# THE LINEAGE DECISIONS OF HELPER T CELLS

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After encountering antigen, helper T (T$_H$) cells undergo differentiation to effector cells, which can secrete high levels of interferon-γ, interleukin-4 (IL-4), IL-10 and other immunomodulators. How T$_H$ cells acquire, and remember, new patterns of gene expression is an area of intensive investigation. The process is remarkably plastic, with cytokines being key regulators. Extrinsic signals seem to be integrated into cell-intrinsic programming, in what is becoming an intriguing story of regulated development. We summarize the latest insights into mechanisms that govern the lineage choices that are made during T$_H$-cell responses to foreign pathogens.

DECISION MAKING IN THE IMMUNE SYSTEM

After engagement of the T-cell receptor (TCR) by the appropriate peptide–MHC complex, which triggers clonal expansion, helper T (T$_H$) cells rapidly undergo programmed differentiation (reviewed in REFS 1,2). This differentiation process can result in highly polarized immune responses in the case of chronic infections, such as parasitic infections. A more heterogeneous response is often shown by analyses of acute immune responses.

Naive T$_H$ cells can differentiate to at least two functional classes of cell during an immune response[3] — T$_H$1 cells, which secrete interferon-γ (IFN-γ), and T$_H$2 cells, which secrete interleukin-4 (IL-4) (reviewed in REFS 1,2). Hereafter, we refer to IFN-γ and IL-4 as effector cytokines. T$_H$1 cells are responsible for cell-mediated immunity, whereas T$_H$2 cells are responsible for extracellular immunity. In terms of the control of parasites, T$_H$1 immunity provides protection against intracellular protozoa, such as *Leishmania* species and *Toxoplasma gondii*, whereas T$_H$2 immunity is associated with protection against intestinal helminths. As well as their protective roles in host defence, both subsets of T$_H$ cell have been implicated in pathological responses. T$_H$1 cells can mediate organ-specific autoimmunity and T$_H$2 cells have been implicated in the pathogenesis of asthma and allergy. The final composition of the T$_H$-cell response to antigen can, therefore, determine whether the outcome of infectious, inflammatory and autoimmune responses is favourable or unfavourable.

The process by which an uncommitted T$_H$ cell develops into a mature T$_H$1 or T$_H$2 cell is a useful model of developmentally regulated gene expression (FIG. 1). There is good evidence to indicate that this differentiation process is highly plastic. Many factors influence the decision to become a T$_H$1 or T$_H$2 cell. The cytokines IL-12 and IL-4, acting through signal transducer and activator of transcription 4 (STAT4) and STAT6, respectively, are key determinants of the outcome (reviewed in REFS 1,2). It has been proposed also that antigen dose, co-stimulators, genetic modifiers and other non-cytokine factors have crucial roles in determining the dominance of a T$_H$-cell
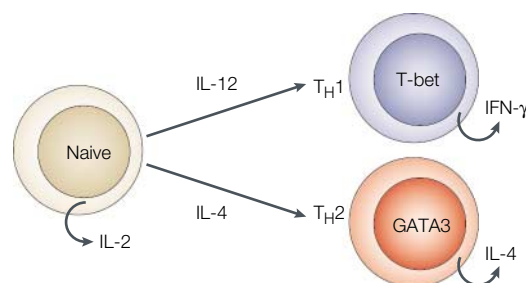
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Figure 1 | **The original instructive model of helper T-cell differentiation.** An uncommitted, naive helper T (T$_H$)-cell precursor can become either a T$_H$1 or T$_H$2 cell under the instructive influence of interleukin-12 (IL-12) or IL-4, respectively. T$_H$1 cells express T-bet and secrete interferon-γ (IFN-γ). T$_H$2 cells express GATA3 and secrete IL-4.
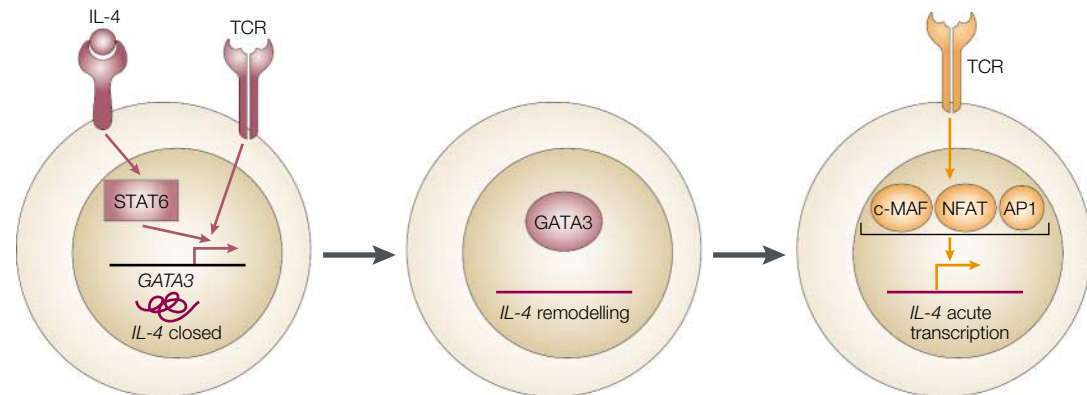
Figure 2 | **Pathways for the induction of expression of IL-4.** Naive helper T (T$_H$) cells stimulated with interleukin-4 (IL-4) and antigen, through the T-cell receptor (TCR), upregulate *GATA3* transcription. The GATA3 protein induces heritable remodelling of the *IL-4* locus, which is characteristic of fully differentiated T$_H$2 cells. Experimental evidence indicates that GATA3 might not have a crucial role in the acute transcription of the *IL-4* gene. Instead, a second step, mediated by signals downstream of the TCR, causes rapid acute transcription of *IL-4*. The mediators of this effect include lineage-specific (such as c-MAF) and non-specific (such as NFAT and AP1) transcription factors. NFAT, nuclear factor of activated T cells.

response. How each signal influences the differentiation process is an area of active investigation and, often, lively controversy.

Many data indicate that certain crucial transcription factors have causal roles in the gene-expression programmes of T$_H$1 and T$_H$2 cells (FIG. 1). For example, the T-box transcription factor T-bet has been shown to have a central role in T$_H$1-cell development[4,5], inducing both transcriptional competence of the locus encoding IFN-γ and selective responsiveness to the growth factor IL-12 (REFS 6,7). By contrast, the zinc-finger transcription factor GATA3 seems to be crucial for inducing some, but perhaps not all, key attributes of T$_H$2 cells — in particular, transcriptional competence of the T$_H$2 cytokine cluster, which includes the genes encoding IL-13, IL-4 and IL-5 (REFS 8–14).

### Box 1 | **Chromatin remodelling**

There is an emerging consensus that the subset-specific expression of genes can be attributed to two types of effect of factors — general locus-activating effects or acute transcriptional effects. The first type of effect is associated with changes in gene structure, such as the repositioning of nucleosomes or decompaction of condensed chromatin fibres, and it is known as epigenetic or (imperfectly) as chromatin remodelling. The term epigenetic implies that the state is transferred from parent to daughter cell — in other words, some of the changes in gene activity that accompany helper T-cell differentiation are heritable. By contrast, the term chromatin remodelling can be used also in reference to acute, reversible, time-dependent changes in the chromatin structure of a promoter. Beyond chromatin remodelling, the acute transcriptional induction of subset-specific genes can be mediated by transcription factors that are either activated or expressed selectively in one subset, but that can induce transcription of remodelled gene targets only.

Chromatin remodelling might be required to induce competence for gene activity or transcription, and it is mediated by histone modifications, of which the best studied are acetylation, methylation and phosphorylation. In addition, histones can be ADP-ribosylated and ubiquitylated. The role of these modifications in gene expression is uncertain. The roles of acetylation, methylation and phosphorylation are complex and might vary depending on the specific histone residue that is being modified. Chromatin remodelling can be indicated by various technical parameters, including increased DNase-I hypersensitivity and histone acetylation.

In this article, we highlight some recent advances in our understanding of this model of developmentally regulated alterations in gene activity. We do not attempt to be encyclopaedic, but we try primarily to discuss and unite the transcriptional and EPIGENETIC mechanisms that act during T$_H$1- and T$_H$2-cell development, as well as mentioning a few topical cytokine-related issues.

### How T$_H$2 cells arise

In terms of effector T-cell differentiation, T$_H$2-cell development was the first process to be linked to the actions of a cytokine. IL-4 was recognized early on to promote the development of the T$_H$2-cell subset[15–18]. Later, this activity was shown to operate through the actions of STAT6 (FIG. 2). The observation that T cells from Stat6-deficient mice have a severe block in T$_H$2-cell development led to the conclusion that STAT6 is necessary for T$_H$2-cell development[19–21].

Some transcription factors are expressed selectively by T$_H$2 cells. c-MAF, a member of the b-ZIP (basic-region leucine-zipper) protein family, was identified initially as a T$_H$2-cell-specific transcription factor that regulates the expression of IL-4 (REF. 22). c-Maf promotes skewing towards T$_H$2-cell development when it is expressed in transgenic mice[23], and c-Maf-deficient mice have a selective defect in IL-4 production[24]. A second, and perhaps further upstream, T$_H$2-cell-specific transcription factor, GATA3, was identified originally as regulating the expression of a broad array of T$_H$2 cytokines[10,11]. The expression of GATA3 is induced rapidly by IL-4, through STAT6, increasing from a low level in naive T cells to a high level in T$_H$2 cells[9] (FIG. 2). Whereas the requirement for STAT6 and c-MAF in T$_H$2-cell development has been confirmed using gene-targeting, this has not been reported so far for GATA3, because this seems also to be an essential factor for normal thymocyte development and embryonic survival[25–27]. GATA3 can transactivate the *IL-5* promoter[11,12,28], whereas c-MAF can transactivate the *IL-4* promoter[12,22]. As we discuss further later, the role of GATA3 in establishing competence of *IL-4* gene activity
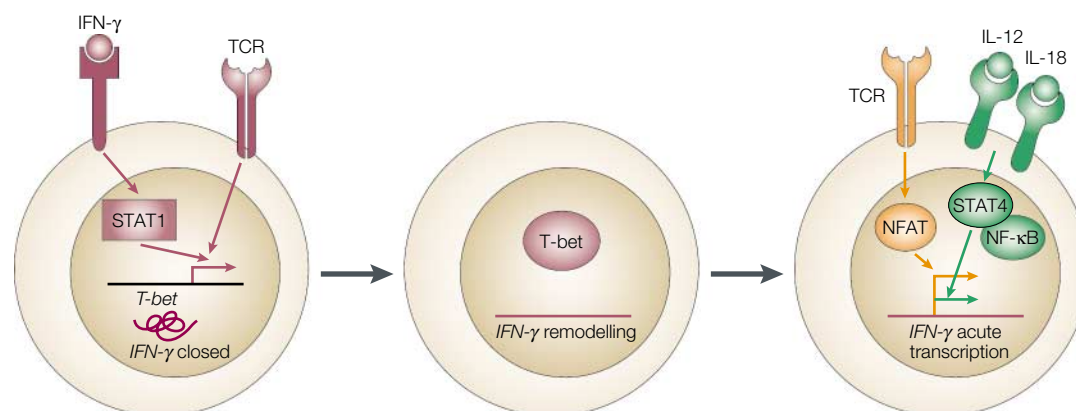
**Figure 3 | Pathways for the induction of expression of IFN-γ.** Naive helper T (T$_H$) cells activated under T$_H$1-inducing conditions are exposed to interferon-γ (IFN-γ) signalling during T-cell receptor (TCR) engagement, leading to the activation of signal transducer and activator of transcription 1 (STAT1). Downstream of STAT1, the expression of T-bet is induced, and T-bet acts in this model to induce remodelling of the repressed *IFN-γ* locus (shown) and induce expression of the interleukin-12 receptor β2-subunit (IL-12Rβ2) (not shown). Subsequently, the committed T$_H$1 cell, which expresses receptors for IL-12 and IL-18, has at least two pathways available to induce the acute transcription of IFN-γ — the TCR signalling pathway or a cytokine signalling pathway that can include combined IL-12 and IL-18 signalling. TCR-induced transcription of the gene encoding IFN-γ is distinguishable pharmacologically from cytokine-induced transcription — the former is sensitive to cyclosporin A (CsA), whereas the latter is CsA resistant. Different nuclear factors might mediate each pathway. NFAT, nuclear factor of activated T cells; NF-κB, nuclear factor-κB.

seems to be related to its ability to induce the remodelling of CHROMATIN[8,12,29] (BOX 1 and FIG. 2).

GATA3 has been shown to promote T$_H$2-cell development strongly, when expressed by a transgene[10] or by a retrovirus[9,28]. Furthermore, GATA3 induces T$_H$2-cell development of *STAT6*$^{-/-}$ T cells[8], including the production of T$_H$2-cell-specific cytokines and expression of c-MAF, which depend normally on STAT6. A putative dominant-negative mutant of GATA3 decreases T$_H$2-cell-mediated pulmonary allergic responses when expressed by a transgene in mice[30]. In addition, antisense oligonucleotide treatment directed against GATA3 can repress T$_H$2-cell responses *in vivo*, which further implies a requirement for GATA3 for T$_H$2-cell development[31].

Several non-T$_H$2-cell-specific transcription factors contribute to the regulation of expression of T$_H$2 cytokines, particularly members of the nuclear factor of activated T cells (NFAT) family (reviewed in REF. 2). Interactions between the different NFAT-family members on T$_H$2-cytokine genes are complex, and the transcription factors can have both positive and negative effects. In general, the role of these factors in T$_H$2-cytokine gene expression seems to be in mediating the acute transcription of inducible cytokines after triggering of a differentiated T$_H$2 cell through the TCR (FIG. 2).

As well as STAT6, MEL18, a Polycomb-group protein, also seems to regulate the expression of GATA3 (REF. 32). Although Polycomb-group proteins are associated generally with the heritable silencing of loci[33], MEL18 seems to act as a positive regulator of *GATA3* transcription. So, in the absence of MEL18, the levels of GATA3 and IL-4 are reduced, and reintroduction of GATA3 using a retroviral vector can correct the defect in IL-4 expression partially[32]. There are at least two potential post-transcriptional regulators of GATA3 — the zinc-finger proteins FOG1 (friend of GATA1) and ROG (repressor of GATA). Overexpression of FOG1

repressed T$_H$2-cell development induced by ectopic GATA3 in *STAT6*$^{-/-}$ T cells and decreased the efficiency of *GATA3* transcriptional autoactivation[34,35]. ROG was isolated as a GATA3-interacting protein; its overexpression decreases GATA3 activity and the production of T$_H$2 cytokines[36].

**How T$_H$1 cells arise**

T-bet was identified recently as a T$_H$1-cell-specific factor that can induce the production of IFN-γ by developing T$_H$2 cells[5]. A member of the T-box family of transcription factors, T-bet seems to be expressed in developing and committed T$_H$1 cells. In addition to its role in inducing the expression of IFN-γ, T-bet seems to be involved in chromatin remodelling of the gene that encodes IFN-γ[6] (FIG. 3), induction of expression of the IL-12 receptor β2-subunit (IL-12Rβ2)[6,37] and stabilizing its own expression, either through an intrinsic auto-catalytic loop or the autocrine effects of IFN-γ signalling[6,37]. Although both CD4$^+$ and CD8$^+$ T cells, as well as natural killer (NK) cells, express T-bet, there seems to be less dependence on T-bet for high-level expression of IFN-γ in CD8$^+$ T cells than in CD4$^+$ T cells or NK cells[4]. Nevertheless, the crucial importance of T-bet for the development of T$_H$1 responses *in vivo* is underscored by the susceptibility of *T-bet*-knockout mice to challenge with *Leishmania major*[4] and their predisposition to allergic airway disease[38].

How T-bet induces expression of IFN-γ is still an area of active investigation. Recently, a genetic interaction was reported between *T-bet* and *HLX* (H2.0-like homeobox 1)[7]. The *HLX* gene seems to be expressed in developing T$_H$1 cells, although its expression is induced at a slower rate than for *T-bet*. Transcription of *HLX* seems to be downstream of T-bet activity, and HLX protein seems to interact with T-bet protein, which mediates the synergistic induction of IFN-γ expression[7].

CHROMATIN
Composed of nucleosomes, which are the basic repeating units of eukaryotic genomes. Nucleosomes consist of 146 base pairs of DNA wound around an octamer of histone proteins.
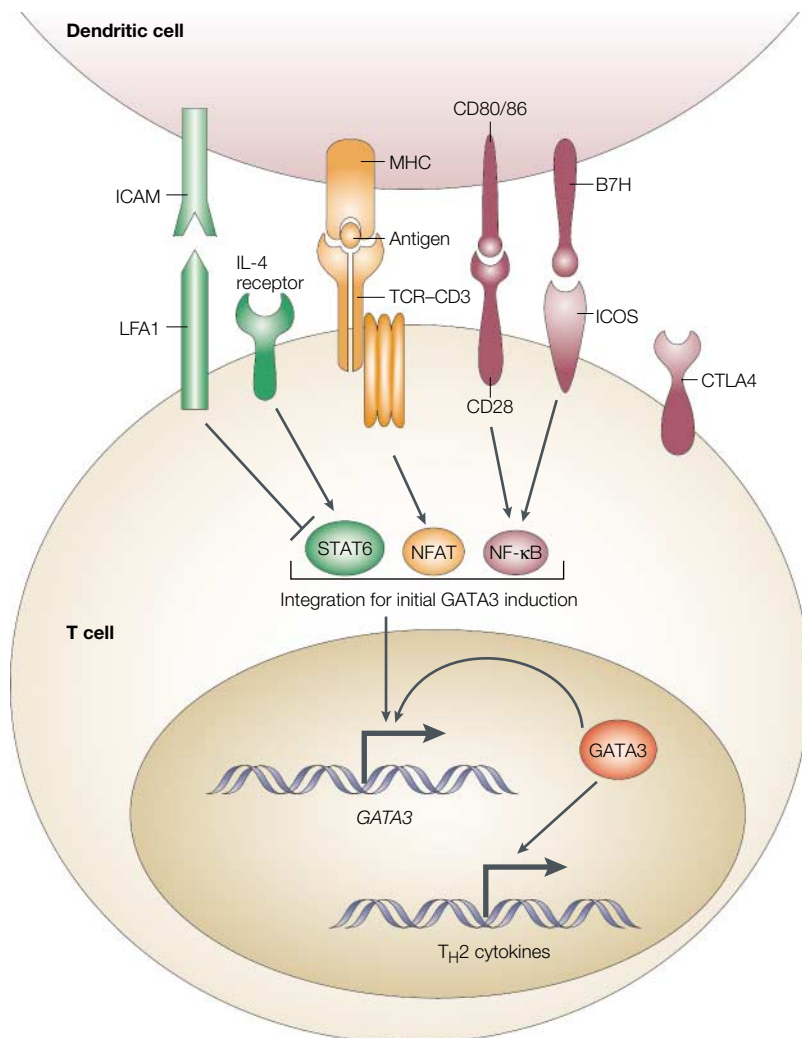
Figure 4 | **Redundant pathways of T$_H$2-cell development — integration at the level of GATA3 transcription.** The observation that T helper 2 (T$_H$2) cells can develop in the absence of signal transducer and activator of transcription 6 (STAT6), both *in vivo* and *in vitro* (see text), might indicate that there are alternative pathways for the induction of expression of GATA3. As GATA3 expression undergoes positive feedback by transcriptional autoactivation, signalling through nuclear factor-κB (NF-κB) by CD28, and perhaps other receptors, might reach the threshold that is necessary for sustainable GATA3 autoactivation and T$_H$2-cell commitment. Strong co-stimulation (in the absence of inhibition by IL-12, IFN-γ or LFA1) might, therefore, provide a STAT6-independent pathway of T$_H$2-cell development. CTLA4, cytotoxic T-lymphocyte antigen 4; ICAM1, intercellular adhesion molecule 1; ICOS, inducible co-stimulator; IFN-γ, interferon-γ; IL-12, interleukin-12; LFA1, leukocyte function-associated antigen 1; NFAT, nuclear factor of activated T cells.

The role of HLX *in vivo* has not been examined fully yet because knockout mice die during embryogenesis[39].

The expression of T-bet seems to be induced readily in naive T cells by IFN-γ signalling, mediated by STAT1 (REFS 37,40) (FIG. 3). Although STAT1 seems to be a crucial factor for the induction of expression of T-bet, type I IFNs, which activate STAT1 also, do not seem to be able to induce *T-bet* transcription[40]. Whether T-bet protein can autoactivate transcription of the *T-bet* gene is less clear. Endogenous expression of T-bet was shown to be induced by ectopic T-bet, which implies that autoactivation does occur[6,7], but a reduction in the level of endogenous *T-bet* transcription in the absence of autocrine IFN-γ signalling[37,40] was observed also, which indicates

GADD-FAMILY PROTEINS
(Growth-arrest and DNA-damage inducible proteins). In response to environmental stresses, these proteins mediate activation of the p38 mitogen-activated protein kinase pathway and are involved in the regulation of growth and apoptosis.

that there is an exocrine mechanism. Because only early time points in development have been examined in each of these studies, it is possible that a shift from a STAT-dependent pathway to an autoactivation pathway could occur at some later time. Finally, as STAT1-deficient cells can be driven to T$_H$1-cell polarization, it is possible either that expression of a low level of T-bet might be independent of IFN-γ signalling, allowing T$_H$1-cell development, or that the requirement for T-bet for T$_H$1-cell development is conditional. The latter possibility is raised by the observation that T-bet-deficient CD4$^+$ T cells from the autoimmune *Lpr* mouse background produce abundant IFN-γ[41], which indicates that there is some flexibility in the requirement for T-bet. So, it is uncertain whether STAT1-independent T$_H$1-cell development arises from an alternative extrinsic signal or an autonomous property of the activated T cell.

**Acute transcription of the gene encoding IFN-γ**
In terminally differentiated T$_H$1 cells, reiteration of IFN-γ expression can occur through two experimentally distinct pathways — TCR ligation or cytokine (IL-12 and IL-18) stimulation. IL-18 augments the production of IFN-γ by differentiated T$_H$1 cells, despite its inability to drive the development of T$_H$1 cells on its own[42]. The combination of IL-12 and IL-18 can induce the production of IFN-γ by differentiated T cells in the absence of signalling through the TCR[42,43] (FIG. 3). The IFN-γ production that is induced is more prolonged and more resistant to treatment with cyclosporin A (CsA) (an inhibitor of NF-AT activation) than that induced by TCR crosslinking[43], which indicates that cytokine-driven IFN-γ production is not coupled to the NFAT pathway. *In vivo*, it seems that IL-12 and IL-18 act synergistically to induce maximum IFN-γ production[44,45].
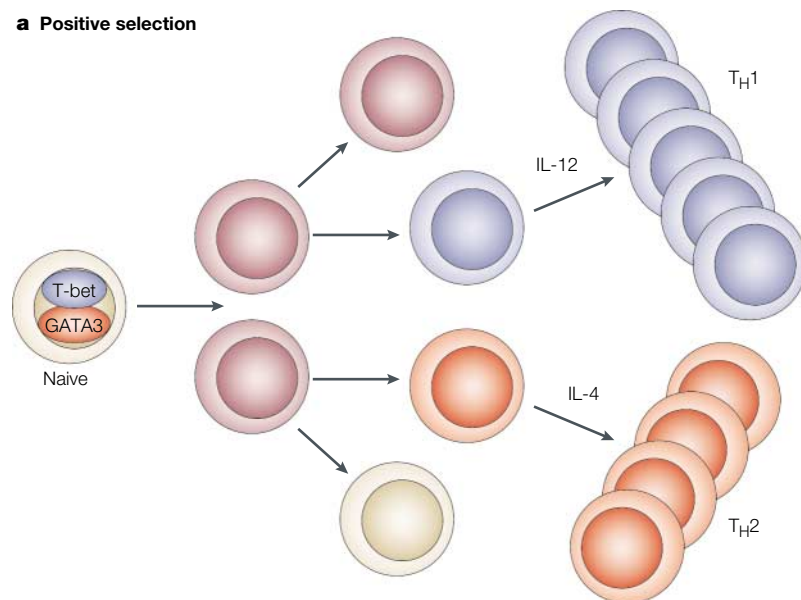
IL-12- and IL-18-induced IFN-γ production correlates with induction of expression of the GADD-FAMILY PROTEINS GADD45β and GADD45γ. Overexpression of GADD45β augments IFN-γ production[46], and absence of GADD45γ decreases IFN-γ production, thereby inhibiting T$_H$1-cell development[47]. The transcriptional details of IL-12- and IL-18-induced IFN-γ production are uncertain still. It is clear that IL-12- and IL-18-induced IFN-γ production depends strongly on STAT4, whereas TCR signalling can induce IFN-γ production, although at a reduced level, in *STAT4*$^{-/-}$ T$_H$1 cells[37]. The other factors that mediate cytokine-induced IFN-γ production — in contrast to CsA-sensitive, anti-CD3 antibody-induced IFN-γ production — are uncertain still, although some evidence indicates that these might be p38 mitogen-activated protein kinase (p38 MAPK)-sensitive factors[46,48], which is consistent with previous suggestions (reviewed in REF. 49).

The secondary, cytokine-dependent pathway of IFN-γ expression that has been described for CD4$^+$ T$_H$1 cells might operate in other cell types. IL-12 and IL-18 have an active role in inducing the production of IFN-γ by CD8$^+$ T cells and NK cells in response to bacterial pathogens[50]. Similarly, there is some indirect evidence that such a bystander pathway might operate in CD4$^+$ T cells *in vivo*. Even after a T$_H$1 response has been initiated, it has been

shown that continuous IL-12 production is required for protection in some experimental infections, including with *T. gondii*[51] and *L. major*[52,53]. It will be of interest, therefore, to determine whether continuous production of IL-12 is required to stabilize the $T_H1$-cell phenotype or the magnitude of the effector response — that is, IFN-γ production. Perhaps consistent with the latter possibility,

deletion of the gene encoding IL-18 results in reduced resistance to some pathogens[44,54], despite the non-essential role of this cytokine in $T_H1$-cell development[42]. So, the extent to which the TCR and cytokine-driven pathways act to induce IFN-γ production *in vivo* during the effector phase of $T_H1$ responses will require additional investigation.

### Recently discovered $T_H1$-cell-promoting factors

The recently discovered cytokine IL-23 is composed of the p40 subunit of IL-12 paired with a unique chain, p19 (IL-23α), that is related distantly to IL-12 p35 (REF. 55). IL-23 binds IL-12Rβ1, but not IL-12Rβ2; it interacts instead with a unique receptor subunit, IL-23R[56]. IL-23 is reported to activate STAT4 (REF. 55), and it might act during both the induction of $T_H1$-cell development and the acute reiteration of IFN-γ production induced by cytokines, in cooperation with IL-18 (as described for IL-12 and IL-18; REF. 46). However, a role for IL-23 in initiating early $T_H1$-cell development has not been established yet. In addition to its effects on T cells, IL-23 might influence dendritic cells (DCs), by enhancing the stimulatory capacity of these antigen-presenting cells (APCs)[57].

Another heterodimeric cytokine, IL-27 (REF. 58), was reported recently, which is composed of EBI3 (Epstein–Barr virus-induced gene 3)[59] and p28, a subunit related to IL-12 p35. IL-27 is produced by APCs; it induces the proliferation of naive T cells selectively, acts with IL-12 to promote IFN-γ production and is the ligand for TCCR (T-cell cytokine receptor, also known as WSX1), a receptor on T cells that is involved in early $T_H1$-cell development[60,61]. The precise mechanisms by which IL-27 and TCCR act in early $T_H1$-cell development are unclear, but IL-27 seems to interact synergistically with IL-12 at an early checkpoint in $T_H1$-cell commitment[62].

### Instruction, selection and parental controls

Most previous studies have been interpreted to support an instructive model of $T_H$-cell differentiation (FIG. 1). In this model, IL-4 carries instructive signals to the T-cell nucleus through a series of intermediate molecules. First, the IL-4 receptor activates STAT6 in the naive T cell. Then, phosphorylated, dimeric STAT6, in the context of a fully activated T cell, efficiently increases the expression of GATA3 from a low to high level. At some point, a feedback of transcriptional autoactivation occurs, which stabilizes GATA3 expression[8,12]. GATA3 acts directly on certain cytokine promoters, as in the case of *IL-5* (REFS 11,12), or indirectly through important *cis* elements, as for the IL-4- and IL-13-encoding cytokine loci[29], to transactivate the promoter or mediate the reversal of chromatin-based repression. In this model, the IL-4 signalling pathway does not provide signals for cell growth or proliferation, but simply instructs the cell to 'open' the $T_H2$-cytokine loci for expression.

*Cytokines might select rather than instruct.* Several questions have, however, arisen concerning models that indicate that the lineage-defining transcription factors T-bet and GATA3 are absent in naive cells, and that the
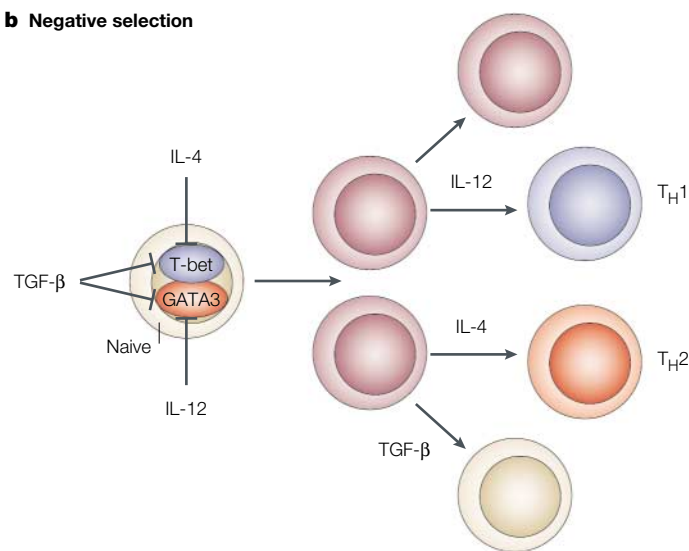


Figure 5 | **Models of secondary roles for cytokines in differentiation. a** | Positive selection. After activation, the expression of T-bet and GATA3 is induced and T cells divide, acquiring a mixed T helper 1 ($T_H1$)- and $T_H2$-lineage profile (indicated as burgandy shading). With further division, lineage commitment progresses, including the selective responsiveness to growth-factor signals. The presence of interleukin-12 (IL-12) will favour the growth of cells that express the IL-12 receptor β2-subunit (IL-12Rβ2) (committed $T_H1$ cells; blue), whereas the presence of IL-4 will favour the growth of cells that express GATA3 (committed $T_H2$ cells; red), which seems to synergize with growth-factor-independent 1 (GFI1), a downstream mediator of signal transducer and activator of transcription 6 (STAT6) signals. **b** | Negative instruction. As cells acquire competence for growth-factor signal transduction, IL-12 silences *GATA3*, IL-4 silences *T-bet*, and transforming growth factor-β (TGF-β) works to repress both transactivators. This instructive effect of cytokines helps to 'lock-in' patterns of differentiation, but it still relies on some prior degree of cytokine-independent lineage commitment. It is speculated that T-bet and GATA3 are active during the initial cell division(s), because their silencing by cytokines might require passage through the cell cycle.
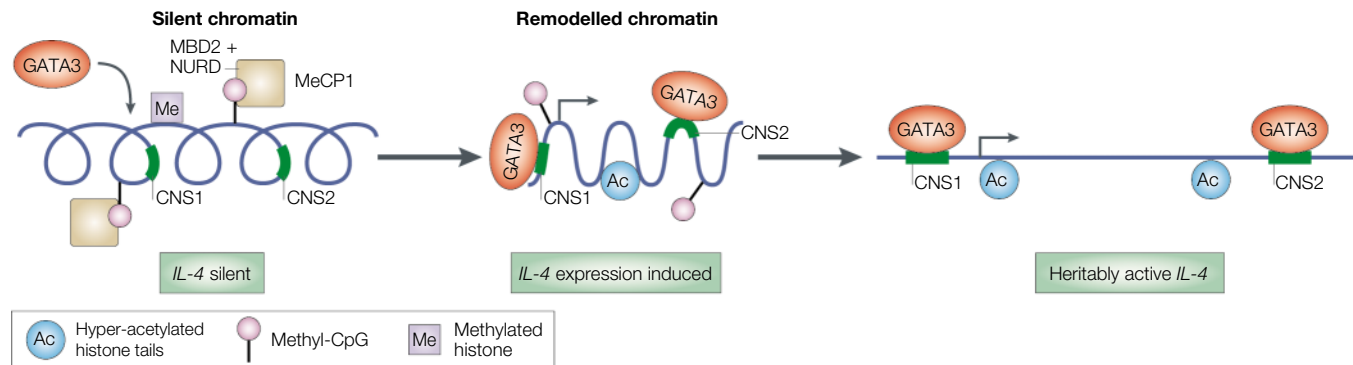
Figure 6 | **Model of orderly derepression of the *IL-4* gene.** The interleukin-4 (*IL-4*) locus is actively repressed in naive CD4+ T cells by molecules that link methylated DNA (MBD2) to repressive chromatin (NURD). Together, MBD2 (methyl-CpG-binding domain protein 2) and NURD (nucleosome remodelling and histone deacetylase) form the MeCP1 complex. The sites of action of this repression probably overlap with crucial *cis*-acting elements in the locus, such as the *IL-4–IL-13* intergenic region (CNS1), the second intron of *IL-4*, and the 3′ enhancer (CNS2). Activation of the locus occurs in a stepwise fashion. First, GATA3 mediates displacement of MBD2, leading to chromatin changes such as histone acetylation, an effect that is concurrent with the induction of *IL-4* transcription. Later, the acquisition of additional epigenetic changes, such as stable CpG demethylation, occurs along the cytokine gene. CNS, conserved non-coding sequence.

induction of their expression is caused by exposure to IL-12 and IL-4, respectively (FIG. 1). One problem lies in the observation that an effector T-cell lineage might have the unique ability to respond to a specific cytokine. Indeed, there is evidence that T$_H$2 cells transduce signals more efficiently through the IL-4 receptor[63] and that T$_H$1 cells selectively express a signalling chain of the IL-12 receptor[64]. So, the ability of a cytokine to stimulate an increased level of transactivator messenger RNA in a T-cell population[65] might be an indirect effect of favouring the growth of T cells that express such mRNA.

Recently, expression of growth-factor independent 1 (GFI1) was described to be induced selectively by STAT6 signalling in T cells and to mediate the clonal expansion of T$_H$2 cells[66]. This result provides a potential explanation for a selective, rather than instructive, component to T$_H$2-cell development. Previously, GATA3 was found to influence mainly T$_H$2-cytokine gene expression rather than cell growth[67], which indicates that there is an instructive effect. However, GFI1 seems to drive clonal expansion of only those T$_H$ cells that co-express GATA3, which indicates a cooperative model that incorporates both instructive and selective components. It is uncertain precisely how the interaction between GATA3 and GFI1 occurs in T$_H$2-cell development. Despite the known requirement of STAT6 for T$_H$2-cell development, *Stat6*$^{-/-}$ T$_H$2 cells have been observed in several systems[8,68,69]. In two systems of pathogen-induced responses, reduced, but detectable, T$_H$2 responses were seen in *Stat6*$^{-/-}$ backgrounds[68,69]. In an *in vitro* system, the ectopic expression of GATA3, introduced by a retrovirus, in *Stat6*$^{-/-}$ T cells induced endogenous expression of GATA3 and triggered full T$_H$2-cell development[8]. However, in none of these systems has the expression of GFI1 been examined.

*Instructions from signals other than cytokines.* As the previous discussion indicates, it is not entirely clear whether instructive or selective models can explain T$_H$2-cell development. The interesting, albeit reduced,

development of T$_H$2 cells in *Stat6*$^{-/-}$ mice could be evidence of an intrinsic mechanism that generates a diversity of committed T$_H$-cell fates in the progeny of a single cell without requiring instructive signals. There could, however, be additional instructive signals, other than IL-4 and STAT6, that give rise to the T$_H$2-cell fate. Redundant signalling pathways, for example, might induce the transcription of *GATA3* directly, independently of STAT6 (FIG. 4). CD28 co-stimulation has been reported to augment the expression of GATA3 (REF. 70), and a role for nuclear factor-κB (NF-κB) has been proposed also[71]. Although these studies did not examine the efficiency of these effects in the absence of STAT6, it is conceivable that strong CD28 co-stimulation, which can activate NF-κB[72], could be a physiological, STAT6-independent pathway to promote T$_H$2-cell development. In fact biasing T$_H$1- or T$_H$2-cell development, caused by some DC subsets[73], has been proposed to be cytokine independent in some cases (reviewed in REF. 74).

As GATA3 has a transcriptional autoactivating property — which leads to the massive upregulation of *GATA3* transcription by GATA3 protein[8,12] — it is possible that this pathway could be effective as an initial source of IL-4 (FIG. 4). It can be speculated that inducible co-stimulator (ICOS), another CD28-family co-stimulatory receptor present on T cells, could regulate the expression of GATA3, because ICOS signalling might overlap partially with CD28 signalling and is known to promote T$_H$2 responses (reviewed in REF. 75). Finally, the LFA1 (leukocyte function-associated antigen 1)–ICAM1 (intercellular adhesion molecule 1) pathway has been reported to regulate the T$_H$1–T$_H$2 balance, such that LFA1 signalling favours T$_H$1-cell development, although this has not been examined in the setting of STAT6 deficiency[76–79]. Regulation of the expression of ICAM1 by DCs could modify the strength of LFA1 ligation, thereby tuning the T$_H$1–T$_H$2 balance (FIG. 4). It is uncertain how LFA1 signalling is integrated with the other T$_H$1 signalling pathways , but it might act in a STAT6-independent manner.

In summary, there are a few candidate proteins that could regulate the expression of GATA3 directly and possibly trigger transcriptional autoactivation of *GATA3*, independently of STAT6. These proteins would also be considered to be instructive signals (FIG. 4).

*Extrinsic meets intrinsic during proliferation.* Despite the existence of these potentially instructive mechanisms, it is still possible that a programmed diversification mechanism operates during early T-cell priming (FIG. 5). One unresolved issue concerning the nature of the T$_H$-cell-fate decision is whether naive T cells themselves adopt new fates or whether they proliferate and produce progeny that acquire new fates[80–85]. The importance of this distinction lies in whether an antigen-specific clonotype must make an exclusive decision to become only T$_H$1 cells or only T$_H$2 cells. If differentiation occurs before proliferation, then naive parental T cells might be forced to make exclusive, binary choices (FIG. 1). By contrast, differentiation during or after proliferation would be consistent with an ability of the naive parental T cell to have many progeny, each with potentially distinct, or multi-lineage, fates (FIG. 5). In this case, achieving polarized differentiation might depend more on the selective growth and survival of particular cell fates (FIG. 5a; positive selection) or the selective repression of particular transcription factors (FIG. 5b; negative instruction) than on signals that give rise to the new cell fate (FIG. 1; conventional, positive instruction).

Just as there seem to be STAT6-independent T$_H$2 cells[8,68,69], STAT4-independent T$_H$1-cell commitment has been described[6,37,40]. A further problem in invoking STAT4 as the most upstream factor of T$_H$1-cell commitment arises from the surprising result that ectopic

IL-12 signalling in developing T$_H$2 cells cannot induce transcriptional competence of the gene encoding IFN-γ[86,87]. Consistent with the notion of positive selection by cytokines (FIG. 5a), STAT4 and STAT6 help mediate cell division and the migration of lymph-node effector cells to peripheral tissues[6,88], perhaps by antagonizing proliferative arrest[89] and counter-regulating lymph-node homing molecules[90,91].

Consistent with the concept of negative instruction (FIG. 5b), the cytokines IL-12 and IL-4 have a prominent role in the silencing of *GATA3* and *T-bet*, respectively[6,8,9,82]. Transforming growth factor-β (TGF-β) seems to be able to silence transcription of both *GATA3* and *T-bet*[82,92–95]. It could be speculated that the ground state (or default) for transcription of *T-bet* and *GATA3* loci is one of activity (FIG. 5) in naive T cells. Evidence for this comes from the finding that silencing of *T-bet* or *GATA3* loci by prohibitive cytokine signals seems to require passage through S-phase of the cell cycle[82], and that cells that do not divide remain multi-potent[82,96]. One potential benefit of such a system of gene regulation would be to provide a default state of diversification at the outset of T$_H$-cell differentiation.

The requirement for cell-cycle progression[81–83] for gene silencing, and its corollary, the ability of clonally related T$_H$ cells to adopt many fates, might a provide a basis for the various extracellular cues that have been described to shape highly pleiotropic outcomes. Indeed, the existence of STAT6-independent T$_H$2-cell commitment[8,68,69] and STAT4-independent T$_H$1-cell commitment[6,37,40], the non-selective transcription of cytokines[96,97] and transactivators[82], and the acetylation of histones[98,99] are all consistent with a degree of intrinsic commitment that might precede cytokine-driven selection or instruction.
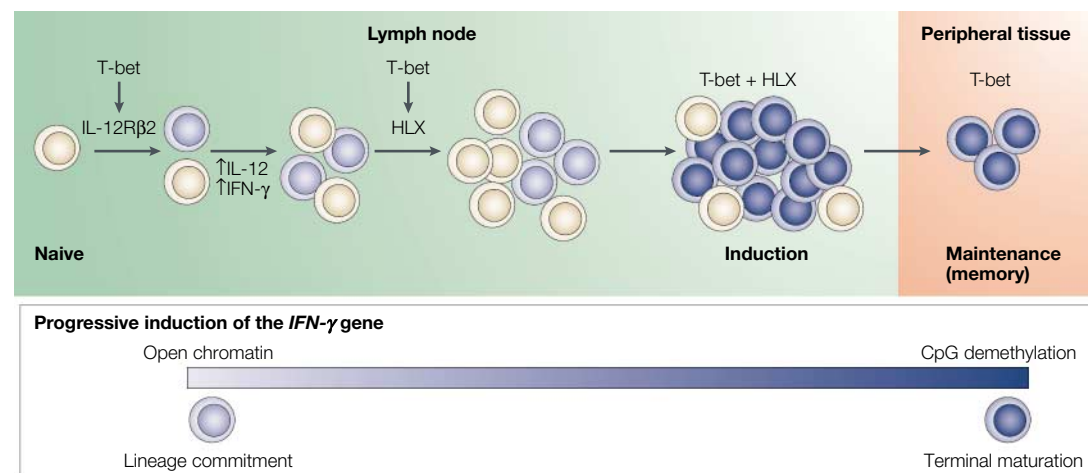


Figure 7 | **Model of stages of T$_H$1-cell induction and maturation.** Hypothetical schema in which the intrinsic regulation of T helper 1 (T$_H$1)-cell development is triggered by T-bet. T-bet induces expression of the interleukin-12 receptor β2-subunit (IL-12Rβ2) and chromatin remodelling of the locus encoding interferon-γ (IFN-γ). Later, T-bet induces expression of HLX (H2.0-like homeobox 1), which cooperates synergistically with T-bet to mediate high-level induction of transcription of the gene encoding IFN-γ. After this stage, the gene encoding IFN-γ undergoes demethylation, probably corresponding to the point at which its transcriptional competence no longer depends strictly on T-bet activity. This might be considered the memory of a differentiated effector cell. It is speculated that the initial cell divisions occur in the lymph nodes, whereas the later stages occur in sites of tissue inflammation. Cell-extrinsic input is provided by IFN-γ signalling, which helps induce expression of T-bet, and IL-12 signalling, which helps T$_H$1 cells to divide and augments transcription of the gene encoding IFN-γ.

**Epigenetic effects — silence is golden**

In the past few years, several lines of evidence have indicated that chromatin structure has an integral role in $T_H$-cell differentiation. The induction of competence for effector-cytokine gene expression seems to involve the derepression of silent chromatin contexts[81,100–102]. In mammals, silencing might involve the concerted actions of epigenetic mechanisms (including DNA methylation), condensation of chromatin and transposition of loci near to heterochromatic nuclear subdomains[103]. Some of these mechanisms are highly interdependent. Proteins that bind methylated CpG, for example, have been shown to recruit histone deacetylases and repressive chromatin-remodelling complexes[104–106]. Conversely, some repressive chromatin- and histone-modifying proteins have been shown recently to influence patterns of DNA methylation[107–109]. The HISTONE CODE has received a great deal of attention as a determinant of transcriptional competence[110]. This is owing to the association of gene activity with specific post-translational modifications of histone tails that differ from the modifications that are associated with gene silencing. How chromatin states (active or silent) are established, maintained, altered and inherited are now fertile areas of investigation.

It is accepted generally that there are various chromatin modifications, lacking in naive T cells, that accompany the acquisition of effector-cytokine gene activity[81,101]. The *IL-4* locus acquires DNASE-I HYPERSENSITIVITY (BOX 1) in $T_H$2 cells, but not in naive $T_H$ or $T_H$1 cells[101]. This acquisition of DNase-I hypersensitivity is genetically downstream of GATA3 expression[8]. Also, the *IL-4* locus becomes demethylated in certain regulatory regions (such as the second intron enhancer)[81], but this has not been shown yet to be a direct effect of GATA3 (REF. 100). Similarly, the gene encoding IFN-γ acquires nuclease hypersensitivity in some of its introns in $T_H$1 cells, but not $T_H$2 cells[6,7,101]. The formation of hypersensitivity site I is genetically downstream of the expression of T-bet[6,7]. Later in differentiation, this region of the gene undergoes demethylation[7]. It is not clear, however, what mediates this effect. Moreover, it has been shown recently that heritably permissive cytokine loci are marked by acetylated histones[98,99], which are a feature of decondensed chromatin.

The role of gene silencing in $T_H$ cells is complicated further by the recent observation that the inactive cytokine loci of naive T cells might undergo a deeper state of silencing in lineages that finally 'forbid' their expression. Using highly sensitive techniques, rapid transcriptional activation of the effector-cytokine genes and localization of these genes to non-centromeric subdomains of the nucleus can be detected in naive CD4$^+$ T cells[96]. In differentiated $T_H$1 and $T_H$2 cells, however, the 'forbidden' cytokine loci seem to become repositioned to centromeres[96] and they can also show evidence of *de novo* CpG methylation[111]. The studies showing non-centromeric location of effector-cytokine loci during their early activity[96,97] might seem to be at odds with the absence of DNase-I hypersensitivity sites[6,7,101,102], hyperacetylated histones[98,99] or demethylation of CpG dinucleotides[81,111], which characterize effector-cytokine

loci in naive T cells. However, this ground state of incomplete gene silencing might indicate simply that there are many levels of chromatin-based repression in the regulation of these lineage-restricted genes.

**How loci are derepressed**

Functional evidence that silencing elements in naive T cells are rate-limiting for the induction of cytokine gene expression was obtained using small-molecule inhibitors of DNA methylation and histone deacetylases[81]. Genetic evidence for silencing effects was obtained recently in mice with a conditional deletion of the maintenance methyltransferase *Dnmt1* in T cells[112]. In addition, mice with a deletion of *Mbd2*, which encodes a methyl-CpG-binding protein that recruits repressive complexes, also have ectopic expression of effector-cytokine genes[100] (FIG. 6). MBD2 links gene silencing to DNA methylation and seems to set the threshold of GATA3 function. Absence of MBD2 markedly increases the efficiency of GATA3-dependent and -independent induction of expression of IL-4 (REF. 100). Indeed, one role of GATA3 as an inducer of expression of IL-4 might be to displace MBD2 from chromatin at the *IL-4* locus, bringing the two opposing regulators — GATA3 the activator and MBD2 the silencer — into genetic and biochemical competition. So, chromatin structure and organization are emerging as important regulatory principles, but it is still controversial how $T_H$ cells develop heritable states of gene activity or silencing.

Several crucial regulatory *cis* elements have been discovered in the *IL-4* locus. The conserved non-coding sequence (CNS) that lies between the *IL-4* and *IL-13* loci, CNS1, has been deleted from transgenic mice harbouring a human $T_H$2-cytokine gene cluster[113], and the endogenous sequence has been deleted also from the mouse loci[114]. Both situations result in a reduction in the level of expression of IL-4. The hypersensitive sites at the 3′ end of the *IL-4* gene, known as V/VA, have been deleted also, leading to a defect in the expression of IL-4 by T cells and mast cells[115]. By contrast, deletion of CNS1 perturbs T-cell production of IL-4 only[114]. So, the chromatin organization of a single locus might be cell-type specific. It seems that the elements contained in CNS1 and the second intron of *IL-4* are sufficient for tissue-specific, chromatin-dependent, GATA3-dependent gene induction[13]. Sites at the 3′ end of the *IL-4* gene might also have a redundant role in promoting $T_H$2-cell-specific activation[13]. Binding of MBD2 has been detected at CNS1 and the second intron of the *IL-4* locus[100].

GATA3 induces changes in chromatin structure of the *IL-4* gene[8,12] that have been associated previously with $T_H$2-cell-specific expression[101]. In addition to altering chromatin structure, GATA3 has been placed genetically upstream of the selective pattern of histone acetylation that is acquired at the *IL-4* locus in $T_H$2 cells[99]. Also, GATA3 seems to influence chromatin activation of the *IL-4* locus by displacing MBD2 (REF. 100) (FIG. 6). This seems to occur at the time of transcriptional induction and before demethylation. Later during differentiation, it seems that the *IL-4* locus undergoes progressive

demethylation in $T_H2$ cells[81,111], although this does not seem to be a proximate effect mediated by GATA3 (REF. 100).

These described actions of GATA3 are reminiscent of the roles of GATA4 and HNF3β (hepatocyte nuclear factor-3β), transcription factors that decompact target genes during liver differentiation[116]. The essential role of GATA3 in the induction of *IL-4* gene expression contrasts with its non-essential role as a conventional transcription factor for the *IL-4* promoter, but a strong transactivator of the proximal *IL-5* promoter[12,117,118]. In addition to the lack of evidence of a role for GATA3 as a
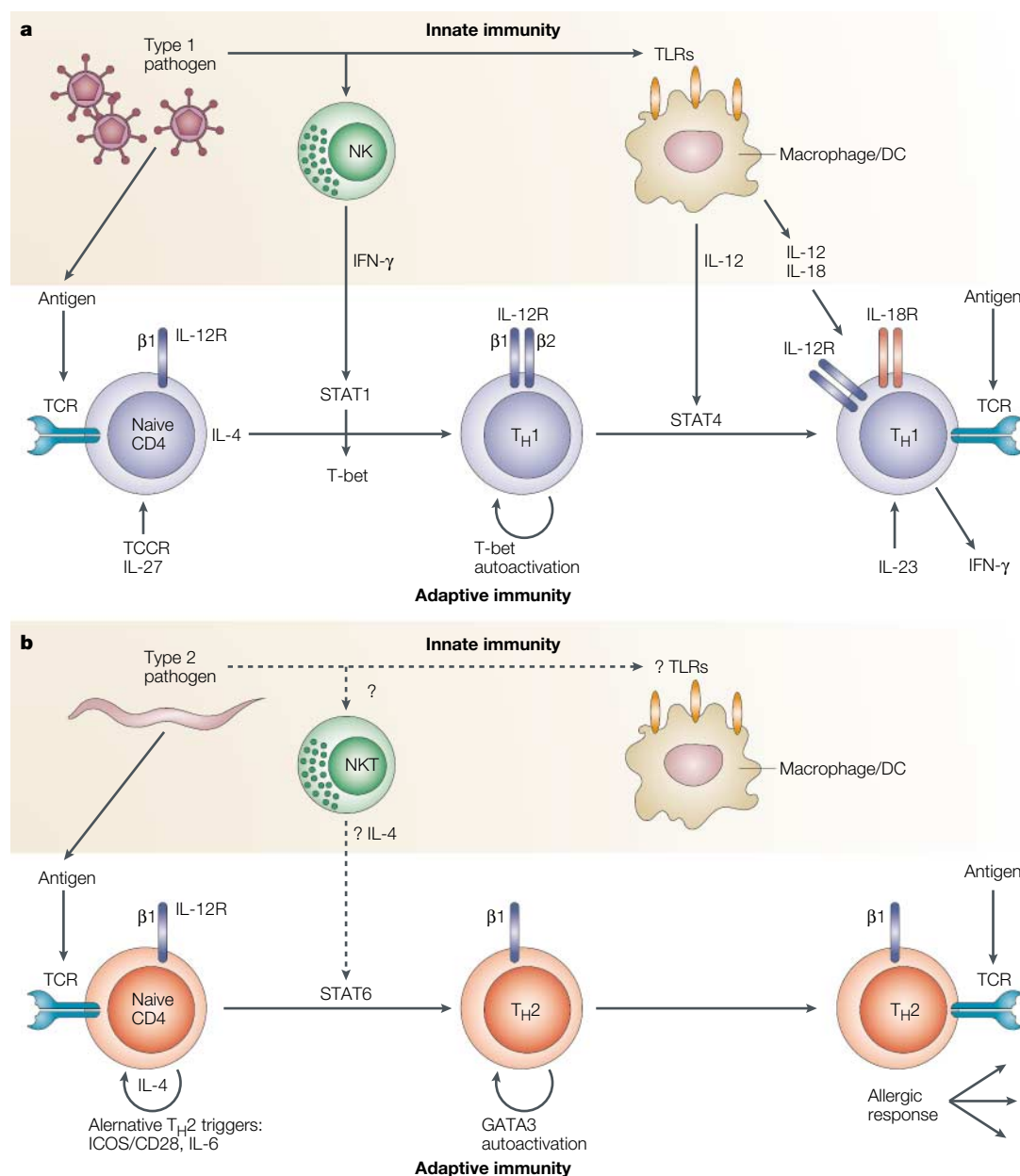


Figure 8 | **Relationships between pathogens, the innate immune system and $T_H$-cell development. a** | T helper 1 ($T_H$1)-cell development is augmented by signals from innate immune responses. The first commitment step results from interferon-γ (IFN-γ) acting through signal transducer and activator of transcription 1 (STAT1), together with T-cell receptor (TCR) signalling, to increase markedly the expression of T-bet by naive T cells. This results in remodelling of the gene encoding IFN-γ to an active status and induces expression of the interleukin-12 receptor β2-subunit (IL-12Rβ2). Next, IL-12 signalling can amplify $T_H$1 responses in two ways. IL-12 can augment the production of IFN-γ directly and can promote expression of the IL-18 receptor (IL-18R), opening up an alternative pathway for IFN-γ production, which might operate for as long as the inflammatory cytokines are produced. DC, dendritic cell; NK cell, natural killer cell. **b** | $T_H$2-cell development could occur in response to an extrinsic source of IL-4 or as a default pathway in the absence of inhibition by innate immune signals. In naive T cells, basal expression of GATA3 is low, but might be sufficient for low-level production of IL-4 by some naive T cells, leading to a cascade of $T_H$2-cell development if no inhibition is encountered. Furthermore, the expression of GATA3 might be under positive regulation by CD28 and inducible co-stimulator (ICOS) signalling, and repression through engagement of leukocyte function-associated antigen 1 (LFA1). So, the priming dendritic cell (DC) might influence $T_H$2-cell commitment through expression of CD80/CD86, PD1 and intercellular adhesion molecule 1 (ICAM1). NKT cell, natural killer T cell. TLR, Toll-like receptor.

classical transactivator of the *IL-4* gene, some evidence indicates that T-bet might also be a remodeller of condensed chromatin structure at the gene encoding IFN-γ, rather than a crucial transactivator of the proximal promoter of this gene. A dominant-negative form of T-bet reduced T$_H$1-cell commitment of naive T cells, but had little effect on the production of IFN-γ by fully differentiated T$_H$1 cells[7]. This indicates that T-bet might not be a classical transcription factor for the *IFN-γ* promoter, a finding that is in line with the non-essential role of T-bet in the expression of IFN-γ by CD8^{+} T cells[4]. This finding indicates also that a key inducer of competence to express a gene might be different from both the factors that maintain heritable competence of gene expression (FIG. 7) and those that mediate acute transcription from that gene (FIG. 3).

### Remembrance of things past

DNA methylation seems to have a pleiotropic role in regulation of the loci encoding IL-4 and IFN-γ. T cells from *Dnmt1*-knockout mice produce excessive amounts of effector cytokines[112]. DNA methylation might have an important role at the start of T$_H$-cell differentiation as a direct inhibitor of factor binding or as a scaffold for silencing complexes[100]. The remodelling of chromatin at the *IL-4* locus and activation of transcription seem to precede the demethylation of this gene[100,111] (FIG. 6). This is true also for the locus encoding IFN-γ[7] (FIG. 7). Together, these data imply that the methyl-mediated repression of gene expression can be separated from the methylation modification itself.

Demethylation seems to correlate with maturation of transcriptional output from a locus[111]. Whether demethylation causes the elevated transcriptional output or is, instead, a modification that 'locks-in' this state is an area of active controversy[119]. The programmed elimination of methyl groups, as arises from interference with maintenance methylation, might simply be a convenient way to imprint the activity of a locus in lymphocytes. The ability to clonally inherit competence for activity of the genes encoding IFN-γ and IL-4, which characterizes terminally differentiated T$_H$1 and T$_H$2 cells, respectively, might be owing to the loss of CpG methylation[81]. Indeed, transcriptional maturation of the gene encoding IFN-γ, which is mediated by the combined actions of T-bet and HLX, seems to precede the onset of demethylation[7] (FIG. 7). Instead, demethylation of the locus encoding IFN-γ seems to correlate most closely with the stage of differentiation at which a dominant-negative form of T-bet no longer antagonizes transcription and chromatin remodelling of the gene encoding IFN-γ[7]. It is uncertain still which factors are responsible ultimately for demethylation of the cytokine loci.

### Where are we now?

Much detail has been added to our picture of T$_H$1- and T$_H$2-cell commitment. To a large extent, the transcriptional and epigenetic changes in cytokine genes that are associated with their selective activation or repression during T-cell activation are the same processes that govern gene expression controlled by tissue- or lineage-specific factors in general. Previous debates about instructive versus selective models have tended to dissipate as examples of both processes in T$_H$2-cell development have been shown to be valid. The original model shown in FIG. 1 has now been modified and extended to include many points of control. FIGURE 8 presents newer models of T$_H$1- and T$_H$2-cell development that incorporate much of the recent transcriptional and signalling advances.

For T$_H$1-cell development, we continue to emphasize the important contribution of the innate immune system, which was recognized initially in 1993 (REF. 120). However, now we understand that both IL-12 and IFN-γ provide important signals for T$_H$1 responses. IFN-γ, derived perhaps from pathogen-activated NK cells, can strongly augment the expression of T-bet during primary T-cell activation through STAT1 signalling, and increased expression of T-bet acts in primary T$_H$1-cell commitment by remodelling at the IFN-γ locus and by driving expression of the IL-12 receptor. IL-12, derived perhaps from pathogen-activated macrophages (or from appropriately activated DCs) amplifies the production of IFN-γ by T cells and drives increased expression of the IL-18 receptor, which opens the door to an alternative pathway for inducing IFN-γ production. Issues that are not completely resolved yet include the mechanisms of action of IL-27 and TCCR in early T$_H$1-cell priming, whether expression of T-bet involves transcriptional autoactivation in a similar manner to that ascribed to GATA3, and the role of IL-23 in pathogen-mediated versus autoimmune responses. In summary, T$_H$1 responses seem to be driven primarily by active signals derived from pathogen-activated innate immune sources, and a strong T$_H$1 response might be maintained only in the presence of such signals. In this manner, when the pathogen has been cleared, signals driving the high-level production of IFN-γ (and its potentially dangerous consequences) should abate.

For T$_H$2-cell development, it is still uncertain whether any active innate signals drive this process. Although certain possible sources, such as NKT cells and basophils, have been considered (reviewed in REF. 121), these do not seem to be necessary. Potentially, it might be that the absence of activation of the innate immune system removes inhibition of T$_H$2-cell development by IFN-γ and IL-12, which allows T$_H$2-cell development to be driven by positive feedback through IL-4 and GATA3. As T$_H$2-lineage commitment in some cells can occur independently of STAT6 activation, it is possible that some alternative signal, such as IL-6 (IFN-β2) or B7H expressed by DCs, might trigger the initial production of IL-4. However, the identity of such alternative stimuli remains an unresolved issue. Importantly, *GATA3* transcription itself seems to be responsive to GATA3 protein, providing an intrinsic signal that maintains T$_H$2-cell commitment at the cellular level. After T$_H$2-cell commitment, GATA3 has downstream effects on several genes, and it is probable that in the near future we will make progress in understanding precisely how these effects occur.

## Concluding remarks

The study of T_H-cell differentiation *in vitro* is anticipated to continue to yield insights into the problem of how cells give rise to alternative fates. This *in vitro* system provides robust conditions to drive polarized differentiation and exciting tools to analyse the developmental genetics and biochemical foundations of heritable states of gene expression. However, we should keep in mind that ultimately these T_H-cell subsets provide protection against pathogens, which require different types of effector mechanism. Future studies will

need to analyse T_H1- and T_H2-cell commitment *in vivo*. The first reagents for such studies have now been reported in the form of cytokine-reporter-knockout mice, in which cytokine gene expression is not perturbed[88,122]. Although *in vitro* analyses have provided information about the signals and factors that act in lineage commitment, we might soon know more about the physiological context — when and where various steps in commitment take place in response to pathogens, and from which APCs T cells receive instructive or selective signals.

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