pre-B migration away from the source of IL-7. An alternative explanation for why IRF-4,8 double-deficient mice cannot produce B cells could come from the observation that withdrawal of IL-7 from the culture medium of IRF-4,8 double-deficient pre-B cells gave robust κ gene rearrangement (Johnson et al., 2008) but no expression of the BCR on the cell surface (Ma et al., 2006; Johnson et al., 2008). This finding could reflect a downstream dependency on IRF-4 or IRF-8, either for efficient transcription of the rearranged light-chain gene itself or of other genes necessary for efficient light-chain expression on the cell surface. The observation that withdrawal of IL-7 induces RAG expression in pre-B cells raises questions as to how the presumed inhibition of RAG expression is circumvented at the earlier pro-B cell stage, where IL-7 is known to support growth and maintenance of the cells. Could it be that a fraction of pro-B cell precursors transiently and perhaps repeatedly enter an IL-7 refractory, nonproliferative phase, where RAG expression is induced? Attenuated IL-7 signaling has been reported in B cells even though they continue to express the IL-7 receptor. If so, it would be interesting to identify the intracellular components responsible for regulating IL-7 signaling in this way. Alternatively, rearrangement of the light-chain genes may require higher amounts of RAG than does that of the heavy-chain genes, and the IL-7 inhibition of RAG expression may be concentration dependent. Finally, in early pre-B cells, IL-7 and pre-BCR signaling may synergize to dampen *Rag* transcription to a greater extent than IL-7 signaling alone.

In conclusion, the work described by Johnson et al. (2008) provides exciting insight into the function of IRF-4 as a regulatory node in the transition of pre-B to immature B cells. One important role of this transcription factor, which is upregulated by pre-BCR signals, consists in the downregulation of the surrogate light-chain genes and the activation of the 3′ κ and λ enhancers, resulting in the initiation of light-chain gene rearrangement and positioning of the loci away from heterochromatin. Another unexpected function of IRF-4 appears to involve the induced expression of chemokine receptors, which could help pre-B cells to move away from IL-7-producing stromal cells. This cell migration would lead to an attenuation of IL-7 signaling and to enhanced binding of E2A to the intronic κ and *Rag* enhancers.

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# Thymus Lineage Commitment: A Single Switch

Stephen M. Hedrick[1,*]
[1]University of California, San Diego, La Jolla, CA 92093-0377, USA
*Correspondence: shedrick@ucsd.edu
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**In this issue of *Immunity*, He et al. (2008) establish the logic and circuitry that determine CD4-CD8 lineage specification. It all comes down to an eighty base pair silencer switch.**

Developmental contingencies are endlessly fascinating. There's something about a circuit that measures small differences and accurately amplifies them into an error-free outcome. Contingencies represent the opposite of chaos and the epitome of robustness. In developmental immunology, none has received more attention than the CD4 versus CD8 T cell lineage commitment, perhaps because it is intertwined with the storied concepts of MHC restriction and thymic selection.

The principles of lineage commitment consist of a basis for the decision, the conceptual underlying logic, and the analog circuitry used the compute an all-or-none result. We understand the first, I propose that an understanding of the second is close at hand, and with recent breakthroughs we have the tools to understand the third. The first is easy; the specificity of the receptor (TCR) determines the outcome of the differentiation pathway—but what is the logic? Do class II- and class I-specific TCRs transmit quantifiably different signals (instruction), or does the developing thymocyte venture

a guess and survive or not depending on its accuracy (stochastic selection)? These two models were initially proposed, each was tested successfully, and each had its proponents. As we might have predicted from similar debates in science, the truth has elements of each.

A clue came from an analysis of MHC class II-deficient mice. Flow cytometry showed the lesion correlated with an absence of mature $CD4^+$ T cells but with one curious finding. Although there were no developing thymocytes that displayed the $CD4^+CD8^-$ phenotype, there was a stubbornly persistent $CD4^+CD8^{lo}$ population. The inference was that this population had abortively attempted the wrong pathway, and this was said to be evidence for selection. The observation was highly influential in that it gave rise to a field devoted entirely to ever-more-detailed analyses of the iconic CD4 versus CD8 bivariant FACS plot. Thymocyte subsets, based on graded CD4 and CD8 expression, were finely dissected into intermediate steps with vectored trajectories, and there arose a new generation of models describing asymmetric signaling requirements and default pathways. Still, none was rigorously tested.

A breakthrough came in the form of the kinetic signaling model, which is a deceptively simple but conceptually distinct variation of all previous notions of CD4-CD8 lineage commitment (Singer, 2002). The idea is that recognition of a ligand by $CD4^+CD8^+$ thymocytes results in positive selection. This, in turn, causes a reduction in the intensity of CD8 expression that can be mapped, in part, to the E8III enhancer active during the $CD4^+CD8^+$ stage but turned off upon positive selection (Hedrick, 2002). The idea is that continuous signaling after positive selection denotes the recognition of MHC class II, whereas signaling that wanes as a result of a loss of CD8 is evidence for the recognition of MHC class I. Evidence in support of this is that expression of CD4 under control of the E8III enhancer, resulting in abortive MHC class II-mediated signaling, causes a lineage-commitment reversal (Sarafova et al., 2005).

That leaves *only* the circuitry. The transcription factor, GATA-3, is necessary for $CD4^+$ T cell development, but in its absence, there is no lineage redirection (Hernandez-Hoyos et al., 2003). The HMG-containing factor, Tox (thymus HMG box), now appears to be essential for the transition from $CD4^+CD8^+$ to the $CD4^+CD8^{lo}$ stage of development, and although no $CD4^+$ T cells arise in its absence, $CD8^+$ T cell development is almost normal (Aliahmad and Kaye, 2008). I suspect that this is an indication that the kinetic-signaling model is still incomplete. The analysis of *Runx1*- and *Runx3*-deficient mice showed that they are necessary for $CD8^+$ lineage development and directly affect the silencer activity of the *Cd4* gene (Taniuchi et al., 2002). The Runx molecules are surely part of the lineage-specification network.

The spectacular discovery of a single transcription factor that is necessary and sufficient for CD4 lineage commitment now forms the basis for a detailed description of the circuitry. One group found, by chance (but with enlightened insight), a mouse mutation, termed HD (T helper deficient), lacking $CD4^+CD8^-$ thymocytes and T cells. Through genetics and analysis by transgenesis, they mapped the mutation to a single base in the DNA binding domain of a transcription factor designated as ThPOK, but known alternatively as c-Krox, Zfp67, and the official symbol, Zbtb7b (zinc finger and BTB domain containing 7B) (He et al., 2005). Another group independently identified this lineage-commitment factor from a differential gene expression analysis of $CD4^+$ and $CD8^+$ T cells (Sun et al., 2005). Zbtb7b is first seen in the $CD4^+CD8^{lo}$ subset of thymocytes, and in HD mice (predicted to be a *Zbtb7b* loss of function), MHC class II-specific thymocytes incorrectly differentiate into the $CD8^+$ T cell lineage. Moreover, enforced expression of *Zbtb7b* in thymocytes causes MHC class I-specific cells to incorrectly differentiate into the $CD4^+$ T cell lineage. Zbtb7b is thus necessary and sufficient for CD4 lineage specification, and from this arises the principle that positive selection is separate from lineage commitment. Work is now centered on identifying the downstream targets of Zbtb7b, and also characterizing its own *cis*-acting transcriptional regulation—the bottoms-up approach taken to another stage (Hedrick, 2002). Along with another recently published article (Setoguchi et al., 2008), work by He et al. (2008) in this issue of *Immunity* at once reinforces the kinetic signaling model as a principle of CD4-CD8 lineage specification and focuses attention on a silencer-enhancer governing *Zbtb7b* expression.

The report by He et al. (2008) is really two related studies. In the first, they sought to establish a basis for their previous observation that Zbtb7b is first expressed at the proposed lineage-commitment step—$CD4^+CD8^{lo}$. The prediction was that strong or extended signaling is the origin this difference, and they showed this by treatment of MHC class II-deficient mice with antibody specific for TCRβ. The result was an induction of Zbtb7b in $CD4^+CD8^{lo}$ cells and the appearance of $CD4^+CD8^-$ thymocytes. This result is an affirmation that continued signaling at this all-important stage, regardless of its origin, is necessary for CD4 lineage commitment. One could say that MHC class II-mediated signaling is stronger at this juncture and therefore instructive, but that misses the essence of the logic. This experiment is also telling in that positive selection requires MHC recognition *because* Lck is entirely bound by CD4 and CD8 (Van Laethem et al., 2007). Given that anti-TCRβ is unlikely to coordinate the inclusion of CD4 (and it attendant Lck), I infer that signaling leading to lineage commitment is different from that required for positive selection.

To distinguish between the requirement for strong or extended signaling at the lineage-commitment stage, He et al. (2008) produced *HD;B2m*$^{-/-}$ mice and analyzed the $CD4^+CD8^{lo}$ population. Placing these cells into culture presumably interrupts TCR-CD4 signaling, and the result was that Zbtb7b disappeared and the cells expressed CD8. Furthermore, Zbtb7b expression was rescued by the addition of antibody specific for CD3. With caveats concerning thymocyte development in culture, this is consistent with the requirement for continued TCR-mediated signaling at this decision stage in order to maintain CD4 lineage commitment. The class of MHC recognition is unimportant; rather, it is only the extension of signaling to maintain Zbtb7b expression (Figure 1).

In the second part, He et al. (2008) analyzed the *cis*-acting regulatory sequences needed for *Zbtb7b* expression. Although expression is mediated by multiple enhancer-like sequences (Figure 1), the most interesting facet of the work was the identification of a 5′ distal sequence that has characteristics of a silencer and enhancer. Deletion of this distal response

element (DRE) caused reporter expression in both CD4 and CD8 lineages, yet, a sequence or sequences in this region also functioned as an enhancer largely restricted to the $CD4^+CD8^{lo}$ stage. This would appear to be further evidence that the $CD4^+CD8^{lo}$ stage is a truly distinct subset along the T cell developmental pathway.

A region of 300 bp near the DRE was found to be conserved in mammals and marsupials and contains motifs predicted to bind Gata, Runx, NFκB, and E-box factors. After deletion mutants were generated and then tested with their DRE-deleted reporter construct, the Gata, Runx, and E-box sites appeared to be irrelevant, and they defined an 80 bp region important for silencing. Analysis of multiple founders was consistent with weak silencing outside the 80 bp region, and thus a contribution by Runx or Gata was not entirely eliminated. Through this elegant, extensive, in vivo analysis, He et al. (2008) appear to have defined a silencer element that is controlled by an unknown transcription factor or factors that embody CD4-CD8 lineage specification. Yet, there is one complication.

In a recent report, Setoguchi et al. (2008) described their continued analysis of Runx as a repressor important for CD4-CD8 lineage specification. A number of transcriptional repressors, including the Runt-Runx family, act by way of a corepressor known as Groucho. In addition to conditional mutations in *Runx1* and *Runx3* previously analyzed, these investigators produced a *Runx1* mutant lacking the conserved VWRPY Groucho-interaction motif at the carboxyl terminus. Analyzing mice with various combinations of *Runx* genes deleted or mutated, they found that *Runx3*-deficient mice with a *Runx1-VWRPY* mutation completely lacked $CD8^+$ T cells—even in the absence of MHC class II genes.

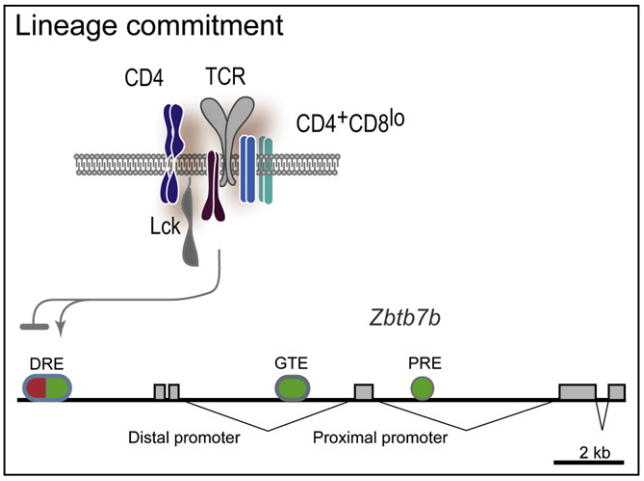


**Figure 1. Lineage Commitment Depends on a *Zbtb7b* Silencer**
The lineage-commitment step takes place subsequent to positive selection at a stage in which cells express a $CD4^+CD8^{lo}$ phenotype. With continued signaling through the TCR, the DRE silencer activity is blocked and enhancer activity activated; Zbtb7b resolves CD4 lineage commitment through repression and activation of lineage-specific genes. Discontinued signaling through the TCR results in Groucho-dependent DRE silencer activity and no Zbtb7b expression, and cells commit to the CD8 lineage. DRE denotes distal regulatory element, GTE denotes general T lymphoid element, and PRE denotes proximal regulatory element.

The investigators used ChIP-on-chip to find sequences in the *Zbtb7b* locus that bind to the Runx binding partner, Cbfβ2. Two were found and one corresponded with the DRE. Setoguchi et al. (2008) similarly found the deletion of this gene element derepressed the *Zbtb7b* locus in CD8 T cells, but differing from the above results, the Runx sites were necessary for efficient silencer activity. On the other hand, these Runx complexes were shown to bind even in cells expressing Zbtb7b, and therefore, Runx binding does not itself confer the specificity of silencing.

Although they differ in a role for Runx-Groucho-mediated repression, the two studies establish very similar and important principles underlying CD4-CD8 lineage specification. *Zbtb7b* is regulated by an enhancer-silencer element in the distalmost region of the upstream regulatory region, and it acts at the critical $CD4^+CD8^{lo}$ stage of development. The element includes Runx sites, but importantly, there is a critical factor yet to be identified. The tools are now at hand to understand CD4-CD8 lineage specification to a degree previously only achieved with saturating mutagenesis screens in simple metazoans. As referred to above, there are probably principles yet to be discovered, or as Groucho himself said, "Those are my principles … if you don't like them I've got others."

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