Alternative Activation of Human Macrophages Is Rescued by Estrogen Treatment In Vitro and Impaired by Menopausal Status

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Context and Objective: During their reproductive years, women are generally protected from cardiovascular disease events by their estrogen-replete status. Our starting hypothesis was that lower estrogen levels after menopause are associated with macrophage activation profiles skewed toward proinflammatory phenotypes.

Research Design and Setting: This was an in vitro and ex vivo study in human blood-derived macrophages.

Subjects: We obtained blood from 12 healthy male donors for the in vitro study and from 5 premenopausal and 8 postmenopausal women for the ex vivo study.

Outcome: We measured macrophage immunophenotypes in the resting state and after activation with M1-associated (lipopolysaccharide [LPS]/interferon-γ [IFN-γ]) or M2-associated (IL-4/IL-13) stimuli and expression of estrogen receptors (ERs) and other transcription factors.

Results: Unpolarized macrophages expressed both ERα and ERβ, and ERα but not ERβ levels were decreased by M1 stimuli. LPS/IFN-γ also induced down-regulation of CD163 and CD206, markers of alternative activation, and increased cell-bound TNF-α and IL-10. These effects were prevented by 17β-estradiol treatment through impaired nuclear factor-κB liberation. In agreement with a role for 17β-estradiol in attenuating the inflammatory response, M1/M2 subpopulations in monocytes and unstimulated macrophages from premenopausal and postmenopausal donors were similar. In contrast, M2 activation appeared to be blunted in macrophages from postmenopausal women, leading to an increased M1/M2 response ratio.

Conclusions: Estrogen treatment prevented LPS/IFN-γ action on human M2 macrophage markers and cytokine production, whereas menopausal estrogen loss was associated with an impaired response to alternative activation, suggesting that these mechanisms affect the cardiovascular risk profile in relation to menopausal status. **(*J Clin Endocrinol Metab* 100: E50–E58, 2015)**

Macrophages are a heterogeneous population of innate myeloid cells involved in health and disease (1), and their activation state can be influenced by a variety of cytokines and microbial products (2). The inflammatory response, particularly macrophages, is involved in the development of atherosclerosis and metabolic disease (3, 4). According to the current framework, macrophages can be polarized into classically (M1) or alternatively (M2)

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Abbreviations: ER, estrogen receptor; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GPER, G protein–coupled estrogen receptor; IFN, interferon; LPS, lipopolysaccharide; NF, nuclear factor; PE, phycoerythrin; STAT, signal transducer and activator of transcription.

activated cells representing 2 polar extremes of signals computed by macrophages (5). The M1 and M2 phenotypes were identified by expression of a variety of markers in different studies (6, 7). Evidence for M1/M2 activation in disease is accumulating (2, 8), yet it lacks defined subsets (9). We recently validated phenotypes that truly reflect circulating monocytes with different inflammatory potential, resemble the corresponding monocyte-derived macrophage populations, and are modulated by disease, specifically type 2 diabetes (10).

During their reproductive years, women are generally protected from disease events by their estrogen-replete status. Although the cardiovascular risk profile worsens in postmenopausal women, the causative mechanisms are incompletely understood (11). Estrogen has a number of effects on the cardiovascular system including the modulation of the inflammatory response and immune cell function (12, 13). These effects are largely mediated through estrogen receptors (ERs), which are expressed in monocytes and macrophages. Expression of ERα is greater than that of ERβ in both monocytes and macrophages, whereas macrophages express higher levels of ERα and lower levels of ERβ than monocytes (14, 15). ERβ transcript and protein are detected in primary monocyte-derived macrophages and are not regulated by estrogen levels in vitro (14, 16). Whereas the prominent contribution of other nuclear receptors to macrophage polarization in different settings is well established (13, 17), the ER expression profile in human macrophages in relation to their activation status has not been investigated. Estrogen has been shown to inhibit alternative activation of tumor-associated macrophages, resulting in attenuated hepatocellular carcinoma progression in mice (18). To the best of our knowledge, however, the ability of 17β-estradiol (E2) to interfere with functional phenotypes of blood-derived macrophages after diverse activation stimuli in relation to menopausal status has not been investigated so far.

Thus, we set out to explore the connection between the molecular designation of macrophage functional phenotypes and modulatory roles for estrogen. Because estrogen may induce an overall inhibitory effect on inflammation by suppressing proinflammatory pathways (12–16), our starting hypothesis was that macrophage functional phenotypes (1) are modulated toward attenuated inflammation by estrogen treatment in vitro and (2) display an enhanced inflammatory potential when macrophages differentiated from the peripheral blood of postmenopausal women are compared with those from women of fertile age. These mechanisms may in part be involved in the protection against cardiovascular disease in premenopausal women. We therefore investigated whether estrogen regulated human macrophage subsets and function in response to different activation stimuli in vitro and ex vivo in relation to menopausal status.

Materials and Methods

Study design/subjects

In vitro study

Deidentified buffy coats from 12 healthy male blood donors aged 18 to 35 years were obtained from the University of Padua Medical Center Transfusion Unit (operating under the supervision of the University of Padua institutional review board). We did not have any interaction with human subjects or protected information, and therefore no informed consent was required. Peripheral blood mononuclear cells from healthy male donors were used for in vitro experiments to limit the influence of endogenous estrogen levels on study endpoints.

Ex vivo study

Selected experiments were performed with blood samples taken from 5 premenopausal and 8 postmenopausal women. The protocol was approved by the University of Padua institutional review board and conducted in accordance with the Declaration of Helsinki as revised in 2000. Participants were recruited from the Division of Metabolic Diseases at Padua University Hospital. All consecutive subjects were deemed eligible, pending provision of informed consent and meeting of inclusion criteria (Supplemental Table 1). Descriptions and comparisons of participating premenopausal and postmenopause women are shown in Supplemental Table 2. These 2 populations were comparable for tested metabolic parameters but not for age. The ex vivo study was designed to extend the findings of the in vitro study to macrophages from female donors in relation to menopausal status.

Cell culture

For generation of human blood-derived macrophages, human peripheral blood mononuclear cells were isolated from buffy coats or freshly heparinized blood by density gradient centrifugation with Histopaque-1077 (Sigma-Aldrich). Cells were seeded at 1×10^6/mL in serum-free RPMI 1640 medium supplemented with 2 mM L-glutamine (Lonza), 100 U/mL penicillin, and 100 μg/mL streptomycin (Invitrogen). After 2 hours, nonadherent cells were removed by repeated washing, and the remaining adherent fraction was cultured for 7 days at 37°C and 5% CO_2 in the presence of 10% fetal calf serum (FCS) (EuroClone) in plastic tissue culture dishes (Corning Inc.). The culture medium was not replaced throughout the culture period, and to allow spontaneous differentiation into unpolarized (M0) macrophages, no further exogenous agent was added.

Protocols of macrophage activation

After replacement of the culture medium, M0 macrophages were polarized toward the M1 phenotype by incubation with lipopolysaccharide (LPS) (1 μg/mL; Sigma-Aldrich) and interferon-γ (IFN-γ) (10 ng/mL; PeproTech) for 48 hours. M2 polarization was obtained by addition of IL-4 (20 ng/mL) and IL-13 (5 ng/mL; both from PeproTech) for 48 hours. M0 cells were also pretreated with 100 nM dexamethasone or E2 (Sigma-Aldrich) overnight where indicated. Concentrations of LPS, IFN-γ, IL-4,

IL-13, dexamethasone, and E2 were chosen from previously published studies on related cells from the literature (5, 10, 14).

Morphological observations

At the end of activation protocols, the morphology of M0, M1, and M2 macrophages was inspected by phase contrast microscopy (×20 or ×40 magnification) using a Nikon Eclipse Ti-S microscope.

Flow cytometry

Surface marker expression

At the end of stimulations, cells were washed and harvested by gently scraping the culture plates with 1 mL of PBS containing 5 mM EDTA and 2% FCS. Purity was assessed by staining with phycoerythrin (PE) anti-CD14 and was not less than 90% after 8 days in culture. Resting and activated macrophages were labeled with fluorochrome-tagged monoclonal antibodies against surface CD68-fluorescein isothiocyanate (FITC), CD80-PE, and CCR2-allophycocyanin to typify the M1 phenotype and against CD206-FITC, CD163-PE, and CX3CR1-PerCP to characterize the M2 phenotype. This panel of M1/M2 markers was selected based on a recent characterization (10). All antibodies were purchased from BD Biosciences. Analyses were performed on a FACSCanto II flow cytometer (BD Biosciences), and data were analyzed using FACSDiva software. Isotype-matched controls were used as a reference. Typically, <2% positive cells were allowed beyond the statistical marker in appropriate controls.

Cell-bound cytokine assays

After spontaneous differentiation, macrophages were stimulated for 6 to 48 hours as indicated in the presence of 10 μM brefeldin (Sigma-Aldrich) to block cytokine secretion. After activation, cells were collected in a round-bottom tube for flow cytometry and processed for immunostaining. Macrophages were fixed with 100 μL of 4% paraformaldehyde solution and permeabilized by addition of 2 mL of Perm Buffer solution (1×, eBioscience) containing 0.1% saponin and 0.009% sodium azide. Cells were centrifuged twice and incubated in 100 μL of Perm Buffer solution in the presence of PE anti-TNFα, FITC anti-IL-1β, PE anti-IL-10, or PE anti-CCL22 (5 μL/1 $\times$ 10^6 cells) for 20 minutes in the dark. To measure monocyte-derived cytokines in whole-blood samples, the monocyte subset was analyzed by gating $CD14^+$ cells.

Gene expression analysis

RNA was extracted using an RNeasy Plus Mini Kit (QIAGEN). cDNA was generated from 200 ng of total RNA (NanoDrop Thermo Scientific) using RevertAid Reverse Transcriptase (Fermentas) and random primers according to the manufacturer's instructions. The relative quantification of the genes of interest was measured by real-time quantitative PCR performed using Max SYBR Green PCR Master Mix for 40 cycles of denaturation (15 seconds, 95°C), annealing (30 seconds, 60°C), and extension (30 seconds, 72°C) on a CFX96 real-time PCR detection system thermocycler (Bio-Rad). Oligonucleotide primers were designed using the online tool for real-time PCR BLAST and obtained from Invitrogen. The primer sequences are shown in Supplemental Table 3. Results were normalized to a *18S* reference and analyzed using the $2^{\Delta\Delta}C_t$ method.

Western blot

After activation, macrophages were washed and harvested in 150 μL of lysis buffer (50 mM Tris/HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 25 mM NaF, 0.5% sodium deoxycholate, 10% SDS, 1 mM EGTA, 1 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 1 mM phenylmethylsulfonyl fluoride, and Complete protease inhibitors from Roche). Cell protein lysates (40 μg) were separated by SDS/polyacrylamide gel, blotted, and probed with specific primary antibodies (1:1000; Abcam). Immunolabeled proteins were detected by using appropriate horseradish peroxidase–conjugated secondary antibodies, and signals were revealed using enhanced chemiluminescence detection (Merck-Millipore). Band intensities were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and densitometry analysis was performed using ImageJ.

Immunocytochemistry

ER expression was also assessed by immunocytochemistry. Macrophages were cultured in 24-well plates, fixed with ice-cold 95% ethanol for 30 minutes at room temperature, permeabilized using 0.1% Triton X-100 for 1 minute, and treated with 0.3% hydrogen peroxide for 30 minutes. Nonspecific reactive sites were blocked using PBS with 2% fetal bovine serum. Macrophages were incubated with rabbit or mouse monoclonal antibody toward human ERα and ERβ (both 1:50), respectively, for 2 hours at room temperature. After washing, cells were stained with anti-mouse or anti-rabbit biotinylated secondary antibody

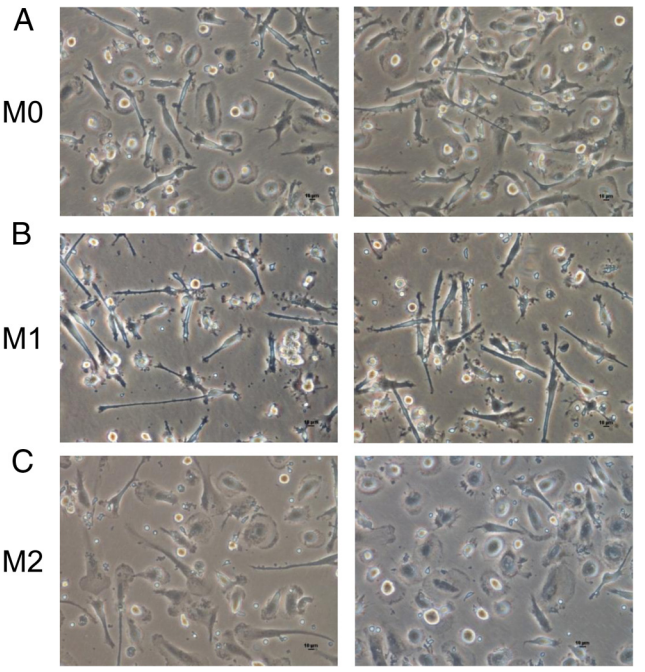

Figure 1. Morphotypes of spontaneously differentiated human macrophages. Human blood monocytes were grown in RPMI 1640 plus 10% FCS to produce monocyte-derived macrophages. These macrophages were incubated for 2 days with culture medium to produce unpolarized macrophages (M0, A), with LPS/IFN-γ to produce M1 macrophages (B), or with IL-4/IL-13 to produce M2 macrophages (C). Phase contrast images indicate the heterogeneous macrophage population and enrichment for the 2 main spindle-shaped and round morphotypes. Representative images were captured from macrophages from the same donor. Magnification, × 20; Nikon Eclipse Ti-S microscope.

(1:1000) for 30 minutes and subsequently incubated with streptavidin (ABC kit; Vector). The reaction was developed by adding a peroxidase substrate kit (Vector). Images were captured using a Nikon Eclipse Ti-S microscope at ×20 and ×40 magnifications and a Digital Sight DS-Fi1 camera.

Statistical analysis

Statistical analyses were performed using GraphPad Prism (version 5; GraphPad Software). Data are expressed as mean values ± SEM. ANOVA followed by a Bonferroni post hoc test was used for comparison between samples. A P value of ≤.05 was considered to be statistically significant.

Results

At the end of differentiation, unpolarized macrophages (M0) under phase contrast microscopy showed 2 dominant and distinct morphotypes (Figure 1), ie, spindle/elongated and round "fried-egg" shaped (19), which represented about 30% and 40% of adherent cells, respectively. The 2 morphotypes could also be distinguished by forward light scatter-side light scatter flow cytometry (data not shown). After classic (LPS/IFN-γ) activation for 48 hours, macrophages were enriched in the long and spindle-shaped morphotype (Figure 1), whereas alternatively (IL-4/IL-13) activated macrophages were largely round-shaped. These observations suggest that polarized activation affected macrophage morphology.

To investigate the potential roles of E2 in this truly heterogeneous population of spontaneously differentiated human monocyte-derived macrophages, we first assessed the expression profile of ER isoforms. Resting macrophages expressed both ER isoforms (Figure 2A). After 6 hours of stimulation with IL-4/IL-13 (M2), a transient increase in ERα mRNA levels was observed (Figure 2B) compared with that in M0. In contrast, after 48 hours of stimulation with LPS/IFN-γ (M1), mRNA levels for ERα were lower with respect to that for M0. At this time point, ERα protein abundance was significantly down-regulated by 23% in M1 and unchanged in M2. ERβ mRNA (1.5 ± 0.5 in M1 and 1.3 ± 0.4 in M2 relative to that in M0, n = 6) and protein levels (Figure 2C) were comparable in all subsets. G protein–coupled estrogen receptor (GPER)-1 mRNA levels were close to the detection limits (ΔC_t GPER-1 − 18S > 20 in M0) and unchanged after activation (0.6 ± 0.5 for M1 and 1.6 ± 0.7 for M2 relative to that for M0, n = 3), suggesting that the effects of estrogen are probably not being mediated via GPER-1. These findings suggest that macrophage ERα expression is decreased by proinflammatory factors.

Next, the effects of E2 on macrophage activation marker expression were investigated. A significant decrease in the fraction of $CD206^+/CD163^+$ (M2) cells was observed after incubation with LPS/IFN-γ compared with that in resting cells (Figure 3). Treatment with 10 nM E2, however, restored the fraction of $CD206^+/CD163^+$ cells, as well as that of $CD206^+$ and $CD163^+$ cells (data not shown), in LPS/IFN-γ–activated but not in unpolarized or IL-4/IL-13–activated macrophages. Pretreatment with E2 did not change the fraction of $CD80^+/CCR2^+$ (M1) cells that was increased by LPS/IFN-γ activation (Supplemental Figure 1). Therefore, E2 was able to prevent LPS/IFNγ-induced down-regulation of alternative activation markers, rather than boosting them. For comparison, treatment with dexamethasone enhanced the fraction of $CD206^+/CD163^+$ macrophages across the board irrespective of the activation status (Supplemental Figure 2).

We then investigated the functional effect of E2 on intracellular cytokine accumulation induced by pro-

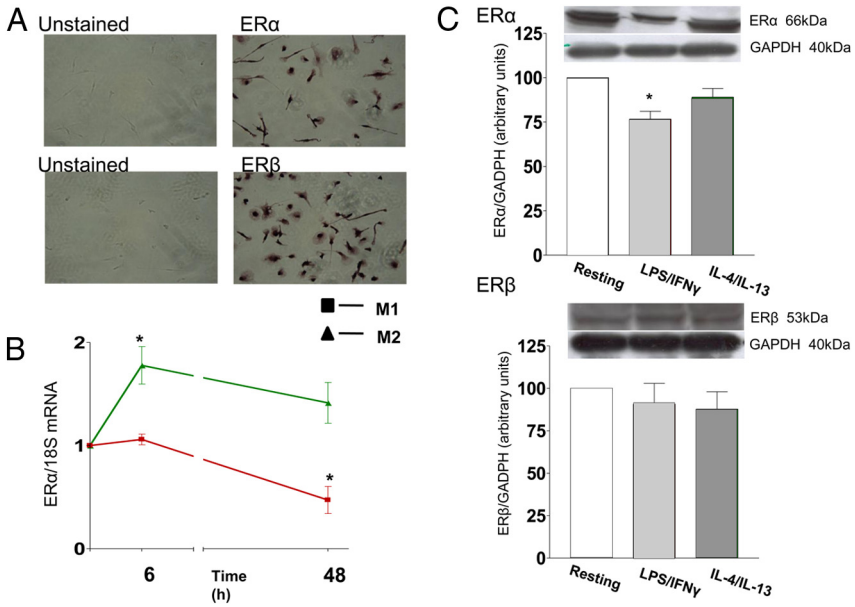

Figure 2. ER expression in spontaneously differentiated human macrophages. A, Immunocytochemical staining of ER isoforms in resting macrophages. After 7 days of spontaneous differentiation from blood monocytes, macrophages were incubated with antibodies against ERα and ERβ or vehicle. Positive staining was revealed using diaminobenzidine, which is reduced by peroxidase in the presence of hydrogen peroxide to form a brown precipitate. Magnification, ×20; Nikon Eclipse Ti-S microscope. B, ERα mRNA levels of LPS/IFN-γ–stimulated (M1) and IL-4/IL-13–stimulated (M2) macrophages were measured by quantitative PCR and normalized to 18S. Results are presented relative to M0, to which we assigned a value of 1. Data are shown as means ± SEM of n = 6 independent experiments. *, $P < .005$ vs resting. C, Immunoblot analysis of protein extracts from M0, M1, and M2 macrophages showing down-regulation of ERα, but not of ERβ, after 48 hours of activation with M1 but not M2 stimuli. GAPDH served as the loading control. Bars represent the means ± SEM of n = 6 independent experiments. *, $P < .005$ vs resting.

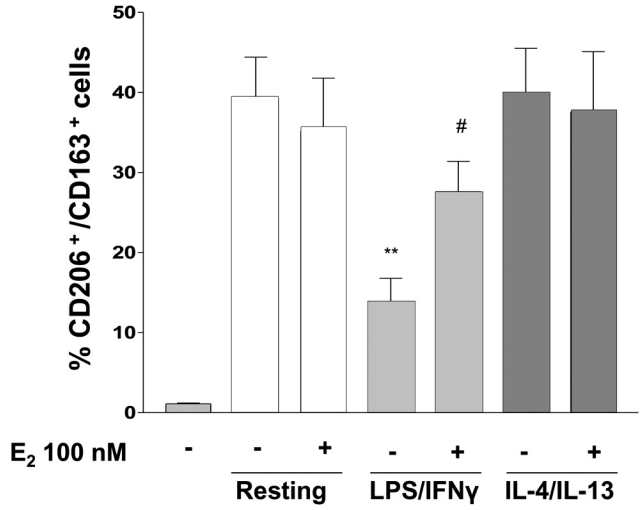

Figure 3. Effect of E2 on the $CD206^+/CD163^+$ (M2) macrophage subset. After 7 days of differentiation, human macrophages were pretreated with vehicle or 100 nM E2 for 16 hours and then activated to M0 (resting), M1 (LPS/IFN-γ), and M2 (IL-4/IL-13) for 48 hours. Cells were then stained with FITC anti-CD206– and PE anti-CD163-specific antibodies. Bars indicate the percentages of double-positive cells and represent the means ± SEM of n = 12 independent experiments. **, $P \leq .005$ vs resting; #, $P \leq .05$ vs untreated.

inflammatory stimuli. Minor fractions of TNF-α^+ (2.5 ± 0.6%) (Figure 4A) and IL-1β^+ macrophages (1.9 ± 0.6%) (Figure 4B) were detectable under resting conditions. The percentage of IL-10^+ cells under resting conditions was slightly higher (5.9 ± 2.3%) (Figure 4C). After 6 hours of activation with LPS/IFN-γ, the fractions of cells staining for cell-bound TNF-α (Figure 4A) and IL-1β (Figure 4B) were significantly increased. Unexpectedly, the percentage of IL-10^+ cells was not significantly increased by IL-4/IL-13, but by LPS activation (Figure 4C) (20, 21). Overnight pretreatment with E2 significantly decreased the percentage of both TNF-α^+ (Figure 4A) and IL-10^+ (Figure 4C) cells but did not affect IL-1β production (Figure 4B) in LPS/IFN-γ–activated macrophages. Treatment with dexamethasone as a reference agent reduced the fraction of cells expressing all of the above cytokines (Figure 4). Neither E2 nor dexamethasone affected LPS/IFN-γ–induced intracellular cytokine accumulation in freshly isolated monocytes (Supplemental Figure 3). Overall, these findings suggest that E2 prevented the effects of proinflammatory agents on polarized macrophage function. We further explored the modulation of CCL22, a Th2-related chemokine, by E2. Activation with IL-4/IL-13 for 48 hours, but not for shorter times (data not shown), led to a significant increase in the relative amount of $CCL22^+$ cells compared with that of resting macrophages (Figure 4D). E2 treatment did not affect IL-4/IL-13–induced CCL22 accumulation in cultured macrophages, suggesting that E2 was ineffective in enhancing the production of alternative activation mediators. Similarly, overnight pretreatment with dexamethasone did not change the percentage of CCL22-expressing cells in the presence of IL-4/IL-13 (Figure 4D).

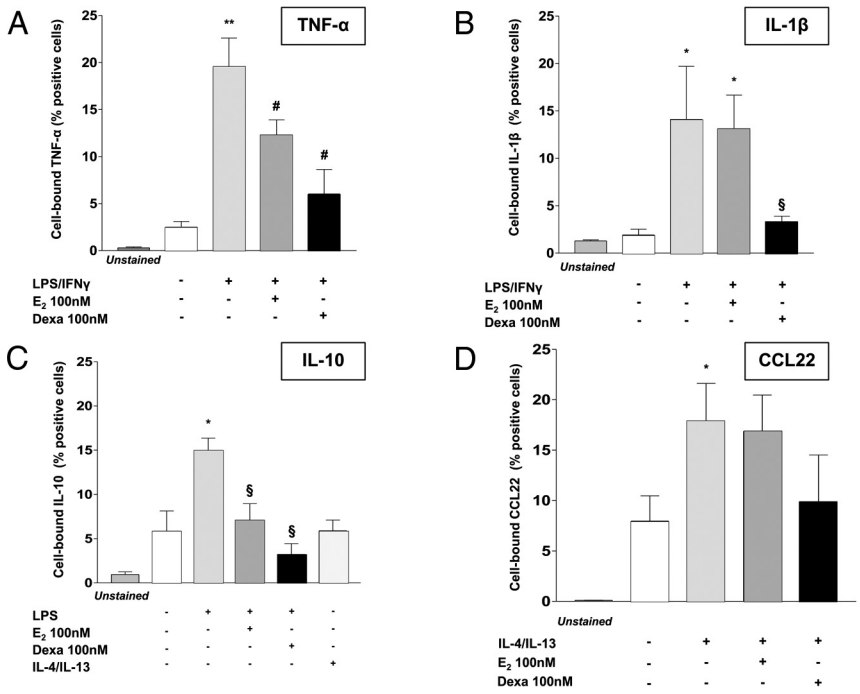

Figure 4. Effect of E2 on intracellular cytokine accumulation in human macrophages. After 7 days of differentiation, human macrophages were pretreated overnight with vehicle,100 nM E2, or dexamethasone (Dexa) as a reference compound for 16 hours and then were stimulated with LPS/IFN-γ for 6 hours (A and B) or LPS (C) or IL-4/IL-13 (C, D) for 24 hours in the presence of 10 μM brefeldin to disrupt cytokine secretion. Before flow cytometry analysis, fixed/permeabilized cells were stained with PE anti-TNF-α-specific (A), FITC anti-IL-1β-specific (B), PE anti-IL-10-specific (C), or allophycocyanin anti-CCL22-specific (D) antibodies. Bar graphs represent the means ± SEM of n = 5 to 8 independent experiments. *, $P < .05$, **, $P < .005$ vs resting; §, $P < .05$ vs untreated; #, $P < .05$ vs resting and vs untreated.

Several major transcriptional pathways have been identified as essential to macrophage subset specification (17). In particular, nuclear factor (NF)-κB is known to be induced under inflammatory stress to promote M1 polarization (22). Both E2 and dexamethasone treatment prevented IκB phosphorylation in the presence of M1-associated stimuli, thereby reducing the phosphorylated IκB/IκB ratio and NF-κB liberation (Figure 5).

The in vitro findings were validated in a pilot ex vivo study using peripheral blood monocytes and differentiated macrophages from premenopausal and postmenopausal donors. The phenotype of circulating monocytes did not significantly

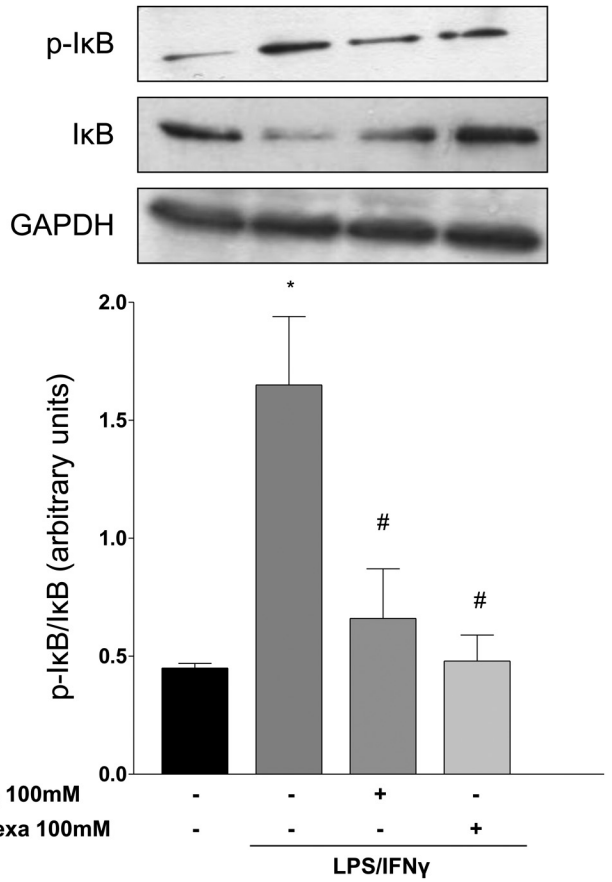

Figure 5. Immunoblot analysis of the phosphorylation of IκB in human macrophages exposed to vehicle,100 nM E2, or dexamethasone (Dexa) as a reference compound for 16 hours and stimulated with LPS/IFN-γ for 30 minutes. Relative quantification of NF-κB and phosphorylated (p)-IκB normalized to total IκB is expressed as the mean ± SEM of 3 independent experiments performed in triplicate. *, $P < .05$ vs control; #, $P < .05$ vs untreated.

differ between the 2 groups in an enlarged donor cohort (Supplemental Figure 4). Similarly, when the phenotypes of unpolarized macrophage cultures were compared, the ratio between the fractions of $CD80^+/CCR2^+$ (M1) and $CD163^+/CD206^+$ (M2) cells did not significantly differ in premenopausal vs postmenopausal women (Figure 6A). Unpolarized cultures were then activated using the in vitro study protocols. Figure 6B shows individual responses to LPS/IFNγ and IL-4/IL-13 activation in macrophages from premenopausal women, with expanded M1 and M2 surface marker–expressing subsets, respectively. Whereas individual responses to LPS/IFN-γ were comparable between the 2 groups, the phenotypic responses to alternative activation in postmenopausal women appeared to be blunted with respect to those seen in premenopausal women (Figure 6C). This resulted in a marked increase in the M1/M2 response ratio of macrophages from postmenopausal vs premenopausal women as calculated by individual percent phenotypic changes in response to M1 (classic) and M2 (alternative) activation, respectively (Figure 6D). These findings suggest that decreased estrogen levels impaired the potential of macrophages to undergo dynamic transition to the alternative functional phenotype in response to microenvironmental cues.

Discussion

Understanding the immune and functional phenotypes of human blood–derived macrophages in relation to estrogen treatment and menopausal status may be relevant for identifying sex-specific cardiovascular disease risk mechanisms and prediction strategies (13, 23). The models used to study human macrophage polarization are limited (24), and gold standard protocols for generating M1/M2 phenotypes in vitro are lacking. An advantage of using human monocyte–derived macrophages that have been spontaneously differentiated in vitro (19) is that they generate a rather heterogeneous starting population that has not been exposed to polarizing factors over the course of monocyte differentiation other than serum by itself, while reflecting the features of plasticity of tissue macrophages. Such heterogeneity is consistent with growing evidence that macrophage populations in vivo tend to contain different subpopulations of differentially activated cells (25).

We found the ER expression profile to be significantly affected by different stimuli. In particular, ERα levels were down-regulated by LPS/IFN-γ activation and transiently up-regulated at the mRNA level by IL-4/IL-13, whereas ERβ and GPER-1 levels were unchanged. To the best of our knowledge, these findings were not reported previously. Phiel et al (26) compared ER gene expression in IFN-γ–activated macrophages with that of monocytes and demonstrated a significant increase in ERα expression in monocyte-derived macrophages compared with that in $CD14^+$ peripheral blood monocytes, whereas ERβ expression was similar. However, ER expression patterns were not determined in unpolarized or alternatively activated macrophages in that study. A significant increase in LPS-induced TNF release has been reported in ERα-deficient mouse macrophages, suggesting that ERα, but not ERβ, mediates the inhibitory effects of endogenous estrogen on proinflammatory cytokine production in innate immune responses (27). Hematopoietic/myeloid-specific ERα deletion in mice induces defects in macrophage function including impaired IL-4–induced alternative activation and maximal phagocytic capacity along with abnormal tissue inflammation and insulin resistance (28), further suggesting that macrophage ERα is involved in the control of the inflammatory response. ERα expression is elevated in wild-type bone marrow macrophages in re-

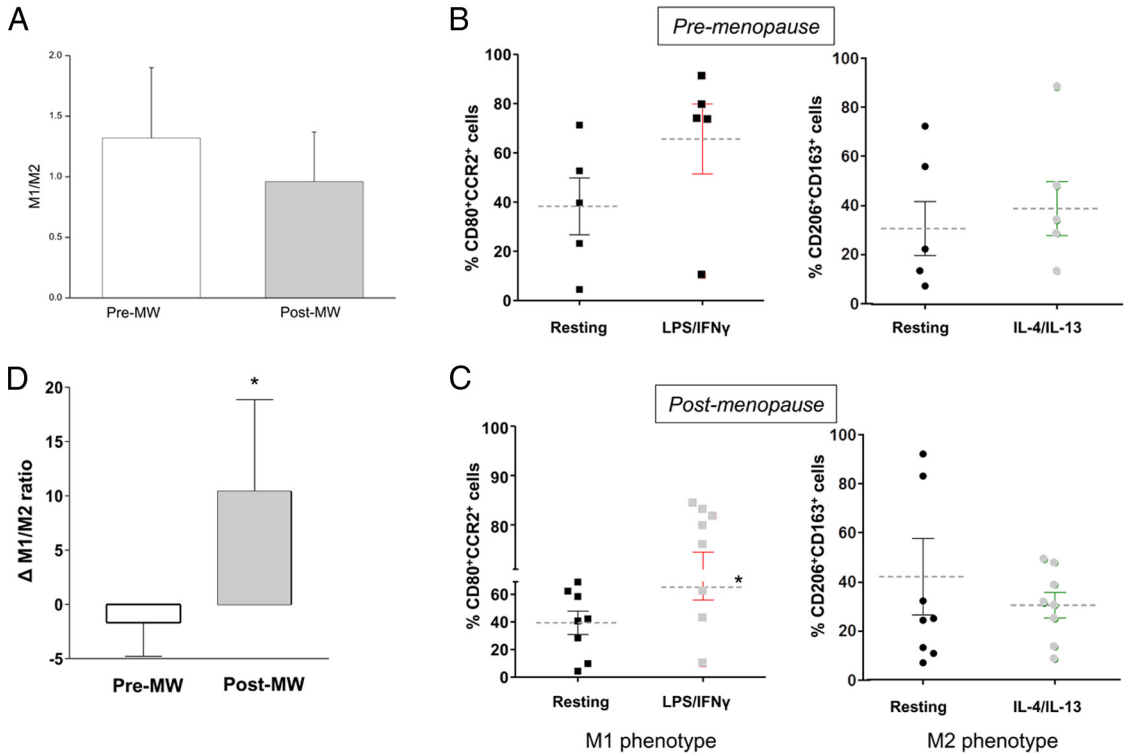

Figure 6. Activation phenotypes of blood-derived macrophages from premenopausal and postmenopausal women. A, Ratio of $CD80^+CCR2^+$ (M1) to $CD206^+CD163^+$ (M2) subsets in unpolarized macrophages derived from monocytes isolated from blood of premenopausal (Pre-MW; n = 5) and postmenopausal women (Post-MW; n = 8) after 7 days of differentiation. Unpolarized macrophages were incubated with antibodies specific for markers of both immunophenotypes as indicated. B, Macrophages from n = 5 premenopausal women were activated with LPS/IFN-γ (M1, left panel) or IL-4/IL-13 (M2, right panel) for 48 hours. Results from individual donors are shown. Dashed lines in the scatter plots indicate mean values ± SEM within each group. C, Macrophages from n = 8 postmenopausal women were activated with LPS/IFN-γ (M1, left panel) or IL-4/IL-13 (M2, right panel) for 48 hours. Results from individual donors are shown. Dashed lines in the scatter plots indicate mean values ± SEM within each group. D, Ratio of $CD80^+CCR2^+$ to $CD206^+CD163^+$ subsets after activation with LPS/IFNγ (M1) or IL-4/IL-13 (M2) for 48 hours with respect to unpolarized macrophages (Δ M1/M2 ratio) from n = 5 premenopausal and n = 8 postmenopausal women. *, $P < .05$.

sponse to estradiol and IL-4 stimulation (28). Consistently, estrogen inhibits alternative activation of the tumor-associated ANA-1 macrophage cell line in vitro, mainly through ERβ (18). Furthermore, alterations in the ERα/ERβ ratio have been demonstrated in different cell types and disease settings including diabetes (29). Hence, ER-mediated estrogen responsiveness appears to be dependent on macrophage activation status.

LPS/IFN-γ activation down-regulated the alternative activation markers CD163/CD206, thereby reducing the ability of the macrophages to bind a diverse array of endogenous and foreign molecules as part of a protective immunoregulatory response (30), and increased intracellular accumulation of TNF-α, IL-1β, and IL-10 as part of profound changes in the transcriptional profile associated with M1 polarization (5). Although IL-10 is regarded as the signature cytokine of alternative activation leading to an anti-inflammatory phenotype (31), we and others (20, 21) observed elevated IL-10 production in LPS/IFN-γ–activated but not in IL-4/IL-13–activated macrophages. The effects of E2 on macrophage cytokine production are known to be highly dependent on the cell differentiation stage, hormone levels, and test model (13). Here we found that E2 treatment in vitro attenuates functional readouts of LPS/IFN-γ activation including TNF-α and IL-10 production, consistent with estrogen interfering with the early-phase activation of LPS signaling via NF-κB through genomic and nongenomic mechanisms (32, 33). In contrast, LPS/IFNγ-induced cell-bound IL-1β production was not modulated by E2, probably because of its inability to interfere with the 2-step inflammasome/IL-1β activation pathway (34, 35). E2 treatment also failed to enhance the IL-4/IL-13–induced alternative phenotype. A recent study showed that E2 pretreatment reduces the number of alternatively activated $CD206^+$ macrophages in mouse liver tumor models (18). The discrepancy from our findings is probably due to key differences in species (mouse vs human), experimental setting (in vivo vs in vitro), tissue microenvironment (tumor-associated vs blood-derived), and ER expression profile.

Besides sharing the above-mentioned effects of E2, dexamethasone pretreatment reduced LPS/IFN-γ–mediated IL-1β intracellular accumulation and enhanced the M2 phenotype expressed as the fraction of $CD206^+$/

$CD163^+$ cells irrespective of activation status. Glucocorticoids are known to lead to macrophage M2 profiles (36, 37). Compared with E2, glucocorticoid treatment induces more markedly transcription factors such as signal transducer and activator of transcription (STAT) 3, which is not activated by E2 (38). The resulting enhancement of physiological cell functions such as migration, chemotaxis, and phagocytosis and neutralization of reactive metabolites through up-regulation of antioxidants enzymes such as sestrin-1 (36, 39) strongly indicates that glucocorticoids do not simply prevent the attenuation of anti-inflammatory phenotypes after LPS stimulation but rather induce a specific phenotype of macrophages with characteristic cellular activities. However, neither estrogen nor dexamethasone attenuated LPS/IFN-γ–induced up-regulation of classic activation markers in our culture system (data not shown).

The in vitro findings were validated in monocytes and monocyte-derived macrophages isolated from premenopausal and postmenopausal women. We found that the M1 ($CD80^+CCR2^+$) and M2 ($CD206^+CD163^+$) monocyte and macrophage subsets were comparable under resting conditions, leading to a similar M1/M2 ratio. This finding is in line with our in vitro study showing that estrogen per se is not a macrophage-polarizing signal. The phenotypic response to M1-associated stimuli was also comparable, whereas the response to M2-associated stimuli was markedly impaired in macrophages from postmenopausal vs premenopausal women. The resulting marked increase in the M1/M2 response ratio is therefore associated with circulating estrogen loss in postmenopausal women. Because of limited cell yields from donors, we did not test the anti-LPS action of E2 or explore additional pathways, including Toll-like receptor signaling. These data, however, suggest that estrogen deficiency at menopause is associated with an increased potential for unresolved inflammatory status. This impaired M2 response may lead to impaired capacity for parasite clearance, tissue remodeling, and resolution of smoldering chronic inflammation in response to a variety of stimuli such as IL-4 or IL-13, IL-1 receptor ligands or exposure to immune complexes plus LPS or IL-10, TGF-β, and endogenous glucocorticoid hormones. In agreement, Pechenino et al (40) compared the whole-heart gene expression profile for aged rats with and without estrogen replacement or with late estrogen replacement, which induced paradoxically a proinflammatory set of gene changes. Previous studies showed that impaired activity of STAT6, which is modulated by estrogen (18), as well as changes in monocyte ER expression patterns (26) and function (41), are likely to contribute to the observed shift in the macrophage activation profile in the postmenopausal group. Recently, the $CD206^+$ (M2) macrophage density in liver sections from ovariectomized female mice was found to be about 2-fold more than that in the control (18), suggesting that estrogen loss fosters alternative activation of tumor-associated macrophages. Whether this applies to the microenvironment of human estrogen-dependent tumors remains to be determined.

Our study is a first step in understanding the relationship between estrogen, menopausal status, and macrophage activation. However, the study has limitations. The current paradigm of macrophage activation has been extended from M1 vs M2 polarization to a spectrum model (40). Therefore, our selected phenotypes do not entirely reflect the complex biology of macrophage activation in the context of cardiovascular disease, although they were validated in patients with type 2 diabetes (10). The pilot ex vivo study had a small number of subjects and only phenotypic endpoints because of the limited volumes of blood taken from each subject. Therefore, further research studies are required to unravel key signaling cascades involved in macrophage activation and the role of estrogen and other second messengers in it. In addition, the observed differences in M1/M2 phenotypes in relation to menopausal status may be due to confounding factors other than estrogen loss, including aging. Nevertheless, to the best of our knowledge, this is the first demonstration for prevention of the LPS-IFNγ–induced fall in alternative activation markers by estrogen in vitro and increased M1/M2 response ratio in response to activating stimuli in macrophages from postmenopausal women, which entails a potential impact on cardiovascular disease risk.

Acknowledgments

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