IL-10 Is Excluded from the Functional Cytokine Memory of Human CD4+ Memory T Lymphocytes

Jun Dong, Claudia Ivascu, Hyun-Dong Chang, Peihua Wu, Roberta Angeli, Laura Maggi, Florian Eckhardt, Lars Tykocinski, Carolina Haefliger, Beate Möwes, Jochen Sieper, Andreas Radbruch, Francesco Annunziato and Andreas Thiel

*J Immunol* 2007; 179:2389-2396;
doi: 10.4049/jimmunol.179.4.2389
http://www.jimmunol.org/content/179/4/2389

**Why The JI?**

- **Rapid Reviews! 30 days** from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Speedy Publication!** 4 weeks from acceptance to publication

*average*

**References**
This article cites 59 articles, 28 of which you can access for free at:
http://www.jimmunol.org/content/179/4/2389.full#ref-list-1

**Subscription**
Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
IL-10 Is Excluded from the Functional Cytokine Memory of Human CD4⁺ Memory T Lymphocytes

Jun Dong, Claudia Ivascu, Hyun-Dong Chang, Peihua Wu, Roberta Angeli, Laura Maggi, Florian Eckhardt, Lars Tykocinski, Carolina Haefliger, Beate Möwes, Jochen Sieper, Andreas Radbruch, Francesco Annunziato, and Andreas Thiel

Epigenetic modifications, including DNA methylation, profoundly influence gene expression of CD4⁺ Th-specific cells thereby shaping memory Th cell function. We demonstrate here a correlation between a lacking fixed potential of human memory Th cells to re-express the immunoregulatory cytokine gene *IL10* and its DNA methylation status. Memory Th cells secreting IL-10 or IFN-γ were directly isolated ex vivo from peripheral blood of healthy volunteers, and the DNA methylation status of *IL10* and *IFNG* was assessed. Limited difference in methylation was found for the *IL10* gene locus in IL-10-secreting Th cells, as compared with Th cells not secreting IL-10 isolated directly ex vivo or from in vitro-established human Th1 and Th2 clones. In contrast, in IFN-γ⁺ memory Th cells the promoter of the *IFNG* gene was hypomethylated, as compared with IFN-γ⁻nonsecreting memory Th cells. In accordance with the lack of epigenetic memory, almost 90% of ex vivo-isolated IL-10-secreting Th cells lacked a functional memory for IL-10 re-expression after restimulation. Our data indicate that *IL10* does not become epigenetically marked in human memory Th cells unlike effector cytokine genes such as *IFNG*. The exclusion of IL-10, but not effector cytokines, from the functional memory of human CD4⁺ T lymphocytes ex vivo may reflect the need for appropriate regulation of IL-10 secretion, due to its potent immunoregulatory potential. The Journal of Immunology, 2007, 179: 2389–2396.

Immune reactions are tightly controlled to avoid excessive activation in the course of pathogen-specific immune responses and to suppress the activation of autoreactive lymphocytes. Different subsets of regulatory T cells represent pivotal players of immune regulation in the immune system (1). Populations of regulatory T cells identified include naturally occurring CD4⁺CD25⁺ T regulatory cells (2), Ag-induced T regulatory type 1 cells (Tr1) (3), and Th3 cells (4), which all display certain abilities to produce the immunoregulatory cytokine IL-10. IL-10 exerts its inhibitory action on macrophages and dendritic cells, thereby regulating effector cell activation, but has also inflammatory effects on B and T cells (5). *Il10*-deficient mice develop severe chronic enterocolitis (6) while tissue- or cell-specific overexpression of *Il10* leads to impaired immune responses (7–10). Tr1 cells secret high levels of IL-10 and low levels of IFN-γ and IL-2 upon activation, but display their regulatory function in an IL-10-dependent manner (11–14). IL-10 production by CD4⁺CD25⁺ regulatory T cells has been reported in different in vivo (15, 16) and in vitro (17) experimental systems. Thus, IL-10 gene expression by regulatory T cell subsets is an essential factor for effective regulation of immune responses.

The immune system needs to act rapidly and specifically to pathogens but also has to control autoimmunity. These actions are partially mediated by effector and regulatory cytokines produced by memory Th cells that memorize the expression of cytokine genes after primary activations triggered by TCR signaling and instructive costimulatory signals, and re-express memorized cytokine genes within a few hours after restimulation via TCR, in the absence of original instructive costimulatory signals (18). Control of effector cytokines expression via networks of transcription factors and epigenetic regulation has been well documented. Epigenetic modifications such as changes in DNA methylation (primarily at the CpG5 position of cytosine in CpG dinucleotides), histone modifications, and chromatin rearrangement within the nucleus influence the accessibility of transcription factors to their DNA binding sites. These associated molecular changes are heritable and provide a basis for memory of gene expression (19–22). Indeed, these mechanisms have been documented for Th1 and Th2 cells in the transcriptional regulation of the *Ifng*/*IFNG* and *Il4* clustered genes (23–30). However, epigenetic regulation of *IL10*, especially by DNA methylation, has remained poorly understood.

The expression of *Il10* is under the control of several transcription factors such as Stat3 (31), Sp1 (32) and Sp3 (33), NF-κB (34), Smad-4 (35), c-Maf (36), and Jun proteins (37). Recent data obtained in mouse on *Il10* gene accessibility according to DNase I hypersensitivity have suggested chromatin remodeling of the *Il10* gene locus. However, some DNase I hypersensitive sites (HSS) were described as both enhancing and silencing *Il10* gene expression (34, 38, 39).

To gain insight into the epigenetic regulation of the *IL10* gene in comparison to the *IFNG* gene in human Th cells, we characterized...
IL-10− IFN-γ−, IL-10+ IFN-γ−, IL-10− IFN-γ+, and IL-10+ IFN-γ+ Th cell subsets isolated directly ex vivo from peripheral blood of healthy adults. Using a bisulfite-modified DNA sequencing approach, we performed semiquantitative assessments of the DNA methylation pattern of the entire IL10 gene locus spanning 9.1 kb upstream and 9 kb downstream of the transcriptional start site that encompasses 88 selected CpGs. We demonstrate the lack of a specific DNA methylation pattern of the IL10 gene in IL-10-secreting Th cells isolated from ex vivo- and in vitro-established Ag-specific human Th1 and Th2 clones. However, we show an unambiguous hypomethylation of the IFNG gene promoter in different IFN-γ-producing Th cell subsets. Thus, contrary to the effector cytokine gene IFNG, expression of the immunoregulatory cytokine gene IL10 is not mainly regulated by DNA methylation. Moreover, IL-10-producing Th cells lack a memory for IL-10 re-expression in vitro. In conclusion, IL-10 is excluded from the functional cytokine memory in human Th cells, preventing the generation of memory Th cells with an inherited program to secrete IL-10, possibly to ensure a limited effect of IL-10 in downregulation of adaptive pathogen-specific immunity.

Materials and Methods

Media and reagents

The media used were RPMI 1640 supplemented with 1% glutamax, 100 U/ml penicillin, 100 μg/ml streptomycin (Invitrogen Life Technologies), and 10% human AB serum (PAA). PMA (5 ng/ml; Sigma-Aldrich) and 1 μg/ml ionomycin (1; Sigma-Aldrich) were used for stimulation. rIL-7 and rIL-15, each 10 μg/ml (R&D Systems), and 5–10 μM 5-azacytidine (Sigma-Aldrich) were applied to in vitro cell cultures. Brefeldin A (5 μg/ml; Sigma-Aldrich) was used to block cytokine secretion. The following Abs were used for FACS analysis: anti-CD69 FITC, anti-IFN-γ PerCP Cy5.5, anti-IL-10 allophycocyanin, anti-CD45RA (allophycocyanin)-CCR7 (FITC; BD Biosciences), and anti-CD45RO (PTT1, mouse IgG1, house conjugate).

Sample collection and preparation

Buffcoats from healthy adult anonymous donors were obtained in accordance with local ethical committee approval. PBMCs were isolated from each buffy coat by density gradient sedimentation using Ficoll-Hypaque (Sigma-Aldrich). Cells were washed twice with PBS before CD4+ T cell separation.

Purification, sorting of CD4+ T lymphocytes

CD4+ T cells were purified from PBMCs by MACS using CD4 microbeads (Miltenyi Biotec). The purity of the sorted population was 95–99%, as determined by FACS Calibur using CellQuest software (BD Biosciences).

Isolation of Th cell subsets secreting IL-10 or IFN-γ or both

Human Th1 and Th2 clones, highly purified CD4+ T lymphocytes or CD4+ T lymphocytes labeled with CD45RO or CD45RA and CCR7 were stimulated with P/I, followed by detection and isolation of IL-10-secreting and IFN-γ+ and/or IL-10-secreting Th cell subsets using single or double cytometric cytokine secretion assays (Miltenyi Biotec) and a FACSDiva (BD Biosciences). In brief, cells were activated at 1 × 10^5 cells/ml. After 4 h of stimulation, cells were washed with ice-cold buffer (PBS with 0.5% BSA and 2 mM EDTA) and labeled with IL-10- or IFN-γ-specific or pre-mixed (1:1) IFN-γ- and IL-10-specific capture matrix in cold medium for 5 min on ice. Subsequently, the labeled cells were diluted in prewarmed medium (≤10^5 cells/ml) and subjected to a 45-min cytokine secretion period at 37°C under slow continuous rotation. The cytokine secretion was stopped by filling up the tube with cold buffer and subsequent incubation on ice for 15 min. Cells were harvested and surface stained with specific detection Abs for IL-10 (allophycocyanin or PE) or IFN-γ (PE) or equal amounts of specific detection Abs for IL-10 (allophycocyanin or PE) and IFN-γ (PE or FITC), IL-10+ and IL-10− or IFN-γ+ and IFN-γ−, or IFN-γ–IL-10+ and IL-10−, IFN-γ+ and IL-10−, and IFN-γ–IL-10+ Th cell subsets were purified (>95%) by cell sorting.

Cytometric bead array (CBA) assay

Supernatants from 48- or 72-h cultures of P/I-stimulated Th cell subpopulations were analyzed using the human Th1/Th2 cytokine CBA kit (BD Biosciences), which allows the simultaneous detection and quantification of soluble IL-2, IL-4, IL-5, IL-10, TNF-α, and IFN-γ in a single sample. In brief, a mixture of 10 μl of each of the six different bead suspensions specific for each cytokine (resolved in FL3 channel) was incubated with 50 μl of sample and 50 μl of PE-conjugated detection Ab (resolved in FL2 channel) for 3 h. Following acquisition of sample data by a FACSCalibur, the results were analyzed using the BD Biosciences CBA analysis software.

In vitro expansion and cytokine profiling of Th cell subsets

Sorted Th cell subsets were seeded in a 96-well plate (2 × 10^4 cells/well). Cytokines were added twice during the expansion. The cytokine profile was assessed at day 7, after 6 h of P/I stimulation (the last 4 h with brefeldin A) and by intracellular cytokine staining for IFN-γ and/or IL-10 and FACS analysis.

Real-time quantitative PCR analysis

Total RNA from the different Th cell subsets was extracted using the Absolutely RNA MicroPrep Kit (Stratagene) and reverse transcribed with TaqMan reverse transcription reagents (Roche Applied Biosystems) according to the manufacturer's recommendations. cDNA was analyzed for the expression of IL10, IFNG, GATA3, Tbet, Sp1, and ubiquitin (UBCH5B) by real-time PCR (primer sequences available upon request) using a LightCycler FastStart DNA Master SYBR Green Kit and a LightCycler (Roche Applied Science). Quantification of target gene expression was calibrated according to the values relative to the expression of ubiquitin gene.

Bisulfite treatment, PCR amplification, and semiquantitative DNA sequencing

Different Th cells were isolated from buffy coats of 30 healthy donors as described above. High molecular weight genomic DNA from each purified cell subset was isolated using a QIamp DNA minikit (Qiagen) as recommended by the manufacturer. Three pools for each subset were analyzed. Each pool consisted of equal amounts of DNA from 10 age- and sex-matched (5 male and 5 female) donors. The average age of the donors are 39.1, 44.7, and 40.0 years for pools 1, 2, and 3, respectively. The DNA methylation status of pooled DNA was analyzed by direct bisulfite-modified DNA sequencing. The bisulfite treatment was performed as described earlier (40). Briefly, heat-denatured genomic DNA was embedded in 2% sodium bisulfite solution (Merck) and then washed with Tris-EDTA buffer. Subsequently, PCR fragments were generated using the bisulfite-treated DNA as template. Primers were designed corresponding to the bisulfite-modified DNA sequence (primer sequences available upon request). PCR products were sequenced from both orientations with the dye terminator chemistry (Applied Biosystems V3.1.) using the PCR primers. The sequencing reaction was performed with an annealing temperature at 55°C and extension at 60°C for 24 cycles. Methylation levels were calculated using the Applied Biosystems trace files and previously described software (41, 42).

Chromatin immunoprecipitation assay (ChIP)

Sorted IL-10+ and IL-10− Th cells were fixed with 1% formaldehyde for 10 min at room temperature. The fixation was stopped with 0.125 M glycine. The chromatin was sheared to 200–1000 bp in length by sonication with five pulses of 10 s at 30% power (Bandelin). The chromatin was incubated with Ab directed against hyperacetylated histone H3 or lysine 4 trimethylated histone H3 (Upstate Biotechnology) overnight, followed by incubation with protein A-MicroBeads (Miltenyi Biotec) for 2 h. Washing steps were performed on μ columns (Miltenyi Biotec) with high salt, low salt, LiCl, and Tris-EDTA buffer sequentially. Chromatin precipitate was eluted with 1% SDS and 0.1 M NaHCO3. Cross-links were reversed by incubation at 65°C for 4 h in the presence of 0.2 M NaCl, and the DNA was purified with NucleoSpin Extract II (Macherey-Nagel). The amount of immunoprecipitated DNA was determined by real-time PCR with LightCycler (Roche Applied Science) using FASTStart SYBR Green Master (Roche Applied Science). The relative amount of DNA was calculated with the crossing point input – crossing point input.

Statistics

The two-tailed Welch t test was performed using GraphPad Prism 4.00. Values of p ≤ 0.05 were regarded as significant. Data are presented as mean of replicates of relative mRNA expression from the same donor.
were obtained only in IL-10-secreting subsets, IL-10 GATA3 (45–47), and levels of transcription factors that have been associated with the various cytokines. IL-10 were cultured for 48 or 72 h and supernatants were analyzed for cytokine secretion pattern of the isolated Th cell subsets, purified cells binding 95% in all experiments (Fig. 1). To confirm the supposed cytokine secretion pattern of the isolated Th cell subsets, purified cells were cultured for 48 or 72 h and supernatants were analyzed for various cytokines. IL-10-10-10-10-10-10 Th cells produced only IL-10 but no IFN-γ, while IL-10-10-10-10-10-10 Th cells produced only IFN-γ, but no IL-10. As expected, Tr1-like IL-10-10-10-10-10-10 Th cells secreted both cytokines, whereas IL-10-10-10-10-10-10 Th cells secreted neither (Fig. 1C).

To further phenotypically analyze ex vivo IL-10-secreting Th cells, an IL-10 secretion assay was performed with CD45RO or CD45RA and CCR7-labeled CD45 cells. As characterized by expression of the CD45A and CD45RO isoforms and homing receptor CCR7, IL-10-secreting Th cells resembled Ag-experienced CD45RA CCR7 central memory Th cells (43) (Fig. 2).

mRNA expression in human IL-10-secreting Th cell subsets

We next performed quantitative real-time PCR analysis to assess mRNA expression of IL10 and IFNG and to evaluate expression levels of transcription factors that have been associated with the regulation of IL10 and IFNG such as Sp1 (32), FoxP3 (44) and GATA3 (45–47), and Tbet (29). Abundant amounts of IL10 mRNA were obtained only in IL-10-secreting subsets, IL-10 IL-10 IL-10 IL-10 Th cells. Notably, GATA3 mRNA expression was significantly higher in IL-10 IL-10 IL-10 IL-10 Th cells (p < 0.05) in comparison to IL-10 IL-10 IL-10 Th cells. Sp1 and FoxP3 were not differentially expressed (data not shown). As expected, IFNG and higher Tbet mRNA expression levels were restricted to IFN-γ-secreting Th cells, except for one sample with lower expression in the IL-10 IL-10 IL-10 Th cells in comparison to the other four donors (Fig. 3). Together, these data demonstrate that IL10 gene expression in human Th cells is regulated at the level of transcription.

Epigenetic status of the IL10 gene

We next assessed the level of DNA methylation of the entire IL10 gene by DNA methylation in CD45 Th cell subsets purified according to IL-10 and/or IFN-γ secretion. Fig. 4A illustrates a schematic map of the 18.1-kb IL10 gene locus spanning 9.1 kb upstream and 9 kb downstream of the transcriptional start site. Thirteen regions of interest (ROI) were analyzed, encompassing 88 CpGs. ROI were selected preferentially in conserved sequences (man and mouse), with priority given to known regulatory regions (promoter, HSS) and CpG density. There are no CpG islands

Results

Distinct cytokine profiles of IL-10 IFN-γ, IL-10 IFN-γ, and IL-10 IFN-γ and IL-10 IFN-γ Th cell subsets

In our initial experiments, we observed that after both polyclonal stimulation with P/I or staphylococcal enterotoxin B and Ag-specific stimulation with CMV-derived Ags, human IL-10 Th cells can be delineated into a subset of only IL-10-secreting cells and a subset of Tr1-like IL-10- and IFN-γ-secreting cells (data not shown). To isolate ex vivo different human Th cell subsets secreting IL-10 or IFN-γ or both, we used a new technology, a double cytokine secretion assay for IFN-γ and IL-10. After stimulation of CD45 T cells with P/I, IL-10 IL-10 IL-10 IL-10 (0.6–3%), IL-10 IL-10 IL-10 IL-10 (5–15%), and IL-10 IL-10 IL-10 IL-10 (80–90%), Th cell subsets were induced (Fig. 1A) and sorted to a purity exceeding 95% in all experiments (Fig. 1B). To confirm the supposed cytokine secretion pattern of the isolated Th cell subsets, purified cells were cultured for 48 or 72 h and supernatants were analyzed for various cytokines. IL-10 IL-10 Th cells produced only IL-10 but no IFN-γ, while IL-10 IL-10 IL-10 IL-10 Th cells produced only IFN-γ, but no IL-10. As expected, Tr1-like IL-10 IL-10 IL-10 IL-10 Th cells secreted both cytokines, whereas IL-10 IL-10 IL-10 Th cells secreted neither (Fig. 1C).
within this locus as predicted by a cpgplot program (http://www.ebi.ac.uk/emboss/cpgplot/). Due to the low frequency of IL-10$^{-}$Th cells and to increase uniformity of all measurements, the same amount of DNA of each Th cell subset (Fig. 1, A and B) from peripheral blood samples of 30 healthy donors were pooled and subjected to bisulfite conversion, PCR amplification, and DNA sequencing.

Strikingly, no methylation pattern specific for IL-10-secreting Th cells could be demonstrated as compared with IL-10-nonsecreting Th cells. Two CpGs of ROI 1, located 9.1 kb upstream of the transcriptional start site, showed low levels of methylation in both IL-10$^{-}$ and IL-10$^{+}$ Th cells. CpGs of ROI 2 and 3 (5.5 and 5 kb upstream) and ROI 12 (3’ end) were almost fully methylated in all cell subsets. CpGs of ROI 4 (2.5 kb upstream) displayed alternating patterns of methylation, without an explicit preference assignable to a certain Th cell subset. Two CpGs in ROI 5 (proximal promoter) were slightly demethylated in IL-10$^{-}$ vs IL-10$^{+}$ Th cells. There was no similar methylation pattern observed for adjacent CpGs. In contrast to the $IL10$ loci conservation, the two CpGs in ROI 5 were not evolutionary conserved between man and mouse. Some CpG sites of ROI 6 (intron I), 7 (intron I and exon II), 8 (exon III), 9 (intron IV), 10 (intron IV and exon V), 11 (3’ untranslated region), and 13 (3’ end) showed low levels of methylation throughout all Th cell subsets analyzed (Fig. 4B). Similar results were obtained for IL-10-secreting Th cells isolated from in vitro-established human Th1 and Th2 clones (Fig. 4C).

Further analysis on histone modifications by histone 3 acetylation (H3Ac; Fig. 4D) and histone 3 lysine 4 trimethylation (H3K4me3; Fig. 4E), which closely correlate with transcriptional activity (48), showed that H3Ac and H3K4me3 associated with activation of $IL10$ not only in ROI 5, but also in ROI 1, 2, 4, and 13 to a lesser extent. Taken together, these data indicate that limited differential demethylation of only two CpGs at the proximal promoter correlated with $IL10$ gene expression. However, there is no methylation pattern correlating with $IL10$ gene expression in any other selected amounts of DNA of each Th cell subset (Fig. 1, A and B) from peripheral blood samples of 30 healthy donors were pooled and subjected to bisulfite conversion, PCR amplification, and DNA sequencing.

FIGURE 3. mRNA expression by IL-10$^{-}$IFN$^{-}$, IL-10$^{-}$IFN$^{+}$, IL-10$^{+}$IFN$^{-}$, and IL-10$^{+}$IFN$^{+}$ Th cell subsets. $IL10$, IFNG, and transcription factors GATA3 and Tbet mRNA levels in ex vivo-differentiated CD4$^{+}$ Th cell subsets from a same donor (as described in Fig. 1, A and B) were quantified by real-time PCR and normalized to human E2 ubiquitin-conjugating enzyme (UBCH5B) mRNA levels. The data shown are derived from five independent experiments.

FIGURE 4. Genomic organization and quantitative DNA methylation and histone modification analyses of the human $IL10$ gene. A, Genomic organization of the human $IL10$ gene and alignment of human and mouse $IL10$ gene loci with DNA sequence identity >50% over at least 100 bp are shown in the histogram plot (59). The selected ROI are labeled below the gene locus. B, The DNA methylation status of three pooled DNA populations of each IL-10$^{-}$IFN$^{-}$, IL-10$^{-}$IFN$^{+}$, IL-10$^{+}$IFN$^{-}$ and IL-10$^{+}$IFN$^{+}$ Th cell subsets and IL-10$^{-}$ and IL-10$^{+}$ cells from Th1 and Th2 clones (C) are shown in rows. DNA methylation levels were determined by signal proportions between C and T peaks in colors shown in columns. Each rectangle in the grid represents a distinct CpG site on the designated ROI. ChIP assay assessing H3Ac (D) and H3K4me3 (E) at indicated ROI in ex vivo-purified IL-10$^{-}$ and IL-10$^{+}$ Th cells. Immunoprecipitated DNA was quantified by real-time PCR and normalized to input. The data shown are from two of three independent experiments.
with IFN-γ-nonsecreting Th cell subsets (IL-10−IFN-γ− and IL-10 IFN-γ− cells; Fig. 5). Particularly, CpG sites at positions −295, −186, −54, +122, and +128 relative to the start of transcription were hypomethylated specifically in IFN-γ− Th cells. Our data emphasize the strong correlation between expression of the IFNG gene and hypomethylation of its promoter.

**Limited IL-10 re-expression in ex vivo-expanded IL-10+ Th cell subsets**

To determine a possible functional relevance of the lack of epigenetic memory of IL10, we analyzed the stability of IL-10 re-expression in comparison to IFN-γ re-expression in ex vivo-isolated IL-10+ and IFN-γ+ Th cell subsets following short-term in vitro cultures. One week after isolation and expansion under neutral conditions with rIL-7 plus rIL-15 (rIL-7/15), re-expression of IL-10 and IFN-γ was assessed after restimulation with P/L. Little induction of IFN-γ was observed in cultured IL-10−IFN-γ− and IL-10+ Th cell subsets, most likely due to a small fraction of pre-Th1 cells (49) in response to homeostatic cytokines rIL-7/15 (Fig. 6A). Strikingly, from both sorted IL-10−IFN-γ− (Fig. 6A) and IL-10+IFN-γ− (Fig. 6B) Th cells, only ~10% re-expressed IL-10. In contrast, in both IFN-γ-secreting subsets, IL-10−IFN-γ− (Fig. 6B) and IL-10+IFN-γ+ (Fig. 6C) Th cells, >90% of the cells maintained IFN-γ expression. To address whether stable IL-10 re-expression is restricted to a special subset of Th cells, IL-10−IFN-γ− Th cells were reisolated following 1 wk of culture of IL-10−IFN-γ− Th cells. After 1 wk of further expansion and stimulation of these reisolated IL-10−IFN-γ− cells, again only ~10% of cells re-expressed IL-10 (Fig. 6A). Thus, ~99% of ex vivo-isolated human IL-10+ Th cells were unable to maintain IL-10 expression upon secondary restimulation, implicating that the immunoregulatory cytokine IL-10 is excluded from the functional cytokine memory of human memory Th cells compared with the effector cytokine IFN-γ.

---

**FIGURE 5.** Correlation between hypomethylation and IFN-γ expression in the IFNG gene promoter, exon I, and intron I in human IFN-γ secreting vs nonsecreting Th cells. A. Genomic organization of the human IFNG gene and alignment of human and mouse IFNG gene loci with DNA sequence identity >50% over at least 100 bp are shown in the histogram plot. The selected ROI are labeled below the gene locus. Overlapping ROI 1, 2, and 3 in the proximal promoter, exon I, and intron I are depicted below the gene locus. ROI 1 consists of CpG sites of −295 and −186; ROI 2, −186, −54 and +122; and ROI 3, −54, +122 and +128 relative to the transcriptional start site. B. Cell types tested and method of CpG methylation quantification are the same as in Fig. 4B. These hypomethylation and hypermethylation patterns were reproducible in IFN-γ+ and IFN-γ− Th cells isolated from at least four individual donors, respectively.

**Hypomethylation pattern of the IFNG gene promoter in IL-10−IFN-γ+ and IL-10−IFN-γ− Th cell subsets**

For comparison, we investigated the methylation status of the IFNG gene focusing on the promoter region in these Th cell populations. IFN-γ+ Th cell subsets (IL-10+IFN-γ+ and IL-10− IFN-γ+ cells) were hypomethylated at the IFNG locus compared

---

**FIGURE 6.** Attenuation of IL-10 re-expression. Ex vivo IL-10+IFN-γ− (A), IL-10+IFN-γ+ (B), and IL-10−IFN-γ+ (C) Th cell subsets were sorted as described in Fig. 1. Purified Th cell subsets were expanded under neutral (rIL-7 plus rIL-15) conditions. After 1 wk, a second-round cytokine secretion assay for IL-10 and IFN-γ was performed to reisolate IL-10+IFN-γ− cells of first-round sorted IL-10+IFN-γ− cells. In parallel, re-expression of IL-10 and IFN-γ was analyzed by intracellular staining (i.c.) following 6 h of P/L restimulation for all of the subsets. Similar results were obtained in three independent experiments. D, Kinetics of IL10 and GATA3 expression. IL-10+ and IL-10− Th cell subsets were reisolated after 1 wk of culture of IL-10+ cells and subjected to real-time PCR analysis. Normalization of IL10 and GATA3 expression was the same as in Fig. 3. One of two independent experiments is shown. E, DNA methylation status of the promoter region of the IL10 gene (ROI 5) in secondary (2°) IL-10+ and IL-10− cell subsets. Method of CpG methylation quantification is the same as described in Fig. 4B. The data shown are representative of four independent experiments.
Transcriptional regulation of secondary IL-10-producing Th cells

To understand the molecular mechanisms underlying the inefficiency of functional IL-10 memory, we next performed quantitative real-time PCR analysis to examine mRNA expression of IL10 and GATA3 by short-term cultured cells. IL-10+ and IL-10− cells were reisolated after 1 wk of culture of IL-10+ Th cells. As expected and similar to the results observed in primary Th cell subsets (Fig. 3), IL10 mRNA expression was restricted to IL-10-secreting Th cells (Fig. 6D). Also, a higher level of GATA3 was detected only in IL-10-secreting Th cells (Fig. 6D). The kinetics of IL10 and GATA3 expression suggest that IL10 gene expression is regulated at the level of transcription and GATA3 is important and IL10 was detected only in IL-10-secreting Th cells (Fig. 6). At day 7, re-expression of cytokine was assessed by P/I stimulation for 6 h in the presence of brefeldin A for the last 4 h. The data shown are representative of three independent experiments.

5-Azacytidine not only induces IL-10 expression in IL-10+ cells but also increases IL-10 re-expression in cultured IL-10+ cells

We next investigated whether IL10 gene expression can be in principle regulated by DNA methylation. To this end, 5-azacytidine was applied to IL-10+ and IL-10−, IFN-γ− and IFN-γ+ Th cell subsets were highly purified following IL-10 and IFN-γ secretion, respectively. rIL-7/15 or IL-7/15 plus 5-azacytidine were applied to cultures at days 0 and 2. At day 7, re-expression of cytokine was assessed by P/I stimulation for 6 h in the presence of brefeldin A for the last 4 h. The data shown are representative of three independent experiments.

Discussion

Epigenetic modification of effector cytokine genes such as Ifng/IFNG and Il4 have been demonstrated in Th cells by changes in DNA methylation, histone deacetylation, and rearrangement of the chromatin within the nucleus. The “poised” state apparent in effector/memory Th cells is thought to allow for rapid secretion of effector cytokines when rechallenged by invading pathogens (52–54). In this study, we show that things are different for the immunoregulatory cytokine IL-10. IL-10-secreting memory Th cells isolated ex vivo did not display a specific DNA methylation pattern as compared with Th cells not secreting IL-10. In contrast, hypomethylation of the IFNG gene promoter strongly correlated with IFN-γ expression in memory Th cells. In accordance with the lack of methylation memory, the majority of ex vivo-isolated IL-10-secreting Th cells lack a functional memory for IL-10 re-expression after restimulation. The unique role of IL-10 with its broad immunoregulatory functions might be a major reason for these differences in expression and regulation. So far, the regulation of IL10 gene expression, particularly in human Th cells representing a major source of IL-10 in the course of adaptive immune responses had remained poorly understood.

Unlike memory Th cell subsets producing effector cytokines such as IFN-γ and IL-4, efficient protocols for the generation of IL-10-secreting cells from naive Th cells have not been established yet or results remain controversial. Moreover, in vivo-generated human IL-10-secreting Th cells are usually rare and cannot be assessed according to characteristic surface markers. Using a new technology, we isolated human Th cell subsets secreting IL-10 or IFN-γ, or both, directly ex vivo from peripheral blood of healthy donors after short-term polyclonal stimulation. To evaluate epigenetic mechanisms that underlie IL10 gene expression, we provide here the first assessments of DNA methylation status of the IL10 gene, encompassing 88 selected CpGs, and the IFNG gene promoter, encompassing 5 CpGs. Our results offer initial evidence that in contrast to the expression of the effector cytokine IFNG, the expression of immunoregulatory cytokine IL10 is not mainly regulated by DNA methylation. Concomitantly, ex vivo IL-10-secreting Th cells lack a functional cytokine memory for IL-10 re-expression after short-term in vitro expansion, as compared with the epigenetically marked IFNG in IFN-γ-secreting Th cells.

Demethylation of cytokine in CpG dinucleotides in regulatory regions often correlates with other epigenetic modifications such as increased nucleosome sensitivity. DNase I HSS, are believed to reflect the “open” chromatin configuration (55). With respect to the regulation of IL10 gene expression, such DNase I HSS have been described in macrophages, dendritic cells, and Th1 and Th2 clones (34, 38, 56). One HSS described as Th2 specific and constitutive coexists with DNA methylation in the promoter region (38) that is similar to our ROI 5. At the level of histone modifications, the relative hyper-H3Ac and H3K4me3 do not completely match the DNA methylation status. It seems that a slight coincidence between H3Ac and H3K4me3 and DNA methylation only exists in the ROI 5 (Fig. 4, D and E), suggesting an intricate crosstalk between different layers of chromatin modifications given by histone acetylation and methylation and DNA methylation that contribute to the regulation of IL10 gene expression.

Of note, GATA-3 (47) that has been implicated in the regulation of Il10 gene expression (45, 46, 57) was significantly up-regulated in IL-10− IFN-γ− vs IL-10− IFN-γ+ Th cells. Furthermore, GATA3 remained up-regulated in reisolated IL-10+ Th cells in comparison to IL-10− Th cells following 1-wk culture of primary IL-10+ Th cells (Fig. 6D), confirming the importance of GATA-3.

![Diagram](http://www.jimmunol.org/DownloadedfrombyguestonJanuary14,2018/2394.Il-10-Memory-in-Primary-Human-Cd4Memory-T-Lymphocytes)
with regard to IL10 gene expression. Conversely, Sp1 is ubiquitously expressed (58) and we did not find a correlation between Sp1 and IL10 gene expression (data not shown). However, we currently cannot exclude the possibility that the limited difference in DNA methylation is conferring transcriptional specificity for undefined transcription factors in human Th cells.

In accordance with the lack of epigenetic memory of IL10 by DNA methylation, human IL10-secreting Th cells displayed a limited memory for IL-10 re-expression after short-term in vitro culture. In contrast, IFN-γ-secreting Th cell subsets were characterized by a specific epigenetic memory for IFNG gene expression. However, when treated with DNA Dnmts inhibitor 5-azacytidine, IL-10 expression was significantly augmented in both ex vivo IL-10+ and IL-10− Th cells after 1 wk of culture to a similar extent as augmented IFN-γ production in treated ex vivo IFN-γ-nonproducers (Fig. 7). Although this shows that in principle expression of the IL10 gene can be subject to regulation by demethylation, the IL10 gene in IL-10+ Th cells directly isolated ex vivo (Fig. 4B) in vitro-established IL-10+ Th cell clones (Fig. 4C) and in IL-10+ Th cells reisolated from in vitro cultures initiated with IL-10+ Th cells (Fig. 6E) is not imprinted by demethylation, in contrast to the IFNG gene (Fig. 5B). Apparently, the expression of the major immunoregulatory cytokine IL10 in human memory Th cells is regulated in a completely different manner compared with an effector cytokine such as IFNG. IL10 gene expression might be determined by a variable mixture of signals rather than by a fixed heritable program acquired during Th cell differentiation as seen for effector cytokines. An epigenetic memory for IL10 gene expression in memory Th cells generated in immune responses specific for exogenous Ags could be dangerous, because it could allow for an immediate and undesired suppression of an immune response.

In summary, in contrast to effector cytokines such as IFN-γ, IL-10 is excluded from the functional cytokine memory of human memory Th cells. Our results indicate that cytokine expression in memory Th cells is differentially regulated for the major immunoregulatory cytokine IL-10 and the effector cytokine IFN-γ, most likely to ensure efficient pathogen-specific recall immune responses.

Acknowledgments

We thank Drs. J. L. Grogan, M. Bros, and J. Li for valuable comments and S. Cottrell and F. Hatam for critical reading of this manuscript. We are grateful to K. Raba, T. Kaiser, L. Reiners-Schramm, R. Cortese, and J. Dietrich for their expert technical assistance.

Disclosures

Our disclosures have no financial conflict of interest.

References


