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Efficient Clearance of Early Apoptotic Cells by Human Macrophages Requires M2c Polarization and MerTK Induction

Gaetano Zizzo,*,† Brendan A. Hilliard,*,† Marc Monestier,†,‡ and Philip L. Cohen*,†,‡

Mer tyrosine kinase (MerTK) is a major macrophage apoptotic cell (AC) receptor. Its functional impairment promotes autoimmunity and atherosclerosis, whereas overexpression correlates with poor prognosis in cancer. However, little is known about mechanisms regulating MerTK expression in humans. We found that MerTK expression is heterogenous among macrophage subsets, being mostly restricted to anti-inflammatory M2c ($CD14^+CD16^+CD163^+CD204^+CD206^+CD209^-$) cells, differentiated by M-CSF or glucocorticoids. Small numbers of $MerTK^+$ "M2c-like" cells are also detectable among circulating $CD14^{bright}CD16^+$ monocytes. MerTK expression levels adapt to changing immunologic environment, being suppressed in M1 and M2a macrophages and in dendritic cells. Remarkably, although glucocorticoid-induced differentiation is IL-10 independent, M-CSF–driven M2c polarization and related MerTK upregulation require IL-10. However, neither IL-10 alone nor TGF-β are sufficient to fully differentiate M2c ($CD16^+CD163^+MerTK^+$) macrophages. M-CSF and IL-10, both released by T lymphocytes, may thus be required together to promote regulatory T cell–mediated induction of anti-inflammatory monocytes-macrophages. MerTK enables M2c macrophages to clear early ACs more efficiently than other macrophage subsets, and it mediates AC clearance by $CD14^{bright}CD16^+$ monocytes. Moreover, M2c cells release Gas6, which in turn amplifies IL-10 secretion via MerTK. IL-10–dependent induction of the Gas6/MerTK pathway may, therefore, constitute a positive loop for M2c macrophage homeostasis and a critical checkpoint for maintenance of anti-inflammatory conditions. Our findings give new insight into human macrophage polarization and favor a central role for MerTK in regulation of macrophage functions. Eliciting M2c polarization can have therapeutic utility for diseases such as lupus, in which a defective AC clearance contributes to initiate and perpetuate the pathological process. *The Journal of Immunology*, **2012, 189: 3508–3520.**

The prompt recognition and removal of dead and dying cells is critical for maintenance of immunologic tolerance and resolution of inflammation. Physiologic mechanisms of apoptotic cell (AC) clearance typically associate with induction of regulatory pathways in phagocytes and release of anti-inflammatory cytokines (1, 2). Such mechanisms have attracted increasing interest over the last decade, and many distinct molecular pathways have been identified. Although there seems to be conspicuous redundancy among these pathways, they differ from each other in regard to several features. These features include dependence on specific nuclear transcription factors (3–6), expression under basal conditions versus inducibility by excess numbers of ACs (5–9), recognition of unmodified versus modified phosphatidylserine on ACs, direct AC recognition versus use of bridging molecules, and, importantly, clearance of early versus late (secondarily necrotic) ACs (9, 10).

Mer tyrosine kinase (MerTK), a member of the TAM (Tyro3, Axl, Mer) subfamily of receptors, is specifically involved in removal of early ACs and recognizes unmodified phosphatidylserine through the bridging molecules protein S and Gas6 (9-12). MerTK is expressed on phagocytes following exposure to ACs and subsequent LXR receptor activation (5). Its functional impairment causes defective AC clearance in the presence of excess ACs (7–9). Furthermore, other critical AC pathways are closely linked with MerTK activity (13–15).

MerTK is expressed in primary and secondary lymphoid organs (7, 8) and is key for maintenance of both central and peripheral tolerance through multiple mechanisms: removal of AC-derived potential autoantigens (8, 16), inhibition of TLR-induced production of proinflammatory cytokines (17–19), and prevention of autoreactive B and T cell expansion (16, 20). The loss of these functions results in lupus-like autoimmunity in MerTK-deficient or LXR-deficient mice (4, 8). In lupus patients, impaired AC clearance is believed to cause the persistence of ACs in various tissues, including lymphoid organs. This may promote the production of autoantibodies against apoptotic material and may lead to delayed and proinflammatory clearance of secondary necrotic cells mediated by autoantibodies (21, 22). In these patients, we recently reported a reduction in plasma levels of the MerTK ligand protein S (23), which may account for a functional defect in AC clearance. MerTK has also a protective role in atherosclerosis, where it enables discrete macrophages to phagocytose cholesterol-laden apoptotic macrophages, thereby preventing secondary necrosis, inflammation, and plaque instability (24). Moreover, MerTK inhibits cholesterol uptake from macrophages themselves (25). In contrast, immunomodulation, cell viability, and resistance to apoptosis promoted by MerTK are

*Section of Rheumatology, Department of Medicine, Temple University, Philadelphia, PA 19140; †Temple Autoimmunity Center, Temple University, Philadelphia, PA 19140; and ‡Department of Microbiology and Immunology, Temple University, Philadelphia, PA 19140

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Address correspondence and reprint requests to Dr. Philip L. Cohen, Section of Rheumatology, Department of Medicine, Temple University, 3322 N. Broad Street, Philadelphia, PA 19140-5185. E-mail address: philco@temple.edu

Abbreviations used in this article: AC, apoptotic cell; DC, dendritic cell; MerTK, Mer tyrosine kinase; MFI, mean fluorescence intensity; Treg, regulatory T cell.

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detrimental in cancer (26), whereby the ligand Gas6, secreted by tumor-associated macrophages, promotes tumor growth and metastasis (27).

Therefore, modulating MerTK activity could be a promising therapeutic approach to various pathologic conditions. Surprisingly, little is known about the mechanisms promoting MerTK expression in humans. Both MerTK and protein S can be upregulated by steroids (28–30), and protein S/MerTK-mediated AC clearance is enhanced by these drugs (12). However, steroids induce many other molecules involved in AC clearance (29, 30). It is, therefore, unclear whether MerTK upregulation by glucocorticoids is due to intrinsic and unique properties of these drugs or to steroid-induced apoptosis, which in turn promotes LXR activation, or to other unidentified mechanisms.

Our study examined how the immunologic microenvironment affects MerTK expression and aimed to identify the human macrophage subsets in which MerTK is clearly expressed and functionally relevant. Macrophages subsets include: M1, secreting IL-12 and promoting Th1 differentiation; M2a, secreting IL-4 and related to Th2 polarization; M2b and M2c, both secreting IL-10 and associated with regulatory T cell (Treg) expansion (31). We found that, in the presence of IL-10, M-CSF differentiates macrophages expressing high levels of MerTK; such macrophages are characterized by an M2c phenotype. Glucocorticoids have analogous effects, with MerTK upregulation occurring as a consequence of steroid-induced M2c polarization; however, this process is IL-10 independent. Remarkably, neither M-CSF alone nor IL-10 alone is able to drive full M2c differentiation and upregulate MerTK significantly. MerTK is also expressed among *M2c-like* $CD14^{bright}$ $CD16^{+}$ circulating monocytes and in minor populations of $CD14^{bright}CD16^{+}CD163^{+}$ cells differentiated among M1 and M2a macrophages. MerTK makes M2c (M-CSF plus IL-10) macrophages and *M2c-like* cells highly capable of clearing ACs, and it significantly enhances IL-10 secretion by M-CSF–driven macrophages following Gas6 ligation. Our data support a broad role for MerTK in M2c macrophage homeostasis and highlight the potential usefulness of polarizing human macrophages with both M-CSF and IL-10 to ensure an optimal, MerTK-mediated clearance of ACs.

Materials and Methods

Cell cultures

Human monocytes from buffy coats of healthy blood donors were isolated by Ficoll-Paque Plus gradient (GE Healthcare) and magnetic separation, using a kit for human monocyte enrichment by negative selection (EasySep, StemCell), according to the manufacturers' instructions. Purity of $CD14^{+}$ cells was 89–94%. $CD14^{+}$ cells were cultured in 24-well plates at 0.8×10^{6} cells/ml for 7–8 d at 37˚C in 5% CO_2 in complete RPMI 1640 medium containing 10% human AB serum, L-glutamine, penicillin, and streptomycin. To prevent formation of clumps induced by autologous serum and to allow optimal differentiation, 10% heat-inactivated FBS was also added. Cells were incubated from day 0 with GM-CSF (100 ng/ml; Peprotech) or M-CSF (50 ng/ml; Peprotech), to differentiate M1 or M2 macrophages, respectively. In some cases, as specified in the text, these or other macrophage polarizing factors were newly added in the last 3 d: for M1, IFN-γ (2.5–10 ng/ml; R&D Systems) or LPS (1 μg/ml; Sigma-Aldrich), or both; for M2, IL-4 (20 ng/ml; Novus Biologicals), IL-10 (1–100 ng/ml; Peprotech), TGF-β (20 ng/ml; Peprotech) or dexamethasone (1–1000 nM; Sigma-Aldrich). Dendritic cells (DCs) were obtained by culturing cells in the presence of GM-CSF (100 ng/ml) and IL-4 (20 ng/ml) for 7–8 d. For experiments in serum-free conditions, cells were cultured at 0.8×10^{6} cells/ml for 3–4 d in X-Vivo15 medium (Lonza), and incubated from day 0 with one or more growth and polarizing factors. For inhibiting IL-10 activity, a purified LEAF purified mouse anti-human IL-10 Ab was used (BioLegend, clone JES3-9D7).

Prior to participation, all subjects gave informed consent to donate their blood samples. The study was approved by the Institutional Review Boards of Temple University.

Analysis of cell-surface molecules by FACS

Phenotype analysis by flow cytometry was performed in freshly isolated and in cultured cells after washing in buffer containing 2% BSA. The following mouse mAbs were used: anti-CD14 (PE-Cy7), anti-HLA-DR (APC), anti-CD163 (APC or PerCP-Cy5.5), anti-CD206 (APC-Cy7), anti-CD209 (PerCP-Cy5.5), anti-CD1a (APC), anti-CD210/IL-10R (PE; BioLegend); anti-CD16 (APC-Cy7; BD Biosciences); anti-CD204 (APC), and anti-MerTK (clone 125518; PE; R&D Systems). MerTK expression was evaluated using appropriate PE-labeled isotype control (BioLegend). Cells were analyzed using FACSCalibur (BD Biosciences) and FlowJo software.

Detection of MerTK expression by Western blot

Cell lysates were obtained in buffer containing 50 mM HEPES, 150 mM NaCl, 10% glycerol, 1% Triton X-100, and freshly added cocktails of protease and phosphatase inhibitors (Sigma-Aldrich). Lysates were resolved on a SDS-PAGE 8% polyacrylamide gel. Proteins, transferred to PVDF membranes (Millipore), were probed with biotinylated goat polyclonal anti-human MerTK (R&D Systems) and rabbit anti–β-actin Abs (Santa Cruz Biotechnology), followed by HRP-conjugated streptavidin (BioLegend) and secondary goat anti-rabbit Ab (Santa Cruz Biotechnology), respectively. Immunoblots were developed and visualized by ECL using Amersham ECL reagents (GE Healthcare). Densitometry of bands, normalized to β-actin expression, was calculated using Image J software.

Induction of apoptosis and phagocytosis assay

Human neutrophils were isolated from Ficoll-Hypaque pellets through dextran erythrocyte sedimentation and lysis of contaminating erythrocytes by incubation with ice-cold ammonium chloride (0.15 M) and potassium bicarbonate (0.01 M) solution. Neutrophils were resuspended at 1×10^{6} cell/ml in 10% FBS-RPMI, labeled with 2.5 μM CFSE (Sigma-Aldrich), and incubated for 20 h at 37˚C in 5% CO_2. The composition of neutrophils routinely obtained after incubation, according to annexin V and propidium iodide (PI) staining, was 66.0 ± 10.2% early ACs (annexin V^{+} PI^{-}), 3.8 ± 2.2% late ACs (annexin V^{+} PI^{+}), 0.3 ± 0.2% necrotic cells (annexin V^{-} PI^{+}).

Apoptotic neutrophils were added for 60 min to cultured monocyte-macrophages at a 5:1 ratio. Flow cytometry was used to quantify percentages of CD14-labeled macrophages that phagocytosed CFSE-labeled ACs, and to calculate phagocytosis index. In some experiments, as specified in the text, phagocytosis activity was assessed separately on $CD14^{dim}$ $CD163^{-}$ and $CD14^{bright}CD163^{+}$ macrophage subsets.

For phagocytosis assays on circulating monocytes, PBMCs were mixed with apoptotic neutrophils at a 1:1 ratio, and incubated in BD Falcon tubes for 4 h at 37˚C in 5% CO_2. Phagocytosis activity was measured on $CD14^{+}$ $CD16^{-}$, $CD14^{bright}CD16^{+}$ and $CD14^{dim}CD16^{+}$ monocyte subsets by flow cytometry.

For inhibition studies, macrophages and circulating monocytes were preincubated with a goat anti-human MerTK Ab (R&D Systems) or goat control IgG (Southern Biotech) for 30 min before adding apoptotic neutrophils. The effects of MerTK block during phagocytosis assay was additionally observed using a Leica TCS SP5 confocal laser scanning microscope, 40×/1.25 numerical aperture (NA) oil objective, labeling apoptotic neutrophils with Hoechst 33342 (0.5 μg/ml; Invitrogen) and staining macrophages with mouse anti-CD14 (BioLegend) and goat biotinylated anti-CD163 (R&D Systems) Abs, followed by secondary APC-conjugated goat anti-mouse Ab and FITC-conjugated streptavidin (BioLegend), respectively.

Gas6, IL-10, and TNF-α detection by ELISA

Gas6 levels were measured in supernatants of cell cultures treated with various cytokines for 4 d in serum-free conditions, using sandwich ELISA according to standard procedure (23). Standard curves were prepared with rhGas6 (R&D Systems). Purified goat anti-human Gas6 Ab (R&D Systems) was used for capture. Biotinylated goat polyclonal anti-human Gas6 Ab (R&D Systems), followed by HRP-conjugated streptavidin (BioLegend), was used for detection.

Secretion of IL-10 and TNF-α was induced by LPS (50 ng/ml; Sigma-Aldrich) ± rhGas6 (1 μg/ml; R&D Systems), in the presence or absence of goat anti-human MerTK Ab (R&D Systems) or goat control IgG (Southern Biotech), from cells cultured with or without M-CSF. IL-10 and TNF-α levels were measured in supernatants using human IL-10 ELISA MAX Standard kit and TNF-α ELISA MAX Standard kit (BioLegend), following the manufacturer's instructions.

In Gas6-producing monocyte-macrophages, the potential effect of endogenously produced Gas6 on TNF-α production was studied. For this purpose, cells were differentiated in the presence of M-CSF and IL-10 and coincubated with either recombinant MerFc (5 μg/ml; R&D Systems) or

a goat anti-human Gas6 Ab (5 μg/ml; R&D Systems) to block Gas6 activity prior to LPS stimulation. Potential variations in TNF-α levels were assessed by ELISA.

Statistical analysis

Data are expressed as mean ± SEM. Statistical significance among different cell treatments was assessed with Student paired *t* test, or one-way repeated measures ANOVA if treatment groups were more than two. Statistical significance was defined as $p < 0.05$. Analysis and graphing were performed using GraphPad Prism software.

Results

MerTK is upregulated during monocyte-to-macrophage differentiation and is further enhanced by M-CSF

Healthy monocytes were sorted from PBMCs through negative selection using magnetic beads and analyzed for MerTK expression at days 0, 1, and 3 by flow cytometry. Freshly isolated monocytes tended to aggregate with platelets, in variable proportions according to individuals; however, platelet-monocyte conjugates were poorly detectable after plating cells (Fig. 1A). Because platelets can also express MerTK (32), we had to rule out their potential contribution to MerTK detection. Therefore, we used the platelet marker CD42b along with CD14 for analyzing MerTK in monocytes, either isolated or conjugated with platelets. Surprisingly, no MerTK expression was clearly found at day 0 in either case, except for rare cells located in the conjugated fraction (Fig. 1B). In cultured monocytes, MerTK was gradually acquired during monocyte-to-macrophage differentiation, and was evident at intermediate stages (day 3) (Fig. 1C, 1D). However, in the absence of growth factors (i.e., CSFs), MerTK could be detected on the cell surface at variable levels, depending on individual experiments. In contrast, in the presence of M-CSF, MerTK upregulation was reproducibly enhanced. GM-CSF, instead, decreased MerTK expression levels (Fig. 1E–G).

Macrophage MerTK expression promptly adapts to changes of immunologic environment

Terminally differentiated macrophages, obtained after 8 d of culture, also upregulated MerTK in the presence of M-CSF, but not GM-CSF (Fig. 2A). We tested whether M-CSF and GM-CSF effects on MerTK expression were reversible. The addition of GM-CSF at day 5 to M-CSF–differentiated macrophages resulted in significant MerTK downregulation at day 8 (Fig. 2A, 2B), while the addition of M-CSF to GM-CSF-driven macrophages tended to increase MerTK expression, yet not significantly (Fig. 2C).

Because GM-CSF and M-CSF are known to drive M1 or M2 macrophage differentiation, respectively (33, 34), we studied the effects of other conventional M1 and M2 stimuli on terminally differentiated macrophages. According to a recent classification, macrophages can be divided into M1 (driven by IFN-γ, LPS, or both), M2a (driven by IL-4 or IL-13), M2b (driven by immune complexes and LPS), and M2c (driven by IL-10, TGF-β, or glucocorticoids) (31). Although M2b and M2c macrophages are elicited by different conditions, both subsets are characterized by IL-10 production, and we focused only on the M2c population, better

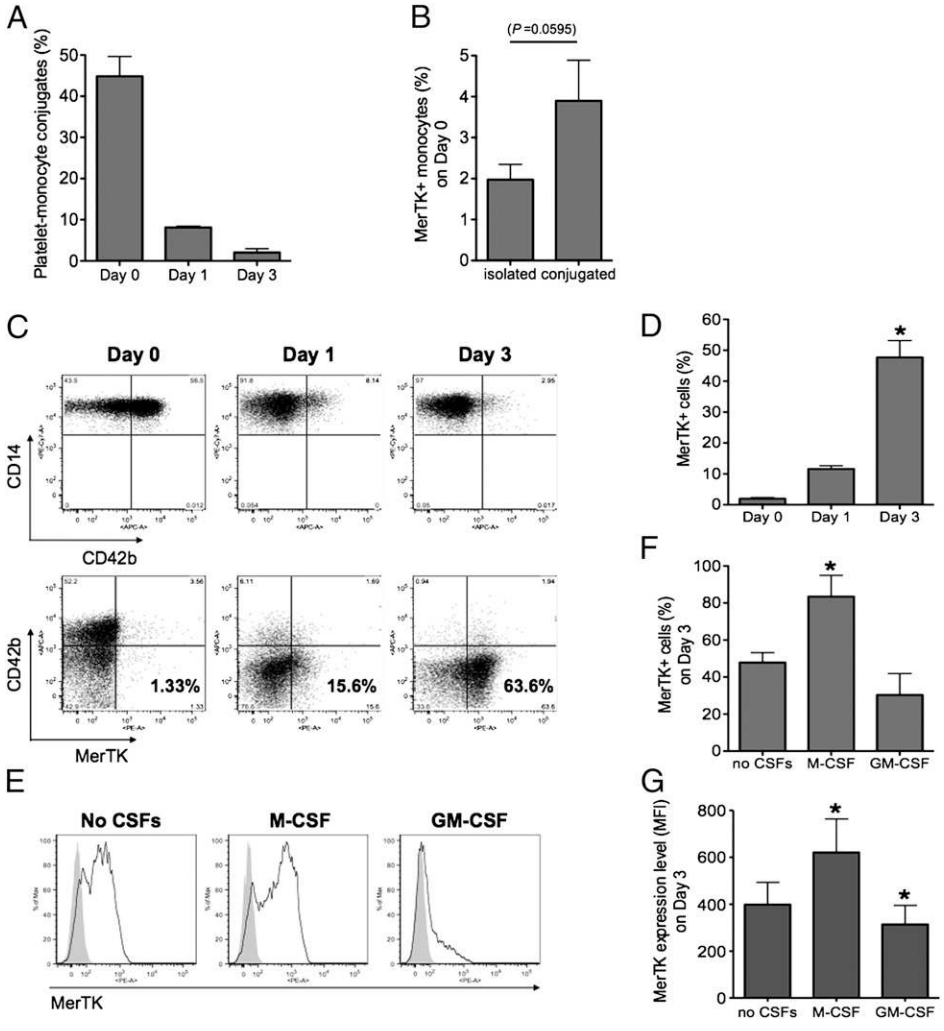

FIGURE 1. MerTK is upregulated during monocyte-to-macrophage differentiation, and is further enhanced by M-CSF. (**A**–**D**) Monocytes were sorted from healthy human PBMCs through negative selection magnetic beads and analyzed by flow cytometry at day 0, and after 1 and 3 d of culture. Monocytes were gated using an anti-CD14 Ab and stained with anti-MerTK Ab. An anti-CD42b Ab was also used to distinguish platelet-monocyte conjugates from isolated monocytes. (**E**–**G**) Monocytes were cultured in complete medium in the presence of M-CSF (50 ng/ml) or GM-CSF (100 ng/ml), or in the absence of CSFs, and analyzed for MerTK expression on day 3. Data shown are representative of three independent experiments. *$p < 0.05$.

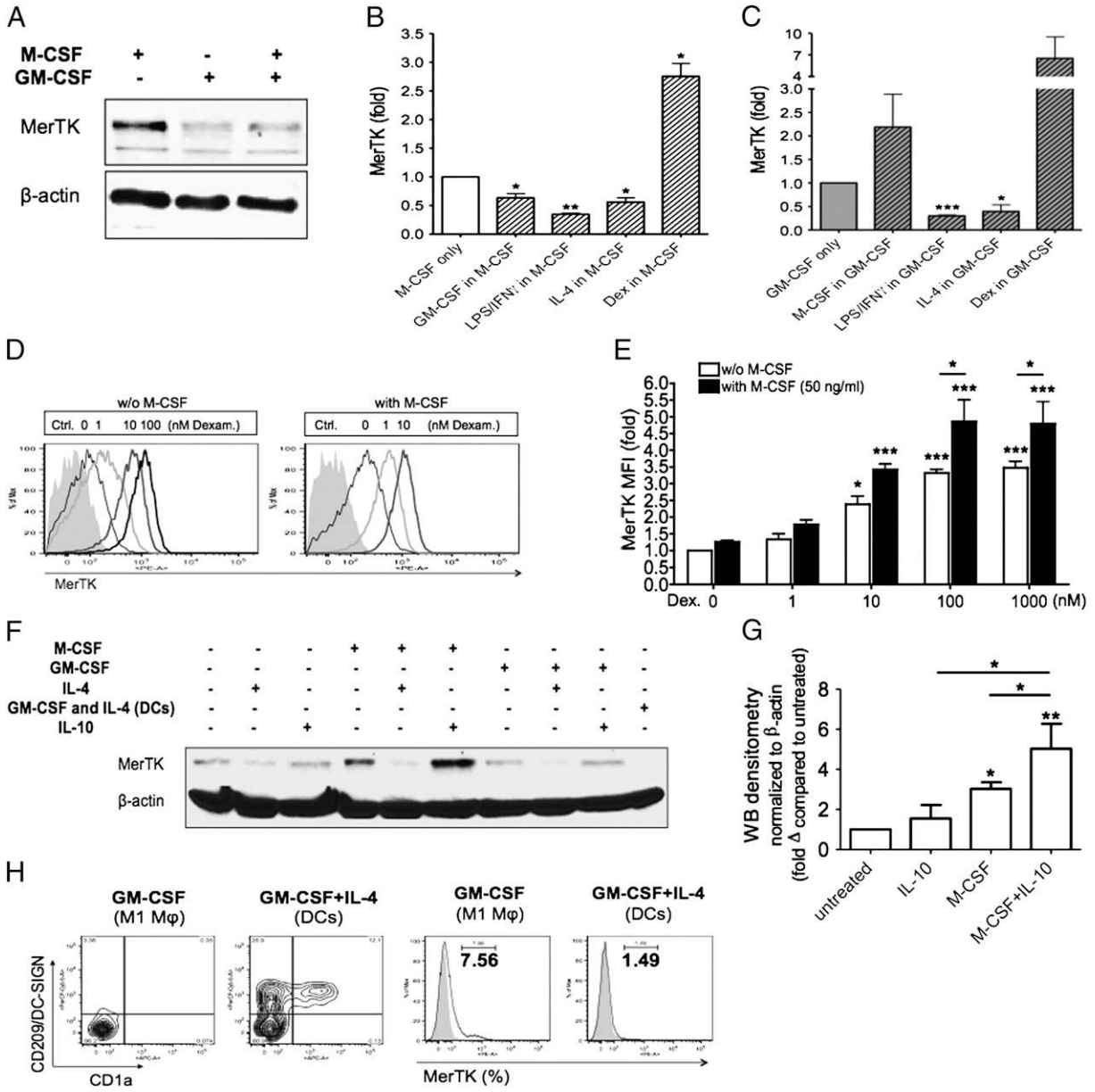

FIGURE 2. Macrophage MerTK expression promptly adapts to changes in immunologic environment. (**A**–**C**) CD14$^+$ cells were cultured in complete medium in the presence of M-CSF (50 ng/ml) or GM-CSF (100 ng/ml). On day 5, cells were treated with GM-CSF (100 ng/ml), M-CSF (50 ng/ml), IFN-γ (10 ng/ml) plus LPS (1 μg/ml), IL-4 (20 ng/ml), or dexamethasone (Dex; 100 nM), for an additional 3 d. MerTK expression was analyzed by Western blot (A), or measured by flow cytometry as mean fluorescence intensity (MFI) fold variation compared with levels obtained with culturing cells with M-CSF alone (B) or GM-CSF alone (C) for 8 d. Data shown are representative of three independent experiments. (**D** and **E**) Cells were incubated with Dex (1–1000 nM) in the presence or absence of M-CSF (50 ng/ml) for 3 d in serum-free medium. MerTK upregulation was measured by flow cytometry as MFI fold increase compared with expression levels in untreated cells. Data shown are representative of four independent experiments. (**F** and **G**) Cells were cultured in complete medium in the presence of M-CSF (50 ng/ml) or GM-CSF (100 ng/ml), or in the absence of CSFs. On day 5, cells were treated with IL-4 (20 ng/ml) or IL-10 (50 ng/ml), for an additional 3 d. DCs were differentiated in the presence of GM-CSF and IL-4 from day 0 for 8 d. MerTK expression was analyzed by Western blot (F). Densitometry of Western blots was performed to quantify MerTK expression following cell treatment with IL-10, M-CSF, or both (G). Densitometry values were normalized to β-actin and are reported as fold variation compared with MerTK expression levels in untreated cells. Data are representative of three independent experiments. (**H**) Cells were incubated with GM-CSF or GM-CSF plus IL-4 for 8 d in complete medium, to differentiate M1 macrophages or DCs, respectively. Cells were stained for CD209 (DC-SIGN), CD1a, and MerTK. MerTK$^+$ cells were quantified as percentages of total cells by flow cytometry. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

described in humans (31). In either GM-CSF– or M-CSF–differentiated macrophages, stimulation at day 5 with either M1 or M2a polarizing factors (IFN-γ ± LPS or IL-4) for 3 d downregulated MerTK. The relative decreases in MerTK expression were similar in GM-CSF– and M-CSF–differentiated macrophages. The glucocorticoid dexamethasone, instead, increased MerTK in both macrophage subsets, although full statistical significance was reached only in M-CSF–differentiated macrophages (Fig. 2B, 2C).

Remarkably, M-CSF positively synergized with dexamethasone and, in the presence of M-CSF, a 10-fold lower dose of dexamethasone (10 nM instead than 100 nM) was sufficient to reach maximal induction of MerTK; moreover, the addition of M-CSF to dexamethasone 100 nM further enhanced MerTK expression, whereas increasing the dose of dexamethasone per se (1 μM) was no longer effective (Fig. 2D, 2E). IL-10 also synergized with M-CSF in upregulating MerTK (Fig. 2F, 2G). In contrast, GM-CSF

and IL-4 combined to abrogate MerTK expression in human peripheral monocyte-derived DCs (Fig. 2F, 2H). Therefore, MerTK is not only inducible by AC exposure (6), but is also finely regulated by cytokines, growth factors, or both. Loss of MerTK in DCs indicates that MerTK is dispensable for AC clearance in these cells, similar to what has been previously reported in mice (35, 36).

MerTK expression is restricted to ($CD163^+CD16^+CD206^+$) M2 macrophages

Because MerTK expression was enhanced under M2 conditions, we examined MerTK expression along with several M2 surface receptors to identify potential markers that could predict the presence of MerTK in human macrophages.

Although CD206 (i.e., mannose receptor) was among the first identified markers for conventional (IL-4–driven) M2 macrophages (37), it is a nonspecific M2 marker in humans, being also upregulated by M-CSF, glucocorticoids (Fig. 3), TGF-β (not shown), and even by GM-CSF, a non-M2 factor. We noted instead that CD209 (earlier known as DC-SIGN) was a reliable and specific marker of exposure to IL-4 (M2a polarization), whereas CD163 (hemoglobin-haptoglobin scavenger receptor) or CD16 (FcγRIIIa), in agreement with a recent report (38), were specific markers of exposure to M-CSF, glucocorticoids, IL-10, or TGF*β (M2c polarization). Remarkably, MerTK showed the same expression pattern as CD163. Macrophages that had low levels of CD163 (M1 and M2a) also showed significant MerTK downregulation. However, CD163 detection was not sufficient to identify $MerTK^+$ cells, as in the case of IL-10–treated cells. What best discriminated the macrophage subset that highly expressed MerTK was the coexpression of CD163 and CD16; alternatively, the same population could be identified by the coexpression of CD163 and CD206. Both M-CSF and dexamethasone were able to induce this specific phenotype ($CD163^+CD16^+CD206^+$; Fig. 3). Therefore, MerTK upregulation by steroids is due to pharmacologic induction of M2c polarization, which reproduces the physiologic effects of M-CSF differentiation.

IL-10 is required for M-CSF to induce M2c differentiation and upregulate MerTK

It was noteworthy that IL-10 could enhance M-CSF effects, although IL-10 per se could not promote further MerTK upregulation compared with medium (Fig. 2F, 2G). We then wondered whether serum-derived bioactive IL-10 present in culture medium (39, 40) might account for the basal M-CSF effects observed. Consistent with this hypothesis, M-CSF upregulated MerTK only in the presence of serum (Fig. 4A). In serum-free medium, the addition of low doses of IL-10 was crucial for M-CSF induction of MerTK expression by the first days of differentiation (Fig. 4B). Conversely, blocking IL-10 in serum-containing medium, through a neutralizing anti–IL-10 Ab, prevented spontaneous increase as well as M-CSF enhancement of MerTK and CD163 expression

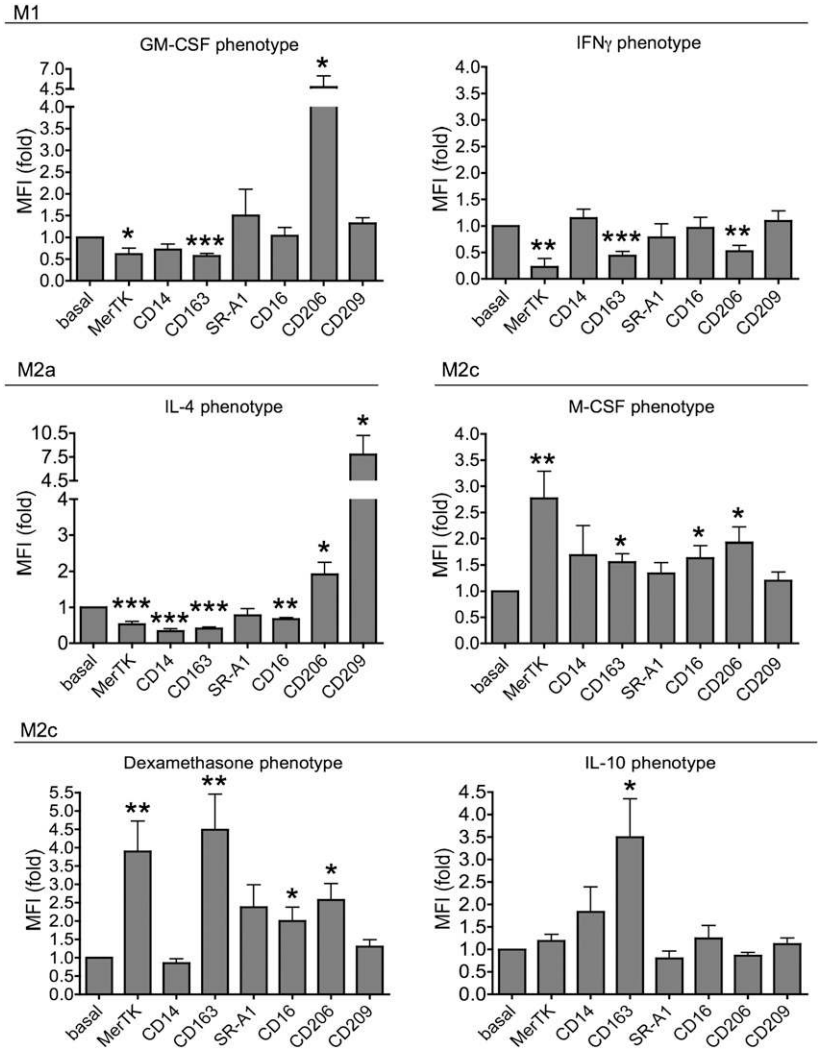

FIGURE 3. MerTK expression is restricted to ($CD163^+CD16^+CD206^+$) M2 macrophages. Macrophages were differentiated from peripheral monocytes for 7–8 d in complete medium, in the presence of GM-CSF (100 ng/ml), IFN-γ (10 ng/ml), IL-4 (20 ng/ml), M-CSF (50 ng/ml), dexamethasone (100 nM), or IL-10 (50 ng/ml). Cells were stained for MerTK, CD14, CD163, CD204/SR-A1, CD16, CD206, and CD209 and analyzed by flow cytometry. Histograms show MFI fold variations compared with levels in untreated cells (basal). Data shown are representative of 8 to 12 independent experiments. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

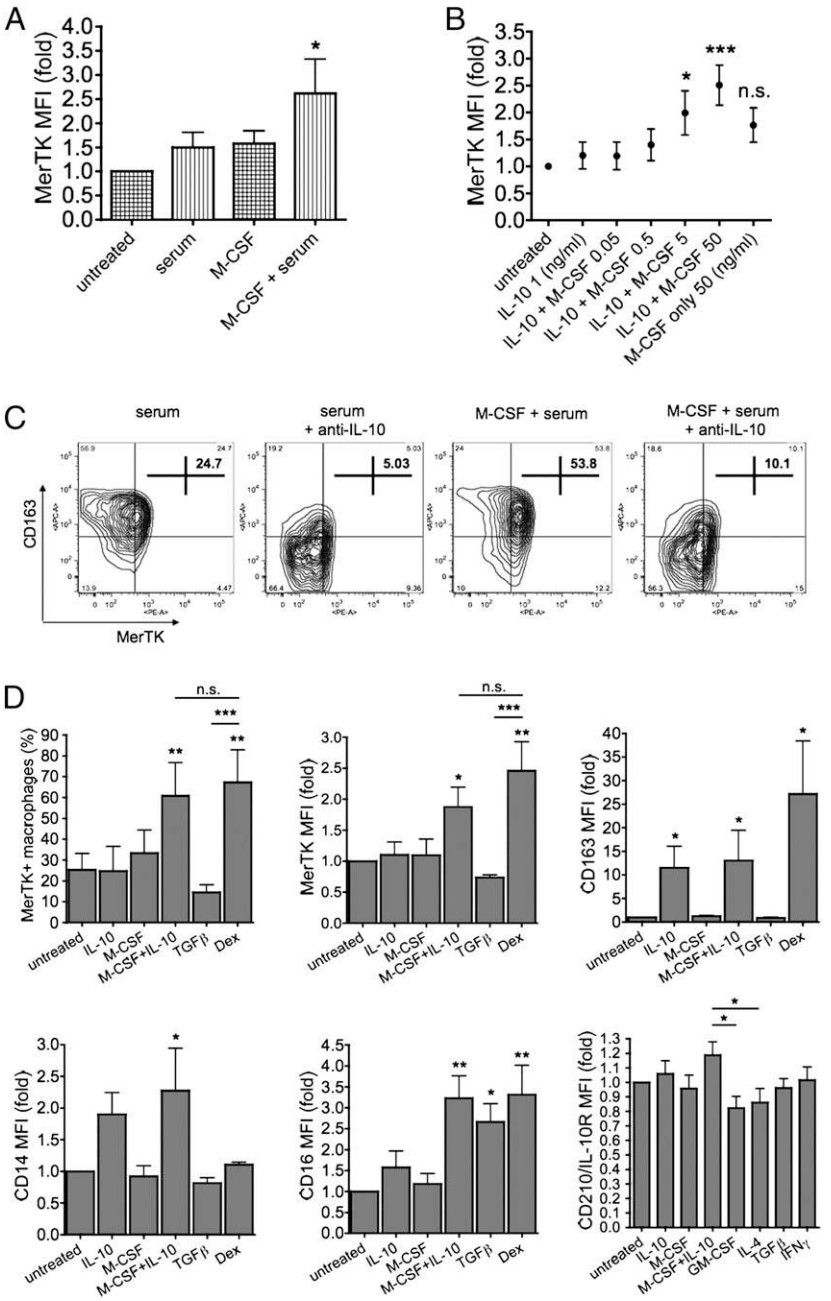

FIGURE 4. IL-10 is required for M-CSF to induce M2c differentiation and upregulate MerTK. (**A**) $CD14^+$ cells were cultured in the presence or absence of M-CSF (50 ng/ml), in either serum-containing or serum-free medium, for 3 d. (**B**) Cells were incubated with IL-10 (1 ng/ml), increasing doses of M-CSF (0.05 to 50 ng/ml), or both. MerTK upregulation was measured by flow cytometry as MFI fold increase compared with expression levels in untreated cells. Data shown are representative of three independent experiments. (**C**) Cells were cultured in serum-containing medium with or without M-CSF (50 ng/ml), in the presence or absence of a neutralizing mouse monoclonal anti-human IL-10 Ab (5 μg/ml; Biolegend, clone JES3-9D7), for 3 d. Cells were stained for CD163 and MerTK, and they were quantified by flow cytometry as percentages of total cells. (**D**) Cells were cultured in serum-free medium in the presence of M-CSF (50 ng/ml), IL-10 (50 ng/ml), M-CSF plus IL-10, TGF-β (20 ng/ml), and dexamethasone (Dex; 100 nM) for 4 d. Cells were stained for MerTK, CD163, CD16, and CD163. MerTK expression is shown as MFI fold variation compared with levels in untreated cells, as well as $MerTK^+$ cell percentages; CD163, CD16, and CD14 MFI fold variations are also reported. Data shown are representative of four independent experiments. The expression of IL-10 receptor (IL-10R, or CD210) was measured after 3-d cytokine stimulations. Data shown are representative of three independent experiments. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

during differentiation (Fig. 4C). In serum-free conditions, neither M-CSF nor IL-10 alone, but only the combination M-CSF plus IL-10 was able to induce MerTK in macrophages, at levels comparable to those reached with dexamethasone. Interestingly, M-CSF plus IL-10 gave a modest but significant increase in IL-10 receptor (CD210) expression compared with GM-CSF or IL-4. More broadly, we found that the combination M-CSF plus IL-10 was critically required for complete M2c differentiation (e.g., CD163 and CD16 upregulation) in serum-free conditions (Fig. 4D).

Instead, dexamethasone upregulated MerTK and induced M2c macrophages even in serum-free medium (Fig. 4D) or, as previously reported for CD163 (41), in the presence of anti–IL-10 Ab in serum-containing medium (not shown). M2c differentiation was, therefore, IL-10 dependent for M-CSF and IL-10 independent for steroids. M2c (M-CSF plus IL-10) macrophages also differed from dexamethasone-treated cells in having significantly higher levels of CD14 (Fig. 4D), a receptor known to be involved not only in LPS recognition, but also in tethering ACs (9).

Although currently classified as an M2c stimulus (31), TGF-β gave a different phenotype, characterized by CD16 induction, but inhibition of MerTK, CD163, and CD14 expression (Fig. 4D). Contrary to IL-10, TGF-β did not upregulate MerTK even in combination with M-CSF and tended to reduce the upregulating effect of M-CSF plus IL-10 (not shown).

MerTK confers to M2c (M-CSF plus IL-10) macrophages enhanced ability to clear early ACs

We aimed to assess the functional importance of MerTK upregulation in M2c macrophages in regard to AC clearance efficiency. For this purpose, we cultured cells for 7 d in complete medium, adding IFN-γ (M1 stimulus), IL-4 (M2a stimulus), M-CSF, and IL-10 (M2c stimuli) or fresh medium only (M0) from day 4. At day 7, we coincubated macrophages with early apoptotic neutrophils (Fig. 5A), at a 1:5 ratio.

We observed a remarkably higher capacity of AC clearance in M2c (M-CSF plus IL-10) macrophages, compared with the other

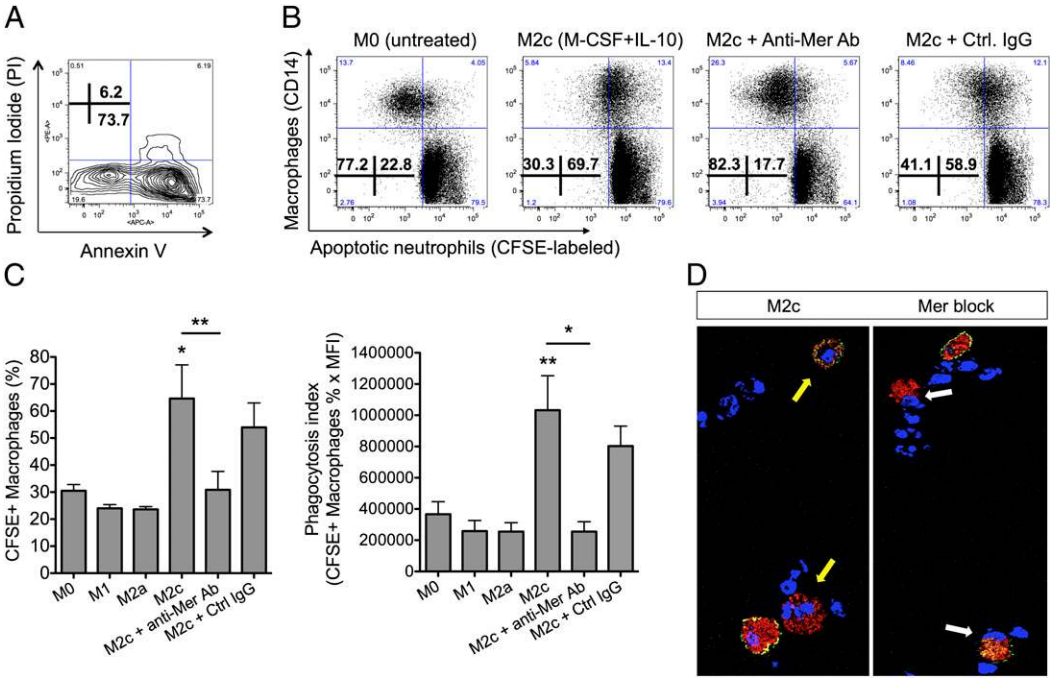

FIGURE 5. MerTK confers to M2c (M-CSF plus IL-10) macrophages an enhanced ability to clear early ACs. (**A**) Early ACs were obtained from incubating human neutrophils, isolated from peripheral blood healthy donors, in 10% FCS-RPMI for 20 h. According to annexin V and propidium iodide (PI) staining, early ACs constituted 65–70% of total neutrophils. (**B** and **C**) CFSE-labeled apoptotic neutrophils were added for 60 min to 7-d differentiated M0 (untreated), M1 (IFN-γ, 2.5 ng/ml), M2a (IL-4, 20 ng/ml) and M2c (M-CSF, 50 ng/ml plus IL-10, 50 ng/ml) macrophages, labeled with a fluorochrome-conjugated anti-CD14 Ab at a 5:1 ratio. M2c macrophages showed significantly enhanced ability to clear early ACs, expressed as higher percentages of phagocytic ($CSFE^+$) macrophages. Preincubation of M2c macrophages with a goat polyclonal anti-human MerTK Ab (5 μg/ml; R&D Systems) for 30 min before addition of apoptotic neutrophils abolished such superiority of M2c cells to phagocytose ACs compared with other macrophage subsets. Blocking MerTK diminished not only the number of $CFSE^+$ macrophages, but also the mean phagocytosis activity per single cell, depicted as CFSE MFI. Altogether, it resulted in a significant decrease of the phagocytosis index, determined by multiplying the percentage of $CFSE^+$ macrophages by the CFSE MFI of phagocytic macrophages. Data shown are representative of three independent experiments. (**D**) By fluorescence microscopy (Leica TCS SP5 confocal laser scanning microscope, 40×/1.25 NA oil objective), M2c macrophages stained for CD14 (red) and CD163 (green) were shown to engulf Hoechst 33342-labeled apoptotic neutrophils (blue; *left panel*, yellow arrows). Preincubation of M2c macrophages with an anti-MerTK blocking Ab inhibited engulfment, but not the physical interaction between macrophages and apoptotic neutrophils (*right panel*, white arrows). $*p < 0.05$, $**p < 0.01$.

subsets. Importantly, such superiority of M2c cells was abrogated in the presence of a blocking anti-MerTK Ab (Fig. 5B, 5C). Using immunofluorescence microscopy, we could observe that blocking MerTK inhibited AC engulfment by M2c macrophages, but not the physical interactions between macrophages and ACs (Fig. 5D) that typically precede phagocytosis of early ACs (10).

M2c markers universally define MerTK$^+$ macrophages prone to AC clearance

We showed above that IFN-γ and IL-4 downregulate MerTK protein expression, which is consistent with previous microarray data (42). Nevertheless, after culturing monocytes for 4 d in the presence of IFN-γ or IL-4, we were able to detect rare $MerTK^+$ cells even among M1 and M2a macrophages. Looking at the phenotype of these cells, we could observe that they were clearly distinguishable from the other macrophages by the selective expression of M2c markers (CD163, CD16, CD14, CD204; Fig. 6A, B). In particular, CD204 expression ratio (i.e., percentage of $CD204^+$ cells among $MerTK^+$ macrophages to percentage of $CD204^+$ cells among total macrophages in culture) was significantly higher in M1 conditions, whereas CD163 and CD16 expression ratios were significantly higher in both M1 and M2a conditions (Fig. 6B). These minor populations of M2c macrophages did not occur as a consequence of IFN-γ or IL-4 inefficacy on some cells, because they were less clearly distinguishable among untreated cells; they were, rather, actively induced by treatment. In contrast to $MerTK^-$ M2a cells, the $MerTK^+$ M2c macrophages occurring among IL-4–treated cells were CD209 negative and brighter for CD206 (Fig. 6C). Thus, MerTK localizes in macrophage subsets that share the phenotype $CD14^{bright}CD16^+CD163^+$ $CD204^+CD206^{bright}CD209^{null}$; $MerTK^+$ M2c cells occur even in non-M2c conditions, as small endogenous populations.

We tested whether such minor populations of M2c cells were more capable of performing AC clearance compared with the major populations of M1 and M2a macrophages. As predicted, $CD14^{bright}$ $CD163^+$ cells showed significantly higher ability to clear apoptotic neutrophils compared with $CD14^{dim}CD163^-$ cells. Moreover, phagocytosis of ACs by $CD14^{bright}CD163^+$ cells, but not by $CD14^{dim}CD163^-$ cells, was significantly inhibited by blocking MerTK (Fig. 6D, 6E).

M2c-like CD14^{bright}CD16$^+$ circulating monocytes use MerTK to phagocytose ACs

We wanted to determine whether the M2c markers were able to predict MerTK positivity and identify efferocytotic cells even among circulating monocytes. We reported above that freshly isolated monocytes analyzed after negative selection did not express MerTK. Nevertheless, we reasoned that negative selection eliminated $CD16^+$ monocytes along with lymphocytes, and that this minor population of more mature ($HLA\text{-}DR^+$) monocytes could represent a counterpart in the circulation of M2c macrophages. Thus, in this set of experiments, we looked at fresh monocytes directly from PBMCs, without magnetic sorting. According to CD14 and CD16 expression, three populations of monocytes were distinguishable: a major subset of $CD14^+CD16^-$ cells, and small numbers of $CD14^{bright}$

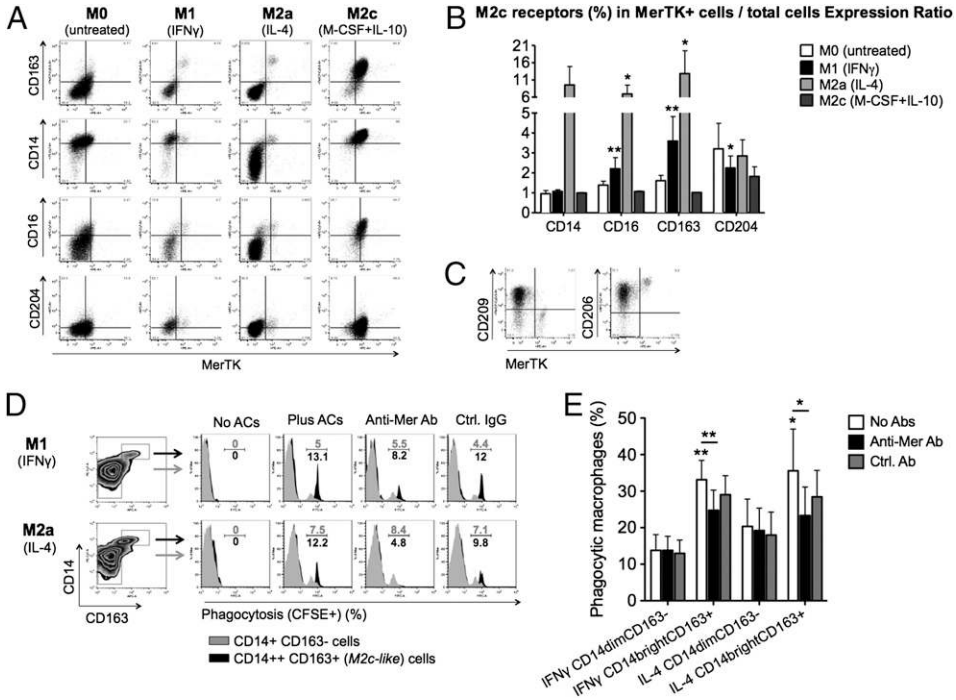

FIGURE 6. M2c markers universally define MerTK$^+$ macrophages prone to AC clearance. (**A**–**C**) CD14$^+$ cells were cultured in serum-free medium in the presence of IFN-γ (10 ng/ml; M1), IL-4 (20 ng/ml; M2a), M-CSF (50 ng/ml) plus IL-10 (50 ng/ml; M2c), or in the absence of cytokines (M0), for 4 d. Cells were stained for MerTK, CD163, CD14, CD16, and CD204. Coexpression of MerTK and M2c surface markers was studied by flow cytometry (A). For each M2c receptor (CD14, CD16, CD163, CD204), an expression ratio was obtained by dividing "percentage of macrophages expressing a given M2c receptor among MerTK+ macrophages" by "percentage of macrophages expressing a given M2c receptor among total macrophages in culture", after differentiation in M0, M1, M2a, or M2c conditions. Frequencies of each M2c receptor (percentages of positive cells) among MerTK$^+$ macrophages and among total macrophages were analyzed for potential significant differences between the two sets of data (B). IL-4–treated cells were also stained for CD209 and CD206 (C). Data shown are representative of four independent experiments. (**D** and **E**) CD14$^+$ cells treated with IFN-γ or IL-4 for 3 d in serum-free medium were coincubated with CFSE-labeled apoptotic neutrophils for 1 h, and then stained for CD14 and CD163. For inhibition studies, cells were preincubated with a goat polyclonal anti-human MerTK Ab (2 μg/ml; R&D Systems) or a goat control IgG (2 μg/ml; Southern Biotech) for 30 min before the addition of apoptotic neutrophils. Percentages of CFSE$^+$ phagocytic macrophages were determined among the major populations of M1 or M2a cells (CD14^{dim}CD163$^-$) and the minor populations of *M2c-like* cells (CD14^{bright}CD163$^+$). Data from one representative experiment (D) and three independent experiments (E) are reported. $*p < 0.05$, $**p < 0.01$.

CD16$^+$ and CD14^{dim}CD16$^+$ cells (Fig. 7A, 7B). We could detect MerTK$^+$ monocytes within the CD14^{bright}CD16$^+$ subset, representing approximately one third of this population (Fig. 7C). Detection of MerTK was not associated with a higher rate of platelet-monocyte conjugates in this subset (Fig. 7D); rather, MerTK positivity was associated with the expression of the M2c scavenger receptors CD163 and CD204 (Fig. 7E). CD204 is, in fact, known to improve AC clearance by signaling via MerTK (13).

To examine the ability of each monocyte subset to phagocytose ACs, we coincubated PBMCs with apoptotic neutrophils for 4 h at a 1:1 ratio; next, we gated on monocytes by flow cytometry for analysis. In agreement with a recent study (43), CD16$^+$ monocytes showed higher capacity of AC clearance compared with CD16$^-$ monocytes. Differences in efferocytosis rates were statistically significant when CD14^{bright}CD16$^+$ cells were compared with CD16$^-$ cells. Although CD14^{dim}CD16$^+$ cells were the most efficient in efferocytosis in two of three experiments, the difference with CD16$^-$ monocytes did not reach full statistical significance (Fig. 7F). Furthermore, only CD14^{bright}CD16$^+$ monocytes were dependent on MerTK activity for phagocytosis of ACs; AC clearance by this subset, in fact, was significantly reduced following pretreatment with a blocking anti-MerTK Ab (Fig 7G, H).

Gas6 is released by M2c macrophages and amplifies IL-10 secretion via MerTK

Gas6 is a major ligand of MerTK; it is produced by several cell types, including macrophages themselves (36, 42). We examined Gas6 levels in the supernatants of serum-free cell cultures to investigate how MerTK regulation was related to Gas6 production. We found that Gas6 was released upon cell stimulation with IL-10, dexamethasone, or IL-4, but not with GM-CSF or IFN-γ, indicating that both M2c and M2a, but not M1, macrophages were Gas6 producers. Similarly to what we observed with MerTK expression, M-CSF alone had no effect on Gas6 production in serum-free conditions, yet enhanced dexamethasone effects. TGF-β was a negative regulator, as shown by its inhibitory effect on IL-4–induced Gas6 secretion (Fig. 8A).

The Gas6/MerTK pathway is known to inhibit production of proinflammatory cytokines (17, 18) and this function can be independent from AC clearance (19). Consistent with previous results (17, 18), we found that in monocyte-macrophages cultured in the absence of CSFs, Gas6 significantly reduced LPS-induced production of TNF-α (Fig. 8B) and tended to increase IL-10 production (Fig. 8C).

Because IL-10 production represents a central functional property of M2c macrophages (31), we hypothesized that Gas6 effects on IL-10 secretion could be more pronounced in cells cultured in the presence of M-CSF. In these conditions, it was possible that Gas6, inducible by IL-10, could signal through MerTK, inducible by M-CSF in an IL-10–dependent manner, to stimulate additional IL-10 secretion. We indeed found that rhGas6 significantly increased IL-10 levels in supernatants of M-CSF–cultured cells stimulated with low doses of LPS, and this effect was prevented by blocking MerTK activation (Fig. 8D). In the

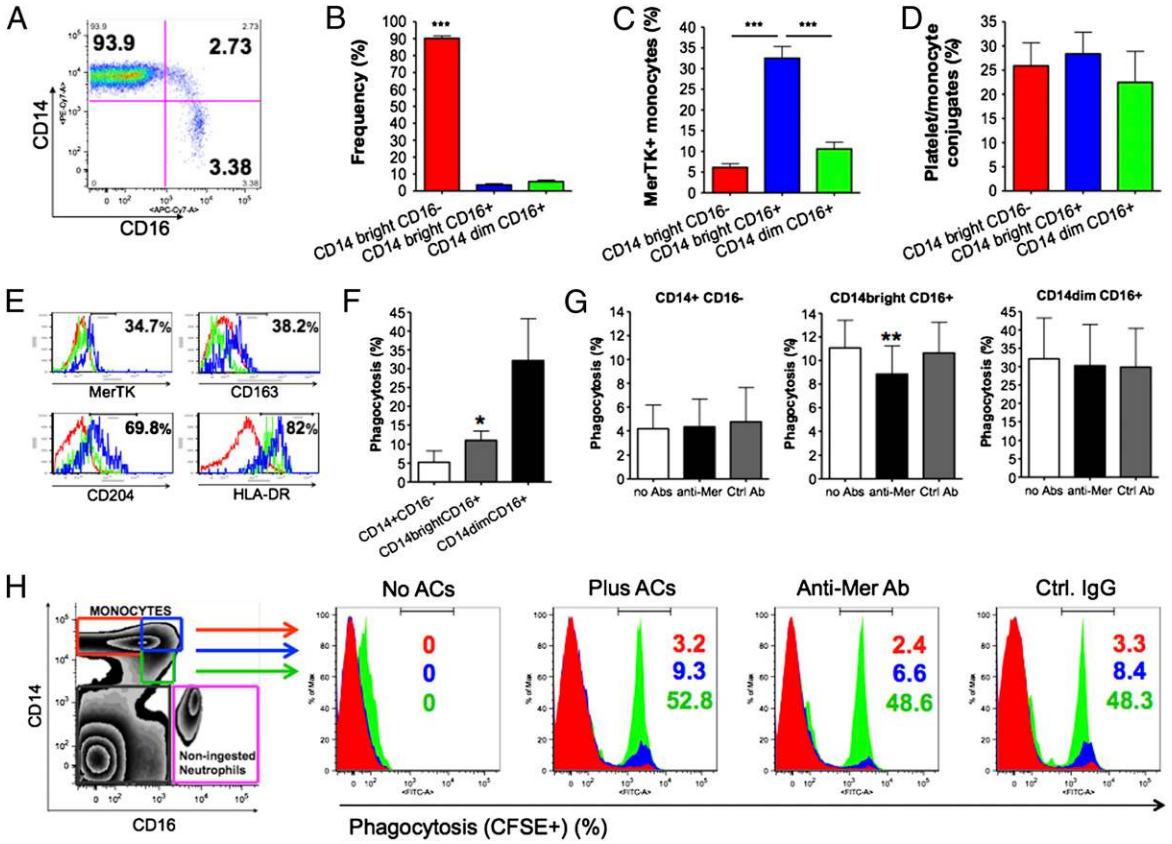

FIGURE 7. *M2c-like* CD14^{bright}CD16^{+} circulating monocytes use MerTK to phagocitose ACs. (**A**–**E**) Freshly isolated monocytes were analyzed by flow cytometry directly from PBMCs, without magnetic sorting, to include also CD16^{+} (HLA-DR^{+}) monocytes. On the basis of CD14 and CD16 expression levels, monocytes were divided into three categories: CD14^{bright}CD16^{-} (red histograms and peaks), CD14^{bright}CD16^{+} (blue) and CD14^{dim}CD16^{+} (green). Platelet-monocyte conjugates were depicted by flow cytometry as events also positive for the platelet marker CD42b in each monocyte subset (D). Percentages in E refer to positivity of CD14^{bright}CD16^{+} cells for the receptors indicated. Data shown are representative of four independent experiments. (**F**–**H**) Freshly isolated PBMCs were coincubated with CFSE-labeled apoptotic neutrophils at a 1:1 ratio for 4 h. For inhibition studies, cells were preincubated with a goat polyclonal anti-human MerTK Ab (2 μg/ml; R&D Systems) or a goat control IgG (2 μg/ml; Southern Biotech) for 30 min before addition of apoptotic neutrophils. Percentages of CFSE^{+} phagocytic monocytes were determined within each monocyte subset. Data from three independent experiments (F, G) and one representative experiment (H) are reported. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

presence of M-CSF, IL-10, and Gas6 were, therefore, able to reciprocally stimulate one another's production. Gas6 release from MerTK^{+} M2c cells can, then, be part of an autocrine loop that amplifies IL-10 secretion and positively regulates homeostasis of M2c macrophages.

In the presence of M-CSF, cell secretion of TNF-α was instead negligible. We tested whether the endogenous production of Gas6 by M2c (M-CSF plus IL-10) cells played a role in inhibiting TNF-α production. However, the addition of either recombinant MerFc or a blocking anti-Gas6 Ab to neutralize Gas6 failed to increase TNF-α levels in supernatants (Fig. 8E).

Discussion

In this study, we examine the expression of the key AC receptor MerTK in human populations of monocytes and macrophages, determining for each subset the ability to clear ACs and the functional relevance of MerTK in this process. Furthermore, we make a novel contribution to characterization of human anti-inflammatory macrophages, in regard to their phenotype, the immunologic factors promoting their differentiation, and the favoring role of the Gas6/MerTK pathway in mediating IL-10 secretion from these cells.

It has been shown previously that MerTK is regulated by metabolic pathways through LXRs, nuclear sensors activated following macrophage exposure to ACs or to other sources of cholesterol, and through PPARs and RXRs, transcription factors activated during macrophage differentiation (4–6). In this study, we show that this molecule is also importantly regulated by specific immunologic factors. In fact, MerTK is not homogenously distributed among human macrophage populations, but is mostly restricted to a discrete subset of IL-10–secreting anti-inflammatory M2 macrophages, recently named M2c (31). This subset is distinguishable from M2a (IL-4–producing and Th2-related) and M1 (IL-12–secreting and Th1-related) macrophages for its specific phenotype, here characterized as CD14^{bright}CD16^{+}CD163^{+}CD204^{+}CD206^{bright}CD209^{null}. M2c polarization is closely associated with MerTK upregulation, and detection of M2c receptors predicts MerTK expression. Remarkably, M2c differentiation is required to obtain macrophages highly capable of clearing ACs. *M2c-like* cells are also detectable among circulating CD14^{bright}CD16^{+} monocytes, and occur even in M1 and M2a differentiating conditions as minority populations; consistently, these latter cells are particularly prone to AC clearance and use MerTK for this purpose. M2c macrophages are able to release Gas6, which can in turn amplify IL-10 secretion in an autocrine manner, via MerTK signaling. MerTK importantly affects AC clearance activity and is critical for homeostasis of M2c macrophages.

Recently, Galvan et al. (15) reported that prolonged macrophage exposure to the complement component C1q, another molecule primarily involved in AC clearance, stimulates expression of MerTK

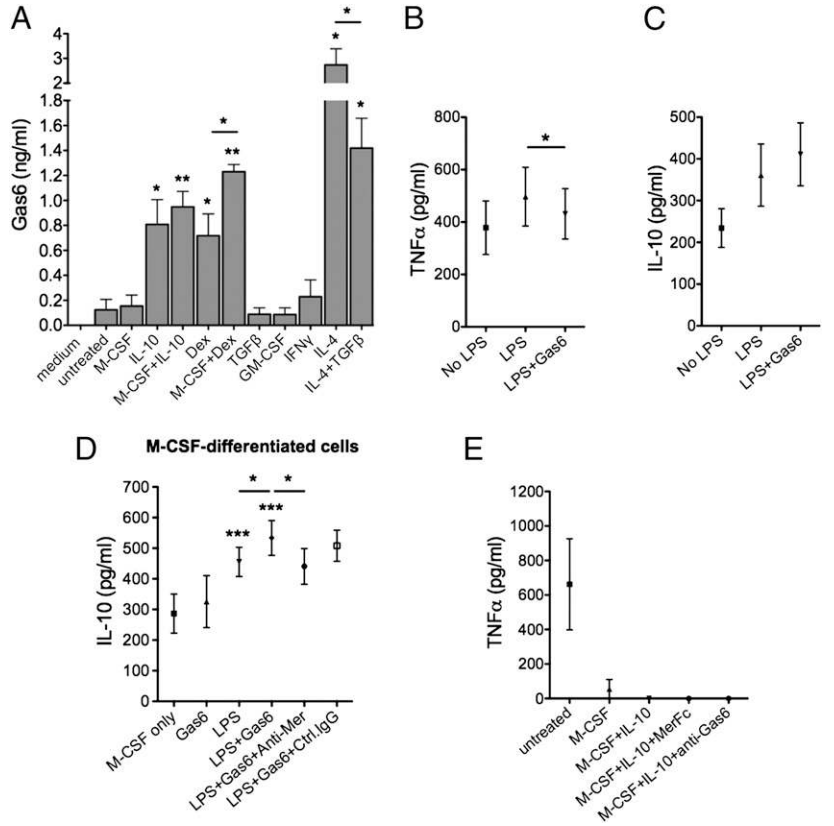

FIGURE 8. Gas6 is released by M2c macrophages and amplifies IL-10 production via MerTK. (**A**) Gas6 was measured by ELISA in supernatants of $CD14^+$ cells cultured in serum-free medium, in the presence of different treatments for 4 d (M-CSF, 50 ng/ml; IL-10, 50 ng/ml; dexamethasone, 100 nM; TGF-β, 20 ng/ml; GM-CSF, 100 ng/ml; IFN-γ, 10 ng/ml; IL-4, 20 ng/ml). Data shown are representative of four independent experiments. (**B** and **C**) $CD14^+$ cells were cultured in serum-free conditions for 3 d in the absence of CSFs and stimulated from day 1 with LPS (50 ng/ml) ± rhGas6 (1 μg/ml) for 48 h; TNF-α and IL-10 levels were measured in supernatants by ELISA. Data shown are representative of three independent experiments. (**D**) Cells were cultured in serum-free conditions for 3 d in the presence of M-CSF (50 ng/ml) and stimulated from day 1 with LPS ± rhGas6 for 48 h; rhGas6 significantly increased LPS-induced IL-10 release in culture supernatants, as assessed by ELISA. IL-10 increase was prevented by blocking MerTK with a goat polyclonal anti-human MerTK Ab (5 μg/ml; R&D Systems) during LPS plus rhGas6 stimulation. Data shown are representative of four independent experiments. (**E**) Cells were cultured in serum-free conditions for 4 d in the presence or absence of M-CSF (50 ng/ml) ± IL-10 (50 ng/ml). On day 1, a recombinant MerFc (5 μg/ml; R&D Systems) or a goat polyclonal anti-human Gas6 blocking Ab (5 μg/ml; R&D Systems) was added to precipitate Gas6 endogenously produced by cultured cells. From day 2, cells were stimulated with LPS for 48 h. Neither MerFc nor anti-Gas6 Ab were able to restore TNF-α secretion, inhibited in M2c (M-CSF plus IL-10) cells. Data shown are representative of three independent experiments. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

and production of Gas6 and C1q itself. Moreover, MerTK was recognized to be essential for C1q-dependent efferocytosis. However, the mechanism accounting for C1q induction of MerTK was not defined. In this regard, it will be interesting to determine whether stimulation with C1q is able to elicit the M2c phenotype. Alternatively, M2c polarization might promote release of C1q, which in its turn could mediate or amplify MerTK upregulation.

M2 differentiation is a key process regulating inflammation and fibrosis (33, 34, 37). The factors controlling this process are just becoming understood. We performed the first systematic study on the effects of cytokines and growth factors on human macrophage phenotype, MerTK expression, and Gas6 secretion. The effects observed are summarized in Table I. Remarkably, MerTK and Gas6 levels follow the expression pattern of CD16 and CD163—molecules identifiable as specific M2c markers—in agreement with a recent study (38). CD206 is highly expressed in these macrophages; however, CD206 is also upregulated in M2a (IL-4–treated) and in GM-CSF-differentiated M1 macrophages. M2a

Table I. Effects of immunologic environment on macrophage phenotype markers, MerTK expression, and Gas6 production

Macrophage Differentiation Factors	CD206	CD209	CD16	CD163	CD14	MerTK	Gas6
IFN-γ	↓	=	=	↓	=↑	↓	=
GM-CSF	↑	=	=	↓	=↓	↓	=
IL-4	↑	↑	↓	↓	↓	↓	↑
TGF-β	↑	=↓	↑	↓	↓	=↓	=↓
IL-10	=	=	=	↑	=↑	=	↑
M-CSF plus IL-10 (or M-CSF plus serum)	↑	=	↑	↑	↑	↑	↑
Glucocorticoids	↑	=	↑	↑	=	↑	↑

↑ indicates increase, ↓ indicates decrease, = indicates "no change" in macrophage expression of phenotype markers, MerTK or Gas6 compared to expression in macrophages obtained in the absence of differentiation factors.

cells are selectively characterized by CD209 expression and downregulate surface expression of MerTK. Nevertheless, contrary to what was reported by others (27), we found that M2a macrophages secrete Gas6, consistent with IL-4 effects on Gas6 gene induction (42); this suggests that IL-4 might induce other membrane or soluble TAM receptors.

Our study provides novel insights into mechanisms of M2c polarization in humans. IL-10, TGF-β, and glucocorticoids are the factors currently classified as M2c stimuli (31). We show that M2c macrophages are differentiated in the presence of steroids or in the presence of M-CSF and IL-10. These two types of stimulation have comparable effects on macrophage polarization. Steroids are known to increase a broad range of molecules involved in AC clearance, including MerTK, MFG-E8, C1q, Axl, ADORA3, and thrombospondin-1 (29, 30). We show in this study that MerTK upregulation by these drugs is closely related to induction of M2c polarization. We report for the first time that steroids stimulate macrophages to secrete Gas6, possibly resulting in enhanced MerTK-dependent clearance independent from Protein S (12, 28). In contrast, we observed that TGF-β gives a different phenotype ($CD163^-CD16^+MerTK^-Gas6^-$), whereas IL-10 gives only a partial phenotype ($CD163^+CD16^-MerTK^-Gas6^+$) that requires M-CSF for its complete expression ($CD163^+CD16^+MerTK^+Gas6^+$).

Incubation of monocytes with M-CSF promotes M2 macrophage differentiation (33, 34). We noted that M-CSF synergizes with either IL-10 or glucocorticoids, thereby identifying M-CSF as a specific M2c factor or cofactor rather than a general M2 cytokine. Additive effects between M-CSF and IL-10 were also previously reported. Specifically, IL-10 increased CD16 (FcγRIII) and CD32 (FcγRII) expression in M-CSF–driven macrophages, thus enhancing FcγR-mediated phagocytosis in these cells (44); IL-10 alone, instead, was only able to upregulate CD64 (FcγRI) (45). Similarly, we found that MerTK expression and MerTK-mediated AC phagocytosis rely on a combined action of M-CSF and IL-10, whereas IL-10 alone is able to induce only Gas6, in agreement with a recent report (27).

Importantly, we demonstrated that IL-10 is not only an enhancer of M-CSF effects, but is essential for M-CSF to act as an M2c factor and upregulate MerTK. We examined the effects of M2c factors in the presence and absence of serum in cell culture medium, as well as in IL-10–blocked serum-containing and IL-10–supplemented serum-free medium. We concluded that M-CSF–driven M2c differentiation is dependent on IL-10 bioactivity, present in serum-containing medium (39, 40) or exogenously added in serum-free conditions, whereas glucocorticoid-induced polarization is IL-10 independent.

In the absence of M-CSF, IL-10 did not upregulate MerTK, which was consistent with previous microarray studies of IL-10 effects on gene regulation in monocytes-macrophages (46, 47). Only Jung et al. (48) observed Mer gene upregulation by IL-10. However, these authors incubated PBMCs rather than purified monocytes in vitro with IL-10, and only afterward sorted monocytes for microarray analysis. This may have underestimated the contribution of T lymphocytes to MerTK upregulation; in particular, T cells are a well-known source of M-CSF (49). Indeed, Jung et al. (48) also reported IL-10 induction of CD16 and CD32, for which M-CSF is required (44). Of interest, Tiemessen et al. (50) reported that Tregs promote anti-inflammatory monocytes-macrophages expressing CD163 and CD206; however, although CD163 expression was dependent on IL-10 released by Tregs, CD206 induction was suggested to be cytokine independent. In this study, we showed that macrophage coexpression of CD163, CD206, and CD16, is inducible by M-CSF, strongly supporting a central role for T cell–derived M-CSF, together with IL-10, in the induction of anti-inflammatory M2c macrophages by Tregs. From this perspective, production of M-CSF by Tregs might counterbalance the well-known production of GM-CSF by proinflammatory Th1 and Th17 lymphocytes (51), with strong and direct repercussions of the Treg versus Th17 balance on M-CSF versus GM-CSF macrophage differentiation.

Interestingly, $MerTK^+$ *M2c-like* cells could be found even among circulating $CD16^+$ monocytes. $CD16^+$ monocytes represent more mature monocytes and can phagocytose ACs via CD36 (43). However, only $CD14^{bright}CD16^+$, but not $CD14^{dim}CD16^+$, monocytes express MerTK and the M2c receptors CD163 and CD204; accordingly, their ability to clear ACs is in part MerTK dependent. This finding is consistent with previous findings that $CD14^{bright}CD16^+$ monocytes are predominantly anti-inflammatory and secrete IL-10, whereas $CD14^{dim}CD16^+$ are proinflammatory and produce TNF-α (52, 53). A role for TGF-β in the development of $CD16^+$ monocytes was also suggested (54). We reported that M-CSF and IL-10 trigger CD16 expression along with CD14, CD163, and MerTK, whereas TGF-β upregulates CD16, but not CD14, CD163, or MerTK; therefore, we propose that $CD14^{high}CD16^+$ and $CD14^{dim}CD16^+$ subsets are selectively elicited by different cytokines.

M-CSF–driven macrophages have already been reported by Xu et al. (55) to have augmented capability of clearing early ACs; however, these authors attributed such ability to enhanced macropinocytosis activity, independent from IL-10. This conclusion was soon challenged by Krysko et al. (10), who demonstrated a major role for macropinocytosis in removing late, rather than early, ACs. In fact, Xu et al. (55) initially showed enhanced AC clearance by M-CSF–driven macrophages, even when late ACs were preponderant compared with early ACs, and suggested the involvement of macropinocytosis in clearance of both early ACs and blebs (i.e., late apoptotic debris). Krysko et al. (10) also argued that Xu et al. did not provide evidence of colocalization of fluid phase tracers with endosomes at microscopy, which is crucial to definitively prove macropinocytosis. Although we do not exclude enhanced macropinocytosis by M-CSF–driven macrophages, we identified MerTK as the major driver of early AC clearance in these cells, dependent on IL-10. In fact, whereas the majority of AC clearance receptors preferentially binds to late ACs (10), MerTK specifically clears early membrane-intact ACs (7, 12). Therapeutic induction of M2c polarization may, therefore, ameliorate the defective clearance of early ACs in diseases such as lupus, in which secondary necrotic cells are believed to be responsible for development of autoimmunity and inflammation (21, 22). This may also be beneficial in atherosclerosis, in which secondary necrosis of cholesterol-laden apoptotic macrophages is associated with plaque instability (24).

In addition, we showed that Gas6 is able to significantly enhance IL-10 secretion via MerTK from M-CSF–cultured cells stimulated with LPS. This finding suggests that M-CSF–driven, IL-10–dependent, M2c macrophages may use this pathway as a positive feedback loop to strengthen and prolongate secretion of IL-10 in the microenvironment, facilitating in this way the reestablishment or the persistence of anti-inflammatory conditions. There are striking parallels with the heme oxygenase 1 (HO-1) pathway, inducible by IL-10 and promoting IL-10 secretion in M-CSF–driven $CD163^+$ (i.e., M2c) macrophages (56). Stimulating Gas6 and MerTK activity in $CD163^+$ macrophages to increase IL-10 production may represent a promising strategy to treat some inflammatory diseases in which $CD163^+$ macrophages and IL-10 production are reduced, such as in atherosclerotic lesions (57, 58). Conversely, blocking the Gas6/MerTK pathway in tumor-associated $CD163^+$ macrophages may be critical to reduce IL-10

production and immune tolerance to cancer (59). The prognostic relevance of TAM receptors in cancer is so far uniquely attributed to the aberrant expression of Axl and MerTK in tumor cells, which may promote cell survival and proliferation in response to Gas6 released by tissue macrophages (26, 27). Because tumor-associated macrophages are characterized by an M2c phenotype (59), it is tempting to speculate that MerTK is overexpressed also in these nonmalignant cells that infiltrate and surround the tumor. In this view, Gas6 might foster tumor growth by also acting on macrophages in an autocrine/paracrine manner; macrophage secretion of anti-inflammatory cytokines mediated by MerTK would ultimately result in suppression of antitumor immune responses.

In conclusion, we provide evidence that M2c polarization is desirable to ensure efficient clearance of early ACs by human macrophages and monocytes, owing to intense MerTK up-regulation. The presence of both M-CSF and IL-10 is needed to differentiate M2c macrophages, implying that T cell release of M-CSF, along with IL-10, can be crucial for induction of anti-inflammatory monocytes-macrophages by Tregs. M-CSF and IL-10 may have therapeutic utility in the treatment of autoimmune diseases and atherosclerosis, in which AC clearance is impaired. In addition, modulating MerTK activity in vivo (e.g., through recombinant Gas6 or activating and inhibitory mAbs) may constitute an effective strategy to attenuate or promote innate inflammation related to autoimmune, metabolic, and tumoral diseases.

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Disclosures

The authors have no financial conflicts of interest.

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