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Dynamic DNA methylation patterns across the mouse and human *IL10* genes during CD4⁺ T cell activation; influence of IL-27

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1. Introduction

IL-10 is an immunoregulatory cytokine that plays a fundamental role in regulating inflammation and instructing adaptive immune responses (Moore *et al.*, 2001). Increasing evidence indicates that precise temporal/spatial regulation of IL-10 is required for maintaining immune homeostasis. In fact, dysregulation of IL-10 has been implicated in the etiology of numerous infectious, autoimmune, and allergic disorders (Couper *et al.*, 2008; Izcue *et al.*, 2009; Hawrylowicz and O'Garra, 2005) as well as cancer (Mosser and Zhang, 2008). IL-10 is expressed by a variety of cell types but T cell sources of IL-10 are often implicated in mediating disease susceptibility *in vivo* (Li and Flavell, 2008).

Cytokine expression patterns are strictly controlled in CD4⁺ subpopulations and define T-helper subsets. It is now clear that cytokine gene loci have explicit epigenetic requirements which support or repress gene expression in the respective subsets (Wilson *et al.*, 2009). Despite restricted expression of the signature T-helper cytokines, most if not all CD4⁺ subsets can acquire the ability to express IL-10 (Saraiva and O'Garra, 2010). Very little is known however, regarding the chromatin structure and epigenetic modifications in the *IL10* gene which govern its complicated expression profiles in the CD4 lineage alone. Nonetheless, evidence from several groups suggest cell type-specific chromatin structure in the mouse *Il10* cluster in the myeloid and lymphoid compartments (Saraiva *et al.*, 2005; Im *et al.*, 2004; Jones and Flavell, 2005; Wang *et al.*, 2005).

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Changes in cytidine phosphate guanosine (CpG) DNA methylation is a key mechanism controlling transcription while establishing stable heritable epigenetic marks (Chen and Riggs, 2005). Demethylated CpG DNA in regulatory regions signals a shift from inert heterochromatin to active euchromatin, whereas DNA methylation leads to gene silencing (Ooi and Bestor, 2008) affecting many biological processes, notably genomic imprinting and X inactivation (Vire *et al.*, 2006). Current studies suggest, that DNA methylation acts in concert with other epigenetic processes (Brenner and Fuks, 2007) and may thereby function as a central signal for the regulation of chromatin structure (Espada and Esteller, 2007). In Th1 and Th2 cells, CpG sites in the *Ifng* and *Il4* loci respectively become demethylated in conjunction with an accumulation of permissive histone modifications during the process of T helper differentiation (Schoenborn *et al.*, 2007; Kim *et al.*, 2007). With respect to *IL10*, Dong *et al.* found no clear correlation between DNA methylation patterns in peripheral IL-10-expressing and non-expressing CD4⁺ T cells in selected conserved non coding sequence (CNS) regions over the human *IL10* cluster (Dong *et al.*, 2007). However, Tsuji-Takayama demonstrated the involvement of CpG DNA methylation, in a specific intronic region of human *IL10*, in gene expression following IL-2 stimulation in T cell lines generated from umbilical cord blood (Tsuji-Takayama *et al.*, 2008).

Given that human IL-10 expression profiles are confounded by host genetic factors (Reuss *et al.*, 2002; Mormann *et al.*, 2004; Gibson *et al.*, 2001; Siebert *et al.*, 2008; Eskdale *et al.*, 1998), we created a functional human IL-10 transgenic mouse model (hIL10BAC) to avoid inter-individual variation while investigating tissue-specific regulation of human IL-10 (Ranatunga *et al.*, 2009). We previously determined that reconstitution of *Il10*^{-/-} mice with the hIL10BAC (*Il10*^{-/-}/hIL10BAC) results in appropriate transgenic human IL-10 expression in myeloid cells prompting the rescue of *Il10*^{-/-} mice from LPS toxicity (Ranatunga *et al.*, 2009). In CD4⁺ T cells however, we observed that human IL-10 was only weakly expressed relative to mouse IL-10. As a result, *Il10*^{-/-}/hIL10BAC mice effectively cleared *Leishmania donovani* infection (much like *Il10*^{-/-} animals) and thus failed to recapitulate pathogen persistence normally observed in *Leishmania donovani*-infected wild type (WT) mice. Furthermore, we found that spleen-derived CD4⁺ T cells cultured with the IL-10-promoting cytokine IL-27 strongly enhanced mouse but not transgenic human IL-10 production.

Since species-specific gene expression can be transferred across species to mice (Bonifer *et al.*, 1990; Welstead *et al.*, 2005) and BACs typically contain the required regulatory regions to support cell-type specific transgene expression (Sparwasser and Eberl, 2007), we used our hIL10BAC mice to gain further insight in the epigenetic regulation of mouse and human *IL10*. We questioned if the low human IL-10 expression in CD4⁺ T cells of hIL10BAC mice relates to resistance of the human *IL10* gene to be demethylated. While human *IL10* was more highly methylated overall in CD4⁺ T cells than mouse *Il10*, it was difficult to discern methylation patterns in naïve and polyclonally activated T cells. However, when CD4⁺ T cells were cultured with IL-27 we identified site-specific reductions in DNA methylation in mouse but not human *IL10* which co-localizes to an intronic region and correlates with IL-10 expression.

2. Materials and Methods

2.1 Mice

hIL10BAC mice (on the C57BL/6 background) were created using a BAC clone of approximately 175Kb (RP11-262N9) from human chromosome 1, which contains the human genes encoding *MAPKAPK2*, *IL10*, and *IL19* (Ranatunga *et al.*, 2009). Mice were housed in specific pathogen free conditions. All experimental procedures were approved by the Johns Hopkins Animal Care and Use Committee.

2.2 Cytokines and antibodies

Recombinant human IL-2 was from the NCI Preclinical Repository. Recombinant mouse and human IL-27 was purchased from R&D Systems (Minneapolis, MN). Purified hamster anti-mouse CD3 ϵ (145-2C11) and CD28 (37.51) were from BD Bioscience (San Diego, CA). Neutralizing anti-IFN- γ (XMG 1.2) mAb was from eBioscience (San Diego, CA) and anti-IL-4 was from the NCI Preclinical Repository. For cell sorting, PE labeled anti-mouse CD4 antibodies were purchased from BD Bioscience and Pe-CyE conjugated anti-mouse CD62L antibodies were from eBioscience.

2.3 Preparation and stimulation of naïve mouse CD4⁺ T cells

Naïve CD4⁺CD62L⁺ T cells were isolated from spleens by negative selection using magnetic beads (R&D Systems). Population purity was usually over 95%. Some of these cells were polyclonally activated with plate bound anti-CD3/CD28 and reactivated with phorbol 12-myristate 13-acetate and ionomycin (P/I). For some experiments cells were cultured under Th0 conditions plus IL-27 as described elsewhere (Stumhofer *et al.*, 2007; Ranatunga *et al.*, 2009) and reactivated with P/I. For methylation analysis of freshly isolated naïve T cells, CD4⁺CD62L⁺ cells were sorted by flow cytometry and were approximately 99% pure. For analysis of human T cells, naïve CD4⁺CD45RA⁺ T cells were sorted from healthy donors and in some cases cultured under Th0 conditions + IL-27 as reported previously (Ranatunga *et al.*, 2009).

2.4 mRNA analyses

Total RNA was isolated by guanidinium-isothiocyanate phenol/chloroform extraction method (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. cDNA was generated using a first strand cDNA synthesis kit (Invitrogen). For gene expression analyses, real-time PCR was performed using Taqman site-specific primers and probes (Applied Biosystems, Foster City, CA) on an ABI 7300 Real time PCR System. Results were normalized to β -2 microglobulin levels. For relative comparisons, IL-10 expression in mouse brain was assigned an arbitrary value of one.

2.5 Bisulfite treatment, PCR amplification, cloning and DNA sequencing

For bisulfite conversion of DNA, the EZ DNA Methylation Kit (Zymo Research, Orange, CA) was used. Genomic DNA was isolated using the ZR Genomic DNA II Kit (Zymo Research) keeping to manufacturer's instructions. Subsequently, PCR products were generated using bisulfite treated DNA as a template. PCR primers were designed based on bisulfite-treated DNA (Table 1) and correspond to regions of interest (ROI) which contained target CpG motifs across the mouse and human *IL10* genes. All primers were analyzed for cross reactivity between mouse and human genomic DNA and confirmed to yield species-specific products. PCR products were purified, using Microcon centrifugation filter devices (Millipore, Billerica, MA), cloned into a pCR2.1-TOPO vector using a Topo TA Cloning kit (Invitrogen). Isolated plasmids were purified with a QIAprep Miniprep kit (Qiagen, Valencia, CA) and used as template for sequencing. For each ROI, a minimum of six to ten clones were analyzed from at least two independent experiments.

2.6 Data analysis

The degree of methylation was assessed by calculating a methylation index (MI) as the average values of $[\text{mC}/(\text{mC}+\text{C})]\times 100\%$ for all putative CpG sites within the region of interest. For statistical analysis, non-parametric Mann-Whitney *t* test was performed using Graph Pad Prism 4.0. Values of ≤ 0.05 were regarded as statistically significant. Data is presented as average of MI from 6–10 clones out of two separate experiments, including standard deviations.

3. Results

3.1 IL-10 expression during T cell activation

We previously reported that relative to mouse IL-10, human IL-10 is weakly expressed in CD4⁺ hIL10BAC T cells. We sought to identify the molecular mechanism(s) which could account for this discrepancy by first examining IL-10 expression in naïve CD4⁺ T cells prior to and under different activation conditions. We analyzed human/mouse IL-10 mRNA expression and protein production in freshly isolated, naïve CD4⁺ T cells, following 3 days of polyclonal activation (α CD3/28), or following 4 days of culture under Th0 conditions in the presence of IL-27 (Figure 1).

In hIL10BAC transgenic mice, freshly isolated, naïve CD4⁺ T cells constitutively express mouse IL-10 mRNA at a relatively low level (Figure 1A). We found an increase in IL-10 mRNA and protein expression after 72hr of polyclonal activation with CD3/CD28, which was further increased after restimulation with P/I for 6hr (Figure 1A–B). After 24hr of stimulation with P/I, mIL-10 mRNA fell to near baseline levels while mIL-10 protein production slightly increased. Transgenic human IL-10 mRNA and protein had a similar expression profile in naïve (mRNA only) and polyclonally activated T cells but at markedly lower levels (Figure 1A–B). As previously reported, differentiation of Th0 cells in the presence of IL-27 resulted in a strong up-regulation of mIL-10 mRNA and protein but only weak induction of hIL-10. Our data suggests the presence of additional or different regulatory constraints of human as compared to mouse IL-10 in splenic CD4⁺ T cells.

3.2 Distribution of CpG DNA sequences across the mouse and human IL10 genes

Since DNA methylation has a well-established role in regulating gene expression, we determined the DNA methylation status of the human/mouse *IL10* genes in splenic CD4⁺ T cells. We first identified the comparative distribution of CpG DNA sequences in and around the mouse and human *IL10* genes, by performing a computer based search for CpG sites from position –528 to +4747 in mouse *Il10* and –520 to +4248 in human *IL10*, including the proximal promoter and the 3' untranslated region (3'UTR). A schematic diagram of the human *IL10* BAC construct is displayed in Figure 2A, which includes *IL10* and its flanking genes. Applying bioinformatic approaches, we aligned the mouse and human *IL10* genes (VISTA Genome Browser, <http://pipeline.lbl.gov/cgi-bin/gateway2>) and show CNS (pink), exons (blue) and UTR (turquoise) in Figure 2B. CNS sites were defined as regions with sequence homology of >75% between human and mouse over a length of at least 200bp (Figure 2B). Known regulatory regions and DNaseI hypersensitivity sites (HSS) are shown above the alignment.

Twelve regions of interest (ROI) were defined in human (49 CpGs assessed) and ten in mouse *Il10* (46 CpGs assessed), using CNS regions and the distribution of CpGs as a guide (Clone manager, Sci-Ed Software) (Figure 2C). All CpGs were analyzed with a few exceptions which were due to technical reasons (noted by grey circles). Of note, two ROI were not analyzed in mouse *Il10* due to a lack of CpGs in ROI 10, and as mentioned, for technical reasons in ROI 11, where only one CpG is present. The 5th exon was not included in this analysis, due to the absence of CpG motifs in mouse and only one CpG site in the human.

Although the total number of CpG sites across the mouse and human *IL10* genes is nearly equivalent (N=49 in mouse and N=50 in human), the distribution of CpGs over the mouse and human *IL10* gene are differentially clustered. However, it is apparent that in some regions the number of CpGs and even the genomic position of single CpGs are conserved between species (red boxes Figure 2C). The numbers of CpG sites were similar or identical in ROI 1 (5 in human vs. 6 in mouse), ROI 5 (4 in both species) and ROI 2 (3 in both

species). Several CpGs are conserved in ROI 1 (−408 and −387, *hIL10*), ROI 5 (+1531, *hIL10*) and ROI 8 (+2543, *hIL10*). Interestingly, all of these are located within highly conserved sequences of the proximal promoter, exon 3 and exon 4. While mouse CpGs are concentrated around ROI 6 to 8 (3rd intron through proximal portion of the 4th intron) where two T cell specific HSS are located, CpGs in human *IL10* are clustered in ROI 9 to 11 (remainder of 4th intron) (Figure 2C).

3.3 Methylation of mouse and human *IL10* in naïve and polyclonally activated CD4⁺ T cells

We sought to gain insight into the dynamics of DNA methylation in *IL10* gene during T cell activation. Thus, we assessed DNA methylation patterns in naïve CD4⁺ T cells and after polyclonal T cell activation followed by overnight resting and 6h re-stimulation with P/I. As shown in Figure 3A, methylation results are presented for the human and mouse *IL10* genes in hIL10BAC CD4⁺ T cells as well as for mouse *Il10* in WT T cells for comparison. Each circle represents the methylation status based on DNA sequencing of individual clones at each locus (open circles = unmethylated, closed circles = methylated).

To facilitate the assessment of overall DNA methylation status, we summarized the methylation profile of all ROI by creating a methylation index (number of methylated CpGs/total number of CpGs in ROI × 100%). By consolidating all measured CpG sites across each gene, we could visualize “gross” levels of DNA methylation in freshly isolated naïve and activated CD4⁺ T cells (Figure 3B). In naïve T cells, we observed significantly greater levels of DNA methylation of the human as compared to the mouse *Il10* genes in hIL10BAC (p=0.002) and WT (p=0.002) mice respectively. Following activation however, these differences became less obvious, and do not correlate comparatively with levels of human and mouse IL-10 expression in these cells (Figure 1). There were no significant differences in methylation of mouse *Il10* between WT and hIL10BAC cells.

To visualize the data in greater detail we generated several different plots to facilitate specific group comparisons. In Figure 3C, the comparisons are of endogenous mouse *Il10* – top, and human *IL10* as a reciprocal image – bottom only from naïve and activated hIL10BAC T cells. Thus, we could examine region-specific differences in DNA methylation within and between the mouse and human *IL10* genes under different activation conditions. In hIL10BAC cells, the effect of polyclonal T cell activation on methylation patterns within the mouse *Il10* gene appeared minimal with the exception of a dramatic increase in methylation observed at ROI 6 (27.8% to 70.8%). A more modest but clear decrease in methylation occurred in ROI 8 (66.7% to 41.7%). The effect of T cell activation on DNA methylation of the human *IL10* gene was more consistent and for much of the gene resulted in a decrease in methylation. However, from ROI 9–12 there was little effect of T cell activation on human *IL10* methylation. While no CpGs were analyzed in mouse ROI 10–11, like human ROI 12, mouse ROI 12 was heavily methylated in naïve and activated T cells.

To better compare methylation status between mouse *Il10* in WT and hIL10BAC cells and human *IL10*, we plotted the data for each group based on T cell activation status (Figure 3D) (naïve – top, polyclonally activated – bottom). Although overall methylation levels of mouse *Il10* between WT and hIL10BAC were not significantly different (Figure 3A), we observed apparent disparities in naïve T cells at specific ROI including ROI 8 (WT – 12.5%, hIL10BAC – 66.7%). This difference however, became less pronounced upon activation. In activated T cells, other differences were identified between mouse *Il10* in activated T cells from WT and hIL10BAC mice. In ROI 2, 5 and 6 we observed substantially lower methylation levels in WT cells (9.4%, 40% and 45.8% respectively) as compared to hIL10BAC cells (36.1%, 80% and 70.8% respectively). Although it is not clear why these differences were observed in these regions only, it may suggest that these sites are preferentially targeted for epigenetic modifications during the early stages of T cell

activation and subject to more variability. Nevertheless, they do not appear to impact mouse IL-10 expression as we previously determined that there were no significant differences in mouse IL-10 expression in naïve and activated CD4⁺ T cells from WT and hIL10BAC mice (Ranatunga *et al.*, 2009). These data suggest the potential importance of region-specific DNA methylation in regulating IL-10 in CD4⁺ T cells.

3.4 Influence of IL-27 on DNA methylation in mouse and human IL10

IL-27 is a key inducer of IL-10 expression in CD4⁺ T cells (Stumhofer *et al.*, 2007; Awasthi *et al.*, 2007; Fitzgerald *et al.*, 2007). While Th0 cells from hIL10BAC mice cultured with IL-27 resulted in large amounts of mouse IL-10 production, we observed little effect on human IL-10 expression (Figure 1A and B) (Ranatunga *et al.*, 2009). Since our previous data suggested region-specific patterns of DNA methylation during polyclonal activation, we determined the effects of IL-27 on DNA methylation in CD4⁺ hIL10BAC T cells. We focused these efforts on specific ROI (ROI 1, 2, 5, 7, 8, and 12) which displayed interesting methylation profiles in naïve and/or CD3/28 activated T cells (Figure 3A bottom sections). To evaluate the influence of IL-27 on DNA methylation status, we plotted the MI for naïve, activated, and IL-27-treated hIL10BAC T cells (endogenous mouse *Il10* – top and human *IL10* – bottom) in Figure 3E. While IL-27 co-culture did not lead to marked methylation changes in the human *IL10* gene, we observed region-specific differences in the methylation patterns in mouse *Il10* as compared to naïve and CD3/28-activated CD4⁺ T cells (Figure 3E). In particular, there was a substantial decrease in CpG methylation relative to naïve T cells specifically in ROI 7 (50% to 13.8%) and 8 (66.7% to 12.5%) in hIL10BAC cells. Given that naïve T cells from WT mice had low levels of methylation at ROI 8 (Figure 3A, D), we cannot determine if the differences in methylation at ROI 8 in hIL10BAC cells was a function of cellular activation or merely a disparity between WT and the hIL10BAC. To further test the consistency of methylation levels at these sites we then examined ROI 7 and 8 in WT T cells treated with IL-27 (as well as ROI 12 as a control) (Supplemental Figure 1). Following IL-27 co-culture, we observed selective DNA demethylation at mouse ROI 7 in WT and hIL10BAC T cells while ROI 8 maintains a low level of methylation in WT cells. ROI 12 remains highly methylated in all conditions.

Although there are fewer CpG sites in the same regions of the human *IL10* gene, ROI 7 and 8 were heavily methylated in all activation states in hIL10BAC T cells. Interestingly, we observed increased methylation in ROI 1 and 2 (proximal promoter and 1st exon) of mouse *Il10* in Th0 cells cultured with IL-27. As noted under other conditions, the 3'UTR region (ROI 12) of both mouse and human *IL10* remained heavily methylated after IL-27 culture (Figure 3E, Supplemental Figure 1). We confirmed human DNA methylation patterns observed in the hIL10BAC at select ROI with naïve, activated, and IL-27-cultured CD4⁺ T cells from peripheral blood mononuclear cells (PBMCs) of two healthy donors (Supplemental Figure 2). Similar to the hIL10BAC, both donors had high methylation levels at ROI 7, 8 and 12 under all T cell activation conditions. These data indicate that IL-27 induces a site-specific reduction in DNA methylation (ROI 7) of the mouse but not the human *IL10* gene, which correlates with IL-10 expression.

3.5 Methylation of conserved CpG motifs in mouse and human IL10

There are 4 highly conserved CpG sites within the mouse and human *IL10* genes (Figure 2C). Two are located in ROI 1 of the proximal promoter (−419 bp and −398 bp of *mIl10*), and the remaining two are in ROI 5 (+1563) and ROI 8 (+2524) (Figures 2 and 4). We questioned if these conserved CpG sequences were also conserved in methylation status in CD4⁺ T cells. By grouping all T cell conditions based on these sites, we found that methylation of the CpGs within ROI 1 were quite similar between mouse (hIL10BAC and WT) and human except that CD3/28 activation selectively reduced human but not mouse

methylation (Figure 4). The methylation pattern in the conserved CpG site in ROI 5 was comparable between mouse and human for the majority of the stimulation conditions. Meanwhile, the conserved site in ROI 8 was selectively reduced in mouse *Il10*. This conserved CpG was even demethylated in naïve T cells from WT mice as noted previously. Altogether, these data suggest a complex state of DNA methylation in the *IL10* gene during CD4⁺ T cell activation that may be region- and stimulation-specific.

4. Discussion

Over recent years, the importance of various epigenetic events which drive CD4⁺ T helper subset differentiation has been established (Fields *et al.*, 2002; Kim *et al.*, 2007; Schoenborn *et al.*, 2007; Wilson *et al.*, 2009; Avni *et al.*, 2002; Aune *et al.*, 2009). Genomic DNA itself is a target of epigenetic modifications and methylated DNA is generally associated with condensed chromatin and transcriptional silencing which may occur at higher rates within and flanking genomic regulatory regions (Kangaspeka *et al.*, 2008). The role of DNA methylation in determining cytokine expression patterns during CD4⁺ T cell differentiation has been well characterized in the *Ifng* and *Il4* loci in Th1 and Th2 cells (Young and Bream, 2007; Wilson *et al.*, 2009). Marked differences in site-specific CpG DNA methylation are predictive of the ability of IFN- γ and IL-4 to be appropriately expressed in Th1 and Th2 subsets respectively (Schoenborn *et al.*, 2007; Kim *et al.*, 2007). In fact, the DNA methylation status of single CpGs (Young *et al.*, 1994) or CpG DNA clusters near regulatory regions has predictive value with respect to IFN- γ and IL-4 expression (Schoenborn *et al.*, 2007; Lee *et al.*, 2002; Kim *et al.*, 2007).

The expression patterns of IL-10 in CD4⁺ T cells however, is far less restricted and IL-10 is recognized to be expressed by subsets of Th1, Th2, Th17, and various T regulatory (Treg) populations (Maynard and Weaver, 2008). This will likely complicate the search for molecular mechanisms which regulate cell type-specific expression of IL-10 not only in T cells, but in the variety of other cells which also produce IL-10. In fact, very little is known about epigenetic regulation of IL-10. A recent study however, failed to identify specific CpG DNA methylation patterns in human IL-10-expressing and non-expressing CD4⁺ T cells (Dong *et al.*, 2007). Nevertheless, in the same study inhibition of DNA methyltransferase (DNMTs) activity led to induction of IL-10 expression. This suggests that IL-10 expression can be influenced by DNA methylation. The involvement of DNA methylation in IL-10 regulation has further been suggested by Tsuji-Takayama *et al.* who demonstrated a correlation between DNA methylation in the 4th intron of human *IL10* and IL-10 expression in response to IL-2 stimulation in T cell lines (Tsuji-Takayama *et al.*, 2008).

Here, we provide a simultaneous analysis of DNA methylation of the mouse and human *IL10* genes during T cell activation. We examined methylation patterns in naïve CD4⁺ T cells in WT and hIL10BAC mice and validated human DNA methylation levels at select ROI using purified, naïve CD4⁺CD45RA⁺ T cells from two human donors (Supplemental Figure 2). Although we detected a drop in *IL10* methylation across the majority of the human gene after T cell activation (Figure 3C), overall DNA methylation levels did not correlate well with the expression of human IL-10. We should note however that this analysis was focused on characterizing the dynamics of DNA methylation patterns during T cell activation and does not distinguish between IL-10⁺ and IL-10⁻ T cell subsets. Nonetheless, our data are in agreement with those of Dong *et al.* who analyzed DNA methylation between IL-10-expressing and non-expressing human T cells and concluded that DNA methylation status does not predict IL-10 expression (Dong *et al.*, 2007).

In contrast, the study by Tsuji-Takayama and colleagues described a region in the 4th intron of the human *IL10* gene which binds Stat5 and displays enhancer activity that appears to

correlate with CpG DNA demethylation in this region (Tsuji-Takayama *et al.*, 2008). Furthermore, they demonstrated methylation-dependent Stat5 binding which indicates the potential impact of DNA methylation on gene expression on a CpG by CpG basis. The enhancer effect of this site was dependent on IL-2 co-stimulation. Although we did not observe activation-dependent changes in human DNA methylation within and adjacent to the 4th intron of the human *IL10* gene (Figure 2 B, C; ROI 9–11), we also did not re-stimulate T cells in the presence of IL-2, which may explain this difference.

In this study, when comparing methylation between mouse and human, the overall MI in mouse *Il10* is significantly lower compared to human *IL10* in hIL10BAC mice (Figure 3B). Even though the MI for mouse *Il10* gene shows little change following polyclonal T cell activation, there is a 25% decrease in methylation in mouse ROI 8 in hIL10BAC mice (Figure 3C). As noted, ROI 8 in WT T cells has low levels of DNA methylation in naïve cells, which complicates the interpretation of stimulation-induced DNA demethylation at mouse ROI 8 (Figure 3D, E). Also some other disparities in methylation of mouse *Il10* were identified between WT and hIL10BAC T cells, specifically at ROI 2, 5 and 6 (Figure 3D). Although these may indicate true differences in DNA methylation patterns, we did not observe differences in mouse IL-10 expression in polyclonally activated T cells between WT and hIL10BAC mice (Ranatunga *et al.*, 2009). It is possible that in preparation for rapid gene transcription following activation, these regions serve as a hub of epigenetic activity and are sensitive to subtle environmental cues.

Recently the IL-12 homolog IL-27 was identified as a key regulator of IL-10 in CD4⁺ T cells (Stumhofer *et al.*, 2007). We found that Th0 cells cultured with IL-27 induced large amounts of mouse, but not human IL-10 in hIL10BAC T cells (Ranatunga *et al.*, 2009) (Figure 1). While IL-27 had very little influence on human *IL10* methylation, we observed dramatic changes in mouse *Il10* which included increases in methylation in the proximal promoter (ROI 1–2) (Figure 3E). However, we found selective demethylation of the region adjacent to intron 4 (ROI 7) which correlates with enhanced mouse IL-10 expression. Although mouse ROI 8 had low levels of methylation in naïve WT T cells, we observed a consistent decrease in methylation in the adjacent mouse ROI 7 in response to IL-27 in both WT and hIL10BAC cells indicating that this region is specifically targeted for DNA demethylation (Supplemental Figure 1). In fact, ROI 7 and 8 co-localize to a region between the 3rd and 4th intron which is adjacent to known regulatory elements mentioned previously (Jones and Flavell, 2005; Grant *et al.*, 2008; Tsuji-Takayama *et al.*, 2008). Studies have suggested this region is a site for epigenetic modifications based on the presence of a DNaseI HS identified in mouse Th cells (+2.98HSS) (Im *et al.*, 2004; Jones and Flavell, 2005) immediately upstream of CNS+3.10) (Grant *et al.*, 2008). Furthermore, the same HS has been identified in other IL-10-expressing cells including bone marrow derived macrophages and DCs as well as T regulatory cells (Saraiva *et al.*, 2005).

It will also be important to define the role of chromatin structure in shaping cell-specific IL-10 expression patterns. Several groups have described cell type- or receptor-specific histone modifications which correlate with the capacity to express IL-10. For example, FcγR cross-linking resulted in ERK-dependent histone phosphorylation and Sp1 recruitment to the IL-10 promoter in macrophages (Lucas *et al.*, 2005). In addition, another group demonstrated that histone deacetylase 11 (HDAC11) negatively regulated the expression of IL-10 in mouse and human APCs by interacting with a distal segment of the promoter *Il10* (Villagra *et al.*, 2009).

Our results suggest that overall, DNA methylation indices across the human and mouse *IL10* genes are poorly predictive of IL-10 expression in CD4⁺ T cells. In the case of mouse *Il10*, there is induction of IL-10 after polyclonal activation despite minimal changes in DNA

methylation (Figures 3, 4). Nevertheless, ROI 7 (and perhaps ROI 8) is a target for decreased methylation. Thus, we have identified a molecular mechanism which may mediate the ability of IL-27 to induce large amounts of IL-10 in mouse CD4⁺ T cells due to site-specific reductions in DNA methylation which co-localize to an intronic region with known regulatory functions. Altogether, our data contribute to a growing literature which suggests that the regulatory mechanisms governing IL-10 expression deviate from those which control the signature cytokine genes in specific CD4⁺ T helper subsets.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1

Figure 1.

Human and mouse IL-10 mRNA and protein expression in naïve CD4⁺ T cells, activated CD4⁺ T cells and Th0 cells cultured with IL-27 from hIL10BAC mice.

A) IL-10 mRNA expression for mouse (light grey) and human (dark grey) in naïve and activated CD4⁺ T (activation for 72 hr with αCD3/28) cells (0 hr) as well as after re-stimulation for 6 and 24 hr. Expression levels of Th0 cells, co-cultured with IL-27 (Th0 + IL-27) of hIL10BAC mice are displayed on the right (naïve T cells in co-culture with IL-27 for 96 hr, then re-stimulation with P/I for 6 hr). mRNA expression is normalized to β2-microglobulin.

B) IL-10 protein secretion for mouse (light grey) and human (dark grey) in activated CD4⁺ T (72 hr α CD3/28) cells as well as after re-stimulation with PMA and ionomycin for 6 and 24 hr. Protein levels of Th0 cells co-cultured with IL-27 (Th0 + IL-27) and re-stimulated with PMA/Iono for 6 and 24 hr of hIL10BAC mice are displayed on the right.

Figure 2.

Map of the *IL10* cluster, CpG distribution and conservation in mouse and human *IL10*

A) Location of mouse and human *IL10* on chromosome 1. A map of the genomic organization of the *IL10* cluster and the human *IL10* BAC cassette.

B) Alignment of the mouse and human *IL10* genes with sequence identity of >75% over at least 200 bp is displayed. Pink peaks denote CNS sites, purple peaks are exons. Red (human) and green (mouse) bars indicate the selected ROI being tested for CpG DNA methylation. Black arrows denote known DNaseI HS. Other known regulatory elements are indicated.

C) Map of CpG motifs within the ROI of mouse and human *IL10*. CpGs, analyzed by bisulfate sequencing are displayed as filled circles. CpGs not analyzed are displayed as open circles. Red boxes display conserved CpGs in mouse and human.

Figure 3B

Figure 3.

DNA methylation patterns in naïve $CD4^+$ T cells, in response to polyclonal T cell activation and in naïve $CD4^+$ T cells, cultured under Th0 conditions in the presence of IL-27.

A) Methylation status for each CpG site. Within any given ROI, each circle represents a single clone sequenced for a specific CpG. The number of CpGs in each ROI are shown horizontally and the number of clones sequenced for each CpG is shown vertically. Closed circles depict a methylated and open circles identify unmethylated CpG sites. Methylation status for human *IL10* (upper panel) and mouse *IL10* (middle panel) in hIL10BAC mice and mouse *IL10* (lower panel) in WT mice in naïve and activated T cells are shown. For selected

ROIs, methylation of *IL10* in naïve CD4⁺ T cells, cultured under Th0 conditions in the presence of IL-27 is shown.

B) Gene-wide methylation index (MI) of *IL10* genes in WT and hIL10BAC mice. MI is defined as the percentage of methylated CpGs out of all CpGs over mouse and human *IL10*. Light grey bars indicate naïve and dark grey bars indicate activated CD4⁺ T cells.

C) Comparison of mouse (top-diamonds) and human (bottom-triangles) *IL10* methylation in naïve (dashed line) and polyclonally activated (solid line) CD4⁺ T cells of hIL10BAC mice. The top and bottom portions are mirror images of one another with 0 in the middle being no methylation on each scale and 100 being equivalent to 100% methylation.

D) Comparison of naïve (top) and polyclonally activated (bottom) methylation patterns of mouse *IL10* in WT (circles) and hIL10BAC mice (diamonds), and human *IL10* (triangles) in hIL10BAC mice.

E) Comparison of mouse (top) and human (bottom) *IL10* MI in naïve (diamonds), polyclonally activated (circles), and Th0 cells cultured with IL-27 (squares) in IL10BAC mice.

Figure 4.

Direct comparison of conserved CpG DNA sequences of the mouse and human *IL10* gene in WT (mouse) hIL10BAC (mouse and human) mice. Top of figures shows the location of conserved CpG sites in mouse and human *IL10*. Below portion shows methylation status at each conserved CpG in naïve, activated and Th0 cells cultured with IL-27 (indicated at the very bottom). The MI for each ROI is shown as a percentage under each result.

Table 1

Primers, used for bisulfate sequencing. Displayed are primer sequences of forward and reverse primers, annealing temperatures for PCR and product sizes. (ROI: Region of interest, Sp.: species, AT: annealing temperature for PCR, bp: product length in base pairs).

Column 1	Species	Forward Primer	Reverse Primer	Tm
ROI1	H	5' – GGAAGGGTTGTTGGGAATTTGAG – 3'	5' – CTCCTTCTCTAACCTCTCTAATAAAC – 3'	55
	M	5' – GGGGTTTTTTTGGGTAATTGAGTG – 3'	5' – AATTTCTTTCTTTTTTTTTTCTTTTCCC – 3'	55
ROI2	H	5' – GGTGAGGGTTAGTTTAGGTTAGGG – 3'	5' – CCTAACCAATAAAATATAAACACCAAC – 3'	56
			5' – CCTAACCAATAAAATATAAACGCCAAC – 3'	
	M	5' – TATGAGGATTAGTAGGGGTTAGTA – 3'	5' – CACCTCCTAATTAACCTTTCAAATATACC – 3'	53
			5' – CACCTCCTAATTAACCTTTTGAATATACC – 3'	
ROI3	H	5' – GAGTGTTTTAGATTGAAAGATTAGT – 3'	5' – CATCACTTAAATCAAATCCTCCTCCTC – 3'	51
	M	5' – GGGATAGAGGTTGGGGGTTGAAGTAGTAT – 3'	5' – CAAAATTAAATCAAAATCCTTCCTCTTAAATC – 3'	51
			5' – CGAAATTAAATCAAAATCCTTCCTCTTAAATC – 3'	
ROI4	H	5' – GGGTATTAAAAAGATCGTATTTTAGT – 3'	5' – CACCAAAAACCTCCCGAAAAAAC – 5'	55
		5' – GGGTATTAAAAAGATTGTATTTTAGT – 3'	5' – CACCAAAAACCTCCCAAAAAAAC – 3'	
	M	5' – GTTGTTAGGGTATTGAATTGATTAT – 3'	5' – CCCTTCTAATTACATTTCTCCCTTTAC – 3'	55
ROI5	H	5' – GGGTATTAAAAAGATTGTATTTTAGT – 3'	5' – CTCATTTACAATACTCTACCAATCTAT – 3'	55
		5' – GTTTTTTTGGGGAGGTTTTTGGTG – 3'		
	M	5' – GTAAAGGGAGAAAATGTAATTAGAAGGG – 3'	5' – TAAATTACCTAATCTCCTATCTA – 3'	55
ROI6	H	5' – ATAGATTGGTAGAGTTAGTTGTAATGAG – 3'	5' – CCGCCCAAAATCTAATTACAAAAAAC – 3'	54
			5' – CCACCAAAATCTAATTACAAAAAAC – 3'	
	M	5' – TAGATAGGAGATTAGGTAATTTA – 3'	5' – CAACTAACACCCAAAAATAAAAAAAC – 3'	51
ROI7	H	5' – AGGATAAGGTTATGTGAAGGTTTGGT – 3'	5' – CCAAATTTATCCAAATACCTTACCTCAC – 3'	55
	M	5' – GTAATGGTTTGGTTTGATGTTTGGT – 3'	5' – ACACCCTTACTCATCTTCAATACCCAC – 3'	55
		5' – GTAAGGGTTTTGGTTTGATGTTTGGT – 3'		
		5' – GTAATGGTTTGGTTTGATGTTTGGT – 3'		
		5' – GTAAGGGTTTTGGTTTGATGTTTGGT – 3'		
ROI8	H	5' – GTGAGGTAAGGTATTTGGATAAATTGG – 3'	5' – TACCATCTATCAAATCCCACACTCTCTCC – 3'	58
	M	5' – GTGGGTATTGAAGATGAGTAAGGGTGT – 3'	5' – CTAAATCCCACACTCCAAATACAAAATAAC – 3'	58
ROI9	H	5' – GGAGAGAGTGTGGGAATTTGATAGATGGTA – 3'	5' – AACTAACATTCTCAAACACCTCCGCAAATACGA – 3'	60

Column 1	Species	Forward Primer	Reverse Primer	Tm
			5' – AACTAACATTCTCAAACACCTCCGCAAATACAA -3'	
			5' – AACTAACATTCTCAAACACCTCCACAAATACGA -3'	
			5' – AACTAACATTCTCAAACACCTCCACAAATACAA -3'	
	M	5' – GTTATTTTGTATTGAGTGTGGAATTAG – 3'	5' – ATTTACCAAATAAAAAATACCCGAAAC – 3'	55
			5' – ATTTACCAAATAAAAAATACCCAAAAC – 3'	
ROI10	H	5' – GCGGAGGTGTTTGAGAATGTTAGTTTT – 3'	5' – CCTAATATTAACACTCTATACAATATCTATCC – 3'	55
		5' – GTGGAGGTGTTTGAGAATGTTAGTTTT – 3'		
ROI11	H	5' – GGTTAGGGTGGTAGGGTAGGGTTTGTTTAG – 3'	5' – CTCCAAACCTCCCCCTAACAAAAAAACATTTC – 3'	60
ROI12	H	5' – GTGATTTTATAGATTTTAGGATATAAATTAGAGG – 3'	5' – ACAATTAAAAAACCCCAACCCAAAAACA – 3'	54
	M	5' – GTAGTGTGTATTGAGTTTGTGGATTTTAGGATT – 3'	5' – CACCATAACAAAAACCCTACAACCTCTC – 3'	55