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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[1]

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IFN-γ is a potent pleiotropic Th1 cytokine, the production of which is tightly regulated during fetal development. Negative control of fetal/neonatal IFN-γ production is generally attributed to the Th1-antagonistic effect of mediators produced by the placenta, but evidence exists of additional and more direct transcriptional regulation. We report that neonatal (cord blood) CD3$^+$/CD45RO$^-$ T cells, in particular the CD4$^+$/CD45RO$^-$ subset, are hypermethylated at CpG and non-CpG (CpA and CpT) sites within and adjacent to the IFN-γ promoter. In contrast, CpG methylation patterns in cord blood IFN-γ-producing CD8$^+$/CD45RO$^-$ T cells and CD56$^+$/CD16$^+$/CD3$^-$ NK cells did not differ significantly from those in their adult counterparts. Consistent with this finding, IFN-γ production by stimulated naive cord blood CD4$^+$ T cells is reduced 5- to 10-fold relative to adult CD4$^+$ T cells, whereas production levels in neonatal and adult CD8$^+$ T cells are of a similar order. Evidence of significant CpA and CpT methylation was not discovered in promoter sequence from other cytokines (IL-4, TNF-α, or IFN-γR α-chain). We additionally demonstrate that overexpression of DNA methyltransferase 3a in embryonic kidney carcinoma cells is accompanied by CpA methylation of the IFN-γ promoter. ***The Journal of Immunology*****, 2002, 168: 2820–2827.**

Interferon-γ has a variety of important roles in the maintenance of immunological homeostasis, in particular in the activation of a range of Th1-associated cellular immune functions that are central to host defense against viral and bacterial infections. However, excessive or prolonged production of IFN-γ can also contribute to the pathogenesis of inflammatory diseases as a direct result of its toxic effects on host tissues or via its role in activation of cytotoxic effector cells such as macrophages (1).

Prime examples of the dualistic effects of this pleiotropic cytokine are its activities in relation to fetal development. At one extreme, IFN-γ plays a key role in implantation via regulation of arteriolar remodeling and decidualization (2). In contrast, excessive production of IFN-γ at the feto-maternal interface, resulting from infections or allogeneic interactions, is a major cause of fetal loss, as a consequence of its deleterious effect on placental integrity (3, 4). These contradictory findings suggest that fine control of IFN-γ gene transcription may be even more important during fetal life than at later ages.

Consistent with this idea, recent research indicates that biasing of adaptive immune function in the fetus toward production of Th1-antagonistic Th2 cytokines is an evolutionary adaptation common to all mammalian species studied, and furthermore, that this biasing is orchestrated principally via selective down-regulation of IFN-γ production (3, 5). The degree of this down-regulation in humans is profound, as neonatal lymphocytes produce 10-fold less IFN-γ than adult cells when optimally stimulated using PMA and Con A (6).

The precise mechanism(s) underlying this Th2 polarization of the fetal response are not fully understood, but production within the placenta of a range of effector molecules, including IL-4, IL-10, PGE_2, and progesterone, which down-regulate Th1 function via preferential up-regulation of Th1-antagonistic Th2 differentiation (3, 7–11), is believed to be central in this process. While these "bystander" mechanisms appear likely to play a significant role in damping IFN-γ production at the feto-maternal interface, it is less plausible to consider that they are alone responsible for controlling this potentially lethal process. Instead, given the fundamental importance of this protective mechanism for fetal survival in the face of the constant danger of Th1-inductive feto-maternal allogeneic interactions during gestation, it is reasonable to hypothesize that evolution may have selected for additional safeguarding mechanisms for control of IFN-γ gene expression during this life phase. A prime candidate mechanism in this context involves methylation of specific CpG sites within the IFN-γ gene, in particular within the promoter region.

A wide body of research (reviewed in Refs. 12–14) indicates that CpG methylation in mammals is an important mechanism by which transcription from specific gene loci is regulated in different cell types. Some recent findings indicate that CpG methylation contributes to regulation of IFN-γ gene transcription in the mouse, and the changes associated with Th1 cell function are also accompanied by chromatin modification. Firstly, a study of naive and memory CD8$^+$ T cells demonstrated uncoupling of IFN-γ transcriptional control using the methylation inhibitor 5-aza-2′-deoxycytidine (15). Clear evidence presented in that study showed that CD8$^+$ T cell clones with an unmethylated IFN-γ promoter produced 1000-fold more IFN-γ message than cells with hypermethylated promoters. In subsequent experiments (16) long term stable inheritance of an unmethylated IFN-γ promoter was demonstrated

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in murine CD44^{high} (memory) CD8$^+$ T cell clones, even after withdrawal of stimulation in culture and return of IFN-γ transcription to undetectable levels. 5-Aza-2′-deoxycytidine treatment has also been demonstrated to restore IFN-γ-production capacity to murine CD4$^+$ Th2 clones (17).

In addition to these observations in mice, increased IFN-γ gene transcription has been correlated with hypomethylation of the proximal promoter region in human Th1 cell clones, and hypermethylation has been demonstrated to inhibit nuclear factor binding to the human IFN-γ promoter (17). Of particular interest here are earlier investigations that used methylation-sensitive restriction mapping to identify the methylation status of a single *Sna*BI site in the proximal IFN-γ promoter in adult human CD4$^+$ and CD8$^+$ cells (18). In addition, treatment of human neonatal naive T cells with 5-aza-2′-deoxycytidine was shown to markedly up-regulate their capacity to produce IFN-γ (19). It is also of interest to note that the down-regulation of IFN-γ gene expression that follows HIV-1 infection of human T cells is associated with CpG hypermethylation in the IFN-γ promoter, and that treatment of infected cells with an antisense DNA methyltransferase construct reverses the hypermethylation and markedly increases IFN-γ production (20).

The study presented below further investigates the hypothesis that epigenetic mechanisms involving DNA methylation play an important role in maintaining the low IFN-γ phenotype characteristic of human neonatal T cells. Our results suggest that an intricate relationship exists between transcription from the human IFN-γ promoter and the effects of DNA methyltransferase activity (levels of CpG and non-CpG methylation), and further, that this relationship differs between IFN-γ-producing cell populations in neonates. In addition, experiments employing embryonic kidney cells expressing DNA methyltransferase 3a (Dnmt3a)[3] demonstrated patterns of CpA methylation in the IFN-γ gene that were comparable to those observed in T cells.

Materials and Methods

Cell isolation and differentiation

The cord blood and adult peripheral blood samples used during this study were collected with prior approval for the study from the Princess Margaret Hospital human ethics committee. PBMC and cord blood mononuclear cells were isolated from whole blood (diluted 50% (v/v) in RPMI medium) over a Lymphoprep gradient at room temperature and 900 relative centrifugal force for 30 min. Purification of cell types of interest was conducted by either immunomagnetic separation employing Dynabeads (Dynal Biotech, Lake Success, NY) or preparative flow cytometry. Magnetic separation using Ab-coated Dynabeads was performed according to the manufacturer's instructions, except that the buffer employed was 1× PBS, 2% (v/v) FCS, 0.6% (w/v) citrate, and gentamicin (50 μg/ml). In addition, cord blood samples were depleted of nucleated red cells with mAb anti-glycophorin A (Immunotech, Westbrook, ME; clone 11E4B7.6). The final CD45RO$^-$ T cells for immediate genomic DNA isolation were prepared by CD45RO depletion (DAKO, Carpenteria, CA; clone UCHL1, CD45RO-FITC; Dynabeads M-450 goat anti-mouse IgG), and CD3 selection (Dynabeads M-450 CD3 pan T). Preparative flow cytometry was performed by standard methodology to >95% purity on cord blood and adult blood mononuclear cells after initial gating on a forward/side scatter profile of medium-sized nongranular lymphocytes.

Bisulfite treatment, PCR, and sequencing

Genomic DNA in the size range of 20–30 kb was prepared from 1–2 × 10^6 isolated T cells using a QIAamp DNA Blood Mini Kit (Qiagen, Valencia, CA). The genomic DNA (~1–2 μg from 1 × 10^6 cells) was bisulfite-treated using established procedures (21). Briefly, 1 μg genomic DNA in 100 μl water was denatured at 37°C for 15 min with 20 μl freshly prepared 1.8 M sodium hydroxide (NaOH). Freshly prepared sodium bisulfite (pH 5 with 10 M NaOH) was added directly (3.1 M final concentration) with hydroquinone (0.5 mm final concentration), and sulfonation was allowed to proceed at 55°C for 18–24 h under mineral oil. The reaction was recovered and diluted in 1 vol ethanol and 1 vol of water and purified again using a QIAamp DNA Blood Mini Kit (Qiagen) before alkali desulfonation (0.3 M NaOH) at 37°C for 15 min. Finally, the bisulfite-treated DNA was ethanol-precipitated as described previously (22) with 20 μg glycogen (Roche, Indianapolis, IN) before resuspension in 20 μl water for PCR amplification.

Three nested sets of forward and reverse primers were designed against coding and noncoding strand bisulfite-treated DNA for each gene of interest. The separate primer sets were tested using the PCR conditions outlined below, and the most efficient primer sets for each gene were preserved for later use (Table I). The IFN-γ promoter (GenBank accession no. J00219) noncoding region primers amplified a 526-bp DNA from nt 21–546, and coding region primers amplified a 550-bp DNA from nt 21–570. The IL-4 promoter region (GenBank accession no. M23442) noncoding primers amplified a 600-bp DNA from nt 621-1220, and coding region primers amplified a 620-bp DNA from nt 611–1,230. The IFN-γ receptor α-chain (IFNGR1) CpG island promoter region (GenBank accession no. AL050337) noncoding strand primers and coding strand primers amplified the same 462-bp DNA from nt 80,872–81,411. The TNF-α promoter region (GenBank accession no. X02910) coding primers amplified a 648-bp DNA from nt 51–698.

The PCR primers against IFN-γ, IL-4, TNF-α, and IFNGR1 promoter were used in PCR at 300 nM with 1.5 U PLATINUM *Taq* DNA polymerase (Invitrogen, Carlsbad, CA), 10–100 ng bisulfite-treated genomic DNA, 1.5 mM $MgCl_2$, and 200 μM dNTPs. The cycling parameters were 96°C for 3 min and 30 s (1 cycle); 96°C for 15 s, 59°C (decreasing by 1°C/cycle) for 30 s, 72°C for 1 min (9 cycles); 96°C for 15 s, 50°C for 30 s, and 72°C for 1 min (increasing by 5 s/cycle; 30 cycles); and 72°C for 7 min (1 cycle). The PCR product DNA was cloned directly into pCR2.1 plasmid using an Original TA Cloning Kit (Invitrogen, San Diego, CA), and plasmid DNA was prepared for automated sequencing using a QIAprep Spin Miniprep Kit (Qiagen). Sequencing of 500 ng plasmid DNA was performed with T7 primer (5′-TAA TAC GAC TCA CTA TAG GG) and BigDye Terminator Ready Reaction Mix (PE Applied Biosystems, Foster City, CA) according to the manufacturer's instructions, except that the cycle-sequencing PCR parameters were varied to compensate for AT-rich bisulfite-treated DNA using an annealing temperature of 45°C and extension at 55°C.

Results

The clustering of CpG dinucleotides is different in the promoters of human IFN-γ, IL-4, TNF-α, and IFNGR1 genes

Fig. 1*A* illustrates the relative positions of CpG dinucleotides in the human IFN-γ promoter compared with the promoters of IL-4, TNF-α, and IFNGR1. The arrangement of symmetrical CpG dinucleotides in the IFN-γ and IL-4 promoters is sparse compared with the CpG island occurring at the IFNGR1 promoter and the intermediate frequency of CpG sites in the promoter of TNF-α. Human studies have shown that a hypomethylated human IFN-γ promoter results in transcriptional up-regulation (18, 23), and, conversely, a hypermethylated IFN-γ promoter can disrupt nuclear factor binding (17, 24). The positions of CpG sites that, when methylated, may be potentially disruptive to nuclear factor binding are identified in Fig. 1*B*.

The human IFN-γ promoter is hypermethylated at CpG and non-CpG sites in cord blood CD3$^+$/CD45RO$^-$ T cells

The IFN-γ promoter methylation status of CD3$^+$/CD45RO$^-$ T cells was investigated in neonatal cord blood and adult peripheral blood. Fig. 2*A* presents a schematic map of the 526-bp IFN-γ promoter region that was examined by sequencing bisulfite-treated and cloned genomic DNA from different randomly selected individuals. Between 9–12 reverse strand sequences were scored for their methylation profile at CpG sites −295, −186, −54, +122, +128, and +171 relative to the start of transcription. During the scoring of methylated CpGs in the IFN-γ promoter from cord blood in particular, a number of apparently methylated cytosines were observed in the context of CpA (positions −290, −254,

[3] Abbreviations used in this paper: Dnmt3a, DNA methyltransferase 3a; HDAC, histone deacetylase; IFNGR1, IFN-γR α-chain.

Table I. *Oligonucleotides used for PCR from bisulphite-treated genomic DNA*[a]

	Details	Sequences	Position
			GenBank J00219
hifng.F3	(nc) IFNγ fwd	5′-TATAAATAAAAAATCAACATTTTACCAAAA	21–50
hifng.R3a	(nc) IFNγ rvse	5′-TTGGTAGTAATAGTTAAGAGAATTTA	546–521
hifng.F3a	(c) IFNγ fwd	5′-TGTGAATGAAGAGTTAATATTTTATTA	21–47
hifng.R2	(c) IFNγ rvse	5′-TTCTACTTCTTTTACATATAAATCCTAACA	541–570
			GenBank M23442
hil4.F3	(nc) IL-4 fwd	5′-CCTCTATACAAAAAAAAAACCCCAAAT	621–647
hil4.R3a	(nc) IL-4 rvse	5′-TATGTTAGTAGGAAGAATAGAGGGG	1,196–1,220
hil4.F2a	(c) IL-4 fwd	5′-TGTTATTTTGTTTTTATGTAGAGAAGGA	611–638
hil4.R2	(c) IL-4 rvse	5′-ATTACCAACACATACTAACAAAAAAAACAA	1,201–1,230
			GenBank X02910
TNF.F3	(nc) TNFα fwd	5′-ACCCAACCTTTCCTAAAACCTCAAA	51–75
TNF.R1a	(nc) TNFα rvse	5′-GTTGTTTTTAGGGGGGGTTTGTAG	675–698
			GenBank AL050337 (rvse complement)
ifngr.F1	(nc) IFNGRI fwd	5′-AAATTCCTCAAATAAAAAAACAAAAACT	32,401–32,428
ifngr.R2a	(nc) IFNGRI rvse	5′-YGYGGTGTTTATTTTAGTTTTGTTTAT	32,914–32,940
ifngr.F1a	(c) IFNGRI fwd	5′-AGGTTTTTTAAATGAAAAAGTAGGGAT	32,401–32,427
ifngr.R2	(c) IFNGRI rvse	5′-CRCRATACCCATCTCAACCCTA	32,919–32,940

[a] Oligonucleotide details include whether the primer was designed against bisulphite-treated coding (c) or noncoding (nc) strand. fwd, forward; rvse, reverse.

−252, −240, −209, and −2) and CpT (positions −169 and −2) sites.

The IFN-γ promoter of cord blood CD3$^+$/CD45RO$^-$ T cells was hypermethylated at the three CpG sites 5′ to the start of transcription (−295, −186, and −54) and at an additional three sites downstream (+122, +128, and +171; Fig. 2*B*). Bisulfite sequence analysis of both DNA strands from the IFN-γ promoter confirmed the symmetrical nature of CpG methylation in DNA sequences from cord blood cells (data not shown). Bisulfite treatment of genomic DNA resulted in DNA strands that were no longer complementary, and as such, an early decision was made in this study to primarily analyze data from the noncoding strand.

Bisulfite sequencing of the IFN-γ promoter from adult CD3$^+$/CD45RO$^-$ T cells revealed variable, but consistently lower, levels of methylation at CpG sites relative to the results for cord blood T cells (Fig. 2*C*). The measured difference in CpG methylation at the IFN-γ promoter of cord blood and adult CD3$^+$/CD45RO$^-$ T cells was highly significant at positions −295, −186, −54, +122, and +128 ($p < 0.01$–0.001) by a Fisher's exact test of adult/cord vs methylated/unmethylated contingency table for each CpG site.

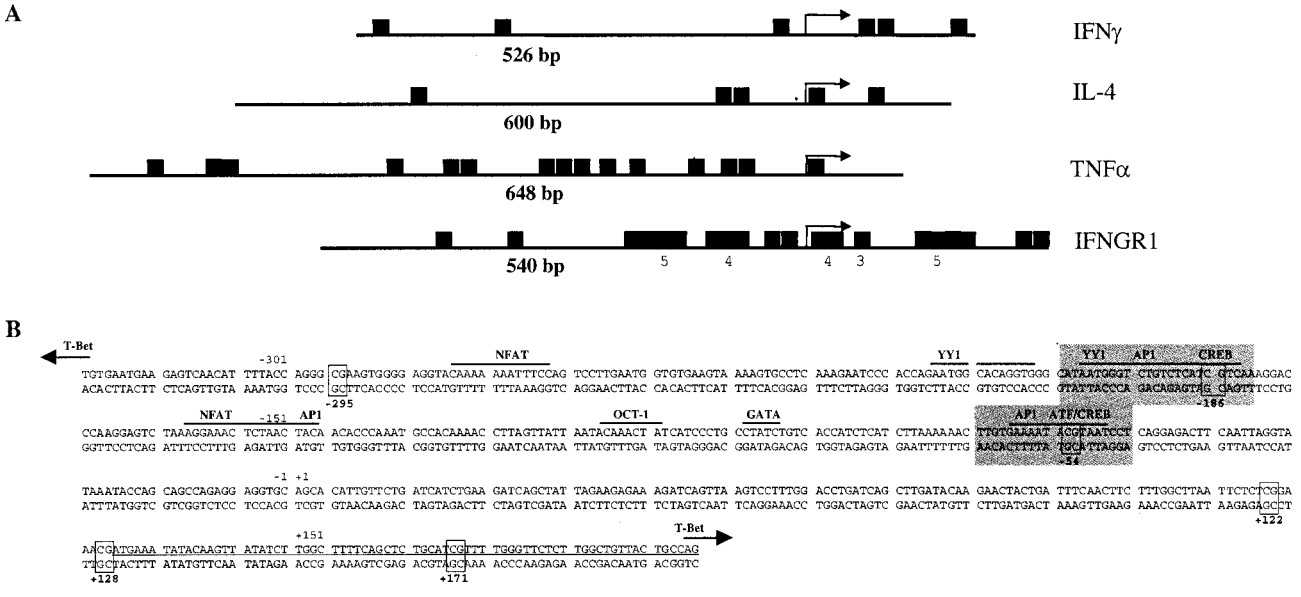

FIGURE 1. Methylated CpG sites can negatively regulate transcription. *A*, The IFN-γ, IL-4, TNF-α, and IFNGR1 promoters were PCR-amplified using bisulfite-treated DNA-specific oligonucleotides (refer to Table I). The primers amplified 526 bp of the IFN-γ promoter from nt 21–546 (GenBank accession no. J00219), 648 bp of the TNF-α promoter from nt 51–698 (GenBank accession no. X02910), 600 bp of the IL-4 promoter from nt 621–1,220 (GenBank accession no. M23442), and 540 bp of the IFN-γ receptor α-chain (IFNGR1) promoter CpG-island from nt 81,411–80,872 (GenBank accession no. AL050337, complement). Filled squares mark the position of CpG dinucleotides, and an arrow marks the start of transcription. The number of closely spaced CpGs is marked below the gene map. *B*, The IFN-γ promoter has an open spatial arrangement of methylated CpG sites (boxed), two of which are sites of particular interest because of transcription factor activity (shaded). The nucleotide position is marked relative to the start of transcription (+/−). Functionally important transcription factor binding sites are marked in bold above their putative binding sites in the DNA sequence (17, 24, 39–41).

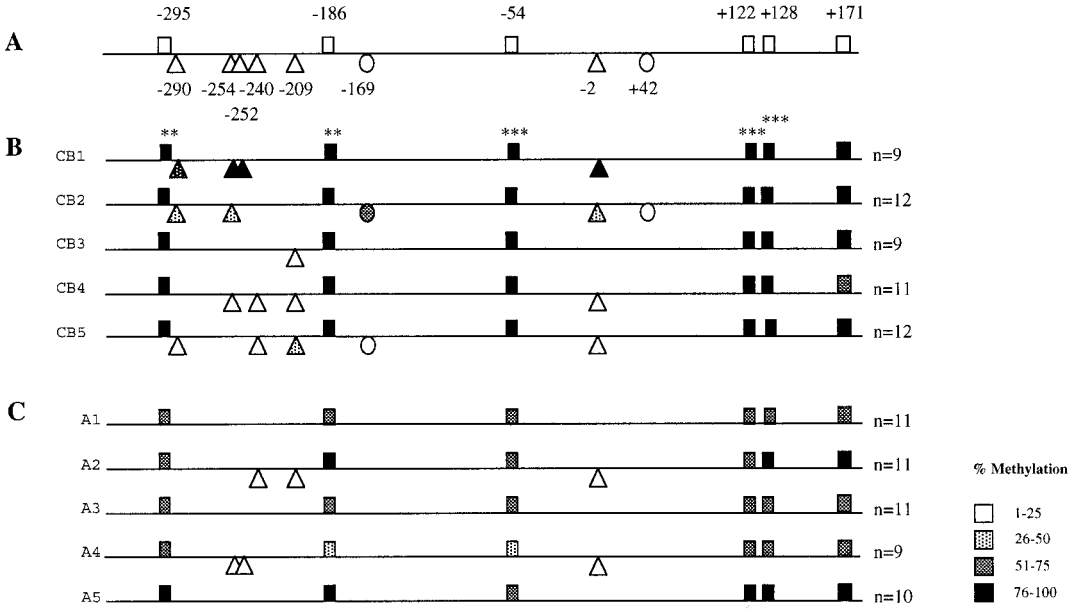

FIGURE 2. IFN-γ promoter methylation at CpG, CpA, and CpT sites in cord blood and adult peripheral blood $CD3^+/CD45RO^-$ T cells. *A*, Primary $CD3^+/CD45RO^-$ T cells were purified using Dynabeads, and 2 μg genomic DNA was isolated and bisulfite-treated overnight. A 526-bp region of the IFN-γ promoter was PCR-amplified from the bisulfite-treated reverse strand using specific oligonucleotides. Methylated cytosines resistant to bisulfite treatment were scored from cloned PCR product in 9–12 sequences for each individual and marked on the gene map of the region as CpG (squares), CpA (triangles), and CpT (circles) sites. *B*, Gene map of the hypermethylated IFN-γ promoter from five different cord blood $CD3^+/CD45RO^-$ T cell preparations. The degree of methylation at each CpG, CpA, and CpT site is depicted by the strength of shading. *C*, Gene map of the hypomethylated IFN-γ promoter from five different adults, and degree of methylation depicted as described in *B*. A 2 × 2 Fisher's exact test contingency table was used to assign *p* values for cord vs adult comparisons (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$) after scoring the nominal variables adult/cord and methylated/unmethylated into continuous data for each CpG site.

The scoring of IFN-γ promoter non-CpG methylation was almost entirely restricted to specific CpA sites (positions −290, −254, −252, −240, −209, and −2) and CpT sites (positions −169 and +42) in the DNA sequence. There were a number of potential CpA and CpT sites in the IFN-γ promoter reverse strand, but clearly, methylation activity appeared specifically at a limited number of them. We measured a highly significant differential in IFN-γ promoter CpA and CpT methylation between cord blood and adult naive T cells ($p < 0.0001$; refer below to Table II), but found little evidence of previously reported non-CpG methylation at CpNpG sites in the IFN-γ promoter (25).

Table II. *Methylation at CpA and CpT sites in the IFN-γ promoter is significantly different between cord blood and adult T cells*[a]

	Sequences	Methylated	Unmethylated
$CD3^+/CD45RO^-$			
Adult	52	10	458
Cord	53	69	408***
$CD4^+/CD45RO^-$			
Adult	45	10	395
Cord	120	51	1029*
$CD8^+/CD45RO^-$			
Adult	33	1	296
Cord	37	2	331
$CD56^+/CD16^+/CD3^-$			
Adult	31	3	276
Cord	31	3	276

[a] CpA methylation (position −292, −254, −252, −240, −209, and −2) and CpT methylation (position −169, −41 and +42) was scored at specific sites in IFN-γ promoter DNA sequence. The number of methylated and unmethylated sites was scored and a Fisher's exact test was used to assign *p* values (*, = 0.056; ***, $p <$ 0.0001).

The human IFN-γ promoter is hypermethylated at CpG and non-CpG sites in $CD4^+/CD45RO^-$ T cells

To further characterize the level of CpG methylation at the IFN-γ promoter of adult and neonatal naive cells we purified $CD4^+$, $CD8^+$ T cells and $CD56^+/CD16^+$ NK cells from selected individuals by preparative flow cytometry and immunomagnetic separation. The analysis of IFN-γ promoter methylation profiles in these purified IFN-γ-producing cell types revealed that $CD4^+$ naive T cells were significantly hypermethylated in cord blood and hypomethylated in adult peripheral blood (Fig. 3*A*). In contrast, there was no significant difference between the IFN-γ promoter CpG methylation status of cord blood and that of adult $CD8^+$ T cells or NK cells (Fig. 3, *B* and *C*). Cytosine methylation at non-CpG sites was also scored for these cell types, employing the sequences analyzed in Figs. 2 and 3, and is illustrated in Table II. Methylation of IFN-γ promoter CpA and CpT sites was observed 1) in the overall naive $CD3^+/CD45RO^-$ T cell population (Fig. 2), and 2) in the purified $CD4^+$ naive T cell population (Fig. 3*A*). Like CpG methylation, non-CpG methylation patterns measured at the IFN-γ promoter were not significantly different between cord blood and adult $CD8^+$ T cells and NK cells.

Comparison of the adult methylation pattern (Fig. 3, □) among the three cell types clearly indicates an active process may be affecting the distribution of methylated CpG sites along an actively transcribing promoter (Fig. 3, *A–C*, compare the hypermethylated +122 and +128 sites proximal to the start of transcription with the demethylated −186 and −54 sites of known nuclear factor binding). It is also clear from these results that cord blood $CD8^+$ T cells and NK cells would be no less inhibited as to their IFN-γ-producing capacity by CpG methylation than would their adult counterparts (Fig. 3, *B* and *C*).

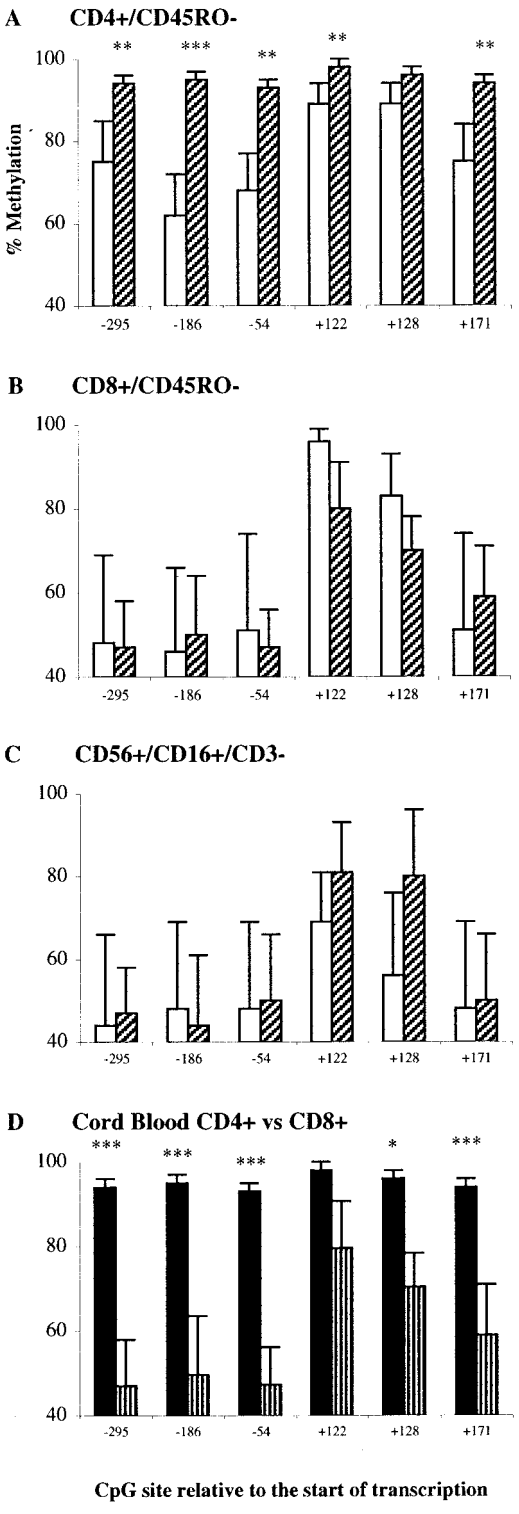

FIGURE 3. Methylation of CpG sites in the IFN-γ promoter is significantly different between cord blood and adult $CD4^+/CD45RO^-$ T cells and between $CD4^+/CD45RO^-$ and $CD8^+/CD45RO^-$ T cells in cord blood. Data shown are the percent methylation for each IFN-γ promoter CpG site. Error bars measure SEM from a two-tailed unpaired *t* test, and a 2×2 Fisher's exact test contingency table was used to assign *p* values for cord vs adult comparisons (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$) after scoring the nominal variables adult/cord and methylated/unmethylated into continuous data for each CpG site. *A*, Cord blood (▨) vs adult (□) $CD4^+/CD45RO^-$ T cells. *B*, Cord blood (▨) vs adult (□) $CD8^+/CD45RO^-$ T cells. *C*, Cord blood (▨) vs adult (□) $CD56^+/CD16^+/CD3^-$ NK cells. *D*, Cord $CD4^+/CD45RO^-$ (■) T cells vs cord blood $CD8^+/CD45RO^-$ (▥) T cells from *A* and *B*.

Bisulfite sequence data from the IL-4, TNF-α, and IFNGR1 genes

We additionally sought to examine CpG methylation in other important cytokine promoters, focusing initially on IL-4 promoter methylation in the cord blood and adult $CD3^+/CD45RO^-$ T cell populations. We sequenced the IL-4 promoter from the same batches of bisulfite-treated DNA that were used to amplify the IFN-γ promoter during the course of our study in Fig. 2. This served two purposes: first, to confirm whether the disparity in IFN-γ promoter CpG and non-CpG methylation observed between cord blood and adult T cells was a consequence of methyltransferase activity and possibly measurable in *trans* at other accessible promoters such as the IL-4 promoter; and second, to quantify the levels of reiterative de novo methylation at CpA and CpT sites in another promoter of interest. Methylation at CpA or CpT sites in the IFN-γ promoter was approximately 7-fold higher in cord blood $CD3^+/CD45RO^-$ T cells than in adult cells (Table II) and 5-fold higher in purified cord blood $CD4^+/CD45RO^-$ T cells than in adults. In contrast, there was little or no evidence of non-CpG methylation in IL-4 promoter sequence (data not shown) from cord blood and adult $CD3^+/CD45RO^-$ T cells.

Coding and noncoding strand IFNGR1 CpG-island (GenBank accession no. AL050337; nt 81,411–80,872) sequences were PCR-amplified and cloned from the same bisulfite-treated genomic DNA used to isolate IFN-γ and IL-4 promoter regions. Of 10 coding strand sequences and nine noncoding strand sequences, no unconverted cytosines were observed at CpA or CpT sites. In addition we also sequenced a 648-bp region of the TNF-α promoter from cord CB1 $CD3^+/CD45RO^-$ T cell bisulfite-treated genomic DNA and found no evidence of methylated CpA or CpT sites in a total 5800 bp of sequence.

The possibility exists that the non-CpG methylation we have observed may have resulted in part from local effects causing resistance to bisulfite treatment. While we cannot entirely rule out this possibility, we believe that the contribution of such factors is likely to be small, for the following reasons. First, the average frequency of unconverted cytosines measured outside putative non-CpG sites in this study varied between $4–9 \times 10^{-4}$/base, and as such was relatively low and equivalent to the error rate of *Taq* polymerase in our system. Second, if a DNA structure resistant to bisulfite treatment caused unconverted hot spots, such sites should consistently generate a false signal in all sequences examined. This was not the case, and CpA/CpT methylation occurred most commonly in neonatal T cell sequences (Table II). Third, findings reported for murine embryonic stem cells show that the relative frequency of non-CpG methylation is CpA>CpT>CpC in genome-wide analysis (26), which correlates with the frequency of these sites we have observed at the IFN-γ promoter, in that most, if not all, non-CpG methylation appears at CpA dimers, and none appears above background levels at CpC dimers. Finally, during the course of bisulfite sequencing we did observe sequences (~1 in 200) that were incompletely bisulfite converted, containing a number of unconverted cytosines. In contrast to the results reported in Fig. 2, those sites of incomplete conversion appeared randomly (data not shown).

Non-CpG methylation at specific CpA and CpT residues in the human IFN-γ promoter parallels Dnmt3a expression in 293 cells

Reports of non-CpG methylation have been sporadic in the literature (25, 27–29) and presumably have only arisen recently because of the detailed sequence analysis possible using PCR-amplified gene elements from bisulfite-converted genomic DNA. Two

studies of particular interest used the method of nearest neighbor analysis to show that in mammals (26), and even insects (30), CpA and CpT methylation is the most common form of non-CpG methylation. In another investigation, overexpression of Dnmt3a in the 293 embryonic kidney carcinoma cell line caused methylation of a chromosomally integrated copy of the EBV nuclear Ag 1 gene at two *Hha*I methylation-sensitive restriction sites (31). Using DNA from the latter model we bisulfite-sequenced the IFN-γ promoter from 293 cells expressing either Dnmt3a or Dnmt3b (31) to investigate whether the CpA and CpT methylation patterns in the promoter could be attributable to the activity of either enzyme. The results demonstrated Dnmt3a expression in 293 cells was accompanied by the appearance of non-CpG methylation, specifically at CpA sites in the IFN-γ promoter noncoding strand (Fig. 4*B*). This effect was not seen in cells that overexpressed Dnmt3b or in control 293 parent cells (data not shown).

Bisulfite treatment of cloned native IFN-γ sequence does not cause unconverted 5-methylcytosine artifacts in the context of CpA or CpT sites

To discount the likelihood that the unusual DNA structure of the IFN-γ promoter caused incomplete bisulfite treatment hot spots and unconverted cytosine in the context of CpA and CpT sites, a control bisulfite experiment was performed using cloned native IFN-γ (data not shown). The native IFN-γ promoter was cloned into pBluescript II SK^+ plasmid, and purified plasmid was bisulfite-treated essentially as described for the genomic DNA samples. The PCR-amplified IFN-γ was then subcloned and sequenced from bisulfite-treated plasmid, instead of the normal bisulfite-treated genomic DNA. Little or no unconverted cytosine was discovered in the context of the CpA or CpT sites that were identified during genomic DNA bisulfite sequencing. Unconverted cytosines arose from incomplete bisulfite treatment in the DNA surrounding CpA and CpT sites as a result of bacterial methylase activity with a similar frequency as it did at the CpA and CpT sites.

IFN-γ expression by cord blood and adult $CD4^+$ and $CD8^+$/$CD45RO^-$ T cells

To demonstrate the differential between IFN-γ production from cord blood and adult $CD4^+$ and $CD8^+$ T cells we optimally stimulated immunomagnetically purified T cells from two neonates and two adults with PMA/ionomycin using standard methods over a 48-h period (Table III). Under these conditions of short term stimulation, where little cell division has occurred, the absolute levels of IFN-γ are greatest from adult $CD8^+$ T cells and lowest from cord blood $CD4^+$ T cells. However, direct comparison of relative levels of IFN-γ production within these T cell populations in the two age groups indicates that while production in cord blood $CD8^+$ T cells is 50–65% of that in their adult counterparts, corresponding production levels in cord blood $CD4^+$ T cells are reduced 5- to 10-fold relative to adult levels, as reported previously (6). This finding parallels our observation of high level IFN-γ promoter methylation in cord blood $CD4^+$ T cells compared with $CD8^+$ T cells (Fig. 3*D*), in contrast to the comparable methylation status of respective T cells in the adult (Fig. 3, *A* and *B*).

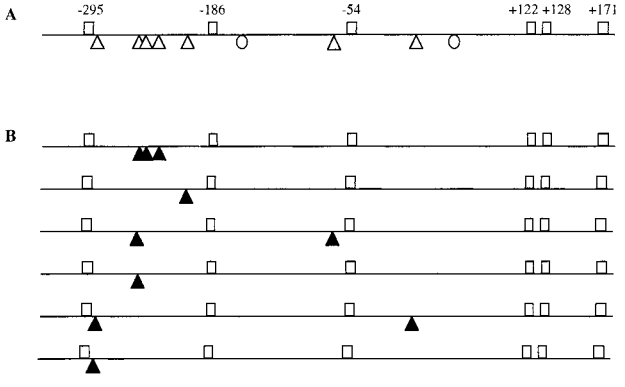

FIGURE 4. The IFN-γ promoter is methylated at CpA sites when Dnmt3a is expressed in 293 cells. *A*, A gene map of the IFN-γ promoter illustrating CpG and non-CpG methylation sites as described in Fig. 2. *B*, Bisulfite sequencing data from the IFN-γ promoter of 293 cells expressing Dnmt3a (28). Filled triangles indicate unconverted cytosine in the context of CpA dimers. Each line of methylation data represents the scored data from a single cloned bisulfite-treated IFN-γ promoter containing methylated non-CpG sites. No unconverted cytosine was discovered in parental 293 cells ($n = 20$) or 293 cells expressing Dnmt3b.

Table III. *IFN-γ production from $CD4^+$ and $CD8^+$/$CD45RO^-$ T cells*[a]

	Cords		Adults	
	CB11	CB9	A1	A4
$CD4^+$/$CD45RO^-$				
Unstimulated	110	520	14,000	7,000
PMA/Ionomycin	8,400	9,300	190,000	330,000
$CD8^+$/$CD45RO^-$				
Unstimulated	26	20	18	16
PMA/Ionomycin	39,000	96,000	360,000	500,000
	(5)[b]	(10)	(2)	(1.5)

[a] IFN-γ levels were measured in picograms per milliliter by time-resolved fluorometry with a Wallac Victor II.

[b] Figures in parenthesis indicate the ratio of $CD8^+$:$CD4^+$ IFN-γ production in each sample.

Discussion

An accumulating body of evidence implicates CpG methylation in the IFN-γ promoter as an important negative transcriptional regulator of IFN-γ production by human T cells. Previous studies using methylation-sensitive restriction mapping have investigated the status of the −54 CpG site in human $CD4^+$ and $CD8^+$ T cells (18, 23). We have extended these studies by the use of a sensitive bisulfite sequencing technique that allows measurement of methylated cytosine in any context over a DNA region of interest. These studies were initially prompted by an observation that murine $CD8^+$ memory T cell clones producing large amounts of IFN-γ message are hypomethylated at the IFN-γ promoter, and that this epigenetic characteristic was preserved upon withdrawal of stimulation and return of message production to basal levels (15, 16). Our present results with human cells are consistent with these murine findings, and further suggest that variations in the degree of CpG methylation in the IFN-γ promoter may underlie developmentally related differences in the capacity to express IFN-γ responses following stimulation. In particular, PMA/ionomycin-stimulated $CD4^+$/$CD45RO^-$ neonatal T cells produce 5- to 10-fold lower levels than their adult $CD4^+$/$CD45RO^-$ counterparts, and this correlates with methylation in the IFN-γ promoter of 94–96% vs 62–74% of CpG sites, respectively.

The mechanisms by which CpG methylation regulates gene transcription is incompletely understood. In particular, there is a paucity of information on what constitutes the threshold level of CpG methylation necessary for silencing of individual genes. However, recent findings implicating chromatin remodeling at both the IFN-γ and IL-4 loci in murine T cells during Th1/Th2 differentiation (32) provide a broad theoretical framework for study of the underlying mechanisms. Thus, it appears that methylation of CpG dinucleotides in a symmetrical fashion attracts

methyl-binding proteins that may modulate transcription factor binding by direct steric hindrance or may act in combination with multiprotein complexes containing histone deacetylase (HDAC) activity to increase the charge on histone and ultimately cause chromatin remodeling. The mere association of methyl-binding proteins to methylated DNA has also been shown to repress transcription without associated HDAC activity and higher order chromatin formation (33). Heterochromatin formation is thus an attractive theoretical mechanism for explaining reduced accessibility and negative regulation of transcription; however, in practice, proving the link between methylation and changes to DNA accessibility can be difficult. Treatment of cells with trichostatin A (HDAC inhibitor) did not, for example, restore high level transcription from a number of tumor suppressor genes, even when the cells had been precultured with the methylation inhibitor 5-aza-2′-deoxycytidine (34, 35).

Our results also suggest the possibility that control of IFN-γ gene activation via promoter methylation may be even more complex than hitherto realized, as non-CpG (notably CpA and CpT) sites may also be involved. There are few precedents for this possibility in the current literature, and reports of non-CpG methylation in mammalian gene sequences have been sporadic and largely restricted to repetitive DNA elements. Densely methylated CpG islands occurring at the ori_{S14} and ori-β origins of replication in Chinese hamster ovary cells are bilaterally methylated at CpN dinucleotides (28) and are conserved in humans (36, 37). Bisulfite sequencing of two regions of pBluescript II SK^+ plasmid that had been transfected into mouse F9 embryonal carcinoma cells and mouse NIH-3T3 fibroblasts revealed methylated CpNpG sites at different cytosines along both DNA strands for each of the two regions of the plasmid (25). In a sequence covering four gene regions, we detected little methylation at these putative CpNpG sites. In another study examining human embryonic fibroblasts, a number of non-CpG methylation sites were identified in the sequence of an L1 retrotransposon (29). The most recent study of importance here was a genome-wide examination of non-CpG methylation in murine embryonic stem cells (26), which showed that methylation occurred at CpA and CpT sites in wild-type embryonic stem cells and different somatic tissues. The same group also identified non-CpG methylation in the genome of *Drosophila* (30).

We hypothesized that the non-CpG methylation we have observed here may be an event activated by specific DNA methyltransferase activity in T cells. Several observations lead us to this suggestion. First, the proportions of CpA and CpT methylations we observe in the IFN-γ promoter of neonatal $CD3^+CD45RO^-$ naive T cells and purified neonatal $CD4^+/CD45RO^-$ T cells were similar, with a bias toward CpA methylation. This observation has also been made in mouse embryonic stem cells (26). Second, our IFN-γ promoter methylation data for CpG sites from adult $CD8^+/CD45RO^-$ T cells demonstrates hypomethylation similar to that observed in mouse $CD44^{high}$ (memory) $CD8^+$ T cells (15), which are also capable of generating high level IFN-γ production upon activation. Third, in the neonates studied here the IFN-γ promoter was hypermethylated in $CD4^+/CD45RO^-$ T cells at all of the six CpG sites, but, conversely, was hypomethylated in $CD8^+/CD45RO^-$ T cells, suggesting that the $CD8^+$ (cytotoxic) T cell component of neonatal host defenses may be relatively competent compared with their $CD4^+$ counterparts. This initial block in $CD4^+$ Th cell function may be a contributing factor to the poor generation of T cell memory against viral infection that is characteristic of the neonate (38).

We have also observed that the differential between IFN-γ promoter methylation at CpG and non-CpG sites in neonatal and adult $CD4^+/CD45RO^-$ T cells was not paralleled at the IL-4 promoter. Lastly, we have demonstrated that Dnmt3a overexpression accompanies IFN-γ promoter CpA methylation in the 293 embryonic kidney carcinoma cell line and thus may be the cause of de novo non-CpG methylation in neonatal $CD3^+/CD45RO^-$ and $CD4^+/CD45RO^-$ T cells. However, caution must be exercised in extrapolation of these findings in 293 cells to primary T cells, as Dnmt3A expression is known to be significantly higher in the transformed cell line (31), and additional studies will accordingly be required to resolve this issue. Nevertheless, our capacity to recapitulate the precise effect observed in unmodified T cells by supplementation of specific enzyme activity in a cell line argues strongly that the non-CpG methylation phenomenon we have observed is not simply a random artifact.

In conclusion, we speculate that the dualistic nature of the functions of IFN-γ in fetal development, viz., an obligatory role in implantation vs potentially catastrophic effects on placental function if produced locally at significant levels later in gestation, has dictated the necessity for particularly fine, yet robust, control of IFN-γ gene expression during this life phase. We further hypothesize that the basis for this fine control involves either regulated or constitutive Dnmt1/3a expression, which, in turn, coordinates a program of reiterative de novo methylation at specific sites in the IFN-γ gene. Current understanding of how this mechanism operates emphasizes the importance of symmetrical CpG methylation in repressing transcription via recruitment of methyl-binding proteins. Therefore, addition of further methyl groups to DNA by reiterative de novo methylation at CpA and CpT sites, as demonstrated above, could enhance the efficiency of transcriptional repression either directly by steric hindrance or via attraction of as yet uncharacterized regulatory proteins. As such, de novo methylation at CpA and CpT sites may represent a rapid, yet transient, method that a cell might employ to modulate epigenetic reprogramming.

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