3. Materials and Methods

3.1. Substances

3.1.1. Substances for whole blood incubation

Saline, sterile and pyrogen-free, 0.9%, Berlin-Chemie, Berlin

Human or rabbit whole blood, heparinized (15 IU/ml)

Pyrogens:

LPS (lipopolysaccharide) from *E. coli* O113: H10 (WHO reference standard), provided by the National Institute for Biological Standards and Controls (NIBSC), Potters Bar, London, a kind gift from Stephen Poole

E. coli O111: B4, provided by Charles River Endosafe, Charleston, South Carolina, USA

E. coli O128, phenol extraction, from Sigma-Aldrich, Steinheim, Germany

Salmonella typhosa, phenol extraction, from Sigma-Aldrich

Salmonella typhimurium, phenol extraction, from Sigma-Aldrich

Shigella flexneri 1A, phenol extraction, from Sigma-Aldrich

Serratia marcescens, phenol extraction, from Sigma-Aldrich

Bordetella pertussis, phenol extraction, from Sigma-Aldrich

Pseudomonas aeruginosa, phenol extraction, from Sigma-Aldrich

LTA (Lipoteichoic acid) from

Bacillus subtilis, provided by Charles River Endosafe

Staphylococcus aureus, in-house preparation (Morath et al., 2001)

3.1.2. ELISA

Buffers:

Coating Buffer:

$NaHCO_3$, Sigma-Aldrich

Distilled water

Blocking Buffer:

NaCl,

Na_2HPO_4,

KCl,

$KHPO_4$, all Sigma-Aldrich

BSA (Bovine Serum Albumine), Fraction V, PAA Laboratories GmbH, Pasching, Austria

Washing Buffer:
NaCl,
Na_2HPO_4,
KCl,
$KHPO_4$,
Tween 20, all Sigma-Aldrich

POD (Peroxidase), Biosource, Camarillo, California, USA
TMB (Tetramethylbenzidine), Sigma-Aldrich
H_2SO_4, Sigma-Aldrich

Recombinant antigens:
Human IL-1β, provided by NIBSC
Human IL-6, provided by NIBSC
Human TNF-α, provided by NIBSC
Human IL-8, Peprotech, Frankfurt am Main, Germany
Rabbit IL-1β, a kind gift from Adrian Bristow, NIBSC
Rabbit IL-6, a kind gift from Adrian Bristow, NIBSC
Rabbit TNF-α, a kind gift from Adrian Bristow, NIBSC
Rabbit IL-8, from BD PharMingen, San Diego, California, USA

Antibodies:
Anti-human IL-1β, capture antibody from Pierce-Endogen, Bonn, Germany
Anti-human IL-1β, detection antibody from Pierce-Endogen
Anti-rabbit IL-1β, capture antibody, a kind gift from Stephen Poole, NIBSC
Anti-rabbit IL-1β, detection antibody, a kind gift from Stephen Poole, NIBSC
Anti-human IL-6, capture antibody from R+D, Minneapolis, USA
Anti-human IL-6, detection antibody from R+D
Anti-rabbit IL-6, capture antibody, a kind gift from Stephen Poole, NIBSC
Anti-rabbit IL-6, detection antibody, a kind gift from Stephen Poole, NIBSC
Anti-human IL-8, capture antibody from Pierce-Endogen
Anti-human IL-8, detection antibody from Pierce-Endogen

Anti-rabbit IL-8, capture antibody from BDPharMingen
Anti-rabbit IL-8, detection antibody from BDPharMingen
Anti-human TNF, capture antibody from Pierce-Endogen
Anti-human TNF, detection antibody from Pierce-Endogen
Anti-rabbit TNF, capture antibody, a kind gift from Stephen Poole, NIBSC
Anti-rabbit TNF, detection antibody, a kind gift from Stephen Poole, NIBSC

3.1.3. WEHI
PBS (Dulbecco´s Phosphate Buffered Saline, w/o Calcium and Magnesium), PAA Laboratories, Pasching, Austria
RPMI 1640, + Ultraglutamine, Bio Whittaker, Cambrex, Verviers, Belgium
Fetal Calf Serum, heat-inactivated, Bio Whittaker, Cambrex
Penicillin-Streptomycin, Bio-Whittaker, Cambrex
Ethanol 96%, Sigma-Aldrich
Trypsin-EDTA, Sigma-Aldrich
Alamar Blue, Sigma-Aldrich
Actinomycin D, Sigma-Aldrich
Cell line, ATCC (American Tissue Culture Collection)
Human recombinant TNF-α, Biosource, Camarillo, California, USA

3.2. Animals
Eleven male Chinchilla rabbits, age 0.5-2 years, 4-8 kg bodyweight, Charles River, USA
Four male Beagle dogs, age 8-11 months, 18-25 kg bodyweight, Altana, Konstanz, Germany

3.3. Equipment
3.3.1.Whole blood incubations
Reaction tubes, sterile, pyrogen-free, 1.5. ml, Eppendorf, Hamburg, Germany
Monovettes, Sarstedt, Li-Heparin, 15 IU/ml, Nümbrecht, Germany
Multifly needle set, Sarstedt, for drawing human and canine blood
21 Gauge needle, for drawing rabbit blood, Terumo, Becton Dickinson, Heidelberg, Germany
Incubator, 37°C, + 5% CO_2, HeraCell, Kendro (Heraeus), Hanau, Germany
Centrifuge, BioFuge fresco, Kendro

Vortexer, Vortex Genie 2, VWR International, Darmstadt, Germany

3.3.2. ELISA

Nunc Maxisorp plates, Nunc Kamstrup, Denmark

U-bottom microtiter plates 96-well, Greiner bio-one, Frickenhausen, Germany

Plate washer, Tecan, Crailsheim, Germany

ELISA plate reader, 450 nm wavelenght, 690 nm correction filter, Tecan

3.3.3. WEHI

U-bottom microtiter plates 96-well, for predilution of samples/standards; Greiner bio-one

Centrifuge, BioFuge fresco, Kendro

Vortexer, Vortex Genie 2, Scientific Industries

Flatbottom microtiter plates, Greiner bio-one

Incubator, 5% CO_2, 95% humidity, Kendro

Photometer, Eppendorf, Hamburg, Germany

3.4. Methods

3.4.1. Whole blood incubation

Blood from the respective animal or human was drawn into a Li-Heparin monovette and agitated gently. For the humans and dogs, a multifly needle set was used and around 7.5 ml of blood were taken from each donor. For rabbit blood, the heparin tube was screwed open. Venipuncture was performed with a 21 Gauge needle in the Vena auricularis, and the blood was slowly running into the tube. On average, 5 to 7 ml could be drawn from each animal every 2 weeks. For the dog whole blood, the vena antebrachii was punctured and the blood was drawn into the heparin tube. The final concentration of heparin was 15 IU/ml for each species.

500 µl of physiological saline was added to each Eppendorf tube. Dose-response curves of the respective pyrogens were being prepared using physiological saline, vortexing each dilution for about 3 seconds before diluting further. 50 µl of the diluted stimulus were added to the incubation. Last, 50 µl of blood were pipetted. The cups were closed and inverted once or twice in order to ensure the complete mixing of the whole blood incubations. The tubes were then placed into an incubator at 37°C overnight where the cytokines were formed.

After the incubations, the Eppendorf cups were again inverted once or twice in order to allow the cytokines to redistribute evenly throughout the volume of the incubations. The cups were then centrifuged at 13.000 rpm for 1 minute and the clear supernatant was taken for measurement in ELISA or WEHI.

3.4.2. ELISAs (see appendix)

The enzyme-linked immunosorbent assay (ELISA) is based on an antigen-antibody reaction. The antibodies bind specifically and concentration-dependent to their respective antigen, in our case the cytokines and IL-8 formed by the monocytes in the blood in response to the pyrogenic stimulus, and the reaction is made visible by an enzymatic reaction.

3.4.2.1. Human ELISAs

Table 1: IL-1β

Table 2: IL-6

Table 3: TNF-α

Table 4: IL-8

3.4.2.2. Rabbit ELISAs

Table 5: IL-1β

Table 6 : IL-6

Table 7: TNF-α

Table 8: IL-8

3.4.3. WEHI

The WEHI (Walter and Elisabeth Hall Institute) assay employs murine cells which undergo apoptosis when in contact with bioactive TNF-α. Since this cytokine is formed by the monocytes in response to a pyrogenic challenge, the supernatants of a whole blood incubation can be used for testing in this bioassay. The cells undergo apoptosis in a dose-dependent manner, and the reaction is not restricted to murine TNF-α, but takes place also with human and canine TNF-α.

The cells were cultivated in RPMI + Ultraglutamine with addition of 10% heat-inactivated Fetal Calf Serum and 1% Penicillin-Streptomycin in an incubator for 3 days. Adherent cells were harvested by discarding their medium and washing twice with warm

PBS. Trypsin-EDTA was added for separating the cells from the plate. After 5-10 minutes in the incubator, the cells were resuspended in new warm cell culture medium. The cells were separated from the bottom of the flask with warm RPMI 1640 and resuspended.
They were then centrifuged for three minutes at 1200 rpm and the supernatant was discarded.
The pellet was resuspended in a defined volume and the cell count was adjusted to 2.5 x 10^5 cells / ml. 100 µl of the cell suspension was added to each well of a microtiter plate (= 2.5 x 10^4 cells/well). The cells were left to adhere for 4 hours in the incubator at 37°C and 5% CO_2. During this time, the cells adhered to the surface of the cell culture plate. Actinomycin D was added to the cells at a final concentration of 1 µg/ml in order to enhance sensitivity to TNF-α about 50-100 fold. The plate was then incubated at 37°C and 5% CO2 for another 30 minutes. Samples and recombinant TNF-α were added at a volume of 10 µl. Samples were diluted on a 96 well plate with cell culture medium from undiluted to 1:128. Recombinant TNF-α was serially diluted from 100 pg/ml to 0.006 pg/ml (1:4). After addition of samples/standards the cell culture plate was incubated for 17 h before 30 µl of medium were removed from the wells intended for lysis control and replaced with 40 µl of ethanol. The plate was incubated for one more hour before 10 µl of Alamar Blue were added to each well and the plate was left in the incubator for 3 hours. The color reaction was measured spectrophotometrically at 560/580 nm. Untreated cells were used to set the basal level of cytotoxicity (i.e. 0% cytotoxicity), cells lysed with ethanol were used to set its maximum level (i.e. 100% cytotoxicity).

Table 9: Pipetting scheme for the WEHI assay (see appendix)

3.5. Statistics

Statistics were performed with GraphPad InStat 3.0 (GraphPad Software, San Diego, USA). Significance was tested by one-way ANOVA and Dunnetts post-test/Dunn´s multiple comparison.