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Differential Expression of IFN Regulatory Factor 4 Gene in Human Monocyte-Derived Dendritic Cells and Macrophages

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In vitro human monocyte differentiation to macrophages or dendritic cells (DCs) is driven by GM-CSF or GM-CSF and IL-4, respectively. IFN regulatory factors (IRFs), especially IRF1 and IRF8, are known to play essential roles in the development and functions of macrophages and DCs. In the present study, we performed cDNA microarray and Northern blot analyses to characterize changes in gene expression of selected genes during cytokine-stimulated differentiation of human monocytes to macrophages or DCs. The results show that the expression of IRF4 mRNA, but not of other IRFs, was specifically up-regulated during DC differentiation. No differences in IRF4 promoter histone acetylation could be found between macrophages and DCs, suggesting that the gene locus was accessible for transcription in both cell types. Computer analysis of the human IRF4 promoter revealed several putative STAT and NF-κB binding sites, as well as an IRF/Ets binding site. These sites were found to be functional in transcription factor-binding and chromatin immunoprecipitation experiments. Interestingly, Stat4 and NF-κB p50 and p65 mRNAs were expressed at higher levels in DCs as compared with macrophages, and enhanced binding of these factors to their respective IRF4 promoter elements was found in DCs. IRF4, together with PU.1, was also found to bind to the IRF/Ets response element in the IRF4 promoter, suggesting that IRF4 protein provides a positive feedback signal for its own gene expression in DCs. Our results suggest that IRF4 is likely to play an important role in myeloid DC differentiation and gene regulatory functions. The Journal of Immunology, 2005, 175: 6570–6579.

Circulating blood monocytes provide a pool of precursor cells that are able to differentiate into macrophages and DCs, the cell types that form the bridge between innate and adaptive immune responses. Macrophages and dendritic cells (DCs) are APCs with overlapping as well as cell type-specific functions. After capturing and processing foreign Ags, activated DCs move to local lymph nodes and present Ags to naïve T cells, thus activating Ag-specific immune responses. Macrophages act as scavengers by directly phagocytosing and destroying infectious agents and other harmful substances. In the course of these events, both macrophages and DCs produce a range of proinflammatory chemokines and cytokines that regulate inflammatory reactions and stimulate the development of innate and adaptive immune responses. At present, the molecular basis for the functional differences between macrophages and DCs is under active investigation.

IFN regulatory factors (IRFs) are a family of transcription factors involved in the regulation of both innate and adaptive immunity (1, 2). By gene knockout studies, IRF1, IRF4, and IRF8 have been found to be essential for the proper differentiation and functions of immune cells. Lymphocyte development requires IRF1 and IRF4 (3–7), whereas IRF7 and IRF8 are needed for monocyte-to-macrophage differentiation (8, 9), and together with IRF1, IRF8 orchestrates the development of Th1 immune responses by regulating the gene expression of IL-12, the major Th1 response-inducing cytokine (10–13). Recently, IRF4 and IRF8 have also been shown to participate in DC differentiation and functions (14–19). IRF1 is ubiquitous in its expression, whereas IRF4 and IRF8 are preferentially expressed in the cells of the immune system (1, 2, 20, 21).

IRF proteins bind to a variety of related DNA elements on their target promoters. An interesting feature of IRF4 and IRF8 is their ability to function as transcriptional activators and repressors (20, 21), depending on the promoter context. This is due to their ability to form homo- and heterodimers with other IRFs and their ability to interact with other transcription factors in a cell type-specific manner (22–26), adding another level of regulation in the control of cell-specific target gene expression.

IRF4, originally thought to follow a lymphocyte-restricted expression pattern (27), has remained one of the least studied members of the IRF family. This holds true even though IRF4 expression has also been detected in murine and human macrophages (28, 29) and in human DCs (19, 30). IRF4 is known to interact with IRF8, a factor playing a key role in regulating the differentiation and functions of the cells of the monocytic lineage. Additionally, both IRF4 and IRF8 are known to form complexes with PU.1 (31), an Ets family transcription factor required for terminal differentiation of myeloid cells (32, 33). To date, no specific inducer of IRF4 expression has been identified in myeloid cells. In the present study, we have focused on factors regulating IRF4 gene expression in human primary monocytes, macrophages, and DCs. We show that IRF4 mRNA expression was regulated by cytokines important for generation of macrophages and DCs from monocytes. DCs were found to express IRF4 mRNA and protein constitutively, and...
STAT and NF-κB transcription factors play an important role in regulating IRF4 expression in DCs. Finally, IRF4 protein bound to a regulatory element in its own promoter, suggesting an autoregulatory loop controlling IRF4 mRNA expression in DCs.

Materials and Methods

Cytokines and cell culture

Monocytes were purified from freshly collected leukocyte-rich buffy coats obtained from healthy blood donors (Finnish Red Cross Blood Transfusion Service). Human PBMCs were isolated by a density gradient centrifugation over Ficoll-Paque gradient (Amersham Biosciences). To obtain DCs, monocytes were further purified as described previously (34). Briefly, mononuclear cells were centrifuged over a Percoll gradient (Amersham Biosciences). Next, the remaining T and B cells were removed using anti-CD3 and anti-CD19 magnetic beads (Dynal). Monocytes were allowed to adhere to plastic 6-well plates (Falcon; BD Biosciences) for 1 h at 37°C in RPMI 1640 medium supplemented with antibiotics and glutamine without FCS (2.5 × 10^5 cells/well). After incubation, nonadherent cells were removed, and the wells were washed with PBS. Monocytes were differentiated into macrophages in macrophage-serum-free medium (Invitrogen Life Technologies) supplemented with antibiotics and recombinant human GM-CSF (10 ng/ml; Biosite). Culture medium (2 ml/well) was replaced every 2 days. Monocyte-derived immature DCs were cultured for 6–7 days in RPMI 1640 medium with 10% FCS, recombinant human IL-4 (20 ng/ml; R&D Systems), and recombinant human GM-CSF (10 ng/ml; Biosite). Fresh medium (1 ml/well) was added every 2 days. Cultured DCs were differentiated into macrophages in macrophage-serum-free medium (Invitrogen Life Technologies) supplemented with antibiotics and recombinant human GM-CSF (10 ng/ml; Biosite). Fresh medium (1 ml/well) was added every 2 days. Cultured DCs were CD1a^+ , CD14^- , CD80^- , CD83^- , and CD86^- , and they showed a typical DC morphology (data not shown). To study the effect of protein synthesis inhibition on gene expression in monocytes, the cells were cytokine stimulated in the presence of cycloheximide (CHX) (100 μg/ml) for 2 h. CHX was added onto the cells 0.5 h before cytokine stimulation. In some experiments, DCs were treated with TNF-α (10 ng/ml; Biosite) for times indicated in the figures.

cDNA microarray analyses

The human ImmunoChip cDNA microarrays (35) were printed on glass slides in the Finnish DNA Microarray Centre at Turku Centre for Biotechnology (www.btk.ttu.fi) according to established protocols (http://microarrays.btk.tu). The ImmunoChip contains ~2000 genes implicated in immune cell activation and differentiation, including cytokines, chemokines and their receptors, transcription factors, and genes involved in signaling, apoptosis, and cell cycle regulation. Samples were labeled with Fluorolink Cy3-dUTP and Cy5-dUTP (Amersham Biosciences) using 16 μl of total RNA for direct labeling during CDNA synthesis and hybridized using loop-design (36). Protocols for labeling and hybridization are available through a web site of the Finnish DNA microarray centre (http://microarrays.btk.tu/public/Resources.shtml). Separate images for Cy3 and Cy5 dyes were acquired using ScanArray 5000 laser-scanning microscope (Packard BioSciences), and gene transcript levels were then determined from the fluorescence intensities of scanned data image files with the Quan-TArray Microarray Analysis software (Packard BioSciences).

Northern blot analysis

Total cellular RNA was isolated as described previously (37). Ten-microgram aliquots were size-fractionated on a 1% formaldehyde-agarose gel, transferred to a nylon membrane (Hybond; Amersham Biosciences), and hybridized with IRF1, IRF4, IRF8, PU.1, Stat1, Stat4, Stat5A, Stat5B, Stat6, NF-κB p50, and NF-κB p65 probes. Ethidium bromide staining of rRNA bands was used to ensure equal RNA loading. The probes were labeled with [α-^32P]dCTP (3000 Ci/mmol; Amersham Biosciences) using the random priming method. The membranes were hybridized in Ultraglyc hybridization buffer (Ambion), washed twice at room temperature, and once at 65°C in 1× SSC/0.1% SDS for 0.5 h each time and exposed to Kodak AR X-Omat films at −70°C using intensifying screens.

DNA affinity-binding and Western blot analysis

Cells were treated with cytokines as indicated in the figures and figure legends. Cells were collected, and whole cell extracts were prepared as described previously (38). Both strands of the DNA elements (Table 1) were synthesized with RanHFl overhangs as spacers, and the upper strand oligonucleotide was 5'-biotinylated (DNA Technology). The oligonucleotides were annealed in 0.5 M NaCl and incubated with streptavidin-agarose beads (Neutrevadin; Pierce) at +4°C for 2 h in a ratio to yield maximum saturation of the beads with the biotinylated oligonucleotide. Protein samples were incubated with agarose beads saturated with the oligonucleotide for 2 h at +4°C. After washing, the bound proteins were released in SDS sample buffer, and equal aliquots were subjected to SDS-PAGE and Western blotting.

For direct Western blot analyses, cells were lysed, and 30-μg protein aliquots were separated on 10% SDS-PAGE (15% for detection of PU.1) using the Laemmli buffer system. Proteins separated on gels were transferred onto Immobilon-P membranes (Millipore). Binding of primary and secondary Abs was performed in PBS (pH 7.4) containing 5% nonfat milk for 1 h at room temperature. Primary Abs against IRF4 (1/1000) (39), IRF8 (60509), PU.1 (sc-352X), Stat4 (sc-486X), Stat5 (sc-835X), Stat6 (sc-981X), p50 (sc-717X), p65 (sc-372X), RelB (sc-226X), and El-Rel (sc-70X; all from Santa Cruz Biotechnology) were used in immunoblotting (all in 1/5000 dilution, except p65 in 1/100). HRP-conjugated anti-guinea pig (P0141; DakoCytomation), anti-goat (P0449; DakoCytomation), and anti-rabbit (P0448; DakoCytomation) IgGs were used as secondary Abs. The protein bands were visualized on HyperMax film using the ECL system (Amersham Biosciences).

Chromatin immunoprecipitation (ChIP)

Detection of histone-H3 acetylation of the IRF4 promoter was done by using a commercial ChIP kit (17-245; Upstate Biotechnology) according to the manufacturer’s instructions. Macrophages and DCs were treated as specified in the kit’s protocol. The primers from the IRF4 gene promoter region used in PCR were 5'-ACG CCT GGC CTT CTA TTG TG-3' (forward) and 5'-TCC ATC TAT TAG GCT GGT GA-3' (reverse). Binding of transcription factors to the IRF4 promoter region in macrophages and DCs was further characterized with ChIP experiments using the following Abs: anti-Stat4 (sc-486), anti-Stat6 (sc-1698), anti-PU.1 (C-terminal, sc-352), and anti-PU.1 (N-terminal, sc-5972) (all Abs from Santa Cruz Biotechnology). The PCR detection (FastStart TaqDNA polymerase; Roche Diagnostics) of the precipitates was done using primers flanking the IRF4-comb binding site: 5'-AGC ATG TCA GAC ACG CAG ACG CAG AG-3' (forward) and 5'-CTTG CCT CGT TTT GCA GAG CGT GT-3' (reverse). Input controls, representing the starting material before the immunoprecipitation step, were included in the PCR, alongside with the immunoprecipitated samples and no-Ab control samples. PCR samples were analyzed by agarose gel electrophoresis on 1.5% gels.

EMSA

Nuclear extracts and nuclear protein/DNA-binding reactions were performed as described previously (40, 41). CIS1 IFN-γ activation site (GAS) (5'-gagc-CCC CGT TTT CTT GGA AAG TTT TGG AAA TCT GT-3') and IRF4/Stat5 (5'-gagc-CCG CCT CGG GCA ACG GCC CC 3') oligonucleotides were obtained from DNA Technology. The probes were labeled by Klenow fill-in. The binding reaction was done at room temperature for 0.5 h. The samples were analyzed by electrophoresis on 6% nondenaturing low-ionic-strength polyacrylamide gels in 0.25× Tris-borate-EDTA. The gels were dried and visualized by autoradiography.

Table 1. Sequences of the DNA elements used in DNA-affinity binding assays

<table>
<thead>
<tr>
<th>Element</th>
<th>Sequence (5′-biotin-ggatcc-)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IRF4combi</td>
<td>GGCCTTCTTCTATTTCTTTTATGAGTGCCAGTGTTCCTCTTAAACCCGGC</td>
</tr>
<tr>
<td>CD68</td>
<td>CCTCCTTTCTGGAAGGGAGAATGGAAGTC</td>
</tr>
<tr>
<td>NF-κBRE1</td>
<td>AAAATGATGAAAATCCCCCTGTGCCA</td>
</tr>
<tr>
<td>NF-κBRE2</td>
<td>TCCGTCTCAGAAGTCCCTCCCTCCC</td>
</tr>
<tr>
<td>Stat6α/B</td>
<td>CCTGCGCCTTGGGCGAAACGCCCCAAGGACAGTCCTGCCGAGGCGC</td>
</tr>
</tbody>
</table>
Results

Expression of IRF4 mRNA is induced in monocytes treated with GM-CSF or IL-4

To study the immediate early events in monocyte differentiation into macrophages or DCs, we stimulated freshly isolated human primary monocytes for 3 h with GM-CSF, IL-4, or their combination. Total cellular RNA was isolated and hybridized to a cDNA microarray (ImmunoChip). Because IRF8 is essential for the development of macrophages and DCs (9, 14–17), we were interested in analyzing IRF gene expression in the early phases of macrophage and DC differentiation. Surprisingly, the expression of the IRF8 gene remained virtually unchanged when monocytes were treated with GM-CSF and/or IL-4 (Table II). The IRF4 gene, instead, was one of the most strongly up-regulated genes in cytokine-stimulated monocytes (Table II). Therefore, the regulation of IRF4 gene expression, a known partner of IRF8, was studied in more detail. First, to verify the results obtained with the cDNA array, monocytes were treated with GM-CSF, IL-4, or their combination for 2 or 4 h, and total cellular RNA was isolated and analyzed by Northern blotting with IRF1, IRF4, IRF8, and PU.1 probes. IRF4 mRNA expression was strongly up-regulated already at 2 h after stimulation with GM-CSF or IL-4 and especially with their combination (Fig. 1A). IRF8 gene expression was transiently up-regulated (at 2 h) by IL-4 alone or in combination with GM-CSF. IRF1 mRNA expression was weakly down-regulated by IL-4.

IRF4 mRNA expression is enhanced during DC differentiation

To further characterize IRF and PU.1 expression during macrophage and DC differentiation, we performed Northern blot analyses on total cellular RNA isolated from monocytes and 3- and 7-day macrophages and DCs. IRF1 was basally expressed in monocytes, but its expression decreased during differentiation (Fig. 1B). IRF8 and PU.1 mRNA expression remained stable throughout macrophage and DC differentiation. In contrast, IRF4 mRNA expression could only be seen in cells committed to DC differentiation (Fig. 1B).

Because there was a marked difference in IRF4 mRNA expression between macrophages and DCs, we analyzed whether this difference was due to accessibility of the IRF4 promoter. We performed ChIP experiments using anti-acetyl-histone H3 Abs to detect possible differences in IRF4 promoter acetylation between macrophages and DCs (Fig. 1C). IRF4 promoter-specific primers produced an amplification product of the expected size both from macrophages and DCs (Fig. 1C), suggesting that there was no major difference in IRF4 promoter histone acetylation that could explain the differential IRF4 mRNA expression between macrophages and DCs.

Comparison of human and mouse IRF4 promoters

To characterize the role of cytokine-activated transcription factors in regulating IRF4 gene expression, we analyzed the IRF4 mRNA expression in human and mouse IRF4 promoters.

Table II.  Fold change values from the cDNA microarray analysis of cytokine-stimulated monocytes

<table>
<thead>
<tr>
<th>Accession</th>
<th>Gene Name</th>
<th>GM-CSF</th>
<th>IL-4</th>
<th>GM-CSF+IL-4</th>
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<tr>
<td>AA478043</td>
<td>IRF1</td>
<td>0.70</td>
<td>-0.01</td>
<td>-0.55</td>
</tr>
<tr>
<td>AA393214</td>
<td>IRF2</td>
<td>0.08</td>
<td>-0.28</td>
<td>-0.24</td>
</tr>
<tr>
<td>AA825491</td>
<td>IRF4</td>
<td>2.10</td>
<td>2.18</td>
<td>2.63</td>
</tr>
<tr>
<td>N30372</td>
<td>IRF5</td>
<td>0.50</td>
<td>0.82</td>
<td>0.68</td>
</tr>
<tr>
<td>AA877255</td>
<td>IRF7</td>
<td>-0.15</td>
<td>-0.14</td>
<td>-0.21</td>
</tr>
<tr>
<td>AA391632</td>
<td>IRF8</td>
<td>-0.30</td>
<td>-0.41</td>
<td>0.64</td>
</tr>
<tr>
<td>AA291577</td>
<td>IRF9</td>
<td>-0.18</td>
<td>-0.14</td>
<td>0.02</td>
</tr>
</tbody>
</table>

FIGURE 1. Characterization of IRF and PU.1 mRNA expression in monocytes and during differentiation of macrophages and DCs. A, Monocytes were stimulated with GM-CSF, IL-4, or their combination for 2 or 4 h. Total cellular RNA was isolated and analyzed by Northern blotting with IRF1, IRF4, IRF8, and PU.1 probes. B, Monocytes were differentiated into 3- and 7-day macrophages (Mφ) and DCs with GM-CSF or GM-CSF+IL-4, respectively. Total RNA was isolated and analyzed as in A. Ethidium bromide staining of ribosomal RNA is shown as a control of equal RNA loading. C, ChIP was used to analyze the status of IRF4 promoter histone H3 acetylation in 7-day Mφ and DCs. The resulting PCR products from the IRF4 promoter region are visualized by agarose gel electrophoresis.
promoter region in detail in silico. The human and mouse IRF4 promoters have partially been characterized previously (42–45). Analysis of the human (1307 bp; compiled of sequences U52683 (43) and BC015752) and mouse IRF4 promoters (1375 bp; bp 1981–3355 of sequence U20949 (42)), with the MatInspector program (46), revealed both similarities and differences in the promoter

FIGURE 2. Sequence analysis of the human IRF4 promoter and comparison of the transcription factor binding sites between human and mouse promoters. The program MatInspector was used to analyze the human (compiled from sequences U52683 and BC015752) and mouse (bp 1981–3355 of sequence U20949) IRF4 promoter sequences for putative transcription factor binding sites. A, Detailed sequence of the human promoter. Previously characterized binding sites are boxed and named as in original publications. The binding sites analyzed in this study are shaded and named IRF4combi, Stat6/kB, NF-kBRE1, and NF-kBRE2. Factor binding sites are typed in boldface. B, Alignment of transcription factor binding sites between human and mouse promoters. Previously characterized binding sites and those relevant to this study are shown in the comparison.

promoter region in detail in silico. The human and mouse IRF4 promoters have partially been characterized previously (42–45). Analysis of the human (1307 bp; compiled of sequences U52683 (43) and BC015752) and mouse IRF4 promoters (1375 bp; bp 1981–3355 of sequence U20949 (42)), with the MatInspector program (46), revealed both similarities and differences in the promoter
elements (Fig. 2). A total of 171 putative transcription factor binding sites in the human and 188 sites in the mouse promoter was found. For more detailed studies, we chose three putative NF-κB binding sites (NF-κBRE1, NF-κBRE2, and Stat6/κB) and two GAS sites (Stat6/κB and IRF4/κB). The Stat6-binding GAS element is adjacent to a NF-κB element and is conserved between human and mouse (Fig. 2A). The IRF4/κB element includes the IRF4 GAS element previously described by us (39), with an extension of 8 nt upstream to include a predicted PU.1/IRF4 binding site.

Cytokine-inducible expression in monocytes

According to the computer analysis (Fig. 2A) and our previous studies (39), there are at least two GAS elements located in the IRF4 promoter. GAS is the target element for STATs that are activated by a number of cytokines, including GM-CSF (activates Stat5) and IL-4 (activates Stat6). We studied cytokine-induced IRF4 gene expression in the presence of CHX to see whether the up-regulation of IRF4 gene expression was a direct effect mediated by activated STATs. CHX was able to inhibit the induction of IRF4 mRNA in response to GM-CSF (Fig. 3A). However, the effect of IL-4 alone or together with GM-CSF could not be completely blocked by CHX, suggesting that IL-4 was able to directly activate transcription of the IRF4 gene. The expression of cytokine-inducible Src homology 2-domain containing protein (CIS; Fig. 3A), whose expression is known to be regulated directly by Stat5 (47, 48), was readily induced by GM-CSF also in the presence of CHX. IL-4 was also able to enhance CIS mRNA expression. We also studied STAT DNA binding in monocytes by EMSA using a GAS element from the CIS1 promoter region and the Stat6-binding element from the IRF4 promoter. Strong DNA binding in response to GM-CSF and GM-CSF + IL-4 was detected with the CIS GAS probe (Fig. 3B). In contrast, a DNA-binding complex was formed on the IRF4 promoter Stat6 GAS element only in response to GM-CSF + IL-4 stimulation.

Multiple transcription factors bind to the IRF4/comb and Stat6/κB GAS-containing elements in DCs

To characterize STAT binding to putative IRF4 promoter GAS elements during differentiation of macrophages and DCs, we prepared whole cell extracts from monocytes and 3- and 7-day macrophages and DCs and conducted DNA affinity-binding experiments. Biotinylated double-stranded oligonucleotides were prepared spanning the putative GAS elements, IRF4/κB and Stat6/κB (Fig. 2A). Proteins binding to these elements were pulled down and identified by Western blotting using STAT-specific Abs (Fig. 4A). The IRF4/comb element bound Stat4 in monocytes and DCs, but only very weakly in macrophages. Stat5 and Stat6 binding was detected only in 7-day DCs. The Stat6/κB element bound Stat6 very strongly in 7-day DCs, whereas Stat5 binding occurred at a lower level (Fig. 4A). Weak Stat4 binding was also detected in DCs. As a whole, the results suggest that Stat6 is the major factor binding to this site, as predicted by the computer analysis.

Interestingly, the MatInspector program recognized a putative IRF4/PU.1 binding site in the IRF4 promoter, adjacent to the Stat4 binding GAS site (IRF4/comb). To test the functionality of this binding site, pull-down experiments followed by Western blot detection with anti-IRF4 and anti-PU.1 Abs were performed. IRF4 bound to this element, but only in 7-day DCs, although IRF4 protein expression was high also in 3-day DCs (Fig. 4B). PU.1 was also found to bind the IRF4/comb element especially in 7-day DCs and, to a lesser extent, in macrophages. Western blotting with the PU.1 Ab also revealed a protein band of smaller size (~25 kDa) with DNA-binding capacity.

We also performed ChIP analyses in differentiated macrophages and DCs using Abs recognizing Stat4, Stat6, and the N terminus and the C terminus of PU.1. In macrophages, only the sample precipitated with the anti-Stat4 Ab yielded an amplification product when primers flanking the IRF4/comb element were used (Fig. 4C). In DCs, Abs against Stat4 and Stat6 as well as both anti-PU.1 Abs were able to precipitate the promoter fragment.

Stat4 mRNA is differentially expressed in macrophages and DCs

Differences in the expression of STAT transcription factors between macrophages and DCs could account for the differential binding of STATs to IRF4 promoter elements in pull-down and ChIP experiments. Because there is no comprehensive data on the expression of STAT genes during primary human monocyte differentiation to macrophages or DCs, we conducted analyses of
steady-state STAT mRNA expression by Northern blotting. Stat1 mRNA expression remained virtually unchanged during differentiation (Fig. 4D). Stat5A and Stat5B mRNAs were more strongly expressed in differentiated macrophages and DCs than in monocytes. The expression of Stat6 mRNA was the highest in monocytes and was somewhat lower in DCs and clearly lower in macrophages. The most significant difference was seen with Stat4 mRNA (Fig. 4D). Monocytes show low-level Stat4 mRNA expression, and in macrophages, Stat4 mRNA was barely detectable. The strongest expression of Stat4 mRNA was seen in 3- and 7-day DCs, where it coincided with IRF4 expression (compare Fig. 1B).

Constitutive mRNA expression and DNA-binding activity of NF-κB proteins are altered during macrophage and DC differentiation

Human and mouse IRF4 promoters contain previously characterized (44, 45) as well as novel putative NF-κB binding sites (Fig. 2). To characterize the NF-κB system in our cell model, we analyzed p50 and p65 mRNA expression by Northern blotting in monocytes, 3- and 7-day macrophages and DCs. The expression of p50 and p65 mRNAs was clearly lower in 7-day macrophages than in other cell types (Fig. 5A). We also studied whether NF-κB family proteins constitutively bound to the Stat6/κB site of the IRF4
promoter. DNA pull-down experiments, followed by Western blotting with Abs specific for NF-κB p50, p65, RelB, and c-Rel, revealed basal binding of NF-κB p50 protein in all cell types (Fig. 5B). In more differentiated cells a higher molecular mass form of p50 protein was found. Basal binding of p65, RelB, and c-Rel was only detected in 7-day macrophages and DCs, and the binding was stronger in DCs compared with macrophages, except for the binding of c-Rel.

**FIGURE 5.** Gene expression and DNA binding of NF-κB transcription factors to IRF4 promoter elements during macrophage and DC differentiation. A, Total cellular RNA was isolated from untreated monocytes and 3- and 7-day macrophages (Mφ) and DCs. Basal NF-κB p50 and p65 mRNA was analyzed by Northern blotting. B, Whole-cell extracts were prepared from monocytes and 3- and 7-day Mφ and DCs. Proteins binding to the Stat6/κB DNA element from the IRF4 promoter were pulled down and analyzed by Western blotting with anti-p50, anti-p65, anti-RelB, and anti-c-Rel Abs.

**FIGURE 6.** TNF-α enhances NF-κB binding to DNA elements and up-regulates IRF4 expression and DNA binding in DCs. A, Whole-cell extracts were prepared from 7-day DCs treated with TNF-α for 0.5 h and untreated control cells. Proteins binding to the Stat6/κB, NF-κBRE1, and NF-κBRE2 DNA elements from the IRF4 promoter (Fig. 2A) were pulled down and analyzed by Western blotting with anti-p50, anti-p65, anti-RelB, and anti-c-Rel Abs. B, DCs were treated with TNF-α for 3 or 6 h or left untreated. Total cellular RNA was isolated and mRNA expression of IRF4, IRF8, and PU.1 was studied by Northern blotting. 0I and 0II represent untreated control cells collected at 3 and 6 h, respectively. C, Whole-cell extracts were prepared from 7-day macrophages (Mφ) and DCs treated with TNF-α for 6 h and untreated control cells. Proteins binding to CD68 promoter IRF/Ets or IRF4combi DNA elements were pulled down and analyzed by Western blotting with anti-IRF4 and anti-PU.1 Abs.
and their binding was weakly enhanced by TNF-α stimulation (Fig. 6A). To study the effects of TNF-α on IRF4 mRNA expression, we stimulated 7-day DCs with TNF-α for 3 or 6 h or left them untreated. Northern blot analyses show that, although constitutive expression of IRF4 and IRF8 mRNAs was clearly detectable in DCs, their expression was also weakly induced by TNF-α stimulation (Fig. 6B). PU.1 expression was not significantly altered.

IRF4 protein was found to bind to the IRF4combi DNA element in DCs (Fig. 4B). To analyze whether TNF-α stimulation also enhanced DNA binding of IRF4, we prepared whole-cell extracts for DNA affinity-binding studies from TNF-α-treated (for 6 h) 7-day macrophages and DCs. We used IRF4combi and CD68 gene IRF/Ets DNA elements to pull down the interacting proteins. The IRF/Ets element from the CD68 gene is known to bind IRF4 together with PU.1 and served as a positive control (49). IRF4 was found to bind to both CD68 (IRF/Ets) and IRF4combi elements in DCs, and its binding was weakly enhanced by TNF-α stimulation (Fig. 6C). Constitutive PU.1 binding was detected in macrophages and DCs, and its binding was not further increased by TNF-α stimulation. In DCs, a 25-kDa DNA-binding PU.1 protein fragment was also detected (Fig. 6C).

**Discussion**

Macrophages and myeloid DCs are important players in the interface between innate and adaptive immune responses. Blood monocytes give rise to both macrophages and DCs, two functionally distinct cell types. The transcription factors that regulate functional differences between macrophages and DCs in both human and mouse have been intensively investigated by microarray analyses (50–56). However, the picture is far from being complete. Previously, we have observed that STAT expression and activation during human monocyte/macrophage differentiation is altered (37, 48). In the present study, we have compared the expression, activation, and functional differences of IRF, STAT, and NF-κB transcription factors between monocyte-derived macrophages and DCs.

Our major observation was that IRF4 gene expression was very high in DCs but was virtually absent in macrophages. It has been shown previously that IRF8 is required for the terminal differentiation of macrophages and DCs (9, 14–17). We observed that IRF8 is constitutively expressed in human macrophages and DCs (Fig. 1). Thus, it is likely that IRF8 requires other interaction partners or that additional transcription factors are involved in regulating the functional differences between macrophages and DCs. We propose here that IRF4, with its DC-specific expression, may be one of these factors that regulate DC differentiation and functions.

We have shown for the first time that in human monocytes, IRF4 gene expression is up-regulated by GM-CSF and IL-4. The effect of GM-CSF was sensitive to protein synthesis inhibition, whereas IL-4 was able to directly activate IRF4 gene expression. The protein synthesis-sensitive factor responsible for mediating GM-CSF-induced IRF4 expression could be AP-1. Growth factors rapidly up-regulate the expression of Fos and Jun, the constituents of AP-1 (Ref. 45 and present study Fig. 2). In contrast, IL-4 may mediate its direct IRF4 gene activation functions via Stat6. Furthermore, in monocytes, the up-regulation of IRF4 expression was transient, and constitutively high IRF4 mRNA and protein expression was seen only in cells committed to DC differentiation.

Analyses on the effects of cytokine stimulation on IRF4 gene expression have been limited, and little is known of the factors regulating its expression in myeloid cells. In human NK and T cells, we have shown that IL-12 or IFN-α induce IRF4 expression, and this occurs via Stat4 (39). IL-4 alone or in synergy with CD40 ligation up-regulates IRF4 expression B cells (23). In the present report, we identified a new GAS element in the IRF4 promoter that bound Stat6 in IL-4/GM-CSF-differentiated DCs. This Stat6-binding element may convey both constitutive and inducible IL-4 responsiveness to the promoter in different cell types. In 7-day fully differentiated immature DCs, Stat6 binding to the Stat6/κB site was strong even though the last GM-CSF/IL-4 stimulus had been given 2 days earlier. This kind of prolonged Stat6 DNA-binding activity has been reported previously in murine B cells (57). Also, Stat6 and NF-κB are known to interact on Stat6/κB DNA elements resembling the IRF4 gene element in different cell types (58–60).

In addition to lymphocytes, Stat4 expression has been found in human and mouse monocytic cells (61, 62). In human and murine monocytes and DCs, IFN-α and IL-12 stimulation, respectively, results in Stat4 activation (61, 62). In the present study, we demonstrate by DNA pull-down and ChIP assays that Stat4 binds to IRF4 regulatory elements. However, we were unable to directly detect phosphorylation of Stat4 in DCs, and IFN-α or IL-12 stimulation that also activate Stat4 did not further enhance IRF4 mRNA expression in DCs (data not shown). Previously, it has been shown that some nonphosphorylated Stat1 and Stat3 is constitutively found in the nucleus in some cell lines and primary human cells (63, 64). Stat4 could also reside in the nucleus in a nonphosphorylated form. Interestingly, constitutive transcription of LMP2 is supported by nonphosphorylated Stat1 together with IRF1 (65). In this report, we provide evidence that Stat4, IRF4, and PU.1 bind constitutively to specific DNA elements in the IRF4 promoter in DCs, pointing out a role for this protein complex in basal IRF4 mRNA expression. It remains to be determined whether interactions between IRFs and nonphosphorylated STAT emerge as a common theme in regulating constitutive expression of some genes.

We also found that IRF4 bound to the IRF4combi promoter element in DCs but not in macrophages, suggesting the possibility of a positive autoregulatory loop. IRF8 gene expression is also positively regulated by its own gene product (66). We demonstrated IRF4 protein expression in 3- and 7-day DCs, but enhanced IRF4 DNA-binding activity was seen only in 7-day DCs. IRF4 requires PU.1 for its interaction partner for binding to IRF/Ets sites (67), and this interaction requires the PEST domain in PU.1 (68). The 25-kDa PU.1 protein fragment detected in 3-day DCs had DNA-binding capacity but was apparently unable to interact with IRF4. Previously, it has been shown that molecular interactions between the DNA-binding domains of PU.1 and IRF4 are not strong enough to support stable complex formation (68–70), accounting for the lack of interaction between these molecules in 3-day DCs. Alternatively, IRF4 protein may require some activation signals that are present in 7-day but not in 3-day DCs.

Functional NF-κB binding sites were also identified in the IRF4 promoter, demonstrating both basal and TNF-α-inducible NF-κB binding. NF-κB p50 and p65 mRNA expression levels were lower in macrophages than in DCs and the same difference applied for constitutive NF-κB DNA-binding activity. Similar results have been obtained by others (71, 72). In addition, we found that the basal RelB binding to IRF4 promoter Stat6/κB element was strong in DCs. In lymphoid cells, constitutively active RelB is known to mediate basal transcription (73, 74). RelB is also essential for the generation of myeloid DCs in the mouse (75). Constitutive expression and DNA-binding activity of RelB may be one of the factors specifically regulating basal IRF4 expression in DCs. Inducible expression of IRF4 mRNA and protein by TNF-α was weak, even though NF-κB p65 DNA binding was strongly induced. This suggests that basal NF-κB activity in DCs is sufficient to enable efficient IRF4 gene expression together with STATs and possibly constitutive expression of other IRFs.
IRF4 itself, and further stimulation of NF-kB phosphorylation of the molecule. Phosphorylation of NF-

IRF4 is linked to GM-CSF-induced in vitro DC differentiation, whereas IRF8 was linked to Fms-like tyrosine kinase 3 ligand-mediated differentiation. In the present study, we conducted a detailed analysis of IRF4 gene promoter elements, revealing functional Stat6, NF-kB, and, interestingly, also IRF4-binding elements that were likely to regulate IRF4 expression in human DCs. Our results also demonstrated differences in the expression and activity of several transcription factor systems, IRF, STAT, and NF-kB, between human macrophages and DCs. Expression of IRF4 was controlled by the concerted action of several factors, some ubiquitous and some following tissue-specific events in the mouse (18). IRF4 was linked to GM-CSF-induced IL-12 p40 production. J. Exp. Med. 186: 1523–1534.


