EPO inhibits NF-κB-inducible immune pathways

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Supplemental Information

Erythropoietin Contrastingly Affects Bacterial Infection and Experimental Colitis by Inhibiting Nuclear Factor-κB-Inducible Immune Pathways

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Supplemental Data

Figure S1
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**Figure S1. Macrophage populations express components of the EPOR signaling complex and are responsive to EPO, related to Figure 1.**

Expression of EPOR (A), ßcR (B) and JAK2 (C) mRNA in different populations of primary immune cells was evaluated by means of quantitative reverse transcription polymerase chain reaction (qRT-PCR). RAW264.7 macrophage-like cells and Ter119+ bone marrow erythroid cells served as references. Results were normalized to Hprt expression and are presented as means ± S.E.M. of 3-6 independent cell preparations.

(D) Thioglycolate-elicited primary peritoneal macrophages were pre-treated with PBS or EPO 30 min before the addition of LPS or solvent. Supernatants were analyzed for cytokine concentrations by ELISA. Data from at least 3 independent experiments were compared by means of Kruskal-Wallis test. Values are depicted as lower quartile, median and upper quartile (boxes) with minimum and maximum ranges. n.d. denotes not detectable.

(E) qRT-PCR analysis of immune response genes, normalized to Hprt expression. Data were analyzed and are presented as above. Statistically significant differences between means of LPS-stimulated cells pre-treated with either PBS or EPO are indicated.

**Figure S2**
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**A**

![Box plot showing gene expression](image)

**B**

![Box plot showing gene expression](image)

**C**

| Protein | Size (kD) |
|---------|-----------|
| TFR1    | 100       |
| Nos2    | 130       |
| Actin   | 42        |

**D**

![Western blot showing protein expression](image)

**E**

![Western blot showing protein expression](image)

**F**

![Western blot showing protein expression](image)

**G**

![Box plot showing gene expression](image)

**H**

![Box plot showing gene expression](image)

**I**

![Box plot showing gene expression](image)
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Figure S2. Effects of EPO on macrophage immune response pathways are independent of TFR1 expression and STAT activation, related to Figure 2.

RAW264.7 macrophage-like cells were pre-incubated with EPO or solvent for 30 min and subsequently stimulated with IFN-γ and LPS. TFR1 (A) and Nos2 (B) mRNA expression was studied by qRT-PCR. Data from at least 3 independent experiments, normalized to Hprt expression, are shown and statistically significant differences between means of stimulated cells pre-treated with either PBS or EPO are indicated.

(C) TFR1 and Nos2 protein expression was studied by means of Western blot. One of 3 representative experiments is shown.

(D-F) RAW264.7 macrophage-like cells were pre-incubated with EPO or solvent for 30 min and subsequently stimulated with IFN-γ and LPS for 60 min. Cytoplasmic proteins were prepared, the presence of phospho-STAT and pan-STAT proteins was detected using specific antibodies. Depicted Western blots for STAT1 (D), STAT3 (E) and STAT5 (F) are representative of 3 independent experiments and of 4 time points studied.

(G-I) Cells were pre-treated with the selective STAT5 inhibitor M573108 and subsequently incubated with EPO, followed by stimulation with IFN-γ and LPS for a total of 24 hours. Expression of Nos2 (G), TNF-α (H) and VEGF (I) mRNA was analyzed by means of qRT-PCR and normalized to the amount of Hprt mRNA. Data of 5 independent experiments are shown and statistically significant differences are indicated.

Figure S3
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Figure S3. EPO modulates serum concentrations and splenic mRNA expression of immune response genes in Salmonella septicemia, related to Figure 3.

(A) Serum samples of the mice described in the legend to Figure 3 were analyzed for concentrations of nitrite and of cytokines, respectively. Data from 8-12 animals per group are shown and statistical significances are indicated. Cytokines concentrations in sera of uninfected mice remained below the reported detection limits of the corresponding ELISA kits. n.d. denotes not detectable.
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(B) Spleen samples of the mice (n=8-12 per group) described in the legend to Figure 3 were used for RNA preparation. The expression of immune response genes was evaluated by qRT-PCR and normalized to Hprt mRNA amounts.

Figure S4
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**Figure S4. Selectivity of inhibitory effects of EPO on lamina propria macrophages, related to Figure 6.**

(A) CD11b⁺ myeloid cells and CD4⁺ T cells (B) isolated from colonic lamina propria from 1 out of 3 experiments summarized in Figure 6A were isolated and analyzed for the expression of immune response genes and NF-κB binding activity by qRT-PCR and a chemi-luminescent transcription factor assay, respectively. Data of 4-8 samples per group were analyzed and statistically significant differences are indicated.

(C) Splenic CD4⁺ T cells were isolated from control mice and stimulated with PMA and ionomycin after pre-treatment with EPO or PBS. Expression of immune response genes was analyzed by qRT-PCR. Data of 4-6 independent experiments were analyzed after normalization to Hprt expression.

**Supplemental Experimental Procedures**

*Cell isolation and culture*

Thioglycolate-elicited primary peritoneal macrophages (Peritoneal MΦ) were harvested as described (Nairz et al., 2009a; Schleicher and Bogdan, 2009). Briefly, C57BL/6 mice were injected intraperitoneally with 4% Brewer’s thioglycolate medium (Sigma). After 3 days, mice were sacrificed by cervical dislocation and cells were isolated by flushing the peritoneal cavity with PBS. Cells were seeded in six-well dishes in complete RPMI. Subsequently, non-adherent cells were removed by extensive washing and adherent peritoneal macrophages were treated and analyzed as specified below. The use of either recombinant human EPO (rhuEPO; purchased from Amgen Inc) or of recombinant murine EPO (rmuEPO; obtained from R&D) yielded very similar results. Thus, rhuEPO was used for subsequent experiments. Corresponding results on Nos2 and cytokine expression were obtained when primary
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peritoneal macrophages or RAW264.7 macrophage-like cells were stimulated with LPS or a combination of IFN-γ and LPS in the presence or absence of EPO for 6 or 24 hours.

For other experiments, cells were pre-treated with 5 μg/mL actinomycin D, 30 μg/mL cycloheximide, 1 μM wortmannin (obtained from Sigma), 10 μM UO126, 100 μM AG490, 10 μM WP1066, 100 μM M573108 (obtained from Merck) or 50 μM TRAF-6 inhibitory peptide (Imgenex). In additional experiments, cells were stimulated with 100 ng/ml MALP-2 (Imgenex), 100 ng/ml PAM2Cys, 100 ng/ml PAM3Cys (Genaxxon), 20 ng/ml rmuTNF-α, 20 ng/ml rmuIL-1β or 20 ng/ml rmuIL-17A (all from R&D).

For isolation of bone marrow derived macrophages (BMDMΦ), mice were euthanized, and the femora and tibiae were flushed with PBS. Bone marrow cells were washed and resuspended in complete DMEM supplemented with 20% L929 cell-conditioned medium (as a source of M-CSF). Cells were subsequently incubated for 7 days at 37°C and cultures were washed vigorously to remove nonadherent cells before harvesting (Nairz et al., 2009a; Schleicher and Bogdan, 2009).

For isolation of Kupffer cells and primary hepatocytes, an optimized protocol was followed exactly as described (Theurl et al., 2008). In brief, Seglen’s perfusion buffer supplemented with 100 μM EGTA (Sigma) and Seglen’s collagenase buffer containing 20 μg/mL Liberase Blendzyme 3 (Roche) were sequentially used for retrograde liver perfusion. Hepatic cell suspensions were filtered through a 100 μm membrane (Falcon). Hepatocytes were separated from non-parenchymal cells by repeated washing and centrifugation. Dead cells were removed by Percoll centrifugation. Kupffer cells were isolated from the first supernatant by enriching them on 17.5% Nycodenz, followed by plastic adhesion and extensive washing.

For isolation of splenic CD4⁺, CD8⁺ and CD11c⁺ cells, spleens were mechanically disrupted under sterile conditions and cell suspensions were filtered through a 70 μm membrane.
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Splenocytes were then further purified as described (Tomita et al., 2008) using the MACS system (Miltenyi Biotech) with immuno-magnetic beads specific for CD4, CD8 and CD11c according to the manufacturer’s instructions.

Colonic lamina propria cells were isolated from the large intestine as described previously (Becker et al., 2004; Hirotani et al., 2005). In brief, the colon was opened longitudinally, cut into small pieces and washed extensively in PBS to remove feces and debris. Following incubation in HBSS containing 1 mM DTT (Sigma), 2.0 mg/ml collagenase and 0.01% DNase (Roche). Suspensions of lamina propria cells were sequentially enriched for CD4+, CD11c+ and CD11b+ cells using the corresponding MACS systems (Miltenyi Biotech) according to the manufacturer’s instructions.

For isolation of Ter119+ erythroid cells, femora and tibiae were flushed with PBS. Cell suspensions were passed through a 70 μm membrane and centrifuged. Immuno-magnetic beads specific for Ter119+ (Miltenyi Biotec) were used for enrichment of bone marrow erythroid cells as recommended by the manufacturer. Purity of all cell preparations was at least 90-95%, as determined by FACS analysis. Purified cells were then directly used for RNA preparation or further incubated in complete medium for subsequent experiments.

Macrophages were stimulated with PBS (controls), 200 ng/mL LPS and/or rmuIFN-γ for 6 or 24 hours, while CD4+ T cells were stimulated with either phorbol myristate acetate (PMA; 4 ng/ml) and ionomycin (Iono; 2 μM) or plate-bound CD3 (1 μg/ml) and CD28 (10 μg/ml) antibodies after pre-treatment with EPO (5 U/ml) or PBS for 30 min. Thereafter, supernatants were harvested and cells were subjected to RNA preparation.

*Salmonella infection in Epor−/−rescued mice*

Genetic ablation of Epor in mice (Epor−/−) is lethal around embryonic day 13 secondary to the lack of erythropoiesis (Kieran et al., 1996; Lin et al., 1996). To study the extra-hematopoietic effects of EPO, Epor−/− mice have been rescued from embryonic lethality by selective
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transgenous expression of human *EPOR* in erythroid cells under the control of the *GATA-1* (for globin transcription factor-1) promoter (Suzuki et al., 2002). *Epor<sup>−/−</sup>* rescued mice thus contain a mouse *GATA-1* minigene encompassing exons IE and II, which drives expression of the full-length human *EPOR* cDNA selectively in erythroid cells, whereas non-erythroid cells are *Epor* deficient. Construction, genotyping and phenotype of *Epor<sup>−/−</sup>* rescued mice has been described in further detail elsewhere (Satoh et al., 2006; Suzuki et al., 2002). *Epor<sup>−/−</sup>* rescued mice (herein termed *Epor<sup>−/−</sup>* mice) were crossed back on a C57BL/6 background for at least 12 generations and were used at 8-14 weeks of age for macrophage preparations or *in vivo* experiments.

*Establishment of TNBS-Colitis*

Colitis was induced in male SJL/J mice at the age of 6-8 wk as described elsewhere with slight modifications (Neurath et al., 2000; Neurath et al., 1995). 1 mg of 2,4,6-trinitrobenzene sulfonic-acid (TNBS; obtained Sigma) was dissolved in 50% ethanol and applied via the rectal route after cutaneous pre-sensitization 7 days before rectal challenge. For sensitization, a 2x2 cm field of the abdominal skin was shaved and 100 μl of 5 mg TNBS in 50% ethanol solution was applied. On the day of challenge, mice were first anesthetized with ketamine and xylacine, subsequently TNBS was administered per rectum using a vinyl catheter positioned 3 cm from the anus. To ensure equal distribution of hapten within the entire colon, mice were kept vertically for 60 seconds after the instillation. Control mice underwent identical procedures but were instilled with 50% ethanol. Care was taken to ensure that the two TNBS-exposed groups to be compared had lost equivalent amounts of weight. TNBS-treated animals whose weight loss was lower than the mean weight loss minus the S.E.M. multiplied by 2 were considered unresponsive to the induction of colitis. Therefore, these mice were excluded from subsequent analysis. Mice were treated i.p. with rhuEPO or PBS as described and the severity of colitis was assessed as detailed.
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Establishment of DSS-Colitis

DSS-colitis was induced following a reported protocol with slight modifications (Becker et al., 2004; Kaser et al., 2008). Briefly, 3% dextran sulfate sodium (DSS; MW 36,000-50,000; obtained from MP Biomedicals) was added to the drinking water and available to the mice *ad libitum*. Control mice received drinking water only. *Epor<sup>−/−</sup>* mice and *Epor<sup>+/+</sup>* littermates on a C57BL/6 background were treated with DSS or water for 7 consecutive days. Thereafter, DSS was replaced by drinking water and all animals were followed-up for another 7 days. On days 7, 8 and 9 after the initiation of DSS treatment, mice were administered rhuEPO (5 U/g body weight) or PBS as a control. Weight was recorded daily for 14 days and mice were monitored twice daily for signs of illness.

Histopathology

Histological examinations of tissues from *in vivo* infection and colitis experiments, respectively, were performed on formalin-fixed tissue sections stained with hematoxylin and eosin (HE) according to a standard protocol. The degree of colonic inflammation on microscopic sections was graded semi-quantitatively as described (Dieleman et al., 1998). Histological scoring was performed by an experienced pathologist blinded to study design and sample identity. Images of HE stainings were acquired using a Nikon-Eclipse 80i microscope equipped with 4x and 10x objectives with 0.10 and 0.25 numerical apertures, respectively. Image acquisition was done using the NIS-Elements BR3 software.

Quantitative real-time PCR

Probes used for Taqman quantitative reverse transcription polymerase chain reaction (qRT-PCR) carried 5’FAM and 3’BHQ1 labels. Sequences of primers and probes spanned exon-intron boundaries and have been listed elsewhere (Izcue et al., 2008; Jono et al., 2004;
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Kullberg et al., 2006; Moschen et al., 2007; Nairz et al., 2009b; Ogata et al., 2006; Tiedt et al., 2008; Zacharioudaki et al., 2009).

**Western blot analysis**

For Western blot analysis, commercially available antibodies were used as follows: a mouse TFR1 antibody (1:1000; Zymed), a mouse Nos2 antibody (1:1000; BD), a rabbit actin antibody (1:1000; Sigma-Aldrich), a rabbit phospho-STAT1 (1:500; Upstate), a rabbit phospho-STAT3 (1:500; Upstate), a rabbit phospho-STAT5 (1:500; Upstate), a rabbit STAT1 (1:500; Santa Cruz), a rabbit STAT3 (1:500; Santa Cruz), a mouse STAT5 (1:500; Santa Cruz), a rabbit phosphor-JAK2 (1:2000; Cell Signaling), a rabbit JAK2 (1:2000; Cell Signaling), a rabbit phosphor-IκB-α (1:1000; Cell Signaling), a mouse IκB-α (1:1000; Cell Signaling), a rabbit phospho-NF-κB p65 (1:1000; Cell Signaling), a rabbit NF-κB p65 (1:1000; Cell Signaling) and appropriate HRP-conjugated secondary antibodies (1:1000; Dako).

**Electro-mobility shift assays (EMSA)**

For the generation of radio-labeled probes representing standard consensus sequences of various transcription factors that are known to bind within the murine NOS2 promoter or to transduce signal down-stream of the EPOR, the following oligonucleotides were used: IRF1 sense 5’-GGAAGCGAAAATGAAATTG-3’, IRF1 antisense 5’-TGAGTCAATTTTCCG-3’; STAT1 sense 5’-CATGTTATGCATATCCTGTAAGTG-3’, STAT1 antisense 5’-CGTGCACTTACAGGAATATGCATA-3’; STAT3 sense 5’-GATCCTTCTGGGAATTCAGATA-3’, STAT3 antisense 5’-GATCTAGGAATTCCAGAAG-3’; STAT5 sense 5’-AGATTTCTAGGAATTCATC-3’, STAT5 antisense 5’-
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CTGGATTGAATTCCTAGAAA-3'; NF-κB sense 5’-AGCTTCAGAGGGACTTTCCGAGG-3’, NF-κB antisense 5’-TCGACCTCTCGGAA AGTCCCCCTCTGA-3'; NF-IL6 sense 5’-AAGCTGCAGATTGCAGCAAATCTGCA-3’, NF-IL6 antisense 5’-CGTGCAGATTGCAGCAAATCTGCA-3’.

For the preparation of double-stranded probes, oligomers were annealed, and overhanging ends were filled with [α-32P]dCTP (Amersham) and the three other non-radio-labeled dNTPs (Pharmacia) using Klenow enzyme (Amersham) as described (Dlaska and Weiss, 1999; Fritsche et al., 2003). For competition studies, a 30-fold excess of unlabeled oligonucleotide probe was added to the nuclear extracts 10 min before addition of the radioactive probe. The DNA binding reactions were performed in the presence of 200 mM HEPES (pH 7.8), 10 mM EDTA, and 10 mM DDT for 20 min on ice. After addition of 87% glycerol as a loading buffer, samples were separated on a 6% non-denaturing polyacrylamide gel. The gel was subsequently dried and exposed for autoradiography.

**Transient Transfections**

The full-length Nos2 promoter (wild-type-Nos2-luc), cloned into the pGL2 basic luciferase reporter gene vector (Fritsche et al., 2003), as well as Nos2-luciferase reporter constructs bearing point mutations at the NF-κB consensus sequences of either the proximal region I (mut-κBI-Nos2-luc), the distal region II (mut-κBII-Nos2-luc), or at both sites (mut-κBI-κBII-Nos2-luc) were used (Morris et al., 2003). The NF-κB luciferase construct pNF-κB-TA-Luc containing 4 consecutive NF-κB consensus sequences (Clontech) was used as described for transfections of primary peritoneal macrophages (Bittorf et al., 2001). Nos2 promoter and NF-κB transactivation activities were determined by the Dual Luciferase system (Promega) according to the manufacturer’s instructions. Firefly luciferase activity was corrected by co-transfection of cells with the constitutively expressed Renilla luciferase vector pRL-SV40.
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Efficacy of transfections was monitored by fluorescence microscopy of cells transfected with 6 μg of pmax-GFP control vector and was 71±7%.

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