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# How are $T_H1$ and $T_H2$ effector cells made?

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Differentiation of $T_H1$ and $T_H2$ effector cells proceeds through several phases: First, naïve $CD4^+$ precursor cells are instructed to differentiate as appropriate to optimally fight the infectious threat encountered. This process is governed by the IL12 and IL4 cytokines, as well as by signaling through the Notch receptor. In response to these signals, transcription is initiated of lineage specific cytokine genes including the *Ifnγ* and *Il4* genes as well as of genes encoding transcriptional regulators, such as T-bet and Gata3. The respective differentiation programs are reinforced by both positive and negative feedback mechanisms. Furthermore, epigenetic modifications of the lineage specific genes result in the emergence of regulatory elements, which control high level lineage restricted expression by both intrachromosomal and interchromosomal associations. Together, these mechanisms ensure stable inheritance of the differentiated fate in the numerous progeny of the original naïve $CD4^+$ T cells.

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## Introduction

Immunity to different classes of microorganisms is orchestrated by separate lineages of effector T helper ($T_H$)-cells [1], which differentiate from naïve $CD4^+$ precursor cells in response to cues provided by antigen presenting cells (APC) [2]. In this review, we will discuss the molecular mechanisms that control induction of the effector lineage as well as the genetic and epigenetic events controlling long term stable expression of the lineage specific cytokine genes. Although multiple different $T_H$-cell lineages have been identified, we will focus here on the $T_H1$-cell lineage, characterized by production of IFNγ and responsible for orchestrating immunity to intracellular microorganisms, and the $T_H2$-cell lineage, producing IL4, IL5, and IL13, which directs responses to parasites [1].

## Cytokine induced $T_H1/T_H2$ induction

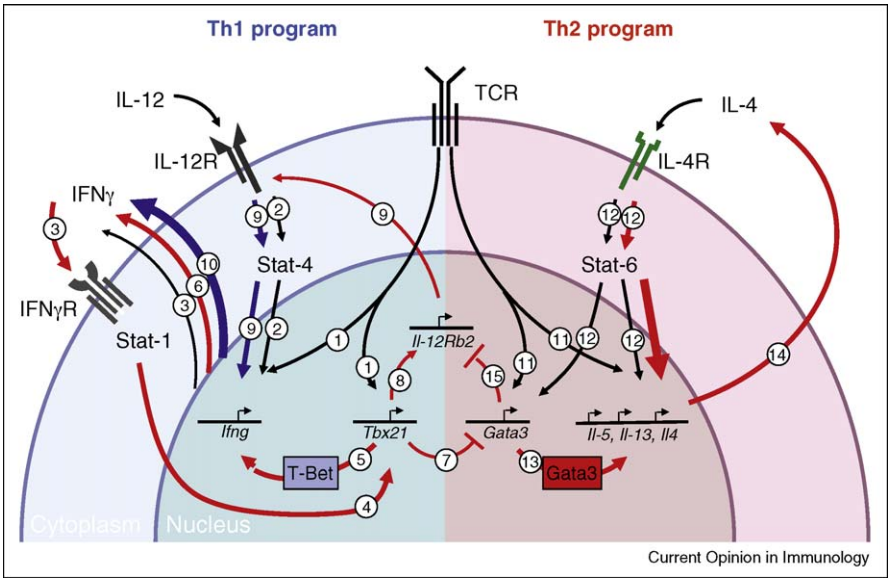
Activation of naïve $CD4^+$ T cells *in vitro* in the presence of exogenously added IL12 skews their differentiation to the $T_H1$-cell lineage (Figure 1). The major effector of IL12 receptor signaling is the transcription factor STAT4, which promotes expression of multiple $T_H1$-cell genes, including the *Ifnγ* gene, at least partly by inducing distinct chromatin modifications [3•]. STAT4 collaborates in this with the transcription factor T-bet [3•]. Expression of T-bet is induced by TCR signaling and strongly elevated by activation of the STAT1 transcription factor, which occurs in a positive feedback loop in response to auto/paracrine produced IFNγ [4]. One of the genes induced by T-bet encodes Runx3 [5,6•] and together Runx3 and T-bet bind to several enhancers and the promoter of the *Ifnγ* gene, further promoting its transcription. Runx3 and T-bet also bind to a silencer in the *Il4* gene, resulting in transcriptional repression of this $T_H2$ gene [5,6•]. Finally, T-bet promotes expression of the IL12 receptor β chain [4], increasing responsiveness to IL12. Among the many functions T-bet performs, the crucial function may be to antagonize Gata3 [7], a transcription factor controlling $T_H2$ differentiation.

Addition of exogenous IL4 to *in vitro* differentiation cultures promotes $T_H2$ differentiation (Figure 1) [8]. This depends on activation of the transcription factor STAT6, which induces expression of Gata3 and may directly transactivate the *Il4* gene [8]. An important function of Gata3 is to reorganize chromatin structure in the so called $T_H2$ locus (see below), encompassing the IL4, IL5 and IL13 genes [8]. In addition, Gata3 opposes $T_H1$ differentiation by inhibiting expression of the IL12 receptor β chain and of STAT4. STAT6 signaling also induces expression of c-Maf, a transcription factor necessary for high-level expression of the *Il4* gene [8].

## Notch ligands are alternative signals for $T_H1/T_H2$ differentiation

Not all $T_H1$ responses require IL12. $T_H1$ responses to certain viruses are not dependent on IL12, for example [9–11]. Also, many $T_H2$ responses *in vivo* are largely independent of IL4 receptor signaling, including those to parasites [12–14,15•]. Alternative $T_H1$ and $T_H2$ instructing signals must therefore exist. Ligands for the Notch pathway are candidate-instructing signals for both lineages.

**Figure 1**




IL12 and IL4 driven T helper differentiation. $T_H1$ induction by IL12: Initial TCR activation induces low-grade expression of the *Ifng* and the *Tbx21* genes (1). Signaling through the IL12 receptor results in STAT4 mediated promotion of *Ifn*γ expression (2). Binding of the IFNγ receptor by low initial auto/paracrine produced IFNγ activates STAT1 (3), which strongly promotes expression of the *Tbx21* gene (4). T-bet then enhances the transcriptional competence of the *Ifn*γ gene (5) leading to increased production of this cytokine (6). In addition, T-bet prevents $T_H2$ differentiation by inhibiting Gata3 (7). Finally, T-bet promotes expression of the IL12 receptor β2 chain (8), resulting in greater IL12 responsiveness (9) and yet further elevated production of IFNγ (10). $T_H2$ induction by IL4: Initial TCR signaling induces low-level expression of the *Il4* and *Gata3* genes (11). IL4 receptor signaling strongly promotes expression of these two genes (12). Gata3 reorganizes chromatin structure in the $T_H2$ locus, encompassing the *Il4*, *Il5*, and *Il13* genes, enhancing their transcription competence (13). Increased IL4 production further enhances $T_H2$-cell differentiation in a feed forward loop (14). Finally, Gata3 prevents the $T_H1$ differentiation program by inhibiting expression of the IL12 receptor β2 chain (15) and of the *Stat4* gene (not depicted). Primary events are indicated with black arrows, secondary events with red arrows, and tertiary events with blue arrows.

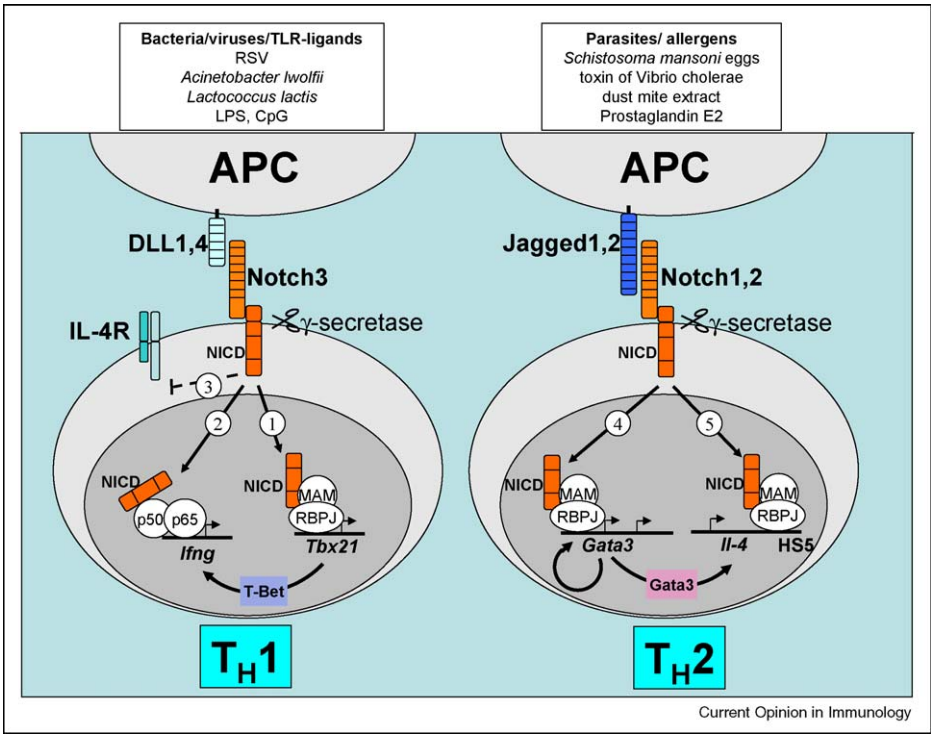
The heterodimeric cell surface receptor Notch consists of an extracellular domain, which is non-covalently associated with a transmembrane polypeptide (Figure 2) [16]. In the canonical signaling pathway, ligand binding by Notch leads to cleavage, which allows the intracellular domain (NICD) to translocate to the nucleus and convert the DNA binding protein RBP-J (also known as CSL) into a transcriptional activator (Figure 2) [16]. Two conserved families of ligands exist, called Jagged and Delta-like (DLL) (Figure 2), which are structurally quite distinct [16]. Jagged and DLL are both capable of inducing the same canonical Notch signaling pathway. However, the different ligands do possess unique functions, although the underlying mechanisms are not understood [17].

## Induction of $T_H1$-cell differentiation by Notch

Expression of DLL ligands (1 or 4) is induced on APC by stimulation with microbial products that promote the ability of APC to induce $T_H1$-cell differentiation (Figure 2) [18,19,20•,21–23]. Furthermore, when overexpressed on APC or when crosslinked as fusion proteins, DLL ligands promote $T_H1$-cell differentiation [18,20•,22,24–26]. Although genetic evidence supporting a requirement for the Notch pathway in $T_H1$ responses is currently lacking [18,27,28], blockade of DLL ligands did reduce $T_H1$ responses *in vivo* [20•,24,25].

T cell specific RBP-J deficiency did not block $T_H1$-cell responses in several studies [18,27,28,29•]. However, since these studies employed IL12 dependent $T_H1$-models, whereas Notch may be required specifically in the absence of IL12 [20•], no definitive conclusion can currently be drawn about the involvement of RBP-J in $T_H1$-cell induction. Consistent with a role for RBP-J in this, Notch activated expression of the *Tbx21* gene (which encodes T-bet) and RBP-J associated with the *Tbx21* gene *in vivo* in a T cell hybridoma (Figure 2) [30], although this could not be confirmed in primary T cells [31•]. A potentially RBP-J-independent mechanism involves the ability of Notch to prolong the nuclear retention of the NFκB family proteins P50 and P65 (Figure 2) [32]. However, since at least P50 has also been reported to induce expression of the $T_H2$ factor Gata3 [33], it is not obvious how these effects on NFκB proteins would specifically translate into selective $T_H1$-cell differentiation. Finally, DLL may inhibit $T_H2$-cell differentiation by interfering with IL4 receptor signaling (Figure 2) [22].

**Figure 2**




Notch mediated $T_H$-cell differentiation. Notch signaling: Two families of Notch ligands exist, called DLL and Jagged. Notch is present as a heterodimer on the surface of the T cells. Binding of ligand to the extracellular domain of Notch results in γ-secretase mediated cleavage of the transmembrane portion of Notch, allowing the intracellular domain (NICD) to translocate to the nucleus, where it forms a trimolecular complex with the DNA binding protein RBP-J and the transcriptional coactivator Mastermind-like (MAM). $T_H1$ (left): Members of the DLL family of Notch ligands are expressed on APC in response to microbial stimuli that promote $T_H1$-cell induction by APC. Notch may promote $T_H1$-cell differentiation by direct transactivation of the *Tbx21* gene (1), leading to expression of T-bet, transactivation of the *Ifn*γ gene via binding to NFκB family members like p50 (2), and indirectly through inhibition of IL4 receptor signaling (3). $T_H2$: Expression of Jagged family members is induced on APC by $T_H2$ promoting microbial and pro-inflammatory stimuli. Notch directly promotes activity of the upstream Gata3 promoter resulting in transcription of the *Gata3* gene (4). Furthermore, Notch regulates transcription of the *Il4* gene by binding to the HS5 enhancer (5). Please note that crucial events, such as TCR signaling have been omitted for clarity.

## Induction of $T_H2$-cell differentiation by Notch

Interestingly, Notch has also been implicated in $T_H2$-cell differentiation. In fact, genetic loss of function studies have shown that Notch is essential for $T_H2$-cell responses under physiologically relevant conditions, such as during parasite infections [18,27,28,29•,34]. The consequence of Notch ligation ($T_H1$ or $T_H2$) may depend on the ligand used to activate Notch. Whereas expression of DLL ligands is associated with $T_H1$ differentiation, expression on APC of Jagged ligands is associated with $T_H2$ responses (Figure 2), and ectopically expressed Jagged1 or crosslinked Jagged1-Fc fusion proteins can promote $T_H2$-cell differentiation [18,25,34–36]. A requirement for Jagged ligands in $T_H2$-cell responses has, however, not (yet) been demonstrated [35,37].

$T_H2$-cell induction by Notch depends on the canonical signaling pathway involving RBP-J. $T_H2$-cell responses are severely diminished in the absence of T cell specific expression of RBP-J or of Notch1 and Notch2, as well as in transgenic mice expressing a dominant negative version of the Notch cofactor MAML1 (Figure 2) in T cells [18,27,28,29•]. Several studies indicated that Notch regulates transcription of crucial $T_H2$-cell genes. Thus, the upstream (but not the downstream) promoter of the *Gata3* gene responds to Notch signaling and both RBP-J and Notch1 associate with a conserved site in this promoter *in vivo* (Figure 2) [29•,31•]. Also, Notch directly activates transcription of the *Il4* gene via conserved RBP-J binding sites in the HS5 enhancer (Figure 2) [18,38]. The fact that IL4 is genetically downstream of Notch explains why exogenously added IL4, such as in traditional cytokine mediated skewing experiments, overcomes the requirement for Notch in $T_H2$-cell differentiation [30,39].

The available data are consistent with the following model (Figure 2): Since Gata3 is necessary for Notch induced $T_H2$-cell differentiation [29•,31•], Notch may promote expression of the Gata3 gene first. Gata3 protein

then remodels the $T_H2$ cytokine locus, rendering the HS5 enhancer accessible to Notch. Ensuing auto/paracrine production of IL4 further enhances $T_H2$ differentiation. In a further positive feedback mechanism, Gata3 protein reinforces expression of the Gata3 gene, and this effect from Gata3 is synergistically enhanced by Notch [31•]. Crucial elements of this model still need to be proven definitively. One important experiment would be to delete the Notch responsive element in the *Gata3* gene to formally determine whether transactivation of *Gata3* by Notch is indeed necessary for $T_H2$ differentiation.

## The emergence of regulatory elements

Although the *Ifn*γ and *Il4* genes are transcribed in naïve T cells within 3–24 h after initial activation [40], only upon terminal differentiation several days later does strong lineage specific expression occur. This elevated and lineage restricted expression requires increased expression of positively or negatively acting lineage specific transcription factors. These factors bind to the gene promoters as well as lineage specific regulatory regions, which are induced during differentiation by chromatin modifying enzymes associated with factors such as T-bet and Gata3.

Regulatory elements have been identified by a combination of bioinformatics and biochemistry. Conserved (between species) non-coding sequences (CNS) indicated the existence of important regulatory elements, and hypersensitivity to digestion by DNAse I revealed the binding of protein factors typically found at regulatory elements. During the differentiation process, many epigenetic modifications are made to these regulatory regions. The histones in the chromatin of cytokine loci of naïve T cells are hypo-acetylated. TCR stimulation induces rapid acetylation on histones H3 and H4 in the cytokine gene promoters, irrespective of polarizing conditions. The lineage specific maintenance of acetylation depends on cytokine dependent STAT signaling and lineage specific transcriptional activators T-bet and GATA3 [41].

## *Cis*-regulatory elements in the *Ifn*γ cytokine locus

Expression of the *Ifn*γ gene is regulated by its *cis* acting promoter and by two intronic regulatory elements (Figure 3) [42]. These elements are not by themselves sufficient to achieve $T_H1$ specific expression. Indeed, additional regulatory elements have been characterized. Two of these, *Ifn*γ-CNS1 and *Ifn*γ-CNS2, are located 5 kb upstream and 18 kb downstream of the initiation codon of the murine *Ifn*γ gene, respectively (Figure 3) [43,44]. Furthermore, studies utilizing bacterial artificial chromosomes containing a human *Ifn*γ transgene suggested that for proper expression yet additional elements are required, which are dispersed in an extended region of over 100 kb around the gene (Figure 3) [41].

In $T_H1$ cells, the CNSs in the *Ifn*γ locus contain dimethylations of histone H3 lysine 4 (H3K4), a stable mark associated with poised or actively transcribed chromatin. Furthermore, H3K9 methylation occurs after T cell activation and is maintained throughout $T_H1$-cell differentiation [45••]. By contrast, this modification is diminished in $T_H2$ cells in a GATA3 dependent manner, and both STAT6 and GATA3 are recruited to the *Ifn*γ locus in $T_H2$ cells [46]. Also recruited is the polycomb group methyltransferase enzyme EZH2, which initiates repressive H3K27 methylation. Correspondingly, trimethyl H3K27 is broadly distributed throughout the locus in $T_H2$ cells. Thus, multiple regulatory regions have now been identified in the *Ifn*γ locus, which undergo dynamic modifications during cellular differentiation.

## *Cis*-regulatory elements in the $T_H2$ cytokine locus

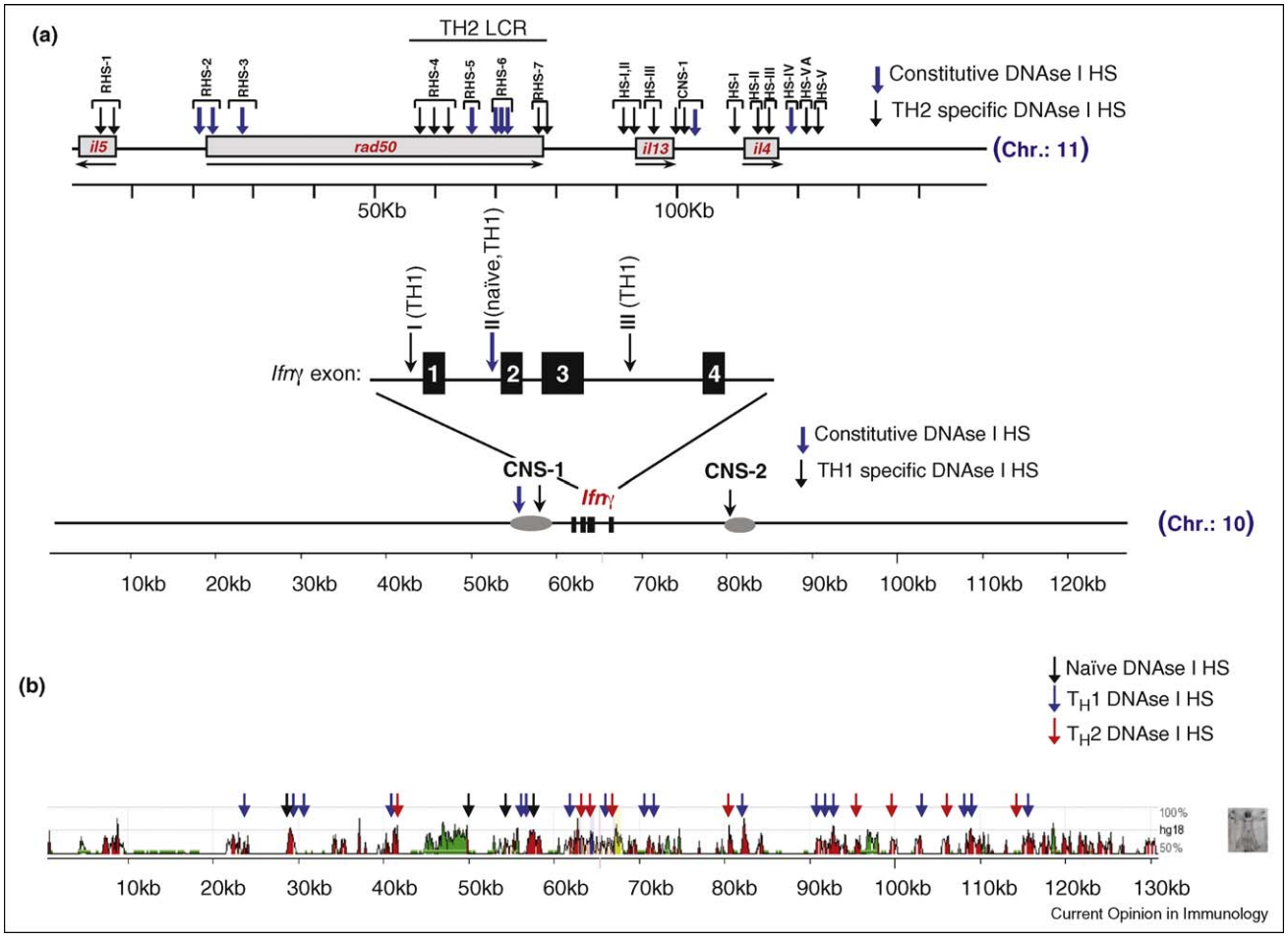
The $T_H2$ cytokine locus comprises the *Il4*, *Il5* and *Il13* cytokine genes and stretches out over 120 kb. Between the *Il5* and *Il13* genes resides the *Rad50* gene, which is constitutively expressed and not coregulated with the $T_H2$ cytokine genes. Multiple DNAse I hypersensitive sites have been identified in the $T_H2$ locus. Some of these flank the *Il4* and *Il13* genes (Figure 3) and together controlled expression of reporter transgenes in a $T_H2$-specific fashion [47,48]. Among these is the 3′ Notch responsive enhancer HS5 [18,38]. Additionally, seven hypersensitive sites exist more distally. Four of these (*RHS4-7*) contain the so called $T_H2$ locus control region (LCR) at the 3′ of the *Rad50* gene (Figure 3). This was shown by the ability of this region to support high level tissue ($T_H2$)-specific expression of linked reporter transgenes, irrespective of any flanking sequences [49,50•], consistent with the operational definition of LCRs.

## Long range intrachromosomal and interchromosomal interactions

As discussed, many of the regulatory regions are located far from the cytokine genes. How then do such elements act on their target genes? Using the so called Chromosome Conformation Capture (3C) assay [51•], it was shown that the *Ifn*γ locus adopts a secondary chromatin configuration where distant regulatory regions come in close proximity with the promoter of the *Ifn*γ gene, depending on the expression profile of the cell (Figure 4) [52]. Similarly, the coordinated regulation of the $T_H2$ cytokine genes involves the generation of chromatin loops that bring together the promoters of the genes, their enhancers and the $T_H2$ LCR (Figure 4) [53]. This LCR regulates the cytokine genes without affecting the constitutively expressed *Rad50* gene, which is looped out of the active chromatin configuration (Figure 4).

Quite unexpectedly, long-range chromatin interactions are not restricted to intrachromosomal regions. The $T_H2$

**Figure 3**



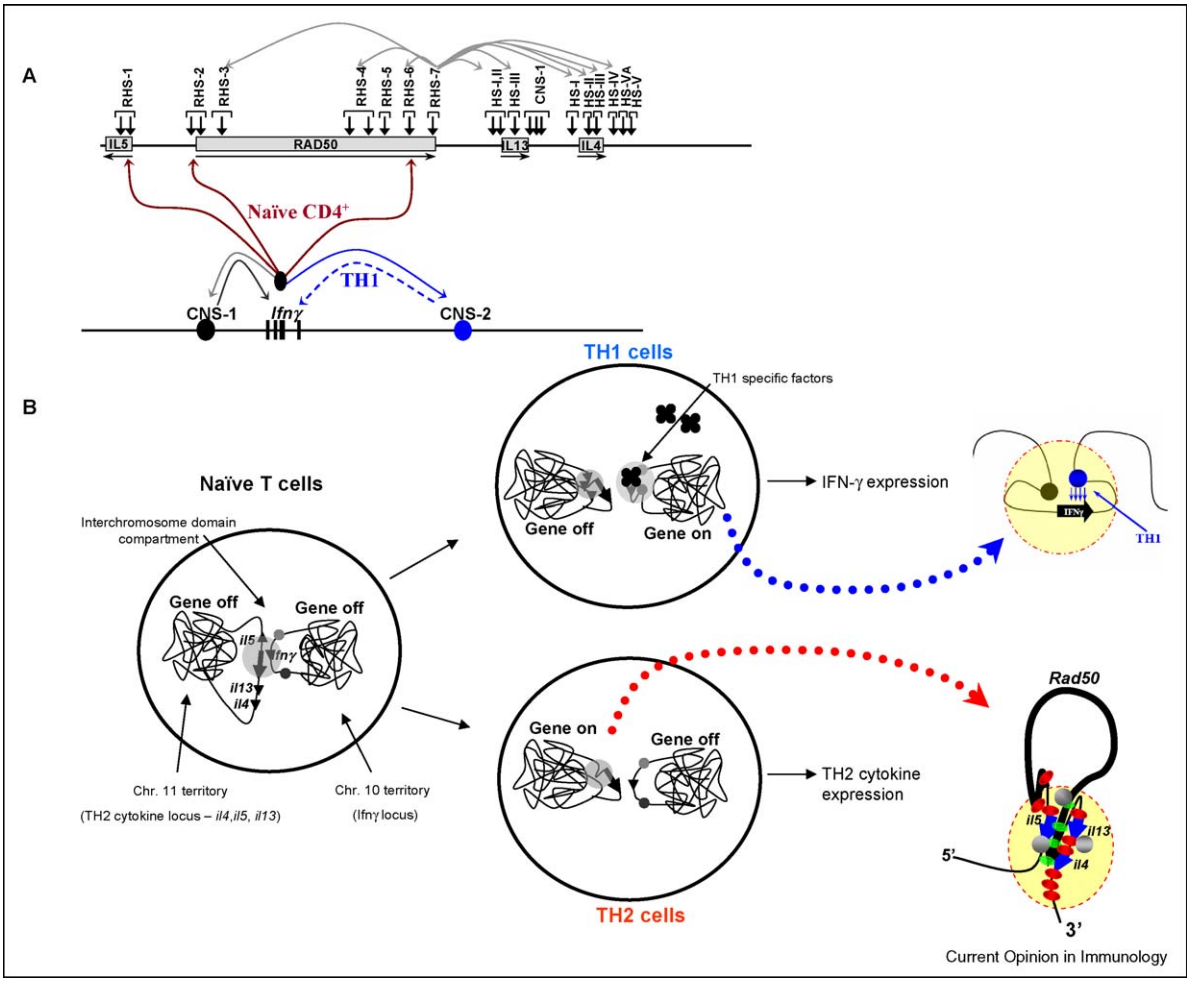

Genomic organization of the $T_H2$ cytokine locus on mouse chromosome 11 and the *Ifn*γ locus on mouse chromosome 10. **(a)** Blue bold arrows indicate constitutive (present in naïve $CD4^+$, $T_H1$, and $T_H2$ cells) DNAse I hypersensitive sites and black arrows indicate DNAse I hypersensitive sites specific in $T_H2$ cells (for the $T_H2$ locus) or $T_H1$ cells (for the *Ifn*γ locus). Vertical boxes on the loci indicate gene exons. **(b)** Evolutionary conserved regions in the IFNγ locus. Cross species conservation analysis of at least 100 bp and 70% DNA homology between mouse and human species for the genomic regions displaying the *Ifn*γ locus on mouse chromosome 10 using the ECR browser (http://ecrbrowser.dcode.org) (Colors depicting the conserved regions: blue-coding regions, yellow-untranslated regions, red-intergenic region, green-transposons and simple repeats, pink-intronic regions). Arrows on top depict DNAse I hypersensitive sites in naïve $CD4^+$ cells (black), $T_H1$ cells (blue), and $T_H2$ cells (red).

LCR and other regulatory elements in the $T_H2$ locus, localized on mouse chromosome 11, colocalize and come in close physical proximity with the *Ifn*γ gene locus, located on mouse chromosome 10 [54•]. Analysis utilizing genetically modified animals suggests a direct mechanism for transcriptional gene regulation of genes by regulatory elements located on different chromosomes. A genetic deletion of the RHS7 DNAse I hypersensitive site of the $T_H2$ LCR that predominantly regulates the expression of the $T_H2$ cytokine gene loci on chromosome 11 could affect the expression profile of the *Ifn*γ gene on mouse chromosome 10 [54•]. Interchromosomal interactions opened a completely new avenue in our understanding of gene regulation. This phenomenon now appears to be a general mechanism employed in multiple genetic systems [40].

## Subnuclear localization of loci in lymphocytes

Given that regulatory elements can control genes on different chromosomes, another layer of control over gene expression involves the subnuclear localization of genes. Genes tend to relocalize from the nuclear periphery to the inner part of the nucleus upon their activation [55]. Silent genes in developing B and T cells are repositioned in the nucleus to pericentromeric heterochromatin showing a direct link between the subnuclear localization of genes and differentiation [56,57]. Regulatory elements such as LCRs, enhancers, or insulators also act by repositioning specific genetic loci to regions with active or silent transcription. Furthermore, sequence specific DNA binding proteins may act by directly repositioning loci to relevant chromatin compartments [58,59].

**Figure 4**




Dynamic interplay of chromosomal interactions in the nucleus of T cells. **(a)** Arrows on top of the $T_H2$ cytokine locus represent the intrachromosomal interactions between the RHS7 DNAse I hypersensitive site of the $T_H2$ LCR and several other genomic regions of the $T_H2$ locus in $T_H2$ cells. Arrows on top of the *Ifn*γ locus depict the intrachromosomal as well as the interchromosomal interactions captured for the *Ifn*γ–$T_H2$ cytokine loci. The arrow with the discontinuous line represents the $T_H1$ specific intrachromosomal interaction between two regions of the *Ifn*γ locus. **(b)** Schematic hypothetical representation of the territories for mouse chromosome 10 and 11. The $T_H2$ cytokine locus is located on mouse chromosome 11 and the *Ifn*γ locus is located on mouse chromosome 10. We hypothesize that the interchromosomal interactions between the $T_H2$ cytokine locus and the *Ifn*γ locus are taking place in the interchromosome domain compartment in naïve $CD4^+$ cells potentially keeping the two loci poised and at the same time transcriptionally silent for rapid expression upon TCR stimulation. During differentiation of naïve $CD4^+$ T cells to $T_H1$ cells the *Ifn*γ gene is mainly regulated through intrachromosomal interactions with regulatory elements in the locus, and in $T_H2$ cells intrachromosomal interactions between the $T_H2$ LCR and the cytokine gene promoters upon the action of $T_H2$ cell lineage specific factors regulate transcription.

Reportedly, the transcriptionally silent *Il4* (in $T_H1$ cells) and *Ifn*γ genes (in $T_H2$ cells) are associated with centromeric domains [60], although this was not confirmed by another group. Instead, it was found that during $T_H1$ differentiation, the *GATA3* and *c-maf* loci were progressively repositioned to centromeric heterochromatin and/or the nuclear periphery, compartments associated with transcriptional repression. Therefore, the loci of the transcriptional regulators of the cytokine genes may reposition in the cell nucleus during T cell differentiation rather than the cytokine gene loci themselves [61].

## Conclusion

Differentiation of naïve $CD4^+$ T helper cells into the $T_H1$-cell and $T_H2$-cell lineages occurs in response to specific information from APC and cytokines. In addition to the cytokines IL4 and IL12, ligands for the Notch pathway are also involved in this process.

TCR stimulation in combination with instructional differentiation signals results in the activation of specific transcription factors that initiate cytokine gene expression and modify chromatin structure of regulatory regions.

These control strong lineage specific expression of the cytokine genes both by acting on genes located within the same chromosome, as well as with genes located on other chromosomes. How such intrachromosomal and interchromosomal interactions control gene transcription is an important question for the future.

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