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Original Paper

Virus-Induced Type I Interferon Deteriorates Control of Systemic *Pseudomonas Aeruginosa* Infection

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Key Words
Pseudomonas aeruginosa • Type I interferon • LCMV • Bacterial superinfection • Lysozyme

Abstract
Background: Type I interferon (IFN-I) predisposes to bacterial superinfections, an important problem during viral infection or treatment with interferon-alpha (IFN-α). IFN-I-induced neutropenia is one reason for the impaired bacterial control; however there is evidence that more frequent bacterial infections during IFN-α-treatment occur independently of neutropenia. ***Methods:*** We analyzed in a mouse model, whether *Pseudomonas aeruginosa* control is influenced by co-infection with the lymphocytic choriomeningitis virus (LCMV). Bacterial titers, numbers of neutrophils and the gene-expression of liver-lysozyme-2 were determined during a 24 hours systemic infection with *P. aeruginosa* in wild-type and *Ifnar*$^{-/-}$ mice under the influence of LCMV or poly(I:C). ***Results:*** Virus-induced IFN-I impaired the control of *Pseudomonas aeruginosa*. This was associated with neutropenia and loss of lysozyme-2-expression in the

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liver, which had captured *P. aeruginosa*. A lower release of IFN-I by poly(I:C)-injection also impaired the bacterial control in the liver and reduced the expression of liver-lysozyme-2. Low concentration of IFN-I after infection with a virulent strain of *P. aeruginosa* alone impaired the bacterial control and reduced lysozyme-2-expression in the liver as well. ***Conclusion:*** We found that during systemic infection with *P. aeruginosa* Kupffer cells quickly controlled the bacteria in cooperation with neutrophils. Upon LCMV-infection this cooperation was disturbed.

Introduction

Viral infection often predisposes to bacterial superinfection. This has been attributed to the reduction of granulocyte-numbers (neutropenia) triggered by high IFN-I-levels in a mouse model with LCMV [1]. The treatment with IFN-α2 is common for patients chronically infected with hepatitis B and C virus [2, 3]. It has been shown that among the treated patients bacterial infections occur more frequently [4]. Neutropenia is also a side effect of the treatment and a recommended reason for dose reduction, because neutrophils are important for bacterial control [3, 5]. However, according to some studies neutropenia does not always correlate with the rate of bacterial infections in those patients [4, 6]. This suggests that there are other IFN-α-dependent factors besides neutropenia impairing the control of bacteria.

Pseudomonas aeruginosa is a multiresistant, gram-negative nosocomial pathogen [2], which accounts for approximately 10 % of clinical infections and carries a high mortality rate [7]. This ubiquitous and predominantly extracellularly living bacterium is a facultative pathogen and usually not invasive in an immunocompetent host. However, in patients with systemic or local immunosuppression, such as AIDS (Acquired Immunodeficiency Syndrome)[8], in transplant recipients or traumatized patients [9], in patients with burn injuries, ventilated patients, and cystic fibrosis patients *P. aeruginosa*-infections can develop into a life-threatening disease [10-12].

It has not yet been shown, which cells finally digest this bacterium. However, neutrophils (granulocytes) are necessary for control of respiratory *P. aeruginosa*-infections [13, 14]. For systemic *P. aeruginosa*-infections the role of neutrophils compared to macrophages is still ill-defined. The effect of IFN-I on a systemic infection with this bacterium remains elusive.

Lysozyme-2 is an important molecule, which exerts a direct antibacterial action against *P. aeruginosa* and other gram-negative and gram-positive bacteria [15]. Murine lysozyme-2, also called lysozyme-M, is closely related to the human lysozyme [15]. *Lysozyme-2* $^{-/-}$ mice were disabled to clear *P. aeruginosa* from their lungs [16], whereas lysozyme-overexpressing mice were protected against a respiratory infection with *P. aeruginosa* [17]. Treatment with human lysozyme could reduce the bacterial burden in lungs during a respiratory *P. aeruginosa* infection of mice [18]. Lysozyme is produced by antibacterial effector cells [15], bronchial epithelial cells [17] and can be induced in hepatocytes [19].

In order to examine further IFN-I induced mechanisms impairing the innate immune defense against *P. aeruginosa* we first characterized the importance of neutrophils and macrophages in the systemic *P. aeruginosa*-infection and then compared different inducers of IFN-I according to their influence on systemic bacterial clearance, neutrophil numbers and lysozyme-2-expression in the liver using wild-type and *Ifnar* $^{-/-}$ mice. We found that neutrophils are essential to clear the bacterium after it has been taken up by macrophages predominantly in the liver. IFN-I, which was induced by a co-infection with the lymphocytic choriomeningitis virus (LCMV), lead to neutropenia and blunted the lysozyme-2-expression. Lower levels of IFN-I released by poly(I:C)-treatment or the bacterial infection itself had no impact on neutrophil numbers, but reduced the gene-expression of liver-lysozyme-2.

Material and Methods

Pathogens

LCMV strain WE was originally obtained from F. Lehmann-Grube (Heinrich Pette Institute, Hamburg, Germany) and was propagated in L929 cells. *P. aeruginosa* (strain Boston 41501; ATCC no. 27853), which was provided by E. Gulbins, Department of Molecular Biology, University of Duisburg-Essen, was plated from a glycerol stock on trypticase soy broth agar (BD Biosciences, no. 212305) and incubated overnight at 37°C. The bacteria were scraped into trypticase soy broth medium (BD Biosciences, no. 221093) and diluted to an optical density (OD, 550 nm) of 0.1. After a growth time of 2 hours at 37°C with shaking at 250 rpm, the bacteria had an OD (550 nm) of 0.8 to 1 and were diluted in phosphate-buffered saline (PBS) for intravenous injection. The *P. aeruginosa* strain F469, provided by B. Tümmler, Department of Pediatrics, Faculty of Medicine, University of Hannover, was processed similarly.

Mice

All animals were housed in groups in individually ventilated cages during the experiments. All mice were bred on the C57Bl/6J background. *Ifnar*$^{-/-}$ mice were used as described before [20]. Male and female mice of age from 6 to 8 weeks were sex- and weight-matched for all experiments.

Mice were infected intravenously with 2×10^6 PFU of LCMV-WE diluted in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 2 % FCS and with 1.3×10^7 to 2×10^8 CFU of *P. aeruginosa* diluted in PBS. The depletion antibodies anti-Ly6G clone RB6-8C5 (Bioxcell, no. BE0075) and clone 1A8 (Bioxcell, no. BE0075-1) were administered intravenously at the doses of 500 µg and 200 µg per mouse, respectively, two days before infection with *P. aeruginosa* (day -2). 200 µg of poly(I:C) was injected intravenously in 100 µl PBS. Macrophages were depleted by intravenous injection of 100 µg of clodronate-encapsulated liposomes 24 hours before bacterial infection [21].

The disease score was determined as follows. 0 (no symptoms) = mice appeared completely healthy and normal, 1 (weak symptoms) = mice showed slightly decreased motility and scrubby fur, 2 (ill) = mice showed decreased motility, scrubby fur and/or pus efflux from the eyes, 3 (morbid) = the motility of mice was strongly reduced and indications of pain occurred. When mice reached the morbid state (score=3) they were considered dead by disease in the survival analysis.

Determination of P. aeruginosa CFUs

Equal pieces of organs were homogenized in 1 ml PBS with a Tissue Lyser II (Qiagen) for 5 min at a speed of 25 s^{-1}. The samples were titrated in PBS, and 60 µl of 3 dilutions ranging from 3 to 1000 were distributed on separate wells of a 6-well plate containing trypticase soy broth agar. After 15-20 hours incubation at 37°C, the colonies were counted.

Antipseudomonal activity assay

25.000 CFU/ml of mid-log-phase *P. aeruginosa* strain Boston 4101 was mixed with 75 µg of human lysozyme (L1667, SIGMA) in activity buffer (10% Trypticase-Soy-Broth in 10 mM potassium phosphate buffer, pH 7.0) in a total reaction volume of 115 µl and incubated for 60 min at 37 °C. Living bacteria were determined by an MTT-assay by incubating the MTT-substrate with the bacteria over night. Results were calculated relative to PBS-treated bacteria. Assays were performed in triplicate on three different days to yield biological replicates.

Flow Cytometry (FACS)

For sample preparation, livers were digested by liberase (Roche) at a concentration of 0.16 mg/ml in PBS at 37°C for 30 min. Spleens were homogenized through a 70-µm mesh (FALCON, No. 352350) in PBS. Antibodies used for all staining at dilutions of 1:100 were anti-Ly6G-eFluor660 (BD, 51-5931), anti-Ly6G-FITC (BD, 11-5931), anti-Ly6C-PerCP (BD, 45-5932) and anti-CD115-PE (BD, 12-1152). The staining was performed for 30 minutes in 50 µl of FACS buffer (PBS, 0.1 % sodium azide, 0.1 % 5 M EDTA, 0.1 % FCS) at 4 °C. For erythrocyte-lysis, BD FACS lysing solution was used according to the manufacturer's instructions, and for non-fixing conditions 1 ml of ammonium-chloride-buffer (5 mM $KHCO_3$, 40 µM EDTA, 0.15 M NH_4Cl; pH = 7.8) was added on 10 µl of blood for 2 minutes, followed by washing with FACS buffer. The analysis was performed on a BD LSR Fortessa™. Dublets and dead cells were excluded from FACS analysis. Absolute cell-numbers were determined using calibration beads (BD).

Histology

Histologic analyses of snap-frozen tissue (Tissue-Tek, no. 4583, Sakura) were performed with the antibodies anti-F4/80-PE (BD, 12-4801), anti-F4/80-APC (BD, 17-4801), anti-F4/80-DyLight™488 (Novus biological, NB600-404G) anti-Ly6C-APC (BD, 17-5932), anti-Ly6G-FITC (11-5931), anti-Ly6G-eFluor660 (BD, 51-5931), anti-*P. aeruginosa* (Abcam, ab68538), anti-Rabbit-Cy3 (Jackson, 111-165-144) and anti-Rabbit-DyLight™594 (Thermo scientific, 35561) antibodies. In short, sections were fixed with acetone, and non-specific binding was blocked with phosphate-buffered saline (PBS) containing 2% FCS for 10 min, followed by various stainings for 45 min at room temperature. All antibodies were diluted 1:100 from their original concentration for staining, except for anti-*P. aeruginosa*, which was diluted 1:5000. Images of stained sections were acquired with a fluorescence microscope (BZ-9000 II analyser, KEYENCE). Confocal microscopy was performed on the Leica TCS SP8 microscope. Quantification was performed with the Cell Counter Plugin from Image J x 2.1.4.5 by evaluating six fields of view per mouse.

Enzyme-linked immunosorbent Assay (ELISA)

The IFN-α-ELISA from serum samples (VeriKine Mouse Interferon Alpha ELISA Kit, PBL interferon source, 42120-2) was performed according to the manufacturer's instructions. Absorbance was determined by the plate reader FLUOstar Omega (BMG Labtech).

Quantitative real-time PCR

For expression analysis organs were homogenized in TRizol reagent according to manufactures instructions (Ambion, no. 15596018). RNA was isolated according to instructions from Peqlab, and cDNA was produced with the QuantiTect Reverse Transcription Kit (Qiagen, no. 205313). Real-time PCR was performed with the SYBR Green PCR Master Mix (Applied Biosystems, no. 4369155), using the primers for Lysz2 (Qiagen, no. QT01555701), IFN-α4 (Qiagen, no. QT01774353) and IFN-β1 (Qiagen, no. QT00249662) in the Light Cycler 480 (Roche). The expression levels of respective genes were normalized to GAPDH (Qiagen, no. QT01658692). Relative expression levels to GAPDH were calculated with the ΔCt-method using the formula $2^{CT(GAPDH) - CT(target)}$.

Statistical analysis

Survival curves and graph`s, as well as other statistic tests, were prepared by the analysis software GraphPad Prism. Normal distribution of values was tested by the Shapiro-Wilks-test [22]. Statistical significance of survival curves was tested by the log-rank(Mantel-Cox)-test. For the comparison of bacterial titers values were tested by the Mann-Whitney-U-test or Student's *t*-test. For multiple comparisons ANOVA, followed by indicated tests for multiple comparisons, was performed.

Ethics Statement

Animal experiments were carried out with approval of the "Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen" (LANUV), Germany (approval numbers: 84-02.04.2013.A242, 84-02.04.2013.A218) in accordance with the German laws for animal protection and with the institutional guidelines at the Ontario Cancer Institute. Animal care and documentation were supervised by the central animal laboratory of the University Hospital Essen, Essen, Germany or in the Ontario Cancer Institute, according to the German laws for animal protection and to the institutional guidelines of the Ontario Cancer Institute.

Results

P. aeruginosa is filtered by Kupffer cells and controlled in cooperation with granulocytes

To get insight into the processes after intravenous infection with *Pseudomonas aeruginosa* we first monitored the dependency of bacterial uptake on macrophages or granulocytes in liver and spleen.

We hypothesized that the liver is the main organ, which takes up *P.* aeruginosa from the blood, like it has been shown for *P. aeruginosa* strain PA103 and listeria [23, 24]. The spleen has with its high amount of macrophages potential to uptake bacteria and was therefore

Table 1. *P. aeruginosa* distribution in liver and spleen 1 hour post infection. The proportion of bacteria interacting with macrophages (M.; F4/80$^+$), granulocytes (Ly6G$^+$) or free (not interacting) bacteria were determined by histological analysis with or without previous macrophage-depletion by clodronate-liposomes. Splenic macrophages were too dense to clearly count internalized bacteria in intersected cells (n. a.: not available); n = 3

organ	treatment	total bacteria	% associated with M. (internalized)	% free	% associated with granulocytes
Liver	PBS-liposomes	58.78 +/- 13.64	89.89 +/- 3.92 (17.75 +/- 1.02)	5.13 +/- 3.50	4.98 +/- 0.50
	Clodronate-liposomes	11.56 +/- 1.40	5.88 +/- 3.53 (0)	34.0 +/- 6.34	37.09 +/- 8.08
Spleen	PBS-liposomes	103.17 +/- 11.43	59.14 +/- 0.70 (n. a.)	18.78 +/- 3.28	22.08 +/- 2.90
	Clodronate-liposomes	240.94 +/- 35.99	36.70 +/- 8.24 (n. a.)	15.33 +/- 0.46	47.97 +/- 9.82

compared to the liver in its uptake capacity. First we assessed the distribution of *P. aeruginosa* relatively to macrophages and granulocytes in histological sections of liver and spleen one hour post infection. Approximately 90 % of *P. aeruginosa* were interacting with macrophages in the liver (Table 1, PBS-liposomes). The content of macrophage-interacting *P. aerugionosa* was lower in spleen (approximately 59 %) due to more interaction with granulocytes and a higher amount of non-interacting bacteria (n = 3, t-test: $p < 0.001$; Table 1; Fig. 1A). Nearly 18 % of total *P. aeruginosa* in the liver was localized inside Kupffer cells (Table 1). The internalization of bacteria was confirmed by 3D-microscopy (Fig. 1B).

To investigate uptake-kinetics we depleted mice for macrophages via clodronate-liposomes or for granulocytes via anti-Ly6G (clone 1A8) or additionally for inflammatory monocytes by anti-Ly6G (clone Rb6-8C5) antibody prior infection with 2 x 10^7 colony-forming-units (CFU) of *P. aeruginosa* strain Boston 41501 (Fig. 1C). The bacteria remained longer in the bloodstream of macrophage-depleted mice compared to control mice (Fig. 1D, left panel), whereas the anti-Ly6G-treatment (clone RB6-8C5, which depleted granulocytes and inflammatory monocytes) had no impact on the uptake of bacteria from the blood (Fig. 1D, right panel).

Moreover the macrophage-depletion resulted in elevated *P. aeruginosa* titers in the spleen and reduced titers in the liver one hour post infection (Fig. 1E, Table 1). This indicates that macrophages were necessary for the uptake of *P. aeruginosa* into the liver, but not into the spleen.

Thus, these observations underline the hypothesis that the interaction of *P. aeruginosa* and macrophages, which lead to uptake of bacteria, is more relevant in the liver than in the spleen.

To address the importance of granulocytes and macrophages for the control of the systemic infection we measured bacterial titers 24 hours post infection in granulocyte depleted and control mice. The specific depletion of granulocytes via anti-Ly6G (clone 1A8) antibody resulted in a failure in bacterial control in spleen and liver (Fig. 1F, upper panel). In this experiment the number of liver granulocytes correlated inversely with the liver-titer of *P. aeruginosa* (Spearman-correlation: anti-Ly6G(1A8)-treated mice: $p = 0.007$, anti-Ly6G(1A8)-treated and untreated mice: $p < 0.001$; data not shown). Similarly depletion of macrophages via clodronate also impaired the bacterial clearance in spleen and liver (Fig. 1F, lower panel). However, the macrophage depletion went along with a strong reduction of granulocytes in liver (Fig. 1F, lower panel) and spleen (data not shown).

The mRNA expression of the anti-microbial effector molecule lysozyme-2 in the liver was strongly induced within 24 hours of *P. aeruginosa*-infection (Fig. 1G), whereas only approximately 40 % of the induction was measured in granulocyte-depleted mice (Fig.

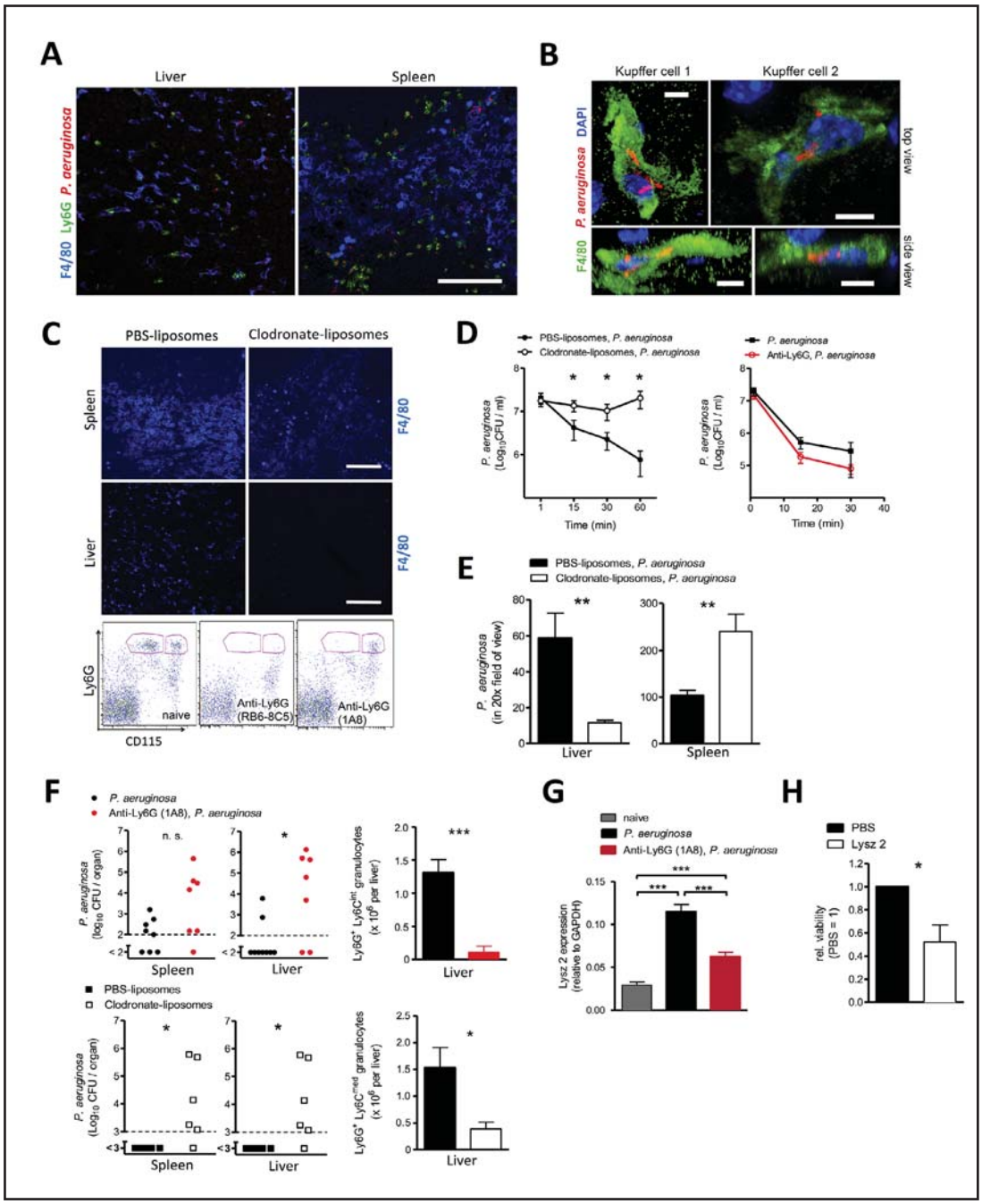

Fig. 1. Kupffer cells take up *P. aeruginosa*, but granulocytes are necessary for bacterial clearance. (A) Representative histology from liver and spleen sections 1 h after an intravenous infection with 2 × 10^7 CFU of *P. aeruginosa* stained for macrophages (F4/80, blue), *P. aeruginosa* (red) and granulocytes (anti-Ly6G, green); scale bar = 100 µm (B) Confocal microscopy from liver section, stained for Kupffer cells (F4/80, green), *P. aeruginosa* (red) and DAPI (blue) 1 h after infection with 4 x 10^7 CFU *P. aeruginosa*. Two 3D-images from two different perspectives each are shown (scale bar = 5 µm). (C) Representative histology from spleen and liver sections 1 h after an intravenous infection with *P. aeruginosa* with an additional intravenous injection of 100 µg of either PBS-liposomes or clodronate-liposomes 24 h prior infection stained for macrophages (F4/80: blue, upper panel); representative FACS-plots of blood from naive mice compared to mice treated 24 h earlier with either 500 µg anti-Ly6G (RB6-8C5) or 200 µg anti-Ly6G (1A8) antibody. $Ly6G^{high}$ $CD115^{int}$ granulocytes are marked in the left, $Ly6G^{high}$ $CD115^{high}$ inflammatory monocytes in the right gate (lower panel); scale-bar = 100 µm (D) *P. aeruginosa* titers in blood 1, 15, 30 and 60 min post infection with 2 × 10^7 CFU of *P.*

aeruginosa. Mice depleted for macrophages via an intravenous injection of 100 µg clodronate-liposomes 24 hours prior infection are compared to a group of mice treated simultaneously with PBS-liposomes (means and SEM, n = 6 from two independent experiments; 2-way ANOVA: interaction of time and treatment: p = 0.003; Wilcoxon-test: 15, 30 and 60 min p = 0.031). Mice pre-treated 48 hours prior bacterial infection with 500 µg anti-Ly6G (RB6-8C5) antibody are compared to mice without pre-treatment (means and SEM, n = 3 - 4; 2-way ANOVA: n. s.) (E) *P. aeruginosa* counts in liver and spleen of macrophage-depleted (clodronate-liposomes) compared to control (PBS-liposomes) wild-type mice, as determined by histological quantification after staining with anti-*P. aeruginosa* (B; means and SD from n = 3; 2-tailed *t*-test; liver: p = 0.004, spleen: p = 0.003) (F) *P. aeruginosa* titers 24 h post infection and liver-granulocyte-numbers 1 h post infection with 2×10^7 CFU of *P. aeruginosa* with or without granulocyte-depletion 48 h earlier via 200 µg anti-Ly6G (clone 1A8) antibody (upper panel, n = 7; 2-tailed Mann-Whitney-U-test; spleen: p = 0.101, liver: p = 0.027, lung: p = 0.212; granulocyte-numbers: means and SD from n = 3, 2-tailed t-test: $p < 0.001$) or with (clodronate-liposomes) or without (PBS-liposomes) macrophage-depletion (lower panel; n = 6; 1-tailed Wilcoxon-Test, liver and spleen: p = 0.031; granulocyte-numbers: mean and SEM from n = 6, 2-tailed *t*-test: p = 0.013) (G) Lysozyme-2 (Lysz2) expression levels in the liver 24 h post infection in wild-type mice with *P. aeruginosa* (2×10^7 CFU) with or without 48 h earlier granulocyte-depletion via anti-Ly6G (clone 1A8) antibody (means and SEM; n = 5 - 7 mice from two independent experiments; one-way ANOVA ($p < 0.001$), followed by a Bonferroni's multiple comparison test) (H) Viability of *P. aeruginosa* after a 60 min treatment with 652 µg/ml recombinant human lysozyme compared to a PBS-treated group (n = 3; 2-tailed Mann-Whitney-U-test, p = 0.031) *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

1G). *In vitro* recombinant human lysozyme had a direct killing effect on *P.aeruginosa* strain Boston 41501 as less bacteria survived during a 60 min lysozyme treatment compared to PBS-treated controls (Fig. 1H) demonstrating the killing-effectiveness of lysozyme against strain Boston 41501.

Together, this data show that neutrophils are critical for the clearance of *P. aeruginosa* after capture of bacteria by macrophages predominantly in the liver and that lysozyme-2 is partially granulocyte-dependent induced in the liver after a systemic *P. aeruginosa*-infection.

Virus-induced IFN-I limits the control of P. aeruginosa, creates neutropenia and blunts the expression of lysozyme-II in the liver

To study the effect of viral infection on the innate immune defense against *P. aeruginosa*, we pre-infected wild-type mice with 2×10^6 plaque-forming units (PFU) of LCMV (strain WE) 24 hours before infection with 4×10^7 CFU of *P. aeruginosa*. The combination of viral and bacterial infection resulted in high levels of IFN-α in the serum 24 hours post infection, whereas no IFN-α was detected with bacterial infection alone (Fig. 2A).

To determine whether virus-induced IFN-I affects the control of *P. aeruginosa,* we infected wild-type and *Ifnar*$^{-/-}$ mice (which lack the receptor for IFN-I) with *P. aeruginosa* in the presence or absence of LCMV pre-infection. In contrast to wild-type mice, *Ifnar*$^{-/-}$ mice, which were pre-infected with LCMV, survived the superinfection with *P. aeruginosa* (Fig. 2B). Examination of bacterial titers 24 hours post *P. aeruginosa*-infection with a lower dose (2×10^7 instead of 4×10^7 CFU) revealed that with LCMV pre-infected *Ifnar*$^{-/-}$ mice cleared *P. aeruginosa* from the body, predominantly from the liver, more efficiently than wild-type mice (Fig. 2C).

The infiltration of Ly6G$^+$ granulocytes into the liver was reduced by 2-fold in the LCMV pre-infected group as quantified from liver-histology one hour after *P. aeruginosa*-infection (Fig. 2D). FACS-analysis of livers from wild-type and *Ifnar*$^{-/-}$ mice showed that this reduction lasted at least for 24 hours and depended on the presence of the IFN-I-receptor (Fig. 2E). A similar LCMV-induced and IFNAR-dependent reduction of granulocyte-numbers was also measured in spleen and blood (data not shown).

The LCMV pre-infection blunted the lysozyme-2 gene-expression completely in wild-type but not in *Ifnar*$^{-/-}$ mice (Fig. 2F).

Together the results show that virus-induced IFN-I exacerbates the systemic *P. aeruginosa*-infection, leads to neutropenia and loss of lysozyme-2-expression in the liver.

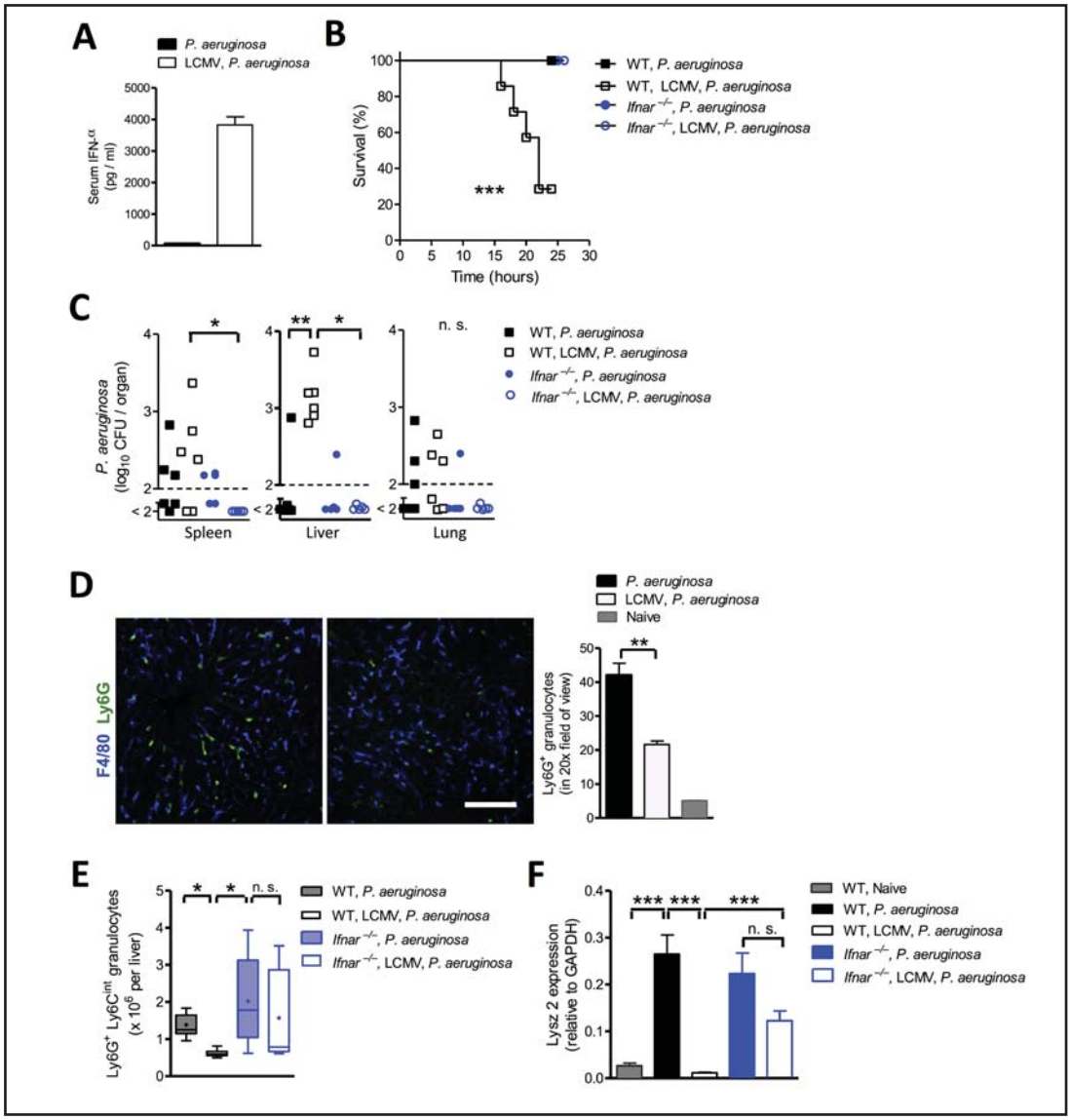

Fig. 2. Virus-induced type I interferon limited the control of *P. aeruginosa*. (A) IFN-α-concentration in serum of wild-type mice 24 h after an intravenous *P. aeruginosa*-infection (2×10^7 CFU) with or without infection 24 h earlier with LMCV-WE (2×10^6 PFU), as measured by ELISA (n=3). (B) Survival of wild-type (WT) and *Ifnar*$^{-/-}$ mice infected intravenously with *P. aeruginosa* (4×10^7 CFU) with or without infection 24 h earlier intravenously with 2×10^6 PFU LMCV-WE (n = 6; Log-Rank-Test, $p < 0.001$). (C) *P. aeruginosa* titers in organs of wild-type (WT) and *Ifnar*$^{-/-}$ mice 24 h after an intravenous *P. aeruginosa*-infection (2×10^7 CFU) with or without infection 24 h earlier with 2×10^6 PFU LMCV-WE (n = 5 - 9; Kruskal-Wallis-Test (liver: $p = 0.004$, spleen: $p = 0.04$, lung: $p = 0.404$) with a Dunn's test for multiple comparisons) (D) Liver-histology from wild-type mice infected i.v. with 2×10^7 CFU of *P. aeruginosa* for 1 h with or without infection 24 h earlier with LMCV-WE (2×10^6 PFU), stained for F4/80 (blue) and Ly6G (green; scale bar = 100 µm) with quantification of granulocytes (mean, SD; n = 3; 2-tailed Student's *t*-test, $p = 0.005$). (E) FACS-analysis of livers from wild-type (WT) or *Ifnar*$^{-/-}$ mice infected intravenously with 2×10^7 CFU of *P. aeruginosa* for 1 h with or without infection 24 h earlier with LMCV-WE (2×10^6 PFU), gated for Ly6G$^+$Ly6C^{int} granulocytes (mean, SD; n = 5 - 6 from two independent experiments; one-way ANOVA (p = 0.015), followed by a Sidak's test for multiple comparisons) (F) Lysozyme-2 (Lysz2) expression levels in the liver 24 h after infection with 2×10^7 CFU of *P. aeruginosa* of wild-type (WT) or *Ifnar*$^{-/-}$ mice with or without a 24 h earlier intravenous infection with 2×10^6 PFU of LCMV-WE (mean, SEM; one-way ANOVA ($p < 0.001$), followed by a Bonferroni's multiple comparison test). *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

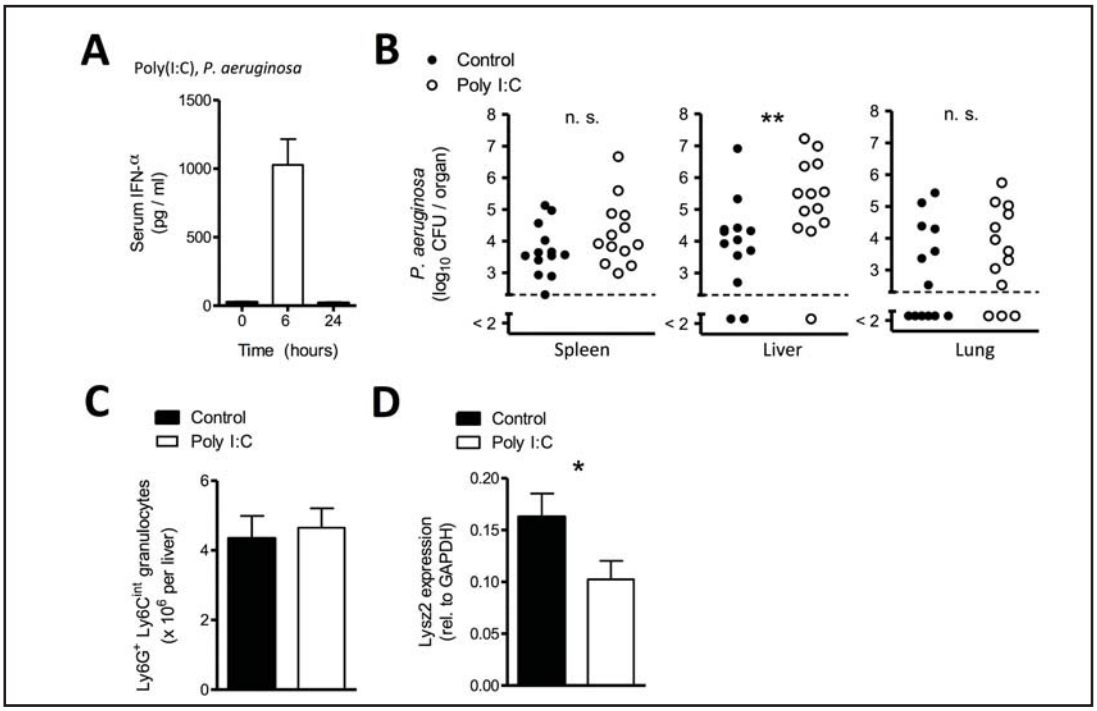

Fig. 3. The influence of poly(I:C) on the innate immune defense against *P. aeruginosa.* Wild-type mice were infected intravenously with 4×10^7 CFU *P. aeruginosa*. 200 µg poly(I:C) was injected 6 h before infection (A) IFN-α-concentration in serum from infected mice at indicated time points after *P. aeruginosa*-infection, as measured by ELISA (n = 3) (B) *P. aeruginosa*-titers in organs of infected mice 24 h post *P. aeruginosa*-infection (2-tailed Mann-Whitney-U test; spleen: p = 0.152, liver: p = 0.004, lung: p = 0.349) (C) FACS-analysis of liver-granulocytes from infected mice 24 h post infection (mean, SEM; n = 6 from 2 independent experiments) (D) Lysozyme-2 (Lysz2) expression levels in the liver 24 h post *P. aeruginosa*-infection (mean, SEM; n = 6 from two independent experiments; 1-tailed *t*-test: $p = 0.031$) *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

Poly(I:C) limits the control of P. aeruginosa and reduces the expression of lysozyme-2 in the liver

Next, we tested whether an alternative induction of type I interferon by a TLR3-ligand such as poly(I:C) could impair the control of *P. aeruginosa* in wild-type mice. The poly(I:C)-pre-treatment induced an IFN-α-level of approximately 1000 pg / ml serum six hours after *P. aeruginosa*-infection (12 hours after poly(I:C)-injection), however no more IFN-α was detectable 24 hours post infection (Fig. 3A). These much lower levels of IFN-α, compared to the viral infection (nearly 4000 pg / ml serum, Fig. 2A), impaired the clearance of *P. aeruginosa* from the liver, whereas spleen and lung showed only tendencies of higher bacterial titers (Fig. 3B).

The amount of granulocytes 24 hours post infection was not altered by the poly(I:C)-injection (Fig. 3C). However, the gene-expression of lysozyme-2 in the liver 24 hours post infection was reduced in poly(I:C)-treated mice (Fig. 3D).

Thus, the data revealed an association of impaired bacteria-elimination from the liver in presence of moderate IFN-I levels with reduced lysozyme-2-expression at normal neutrophil-numbers in the liver.

Type I interferon induction by virulent P. aeruginosa contributes to pathogenicity

Next we studied whether IFN-I, which is induced by the *P. aeruginosa* infection itself, can have a deteriorating effect on the bacterial clearance as well. Therefore we included the more virulent strain *P. aeruginosa* F469. It is one of the most common isolated strains in patients and was previously determined to be highly virulent in an acute murine airway infection model [25]. Also in our systemic infection this strain had a higher disease score

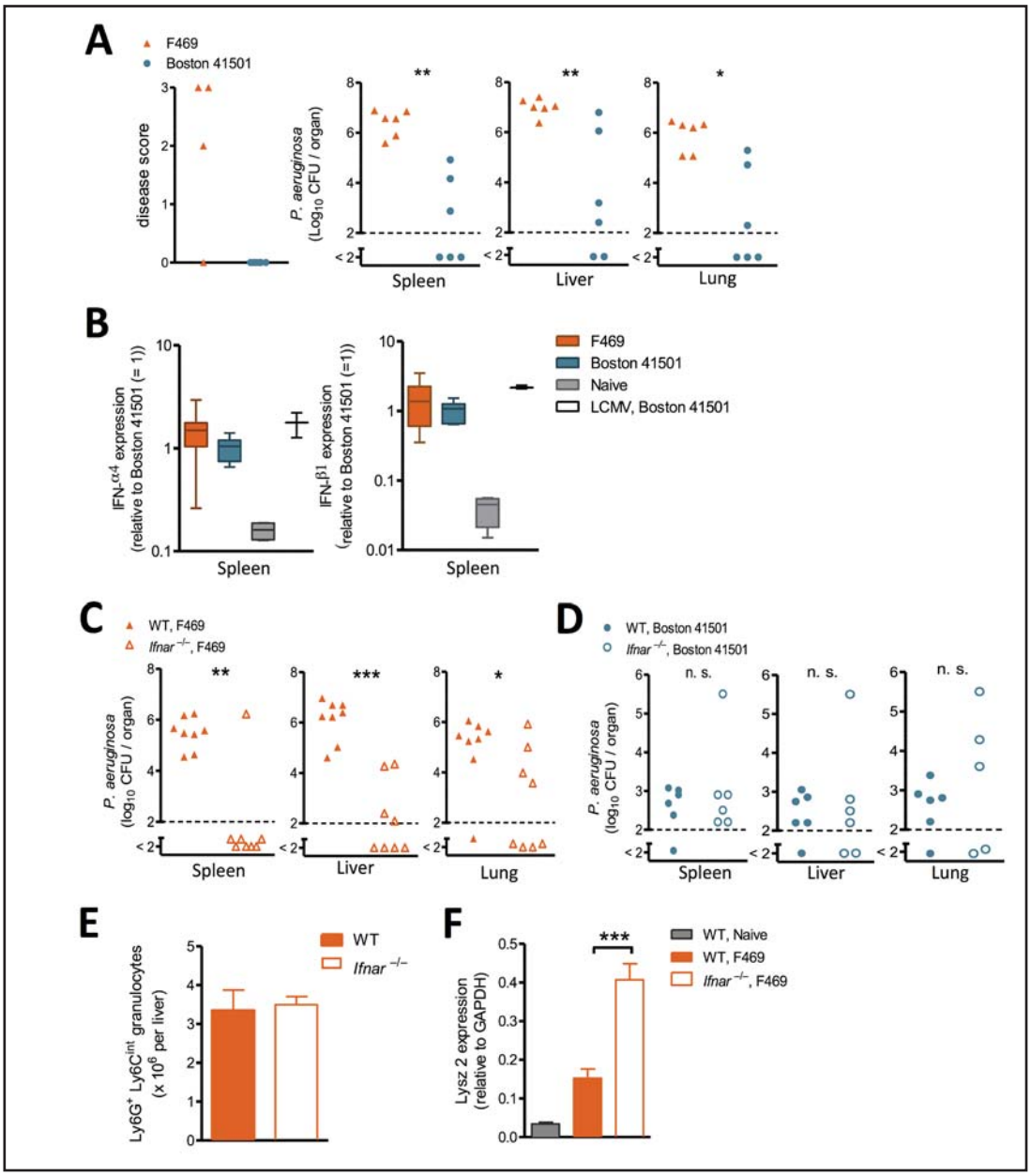

Fig. 4. The impact of bacteria-induced IFN-I on the innate immune defense against *P. aeruginosa.* (A) Virulence of *P. aeruginosa* strains F469 and Boston 41501 (3 = morbid, 2 = ill, 1 = weak symptoms) and organ-titers 24 h after an intravenous infection with 2 × 10^7 CFU of the indicated strain (n = 6 from two independent experiments; liver: 2-tailed *t*-test $p = 0.006$; 2-tailed Mann-Whitney-U test: spleen: $p = 0.002$, lung: $p = 0.012$) (C) gene-expression of IFN-α4 and IFN-β1 relative to GAPDH in the spleens of mice infected with either *P. aeruginosa* strain Boston 41501 or F469 for 24 h. Values were normalized to strain Boston 41501 (=1); F469 and Boston 41501: n = 8; naive: n = 4, LCMV, Boston 41501: n = 3 (C,D) *P. aeruginosa* titers in organs of wild-type (WT) and *Ifnar*$^{-/-}$ mice 24 h post infection with (C) 1.3 × 10^7 CFU of the virulent strain F469 (n = 8; 2-tailed Mann-Whitney-U-test; spleen: $p = 0.007$, liver: $p < 0.001$, lung: $p = 0.027$) or with (D) 4 × 10^7 CFU of strain Boston 41501 (2-tailed *t*-test: n. s.) (E) FACS-analysis of granulocyte-numbers in the liver 24 hours post infection of wild-type (WT) or *Ifnar*$^{-/-}$ mice with 1.3 x 10^7 CFU *P. aeruginosa* strain F469 (n = 3) (F) Lysozyme-2 (Lysz2) expression relative to the internal control gene GAPDH in the liver 24 h after infection of wild-type (WT) or *Ifnar* $^{-/-}$ mice infected with 1.3 × 10^7 CFU of *P. aeruginosa* strain F469 compared to naive mice (n = 8 from two independent experiments; 2-tailed *t*-test, p < 0.001). *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

than the strain Boston 41501, which was associated with higher bacterial titers in the organs 24 hours post infection (Fig. 4A). Both strains induced gene-expression of IFN-α4 and IFN-β1 but with more variance in F469-infected mice (Fig. 4B). However, in neither of the infections any IFN-α could be detected in the serum via ELISA (data not shown). We tested Boston 41501 at a higher dose (4 x 10^7 CFU) and F469 at moderate dose (1.3 x 10^7 CFU) in *Ifnar* $^{-/-}$ mice to see, if IFN-I might induce higher bacterial loads 24 hours post infection. Indeed *Ifnar* $^{-/-}$ mice were able to clear F469 from the organs, in contrast to wild-type mice (Fig. 4C). There was no difference in titers of Boston 41501 between wild-type and *Ifnar* $^{-/-}$ mice (Fig. 4D). We measured the granulocyte-numbers and gene-expression of lysozyme-2 in the liver 24 hours after infection in wild-type and *Ifnar* $^{-/-}$ mice with F469. We detected no difference in the amount of granulocytes in the liver between the two mouse-lines (Fig. 4E), whereas the gene-expression of liver-lysozyme-2 was approximately 3-fold higher in *Ifnar* $^{-/-}$ mice compared to wild-type mice (Fig. 4F).

Taken together these data show that IFN-I-signaling during infection with a virulent strain of *P. aeruginosa* can be detrimental for bacterial clearance and can lead to weaker expression of liver lysozyme-2 in presence of normal amounts of neutrophils in the liver.

Discussion

In this study we characterized the role of neutrophils and macrophages in mechanisms performing systemic clearance of *Pseudomonas aeruginosa*. The results show that neutrophils play a critical role in the elimination of *P. aeruginosa* from organs, as a specific depletion of neutrophils lead to increased bacterial titers predominantly in the liver 24 hours after infection. However, the initial uptake of the bacteria from the blood was managed by macrophages, predominantly by the Kupffer cells in the liver. We could observe some internalization of *P. aeruginosa* by these macrophages. Such an internalization was previously described in an *in vitro* system with Kupffer cells [26]. The depletion of macrophages caused impaired *P. aeruginosa*-elimination. However, this effect might be a consequence of less infiltrating granulocytes. A loss of macrophages leads to a lower production of granulocyte-recruiting cytokines, as shown in a bacterial lung-infection model [27]. The gene-expression of the important anti-bacterial molecule lysozyme-2 was clearly induced in the liver by the systemic *P. aeruginosa*-infection. The reduced lysozyme-2-expression after granulocyte-depletion might reflect the missing granulocytes, which are strongly expressing lysozyme-2 [28]. However, the lysozyme-2-expression in the depleted mice was still higher than in naive mice, a finding, which suggests that other liver-cells were induced to express lysozyme-2.

Further we analyzed the effects of IFN-I induced by different stimulators on the antibacterial defense during the systemic *P. aeruginosa*-infection. We found that a high systemic concentration of IFN-I induced by LCMV pre-infection conferred a severe outcome of the systemic *P. aeruginosa*-infection and limited the number of granulocytes in the liver. In line with this it has been shown that LCMV-induced IFN-I leads to apoptosis of granulocytes [1]. Consistent with these findings is the observation of neutropenia in patients with viral infections such as influenza [29] and acute HIV [30]. In patients with cystic fibrosis (CF), where *P. aeruginosa* infection is a frequent and serious complication [31], enhanced viral infections have been described [32] suggesting that also in CF-patients virus induced neutropenia could contribute to an uncontrolled *P. aeruginosa* infection. Hepatitis C and B patients treated with IFN-I also exhibit reduced numbers of neutrophils [33]. The concentration of serum-IFN-α during IFN-α-treatment determined in one Hepatitis-C-patient over several months did not exceed 130 pg/ml [34]. Dependent on the dose of IFN-α-treatment the blood IFN-α levels range from 50 to 2000 pg/ml [35]. In our system lower concentrations of IFN-I, like approximately 1000 pg/ml induced by poly(I:C) or not detectable levels induced by the bacterial infection alone, did not lead to neutropenia.

The gene-expression of lysozyme-2 in the liver was blunted by IFN-I-signaling during the LCMV-co-infection, slightly reduced by poly(I:C)-treatment and by IFN-I-signaling

during infection with the virulent *P. aeruginosa*-strain F469. However, our findings do not clearly elucidate which cells predominantly express lysozyme-2 or whether its expression is affected cell intrinsically by IFN-I or indirectly by IFN-I-induced cell-migration. Also possible reducing effects on the lysozyme-2-expression caused by higher bacterial loads in the liver cannot be excluded by this study. LPS activates NF-kappaB, a transcription factor stimulating lysozyme-expression as shown in chicken-cells [36]. Recently it was discovered that the IFN-I-induced gene *setdb* is responsible for methylation of promoters of several NF-kappaB regulated antibacterial genes, which leads to their suppression [37]. Even though we could not measure a higher induction of gene-expression of IFN-α4 and IFN-β1 in the spleen or ISG's in the liver (ISG15, OAS; data not shown) by the strain F469 compared to Boston 41501, we anyway think a higher production of IFN-I by F469 could explain the deteriorating effect of IFN-I on the immune defense against the strain F469 but not the strain Boston 41501, but therefore protein levels of IFN-I in the liver should be compared. *P. aeruginosa* induces IFN-I via the LPS-TLR4-TRIF pathway [38] and can be influenced by exotoxin A via IRF1 [39]. The genome of strain Boston 41501 was just recently published [40]. A detailed comparison of the genomes could give additional hints for the cause of potential differences in IFN-I signaling.

Lysozyme-chloride has already reached clinical attention. The here described association between reduced lysozyme-expression and the course of *P. aeruginosa*-infection suggests that a treatment with human lysozyme could compensate the loss of endogenous lysozyme-production and thereby improve the outcome of *P. aeruginosa* bloodstream-infections in patients.

In conclusion, we described Kupffer cells as important cells to filtrate *P. aeruginosa* during a systemic infection and the cooperation with granulocytes as essential for the control of the bacteria. Furthermore we found that the induction of high systemic levels of IFN-I by LCMV exacerbate *P. aeruginosa* infection, decrease the number of blood granulocytes and blunt the gene-expression of liver-lysozyme-2. We further conclude that lower levels of IFN-I induced by poly(I:C) or the bacterial infection itself impair the clearance of *P. aeruginosa* in association with a down-regulation of lysozyme-2 in the liver. These findings may help to understand processes that occur in lethal infections with *P. aeruginosa.*

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Disclosure Statement

The authors have no financial conflict of interest.

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