Published on May 10, 2004 as DOI:10.1189/jlb.1203617

TGF-β regulation of human macrophage scavenger receptor CD163 is Smad3-dependent

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Abstract: Tight regulation of the inflammatory response is essential for the maintenance of physiologic homeostasis. A potentially important mediator of this process is CD163, a macrophage-specific member of the scavenger receptor cysteine-rich family. CD163 surface expression is up-regulated by glucocorticoids and the anti-inflammatory cytokine interleukin-10, and CD163 is shed acutely from the cell surface in response to lipopolysaccharide. We now demonstrate that transforming growth factor-β (TGF-β) markedly reduces expression of CD163. Treatment of primary human monocytes with TGF-β inhibited basal as well as dexamethasone-induced CD163 mRNA and protein expression. De novo protein synthesis was not required for this inhibition, suggesting that TGF-β regulates CD163 expression transcriptionally. To delineate this transcriptional regulation, a 2.5-kb fragment of the CD163 promoter was isolated. This promoter was inhibited by TGF-β, and suppression was dependent on Smad3 expression. These results define a novel function for TGF-β and implicate an important role for CD163 in the host response to inflammation. *J. Leukoc. Biol.* 76: 000–000; 2004.

Key Words: *cytokine · inflammation · glucocorticoid · signal transduction*

INTRODUCTION

Macrophages are critically involved in many inflammatory disease states, including rheumatoid athritis (reviewed in ref. [1]), Crohn's disease (reviewed in ref. [2]), and spondyloarthropathy (reviewed in ref. [3]). In these disease settings, activated macrophages elaborate a variety of cytokines, growth factors, proteolytic enzymes, and receptors that modulate tissue damage and repair. Increased expression of one of these receptors, the hemoglobin/haptoglobin scavenger CD163, has been shown to be correlated with resolution of these disease states [4–7].

Exclusively expressed on macrophages and monocytes, CD163 is a member of the scavenger receptor cysteine-rich family of glycoproteins [8, 9]. Recent data indicate that CD163 mediates the endocytosis of hemoglobin complexed with haptoglobin, suggesting that CD163 regulates cellular homeostasis by attenuating the inflammatory consequences of hemolysis [10]. High levels of CD163 "bright" macrophages are detected in healing tissues [11] and have been reported to release an incompletely characterized anti-inflammatory mediator [12]. In addition, glucocorticoids (GC) and the anti-inflammatory cytokine interleukin-10 (IL-10) induce markedly higher expression of CD163 [13, 14]. Collectively, these data suggest a functional role for CD163 as a mediator against systemic inflammation.

The pleiotropic cytokine transforming growth factor-β (TGF-β) mediates a wide variety of effects on cellular differentiation, activation, and proliferation (reviewed in refs. [15, 16]). Most cell types, including macrophages and monocytes, produce TGF-β, which regulates such diverse functions as monocyte activation, cytokine production, host defense, and chemotaxis [17–19]. Recent reports impute diverse roles to TGF-β in the regulation of inflammation. In addition to its role as a macrophage-deactivating agent, TGF-β can function as a macrophage activator, regulating cytokine production and mediating host defense [15, 20].

Signal transduction initiated by TGF-β confers specificity to these responses [21–24]. Binding of TGF-β to its cognate receptor activates the receptor kinase, which induces phosphorylation and activation of members of the Smad family of signaling molecules (reviewed in ref. [25]). Once phosphorylated, the receptor-regulated or pathway-restricted Smads (Smad2 and Smad3) associate with the common-mediator Smad (Smad4; reviewed in refs. [26–28]). The resulting heteromeric protein complex translocates to the nucleus, where it regulates expression of TGF-β target genes by directly binding to DNA and/or by interaction with other transcription factors, coactivators, or corepressors [29, 30]. The TGF-β response may also be modulated by the inhibitory Smads (Smad6 and Smad7), which interfere with the activation of the receptor-regulated Smads [31–33]. The complexity of biological responses elicited by TGF-β is a reflection of the interplay among these signaling mediators.

In this report, we identify a surprising and novel role for TGF-β as a negative regulator of CD163 expression in human macrophages. CD163 mRNA and protein levels are markedly reduced by TGF-β. Smad3 (but not Smad2) is required for TGF-β-mediated transcriptional inhibition of CD163 message. We also demonstrate that TGF-β partially attenuates the dexamethasone (Dex)-induced up-regulation of CD163 gene expres-

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Received December 5, 2003; revised March 30, 2004; accepted April 7, 2004; doi: 10.1189/jlb.1203617.

sion. These studies further advance our understanding of the molecular mechanisms, which govern CD163 gene regulation and thereby enhance our understanding of the inflammatory response.

MATERIALS AND METHODS

Isolation of human mononuclear cells and cell culture

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized whole blood by using Ficoll-Hypaque (d=1.077) [34]. Monocytes were purified from mononuclear cell fractions as described by Mentzer et al. [35]. Primary human monocytes and mouse RAW264.7 cells (obtained from American Type Culture Collection, Manassas, VA) were cultured in Hepes-buffered RPMI-1640 medium (Cellgro, by Mediatech, Herndon, VA) supplemented with 10% heat-inactivated fetal calf serum (FCS; HyClone, Logan, UT) and 20 μg/ml gentamycin sulfate (Sigma Aldrich, St. Louis, MO) at a density of 2.5×10^6 cells/ml. As indicated, cells were treated with 200 nM Dex (Steraloids, Wilton, NH) and/or 5 ng/ml TGF-β1 (PeproTech, Rocky Hill, NJ). For experiments involving the inhibition of protein synthesis, 10 μg/ml cycloheximide (CHX; Sigma Aldrich) was added to cells 2 h before treatment with TGF-β.

Preparation of subcellular fractions and immunoblotting

Cytosolic lysates were prepared using a method characterized by its lack of contamination by nuclear proteins [36]. Briefly, cells were washed twice in 1× phosphate-buffered saline (PBS) and then lysed by gentle resuspension in 1% Triton X-100 lysis buffer (50 μL/2×10^7 cells) consisting of 10 mM PIPES, pH 6.8, 100 mM KCl, 2.5 mM $MgCl_2$, and 300 mM sucrose. Samples were incubated for 3 min on ice and then centrifuged for 5 min at 500 *g*. The supernatant was aliquoted and stored at −80°C as the cytosolic fraction. The pellet was resuspended in lysis buffer and then spun through a 30% sucrose cushion. The nuclear pellet was resuspended in 0.5 nuclear pellet volume in low salt buffer consisting of 10 mM Tris-HCl, pH 7.6, 20 mM KCl, 1.5 mM $MgCl_2$, 0.5 μM dithiothreitol, 0.2 mM EDTA, 25% glycerol, 2 mM Pefabloc, and 1 μg/ml each leupeptin and pepstatin A. While vortexing, 1.5 nuclear pellet volume of high-salt buffer (containing 0.5 M KCl) was added drop-wise. Samples were incubated with agitation for 30 min at 4°C and then centrifuged for 30 min at 12,000 *g* at 4°C. The supernatant was aliquoted and stored at 80°C as the nuclear fraction. Protein content was quantified with bicinchoninic acid assay (Pierce, Rockford, IL). Total cytosolic (25 μg) and nuclear (10 μg) proteins were resolved by 7.5% or 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; as indicated) under nonreducing conditions and then electrotransferred to polyvinylidene difluoride membrane in tris-glycine buffer with 20% methanol. The membranes were washed in PBS, 0.05% Tween 20, and blocked in 5% nonfat milk overnight at 4°C. Membranes were then probed with a mouse monoclonal antibody (mAb) directed against CD163 (Mac 2-158, Maine Biotechnology Services, Portland), followed by goat anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibody (Bio-Rad, Hercules, CA). As indicated, blots were probed with anti-glyceraldehyde 3-phosphate dehydrogenase (anti-GAPDH) mAb (6C5, American Research Products, Belmont, MA), followed by goat anti-mouse HRP-conjugated secondary antibody to control for loading. Activated Smad2 and -3 were detected with a phospho-specific rabbit polyclonal anti-Smad2 antibody (Cell Signaling Technology, Beverly, MA), followed by goat anti-rabbit HRP-conjugated secondary antibody. This antibody detects endogenous levels of Smad2, only when dually phosphorylated at Ser463 and Ser465, and cross-reacts with phosphorylated Smad3 at its equivalent site. To control for loading of nuclear lysates, membranes were stripped and hybridized with rabbit polyclonal anti-cyclin-dependent kinase 2 (anti-Cdk2) antibody (M2, Santa Cruz Biotechnology, Santa Cruz, CA). Reactive antigens were visualized with Supersignal chemiluminescence substrate (Pierce).

Isolation of RNA and real-time polymerase chain reaction (PCR)

Total cellular RNA was extracted from elutriated human monocytes with the RNeasy mini kit (Qiagen, Valencia, CA). To eliminate genomic DNA contamination, all RNA samples were treated with RNase-free DNase 1 (Qiagen). RNA purity was verified by formaldehyde/agarose gel electrophoresis. First-strand cDNA was synthesized with oligo (d)T_{12-18} template primer and SuperScript II Moloney murine leukemia virus-reverse transcriptase (RT) kit (Invitrogen, San Diego, CA). cDNA (12 ng per well) was transferred into iCycler 96-well plates (Bio-Rad), and SYBR Green PCR master mix (Applied Biosystems, Foster City, CA) was added in accordance with the manufacturer's instructions. The primer pairs used for amplification of CD163 were (5′–3′) CGA GTT AAC GCC AGT AAG G and GAA CAT GTC ACG CCA GC and those for β-actin were (5′–3′) CCT ATG TGG GCG ACG AG and GCG CAG CTC ATT GTA GAA G. Amplification was performed on an iCycler with an optical unit (Bio-Rad) that permits real-time monitoring of increased PCR product concentration based on incorporation of the fluorescent double-stranded DNA dye SYBR Green. The number of cycles required to generate a detectable product above background was measured for each sample [designated as threshold cycle (C_t)]. These cycle numbers were then used to calculate the fold differences in initial mRNA levels in each reaction. This was accomplished by determining the cycle number difference for β-actin (housekeeping control) in the untreated, control sample and experimental sample. This difference was designated $\Delta C_{t\ actin}$. Likewise, the cycle number difference for CD163 expression was calculated for the untreated control sample and each experimental sample ($\Delta C_{t\ CD163}$). The cycle number difference for the gene of interest (CD163) was corrected for differences in total RNA in control and treated samples by subtracting $\Delta C_{t\ actin}$ from $\Delta C_{t\ CD163}$, yielding a new value designated ΔB. The expression ratio for CD163 was then calculated as $2^{-(\Delta B)}$. The thermal profile for SYBR PCR consisted of 5 s of initial denaturation at 95°C followed by 40 cycles of 15 s at 95°C, 45 s at 63°C, and 15 s at 72°C. Amplicon accumulation was measured during the extension phase. All reactions were performed in triplicate. Data were analyzed using the iCycler analysis software, version 2.3 (Bio-Rad), and melting curve analysis was performed to ensure the specificity of each amplification.

DNA constructs

The human CD163 promoter region from –2471 to +43 was subcloned into the pGL3 basic vector to generate CD163-luciferase (LUC). The plasminogen activator inhibitor-1 (PAI-1) luciferase construct was the gift of Harvey Lodish [37], and the Smad3 and Smad2 dominant-negative constructs, Smad3ΔC and Smad2ΔC-F, were kindly provided by Rik Derynck (described in ref. [28]).

Transient transfection and luciferase assay

RAW264.7 cells, plated 18 h before transfection at a density of 2×10^5 cells per well on 24-well tissue-culture dishes, were transfected using Lipofectamine 2000 (Invitrogen). Transfection efficiency was normalized to *Renilla* activity by cotransfection of 40 ng pRL-TK expression vector (Promega, Madison, WI). Cells were incubated with 0.8 μg reporter plasmid (CD163-LUC or PAI-LUC) and Smad3ΔC or Smad2ΔC–F, and the total amount of DNA was normalized using empty vector (pRK5). Cells were treated with TGF-β (5 ng/ml) 24 h post-transfection and harvested with passive lysis buffer (Promega) 12 h after stimulation. Luciferase activity was determined using the dual-luciferase reporter assay system (Promega), and LB9507 luminometer (EG and G Berthold). Data were analyzed in triplicate, and each transfection was repeated at least three times. Data are represented as relative light units.

Flow cytometric analysis

Monocytes or PBMC were washed with 1× PBS, bovine serum albumin (2 mg/ml), and 0.1% sodium azide and were incubated with human immunoglobulin G (IgG; 4 mg/ml; Sigma Aldrich) to block Fc receptor-specific binding to mAb and 60 μg/ml purified or biotinylated mouse IgG1 mAb Mac 2–158 (Maine Biotechnology Services) or an IgG1 isotype control (Caltag Laboratories, S. San Francisco, CA). Cells were then washed and stained with goat anti-mouse $F(ab')_2$ fluorescein isothiocyanate secondary antibody (Caltag) or streptavidin R-phycoerythrin (Caltag). After incubation with secondary antibody, cells were washed and fixed with 2% methanol-free formalin in 1× PBS, followed by analysis with a Becton Dickinson (San Jose, CA) FACSCalibur flow cytometer.

RESULTS

CD163 surface expression is markedly diminished by treatment with TGF-β

Previous studies have demonstrated that IL-6 [38], GC [13], and the anti-inflammatory cytokine IL-10 [14] up-regulate expression of CD163. In contrast, macrophages treated with interferon-γ (IFN-γ) and tumor necrosis factor α exhibit reduced CD163 levels [38]. Given the predominantly anti-inflammatory role imputed to TGF-β in the modulation of inflammation and immune function, this study was undertaken to test the hypothesis that TGF-β would enhance CD163 expression. To examine this question, human mononuclear cells were treated with TGF-β for 2, 24, and 48 h, and CD163 surface expression was analyzed by flow cytometry (**Fig. 1**). In contrast to our expectations, CD163 expression on monocytes was inhibited threefold following 24 h treatment with 5 ng/ml TGF-β and remained depressed at 48 h of culture. No effect on CD163 surface expression was observed within 2 h. This indicates that the TGF-β-mediated suppression of CD163 expression was not a result of acute shedding of surface CD163, as occurs following treatment with lipopolysaccharide (LPS) [39] or phorbol ester [40].

TGF-β suppresses CD163 mRNA and cytosolic protein levels

Given the decline in CD163 surface expression observed with TGF-β treatment, experiments were performed to elucidate the mechanism of this down-regulation. Initial studies focused on examination of mRNA expression to determine whether transcriptional suppression by TGF-β could account for the observed inhibition. Time-course studies were performed in which monocytes were treated with TGF-β for 8, 12, and 24 h, and RNA was extracted, reverse-transcribed, and analyzed by real-time PCR. As demonstrated in **Figure 2**, maximal suppression of CD163 mRNA expression was observed after 12 h of incubation with TGF-β, although CD163 mRNA levels were

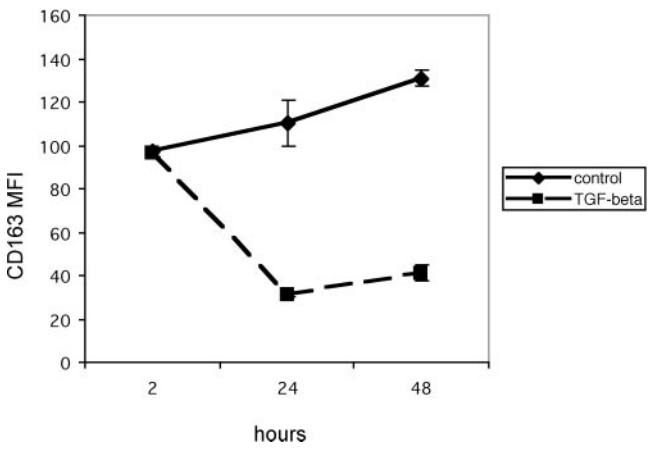

Fig. 1. Inhibition of mononuclear cell-surface CD163 by TGF-β. Mononuclear cells were cultured in the presence or absence of TGF-β (5 ng/mL) for 2, 24, or 48 h. Cells were stained for surface CD163 expression. Values are mean ± SD of triplicates. Data are representative of three experiments. MFI, Mean fluorescence intensity.

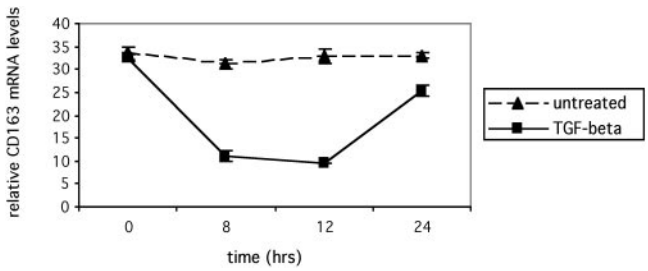

Fig. 2. Basal monocyte CD163 mRNA is inhibited by TGF-β treatment. Elutriated human monocytes were incubated with and without TGF-β (5 ng/ml) for 0–24 h as indicated. Total RNA was extracted, and RT was performed with oligo dT priming, + and − RT. Real-time PCR analysis of relative CD163 mRNA expression was performed in duplicate using CD163-specific primers and β-actin control primers. Samples incubated in the absence of RT were analyzed as well to control for genomic DNA contamination. Values are ± SD of triplicates and are representative of three experiments.

significantly inhibited by 8 h of exposure. CD163 mRNA levels of TGF-β-treated monocytes appeared to rebound but were not comparable with those observed in untreated controls at the 24-h time-point (Fig. 2). To determine if the reduction in CD163 mRNA levels were reflected in inhibited, intracellular protein expression, monocytes were subjected to the same time-course of TGF-β treatment described for examination of mRNA expression. Cytosolic lysates were then prepared and analyzed by immunoblotting. As demonstrated in **Figure 3**, CD163 protein expression was significantly reduced after 12 h and undetectable after 24 h of TGF-β treatment. These data corroborated the surface expression results described in Figure 1.

TGF-β blunts Dex-induced up-regulation of CD163 expression

We and others [13, 14] have reported increased CD163 RNA and protein synthesis in monocytes, which have been cultured with Dex. To investigate whether TGF-β influences the ability of Dex to increase CD163 RNA expression, quantitative real-time PCR analysis was performed on RNA isolated from monocytes cultured in the presence of TGF-β and Dex. As indicated in **Figure 4A**, CD163 mRNA levels in monocytes treated with

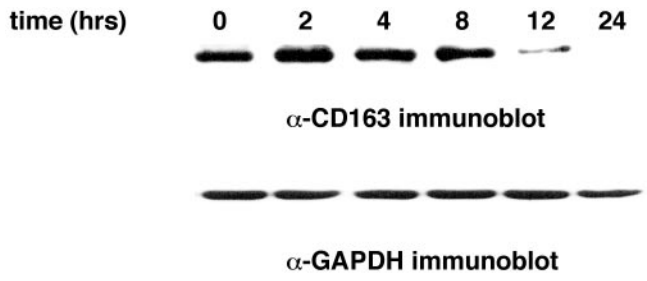

Fig. 3. TGF-β treatment decreases CD163 protein expression. Elutriated monocytes were cultured with 5 ng/ml TGF-β for times indicated in RPMI/10% FCS. Cytoplasmic lysates were prepared, and 25 μg total protein from each treatment was run on 7.5% SDS-PAGE under nonreducing conditions. Cytoplasmic lysates were assayed for CD163 and GAPDH (loading control) expression by immunoblot analysis with Mac2-158 and 6C5 antibodies, respectively. Data are representative of six experiments.

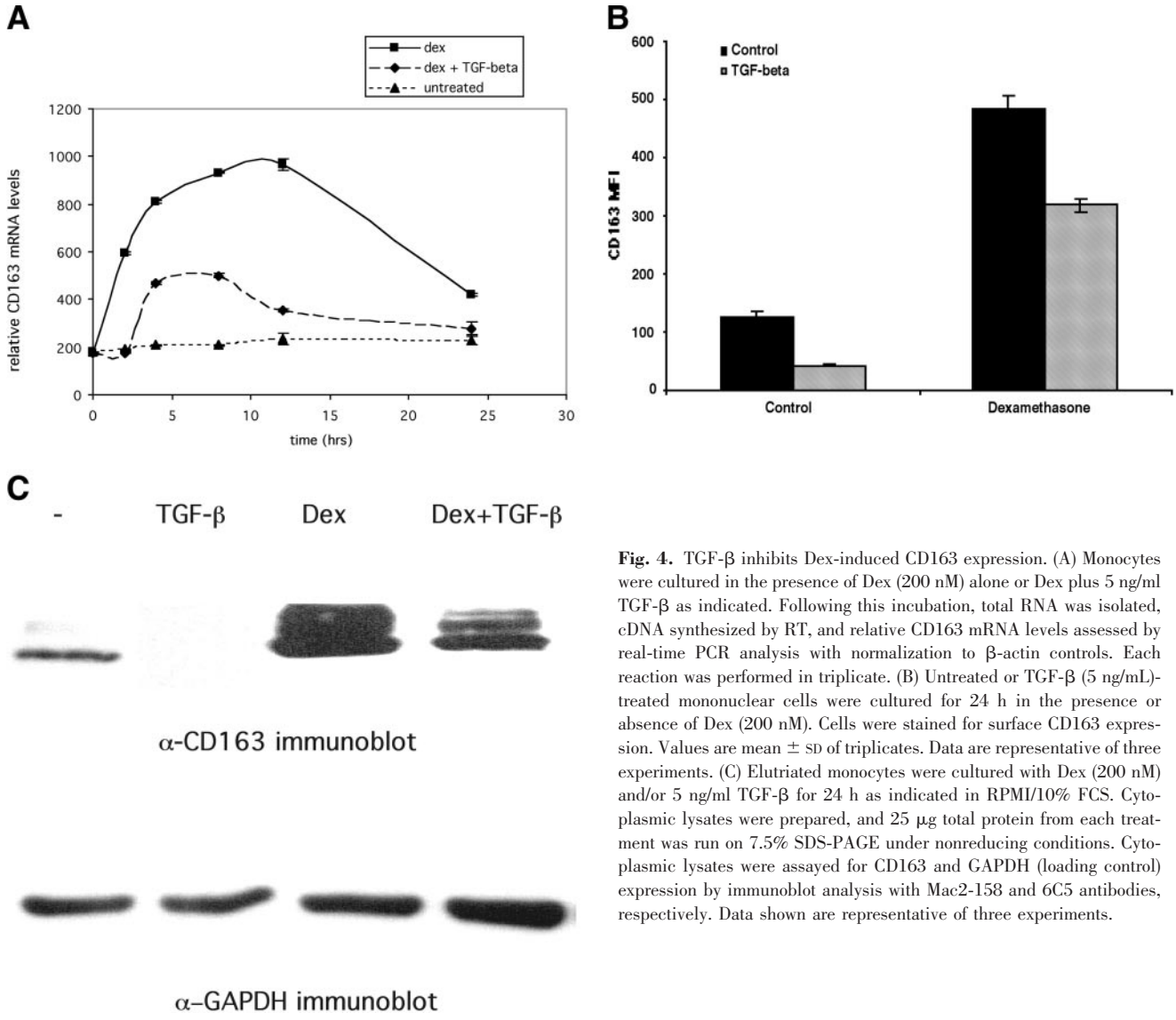

Fig. 4. TGF-β inhibits Dex-induced CD163 expression. (A) Monocytes were cultured in the presence of Dex (200 nM) alone or Dex plus 5 ng/ml TGF-β as indicated. Following this incubation, total RNA was isolated, cDNA synthesized by RT, and relative CD163 mRNA levels assessed by real-time PCR analysis with normalization to β-actin controls. Each reaction was performed in triplicate. (B) Untreated or TGF-β (5 ng/mL)-treated mononuclear cells were cultured for 24 h in the presence or absence of Dex (200 nM). Cells were stained for surface CD163 expression. Values are mean ± SD of triplicates. Data are representative of three experiments. (C) Elutriated monocytes were cultured with Dex (200 nM) and/or 5 ng/ml TGF-β for 24 h as indicated in RPMI/10% FCS. Cytoplasmic lysates were prepared, and 25 μg total protein from each treatment was run on 7.5% SDS-PAGE under nonreducing conditions. Cytoplasmic lysates were assayed for CD163 and GAPDH (loading control) expression by immunoblot analysis with Mac2-158 and 6C5 antibodies, respectively. Data shown are representative of three experiments.

Dex and TGF-β were reduced almost 70% when compared with those cultured in the presence of Dex alone. Moreover, maximal suppression of CD163 mRNA levels by TGF-β occurred at 12 h, coincident with maximal induction of this message by Dex. TGF-β also attenuated the ability of Dex to up-regulate CD163 protein synthesis. As seen in Figure 4B, flow cytometric analysis indicated that expression of surface CD163 was reduced ~40% in monocytes cultured with TGF-β and Dex when compared with those treated with Dex alone. Similar results were obtained by immunoblot analysis of cytosolic lysates (Fig. 4C).

TGF-β-mediated inhibition of CD163 expression is Smad3-dependent

To determine whether de novo protein synthesis was necessary for the suppression of CD163 expression by TGF-β, we investigated the effect of CHX on CD163 mRNA levels. Primary human monocytes were treated with TGF-β in the presence or absence of CHX (10 μg/ml, 2 h pretreatment) for 12 h. Total RNA was isolated and subjected to analysis by real-time SYBR PCR. Levels of the housekeeping gene, β-actin, remained relatively constant across the data points (**Fig. 5**). In contrast, in the presence of CHX, TGF-β treatment reduced CD163 mRNA levels, indicating new protein synthesis is not required for the inhibition of CD163 expression.

As these results imply that TGF-β directly down-regulates CD163 expression, we next investigated the mechanism responsible for mediating this inhibition. Activation of TGF-β receptors precipitates downstream signaling events regulated by the phosphorylation and subsequent nuclear translocation of Smad proteins [25–30]]. Furthermore, studies conducted by Werner et al. [41] have demonstrated (using RAW 264.7 cells) that TGF-β regulation of macrophage activation is mediated by Smad3. Based on these data, we examined the activation and localization of receptor-regulated Smad2 and Smad3 in response to TGF-β treatment. Monocytes were treated with TGF-β (5 ng/ml) for the times indicated, nuclear fractions were isolated from these cells, and the lysates were analyzed by immunoblot analysis using an antibody that binds activated Smad2 and Smad3 (**Fig. 6**). As demonstrated in Figure 6, Smad2 and -3 were rapidly (15 min) and transiently phosphorylated by TGF-β and subsequently translocated to the nucleus.

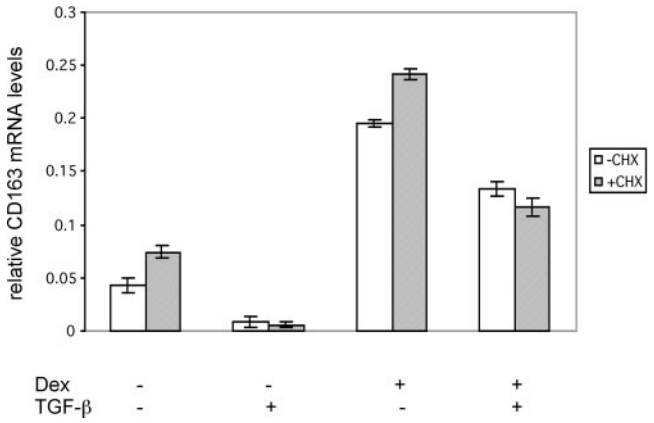

Fig. 5. Repression of CD163 mRNA expression does not require de novo protein synthesis. Elutriated monocytes were cultured with TGF-β (5 ng/ml) for 12 h and/or Dex (200 nM) in the presence or absence of CHX (10 μg/ml, 2 h before TGF-β treatment). Total RNA was isolated and subjected to real-time RT-PCR analysis. Data are expressed as light units relative to β-actin expression and are plotted ± SD of triplicates. Data are representative of three experiments.

To elucidate the signaling effectors necessary for TGF-β inhibition of CD163 gene expression, we performed transient overexpression studies using Smad3 and Smad2 dominant-negative constructs (Smad2ΔC and Smad3ΔC). These constructs each contain a C-terminal deletion, which abrogates Smad2 or Smad3 phosphorylation and activation [28]. We hypothesized that if a given Smad protein were mediating the inhibition of CD163 expression by TGF-β, then overexpression of its dominant-negative construct would mitigate this effect. To confirm proper function of our expression plasmids, we used the PAI-1 promoter as control (**Fig. 7**). As expected, luciferase activity was increased by TGF-β in cells transfected with the PAI promoter construct, and this induction was markedly reduced by cotransfection with Smad3ΔC but not by Smad2ΔC, as has been previously reported [28, 37]. As demonstrated in Figure 7, TGF-β reduced luciferase activity in cells transfected with the full-length CD163 promoter-luciferase construct. Smad3 was important in mediating this effect, as cotransfection of the Smad3 dominant-negative construct with the full-length CD163 promoter partially antagonized the inhibitory action of TGF-β. In contrast, transfection of RAW264.7 cells with Smad2ΔC did not ameliorate TGF-β inhibition of CD163-driven luciferase expression. Collectively, these data identify Smad3 as a critical downstream effector in the inhibition of CD163 expression mediated by TGF-β.

DISCUSSION

The inflammatory response is governed by the sequential release of mediators and the recruitment of circulating leukocytes, which in turn stimulate the further release of mediators. A key player in this process is TGF-β, which has been reported to possess pro- and anti-inflammatory properties, contingent on its cellular milieu (reviewed in ref. [20]). The significance of this cytokine in the regulation of inflammation is illustrated by the phenotype of TGF-β1-deficient mice. Targeted disruption of TGF-β1 in mice is characterized by the generation of an early post-natal wasting syndrome that is accompanied by a massive multifocal inflammatory cell response and tissue necrosis [42]. Levels of circulating monocytes are elevated in these animals [42], and inflammatory infiltrates include large macrophages [42]. Based on these studies, which confirm a major role of TGF-β as an in vivo immune/inflammatory suppressor, we hypothesized that TGF-β would up-regulate expression of the human hemoglobin/haptoglobin scavenger receptor CD163, a molecule associated with the resolution of inflammation [3–5]. To the contrary, we have identified a surprising and novel function of this pleiotropic cytokine as a negative regulator of CD163. Surface and intracellular levels of CD163 were suppressed in TGF-β-treated monocytes. It is intriguing that expression of the ED2 antigen, recently identified as the rat CD163 homologue, is also down-regulated in response to TGF-β in HS-P macrophage-like cells [43].

In addition to the full-length moiety, a soluble form of CD163 has been identified as an abundant component of plasma [44]. Soluble CD163 can be generated via shedding of the cell-surface protein in response to phorbol 12-myristate 13-acetate [40] and LPS [39], and elevated plasma CD163 has been associated with inflammatory conditions [44–46]. As recent reports indicate that soluble CD163 may inhibit human T lymphocyte activation and thereby facilitate suppression of the inflammatory response [47], studies were undertaken to determine whether reduced cell-surface expression in response to TGF-β was a result of shedding. It is intriguing that although surface expression of CD163 was inhibited by TGF-β, soluble CD163 was not increased (data not shown). Thus, lower surface CD163 expression of TGF-β-treated cells was not the result of shedding.

We have also shown that TGF-β dramatically attenuates Dex-induced expression of CD163. This effect of TGF-β is in distinct contrast to that of many proinflammatory cytokines, which do not affect or actually enhance GC-induced CD163 regulation [38]. For example, LPS and IFN-γ, which decrease CD163 expression when used alone, act synergistically with Dex to increase CD163 levels above those induced by Dex alone [38]. To our knowledge, this is the first demonstration that a cytokine can inhibit the up-regulation of CD163 expression by Dex. Moreover, the kinetics by which TGF-β modulates CD163 mRNA expression closely mirrors those observed with

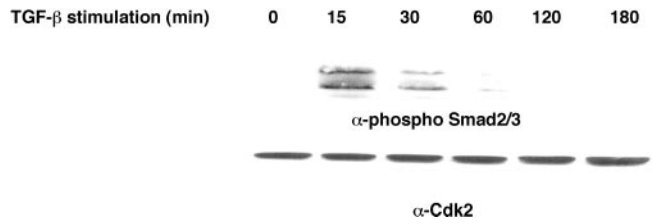

Fig. 6. Smad2 and -3 are rapidly and transiently phosphorylated by TGF-β. Primary human monocytes were treated with 5 ng/ml TGF-β for the times indicated. Nuclear (10 μg) lysates were separated on 10% SDS-PAGE and analyzed by immunoblot for expression of activated Smad2/3 with phospho-specific anti-Smad2/3 antibody. Membrane was stripped and reprobed with anti-Cdk2 polyclonal antibody to control for loading of nuclear lysates. Data are representative of six experiments.

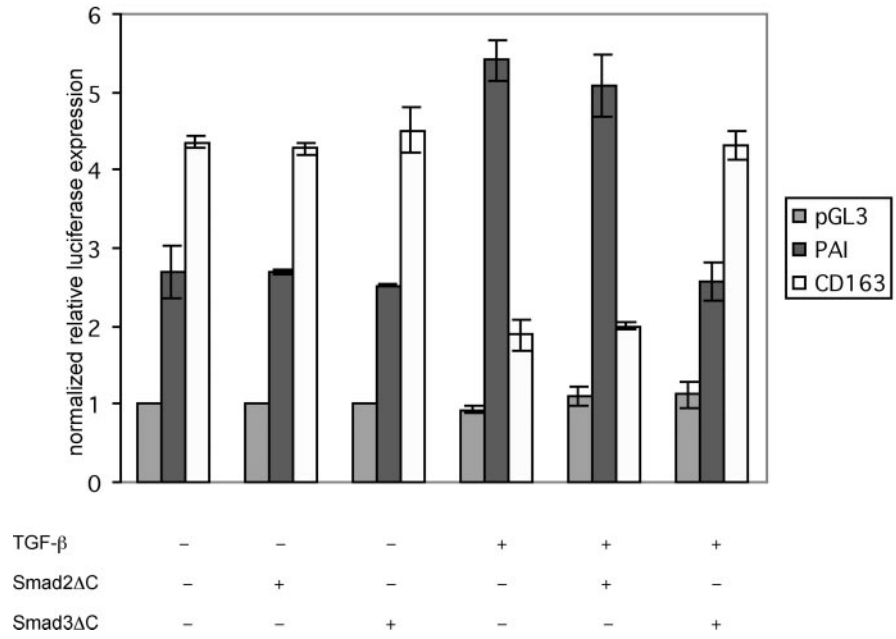

Fig. 7. Dominant-negative Smad3 inhibits TGF-β-mediated suppression of the CD163 promoter (–2471/+43), which with PAI-LUC and pGL3-LUC (empty vector control) were transiently transfected into RAW264.7 cells and were cotransfected with Smad3ΔC or Smad2ΔC as indicated. Cells were harvested for luciferase activity 12 h after treatment with TGF-β (5 ng/ml). Firefly signal was normalized to *Renilla* expression, and expression of pGL3-LUC in the absence of treatment was arbitrarily set to one. All values for PAI-LUC and CD163-LUC are graphed relative to their respective pGL3-LUC control (expression of control set to one), and data are graphed as mean ± SD. Data are representative of three experiments.

other mediators. For example, Buechler et al. [38] reported sustained inhibition of monocyte CD163 mRNA levels after 8 and 25 h of LPS treatment. In the present study, we demonstrated maximal inhibition of CD163 mRNA expression following 8 h of TGF-β treatment.

Many effector functions of TGF-β are transduced through Smads, a novel family of signaling proteins [48–50]. Following TGF-β receptor ligation, heteromeric Smad complexes translocate into the nucleus and act as transcription factors for TGF-β-responsive genes [50]. The subcellular localization of these complexes is critical for mediating transcriptional regulation. For example, oncogenic ras, through mitogen-activated protein kinase (MAPK), alters Smad2 and Smad3 phosphorylation in a manner that precludes their entry to the nucleus [51]. Notably, activated Smad2 and Smad3 were detected in our nuclear lysates, indicating that the TGF-β-induced transcriptional repression of CD163 was not the consequence of impeded nuclear translocation.

Numerous studies have documented that TGF-β-induced transcriptional activation occurs via cooperation of Smads with sequence-specific transcription factors and that Smad corepressors reduce this activation (reviewed in ref. [52]). In contrast, relatively little is known about Smad-mediated transcriptional repression. It is clear, however, that each Smad protein modulates a distinct repertoire of transcriptional responses. Therefore, despite their structural similarity, Smad2 and Smad3 are differentially required for the regulation of particular genes. For example, Smad3 has been implicated in the transcriptional repression of matrix metalloproteinase 1 (MMP-1) and MMP-12 [53, 54], and Smad2 has been identified as the mediator of E-selectin transcriptional inhibition [55]. Our findings support this unique pattern of regulation, as we demonstrate an essential role for Smad3, but not Smad2, in the inhibition of CD163 expression by TGF-β.

Transcriptional regulation of gene expression by Smad proteins is also context-dependent. In epithelial cells, TGF-β/Smad3 signaling inhibits transcription by the androgen receptor [56] and also attenuates the GC-dependent increase in haptoglobin transcription [57, 58]. In mesenchymal cells, Smad3 represses the functions of core binding factor-α 1 in osteoblastic differentiation [59] and of myogenic basic helix-loop-helix transcription factors [60]. Furthermore, Werner et al. [41] identified Smad3 as the critical effector of TGF-β-mediated inhibition of macrophage activation. It is notable that Smad3, unlike Smad2 and Smad4, is expressed in a tissue-specific manner, and the highest level of expression is detected in the spleen and thymus [61]. Moreover, the chemotactic response to TGF-β is impaired in Smad3-deficient macrophages [62]. Taken together, these observations are consistent with a role for Smad3 in immune regulation.

Smad3-mediated repression of CD163 gene expression may involve direct Smad/DNA interactions or Smad modulation of transcription factor-binding through protein–protein interactions. In this regard, our analysis of the CD163 promoter has identified two putative Smad-binding sites (CAGA boxes, at approximately –1137 and –567). Alternatively, it is possible that Smad3 engages in complex formation with positive *trans*-acting factors associated with the CD163 promoter, displacing constitutive and GC-induced transcriptional activators. This model is supported by work conducted by Yu et al. [57], in which Dex treatment of epithelial cells increased haptoglobin mRNA expression through induction of two isoforms of CAAT/enhancer-binding protein (C/EBP). Treatment of these cells with TGF-β attenuated this induction by reducing the ability of C/EBP to bind the haptoglobin promoter [57]. Binding sites for C/EBP (–53; –46) as well as activated protein 1 (AP-1; –144; –138) and nuclear factor (NF)-κB (–546) are located within the CD163 promoter. Previous studies with macrophages have suggested that activated Smad3 competes with AP-1 and NF-κB for the transcriptional coactivator p300/CBP, which has been implicated in Smad signaling and is required for NF-κB transactivation [53, 54, 63]. Under these conditions, sequestration of p300 by Smad3 would reduce p300 availability for interaction with NF-κB, resulting in repression of NF-κB-

driven transcriptional responses. Such competition may provide the molecular basis for Smad3-mediated inhibition of CD163 gene expression.

Although our experiments clearly indicate that TGF-β inhibition of CD163 expression is at least partly at the level of transcription, our results do not exclude the possible contributions of post-transcriptional and translational regulation as well. The reduced levels of CD163 mRNA observed in TGF-β-treated monocytes may also be the consequence of decreased message stability or altered rates of translation. The kinetics of the decline in CD163 message levels observed in TGF-β-treated cells is consistent with the inhibition of protein expression. Although CD163 mRNA levels appear to rebound at 24 h of treatment with TGF-β (Fig. 2), they are still below levels in untreated controls. This reduction in mRNA expression, coupled with a post-transcriptional inhibition of CD163, may fully account for the effect of TGF-β on CD163 expression. In fact, our data suggest a transcription-independent mechanism in the regulation of CD163 surface expression in untreated monocytes. As demonstrated in Figure 1 (24 and 48 h time-points), CD163 surface expression increases as a function of monocyte maturation and in vitro culture [14], yet mRNA expression of CD163 in untreated monocytes is stable (Fig. 2). Possible mechanisms of this augmented expression include increased stabilization of CD163 message, increased rates of translation, or decreased spontaneous shedding.

It is interesting that treatment of phorbol-ester-differentiated THP-1 macrophages with TGF-β decreased mRNA and surface expression of the class B scavenger receptor CD36 [64]. These studies demonstrated that inhibition of CD36 expression was mediated by TGF-β-induced stimulation of MAPK signaling pathways [64]. As CD163 is also a class B scavenger receptor, we investigated whether TGF-β might be down-regulating expression of CD163 through a similar mechanism. In contrast to results obtained with CD36, however, we were unable to determine any effect of MAPK signaling on TGF-β-mediated repression of CD163 expression (data not shown).

Our data unambiguously establish that TGF-β negatively regulates CD163 mRNA and protein expression in cultured human monocytes. Although classically characterized as an anti-inflammatory modulator, recent studies have highlighted the bidirectional effect of TGF-β on monocyte/macrophage function. In this regard, work conducted by Slavin et al. [65] has implicated a proinflammatory role for TGF-β in the mediation of wound repair. In addition, TGF-β has been shown to influence the activity of other cytokines by modulating receptor and receptor-antagonist expression. For example, TGF-β induces monocyte synthesis of IL-1, which then stimulates expression of IL-1 receptor antagonist [66, 67]. Thus, the function of TGF-β is largely context-dependent, influenced by the state of cellular differentiation, the local cytokine milieu, and circulating levels of this cytokine itself. The results of our study must therefore be interpreted in this larger context.

Moreover, it is entirely possible that the effects of TGF-β on CD163 expression are consistent with anti-inflammatory activity attributed to CD163. In this regard, the timing of the inflammatory response and its resolution must be considered. Constitutive expression of CD163 by resting monocytes is relatively low, but it is markedly up-regulated by the inflammation-associated surge in cortisol and IL-10 [45]. Conversely, resting monocytes express ~400 high-affinity type I/II TGF-β receptors, rendering them exquisitely sensitive to low levels of TGF-β [68, 69]. As levels of TGF-β increase during the inflammatory process, TGF-β receptor expression on activated monocytes and macrophages plummets, leading to a subsequent decline in sensitivity to TGF-β [20, 70]. Thus, although TGF-β may suppress CD163 expression during the early stages of inflammation, it is likely that the up-regulation of CD163 by IL-10 and GC would predominate during the resolving stages.

In summary, we have demonstrated that TGF-β mediates suppression of CD163 expression. Transcriptional regulation of CD163 by TGF-β is dependent on Smad3 signal transduction. We further show, for the first time, attenuation of Dex-induced CD163 expression. Collectively, these data implicate a role for TGF-β in the regulation of CD163 gene expression and thereby, may lead to an enhanced understanding of inflammatory control mechanisms.

ACKNOWLEDGMENTS

National Institutes of Health Grants AI051547, DK07508, and AI51877 supported this work.

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