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Abstract

Erythropoietin (EPO) is the main hormone that promotes proliferation and differentiation of erythroid progenitor cells via binding to its surface receptor (EPO-R). Recent studies suggest that this hormone may affect also other cell types, besides the red blood cell lineage. We have previously demonstrated that the immune system is a target of EPO; however, the direct target cells of EPO, as well as the molecular mechanisms underlying its role as an immunomodulator, are unknown. Here we present evidence for functional effects of EPO on dendritic cells (DCs), which are known to initiate the immune response. In-vivo experiments in EPO-injected mice and in transgenic mice over-expressing human EPO showed an increased splenic DC population with a higher cell surface expression of CD80 and CD86. Further analysis based on mouse models, showed that DCs derived in-vitro from bone marrow (BM-DCs) express EPO-R mRNA. In-vitro stimulation of these DCs with recombinant human EPO enhanced viability, upregulated CD80, CD86 and MHC class II and augmented the secretion of IL-12. Biochemical analysis of EPO mediated signaling in the BM-DCs showed activation of the AKT, MAPK and NF-kappaB pathways. EPO stimulation of the BM-DCs led to Tyr-phosphorylation of STAT3. The inability to detect EPO mediated activation of STAT5 in the BM-DCs, suggests that in DCs, STAT3 may play a more important role than STAT5 in EPO-R signaling. Taken together, our data support the premise that DCs are direct targets of EPO, thereby providing an insight to the immunomodulatory functions of EPO.
Non-Erythroid Activities of Erythropoietin: Functional Effects on Murine Dendritic Cells

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**Abstract**

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Here we present evidence for functional effects of EPO on dendritic cells (DCs), which are known to initiate the immune response. *In-vivo* experiments in EPO-injected mice and in transgenic mice over-expressing human EPO showed an increased splenic DC population with a higher cell surface expression of CD80 and CD86. Further analysis based on mouse models, showed that DCs derived *in-vitro* from bone marrow (BM-DCs) express EPO-R mRNA. *In-vitro* stimulation of these DCs with recombinant human EPO enhanced viability, upregulated CD80, CD86 and MHC class II and augmented the secretion of IL-12. Biochemical analysis of EPO mediated signaling in the BM-DCs showed activation of the AKT, MAPK and NF-κB pathways. EPO stimulation of the BM-DCs led to Tyr-phosphorylation of STAT3. The inability to detect EPO mediated activation of STAT5 in the BM-DCs, suggest that in DCs, STAT3 may play a more important role than STAT5 in EPO-R signaling. Taken together, our data support the premise that DCs are direct targets of EPO, thereby providing an insight to the immunomodulatory functions of EPO.

**Key words:** EPO-R, Co-stimulatory molecules, Signal transduction, STAT
Introduction

Erythropoietin (EPO) is the key regulator of erythropoiesis. Secreted mainly by the kidney, EPO promotes the survival, proliferation, and differentiation of committed erythroid progenitor cells (Sasaki et al., 2000). Recombinant human EPO (rHuEPO) has thus been adopted for the treatment of various types of anemia (Cheer and Wagstaff, 2004; Gassmann et al., 2003; Mittelman, 1996). EPO acts on its receptor (EPO-R) that belongs to the cytokine receptor superfamily (Youssoufian et al., 1993). Binding of EPO to the EPO-R initiates the activation of several signaling cascades, including JAK2/STAT5, MAPK and PI3K pathways (Jelkmann et al., 2008).

The detection of EPO-Rs in cells other than erythroid progenitors, such as polymorphonuclear leukocytes, megakaryocytes, endothelial, myocardial and neural cells (Anagnostou et al., 1994; Brines and Cerami, 2005; Buemi et al., 2003; Fraser et al., 1989; Jaquet et al., 2002; Sela et al., 2001) showed that besides its role in erythropoiesis, EPO has additional biological functions. It should be noted that alongside the beneficial effects of EPO, a concern that EPO treatment for anemia might adversely affect the prognosis in certain cases of solid tumor cancers (Aapro et al., 2008; Henke et al., 2006; Longmore, 2007) has been raised. In that respect although EPO-Rs were found in certain solid tumors (Acs et al., 2003; Arcasoy et al., 2002; Yasuda et al., 2003), their functionality is still controversial (Jelkmann et al., 2008; Jeong et al., 2008; Laugsch et al., 2008).

Our previous studies have demonstrated EPO-associated anti-neoplastic activities, which were related to EPO's action on the immune system (Mittelman et al., 2004; Prutchi-Sagiv et al., 2006). These studies were led by the observation that multiple myeloma
patients treated with rHuEPO for their anemia, exhibited prolonged survival and improved quality of life (Baz et al., 2007; Mittelman et al., 1997; Mittelman et al., 2004). EPO treatment in MM patients was associated with effects on given immunological parameters and functions, including normalization of the CD4:CD8 cell ratio, as well as enhanced phytohemagglutinin-mediated T cell activation and proliferation (Prutchi-Sagiv et al., 2006).

Studies in mouse models of MM lent further support to the concept regarding EPO antineoplastic activity, and indicated that this activity was mediated by the CD8 lymphoid population (Mittelman et al., 2001). More recently we noted EPO effects also on the humoral immune response of antigen-injected mice in both EPO-treated mice and tg6 mice (Ruschitzka et al., 2000; Vogel et al., 2003) – transgenic mice over-expressing human EPO (HuEPO) (Katz et al., 2007). Whereas these findings strongly suggest that EPO is an immunomodulator, the cellular mediators and underlying mechanisms are not understood, especially since EPO-Rs were not detected on lymphocytes (Prutchi-Sagiv et al., 2006) and unpublished data).

To examine the possibility that EPO acts on lymphocytes via other cells that may express the EPO-R, we focused on dendritic cells (DCs), the most potent antigen presenting cells that facilitate the initial activation of the adaptive immune response (Banchereau et al., 2000) and induce specific T-cell anti-tumor responses and tumor destruction (Fong and Engleman, 2000). Here we present data in mouse models demonstrating an in-vivo EPO-associated increase in splenic DCs (SDCs), as well as enhanced expression of co-stimulatory molecules on these cells. Our current study demonstrates that EPO directly
targets mouse DCs, as manifested by the effect of EPO \textit{in-vitro} on viability, signaling, phenotype and cytokine secretion of bone marrow-derived DCs (BM-DCs).

These findings, as well as our data on human DCs (Prutchi-Sagiv et al., in press), support the idea that EPO acts as an important immunomodulator, and that it performs these actions at least partially \textit{via} a direct action on the DCs.
Materials and methods

Mice - Female wild-type (wt) mice of the inbred strain C57BL/6, aged 8-12 weeks were obtained from the Tel-Aviv University Animal Breeding Center. These were employed for BM-DC generation and for the EPO-injected mouse model. The transgenic mouse over-expressing EPO (tg6) has been previously described (Heinicke et al., 2006; Katz et al., 2007; Ruschitzka et al., 2000). Female tg6 mice and their wt littermates were used at the age of 3–5 months. Mice were used for the experimental procedures conducted according to the Institutional Animal Care and Use Committee of the Tel-Aviv University (permit M-06-119).

Reagents – rHuEPO - GMP-manufactured sterile syringes of rHuEPO (Eprex®) as used for patient care were kindly provided by Janssen Cilag, Israel, and used throughout this study, thus excluding the presence of toxins in the rHuEPO preparation. Recombinant murine GM-CSF (Prospec) was reconstituted in sterile double distilled water (DDW) at 5 mg/ml stock and kept at -20°C. LPS from the *E. coli* strain 0127:B8 (Sigma) was reconstituted in sterile DDW at 1 mg/ml stock and kept at -20°C. Tyrophostin AG-490 (Sigma) was reconstituted in DMSO at 0.1 M and kept in the dark at -20°C.

rHuEPO injections - C57BL/6 mice were injected subcutaneously three times every other day during a week, with 180 U rHuEPO or with diluent (negative control) as described (Katz et al., 2007).

Preparation of splenocytes - Mice were sacrificed, and their spleens were incubated with 1 mg/ml fresh collagenase D (Sigma) in RPMI for 30 min at 37°C. Note that the tg6 spleen was enlarged (Vogel et al., 2003). The spleens were then immersed and forced through 200-m pore-size wire mesh, using the plunger from a 5 ml syringe to obtain a
single cell suspension. The cells were pelleted by centrifugation, and erythrocytes were lysed by hypotonic shock (10 s in sterile DDW), followed by the addition of 0.1 volume of ×10 HBSS.

**Generation of DCs from bone marrow** – Preparation of DCs was based on the protocol described (Lutz et al., 1999). Briefly, bone marrow cells were isolated from femurs and tibias of female mice. The cells were then incubated for 10 days in RPMI culture medium supplemented with GM-CSF (20 ng/ml), and refreshed every 2-3 days. The DC population (the non-adherent cells), as assessed by expression of the MHC class II and CD11c surface molecules, was typically 80% pure. The cells were further purified (~99%) on the basis of CD11c expression, using a FACS Aria cell sorter (BD). For phenotype, flow cytometry analysis and cytokine detection BM-DCs were washed and treated for 24 h in the presence or absence of 0.05 µg/ml LPS and/or 20 U/ml rHuEPO. Cells were then stained with Trypan Blue and counted in a light microscope, or prepared for flow cytometry analysis. Supernatants were collected for cytokine detection.

**Flow cytometry analysis** – BM-DCs or splenocytes were incubated with the corresponding antibodies for 30 min at 4°C and were washed with PBS. Cells were then analyzed on a FACSort Flow Cytometer (BD). The following fluorescence-conjugated antibodies were used: FITC CD11c, PE MHC class II (I-A/I-E), PE-Cy5 CD80, PE-Cy5 CD86 and Isotype controls (eBiosince). Ten thousand total events were collected and gated for analysis on live cells as determined by forward and side scatter. Results were analyzed using WinMDI software.

**Cytokine detection** – The levels of IL-12 p70 (IL-12) and IL-6 in the media were determined by ELISA (Peprotech) according to the manufacturer’s instructions.
RT-PCR – Total RNA was isolated from pure 10 day old untreated BM-DCs using TRIzol reagent (Invitrogen), according to the manufacturer’s instructions. Reverse transcription was performed using MMLV Reverse Transcriptase (Promega), from 1-2 µg of starting total RNA. Following cDNA synthesis, PCR was preformed using Taq polymerase (Biolabs), for EPO-R or β actin transcripts. Oligonucleotide primers derived from the murine EPO-R and β actin sequences were as follow: EPO-R: forward -5’ CGGGGTACCATTGGACAAACTC 3’, reverse – 5’ CAGGCTACATGACTTTCG TGACTC 3’; actin: forward - 5’ TTCTTTTGCACTCCTTCGT TGCCG 3’, reverse – 5’ TGGATGGCTACGTACATGGCTGGG 3’.

Biochemical analysis of EPO-R signaling in BM-DCs - Cells were starved for 1 h at 37°C by incubating them in RPMI medium without any supplements. In some experiments the JAK2 inhibitor AG-490 (0.1 µM) was added. Subsequently, 50 U/ml rHuEPO, 0.05 µg/ml LPS or 50 ng/ml GM-CSF were added to the cells at given time points. Phosphatase inhibitors (2 mM ZnCl₂, 2mM vanadate, 50 mM NaF, 20 mM Na₄P₂O₇) were then added on ice and cells were pelleted for cell extraction. Cytoplasmic and nuclear cell extracts were prepared as follows. Cell pellets were resuspended in buffer A - 0.1 mM EDTA, 10 mM KCl, 10 mM HEPES (pH 7.9), to which protease inhibitor cocktail (CompleteTM protease inhibitors; Roche Diagnostics) was freshly added and incubated on ice for 10 min. Cells were lysed by addition of 0.75% Nonidet P-40 and incubated for 5 min on ice, with repeated vortexing. The lysate was centrifuged at 5,000 rpm for 5 min to pellet the nuclei, and the supernatant (cytosolic extract) was stored at -80°C. The nuclear pellets were first washed in cold PBS and then resuspended in buffer C - 1 mM EDTA, 0.4 M NaCl, 20 mM HEPES (pH 7.9), with fresh addition of
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 15 min. The supernatants were recovered as nuclear extracts and stored at -80°C. Whole cell extracts were prepared as described (Cohen et al., 2004). 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, 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.

All supernatants were separated on 10% SDS-PAGE and transferred onto a nitrocellulose membrane filter. The nitrocellulose membrane was incubated with the following antibodies: phosphoERK1/2 [Sigma Chemicals]; phosphoSTAT5 (Tyr694), STAT5, phosphoSTAT3 (Tyr705), STAT3, phosphoAKT (Ser473), AKT [Cell Signaling]; ERK2, IκBα (C-21), histone H1 (AE-4) [Santa Cruz], Actin [Chemicon], p65 (Rel A) Ab-1 [Labvision] followed by labeled polymer-HRP anti-rabbit or anti-mouse antibodies [Dako].

**Statistical analysis** - Comparison of the data obtained for EPO and control treatments was performed using Student's t-test. A P-value of less than 0.05 was defined as statistically significant.
Results

In-vivo effects of EPO manifested on splenic DCs

We first determined whether DCs are in-vivo targets of EPO. Mice treated with rHuEPO as well as tg6 mice over-expressing EPO were used as experimental models to establish the effects of EPO on SDCs. Analysis of the total splenocyte population showed an increase in the number and in the percentage of SDCs, as represented by high surface expression of CD11c, in both, EPO-injected and in tg6 mice (Fig. 1A-C). Notably, the majority of SDCs in all mice were MHC class II positive. The expression of MHC class II on these cells was not affected by EPO (Fig. 1A and B).

To further address the effect of EPO on SDCs, we investigated the phenotype of the CD11c++ cell population by analyzing the co-stimulatory molecules CD80 and CD86 expressed on these cells. As shown in Fig. 2 in both, EPO-injected (Fig. 2A) and in tg6 (Fig. 2B) mice, the percentage of SDCs expressing CD80 and CD86 (Fig. 2C), as well as the cell surface expression levels of these molecular markers (Fig. 2D) were enhanced compared to their control counterparts. The stimulatory effects of EPO on SDCs were also observed in LPS-injected mice (data not shown). Our data thus reflect the impact of EPO on splenic DCs in-vivo. Hence, the subsequent experiments were designed to determine whether DCs are direct targets of EPO.

EPO-R expression in DCs from bone marrow

In order to determine whether EPO affects murine DCs directly, we employed the well-established model of BM-DCs (Lutz et al., 1999). This approach enabled the production of a large population of DCs in-vitro for subsequent analysis. Initially, we determined the
expression of EPO-R transcripts in the BM-DCs. mRNA was prepared from BM-DCs, based on their surface expression of CD11c. The mRNA was reverse-transcribed and subjected to PCR analysis, using specific primers derived from the EPO-R extracellular domain, yielding a 300bp fragment; actin specific transcripts were used as control. Our results thus establish the presence of EPO-R transcripts in DCs from bone marrow (Fig. 3, lane 2).

**In-vitro effects of EPO on viability and phenotype of DCs from bone marrow**

In order to properly function as antigen presenting cells, DCs must initially undergo a process of maturation (Banchereau et al., 2000). This process includes upregulation of MHC class II and co-stimulatory molecules, and induction of cytokine secretion (Pearce et al., 2006). In order to evaluate the effects of EPO on these processes, *in-vitro* derived BM-DCs were subjected to rHuEPO (20 U/ml) and/or LPS (0.05 µg/ml) treatment for 24 h. Viability of the BM-DCs was analyzed by Trypan Blue exclusion test and their phenotype assessed by the FACS analysis of cell-surface expression of co-stimulatory molecules CD80 and CD86, and MHC class II.

Figure 4A shows that stimulation with EPO for 24 h improved viability of BM-DCs, as compared to the control non-treated (NT) cells. Notably, under these conditions, EPO treatment also elevated cell surface CD86 and MHC class II but did not affect the expression of the CD80 (Fig 4B). We therefore questioned whether stimulation of BM-DCs using EPO together with LPS affects the cell phenotype and survival. Accordingly, BM-DCs were cultured for 24 h in the presence of LPS alone or LPS together with 20 U/ml rHuEPO. While survival of the BM-DCs following LPS or LPS + EPO treatments
were similar (Fig. 4A), BM-DCs treated with LPS + EPO displayed higher cell surface levels of CD80, CD86 and MHC class II as compared to cells treated with LPS alone (Fig. 4B).

**In-vitro effects of EPO manifested in increased secretion of IL-12 but not of IL-6**

We next examined whether EPO treatment affects the production of cytokines by DCs. Activated DCs secrete a variety of cytokines that influence the outcome of the immune response (Banchereau et al., 2000). IL-12 is essential for the development of the Th1 response and natural killer cell activation (Banchereau and Steinman, 1998; Kaisho and Akira, 2001). IL-6 secretion enhances the Th2 response and has pleiotropic effects on the immune system (Park et al., 2004). Accordingly, we evaluated the secretion of IL-12 and IL-6 by BM-DCs in response to EPO in the absence or presence of LPS. The levels of IL-12 and IL-6 in the culture media of cells after 24 h incubation were analyzed by ELISA. BM-DCs treated with EPO demonstrated increased levels of IL-12 in the culture medium as compared to the control cells, cultured in medium only (Fig. 5A). Treatment of the BM-DCs with LPS augmented the levels of both IL-12 and IL-6 (Fig. 5B). Despite the higher absolute levels of secreted cytokines in the presence of LPS, the combination of EPO and LPS augmented IL-12 (Fig. 5B). Notably, addition of EPO did not affect IL-6 secretion in both, the non-treated (NT) cells and in the LPS treated cells (Fig. 5).

**EPO leads to activation of multiple signaling pathways in DCs from bone marrow**

We questioned whether the EPO treatment of BM-DCs activates signal transduction pathways that participate in DC activation (Lindner et al., 2007; Rescigno et al., 1998)
and thus focused on the NF-κB, MAPK and AKT pathways. BM-DCs were stimulated with 50 U/ml rHuEPO or with 0.05 μg/ml LPS (positive control) for the indicated time periods and cell lysates were subjected to immunoblot analysis. EPO treatment induced Tyr phosphorylation of ERK1/2 and of AKT (Fig. 6A and B, respectively). EPO treatment also decreased the levels of cytosolic IκB (Fig. 6C) and increased p65 levels in the nucleus (Fig. 6D). We therefore conclude that EPO-R activation in BM-DCs results in the activation of the MAPK, AKT and NF-κB pathways (Fig. 6).

**EPO preferentially activates STAT3 in BM-DCs**

JAK2/STAT5 play a key role in initiating EPO-R activation and subsequent recruitment of signaling modules to the receptor (Richmond et al., 2005; Witthuhn et al., 1993). Unexpectedly, EPO-induced stimulation of BM-DCs did not show STAT5 phosphorylation (Fig. 7A, lane 3). In order to determine whether EPO-mediated activation seen in Fig. 6 is mediated by JAK2, we added the JAK2-specific inhibitor AG-490 (Meydan et al., 1996). BM-DCs were pre-incubated with or without AG-490 for 1 h and were stimulated with either EPO or with GM-CSF as a control for JAK2 mediated signaling (Taga and Kishimoto, 1995). Incubation with AG-490 inhibited GM-CSF-mediated STAT5 and STAT3 phosphorylation (Fig. 7A and B, lanes 5 and 6), thus confirming the inhibitory effect of AG-490 on JAK2 activity in BM-DCs. Non-treated and LPS treated cells were not affected by AG-490 (Fig. 7, lanes 1 and 2 and data not shown).

Of note, although EPO-driven STAT5 phosphorylation was not detected, EPO stimulation did lead to STAT3 phosphorylation (Fig. 7B, lane 3, and Fig. 7D).
Importantly, AG-490 inhibited this STAT3 phosphorylation induced by EPO and by GM-CSF (Fig. 7B). AG-490 also inhibited the MAPK and AKT phosphorylation following EPO and GM-CSF treatments (Fig. 7C and data not shown).

We thus conclude that EPO-driven signaling in BM-DCs is mediated by JAK2 and can lead to the activation of signaling pathways. Downstream activation of these pathways can thus prevent cell death through cell survival mechanisms, and can also alter DC phenotype by modulating the expression of cell surface molecules and influencing cytokine secretion (Banchereau and Steinman, 1998).
Discussion

In this study we prove the hypothesis that DCs are targets of EPO. In-vivo studies focused on SDCs, and in-vitro experiments were performed using MB-DCs. Our data revealed EPO-R expression on DCs and EPO effects, both in-vivo and in-vitro that manifest in cell viability and function. Further molecular analyses indicated that EPO led to the activation of signaling pathways in BM-DCs.

We centered on DCs as potential candidates that mediate the effects of EPO on lymphocytes, shown in the enhancement of both the cellular and humoral components of the immune system (Katz et al., 2007; Mittelman et al., 2001). Notably, DCs are the most potent APCs and are therefore capable of diverting the entire immune response according to the stimulatory signals that they receive (Katz et al., 2007; Mittelman et al., 2001). Furthermore, DCs are inhibited in MM patients by cytokines that are secreted by the neoplastic plasma cells (Brown et al., 2001). This phenomenon is one of the major defects of the immune system observed in MM patients. Another indicator for a possible role of EPO on DCs is the finding that, in addition to its anti-neoplastic effects in MM, EPO also has immunomodulatory effects that reduce immunosuppression in MM (Prutchi-Sagiv et al., 2006).

We determined effects on viability and phenotype in SDCs, in EPO-injected and in tg6 mice, supporting the notion that DCs are relevant targets of EPO. The percentage of SDCs of the total splenocytes was 1.4 fold higher in the EPO-injected mice and 2.3 fold higher in the tg6 mice. This increase was evident only in the SDC’s number, and not in the number of total splenocytes. We have previously shown a decrease in the percentage of the CD4⁺ lymphocyte population of the total splenocytes in both EPO-injected and in
tg6 mice (Katz et al., 2007). Though the decrease in CD4+ lymphocytes is higher than the increase in the SDCs, the latter EPO-associated effect may, at least partially, account for the decrease in CD4+ lymphocytes.

The splenic DC population is comprised of a variety of DC subtypes that arise from both myeloid and lymphoid origins (Banchereau et al., 2000). We could not detect any EPO-associated changes in the myeloid and lymphoid subpopulations, as determined by FACS analysis of CD11b and CD8α, respectively (data not shown). Further analysis of the SDCs' phenotype showed enhanced expression of the co-stimulatory molecules CD80 and CD86 in both EPO-injected and in tg6 mice. The EPO-associated increase of SDCs and the upregulation of co-stimulatory molecules were more profound in the tg6 mice. Similarly, EPO-associated effects on splenocyte proliferation, previously reported by us (Katz et al., 2007), were higher in tg6 mice, as compared to EPO-injected mice. The magnitude of these effects in the tg6 mice emphasizes the contribution of the extent of exposure to EPO.

For the in-vitro studies we determined the effect of EPO on myeloid-derived BM-DCs obtained from GM-CSF treatment (Inaba et al., 1992; Lutz et al., 1999). The choice of these cells was based on the reported EPO-R expression on myeloid-derived populations – erythroid progenitors and endothelial cells (Anagnostou et al., 1994; Sasaki et al., 2000). Evidence for a direct impact of EPO on DCs is based on our findings that the in-vitro expanded DCs derived from bone marrow, express EPO-R mRNA. Analysis of the EPO-R in DCs was performed by RT-PCR, due to the low levels of endogenous EPO-R (Broudy et al., 1991) and the inability to detect the EPO-R protein using the anti-murine
EPO-R antibodies (Cohen et al., 2004) routinely employed in our studies (data not shown).

Our results show that EPO-R stimulation in BM-DCs leads to upregulation of co-stimulatory molecules and of MHC class II, and enhances secretion of IL-12. We thus postulate that EPO improves BM-DC functionality and maturation. In this context, the leptin receptor, a member of the cytokine receptor superfamily, was shown to induce maturation of BM-DCs (Lam et al., 2007).

The increase in SDC number causally related to EPO is in line with the in-vitro effects of EPO on BM-DC viability. Notably, EPO (administration or over-expression) in-vivo was sufficient to change the SDC phenotype, while the change in the phenotype of BM-DCs was seen only following a synergistic stimulation of EPO and LPS.

EPO effects observed in the in-vivo mouse models of SDCs were more profound than those observed in the in-vitro models of DCs. This may be attributed to several causes: first, the DCs from bone marrow were stimulated for 24 h, while the injected mice received rHuEPO treatment for a week, and the tg6 mice had a constitutive exposure to high EPO plasma levels. Second, other known and unknown EPO-R-bearing cells may also play a role in activation of the SDCs. For example, activation of endothelial cells by EPO (Marzo et al., 2008) may lead to DC migration and enhanced survival. Third, the mice are exposed to different ongoing environmental stimuli, which may affect the basic status of the DC population. In that respect, it should be noted that EPO treatment in a murine model of multiple sclerosis reduced the number of reactive DCs in the lymph nodes (Yuan et al., 2008).
After exploring both the in-vivo and in-vitro effects of EPO, we sought to determine the molecular mechanisms underling EPO-R activation in DCs. We showed that EPO-R-stimulation in the BM-DCs activated the MAPK, AKT and NF-κB pathways. These pathways are crucial for the maturation and survival of DCs (Rescigno et al., 1998). EPO-R signaling is known to activate survival and proliferation in other EPO-R bearing cells. For example, EPO stimulation of erythroid progenitors leads to STAT, MAPK and AKT activation, thus inducing cell proliferation (Richmond et al., 2005). EPO-R activates AKT signaling, which leads to the activation of enthothelial cells and to NO secretion by them (Marzo et al., 2008). MAPK and NF-κB play a central role in EPO-R signaling in astrocytes (Lee et al., 2004). The enhanced BM-DC viability conferred by EPO, could thus result from activation of the above mentioned cell-survival signaling pathways.

Inhibition of EPO-driven signaling by AG-490, rules out the possibility that EPO activates DCs through either toxin residues or unspecific glycoprotein stimulation. The inability to detect STAT5 activation could result from low levels of STAT5 phosphorylation, undetected by the phosphoSTAT5 antibody. Another possibility is that in DCs, JAK2-mediated signaling through EPO-R might not involve STAT5 activation. This is reinforced by the detection of phosphoSTAT3 following EPO treatment. Our results suggest that in DCs, STAT3 may play a more important role than STAT5 in EPO-R signaling. STAT3 was shown to be activated by EPO-R signaling (Richmond et al., 2005), yet via a distinct mechanism from STAT5 (Kirito et al., 2002). While STAT3 activation is not crucial for EPO-R activity (Menon et al., 2006), and its role in erythropoiesis is still not clear, it plays a prominent role in development and maturation
of DCs (Laouar et al., 2003; Tian et al., 2007). In that respect, STAT5 signaling is associated with inhibition of DC development (Esashi et al., 2008).

Our data implies that in DCs, EPO-R activation may not lead to classical EPO-mediated signaling. It has been shown that non-hematopoietic tissues can express an atypical EPO-R beta-subunit heteroreceptor (Brines et al., 2004) that exhibits a non-classical EPO-mediated signaling (Arcasoy, 2008). Other studies have demonstrated that the classic homodimeric EPO-R is essential for non-hematopoietic effects of EPO (Um et al., 2007). The precise molecule entity of the DC EPO-R thus remains to be resolved.

Taken together, the current data, along with our recent findings, that human monocyte-derived DCs express the EPO-R and that their function is modulated by EPO (Prutchi-Sagiv et al., in press), imply that DCs are indeed an EPO target. We propose that the immunomodulatory effects of EPO, detected in MM and in inflammation models, can be explained, at least partially, by EPO directly activating the DCs.

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normal but functionally defective as they fail to up-regulate CD80 (B7-1) expression after huCD40LT stimulation because of inhibition by transforming growth factor-beta1 and interleukin-10. *Blood* 98, 2992-8.


Figure Legends

Figure 1. EPO \textit{in-vivo} increases the percentage of SDCs
Spleen cells were isolated by collagenase D treatment and analyzed for expression of CD11c and MHC class II surface molecules. Flow cytometry analysis of splenocytes from (A) C57BL/6 mice injected three times with 180 U rHuEPO every other day during a week, or with diluent as a negative control, and from (B) tg6 and their wt C57BL/6 wild-type littermates. (C) Summary of the number and percentage of CD11c positive splenocytes. Table represents the mean ± S.E.M of 3 independent experiments, each depicting 3 female mice per experimental group, **p<0.01 for EPO/tg6 \textit{vs} diluent/wt respectively.

Figure 2. EPO \textit{in-vivo} enhances expression of co-stimulatory molecules on SDCs
CD11c positive splenocytes were analyzed for CD80 and CD86 expression. Surface expression of these molecules is represented as black or gray histograms. Isotype controls are represented by broken line histograms. Analysis of SDCs from (A) rHuEPO injected mice and diluent injected mice - black and gray histograms respectively, and from (B) tg6 and their wt littermates - black and gray histograms respectively. (C and D) Summary of CD80 and CD86 expression depicting: (C) percentage of cells expressing the markers and (D) mean fluorescence intensity (MFI) of these markers. Graphs represent the mean ± S.E.M of 3 independent experiments, each analyzing 3 female mice per experimental group, *p<0.05, **p<0.001.

Figure 3. EPO-R expression in BM-DCs
EPO-R mRNA from murine BM-DCs (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 – Positive control (Pos. co.) - cDNA of BaF/3 cells transfected with murine EPO-R (Cohen et al., 2004). Lane 2 – cDNA of BM-DCs. Lane 3 – Negative control (Neg. co.) - cDNA of MBA cells (Benayahu et al., 1995).

**Figure 4. EPO in-vitro affects viability and phenotype of BM-DCs**

BM-DCs were treated in-vitro with or without 0.05 µg/ml LPS and/or 20 U/ml rHuEPO for 24 h. (A) Following 24 h treatment, BM-DCs were stained with Trypan Blue to assess cell viability. Cells were counted using a light microscope. NT – non-treated. (B) Summary of flow cytometry analysis of BM-DCs' MFI of CD80, CD86 and MHC class II expression following 24 h treatment. Graphs represent the mean ± S.E.M of 3 independent experiments. *p<0.05, **p<0.01 for NT cells vs treated cells, †p<0.05 for LPS+EPO-treated cells vs LPS-treated cells.

**Figure 5. EPO in-vitro increases secretion of IL-12 but not of IL-6 by BM-DCs**

BM-DCs were treated in-vitro in the absence (A) or presence (B) of 0.05 µg/ml LPS with or without 20 U/ml rHuEPO for 24 h. Cell culture supernatants were analyzed for the presence of IL-12 and IL-6 by ELISA. Graphs represent the mean cytokine concentration ± S.E.M. of at least 3 independent experiments, **p<0.01 for NT cells vs EPO-treated cells, ††p<0.01 for LPS+EPO-treated cells vs LPS-treated cells.

**Figure 6. Stimulation of BM-DCs with EPO activates multiple signaling pathways**

BM-DCs were treated in-vitro with 50 U/ml of rHuEPO or 0.05 µg/ml LPS (positive control) for the indicated time periods. Cell lysates of the cytosolic (A, B and C) and nuclear (D) fractions were subjected to immunoblot analysis with the indicated antibodies. (A) MAPK signaling was determined by ERK1/2 phosphorylation. Total
ERK2 levels are depicted for normalization. (B) AKT signaling was assessed by AKT phosphorylation. Total AKT levels are depicted for normalization. (C and D) NF-κB signaling was detected by (C) cytosolic IκB levels and (D) nuclear p65 levels. Cytosolic actin and nuclear histone are depicted for normalization, respectively. Graphs represent the mean ratio (EPO-treated/non-treated) ± S.E.M of at least 3 independent experiments, *p<0.05.

**Figure 7. EPO preferentially activates STAT3 in BM-DCs**

BM-DCs were pre-incubated with or without 0.1 μM of the JAK2 inhibitor AG-490 for 1h. Cells were then treated for 30 min with 50 U/ml of rHuEPO, or 50 ng/ml of GM-CSF. Immunoblot analysis was performed on the protein lysates, using the indicated antibodies, exp-exposure (A) STAT5 signaling was addressed by STAT5 phosphorylation; STAT5 levels are depicted for normalization. (B) STAT3 signaling was determined by STAT3 phosphorylation; STAT3 levels are depicted for normalization. (C) MAPK signaling was assessed by ERK1/2 phosphorylation; ERK2 levels are depicted for normalization. Immunoblots represent one of at least 3 independent experiments, displaying similar results. (D) Summary of EPO-mediated STAT3 phosphorylation. Graph represents the mean ratio (EPO-treated/non-treated) ± S.E.M of at least 3 independent experiments, *p<0.05.