Molecular Immunology 48 (2010) 73–81

Contents lists available at ScienceDirect

Molecular Immunology

journal homepage: www.elsevier.com/locate/molimm

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

Christian M. Hedrich[1], Amritha Ramakrishnan, Djeneba Dabitao, Fengying Wang, Dilini Ranatunga, Jay H. Bream*

Johns Hopkins Bloomberg School of Public Health, Department of Molecular Microbiology and Immunology, N. Wolfe Street, E5410, Baltimore, MD 21205, USA

ARTICLE INFO

Article history:
Received 6 August 2010
Received in revised form 7 September 2010
Accepted 14 September 2010
Available online 16 October 2010

Keywords:
Cytokine
Gene regulation
T cell
DNA methylation
IL-10
IL-27

ABSTRACT

IL-10 plays a critical role in controlling inflammation and the anti-inflammatory functions of IL-10 are regulated based on its coordinated expression from various cellular sources, most notably T cells. Although nearly all CD4+ subpopulations can express IL-10, surprisingly little is known about the molecular mechanisms which control IL-10 induction, particularly in humans. To examine the regulation of human IL-10 expression, we created the hIL10BAC transgenic mouse. As previously reported, we observed conservation of myeloid-derived IL-10 expression but found that human IL-10 was only weakly expressed in splenic CD4+ T cells from hIL10BAC mice. Since DNA methylation is an important determinant of gene expression profiles, we assessed the patterns of DNA methylation in the human and mouse *IL10* genes in naïve and activated CD4+ T cells. Across mouse and human *IL10* there were no obvious patterns of CpG methylation in naïve CD4+ T cells following polyclonal activation. Overall however, the human *IL10* gene had significantly higher levels of DNA methylation. Interestingly, coculture with the IL-10-inducing cytokine IL-27 lead to a site-specific reduction in methylation of the mouse but not human *IL10* gene. Demethylation was specifically localized to an intronic site adjacent to a known regulatory region. Our findings indicate that while the mouse and human *IL10* genes undergo variable changes in DNA methylation during CD4+ T cell activation, IL-27 appears to influence DNA methylation in a particular intronic region thus associating with IL-10 expression.

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 suggests 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).

Change 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 respec-

* Corresponding author. Tel.: +1 410 502 2511; fax: +1 410 955 0105.
E-mail address: jbream@jhsph.edu (J.H. Bream).
[1] Current address: Children's Hospital Dresden, Department of Pediatric Rheumatology and Immunology, University Medical Center Carl Gustav Carus, Technical University Dresden, Fetscherstr. 74, D-01307 Dresden, Germany.

0161-5890/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.molimm.2010.09.009

tively 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 175 kb (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 CD3ε (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 were 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 Sysytem. 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 $[{}^{m}C/({}^{m}C+C)] \times 100\%$ 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 are presented as average of MI from 6 to 10 clones out of two separate experiments, including standard deviations.