP⁺ cells were obtained from in vitro-derived $Rag2^{-/-}$ DN3 or $Rag2^{-/-}Id3^{-/-}$ DN3 cells retrovirally transduced to express TCR- β , $\gamma\delta$ -TCR, neither, or both, and cultured for 6 d with OP9-DL or OP9-Ctrl cells (as indicated) and were stimulated for 36 h (P+I, PMA+Iono) or placed in culture without stimulation (Unstim). Levels of IFN- γ in culture supernatants were quantified by an antibody-capture ELISA. Data are derived from at least three independent experiments. * $P \leq 0.05$; ** $P \leq 0.01$.

DN3 counterparts (Fig. S2B). This finding did not seem to be a consequence of increased *Id2* expression in Id3-deficient cells. However, despite retention of $\gamma\delta$ -identity in the absence of Id3, $Rag2^{-/-}Id3^{-/-}$ DN3 cells showed a greater induction of germ-line *Tcra* transcripts in the presence of Notch signals (Fig. S2).

$\gamma\delta$ -TCR Ligand Strength Affects the Maturation Status of $\gamma\delta$ -TCR-Bearing $Rag2^{-/-}$ DN3 Cells. Results from our previous work (8) and Fig. 2 highlight the necessity for Id3 in inducing strong signals downstream of $\gamma\delta$ -TCR and mediating maturation along the $\gamma\delta$ -lineage. However, the role of ligand involvement in mediating strong signals remains controversial, particularly in light of recent evidence supporting a ligand-independent development of IL-17-producing $\gamma\delta$ -T cells (27). In light of this, we sought to further examine the mechanistic basis behind how weak and strong signaling is sensed, by addressing whether ligand engagement plays a necessary role for the induction of a signal strength differential between pre-TCR and $\gamma\delta$ -TCR. In H2^b mice (or in H2^k OP9 cells), the KN6 $\gamma\delta$ -TCR recognizes the nonclassical MHC class I molecule T22, in association with β 2-microglobulin. In H2^d mice, the T22^d gene is defective (28), thus another β 2M-associated molecule, T10 (also known as T9), which is closely related to the T22 gene, interacts weakly with KN6 TCR in these mice. T10^d and T22^b proteins differ by four residues in the a1I and a2 domains (28), and whereas T10 is able to mediate positive selection of KN6 cells, it is characterized as a “weak” ligand that fails to induce negative selection and fails to activate mature, peripheral KN6 TCR-Tg cells (28–31). Considering this finding, we generated BALB/c (H2^d) stromal cell lines expressing the weak KN6 $\gamma\delta$ -TCR ligand to determine whether it could affect $\alpha\beta$ - versus $\gamma\delta$ -lineage fate decisions.

H2^d stromal cells from BALB/c (B/c) mice, with or without ectopic Dll4 expression (32), B/c-DL, or B/c-Ctrl, respectively, were used for cocultures with $Rag2^{-/-}$ DN3 cells transduced to express a pre-TCR, $\gamma\delta$ -TCR, or both. As expected, the differentiation of pre-TCR- (TCR- β -transduced) expressing DN3 cells was not impaired on B/c-DL cells, as cells proliferated robustly and developed to the DP stage in a Notch ligand-dependent fashion (Fig. 3A and Fig. S3A and B). Interestingly, absence of T22^b on B/c-DL cells reduced the ability of $\gamma\delta$ -TCR-only DN3 cells to develop along the $\gamma\delta$ -lineage, as a greater percentage of $\gamma\delta$ -TCR-only DN3 cells developed into $\alpha\beta$ -lineage DP cells, compared with cocultures with B/c-DL cells ectopically expressing T22^b (B/c-DL^{+T22}) (Fig. S3C) in which a lower frequency of DP cells was observed (Fig. 3A). Additionally, provision of both TCRs led to a lower percentage of DP cells in B/c-DL4 cocultures compared with $\gamma\delta$ -TCR-only DN3 cells, suggesting that in the presence of the weak $\gamma\delta$ -TCR ligand, T10^d, additional signals derived from the pre-TCR appear to enforce a $\gamma\delta$ -lineage outcome. Moreover, the generation of DP cells was further decreased by TCR- β / $\gamma\delta$ -expressing cells in the presence of T22^b (Fig. 3A). Thus, by increasing the TCR-ligand affinity from the endogenous T10^d to T22^b, the propensity to yield DP cells is reduced by $\gamma\delta$ -transduced cells, and this is further reduced when coexpressing a pre-TCR.

Not only did we observe a reduction toward the $\alpha\beta$ -lineage outcome, but equally important, we noted an increase in CD73⁺

($\gamma\delta$ -lineage) cells (33, 34) when B/c-DL^{+T22} cells were used for the cocultures, and this was further increased with the addition of TCR- β (Fig. 3B). Of note, TCR- β / $\gamma\delta$ -expressing DN3 cells differentiated along the $\gamma\delta$ -lineage with similar frequency and cellularity, regardless of whether they were cultured on B/c-DL or OP9-DL cells (Fig. S4). Nevertheless, TCR- β / $\gamma\delta$ -expressing cells, in comparison with $\gamma\delta$ -TCR-only cells, showed higher levels of CD73 expression, and a trend to lower levels of CD24 expression, in B/c-DL cocultures (Fig. S4B). However, on B/c-Ctrl cells, $\gamma\delta$ -TCR-expressing cells behaved like their vector-transduced counterparts in that they remained as DN3 and failed to thrive in the absence of Notch signals (Fig. 3 and Figs. S3 and S4).

To further elucidate whether the CD24^{hi} CD73⁺ CD4⁺CD8⁺ population, which make up the majority of cells within $\gamma\delta$ -TCR-expressing $Rag2^{-/-}$ DN3 B/c-DL cocultures, are progressing toward the $\gamma\delta$ -lineage, we assessed for the expression of $\gamma\delta$ -lineage-biased genes. Surprisingly, provision of weak ligand (T10^d) did not prevent $\gamma\delta$ -TCR-transduced DN3 cells from inducing expression of *Crem*, *Nurr1*, and *Rgs1*, nor did it lead to increased TCR- α germ-line transcription (Fig. S4D).

Fig. 3. Provision of T22^b expression and TCR β enhances the development of $\gamma\delta$ -TCR-expressing DN3 cells toward the $\gamma\delta$ -lineage. (A) Development of in vitro-derived $Rag2^{-/-}$ DN3 cells retrovirally transduced to express TCR- β , $\gamma\delta$ -TCR, or both, and cultured for 5.5 d with B/c-DL cells expressing T22^b or MCherry alone. Flow cytometric analysis of cell surface expression for CD4 and CD8 (A) and CD73 and FSC (B). Data are derived from at least two independent experiments.

To directly determine lineage outcomes in TCR- $\beta/\gamma\delta$ -expressing cells, we performed single-cell and limiting dilution analyses of *Rag2*^{-/-} DN3 cells transduced to express TCR- β or $\gamma\delta$ -TCR, cultured on OP9-DL or B/c-DL stromal cells, and measured their $\alpha\beta$ - or $\gamma\delta$ -lineage precursor frequency (1). Table 1 shows that both stromal cells supported a near-perfect capacity to yield DP cells from TCR- β -expressing cells, with frequencies approaching unity. Importantly, the KN6 $\gamma\delta$ -TCR-expressing cells showed a low DP precursor frequency as expected (1), but when cultured on the weaker TCR-ligand expressing cells (B/c-DL), this frequency was more than doubled (from 1/116–1/48). A more striking change was seen with TCR- $\beta/\gamma\delta$ -expressing cells, which showed a dramatically lower frequency in generating DP cells, with a fivefold reduction seen on B/c-DL cells (1/230; $P < 0.05$). A similar effect, but in reverse, was seen in $\gamma\delta$ -lineage outcomes, with a twofold increase in TCR- $\beta/\gamma\delta$ -expressing cells compared with $\gamma\delta$ -only cells cultured on B/c-DL cells (Table 1).

Taken together, these data suggest that a weak ligand leads to an increase in differentiation of $\gamma\delta$ -TCR-only DN3 cells into DP cells, but the diversion to the $\alpha\beta$ fate is incomplete, because these cells continue to express $\gamma\delta$ -lineage-biased genes. Furthermore, coexpression of TCR- β with $\gamma\delta$ -TCR in *Rag2*^{-/-} DN3 cells cultured on B/c-DL reduces their ability to adopt the $\alpha\beta$ -lineage fate, and increases the propensity of these cells to develop along the $\gamma\delta$ -lineage, rather than hindering it.

$\gamma\delta$ -TCR Ligand Strength Affects the Maturation Status of $\gamma\delta$ -TCR-Bearing *Rag2*^{-/-} DN3 Cells. We have already observed that, in the presence of Notch signaling, Id3-deficiency decreased the percentage of $\gamma\delta$ -TCR-only DN3 cells that matured along the $\gamma\delta$ -lineage (8), and this effect was reduced in TCR- $\beta/\gamma\delta$ -expressing cells (Fig. 2). As expected, in the absence of Id3 and strong $\gamma\delta$ -TCR ligand, differentiation of pre-TCR-expressing DN3 cells progressed to the DP stage (Fig. 4A and C). Interestingly, in the presence of weak ligand (B/c-DL), $\gamma\delta$ -TCR-expressing Id3-deficient DN3 cells did not further divert their lineage choice to become $\alpha\beta$ -lineage DP cells (Fig. 4A). However, maturation was more severely impaired by the combined loss of both Id3 and strong ligand, as the already decreased CD24 down-regulation seen with Id3-deficiency was even more pronounced when combined with the weak $\gamma\delta$ -TCR ligand expressed on B/c-DL cells and CD73 induction was completely abrogated (Fig. 4B). $\gamma\delta$ -TCR-expressing *Rag2*^{-/-} DN3 cells behaved much like their vector-transduced counterparts in that they remained DN, did not down-regulate CD24, up-regulate CD73, or survive in the absence of Notch signaling (Fig. 4C). This finding supports the notion that Notch is required for the differentiation of $\gamma\delta$ -TCR-expressing DN3 cells in the absence of Id3 and a strong $\gamma\delta$ -TCR ligand.

Table 1. Limiting dilution analysis of precursor potential of TCR-transduced DN3 cells

	$\alpha\beta$ -Lineage*		$\gamma\delta$ -Lineage*	
TCR/stromal cell	1/freq	(95% C.L.)	1/freq	(95% C.L.)
TCR- β /OP9-DL4	1.05	(0.73–1.52)	Nil	N/A
TCR- β /B/c-DL4	1.02	(0.72–1.46)	Nil	N/A
TCR- $\gamma\delta$ /OP9-DL4	116.23	(49.97–270.39)	1.35	(0.95–1.91)
TCR- $\gamma\delta$ /B/c-DL4	47.56	(26.81–84.35)	3.38	(2.40–4.77)
TCR(β + $\gamma\delta$)/OP9-DL4	353.00	(84.36–1,477.09)	1.39	(0.98–1.97)
TCR(β + $\gamma\delta$)/B/c-DL4	233.99	(72.38–756.47)	1.67	(1.18–2.36)

* $\alpha\beta$ -Lineage outcome was determined by the detection of CD4 and CD8 expressing cells, with >50 cells per well analyzed. $\gamma\delta$ -Lineage outcome was determined by the presence of CD4 and CD8 DN cells that expressed high levels of CD73, with >30 cells per well analyzed. C.L., confidence limits for $P \leq 0.05$; N/A, not applicable. Limiting dilution analyses coculture conditions and TCR used for transductions of *Rag2*^{-/-} DN3 cells are indicated in *SI Methods*. Numbers in bold represent frequencies of respective lineage outcomes for KN6-transduced DN3s exposed to weak ligand in the absence and presence of TCR- β , respectively.

Fig. 4. Provision of a weak $\gamma\delta$ -TCR ligand with loss of Id3 does not promote $\alpha\beta$ -lineage choice in $\gamma\delta$ -TCR-expressing DN3 cells, both in the presence and absence of Notch signals. (A and B) Development of culture-derived *Rag2*^{-/-}*Id3*^{-/-} DN3 cells retrovirally transduced to express TCR- β , $\gamma\delta$ -TCR, neither, or both, and cultured for 6 d with OP9-DL4 and B/c-DL4. Flow cytometric analysis of cell-surface expression for CD4 and CD8 (A), and CD24 and CD73 (B) are shown for GFP⁺ YFP⁺-gated cells; C shows the corresponding fold expansion in cellularity. (D) Quantitative RT-PCR analysis of $\gamma\delta$ -biased genes: *Crem*, *Nurr1*, and *Rgs1*, and *Trac*, in transduced *Rag2*^{-/-}*Id3*^{-/-} DN3 cells cultured for 6 d on the indicated cells, with mRNA levels normalized to β -actin. β , $\gamma\delta$, and $\beta/\gamma\delta$ represent TCR- β /Miy-, MigR1/TCR- $\gamma\delta$ -, and TCR- β /TCR- $\gamma\delta$ -transduced DN3 cells, respectively. Data are derived from at least three independent experiments. ** $P \leq 0.01$.

To further characterize the identity of CD24^{hi} CD73⁻ CD4⁺CD8⁻ cells within the $\gamma\delta$ -TCR-expressing *Rag2*^{-/-}*Id3*^{-/-} DN3 B/c-DL cocultures, we assessed cells for the expression of $\gamma\delta$ -lineage-biased genes. Notably, we found that the provision of weak signal in combination with Id3-deficiency failed to induce the expression of *Nurr1*, and *Rgs1* in $\gamma\delta$ -TCR-only transduced DN3 cells, but did so in TCR- $\beta/\gamma\delta$ -transduced cells, suggesting that they had received sufficient signals to induce the expression of these $\gamma\delta$ -lineage-biased genes. Nevertheless, *Crem* expression was similarly induced in cultures containing a strong or weak ligand, and *Trac* expression remained low under both conditions.

Taken together, these data suggest that decreasing TCR signal strength by combining low-affinity ligand with Id3-deficiency does partially divert $\gamma\delta$ -TCR-expressing DN3 cells away from a $\gamma\delta$ -lineage fate; however, coexpression of the $\gamma\delta$ -TCR with the pre-TCR signals restores adoption of the $\gamma\delta$ -lineage fate.

Discussion

We have addressed the question of how TCR signals influence DN3 thymocytes to adopt the $\alpha\beta$ - or $\gamma\delta$ -lineage fate. Specifically, we investigated whether weak $\gamma\delta$ -TCR signals can be transformed into effective inducers of $\gamma\delta$ -fate specification when combined with pre-TCR signals, which are normally associated with adoption of the $\alpha\beta$ fate. Here, we find that DN3 cells that

simultaneously express a TCR- β and $\gamma\delta$ -TCR choose a $\gamma\delta$ T-cell fate, in agreement with the principles of the signal strength model. We also confirm the importance of Id3 for the maturation along the IFN- γ -producing $\gamma\delta$ -lineage (8).

Previously, Pereira and colleagues found that TCR- $\beta/\gamma\delta$ transgenic mice generated similar numbers of $\alpha\beta$ cells as those transgenic for TCR- β only, and similar numbers of $\gamma\delta$ cells as those transgenic for $\gamma\delta$ -TCR only (22). However, the timing of TCR expression in the thymocytes of these transgenic mice was premature, and the $V\gamma$ transgene included the flanking DNA sequences encoding a putative silencer element, both of which could affect development along the $\gamma\delta$ -lineage (35). To overcome these issues, we used an in vitro T-cell differentiation system that allows us to temporally regulate TCR expression precisely, as well as a TCR- γ gene used that was cloned without flanking sequences. Finally, we used *Rag2*^{-/-} DN3 cells to prevent the expression of TCR variants before, with, or instead of, the rearranged TCRs provided by retroviral transduction.

Here, we find that $\alpha\beta$ - versus $\gamma\delta$ -lineage choice in $\gamma\delta$ -TCR-expressing DN3 cells is independent of TCR- β coexpression, in agreement with the findings published by Pereira's group (22). However, unlike this previous study, we find that the majority of $\gamma\delta$ -TCR-expressing DN3 cells, in the presence of a strong ligand, develop along the $\gamma\delta$ T-cell lineage and remain DN, instead of along the $\alpha\beta$ -lineage to gain expression of CD4 and CD8. This difference is an important distinction, as it affects how the results are interpreted. The finding that the majority of $\gamma\delta$ -TCR-expressing DN3 cells differentiate along the $\gamma\delta$ -lineage is in agreement with previously published results (1, 11), and reflects the idea that strong signals, such as those transmitted from the $\gamma\delta$ -TCR in the presence of ligand, promote the $\gamma\delta$ -lineage fate and oppose an $\alpha\beta$ choice.

In the context of the signal-strength model for $\alpha\beta$ - versus $\gamma\delta$ -lineage bifurcation, and in contrast to the instructional model, one would predict that coexpression of a TCR- β in $\gamma\delta$ -TCR-expressing DN3 cells would not impact lineage choice. In principle, the addition of TCR- β could only increase the total strength of TCR signal received by the DN3 cell at the bifurcation point, and drive lineage choice to the $\gamma\delta$ -fate. In support of this notion, we find that TCR- β coexpression with $\gamma\delta$ -TCR increases CD24 down-regulation and CD73 up-regulation.

Importantly, differentiation is not the only consequence of TCR-selection at the DN3 developmental checkpoint. Another critical outcome is proliferation, and this could, in theory, be differentially regulated in TCR- β , $\gamma\delta$ -TCR, and TCR- $\beta/\gamma\delta$ -expressing DN3 cells. Here, we find that in certain situations, such as in the presence of a weak ligand, TCR- $\beta/\gamma\delta$ -expressing *Rag2*^{-/-} DN3 cells proliferate more extensively than their $\gamma\delta$ -TCR-only-expressing counterparts. This increase in proliferation may indicate that, although the TCR- β chain cannot drive differentiation along the $\alpha\beta$ -lineage when coexpressed with a $\gamma\delta$ -TCR, it can promote some $\alpha\beta$ -like proliferation, suggesting that survival, differentiation, and proliferation can be separated and distinctly driven by different TCRs (36).

Recent insights into the molecular basis for $\alpha\beta$ - versus $\gamma\delta$ -lineage choice have culminated in support of a signal strength model to dictate lineage fate. However, it remains unclear whether differential signal initiation mechanisms lead to changes in quantitative strength. It is now widely accepted that the pre-TCR is capable of cell-autonomous signaling (37–39), which combined with its low expression levels suggests that it emits either weak or transient signals. Although ligands have been characterized for some $\gamma\delta$ -TCR subsets (40, 41), the role of ligand engagement for $\gamma\delta$ T-cell development is much less clear. Removal of $\gamma\delta$ -TCR ligand reduces transcription of *Egr1* and *Egr3*, impairs $\gamma\delta$ -lineage development and maturation, and simultaneously promotes $\alpha\beta$ -lineage commitment to the DP stage (7). However, recent evidence suggests that IL-17-producing $\gamma\delta$ -T cells may develop independently of ligand availability, but still induce of ERK1 and ERK2 activation through their TCR

(27). Nevertheless, ligand-independent $\gamma\delta$ -TCR-derived signals would appear to be stronger than those from the pre-TCR to drive $\gamma\delta$ -lineage commitment.

Here, we attempt to clarify the role of $\gamma\delta$ -TCR ligand at the $\alpha\beta$ - versus $\gamma\delta$ -bifurcation point by providing KN6 $\gamma\delta$ -TCR-expressing T cells with a weak or strong ligand, via culture with BALB/c stromal cells expressing endogenous T10^d or ectopic T22^b, respectively. Our results show that provision of weak $\gamma\delta$ -TCR ligand does not lead $\gamma\delta$ -TCR-expressing DN3 cells to fully abandon a $\gamma\delta$ -fate and develop as $\alpha\beta$ -lineage DP cells, but appears to affect the ability of $\gamma\delta$ -TCR-bearing cells to mature into the CD24^{lo} CD73⁺ stage. Nevertheless, provision of a strong ligand led to enhanced CD73 expression and a decreased appearance of DP cells. Weak ligand may be sufficient to drive differentiation into the $\gamma\delta$ -lineage as retroviral transduction of $\gamma\delta$ -TCR leads to high levels of $\gamma\delta$ -TCR expression on DN3 cells, and weak signaling through each individual $\gamma\delta$ -TCR may additively equate to a sufficiently strong signal received by the cell that is capable of inducing $\gamma\delta$ -lineage commitment and expression of $\gamma\delta$ -biased genes. Because a fair proportion of $\gamma\delta$ cells express the pre-TCR, it might be of interest to speculate as to the character of their receptors; perhaps these are TCRs transducing weak signals that need the pre-TCR to enforce the $\gamma\delta$ fate. Thus, low affinity $\gamma\delta$ -TCR or those that do not encounter ligand may develop into $\gamma\delta$ -T cells because of the presence of a pre-TCR, which would add to the overall signal strength.

It remains to be determined whether differences in CD24 and CD73 expression can accurately reflect the maturation status of a $\gamma\delta$ -T cell, or mark the bifurcation between cells committing to the $\alpha\beta$ - versus $\gamma\delta$ -lineage (33). Expression of $\gamma\delta$ -biased genes in CD24^{hi} DN $\gamma\delta$ -TCR⁺ *Rag2*^{-/-} *Id3*^{-/-} DN3 cells, or DN3 cells from the B/c-DL cocultures, suggests that these cells have indeed chosen the $\gamma\delta$ -lineage. Thus, CD24 expression may not be a reliable marker of lineage commitment, unlike CD4 and CD8. CD24^{hi} CD4⁺ CD8⁺ reliably marks $\alpha\beta$ -lineage DP cells, but to clearly define the lineage of CD24^{hi} CD4⁺ CD8⁺ and CD24^{lo} CD4⁺ CD8⁺ cells requires further analysis of their gene-expression profile.

TCR-selection at the DN3 stage can be Notch-dependent or -independent, and this decision is based on the final lineage choice, which can be based on the strength of TCR signals, as measured by Id3 induction (8). In this case, strong TCR signals that induce high levels of Id3 are able to suppress E protein activity beyond the threshold required for passage across $\gamma\delta$ -selection, independently of Notch signaling. Conversely, the weak signals that emanate from a pre-TCR require concurrent Notch signals to achieve down-regulation of E2A to levels that allow for successful traversal of the β -selection checkpoint. No DP cells appear in $\gamma\delta$ -TCR-expressing *Rag2*^{-/-} DN3 cocultures without Notch signaling, even in the presence of pre-TCR expression. Interestingly, we also find that provision of a weak $\gamma\delta$ -TCR ligand alters the requirement for Notch signaling in $\gamma\delta$ -TCR-expressing cells. Here, $\gamma\delta$ -TCR-expressing DN3 cells can no longer survive, differentiate, and proliferate in the absence of Notch signaling (B/c-Ctrl cells), in support of the notion that Notch-independence at this stage of development requires strong signals from the TCR.

Taken together, these data confirm previous reports that the strength of TCR signal dictates lineage choice. More importantly, these data provide novel insights into the lineage decisions of a DN3 cell that expresses both a TCR- β and $\gamma\delta$ -TCR. In this case, TCR- β coexpression with $\gamma\delta$ -TCR appears to provide additive TCR signals, and further promote $\gamma\delta$ -lineage selection, maturation, and function. Further work in this area may be needed to address the differential ligand, differential maturation (as determined by CD24 and CD73), and differential TCR signal strength requirements for the functional maturation of $\gamma\delta$ T cells.

Methods

Mice, retroviral transductions of *Rag2*^{-/-} DN3 cells, cocultures of hematopoietic stem cells on OP9-DL1 cells, quantitative real-time PCR for mRNA

analysis, generation of BALB/c stromal cell lines, T-cell stimulation assays, and statistical analysis are described in *SI Methods*. For flow cytometry, all single-cell suspensions were stained with commercially available antibodies (BD Biosciences and e-Biosciences) and analyzed with a BD-LSRII flow cytometer, using Flowjo software (Treestar). Dead cells were excluded from the analyses using DAPI gating.

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