IL-10-Conditioned Dendritic Cells, Decommissioned for Recruitment of Adaptive Immunity, Elicit Innate Inflammatory Gene Products in Response to Danger Signals[1]

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Abstract

Dendritic cells (DCs) are the professional APCs of the immune system, enabling T cells to perceive and respond appropriately to potentially dangerous microbes, while also being able to maintain T cell tolerance toward self. In part, such tolerance can be determined by IL-10 released from certain types of regulatory T cells. IL-10 has previously been shown to render DCs unable to activate T cells and it has been assumed that this process represents a general block in maturation. Using serial analysis of gene expression, we show that IL-10 pretreatment of murine bone marrow-derived DCs alone causes significant changes in gene expression. Furthermore, these cells retain the ability to respond to Toll-like receptor agonists, but in a manner skewed toward the selective induction of mediators known to enhance local inflammation and innate immunity, among which we highlight a novel CXCR2 ligand, DC inflammatory protein-1. These data suggest that, while the presence of a protolerogenic and purportedly anti-inflammatory agent such as IL-10 precludes DCs from acquiring their potential as initiators of adaptive immunity, their ability to act as initiators of innate immunity in response to Toll-like receptor signaling is enhanced.

Introduction

Dendritic cells (DCs)[3] are not only recognized as important initiators of immunity, characteristically unique in their ability to stimulate naive T cells, but also as key determinants of the type of immunity

initiated. They have variously been termed “sensors of infection” and “plastic”, reflecting their ability to convey context of Ag encounter to direct adaptive immunity (1, 2), while “immature” DCs have been implicated in mediating peripheral tolerance (3, 4). Under experimental conditions, a number of agents have been reported to modulate the immunostimulatory potential of DCs (3, 5). Modulation by IL-10 results in DCs that are no longer capable of presenting Ag for immunity, but induce Ag-specific anergy in both $CD4^+$ and $CD8^+$ T cells (5, 6, 7, 8).

Classic maturation of DCs in response to inflammatory stimuli involves extensive reprogramming, manifest as a temporally coordinated cascade, facilitating the conversion of cells specialized for high Ag uptake, but exhibiting poor T cell stimulatory properties, to cells that no longer sample Ag, but are highly efficient T cell stimulators (2, 9). This conversion is accompanied by changes in migration priorities, initially facilitating recruitment and then release of DCs from the site of inflammation, and subsequently homing of Ag-bearing mature DCs to lymphoid tissue. Inflammatory mediators produced early in the response at the site of activation are replaced at later time-points by mediators promoting DC:T cell interactions (10). IL-10 does not effect the capacity of immature DCs for Ag uptake, but does prevent the chemokine receptor switch required for maturation-induced lymphoid homing, and alters Ag presentation, preventing up-regulation of MHC class II, as well as costimulatory and adhesion molecules, impeding acquisition of T cell stimulatory capacity (5, 7, 11, 12). Given these observations, it has been suggested that IL-10 mediates its immunosuppressive effects on DCs by blocking normal maturation (7, 11). An alternative proposal is that DCs are actively “conditioned” by IL-10 to modify the quality of any subsequent response to stimuli (13).

In this study, we have used serial analysis of gene expression (SAGE) (14) to identify genes regulated following LPS-induced maturation of murine bone marrow-derived DCs (BMDCs), and to investigate changes in this regulation effected by pretreating the cells with IL-10. This analysis has facilitated the identification and cloning of a novel LPS-induced chemokine, the functional characteristics and induction of which are consistent with activity during the early proinflammatory phase of DC maturation. Pretreatment with IL-10 did not hinder the induction of this, or other related chemokines. SAGE library comparisons indicate that IL-10 conditions BMDCs to acquire a state from which, although compromised in their ability to present Ag for immunity, they remain competent to generate innate immune responses to microbial danger signals.

Materials and Methods

Cells

BMDCs were generated using an adaptation of Inaba et al. (15). Briefly, CBA/Ca marrow was sieved through a 70-μm nylon mesh in R10 medium (RPMI 1640, 10% FCS, 2 mM l-glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin, 5×10^{-5} M, 2-ME), red cells lysed using ammonium chloride, and cells plated at 7.5×10^6 per 10 cm plate (Corning, Corning, NY), supplemented with ~25 ng/ml murine recombinant GM-CSF, supplied as culture supernatant. Medium was replaced on days 3 and 6 and BMDCs harvested on day 7 by gentle pipetting. For maturation, 1 μg/ml LPS (Sigma-Aldrich, St. Louis, MO), 2 μM 5′-TCCATGACGTTCCTGATGCT-3′, CpG, or 5′-TCCATGAGCTTCCTGATGCT-3′, GpC (control) were added late on day 6 and cells harvested at the required time-points. IL-10 was added at 20 ng/ml (R&D Systems, Minneapolis, MN) from day 6 and cells harvested on day 9, with or without inclusion of 1 μg/ml LPS for the final 18–20 h. Control BMDC were also harvested on day 9. Surface phenotype was assessed by flow cytometry using mAbs; N418 (CD11c), 17-3-3S ($H\text{-}2E^k$), FGK-

45 (CD40), YN1.1 (CD54), 1G10 (CD80), 2D10 (CD86), and 28-8-6S (H-2K^k)(BD PharMingen, San Diego, CA). For allogeneic MLR, mitomycin C-treated CBA/Ca BMDCs were titrated into cultures of nylon wool-purified C57BL/10 splenic T cells, incubated for 3 days, and pulsed with 3H-TdR 24 h before harvesting. Capacity to process and present soluble Ag was assessed as the ability to stimulate IL-2 release from the T cell hybridoma 2G7.1, specific for hen egg-white lysozyme$_{1–18}$, in the context of H-2Ek (16).

Bone marrow macrophages were from 129/1CR mice (17), peritoneal exudates from BALB/c mice, with or without 1 ml Biogel P-100 polyacrylamide beads, 100–200 mesh (Bio-Rad, Hercules, CA) injected i.p. 4–5 days before lavage. Resident cells were plated at 1×10^6 per well in six-well plates, elicited cells at 1×10^7 per well in 9 cm dishes in Optimem (Invitrogen, San Diego, CA), supplemented with 2 mM l-glutamine, 50 U/ml penicillin, 50 µg/ml streptomycin, and cultured for 24 h with or without 1 µg/ml LPS, washed with PBS, and directly lysed in the culture dish for RNA isolation.

SAGE

Libraries were generated, using the anchor enzyme *Nla*III (New England Biolabs, Beverly, MA), the tagging enzyme *Bsm*FI (New England Biolabs) and biotinylated SAGE primers (14, 18). Poly(A)$^+$ mRNA was isolated from ~100 µg total RNA, and cDNA generated using a Roche Diagnostics kit (Basel, Switzerland). Concatenated ditags were cloned to the *Sph*I site of pGEM3Zf (Promega, Madison, WI). Amplified inserts were precipitated using polyethylene glycol, sequenced using the DYEnamic ET dye terminator kit (Amersham Pharmacia Biotech, Piscataway, NJ) and analyzed on a MegaBACE1000 capillary sequencer (Amersham Pharmacia Biotech). SAGE 3.04 beta software (K. W. Kinzler, Johns Hopkins Oncology Center, Baltimore, MD) was used to extract tag sequences. Automated hierarchical assignments were generated using Unigene full mapping files and a custom search algorithm, with tags matching 9 of 10 bases of any other tag occurring at >10-fold frequency within the entire dataset or any linker excluded, and custom software was used for statistical analysis (19).

RACE

Total RNA from CBA/Ca LPS-treated BMDCs was reverse transcribed using a SMART cDNA synthesis kit (BD Biosciences Clontech, Palo Alto, CA), primed using 5′-GACTCGAGTTGACATCGAGG(T)$_{20}$V-3′. 5′-GACTCGAGTTGACATCGAGG-3′ and 5′-CATGAAATATCAAC-3′ were used for 3′ RACE, (30 s at 94°C, 30 s at 39°C, 30 s at 72 C) for 35 cycles. 5′RACE was performed using the SMART II CDS primer, 5′-AAGCAGTGGTAACAACGCAGAGT-3′ and a series of nested or individual, reverse primers; 5′-TTAAACTTCTCCATTCTAAGACAT-3′ nested with 5′-AGGCATTAAATAGTCACTGTGCC-3′; 5′-AGACACCGTTGGGATGGATCGCT-3′, nested with 5′-TCAGCTGGACTTGCCGCTCTTCA-3′; and 5′-GTCAAGCTCTGGATGGTCTCAAAA-3′. Amplifications were performed using Pfu turbo DNA polymerase (Stratagene, La Jolla, CA), “A-tailed” using Biotaq DNA polymerase (Bioline, London, U.K.) and cloned to pGEM-T Easy (Promega). Inserts were identified by colony PCR, polyethylene glycol precipitated, and sequenced to generate a full-length cDNA contig.

Genomic cloning

A mouse embryonic stem cell (129/Sv × 129/Sv-CP) genomic λ library was screened using an ~840 bp cDNA probe, amplified using primers 5′-GTGCTGCACTGGTCCTGCT-3′ and 5′-AGGCATTAAATAGTCACTGTGCC-3′, and labeled using Ready-To-Go DNA labeling beads (Amersham

Pharmacia Biotech). All hybridizations were performed in 50% formamide, 6× SSC, 10× Denhardts, 0.5% SDS, in the presence of 100 μg/ml denatured herring sperm DNA (Sigma-Aldrich), rotating at 42°C in a Techne Hybridizer HB-1D (Techne Laboratories, Cambridge, UK). Membranes were washed to 0.2 × SSC, 0.1% SDS at 65°C. Three rounds of screening yielded nine clones. An ~7 kb *Xba*I fragment was subcloned to pGEM3zf (Promega) and sequenced using extending primers.

Real-time PCR

DNase I-treated RNA, prepared using the SV total RNA isolation kit (Promega), was reverse transcribed using Stratagene's ProSTAR first strand RT-PCR kit. DC inflammatory protein (DCIP)-1 primers, 5'-GGAAAGGAGGAAGCCCCTC-3' and 5'-AACAAGCAGGTAAAGACACATCCA-3', and probe, 5'FAM-ACCTGCATTCTAAATCAGAGAAAAGCGATCCA-3', macrophage inflammatory protein (MIP)-2 primers, 5'-ACCAAAGAGAAAGAAAAAAACAAACAG-3' and 5'-CTCAGACAGCGAGGCACATC-3' and probe 5'FAM-ACCCGGGAAGCCTGGATCGTACC-3', CCR7 primers, 5'-GCTGCGTCAACCCTTTCTTG-3' and 5'-ACCGACGCGTTCCGTACAT-3' and probe 5'FAM-TTCATCGGCGTCAAGTTCCGC-3', CCL5 primers, 5'-CATATGGCTCGGACACCACTC-3' and 5'-CGACTGCAAGATTGGAGCAC-3' and probe 5'FAM-CTGCTGCTTTGCCTACCTCTCCCTCG-3' and HPRT primers, 5'-GACCGGTCCCGTCATGC-3' and 5'-TCATAACCTGGTTCATCATCGC-3' and probe, 5'VIC-ACCCGCAGTCCCAGCGTCGTG-3', were designed using Primer Express software (Applied Biosystems, Foster City, CA). Probes (Applied Biosystems) were labeled with a 5' flurogenic dye and a 3' quencher (TAMRA). Primers (MWG Biotech, Ebersburg, Germany) were HPSF purified. Multiplex reactions were performed using TaqMan Universal PCR MasterMix (Applied Biosystems) and a Prism 7700 sequence detector system (Applied Biosystems). Standard curves generated from LPS-treated BMDC cDNA were used to calibrate the threshold cycle value on each 96-well plate. Samples, analyzed in triplicate, are represented as a mean normalized ratio to HPRT (mean (test/HPRT) × 100). Limits of detection were defined as the mean normalized ratio at 40 cycles and used to position the axes of data plots.

N-terminal sequence analysis of DCIP-1-Fc fusion protein

DCIP-1, amplified using primers 5'-CTTCGCACAAGCTTCCGCGCTCCA-3' and 5'-TTTCCCGGGATCCGAGGACTTGCGG-3' (introducing *Hind*III and *Bam*HI sites, underlined), was cloned in-frame with human IgG1-Fc, in pCDM8 (**20**). Supernatants were harvested 72 h after DEAE-dextran transfection of COS-7 cells and fusion protein purified by adsorption to protein A-Sepharose beads. N-terminal protein sequence analysis was performed by M. A. C. Willis, (Department of Biochemistry, Oxford University, Oxford, U.K.).

Synthetic chemokines

Mature DCIP-1 (Ala^{28}-Ser^{100}) was chemically synthesized using solid-phase F-moc chemistry on a 433A automated peptide synthesizer (Applied Biosystems). Purification and folding were essentially as described (**21**). Purity and molecular integrity of the folded protein were confirmed by analytical HPLC and electron-spray mass spectrometry (>95% purity, experimentally determined m.w., 7910.1). Murine IP-10, also produced synthetically, exhibited the same specific activity in binding and functional assays as commercial IP-10. KC and MIP-2 were purchased from R&D Systems.

Generation of murine CXCR2 transfectants

Murine CXCR2 was amplified from C57BL/6J genomic DNA using 5′-GCAGGATCCAGTTTACCTCAAAGATGGGAGAGTTC-3′ and 5′-ACAGAATTCAGAGGGTAGTAGAGGTGTTTGCTGA-3′ (introducing *Bam*HI and *Eco*R1 cloning sites, underlined, and removing an *Eco*RI site, double underlined), and cloned to pcDNA3.1 (Invitrogen), modified to carry the elongation factor-1α promoter. Stable RBL-1 transfectants were generated by electroporation, with single cell clones selected by chemotaxis to human growth-related oncogene (GRO)α, MIP-2, DCIP-1, and KC, and maintained in 0.8 mg/ml G418.

Chemotaxis assays

Assays were performed using 24-well Biocoat Transwell culture inserts (Costar, Cambridge, MA; 3 μm) (**22**). For RBL-1 stable transfectant clones, naked inserts were used. For human neutrophils the inserts were coated with 2×10^5 ECV304 cells. Neutrophil chemotaxis was performed using the granulocyte fraction isolated from fresh blood by percoll density centrifugation (**22**). Briefly, 1×10^6 cells in 100 μl of chemotaxis media (RPMI 1640, 0.5% BSA) were dispensed in the upper transwell and 600 μl of chemotaxis media with or without chemokine placed in the bottom well. Incubation was at 37°C for 5 h for cell transfectants, or 30–60 min for human neutrophils. Cells that migrated to the bottom well were counted using FACS, with numbers expressed as relative FACS counts, obtained by acquiring events for a set time of 30 s.

Peritoneal recruitment assay

Lavages were performed 90 min after 200 μl i.p. injections of 5 μg/ml chemokine in PBS or PBS alone. Cytospins, 5×10^5 cells/slide, were stained with Wright-Giemsa stain (Fisher Diagnostics, Pittsburgh, PA). The percentage of macrophages, mononuclear cells, and neutrophils were determined by counting eight random high power fields (×40 magnification; total area, 0.5 mm^2) and related to total numbers in the lavage.

Results

SAGE comparison of untreated and LPS-treated BMDCs

Murine BMDCs cultured for 7 days in GM-CSF exhibit surface and functional phenotypes characteristic of immature DCs, while inclusion of LPS for the final 18–20 h generates cells with characteristics typical of mature DCs; increased surface expression of MHC class II and costimulatory molecules, diminished capacity to take up and present new Ag, and enhanced capacity to stimulate primary T cells in an allogeneic MLR (Fig. 1⇓) (**9**). SAGE libraries were generated from both untreated (21,789 tags) and LPS-treated (13,085 tags) BMDC (GEO, GSM3833, and GSM3832), with surface phenotype used to verify the degree of homogeneity (>90% $CD11c^+$) and relative states of maturation of each starting cell population. Statistical comparison of tag frequencies revealed 74 tags differentially up-regulated in the untreated population and 28 tags up-regulated in the LPS-treated population (Fig. 2⇓).

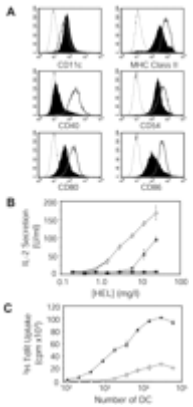

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FIGURE 1.

Characterization of BMDCs. *A*, Surface phenotype of untreated (filled histograms) and LPS-treated (open histograms) BMDCs, determined by flow cytometry. Broken lines represent background staining obtained using irrelevant species- and isotype-matched control Abs. *B*, Processing and presentation of soluble HEL by immature (○) and LPS-treated (▲) BMDCs, assessed by their ability to stimulate IL-2 release from the T cell hybridoma 2G7.1, determined by ELISA. To control for the effects of endocytosis, immature BMDCs fixed with paraformaldehyde were included (▪). *C*, Relative capacity of immature (○) and LPS-treated (▲) BMDCs to stimulate T cell proliferation, determined using allogeneic MLR.

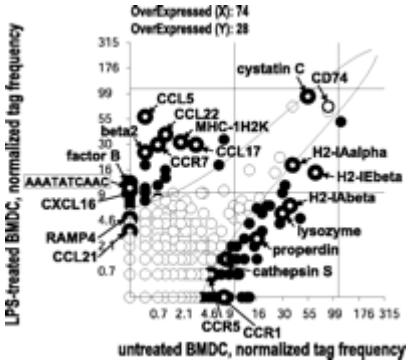

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FIGURE 2.

Scatter-plot comparison of untreated and LPS-treated BMDC SAGE libraries. Individual tag frequencies normalized to 10^4 tags are plotted on logarithmic scales. Tags lying outside the marked area are differentially expressed (95% confidence of >1.5-fold difference) and represented as •. For the purpose of clarity of presentation ~1000 nondifferential tags, mostly mapping to house-keeping and ribosomal protein genes, were removed from the analysis. Annotated transcripts were identified using the SAGE tags; MHC-IH2K GATTGAGAAT (2.3 vs 33.6), β-2M (beta2) TTTTCAAAAA (0.5 vs 27.5), H2-IAα GAAGAAGTGG (39.9 vs 20.6), H2-IEβ TCCTGCTTGG (70.2 vs 16.8), H2-IAβ GCACTATTGT (37.6 vs 7.6), cystatin C CCTTGCTCAA (58.7 vs 97.1), Ii, CD74 GTTCAAGTGA (90.4 vs 80.2), cathepsin S ATAGCCCCAA (8.3 vs 1.5), RAMP4 TAATTTACCT (0 vs 5.4), lysozyme TGTCAGTCTG (31.2 vs 6.1), CCL5 AAGATCTCTG (0.5 vs 63.4), CCL17 GTTTCAACAT (3.7 vs 32.1), CCL21 GCGCCCTTCC (0 vs 3.8), CCL22

TCCTGAAGGT (1.4 vs 40.5), CXCL16 CTGTCTTGAG (0 vs 9.9), CCR1 GGCCCAATAA (7.8 vs 0), CCR5 CAGTTTCGGA (6 vs 0.8), CCR7 TTCTGCTTTT (0.9 vs 32.9), complement factor B CAAAGGAGAT (0 vs 13), properdin AACTGAGGGG (17.9 vs 3.1), with tag frequencies indicated in brackets (untreated vs LPS-matured).

The relative distribution of gene-tags corresponding to transcripts associated with well-documented features of DC maturation provided validation for the integrity of the libraries (Fig. 2⇑). SAGE tags corresponding to MHC class I α and β_2 microglobulin (β-2M) chains were increased, 14.6- and 55-fold respectively, in the LPS-matured library, consistent with delayed neo-synthesis (**23**), while MHC class II α- and β-chain tags were relatively low, consistent with synthesis as a rapid transient burst, peaking as early as 1 h post stimulation, and having resolved by the 18–20 h SAGE time-point (**24**). The invariant chain (Ii, CD74) tag was abundant in both libraries. Although this tag cannot distinguish between Ii-p31 and Ii-p41 isoforms, the ratio of these isoforms has previously been reported to be unchanged at the protein level on DC maturation (**25**). The tag for cystatin C, an endogenous inhibitor of the Ii processing enzyme cathepsin S, was also abundant in both libraries. Although implicated in retention of MHC class II molecules in immature DCs (**25**), increased cathepsin S activity on maturation has been shown to be independent of changes in cystatin C expression or subcellular distribution (**26**). The tag for ribosomal-associated membrane protein 4 (RAMP4) occurred at a frequency of 5.3 per 10^4 in the mature library. Although not differential by statistical criteria at the current library sizes, this tag was not represented in the immature library. RAMP4 has been reported to interact with nascent Ii, controlling glycosylation and transport into the endoplasmic reticulum, and possibly impacting on the cell's capacity to present Ag (**27**).

Expected changes in migratory properties of maturing DCs were also reflected in the SAGE data (Fig. 2⇑) (**10**). Tags for chemokine receptors CCR1 and CCR5, which mediate recruitment and retention of DCs at inflamed sites, were differentially represented in the immature library, while the tag for CCR7, which mediates homing to lymphoid organs in response to the cognate ligands CCL19 and CCL21, was increased in the LPS-matured library. Tags for CCL21, which also attracts CCR7-bearing naive T cells, and for CCL17, CCL22, and CXCL16, which attract recently activated and memory T cells expressing the cognate receptors CCR4 and CXCR6, were differentially expressed in the LPS-matured library, consistent with promoting DC:T cell interactions required for recruitment of an appropriate adaptive effector response.

SAGE reveals an unassigned tag restricted to LPS-matured BMDCs

SAGE tag, AAATATCAAC, occurred at a frequency of 12.2 per 10^4 in the LPS-treated library, but was not represented in the untreated library (Fig. 2⇑). Furthermore, it was not represented in SAGE libraries derived from fibroblasts (www.sagenet.org), an embryonic stem cell line (GSM3829), various T cells (GSM3677–79, GSM3681–86), and lymph nodes (GSM3680 and GSM3687). Using the SAGEmap tag-to-gene mapping resource (**28**), which uses UniGene cluster information in conjunction with GenBank information, it was not possible to identify any gene-match for this tag.

RACE extension of SAGE tag AAATATCAAC reveals a novel ELR$^+$, CXC chemokine

The SAGE tag-derived primer 5′-CATGAAATATCAAC-3′, generated a 234 bp 3′ RACE product, containing a typical polyadenylation sequence, AATAAA, located 12 bp upstream of a putative poly-A tail. Extension of this sequence using 5′RACE provided an ∼1 kb full-length cDNA sequence

(AY311403), encoding a previously undescribed ELR$^+$, CXC chemokine. The extent of the 27 aa leader peptide sequence was confirmed by sequence analysis. The chemokine is ~82% identical to MIP-2 and we have termed it dendritic cell inflammatory protein-1, DCIP-1. (A single polymorphic amino acid substitution, E_{49} to K_{49}, was detected in the *Mus spretus* strain, AY311405).

In rodents, the murine KC and MIP-2, and the rat cytokine-induced neutrophil chemoattractant (CINC) proteins, are the closest relatives of the human GRO proteins, and these molecules have previously been assigned as a subgroup of ELR$^+$, CXC chemokines (**29**). By homology DCIP-1 represents a new member of this subgroup and likely represents the previously undescribed murine homologue of CINC-2 (~80% identical); no mRNA species equivalent to the 3′ alternatively spliced β form of CINC2 (**30**) has yet been identified. The gene (AY311404) is organized as 4 exons and 3 introns, as are genes encoding other chemokines of this subgroup. Sequence overlap with the genomic clone AF349465, encompassing three other CXC chemokine genes CXCL5, CXCL7, and CXCL4, positioned the DCIP-1 gene within the MIP-2 and KC containing proinflammatory gene cluster on murine chromosome 5, ~12.4 kb downstream of CXCL4. A TATA-box and a putative NF-κB binding site, positioned −32 to −27 and −75 to −66, respectively, from the transcription start site (assigned by 5′RACE), are both highly conserved within promoters of other members of this subgroup, with the NF-κB binding site important for the induction of these genes in response to inflammatory stimuli, including LPS (**31**, **32**, **33**, **34**).

The DCIP-1 gene has now been computationally annotated on the mouse genome, XM_284097, although this entry is truncated compared with the sequence described here and does not include the SAGE tag sequence.

DCIP-1 interacts with CXCR2 and mediates neutrophil chemotaxis

DCIP-1-mediated chemotaxis of murine CXCR2 transfected RBL-1 cells, displaying a chemotactic maximum of ~10 nM, as compared with 5 nM for MIP-2 and KC, while no chemotaxis was observed in response to the control chemokine IP-10, a ligand for CXCR3 (Fig. 3⇓*A*), or by murine CXCR3-, CCR8-, or CCR6-transfected RBL-1 cells (data not shown). DCIP-1 also generated calcium flux (EC50 75–100 nM) in K293/Gal6 and CHO/Gal6 CXCR2 transfectants (data not shown), and mediated potent recruitment of neutrophils, but not macrophages or mononuclear cells in vivo (Fig. 3⇓*B*). In addition to functioning as a ligand for murine CXCR2, DCIP-1 also mediated chemotaxis of human neutrophils with potency and efficacy equivalent to that of human IL-8 (Fig. 3⇓*C*).

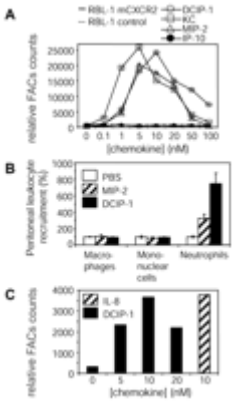

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FIGURE 3.

DCIP-1 is a ligand for CXCR2 and mediates chemotaxis of neutrophils. *A*, Chemotaxis of murine CXCR2-transfected and untransfected RBL-1 cells in response to chemokines DCIP-1, KC, MIP-2, and IP-10. This is a representative experiment with each data point performed in duplicate. *B*, Peritoneal recruitment of macrophages, mononuclear cells, and neutrophils in response to DCIP-1 or MIP-2, represented as a percentage of recruitment in response to PBS alone (assigned as 100%). Data represents mean results obtained from five mice. *C*, Chemotaxis of human neutrophils in response to indicated concentrations of DCIP-1 or 10 nM human IL-8.

DCIP-1 mRNA induction kinetics are consistent with an early proinflammatory function

DCIP-1 mRNA levels increased rapidly, within 30 min of addition of LPS to BMDC cultures (Fig. 4⇓), consistent with an early role in establishing inflammation at sites of activation. MIP-2 mRNA peaked after ~1 h, while DCIP-1 reached a maximum at ~2–4 h. Both mRNAs decayed only very slowly to the 20 h time-point, compatible with the differential DCIP-1 expression detected by SAGE. RANTES (CCL5) and CCR7 transcript levels both increased less rapidly than DCIP-1 and MIP-2 and remained high to the 20-h time-point consistent with previous reports (**10**, **35**).

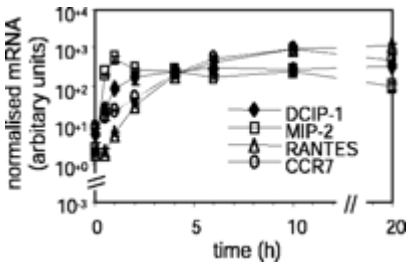

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FIGURE 4.

Real-time PCR analysis of DCIP-1, MIP-2, RANTES, and CCR7 over a time-course of LPS-induced maturation of BMDCs. A single time-point at 20 h indicated no change in the levels of mRNA in the absence of LPS stimulation (not shown). This data is representative of at least three independently derived experiments. Error bars indicate the SD of PCR triplicates with data represented as the mean normalized ratio of the triplicates to HPRT.

Induction of DCIP-1 mRNA is not restricted to BMDCs and is not prevented by pretreatment with IL-10

Real-time PCR analysis confirmed SAGE data indicating that DCIP-1 mRNA is not expressed by Th1, Th2, or Treg clones (**36**) or by the fibroblast cell line NIH3T3. It further demonstrated that DCIP-1 is not expressed by the T cell line EL4 or the B cell lymphoma line A20, with or without PMA stimulation, the untreated B cell line CH27, or the macrophage cell line RAW, untreated or treated with LPS. Expression was not detected in muscle, kidney, brain, thymus, or skin (data not shown). DCIP-1 mRNA was, however, generated in response to LPS by primary macrophage populations, in particular by elicited peritoneal macrophages (Fig. 5⇓*A*). It was also generated by BMDCs in response to the alternative inflammatory stimulus, unmethylated CpG (Fig. 5⇓*B*). BMDCs pretreated with the anti-inflammatory cytokine IL-10 retained, and indeed appeared enhanced in their ability to

generate DCIP-1 mRNA in response to LPS (Fig. 5⇓*C*). In each case, the relative expression of DCIP-1 mRNA mirrored that of the related chemokine MIP-2 (Figs. 4⇑ and 5⇓).

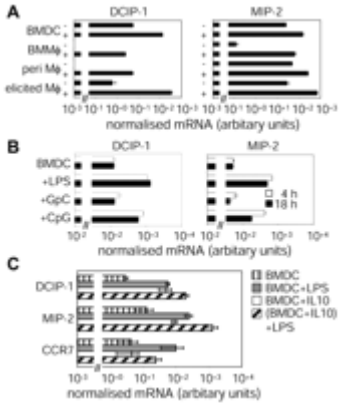

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FIGURE 5.

DCIP-1 expression in BMDC and macrophage populations in response to inflammatory stimuli. Induction of DCIP-1 and MIP-2 mRNA assessed in, *A*, BMDCs, bone marrow, resident peritoneal and Biogel elicited peritoneal macrophages with (+) and without (−) the addition of 1 μg/ml LPS, and in *B*, BMDCs in response to LPS (1 μg/ml) and the alternative inflammatory, CpG (2 μM), or nonstimulatory GpC (2 μM), oligonucleotides, following 4 and 18 h exposure. Data is represented as the mean normalized ratio to HPRT, with error bars indicating the SD of PCR triplicates, and in each case is representative of at least two independent cell preparations. *C*, DCIP-1, MIP-2, and CCR7 mRNA levels were assessed in 9 day BMDCs in response to LPS with and without preexposure to IL-10. Values represent the mean normalized ratio to HPRT of two independently derived panels of cells analyzed in the same real-time PCR experiment, and is representative of at least three independent experiments. Error bars indicate the SD of six PCR.

SAGE comparisons indicate that IL-10 conditions BMDC to an alternative state of activation

IL-10 is generally considered to limit immune and inflammatory responses (**37**). While inclusion of IL-10 in BMDC cultures does not impair phagocytic ability it does impair APC function (**11**), and BMDC treated with IL-10 were unable to acquire the ability to stimulate naive T cells in MLC, even following the addition of LPS (Fig. 6⇓*A*). The surface phenotype of IL-10-treated cells was reminiscent of untreated BMDCs, expressing moderate amounts of MHC II and CD86 and low levels of CD80, but in contrast to the untreated cells, these surface molecules were not up-regulated following exposure to LPS (Fig. 6⇓*B*). The effects of IL-10 were found to be time and concentration dependent, with maximal effects observed following culture with 20 ng/ml recombinant IL-10 for 72 h, with no adverse effect on cell viability observed by trypan blue exclusion (data not shown).

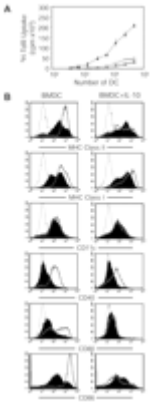

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FIGURE 6.

Characterization of BMDCs conditioned by exposure to IL-10. *A*, Relative capacities of 9 day, untreated (○), IL-10-treated (△), LPS-treated (▲), and IL-10-treated, LPS-treated (•) BMDCs to stimulate T cell proliferation, determined by allogeneic MLR. *B*, Surface phenotype of 9 day, untreated (filled histograms) and LPS-treated (open histograms) BMDCs, with and without exposure to IL-10, determined by flow cytometry. Broken lines represent background staining obtained using irrelevant species- and isotype-matched control Abs.

Comparison of SAGE libraries generated from untreated (21,789 tags), LPS-treated (13,085 tags), IL-10-conditioned (30,455 tags), and IL-10-conditioned, LPS-treated BMDCs (31,135 tags) (GEO, GSM3832–35), demonstrated marked global differences in gene expression. Although 58 genes were repressed or down-modulated in BMDCs following IL-10 treatment, 75 were up-regulated, of which 9 were not represented in other immunologically related and unrelated libraries (GSM580, GSM367, GSM3677–87, and GSM3824–37). Of the overall changes seen in response to IL-10, 31 tags were in common with 73 changes seen in response to LPS alone (the number of statistically differential genes vary from those in Fig. 2⇑ due to the inclusion of larger numbers of libraries). Following LPS treatment of IL-10 conditioned cells, 41 of the 73 overall changes associated with maturation in response to LPS alone were still present, while 71 additional genes were statistically up- or down-modulated.

LPS-induction of CCR7, which was marked in response to LPS alone, and induction of tags associated with chemokines CCL17, CCL21, and CCL22s that promote various DC:T cell interactions, were hampered by IL-10 (Table I⇓ and Fig. 5⇑), indicating interference with mechanisms recruiting adaptive immunity, while the tag for programmed death ligand (PD-L)-1, involved in mediating the inability of IL-10 treated DCs to present Ag to T cells (**38**), was increased. In contrast, real-time PCR (Fig. 5⇑) indicated that IL-10 conditioning of BMDCs does not hinder LPS-induced expression of inflammatory molecules such as DCIP-1 and MIP-2, and SAGE tags for the genetically linked inflammatory chemokines CXCL4 and CXCL5, and the proinflammatory cytokine IL-1 were also increased (Table I⇓). Further, a decrease in tags for the decoy receptor molecule, IL-1R2, in response to IL-10 alone, indicated increased sensitivity to IL-1, although a counter increase in tags for the IL-1R antagonist was also apparent (**39**). An increase in tags for membrane-bound and soluble innate pattern recognition molecules, including macrophage receptor with collagenous structure (**40**), CD14, TLR2 (**41**), galectin-3 (**42**), and components of the alternative pathway of complement (**43**), in

response to LPS, combined with a general increase in degrading lysosomal activity, suggest that in addition to promoting inflammation, exposure to IL-10 also promotes removal of pathogens via phagocytosis, consistent with observations of increased Ag uptake by IL-10-treated DCs in the presence of bacteria (11).

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Table I.

Comparison of SAGE tag frequencies in immature and LPS-matured BMDC libraries following IL-10 conditioning, normalized to per 10^4 tags[a]

Although IL-10-conditioned DCs retain and indeed appear enhanced in their capacity to generate at least a subset of proinflammatory mediators in response to LPS, a coordinated increase in the production of protective anti-inflammatory agents, such as heme oxygenase-1 (44), and arginase-1, which competes with inducible NO synthase for the shared substrate arginine, down-regulating synthesis of NO (45) (Table I⇑), is also seen. Inflammatory responses reflect a complex network of chemokine/protease interactions and the substantial increase in tags derived from lysosomal proteases, in particular cathepsins D, S, L, B, and C, following IL-10 treatment, could reflect alterations in this network, modifying subsequent cell recruitment and activation in response to inflammatory stimuli (46, 47, 48). IL-10 mediated unique acquisition of the SAGE tag for CXCL7 (platelet basic protein), to a frequency of 64 per 10^4 (Table I⇑). Differential rates of processing of various inactive precursor forms of this chemokine result in pro- and anti-inflammatory effects, both mediated by neutrophil activating protein-2, with excessive accumulation of neutrophil activating protein-2 resulting in functional desensitization via down-regulation of CXCR2 (49). The accumulation of CXCL7 tags in response to IL-10 was reversed on subsequent addition of LPS (Table I⇑).

Discussion

We have described the identification and cloning of a novel CXC chemokine molecule, DCIP-1, using SAGE libraries derived from immature and LPS-matured BMDCs, and provide comparative SAGE and real-time PCR data indicating that the immune responsiveness of BMDCs is conditioned by IL-10 to promote local innate effector mechanisms, while inductive mechanisms capable of recruiting adaptive immunity are down-graded.

DCIP-1 is an ELR^+, CXC chemokine which, by homology, belongs to the subgroup of proinflammatory, neutrophil-attracting chemokines comprising murine MIP-2, KC, rat CINC, and the human GRO proteins, and would appear to represent the murine homologue of rat CINC2. It functions as a ligand for CXCR2 mediating selective recruitment of murine neutrophils in vivo, and can also cross species to mediate chemotaxis of human neutrophils in vitro (Fig. 3⇑). The kinetics of DCIP-1 mRNA induction in response to LPS are consistent with a role for this novel chemokine during the early proinflammatory phase of DC maturation (Fig. 4⇑).

DCs not only initiate immune responses, but also determine whether they are driven toward appropriately skewed immunity, or alternatively toward unresponsiveness or tolerance (1, 2, 3, 4). IL-10 is among a number of agents reported to modulate DCs for the generation of Ag specific anergy (5, 6, 7, 8), and it has been variously considered that it may be acting either by inhibiting

normal maturation (7, 11), or by conditioning DCs to acquire an alternative activation state (13). From a global perspective, the changes in gene expression observed by comparative SAGE analysis are supportive of a mechanism more subtle than simple inhibition of maturation. As a consequence of IL-10 treatment, 31 tags varied in common with changes seen in the response of BMDCs to LPS, suggesting at least some form of partial activation, although 102 tags were also modulated independently. Following LPS treatment of these cells, while 41 of 73 changes associated with LPS maturation in the absence of IL-10 were retained, again suggesting that aspects of the normal response to LPS have been preserved, 71 additional tags were modulated. These changes are persuasive that IL-10 actively drives DCs to acquire a modified state from which to respond to inflammatory stimuli.

IL-10 has no effect on the ability of BMDCs to pick up and process Ag (11), a classic function of immature DCs, but prevents them from acquiring the ability to effectively present this Ag to naive T cells, a feature which has been attributed to failure to up-regulate MHC class II and costimulatory molecules (7, 11) (Fig. 6⇑). An important contribution to this IL-10-mediated decrease in stimulatory capacity is provided by the interaction of PD-L1 and PD-L2 with their receptor programmed death receptor-1 (38), and is reflected in the SAGE data (Table I⇑). Although a fragment of Ii-p41 has been shown to inhibit the Ii processing enzyme cathepsin L (50), the impact of a 3.3-fold increase in CD74 in response to IL-10, which is reversed on subsequent exposure to LPS, (Table I⇑), is uncertain. Despite an increase in cathepsin S and L SAGE tags in response to IL-10 (Table I⇑), lysosomal acidification, rather than changes in individual protease levels, is considered to be the critical determinant controlling transport of peptide:MHC class II complexes to the cell surface (26, 51). In contrast to MHC II, tags for MHC class I and β-2M were elevated in response to IL-10, and on addition of LPS attained levels equivalent to those generated by LPS alone, consistent with surface expression data (Table I⇑ and Fig. 6⇑).

IL-10 blocks the maturation-induced switch from inflammatory chemokine receptors, such as CCR1 and CCR5, to the lymphoid homing receptor CCR7, inhibiting migration to the lymph nodes and recruitment of T cells (12, 52). This block in CCR7 induction was apparent by SAGE, which also indicated a similar block in induction of the T cell attracting chemokines CCL17, CCL21, and CCL22 (Table I⇑). The slight increase in CCR7 expression revealed by real-time PCR, was also apparent in previous reports, but did not prevent the inhibitory effect of IL-10 on migration (12, 52). CCR1 and CCR5 tag levels were not reduced following IL-10 conditioning (Table I⇑). These receptors remain at the cell surface, but are functionally uncoupled (12), acting as scavengers to reduce local ligand levels, an effect reinforced by reduction in LPS-induced synthesis of ligands, such as CCL3 and CCL4 (12), which are also substrates for the elevated protease cathepsin D (48), and CCL5 (Table I⇑).

In contrast to the down-regulation of CC inflammatory chemokines that mediate recruitment of immature DCs and also activated T cells to sites of inflammation, IL-10 conditioning of BMDC does not impair LPS-induced synthesis of neutrophil attracting CXC inflammatory chemokines, such as DCIP-1 and MIP-2, or the proinflammatory cytokine IL-1 (Fig. 5⇑ and Table I⇑). These data contrast with previous reports of the anti-inflammatory effect of IL-10 in in vivo models (37, 53, 54), and probably reflect the critical importance of context in cytokine behavior. Indeed, the effects of IL-10 in vivo, and on individual cell types, are not always anti-inflammatory (55, 56, 57). Down-modulation of the IL-1R2 decoy receptor in response to IL-10 is suggestive of priming for IL-1 responsiveness, while the general increase in degrading lysosomal activity, in association with LPS-induced

expression of membrane bound and soluble pattern recognition molecules, is indicative of enhanced phagocytic bacterial clearance (Table I⇑). The increase in inflammatory potential following exposure to IL-10 appears to be tempered by a coordinated increase in the production of anti-inflammatory agents, such as heme oxygenase-1, reportedly responsible for IL-10-mediated protection of mice from LPS-induced septic shock (**44**), arginase-1, IL-1R antagonist, and potentially CXCL7 (Table I⇑) (**39**, **45**, **49**).

It would appear that while classic maturation of BMDCs indeed does not occur in response to bacterial stimuli following pretreatment with IL-10, this is not simply due to a block in maturation, since aspects of the "normal" response are retained. The IL-10-treated cells described here are phenotypically distinct from the "semi-mature" DCs implicated in maintaining peripheral tolerance (**58**), but they could also be regarded as a type of "semi-mature" cell, in which the ability to activate and recruit T cells in response to LPS-signaling is constrained, while the ability to promote local inflammation and bacterial clearance is enhanced. How these observations relate to particular subsets of DCs and biological scenarios in vivo remains to be established. However, if the full clinical potential of future DC-based therapeutics is to be realized (**5**, **59**), it will also be important to appreciate the complete immune-responsive spectrum of ex vivo manipulated populations. SAGE provides a versatile approach to characterizing these cells, providing both for the identification of novel molecules, potentially representing new therapeutic targets in their own right, and as an indicator of key changes in cellular processes.

Acknowledgments

We thank S. Humm and S. Cartland for technical assistance.

Footnotes

1 This work was supported by grants from the Medical Research Council, U.K., and Millenium Pharmaceuticals.

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3 Abbreviations used in this paper: DC, dendritic cell; BMDC, murine bone marrow-derived DC; SAGE, serial analysis of gene expression; TLR, Toll-like receptor; MIP, macrophage inflammatory protein; GRO, growth-related oncogene; DCIP-1, DC inflammatory protein-1; RAMP4, ribosomal-associated membrane protein 4; CINC, cytokine-induced neutrophil chemoattractant; PD-L, programmed death ligand; β-2M, β_2-microglobulin; Ii, invariant chain.

Received September 8, 2003.

Accepted December 4, 2003.

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