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IL-17 Stimulates Differentiation of Human Anti-Inflammatory Macrophages and Phagocytosis of Apoptotic Neutrophils in Response to IL-10 and Glucocorticoids

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Exposure of human monocytes/macrophages to anti-inflammatory agents, such as IL-10 or glucocorticoids, can lead to two separate fates: either Fas/CD95-mediated apoptosis or differentiation into regulatory and efferocytic M2c (CD14^{bright}CD16^{-}CD163^{-}Mer tyrosine kinase^{+}) macrophages. We found that the prevalent effect depends on the type of Th cytokine environment and on the stage of monocyte-to-macrophage differentiation. In particular, the presence of IFN-γ (Th1 inflammation) or the prolonged exposure to IL-4 (chronic Th2 inflammation) promotes apoptosis of monocytes/macrophages and causes resistance to M2c differentiation, thus provoking impaired clearance of apoptotic neutrophils, uncontrolled accumulation of apoptotic cells, and persistent inflammation. In contrast, the presence of IL-17 (Th17 environment) prevents monocyte/macrophage apoptosis and elicits intense M2c differentiation, thus ensuring efficient clearance of apoptotic neutrophils and restoration of anti-inflammatory conditions. Additionally, the Th environment affects the expression of two distinct Mer tyrosine kinase isoforms: IL-4 down-regulates the membrane isoform but induces an intracellular and Gas6-dependent isoform, whereas IFN-γ downregulates both and IL-17 upregulates both. Our data support an unexpected role for IL-17 in orchestrating resolution of innate inflammation, whereas IFN-γ and IL-4 emerge as major determinants of IL-10 and glucocorticoid resistance. The Journal of Immunology, 2013, 190: 5237–5246.

Innate inflammation is the first line of defense against microbes. Once the pathogen is removed or neutralized, a variety of mechanisms act to downregulate innate inflammation and protect tissues from uncontrolled chronic injury. Such mechanisms include apoptosis of monocytes/macrophages (1–4), switch of proinflammatory (classically activated) into regulatory (alternatively activated) macrophages (5–7), and apoptosis of activated neutrophils with subsequent phagocytosis (8, 9).

Recent data highlight that a discrete subset of alternatively activated (M2) macrophages, induced by glucocorticoids or by M-CSF plus IL-10 and called “M2c,” are importantly involved in resolution of innate inflammation, thanks to their unique ability to phagocytose early apoptotic neutrophils (ANs) and to release anti-inflammatory cytokines (5, 10–13). In particular, the upregulation of Mer receptor tyrosine kinase (MerTK) by these macrophages is crucial for intense efferocytosis and IL-10 production following apoptotic cell recognition and/or Gas6 stimulation (10–12, 14). Besides turning activated monocytes/macrophages into regulatory cells, another way to silence proinflammatory monocytes/macrophages is to induce their apoptosis. Some studies have shown that glucocorticoids and IL-10 also have proapoptotic effects on monocytes/macrophages (1–4). However, increased macrophage apoptosis may interfere with phagocytosis of ANs, and so contribute to further accumulation of apoptotic bodies. As such, macrophage apoptosis can be detrimental for resolution of innate responses; instead, it can stimulate abnormal adaptive responses and development of autoimmunity (15).

Optimal resolution of innate inflammation also depends on the fate of dying neutrophils recruited into inflamed tissues. In particular, early apoptosis of activated/aged neutrophils promotes resolution by inhibiting further neutrophil oxidative burst and the release of proinflammatory mediators, and by eliciting regulatory pathways in efferocytic macrophages (8–10). Regulatory pathways can also be induced by neutrophil release of ectosomes, which stimulate macrophage production of anti-inflammatory cytokines following MerTK-mediated recognition of phosphatidylserine exposed on their surface (16). In contrast, some alternative fates of activated neutrophils can fuel ongoing inflammation, such as late apoptosis (secondary necrosis) and a newly described form of programmed cell death called NETosis (9, 17). Both secondary necrosis and NETosis generate extracellular release of chromatin and proteases, which may act as danger signals and supply antigenic material fostering chronic activation of immune system, uncontrolled tissue damage, and development of autoimmunity (18–25). Late apoptosis results from impaired clearance of early membrane-intact apoptotic cells (19, 26), as occurs in the presence of macrophages with low or absent MerTK expression (10, 18). Whether a prompt clearance of early ANs can also have a role in preventing NETosis remains to be determined. Altogether, multiple mechanisms aimed at resolving innate inflammation use MerTK to enhance regulatory activity of anti-inflammatory macrophages...
and to transmit regulatory signals from activated and dying neutrophils to surrounding macrophages. M2c polarization and MerTK induction lead to noninflammatory clearance of activated neutrophils and homeostatic control of innate immunity, whereas macrophage apoptosis and impaired clearance of early ANs exacerbate immune responses.

Activation of innate immune cells during disease occurs within a discrete immunopathological context, depending on polarization of adaptive immune response. In the present study, we investigated the specific influences of Th cytokines on macrophage responsiveness to M2c polarizing agents (IL-10 and glucocorticoids) in terms of monocyte/macroage apoptosis, M2c macroage differentiation, MerTK induction, and phagocytosis of ANs. We found that IL-17 strongly amplifies M2c differentiation and MerTK-dependent clearance of neutrophils, whereas IFN-γ and chronic exposure to IL-4 induce Fas/CD95-mediated apoptosis of monocytes/macrophages and uncontrolled accumulation of apoptotic neutrophils. Furthermore, the Th environment affects differential expression of surface and intracellular MerTK isoforms. Results suggest that the Th17 environment promotes successful resolution of innate inflammation by glucocorticoids and IL-10, whereas Th1 and chronic Th2 diseases may be resistant to therapies aimed at control of innate immunity.

Materials and Methods

Cell cultures

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 Technologies) according to the manufacturer’s instructions. Purity of CD14+ cells was >90%, as assessed by flow cytometry. CD14+ cells were cultured for 8 d in 24-well plates at 0.8 × 10^6 cells/ml in X-VIVO 15 (StemCell Technologies), according to the manufacturers’ instructions. Monocytes from buffy coats of healthy blood donors were isolated by 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 1640, labeled with 2.5 μM CFSE (Sigma-Aldrich), and incubated for 20 h at 37°C in 5% CO₂. Macrophage culture medium included: RPMI 1640 (Pierce), or goat anti-rabbit Abs (Santa Cruz Biotechnology). Immuno-}

blots were developed and visualized by ECL using Amersham ECL reagents (GE Healthcare).

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 1640, labeled with 2.5 μM CFSE (Sigma-Aldrich), and incubated for 20 h at 37°C in 5% CO₂. Macrophage/microage-conditioned annexin V (BD Biosciences) and propidium iodide (PI; Sigma-Aldrich) were used to measure apoptosis by flow cytometry. The composition of neutrophils routinely obtained after incubation was 71.7 ± 14.1% for early apoptotic cells (ACs) (annexin V+PI), 4.3 ± 2.0% for late ACs (annexin V+PI+), and 0.4 ± 0.2% for necrotic cells (annexin V+P). ANs were added for 30 min to cultured macrophages at a 5:1 ratio. Flow cytometry was used to quantify percentages of CD14-labeled macrophages that phagocytosed CFSE-labeled ACs. For inhibition studies, macrophages were preincubated with a goat polyclonal anti-human MerTK Ab (5 μg/ml; R&D Systems) or a goat polyclonal control IgG (5 μg/ml; SouthernBiotech) for 30 min before adding ANs.

Fluorescence microscopy

Cultured macrophages were stained in 24-well plates using an FITC-conjugated anti-CD95 Ab (BioLegend) and a biotinylated mouse monoclonal anti-CD16 Ab (AnCell), followed by incubation with Cy3-conjugated streptavidin (BioLegend). Cells were viewed under a Leica DMIRE2 inverted fluorescence microscope, which was controlled by Slidebook software.

ELISA

Gas6 levels were measured in supernatants of 4-d cell cultures in serum-free conditions using sandwich ELISA according to standard procedures (27). 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.

Statistical analysis

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

Results

Macrophage response to IL-10: IL-17, but not IFN-γ or IL-4, elicits M2c differentiation and MerTK-dependent effecorystosis

We examined the susceptibility of untreated (M0), IFN-γ-conditioned (M1), IL-4-conditioned (M2a), and IL-17-conditioned (hereafter called “M17”) macrophages to switch into M2c (CD14bright CD163^{MerTK+}) cells in response to IL-10. We found that the IL-10 effects on macrophage polarization were significantly enhanced by IL-17. Although detectable, IL-10 effects were weak and/or did not reach full statistical significance in the absence of IL-17. Along with a potent induction of CD163, the combination of IL-17 plus IL-10 induced the highest levels of CD14 and was able to upregulate MerTK (Fig. 1A–D). In contrast, both IFN-γ and IL-4 inhibited M2c response, preventing IL-10 induction of CD163, CD14, and MerTK on the cell surface (Fig. 1A–D). Analogous results were obtained whether IL-10 was added from day 5 to already differentiated M1, M2a, or M17 macrophages, as a polarizing agent (Fig. 1A), or from day 0 to cultured monocytes together with IFN-γ, IL-4, or IL-17, as a differentiating agent (Fig. 1B–D). In the presence of IL-17, IL-10 also induced greater secretion of the MerTK ligand Gas6 compared with IL-10 alone (Fig. 1E). In accord with our previous results (10), IL-4 induced high levels of Gas6, but in this case the addition of IL-10 exerted no additive effect (Fig. 1E). Subsequently, we analyzed the ability of M0, M1, M2a, and M17 macrophages to phagocytose ANs...
following exposure to IL-10. Consistent with the MerTK expression pattern, IL-10 significantly increased phagocytosis of ANs only in M17 cells, and this effect was abrogated in the presence of an anti-MerTK blocking Ab (Fig. 1F).

Detection of phenotype markers and quantification of phagocytosis were not altered by macrophage autofluorescence, which was absent or negligible in our experimental conditions (Supplemental Fig. 1).

These data indicate that IL-17, but not IFN-γ or IL-4, potentiates the anti-inflammatory response of human macrophages to IL-10, resulting in more intense M2c polarization, greater secretion of Gas6, and enhanced clearance of ANs via MerTK.

**FIGURE 1.** Macrophage response to IL-10: IL-17, but not IFN-γ or IL-4, elicits M2c differentiation and MerTK-dependent efferocytosis. (A) Human monocytes were sorted from healthy PBMCs through negative selection magnetic beads and cultured in complete medium without cytokines (M0 differentiation) or in the presence of IFN-γ (10 ng/ml; M1 differentiation), IL-4 (20 ng/ml; M2a differentiation), or IL-17 (100 ng/ml; M17 differentiation). On day 5, cells were treated with IL-10 (50 ng/ml) for an additional 3 d (M2c polarization). Gray histograms show cells cultured with IL-10; white histograms show cells cultured without IL-10. Expression of the M2c markers CD14, CD163, and MerTK was measured by flow cytometry. (B–E) To measure autocrine production of Gas6 along with surface expression of M2c markers, cells were cultured for 4 d in serum-free medium with IFN-γ, IL-4, or IL-17 in the presence or absence of IL-10. Expression levels of CD14 (B), CD163 (C), and MerTK (D) were assessed by flow cytometry and depicted as percentages of CD14 bright cells or mean fluorescence intensity (MFI) fold variation compared with levels obtained with culturing cells without IL-10. Gas6 levels (E) in culture supernatants were quantified by ELISA. Data are representative of four independent experiments. (F) Early ANs were obtained by incubating healthy human neutrophils in 10% FBS/RPMI 1640 for 20 h. CFSE-labeled ANs were added for 30 min, at a 5:1 ratio, to CD14-labeled macrophages differentiated for 7–8 d in complete medium without cytokines or in the presence of IFN-γ, IL-4, or IL-17, with or without IL-10 in the last 3 d. For inhibition studies, macrophages were preincubated with a goat polyclonal anti-human MerTK Ab (5 μg/ml; R&D Systems) for 30 min before the addition of ANs. Data are representative of four independent experiments. Pooled data are represented as mean values ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001.

IL-4 and IL-10 are both considered major anti-inflammatory cytokines. However, they induce different types of M2 macrophages, namely M2a for IL-4 (CD206*CD209*) and M2c for IL-10 (CD163*) (5, 10, 28). We examined the effects of combining IL-4 and IL-10 on macrophage phenotype. We found that IL-4 is dominant over IL-10. The great majority of cells incubated with both cytokines were in fact CD209*, including a minor population of double-positive CD209*CD163+ cells, whereas very few cells were single positive for CD163 (Fig. 2A). In the co-presence of IL-4 and IL-10, CD209 expression was even enhanced compared...
with cells treated with IL-4 alone, whereas CD206 was unchanged (Fig. 2B, 2C); conversely, membrane expression of the M2c receptors CD163, CD14, and MerTK was significantly decreased compared with IL-10 alone (Fig. 2B, 2C). As we showed above (Fig. 1E), both IL-10 and IL-4 stimulated Gas6 secretion. This is in apparent contradiction with IL-4 inhibition of the surface 200-kDa MerTK isoform (11). Because prolonged Gas6 stimulation was recently found to induce a poorly glycosylated intracellular isoform with lower molecular mass (150 kDa) (29), we investigated whether IL-4, as well as IL-10, might still upregulate the Gas6-dependent isoform. To facilitate the potential detection of this minority glycoform, we increased exposure time of Western blots (up to 30 min); additionally, we cultured cells with macrophage growth factors (M-CSF or GM-CSF) to prolong times of culture and therefore Gas6 exposure. In particular, because GM-CSF downregulates MerTK (10), it was used to decrease MerTK background levels at baseline to increase the contrast for potential new bands upon IL-4 or IL-10 treatment. Ultimately, we were able to clearly detect IL-4–specific induction of the 150-kDa MerTK isoform; IL-10 was able to upregulate both 200- and 150-kDa isoforms (Fig. 2D). The 150-kDa isoform was not detectable on the cell surface, as assessed by flow cytometry (Fig. 2E). These studies show that IL-4 exerts dominant effects on the M2 macrophage phenotype, impeding IL-10 induction of M2c cells. Although M2a macrophages do not express MerTK on their surface, they still express Gas6 and an intracellular 150-kDa MerTK glycoform.

**Early response to glucocorticoids: IL-17 and IL-4, but not IFN-γ, allow differentiation of M2c MerTK-dependent efferocytic macrophages**

We examined the susceptibility of M0, M1, M2a, and M17 macrophages to switch into M2c cells in response to glucocorticoids. For this purpose, we cultured monocytes in the presence or absence of IFN-γ, IL-4, or IL-17 with or without dexamethasone from day 0. We found that dexamethasone induced an M2c phenotype in all cell populations except for M1 macrophages. IFN-γ lowered expression levels of CD14 (Fig. 3A) and significantly suppressed the M2c marker CD16/FcγRIII (Fig. 3B). Lower expression of other M2c receptors (e.g., CD204, CD163) was also observed (not shown). Additionally, IFN-γ–treated cells were refractory to glucocorticoid upregulation of MerTK (Fig. 3C) and Gas6 (Fig. 3D). By Western blot, we observed dexamethasone-specific induction of the 200-kDa MerTK isoform and confirmed IFN-γ inhibition of MerTK (Fig. 3C). Lower expression of other M2c receptors (e.g., CD204, CD163) was also observed (not shown). Additionally, IFN-γ–treated cells were refractory to glucocorticoid upregulation of MerTK (Fig. 3C) and Gas6 (Fig. 3D). By Western blot, we observed dexamethasone-specific induction of the 200-kDa MerTK isoform and confirmed IFN-γ inhibition of MerTK (Fig. 3C). In line with higher levels of Gas6 in supernatants (Fig. 3F), cell treatments with dexamethasone in the presence of IL-4 or IL-17 resulted in the upregulation of the intracellular 150-kDa MerTK isoform (Fig. 3E). Subsequently, we tested dexamethasone effects on phagocytosis of ANs by M0, M1,
M2a, and M17 macrophages. For inhibition studies, we used a goat polyclonal anti-MerTK neutralizing Ab whose specificity was preliminarily verified by comparing its effects with those exerted by a goat polyclonal control IgG (Fig. 4A, 4B). Consistent with IFN-γ resistance to glucocorticoid induction of MerTK (Fig. 3), cells cultured with IFN-γ plus dexamethasone showed significantly

FIGURE 3. Early response to glucocorticoids: IL-17 and IL-4, but not IFN-γ, allow M2c differentiation and MerTK/Gas6 induction. Monocytes were cultured for 4 d in serum-free medium with or without dexamethasone from day 0 (100 nM; M2c differentiation) in the presence or absence of IFN-γ (10 ng/ml), IL-4 (20 ng/ml), or IL-17 (100 ng/ml). Expression levels of CD14 (A), CD16 (B), and MerTK (C) were measured by flow cytometry and depicted as mean fluorescence intensity (MFI) fold variation compared with untreated cells. Gas6 levels (D) in culture supernatants were measured by ELISA. Expression of 200- and 150-kDa MerTK isoforms (E) was assessed by Western blot. Data are representative of four independent experiments. Pooled data are represented as mean values ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001.

FIGURE 4. Early response to glucocorticoids: IL-17 and IL-4, but not IFN-γ, allow differentiation of MerTK-dependent efferocytic macrophages. (A–D) CFSE-labeled ANs were added for 30 min, at a 5:1 ratio, to CD14-labeled macrophages differentiated for 7–8 d in complete medium with or without dexamethasone from day 0 (100 nM; M2c differentiation) in the presence of absence of IFN-γ (10 ng/ml), IL-4 (20 ng/ml), or IL-17 (100 ng/ml). For inhibition studies, macrophages were preincubated with a goat polyclonal anti-human MerTK Ab (5 μg/ml; R&D Systems) for 30 min before the addition of ANs. (A and B) Specificity of anti-MerTK Ab was preliminarily assessed by comparing its effects with those exerted by a goat polyclonal control IgG (5 μg/ml; SouthernBiotech). All data shown are representative of four independent experiments. Pooled data are represented as mean values ± SEM. *p < 0.05, **p < 0.01.
lower clearance of ANs compared with macrophages differentiated in the presence of dexamethasone alone or dexamethasone plus IL-4 or IL-17 (Fig. 4C, 4D). Moreover, glucocorticoid induction of efferocytosis by M1 cells was not dependent on MerTK. In contrast, dexamethasone effects on efferocytosis by M0, M2a, and M17 were significantly reduced by the addition of an anti-MerTK blocking Ab (Fig. 4C, 4D). These data indicate that a Th1 environment promotes monocyte resistance to differentiation into anti-inflammatory and efferocytic macrophages in response to early glucocorticoid treatment. In contrast, Th2 or Th17 conditions allow optimal glucocorticoid differentiation of monocytes into MerTK+ M2c cells.

Late response to glucocorticoids: IL-17, but not IFN-γ or IL-4, promotes MerTK-dependent efferocytosis by terminally differentiated macrophages

The first 24 h of culture are critical for glucocorticoid-mediated augmentation of AC clearance by macrophages (30). We observed, in fact, that the addition of dexamethasone at later stages of macrophage differentiation (from day 3 or day 5) gave a weaker stimulation of efferocytosis compared with dexamethasone treatment from day 0; phagocytic activity progressively decreased as glucocorticoid treatment was delayed (Fig. 5A). Strikingly, in the presence of IL-17, the glucocorticoid promotion of efferocytosis was instead preserved over time. When dexamethasone was given from day 5, IL-17 was able to rescue dexamethasone induction of highly efferocytic (CFSE	ext{bright}) cells, causing an effect analogous to what was seen with early glucocorticoid treatment (Fig. 5A, 5B). In contrast, in the absence of IL-17, CFSE	ext{bright} macrophages were significantly fewer than with early glucocorticoid treatment (Fig. 5B). Addition of dexamethasone from day 5 did not result into a clearcut augmentation of total efferocytic (CFSE+) macrophages (Fig. 5C, 5D). Similar results were obtained in the presence of IFN-γ (Fig. 5C, 5D). IL-4 facilitated glucocorticoid-induced efferocytosis, yet this was independent of MerTK activity (Fig. 5C, 5D). Remarkably, IL-17–differentiated macrophages exhibited the greatest response to dexamethasone, and M17 phagocytosis of ANs was significantly abrogated by blocking MerTK (Fig. 5C, 5D). These results indicate that a Th17 environment uniquely disposes...
terminally differentiated macrophages to highly efficient clearance of ANs in response to glucocorticoid treatment by rescuing and amplifying M2c differentiation and MerTK induction.

To exclude that our results might have been conditioned by potential endotoxin contamination of reagents, we conducted control experiments looking at the effects of IL-10 and dexamethasone on macrophage phenotype and phagocytosis of ANs in the presence or absence of LPS. LPS did not significantly affect macrophage response to IL-10 and dexamethasone, added either on day 0 or on day 5 (Supplemental Fig. 2).

Response to IL-10 and glucocorticoids: IL-17, but not IFN-γ or IL-4, protects macrophages from CD95 upregulation and apoptosis

We explored the effect of IFN-γ, IL-4, or IL-17 on monocyte susceptibility to apoptosis following exposure to glucocorticoids or IL-10. Resistance of IFN-γ–cultured cells to glucocorticoid induction of MerTK and M2c markers such as CD16 was associated with unchanged cell morphology upon treatment, as indicated by unmodified forward and side scatter at flow cytometry (Fig. 6A). In the presence of IFN-γ, indeed, either glucocorticoids or IL-10 significantly induced Fas/CD95 expression (Fig. 6B) and apoptosis, as assessed by increased proportions of CD95+annexin V+ cells (Fig. 6C). PI staining, indicative of cell death by necrosis, was instead negative (not shown). Dexamethasone upregulation of CD95 was inversely related to MerTK induction (Fig. 6D, 6E). Specifically, the presence of IFN-γ allowed CD95 upregulation and prevented MerTK expression, whereas the presence of IL-17 allowed MerTK upregulation and prevented CD95 expression (Fig. 6D–F). The presence of IL-4 variably influenced glucocorticoid responses depending on the stage of macrophage differentiation: early dexamethasone administration allowed MerTK upregulation (Fig. 6D, 6E), whereas late administration increased CD95 expression and prevented MerTK induction (Fig. 6E). As for MerTK, an inverse relationship with CD95 expression was found for the expression pattern of M2c markers such as CD16 (Fig. 6F). These data indicate that M2c macrophage differentiation and macrophage apoptosis upon glucocorticoid or IL-10 stimulation are mutually exclusive responses, and that the final effect is primarily influenced by the Th cytokine environment and by the timing of cell stimulation. Th1 and protracted Th2 environments program monocytes/macrophages to respond to anti-inflammatory treatments by undergoing apoptosis, thereby preventing MerTK expression and phagocytosis of ANs. In contrast, the Th17 environment constantly protects macrophages from apoptosis and elicits active resolution of macrophage inflammation with noninflammatory clearance of ANs. Our principal findings regarding cytokine and glucocorticoid effects of macrophage differentiation and phagocytosis are summarized in Table I.

Discussion

Macrophages are, at once, the main effectors and regulators of innate inflammation. Owing to macrophage plasticity, intervention of anti-inflammatory agents in the microenvironment can promptly

FIGURE 6. Response to IL-10 and glucocorticoids: IL-17, but not IFN-γ or IL-4, constantly protects macrophages from CD95 upregulation and apoptosis. (A) Cells were cultured for 4 d in serum-free medium with or without dexamethasone from day 0 (100 nM; M2c differentiation) in the presence or absence of IFN-γ (10 ng/ml). Panels representing CD16 and MerTK expression, along with panels representing side scatter (SSC) and forward scatter (FSC) are shown. Data shown are representative of four independent experiments. (B–D) Cells were cultured for 3 d in complete medium in the presence or absence of IFN-γ (10 ng/ml; M1 differentiation), IL-4 (20 ng/ml; M2a differentiation), or IL-17 (100 ng/ml; M17 differentiation) with or without IL-10 (50 ng/ml) or dexamethasone (100 nM) from day 0. Apoptosis of monocytes/macrophages was assessed by flow cytometry using CD95 (B–D), annexin V (C), and PI (not shown) stainings. Data are representative of four independent experiments. (E and F) Macrophages were differentiated for 8 d in complete medium in the presence or absence of IFN-γ, IL-4, or IL-17; dexamethasone was added to some cell cultures from day 5. Expression of markers of macrophage apoptosis (CD95) and M2c polarization (MerTK, CD16) was assessed by Western blot (E) and fluorescence microscopy (original magnification ×40) (F). Pooled data are represented as mean values ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001.
shift macrophages from proinflammatory to regulatory suppressive cells (10, 31). In this study, we show that the response of human monocytes and macrophages to anti-inflammatory mediators is dramatically influenced by polarization of the coexisting adaptive response. The presence in the microenvironment of the Th cytokines IFN-γ, IL-4, or IL-17 has crucial effects on the overall outcome of monocyte/macrophage exposure to IL-10 and glucocorticoids in terms of regulatory M2c macrophage differentiation, macrophage phagocytosis of ANs, or monocyte/macrophage apoptosis. Owing to dynamic changes of immunological environment during in vivo inflammation, the study was conducted on well-determined macrophage subsets obtained after in vitro polarization. The main findings are summarized in Table 1.

We found that the IFN-γ/Th1 environment makes monocytes/macrophages highly resistant to M2c polarization and phagocytosis of ANs. In this regard, Heasman et al. (32) previously reported inhibition of glucocorticoid augmentation of apoptotic cell clearance; however, these authors failed to elucidate the mechanisms of such phenomenon. In this study, we determined that IFN-γ potently inhibits glucocorticoid and IL-10 differentiation of human monocytes into regulatory M2c macrophages (CD14\(^{high}\)CD16\(^{high}\)CD163\(^{+}\) MerTK\(^{+}\)Gas6\(^{+}\)) macrophages; similarly, terminally IFN-γ-differentiated M1 macrophages do not switch into M2c cells upon treatment. M2c macrophages are, in fact, a subset of alternatively activated macrophages (5) that we recently characterized as highly specialized in the clearance of early (membrane-intact) apoptotic cells due to intense MerTK upregulation (10). ANs maintain their intact membranes longer than most other apoptotic cells (9) and, consistently, MerTK has been shown to play a central role in their clearance (33).

In the presence of IFN-γ, monocytes/macrophages show abnormal responses to IL-10 and glucocorticoids; instead of differentiating into M2c cells, they upregulate the proapoptotic receptor Fas (CD95) and undergo apoptosis. Increased apoptosis may explain impaired M2c differentiation and effecroycosis; alternatively, because MerTK exerts an important antiapoptotic role in macrophages (34), it is also possible that defective MerTK expression promotes apoptosis.

The IL-4/Th2 environment allows glucocorticoids to differentiate monocytes into M2c macrophages; however, terminally IL-4-differentiated M2a macrophages are refractory to switching into M2c cells in response to either glucocorticoids or IL-10. Similarly to IFN-γ, chronic exposure to IL-4 results, instead, in downregulation of MerTK and M2c receptors and increased expression of CD95.

By inducing monocyte/macrophage apoptosis and preventing phagocytosis of ANs, IFN-γ and IL-4 may promote autoimmune responses. In fact, coincubation of serum from lupus patients with healthy macrophages induces macrophage apoptosis (26), and eliciting macrophage apoptosis exacerbates autoimmunity in lupus models (15). Additionally, in lupus and other chronic inflammatory diseases, impaired clearance of early ANs accounts for late apoptosis, tissue damage, and aberrant immune activation (17–20, 24, 26).

Th1 and chronic Th2 inflammation may represent major determinants of macrophage resistance to the anti-inflammatory effects of IL-10 and glucocorticoids. In the presence of IFN-γ and IL-4, IL-10 may have roles different from M2c polarization and immune suppression: in Th1 diseases and lymphoproliferative disorders, as well as in organ rejection, IL-10 may cooperate with IFN-γ in promoting expansion of B and/or NK cells (7); in chronic Th2 diseases and fibrotic disorders, IL-10 may enhance IL-4 induction of remodeling M2a macrophages (35, 36).

Resistance to glucocorticoids is frequently observed in clinical practice (37). Several IFN-γ-mediated pathologies, including atherosclerosis, acute respiratory distress syndrome, or many cases of inflammatory bowel disease, have poor or no response to glucocorticoids (38–40); similarly, refractory or relapsing forms of vasculitis show persistent elevation of IFN-γ levels in tissues despite glucocorticoid treatment (41). IL-4 overexpression is involved in glucocorticoid resistance of several chronic Th2 diseases characterized by impaired apoptotic cell clearance and fibrotic remodeling, such as chronic asthma, chronic obstructive pulmonary disease, and idiopathic pulmonary fibrosis (20, 21, 37, 42, 43); also, glucocorticoid-resistant asthma is associated with local production of IFN-γ (44). Our data provide new insights into the mechanisms of IL-10 and glucocorticoid resistance in these diseases, showing that IFN-γ and IL-4 strongly inhibit the expansion of M2c regulatory macrophages, thus preventing efficient effecrocyosis and restoration of anti-inflammatory conditions. From this view, glucocorticoid-resistant airway fibrotic remodeling may reflect refractoriness of fibrogenic M2a macrophages to M2c polarization.

Strikingly, the IL-17/Th17 environment significantly amplifies the anti-inflammatory effects of IL-10 and sustains macrophage susceptibility to glucocorticoids. Facilitation of M2c polarization by IL-17 is associated with macrophage protection from CD95.
upregulation and apoptosis; this is consistent with the previous demonstration in a mouse model of allergen-induced airway inflammation that IL-17 inhibits CD95 expression and prolongs survival of bronchoalveolar macrophages (45). To date, IL-17 has been identified as a proinflammatory cytokine importantly involved in immune responses against infections (46), in chronic inflammation, and in autoimmunity (47, 48). Nonetheless, recent studies suggest that IL-17 effects are more complex than what was initially thought. In fact, IL-17 not only stimulates granulopoiesis and neutrophil recruitment to sites of inflammation (48–50), but it also promotes neutrophil apoptosis (51–53) and macrophage phagocytosis of ANs (53). Thus, IL-17 could orchestrate multiple steps in acute inflammation, including its resolution once the triggering agent has been neutralized. Silverpil et al. (53) recently reported that IL-17 is able to stimulate phagocytosis of ANs by murine bronchoalveolar macrophages. In humans, instead, we found that IL-17 is not responsible per se for phagocytosis, but it significantly amplifies and prolongs IL-10 and glucocorticoid induction of phagocytosis. It is noteworthy, however, that Silverpil et al. (53) primed macrophages with LPS before IL-17 administration; therefore, autocrine production of IL-10 induced by LPS (54) could have allowed macrophage exposure to IL-17 and IL-10 in combination. Intervention of IL-10 in the microenvironment is probably required to convert IL-17 from a proinflammatory to a regulatory molecule. The coexistence of IL-17 and IL-10, in fact, disposes innate immune cells to restore anti-inflammatory conditions. IL-10 alone is poorly effective on otherwise untreated M0 macrophages, whereas IL-17 enables M17 macrophages to respond to IL-10 efficiently, in terms of M2c polarization, MerTK expression, Gas6 production, and M2c-related phagocytosis of ANs via MerTK. Our findings are consistent with previous data reporting the efficient role of IL-10 in suppressing production of proinflammatory cytokines by macrophages exposed to IL-17 (55). The presence of both IL-17 and IL-10 in the milieu can be attributed to multiple cell sources, including both T cells and macrophages. In fact, Th17 cells differentiated in the presence of high levels of TGF-β and/or low levels of IL-23 are able to secrete both cytokines (56); moreover, IL-10 inhibits T cell production of IFN-γ, but it does not suppress IL-17 production by Th17 cells (57), thus favoring coexistence of IL-17. Finally, IL-17 stimulates IL-10 production by macrophages themselves (58).

The facilitating role of IL-17 on the clearance of ANs may constitute an important feedback loop controlling the effects of IL-17 on granulopoiesis and acute inflammation. Consistent with this view, the activation of liver X receptors by ANs was recently reported in mice to elicit efficient clearance of senescent neutrophils via MerTK and to suppress the IL-23/IL-17 cytokine cascade (33). Additionally, we suggest that IL-17 could modulate innate immune responses by switching proinflammatory forms of neutrophil programmed cell death into regulatory ones. It is noteworthy, in fact, that recognition of late apoptotic blebs by APCs stimulates T cell production of IL-17 (58); moreover, IL-17 is released by neutrophils themselves during NETosis (25). By eliciting macrophage phagocytosis of early ANs, IL-17 may eventually redirect the fate of dying neutrophils toward resolution of innate inflammation and tissue homeostasis.

Our findings may have therapeutic implications. Th17-mediated disease flares can promptly respond to glucocorticoid therapy, whereas Th1 and chronic Th2 inflammation may cause glucocorticoid resistance. Detection of high levels of IFN-γ and IL-4 in inflamed tissues may suggest alternative anti-inflammatory (“steroid sparing”) treatments or the use of drugs that can reverse glucocorticoid resistance (e.g., MAPK inhibitors, vitamin D, theophylline, antioxidants) (37).

In the course of this study, we observed that different Th cytokine environments promote macrophage expression of different MerTK isoforms. Graham and colleagues (29) recently found that Gas6 induces an intracellular 150-kDa MerTK glycoform. In accord with Gas6 upregulation by IL-4, we observed that M2a macrophages specifically express the 150-kDa MerTK isoform. This observation helps us to interpret our previous finding that IL-4 stimulates Gas6 production by M2a macrophages but does not upregulate MerTK on their membrane (10). Consistent with the higher levels of Gas6 produced by glucocorticoids in the presence of IL-17 compared with glucocorticoids alone, the 150-kDa MerTK isoform was also detectable in the presence of IL-17. The intracellular MerTK glycoform is not involved in membrane recognition of apoptotic cells, but it might play a role in gene modulation, as suggested by its capability to translocate to nucleus (29). Further investigation will be needed to better define its role.

The cytokines tested in the present study were used at standard doses, on the basis of previous similar in vitro studies (ranging from 10 to 100 ng/ml). For many of these cytokines, previous in vivo studies have determined their plasma levels, generally lower than the concentrations used in this study (in the range of picograms per milliliter). However, plasma levels do not reflect the local tissue levels reached in sites of inflammation. We chose concentrations of cytokines to reflect their peak bioactivity and to detect and compare their full effects.

Although macrophage autofluorescence might complicate flow cytometry analysis, we did not clearly observe autofluorescent cell populations. Accordingly, previous data reported that autofluorescence is low or negligible in monocytes/macrophages cultured in vitro for a few days (59). Macrophage autofluorescence is instead a characteristic of resident tissues macrophages (e.g., murine bone marrow and peritoneal macrophages, human alveolar macrophages) and might be in part due to in vivo uptake of remnants and environmental particles (60).

In conclusion, different Th cytokine environments determine different responses of innate immunity to anti-inflammatory treatments. Our data highlight the unexpected role of IL-17 in orchestrating resolution of innate inflammation, by amplifying and protracting macrophage susceptibility to M2c differentiation and phagocytosis of ANs. In contrast, IFN-γ and IL-4 limit the expansion of regulatory macrophages and might elicit autoimmunity owing to induction of apoptotic macrophages and uncontrolled accumulation of ANs.

Disclosures

The authors have no financial conflicts of interest.

References

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Supplementary Figure 1. Poor autofluorescence of cultured monocytes-macrophages. (A-B) Cells were cultured for 4 days in serum-free medium. Cells were analyzed unstained, to detect autofluorescent cells, and after incubation with fluorochrome-conjugated antibodies against surface antigens (MerTK, CD163, CD204, CD16, CD14), as positive controls. Data are representative of four independent experiments. Pooled data are represented as mean values ± SEM. (C) Cells were cultured for 8 days in complete medium. CD14-labeled macrophages were then analysed alone, to detect FITC autofluorescent cells, or after co-incubation with CFSE-conjugated (FITC-labeled) ANs, as positive control. A representative experiment of three is shown.

Supplementary Figure 2. Effects of LPS on macrophage response to IL-10 and glucocorticoids. (A-B) Cells were cultured for 4 days in serum-free medium with or without LPS (10 μg/ml), and in the presence or absence of IL-10 (50 ng/ml) or dexamethasone (100 nM). Expression of MerTK and CD163 was analyzed. (C-D) Macrophages were differentiated for 8 days in complete medium and treated with IL-10 ± LPS from day 5 or with dexamethasone ± LPS from day 0 or from day 5. CFSE-labeled ANs were then added for 30 minutes, at a 5:1 ratio, to CD14-labeled macrophages. Rate of macrophage phagocytosis of ANs was calculated. All data shown are representative of three independent experiments. Pooled data are represented as mean values ± SEM. *P <0.05; ***P <0.001; n.s., not significant.