Supplementary Data

Results

In vitro neutralization of inflammatory mediators induced by bacterial immune stimulatory molecules

The ability of compound Pep19-2.5 to suppress inflammatory reactions of cells exposed to microbial stimuli in vitro was investigated on human mononuclear cells (MNC) or stromal m-IC_{cl2} cells. For this purpose, each pathogenicity factor (PF) was incubated with Pep19-2.5 and then levels of cytokines induced by the PF-peptide mixtures in the cells were compared with those induced by the PF alone. The secretion of the pro-inflammatory cytokines tumor-necrosis factor (TNF) α, macrophage inflammatory protein (MIP-2) and reactive oxygen species (ROS) was monitored (Fig. S1). Highly purified LPS Ra isolated from *Salmonella enterica subsp. enterica* sv Minnesota was utilized, since this molecule is known to be the main bacterial stimulus of Gram-negative bacteria (1; 2).

Pep19-2.5 inhibited in a dose-dependent manner the LPS-induced secretion of TNF-α (Fig.S1A), PGE2, IL-6 and IL-8 (data not shown) by MNC. Similarly, the LPS-induced expression and secretion of the chemokine MIP-2 by m-IC_{cl2} cells was almost abrogated in the presence of 5 μg/ml Pep19-2.5 (Fig.S1B). Cells that were exposed to the LPS-Pep19-2.5 mixture had levels of MIP-2 mRNA similar to cells incubated in the absence of LPS, demonstrating that the presence of the peptide does not interfere with cytokine quantification but rather that inhibition of MIP-2 is due to the peptide LPS-neutralizing activity (Fig. S1C).

ROS expression represents a warning signal for the immune system. To examine whether the peptide could influence LPS-induced cell production of ROS by human MNC, the kinetics of ROS production upon addition of LPS alone or incubated with Pep19-2.5 was monitored using a ROS-dependent chemiluminescent assay. As shown in Fig. S1D, presence of the peptide at a 10:1 ratio with respect to LPS resulted in a nearly complete neutralization of the

LPS stimulatory effect. As shown for the various inflammatory mediators, the addition of Pep19-5.2 inhibited their expression and secretion in a dose-dependent fashion.

In addition to LPS from *S. enterica* Minnesota, Pep19-2.5 neutralized TNF-α expression by MNC induced with LPS from phylogenetically unrelated bacterial species (Tab. S1). Using the same methodology, we found that Pep19-2.5 had a broad spectrum of anti-inflammatory activity, since the peptide also counteracted with high efficiency TNF-α expression induced by amphiphilic immune stimuli derived from Gram-positive organisms and *Mycobacterium*. It should be noted that Pep19-2.5 did not inhibit the immune-stimulatory potential of the synthetic TLR2 agonist Pam_3CSK_4, a shortened variant of MALP-2 and therefore a non-natural molecule.

Methods

Stimulation of human mononuclear cells and mouse intestinal epithelial cells by LPS

Mononuclear cells (MNC) were isolated from heparinized blood of healthy donors as described previously (3). The cells were resuspended in medium (RPMI 1640) and their number was equilibrated at $5x10^6$cells/ml. For stimulation, 200 µl MNC ($1x10^6$cells) were transferred into each well of a 96-well culture plate. LPS Ra (or other pathogenicity factors summarized in Table 1, supplementary data) were incubated with the peptide for 30 min at 37 °C, and added to the cells at 20 µl per well. The cultures were incubated for 4 h at 37 °C under 5 % CO_2. Supernatants were collected after centrifugation of the culture plates for 10 min at 400 x g and stored at -20 °C until determination of TNF-α, carried out in a Sandwich ELISA using a monoclonal antibody against TNF-α from BD Biosciences (San Diego, USA).

m-IC_{cl2} cells were seeded into 48 well plates and grown to confluency for 6 days as previously described (4). *E. coli* K12 D31m4 LPS was added to the cells at the concentration

of 1 ng/mL in the presence or absence of various concentrations of Pep19-2.5. Cell supernatants were harvested after 4 hours and stored at -20 °C until determination of the MIP-2 concentration by ELISA from Nordic Biosite (Taby, Sweden). Cells were washed in PBS and resuspended in TRIZOL (Invitrogen, Carlsbad, USA). RNA was extracted following the manufacturer's instructions. cDNA was synthesized using 5 µg of total RNA with the RevertAid Reverse transcriptase (Fermentas, St. Leon-Rot, Deutschland) and SYBR green based real time PCRs were performed using specific mouse *Mip-2* and mouse *Gapdh* primers (5; 6).

LPS-induced chemiluminescence (CL) of human mononuclear cells

MNC were isolated from human blood as described (3). MNC were suspended in a modified RPMI-medium (RPMI-1640-medium without phenol red and sodium bicarbonate but containing 20 mmol /l HEPES [Biochrom, Berlin, Germany]) and placed in CL-reaction vials each containing 400,000 MNC in 280 µl medium. Then the vials were incubated for at least 60 min before CL measurement at 37 °C. Thereafter the vials were put in the 6-channel luminometer (Biolumat LB9505, Berthold Technologies, Bad Wildbad, Germany) and 10 min prior to the CL measurements luminol (5-amino-2,3-dihydro-1,4-phthalazinedione, Sigma, Taufkirchen, Germany) was added (10 µl per vial of a 2 mg/ml solution) as the chemiluminescence mediating compound. Then after addition of 6 µl of medium (unstimulated control), of LPS, and of the LPS:peptide mixtures, respectively, the chemiluminescence of 6 vials was recorded in parallel for 60 min whereby the vials were always kept at 37 °C in the luminometer. The data are shown as cumulated CL-activity above background of unstimulated MNC-CL-activity as RLU per 10 min, 20 min, and 50 min (cf. Fig. 2D).

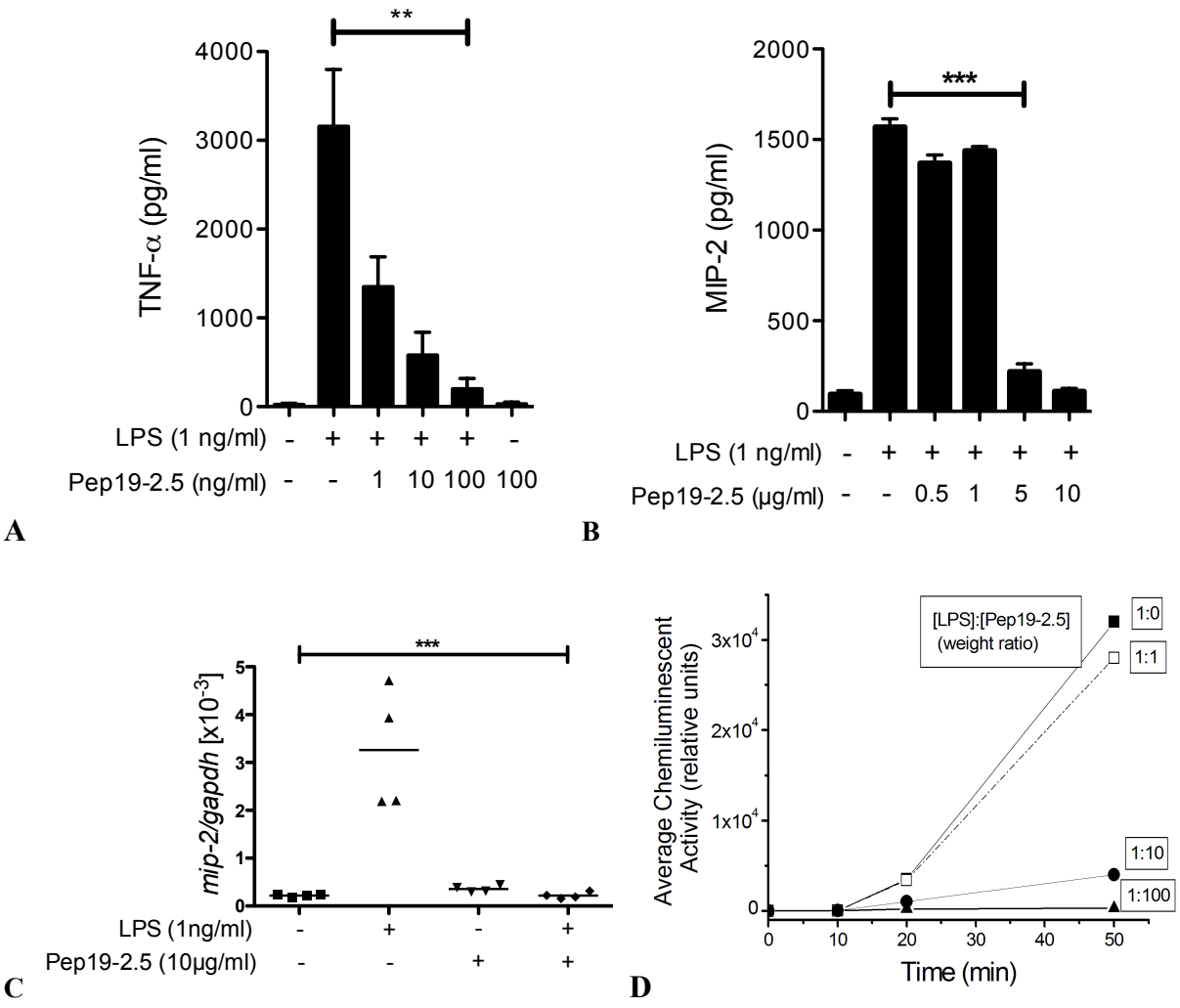

Figure S1: In vitro inhibition of LPS-induced inflammation by Pep19-2.5. TNF-α expression by human MNC after 4 h of stimulation with the indicated mixtures of LPS and Pep19-2.5 (**A**). Production of either MIP-2 protein (**B**) or MIP-2 mRNA (**C**) by m-IC_{cl2} cells after 4 h of stimulation with the specified combinations of LPS and Pep19-2.5. Release of reactive oxygen species (ROS) from human MNC detected by luminol-aided chemiluminescence (CL) (**D**). Depicted is the mean +/- SEM of 4 independent experiments (**A**) or one representative experiment of four (**B**). For statistic analysis, data were log-transformed and groups were compared by use of One-Way Repeated Measure ANOVA (* $p<0.05$, **: $p<0.01$).

Pathogenicity factor, Receptor	**Source**	**Related Disease or ecological niche.**	**Inhibition (%)**
LPS, TLR4 (7)			
Brucella abortus	Universidad de Navarra, Pamplona	Brucellosis (cattle)	> 98
Chromobacterium violaceum	Institute of Fermentation, Osaka	Sepsis	> 90
Citrobacter freundii	Forschungszentrum Borstel, U. Zähringer	Nosocomial infections	> 98 (10 ng)
Escherichia coli (rough and smooth type)	Forschungszentrum Borstel	Gut commensal, sepsis	> 90
Klebsiella pneumonia (Lipid A)	Forschungszentrum Borstel	Gut commensal, pneumonia, sepsis	> 98 (100 ng)
Phospholipid ER803022 (synthetic (8))	EISAI Co., Andover, Ma	n.a.	> 98
Proteus mirabilis	Forschungszentrum Borstel	Urinary tract infection, sepsis	> 98 (10 ng)
Rhizobium trifolii	Forschungszentrum Borstel	Symbiont of trefoil	> 98
Salmonella abortus equi	Forschungszentrum Borstel	Salmonellosis (horse, donkey)	> 90
Salmonella enterica Minnesota	Forschungszentrum Borstel	Salmonellosis, sepsis	85
Yersinia pestis	Universidad de Navarra, Pamplona	Plague	70 (10 ng)
Lipopeptides, TLR2 (9)			
Bacillus subtilis (lipoteichoic acids)	Sigma-Aldrich Co.	Soil	> 98 (10 ng)
FSL-1 (fibroblast-stimulating factor, synthetic, *Mycoplasma*)	EMC Microcollections, Tübingen	Pneumonia	> 90 (10 ng)
MALP-2 (macrophage-activating lipoprotein, synthetic, *Mycoplasma*)	EMC Microcollections, Tübingen	Pneumonia	50
Pam3CSK4 (synthetic)	EMC Microcollections, Tübingen	n.a.	0
Staphylococcus aureus	Universität zu Lübeck	Nosocomial	> 98

	infections

Table S1: Neutralization efficiency from Pep19-2.5 of various bacterial immune stimuli.

Pep19-2.5 mediated inhibition of TNF-α expression by human MNC upon incubation with various stimulatory molecules purified or derived from bacteria. If not specified, data relate to a stimulus concentration of 1 ng/ml at a stimulus:peptide ratio of 1:10 weight %. (n.a. not applicable)

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