Thrombopoietin modulates the proliferation, migration and cytokine profile of decidual cell subsets during early gestation

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ABSTRACT: During early gestation, a considerable increase in different leukocyte subsets can be observed in the decidualized endometrium concomitantly to the invasion of cytotrophoblast cells (CTB). To date, it is still in question which factors induce this accumulation of immune cells and whether it is evoked by an in situ proliferation or by a migratory process. Studies on hepatoblastoma cells identified thrombopoietin (TPO) as a novel factor, which elicits dose-dependent chemotactic and chemokinetic effects. However, the impact and function of TPO on decidual cells has not been clarified yet.

This study analyses the expression and function of TPO and its receptor c-Mpl in decidua during early gestation. Applying western blot analysis, we detected that TPO is expressed by decidual immune cells (uNK cells and CD14+ monocytes) as well as CTB and decidual stromal cells (DSCs). Expression of the different isoforms of c-Mpl was found in uNK cells, CD14+ monocytes and DSC. Studying the signalling pathway proteins in the uNK cells, an activation of STAT3/Tyr by TPO, was detected. The investigation of the proliferative effects of TPO on the decidual cell subsets revealed that TPO enhances the proliferation of uNK cells and CTB. No change of the proliferative activity after TPO incubation was found in DSC and even a decrease in CD14+ monocytes. In addition, TPO was observed to induce significantly the migratory activity of uNK cells, CD14+ monocytes and CTB. Investigating the effects of TPO on the cytokine profile of the isolated decidual cells, we observed a decrease in the secretion of IL-8, IL-10 and IL-1β of isolated uNK cells, CD14+ monocytes and CTB, although these changes did not reach statistical significance. Thus, we here identified TPO as a novel factor modulating the proliferation, migration and possibly cytokine secretion of decidual cell subsets.

Key words: early pregnancy / decidua / TPO / migration / immune cells

Introduction

Upon establishment of early pregnancy, human uterine mucosa is characterized by a remarkable increase of a broad range of immune cell populations. Apart from the regulation through hormonal factors, soluble mediators such as chemokines are likely to orchestrate this migratory and proliferative activity of immune cells (Norwitz et al., 2001; Dominguez et al., 2003). Chemokines are small polypeptides that are able to mediate leukocyte migration through specific receptors and to induce chemotaxis along a concentration gradient (Jones et al., 1997). There is growing evidence that chemokines may contribute to the accumulation of leukocyte subsets in the decidual tissue during early gestation (Jones et al., 2004). Thus, a simultaneous rise in uterine natural killer cells (uNK cells) and an increasing expression of granulocyte attracting proteins like interleukin-8 (IL-8) is observed within the decidual tissue (Loke and King, 1995; Koopman et al., 2003; Segerer et al., 2009). In addition, monocyte-attracting factors such as the monocyte chemotactic protein-1 (MCP-1) were detected in endometrial and decidual tissue samples which might be responsible for the presence of remarkable amounts of CD14+ monocytes during the menstrual cycle and early human pregnancy (Rieger et al., 2004).

In our previous studies, which focused on the investigation of the expression of cytokines and chemokines in the endometrial and decidual tissue, we detected a significantly higher expression of the
hematopoietic growth factor thrombopoietin (TPO) in the decidual tissue compared with non-pregnant endometrium (Segerer et al., 2009). To date, this growth factor was principally described as a potent stimulator of megakaryocytopoiesis and of hematopoietic cell lineages (Kaushansky et al., 1994; Wendling et al., 1994). TPO is able to ensure an adequate thrombopoiesis by controlling the proliferation and differentiation of megakaryocyte progenitor cells (de Sauvage et al., 1994; Kaushansky et al., 1995a, b). The major sources of TPO production known so far are the liver, the kidney, the bone marrow and the spleen (Shimada et al., 1995; Nomura et al., 1997).

TPO mediates its signalling through c-Mpl, a receptor sharing structural features of the receptors for granulocyte-colony-stimulating factor (Miyakawa et al., 2000). Dimerization of c-Mpl induced by TPO binding is followed by the activation of several intracellular signalling pathways, including JAK tyrosine kinases and members of the STAT family (Drachman et al., 1999). To date, several isoforms of unknown function have been detected for both mouse and human c-Mpl, which could play an important role in the modulation of TPO response (Coers et al., 2004; Saur et al., 2010). Previous studies demonstrated that not only megakaryocytes are expressing c-Mpl but also erythroid, granulocyte–macrophage progenitor cells and platelets (Methia et al., 1993; Kaushansky et al., 1996).

Experiments on malignant cell lines demonstrated a potent chemotactic effect of TPO (Romanelli et al., 2006). In this study, TPO was shown to increase the migration of hepatoblastoma cells in a dose-dependent manner. We therefore speculated that TPO could represent a novel factor exerting chemotactic effects on decidual cell subsets as well as on cytrophoblast cells (CTB). To verify this hypothesis, we first identified the decidual cell subsets expressing TPO and its specific receptor c-Mpl. Subsequently, we investigated the ability of TPO to act as a growth factor or chemokine on uNK cells, CD14+ decidual monocytes and isolated invasive CTB. Observing that it is especially uNK cells which are affected by TPO, we examined the intracellular signalling pathway of TPO in uNK cells.

Materials and Methods

Tissue specimens

First-trimester decidual tissue was obtained from healthy women undergoing legal therapeutic abortion of a normal pregnancy (6–9 weeks of gestation). Fragments of decidual tissue were dissected free of embryonic tissues and washed twice with phosphate-buffered saline (PBS). Subsequently, tissue samples were used either directly for cell isolation, fixed in 4% buffered formalin, processed in routine paraffin embedded or snap frozen in liquid nitrogen and stored at −80°C. Sections of frozen tissue samples were evaluated after routine hematoxylin–eosin and cytokeratin staining to detect samples without signs of necrosis and inflammation but containing extravilious trophoblast cells. Corresponding tissue was used for protein extraction. The institutional Ethics Committee approved all these investigations and women gave informed consent (ethical approval number: 07/77).

Single cell isolation

For isolation of decidual cells, native decidua was thoroughly washed in PBS, blood clots were completely removed. The tissue was fragmented into pieces of ~1 mm³. Those were consecutively digested for 20 min at 37°C under slight agitation in PBS with 200 U/ml hyaluronidase (Sigma, Taufkirchen, Germany), 1 mg/ml collagenase type I (Biochrom, Berlin, Germany), 0.2 mg/ml DNASE I (2500 U/mg; Sigma) and 1 mg/ml bovine serum albumin/fraction V (Sigma). The generated cell suspension was passed through a sterile stainless steel of 50-µm wire mesh and washed once in PBS. Mononuclear cells were separated by a density gradient at 400g (Leucocyte separation medium, PAA, Cölbe, Germany). After washing twice in PBS, cells were immediately used for specific cell isolation.

Positive selection of CD56+CD16- uNK cells was performed in a two-step procedure applying the CD56+CD16-isolation kit as described in Segerer et al. (2012) (order number: 130-092-661, Miltenyi, Bergisch Gladbach, Germany; clone Leu-M3, Miltenyi). Selection of CD14+ monocytes/immature DC was performed by labeling with anti-CD14 Microbeads (clone AF12-7H3; Miltenyi), according to the manufacturer’s instructions. Invasive CTB are shown to be positive for c-erb B2 (Jokhi et al., 1994) and thus isolated using an anti c-erb B2 antibody (order number: 130-090-482, Mouse IgG1; Miltenyi; Supplementary data, Fig. S1). We found decidual stromal cells to be highly and specifically positive for a fibroblast specific antibody (order number: 130-050-601, Anti-Fibroblast MicroBeads, IgG2a; Miltenyi) and used this surface marker for their enrichment. Labeled cells were passed through a positive selection column by a magnetic-assisted cell sorting (MACS, Miltenyi) separator.

Purity of enriched cell fractions was analyzed by flow cytometry and in all cases >90% of the specific cell type was detected (a representative example is shown in Engert et al., 2007 and Supplementary data, Fig. S2). Cells were subsequently used for cell culture experiments or stored at −80°C until RNA/protein isolation.

As control cell for our experiments, HUVEC cells (purchased from Cell Lines Service Heidelberg, Germany) as well as thrombocytes (obtained as aliquot from thrombocyte donations at the Department of Haemotherapy and Transfusion Medicine, University of Wuerzburg) were utilized.

Blood monocyte derived dendritic cell preparation

Specimens were obtained from healthy human donors donating thrombocytes. White blood cells were collected in the separation chambers of the Cobe Trima Leukapharesis system at the Department of Immunohematology and Transfusion Medicine, University of Wuerzburg. Cells were isolated and cultured as described by Romani et al. (1996). In brief, after dilution with an equal volume of PBS, citrate-buffered buffy coats were distributed over a density gradient (density 1.077 g/ml, leukocyte separation medium, PAA) and centrifuged at 400 g for 30 min at room temperature (RT). Peripheral blood monocytes cells (PBMCs) were collected from the interface, washed twice with PBS, resuspended in AIM-V-Medium (Invitrogen), and incubated for 1 h at 37°C with gentamycin (20 µg/ml; Biochrom). To remove non-adherent cells, the dishes were thoroughly washed several times with PBS. The remaining adherent cell fraction was used for the generation of monocyte-derived DC by culturing them with 800 U/ml purified recombinant human granulocyte macrophage colony-stimulating factor (GM-CSF; Leukomax, Bayer, Brussels, Belgium) and 250 U/ml IL-4 (Cellgro, Freiburg, Germany) for 5 days in RPMI 1640 (Biochrom) with 10% FCS (PAA) and 20 µg/ml gentamycin (Biochrom).

Western blot analysis

The frozen cell pellets (corresponding to 1 x 10⁸ cells) were lysed in 150-µl RIPA buffer (Invitrogen, Karlsruhe, Germany) containing proteinase inhibitors (Complete, Roche, Mannheim, Germany), phosphatase inhibitors (Phosphatase Inhibitor Cocktails Set II, Calbiochem, Germany) and 2 mM DTT (dithiothreitol; Invitrogen, Karlsruhe, Germany). The mixture
was incubated on ice for 30 min and remixed every 10 min. To separate the proteins from the cell debris, the mixture was then centrifuged at 14,000 g for 10 min using a QIAshredder spin column (QIAGEN, Hilden, Germany). Afterwards, the cell lysates were diluted at 1:5 in 5 × loading buffer (Fermentas, St. Leon-Roth, Germany), denatured at 95 °C for 5 min and cooled down on ice.

The solubilized proteins were isolated on a 10% polyacrylamide gel (SDS-PAGE), and then blotted onto a nitrocellulose membrane for 90 min at 10 V using a semi-dry transfer unit (Peqlab, Erlangen, Germany). After blocking with a solution of 5% non-fat milk powder in 0.05% PBS/Tween 20, target proteins were incubated for 18 h with the goat-anti-TPO or mouse-anti-c-Mpl antibody (R&D Systems, Wiesbaden, Germany) diluted 1:500 in 2% non-fat milk and PBS/Tween. Afterwards the membrane was incubated with a horseradish peroxidase-conjugated donkey-anti-goat or goat-anti-mouse antibody, respectively (KPL, Wedel, Germany) as secondary antibodies diluted at 1:750 in 2% non-fat milk and PBS/Tween. Home-made enhanced chemiluminescence (ECL; Labjournal, 2007; http://www.laborjournal.de/rubric/tricks/tricks/trick81.html) was used as substrate and luminescence was detected on an X-ray film (Fuji, Düsseldorf, Germany). For internal loading control, the antibodies were Cα-actin, using a mouse-anti-α-actin antibody (1:10,000; Abcam, Cambridge, UK) followed by goat-anti-mouse POD conjugate (1:7,500; KPL) as secondary antibody. For the interpretation of results, the protein bands were quantified using the Image J Program (NIH).

**Immunohistochemistry**

For the detection of c-Mpl-positive uNK cells, immunohistochemical stainings were performed. Sections (2–3 μm) from paraffin-embedded decidua tissue were placed onto 3-amino-propyltrioxy-silane (Roth, Karlsruhe, Germany) coated slides, dehydrated in graded ethanol and in TRIS-buffered saline (TBS; 25 mM TRIS/HCl, pH 7.4, 137 mM NaCl, 2.7 mM KCl). Antigens were presented by heat pretreatment (boiling in 0.01 M of sodium citrate buffer (pH 6.0) for 10 min in a microwave oven at 750 W). In order to reduce non-specific background staining, tissue sections were blocked by human immunoglobulin (Bergiglobin; Centeon, Marburg, Germany) diluted 1:20 in PBS. The sections were then incubated successively with rabbit-anti-human polyclonal TPO-R antibody (Sigma, Germany) diluted at 1:100 and mouse-anti-human CD56 antibody (Monosan, Germany, clone 123C3) at 1:50 overnight at 4 °C. Primary antibodies were then bound with a combination of the Cy2-labeled goat-anti-mouse antibody (Dianova, Hamburg, Germany) at 1:400 and the Cy3-labeled goat-anti-rabbit antibody (Dianova) at 1:500 for 45 min at RT in the dark.

After a final wash in PBS, stained sections were embedded in anti-fade-mounting medium (DAKO) and analyzed using Zeiss LSM 510 Meta confocal microscope, equipped with 40 × oil objective (NA 1.4) and laser lines 488 and 543 nm at the single-track option to avoid cross reaction of the fluorescence.

**Incubation of isolated uNK cells, CD14+ monocytes and CTB with TPO**

Human recombinant TPO (R&D Systems, Wiesbaden, Germany) was added at 100 ng/ml to the cultured cells. This concentration was selected based on the literature to obtain optimal results (Kubota et al., 1996). In advance, concentration–response experiments were performed whereby significant effects were observed at 100 ng/ml. For analysis, the cells were harvested after 12, 24 or 48 h of culture for the different experiments.

**Cell signalling**

For the investigation of cell signalling pathway, western blot analysis was applied as described above. Isolated uNK cells were incubated with or without 100 ng/ml TPO for 24 h. Thereafter, extracts from treated/un-treated uNK cells were analyzed by western blot using phopho-STAT1 antibody, phopho-STAT2 antibody, Phopho-STAT3 (Tyr 705) antibody, phopho-STAT3 (Ser) antibody, phopho-STAT5 antibody and phopho-STAT6 antibody all purchased by Cell Signalling Technology (Denver/USA). As recommend by the purchaser, Hela cells were taken as internal positive control and stimulated with IL-4 (100 ng/ml, Cell Grow, Freiburg, Germany) or IFN-alpha (100 ng/ml, Cell Grow), respectively, for 24 h.

**Proliferation assay**

Isolated uNK cells, CD14+ monocytes, DSC and CTB (1 × 10⁴) were incubated with or without increasing concentrations of TPO (0, 1, 10 and 100 ng/ml) in serum-free RPMI supplemented with 10% FCS for 12–48 h. Proliferation was determined with a colorimetric non-radioactive WST-8 proliferation assay for quantification of cell proliferation and cell viability (Biozol, Eching, Germany). The test is based on the cleavage of the tetrazolium salt WST-8 by mitochondrial dehydrogenases in viable cells. Detection was performed by absorption spectroscopy in an ELISA reader at 420–480 nm.

**Migration assay**

Migration of uNK cells, CD14+ monocytes or CTB was measured in Transwells Chambers with 8-μm pore size (Becton Dickinson, Heidelberg, Germany). Six independent sets were performed each. The lower well was filled with 750 μl of serum reduced medium (RPMI with 1% FCS) with or without 100 ng/ml TPO. Cells (2 × 10⁴) were placed in the upper well. Migration was assessed overnight. Cells reaching the lower surface were fixed and stained with hematoxylin. Migration was quantified by performing cell counts on 10 randomly selected fields at ×400 magnification.

**Analysis of cytokine secretion by cytometric bead array**

To test the secretion of IL-6, IL-8, IL-10 and IL-1β after TPO exposure, culture supernatants of isolated uNK cells, CD14+ monocytes and CTB (10⁶ each) were applied for a cytometric bead assay according to the manufacturer’s instructions (Bender Med Systems, Wien, Austria). Cells were cultured with or without 100 ng/ml TPO overnight in RPMI 1640 (Biochrom) supplemented with 10% fetal calf serum (FCS; PAN, Aidenbach, Germany) and gentamycine (20 μg/ml; Biochrom). Briefly, bead populations with distinct fluorescence intensities coated with capture antibody proteins were first mixed with PE-conjugated detection antibodies and re-combinant standards or test samples, and then incubated to form sandwich complexes. Beads specific for IL-6, IL-8 IL-10 and IL-1β were detected by FACScan flow cytometer (BD Biosciences, Heidelberg, Germany). Results were analyzed using the software FlowCytomix Pro 2.2.1 (Bender Med Systems).

**Data analysis**

In total, 3–12 independent experiments were analyzed for each test system. Since the decidual cell donors did not conform to a normal distribution pattern as assessed by the Shapiro–Wilkins test for normality, we used the non-parametric Mann–Whitney U-test for the comparison of proliferative and migratory activity as well as cytokine secretion. Statistical analyses refer to comparisons between TPO pretreatment and the
corresponding untreated control cells. P-values of \(<0.05\) were considered as significant (Graph-Pad Software, San Diego, USA).

## Results

### TPO and its distinct receptor c-Mpl are expressed by decidual cell subsets

Western blot experiments revealed that TPO at its different isoforms (Kubota et al., 1996) is expressed by uNK cells, CD14+ monocytes, CTB, DSC and PBMC (Fig. 1A). Investigating the expression of c-Mpl, we found that alike TPO, c-Mpl is expressed at its isoforms (80/85 kDa: Saur et al., 2010; 93 kDa: Coers et al., 2004; 71 kDa Abcam Antibodies, Cambridge, UK) by uNK cells, CD14+ monocytes, PBMC and also by DSC. Reduced expression was found in CTB. Thromobocytes and HUVEC cells were chosen as internal positive controls (Methia et al., 1993) (Fig. 1A).

To support the localization of the TPO receptor in uNK cells, an immunohistochemical staining was added (Fig. 1B).

### TPO activates molecules of the STAT pathway in uNK cells

Activation of c-Mpl initiates multiple intracellular pathways like the STAT pathway in platelets which controls specific gene expression and pro- or anti-apoptotic effects. We therefore tested the activation of this intracellular pathway in uNK cells which were found to express c-Mpl in all samples tested. Using western blot, we detected that phosphorylated STAT1, STAT2 and STAT3/Ser were expressed in isolated uNK cells even prior to TPO exposure. An increased expression after TPO exposure was only detected for phosphorylated STAT3/Tyr (Fig. 2). Investigating the expression of phosphorylated STAT6 with or without TPO, we did not find any expression (data not shown).

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**Figure 1** Expression of TPO and TPO receptor isoforms (c-Mpl) in decidual cell types. (A) After isolation of decidual cells by either anti-CD56, anti-CD14, anti-c-erbB and anti-fibroblast MicroBeads, the expression of TPO and its corresponding receptor were investigated by western blot. The different isoforms of c-Mpl (71, 80/85 and 93 kDa) and TPO (34/36, 46 and 66 kDa) could be detected in the different decidual cell subsets. HUVEC cells and thrombocytes were chosen as internal positive controls. uNK, uterine natural killer cells; CD14, CD14+ decidual monocytes, CTB, cytotrophoblasts; DSC, decidual stromal cells; PBMC, peripheral blood monocytes. (B) Immunohistochemical detection of c-Mpl expression in CD 56+ NK cells in the decidual tissue.
Proliferative activity of decidual immune cells is affected by TPO

It is a well known fact, that TPO represents a growth factor controlling the proliferation and differentiation of megakaryocytes (Methia et al., 1993; Akkerman, 2006). Since a significant increase in immune cells can be detected in the decidual tissue with the onset of pregnancy (Methia et al., 1993; Gomez-Lopez et al., 2010), we tested whether this increase could be evoked by an in situ proliferation of decidual immune cells after TPO exposure. Thus, we analyzed the proliferative activity of uNK cells, CD14+ monocytes, DSC and CTB after incubation for 12–48 h with TPO at four different concentrations, whereby significant effects were shown at 100 ng/ml (Fig. 3). Here we detected that TPO significantly increased the proliferation rate of isolated uNK cells and CTB at 100 ng/ml, whereas CD14+ monocytes remained at stable levels or even decreased with increasing concentrations. Proliferative activity of DSC was not altered by exposure to TPO (Fig. 3). Effects were already observed after 12 h and did not change significantly increasing the incubation period (48 h, see Supplementary data, Fig. S3).

Migratory activity of uNK, CD14+ monocytes and CTB is significantly increased by TPO

Studies by Romanelli et al. (2006) have reported an increase of the cell migration of hepatoblastoma cells after TPO exposure. We therefore studied the chemotactic effect of TPO on isolated uNK cells, CD14+ monocytes and CTB. Here, we detected that TPO was able to increase significantly the migratory activity of these three cell subsets (Fig. 4). Thus, we revealed that TPO could act as a potent chemokine of uNK cells, CD14+ monocytes and CTB.

Secretion of pro- or anti-inflammatory cytokines is altered by TPO

The decidual micromilieu is characterized by a special composition of chemokines and cytokines. We therefore analyzed the secreted cytokines of isolated uNK cells, CD14+ monocytes and CTB (10⁶ each) after incubation with TPO for 24 h. On the one hand, the secretion of the selected cytokines differed concerning the quantity produced by either uNK, CD14+ monocytes or CTB. Thereby, a predominant secretion of IL-8 was observed by uNK cells (4577 ± 338 pg/ml). The production of IL-6 (10 220 ± 2852 pg/ml), IL-10 (12 870 ± 1384 pg/ml) and IL-1beta (1891 ± 133 pg/ml) was dominated by CD14+ monocytes. Exposing these cell subsets to TPO an overall reduction in the levels of IL-6, IL-8, IL-10 and IL-1beta (except for IL-6, whose secretion was increased in CTB) was observed, although effects were not significant due to the large standard deviation. To summarize, we observed that TPO affects the release of cytokines in uNK cells, CD14+ monocytes and CTB, although not in a significant manner (data not shown).

Discussion

Clinical studies measuring the circulating serum levels of cytokines, chemokines and growth factors in pregnant women demonstrated an increased risk of miscarriage in pregnancies with elevated TPO levels (Whitcomb et al., 2008). In these investigations, TPO, the primary regulator of the megakaryocyte lineage and factor promoting the expression of vascular endothelial growth factor was speculated to mimic a hypoxic status at the feto-maternal interface and thus to impact placentation (Kiriti et al., 2005; Whitcomb et al., 2008).

To date, little is known about the expression of TPO and the function of this growth factor at the feto-maternal interface. This is the first study illustrating that TPO is constitutively expressed by uNK cells CD14+ monocytes, CTB and DSC. In addition, the corresponding highly specific TPO receptor c-Mpl has been detected at its several isoforms (Coers et al., 2004; Saur et al., 2010) in uNK cells, in CD14+ monocytes, in DSC and at lower levels in CTB. This observation is in line with the detection of the expression of c-Mpl and its signal transduction molecules in non-hematopoietic cells (Morella et al., 1995).

As it is well established that TPO is able to control the proliferation and differentiation of megakaryocyte progenitor cells (de Sauvage et al., 1994; Kaushansky et al., 1995b), we firstly investigated the effects of TPO on the proliferative activity of the decidual immune cells (uNK cells and CD14+ monocytes) and on CTB and DSC. To our surprise, TPO was able to act as a growth factor on uNK cells and CTB where best effects could be observed at 100 ng/ml, but exhibited adverse effects on CD14+ monocytes. Here, the proliferative activity decreased with increasing concentrations. In DSC, proliferation was not significantly affected. Previous studies on hepatoblastoma cells showed that TPO was not able to increase the proliferation of these malignant cells when incubated in serum-deprived conditions with 100 ng/ml TPO (Romanelli et al., 2006). However, acting in different ways on uNK cells or CD14+ monocytes, CTB and DSC, it could be speculated that TPO affects the different isoforms of c-Mpl in the decidual cell subsets or activates different intracellular pathways which leads to a modified TPO response.
Activation of c-Mpl by TPO results in receptor homodimerization and subsequent activation of the JAK2 and Tyk2 Janus kinases (Parganas et al., 1998; Drachman et al., 1999). Key downstream targets of these Jak kinases are the signal transducers and activators of transcription (STAT). In human platelets, phosphorylation of STAT1, STAT3 and STAT5 has been demonstrated following the binding of TPO on c-Mpl (Schulze et al., 2000). To date, nothing is known about the signal transducing molecules of c-Mpl in decidual cell subsets. Detecting c-Mpl in all isolated uNK cell samples, we exemplarily examined those transducing molecules in uNK cells. Here, we found that exposure of uNK cells to TPO resulted in a phosphorylation of the STAT3/Tyr pathway. This observation would be in line with the description of molecules of the STAT pathway in uNK cells (Poehlmann et al., 2005). For the STAT1, STAT2 and STAT3/Ser pathway of the isolated uNK cells, we did not find a substantial increase after exposure to TPO in vitro. It could be speculated that these pathway molecules have already been phosphorylated in vivo in isolated uNK cells and thus exhibit a pre-activated status.

Studies of Romanelli et al. (2006) demonstrated a potent chemotactic role for TPO. In view of the complex migratory activities of maternal immune cells and fetal cytrophoblasts in the decidual tissue, we analyzed the ability of TPO to act as a chemokine on decidual cells.

Figure 3 TPO increases the proliferation of uNK and CTB but decreases that of CD14+ monocytes. No effects were found for DSC. Cells were incubated without or with 0, 1, 10 or 100 ng of TPO for 12 h. Proliferation was determined with a colorimetric non-radioactive WST-8 proliferation assay for quantification of cell proliferation and cell viability whereby as a reference point, the initiation of the culture has been chosen. n = 12.

Figure 4 TPO induces the migratory activity of uNK, CD14+ monocytes and CTB. Migration of the cells was measured in Transwell Chambers and cell numbers per 10 high power fields (×400 magnification) were counted. TPO was able to increase significantly the migratory activity of uNK, CD14+ monocytes and CTB (*P < 0.05, untreated cells versus cells exposed to TPO; n = 10).
Consistent with the results on hepatoblastoma cells, we ascertained that TPO can stimulate the migratory activity of uNK cells, CD14+ monocytes and isolated CTB (Romanelli et al., 2006). Thus, we propose that TPO may support the placentation process by promoting the migration of CTB. Additionally, it seems to stimulate the increase of immune cells at the feto-maternal interface which may contribute to the generation of a micromilieu optimal for the acceptance of the semiallogenic fetus.

The decidual microenvironment is dominated by a distinct equilibration of pro- and anti-inflammatory factors. While pro-inflammatory factors like IL-8 are thought to support placentation (Bischof et al., 2000), anti-inflammatory cytokines (e.g., IL-10) rather induce tolerance by stimulating regulatory T-cells (Robertson et al., 2000). We therefore speculate that TPO may contribute to the balance of the cytokine milieu by modulating the secretion of cytokines by the decidual cell subtypes.

In conclusion, we have identified TPO as a novel factor influencing the functional role of decidual immune cells as well as cytotrophoblasts which may modulate their migratory and proliferatory effects.

**Supplementary data**

Supplementary data are available at http://molehr.oxfordjournals.org/.

**Authors’ roles**

S.E.S. drafted the manuscript, set up the experiments, participated in data collection, analyzed and interpreted the results. F.M. and A.B. carried out the western blot analysis, the migration and proliferation assays. N.M. and M.K. were performing the immunoocytochemical stainings. J.D. participated in editorial support; L.R. revised the article. U.K. participated in the design of the study, data analysis, provided images and figures and revised the article for intellectual content. All authors read and approved the final manuscript.

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**Conflict of interest**

None declared.

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