

Effects of anti-inflammatory vagus nerve stimulation in endotoxemic rats on blood and spleen lymphocyte subsets

S. Mihaylova · H. Schweighöfer ·
H. Hackstein · B. Rosengarten

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Abstract

Background Anti-inflammatory cytokine effects of vagus nerve stimulation in sepsis syndromes are well established. Effects on immune cells are less clear. Therefore, we studied changes in peripheral and spleen leukocyte subsets in an endotoxic rat sepsis model.

Methods Ventilated and sedated adult male SD rats received 5 mg/kg b.w. lipopolysaccharide intravenously to induce endotoxic sepsis. Controls and a group with both-sided vagotomy were compared to animals with both sided vagotomy and left distal vagus nerve stimulation. 4.5 h after sepsis induction immune cell counts and types in the peripheral blood and spleen were determined [T-lymphocytes (CD3+), T-helper cells (CD3+ CD4+), activated T-helper cells (CD3+ CD4+ CD134+), cytotoxic T-cells (CD3+ CD8+), activated cytotoxic T-cells (CD3+ CD8+ CD134+), B-lymphocytes (CD45R+ CD11c^{neg}-dim), dendritic cells (CD11c+ OX-62 +), natural killer cells (CD161+ CD3^{neg}) and granulocytes (His48 +)] together with cytokine and chemokine plasma levels (IL10; IFN- γ , TNF- α , Cxcl5, Ccl5).

Results Blood cell counts declined in all LPS groups. However, vagus nerve stimulation but not vagotomy activated cytotoxic T-cells. Vagotomy also depleted natural

killer cells. In the spleen, vagotomy resulted in a strong decline of all cell types which was not present in the other septic groups where only granulocyte numbers declined.

Conclusion Vagotomy strongly declines immune cell counts in the septic spleen. This could not be explained by an evasion or apoptosis of cells. A marginalisation of spleen immune cells into the peripheral microcirculation might be therefore most likely. Further studies are warranted to clear this issue.

Keywords Vagus nerve stimulation · Endotoxic shock · Lymphocytes · Splenocytes · Anti-inflammation · Rat

Introduction

Sepsis and septic shock are the leading causes of death on all intensive care units [1, 2]. In the USA, the annual incidence was estimated to be more than 750,000 cases from which approximately 200,000 patients die [2]. In developed countries, the average incidence is about 300/100,000 p.a. with a near to exponential increase in the elderly [3, 4]. The lethality of the disease is still high, reaching 30–40 % in sepsis sufferers, and increasing to 50–60 % in patients with septic shock [3]. Longer life expectancy, higher number of patients with disturbed or depressed immune system and increasing antibiotic resistance of microbes will be the factors which will increase the number of septic patients in the next years. Currently, sepsis is the third leading cause of death in developed countries [3, 5].

Sepsis is defined by an infection, which is followed subsequently by an inflammatory host response [6]. Nowadays, it is widely accepted that the excessive activation of the immune system has a more detrimental effect than the

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S. Mihaylova · H. Schweighöfer · B. Rosengarten (✉)
Departments of Neurology, Justus-Liebig University Giessen,
Klinikstrasse 33, 35392 Giessen, Germany
e-mail: bernhard.rosengarten@neuro.med.uni-giessen.de

H. Hackstein
Departments of Clinical Immunology and Transfusion Therapy,
Justus-Liebig University Giessen, Klinikstrasse 33,
35392 Giessen, Germany

original infection itself. The inflammation causes tissue damage, multi-organ failure, and mortality. The first immune response is mainly triggered by the innate immune system [7]. Within minutes to hours, pro-inflammatory cytokine levels increase excessively followed by strong leukocyte activation. Preventing from an excessive immune activation effectively improved outcome [8–11]. The anti-inflammatory effects of vagus nerve stimulation (VNS) led to the concept of a “cholinergic anti-inflammatory reflex” of the vagus nerve [8, 12, 13]. VNS reduced the pro-inflammatory cytokine levels and improved the outcome in septic mice, whereas vagotomy (VGX) had the opposite effects [12, 14]. The detailed pathway of the reflex is still not clear. However, it was shown that acetylcholine, the alpha-7 subunit of the nicotinic acetylcholine receptor and the lymphocytes in the spleen are essential components of the anti-inflammatory reflex [13]. Whereas the cytokine effects of VNS are well established, the effects on the immune cells are less clear. Recently, it was shown that VNS was only effective within the first 6 h after sepsis induction and that T-lymphocytes were mainly responsible for its anti-inflammatory action [15–18]. However, the effect of VNS or VGX on the leukocyte subsets at this early stage of a sepsis syndrome was not investigated previously. We performed an extensive analysis of leukocyte subsets in blood as well as in spleen samples. Studies were performed 4.5 h after rats have been subjected into a non-septic or a septic group using an established endotoxin sepsis model. Each group was further divided into groups without vagus nerve intervention, VGX or vagotomy with left-sided VNS.

Materials and methods

All procedures performed on the animals were in strict accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals and approved by the local Animal Care and Use Committee. The study was approved by the Institutional Review Board for the care of animal subjects.

Animal experiments

Adult male Sprague–Dawley rats (290–320 g) were purchased from the Harlan Laboratories (Harlan, Rossdorf, Germany). They were housed five in a cage with food and water available ad libitum and were maintained on a 12-h light/dark cycle (lights on at 7 a.m.). For the experiments, rats were initially anaesthetised with isoflurane, tracheotomized, paralysed with pancuronium bromide (0.2 mg/kg/h) and mechanically ventilated (Harvard Rodent Ventilator; Harvard, South Natick, Massachusetts, USA). The right femoral artery and vein were

cannulated for blood pressure recording, blood sampling, and drug administration. Rectal body temperature was maintained at 37 °C using a feedback-controlled heating pad.

Isoflurane anaesthesia was discontinued and replaced by an intravenous application of α -chloralose (80 mg/kg; Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany). A washout period from isoflurane anaesthesia of 60 min was allowed in all rats. Supplementary doses of chloralose (30 mg/kg) were given every hour. During chloralose anaesthesia, the animals were ventilated with a 1:1 mixture of nitrogen and oxygen. Arterial blood gas analyses and pH were measured repeatedly as needed and at least every 30 min (Blood gas analyzer model Rapidlab 348, Bayer Vital GmbH, Fernwald, Germany). Glucose and lactate levels were measured simultaneously (Glukometer Elite XL, Bayer Vital GmbH, Fernwald, Germany; Lactate pro, Arkray Inc. European Office, Düsseldorf, Germany), while glucose concentration was maintained at >60 mg/dl. A moderate volume therapy of 1.2 ml/h of 0.9 % NaCl was given to replace renal and perspirative fluid losses.

Study design

Sixty rats were assigned to two main groups (non-septic vs. sepsis) with each three subgroups (no vagal stimulation, VGX, VNS). Therefore, each group consisted of ten rats. Sepsis was initiated by intravenous application of 5 mg/kg body weight lipopolysaccharide (LPS, from *Escherichia coli*, O111:B4, Sigma-Aldrich Chemie GmbH, Germany) dissolved in 0.5 ml 0.9 % NaCl. Control groups received a similar volume of 0.9 % NaCl without toxin. Volumes were given slowly within 5 min. The endotoxinemic and control groups were further divided into three subgroups each. In the first subgroup, the vagus nerves were bilaterally surgically dissected (specified as the following groups: VGX; LPS VGX). In the second subgroup, the vagus nerves were also bilaterally surgically dissected but then the distal trunk of the left vagus was prepared for electrical stimulation (specified as the following groups: VNS; LPS VNS). Allowing constant stimulation, the distal trunk of the nerve was placed into a special stimulation clamp (HSE, March-Hugstetten, Germany). A stimulation block had a duration of 10 min applying electrical pulses of 2 mA, 0.3 ms pulse width, and 2 Hz repetition frequency. The first stimulation was undertaken right before application of the LPS/vehicle. Thereafter, stimulation blocks were repeated every 45 min until the end of experimentation. Because most fibres of the right nerve innervate the heart and to avoid cardio-depressive side effects the left N. vagus was chosen for stimulation. The last two subgroups underwent neither VNS nor VGX and served as sham groups without dissecting the vagus nerve (specified as the following groups: SHAM; LPS SHAM). Experiments were

performed up to 4.5 h after LPS/vehicle administration. The reason for choosing the time window was that afterwards the blood pressure falls below the lower limit of cerebral autoregulation, thus leading to additional neuronal dysfunction due to insufficient blood supply [19]. Blood samples and spleens were obtained before the rats were killed. Plasma samples were aliquoted and stored at -80 °C for ELISA.

Flow cytometry

Spleen was harvested into a tissue culture dish and teased apart into a single cell suspension by gentle pressing with the plunger of a syringe. Afterwards, cells were collected in a flow cytometry staining buffer and passed through a cell strainer. Later, cell suspension was centrifugated for 4–5 min at 300–400 g. Supernatant was discarded and the cell pellet was resuspended. After cell count and viability check cells were centrifugated. Afterwards they were resuspended in appropriate volume of flow cytometry staining buffer to a final concentration of about 1 × 10⁷ cells/ml. Cellular phenotyping was performed on a FACS CantoII flow cytometer (Becton–Dickinson, San Jose, CA, USA). The following fluorochrome-labelled monoclonal antibodies conjugated to FITC, PE, PerCP, APC were used for surface staining according to the manufacturer’s instructions: CD3e, CD4, CD8a, CD11c, CD45R, CD134, CD161a, His48, OX-62 (all mabs from BD Biosciences, Germany). Absolute leukocyte numbers were determined by using a Neubauer counting chamber or sysmex KX 21-N cell counter (Sysmex, Norderstedt, Germany). Erythrocytes were lysed in heparin-anticoagulated blood samples and spleen cell suspensions prior to flow cytometry analysis using BD FACS Lysing Solution (BD Biosciences) according to the manufacturer’s instructions. The following leukocyte subpopulations were quantitated by flow cytometry according to surface marker expression: T-lymphocytes (CD3+), T-helper cells (CD3+ CD4+), activated T-helper cells (CD3+ CD4+ CD134+), cytotoxic T-cells (CD3+ CD8+), activated cytotoxic T-cells (CD3+ CD8+ CD134+), B-lymphocytes (CD45R+ CD11c^{neg}-dim), dendritic cells (DC; CD11c+ OX-62+), natural killer cells (NK; CD161+ CD3^{neg}) and granulocytes (His48+).

ELISA

Cytokine and chemokine concentrations were analysed using ELISA. IL10, IFN γ and TNF α were analysed with specific OptIA ELISA sets (BD, San Diego, USA). Cxcl5/Lix was analysed with the Cxcl5 Duo Set (R&D, Minneapolis, USA), Ccl5/RANTES was analysed using the RANTES-Single analyte ELISArray Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions.

Table 1 Group averaged (*n* = 10) data for RR, glucose, lactate, pH, pCO₂, and haemoglobin

	Mean RR (mm/Hg)		Glucose (mg/dl)		Lactate (mmol/l)		pH		pO ₂ (mmHg)		pCO ₂ (mmHg)		Haemoglobin (g/l)	
	Begin	End	Begin	End	Begin	End	Begin	End	Begin	End	Begin	End	Begin	End
SHAM	116 ± 20	108 ± 20	63 ± 8	84 ± 21	0.8 ± 0.05	0.9 ± 0.1	7.49 ± 0.06	7.47 ± 0.03	250 ± 18	246 ± 29	36 ± 3	38 ± 4	140 ± 10	120 ± 20**
VGX	115 ± 13	115 ± 22	98 ± 30	98 ± 18	0.8 ± 0.03	0.9 ± 0.1	7.45 ± 0.05	7.50 ± 0.06	265 ± 19	250 ± 23	41 ± 8	33 ± 4	140 ± 10	130 ± 10*
VNS	109 ± 10	93 ± 21	71 ± 15	105 ± 49	0.9 ± 0.2	1.3 ± 0.5	7.52 ± 0.06	7.51 ± 0.04	259 ± 19	248 ± 5	37 ± 5	36 ± 5	130 ± 10	100 ± 20*
LPS SHAM	123 ± 11	90 ± 18 ^s	65 ± 13	69 ± 26	0.8 ± 0.1	1.9 ± 0.5 ^s	7.47 ± 0.05	7.45 ± 0.03	250 ± 14	233 ± 30	40 ± 4	35 ± 3*	150 ± 10	120 ± 10 ^s
LPS VGX	117 ± 9	97 ± 10**	82 ± 22	129 ± 90	0.8 ± 0.1	2.2 ± 0.7 ^s	7.45 ± 0.07	7.37 ± 0.08**	272 ± 13	249 ± 26	41 ± 6	39 ± 4	150 ± 10	120 ± 20**
LPS VNS	119 ± 13	90 ± 14***	85 ± 25	65 ± 20	0.9 ± 0.1	1.8 ± 0.6***	7.48 ± 0.4	7.43 ± 0.1	251 ± 22	233 ± 21	38 ± 2	35 ± 5	130 ± 10	100 ± 20***

Data are given as the mean ± standard deviation (SD) together with statistical results of a paired *t* test. Significance is given as * *p* < 0.05; ** *p* < 0.01; *** *p* < 0.001; ^s *p* < 0.0001 compared with baseline

RR blood pressure, pO₂ partial pressure of oxygen, pCO₂ partial pressure of carbon dioxide, SHAM sham surgery, LPS lipopolysaccharide, VGX bilateral vagotomy, VNS vagus nerve stimulation

Table 2 Data from cytokine and chemokine measurements of plasma samples as group averaged data ($n = 10$)

	TNF- α [pg/ml]	IFN- γ [pg/ml]	IL 10 [pg/ml]	IL 6 [pg/ml]	Ccl-5/Rantes [pg/ml]	Cxcl-5/Lix [pg/ml]
SHAM	40 \pm 10	50 \pm 40	300 \pm 60	110 \pm 30	750 \pm 95	140 \pm 80
SHAM LPS	140 \pm 20*	690 \pm 160*	1.700 \pm 510**	160.000 \pm 20.000 ^s	4.100 \pm 620**	35.000 \pm 1.800 ^s
LPS + VGX	200 \pm 30**	850 \pm 200**	2.400 \pm 400***	170.000 \pm 20.000 ^s	4.500 \pm 310**	27.000 \pm 3.300 ^s
LPS + VNS	160 \pm 30*	500 \pm 140*	2.300 \pm 420***	140.000 \pm 20.000 ^s	5.300 \pm 1.200***	28.000 \pm 2.700 ^s

Data are given as mean \pm sem. Significance of the ANOVA and the Fisher post hoc tests are given as: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ^s $p < 0.0001$ compared to sham animals

SHAM sham surgery, LPS lipopolysaccharide, VGX bilateral vagotomy, VNS vagus nerve stimulation

Table 3 Effects of VGX or VNS on leukocyte subset numbers (mean \pm SD) from blood or spleen given as group averaged data ($n = 10$) for the non-septic groups

Cell-type	Surface marker	Sham (A)	VGX (B)	VNS (C)	Statistics: ANOVA and Fisher post hoc test
Blood data					
Total leukocytes [μ l]		8.800 \pm 1.200	5.700 \pm 1.100	8.300 \pm 2.000	$p < 0.01$ A–B; $p < 0.005$; B–C: $p < 0.01$
T-lymphocytes [μ l]	CD3+	1.100 \pm 400	800 \pm 300	1.000 \pm 700	n.s.
B-lymphocytes [μ l]	CD45RA+	800 \pm 400	300 \pm 200	500 \pm 300	$p < 0.05$ A–B; $p < 0.01$
Cytotoxic T-cells [μ l]	CD3+ CD8+	400 \pm 100	300 \pm 100	300 \pm 200	n.s.
T-helper cells [μ l]	CD3+ CD4+	700 \pm 300	500 \pm 200	600 \pm 500	n.s.
Activated cytotoxic T-cells [% of total CTL]	CD3+ CD8+ CD134+	17 \pm 6	23 \pm 16	45 \pm 17	$p < 0.001$ A–C; $p < 0.001$; B–C: $p < 0.005$
Activated T-helper cells [% of total T-helper cells]	CD3+ CD4+ CD134+	9 \pm 3	8 \pm 8	14 \pm 12	n.s.
DC-cells [μ l]	CD45RA– CD11c+ Ox62+	8 \pm 5	5 \pm 4	5 \pm 4	n.s.
NK-cells [μ l]	CD3–CD161a+	40 \pm 21	80 \pm 34	150 \pm 47	$p < 0.005$ A–C; $p < 0.001$; B–C: $p < 0.01$
Granulocytes [μ l]	His 48+	4.900 \pm 700	2.600 \pm 500	3.500 \pm 800	$p < 0.001$ A–B; $p < 0.0005$; A–C: $p < 0.01$
Thrombocytes [μ l]		640.000 \pm 170.000	600.000 \pm 100.000	690.000 \pm 120.000	n.s.
Spleen data					
Total leukocytes [mg]		270.000 \pm 60.000	300.000 \pm 80.000	380.000 \pm 30.000	$p < 0.01$ A–C; $p < 0.005$; B–C: $p < 0.01$
T-lymphocytes [mg]	CD3+	84.000 \pm 20.000	77.000 \pm 26.000	105.000 \pm 15.000	n.s.
B-lymphocytes [mg]	CD45RA+	138.000 \pm 20.000	92.000 \pm 2.400	147.000 \pm 25.000	$p < 0.005$ A–B; $p < 0.01$; B–C: $p < 0.005$
Cytotoxic T-cells [mg]	CD3+ CD8+	26.000 \pm 8.000	27.000 \pm 8.000	29.000 \pm 3.000	n.s.
T-helper cells [mg]	CD3+ CD4+	50.000 \pm 13.000	43.000 \pm 16.000	60.000 \pm 10.000	n.s.
Activated cytotoxic T-cells [% of total CTL]	CD3+ CD8+ CD134+	4 \pm 3	3 \pm 2	4 \pm 2	n.s.
Activated T-helper cells [% of total T-helper cells]	CD3+ CD4+ CD134+	9 \pm 4	8 \pm 5	5 \pm 2	n.s.
DC-cells [mg]	CD45RA– CD11c+ Ox62+	260 \pm 100	110 \pm 70	110 \pm 40	$p < 0.05$ A–B; $p < 0.01$; A–C: $p < 0.01$
NK-cells [mg]	CD3– CD161a+	5.800 \pm 1.100	8.000 \pm 2.000	10.000 \pm 3.000	$p < 0.01$ A–C; $p < 0.005$
Granulocytes [mg]	His 48+	15.000 \pm 7.000	20.000 \pm 20.000	27.000 \pm 13.000	n.s.

In non-septic rats, VGX resulted in a significant lowering of leukocyte, B-lymphocyte as well as granulocyte counts in blood. VNS significantly increased NK-cells and activated CTL in blood. Regarding the spleen VNS increased the number of leukocytes and NK-cells, whereas VGX reduced the B-lymphocytes. DCs were reduced by both VGX and VNS in spleen. Compared to SHAM, *bold values* indicate a significant reduction, *bold italics* a significant induction of cell numbers SHAM sham surgery, VGX bilateral vagotomy, VNS vagus nerve stimulation

Table 4 Effects of VGX or VNS on leukocyte subset numbers from blood or spleen given as group averaged data (*n* = 10) for the LPS groups

Cell-type	Surface marker	Sham (A)	LPS (B)	LPS + VGX (C)	LPS + VNS (D)	ANOVA (<i>p</i> value)	Fisher post hoc test
Data from blood							
Total leukocytes [μl]		8.800 ± 1.200	3.600 ± 0.900	2.300 ± 0.600	4.700 ± 1.300	<0.0001	A-B: <i>p</i> < 0.0001; A-C: <i>p</i> < 0.0001; A-D: <i>p</i> < 0.0001; C-D: <i>p</i> < 0.005
T-lymphocytes [μl]	CD3+	1.100 ± 400	590 ± 210	500 ± 60	460 ± 140	<0.0005	A-B: <i>p</i> < 0.001; A-C: <i>p</i> < 0.0005; A-D: <i>p</i> < 0.0001
B-lymphocytes [μl]	CD45RA+	800 ± 400	300 ± 100	190 ± 50	300 ± 200	<0.005	A-B: <i>p</i> < 0.005; A-C: <i>p</i> < 0.001; A-D: <i>p</i> < 0.005
Cytotoxic T-Cells [μl]	CD3+ CD8+	400 ± 100	190 ± 90	140 ± 20	140 ± 50	<0.0001	A-B: <i>p</i> < 0.001; A-C: <i>p</i> < 0.0001; A-D: <i>p</i> < 0.0001
T-helper cells [μl]	CD3+ CD4+	700 ± 300	350 ± 120	310 ± 36	280 ± 90	<0.0005	A-B: <i>p</i> < 0.0005; A-C: <i>p</i> < 0.001; A-D: <i>p</i> < 0.001
Activated cytotoxic T-cells [% of total CTL]	CD3+ CD8+ CD134+	17 ± 6	55 ± 21	33 ± 14	61 ± 7	<0.001	A-B: <i>p</i> < 0.001; A-D: <i>p</i> < 0.005; B-C: <i>p</i> < 0.05; C-D: <i>p</i> < 0.01
Activated T-helper cells [% of total T-helper cells]	CD3+ CD4+ CD134+	9 ± 3	9 ± 4	6 ± 4	9 ± 3	n.s.	
DC-Cells [μl]	CD45RA- CD11c+ Ox62+	8 ± 5	3 ± 3	4 ± 2	2 ± 3	<0.05	A-B: <i>p</i> < 0.01; A-C: <i>p</i> < 0.05; A-D: <i>p</i> < 0.01
NK-Cells [μl]	CD3- CD161a+	40 ± 21	35 ± 14	15 ± 5	25 ± 12	<0.05	A-C: <i>p</i> < 0.05; B-C: <i>p</i> < 0.05
Granulocytes [μl]	His 48+	4.900 ± 700	900 ± 560	570 ± 240	1.000 ± 700	<0.0001	A-B: <i>p</i> < 0.001; A-C: <i>p</i> < 0.0001; A-D: <i>p</i> < 0.0001
Thrombocytes [μl]		640.000 ± 170.000	340.000 ± 160.000	470.000 ± 20.000	310.000 ± 90.000	<0.005	A-B: <i>p</i> < 0.001; A-C: <i>p</i> < 0.05; A-D: <i>p</i> < 0.001
Data from spleen							
Total Leukocytes [mg]		270.000 ± 60.000	280.000 ± 60.000	140.000 ± 60.000	270.000 ± 70.000	<0.01	A-C: <i>p</i> < 0.005; B-C: <i>p</i> < 0.005; C-D: <i>p</i> < 0.01
T-lymphocytes [mg]	CD3+	84.000 ± 20.000	73.000 ± 21.000	38.000 ± 17.000	82.000 ± 23.000	<0.05	A-C: <i>p</i> < 0.005; B-C: <i>p</i> < 0.05; C-D: <i>p</i> < 0.005
B-lymphocytes [mg]	CD45RA+	138.000 ± 20.000	101.000 ± 26.000	68.000 ± 39.000	101.000 ± 29.000	<0.05	A-C: <i>p</i> < 0.005
Cytotoxic T-cells [mg]	CD3+ CD8+	26.000 ± 8.000	26.000 ± 6.000	11.000 ± 5.000	30.000 ± 9.000	<0.01	A-C: <i>p</i> < 0.01; B-C: <i>p</i> < 0.01; C-D: <i>p</i> < 0.001
T-helper cells [mg]	CD3+ CD4+	50.000 ± 13.000	37.000 ± 10.000	10.000 ± 5.000	40.000 ± 13.000	<0.05	A-C: <i>p</i> < 0.005; C-D: <i>p</i> < 0.05
Activated cytotoxic T-cells [% of total CTL]	CD3+ CD8+ CD134+	4 ± 3	4 ± 1	4 ± 1	4 ± 1	n.s.	

Table 4 continued

Cell-type	Surface marker	Sham (A)	LPS (B)	LPS + VGX (C)	LPS + VNS (D)	ANOVA (p value)	Fisher post hoc test
Activated T-helper cells [% of total T-helper cells]	CD3+ CD4+ CD134+	9 ± 4	9 ± 3	5 ± 1	5 ± 1	n.s.	
DC-Cells [mg]	CD45RA- CD11c+ Ox62+	260 ± 100	160 ± 110	60 ± 50	130 ± 40	<0.05	A-C: <i>p</i> < 0.005
NK-Cells [mg]	CD3- CD161a+	5.800 ± 1.100	5.600 ± 2.000	2.800 ± 800	6.800 ± 2.500	<0.05	A-C: <i>p</i> < 0.05; B-C: <i>p</i> < 0.05; C-D: <i>p</i> < 0.005
Granulocytes [mg]	His 48+	15.000 ± 7.000	9.000 ± 5.600	5.600 ± 2.400	11.000 ± 7.000	<0.0001	A-B: <i>p</i> < 0.0001; A-C: <i>p</i> < 0.0001; A-D: <i>p</i> < 0.0001

Sepsis resulted in blood to a decline in cell numbers of leukocytes, T- and B-lymphocytes, CTLs, T-helper cells, granulocytes, DCs as well as thrombocytes. Additionally, NK-cells decreased in the LPS + VGX group. Sepsis activated CTLs. LPS + VGX, but not LPS + VNS inhibited CTL activation and lowered NK-cell count. Comparing LPS + VNS and VGX + VNS, VNS had a strong-activating effect on CTLs and mitigated the decline in leukocyte count. In spleen, we found no changes in the LPS or LPS + VNS group as compared to SHAM group. However, VGX had a strong negative effect on cell counts of all cell types. Compared to SHAM, *bold values* indicate a significant reduction, *bold italic values* a significant induction of cell numbers. SHAM sham surgery, LPS lipopolysaccharide, VGX bilateral vagotomy, VNS vagus nerve stimulation

Statistics

Groups were compared using a one-way ANOVA test. When significant, a Fisher post hoc test was used to compare between the groups. Statistics were performed differently for the non-septic and septic groups. Significance was inferred at *p* < 0.05.

Results

General results

Whereas the non-septic rats showed stable physiological data for the entire experimental condition all LPS subjected rats developed typical signs of a progressive sepsis syndrome up to 4.5 h after endotoxin injection. Septic rats showed increased lactate levels without difference among the sepsis groups. The pH levels in septic rats tended to be lower at the end of experiments and were significantly lowered in the LPS + VGX group. Table 1 shows the results for the mean arterial blood pressure, glucose, lactate, pH, pO₂, pCO₂ and haemoglobin for the different groups. Due to a moderate volume therapy, the haemoglobin level decreased about 20 g/l in all groups. Stimulation of the left N. vagus did not result in relevant changes of blood pressure.

Analysis of chemokine and cytokine levels

Pro- as well as anti-inflammatory cytokine levels increased excessively in all LPS-treated groups (Table 2). Although a trend to lower values in the LPS + VNS group and higher values in the LPS + VGX group appeared, differences did not reach significance. Also, there were no significant differences regarding the chemokine levels of Cxcl-5 and Ccl-5 within the LPS groups.

Subset analysis of leukocytes

Non-septic condition (Table 3)

Vagus nerve stimulation induced relative numbers of activated cytotoxic T-cells and NK-cells in blood and total leukocyte counts as well as NK-cells in spleen. Vagotomy decreases total leukocyte counts, B-lymphocytes and granulocytes in blood as well as B-lymphocytes in spleen. However, DC-cells in spleen were reduced by vagotomy as well as vagus nerve stimulation.

Septic condition (Table 4)

In blood, sepsis led to a significant depletion of nearly all leukocytes but a strong activation of the remaining CTL in

blood. DC-cells and granulocytes also significantly declined in numbers. Only Nk-cells remained constant. Vagus nerve stimulation resulted in similar findings differing in an even stronger shift to activated CTL. This activation was lacking in the LPS + VGX group. Also, Nk-cells significantly dropped only in the LPS + VGX group. No shift in activation was found for the T-helper cells.

In the spleen, only a decline in granulocyte numbers was seen in the LPS and LPS + VNS group. Vagotomy showed a strong decline in all cell types. No significant change in the activation pattern was found neither for the activated CTL or T-helper cell subsets.

Discussion

A decline of blood leukocytes is a frequent and early finding in sepsis or severe trauma and was often associated with immune paralysis [20–23]. However, in the last years a more detailed picture emerged. Changes in cell counts vary in regard to the time point and route of infection also differing in response of a viral or bacterial origin of infection [1, 24, 25]. A bacterial infection typically induces an initial decrease of all leukocytes due to a marginalisation of leukocytes in the peripheral vascular territories before later on apoptosis of distinct immune cells occur. The T-helper cells commonly normalise within 1 week, whereas the CTLs show a slower recovery [26]. Several experimental and clinical investigations demonstrated a reduction of CTLs to be accompanied by an improved outcome [27–29]. In line with the literature, we found a strong decline of leukocytes 4.5 h after a LPS challenge affecting both the CTL and T-helper population in a similar manner and independent from the intervention. An activation of CTLs was reported to improve the outcome of human sepsis [26]. A reduced activation was assumed with a less-efficient immune-response-worsening outcome [30, 31]. Increased numbers of activated CTLs had also a beneficial effect [32]. VNS, therefore, seemed to have a beneficial effect since number and activity degree increased in septic as well as in non-septic conditions. Regarding the T-helper cells, it was shown that they transform within 48 h into regulatory T-cells (CD3+ CD4+ CD25+) and cytokine-releasing (CD3+ CD4+ CD25–) cells [33]. Under VGX, the cytokine-releasing T-cells tended to a stronger pro-inflammatory response 48 h after in vitro stimulation, whereas the pro-inflammatory responses declined under cholinergic medication [33]. However, it should be noted that additional markers (e.g. Foxp3) are necessary for unambiguous identification of regulation T-cells. In the present study, the time window was too short to contribute to the further transformation of T-helper cells [33, 34].

The role of NK-cell reduction is also still under debate. Whereas a decline in NK-cells resulted in an increased risk for pulmonary infection in stroke sufferers [35], others found a detrimental role of NK-cells under inflammatory conditions [27, 36]. VGX lowered NK-cell counts in blood and spleen under septic conditions, whereas LPS + VNS or LPS did not affect cell counts.

The present rapid decline in cell numbers in the spleen under VGX cannot readily be explained by apoptosis. Apoptosis of leukocytes starts to influence cell numbers approximately 16 h after sepsis induction [37]. Because blood cell counts in the LPS + VGX group did not change accordingly, a marginalisation of splenocytes in the peripheral vasculature might be speculated. A recent study demonstrated a strong leukocyte adherence in the peripheral arterial walls several hours after an LPS challenge [38]. Further studies are warranted to prove this hypothesis.

An increased activity of the vagus nerve under septic conditions was reported in the literature [39] and might best explain the close to congruent findings in the LPS and LPS + VNS groups.

Besides the well-known effects on the cytokine levels, we presented for the first time data on the leukocyte subset numbers in the peripheral blood and spleen. Here, we found vagotomy to decrease numbers of immune cells compared to controls and vagus nerve stimulation. Unfortunately, we did not collect data from the organ vasculature to study the marginalisation of the immune cells. Further studies are warranted to investigate this issue in more detail.

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