

CpG methylation patterns in the IFN γ promoter in naive T cells: Variations during Th1 and Th2 differentiation and between atopics and non-atopics

White GP, Hollams EM, Yerkovich ST, Bosco A, Holt BJ, Bassami MR, Kusel M, Sly PD, Holt PG. CpG methylation patterns in the IFN γ promoter in naive T cells: Variations during Th1 and Th2 differentiation and between atopics and non-atopics. *Pediatr Allergy Immunol* 2006.

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Interferon- γ (IFN γ) gene expression is tightly regulated in early life, and exaggerated negative control of IFN γ production in CD4 $^{+}$ T cells has been associated with risk for subsequent development of atopy. Recent studies have demonstrated hypermethylation of CpG sites in the IFN γ promoter in neonates, a mechanism which in mice leads to strong suppression of IFN γ gene transcription. In the present study, the methylation status of six CpG sites in the proximal promoter of the human IFN γ gene was determined by bisulphite sequencing. Cell populations studied were Th1 or Th2 polarized cell lines derived from neonatal and adult CD4 $^{+}$ /CD45RA $^{+}$ T cells, CD4 $^{+}$ and CD8 $^{+}$ naive T cells from cord blood of children followed to outcome age 2 for assessment of atopy status, and CD4 $^{+}$ and CD8 $^{+}$ naive T cells from 6 yr old and adult atopics and controls. We demonstrate that *in vitro* differentiation of CD4 $^{+}$ T cells down the Th1 pathway (but not the Th2 pathway) is accompanied by progressive demethylation of CpG sites in the IFN γ promoter, which is most marked in neonatal cells. Atopy development by age 2 was not associated with variations in methylation patterns in cord blood T cells. However, IFN γ promoter methylation was reduced in CD8 $^{+}$ T cells from atopic children in the age range in which hyperproduction of IFN γ as recently been identified as a common feature of the atopic phenotype. The findings demonstrate the potency of IFN γ promoter methylation as a mechanism for control of human IFN γ gene expression, particularly during early life. Differential regulation of IFN γ promoter methylation in T cells may be an important contributory factor in atopy development in childhood, and this possibility warrants further detailed investigation.

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Key words: IFN γ ; methylation; helper T cell; CpG; atopy

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Accepted 14 July 2006

Recent evidence indicates that DNA methylation is an important mechanism by which the Interferon- γ (IFN γ) promoter is developmentally regulated. Fine control of this pleiotropic cytokine is vital to modulate its essential functions in somatic remodelling and correct decidualization during normal pregnancy (1), and to protect against the potentially toxic effects of uncontrolled

IFN γ production at the foetal/maternal interface which is a common cause of foetal loss (2). As a result, immune function in the developing foetus is normally maintained in a relatively Th2-skewed state (2). However, subsequent survival in the microbiologically hostile extrauterine environment necessitates upregulation of Th1 function post-natally, in order to protect against

infectious diseases, and also to protect against development of Th2-polarized immunity to environmental allergens (3, 4). Moreover, variations in the kinetics of this developmental process have been suggested as a determinant of the level of risk for allergic sensitization (5).

Control of IFN γ gene transcription may thus play a central role in the aetiology of allergic disease, but the relevant mechanisms are poorly understood. One potential control mechanism involves methylation of specific sites within the IFN γ gene. Cytosine methylation has been detected in animal genomes at CpG, CpA and CpT dimers (6–8), and methylation as an epigenetic control mechanism has been demonstrated to regulate transcription at several gene loci. In particular, the IFN γ gene promoter of both mice and humans has been the subject of a number of studies investigating CpG methylation and epigenetic control of expression (8–15).

We have recently reported (8) that a relatively hypermethylated CpG profile exists in the proximal IFN γ promoter of freshly isolated human neonatal CD4⁺CD45RA⁺ (naive) T cells compared with naive adult CD4⁺CD45RA⁺ T cells. In contrast, neonatal CD56⁺ NK cells and CD8⁺ T cells were less methylated, particularly at the three CpG sites immediately upstream of the transcriptional start site (–295, –186 and –54), and differed little from their adult counterparts.

In this report we have used bisulphite-sequence analysis to determine the time-dependent changes which occur in CpG methylation within the proximal promoter of the IFN γ gene in adult and neonatal CD4⁺CD45RA⁺ T cells differentiated *in vitro* under Th1 vs. Th2 polarizing conditions. We have additionally sought to explore potential differences between levels of CpG methylation at these sites in atopics and non-atopics, which are perceived to represent the two opposite ends of the IFN γ response spectrum.

Materials and methods

Study subjects

Adult volunteers and 6 yr olds were tested for atopic status via skin prick test (SPT) reactivity to a panel of inhalant and food allergens, and classified as atopic if they displayed wheals ≥ 3 mm diameter in response to ≥ 1 allergen. The 6 yr olds (16) and neonates (17) were part of two prospective cohort studies aimed at following atopy development in early childhood. The studies were all approved by our institutional human ethics committee.

Cell preparation

Blood was collected into preservative-free heparin, and peripheral blood mononuclear cells prepared by gradient sedimentation. All samples were cryobanked for subsequent laboratory studies (17). In some studies, pre-purification of CD45RA⁺CD4⁺ or CD45RA⁺CD8⁺ T cells was undertaken employing Dynabeads (Dynal, Oslo, Norway) as detailed earlier (18).

Cell cultures

Enriched CD45RA⁺CD4⁺ T cells from cryo-preserved samples were polarized employing the methods detailed in (18). Th1 polarization was achieved by polyclonal expansion in the presence of IL-12 plus MoAb anti-IL-14, or in IL-4 plus anti-IL-12 for Th2 polarization. Cytokine responses were elicited by stimulation for 24 h with 10 ng/ml PMA (Sigma, Castle Hill, Australia) plus 400 ng/ml Ionomycin (Sigma). A second cycle of stimulation was then undertaken as previously (18), and cytokine production again determined.

Cytokine assays

IL-4 and IFN γ levels in culture supernatants were determined by enzyme-linked immunosorbent assay (ELISA), employing a time resolved fluorescence readout system, as previously (17).

Assessment of CpG methylation

CpG methylation levels were determined by scoring unconverted cytosines in polymerase chain reaction (PCR) products amplified from bisulphite-treated genomic DNA. Briefly, genomic DNA was extracted from T cells using a QIAamp DNA blood mini kit (QIAGEN, Valencia, CA, UAS), and 1–2 μ g of genomic DNA from 1×10^6 cells was bisulphite-treated following the protocol of Clark *et al.* (19) as detailed previously (8). A 526 bp region of the IFN γ promoter (nt 21–546, GenBank accession no. J00219) was amplified from the non-coding strand of 10–100 ng bisulphite-treated DNA using 300 nM primers (Fwd: 5'-TATA-AATAAAAAATCAACATTTTACCAAAA-3'; Rev: 5'-TTGGTAGTAATAGTTAAGAGAATT-3'), 1.5 U PLATINUM Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA), 1.5 mM MgCl₂ and 200 μ M dNTPs. Cycling parameters consisted of heating at 96°C for 3.5 min, nine cycles of 96°C for 15 s, 59°C (decreasing by 1°C/cycle) for 30 s and 72°C for

1 min, followed by 30 cycles of 96°C for 15 s, 50°C for 30 s and 72°C for 1 min (increasing by 5 s/cycle), with a final incubation at 72°C for 7 min. PCR products were cloned into pCR2.1 using a TOPO TA cloning kit (Invitrogen), and plasmid DNA from 10 transformed clones per sample was purified using a QIAprep spin miniprep kit (QIAGEN) and sequenced at the Australian Genome Research Facility (Brisbane, Queensland, Australia) using an M13Rev primer (5'- CAGGAAACAGCTATGAC-3'). For experiments involving comparisons between groups stratified by age and/or atopic status, subject sample sizes of 5–10 were employed, yielding 50–100 observations per analysis.

Results

Fig. 1 illustrates results from experiments involving Th1 or Th2 polarization of adult and naive CD4⁺ CD45RA⁺ T cells. Data are shown for polyclonal cytokine responses after one or two cycles of polarization. Th1 polarization resulted in log-scale higher rates of IFN γ production than was observed in Th2 polarized lines, derived from either adult or cord T cells.

CpG methylation levels were determined in the proximal IFN γ promoter in cells samples from these cultures, by scoring unconverted cytosines in PCR products amplified from bisulphite-treated genomic DNA. A total of six CpG sites were examined, three upstream from the start of transcription (–295, –186 and –54) and three downstream (+122, +128 and +171).

Differentiation of CD4⁺CD45RA⁺ T cells under Th1- or Th2-polarizing conditions resulted in radically different methylation profiles in the proximal IFN γ promoter of neonatal cells measured at D0, D6 and D15 (Fig. 2; Cord). At D0 all of the CpG sites, both upstream and downstream

of the transcriptional start site, were methylated in $\geq 90\%$ of the cells in the three neonates that formed this part of the study. In particular, $\geq 98\%$ of the sites upstream from the transcription start site were methylated. With Th1-polarization at D6 approximately 60% of neonatal CD4⁺ T cells became demethylated at upstream CpG sites –295, –186 and –54 compared with 20–30% at the three downstream sites (Fig. 2). The effects of Th2 polarization upon these same cells were notably different as the majority of sites remained totally CpG methylated at D6, and remained so during a second cycle of Th2 polarization (D15 in Fig. 2).

Fig. 3 illustrates age-dependent changes in IFN γ promoter methylation patterns in naive CD4⁺ and CD8⁺ T cells, contrasting neonates, 6 yr olds and adults. Age-dependent changes were restricted to CD4⁺ T cells, and were statistically significant at the –54 and +122 sites.

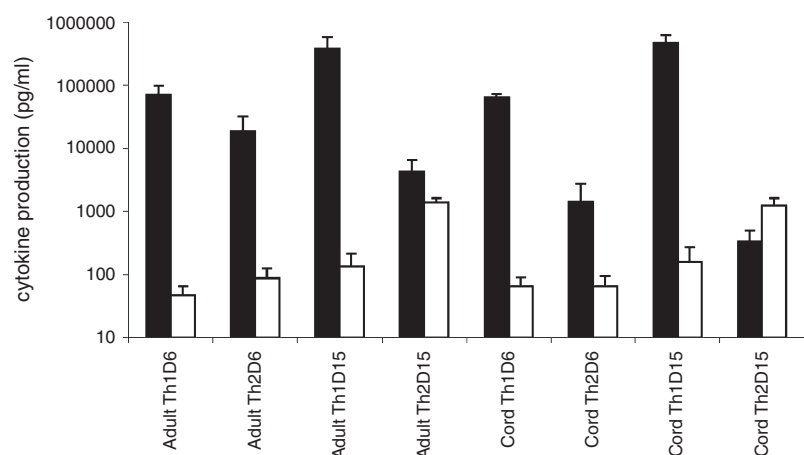
In the experiments in Fig. 4, IFN γ promoter methylation levels were determined in naive CD4⁺ and CD8⁺ T cells from cord blood from samples in a prospective cohort study, contrasting subjects who demonstrated sensitization to ≥ 1 allergen by age 2 yr vs. those who remained atopy free. No significant differences were observed between the two groups.

Fig. 5 contrasts IFN γ promoter methylation in T cells from atopic and non-atopic 6 yr olds and adults. It can be seen that promoter methylation levels at the –186 and –54 sites are constitutively lower in CD8⁺ T cells of atopic children at both ages, and the differences attain statistical significance at the –186 site.

Discussion

In the initial phase of this study we employed an established protocol to drive the *in vitro*

Fig. 1. Cytokine production in Th1-polarized and Th2-polarized cell lines. Purified adult and neonatal CD4⁺ CD45RA⁺ T cells were cultured under Th1 or Th2 polarizing conditions for 3 days followed by a 3 day rest in medium; a second cycle of polarization was then performed over a 6 day period followed by a 3 day rest; cell samples taken at days 6 and 15 were stimulated with PMA/Ionomycin for determination of levels of IFN γ (black bars) and IL-4 (open bars) production. Data shown are mean $\pm$ SEM for three experiments.



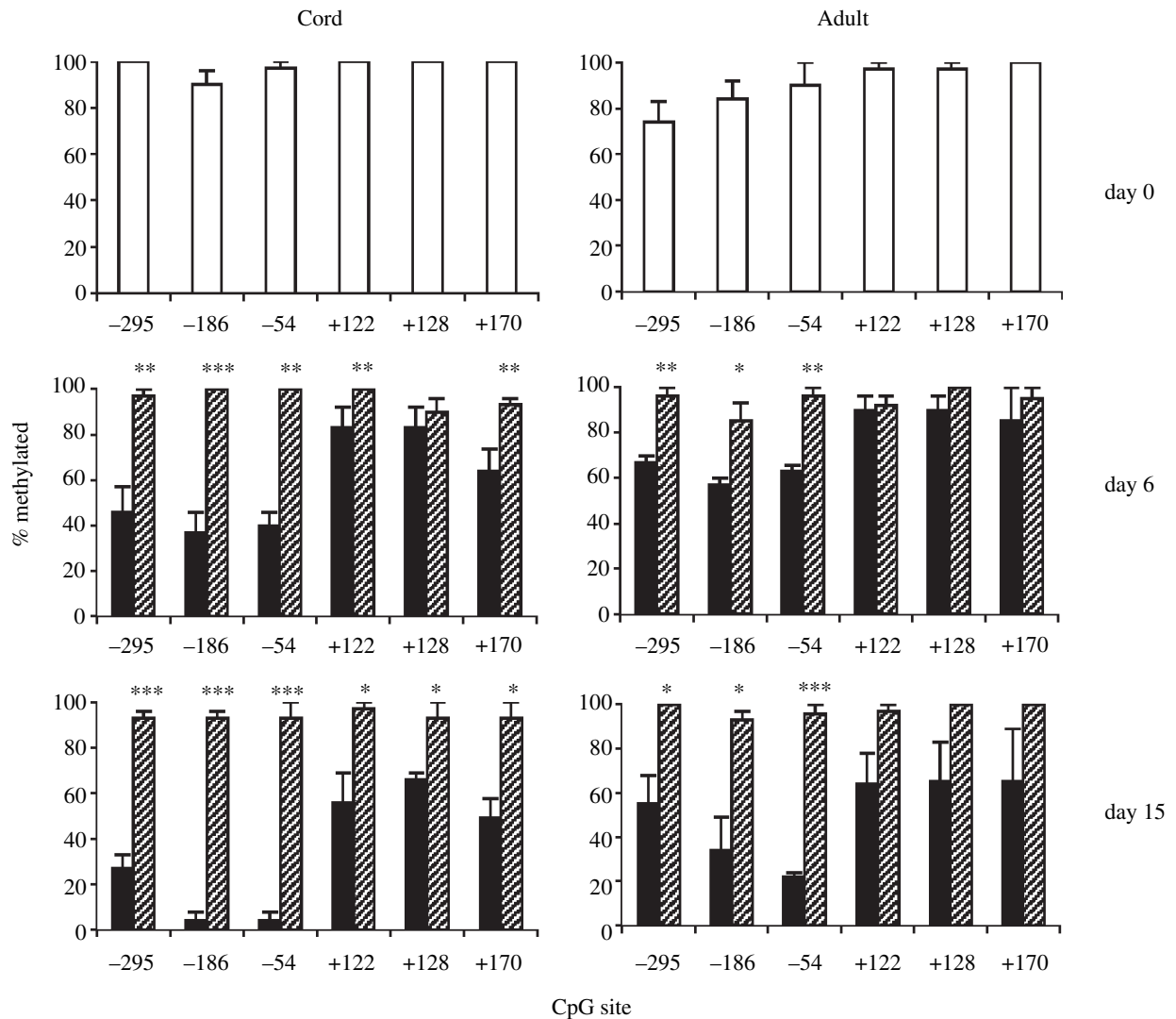


Fig. 2. CpG methylation with the IFN γ promoter during Th1/Th2 polarization. Data shown are mean per cent methylation $\pm$ SEM at CpG sites upstream (-) and downstream (+) of the transcription start site (0) in cell lines grown under Th1 polarizing conditions (black bars) vs. Th2 conditions (hatched bars). Data shown are from a representative experiment utilizing 10 transformed clones from three subjects in each group at each time-point. Methylation in Th1 line > Th2 line: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ by t -test.

differentiation of naive CD4⁺ T cells, involving polyclonal stimulation of CD4⁺CD45RA⁺ T cells in the presence of either IL-12 plus mAb anti-IL-4 (Th1-polarizing conditions) or IL-4 plus mAb anti-IL-12 (Th2-polarizing conditions), and subsequently examined whether the different growth conditions resulted in changes in the methylation state of the IFN γ promoter. In this system T-cell expansion in IL-2 was considerable under both Th1 and Th2-polarizing populations after D6 and D15 (not shown), and determination of levels of IFN γ and IL-4 cytokine in culture supernatant after short term restimulation with PMA/Ionomycin provided a robust index of polarization, in particular for the Th1 lines.

As reported earlier (8), CpG methylation was initially less marked at the upstream sites in the IFN γ promoter in adult CD4⁺ CD45RA⁺ T cells relative to cells from neonates. Differentiation of T cells from both age groups resulted in significantly different CpG methylation profiles under Th1- vs. Th2-polarizing conditions, in particular rapid demethylation of CpG sites in the IFN γ -polarizing Th1 lines vs. maintenance of methylation in the Th2 lines. Moreover, the levels of demethylation ultimately achieved in Th1 lines derived from neonatal CD4⁺ CD45RA⁺ T cells typically exceeded those derived from adults.

These differences between *in vitro* differentiation of adult and neonatal T cells may reflect the

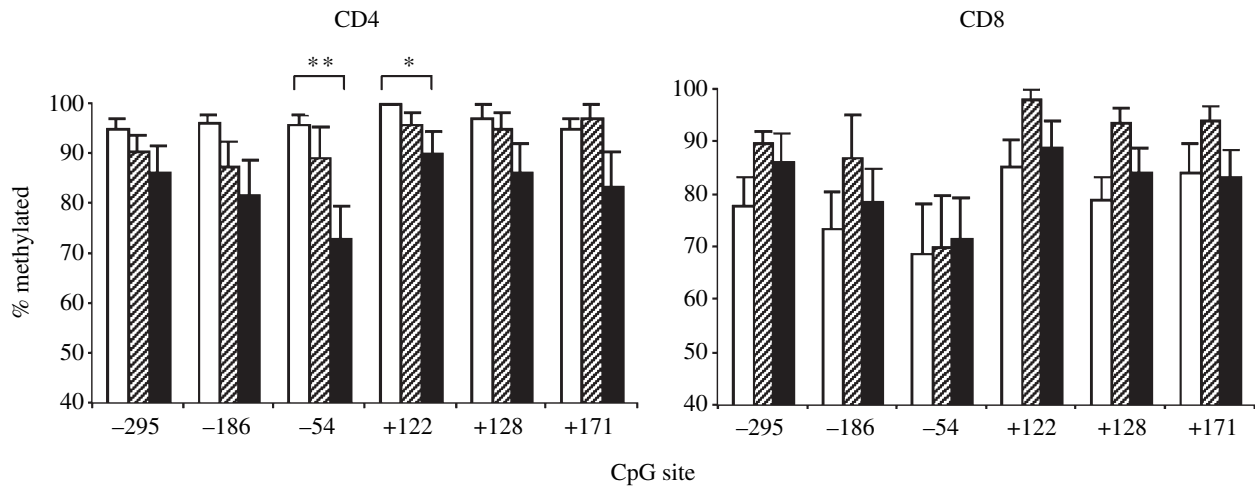


Fig. 3. Age dependent fluctuations in IFN γ promoter CpG methylation. Data shown are mean per cent methylation $\pm$ SEM in the IFN γ gene in neonates (open bars), 6 yr olds (hatched) and adults (black). In CD4 $^{+}$ T cells, methylation in neonates > adults: * p < 0.05; ** p < 0.01 by t -test.

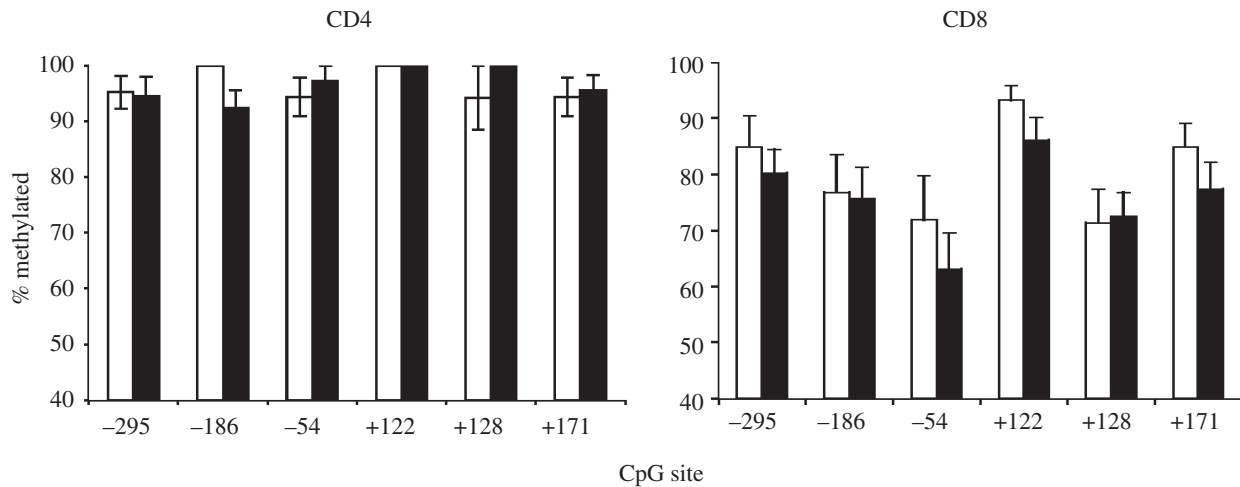


Fig. 4. Neonatal IFN γ promoter CpG methylation in relation to atopy development. Data shown are mean per cent methylation $\pm$ SEM in the IFN γ gene in neonates who subsequently developed atopic disease at age 2 yr (black bars) vs. those who did not (open bars). Inter-group differences were not statistically significant.

predominance within the latter population of recent thymic emigrants (RTE) which differ phenotypically from adult naive cells in a variety of respects, such as requirements for cytokine survival factors during *in vitro* proliferation (20). The present results suggest that Th1 clones which arise during the neonatal period will display lower levels of IFN γ promoter CpG methylation, which potentially equates to less constrained IFN γ gene expression, relative to Th1 clones arising at later stages. This finding has theoretical implications in relation to subsequent expression of allergen-specific Th-cell memory i.e. that early post-natal Th1 priming may lead to more vigorous IFN γ responses in later life, relative to Th1 responses primed at later ages. Follow-up studies are in progress to examine this possibility.

The balance between IFN γ and IL-4 production is currently believed to be an important determinant of risk for inflammatory diseases, in particular atopy, which has been associated with reduced production of Th1 cytokines exemplified by IFN γ (3). Earlier studies from our laboratory have identified the kinetics of post-natal maturation of IFN γ response capacity as an important factor in determining susceptibility to both allergic and infectious diseases (3, 4). As part of these investigations we have demonstrated differences in capacity to produce IFN γ *in vitro* in response to mitogens or specific antigens. These studies suggest that during infancy, the IFN γ response capacity of subjects who subsequently develop atopic sensitization is initially reduced relative to the rest of the population (3, 5, 21, 22),

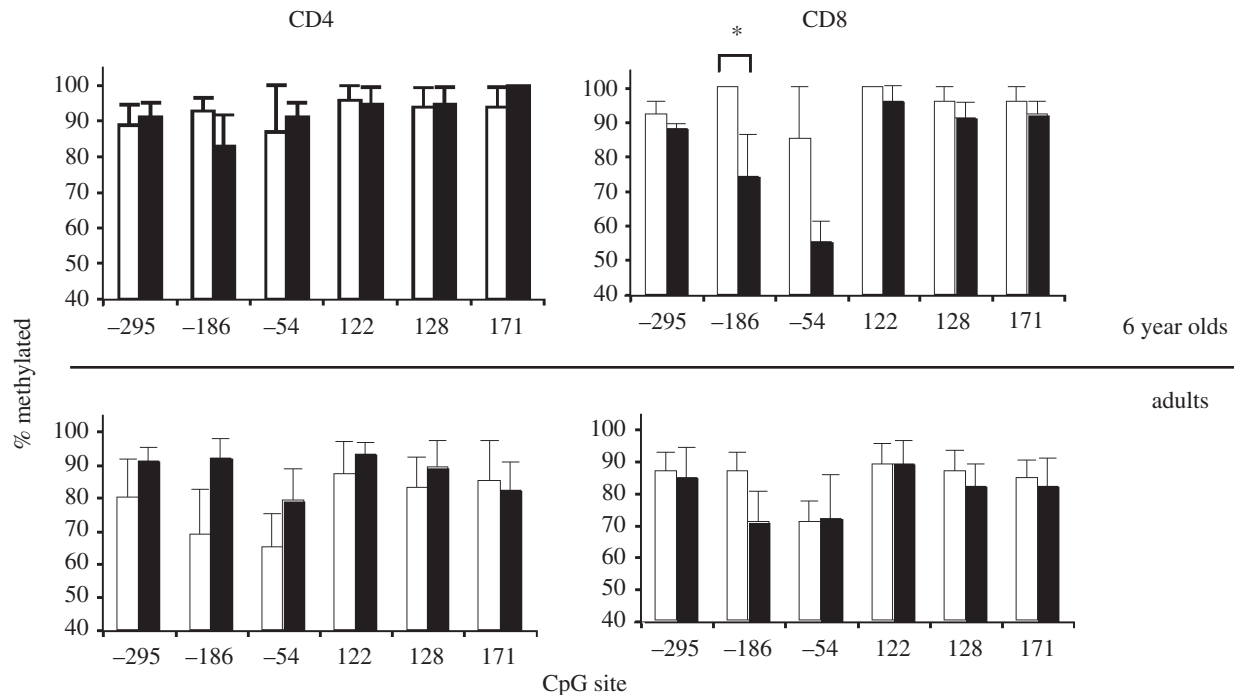


Fig. 5. IFN γ CpG promoter methylation in atopy vs. non-atopy. Data shown are mean per cent methylation $\pm$ SEM in naive CD4 $^{+}$ and CD8 $^{+}$ T cells from groups of 6 yr olds and adults, stratified on the basis of atopic status. Note the significant reduction in CD8 $^{+}$ CpG methylation at the -186 site in 6 yr olds ($p < 0.05$).

and diminished negative feedback from IFN γ during initial immune responses against allergens may favour the outgrowth of atopy-associated Th2-memory cells. However, recent data suggests that with maturation, the responses of atopic children to allergens can further develop into a 'mixed' Th1/Th2 cytokine phenotype, and during this process the IFN γ component of these responses frequently rebounds to exceed that of non-atopics (23, 24). Hyperproduction of IFN γ , in particular by CD8 $^{+}$ T cells, has recently been linked with severe symptomatology in a subset of atopic children (25). These findings prompted the studies in Figs 3–5.

In Fig. 3, we compared T-cell IFN γ promoter methylation patterns in a group of unselected newborns, 6 yr olds and adults, again focusing on the CpG sites in the proximal promoter. Methylation profiles were compared between purified CD4 $^{+}$ CD45RA $^{+}$ and CD8 $^{+}$ CD45RA $^{+}$ T cells. In confirmation of our recent report (8), methylation patterns in naive CD8 $^{+}$ T cells were not developmentally regulated to a comparable extent to naive CD4 $^{+}$ T cells, in which methylation at all sites was strongly age related. The progressive demethylation of CpG sites in the IFN γ promoter in naive CD4 $^{+}$ T cells infers some form of time-dependent ongoing post-thymic maturation amongst uncommitted

CD4 $^{+}$ T cells. It is conceivable that this maturation process is a direct result of the slow turnover of uncommitted CD4 $^{+}$ T cells at the periphery, a process which may be driven by common γ -chain cytokines such as IL-7 (20), and/or to the progressive replacement with age of RTE within the circulating CD4 $^{+}$ T cell compartment by adult-equivalent naive CD4 $^{+}$ T cells.

We next questioned whether the IFN γ promoter methylation status of neonatal T cells may be predictive of their subsequent development of atopy, employing cord blood samples collected and cryobanked as part of a prospective cohort study on asthma development in children at high risk of atopy. Accordingly two groups of cryobanked cord blood mononuclear cells were selected for analyses on the basis of atopic status at age 2 yr. As shown in Fig. 4, while mean levels of IFN γ promoter methylation varied somewhat between the groups who did/did not develop atopy, none of the differences at individual CpG sites attained statistical significance. Restratification of the subjects on the basis of atopic outcome at age 5 yr (data not shown) also did not reveal significant differences.

We finally addressed the question of whether the known differences in Th1/Th2 balance between established atopics and non-atopics are

associated with overt differences in CpG methylation patterns in the IFN γ promoter. The results of parallel analyses of eight groups of 50 individual IFN γ promoter sequences from CD45RA⁺CD4⁺ and CD45RA⁺CD8⁺ T cells from atopic vs. normal adults and young children are illustrated in Fig. 5. While significant inter-group differences were not detected in the CD4⁺ analyses, sequences from naive CD8⁺ T cells from 6 yr olds displayed lower mean levels of CpG methylation at two of the upstream sites in the IFN γ promoter. One interpretation of this finding is that CD8⁺ T cells from atopic children may be more susceptible to triggering of high level IFN γ responses at this age. This suggestion is consistent with recent *in vitro* findings from our group (23) and others (24, 25) and is of interest in the context of reports from several groups [e.g. (25, 26)] implicating hyperproduction of IFN γ with symptomatology such as airways hyperresponsiveness. The precise mechanism(s) by which IFN γ exerts these effects remains to be defined, and may involve enhancement of Th2-triggered effector responses (e.g. eosinophil activation/survival) or direct toxic effects of the IFN γ molecule itself.

We acknowledge that the sample size in the present study and its restriction to *in vitro* models precludes drawing firmer conclusions regarding the biological significance of the findings relating to these IFN γ promoter methylation patterns. However, taken together with findings above from Th1/Th2 polarized cell lines, these data collectively suggest that more detailed investigations on the role of CpG methylation mechanisms in IFN γ regulation in atopic (and other inflammatory) diseases, is warranted. It would also be of interest to investigate the relationship between CD4⁺CD45RA⁺ and/or CD8⁺CD45RA⁺ IFN γ promoter methylation patterns in cord blood and subsequent viral infections during infancy, given that susceptibility to pathogens such as RSV during infancy appears related to the kinetics of post-natal development of Th1 competence (4, 22). Additionally, SNPs have been identified in the promoter of the IFN γ gene in close proximity to CpG sites (27, 28), and it would be of interest to determine whether these mutations modulate CpG methylation/demethylation during IFN γ gene activation. The nature of the molecular mechanisms involved in control of the methylation process itself are poorly understood and merit more detailed study, including the question of how environmental stimuli impact on these mechanisms.

It is also pertinent to note that the -54 and -186 CpG sites in the IFN γ promoter are known

to play a significant role in attracting AP1-CREB-ATF DNA binding complexes (29). Given the evidence from a variety of other systems (30), it is likely that CpG methylation at these sites may play a key role in regulation of this important function, and that hypermethylation may inhibit complex formation. Additionally, strong evidence exists that one of the anti-inflammatory effects of corticosteroids, which are widely used to treat allergic diseases, is mediated by their disruption of transcription factor/DNA complex formation in this region of the IFN γ gene, resulting in negative regulation of IFN γ transcription (29). It would be of interest to determine whether the steroid effects are in part mediated via changes in the methylation patterns in the IFN γ promoter, and this possibility merits investigation in followup experiments.

References

1. ASHKAR AA, DI SANTO JP, CROY BA. Interferon gamma contributes to initiation of uterine vascular modification, decidual integrity, and uterine natural killer cell maturation during normal murine pregnancy. *J Exp Med* 2000; 192: 259–69.
2. WEGMANN TG, LIN H, GUILBERT L, MOSMANN TR. Bidirectional cytokine interactions in the maternal-fetal relationship: is successful pregnancy a Th2 phenomenon? *Immunol Today* 1993; 14: 353–56.
3. HOLT PG, MACAUBAS C. Development of long term tolerance versus sensitisation to environmental allergens during the perinatal period. *Curr Opin Immunol* 1997; 9: 782–7.
4. HOLT PG, SLY PD. Interactions between RSV infection, asthma, and atopy: unraveling the complexities. *J Exp Med* 2002; 196: 1271–5.
5. HOLT PG, CLOUGH JB, HOLT BJ, et al. Genetic 'risk' for atopy is associated with delayed postnatal maturation of T-cell competence. *Clin Exp Allergy* 1992; 22: 1093–9.
6. LYCO F, RAMSAHOYE BH, JAENISCH R. DNA methylation in *Drosophila melanogaster*. *Nature* 2000; 408: 538.
7. RAMSAHOYE BH, BINISZKIEWICZ D, LYCO F, CLARK V, BIRD AP, JAENISCH R. Non-CpG methylation is prevalent in embryonic stem cells and may be mediated by DNA methyltransferase 3a. *Proc Natl Acad Sci U S A* 2000; 97: 5237–42.
8. WHITE GP, WATT PM, HOLT BJ, HOLT PG. Differential patterns of methylation of the IFN γ promoter at CpG and non-CpG sites underlie differences in IFN γ gene expression between human neonatal and adult CD45RO⁺ T-cells. *J Immunol* 2002; 168: 2820–7.
9. FITZPATRICK DR, SHIRLEY KM, McDONALD LE, BIELEFELDTOMANN H, KAY GF, KELSO A. (IL-3) genes in newly activated primary CD8(+) T lymphocytes – regional IFN-gamma promoter demethylation and mRNA expression are heritable in CD44(High)CD8(+) T Cells. *J Exp Med* 1998; 188: 103–17.
10. FITZPATRICK DR, SHIRLEY KM, KELSO A. Cutting edge: stable epigenetic inheritance of regional IFN-gamma promoter demethylation in CD44(high)CD8(+) T lymphocytes. *J Immunol* 1999; 162: 5053–7.

11. KATAMURA K, FUKUI T, KİYOMASU T, et al. IL-4 and prostaglandin E2 inhibit hypomethylation of the 5' regulatory region of IFN-gamma gene during differentiation of naive CD4+ T cells. *Mol Immunol* 1998; 35: 39–45.
12. KİYOMASU T, KATAMURA K, UENO H, et al. Hypomethylation of the proximal and intronic regulatory regions of the IFN-gamma gene is not essential for its transcription by naive CD4(+) T cells cultured with IL-4. *Immunol Lett* 1999; 69: 239–45.
13. MELVIN AJ, MCGURN ME, BORT SJ, GIBSON C, LEWIS DB. Hypomethylation of the interferon-gamma gene correlates with its expression by primary T-lineage cells. *Eur J Immunol* 1995; 25: 426–30.
14. PENIX LA, SWEETSER MT, WEAVER WM, HOEFFLER JP, KERPPOLA TK, WILSON CB. The proximal regulatory element of the interferon-gamma promoter mediates selective expression in T cells. *J Biol Chem* 1996; 271: 31964–72.
15. YOUNG HA, GHOSH P, YE J, et al. Differentiation of the T helper phenotypes by analysis of the methylation state of the IFN-gamma gene. *J Immunol* 1994; 153: 3603–10.
16. ODDY WH, HOLT PG, SLY PD, et al. Association between breast feeding and asthma in 6 year old children: findings from a prospective birth cohort study. *BMJ* 1999; 319: 815–9.
17. SHARP MJ, ROWE J, KUSEL M, SLY PD, HOLT PG. Specific patterns of responsiveness to microbial antigens staphylococcal enterotoxin B and purified protein derivative by cord blood mononuclear cells are predictive of risk for development of atopic dermatitis. *Clin Exp Allergy* 2003; 33: 435–41.
18. MACAUBAS C, LEE PT, SMALLACOMBE TB, et al. Reciprocal patterns of allergen-induced GATA-3 expression in peripheral blood mononuclear cells from atopics versus non-atopics. *Clin Exp Allergy* 2002; 32: 97–106.
19. CLARK SJ, HARRISON J, PAUL CL, FROMMER M. High sensitivity mapping of methylated cytosines. *Nucleic Acids Res* 1994; 22: 2990–7.
20. HASSAN J, REEN DJ. Human recent thymic emigrants-identification, expansion and survival characteristics. *J Immunol* 2001; 167: 1970–76.
21. PRESCOTT SL, MACAUBAS C, SMALLACOMBE T, HOLT BJ, SLY PD, HOLT PG. Development of allergen-specific T-cell memory in atopic and normal children. *Lancet* 1999; 353: 196–200.
22. ROWE J, MACAUBAS C, MONGER T, et al. Heterogeneity in diphtheria-tetanus-acellular pertussis vaccine-specific cellular immunity during infancy: relationship to variations in the kinetics of postnatal maturation of systemic Th1 function. *J Infect Dis* 2001; 184: 80–8.
23. HOLT PG, RUDIN A, MACAUBAS C, et al. Development of immunologic memory against tetanus toxoid and pertactin antigens from the diphtheria-tetanus-pertussis vaccine in atopic versus nonatopic children. *J Allergy Clin Immunol* 2000; 105: 1117–22.
24. SMART JM, KEMP AS. Increased Th1 and Th2 allergen-induced cytokine responses in children with atopic disease. *Clin Exp Allergy* 2002; 32: 796–802.
25. MAGNAN AO, MELY LG, CAMILLA CA, et al. Assessment of the Th1/Th2 paradigm in whole blood in atopy and asthma. Increased IFN-gamma-producing CD8(+) T cells in asthma. *Am J Respir Crit Care Med* 2000; 161: 1790–6.
26. HEATON T, ROWE J, TURNER S, et al. An immunoepidemiological approach to asthma: identification of in-vitro T-cell response patterns associated with different wheezing phenotypes in children. *Lancet* 2005; 365: 142–9.
27. BREAN JH, PING A, ZHANG X, WINKLER C, YOUNG HA. A single nucleotide polymorphism in the proximal IFN-gamma promoter alters control of gene transcription. *Genes Immun* 2002; 3: 165–9.
28. CHEVILLARD C, HENRI S, STEFANI F, PARZY D, DESSEIN A. Two new polymorphisms in the human interferon gamma (IFN-gamma) promoter. *Eur J Immunogenet* 2002; 29: 53–6.
29. YE J, GHOSH P, CIPPITELLI M, et al. Characterization of a silencer regulatory element in the human interferon-gamma promoter. *J Biol Chem* 1994; 269: 25728–34.
30. ROBERTSON KD, WOLFFE AP. DNA methylation in Health and Disease. *Nat Genet* 2000; 1: 11–19.