TGF-β1 Suppresses Myeloid Fcγ Receptor Function by Regulating the Expression and Function of the Common γ-Subunit

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TGF-β1 Suppresses Myeloid Fcγ Receptor Function by Regulating the Expression and Function of the Common γ-Subunit

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We have previously reported that FcγR-mediated function in myeloid cells is a tightly regulated event that is influenced by the cytokines present in the milieu. TGF-β1 is an immunosuppressive cytokine with pleiotropic effects on immune responses; however, the molecular mechanism by which TGF-β suppresses immune responses is poorly understood. In this study, we have analyzed the effect of TGF-β on FcγR-mediated activation of myeloid cells. We report that TGF-β1-treated THP-1 human myeloid cells displayed reduced ability to phagocytose IgG-coated particles. Because FcγR expression is modulated by cytokines, we analyzed expression levels of FcγRI, FcγRIIa, FcγRIIb, and FcγRIIIa in cells cultured with or without TGF-β1 and found that total protein levels of the FcγR were not reduced, surface expression of FcγRI and FcγRII was lower in cells cultured with TGF-β1. Concomitantly, there was a dose-dependent reduction in the expression of the FcγR-associated γ-subunit. This suppressive effect of TGF-β1 was likewise observed in bone marrow-derived murine myeloid cells and human monocytes. Importantly, TGF-β1 also significantly reduced the production of monocyte chemoattractant protein-1 induced by immobilized IgG, which would further reduce monocyte recruitment to the site of inflammation. In contrast, human alveolar macrophages were refractory to this effect, expressing low levels of TGF-β type II receptors compared with peripheral blood monocytes from the same donor. These data provide insight into the regulation of immune responses by TGF-β1 and demonstrate the selectivity of these effects. The Journal of Immunology, 2003, 170: 4572–4577.
γ-subunit is suppressed in a dose-dependent manner. Third, the suppressive effect of TGF-β on the γ-subunit and the signaling properties of the associated FcyR are suppressed both in murine bone marrow-derived myeloid cells and in human peripheral blood monocytes (PBM). TGF-β1 also substantially reduced monocyte chemotactant protein-1 (MCP-1) production from monocytes stimulated with immobilized IgG, suggesting a mechanism used by TGF-β1 to reduce leukocyte recruitment to the lung. Finally, the suppressive effect of TGF-β1 was not seen in human alveolar macropages derived from the same donors. Further analysis of this dichotomy in TGF-β1 effect revealed that alveolar macrophages express very low levels of TGF-β type II receptors in comparison with PBM from the same donor, indicating that the effect of TGF-β is specific, based on cellular context.

Materials and Methods

Cells, Abs, and reagents

THP-1 cells were obtained from American Type Culture Collection (Manassas, VA) and maintained in RPMI supplemented with 10% FBS. Murine bone marrow-derived myeloid cells immortalized by retroviral infection were a kind gift from B. G. Neel (Harvard Medical School, Boston, MA) (15). Murine myeloid cells were maintained in DMEM supplemented with 10% FBS and 20% L cell-conditioned medium. Fab(1/2) of anti-FcγRII Abs 32.2, anti-FcγRIIIa Ab IV-3, and anti-FcγRIIIb Abs 3G8 were obtained from Medarex (Annandale, NJ). Rabbit polyclonal Abs specific for FcγRI, FcγRIIa, and FcγRIIb have been described elsewhere (16, 17). The anti-γ-subunit mAb 4DB was a generous gift from J. Kochan (Hoffmann-La-Roche, Nutley, NJ). The rabbit polyclonal anti-γ-subunit Ab and the anti-TGF-β type II receptor Ab were obtained from Upstate Biotechnology (Lake Placid, NY). Anti-phosphotyrosine and anti-phosphoAkt Abs were obtained from Cell Signaling Technology (Beverly, MA). Anti-β-actin Ab was from Sigma-Aldrich (St. Louis, MO).

Isolation of PBM

Monocytes were isolated from heparinized blood of volunteers, as previously described (18). Polymyxin B (2 ng/ml) was used in PBS and RPMI unless specified to protect against LPS contamination. Briefly, monocytes were enriched by diluting buffy coats 1/1 with sterile PBS and then layering onto 15 ml of Histopaque. Cells were centrifuged at 18°C for 20 min without braking at 4000 rpm (800 × g). Mononuclear (interface layer) cells were pooled into larger tubes, washed twice with fresh endotoxin-free RPMI, and centrifuged at 4°C at 1400 rpm (400 × g) for 10 min. Cells were then resuspended in RPMI containing 10% FBS at a density of 5 × 10^6 cells/ml in polypropylene tubes. Tubes were gently rocked at 4°C for 1 h to promote clumping of monocytes. After 1 h, cells were carefully layered 1/1 over 100% FBS for 15 min on ice, allowing the monocytes to sink to the bottom of the tubes. Cells were collected from the bottom of the tubes washed in FBS-free RPMI. Using light microscopy and Diff-Quick (Dade Diagnostics, Aquada, Puerto Rico) staining, there was an average of 67% monocytes, 32% lymphocytes, and <1% neutrophils in the preparations. Cell viability was >98%, as determined by trypan blue exclusion.

Isolation of CD14-positive PBM

For analysis of TGF-β type II receptor expression, CD14-positive monocytes were used. PBMcs were first isolated by density-gradient centrifugation over Histopaque (Sigma-Aldrich). Monocytes were then purified from the PBMcs by negative selection using the MACS Monocyte Isolation Kit (Miltenyi Biotec, Auburn, CA). Briefly, PBMcs were first treated with FcR blocking reagent (human IgG [hiG]), followed by a hapten-Ab cocktail (cocktail of hapten-conjugated CD3, CD7, CD19, CD45RA, CD56, and anti-IgE mAbs). The labeled cells were further treated with MACS magnetic microbeads that were conjugated to a anti-hapten Ab. The cells were then passed over a MACS column, and the effluent was collected as the negative fraction representing enriched monocytes. The monocytes thus purified were subsequently analyzed for purity by double labeling with CD14 PE and CD45 FITC Abs, followed by flow cytometry. Data from 10,000 cells indicated that the isolated monocytes were >97% CD14 positive. Cell viability was greater than 98%, as determined by trypan blue exclusion.

Preparation of human alveolar macrophages

Macrophages were obtained from healthy donors by bronchoalveolar lavage after signing an Institutional Review Board-approved consent. Cells were washed twice with PBS and counted and analyzed by Diff-Quick staining for purity. Cell preparations were >95% positive for macrophages. The cells were resuspended in RPMI 1640 medium with 5% FBS at 5 × 10^6 cells/ml. Cell viability was greater than 98%, as determined by trypan blue exclusion.

Cell lysis, immunoprecipitation, and immunoblotting

For immunoprecipitations, 10^7 cells/sample were lysed (25 mM HEPES, pH 7.5, 20 mM Na_2HPO_4, 100 mM NaF, 4 mM EDTA, 1% Triton X-100, 5 mM Na_2VO_4, 10 μg/ml aprotinin and leupeptin, 2 mM PMSF) and incubated overnight with Ab of interest and protein G-agarose beads or Sepharose beads coupled to F(ab′)_2 of goat anti-mouse IgG Abs for FcγR immunoprecipitations with F(ab′)_2. Abs. The immune complexes were separated by SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted with the Ab of interest and developed by ECL. For whole cell lysates (WCL), protein content was normalized between samples. 15 μg of protein was loaded in each lane, and immunoblotting was performed, as described above. The ECL signal was quantitated using a scanner and a densitometry program (Scion Image; Scion, Frederick, MD), as described previously (19). To quantitate the phosphotyrosine signal in the activated samples, we first subtracted background, normalized the signal to the amount of precipitated protein, and plotted the values obtained as fold increase of phosphotyrosine signal in activated samples over resting samples.

Preparation of IgG-coated SRBCs

SRBCs (Colorado Serum, Denver, CO) were washed in PBS and labeled overnight with PKH-26 Red, as per the manufacturer’s protocol, washed in PBS, and incubated with a subagglutinating dose of rabbit anti-SRBC IgG (Diamedix, Miami, FL) at 37°C for 1 h. Unbound IgG was removed by washing the cells with PBS.

Phagocytosis assay

Phagocytosis assays were performed, as previously described (20). Briefly, THP-1 cells or PBM cultured overnight with or without TGF-β were incubated with the SRBC described above. To measure phagocytic index (the number of SRBC ingested by three or more SRBC), samples were incubated for 1 h at 4°C. In parallel, monocyte cells were also incubated with SRBC that had not been opsonized with IgG. No phagocytosis or rosetting activity was seen in the latter samples.

Flow cytometry analysis

To analyze surface expression of FcγR, cells cultured with or without TGF-β were labeled with F(ab′)_2 of mAb 32.2 that specifically recognizes FcγRI, F(ab′)_2 of mAb 3G8 that recognizes FcγRIIa, or Fab of the anti-FcγRIIa mAb IV.3, followed by a FITC-conjugated secondary Ab. Receptor levels were analyzed by flow cytometry and expressed graphically as percentage of mean fluorescence intensity (MFI) of the TGF-β-treated cells in comparison with untreated cells.

For experiments analyzing TGF-β type II receptor surface expression, cells were labeled with either TGF-β type II receptor Ab or normal rabbit IgG, followed by FITC-labeled secondary Ab. To ensure specific binding of anti-receptor Ab, FcR on the cells were blocked with 100 μg/ml hIgG.

ELISA determination of human MCP-1 production from stimulated PBM

PBM isolated as described above were counted, washed, and resuspended to 5 × 10^6 cells/ml for all cytokine experiments. Before cell culture in flat-bottom plates, cells were treated with or without 5 ng/ml active TGF-β for 2 h at 37°C before culture on flat-bottom plates, as described below. A total of 1.25 × 10^5 cells in 250-μl replicates was cultured on sterile flat-bottom Immulon IV plates (Fisher, Pittsburgh, PA) coated with either PBS alone, endotoxin-free intact hIg at 100 μg/ml, or F(ab′)_2, of hIgG at 100 μg/ml. After 18 h in culture, supernatants of replicates were harvested in a sterile fashion, centrifuged to remove dead cells, and analyzed by ELISA using a human MCP-1 OPT-EIA detection kit (BD Pharmingen, San Diego, CA). Supernatants were thawed, and dilutions were made in assay diluent (BD Pharmingen) in accordance with recommendations of the OPT-EIA ELISA system. Immulon IV flat-bottom ELISA plates were pre-coated with specific capture Ab against human MCP-1 using recommended dilutions. Human rMCP-1 was titrated on each plate for standardization.
following kit instructions. Subsequent washing, detection, and developing steps were accomplished following specific kit instructions. After 20 min of developing in the dark and stopping with 50 µl 2 N H2SO4, plates were read at 450 nm for quantification of MCP-1 production.

Results

TGF-β1 reduces phagocytic efficiency of THP-1 cells

To assess the influence of TGF-β1 on FcγR-mediated phagocytosis, THP-1 cells were cultured overnight with or without 5 ng/ml TGF-β1. The cells were then incubated for 1 h with IgG-coated SRBC either at 4°C to assess binding ability, or at 37°C to measure phagocytosis. A total of 200 cells was analyzed in each experiment. We found that the phagocytic ability of cells cultured with TGF-β1 was significantly diminished (Fig. 1A) in comparison with the phagocytic ability of cells that were cultured in the absence of TGF-β1. Interestingly, THP-1 cells cultured in the presence or absence of TGF-β1 were similar in their ability to bind IgG-coated SRBC (Fig. 1B). These findings suggest that the FcγR-mediated signaling ability of THP-1 cells is diminished by TGF-β1.

TGF-β1 does not influence FcγR protein expression in THP-1 cells

We and others have demonstrated that anti-inflammatory cytokines such as IL-4 diminished phagocytosis of IgG-containing immune complexes by altering the expression levels of FcγR (19, 21). Specifically, the expression of the inhibitory receptor FcγRIIb was significantly increased in monocytes cultured with IL-4. Based on these observations, we next asked whether the suppressive effect of TGF-β1 on phagocytic efficiency could be explained by altered expression of FcγR. Our prediction was that TGF-β1-treated cells would either express enhanced levels of the inhibitory FcγRIIb, or decreased levels of activating FcγR. Thus, we analyzed by Western blotting the protein expression of FcγRIb, FcγRIIA, FcγRI, and FcγRIII in THP-1 cells cultured with or without TGF-β1. Results indicated that total cellular protein levels of the inhibitory FcγRIIb were not significantly altered by the presence of TGF-β1 (Fig. 2A). Likewise, there was no decrease in the expression levels of the activating FcγR, FcγRI (Fig. 2C), FcγRIIA (Fig. 2B), or FcγRIIIa (Fig. 2D). All of these receptors are glycosylated molecules, which accounts for the diffuse nature of the bands seen in the figure.

TGF-β1 suppresses the common γ-subunit expression in THP-1 cells

To test whether the FcγR signaling components were intact in TGF-β1-treated cells, we then analyzed expression levels of the common γ-subunit, which is critical for FcγRI and FcγRIIa signaling. Thus, we analyzed, by Western blotting, the protein expression of the γ-subunit in THP-1 cells cultured overnight with or without TGF-β1. As seen in Fig. 3A, γ-subunit expression was decreased by TGF-β1 in a dose-dependent manner. We next asked whether the decreased expression of γ-subunit was accompanied by a decrease in the surface expression of FcγR because earlier reports indicated that the γ-subunit is critical for surface expression of both FcγRI and FcγRIIa (11). For these experiments, THP-1 cells cultured with or without TGF-β1 were labeled with F(ab’), of mAb 32.2 that specifically recognizes FcγRI, mAb 3G8 that recognized FcγRIIa, or the anti-FcγRIIa mAb IV.3, followed by a FITC-conjugated secondary Ab. Receptor levels were analyzed by flow cytometry and expressed graphically as percentage of MFI of the TGF-β1-treated cells in comparison with untreated cells. Results shown in Fig. 3B indicate that surface expression of FcγRIIa was not significantly influenced by TGF-β1. However, there was a significant decrease in the surface expression of FcγRI and FcγRIII in THP-1 cells cultured in the presence of TGF-β1. These results suggest that the suppressive effect on TGF-β1 on FcγR-mediated phagocytosis is due to a decrease in the expression of the γ-subunit.
TGF-β suppresses γ-subunit expression and FcγR-mediated signaling events in murine myeloid cells

To test whether the suppressive effect of TGF-β1 on the γ-subunit expression in human myeloid cell line could be extended to murine cells, we assessed TGF-β1 effects in murine bone marrow-derived myeloid cell line. In this study, the murine myeloid cells were cultured overnight with and without TGF-β1, and the following analyses were performed. First, we analyzed the influence of TGF-β on γ-subunit expression by Western blotting. As indicated in Fig. 4A, murine myeloid cells, much like THP-1 cells, displayed decreased levels of γ-subunit upon TGF-β treatment. Second, we analyzed the signaling events initiated by clustering FcγR with the mAb 2.4G2 that clusters both FcγRIIa and FcγRIII. Results indicated that concomitant with a decrease in γ-subunit expression, cellular signaling events were also decreased in cells cultured with TGF-β. Specifically, overall tyrosine phosphorylation induced by FcγR clustering was diminished in TGF-β-treated cells (Fig. 4B). Likewise, phosphorylation of the survival factor Akt was also diminished in cells cultured with TGF-β1 (Fig. 4C). These results suggest that TGF-β suppresses FcγR-mediated signaling events by suppressing the expression of the γ-subunit in murine myeloid cells.

TGF-β1 suppresses γ-subunit expression in human PBM, but not in human alveolar macrophages

To ensure that the effect of TGF-β1 on the expression of γ-subunit was not limited to transformed cell lines, similar experiments were performed in primary cells. Thus, human PBM and human alveolar macrophages obtained from the same volunteers were cultured overnight with or without TGF-β1. Expression level of the γ-subunit was assessed by Western blotting WCL from the above cells normalized for protein content. As seen in Fig. 5A (upper panel), PBM cultured in the presence of TGF-β1 displayed decreased expression of the γ-subunit. The lower panel (Fig. 5A) is a Western blot of the same membrane with Ab to β-actin to demonstrate equal loading of protein in both lanes. Surprisingly, TGF-β1 did not have a suppressive effect on γ-subunit expression in human alveolar macrophages (Fig. 5B, upper panel). Likewise, there was no change in the surface expression of the FcγR (data not shown). Further analysis by Western blotting using anti-TGF-β type II receptor Abs revealed that alveolar macrophages, unlike PBM from the same donor, express very little TGF-β type II receptors (Fig. 5C). Analyzing surface-expressed TGF-β type II receptors by flow cytometry, we were not able to detect any expression on alveolar macrophages (Fig. 5D). These results suggest that the effects of TGF-β1 are cell specific.

TGF-β suppresses FcγR-mediated MCP-1 production in human PBM

We next assessed the functional capacity of human PBM cultured with or without TGF-β1. First, we compared FcγR-mediated production of MCP-1 in PBM cultured overnight on hIgG-coated plates either in the presence or absence of 5 ng/ml TGF-β1. As controls, PBM were also cultured in plates coated with F(ab′)2 of hIgG or in noncoated plates. Cell supernatants were collected 24 h later and analyzed by ELISA for the amounts of MCP-1 produced. PBM cultured in IgG-coated plates, but not those cultured in noncoated plates or plates coated with F(ab′)2 of hIgG, produced MCP-1, which was significantly reduced by TGF-β1 (60–80% reduction; p value 0.01) (Fig. 6). Likewise, PBM cultured in the presence of TGF-β1 displayed reduced phagocytic ability (25–30%) in comparison with PBM cultured in the absence of TGF-β1 (data not shown). These results indicate that TGF-β suppresses PBM responses to immune complexes and can reduce the production of chemokines that may recruit additional monocytes.

Discussion

In this study, we have analyzed the influence of TGF-β1 on expression and function of FcγR in human monocytes. Contrary to our initial hypothesis that the suppressive effect of TGF-β1 on FcγR-mediated functions would be due to altered FcγR expression, we found that TGF-β1 reduced protein expression of the common γ-subunit. This finding has potentially far-reaching implications because the γ-subunit is required for the proper surface expression and functioning of a number of immune receptors, including the IgE receptor FcεRI (22, 23), TCR (24–27), FcαR (28), and the platelet receptor gpVI (29). Although this suppressive effect of TGF-β1 on the γ-subunit provides an explanation for the pleiotropic effects of TGF-β, surprisingly, we found that alveolar macrophages could remain refractory to TGF-β1 effects by expressing low levels of TGF-β type II receptors. These results indicate that the effects of TGF-β are specific and depend on cellular context. Indeed, Wahl and colleague (30) previously reported that in vitro differentiation of PBM with γ-IFN or LPS results in the gradual loss of expression of TGF-β type II receptors.

The data presented in this study demonstrate that the suppressive effect of γ-subunit can be extended to murine myeloid cells as well. Preliminary experiments to identify similar effects in human NK cells, which are reported to display reduced Ab-dependent cellular cytotoxicity in response to TGF-β1, revealed that while the suppression of γ-subunit could be observed in one of the three donor samples tested, surface expression of FcγRIII remained unchanged (data not shown), perhaps due to the presence of the...
Further investigation is needed to resolve these issues. Likewise, the exact mechanism by which TGF-β suppresses γ-subunit expression is not clear. Studies are underway in our laboratory to determine the latter.

The significance of the γ-subunit is perhaps most effectively demonstrated in genetically altered mice that lack expression of the γ-subunit. Ravetch and colleagues (11) elegantly demonstrated

**FIGURE 4.** TGF-β suppresses γ-subunit expression and function in murine myeloid cells. Murine myeloid were cultured overnight with or without 20 ng/ml of TGF-β1. A, γ-subunit expression was assessed by immunoblotting protein-matched WCL with rabbit polyclonal anti-γ-subunit Ab. The numbers below the panel indicate band intensity. R, WCL from resting (R) cells and cells activated for 5' by clustering FcγRIII/II with mAb 2.4G2 and mouse anti-rat IgG secondary Ab were analyzed for overall tyrosine phosphorylation by immunoblotting with anti-phosphotyrosine Ab. C, WCL from resting and activated cells, as described above, were analyzed for Akt activation by immunoblotting with anti-phosphoAkt Ab (upper panel). The same membrane was reprobed with anti-Akt Ab (middle panel). Lower panel, Graphical representation of Akt phosphorylation, as determined by densitometry analysis of band intensities in the Western blots above. To quantitate phosphotyrosine signal in the activated samples, we first subtracted background, normalized the signal (upper panel) to the amount of precipitated protein (lower panel), and plotted the values obtained as fold increase of phosphotyrosine signal in activated samples over resting samples. These results are representative of three independent experiments.

**FIGURE 5.** TGF-β1 suppresses γ-subunit expression in human PBM, but not in alveolar macrophages from the same donor. PBM (A) and alveolar macrophages (B) were cultured overnight with or without 5 ng/ml TGF-β1. Protein-matched lysates were analyzed by immunoblotting for the expression of the γ-subunit (upper panels) and β-actin (lower panels). C, Protein-matched lysates of PBM and alveolar macrophages were analyzed by Western blotting for TGF-β type II receptors (upper panel) and β-actin (lower panel). D, PBM and alveolar macrophages were analyzed for TGF-β type II receptor expression by flow cytometry. Shaded histograms represent labeling with control Ab and clear histograms with TGF-β type II receptor Ab. These results are representative of three independent experiments.

**FIGURE 6.** TGF-β1 down-regulates FcγR-mediated production of MCP-1. Human PBM were cultured with or without 5 ng/ml TGF-β1 in plates that were either not coated or plates coated with F(ab')2 of hlgG or intact hlgG. Cell supernatants were analyzed by ELISA for MCP-1. The amount of MCP-1 produced by untreated PBM cultured on IgG-coated plates was set as 100%. The graph represents the mean and SD of three independent experiments. The p value for percentage of MCP-1 production by untreated vs TGF-β1-treated PBM, plated on intact hlgG, was 0.01. Statistical analysis was performed with a paired, two-tailed Student’s t test.
that γ-subunit-deficient animals are severely impaired in their ability to respond to immune complexes. These animals display attenuated ADCC compared with wild-type animals. Likewise, mast cell response to IgE Ag is lost in γ-subunit-deficient animals. In experiments presented in this work, we found that monocyte FcγR-mediated functions were impaired, concomitant with a decrease in γ-subunit expression, in cells cultured in the presence of TGF-β1. The suppressive effect on MCP-1 production was much more pronounced than the effect on phagocytosis. This dichotomy in the suppressive effect of TGF-β1 on these two functions can perhaps be explained by the fact that while phagocytosis proceeds only a partial effect on phagocytosis because Fcγ subunit of the IgE receptor.

Of note, reduced MCP-1 production would lead to reduced recruitment of circulating monocytes to an area of inflammation in the lung and would thus reduce monocyte-derived inflammatory cytokines. In the lung, which is recognized to be an immune-suppressive environment, this mechanism may further reduce the susceptibility to monocyte-derived inflammation in response to inhaled Ags. Although reduced production of MCP-1 may protect the lung from monocyte-derived inflammation, it is also important to note that alveolar macrophages maintain their ability to respond immunologically due to reduced expression of TGF-β1. Thus, the activity of γ-subunit has only a partial effect on phagocytosis because FcγRIIA does not rely on the γ-subunit for signaling.

In summary, our data suggest that TGF-β regulates inflammatory cytokine production and responsiveness to immobilized IgG through direct effects on γ-chain expression. This observation may enable modulation of immune responsiveness in inflammatory diseases.

References