



Published in final edited form as:

*Semin Immunol.* 2010 August ; 22(4): 222–227. doi:10.1016/j.smim.2010.04.003.

## Disorderly conduct in $\gamma\delta$ versus $\alpha\beta$ T cell lineage commitment

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### Abstract

The mechanism of T cell precursor commitment to the  $\gamma\delta$  or  $\alpha\beta$  T cell lineage remains unclear. While TCR signal strength has emerged as a key factor in lineage commitment based on TCR transgenic models, the entire TCR repertoire may not possess the same discriminatory power. A counterbalance to the TCR as the lineage determinant is the pre-existing heterogeneity in gene expression among precursors, which suggests that single precursors are unlikely to respond homogeneously to a given instructive signal.

### Keywords

T cell development; T cell lineage commitment; T cell receptor; gammadelta T cells; WNT signaling

## 1. Introduction

Binary cell fate decision processes underpin the production of functionally distinct cell lineages of the mammalian immune system. For precursors committed to the T cell lineage, the earliest cell fate decision generates two types of T cells distinguished by the expression of either an  $\alpha\beta$  T cell receptor (TCR) or a  $\gamma\delta$  TCR on their cell surface. The resolution of the cell fate decision process has an indisputable order, leading to eventual commitment to either the  $\gamma\delta$  or  $\alpha\beta$  T cell lineage. However, whether the process itself is orderly, following a linear progression with defined checkpoints, or disorderly, with initial stochastic variations in gene expression and plasticity in precursors that attain lineage specificity as a function of a developmental clock, is poorly understood.  $\gamma\delta$  T cells are prototypic innate-like lymphocytes that share some phenotypic and functional similarities with NK and NKT cells. They are found predominantly in mucosal interfaces with the outside world, such as the skin, gut, and reproductive tract. How  $\gamma\delta$  T cells are generated in the thymus, and how they acquire the unique homing and functional capacities that set them apart from the conventional adaptive T cells expressing  $\alpha\beta$ TCRs remains a challenging question.

## 2. Two models of lineage commitment

$\alpha\beta$  and  $\gamma\delta$  T cells are derived from T cell lineage-committed double-negative 2 (DN2) precursors ( $CD3^-CD4^-CD8^-CD25^+CD44^+c-kit^+$ ). TCR gene rearrangements are initiated

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during the DN2 to DN3 ( $CD3^-CD4^-CD8^-CD25^+CD44^-$ ) transition [1,2] and productively rearranged *Tcrp* and *Tcrd* genes are necessary for  $\gamma\delta$  T cell differentiation, whereas successful *Tcrb* gene rearrangement alone is sufficient for DN3 cells to develop into immature  $\alpha\beta$  lineage  $CD4^+CD8^+$  double positive (DP) thymocytes. Functional TCR $\beta$  chains pair with the invariant preTCR $\alpha$  (pT $\alpha$ ) chain to form the preTCR complex. Over the years, two evolving models have occupied the field of  $\gamma\delta/\alpha\beta$  lineage commitment, centered on the possible role of the TCR as an on/off switch in the lineage decision process. The TCR instructive model proposes that the type of TCR, by virtue of different strengths of signaling, specifies the fate of bipotential precursor cells: relatively strong TCR signaling in DN2 and DN3 thymocytes specifies  $\gamma\delta$  T cell lineage fate, while signaling of lower intensity or duration wires precursors to become DP thymocytes [3,4]. In contrast, the stochastic or TCR permissive/selective model proposes that a functional TCR simply permits the maturation of precursor cells that are already lineage-biased [5,6]. The stochastic model also predicts that DN2 cells are not a uniform population that experiences quantitative variations in TCR signals, but rather a heterogeneous mixture of sub-populations marked by variations in gene expression that, once reinforced and fixed, become lineage-restricted [7]. In this scenario, while either preTCR or  $\gamma\delta$ TCR expression is sufficient to permit the continued differentiation of DN precursors,  $\alpha\beta$  lineage precursors expressing a mismatched functional  $\gamma\delta$ TCR will not mature since *Tcrp* gene transcription is silenced in DP thymocytes [8]. Conversely,  $\gamma\delta$  lineage precursors expressing a mismatched preTCR will not develop further since termination of *Rag1/2* expression in TCR-signaled cells will prevent functional TCR $\alpha$  chain production [9–11], and the absence of a  $\gamma\delta$ TCR or  $\alpha\beta$ TCR will lead to the death of thymocytes.

Recently, direct probes of TCR signaling and of the molecular heterogeneity of early T cell precursors have yielded potential deterministic factors of T cell lineage commitment. This review will present a case against an instructive role for the TCR in  $\gamma\delta/\alpha\beta$  T cell lineage commitment and summarize evidence favoring the stochastic model.

### 3. Potential issues with the signal strength model

The recent conceptual framework of the instructive model consists of documented differences in signaling by the preTCR and  $\gamma\delta$ TCR [12], and the precedent that other T cell lineage and subset differentiations are thought to be controlled by TCR signal strength or duration [13, 14]. Convincing experimental support for the quantitative instructive model of  $\gamma\delta/\alpha\beta$  T cell lineage commitment was provided by the laboratories of Love [3] and Wiest [4] using  $\gamma\delta$ TCR transgenic (Tg) mice with altered signaling capacity. As detailed by others in this issue, in essence, decreasing the overall signal strength transmitted in T cells of two  $\gamma\delta$ TCR Tg mouse lines consistently generated more  $CD4^+CD8^+$   $\alpha\beta$  lineage DP thymocytes, whereas increasing the TCR signaling capacity led to fewer DP cells (and sometimes more  $\gamma\delta$  T cells). Using KN6  $\gamma\delta$ TCR Tg mice, which recognize the  $\beta 2m$ -dependent non-classical MHC class Ib molecule T22, it was shown that decreasing the signal strength through restriction of ligand availability resulted in the generation of  $\alpha\beta$  lineage DP cells [4]. However, in an alternate transgenic model of  $\gamma\delta$ TCR interaction with T22 (G8) the ligand is not essential for the production of mature ( $CD24^{lo}$ ) Tg $^+$  thymocytes; rather it reduces the number of Tg $^+$  thymocytes by negative selection [15]. Further, more recent analysis in non-transgenic mice has shown that  $\gamma\delta$  T cells reactive to T22 and T10 (which is highly homologous to T22) do arise in *B2m* $^{-/-}$  mice, which lack the ligands, and they remain  $CD4^-CD8^-$  with no down-regulation of the TCR, and show no skewing toward the  $\alpha\beta$  lineage [16]. Hence, these discordant results indicate the existence of clonal variations in the requirement for  $\gamma\delta$ TCR signaling, and that the modulatory effects associated with select  $\gamma\delta$ TCRs may not be experienced by most developing  $\gamma\delta$  cells. Finally, while these data show that signal strength can be an important parameter of cell lineage determination in TCR Tg mice, these studies still cannot distinguish between altering the initial

lineage choice and confirming a lineage choice made previously through a stochastic mechanism.

A non-TCR Tg approach to assess the importance of TCR in T cell lineage commitment involves differentiation of precursors on OP9 stromal cells expressing the Notch ligand Delta-like-1 (OP9-DL1) in which TCR signaling can be manipulated. On OP9-DL1 cells, single  $\alpha\beta$  lineage cells that have traversed through the  $\beta$  selection checkpoint by virtue of preTCR expression, known as DN3b cells, do not give rise to DP cells if signaled through the CD3 complex [17]. Instead, nearly all cells develop into CD4<sup>-</sup>CD8<sup>-</sup> thymocytes, a portion of which express the  $\alpha\beta$ TCR (or in a minority of cells, the  $\gamma\delta$ TCR). Similar DN  $\alpha\beta$ TCR<sup>+</sup> thymocytes are especially prevalent in TCR $\alpha\beta$  transgenic mice and have been shown to belong to the  $\gamma\delta$  T cell lineage [18,19]. Hence, these *in vitro* studies suggested that lineage maturation is impacted by TCR signaling and that single preTCR<sup>+</sup> DN3b cells that have been seeded and presumably weakly signaled by the preTCR (in some cases for as many as 8 days) are not lineage fixed. In mice and in fetal thymic organ cultures, the transit time between DN3 to DP thymocytes is relatively short (3 days or less, [20]). The reason for the extended duration for DN3b maturation in the culture system and the extent of gene expression variations in precursors arising from the delay are unknown. This uncertainty, combined with the heightened sensitivity of DP cells to TCR signaling *in vitro* [21], raises the possibility that DN TCR<sup>+</sup> cell generation at the apparent expense of DP cells is not lineage conversion *per se*; but rather it can be the result of DP cell death during culture with anti-CD3 antibody, and consequent selective maintenance and expansion of rare DN TCR<sup>+</sup> cells. Additionally, in reciprocal experiments where  $\gamma\delta$  T cells are generated in OP9-DL1 cultures by strong signaling (TCR $\delta$  crosslinking), the cells that develop are not “conventional”  $\gamma\delta$  T cells, but instead resemble NKT cells ( $\gamma\delta$ NKT cells discussed in section 4). Also troubling, cell surface levels of CD5, which normally correlate with signal strength, were diminished rather than enhanced on cells that received TCR $\delta$  crosslinking. Finally, when transferred into mice, all of the OP9-DL1 culture-derived  $\gamma\delta$  T cells homed to the gut epithelium and few, if any, were found in the spleen and lymph nodes [17]. From these results it is not clear whether the cells generated by TCR crosslinking in this system are representative of normal T cell development *in vivo*.

Lastly, in all these transgenic and *in vitro* studies, a problematic and unexplained observation against the instructive model is that normal polyclonal as well as monoclonal transgenic  $\gamma\delta$ TCRs can drive the production of both DP and  $\gamma\delta$ TCR<sup>+</sup> thymocytes in basal states, without any experimental manipulations of TCR signaling. At the population level, both  $\gamma\delta$ TCR<sup>+</sup> and  $\alpha\beta$  lineage DP thymocytes are generated in mice lacking *Tcrb* (and hence the preTCR). The latter subset is absolutely dependent on the  $\gamma\delta$ TCR for DN-to-DP progression [22,23], but once at the DP stage *Tcrg* gene transcription is silenced and further maturation ceases. Expression of a transgenic TCR $\gamma$  chain in *Tcrb*<sup>-/-</sup> mice greatly enhances DP cell generation, but has no significant effect on  $\gamma\delta$ TCR<sup>+</sup> thymocyte production [23]. Based on similar total numbers and proliferative rates of  $\gamma\delta$ TCR<sup>+</sup> and DP thymocytes in *Tcrb*<sup>-/-</sup> mice [23], about half of  $\gamma\delta$ TCRs would appear to be weak signalers that specify  $\alpha\beta$  T cell fate. Hence, at the population level, a large number of  $\gamma\delta$ TCRs appear to lack the capacity to generate lineage discriminatory signals. Conversely, it could be argued that the preTCR does not exclusively trigger precursor cells to differentiate into  $\alpha\beta$  DP cells, as suggested by the finding that ~15% of  $\gamma\delta$  TCR<sup>+</sup> cells harbor a functional TCR $\beta$  chain [24].

One way out of this quandary for the instructive model is to introduce an element of stochasticity into the TCR/ligand-based interaction upon which instruction of a progenitor cell occurs. Thus, a given  $\gamma\delta$ TCR can experience several possible outcomes. One is failure (within a prescribed developmental window) to transmit a cognate ligand-based signal, leading to dependence on weak tonic signals (from dimerization [16] or mere delivery of the TCR to the cell surface) for survival and differentiation to DP cells. Alternatively, the  $\gamma\delta$ TCR may transmit

a strong signal in response to non-cognate intrathymic ligands. For instance, some T22-specific  $\gamma\delta$ T cells, such as the KN6 TCR, may mature into  $\gamma\delta$ T cells in response to strong signals from interaction with non-cognate MHC-like ligands (some of which must be  $\beta 2m$  independent, as T10/22-reactive  $\gamma\delta$  T cells are produced in  $B2m^{-/-}$  mice). Finally, the  $\gamma\delta$ TCR may respond to a different set of non-cognate ligands with extremely high affinity leading to cell death (for example, the G8  $\gamma\delta$ TCR recognizes T22 with  $\sim 100$  times higher affinity than KN6 and is subject to negative selection [15,25]). Experimentally decreasing signal strength will increase the generation of DP cells, perhaps at the expense of  $\gamma\delta$  T cells, with the caveat that those encounters that normally negatively select  $\gamma\delta$  T cells can be tuned lower to positively select  $\gamma\delta$  T cells. Hence, the quantitative instructive model can be upheld, *ad hoc*, by invoking stochastic encounters with a variety of potential ligands that elicit a spectrum of signal strengths, even from a single  $\gamma\delta$ TCR.

However, a similar speculative logic can turn these observations to support the stochastic model.  $\alpha\beta$  T cell lineage-biased precursors expressing the  $\gamma\delta$ TCR can become DP cells with the similar conditions as above, most important being that DP cells will be negatively selected for those experiencing the stochastic TCR signaling at the higher end of spectrum. Turning down the capacity to signal will increase DP cell numbers by increasing the negative selection signal threshold and thereby decreasing the frequency of interactions that result in negative selection. It is also possible that  $\gamma\delta$ TCRs expressed in  $\alpha\beta$  lineage committed cells prior to the DP stage (particularly prevalent in  $\gamma\delta$ TCR Tg mice) will lead to cell death. This type of transgenic artifact has been demonstrated in the H-Y  $\alpha\beta$ TCR Tg system, where the decrease in DP thymocyte numbers has been convincingly shown to arise from the premature expression of the  $\alpha\beta$ TCR transgene in DN cells [26]. By attenuating the ability of  $\gamma\delta$ TCRs to signal, the death of DPs may be prevented, thereby increasing the size of the DP compartment. At the same time,  $\gamma\delta$ TCRs expressed on  $\gamma\delta$  lineage biased cells with impaired signaling capacity (below the tonic level) may result in aborted differentiation, resulting in a reciprocal loss in  $\gamma\delta$  cell numbers.

#### 4. TCR signal strength and $\gamma\delta$ T cell subset generation

$\gamma\delta$  T cells can be segregated based on *Tcr* gene usage, and the subsets thus defined exhibit distinct activation profiles and effector functions. These  $\gamma\delta$  T cell subsets have different TCR signaling requirements for development and maintenance [16,27,28]. Whether these differences are relevant when  $\gamma\delta$ TCRs are first expressed on DN precursor cells is unknown. However, the very distinct gene expression profiles of  $\gamma\delta$  T cell subsets (for example, previously identified  $\gamma\delta$  lineage biased genes such as *Rgs2*, *Crem* and *Bcl2a1* [29] are primarily expressed in  $V\gamma 1.1^+V\delta 6.3^+$  NKT cells, unpublished and Immgen.org), the attendant acquisition of unique effector functions from the earliest stages of  $\gamma\delta$ TCR<sup>+</sup> thymocyte maturation, and the unknown nature of ligands that participate in speculative  $\gamma\delta$  T cell subset-specific selection processes minimally warrant caution against assuming that a relatively narrow spectrum of signaling (*i.e.*, strong) is imparted by different  $\gamma\delta$ TCRs.

PLZF (*Zbtb16*) is expressed exclusively in innate-like  $\alpha\beta$ NKT, MAIT (innate-like cells that are found in the gut lamina propria), and  $\gamma\delta$ NKT cells [30–33].  $\gamma\delta$ NKT cells express a specific TCR consisting of  $V\gamma 1.1$  paired with  $V\delta 6.3$  [34]. Like  $\alpha\beta$ NKT cells, they are found in the thymus and peripheral lymphoid organs, but they are most abundant in the liver [35,36]. Based on the selective expansion of  $\gamma\delta$ NKT cells in mice deficient in *Itk* [37,38], *Id3* [33], and with a mutation in *Slp76* [33] and *Lat* [39] (and likely in *Cd3z*<sup>-/-</sup> mice [40]), it appears that decreasing the TCR signal strength expands the  $\gamma\delta$ NKT cell lineage. While one study showed a reduction in the number of other  $V\gamma$  expressing cells in *Id3*<sup>-/-</sup> mice [41], we and others [33,42,43] have not noted any differences in the number of  $\gamma\delta$  T cells other than  $V\gamma 1.1^+V\delta 6.3^+$  T cells in the mutant mice, indicating that there was no skewing away from the

$\gamma\delta$  lineage as a whole. Interestingly, while approximately 70% of *wt*  $\gamma\delta$ NKT cells express PLZF, nearly all  $\gamma\delta$ NKTs from *Id3*<sup>-/-</sup> [33], *Itk*<sup>-/-</sup> (unpublished) and *Slp-76* [33] mutant mice express PLZF, even though all these models result in reduced TCR signaling. In a parallel fashion, *Itk* and *Slp76* mutations led to an increased production of innate-like CD8<sup>+</sup> or CD4<sup>+</sup>  $\alpha\beta$  T cells [44,45]. Therefore, the prediction that decreasing the TCR signal strength globally will generate more  $\alpha\beta$  T cells at the expense of  $\gamma\delta$  T cells at the population level is clearly not supported by the data. Rather, decreasing TCR signal strength appears to generate more innate-like lymphocytes expressing the  $\alpha\beta$  or  $\gamma\delta$ TCR.

Another interpretation of these data in the context of the TCR signal strength model is that relatively strong TCR signals generate  $\gamma\delta$ NKT cells, and that a negative selection threshold exists for  $\gamma\delta$ NKT cells such that too much signal normally deletes them. Consistent with this possibility, *Id3*<sup>-/-</sup> mice exhibit defects in negative selection in TCR Tg mice [46]. However, while  $\gamma\delta$ TCR-triggering *in vitro* (strong signal, using anti-TCR $\delta$  chain Ab in the OP9-D11 culture system) exclusively generates PLZF<sup>+</sup> T cells [32], so far it has not been possible to delete these cells using stronger  $\gamma\delta$ TCR triggering, if such a condition exists. Furthermore, consistent with a pre-existing heterogeneity in signal interpretation, only about half of  $\gamma\delta$  T cells in *Vgl.1/Vd6.4* (*Vd6.3* allele of DBA/J mice, [34]) TCR transgenic mice are PLZF<sup>+</sup> [32], despite the fact that the cells all have an identical TCR and should receive the same signal strength. If signal strength is not determining PLZF expression, it seems likely that other molecular differences among  $\gamma\delta$ NKT cells may determine the proportion of cells that express PLZF. Alternatively, the *ad hoc* hypothesis that a clonal  $\gamma\delta$ TCR can transmit varied signals can be invoked to explain this observation.

In addition to these contradictions with the signal strength model, there are several other confounding issues for the role of TCR signal strength in  $\gamma\delta$ NKT cell generation. First,  $\gamma\delta$ NKT cell development is dependent on SAP [32,33], which is required for the development of  $\alpha\beta$ NKT [47–49] cells and other innate-like  $\alpha\beta$  T cells [50] via SLAM protein interactions, but not for “conventional”  $\alpha\beta$  or  $\gamma\delta$  T cells [51]. Hence, there are certain to be qualitative differences in signaling, and intrathymic cellular interactions, regulating the generation of distinct T cell subsets. Second, genes that specify lineage function such as *Plzf*, are expressed early in DN1/2 cells (Immgen.org), potentially programming lineage function prior to TCR expression. Third, although PLZF is only thought to be functional in NKT cells, *Plzf*<sup>-/-</sup> mice have reduced numbers of total  $\gamma\delta$  thymocytes [33], not just the  $\gamma\delta$ NKT cells, suggesting that PLZF expression in early precursor cells may be developmentally relevant or that PLZF<sup>+</sup> cells impact other T cells in trans. Fourth, the nature of intrathymic  $\gamma\delta$ TCR ligands positively or negatively selecting  $\gamma\delta$  T cells or  $\gamma\delta$ NKT cells is, for the most part, unknown, making it difficult to design experimental probes to test the model.

In summary, it is clear that decreasing TCR signal strength has a profound impact on the generation of one subset of  $\gamma\delta$  T cells that belong to the NKT cell lineage and on innate-like  $\alpha\beta$  T cells, while having no apparent effects on the generation of other “conventional”  $\gamma\delta$  and  $\alpha\beta$  lineage cells at the population level. Hence, the  $\gamma\delta$ TCR signal spectrum is not digital and has the potential capacity to shape the  $\gamma\delta$ TCR repertoire. It is very plausible that the  $\gamma\delta$ NKT cell subset expansion in the TCR signaling mutant mice arises from qualitative differences in signaling or clonal variations in lineage potential that interpret the same TCR signals distinctly. Identification of ligands that impact different  $\gamma\delta$  T cell subsets will be necessary to definitively resolve this issue.

## 5. Evidence that $\gamma\delta/\alpha\beta$ lineage choice is stochastic

### 5.1 DN2 precursors are molecularly heterogeneous

The body of evidence that TCR lineage commitment is stochastic is also not fully formed. Molecular heterogeneity at the TCR<sup>-</sup> DN2 stage is a necessary condition of the stochastic model that has been proven to exist. Cytokine receptors (IL-7R $\alpha$  [7], Activin RII, unpublished), preTCR $\alpha$  [52], transcription factors (SOX13 [53] and SOX5, unpublished), and the components of the WNT signaling pathway (AXIN2, [54] and unpublished) are a few of the factors differentially expressed in single DN2 cells. There are likely many more, given that several genes that are uniquely expressed in  $\gamma\delta$  cells are potentially only expressed in a subset of DN2 cells. The most direct proof of the stochastic model is the purification of putative lineage committed precursors that can give rise to only  $\gamma\delta$  or  $\alpha\beta$  T cells. Given the paucity of cell surface markers that can be used to segregate DN2 cells the first step is to generate a panel of mice containing DN2 cells that are differentially marked, in most cases by placing a reporter gene (*Gfp* or *LacZ*, for example) under the control of promoters that are variably active among single DN2 cells. Studies using these mice are beginning to yield evidence pointing toward a pre-existing T cell lineage bias prior to TCR expression.

### 5.2 Developmental potential of single DN2 cells

Ciofani *et. al* provided the first evidence at the single cell level that the major point of lineage commitment occurs at the DN2/DN3 stage [55]. They sorted single DN1, DN2, and DN3 cells and tested their developmental potential using the OP9-DL1 culture system. The results showed that DN1 cells were not committed to either the  $\alpha\beta$  or  $\gamma\delta$  lineage, as almost all single cells were capable of giving rise to both  $\alpha\beta$  and  $\gamma\delta$  T cells. In contrast, over half of DN2 cells were already committed to one T cell lineage: from the fetus ~60% of DN2 cells were capable of generating only  $\alpha\beta$  T cells, while ~3% generated only  $\gamma\delta$  T cells; in comparison ~25% and ~20% of adult DN2 cells were capable of generating only  $\alpha\beta$  and  $\gamma\delta$  T cells, respectively [55]. While the results from the adult DN2 cells support the existence of lineage committed cells prior to TCR gene rearrangement, they also point to a difference in developmental potential between fetal and adult DN2 cells. It has been noted previously that adult and fetal T cell development requirements are distinct [56,57]. In addition, it is possible that the OP9-DL1 culture system, which provides high levels of Notch signaling and IL-7, distinctly influences fetal versus adult T cell development from DN2 cells.

While the success rate of the gene rearrangement process (one productive TCR gene rearrangement required for the preTCR versus two for  $\gamma\delta$  T cells) alone has been proposed to account for the  $\gamma\delta$  and  $\alpha\beta$  T cell precursor frequencies in the culture, this parameter cannot account for the adult DN2 developmental potential, as the single cells generated only  $\alpha\beta$  or  $\gamma\delta$  T cells with nearly equal frequencies [55]. It also can not explain the surprising observation that fetal DN2 cells are exceedingly poor at  $\gamma\delta$ TCR-mediated lineage determination in the OP9 system, given that in the fetus there is a temporal order to TCR gene rearrangements with the *Tcrg* and *d* loci rearrangement occurring prior to the *b* loci (also evident in adults, [1,2]), which significantly alters the rate of generation of productive  $\gamma\delta$ TCR versus preTCR. *In vivo*, if the competitive advantage is a key element of the TCR-mediated lineage instruction, then removing the advantage by introducing a functional TCR $\gamma$  transgene (in otherwise *wt* mice) expressed early in DN2/DN3 cells should decrease the size of the  $\alpha\beta$  T cell pool. This, too, was not observed, as the numbers of DP thymocytes were unaltered while  $\gamma\delta$  cell numbers increased by 2–3 fold in *Tcrg* Tg mice [23]. Results from other manipulations with similar intent, including *Tcrb* Tg mice [58,59], were suggestive of two pools of cells that were generated in parallel from two sources of precursor cells rather than from a common source with two mutually exclusive, competing fates. Hence, the TCR gene rearrangement process *per se* as the primary driver of precursor cell fate cannot be easily sustained.

Despite the uncertainty pertaining to how single precursor cells can give rise to only  $\gamma\delta$  or  $\alpha\beta$  cells, the single precursor cell assay *in vitro* indicated that lineage commitment in adults occurs partly at the DN2 stage, prior to TCR expression, and is completed by the DN3 stage at which point most cells are  $\alpha\beta$  lineage restricted.

### 5.3 Developmental potential of DN2 cells segregated by IL-7R

About half of DN2 cells express relatively high levels of IL-7R on their cell surface. Intrathymic injection of IL-7R<sup>hi</sup> DN2 cells favored  $\gamma\delta$  T cell development, while injection of IL-7R<sup>lo</sup> DN2 cells favored  $\alpha\beta$  T cell lineage development, showing that lineage biased precursors do exist prior to TCR expression [7]. Given that IL-7R signaling has a profound and selective influence on *Tcr* locus rearrangement [60] and expression [61,62], it was possible that a difference in TCR rearrangement efficiency could be responsible for the biased development of precursors distinguished by IL-7R expression. However, even when functional *Tcr* gene transcription was enforced equally in both DN2 subsets, the lineage bias was observed [7]. While rearranged *TcrVd5* transcripts were detectable in the IL-7R<sup>hi</sup> DN2 cell subsets, this is a rare event (<1% based on results in [1]). These findings suggested that DN2 cells are not a homogeneous population and prompted studies to identify other genes of developmental-relevance that are differentially expressed among DN2 cells.

### 5.4 Developmental potential of Sox13<sup>+</sup> DN2 cells

*Sox13* was the first  $\gamma\delta$  lineage-restricted gene identified and is also a candidate T cell lineage fate determinant as it is expressed in half of DN2 cells as determined by single cell (sc) RT-PCR [53]. As  $\gamma\delta$  T cells mature in the thymus and periphery, *Sox13* expression decreases, a pattern that is shared by many  $\gamma\delta$  lineage-restricted transcription factors (Immgen.org). While all CD24<sup>+</sup>  $\gamma\delta$  immature thymocytes express *Sox13*, as assessed by scRT-PCR, *Sox13* expression is quantitatively heterogeneous in  $\gamma\delta$  T cell subsets defined by the V gene usage, with V $\gamma$ 2<sup>+</sup> cell subsets having the highest relative expression in the thymus and periphery (unpublished and Immgen.org). NK1.1<sup>+</sup>PLZF<sup>+</sup>  $\gamma\delta$ NKT cells do not express *Sox13*, a finding consistent with a previous report [17]. This complex pattern of *Sox13* expression has caused some to suggest that it is not a pan  $\gamma\delta$  T cell marker and not essential for all  $\gamma\delta$  T cells. As alluded to in section 4, it is not at all clear that peripheral  $\gamma\delta$  T cells can be defined as one lineage since gene expression profiles of different  $\gamma\delta$  T cell subsets are as different from each other as they are from the expression profiles of  $\alpha\beta$  lineage CD8<sup>+</sup> thymocytes (Immgen.org and unpublished). Nevertheless, *Sox13* expression in all CD24<sup>+</sup>  $\gamma\delta$  thymocytes still positions it as the best candidate molecule to influence T cell lineage commitment prior to TCR expression. SOX13 may function like NOTCH signaling, which is critical for DN precursor differentiation, but not essential for all peripheral  $\alpha\beta$  T cell subsets. Once the lineage is determined, *Sox13* may have additional roles in maintaining specific  $\gamma\delta$  T cell subsets. One possible model is that while *Sox13* expression is necessary for  $\gamma\delta$  T cell development, the relative amount of *Sox13* and/or the presence of alternative factors regulates the generation of distinct  $\gamma\delta$  T cell sublineages defined by V gene usage.

In mice, *Sox13* promotes  $\gamma\delta$  T cell development at the expense of the  $\alpha\beta$  T cell lineage. Consistent with the noted bias toward  $\gamma\delta$  T cell generation, IL-7R<sup>hi</sup> DN2 cells express more *Sox13* than IL-7R<sup>lo</sup> cells [53]. SOX13 belongs to the SRY-related High Mobility Group (HMG) transcription factor family that contains 25 members. Among the HMG proteins are TCF1 (encoded by *Tcf7*) and LEF1, which are absolutely essential for  $\alpha\beta$  T cell development [63, 64], and TOX, which is required for CD4<sup>+</sup>  $\alpha\beta$  T cell differentiation from DP thymocytes [65]. SOX13 binds to TCF1 and LEF1 and alters their function in regulating gene transcription [53]. *Sox5*, a close relative of *Sox13*, is also expressed in  $\gamma\delta$  T cells. However, based on analyses of *Sox5*-reporter mice (from V. Lefebvre, Lerner Research Institute, Cleveland) only ~15% of  $\gamma\delta$  T cells express *Sox5*. In DN2 cells, only 5–10% express *Sox5* (unpublished), again indicating

molecular heterogeneity in the precursor cell subset. Currently, *Sox13*-reporter mice are being generated and a definitive test of SOX13<sup>+</sup> DN2 cells as the committed precursors of  $\gamma\delta$  cells should be forthcoming.

### 5.5 Developmental potential of DN2 cells segregated by WNT signaling

The most compelling finding so far is that the expression of WNT signaling target genes is also heterogeneous in DN2 cells. The canonical WNT signaling pathway involves  $\beta$ -catenin-TCF1/LEF1 complex-mediated gene regulation. As TCF1 is clearly necessary for normal T cell development [63,64], the relatively normal hematopoietic lineage development in mice reconstituted with  $\beta$ -Catenin/ $\gamma$ -Catenin double deficient progenitors was a surprise [54,66]. The implications of this observation for the assumed importance of canonical WNT signaling for T cells are unclear since a redundant factor(s) may exist for Catenins in lymphocytes. Consistent with this possibility, mice deficient in other dedicated downstream signaling molecules of canonical WNT signaling have severe defects in T cell development (unpublished), indicating that WNTs are in fact essential. AXIN2 is a component of the  $\beta$ -catenin destruction complex that is induced by canonical WNT signaling [54,67]. DN2 cells can be divided into AXIN2<sup>neg</sup> (25–30%) and AXIN2<sup>hi</sup> (30–40%) populations. Initial results from the examination of the T cell lineage potential pre-existing in DN2 cells distinguished by AXIN2 expression suggest that DN2 cells that can exclusively give rise to  $\gamma\delta$  T cells can be identified and purified. These results support the contention that the canonical WNT signaling regulated by SOX13-TCF1 complexes is a high value candidate for influencing T cell lineage commitment prior to TCR expression

Other  $\gamma\delta$  T cell lineage-specific gene reporter mice are in production and simultaneous use of multiple markers to track the precursor-product relationship should permit a thorough testing of the stochastic model of T cell lineage commitment. While these data support the idea that heterogeneous expression of transcription factors and other genes at the DN2 stage significantly impacts the outcome of  $\gamma\delta/\alpha\beta$  lineage commitment, the issue of T cell lineage commitment can only be definitively resolved by a rigorous test of molecularly distinct T cell precursor populations *in vivo*. This ultimate proof has not yet been generated, but with the advent of more versatile flow cytometry methods using multiple reporter gene knock-in mice combined with *in vitro* single cell clonal assays, it is becoming possible to dissect DN2 cells into multiple distinct populations based on the expression of molecular effectors of key pathways such as WNT signaling and track their cell lineage fate.

## 6. Conclusion

While favorable momentum for the TCR signal strength model of T cell lineage commitment clearly exists, many questions remain, and definitive tests for the existence of T cell sublineage-committed cells have yet to be completed. While it was appreciated that  $\gamma\delta$  T cells segregated based on TCR V $\gamma/\delta$  gene segment usage are equipped with distinct functions that are programmed during intrathymic differentiation, a recent comprehensive interrogation of  $\gamma\delta$  sublineage gene expression patterns has revealed extensive differences in gene expression among the different  $\gamma\delta$  subsets, with at least one subset (PLZF<sup>+</sup>V $\gamma$ 1.1<sup>+</sup>V $\delta$ 6.3<sup>+</sup>) sharing characteristics with the  $\alpha\beta$ NKT cell lineage. This finding questions the very nature of the  $\gamma\delta$  T cell lineage and challenges the prevailing assumption that distinct TCR signal strengths or master cell fate regulators generate and define all  $\gamma\delta$  T cells.

## Acknowledgments

The authors have been supported by a grant from NIH/NCI (CA100382). We thank members of the lab for discussions and M. Bix for critical comments on the manuscript.



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