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Transcriptional Activators of Helper T Cell Fate Are Required for Establishment but Not Maintenance of Signature Cytokine Expression

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The stability of helper T cell fates is not well understood. Using conditional introduction of dominant-negative factors, we now show that T-bet and GATA-3 are far more critical in establishment than maintenance of IFN-γ and IL-4 activity during Th1 and Th2 maturation, respectively. We also show that a genetic interaction between T-bet and its target Hlx seems to be required for Th1 maturation, but that Hlx may also be dispensable for maintenance of a transcriptionally permissive ifng gene. In parallel to progressive activator independence in the permissive lineage, the ifng gene becomes more recalcitrant to switching as the forbidden lineage matures. T-bet plus Hlx can disrupt ifng silencing when introduced into developing Th2 cells, but they fail to perturb ifng silencing in mature Th2 cells. In contrast, a hypermorphic allele of T-bet can reverse silencing of the ifng gene in mature Th2 cells. These results suggest that signature gene activity of helper T cells is initially plastic but later becomes epigenetically fixed and offer an initial strategy for inducing mature cells to switch their fate. The Journal of Immunology, 2005, 175: 5981–5985.
identify Hlx as a Th1-specific transcript by microarray (7). Th1 clone 9.1-2 protects BALB/c mice from *Leishmania* infection (17).

**Retroviral gene transduction**

Bicistronic retrovirus (RV) vectors were constructed as described previously (7). In some experiments, Hlx bicistronic RV with truncated hCD8 was used to mark cells (18). Construction of DN Hlx, DN GATA-3, and T-bet-VP16 were performed with PCR mutagenesis. For DN Hlx, the central portion of the Hlx homeoprotein (aa 266–346), including the DNA binding domain, was fused to the amino-terminal repression domain of the *Drosophila* engrailed repressor (dEnR; aa 1–299). For DN GATA-3, the central portion of the protein (aa 259–385), including the zinc finger-containing DNA binding domain (aa 263–341), was fused to the amino-terminal repression domain of dEnR (aa 1–299). For T-bet-VP16, the carboxy-terminal region of T-bet was deleted, and the NH2-terminal region, including the T-box (aa 1–371) of T-bet, was fused to the transactivation domain of HSV protein VP16 (aa 410–490). Infection of T cells was performed as described (7).

**Real-time PCR and Northern blotting**

RT-PCR assays were performed as described (7) using an ABI Prism 7900 BioDetecter (Applied Biosystems). All PCR data are levels of test gene over hypoxanthine phosphoribosyltransferase levels, with the lowest value standardized at 1. Northern blotting was performed as described (16).

**Mammalian two-hybrid assay**

Full-length T-bet and Hlx were fused to Gal4 DNA binding domain and VP16 trans-activation domains, respectively (CheckMate Mammalian Two Hybrid assay kit; Promega). pGal4-T-bet, VP16-Hlx, and controls were cotransfected into 293T cells with a plasmid-encoding luciferase driven by five Gal4 response elements (pG5Luc). Forty-eight hours after transfection, Firefly and *Renilla reniformis* luciferase were measured using the Dual-Glo luciferase assay system (Promega).

**Results**

**T-bet and Hlx physically and genetically interact during Th1 maturation**

Attempts to dissect the genetic program that culminates in Th1 cell maturation led to the identification of the homeobox gene, Hlx (for H.2.0-like homeobox) as a Th1-specific transcription factor that appeared to drive maturation of IFN-γ activity in cooperation with T-bet (7). Hlx mRNA was found to be preferentially expressed in Th1 cells (Fig. 1A), as suggested (7, 19), but is strictly dependent on (Fig. 1B) and quantitatively regulated by (Fig. 1C) T-bet. This is the first gene ablation evidence to directly demonstrate that T-bet is essential to induce Hlx, as prior evidence relied on overexpression of wild-type and DN factors (7). Once T-bet induces Hlx, they cooperate to induce maturation of IFN-γ activity in a cell-intrinsic manner. This can be modeled in developing Th2 cells transduced with either T-bet alone or Hlx plus T-bet, all cultured within the same well (Fig. 1D). This effect is best demonstrated by the dramatic increase in amount of IFN-γ produced per cell, as reflected in mean fluorescence intensity (MFI), or by secretion of IFN-γ from sorted cells (Fig. 1D). Similar results were obtained with enriched naive cells from D011 TCR transgenic *Rag2*−/− mice (data not shown).

To test whether the genetic interaction between T-bet and Hlx that results in synergistic induction of IFN-γ might be mediated by a physical interaction, we used the mammalian two-hybrid assay (20), which monitors the ability of two proteins to bring tethered trans-activation and DNA binding domains in proximity. Cotransfection of Gal4-T-bet and VP16-Hlx (Fig. 1E) resulted in a specific and significant increase in luciferase activity (Fig. 1F). T-bet and Hlx might, therefore, physically interact to mediate their synergistic effects on transcription (Fig. 1D), a behavior described in other T-box/homeobox factor partnerships (21–23).

**Hlx may be required for establishment but not maintenance of IFN-γ activity**

The foregoing data support a model of feed-forward gene induction, whereby T-bet induces Hlx, which then cooperates with its inducer, T-bet, to promote IFN-γ maturation in a cell-intrinsic manner. Hlx gene deletion results in embryonic lethality (24). To address the requirement for Hlx in mediating IFN-γ production, we, instead, used a strategy that has been used to successfully antagonize the function of other homeodomain proteins (25, 26). We constructed a RV consisting of the Hlx DNA binding domain and of Herpes simplex VP16 trans-activation domain to Hlx (VP16Hlx). Cotransfection of 293T cells with the Gal4-responsive luciferase reporter (pG5Luc) and indicated expression plasmids was followed by evaluation of relative luciferase activity at 48 h.

FIGURE 1. T-bet and Hlx genetically and physically interact. A–C, Hlx is a Th1 gene, downstream of T-bet. A, Naive CD4+ T cells were stimulated in Th1 or Th2 conditions for 4 days before Northern analysis for indicated transcripts. B, Northern blot of Hlx mRNA from T-bet−/+ and T-bet+/+ cells cultured in Th1 conditions for 7 days. C, Hlx mRNA levels were analyzed by real-time RT-PCR from cells stimulated in Th1 conditions for 7 days. D, Hlx enables maturation of T-bet-mediated IFN-γ expression. Flow cytometry panels (left): cells were stimulated in Th2 conditions (plus anti-IFN-γ) and transduced after 24 h with empty GFP and empty hCD8 RVs (left) or T-bet GFP and Hlx hCD8 RVs (right). After 4 days, cells were restimulated, and IFN-γ staining was assessed. Only CD4+ GFP+ events are depicted. Horizontal and vertical values are the percentage of IFN-γ+ cells and the MFI of the transduced cells, respectively. Bar graph (right): cells were stimulated in Th2 conditions (plus anti-IFN-γ) and transduced with indicated RVs after 24 h. Cells were sorted and restimulated with immobilized anti-CD3 after 1 wk of primary culture. After an additional 48 h, IFN-γ secretion was measured by two-site ELISA. E and F, T-bet and Hlx interact in a two-hybrid assay. E, Schematic of fusion of yeast Gal4 DNA binding domain to T-bet (Gal4T-bet) and of Herpes simplex VP16 trans-activation domain to Hlx (VP16Hlx). F, Cotransfection of 293T cells with the Gal4-responsive luciferase reporter (pG5Luc) and indicated expression plasmids was followed by evaluation of relative luciferase activity at 48 h.
therefore be important follow-up approaches to test the validity of the levels of Hlx in two other Th1 clones drops below the level of amounts of T-bet in Th1 clones (Fig. 3). Developing Th1 cells transduced with indicated RVs were sorted and restimulated with immobilized anti-CD3 after 4 days. After an additional 48 h, IFN-γ secretion was measured by two-site ELISA. 

C. Developing Th1 cells transduced with indicated RVs were sorted and restimulated with real-time RT-PCR 4 days after initial stimulation. E. Antagonism of Hlx or T-bet does not impair IFN-γ production in more mature Th1 cells. DO11 transgenic T cells were activated, and restimulated weekly in Th1 conditions. 

Using a long-term Th1 clone, we had previously suggested that T-bet might not be required for maintenance of IFN-γ activity (7). We wished to further test this model by asking whether T-bet or Hlx is responsible for maintaining the heritably permissive state of IFN-γ activity in newly maturing Th1 cells. We therefore performed a kinetic analysis. DO11.10 transgenic helper T cells were activated with APC/peptide in Th1-polarizing conditions. Cells were transduced with control, DN Hlx, or DN T-bet RVs at days 2, 9, 16, or 23, and IFN-γ expression was analyzed 5 days after transduction. As Th1 maturation progressed, expression of IFN-γ became recalcitrant to the effects of the DN factors (Fig. 2E). Antagonism of Hlx was still evident after 2 days, as reflected in reduced MFI of IFN-γ staining, but was negligible after 1 wk. Co-transduction of DN T-bet and DN Hlx also had no effect on the ability to reiterate IFN-γ activity (data not shown). Thus, establishment and maintenance of IFN-γ activity in Th1 cells are experimentally distinguishable by their sensitivity and recalcitrance to DN transcription factors, respectively.

The DN T-bet construct may influence multiple targets (iil2rb2, ifng, hlx, tbet). Conditional gene deletion or RNA interference will therefore be important follow-up approaches to test the validity of the model that T-bet and Hlx exhibit a hit-and-run behavior in activating the ifng gene. Despite the relatively high levels of Hlx during the first few days of Th1 priming (Refs. 7, 19; Fig. 3A), it has been shown that the levels of Hlx fall in primary Th1 cell cultures after the first few days (19). It was also reported that levels of Hlx in a Th1 clone were significantly lower than in primary Th1 cultures (19). Despite the persistent expression of substantial amounts of T-bet in Th1 clones (Fig. 3A), we also found that the levels of Hlx in two other Th1 clones drops below the level of early Th1 cultures (Fig. 3, A and B). Analysis of two different Th1 clones revealed that Hlx levels may drop slightly below developing Th1 cells or may even fall to levels below a Th2 clone (Fig. 3, A and B), despite maintaining heritable IFN-γ activity (Fig. 3C).

The lack of defect in IFN-γ activity from delayed introduction of DN Hlx (Fig. 2E) is thus quite consistent with the ability to experimentally separate Hlx expression from heritable IFN-γ activity in a mature Th1 clone. The persistent expression of T-bet without Hlx in a Th1 clone is also consistent with the apparent lack of a sufficient or necessary role for Hlx in IL-12Rβ2 expression (Ref. 7; Fig. 2D). Although IL-12Rβ2 expression continues to remain dependent on T-bet in mature Th1 cells (7), the absence of Hlx in such cells would not be predicted to be of consequence, based on the negligible effect of overexpression or antagonism of Hlx on the IL-12Rβ2 target (Ref. 7; Fig. 2D).
The preceding results suggest there is a limited window during Th1 and Th2 differentiation in which the activity of the *ifng* and *il4* genes is still plastic, capable of being antagonized by interference with T-bet/Hlx and GATA-3. During the progression of naive to mature helper T cells, forbidden effectors cytokine genes undergo even deeper silencing, through mechanisms such as centromeric repositioning of the genes or de novo DNA methylation (27, 28). Likewise, introduction of inducing *trans*-acting factors into the forbidden lineages induces the forbidden cytokine gene with diminishing efficiency as cells mature (4, 29). We also found that activation of the *ifng* gene in mature Th2 cells is progressively restricted (data not shown). We therefore fashioned a putative hypomorphic form of T-bet to test its effect on the silencing of the *ifng* gene in mature Th2 cells. T-bet-VP16 (Fig. 5A) has the carboxy-terminal *trans*-activation domain of T-bet replaced by the HSV VP16 *trans*-activation domain (30). This domain was chosen because of the ability of VP16 to recruit both histone acetyltransferases and ATP-dependent nucleosome remodeling complexes, the two major enzymatic activities responsible for chromatin remodeling-dependent gene induction (31).

Cells were stimulated in Th2 conditions for 3 wk and transduced with control, T-bet, T-bet plus Hlx, or T-bet-VP16 RVs. As assessed by intracellular cytokine staining (Fig. 5B) and ELISA (Fig. 5C), expression of T-bet or T-bet plus Hlx resulted in only modest increases in IFN-γ expression in mature Th2 cells. T-bet-VP16, however, could induce substantial secretion of IFN-γ and converted the majority of transduced cells into brightly staining, IFN-γ-positive cells. None of the interventions significantly repressed IL-4 expression (data not shown). The dual expression of IL-4 and IFN-γ also suggested that we were not simply providing a selection mechanism for contaminating Th1 cells, but rather de-repressing IFN-γ in maturing Th2 cells. Thus, mature Th2 cells become recalitrant to the inducing factors, T-bet plus Hlx, which can mediate efficient IFN-γ maturation in developing Th2 cells. By targeting strong activators, however, the progressive restrictions that are imposed on the forbidden cytokine gene may be capable of being more efficiently overcome.

**Discussion**

We now show that an activator of heritable patterns of gene expression might be conditionally critical, being essential for the establishment but not necessarily the maintenance of cytokine gene activity. Using a standard genetic approach that has successfully antagonized the function of other homeodomain proteins (25, 26), we provide the first loss-of-function experiments with Hlx. These studies offer initial evidence that Hlx is indeed required to cooperate with T-bet to establish a heritably permissive state of the *ifng* gene. Moreover, our data show for the first time that Hlx and T-bet can physically interact in a mammalian two-hybrid assay, suggesting a potential molecular mechanism for their synergistic activation of the *ifng* gene. Later, the active state of the *ifng* gene becomes insensitive to the loss of both T-bet and Hlx activity (Fig. 6). The concept that a transcriptional activator that is essential to induce a state of gene activity may not be required to maintain that gene activity is an important, yet poorly understood, feature of gene regulation in helper T cells that will require further study.

In parallel with this progressive stability of activity in the Th1 lineage, the *ifng* gene becomes progressively more difficult to activate by ectopic T-bet and Hlx in maturing Th2 cells. A similar behavior may be occurring in the *il4* locus (Fig. 6), as GATA-3 seems to become progressively dispensable for IL-4 activity in maturing Th2 cells, and the *il4* gene becomes progressively more difficult to *trans*-activate in maturing Th1 cells (29). Although we have not yet been able to silence a heritably active cytokine locus, we offer initial evidence that derepressing a more permanently silenced locus might be achieved with strategies that target strong chromatin activators to the locus (Fig. 5). The success of the T-bet-VP16 construct in achieving substantial induction of IFN-γ provides novel evidence for the viability of genetic strategies to intervene during disease processes mediated by fully differentiated helper T cells.

It is curious that within the same cell type, a single activator might become dispensable for one target but remain continually...
and Hlx. Later in Th2 maturation, the plasticity gives way to stability in reverse heritable silencing. At the stage of stability, the hyperagonistic T-bet VP-16 can or is more accurately genetic, maintained by truly epigenetic, maintained by self-propagating chromatin marks, defined. We do not yet know whether the stability we observe is mechanisms responsible for locking in the activity or silencing of cytotoxic differentiation.

Disclosures

The authors have no financial conflict of interest.

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