

Volume 12, issue 2, June 2001 | Recherches

Expression of thrombopoietin by umbilical vein endothelial cells

Abstract

Current data suggest that the primary source of thrombopoietin (TPO) is the liver. Extra-hepatic sites for TPO production have been demonstrated essentially through the study of the expression of TPO mRNA. In this work, we report that TPO is expressed at low levels by endothelial cells (EC) derived from the umbilical vein (HUVEC). Both TPO mRNA and the protein are expressed and the protein is functional as assessed by biological assay. Expression of TPO by HUVEC may be useful to study the regulation of the production of this cytokine and to understand the apparent specific interactions between mature megakaryocytes and EC in the bone marrow.

Full text

INTRODUCTION

Thrombopoietin (TPO) is a primary regulator of megakaryocytopoiesis. Impairing either TPO production or the expression of the TPO receptor in mice leads to reduction of platelet production to about 15% of normal basal levels in peripheral blood [1, 2]. Overexpression of TPO in mice or administration of recombinant TPO to animals or humans leads to a dose-dependent increase in the platelet counts [3-6]. TPO is not only an essential cytokine for the maturation of megakaryocytes, it also stimulates the expansion of megakaryocyte progenitors [7-9]. Biological activities of TPO have also been demonstrated on non-megakaryocyte lineages. TPO alone or in combination is able to stimulate the growth of human erythroid, myeloid and multipotential progenitors [10-17]. Little is known about the regulation of the production of TPO. One possibility is that TPO gene expression is unchanged and that the blood levels of TPO are dependent on the platelet or megakaryocyte mass, *via* c-mpl receptor uptake and degradation [18-30]. Regulation of gene expression by the platelet mass [31], or mediators such as hepatocyte growth factor [32], TGF-beta1 [33] or the content of platelet alpha-granules [34] has also been shown. TPO is expressed by the liver and extrahepatic tissues such as kidney, spleen, bone marrow stromal cells, fetal and neonatal liver, spleen, bone marrow, kidney and lung [35-42]. In mouse, the expression of TPO mRNA has been demonstrated in liver, kidney, skeletal muscle, spleen, bone marrow, brain and testis [43-47]. Tight morphological associations between maturing megakaryocytes and endothelial cells (EC) in the bone marrow may suggest that these cells are a source of factors promoting megakaryocyte differentiation. In this study, we examined the expression of TPO by the human umbilical vein endothelial cell (HUVEC).

MATERIALS AND METHODS

Human umbilical vein endothelial cells (HUVEC)

Primary cultures of HUVEC were obtained from freshly collected umbilical cords. 0.1% w/v collagenase (Boehringer Mannheim, Meylan, France) was used for treatment of the umbilical vein instead of 0.2%, as originally described [48]. Standard medium for growing HUVEC was DMEM supplemented with 10% heat-inactivated fetal calf serum (FCS), L-glutamine (2 mM), penicillin (100 mug/ml),

and streptomycin (50 mug/ml). Cells were grown to confluence in flasks coated with 100 mug/ml collagen I (Jacques Boy Institute, Paris, France). Cells were routinely used between the second and fifth passages.

RT-PCR

Total RNA was extracted from HUVEC using RNAble reagents (Eurobio, Les Ulis, France). Twenty micrograms of total RNA was reverse transcribed using superscript RT (GibcoBRL, Life Technologies, Cergy-Pontoise, France), in a 50 mul reaction volume. Reverse transcription reaction was run with MMLV enzyme for 1 hour at 37° C on RNA denatured for 5 min at 65° C, in the presence of oligo(dT). The PCR reaction was performed in a 50 mul reaction volume using 5 mul of the solution from the reverse transcription step, sense (5'-AA[GTGGCAGCA-GGGATTCAG]AGC-3') and antisense (5'GTAGG[GGTGGGCGTTGGAGCAG]-3') oligonucleotide primers (42, long or short oligos (brackets) giving the same results), and 0.5 U Taq DNA polymerase (Eurogentec, Seraing, Belgium). The PCR was run for 40 cycles, RT-PCR starting with 5 mug total RNA gave the same results. The PCR procedure included denaturation at 95° C for 1 min, annealing at 67° C for 1 min and extension at 72° C for 1 min. The PCR product was analyzed on ethidium bromide-stained 1% agarose gel. Identification of TPO was confirmed by sequencing of the RT-PCR product.

Northern-blot analysis

Total RNA was extracted from HUVEC cells as described above. Twenty micrograms of total RNA was denatured using formamide and formaldehyde, and run on denaturing gel containing formaldehyde. Blots were hybridized overnight with either the [alpha-32P]-labeled 347bp RT-PCR product or the 450bp *EcoR I/BamH I*-digested-fragment from pME18S plasmid containing the full length TPO cDNA (kindly provided by Dr. W. Vainchenker, Institut Gustave Roussy, Villejuif, France).

Western-blot analysis

HUVEC cells were grown at confluence for 48 hours in standard medium, then washed 5 times and cultured for 8-10 hours in DMEM alone plus antibiotics and L-glutamine (without serum) at 37° C, 5% CO_2. The supernatant was collected,

centrifuged at 3,000 g for 5 min, and concentrated up to 50-fold by freeze-drying, and then the proteins were precipitated with 10% trichloroacetic acid. Alternatively, supernatants were concentrated using filter devices (Millipore, Bedford, MA, USA). Concentrated culture supernatant was subjected to 9% polyacrylamide SDS-PAGE under reducing conditions and electrotransferred to a nylon membrane (Schleicher and Schuell). The membrane was incubated for 1 hour at room temperature in blocking solution (TBS-T (20 mM Tris-HCl/140mM NaCl/0.1% (v/v) Tween 20) + 5% (w/v) non-fat dried milk). The membrane was then incubated overnight at 4° C in blocking solution, containing the polyclonal antibody anti-human TPO (cat # AF-288-NA, R&D Systems, Oxon, United Kingdom). After three washes in TBS-T, the membrane was incubated for 1 hour at room temperature in blocking solution, containing a peroxidase conjugated anti-goat/sheep mAb (Sigma, Saint-Quentin Fallavier, France). Blots were revealed using a chemiluminescence detection kit (Amersham, Les Ulis, France).

ELISA

TPO was measured in cell culture supernatants by ELISA (R&D Systems). The lowest level of detection according to the manufacturer is 15 pg/mL. The first standard point in the assay is 31.2 pg/mL.

BaF3/MPLR cell line proliferation assays

HUVEC were grown to 90% confluence for 48 hours. After incubation, the conditioned media were collected, centrifuged at 8,400 g for 5 min and concentrated ten fold by freeze-drying. Parental BaF3 or BaF3 cells transfected with the murine c-mpl receptor (BaF3/MPLR), kindly donated by F. Wendling (Institut Gustave Roussy, Villejuif), were washed, plated in 96-well plates at 8,000 cells per well in 100 mul of serial dilutions of either HUVEC-conditioned medium or medium only, for control, and grown at 37° C, 5% CO_2. In control experiments, recombinant human TPO (carrier free, R&D cat # 288-TP) was used at a concentration of 10 ng/mL and neutralizing polyclonal anti-TPO antibody (R&D cat # AF-288-NA) was used at a concentration of 1 mug/mL. After 72 hours, cell proliferation was measured by a colorimetric assay using a tetrazolium salt/MTS kit (Promega, Lyon, France) following the manufacturer's instructions.

RESULTS AND DISCUSSION

There is substantial evidence that TPO is the major physiological regulator of platelet production *in vivo*. As an inverse correlation between the platelet count and circulating TPO concentrations has been found in many experimental models and in clinical situations, it has been proposed that the concentration of TPO was dependent on the platelet and megakaryocyte mass [18-31]. Binding of TPO to its receptor c-mpl on platelets and megakaryocytes could function as a titration of TPO and explain this inverse correlation. There are however discordant results. Inverse correlation between the platelet mass and circulating TPO concentrations is not always found [30], and there is a lack of elevated serum TPO levels in NF-E2 knockout mice that are greatly thrombocytopenic [49]. Alternatively, TPO mRNA levels have been shown to be modulated by the platelet mass [31], or mediators such as hepatocyte growth factor [32], TGF-beta1 [33], and proteins of the platelet alpha-granules [34]. A cell model allowing the study of the regulation of the TPO expression could help us to understand how its production is controlled. In the present study, we show that TPO is expressed by HUVEC in basal conditions. The TPO mRNA can be detected by RT-PCR (Figure 1A) and Northern blotting analysis (Figure 1B). Two mRNA can be detected at 1.8 kb and 4.4 kb, using either RT-PCR product or 450 bp EcoR I/BamH I-digested fragment from pME18S plasmid, the 1.8 kb message being expressed with higher intensity than the 4.4 kb message. However, expression of the TPO mRNA is low in HUVEC. Western blot analysis is very difficult due to the low production level of TPO in basal conditions, which are below detection limits. To fall within detection limits, we used concentrated supernatants of HUVEC that had been grown in the absence of serum, because BSA gives non-specific bands. Different bands are revealed by the R&D polyclonal antibody. A diffuse species at an Mr of 55-65 kDa, that migrates to the top of the recombinant TPO band can be detected. There are two higher species, migrating at an Mr of approximately 70-72 kDa and 90-100 kDa (Figure 2). We cannot exclude the possibility that the low Mr species are proteolytic products deriving from the high Mr species. Indeed, TPO protein is known to be highly susceptible to proteolysis [50]. A precise analysis of TPO production by Western blot in basal conditions would require more sensitive detection techniques. According to the low TPO expression, measurement of the protein levels in cell culture supernatants requires the concentration of the supernatants. ELISA measurements indicate that HUVEC produce TPO at a rate of approximately 15-30 pg/mL/48 hours/10^6 cells, but this is at the lower end of the ELISA detection limits. In kinetics experiments, the protein becomes detectable at day 4 of coculture, with concentrations up to 70-80 pg/mL at day 4-5 of coculture (data not shown). TPO that is produced by HUVEC allows the

growth of BaFMPLR cells, suggesting that it has biological activity (Figure 3). However, incomplete neutralization with the antibody may suggest that TPO might not be the only cytokine that is produced by HUVEC supporting the growth of BaFMPLR cells. In cocultures of EC with $CD34^+$ progenitor cells, TPO may be responsible for the reported support of the platelet lineage by the endothelial layer [51-57]. TPO is probably not the only cytokine involved in the support of megakaryocytopoiesis by EC, as other megakaryocytopoiesis-promoting cytokines such as GM-CSF, SCF, IL-6 and LIF are also produced by the EC. The very low levels of TPO production by HUVEC make the study of the regulation of its production at a cell level difficult. However, production of TPO by EC may be a significant contribution to the circulating TPO because of the large number of EC in the body. EC from sinusoid capillaries in the mouse liver have been recently shown to express TPO [44]. The production of cytokines involved in megakaryocyte proliferation and differentiation by the EC may also be important in the interactions between megakaryocytes and EC of the bone marrow sinusoids as megakaryocytes have been shown to mature upon contact with EC [58, 59], and as EC are able to support megakaryocytopoiesis [51-57].

CONCLUSION

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Figures

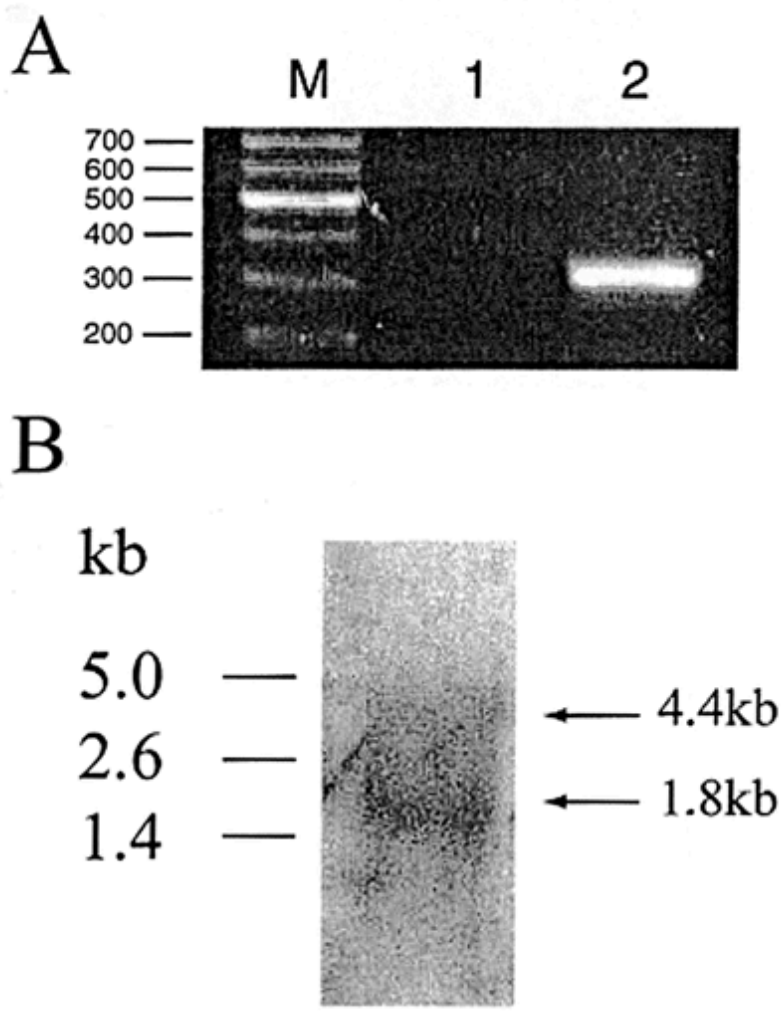


Figure 1

Detection of TPO mRNA in HUVEC by RT-PCR and Northern blot analysis.

A: RT-PCR was performed on 20 mug of total RNA extracted from HUVEC and amplified by PCR using specific primers for TPO as described in Materials and Methods. M: Left: DNA molecular weight markers; lane 1: negative control (PCR reaction without first strand cDNA template); lane 2: PCR product of 347 bp. **B:** Northern blot analysis. Forty mug of total mRNA extracted from HUVEC was run on a formaldehyde gel and blotted. The blot was hybridized with a probe corresponding to RT-PCR product obtained in panel **(A)**, lane 2. RNA molecular weight markers are as shown on the left. TPO mRNAs are shown by arrows on the right.

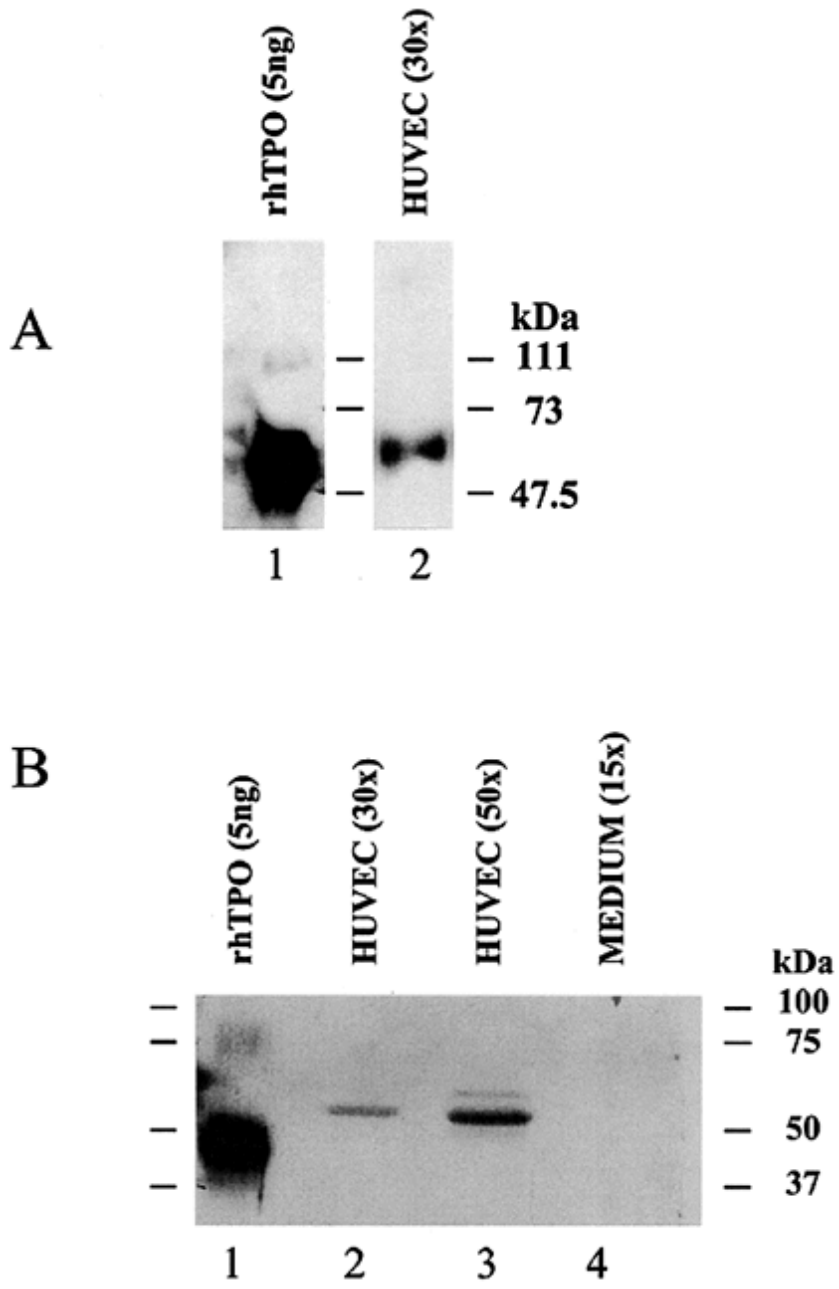


Figure 2

Production of TPO protein by HUVEC.

Western blot analysis. Panel **A:** lane 1: human recombinant TPO protein (5 ng); lane 2: conditioned medium from HUVEC concentrated 30-fold. Panel **B:** lane 1, human recombinant TPO protein (5 ng); lane 2, conditioned medium from HUVEC concentrated 30-fold; lane 3, conditioned medium from HUVEC concentrated

50-fold; lane 4, medium only concentrated 30-fold (DMEM plus antibiotics and L-glutamine).

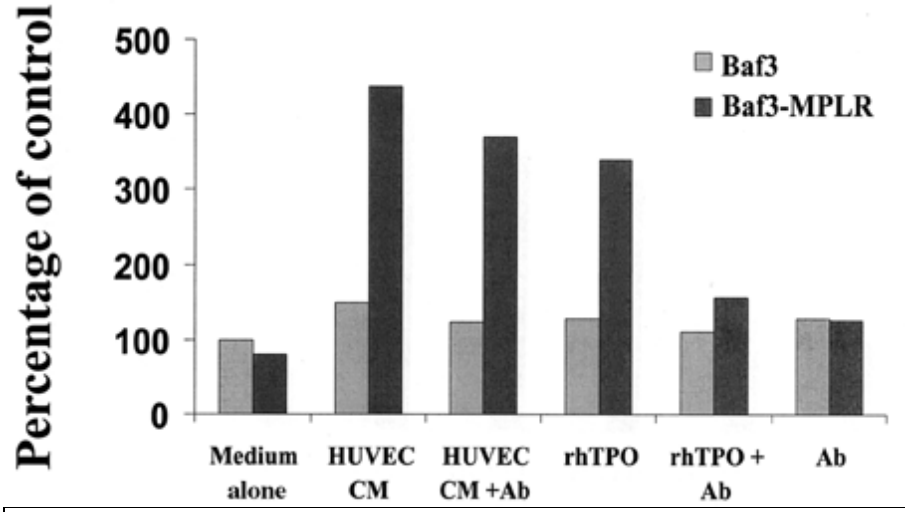


Figure 3

Effect of HUVEC-conditioned media on BaF3/MPLR cell proliferation.

BaF3/MPLR and the parental BaF3 cells were cultured (8,000 cells/well) in the presence of medium alone (Medium) or HUVEC conditioned medium (HUVEC CM) as described in Materials and Methods. Results are normalized to the proliferative rate obtained with parental BaF3 cells grown in the presence of medium alone and used as control. (Average of two experiments). RhTPO: recombinant human TPO used at 10 ng/mL final concentration. Ab: polyclonal anti-TPO antibody used at a final concentration of 1 mug/mL.

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Fibronectin increases the migration induced by stromal cell-derived factor-1a (SDF-1a) in pre-B acute lymphoblastic leukemia cells

E. Sbaa-Ketata, M. Vasse, B. Lenormand, P. Schneider, C. Soria, J.P. Vannier
acute lymphoblastic leukemia, SDF-1a, CXCR-4, VLA-4, VLA-5, fibronectin.

Hematopoietic growth factors in patients receiving intensive chemotherapy for malignant disorders: studies of granulocyte-colony stimulating factor (G-CSF), granulocyte-macrophage colony stimulating factor (GM-CSF), interleukin-3 (IL-3) and Flt-3 ligand (Flt3L)

O. Bruserud, B. Foss, H. Petersen
chemotherapy, acute leukemia, G-CSF, GM-CSF, IL-3, Flt3L.

Can interleukin-2 reverse anthracyclin chemoresistance in metastatic soft tissue sarcoma patients. Results of a prospective phase II clinical trial

G. Gravis, M. Mousseau, J.Y. Douillard, T. Dorval, M. Fabbro, B. Escudier, L. Mignot, P. Viens
immunotherapy, soft tissue sarcoma, interleukin-2, chemoresistance, anthracyclin.

Activation of two distinct anti-proliferative pathways, apoptosis and p38 MAP kinase-dependent cell cycle arrest, by tumor necrosis factor in human melanoma cell line A375

T. Hattori, H. Hayashi, T. Chiba, K. Onozaki
melanoma, tumor necrosis factor, apoptosis, cell cycle arrest, p38 MAPK.

Impaired interleukin-5 (IL-5) production by T cells as a prognostic marker of disease progression in human immunodeficiency virus type 1 (HIV-1)-infected children

S. Resino, S. Sanchez-Ramon, J.M. Bellon, J.L. Jimenez, M.A. Munoz-Fernandez
HIV-infected children, cytokine production, interleukin-5, PBMC, prognostic markers.

Cytokine-mediated inflammatory hyperalgesia limited by interleukin-13

B.B. Lorenzetti, S. Poole, F.H. Veiga, F.Q. Cunha, S.H. Ferreira
inflammatory hyperalgesia, interleukin-13, tumour necrosis factor alpha, interleukin-1b, interleukin-8, prostaglandin E2.

Expression of thrombopoietin by umbilical vein endothelial cells

A. El Andaloussi, P. Duchez, J. Rieffers, J. Ripoche, C. Grosset
thrombopoietin, umbilical vein endothelial cells.

Production of cytokines by immature erythroid cells derived from human embryonic liver

S.V. Sennikov, S.V. Krysov, T.V. Injelevskaya, A.N. Silkov, V.A. Kozlov
erythroid progenitor, IL-1b, IL-2, IL-4, IL-6, fetal liver.

Inflammatory cytokine production in interferon-g-primed mice, challenged with lipopolysaccharide. Inhibition by SK&F 86002 and interleukin-1b-converting enzyme inhibitor

S. Spinelle-Jaegle, P. Devillier, S. Doucet, S. Millet, C. Banissi, A. Diu-Hercend, E. Ruuth
interferon-g, lipopolysaccharide, interleukin-1b, cytokine, interleukin-1b-converting enzyme inhibitors.

The effect of Sambucol, a black elderberry-based, natural product, on the production of human cytokines: I. Inflammatory cytokines

V. Barak, T. Halperin, I. Kalickman
Sambucol, inflammatory cytokines, IL-1b, TNF-a, IL-6, IL-8.

Interferon (IFN)-g and Herpes simplex virus/tumor necrosis factor-a synergistically induce nitric oxide synthase 2 in macrophages through cooperative action of nuclear factor-kB and IFN regulatory factor-1

S.R. Paludan, L. Malmgaard, S. Ellermann-Eriksen, L. Bosca, S.C. Mogensen
macrophages, NOS2, Herpes simplex virus, NF-kB, IRF-1.

Negative regulation of onconstatin M signaling by suppressor of cytokine signaling (SOCS-3)

F. Magrangeas, O. Boisteau, S. Denis, Y. Jacques, S. Minvielle
cytokine signaling, inhibition, Jak-STAT pathway.

Increased expression of interleukin-8 and aminopeptidase N by cell-cell contact: interleukin-8 is resistant to degradation by aminopeptidase N/CD13

A. Kehlen, I. Egbert, K. Thiele, K. Fischer, D. Riemann, J. Langner
cell co-culturing, leucine aminopeptidase, plasma membrane preparations, CD13, proteolysis of IL-8, interleukin-8.

Functional characterization of the mouse lymphotoxin-beta receptor promoter

P. Muller, D.N. Männel, T. Hehlgans
LTbR-promoter, transcriptional activation, dexamethasone.

Glucose potentiates interleukin-1b (IL-1b)-induced p38 mitogen-activated protein kinase activity in rat pancreatic islets of Langerhans

A.M.E. Sprinkel, N.A. Andersen, T. Mandrup-Poulsen
interleukin-1b, p38, ERK1/2, JNK, nitric oxide, diabetes mellitus.

Sustained parasite burden in the spleen of Leishmania infantum-infected BALB/c mice is accompanied by expression of MCP-1 transcripts and lack of protection against challenge

D. Rousseau, S. Demartino, F. Anjuère, B. Ferrua, K. Fragaki, Y. Le Fichoux, J. Kubar
murine leishmaniasis, acquired immunity, inflammation, chemokines, spleen cell subpopulations.

Effects of cytokines and prolactin on the replication of Toxoplasma gondii in murine microglia

N. Benedetto, A. Folgore, C. Romano-Carratelli, F. Galdiero
T. gondii, cytokine, PRL, MGC.

Strip test for bedside detection of interleukin-6 in cervical secretions is predictive for impending preterm delivery

H. Trébéden, F. Goffinet, G. Kayem, F. Maillard, E. Lemoine, D. Cabrol, B. Weill, F. Batteux
cytokine, IL-6, prematurity, cervicovaginal secretions, strip test.

Serum levels of interleukin-18 in patients with uncomplicated Plasmodium falciparum malaria

D. Torre, M. Giola, F. Speranza, A. Matteelli, C. Basilico, G. Biondi
interleukin-18, malaria, Plasmodium falciparum, gamma interferon.

The ecdysone inducible gene expression system: unexpected effects of muristerone A and ponasterone A on cytokine signaling in mammalian cells

S. Constantino, R. Santos, S. Gisselbrecht, F. Gouilleux
ecdysone, PI 3-kinase, AKT, survival.

Presence of estrogen receptors in human myeloid monocytic cells (THP-1 cell line)

M. Cutolo, B. Villaggio, A. Bisso, A. Sulli, D. Coviello, J.M. Dayer
THP-1 cells, macrophages, estrogen receptors, estrogens, monocytes.

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