

REVIEW ARTICLE

Epigenetic regulation of Th2 cytokine expression in atopic diseases

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Abstract

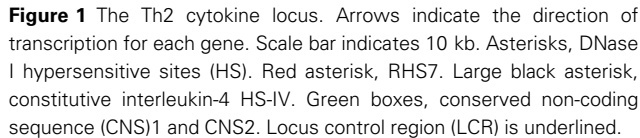
The immune response to allergens starts with stimulation of a naïve T helper (Th) cell and its differentiation into a Th2 cell, expressing the cytokines interleukin (IL)-4, IL-5 and IL-13 responsible for the allergic response. The initial pattern of cytokine expression is retained during restimulation and division of the Th2 cell to create a population of specific allergen-responsive memory Th2 cells. Both, the coordinate cytokine expression and the inherited cytokine memory are specified by epigenetic mechanisms. Th2-specific changes in chromatin configuration at the Th2 locus act locally to open DNA, allowing recruitment of transcriptional machinery and rapid induction of cytokine expression. Induction of the transcription factor GATA3 is critical to this process. Loss of DNA methylation at the Th2 locus during differentiation from a naïve Th cell correlates to increased histone acetylation, consistent with the expression of IL-4, IL-5 and IL-13. The silencing of the Th2 locus in Th1 cells was associated with repressive histone methylation. These data indicate the formation of a 'poised' chromatin configuration at the Th2 locus that in combination with specific transcription factors specifies the cytokine repertoire in daughter cells and allows the immediate, rapid induction of cytokines by those cells in response to allergen.

Introduction

Allergic reactions, asthma and immunity to helminths are all immune responses that are highly dependent on the activities of a specific CD4⁺ T-cell subset termed the Th2 cell. This normally rare CD4 T-cell subset appears soon after sensitization and response to environmental or worm allergens and differs completely from the Th1 cell that appears in immune responses to viruses and bacteria. The phenotype of the fully differentiated Th2 cell is characterized by the coordinated production of the cytokines IL-4, IL-5 and IL-13, which, in conjunction with other inflammatory mediators direct the activation and tissue accumulation of eosinophils, basophils and B cells switching to IgE, which are all hallmarks of the allergic/atopic immune response. In allergy and asthma, the normal Th1/Th2 balance is skewed, with an exaggerated Th2 response to environmental allergens leading to eosinophil infiltration, inflammation and the symptoms of atopy/asthma. This increased Th2 cytokine expression could result from either

loss of normal silencing or abnormal activation, or a combination of the two. The prenatal cytokine milieu is predominantly Th2, with the Th1 arm maturing postnatally. Defects in Th2 silencing during this postnatal period, leading to continued inappropriate expression of Th2 genes could be associated with the development of, or susceptibility to, atopy and asthma (1). Alternatively, exposure to a variety of environmental stimuli, including diesel exhaust, wood smoke and tobacco, all known to increase incidence and severity of asthma and allergy, also increases Th2 cytokine expression (2, 3). The ability to manipulate or suppress expression of Th2 cytokines would alleviate the symptoms of these diseases.

The genes encoding IL-4, IL-5 and IL-13 are all located in the same chromosomal region, 5q31.1 in humans and chromosome 11 in the mouse (Figure 1). Cytokine expression is accompanied by increased expression of the Th2-associated transcription factor GATA3 and progressive epigenetic changes in the chromatin structure, including



Heritable chromatin remodelling at the Th2 locus

The first direct evidence that epigenetic changes might be responsible for acquisition of a heritable cytokine expression pattern came in 1998 in a study by Agarwal and Rao (4) who showed that changes in chromatin structure are reflected in transcription of the IL-4, IL-13 and IFN- γ genes in differentiated CD4+ T cells and that these changes parallel the ability of Th1 and Th2 cells to inherit a pattern of rapidly inducible transcription of these cytokine genes. Chromatin remodelling at the IL-4 locus was assayed using the DNase I hypersensitivity assay. In transcriptionally silent areas of the chromosome DNA is highly compacted into cells by bundling around histone proteins and tightly wound into heterochromatin. In this form, it is resistant to enzymatic digestion by DNase I. When DNA is in the active chromatin configuration, termed euchromatin, transcription

In addition to changes in gross chromatin configuration, Th2 clones derived from these cultures displayed extensive loss of DNA methylation at the IL-4 locus compared with Th1 clones and naïve Thn cells. Methylation of DNA occurs at cytosine residues, particularly those followed by a guanine, the so-called CpG dinucleotide. Hypermethylation of CpG in promoter and regulatory regions is a characteristic of silenced genes, and the IL-4 locus was extensively hypermethylated in Th1 and Thn cells compared with Th2 cells (5). Changes in chromatin configuration were predominantly conserved between the human and the mouse loci, with differences seen mainly in the extent of DNA methylation across the locus. Thus, differentiation of naïve cells under polarizing conditions led to a Th2-specific DNA demethylation associated with transcriptional competence at the Th2 cytokine locus.

The observed epigenetic modification of the chromatin structure during differentiation of CD4⁺ Th2 cells provides an excellent model for explaining several aspects of Th2 differentiation. The development of an open chromatin configuration at specific Th2 cytokine gene loci allows for an acute response to secondary allergen stimulation. The structural changes to chromatin allows the Th2 cytokine genes to be directly and rapidly accessed by specific transcription factors, leading to a faster production of cytokines, which contributes to the speed of a memory response. Also, because epigenetic changes are heritably retained, a mechanism is created that enables specific Th2 cytokine memory to be formed and maintained in individual allergen-specific memory Th2 cells.

The locus control region and other *cis*-acting sites in chromatin remodelling of the Th2 locus

Chromatin remodelling has also been proposed as the mechanism responsible for coordinate regulation of the entire Th2 cytokine locus. Similar to the events at the *IL-4* gene, the appearance of DNase I HSs near the *IL-13* gene correlated to a transcriptionally competent locus in Th2

cells indicating that both *IL-4* and *IL-13* share a common repressed chromatin environment under Th1 conditions and a common accessible chromatin configuration after Th2 polarization (6). Expression of the *RAD50* gene, which lies within the Th2 locus between *IL-13* and *IL-5*, was also found to be significantly higher in Th2 than in Th1 clones, although *RAD50* is constitutively expressed, not regulated in parallel with the Th2 cytokines and not directly activated by α CD3 T-cell stimulation. This indicates that Th2 polarization results in the increase of the region of accessible chromatin, which in turn increases chances of transcription from nearby alleles.

The mechanisms that coordinate the regulation of the Th2 locus have been shown to involve a locus control region (LCR), initially identified using bacterial artificial chromosome transgenic mice. Deletion analysis of the Th2 locus defined a 25-kb fragment in the 3' end of *RAD50* gene as the region responsible for coordinate expression of the Th2 cytokines (7). Deletion of this 25-kb fragment in the transgene caused loss of expression from the *IL-4* and *IL-13* alleles, but surprisingly not the *IL-5* allele (8), possibly because it is located 5' end of *RAD50*. In naïve cells the Th2 LCR DNA is hypermethylated and contains no DNase I HS. However, Th2 differentiation leads to demethylation and the appearance of four HSs within the LCR. One of these (RHS7, Figure 1), has been found to be essential for mediating the coordinate regulation of Th2 expression while excluding *RAD50* (9). Using chromosome conformation capture technique it was shown that the LCR allows the promoters of the Th2 cytokines genes to be physically brought into close spatial proximity with each other. This allows the simultaneous initiation of cytokine transcription, while looping out the *RAD50* promoter, preventing transcription of *RAD50* (see Ansel *et al.* (10) and Lee *et al.* (11) for in-depth reviews on locus accessibility and intra-chromosomal interactions). This process has recently been found to also require SATB1, a long-range chromatin organizing protein that is essential for Th2 cytokine expression (12), giving another level of control of the Th2 locus.

Further to the LCR, additional *cis*-acting regulatory sequences exist in the TH2 locus to modulate chromatin accessibility and cytokine expression. A conserved non-coding sequence (CNS) CNS1 (also called HSS1) located between the *IL-4* and *IL-13* genes (Figure 1, green box 1) is involved in coordinate regulation of *IL-4*, *IL-13* and *IL-5* (13). Transgenic mice with the CNS1 sequence deleted exhibit a slower induction of *IL-4* mRNA, with reduced expression of *IL-4*, *IL-5* and *IL-13* protein on Th2 polarization. The enhancer activity of CNS1 was T-cell dependent and did not function in mast cells, which also express *IL-4* and *IL-13*. In addition, CNS1 was necessary for heterochromatin formation at the *IL-4* locus during Th1 polarization (14). Several other regions of the Th2 locus

have also been shown to be important for chromatin remodelling. One example of this is CNS2, which is found within HS-V, a Th2-specific HS found in both resting and activated Th2 cells. Adjacent to this is HS-Va, a Th2-induced site. Upon stimulation, Va displayed a striking increase in accessibility to restriction enzyme digestion (REA), an alternate measure of chromatin accessibility. REA was found to be twofold to threefold greater in *IL-4* producers.

The specificity of epigenetic regulation of the Th2 locus can be seen by examination of other cell types, which produce *IL-4/IL-13*. Mast cells were found to have two constitutive HSs in *IL-13*, but lacked two inducible HSs, including CNS1, at the *IL-4* locus. This explains the preference of mast cells for making *IL-13* over *IL-4* (15) and why deletion of CNS1 did not impair cytokine responses in mast cells (13).

Additional mechanisms influencing chromatin remodelling at the Th2 locus

Initial studies of the *IL-4* locus indicated that loss of DNA methylation at the *IL-4* gene was strongly associated with changes in chromatin and transcriptional competence (16), but did not explore the role of DNA demethylation further. It could be necessary for either (i) establishing initial transcription of *IL-4*, (ii) chromatin remodelling or (iii) ensuring retention of memory in established Th2 cells. Using a polymerase chain reaction assay for methylation (17) it was shown that the *IL-4* promoter was not strongly methylated in Th2, Thn or, surprisingly, in Th1 cells where the *IL-4* gene was silent. However, exon1 of *IL-4* was highly methylated under Th1 conditions and demethylated under Th2 conditions, providing a basis for the silencing observed after Th1 polarization. However, the mechanics of demethylation requires DNA replication, while cytokine mRNA and Th2-specific HS (4) could be detected as early as 24–28 h after Th2 stimulation, before DNA replication and demethylation could occur (18). By 4 days post-stimulation in Th2 conditions some demethylation (5–10%) of the transcriptional initiation site and intron 1 was observed, with significant cytokine expression. Clearly, loss of methylation was not an absolute requirement for initiating transcription or the modulation of chromatin structure. This is not unprecedented — while DNA methylation is associated with silenced genes, reactivation of gene expression does not always require demethylation (19). However, levels of methylated DNA could possibly control the overall level of transcripts produced. To test this theory, Thn cells were differentiated under Th2 conditions, restimulated after 18 days and CD4⁺ cells were then sorted into *IL-4*-hi and *IL-4*-lo expressors. Methylation analysis showed that *IL-4*-hi cells were significantly less methylated than *IL-4*-lo cells. Again, similar to the studies described above, methylation at

the IL-4 locus CD4⁺ cells did not prevent IL-4 expression. However, transcription from methylated DNA was less efficient and the extent of demethylation correlated to increased IL-4 expression. Guo *et al.* (20) published similar results using the G4 mouse model, where the *GFP* gene is knocked into the IL-4 locus, resulting in parallel GFP and IL-4 expression. Examination of the CpG methylation status of the IL-4 and IL-13 genes in Th2 clones expressing either 3% or 30% GFP (IL-4) showed that progressive loss of DNA methylation was generally associated with higher GFP (IL-4) expression.

Equally important in regulation of chromatin conformation are the DNA-associated histone proteins. Modification of the lysine residues in each of four histone proteins by acetylation, methylation, phosphorylation, sumoylation and ubiquitinylation all impact differently on chromatin configuration and transcriptional competence by changing the affinity of the histones for DNA. Lower affinity interaction between histone and DNA results in relaxed, open configuration, while increasing affinity condenses chromatin. These multiple modifications occur in a complex combinatorial pattern, the 'histone code', to specify chromatin configuration (21). However, the modification most commonly associated with open, transcriptionally competent chromatin is acetylation, particularly of key lysine residues in histone 3 (H3K9, H3K14).

Several studies (22–24) have shown that Th2 differentiation is accompanied by alterations in the acetylation state of histones at cytokine gene loci. In the G4 model described above, histone acetylation generally increased with decreased DNA methylation and increased IL-4/GFP expression. Fields *et al.* (23) reported profound increases in histone acetylation at both IFN- γ and IL-4 loci during Th1/2 differentiation and found that these changes were locus and lineage specific. Although initial TCR activation led to the induction of both IFN- γ and IL-4 mRNA transcripts associated with histone acetylation at both loci in both cell types, by day 3 post-activation, lineage-specific polarization became evident. Increased H3 acetylation at the IL-4 locus in Th2 cells was associated with increased expression and the concomitant decrease in histone acetylation and expression of IFN- γ in these Th2 cells. Increased histone acetylation was associated with binding of the histone acetyltransferase p300/CBP to the CNS1 region within the locus (25). Hyperacetylation generally indicates that a gene is poised, ready to allow transcription, but the absence of acetylation does not necessarily infer gene silencing. When Th1 or Th2 cells are each re-activated under the opposite polarizing regime, they show an increase in histone acetylation of the formerly hypoacetylated locus, with no change in acetylation at the originally activated locus (24). Most of the Th1 to Th2 repolarized cells expressed both IFN- γ and IL-4. Similarly, Th2 to Th1 repolarized cells maintained IL-4 expression while acquiring

a highly acetylated IFN- γ locus and increased IFN- γ expression. This shows that while histone acetylation is stably heritable and may play a role in gene expression, it is reversible and is not enough in itself to fix polarization towards the Th1 or Th2 condition.

Histone methylation adds another layer of complexity to regulation of chromatin structure, as both lysine and arginine residues can be methylated, and lysine can be methylated three times. It has been suggested that histone methylation might be a better candidate for fixing cytokine memory in Th1/Th2 cells, as methylation turns over very slowly *in vivo* (26). Dimethyl H3K4, a poised/active mark, was shown to increase across the Th2 cytokine locus in Th2 cells, correlating to increased acetylation and cytokine expression (27). However, this modification was also shown to increase at the IFN- γ locus as well. The increased H3K4-dimethyl at the IFN- γ locus was lost over long-term culture (>28 days) (28), presumably representing loss of plasticity in the Th1/Th2 system over time and was correlated to heterochromatin formation at the IFN- γ locus after prolonged culture (29). Similarly, markers of repressed gene expression, H3K27 dimethyl and trimethyl were essentially absent from the IL-4 promoter while found at high level across the IFN- γ locus in Th2 cells (30). These repressive histone methylation marks might yet be the definitive controllers of chromatin structure at the Th2 locus and of Th1/Th2 polarization.

Transcription factor regulation of chromatin accessibility

The process of modifying chromatin is initiated by the binding of a specific protein to a specific DNA sequence and recruiting the appropriate modification enzymes to the locus. A key transcriptional pathway in Th2 cytokine expression is signalling mediated by IL-4, leading to STAT6 activation and induction of the transcription factor GATA3.

GATA3 was initially described as a Th2 determining factor after being identified as a dominant species present in a cDNA comparison using representational differential analysis between Th1 and Th2 genes (31). In this study, GATA3 was found to be expressed in naïve CD4⁺ cells and then specifically increased in Th2 cells. This led to the idea that GATA3 expression may be required to maintain Th2 phenotype. The significance of GATA3 in the Th2 allergic response is underscored by genetic analysis, indicating that specific polymorphisms in the GATA3 gene are associated with increased risk of asthma (32), atopic eczema (33) and allergic rhinitis (34).

As the loss of GATA3 leads to embryonic lethality (35) an antisense GATA3 construct was introduced to stable Th2 clones. This decreased expression of GATA3 and severely blocked the ability of transfected cells to express TH2 cytokines upon restimulation, indicating that continued

GATA3 expression was required for maintenance of Th2 expression. Conversely, the constitutive overexpression of GATA3 caused transactivation of the IL-4 promoter in a reporter construct model system and led to abnormal expression of Th2 cytokines in Th1 clones, while not appreciably altering the level of IFN- γ produced. This is consistent with the histone acetylation in repolarized Th1/Th2 cells described earlier. GATA3 was further shown to be critical for expression of other Th2 cytokines, as mutation of the GATA3 binding site in the IL-5 promoter abolished the ability of established Th2 cells to produce IL-5 (36) after stimulation, as did mutation of the GATA3 DNA-binding domain. Other GATA3 binding elements have been detected in Th2-associated genes (31, 36, 37), raising the possibility that GATA3 induces the epigenetic modifications that occur during polarization. It was determined that retroviral expression of GATA3 was sufficient to induce limited remodelling of the Th2 locus, opening the Th2-specific DHS2 site (38). Mutation of the DNA-binding domain of GATA3 indicated that interaction with the IL-4/IL-13 intergenic region was necessary for HS opening in Th2 cells. In addition, GATA3 was shown to bind the CNS2 element downstream of the *IL-4* gene and was essential for mediation of chromosomal interaction and coordinated cytokine expression by the LCR, all indicating that GATA3 acts at the chromatin level to promote Th2 differentiation (39). This was confirmed by Lee *et al.* (40) who showed that GATA3 exerts its effects through the IL-4 distal enhancers DHS2 and IE, a process later shown to be independent of STAT6 (41). Further, IL-10 is part of the Th2 cytokine response, but is not located within the Th2 locus. Shoemaker *et al.* showed that GATA3 is directly responsible for remodelling the IL-10 locus, with increased histone acetylation and expression independent of IL-4 (42).

The role of GATA3 in repressing the development of Th1 cell was also examined (43). GATA3 was retrovirally expressed into naïve cells, which were then differentiated under Th1 conditions. This led to a complete repression of IFN- γ production and a modest induction of IL-4. As it had also previously been shown that IL-4 has inhibitory effects on Th1 development (44), the experiment was repeated in IL-4^{-/-} mice, where expression of GATA3 was sufficient to inhibit Th1 development, showing independence from IL-4 initiated signals.

Further investigation established that the inhibition was not reliant on exogenous Th2 cytokines. A possible mechanism for this phenomenon had previously been observed (45) where extinction of IL-12 signalling in the early phase of differentiation resulted from a selective loss of IL-12R β 2 subunit expression. Expression of GATA3 in naïve Thn cells was sufficient to repress IL-12R β 2 mRNA expression, leading to a complete lack of IFN- γ production after Th1 polarization. However, GATA3 retroviral transduction 8 days after Th1 polarization did not significantly

alter IFN- γ production upon restimulation. This indicates a possible temporal restraint on the capacity of GATA3 to induce Th2 polarization, perhaps because of the inability of GATA3 to reverse an established chromatin configuration. Several other studies (24, 31, 38) have shown a significant plasticity in Th1 polarized cells; however, this appears to be because of exogenous cytokines, which can exert a GATA3 independent effect on Th1 polarization.

Other transcription factors have been shown to have similar roles in opening regions of chromatin in the Th2 locus. In particular, NFAT has been shown to bind to the Th2 locus. Treatment of Th2 cells with the NFAT inhibitor cyclosporin A inhibited the opening of HS-Va, confirming the role of NFAT in regulating a transcriptionally competent Th2 locus (46). Then there are transcription factors with secondary roles in cytokine production, such as cMAF, which mediates IL-4 synthesis, but is not essential (47). If the factors regulating chromatin accessibility are present in naïve T cells only at limiting levels, the situation could arise whereby individual alleles are independently remodelled depending on the availability of factors. Limited availability could possibly explain the phenomenon of monoallelic expression, which has been observed *in vitro* for IL-4 (46).

Prospects for epigenetic treatment

Traditionally, epigenetic approaches to therapy have tried to reverse gene silencing using inhibitors of DNA methylation, such as 5-aza-2-deoxycytidine, histone deacetylase inhibitors, or combinations of the two approaches. A large number of HDAC inhibitors are in development for clinical use, but their potential utility in Th2 disease is not clear. Increased histone acetylation is important in the Th2 response, as lung macrophages from asthmatic subjects have increased histone acetyltransferase (HAT) activity with some decrease in histone deacetylase (HDAC) activity, while corticosteroid treatment reduced HAT activity to normal levels (48). Similarly, *ex vivo* use of the HDAC inhibitor trichostatin A (TSA) on normal human peripheral blood mononuclear cells led to increased GATA3 and Th2 cytokine expression (49). In contrast, TSA *in vivo* decreased IL-4 and IL-5 expressions in a murine asthma model (50), indicating the complexity of epigenetic regulation. Once the phenomenon of increased acetylation at the Th2 locus is better understood, silencing the Th2 cytokines might better be achieved with the inhibitors of histone acetylation currently under development (51).

Conclusion

Epigenetic modifications at the Th2 locus are responsible for the establishment, maintenance and inheritance of the Th2 phenotype, while altered epigenetic regulation is potentially important for the exaggerated Th2 response

seen in atopic diseases, such as asthma and allergy. Environmental stimuli that influence the incidence and severity of allergy and asthma have been shown to alter the epigenetic status of the Th2 locus. With the continued development of pharmacological agents that modify chromatin, epigenetics might one day become a therapeutic approach for the treatment of the inflammatory cell pathology in asthmatic and allergic patients.

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