

Role of the Cathelicidin-Related Antimicrobial Peptide in Inflammation and Mortality in a Mouse Model of Bacterial Meningitis

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Key Words

Antimicrobial peptides · Bacterial meningitis · Cathelicidin · CRAMP · Glial cells · Innate immunity · *Streptococcus pneumoniae*

Abstract

Antimicrobial peptides (AP) are important components of the innate immune system, yet little is known about their expression and function in the brain. Our previous work revealed upregulated gene expression of cathelicidin-related AP (CRAMP) following bacterial meningitis in primary rat glial cells as well as bactericidal activity against frequent meningitis-causing bacteria. However, the effect of cathelicidin expression on the progression of inflammation and mortality in bacterial meningitis remains unknown. Therefore, we used CRAMP-deficient mice to investigate the effect of CRAMP on bacterial growth, inflammatory responses and mortality in meningitis. Meningitis was induced by intracerebral injection of type 3 *Streptococcus pneumoniae*. The degree of inflammation was analyzed in various brain regions by means of immunohistochemistry and real-time RT-PCR. CRAMP deficiency led to a higher mortality rate that was associated with increased bacterial titers in the cerebellum, blood and spleen as well as decreased meningeal neutrophil

infiltration. CRAMP-deficient mice displayed a higher degree of glial cell activation that was accompanied by a more pronounced proinflammatory response. Taken together, this work provides insight into the important role of CRAMP as part of the innate immune defense against pathogens in bacterial CNS infections.

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Introduction

Bacterial meningitis is a neurological emergency that is still associated with a high mortality rate and long-term sequelae. *Streptococcus pneumoniae* is the leading cause of meningitis in adults [1–3]. Despite advances in medical management, childhood vaccination and the development of effective antimicrobial agents, mortality ranges from 16 to 37% [4], and 1 of 3 survivors suffer from neurological sequelae such as epileptic seizures, hearing loss, cognitive impairment and focal neurological deficits [5, 6]. Therefore, advances in its treatment are urgently needed. During the past decades, experimental animal

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models have shown that the outcome of bacterial meningitis is related to the severity of inflammation in the subarachnoid space. This reaction is characterized by an intensive inflammatory response of the meninges and the subarachnoid space which results in a breakdown of the blood-brain barrier. Consequently, large numbers of neutrophils are recruited to the cerebrospinal fluid, which liberate proinflammatory and chemotactic agents, thereby exaggerating and perpetuating the inflammatory reaction. Among the factors released by neutrophils are numerous cytotoxic agents such as oxidants that can cause collateral damage of the brain tissue [7].

Glial cells such as astrocytes and microglia are also involved in pathogen recognition and the activation of innate immune responses. They are the key regulators of innate immune responses within the brain because of their ability to release various factors supporting the immune defense of the host and helping to coordinate the activation of different immune cells [8]. Astrocytes are furthermore required for structural support and the maintenance of the blood-brain barrier. Microglial cells are considered to be CNS-resident macrophages and sensor cells that function as principal innate immune effector cells [9].

The activation of the innate immune system of the CNS leads to the synthesis of antimicrobial peptides (AP) [10], which have distinct anti-infective and proinflammatory properties [11]. Our own previous investigations revealed increased levels of the AP cathelicidin LL-37 and psoriasin in the cerebrospinal fluid of patients suffering from bacterial meningitis [12, 13]. In humans, one gene is known for cathelicidin. The mature peptide LL-37 is cleaved from a precursor peptide [14]. Furthermore, bacterial infection and the stimulation of glial cells by bacterial supernatants and virulence factors resulted in increased expression of the rat cathelicidin-related AP (CRAMP) by astrocytes and microglia as well as meningeal cells *in vivo* and *in vitro* [12, 15]. CRAMP and rat CRAMP are the active peptides from the only known cathelicidin gene present in mice [16] and rats [17], respectively.

This study was designed to determine the role of CRAMP in inflammation and mortality in a mouse model of pneumococcal meningitis. We determined bacterial growth and mortality of infected CRAMP-deficient and wild-type (WT) mice and characterized the inflammatory response by evaluating neutrophil infiltration, glial cell activation and cytokine/chemokine expression by means of immunohistochemistry and real-time RT-PCR. Furthermore, basal gene expression of inflammatory candidates was determined by PCR array analysis in CRAMP-deficient and WT mice.

Materials and Methods

Mouse Model of Experimental Pneumococcal Meningitis [18]

Male CRAMP-WT and CRAMP-deficient (knockout, KO) mice (weight 19–23 g, age 2–3 months; CRAMP-WT mice were backcrossed on the C57BL/6J background for at least 10 generations [19]) were anesthetized with ketamine (100 mg/kg) and xylazine (20 mg/kg), infected by injecting 10^4 colony-forming units (CFU) of an *S. pneumoniae* type 3 strain in the subarachnoid space through the right frontolateral skull. Control animals without infection were injected with 10 μ l of a sterile saline solution. All infected mice developed clinical signs of infection within 24 h. The clinical state and survival time was closely monitored. In a second set of experiments with the same infection regime, male CRAMP-KO and -WT mice were infected and sacrificed 36 h after induction of meningitis without treatment with antibiotics and were either perfused with 4% formalin for immunohistochemical analysis or with 0.9% NaCl solution for RNA isolation. Bacterial titers were evaluated 36 h after infection in tissue homogenates of the cerebellum and spleen and in blood samples by plating 10-fold dilutions on blood agar plates and incubation for 24 h at 37°C with 5% CO₂ (detection limit 10^2 CFU/ml in tissue homogenates and 10^3 CFU/ml in blood samples) [20]. All animal experiments were approved by the Animal Care Committee of the University Hospital of Aachen and by the District Government in Recklinghausen, North Rhine-Westphalia, Germany.

Meningeal Inflammation Score

Meningeal inflammation was estimated by the invasion of granulocytes into the frontal interhemispheric region, the whole hippocampal fissure (both sides), 3 superficial meningeal regions over the convexities and the third ventricle (complete distribution). One high-power field (diameter: 250 μ m) was scored in each region: no granulocytes = 0; <10 granulocytes = 1; 10–50 granulocytes = 2, and >50 granulocytes = 3. The scores of the individual regions were added (range of the score: 0–21) [21].

RNA Isolation and Real-Time RT-PCR and PCR Array

Total RNA was isolated using the peqGold Trifast reagent (Peqlab, Erlangen, Germany) according to the manufacturer's instructions. RNA samples were reverse transcribed by Moloney murine leukemia virus reverse transcriptase (Fermentas, Burlington, Ont., Canada) and random hexamer primers (Invitrogen, Darmstadt, Germany). The cDNA products were used immediately for SYBR green (Applied Biosystems, Darmstadt, Germany) real-time RT-PCR. Gene expression was monitored using the StepOne Plus apparatus (Applied Biosystems) according to manufacturer's protocol [22]. Relative quantification was performed using the Δ Ct method which results in ratios between target genes and a housekeeping reference gene (TATA box binding protein). The primer for glial fibrillary acid protein (GFAP), integrin α M (Itgam) and β -defensin 4 (Defb4) were provided by Qiagen (QT00101143, QT00156471 and QT00257656; QuantiTect Primer Assay; Qiagen, Hilden, Germany). The primers for caspase-1, caspase-4, interleukin (IL)-1 β , IL-6 and tumor necrosis factor- α (TNF- α) were manufactured by Eurofins MWG Operon (Ebersberg, Germany; please refer to table 1 for primer sequences). The specificity of the amplification reaction was determined by melting curve analysis. For PCR array analysis, we used the RT2 Profiler PCR Array Mouse Innate and Adaptive Immune Response (PAMM-052A; Qiagen) according to manufacturer's protocol.

Table 1. Primer sequences for real-time RT-PCR gene analysis

Primer		Sequence	Annealing temperature, °C
mTBP	forward	5'-AGAACAATCCAGACTAGCAGCA-3'	58
	reverse	5'-GGGAAC TTCACATCACAGCTC-3'	
mCaspase-1	forward	5'-GCTTCAATCAGCTCCATCAGC-3'	57
	reverse	5'-CAGTCAGTCCTGGAAATGTGCC-3'	
mCaspase-4	forward	5'-TAGACTCATTTCTGCTTCCGG-3'	57
	reverse	5'-ATATCTCGTCAAGGTTGCCCG-3'	
mIL-1 β	forward	5'-TCTGGGATCCTCTCCAGCCAAGC-3'	58
	reverse	5'-AAGCAGCCCTTCATCTTTTGGGGT-3'	
mIL-6	forward	5'-TGCAAGAGACTTCCATCCAGTTGCC-3'	59
	reverse	5'-AAGCCTCCGACTTGTGAAGTGGT-3'	
mTNF- α	forward	5'-AGGGGCCACCACGCTCTTCT-3'	59.5
	reverse	5'-TGTTTTGCTACGACGTGGGCT-3'	

Immunohistochemistry

Formalin-fixed and paraffin-embedded 5- μ m whole coronary brain sections were examined. For immunofluorescence staining, sections were deparaffinized, pretreated for 3 \times 7 min with microwaves in citric acid buffer, permeabilized with 0.1% Triton X in PBS for 10 min at room temperature and after blocking with 1.5% BSA in Tris incubated with either monoclonal mouse anti-GFAP (1:250; ab10062; Abcam, Cambridge, UK) or polyclonal goat anti-ionized calcium binding adaptor molecule 1 (Iba1; 1:100; ab5076; Abcam) overnight at 4°C. This was followed by incubation with the biotinylated secondary antibody (1:400; DAKO, Hamburg, Germany) and peroxidase-labeled streptavidin-biotin staining. For staining, aminoethyl carbazole was used. After counterstaining with hematoxylin, the slides were finally mounted with Aquatex (Boehringer, Mannheim, Germany). For neutrophil granulocyte staining, slices were stained with naphthol AS-D chloroacetate esterase (91-C Kit, Sigma-Aldrich, Taufkirchen, Germany) according to the manufacturer's protocol. As a negative control, primary antibodies were omitted.

Quantification of Immunoreactive Cells

Sections were examined blinded using objectives from $\times 2$ up to $\times 60$. Only immunoreactive cells within the granule cell layer and subgranular zone of the dentate gyrus were counted. An analysis software imaging system (microscope BX51; Olympus; software AnalySIS 3.2; Soft Imaging System GmbH, Münster, Germany) was used to measure the area of the dentate granule cell layer. The densities of immunolabeled cells were expressed as the number of marked cells per square millimeter of the area measured. The density of labeled cells was evaluated in 3 coronal sections from each mouse. Immunohistochemical data were expressed as medians and interquartile ranges. For statistical comparisons, the nonparametric Mann-Whitney U test was used.

Statistical Analysis

Survival time was expressed in hours and evaluated by generating a Kaplan-Meier plot that was statistically analyzed using the log-rank test. Bacterial titers were converted into log CFU/ml and compared by nonparametric U test. All real-time RT-PCR experiments were performed in duplicate. The values were expressed as means \pm SEM. For statistical comparisons, ANOVA followed by a Bonferroni multiple comparison test was used. A value of $p < 0.05$ was considered statistically significant. For statistical calculation, GraphPad Prism 5.0 was used (GraphPad Software, San Diego, Calif., USA).

Results

CRAMP Deficiency Resulted in Increased Bacterial Burden, Decreased Neutrophil Infiltration and Increased Mortality in Pneumococcal Meningitis

In a first set of experiments, mortality in pneumococcal meningitis was investigated. The survival time after infection was significantly shorter in CRAMP-deficient (KO) compared to WT mice (47.1 \pm 8.8 vs. 68.6 \pm 10.3 h, $p = 0.0001$; Mann-Whitney U test; fig. 1a). Fifty hours after infection, 94.1% of the WT animals were still alive compared to 33.3% of CRAMP-KO mice (fig. 1b). In addition, we determined bacterial titers in homogenates of the cerebellum and spleen and in blood samples from a separate set of CRAMP-KO and -WT mice 36 h after infection (no treatment with antibiotics). The results showed that CRAMP-KO animals had a statistically significantly higher bacterial burden in all investigated tis-

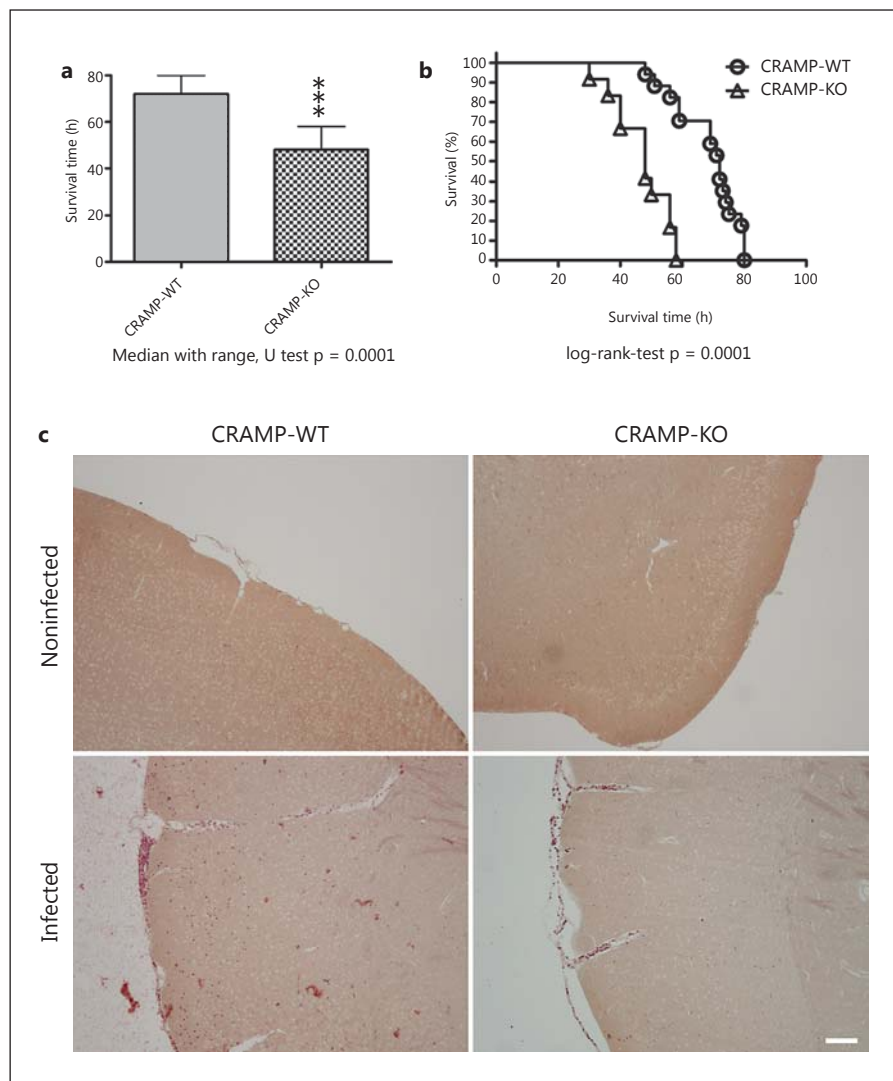


Fig. 1. Mortality in pneumococcal meningitis. CRAMP-WT and CRAMP-KO mice were infected by injection of 10^4 CFU of type 3 *S. pneumoniae* in the subarachnoid space. **a** Comparison of survival times revealed that CRAMP-WT mice lived significantly longer (CRAMP-WT, $n = 17$; CRAMP-KO, $n = 13$; medians and ranges; *** $p = 0.0001$; Mann-Whitney U test). **b** Mortality was significantly increased in CRAMP-KO animals (Kaplan-Meier curve; $p = 0.0001$, log-rank test). **c** Detection of neutrophil granulocytes by the naphthol AS-D chloroacetate esterase reaction in the meninges and parenchyma revealing neutrophil infiltration in CRAMP-WT and -KO mice 48 h after pneumococcal infection. The figures show representative results from 1 of 3 mice per group (scale bar = 100 μm).

Table 2. Bacterial titers after pneumococcal meningitis

	Bacterial titer, log CFU/ml		
	cerebellum	blood	spleen
CRAMP-WT	5.7 (5.1/6.2)	4.28 (3.8/5.39)	4.92 (2.9/5.2)
CRAMP-KO	6.54 (6/7.5)*	6 (5.1/7)*	5.5 (5.1/6)*

Data are presented as medians (25th/75th percentiles). Please note significantly increased bacterial titers in infected CRAMP-KO compared to infected CRAMP-WT mice. $n = 8$ /group; * $p = 0.0117$ (cerebellum), 0.0113 (blood) and 0.0384 (spleen); Mann-Whitney U test.

sue compartments compared to WT mice (table 2). Furthermore, neutrophil granulocyte invasion was histochemically evaluated by chloroacetate esterase staining. Forty-eight hours after infection, WT mice displayed a higher degree of neutrophil granulocyte infiltration than CRAMP-deficient mice (fig. 1c). To verify the results, meningeal inflammation was determined by a semiquantitative score: a strong granulocytic invasion of the meninges was observed in all infected mice 48 h after inoculation, whereas infiltration of granulocytes was absent in noninfected animals. For CRAMP-WT mice, the meningeal inflammation score was 14 ± 1.6 ($n = 6$), whereas for CRAMP-KO mice the score was 9 ± 2.5 ($n = 6$). The difference between infected CRAMP-KO and -WT mice was significant ($p = 0.0357$, Mann-Whitney U test).

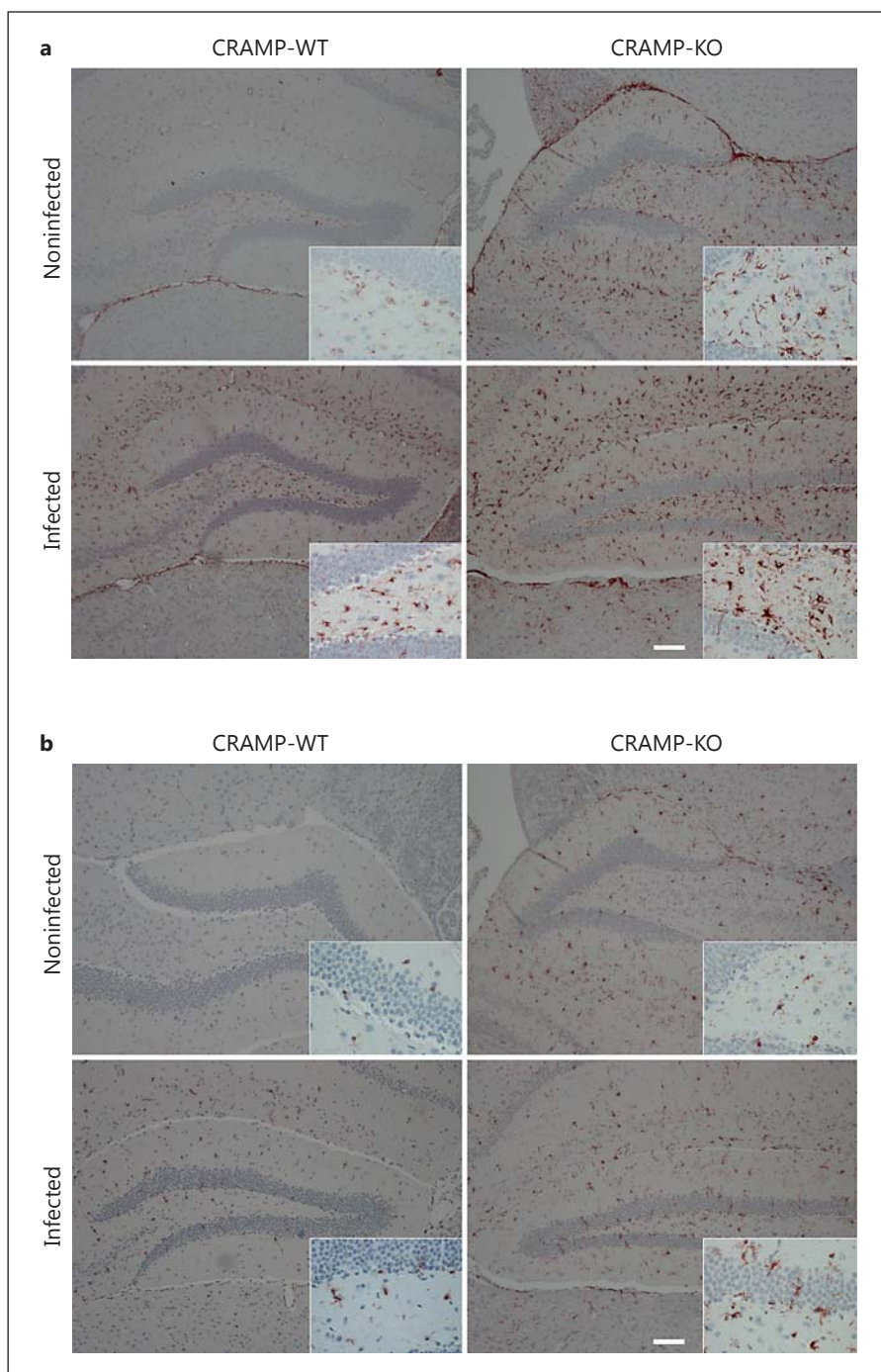


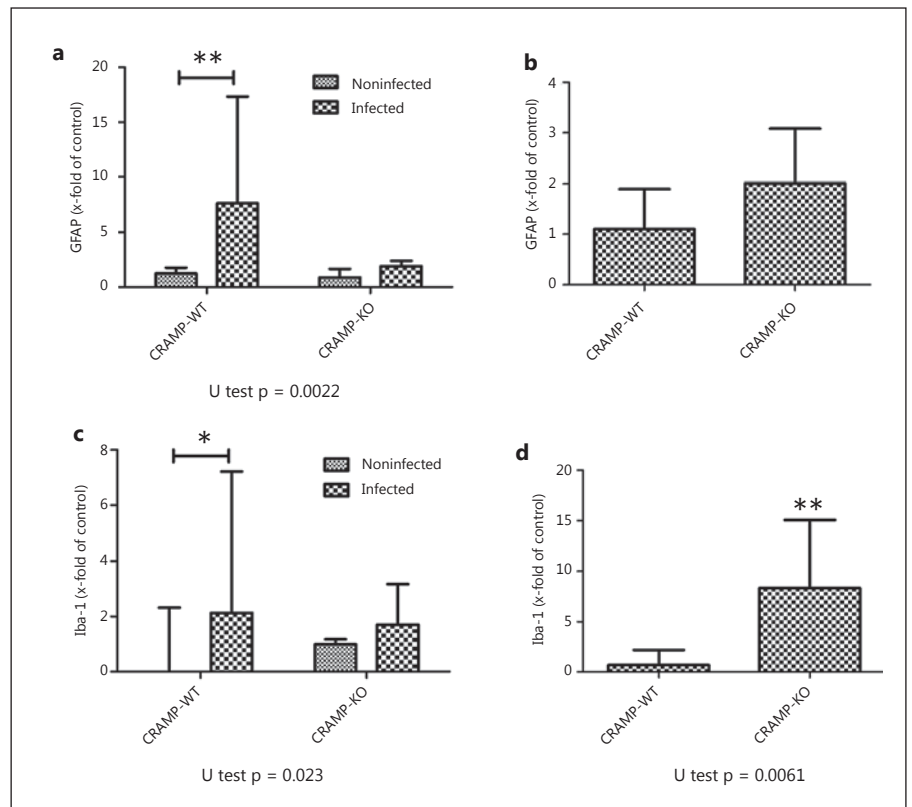
Fig. 2. Glial cell activation in the hippocampus after pneumococcal meningitis. Coronal brain sections stained with anti-GFAP to discover astrocytes (**a**) or anti-Iba-1 to identify activated microglial cells (**b**) 36 h after infection with *S. pneumoniae*. The figures show representative results from 1 of 3 independent experiments (scale bar = 100 μ m). A detailed section was included. Please note that noninfected CRAMP-KO mice showed a stronger endogenous GFAP and Iba-1 immunoreactivity compared to noninfected WT mice.

Increased Glial Cell Activation following Pneumococcal Meningitis in CRAMP-Deficient Mice

Thirty-six hours after infection, the degree of glial cell activation was determined by staining with GFAP, a marker of astrocytes, and Iba-1, a marker for activated microglia, in the hippocampal formation as a clearly definable

brain region especially involved in the pathogenesis of bacterial meningitis. As shown in figure 2, the hippocampal formation of noninfected WT mice showed only very few GFAP- or Iba-1-positive cells, whereas infection with *S. pneumoniae* resulted – as expected – in a strong increase in the number of GFAP- or Iba-1-immunoreactive cells.

Fig. 3. Quantification of glial cells in the dentate gyrus of the hippocampus after pneumococcal meningitis. GFAP- and Iba-1-immunoreactive cells were quantified per mm² area of the subgranular and granule cell layer of the dentate gyrus (see results) of infected and healthy CRAMP-KO and -WT mice 36 h after induction of meningitis. Immunoreactive cells were normalized to noninfected controls and fold increases are presented in the graph (a, c). Data from 4 mice/group are presented as medians with ranges/mm². Density of GFAP (b)- and Iba-1 (d)-immunoreactive cells within the subgranular and granule cell layer of the dentate gyrus of infected CRAMP-KO mice were normalized to infected -WT mice. Previously, basal expression of astrocytes or microglial cells of the uninfected mice was subtracted. Comparison revealed significantly more activated microglia in CRAMP-KO mice in pneumococcal meningitis (* p < 0.05, ** p = 0.0061, infected vs. noninfected mice, Mann-Whitney U test).



Interestingly, healthy CRAMP-KO mice already displayed a high basal rate of GFAP-positive cells in the hippocampal formation in comparison to CRAMP-WT animals. Quantification of astrocytes in the subgranular zone and the granule cell layer of the dentate gyrus revealed significantly more astrocytes (n/mm²) in CRAMP-WT mice exposed to *S. pneumoniae* than in NaCl-treated WT mice [noninfected CRAMP-WT 1.24 (0.13/1.75)/mm² vs. infected WT 7.65 (6.88/11.81)/mm²; medians (25th/75th percentiles), Mann-Whitney U test, p = 0.0022; fig. 3a]. Induction of bacterial infection in the CRAMP-KO mice did not result in a significant increase in GFAP-immunoreactive cells in comparison to noninfected CRAMP-KO [noninfected CRAMP-KO 0.87 (0.63/1.5)/mm² vs. infected KO 1.9 (1.38/2.29)/mm²; medians (25th/75th percentiles), Mann-Whitney U test, p = 0.067; fig. 3a]. Next, the density of GFAP-immunoreactive cells of the dentate gyrus of infected CRAMP-KO mice was normalized to infected WT mice. Previously, basal expression of astrocytes of uninfected mice was subtracted. The results showed no significant difference between infected WT and infected CRAMP-KO mice [2.01 (0.84/2.88)-fold increase; median (25th/75th percentile), p = 0.3810, Mann-Whitney U test; fig. 3b].

Similarly to astrocytes, the noninfected CRAMP-KO mice had already a higher basal rate of microglial cell activation within the hippocampal formation in comparison to WT mice (fig. 3a). As expected, the number of microglial cells strongly increased in WT mice in the hippocampal formation 36 h after infection [noninfected CRAMP-WT 0.0 (0.0/2)/mm² vs. infected WT 2.13 (1.85/4.09)/mm²; median (25th/75th percentile), Mann-Whitney U test, p = 0.023; fig. 3c]. The increase following infection with *S. pneumoniae* was not significant [noninfected CRAMP-KO 0.99 (0.85/1.16)/mm² vs. infected CRAMP-KO 1.71 (0.94/1.53)/mm²; medians (25th/75th percentiles), p = 0.1143, Mann-Whitney U test; fig. 3c]. However, comparing only the infected CRAMP-KO mice normalized to infected WT mice (basal expression of microglial cells of the uninfected mice was subtracted), the density of Iba-1-immunoreactive cells was significantly increased in the CRAMP-deficient mice [8.34 (3.56/13.56)-fold increase in infected CRAMP-KO mice; median (25th/75th percentile), p = 0.0061; fig. 3d].

We analyzed mRNA expression of GFAP (as a marker for activated astrocytes) as well as *Irgam* (or CD11b [23], as a marker of microglial cell activation) in the cerebel-

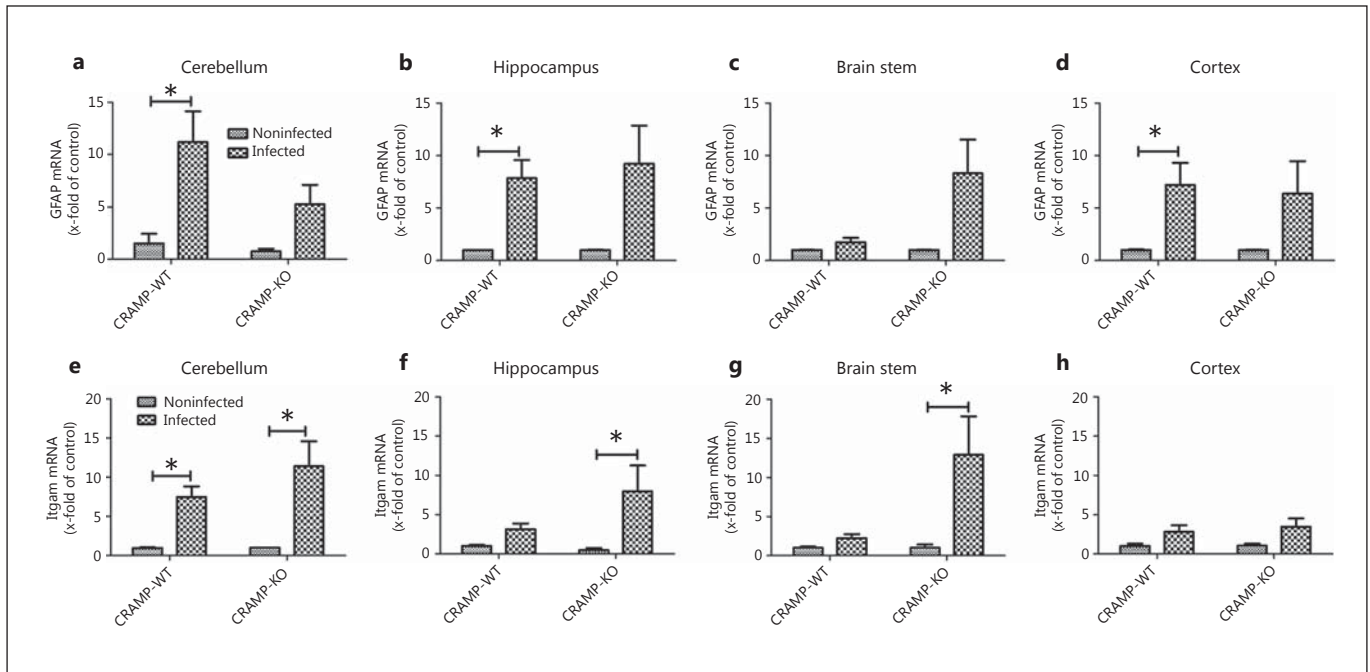


Fig. 4. Gene expression of glial cell markers after pneumococcal meningitis. Analysis of mRNA expression of the astrocyte marker GFAP (**a–d**) and the activated microglial marker Itgam (**e–h**) in the cerebellum, hippocampal formation, brain stem and frontal cortex of CRAMP-KO and -WT mice 36 h after induction of pneu-

mococcal meningitis by real-time RT-PCR. Data were assessed in 6 independent experiments in duplicate. * $p < 0.05$ infected CRAMP-WT vs. CRAMP-KO mice (ANOVA followed by a Bonferroni test).

lum, hippocampal formation, brain stem and frontal cortex of CRAMP-KO and -WT mice 36 h after infection with *S. pneumoniae*. As shown in figure 4a–d, WT mice showed a significant increase in GFAP mRNA expression in the cerebellum, hippocampal formation and frontal cortex (11.2 ± 3 , 7.8 ± 1.7 and 7.2 ± 2.1 times, respectively; all $p < 0.05$; fig. 4a, b, d), whereas no significant increase in GFAP mRNA expression was detected in any region of CRAMP-KO mice. In WT mice, Itgam mRNA expression was only significantly increased in the cerebellum (7.5 ± 1.4 times, $p < 0.05$; fig. 4e), whereas CRAMP-deficient mice showed a significant increase in activated microglial cell gene expression in the cerebellum, hippocampus and brain stem (11.4 ± 3.1 , 8 ± 2.9 and 12.9 ± 4.9 times, respectively; $p < 0.05$; fig. 4e–g).

Comparison of General Immune Response after Bacterial Meningitis in the Hippocampal Formation of CRAMP-KO and -WT Mice by PCR Array and Real-Time RT-PCR

In a next set of experiments, we analyzed mRNA expression of genes generally involved in innate and adap-

tive immune responses in the hippocampal formation of CRAMP-KO and -WT mice 36 h after infection with *S. pneumoniae*. As shown in online supplementary figure 1A (for all online supplementary material, see www.karger.com/doi/10.1159/000353645), 24 genes showed increased expression while 4 genes were downregulated in infected WT mice compared to noninfected WT mice. In 59 genes, the expression rate did not change. Table 3 shows the genes with a change in the expression (increase or decrease). However, the differences between infected and noninfected WT animals were not significant ($n = 3$). In CRAMP-deficient mice, infection resulted in an increased expression of 33 genes, whereas the expression of only 1 gene was decreased. In 14 genes, mRNA expression was significantly increased compared to noninfected mice. Altogether, the results showed a stronger immune response in CRAMP-KO mice than -WT mice after pneumococcal infection.

To verify the results of the PCR array, we evaluated gene expression of candidates both in the hippocampal formation as well as in the cerebellum, brain stem and frontal cortex from CRAMP-deficient and -WT mice.

Table 3. Results of PCR array mouse innate and adaptive immune responses

Gene	x-fold of control	
	CRAMP-WT	CRAMP-KO
Adora2a (adenosine A2A receptor)	5.5895	4.4838
Casp1 (caspase-1)	4.3058	4.314*
Casp 4 (caspase-4)	22.9138	38.8959*
Ccl2 (CCL2)	106.4495	2,875.7481
Ccr3 (C-C chemokine receptor type 3)	–	4.0441
Cd14	34.4855	29.3627
Clec7a (C-type lectin domain family 7 member A/dectin)	–	5.7234*
Cybb (cytochrome b-245)	14.0473	29.3954*
Defb4	–	4.1689
Dmbt1 (deleted in malignant brain tumors 1)	–	4.0212
Hmox1 [heme oxygenase (decycling) 1]	5.4103	5.4396
Ifnb1 (interferon- β 1)	9.1583	4.0817
Il10 (IL-10)	4.4772	4.7127
Il1a (IL-1 α)	11.614	14.019
Il1b (IL-1 β)	52.6794	66.8595
Il1f9 (IL-1 family member 9)	24.1392	25.3921
Il1r2 (IL-1 receptor, type II)	8.5841	6.4109
Il1rn (IL-1 receptor antagonist)	6.6392	9.6017
Il6 (IL-6)	55.0987	48.4762
Irf1 (interferon regulatory factor 1)	6.3225	12.0827*
Lyz1 (lysozyme 1)	5.4183	13.1175
Myd88 [myeloid differentiation primary response gene (88)]	–	6.5557*
Ncf4 (neutrophil cytosolic factor 4)	5.3611	9.4759*
Nfkbia [nuclear factor of κ light polypeptide gene enhancer in B-cells inhibitor, alpha (http://www.genenames.org/data/hgnc_data.php?hgnc_id=7797)]	4.1035	–
Nlrc4 (NLR family CARD domain-containing protein 4)	–	5.5342*
Pglyrp1 (peptidoglycan recognition protein 1)	–	4.5003*
Pglyrp3 (peptidoglycan recognition protein 3)	4.4648	–
Ptafr (platelet-activating factor receptor gene)	–	11.5002*
Serpine1 (plasminogen activator inhibitor-1)	18.6022	23.5001*
Tlr1 (TLR1)	–	5.0086*
Tlr2 (TLR2)	7.9732	26.2452
Tlr8 (TLR8)	–	6.2569*
Tlr9 (TLR9)	–	4.1669
Tnf (TNF- α)	25.0953	82.921
Trem1 (triggering receptor expressed on myeloid cells 1)	7.759	8.4433
Fn1 (fibronectin 1)	–18.7371	–
Ifngr2 (interferon- γ receptor 2)	–33.3823	–
Nos2 (nitric oxide synthase 2)	–13.9407	–
Serpina1a [serine (or cysteine) peptidase inhibitor member 1A]	–	–4.5967

Expression levels of different genes in the hippocampal formation of CRAMP-KO and -WT mice. Only genes with increased or decreased expression rates are listed. – = No change in expression. Each group represents the analysis of 3 mice. Data are presented as means. * $p < 0.05$ vs. noninfected controls (t test).

First we examined gene expression of caspase-1, which proteolytically cleaves other precursors of inflammatory cytokines IL-1 β and IL-18 into active mature peptides [24]. As shown in figure 5a–d, expression analysis revealed only a low but significant increase in infected WT

mice (maximum in the cerebellum; 4.4 ± 0.7 induction compared to noninfected CRAMP-WT; $p < 0.05$) whereas the increase in the expression in three of four brain regions of the CRAMP-deficient mice was statistically significant with a maximum increase in the frontal cortex

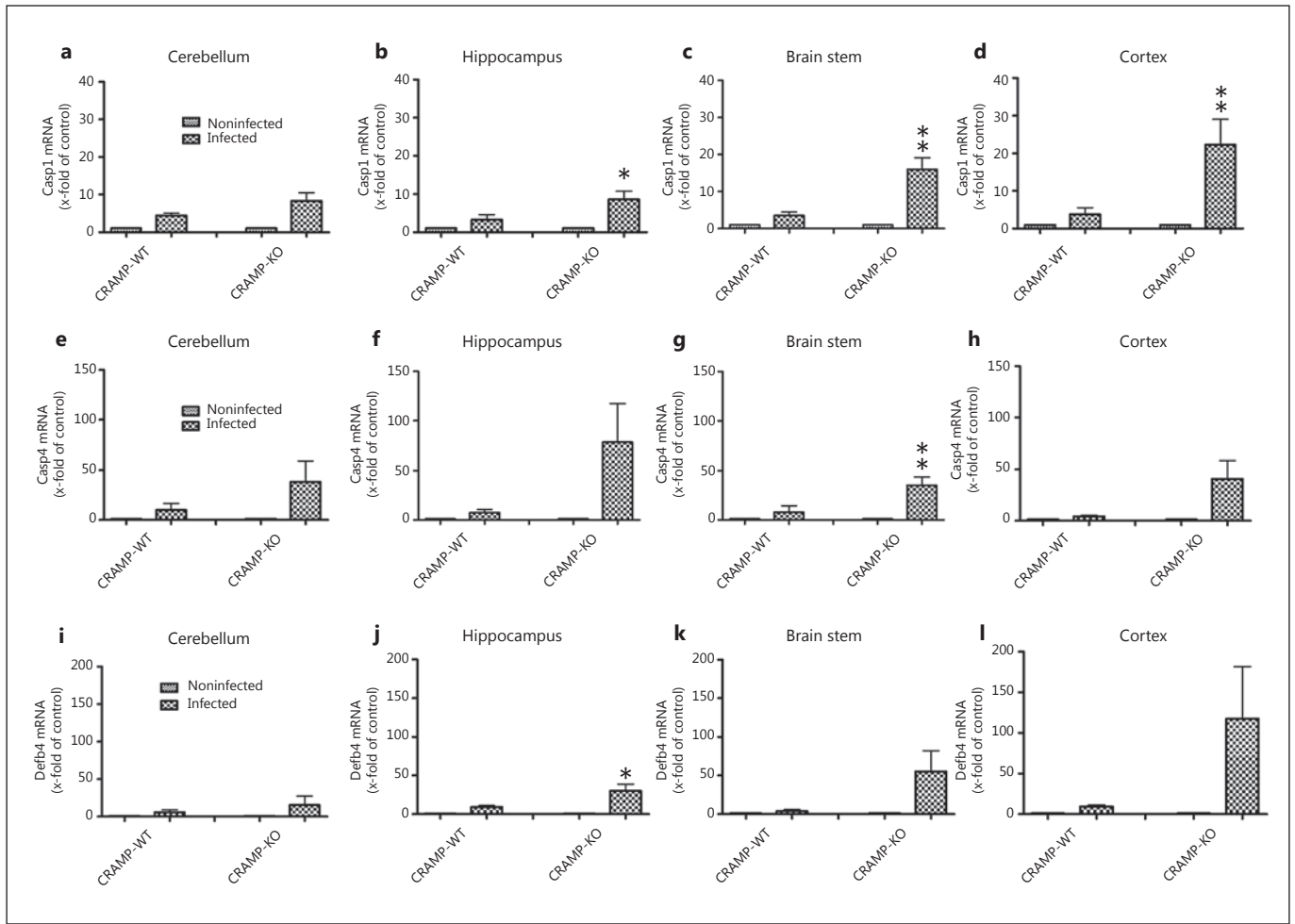


Fig. 5. Expression of different inflammation markers after pneumococcal meningitis in different brain regions. 36 h after subarachnoid infection with *S. pneumoniae*, mRNA expression levels of caspase-1 (a–d), caspase-4 (e–h) and Defb4 (i–l) were determined in the cerebellum, hippocampal formation, brain stem and cortex of CRAMP-KO and -WT mice by real-time RT-PCR. Please

note the stronger increase in expression in infected CRAMP-KO compared to infected-WT mice. Data were assessed in 6 independent experiments in duplicate. * $p < 0.05$, ** $p < 0.01$, infected CRAMP-WT vs. infected CRAMP-KO mice (ANOVA followed by a Bonferroni test). Casp1/4 = Caspase-1/4.

(22.4 ± 6.6 -fold induction compared to noninfected CRAMP-KO mice, $p < 0.05$ between infected CRAMP-WT and -KO mice). Similarly, gene expression of the cysteine protease caspase-4 was only slightly increased by infection in all four brain regions in WT mice (fig. 5e–h; maximum in the cerebellum: 9.8 ± 5.1 increase in induction; nonsignificant compared to noninfected CRAMP-WT mice), whereas in CRAMP-KO mice the expression increase was more pronounced with the gene expression rate reaching statistical significance within the brain stem (35.3 ± 8.2 -fold increase compared to non-infected CRAMP-KO mice, $p < 0.05$ CRAMP-WT vs. -KO mice;

fig. 5g). Furthermore, table 3 shows that in the hippocampal formation of CRAMP-deficient mice Defb4 expression was increased. Defb4 is the mouse homologue of the human Defb2 [25]. To verify the hypothesis that Defb4 compensates the lack of CRAMP, we compared Defb4 expression in the different brain regions of CRAMP-deficient and -WT mice. While in WT mice Defb4 expression was only slightly increased in the hippocampal formation and frontal cortex with maximum expression in the cortex (9.5 ± 2 -fold increase vs. noninfected WT mice; $p < 0.05$; fig. 5l), CRAMP-deficient mice displayed a strong increase in Defb4 expression in all pathological

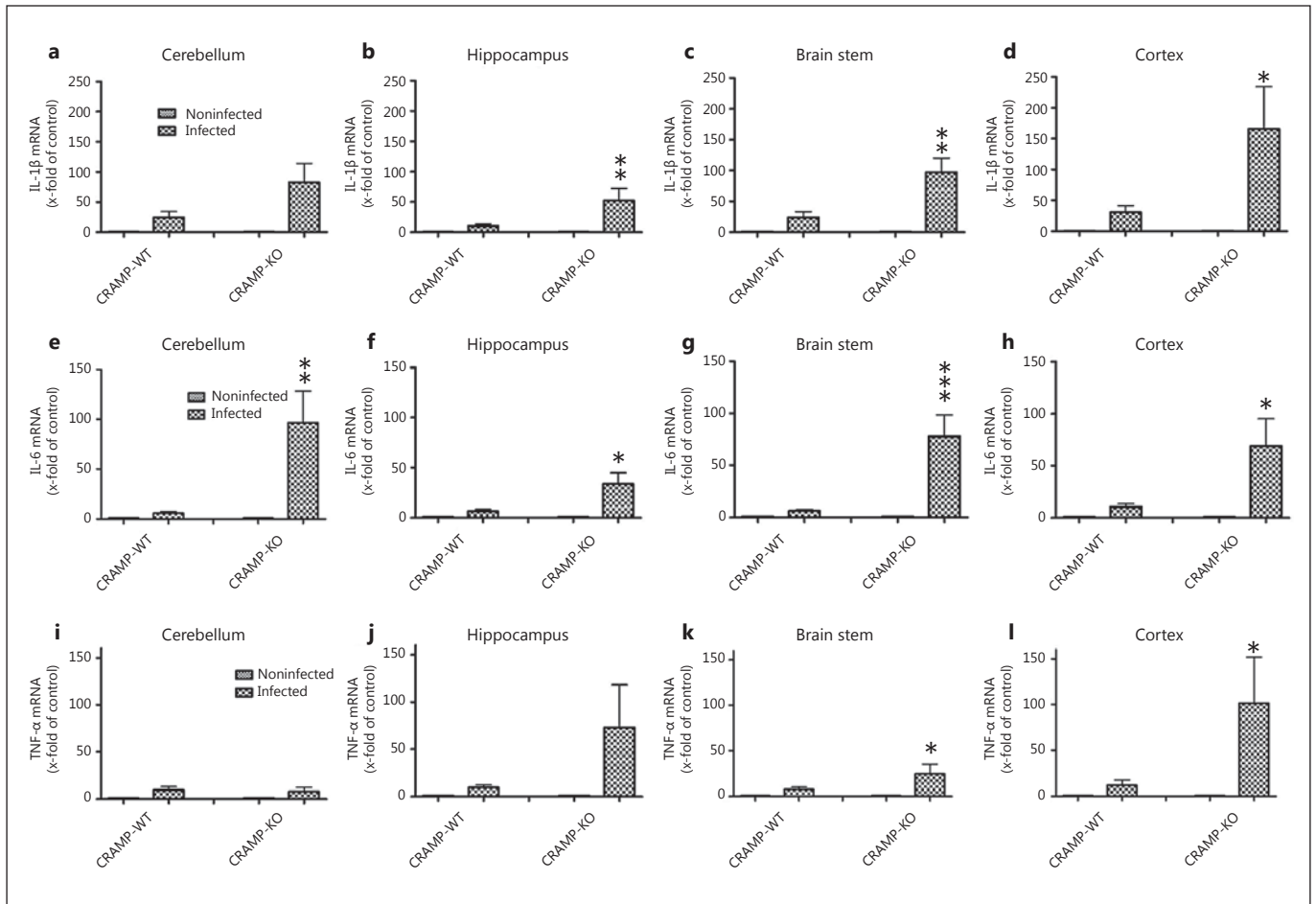


Fig. 6. Expression of proinflammatory cytokines after pneumococcal meningitis in different brain regions. 36 h after subarachnoid infection with *S. pneumoniae*, mRNA expression levels of IL-1 β (a–d), IL-6 (e–h) and TNF- α (i–l) were determined in the cerebellum, hippocampal formation, brain stem and cortex of CRAMP-KO and -WT mice by real-time RT-PCR. Please note the stronger

increase in expression in infected CRAMP-KO compared to infected WT mice. Data were assessed in 6 independent experiments in duplicate. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, infected CRAMP-WT vs. infected CRAMP-KO mice (ANOVA followed by a Bonferroni test).

brain regions studied. The increase in the hippocampal formation was significant compared to infected WT mice (30.3 ± 8.5 -fold increase vs. noninfected CRAMP-deficient mice, $p < 0.05$ CRAMP-WT vs. -KO mice).

Next, we compared the expression of the proinflammatory cytokines IL-1 β , IL-6 and TNF- α , which play an important role during the course of pneumococcal meningitis. The infection resulted in a strong increase in IL-1 β mRNA expression in both strains with highest increases in the cerebellum and cortex of CRAMP-WT mice (24.2 ± 10 - and 30.2 ± 11 -fold induction of expression vs. noninfected WT mice, respectively; $p < 0.05$; fig. 6a–d), whereas the expression within the hippocampus, brain

stem and cortex was significantly higher in CRAMP-deficient mice compared with infected WT mice (52.3 ± 19 -, 97.1 ± 23 - and 166 ± 68 -fold increase in expression, $p < 0.05$ or $p < 0.01$ between infected CRAMP-WT and -KO mice). The expression of both IL-6 and TNF- α were increased in both strains but generally more pronounced in CRAMP-deficient mice. The maximum increase in both IL-6 and TNF- α mRNA expression in the WT mice was detected in the cortex (10.4 ± 2.2 -fold induction in expression vs. noninfected WT mice; $p < 0.01$; fig. 6h; 12.1 ± 4.2 -fold induction in expression compared to noninfected WT mice; $p < 0.05$; fig. 6l). In infected CRAMP-deficient mice, the maximum increase in ex-

pression was reached for IL-6 in the cerebellum (96.5 ± 31.7 -fold increase in expression vs. noninfected CRAMP-KO mice, $p < 0.01$ between infected CRAMP-WT and -KO mice; fig. 6e) and for TNF- α in the cortex (101.7 ± 50.4 -fold increase in expression compared to noninfected CRAMP-KO mice, $p < 0.05$ between infected CRAMP-WT and -KO mice; fig. 6f).

Discussion

In the present study, the consequences of the lack of CRAMP were investigated in a very well-established and -characterized mouse model of pneumococcal meningitis [18]. CRAMP deficiency resulted in a higher mortality rate that was associated with increased bacterial burden and decreased neutrophil granulocyte infiltration in the CNS as well as in distinct changes in inflammatory immune reactions. Our results suggest that CRAMP is an important component of innate immune responses in the defense of the CNS against *S. pneumoniae* invasion and that the lack of this protein results in an unfavorable outcome. These results are supported by other studies using infection models in CRAMP-deficient mice. Chromek et al. [26] showed that cathelicidin protects the urinary tract of children against invasive bacterial infection by *Escherichia coli* and Huang et al. [27] reported an increased susceptibility to *Pseudomonas aeruginosa* keratitis in CRAMP-deficient mice. Reduced bacterial clearance and delayed neutrophil influx were observed in CRAMP-KO mice infected with *P. aeruginosa* [28]. Mice lacking CRAMP were more susceptible to meningococcal infection and exhibited increased bacterial growth in the blood, liver, and spleen [29]. In a previous work, we emphasized the importance of AP in CNS infections by revealing increased synthesis of the human cathelicidin LL-37 and S100 protein psoriasin in the cerebrospinal fluid of patients suffering from bacterial meningitis [12, 13]. In addition, our in vitro studies revealed an increase in rat CRAMP in glial and meningeal cells after exposure to bacterial components [12, 15]. Other work showed a direct antimicrobial activity of cathelicidins against *S. pneumoniae* and *Neisseria meningitidis* as the most important meningitis pathogens [29, 30]. With respect to insertion into membrane bilayers, the cathelicidins form pores in the bacterial membrane which resulted in increased permeability with subsequent rapid cell death. A second mechanism for bacterial killing is the binding of intracellular targets to influence bacterial proteins (e.g. the activation of autolytic enzymes, inhibition of cell wall biosyn-

thesis and synthesis of DNA, RNA and protein). Another important feature of these peptides is their capacity to bind and neutralize bacterial lipopolysaccharides in vitro, which may account for the ability of exogenously administered peptides to protect against sepsis in vivo [31]. Taken together, AP represent an important part of the first-line defense against invading bacteria in the CNS such as the important pathogen *S. pneumoniae*. In addition to its antimicrobial activity, CRAMP also acts as a potent chemoattractant for myeloid cells engaging FPR2 which may be a contributing factor to reduced neutrophil infiltration in CRAMP-deficient mice observed in this study [32, 33]. Furthermore, CRAMP enhanced neutrophil host defense via an increase in IL-8 expression under the control of MAPK p38 and extracellular signal-regulated kinase, and stimulated the generation of reactive oxygen species time and dose dependently. Furthermore, CRAMP exhibits its immunoregulatory properties by stimulating IL-6 cytokine production and glial cell activation and its potential neuroprotective properties via the induction of neurotrophic factors [34, 35].

In general, the meningitis-induced systemic inflammatory reaction is dependent on the release of both bacterial toxins and its interaction with host pattern recognition receptors as well as host-derived cytotoxic agents. This physiologic process can be harmful because it results in the recruitment of neutrophils that are considered an important part of the host-derived detrimental effects by releasing cytotoxic agents that may lead to direct brain tissue damage [7]. However, our results showed decreased neutrophil infiltration in infected KO mice associated with increased bacterial titers and an increased inflammatory reaction.

Several studies have demonstrated the importance of glial cells in the development and regulation of inflammatory reactions following the infection of the CNS or in neurodegenerative diseases [36, 37]. The brain is protected from penetrating pathogens by the blood-brain barrier as well as by glial cells such as astrocytes and microglia. Astrocytes belong to the well-characterized innate immune neuroglia, and in addition to many other roles, their main function is the synthesis and regulation of inflammatory cytokines such as IL-1 β , IL-6 and different chemokines [38] as well as the synthesis of anti-inflammatory cytokines [39]. The mediatory functions of astrocytes are compromised during inflammatory reactions and thereby potentially worsening outcome [36]. Microglial cells are the macrophages of the brain. Microglial cells are being activated after the invasion of pathogens into the brain and similar to astrocytes release various media-

tors such as cytokines and chemokines to activate other immune cells. They can also function as antigen-presenting cells in the brain for invading immune cells such as T lymphocytes [9]. In bacterial meningitis, increased activation of astrocytes and microglia may aggravate neuronal cell death in the hippocampal formation by both induction of apoptosis and necrosis [40]. However, our findings revealed a strong increase in the astrocyte marker GFAP in infected WT mice, whereas the CRAMP-deficient animals displayed an already increased basal rate of astrocyte activation with only small further increase under pathological conditions. Comparison of infected CRAMP-deficient and -WT mice revealed that the density of GFAP-immunoreactive cells was about the same in both groups. This shows that CRAMP deficiency results per se in a higher activation state of astrocytes whose extent is comparable to that of 'physiological inflammation' after bacterial invasion. Similar results were obtained for microglial activation in healthy animals: CRAMP deficiency alone resulted in a higher degree of Iba-1 immunoreactivity in comparison to noninfected controls. In contrast to the observations of astrocytes, the density of Iba-1-positive cells/Itgam gene expression of infected CRAMP-deficient mice was significantly higher than in WT mice suggesting enhanced microglial activation by CRAMP deficiency after pneumococcal meningitis. The observation of enhanced endogenous glial cell activation in CRAMP-deficient mice in comparison to WT mice was not published in other studies yet and therefore needs further investigation.

The hippocampal formation is one of the most vulnerable brain regions in general and in bacterial meningitis in particular. Neuronal damage within the dentate gyrus is due to both bacteremia and inflammatory reactions and closely related to long-term sequelae, such as memory impairment [41, 42]. Therefore, we compared the general immune response after pneumococcal meningitis between CRAMP-deficient and -WT mice. Using a PCR array for markers of innate and adaptive immune responses, we found increased expression of the pattern recognition receptors Toll-like receptors (TLR) 1, 2, 8 and 9 in the hippocampal formation of infected CRAMP-deficient mice. TLR play a major role in triggering the immune response. They are able to detect a variety of pathogens and initiate inflammatory immune responses [9]. Interestingly, TLR1 and TLR2 are involved in the detection of components of Gram-positive bacteria such as *S. pneumoniae* while TLR8 and TLR9 are responsible for the detection of ssRNA and dsDNA from various pathogens [43]. Previous findings suggest an involvement of TLR9

in innate immune responses after pneumococcal meningitis by influencing CRAMP expression in mice [44]. In addition, an important signaling protein of the TLR pathways, the adapter protein MyD88, was increased only in the infected CRAMP-deficient mice. The increase in TLR receptor expression was accompanied by an increase in proinflammatory cytokine expression. Recent studies underlined the importance of TNF- α , for example, for inflammatory processes [45], but also for neuronal apoptosis in the hippocampus [46]. The higher expression rate of TNF- α in infected CRAMP-deficient mice may be regarded as the expression of a stronger immune reaction in those animals and may have influenced or even caused the worse outcome in the form of higher mortality. The generally stronger inflammatory response was also accompanied by increased expression of caspase-1 and -4. As a third class of caspases, they are involved in inflammation [47]. After the recognition of pathogens, caspase-1 molecules group together to form an oligomeric inflammasome [48]. The activated caspase-1 is now able to process proIL-1 β and is thereby involved in immune responses [24]. Our findings showed a strong increase of IL-1 β expression in infected CRAMP-deficient compared to the -WT mice associated with increased caspase-1 expression. Furthermore, caspase-4 – as well as caspase-1 – has immunomodulatory effects. It is believed that caspase-4 is required to splice caspase-1 and thus to activate it [49]. Taken together, all these changes in the inflammatory reaction caused by CRAMP deficiency accompanied the unfavorable outcome. However, it cannot be concluded from these data whether all these changes in the inflammatory cascade are only caused by the lack of CRAMP alone or whether the higher bacterial burden may also in part be responsible for these changes.

Interestingly, the PCR array and real-time RT-PCR revealed increased expression of the AP Defb4 in infected CRAMP-KO mice. Mouse Defb4 is the homologue of the human β -defensin 2 [25]. Defb4 acts synergistically with antimicrobial lysozyme against bacteria [50]. Lysozyme is an enzyme which hydrolyses the sugar backbone of peptidoglycans in the cell wall of Gram-positive bacteria and thus disassembles the highly immunogenic peptidoglycan into less inflammable components [51]. In patients suffering from pneumococcal meningitis, the amount of lysozyme is increased [52]. Our results showed a stronger increase in infected CRAMP-deficient compared to -WT mice. We propose that by increasing Defb4 expression, the lack of CRAMP may be compensated. The lack of CRAMP resulted in an increased number of bacteria and an increased inflammatory response. This might be a

consequence of the imbalance in the innate immune system due to the lack of CRAMP which results in an imbalance in pro- and anti-inflammatory mediators. It can be hypothesized that CRAMP physiologically exerts anti-inflammatory effects. Additional studies need to explore this topic in further detail.

In conclusion, our results reveal the importance of the CRAMP in host defense in a mouse model of pneumococcal meningitis. Although pneumococci are pathogens for rodents [18, 53], the immune response in the mouse is partly different from the immune response in humans [54]. These limitations must be considered in the interpretation of the results. However, despite these limitations, our results provide interesting insights into the function of the innate immune system in the course of

bacterial meningitis. A balanced immune response includes the presence of soluble components of innate immunity such as AP. Thus, the lack of APs leads to greater damage and increased mortality. As a next step, adjunctive treatment of bacterial meningitis with CRAMP will help to evaluate the potential therapeutic use of these peptides in bacterial infections.

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Erratum

In the article by Merres et al., entitled 'Role of the cathelicidin-related antimicrobial peptide in inflammation and mortality in a mouse model of bacterial meningitis' [J Innate Immun 2014;6:205–218, DOI: 10.1159/000353645], the authors' contributions footnote should correctly read 'J.M. and J.H. contributed equally as first authors; S.C.T. and L.-O.B. contributed equally as senior authors'.