Macrophages as novel target cells for erythropoietin

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ABSTRACT

Background

Our original demonstration of immunomodulatory effects of erythropoietin in multiple myeloma led us to the search for the cells in the immune system that are direct targets for erythropoietin. The finding that lymphocytes do not express erythropoietin receptors led to the hypothesis that other cells act as direct targets and thus mediate the effects of erythropoietin. The finding that erythropoietin has effects on dendritic cells thus led to the question of whether macrophages act as target cells for erythropoietin.

Design and Methods

The effects of erythropoietin on macrophages were investigated both *in-vivo* and *in-vitro*. The *in-vivo* studies were performed on splenic macrophages and inflammatory peritoneal macrophages, comparing recombinant human erythropoietin-treated and untreated mice, as well as transgenic mice over-expressing human erythropoietin (tg6) and their control wild-type counterparts. The *in-vitro* effects of erythropoietin on macrophage surface markers and function were investigated in murine bone marrow-derived macrophages treated with recombinant human erythropoietin.

Results

Erythropoietin was found to have effects on macrophages in both the *in-vivo* and *in-vitro* experiments. *In-vivo* treatment led to increased numbers of splenic macrophages, and of the splenic macrophages expressing CD11b, CD80 and major histocompatibility complex class II. The peritoneal inflammatory macrophages obtained from erythropoietin-treated mice displayed increased expression of F4/80, CD11b, CD80 and major histocompatibility complex class II, and augmented phagocytic activity. The macrophages derived *in-vitro* from bone marrow cells expressed erythropoietin receptor transcripts, and *in-vitro* stimulation with erythropoietin activated multiple signaling pathways, including signal transducer and activator of transcription (STAT)1 and 5, mitogen-activated protein kinase, phosphatidylinositol 3-kinase and nuclear factor kappa B. *In-vitro* erythropoietin treatment of these cells up-regulated their surface expression of CD11b, F4/80 and CD80, enhanced their phagocytic activity and nitric oxide secretion, and also led to augmented interleukin 12 secretion and decreased interleukin 10 secretion in response to lipopolysaccharide.

Conclusions

Our results show that macrophages are direct targets of erythropoietin and that erythropoietin treatment enhances the pro-inflammatory activity and function of these cells. These findings point to a multifunctional role of erythropoietin and its potential clinical applications as an immunomodulating agent.

Key words: erythropoietin receptor, pro-inflammation, macrophages, signal transducer and activator of transcription (STAT).

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Introduction

Erythropoietin (EPO), the erythropoietic hormone, is the main treatment option for several types of anemia, including those related to cancer. The function of EPO is regulated by activation of its receptor on erythroid progenitors, thereby promoting anti-apoptotic and survival signaling pathways. Recent studies have demonstrated that the functions of EPO are not limited to erythroid cells, and that EPO displays non-erythroid effects that are also related to anti-apoptotic processes, such as in heart failure improvement and neuroprotection.[1] Ongoing studies in our laboratory have demonstrated anti-neoplastic, immunomodulatory functions of EPO which were primarily observed in patients with multiple myeloma receiving recombinant human EPO (rHuEPO) treatment for their anemia. This phenomenon was revealed unexpectedly when the rHuEPO treatment given to these patients not only elevated their hemoglobin levels, but also prolonged their survival and improved their quality of life.[2] Further studies showed that rHuEPO treatment of patients with multiple myeloma was actually associated with a marked improvement of various immunological parameters and functions.[3] Analysis of murine multiple myeloma models reinforced these data and pointed to anti-neoplastic activities of EPO, mediated by improved CD8 lymphocyte functions.[4,5] In parallel, when assessing the humoral compartment of the immune system, we found that EPO treatment resulted in increased immunoglobulin production in antigen-injected mice, and enhanced lipopolysaccharide-mediated splenocyte proliferation.[6]

Since EPO-receptors were not detectable in lymphocytes,[3] we considered the possibility that other cells that do express the receptor may act as mediators of EPO function. Our search for possible mechanisms of the immunomodulatory effects of EPO led us to the novel discovery that dendritic cells express the EPO-receptor, and that stimulation with EPO enhances their survival and function.[7,8] Studies in murine models showed that EPO increased the splenic dendritic cell population with a higher cell surface expression of the co-stimulatory molecules CD80 and CD86.[8] Further analysis carried out on bone marrow-derived dendritic cells led to the findings that the cells expressed EPO-receptor mRNA and that *in-vitro* stimulation of these cells enhanced the cells' viability, up-regulated CD80, CD86 and MHC class II expression, augmented the secretion of interleukin (IL)-12 and activated multiple signaling pathways.[8]

Based on these findings, we hypothesized that macrophages are also potential targets of EPO. This would be an important discovery, since macrophages and dendritic cells have common progenitors,[9] and macrophages are also powerful antigen-presenting cells and serve as key effectors of the innate immune response.[10,11] The current study was designed to determine whether macrophages express EPO-receptors and whether they are affected directly by EPO. We focused on examining the effects of EPO on macrophage phenotype and functions, under both *in-vivo* and *in-vitro* experimental conditions.

Design and Methods

Mice

Female mice of the inbred C57BL strain, aged 8-12 weeks, were obtained from the Tel-Aviv University Animal Breeding Center. They were used to generate bone marrow-derived macrophages and for the EPO-injected mouse model. The transgenic mice over-expressing HuEPO (tg6) have been previously described.[6,12] Female tg6 mice and their wild-type (wt) littermates, aged 3–5 months, were used for experiments with this murine model. Mouse handling and the experimental procedures were approved by the Institutional Animal Care and Use Committee of Tel-Aviv University (number M-07-068).

Reagents

GMP-manufactured sterile syringes of rHuEPO (Eprex®) as used for patient care were kindly provided by Janssen Cilag, Israel, and employed throughout this study, thus ensuring the critically assured absence of toxins in the rHuEPO preparation. Lipopolysaccharide from the *E. coli* strain 0127:B8 (Sigma) was reconstituted in sterile double distilled water at 1 mg/mL stock solution and kept at -20°C. Anti-human EPO antibody (MAB287; R&D systems, USA) was reconstituted in phosphate-buffered solution (PBS) at 1 mg/mL solution and kept at -20°C.

Preparation of murine splenocytes

The mice were sacrificed by cervical dislocation and their spleens were immersed and forced through a 200-m pore-size wire mesh using the plunger from a 5 mL syringe to produce a single cell suspension. The cells were sedimented by centrifugation, and erythrocytes were lysed by hypotonic shock (10 s in sterile double-distilled water), followed by the addition of 0.1 volume of ×10 Hanks' balanced salt solution. Splenocytes were then prepared for flow cytometry analysis.

Isolation of macrophages from the peritoneal exudate

To induce non-specific inflammatory exudates, mice were injected intraperitoneally with 1 mL of 3% thioglycollate (Sigma, Israel). These mice were sacrificed 3 days later and all the peritoneal exudate cells were obtained by injecting 10 mL of ice-cold PBS into the peritoneal cavity. After a gentle massage, the fluid was harvested and the cells were centrifuged and washed before further use.

Generation of bone marrow-derived macrophages

Preparation of bone marrow cell cultures was based on the protocol described by Boltz-Nitulescu *et al.*[13] Briefly, bone marrow cells were isolated from femora and tibiae of the experimental mice. The cells were then incubated for 7 days in Dulbecco's modified Eagle's medium supplemented with 30% L-conditioning medium that was replenished on the 4th day. The non-adherent cells were purged and the macrophages (adherent cells) were collected by treatment with 15.5 mM EDTA in PBS saline for 10 min. The population of bone marrow-derived macrophages, as assessed by expression of CD11b surface molecule, was typically 99% pure. The bone marrow-derived macrophages were washed, and cultured for 24 h in Dulbecco's modified Eagle's medium and collected by 15 mM EDTA treatment. For flow cytometry analysis and phagocytosis, the bone marrow-derived macrophages were cultured in the presence or absence of rHuEPO. For cytokine detection, 0.05 μg/mL lipopolysaccharide was added to the cultures in the presence or absence of rHuEPO.

Flow cytometry analysis

The following fluorescence-conjugated antibodies were used: PE CD11b (Miltenyi Biotech Inc.), FITC F4/80, PE MHC class II (I-A/I-E), PE-Cy5 CD80 (eBioscience) and the relevant isotype controls. Bone marrow-derived macrophages or splenocytes

were incubated with the antibodies for 30 min at 4°C and washed with PBS. The cells were then analyzed on a FACSort flow cytometer (Becton-Dickinson). Ten thousand total events were collected and gated for analysis on live cells as determined by forward and side scatter. The results were analyzed using WinMDI software.

Phagocytosis of fluorescently labeled E. coli by macrophages

Peritoneal inflammatory macrophages or bone marrow-derived macrophages were seeded in 6-well plates (1×10^6 cells per well) for 2 h at 37°C to allow cell adhesion. *E. coli* was heat-killed by incubation at 65°C for 1 h and labeled with FITC (Sigma), 0.2 mg/mL in 0.1 M $NaHCO_3$ (pH 9.0) for 1 h at 37 °C. FITC-labeled *E. coli* (10×10^7 colony-forming units) were added to the cells (10 bacteria per macrophage). After incubation for 1 h at 37°C or at 4°C (to determine the fluorescence background), phagocytosis was stopped by immediate transfer of the cells to 4°C and washing with ice-cold PBS. The cells were treated with 0.1% crystal violet to quench extracellular fluorescence, and then fixed with 2% paraformaldehyde. They were collected by scraping with a rubber policeman and analyzed using a FACSort flow cytometer (Becton-Dickinson). The results are represented as a phagocytosis index, defined as the percentage of cells with internalized *E. coli* minus the background fluorescence, multiplied by the mean fluorescence intensity.

Reverse transcription and polymerase chain reaction analysis

All the procedures were performed according to the manufacturer's instructions. Total RNA was isolated from 7-day cultures of pure untreated bone marrow-derived macrophages using the TRIzol reagent (Invitrogen). Reverse transcription was performed using MMLV Reverse Transcriptase (Promega) from 1-2 μg of total RNA. Following cDNA synthesis, polymerase chain reaction was performed using Taq polymerase (Biolabs) for EPO-receptor or β actin transcripts, using oligonucleotide primers derived from the murine EPO-receptor (forward and reverse primers derived from EPO-receptor exons 1 and 2, respectively) and β actin sequences: EPO-receptor forward -5' ATGGACAAACTCAGGGTGC 3', reverse – 5' CTAGGAGCAGGCCACATA 3'; actin forward - 5' TTCTTTGCAGCTCCTTCGTTGCCG 3', reverse – 5' TGGATGGCTACGTACATGGC TGGG 3'.

Bone marrow-derived macrophages for biochemical analysis

The cells were starved for 1 h at 37°C by incubating them in Dulbecco's modified Eagle's medium without any supplements, after which 50 U/mL rHuEPO was added to the cells at different time points, as specified. In order to neutralize EPO activity with anti-human EPO antibody, 50 U/mL rHuEPO and 10 μg/mL of the antibody were incubated for 1 h at 37°C, prior to their addition to the cells. Phosphatase inhibitors (2 mM $ZnCl_2$, 2 mM vanadate, 50 mM NaF, and 20 mM $Na_4P_2O_7$) were then added on ice, and the cells were scraped off and collected. The cells were centrifuged at 14,000 rpm and extracted as described below.

Cytoplasmic and nuclear cell extracts were prepared as followed. The cell pellets were resuspended in buffer A - 0.1 mM EDTA, 10 mM KCl, and 10 mM HEPES (pH 7.9), to which a protease inhibitor cocktail (CompleteTM protease inhibitors; Roche Diagnostics) was freshly added, and incubated on ice for 10 min. The cells were lysed by adding 0.75% Nonidet P-40, and the lysate was then kept on ice for 5 min with repeated vortexing. The lysate was centrifuged at 5,000 rpm for 5 min to pellet the nuclei, and the supernatant (cytoplasmic extract) was stored at –80°C. The nuclear pellets were first washed in cold PBS and then resuspended in buffer C consisting of 1 mM EDTA, 0.4 M NaCl, and 20 mM HEPES (pH 7.9), with fresh addition of a protease inhibitor cocktail as mentioned above, and incubated for 15 min on ice with repeated vortexing. The suspensions were clarified by centrifugation at 14,000 rpm for 10 min. The supernatants were recovered as nuclear extracts and stored at –80°C.

Whole cell extracts were prepared as described elsewhere.[14] The cells were lysed at 4°C in lysis buffer (50 mM Tris pH 7.4, 1% Triton X-100, 5 mM iodoacetamide, 5 mM EDTA, and 150 mM NaCl) containing phosphatase and protease inhibitors. Lysates were spun at 14,000 rpm for 10 min and supernatants were recovered and stored at –80°C.

Immunoblotting

All supernatants were separated by 10% sodium dodecylsulfate polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane filter. The nitrocellulose membrane was incubated with the following antibodies: phosphoERK1/2 (from Sigma Chemicals); phosphoSTAT5 (Tyr694), STAT5, phosphoSTAT3 (Tyr705), STAT3, phosphoSTAT1, STAT1, phosphoAKT (Ser473), and AKT (from Cell Signaling); ERK2, and histone H1 (AE-4) (from Santa Cruz), actin (from Chemicon), and p65 (Rel A) Ab-1 (from Labvision) followed by labeled polymer horseradish peroxidase anti-rabbit or anti-mouse antibodies (from Dako).

Cytokine detection

Supernatants from bone marrow-derived macrophage cultures were collected after 24 h of incubation with lipopolysaccharide in the presence or absence of rHuEPO, and then analyzed for IL-12 p70 and IL-10. Cytokine levels were determined by enzyme-linked immunosorbent assays (Peprotech) according to the manufacturer's instructions.

Nitric oxide secretion

Supernatants from bone marrow-derived macrophages were harvested after 24 h of culture, and levels of nitric oxide were measured as the nitrite concentration by mixing equal volumes of culture supernatants (40 μL) and Griess reagent (Sigma). Nitrite concentrations were determined by comparing the absorbance values (at 540 nm) of the test samples with a standard curve generated by serial dilutions of 100 μM sodium nitrite.

Statistical analysis

Comparisons between data resulting from the control treatment *versus* data from EPO treatment were performed using Student's t-test. *P* values less than 0.05 were considered to indicate statistical significance.

Results

Since macrophages are a highly heterogeneous population, with multiple functions and diverse phenotypes,[15] we chose to explore the effects of EPO on macrophages that are steady-state residents in the spleen and can be easily identified, as well as macrophages induced by thioglycollate in the peritoneum and those generated *in-vitro* from bone marrow progenitors. The *in-vivo* effects of EPO were examined in two experimental models. The

first model consisted of rHuEPO-injected mice, with diluent-injected mice serving as controls. The second model consisted of the tg6 transgenic mice over-expressing human EPO,[6,12] with their wild-type littermates serving as controls. Mice spleens were harvested and phenotyped by flow cytometry analysis.

In vivo *effects of erythropoietin on splenic macrophages*

Splenic macrophages were identified by expression of the pan macrophage marker F4/80, which is associated with macrophage differentiation.[9,16] Further analysis of the F4/80-positive cells was performed to assess the expression of CD11b, a marker of macrophage activation, and MHC class II and CD80, markers of antigen presentation.[11,17] The proportion of cells expressing the F4/80 cell surface marker indicated that both the rHuEPO-injected mice and, to a greater extent, the tg6 mice, exhibited a significant increase in the splenic macrophage population compared to their respective control groups (Figure 1A and 1B). Moreover, we found increased proportions of splenic macrophages (F4/80$^+$ cells) expressing both CD80 and MHC class II markers (Figure 1C) in the rHuEPO-injected and the tg6 mice. However, a significant increase in the number of splenic macrophages expressing CD11b was observed only in the tg6 mice (Figure 1C), suggesting that EPO affects this population in a dose-dependent manner. Of note, we did not detect any difference in the expression of CD68 macrophage activation molecule in relation to the excessive availability of EPO compared to the relevant controls (*data not shown*). These results suggest an effect of EPO on macrophage differentiation, as reflected by the increase in the F4/80$^+$ cell population. Furthermore, EPO was found to influence the macrophage phenotype towards a pro-inflammatory cell type, as demonstrated by the enhancement of CD11b, CD80 and MHC class II positive populations.

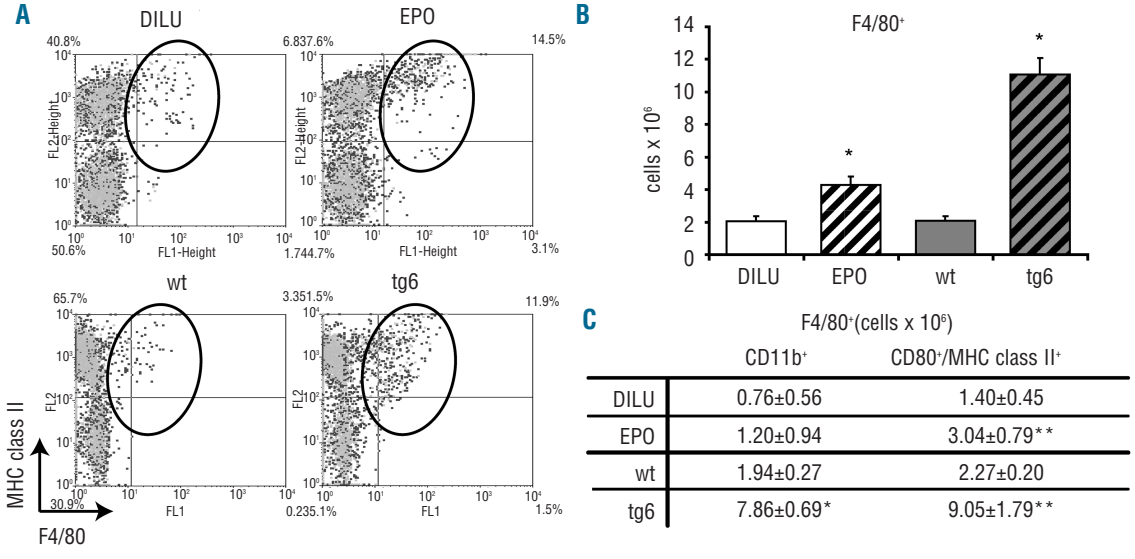
In vivo *effects of erythropoietin on peritoneal inflammatory macrophages*

In order to determine whether EPO can affect the function of inflammatory macrophages, we assessed activated peritoneal macrophages induced by thioglycollate administration to the peritoneum in both rHuEPO-injected and tg6 mice. The peritoneal macrophages were identified by CD11b expression. The cell surface expression of CD11b, F4/80, MHC class II and CD80 molecules increased significantly in both the rHuEPO-injected and tg6 mice compared to in their control counterparts (Figure 2A and 2B), indicating that excessive availability of EPO led to an enhanced activation status of the macrophages. We then investigated the phagocytic ability of the macrophages using the functional assay of uptake of *E. coli* bacteria. Both experimental models showed enhanced macrophage phagocytic activity of *E. coli* bacteria (Figure 2C). It would, therefore, appear that EPO may lead to improved activation and function of peritoneal inflammatory macrophages. Thus, excessive availability of EPO resulted in enhancement of the pro-inflammatory phenotype and function of both splenic steady-state and inflammatory-induced peritoneal macrophages. The results of these *in-vivo* models showed that macrophages are, indeed, influenced by EPO.

In vitro *studies on bone marrow–derived macrophages*

Erythropoietin receptor expression and activation in bone-marrow–derived macrophages

We then studied macrophages derived *in-vitro* from the mouse bone marrow cultures in order to determine



	CD11b$^+$	CD80$^+$/MHC class II$^+$
DILU	0.76±0.56	1.40±0.45
EPO	1.20±0.94	3.04±0.79**
wt	1.94±0.27	2.27±0.20
tg6	7.86±0.69*	9.05±1.79**

Figure 1. *In-vivo* effects of erythropoietin (EPO) manifested in splenic macrophages. Flow cytometry analysis of splenocytes from C57BL/6 mice injected three times (every other day during 1 week) with 180 U rHuEPO (EPO) or with the diluent (DILU) as a negative control, and from tg6 mice and their wild type (wt) littermates. (A) Density plots for F4/80 and MHC class II expression. (B) Summary of the number of splenic macrophages: F4/80$^+$ splenocytes. (C) Summary of the number of macrophages: F4/80$^+$ splenocytes also expressing CD11b and CD80/MHC class II. The graph and table represent the mean ± S.E.M of three independent experiments, each including three female mice per experimental group, **P*<0.05, ***P*<0.01 for EPO/tg6 *versus* diluent/wt, respectively.

whether EPO can affect macrophages directly. We first investigated whether bone marrow-derived macrophages expressed the EPO-receptor, and subsequent reverse transcriptase polymerase chain reaction analysis demonstrated that EPO-receptor mRNA is indeed expressed in these cells (Figure 3A). We further investigated EPO-receptor-associated signaling in the bone marrow-derived macrophages. Activation of the signal transducer and activator of transcription (STAT) pathways, specifically STAT5, is an essential step in the EPO-receptor signaling cascade.[18] We, therefore, tested whether EPO treatment has any effect on phosphorylation of the STAT proteins. Bone marrow-derived macrophages were treated *in-vitro* with rHuEPO and subjected to immunoblot analysis. Following *in-vitro* EPO treatment, we detected STAT5, STAT3 and STAT1 phosphorylation in the bone marrow-derived macrophages (Figure 3B). Involvement of the STAT pathways, specifically STAT5, implies that EPO-receptor is activated following EPO treatment.

We then examined whether EPO treatment *in-vitro* can also trigger signaling pathways that are known to participate in macrophage activation, including MAPK, PI3K and NFκB.[19] As shown in Figure 3C, *in-vitro* EPO treatment of bone marrow-derived macrophages resulted in phosphorylation of ERK2 and AKT and translocation of p65 to the nucleus. The inhibition of EPO-mediated STAT5 activation by the anti-human EPO antibody ensured the EPO specificity of the above mentioned signaling pathways (Figure 3D). It should be noted that background stimulation in the presence of antibody alone could derive from Fc-mediated activation of the bone marrow-derived macrophages which harbor an Fc receptor. The specificity of the antibody towards EPO was verified by the lack of effect on granulocyte-monocyte colony-stimulating factor-mediated activation of STAT5 (*data not shown*).

We were, therefore, able to conclude that exposure of bone marrow-derived macrophages to EPO results in activation of the MAPK, PI3K and NFκB signaling pathways.

Effects of erythropoietin on the phenotype and function of bone-marrow–derived macrophages

We examined whether *in-vitro* EPO treatment can affect the function of bone marrow-derived macrophages. Bone marrow-derived macrophages were cul-

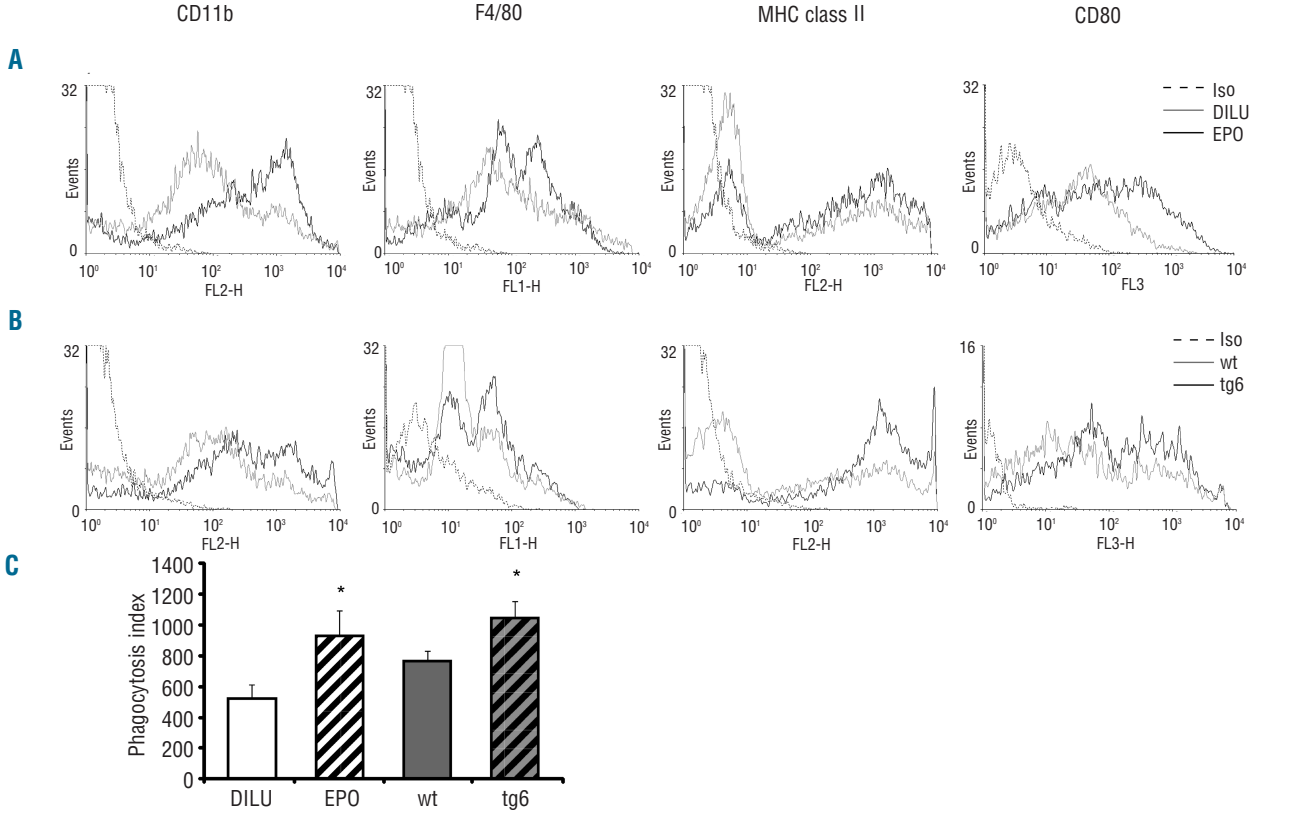


Figure 2. *In-vivo* effects of erythropoietin (EPO) on inflammatory macrophages. Flow cytometry analysis of thioglycollate-induced peritoneal macrophages from C57BL/6 mice injected three times (every other day during 1 week) with 180 U rHuEPO (EPO) or with diluent (DILU) as a negative control, and from tg6 mice and their wild-type (wt) littermates. (A and B) Peritoneal macrophages (identified by CD11b expression) were analyzed for CD11b, F4/80, CD80 and MHC class II cell surface expression. Surface expression of these molecules is represented as black or gray histograms. Isotype controls (Iso) are represented by broken line histograms. Histograms represent one of at least three independent experiments, displaying similar results. (A) Data from rHuEPO-injected mice and diluent injected mice are represented by black and gray histograms, respectively. (B) Data from tg6 mice and their wt littermates are represented by black and gray histograms, respectively. (C) Analysis of thioglycollate-induced peritoneal macrophages incubated with FITC-labeled *E. coli* bacteria. Phagocytosis index = (% of FITC-positive cells) × (mean fluorescence intensity). **P*<0.05 for EPO/tg6 *versus* diluent/wt, respectively. The figure presents data from a total of three experiments.

tured *in-vitro* with or without rHuEPO for 24 h. *E. coli* phagocytosis and nitric oxide secretion assays were performed in order to assess the innate functions of the macrophages. Macrophage activation was assessed by phenotype analysis based on CD11b, F4/80 and CD80 expression. The bone marrow-derived macrophages treated with EPO displayed enhanced phagocytosis and nitric oxide secretion (Figure 4). In addition, EPO treatment of the bone marrow-derived macrophages resulted in up-regulated cell surface expression of CD11b, F4/80 and CD80 (Figure 5). These results reinforce the findings on the *in-vivo* effects of EPO, in which excessive availability of EPO led to similar effects on macrophage phenotype and activity. However, the *in-vitro* experiments on bone marrow-derived macrophages failed to show any effect on MHC class II expression, which had been up-regulated under the *in-vivo* experimental conditions (*data not shown*).

We next determined the cytokine secretion profile of macrophages as an indication of function by analyzing the bone marrow-derived macrophage secretion of IL-12 and IL-10, which represent opposing immunological functions. Specifically, IL-12 is a pro-inflammatory cytokine related to the development of a Th1 type response, whereas IL-10 has anti-inflammatory activities and may lead to a Th2 type response.[17,19] Cytokine secretion by bone marrow-derived macrophages was determined in cultures treated with lipopolysaccharide in the presence or absence of rHuEPO. EPO treatment of the lipopolysaccharide-stimulated bone marrow-derived macrophages resulted in increased IL-12 secretion and reduced IL-10 secretion (Figure 6).

Taken together, our *in-vitro* results showed that macrophages are indeed direct targets of the immunomodulatory effects of EPO. These effects, which stem from the interaction between EPO and macrophages, indicate that EPO enhances the activation and function of macrophages towards a pro-inflammatory response.

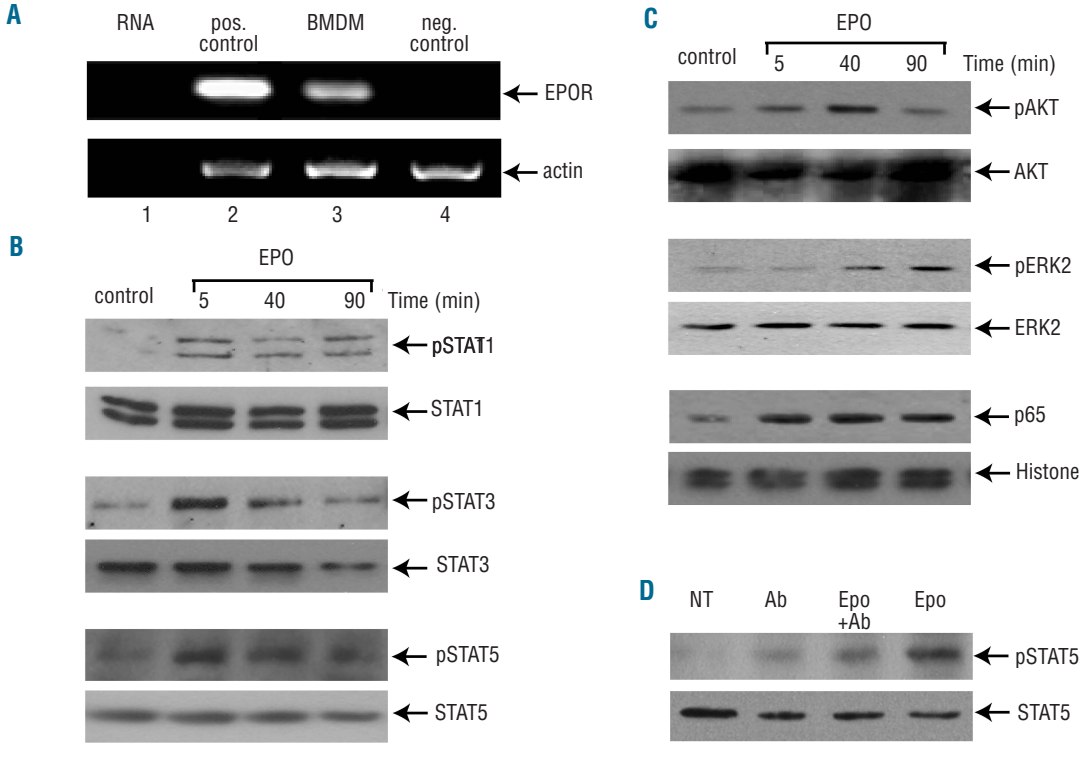


Figure 3. Erythropoietin receptor (EPO-R) expression and activation in bone marrow-derived macrophages (BMDM). (A) EPO-R expression in BMDM. EPO-R mRNA from murine BMDM (99% purity) was reverse-transcribed and subjected to PCR analysis for murine EPO-R and actin transcripts using oligonucleotide primers, thus yielding 300 and 457 bp fragments, respectively. Lane 1: RNA of BMDM. Lane 2: Positive control. cDNA of BaF/3 cells transfected with murine EPO-R. Lane 3: cDNA of BMDM. Lane 4: Negative control. cDNA of MBA cells.[35] (B and C) Stimulation of BMDM with EPO activates multiple signaling pathways. BMDM were treated *in-vitro* with 50 U/mL of recombinant human EPO (rHuEPO) for the indicated time periods. (B) Cell lysates of the cytosolic fraction were subjected to immunoblot analysis with the indicated antibodies. STAT5, STAT1 and STAT3 signaling was determined by phosphorylation. Total STAT levels are depicted for normalization. (C) Cell lysates of the cytosolic (ERK and AKT immunoblots) and nuclear fractions (p65 immunoblot) were subjected to immunoblot analysis with the indicated antibodies. MAPK and PI3K signaling was determined by ERK1/2 and AKT phosphorylation, respectively. Total ERK2 and AKT levels are depicted for normalization. The NFκB signaling was detected by nuclear p65 internalization. Nuclear histone is depicted for normalization. All micrographs represent one of at least three similar independent experiments. (D) Neutralization of EPO mediated signaling by anti-human EPO antibody. BMDM were treated *in-vitro* for 10 min with 50 U/mL rHuEPO in the presence or absence of 10 μg/mL of anti human EPO antibody, or with antibody alone. Cell lysates of the cytosolic fraction were subjected to immunoblot analysis with the indicated antibodies. pSTAT5 and total STAT5 levels are depicted, NT - non treated; Ab - anti-human EPO antibody. Immunoblots represent one of at least three independent experiments, displaying similar results.

Discussion

We had hypothesized that macrophages are potential targets of EPO. The results of the present study supported our hypothesis by showing that macrophages are affected by EPO and that they are direct target cells of EPO. Moreover, we demonstrated that EPO treatment enhances the pro-inflammatory phenotype and function of different macrophage populations, including steady-state splenic resident macrophages, as well as thioglycollate-induced peritoneal macrophages and the *in-vitro* established bone marrow-derived macrophages. The decision to focus on the immunomodulatory functions of EPO arose from our previous studies in which we showed that EPO treatment of patients with multiple myeloma as well as murine models of myeloma was associated with improved immunological functions.[2-6] Those studies, in combination with observations in non-malignant models[6] of the immune system, provided evidence of the effect of EPO on both the cellular and the humoral compartments of the immune system. Specifically, EPO treatment was associated with enhanced lymphocyte activity of both T and B cells.[3,4,6] However, EPO-receptor expression or direct EPO stimulation on lymphocytes could not be demonstrated to date.

Macrophages and dendritic cells both develop from common progenitors and share similar surface markers.[20,21] Together with their many functions related to the innate immune response, macrophages also display antigen-presenting activities, although they are less potent than dendritic cells,[17] which have also been viewed as a subtype of macrophages.[22]

Previous studies have described the effects of EPO on certain macrophage populations. The tg6 mice have been reported to have increased populations of liver and duodenal mucosa macrophages,[23,24] and the liver macrophages in tg6 mice also had enhanced erythrophagocytic activity.[24] Expression of EPO-receptor was shown in wound-healing macrophages, and EPO enhanced the activity of these macrophages.[25] We employed a variety of experimental models that had excessive availability of EPO in the search for possible effects of EPO on macrophages. These models included *in-vivo* administration of EPO, transgenic mice over-expressing HuEPO, as well as direct *in-vitro* exposure of macrophages to EPO. The EPO-driven increase in splenic macrophage levels (CD11b, CD80 and MHC class II expression) could result from enhanced proliferation of macrophage progenitors or the prolonged viability of the existing macrophages. We observed similar effects of EPO on the splenic dendritic cell population in a previous

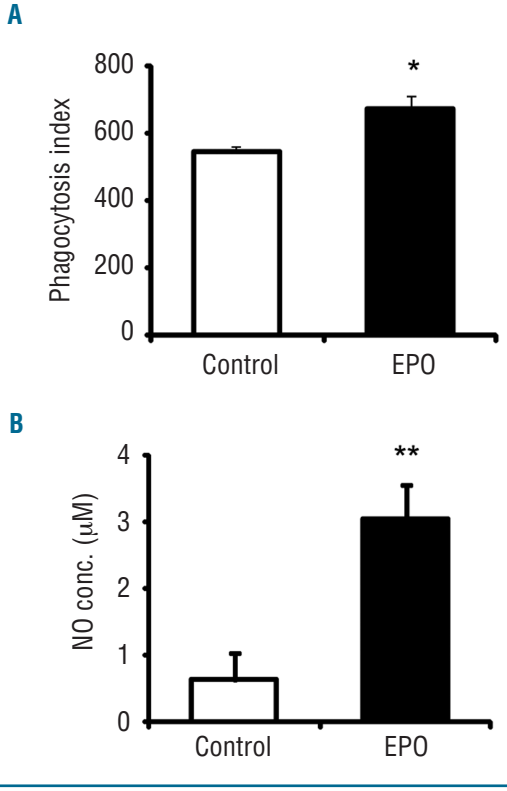


Figure 4. Erythropoietin (EPO) *in-vitro* leads to enhanced innate functions of bone marrow-derived macrophages (BMDM). BMDM were cultured *in-vitro* for 24 h with 5 U/mL rHuEPO (EPO) or without (control). (A) Cells were washed and incubated with FITC-labeled *E. coli* bacteria, and then subjected to FACS analysis. Phagocytosis index = (% of FITC positive cells) × (mean fluorescence intensity). (B) Supernatants were collected and nitric oxide (NO) secretion was detected using Griess reagent. Graphs represent the mean ± S.E.M of three independent experiments. *P<0.05, **P<0.01 for EPO *versus* control.

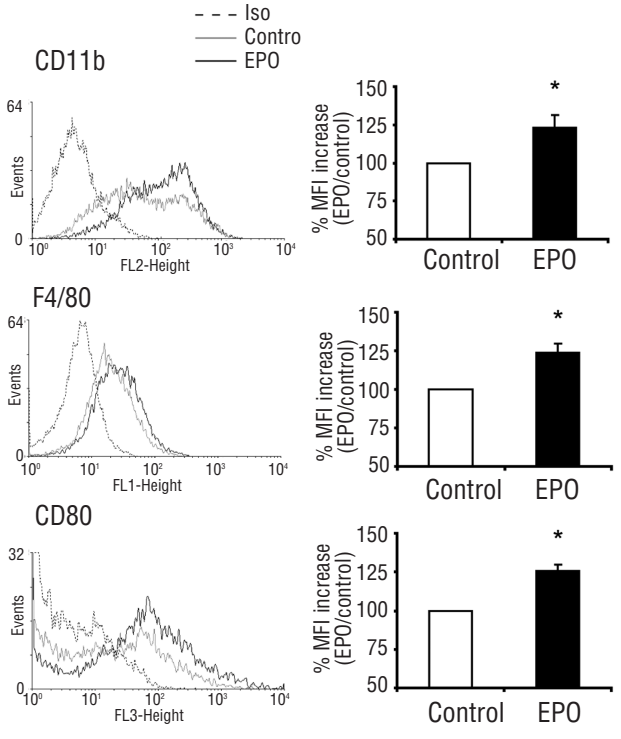


Figure 5. Erythropoietin (EPO) *in-vitro* affects the marrow-derived macrophage (BMDM) phenotype. BMDM were cultured *in-vitro* with (EPO) or without (control) 5 U/mL rHuEPO for 24 h. Flow cytometry analysis of BMDM of CD11b, F4/80 and CD80. Surface expression of these molecules is represented as black (EPO treated) or gray (control) histograms. Isotype controls (Iso) are represented by broken line histograms. Graphs represent the mean percentage of mean fluorescence intensity (MFI) increase of EPO-treated cells *versus* control cells of three independent experiments. *P<0.05 for EPO *versus* control.

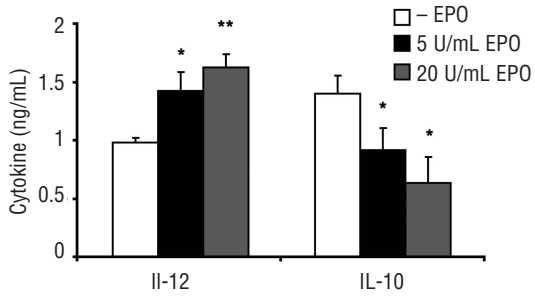


Figure 6. Erythropoietin (EPO) *in-vitro* leads to increased secretion of IL-12 and decreased IL-10 secretion by marrow-derived macrophages (BMDM). BMDM were cultured *in-vitro* with 0.05 mg/mL lipopolysaccharide in the presence or absence of rHuEPO for 24 h. Cell culture supernatants were analyzed for the levels of IL-12 and IL-10, by ELISA. Graphs represent the mean cytokine concentration ± S.E.M. of at least three independent experiments. **P*<0.05, ***P*<0.01 for EPO-treated cells *versus* non-EPO-treated cells.

study,[8] suggesting that the activities of EPO could be related to an effect on common dendritic cell and macrophage progenitors. Notably, the effects of EPO on both macrophages and dendritic cells were more profound in the tg6 mice, probably due to the continuous exposure to high EPO levels.

The effect of EPO on macrophages was also evident in the peritoneal inflammatory macrophages. Here, EPO treatment enhanced macrophage activation as shown by the augmented expression of CD11b, F4/80, CD80 and MHC class II and improved phagocytic function. Notably, unlike in the case of splenic macrophages, EPO treatment was not associated with an increase of the total number of peritoneal macrophages (*data not shown*). As such, these findings suggest that EPO modulates peritoneal macrophage activity, but not migration of the macrophages towards the inflamed peritoneum.

The *in-vitro* administration of EPO enabled us to determine whether EPO can affect macrophages directly, as well as to assess EPO-driven cellular events. Bone marrow-derived macrophages were chosen for the *in-vitro* studies based on the polymerase chain reaction analysis that showed mRNA expression of EPO-receptor. The polymerase chain reaction method was selected for this analysis because of the low levels of endogenous cellular EPO-receptor[26] and the fact that the anti-murine EPO-receptor antibodies that we routinely use in our studies[14] could not detect the EPO-receptor protein (*data not shown*).

The functionality of the bone marrow-derived macrophage EPO-receptor was inferred by the EPO-mediated activation of the STAT5, STAT3 and STAT1 pathways in these cells. The STAT5 pathway is considered to be the central signaling pathway activated by the EPO-receptor and crucial for erythropoiesis.[18] The role of STAT1 in erythropoiesis is less clear,[27] although it does play an essential part in macrophage pro-inflammatory activation.[1] Interestingly, we detected EPO-driven activation of STAT3, but not STAT1 or STAT5, in dendritic cells.[8] STAT3, like STAT1, is not essential for erythropoiesis,[27] but it does play a role in dendritic cell development.[28] Thus, we assume that EPO-mediated activation of the different STAT subtypes is determined by the cell type and/or environment, enabling STAT3 activation in dendritic cells and STAT1, STAT3 and STAT 5 activation in macrophages.

Following EPO treatment, the bone marrow-derived macrophages also exhibited activation of the PI3K, MAPK and NFκB pathways. The activation of these pathways is pivotal in macrophage inflammatory functions, including cytokine secretion and phagocytosis.[29,30] These pathways are also essential in the EPO-receptor activation of both erythroid[18] and non-erythroid cells.[31,32] Notably, EPO treatment that activated signaling pathways did not enhance macrophage survival or proliferation (*data not shown*).

In-vitro EPO-mediated activation of the bone marrow-derived macrophages led to a pro-inflammatory phenotype and function of the cells. This was evident from the increased expression of F4/80, CD11b and CD80, elevated phagocytosis and increased nitric oxide secretion following EPO treatment. Furthermore, the EPO-dependent elevated IL-12 and decreased IL-10 secretion suggest that EPO treatment not only enhances the pro-inflammatory status of the macrophages, but that it can also shift the macrophage typing to M1. Importantly, M1 macrophages are considered as being effector cells that enhance the Th1 response, and they can enable anti-neoplastic activity.[17] Thus, it is possible that the anti-neoplastic activity of EPO, as previously seen in patients with multiple myeloma[4] as well as in murine experimental models,[5] is also regulated by macrophages shifting to an M1 type following EPO activation.

The results of the current study support our earlier findings on dendritic cells and on multiple myeloma in emphasizing the role of EPO as an immunomodulator. This is especially relevant in the light of the ongoing safety concerns regarding EPO treatment. Although EPO-receptors have been reported to be expressed in various types of solid tumors,[31] their functionality remains controversial.[33,34]

In conclusion, we demonstrated that EPO can directly affect macrophages, leading to pro-inflammatory activity and function. We propose that the immunomodulatory activities of EPO are mediated, at least in part, by macrophages and dendritic cells.

Authorship and Disclosures

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