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Identification of a Macrophage-Specific Chromatin Signature in the IL-10 Locus

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The molecular mechanisms that regulate expression of the immunosuppressive cytokine IL-10 remain poorly understood. In this study, by measuring sensitivity to DNase I digestion, we show that production of IL-10 by primary mouse bone marrow-derived macrophages stimulated through pattern recognition receptors is associated with chromatin remodeling of the IL-10 locus. We also demonstrate that the IL-10 locus is remodeled in primary TH2 cells and IL-10-producing regulatory T cells that have been differentiated in vitro. Strikingly, a novel DNase I-hypersensitive site (HSS-4.5) was identified in stimulated macrophages, but not in T cells. We show that hyperacetylated histones were recruited to this site in stimulated macrophages. Furthermore, HSS-4.5 is highly conserved and contains a putative NF-κB binding site. In support of a function for this site, NF-κB p65/RelA was recruited to HSS-4.5 in vivo and its activation was required for optimal IL-10 gene expression in LPS-stimulated macrophages.

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Interleukin-10 is a key factor in regulation of effector cells and inflammation via its inhibitory action on macrophages and dendritic cells (DC) (reviewed in Ref. 1). IL-10 is produced by a variety of cells, including macrophages, DC, B cells, mast cells, and T cells, mainly by effector Th2 cells and some regulatory T cells (Treg) (2). The absence of IL-10 results in spontaneous development of inflammatory bowel disease (3) and increased susceptibility to pathology caused by uncontrolled responses to infectious pathogens (reviewed in Refs. 1 and 2). IL-10 may also act as a negative feedback regulator of immune responses to inhibit pathology in chronic infectious diseases by inhibiting IL-12 and TNF secretion, thus limiting Th1 responses and favoring survival of pathogens (2).

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The transcription factors SV40 promoter factor (Sp) 1 (4), Sp3 (5), CCAAT/enhancer-binding protein β (6, 7), IFN regulatory factor 1, and Stat3 (8) have been proposed to transactivate the IL-10 promoter in both mouse and human cell lines. In primary cells, there is evidence for a role of Smad-4 (9) and Jun proteins (10) in regulating the IL-10 gene in Th1 cells and Th2 cells, respectively. Finally, the protooncogene c-Maf has been shown to play a role in the transcriptional regulation of IL-10 in macrophages, although on its own c-Maf is not sufficient to induce IL-10 gene expression (11). However, many of the molecular mechanisms that regulate the expression of the IL-10 gene remain to be elucidated.

During early events leading to gene expression, condensed chromatin is remodeled, becoming accessible to nuclear factors that enhance or silence gene expression. This has been extensively studied in the immune system, e.g., in control of CD8 and CD4 expression during T cell development (reviewed in Ref. 12) and in expression of IFN-γ and IL-4/IL-5/IL-13 by Th1 and Th2 cells, respectively (reviewed in Refs. 13 and 14). Recent studies have suggested that expression of IL-10 by differentiated T cells is also dictated by changes in the structure of the chromatin at the IL-10 locus (10, 15). However, the contribution of chromatin remodeling for the expression of IL-10 in macrophages or other APCs remains so far unknown.

The NF-κB family of transcription factors has a major role in mediating inflammatory and immune responses and in promoting cell survival (reviewed in Ref. 16). The NF-κB/Rel family is composed of five different members, p50 (NF-κB1), p52 (NF-κB2), p65 (RelA), RelB, and c-Rel, that recognize a common DNA sequence motif. These proteins are present in resting cells as inactive complexes sequestered in the cytoplasm by interacting with the inhibitory protein IκB. A wide variety of signaling pathways, such as pattern recognition receptors, lead to the degradation of IκB by the proteosome, which allows release and nuclear translocation of the NF-κB dimers and rapid reprogramming of gene expression (reviewed in Ref. 17). The expression of several cytokines, such as IL-12p40 (18, 19) and IFN-β (20), is regulated via the NF-κB pathway. The role of the NF-κB family of transcription factors in the control of IL-10 expression is largely unknown.

In this study, we show for the first time that chromatin at the IL-10 locus is remodeled in mouse IL-10-producing primary bone marrow (BM)-derived macrophages (BM-macrophages) and DC, as well as in vitro-derived IL-10-producing Treg (IL-10-Treg). We describe a macrophage/DC-specific DNase I-hypersensitive site (HSS), upstream of the IL-10 promoter, which is absent in T cells. This site contains a highly conserved NF-κB motif and we show in vivo binding of hyperacetylated histones and NF-κB to this site in IL-10-expressing cells.
Materials and Methods

Animals

BALB/c mice were bred and maintained under specific pathogen-free conditions at the National Institute for Medical Research.

Reagents

Reagents for T cell preparation have been described previously (21, 22). LPS (Salmonella minnesota) was purchased from Alexis, CpG1668 from Invitrogen Life Technologies, zymosan A (ZyA) from Invivogen, and BAY11-7082 IKK inhibitor from Calbiochem. Abs for Western blot were obtained from Cell Signaling and for chromatin immunoprecipitation (ChIP) from Santa Cruz Biotechnology.

Isolation of T cell subsets and generation of IL-10 T_{Reg}

CD4^{+} T cells enriched from spleen cell suspensions were purified as CD4^{+} CD62L^{hi}CD45RB^{hi} naive T cells (>98\% of CD4^{+} CD62L^{hi} T cells (>99\%) by MoFlo flow cytometer (DakoCytomation). Th2 cells and IL-10-T_{Reg} were derived in vitro in an APC-independent manner as described elsewhere (21, 22).

Generation of BM-macrophages and DC

BM cells were isolated by flushing femurs and tibia with culture medium conditioned medium containing M-CSF as described previously (23) or into DC (data not shown) in the presence of GM-CSF (50 ng/ml; gift from DNAX Research Institute).

Real-time quantitative PCR

cDNA was synthesized and analyzed by real-time PCR using specific oligonucleotides as described previously (21, 22). Target gene mRNA expression was quantified using SYBR green (Applied Biosystems) and normalized to the ubiquitin mRNA levels.

Cytokine measurement by intracellular staining (ICS) and ELISA

T cells were stimulated with PMA (50 ng/ml) and ionomycin (I; 500 ng/ml) in the presence of brefeldin A (10 \mu M), or ZyA (200 \mu g/ml) for 24 h and Th2 cells with PMA/I for 48 h, and IL-10 and IL-12p40 secretion was assessed by ELISA (21).

DNase I hypersensitivity assay and Southern blot

The DNase I assay was performed as described previously (24). Macrophages were stimulated with LPS (1 \mu g/ml), CpG (1 \mu M), or ZyA (200 \mu g/ml) for 24 h and Th2 cells with PMA/I for 48 h, and IL-10 and IL-12p40 secretion was assessed by ELISA (21).

DNase I hypersensitivity assay and Southern blot

The DNase I assay was described previously (24). IL-10 probes used in Southern blot were PCR-amplified using genomic DNA as template and specific oligonucleotides (5’ probe, 5’-GCAATTTGAATAAG CACACCCCAAG-3’ and 5’-CCCTTGAAGTTAACCTATGTAGC-3’; 3’ probe, 5’-GAGTCTGCTACAAAGGCAGACAAAC-3’ and 5’-GAATGGCATCACAGAAAGAACACTTC-3’ and 5’-GATGGCATCACAGAAAGACCTTC-3’ and 5’-GCATTGATGCA CATATAACATACAC-3’) were PCR-amplified and cloned into pGL3-Basic (Promega). The inserts were checked for the absence of mutations.

ChIP assay

ChIP assays were performed on LPS (1 \mu g/ml)- or CpG (1 \mu M)-stimulated macrophages as described previously (26). Chromatin was sheared by sonication and precleared for 2 h with salmon sperm-blocked protein A beads and washed in low salt buffer. Purified DNA was used as template for PCR with specific oligonucleotides (HSS-4.5, 5’-CTGAGGC and 5’-GTTGCCCAGGGTACAGAA-3’ and 5’-GAATTTGACATCTTCATCAAC-3’ and 5’-GCTGGGTCTTGAGCCTCTTCTGG-3’; 3’-RV fragment of the IL-10 locus). Indicated are the IL-10 exons (A, 21, 22).

Luciferase reporter assays

The IL-10 promoter (1.5 kb; 5’-GCTGGGTCTTGAGCCTCTTCTGG-3’ and 5’-CTGAGGC and 5’-GTTGCCCAGGGTACAGAA-3’ and 5’-GAATTTGACATCTTCATCAAC-3’ and 5’-GCTGGGTCTTGAGCCTCTTCTGG-3’) and HSS-4.5 (1 kb; 5’-GCTGGGTCTTGAGCCTCTTCTGG-3’ and 5’-CTGAGGC and 5’-GTTGCCCAGGGTACAGAA-3’ and 5’-GAATTTGACATCTTCATCAAC-3’ and 5’-GCTGGGTCTTGAGCCTCTTCTGG-3’), then left untreated or stimulated with PMA/I (21, 22) or stimulated with 100 ng/ml LPS. Cells lysates were prepared and analyzed as described elsewhere (25).

Effect of chromatin remodeling and NF-κB on IL-10 expression

To determine whether changes in the chromatin structure at the IL-10 locus accompany differentiation of T cells into IL-10-T_{Reg} cells, we compared naive (CD4^{+} CD62L^{hi}CD45RB^{hi} T cells, which produce no IL-10 upon stimulation, to a relatively homogeneous population of IL-10-T_{Reg} cells generated in vitro in the presence of vitamin D_{3} and dexamethasone (21, 22), which produce high levels of IL-10 upon restimulation (Fig. 1, A and B). An EcoRV

FIGURE 1. The chromatin at the IL-10 locus is remodeled in IL-10-T\textsubscript{Reg} cells. A, IL-10 production by naive T cells and IL-10-T\textsubscript{Reg} cells was assessed by ICS upon stimulation with PMA/I. Both naive T cells and IL-10-T\textsubscript{Reg} produced little to no IL-2, IL-4, and IFN-\gamma (data not shown) (21, 22). B, IL-10 mRNA transcription in unstimulated (0 h) or PMA/I-stimulated (6 h) naive T cells and IL-10-T\textsubscript{Reg} cells (with anti-CD3/anti-CD28 stimulation similar results were observed, data not shown), determined by real-time RT-PCR and normalized to ubiquitin mRNA. C, DNase I profile at the IL-10 locus in naive and IL-10-T\textsubscript{Reg} cells. Arrows indicate the parental and the HSS fragments obtained after DNase I digestion. Similar results were obtained using a 3’ probe. D, Schematic of the studied EcoRV fragment of the IL-10 locus. Indicated are the EcoRV restriction sites relative to the IL-10 transcription start site (+1), the IL-10 exons (E1-E5), the position of the Southern blot probe and of the detected DNase I HSS.

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fragment containing the IL-10 gene and 7 kb upstream of the start site of transcription was used to compare the DNase I hypersensitivity profile at the IL-10 locus in naive and IL-10-TReg. To address the changes of contributions in the chromatin structure at the IL-10 locus in the regulation of IL-10 expression in T cells, we concentrated our investigation in the DNA region located upstream of the IL-10 gene that contains some sites also covered in the study by Im et al. (15). Additional sites in the IL-10 locus that were not included in our study were recently reported by Im et al. (15) and Wang et al. (10).

In naive T cells, the IL-10 locus was inaccessible to digestion by DNase I, suggesting a condensed and transcriptionally inactive form of chromatin (Fig. 1C). However, HSS were detected at the Thy1 locus, consistent with the constitutive expression of Thy1 (data not shown). DNase I HSS were identified in IL-10-TReg (Fig. 1C) and named according to their distance in kb from the IL-10 transcription start site (+1), following the nomenclature proposed by E. A. Jones and R. A. Flavell (personal communication). HSS-0.12 (Fig. 1C), that mapped to the IL-10 promoter, and two other DNase I HSS, HSS + 1.65 (in intron 3), and HSS + 2.98 (in intron 4) were detected (Fig. 1C). Additionally, a 4 kb band (Fig. 1C) and a HSS in intron 1 (HSS + 0.92, data not shown) were variably observed. The latter was also consistently observed by E. A. Jones and R. A. Flavell (personal communication). IL-10-producing T cells differentiated in vitro in the presence of vitamin D₃ or dexamethasone alone showed a HSS pattern similar to that of IL-10-TReg (data not shown) despite differences in their levels of IL-10 secretion, suggesting that another layer or regulation must be involved.

IL-10-producing BM-macrophages, DC, and differentiated Th2 cells show changes in chromatin structure at the IL-10 locus

IL-10 is also produced by BM-macrophages stimulated in vitro with ligands for pattern recognition receptors, LPS, CpG, and ZyA and by Th2 cells upon restimulation (Fig. 2, A and B). We consistently detected a novel HSS (HSS-4.5), present in BM-macrophages, upstream of the IL-10 promoter that was absent in differentiated Th2 (Fig. 2C) and IL-10-TReg cells (Fig. 1C). HSS-4.5 was also found in stimulated BM-derived DC, which produce IL-10 when stimulated with LPS, CpG, or ZyA (data not shown), suggesting a common mechanism for the regulation of IL-10 in macrophages and DC. Unstimulated macrophages showed some extent of chromatin remodeling at the IL-10 locus, suggesting that mature BM-macrophages may be poised to express IL-10 or that they may have been activated by experimental handling. As shown in Fig. 2C, HSS + 1.65, HSS-0.12, and HSS-2 were detected in macrophages and HSS + 2.98, HSS-0.12, and HSS-2 in Th2 cells. HSS + 2.98 was often also detected in stimulated macrophages, as was HSS + 1.65 in Th2 cells (data not shown). Thus, whereas common HSS exist in all types of IL-10-producing cells that were analyzed, macrophages and DC have an additional HSS observed upon stimulation through a number of different pattern recognition receptors that induce IL-10 production, which is absent in Th2 and IL-10-TReg cells.

The novel macrophage/DC HSS at the IL-10 locus is a putative regulatory site

Changes in histone acetylation of cytokine genes during Th cell differentiation have been described elsewhere (27, 28) and shown to coincide with regulatory regions that control the expression of the IFN-γ and IL-4 genes in Th1 and Th2 cells. We thus examined the in vivo acetylation profile at HSS-4.5 in resting vs LPS- or CpG-stimulated BM-macrophages by ChIP using specific Abs for acetylated residues in the histones H3 and H4 and specific oligonucleotides for HSS-4.5 and for the IL-10 promoter (HSS-0.12) in the PCR amplification step. Hyperacetylated histones were enriched both at HSS-4.5 and at the IL-10 promoter in LPS- or CpG-stimulated macrophages (Fig. 3A), consistent with transcriptional activity and the ability of these cells to produce IL-10.

To address the functional activity of HSS-4.5 in vitro, we constructed an IL-10 promoter (1.5 kb 5′ of the IL-10 transcription start site) luciferase reporter gene. One kilobase of DNA containing HSS-4.5 was cloned either upstream or downstream of the IL-10 promoter linked to the luciferase gene. The constructs were transiently transfected into the cell line 68-41 and luciferase activity was measured in resting vs PMA/I-stimulated cells. Consistent with an involvement of this site in IL-10 gene regulation, we

FIGURE 2. An additional HSS detected at the IL-10 locus in macrophages is not shared by Th2-differentiated cells. A, IL-10 production by BM-macrophages and Th2 cells upon stimulation with TLR ligands or PMA/I, respectively, as assessed by ELISA or by ICS. B, IL-10 mRNA transcription by unstimulated (Medium) or stimulated (6 h) BM-macrophages and Th2 cells, determined as in Fig. 1B. C, DNase I profile at the IL-10 locus in BM-macrophages and Th2 cells. Macrophages were left unstimulated (Medium) or stimulated for 24 h with LPS, CpG, or ZyA. Th2 cells were used after 3 wk of polarization. The arrows indicate the parental fragment and the HSS detected after DNase I digestion. Similar results were obtained using a 3′ probe. D, Schematic of the EcoRV IL-10 fragment as indicated in Fig. 1D.
detected a 2- to 3-fold enhancement of the luciferase activity in the presence of HSS-4.5 (Fig. 3B).

The NF-κB subunit p65 is inducibly recruited to HSS-4.5 in vivo in LPS-stimulated BM-macrophages

Bioinformatic approaches have been successfully used to identify conserved interspecies noncoding sequences, often associated with regulatory elements (29). We compared the DNA sequence across the 150 kb of the mouse and human chromosome 1 region containing the IL-10 genomic locus using rVISTA (http://pga.lbl.gov/rVISTA.html) software (30). Consistent with a putative regulatory role for HSS-4.5, we found that this site overlapped with a highly conserved noncoding region and contained a conserved NF-κB-binding motif (Fig. 3C). We performed a ChIP assay using a specific Ab to the activating subunit of NF-κB p65/RelA and primers to amplify the HSS-4.5 site and found that p65 was inducibly recruited to HSS-4.5 in BM-macrophages in vivo (Fig. 3D).

NF-κB activation is required for optimal IL-10 gene expression in LPS-stimulated macrophages

To further investigate whether the NF-κB signaling pathway was involved in the regulation of IL-10 gene expression by BM-macrophages, we treated these cells with the IKK inhibitor BAY11-7082 and subsequently stimulated the cells with LPS. A dose-dependent decrease in IL-10 mRNA expression and protein secretion were observed in the presence of BAY11-7082 (Fig. 4, A and B). As a positive control, we measured the level of secreted IL-12p40 protein in response to LPS. IL-12p40 was diminished in the presence of the inhibitor (Fig. 4A), consistent with the established role of NF-κB in regulating transcription of IL-12p40 (18, 19). Western blotting of cell lysates showed that BAY11-7082 partially blocked LPS-induced IkBα degradation and inhibited MEK phosphorylation, but did not affect p38 phosphorylation (Fig. 4C), confirming its specificity for IKK-regulated signaling pathways as has been shown previously (25).

Discussion

In this report, we have analyzed the chromatin accessibility at the IL-10 locus upstream of the IL-10 gene transcriptional starting site in mouse primary BM-macrophages. Recent studies have addressed the regulation of IL-10 gene expression at the chromatin level in T cells, particularly in DNA sequences located downstream of the transcription starting site (10, 15). By investigating a distinct genomic region within the IL-10 locus, we have identified a novel DNase I HSS (HSS-4.5) in activated BM-macrophages and DC that is absent in T cell populations poised to produce IL-10. Chromatin at HSS-4.5 was decondensed and hyperacetylated in BM-macrophages, suggesting a transcriptionally active element. In further support for a regulatory role for HSS-4.5, interspecies sequence comparisons revealed a high level of conservation in this region.

We also showed that common HSS exist between all types of IL-10-producing cells, including BM-macrophages, BM-DC, Th2 cells, and IL-10-TReg cells. The identified common sites include a HSS located on the IL-10 promoter (HSS-0.12) and two intronic sites (HSS-1.65, in intron 3, and HSS-2.98, in intron 4). In a recent study by Im et al. (15), HSS + 1.65 and HSS + 2.98 were detected only in committed Th1 cells, which the authors suggested did not produce IL-10, and they therefore concluded that these HSS play a role in silencing IL-10 expression. This does not seem to be the case in our

FIGURE 3. HSS-4.5 is enriched in hyperacetylated histones in activated macrophages and contains a conserved NF-κB-binding motif. A, Hyperacetylated histones are recruited to HSS-4.5 and to the IL-10 promoter. BM-macrophages were left unstimulated (−) or stimulated for 6 h with LPS or CpG, and chromatin complexes were immunoprecipitated with mAbs to acetylated residues in histones H3 (AcH3) and H4 (AcH4) or with a control Ab (Ctrl Ab). Specific oligonucleotides were used to amplify by PCR HSS-4.5 and the IL-10 promoter using the immunoprecipitated or untreated (Input) chromatin as template. The EcoRV fragment of the IL-10 locus is represented and the positions of the oligonucleotides used in the PCR are indicated. B, HSS-4.5 enhances the IL-10 promoter activity. The 68-41 cells were transiently transfected with the indicated constructs and cell lysates were prepared 12 h later from unstimulated or PMA/I-stimulated cells. Firefly luciferase activity was measured and normalized to Renilla luciferase activity. Represented is the fold induction in luciferase activity, calculated by dividing the luciferase activity obtained upon stimulation with PMA/I by the luciferase activity obtained in unstimulated cells. The promoter enhancement value refers to the ratio between the fold induction obtained for the combined HSS-4.5/promoter constructs and the promoter control. C, Mouse vs human comparison of the IL-10 genomic locus using rVISTA shows a conserved noncoding hot spot coinciding with HSS-4.5. Represented are the mouse sequence (y-axis) and the percentage of identity to humans (y-axis), the IL-10 exons, and the predicted conserved noncoding sequences (>75% homology and >100 bp long). Alignment of the mouse and human nucleotide sequence at HSS-4.5 showed a conserved NF-κB-binding motif. D, Chromatin from unstimulated (−) or LPS-stimulated macrophages was immunoprecipitated with a p65 Ab or with a control Ab (Ctrl Ab). Specific oligonucleotides were used to amplify by PCR HSS-4.5 from the immunoprecipitated or total (Input) chromatin as in A.
The observation that the chromatin at the IL-10 locus is similarly remodeled in several cell types that express various levels of IL-10 (that is low in IL-10-producing Th1 cells, higher in BM-macrophages, and very high in Th2 and IL-10-TReg cells) favors an essential role for cell-specific enhancers or silencers in regulating IL-10 expression. The findings that the essential activity of the IL-10 promoter is restricted to a single Sp1/Sp3 site (4, 5) and the fact that both Sp1 and Sp3 are ubiquitous transcription factors also uncover a potential important role for distal elements in the IL-10 locus. In this report, we demonstrate that NF-κB may play such a role in macrophages, at least partially via HSS-4.5. The absence of this site from T cells suggests that, depending on the cell type, other transcription factors may be involved. In this context, it has been recently shown that in Th2 cells Jun proteins bind to a strong HSS in the IL-10 locus, enhancing the expression of IL-10 (10).

Our initial study of the molecular mechanisms that regulate the IL-10 gene expression in BM-macrophages suggested that a first layer of regulation consists of changes in the chromatin structure at the IL-10 locus, and that one player in a second layer of regulation enhancement of IL-10 transcription is provided by NF-κB.

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Disclosures
The authors have no financial conflict of interest.

References


