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A Common Single Nucleotide Polymorphism in the CD14 Promoter Decreases the Affinity of Sp Protein Binding and Enhances Transcriptional Activity

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CD14 is a pattern recognition receptor that plays a central role in innate immunity through recognition of bacterial lipopolysaccharides, primarily LPS. Recently, our group has identified a common single nucleotide polymorphism, $-159C\rightarrow T$, in the CD14 proximal promoter. Homozygous carriers of the T allele have a significant increase in soluble CD14, but a decreased total serum IgE. This epidemiologic evidence led us to investigate the molecular basis for the effects of CD14/$-159C\rightarrow T$ on CD14 regulation in monocytes and hepatocytes, the two major cell types known to express this gene in vivo. EMSA analysis showed that the T allele results in decreased affinity of DNA/protein interactions at a GC box that contains a binding site for Sp1, Sp2, and Sp3 transcription factors. In reporter assays, the transcriptional activity of the T allele was increased in monocytic Mono Mac 6 cells, which express low levels of Sp3, a member of the Sp family with inhibitory potential relative to activating Sp1 and Sp2. By contrast, both alleles were transcribed equivalently in Sp3-rich hepatocytic HepG2 cells. Our data indicate that the interplay between CD14 promoter affinity and the [Sp3]:[Sp1 + Sp2] ratio plays a critical mechanistic role in regulating transcription of the two CD14 alleles. Variation in a key gene of innate immunity may be important for the pathogenesis of allergy and inflammatory disease through gene-by-gene and/or gene-by-environment interactions.

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ARIN, a single-copy gene, CD14 protein is found in two distinct forms: a 50- to 55-kDa glycosylphosphatidylinositol-anchored membrane molecule, membrane CD14 (mCD14), expressed primarily on the surface of monocyte/macrophages and neutrophils (6), and a soluble form lacking the glycosylphosphatidylinositol anchor (7). Soluble CD14 (sCD14) appears to derive from monocytes (8) as well as the liver (9, 10) and is found in normal serum at microgram concentrations (7). Both mCD14 and sCD14 are critical for LPS-dependent signal transduction. LPS-responsive cells that lack mCD14, such as endothelial cells, epithelial cells, and astrocytes, become sensitive to low concentrations of LPS in the presence of sCD14 (11).

Regulation of CD14 gene expression appears to be important in several disease states. Increased serum levels of sCD14 are associated with high mortality in Gram-negative septic shock (12). Consistent with these findings, CD14 knockout mice were found to be at least 10-fold more resistant to LPS-induced shock than wild-type controls (13). In addition, inflammatory infectious diseases such as atopic dermatitis (14), HIV infection (15), and malaria (16), as well as extensive tissue damage in polytrauma and severe burns (17), are marked by elevated sCD14 in the circulation.

This compelling epidemiologic evidence led us to investigate whether CD14 expression is influenced by genetic variation. Recently, our group has identified a single nucleotide polymorphism (SNP) in the proximal CD14 promoter at position $-159$ (18). CD14/$-159C\rightarrow T$ is very frequent among both Hispanic and non-Hispanic white populations, with approximately one-half of all chromosomes carrying the C allele and one-half carrying the T allele. Interestingly, homozygous carriers of the T allele have a significant increase in serum levels of sCD14 and a concomitant decrease in total serum IgE (18), suggesting that CD14 may play a role in the regulation of IgE synthesis and IgE-mediated diseases such as allergy and asthma. In contrast, the association between CD14/$-159C\rightarrow T$ and risk for myocardial infarction recently described in at least three different populations (19–21) eloquently highlights the far-reaching effects that genetic variation in CD14 may have on the pathogenesis of cardiovascular diseases. In this context, the role of CD14 in inflammation and/or its ability to bind and transport lipids, including cholesterol (22), may be particularly relevant. Altogether, these findings suggest that CD14/$-159$ may affect the regulation of CD14 gene expression, thus modulating the
impact that CD14-mediated events have on innate and adaptive immune responses (23).

These findings warranted studies aimed at providing a molecular basis for the effects of CD14/−159C→T on the regulation of CD14 gene expression. We developed a reporter system to assess the transcriptional activity of the two allelic variants of the CD14 proximal promoter. We found a monocyte-specific increase in transcription from the T allele that was associated with a decreased affinity in DNA/Sp protein interactions at a GC box that includes the polymorphic nucleotide.

Materials and Methods

Cells

The human monocyctic Mono Mac 6 cell line was generously provided by Dr. H. W. Ziegler-Heitbrock (University of Munich, Munich, Germany) and was cultured as described (24). Human hematopoietic HepG2 cells (HB8065) were obtained from the American Type Culture Collection (Manassas, VA). Primary human monocytes were isolated by counter electromigration from normal volunteers as previously described (25). All cell culture reagents were endotoxin free as determined by the Limulus amebocyte lysate assay (limits of detection 3–6 pg/ml; BioWhittaker, Walkersville, MD).

Construction of CD14 reporter vectors

Fig. 1A shows the sequence of the CD14 5′-flanking region that contains the proximal promoter (26, 27) and CD14/−159C→T (Fig. 1A, bold and underlined). The −159/C and −159/T luc reporter constructs contain a 304-bp insert (position −232±72; GenBank accession no. U00699) and were created by PCR amplification of genomic DNA from individuals homozygous for either C or T at position −159 using primers 5′-GG GCC TCT TGC AAG-3′ (Stratagene, La Jolla, CA) via Clal/SacI and then directionally cloned into the KpnI and SacI sites of the promotorless, enhancerless luciferase reporter plasmid, pGL3-Basic (Promega, Madison, WI). Constructs were verified by sequence analysis and were prepared for transfection using the EndoFree Plasmid Mega kit (Qiagen, Valencia, CA).

Oligonucleotides and probes for EMSA

EMSA

EMSA was performed using 22-bp oligonucleotides (22C or 22T) corresponding to the −167/−146 region in the CD14 promoter that encompasses CD14/−159C (Fig. 1B). Mutant oligonucleotides were generated by introducing three nonoverlapping blocks of 5-bp transversions into wild-type oligonucleotides (Fig. 1C). In addition, we used oligonucleotides containing binding sites for Sp1 (5′-ATTCCATGCCGGCAGCCAG-3′) and STAT6 (5′-CTGTCTTTGGGAAGTC-3′; consensus sequences are underlined). Single-stranded oligonucleotides were obtained from Life Technologies. Complementary oligonucleotide pairs were annealed and isolated as previously described (28). When used as EMSA probes, double-stranded oligonucleotides (50 ng) were end-labeled using [γ-32P]ATP (3000 Ci/ 

Nuclear extract preparation

Mono Mac 6 cells (107 cells) were washed with ice-cold PBS, resuspended in 1 ml of ice-cold hypotonic buffer (29) with protease inhibitors (1 mM PMSF, and 3 γ-32P]ATP (3000 Ci/ 

EMSAS

After preliminary data indicated that Sp family proteins bind differentially to the 22C and 22T oligonucleotides, EMSA conditions were optimized for Sp protein/DNA interactions as follows: nuclear extracts (5–10 μg) were incubated for 20 min on ice with binding buffer (20 mM HEPES-HCl (pH 7.6), 100 mM NaCl, 12.5% glycerol, 14 mM 2-ME, 1 mM EDTA, 0.5% Nonidet P-40, and poly(dI-dC) × poly(dI-dC) (1 μg; Amersham Pharmacia Biotech, Piscataway, NJ) in the presence or absence of oligonucleotide competitors. A 32P-labeled probe (30 MB, 100,000–150,000 cpmlane) was then added to the binding reaction (final volume, 25 μl) for an additional 20-min incubation on ice. The binding reactions were run on a 2.2% poly-
For Ab supershift assays, polyclonal antisera (4 μg) were added to nuclear extracts for 20 min on ice before incubation with the probe. Polyclonal antisera specific for human Sp2 (rabbit K-20), Sp3 (goat n-20), Sp4 (rabbit V-20), and Fra1 (rabbit R-20) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal antisera to Sp1 was purchased from Geneka. Recombinant Sp1 protein was obtained from Pro-mega. An AP2 EMSA kit (including AP2 oligonucleotide (5’-CCACAAACGACCGATTGCGGGCGGT), AP2 mutant oligonucleotide (5’-CCACAAACGACCGATTGCGGGCGGT), JEG-3 nuclear extracts, binding buffer, and anti-AP2-Ab) was obtained from Geneka and was used for the detection of AP2/DNA interactions.

Results

CD14/–159T has enhanced transcriptional activity in reporter assays

The transcriptional activity of the CD14/–159C and T variants was compared by transiently transfecting the −159/Chuc and −159T/luc reporter constructs into monocytic Mono Mac 6 cells. Luciferase activity in cell extracts was assessed 6 h post-transfection and was expressed as fold increase in the activity of the CD14 reporter constructs compared with the empty vector, pGL3-Basic. The data presented in Fig. 2 indicate that, consistent with previous reports (26), the wild-type CD14 promoter (−159C/luc) had strong basal transcriptional activity. Importantly, the C→T SNP at −159 (−159T/luc) resulted in a 32% increase in constitutive gene expression. This difference in activity was significant (p < 0.0001) and consistent in 14 independent transfections.

LPS stimulation enhances CD14 expression in Mono Mac 6 cells, as assessed by quantitation of CD14 mRNA (Ref. 32 and T. D. LeVan, unpublished observations). Therefore, we compared the activity of −159C/luc and −159T/luc in Mono Mac 6 cells stimulated with LPS (100 ng/ml) for 6 h. LPS induced a 49% increase in the activity of −159C/luc and −159T/luc, which was similar to the increase observed for CD14 mRNA under the same conditions (n = 8, data not shown). Most importantly, a 20% difference (p < 0.003) between the expression of −159C/luc and −159T/luc was still apparent after LPS stimulation. These data show that the CD14/−159T allele is transcribed more efficiently in both unstimulated and LPS-stimulated monocyctic cells.

Proteins of the Sp family bind the polymorphic GC box in the CD14 promoter

To understand the mechanism(s) responsible for the increased transcriptional activity of the CD14/–159T allelic variant, the sequence of the CD14 promoter encompassing the SNP was analyzed for the presence of potential transcription factor binding sites (33). A putative Sp protein-binding GC box and an AP2 motif were predicted to span CD14/–159 (Fig. 1B). Therefore, EMSA analysis was performed to assess the ability of AP2 and Sp proteins to bind the C and/or T allelic variants using 22-bp oligonucleotides (22C and 22T, position −167/–146) that include both the GC box and the AP2 site. AP2 complexes were undetectable using nuclear extracts from AP2-expressing JEG-3 cells (Ref. 34 and data not shown), thus ruling out an interaction between the polymorphic region of the CD14 promoter and AP2 proteins.

Transcription factors of the Sp family activate a wide subset of mammalian genes containing GC box promoter elements (reviewed in Ref. 35). Currently, this family consists of four proteins designated Sp1–4. The GC-rich region encompassing the −159 polymorphic site (Fig. 1B) represents a potential Sp protein binding motif (36–38). Of note, the presence of a T at −159 decreases the homology between the CD14 promoter GC box and the Sp consensus sequence.

Sp protein/DNA interactions were investigated by EMSA. Fig. 3 (top) shows that incubation of a 22-bp probe containing a high-affinity binding site for Sp1 with nuclear extracts from HeLa cells that express high levels of Sp proteins (39) resulted in the formation of three specific complexes, all of which were disrupted by the addition of a 100-fold molar excess of unlabeled Sp1 oligonucleotide, but not by an unrelated STAT6 oligonucleotide. Complex I appeared to contain Sp1 and Sp2 because it was supershifted by Sp1- or Sp2-specific antisera. Complexes II and III were identified by Ab supershifts as containing two Sp3 species of different mobility (40). None of the complexes contained Sp4 as assessed by supershifting using Sp4-specific antibody (data not shown). A control antisera to Frl1 had no effect on the migration of the complexes. Notably, all three specific complexes generated by the Sp1 probe were competed by unlabeled 22C and 22T oligonucleotides, suggesting that Sp proteins may bind at, or in the vicinity of, CD14/–159.

To demonstrate more directly the interaction between Sp proteins and the CD14 promoter, EMSAs were conducted using 22C or 22T oligonucleotide probes and HeLa cell nuclear extracts. Three specific complexes, virtually identical in mobility to those detected with the Sp1 probe, were observed (Fig. 3, center and bottom). Indeed, all three complexes were competed specifically by an Sp1 oligonucleotide and contained Sp1 (Fig. 3, complex I), Sp2 (Fig. 3, complex I), and Sp3 (Fig. 3, complexes II and III), as identified by supershift assays. In addition, recombinant Sp1 protein was found to interact with the 22C and 22T as well as Sp1 probes (data not shown). Thus, our results demonstrate that the transcription factors Sp1, Sp2, and Sp3 can bind the CD14 promoter region that contains CD14/–159C→T. Importantly, the competition curves with 22C and 22T oligonucleotides provided the first evidence to suggest a difference in affinity of the two allelic variants for Sp family members.

To further dissect the impact of −159C→T on CD14 promoter/protein interactions, we performed mutational analysis to assess how different regions of the GC box contribute to Sp protein binding. Three mutants, each containing a block of five transversions, were generated on the 22C backbone (Fig. 1C) and were used as competitors in EMSA analysis. Fig. 4 shows that Mut2 (in which the inner core of the GC box had been disrupted) was severely impaired in its capacity to compete 22C probe binding to HeLa nuclear proteins. By contrast, Mut1 (which includes CD14/–159) and Mut3 retained substantial protein binding ability. We conclude that the core of the GC box in the CD14 proximal promoter is essential for Sp protein binding, whereas the polymorphic region immediately upstream may contribute to the overall affinity of Sp protein/DNA interactions.

![FIGURE 2. CD14/–159T has enhanced transcriptional activity in reporter assays. Mono Mac 6 cells were transiently transfected with −159C/luc, −159T/luc, or pGL3-Basic (20 μg). Luciferase activity was normalized for transfection efficiency using a control plasmid pRL-CMV (25 ng) and protein concentration. Results are reported as fold increase in RLA of the CD14 reporter constructs compared with the empty control vector pGL3-Basic. The figure shows the mean ± SEM of results obtained in 14 experiments. * p < 0.0001 as determined by a paired t test.](http://jimmunol.org/content/jimmunol/171/9/5840/F1.large.jpg)
transcription factors of the Sp family bind the GC box in the CD14 promoter. Nuclear extracts (5 μg) from HeLa cells were preincubated with competitors or Abs (4 μg) specific for Sp family members or Fra1 for 20 min on ice, and were then incubated with different oligonucleotide probes: Sp1 (top), 22C (center), and 22T (bottom). The EMSA blots were exposed for 16 h at room temperature (Sp1 probe), for 48 h at −80°C (22C probe), or for 60 h at −80°C (22T probe). Arrows indicate specific complexes containing Sp family proteins: Sp1 and Sp2 (complex I) and Sp3 (complexes II and III). A representative experiment of five is shown for each panel.

FIGURE 3. Transcription factors of the Sp family bind the polymorphic GC box in the CD14 promoter. Nuclear extracts (5 μg) from HeLa cells were preincubated with competitors or Abs (4 μg) specific for Sp family members or Fra1 for 20 min on ice, and were then incubated with different oligonucleotide probes: Sp1 (top), 22C (center), and 22T (bottom). The EMSA blots were exposed for 16 h at room temperature (Sp1 probe), for 48 h at −80°C (22C probe), or for 60 h at −80°C (22T probe). Arrows indicate specific complexes containing Sp family proteins: Sp1 and Sp2 (complex I) and Sp3 (complexes II and III). A representative experiment of five is shown for each panel.

Nuclear extracts from HeLa cells represented a useful tool to characterize Sp protein/CD14 promoter interactions because they contain high levels of Sp1–3. However, to correlate the functional data from transient transfections with the results provided by EMSA analysis, we needed to test nuclear extracts from the monocytic cell line used in reporter assays, Mono Mac 6. Fig. 5 (left) shows that incubation of Mono Mac 6 nuclear extracts with an Sp1 probe resulted in the formation of three specific complexes of mobility similar to that observed using HeLa nuclear extracts (see Fig. 3), even though complex III was barely detectable. All complexes were disrupted by the addition of a 100-fold excess of unlabeled Sp1 oligonucleotide, but not by an unrelated STAT6 oligonucleotide. Ab-mediated supershifting indicated that complex I contained Sp1 and Sp2 proteins. Complex II and the faint complex III were completely abrogated by anti-Sp3 Abs. When EMSA analysis was performed using Mono Mac 6 nuclear extracts and a 22C probe, a similar pattern of protein/DNA interactions was detected (Fig. 5, center).

Finally, nuclear extracts from primary peripheral blood monocytes isolated by elutriation were tested for their ability to bind the region of interest in the CD14 promoter. Fig. 5 (right) shows that the 22C probe was able to bind Sp1, Sp2, and Sp3, resulting in the formation of complexes I, II, and III. All three complexes were specifically abrogated by Sp1 as well as by 22C and 22T oligonucleotides, with competition and Ab supershift patterns similar to those obtained with HeLa and Mono Mac 6 nuclear proteins. Overall, our results confirm a similar pattern of Sp1–3/CD14 promoter interactions for HeLa cells, Mono Mac 6 cells, and freshly isolated human monocytes, except for the low expression of the high mobility form of Sp3 in Mono Mac 6 cells.

Sp proteins have enhanced affinity for the −159C variant of the CD14 promoter GC box

The finding that Sp1, Sp2, and Sp3 bound both the 22C and 22T oligonucleotides indicated that there was no qualitative difference in the ability of Sp proteins to interact with the two allelic variants of the CD14 promoter. In contrast, the 22C and 22T oligonucleotides appeared to differ in their capacity to compete for Sp protein binding (see competition lanes in Figs. 3 and 5). Therefore, we sought to assess whether the presence of a C or a T at −159 resulted in a difference in the affinity of Sp proteins for the CD14 promoter. To this purpose, nuclear extracts from HeLa cells were incubated with an Sp1 probe and an increasing molar excess of Sp1, 22C, or 22T oligonucleotide competitor. The intensity of the resulting bands was quantitated by scanning densitometry, and the percentage of binding relative to uncompeted probe was plotted against the molar excess of competitor added to each sample (Fig. 6, top). The mean ± SEM of three independent experiments is shown for each competitor in each panel. As expected, an unlabeled Sp1 motif competed Sp protein binding quite effectively even when added at a 25-fold molar excess. By contrast, 22C and 22T bound less avidly and, furthermore, markedly different affinities were consistently observed for the two oligonucleotides. Indeed, a 100-fold molar excess of 22C was required to obtain the same degree of competition detected using 22C at 50-fold molar excess. These results indicate a preference for Sp protein binding to the CD14/−159C allelic variant. Of note, a similar pattern of competition was observed using nuclear extracts from freshly isolated human monocytes (Fig. 6, center).

Functional role of the [Sp3]:[Sp1 + Sp2] ratio in the regulation of CD14 transcription

An apparent paradox emerged from our results described above: higher transcriptional activity of the CD14/−159T allele was associated with DNA/protein interactions of lower affinity. However,
our results also demonstrated that the nuclear factors binding to CD14-159C→T are members of the Sp family, which includes proteins with different functional properties. Indeed, Sp1 has been shown to function as a transcriptional activator, whereas Sp3 is characterized usually as a repressor (reviewed in Ref. 35). Therefore, we reasoned that the higher transcriptional activity of the T allele in monocytes may be due to its decreased affinity for Sp3-containing complexes with inhibitory potential. As a corollary, monocytes would be expected to express low and possibly limiting levels of Sp3 relative to Sp1 and Sp2. By the same token, an excess of Sp3 should overcome affinity-related transcriptional differences between the two CD14 alleles.

To test our hypothesis, we took advantage of the existence of another major cell type that expresses CD14 in vivo. The hepatic cell line HepG2, as well as primary hepatocytes, shows moderate but readily detectable constitutive levels of CD14 transcription (10, 41). In preliminary experiments, we used EMSA analysis to determine the relative expression of Sp family members in HepG2 cells. Fig. 7A shows that HepG2 nuclear extracts formed three major complexes with an Sp1 or 22C probe. Complex I appeared to contain Sp1 and Sp2 because it was supershifted by Sp1- and Sp2-specific antisera. Complexes II and III contained Sp3 because the bands disappeared upon addition of an Sp3-specific Ab. As expected, all complexes were specifically competed by 22C and 22T oligonucleotides, but not by an unrelated oligonucleotide (STAT6). Of note, binding studies using the two allelic variants of the CD14 promoter and HepG2 nuclear extracts again showed a marked decrease in the affinity of the T allele (Fig. 6, bottom). Even more importantly, upon densitometric analysis of the complexes formed by Sp factors, we noted that the relative intensity of the Sp3-containing bands was dramatically increased in HepG2 cell EMSAs relative to monocyteic cells. Indeed, the ratio of Sp3 (complexes II and III) to Sp1 and Sp2 (complex I) was 2:1 in HepG2 cells, whereas an inverted 1:2 ratio was observed in both Mono Mac 6 cells and freshly isolated monocytes (Fig. 7B).

Because HepG2 and Mono Mac 6 cells exhibit opposite patterns of Sp family member expression, these two cell lines represent an ideal in vivo model to test whether increased transcriptional activity of the T allele correlates with a decreased sensitivity to Sp3-dependent inhibition. We therefore compared the activities of the −159I17luc and −159C1luc reporter vectors in Mono Mac 6 and HepG2 cells. The results of these experiments are shown in Fig. 7C, and the experiments provided information that supports our hypothesis. On the one hand, the increased activity of the T allele was lost in Sp3-rich HepG2 cells when compared with Mono Mac 6 cells. Thus, an excess of Sp3 overrode the difference in affinity created by the polymorphism. In addition, the overall transcriptional activity of the CD14 reporter vectors was diminished in HepG2 cells, probably because of Sp3-mediated repression through other Sp binding motifs known to exist in the proximal CD14 promoter (26). Taken together, these findings suggest that the interplay between CD14 promoter affinity and the [Sp3]:[Sp1 + Sp2] ratio plays a critical mechanistic role in regulating CD14.

FIGURE 5. Sp proteins from Mono Mac 6 cells and primary human monocytes bind the polymorphic GC box in the CD14 promoter. Nuclear extracts from Mono Mac 6 cells (10 µg, left and center) and primary human monocytes (5 µg, right) were preincubated with competitors or Abs (4 µg) against Sp1, Sp2, Sp3, or an unrelated protein, Fra1, for 20 min on ice. Sp1 (left) and 22C (center and right) oligonucleotide probes were then added. EMSA blots were exposed overnight at −80°C (left), 5 days at −80°C (center), and overnight at −80°C (right). The arrows indicate specific complexes containing Sp family proteins: Sp1 and Sp2 (complex I) and Sp3 (complexes II and III). A representative experiment of three is shown for each panel.

FIGURE 6. Sp proteins have enhanced affinity for CD14-159C. Nuclear extracts (5 µg) from HeLa cells (top), primary human monocytes (center), and HepG2 cells (bottom) were preincubated with an increasing molar excess of competitors before addition of an Sp1 probe. EMSA blots were analyzed by densitometry, and results were expressed as percent binding relative to the uncompetited Sp1 probe. Each panel shows the mean ± SEM of densitometric measurements from three experiments.
CD14 is involved in LPS signal transduction and therefore is essential both for interfacing the innate immune system with microbial pathogens and for directing adaptive immune responses along specific T helper differentiation pathways (5). The association between naturally occurring CD14 promoter genotypes and levels of serum sCD14 recently observed by our group in vivo (18), and the concomitant association with total serum IgE detected in the same population, reiterates the potential role of CD14 as a candidate gene for asthma and allergy. Indeed, our preliminary analysis indicates that subjects homozygous for CD14/−159T are more responsive to LPS inhalation and at the same time are less prone to become atopic (T. D. LeVan, R. Rylander, and O. Michel, manuscript in preparation). In contrast, the association between the same SNP and risk for coronary artery disease (19–21) underscores the importance of CD14 in inflammatory processes.

Because of the central role of CD14 in innate immunity, the regulation of CD14 gene expression has been extensively investigated. A 227-bp region upstream of the major transcription initiation site was found to be sufficient to drive maximal reporter activity in transient transfection assays (26). This region contains binding sites for Sp1 and members of the CCAAT/enhancer-bind-

**Discussion**

More and more reports are becoming available that describe novel polymorphisms in disease-related genes, and associations between these SNPs and specific phenotypes. However, few mechanistic studies have so far addressed the functional impact that SNPs in regulatory regions have on the control of gene expression (45–48). A common theme of these studies, as well as in ours, is that, unlike conventional vectors containing artificial mutations, most reporter constructs that model naturally occurring polymorphic variants exhibit relatively modest differences in transcriptional activity when compared with their wild-type counterpart in transient transfection assays. However puzzling, this paradigm is consistent with the basic notion that these instances of genetic variation may be insufficient to cause disease when taken individually. However, they may induce significant modulation of gene transcription, and more specifically, in determining the differential activity of the two allelic variants of the CD14 promoter.

**FIGURE 7.** Analysis of CD14 transcription in HepG2 cells. A. CD14 promoter/Sp protein interactions. An Sp1 (left) or 22C (right) probe was used with nuclear extracts (5 μg) from the hepatocytic cell line, HepG2. Extracts were preincubated with competitors or Abs (4 μg) for 20 min on ice before addition of the probe. The arrows indicate specific complexes containing Sp family proteins: Sp1 and Sp2 (complex I) and Sp3 (complex II and III). B. [Sp3]:[Sp1 + Sp2] ratio in CD14-expressing cells. The ratio was determined by densitometric analysis of Sp3- and Sp1/Sp2-containing complexes in the same lane of an EMSA gel. EMSA analysis was performed as described in Materials and Methods using an Sp1 probe and 5 μg of nuclear proteins from HepG2 cells (n = 4), primary monocytes (Mφ, n = 3), and Mono Mac 6 cells (n = 4). C. Reporter analysis of CD14 promoter variants. Mono Mac 6 and HepG2 cells were transiently transfected with −159C/luc, −159T/luc, or pGL3-Basic (20 μg). Luciferase activity was normalized for transfection efficiency using a control plasmid pRL-CMV (25 ng) and protein concentration. Results are reported as fold increase in the RLA of the CD14 reporter constructs compared with the empty control vector pGL3-Basic. The figure compares the mean ± SEM of results obtained in 14 experiments with Mono Mac 6 cells (MM6) and three experiments with HepG2 cells.

Basal promoter activity was strictly dependent on the integrity of Sp1 and C/EBP motifs located at position −110 and −135, respectively. The same C/EBP site was also involved in the TGF-β/Vit D3-dependent activation of CD14 expression (42). More recently, in vivo studies with transgenic mice have shown that an 80-kb human genomic fragment that includes the CD14 coding region contains critical regulatory elements necessary to direct tissue-specific CD14 expression in monocytes and hepatocytes (9, 41). Within this fragment, a 0.7-kb enhancer located −6 kb 5′ of the CD14 transcription initiation sites appears to be essential for CD14 expression in hepatocytes, while additional upstream elements are required to direct transcription in monocytes (41).

Our present data show that a common SNP in the proximal CD14 promoter results in increased transcriptional activity. A reporter vector containing CD14/−159T was more active than the C allele variant in transient transfection assays using CD14-expressing monocytic cells. This increase in activity was paralleled by a decreased affinity of the interactions between the Sp1, 2, and 3 proteins and the polymorphic GC box in the CD14 promoter. All Sp family members contain a highly conserved DNA binding domain and bind the same consensus sequence, but show promoter context-related differences in their transactivating properties (35). It is generally believed that the function of Sp protein-dependent promoters is regulated by the relative ratio between activating and repressing members of the Sp family. In this scenario, the −159/ C→T polymorphism increases transcription by lowering the affinity of the CD14 regulatory region for Sp3, a factor known to inhibit the activity of a number of promoters (40, 43, 44). This line of reasoning is strongly supported by the comparison of the transcriptional activities of the C and T allele in two cell lines that exhibit opposite patterns of Sp1/Sp2 and Sp3 expression. Indeed, transcription of the T allele was increased in the monocytic cell line Mono Mac 6 in which Sp3 expression is low. By contrast, in Sp3-rich hepatocytic HepG2 cells, the CD14/−159C→T transcriptional difference was lost. Thus, the affinity of the promoter GC box and the ratio between activating and repressing Sp family members play a complex but critical role in regulating the transcription of the two allelic variants of the CD14 promoter.

In our population studies, levels of serum sCD14 were higher in the CD14/−159T homozygotes (18), and as described in this study, the CD14/−159T reporter construct was transcriptionally more active than the C allele variant in monocytic cells but not in hepatocytes. Yet sCD14 is known to be secreted by both monocytes (8) and hepatocytes (9, 10). Thus, it is possible that genetic variation in the CD14 proximal promoter may affect its ability to engage in long-range interactions with liver-specific, promoter-distal elements such as the enhancer situated −6 kb upstream of the CD14 transcription start site (41). These elements are not included in the reporter constructs analyzed in this study.
expression when they become involved in gene-by-gene and/or gene-by-environment interactions. Thus, complex phenotypes and/or polygenic diseases would not be expected to emerge as all-or-none events, but rather to result from a combination of small quantitative effects. This may be especially true of innate immunity, the genes for which are highly conserved across species and therefore are not likely to tolerate dramatic shifts in their functional patterns. Therefore, we envision a scenario in which a constellation of variations in critical genes nonself from noninfectious self.

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