

A Comparison of Neutrophil Secretion of Cytokines and NET Formation in Response to Lipopolysaccharide and Group B *Streptococcus* (GBS) in Mice and Rats in vitro

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Introduction: Neutrophil granulocytes (PMNs) play a crucial role in initial pathogen defense, in part through the release of neutrophil extracellular traps (NETs). These web-like structures are essential for pathogen clearance and preventing dissemination but can also elicit excessive inflammation resulting in tissue damage.

Purpose: Here, we aimed to investigate the link between NETosis and inflammation induced by gram-negative (lipopolysaccharide, LPS) and gram-positive (Group B *Streptococcus* lysates, GBS) bacterial mimetics. As mice and rats are common models to investigate inflammation and sickness responses, we aimed to compare the inflammatory reaction of neutrophils derived from these two species.

Methods: Bone marrow-derived PMNs from adult mice and rats were cultured after isolation by negative magnetic bead selection. NET formation was evaluated through immunofluorescence detecting the NET markers myeloperoxidase (MPO) and DNA/histone-1-complexes. The secretion of pro-inflammatory cytokines (TNF- α and IL-6) in the supernatants were analyzed using bioassays.

Results: We revealed a significant increase in NET formation after GBS treatment in both rat and mouse PMN cultures, whereas LPS had minimal effects on NET induction. Gram-negative and gram-positive inflammatory conditions increased TNF- α and IL-6 secretion in rat PMN cultures. For mouse PMNs, TNF- α levels were increased after stimulation with LPS, while IL-6 was only elevated upon treatment with the highest dose of LPS (20 μ g/mL) applied. GBS did not significantly alter IL-6 and TNF- α secretion in mouse PMN cultures.

Conclusion: These findings suggest that PMNs derived from mice and rats exhibit species-specific distinct responses to inflammatory stimuli. GBS induced NET formation in both rat and mouse PMNs. However, the main difference in rat and mouse PMNs inflammatory response was observed regarding GBS- and LPS-induced secretion of TNF- α and IL-6, suggesting a greater cytokine responsiveness in rat PMNs relative to mouse PMNs.

Keywords: group B *streptococcus* (GBS), lipopolysaccharide, neutrophil granulocytes, neutrophil extracellular traps, tumor necrosis factor α , interleukin 6

Introduction

Infections with *Streptococcus agalactiae*, also known as Group B *Streptococcus* (GBS), are a common cause of stillbirths, premature delivery, neonatal sepsis and meningitis.^{1,2} The inflammatory potential of GBS is not solely

mediated by viable, virulent bacteria but also by the interaction of bacterial lysates with the innate immune system as recently shown in a study using live and heat-killed GBS with blood-derived human PMNs.³ Neutrophil granulocytes (PMNs) are of special interest in the context of pregnancy-related GBS-associated pathologies due to their ability to form neutrophil extracellular traps (NETs) when activated.⁴ During this process, PMNs release decondensed nuclear chromatin decorated with histones and granule-derived antimicrobial proteins, such as myeloperoxidase (MPO), into the extracellular space to entrap and kill pathogens.^{5,6} While NETs play an important role in protecting the organism from pathogens, studies in mouse models have demonstrated that they can also cause tissue damage and contribute to the pathogenesis of certain diseases, including sepsis-associated encephalopathy,⁷ bacterial meningitis and neuroinflammation.^{8,9} It was also shown that NETs seem to play a role in lipopolysaccharide-induced depression-like behaviors in mice as defined by several behavioral tests including the open field test.¹⁰ Thus, NETs may represent a potential therapeutic target for inflammation-induced depression.¹⁰ Several in vivo and in vitro models are available to study the mechanisms of NET formation and the pathophysiological role of PMN function in GBS-associated disease. While mice have been widely used to study the role of NETs in health and disease,^{9–11} rat models^{12,13} are of particular interest in the context of GBS-associated disease,¹⁴ as they are commonly used to study behavioral changes, sickness responses and neurodegenerative diseases.¹⁵ Moreover, significant differences exist between murine and human PMNs, regarding their receptors, signaling pathways, granule proteins, cytokine repertoire and other relevant characteristics.¹⁶ However, knowledge on the difference in PMN functions in mice and rats is still lacking. Thus, a direct comparison between rat and mouse PMNs is essential, as species-specific differences in innate immune responses, including NET formation and cytokine release, may substantially influence the translational relevance and interpretation of experimental findings derived from these commonly used animal models.¹⁷ Here, we applied a negative immunomagnetic selection protocol to isolate bone marrow-derived rat and mouse PMNs and investigated their responses to GBS and LPS stimulation, focusing on NET formation and the secretion of the pro-inflammatory cytokines TNF- α and IL-6. LPS was included as a well-established gram-negative inflammatory stimulus^{10,16} and served as a positive control for cytokine induction.

Materials and Methods

Animals

Young adult (14–20 weeks old), immunocompetent Wistar rats and C57BL/6J mice of both sexes were bred in-house, group-housed, and maintained under specific pathogen-free conditions. The age range of animals was chosen according to previous studies for rats and mice.^{18,19} Room temperature was maintained at 22°C $\pm$ 1 °C, with a relative humidity of 50% $\pm$ 5%, and artificial lighting was set to a 12 h cycle from 7:00 to 19:00. Original breeding animals were sourced from Charles River WIGA (Sulzfeld, Germany). Animals had access to drinking water and standard laboratory chow ad libitum.

Isolation of Bone Marrow-Derived PMNs

To obtain PMN cultures, the femora and tibiae were separated from the hind limbs and the isolation of PMNs was conducted as previously shown.²⁰ The negative magnetic selection was carried out using the EasySep™ Mouse Neutrophil Enrichment Kit (Stemcell Technologies Inc., Vancouver, Canada) to obtain mouse PMN cultures or the custom-made EasySep™ Rat Neutrophil Enrichment Kit (Stemcell Technologies Inc., Vancouver, Canada) to obtain rat PMN cultures, in accordance with the manufacturer's protocol. The number of isolated cells was determined with a hemocytometer (Neubauer-Improved, Merck, Darmstadt, Germany). The cells were diluted in RPMI 1640 (Capricorn Scientific, Ebsdorfergrund, Germany) supplemented with 2 mM L-glutamine (Thermo Scientific, Dreieich, Germany) to a concentration of 6.7×10^5 cells/mL and 2×10^5 PMNs per well were seeded onto glass coverslips coated with poly-L-lysine (1 mg/mL H₂O; Sigma Aldrich, Munich, Germany). These coverslips were fixed to reusable flexiPERM®-micro-12 chambers (Sarstedt, Nümbrecht, Germany).

Preparation of Heat-Inactivated Group B *Streptococcus* Lysates

Four colonies of *S. agalactiae* ATCC BAA-1176 (Lot. 60629637), Type III of fresh overnight cultures on sheep blood agar (PB5008A; Oxoid, Wesel, Germany) were picked and inoculated in Todd-Hewitt-bouillon (11708962; BD Biosciences, Heidelberg, Germany). Suspensions were incubated at 37 °C, 5% CO₂ until the early exponential growth phase of OD₅₇₀ of 0.1 was reached. Bacteria were washed 2 times with PBS and resuspended in RPMI (11835063; Gibco, Merck, Darmstadt, Germany) to an OD₅₇₀ of 1.4, representing 5×10⁸ CFU/mL which was determined by cell count after serial dilutions. Aliquots of 0.5 mL were heat-inactivated for 45 minutes at 80 °C while being shaken at 500 rpm, before being flash-frozen in liquid nitrogen. Bacterial lysates were stored at −80 °C.

Induction of Neutrophil Extracellular Traps (NETs)

PMN cultures derived from either male or female animals were treated separately with lipopolysaccharide (LPS, O111:B4, L2630, Sigma-Aldrich, Munich, Germany) or heat-inactivated Group B *Streptococcus* lysates (GBS; *S. agalactiae* ATCC BAA-1176, Type III in RPMI, 5.4×10⁸ CFU/mL, LOT #11/2024, Institute of Biochemistry, University of Veterinary Medicine Hannover) to induce the formation of NETs. To study the dose-dependent effect of NET formation, various concentrations were applied: LPS at 1, 10 and 20 µg/mL and GBS at 1, 10, 100 and 200 multiplicity of infection (MOI). As a negative control for NET formation, PBS was applied, while β-methylcyclodextrine (CD, 25 nM; Sigma-Aldrich, Munich, Germany) was used as a positive control.²¹ Following stimulation, PMN cultures were incubated for 1, 2, 3 and 4h at 37°C and 5% CO₂ to assess the time-dependent effects of NET formation. Following incubation, the supernatants were collected and stored at −80 °C for further cytokine analysis, while coverslips were detached from the chamber and fixed with 4% paraformaldehyde (PFA; Sigma-Aldrich, Munich, Germany) for 15 min.

Quantification of NETs

For the immunodetection of NET components, the fixed PMN cultures were incubated with 400 µL of blocking buffer (PBS, 0.5% TritonX-100, 5% normal donkey serum) for 20 min at room temperature. Primary antibodies diluted in blocking buffer were applied for 1 h at room temperature: rabbit anti-human myeloperoxidase (1:1000; A0398, Dako, Glostrup, Denmark), mouse anti-DNA-Histone 1 (1:2000; MAB3864, Millipore, Burlington, USA) as previously shown.²² After 3 washing steps with PBS, the secondary antibodies, goat anti-mouse Cy3 (1:1000; 115–165-003, Jackson, Newmarket UK) and donkey anti-rabbit Alexa Fluor™ 488 (1:500; A21206, Thermo Scientific, Dreieich, Germany) diluted in blocking buffer were applied for 45 min. Nuclei were stained with DAPI (1µg/mL, MoBiTec, Göttingen, Germany) for 15 min, and coverslips were mounted with CitiFluor™ AF1 mounting medium (Electron Microscopy Sciences, Hatfield PA, USA) onto microscope slides.

Using a fluorescent microscope (Leica DM6 B-Z, Leica Microsystems, Wetzlar, Germany), pictures were taken at 20x magnification in five pre-determined locations to ensure a uniform and unbiased representation of NET formation. Quantification was carried out using ImageJ (Fiji, National Institutes of Health, Bethesda, USA). To assess the total number of cells, the channels were separated and the DAPI channel (blue) was used for cell counting. After conversion to the 8-Bit format, the background threshold and minimal particle size were determined, and DAPI-stained cells were counted automatically. For subsequent manual counting of all the NET-positive cells, the ImageJ Cell Counter plugin was used to label each individual cell. To categorize PMNs as NET-positive, they had to exhibit the typical signs of NET formation, such as nuclear decondensation, loss of shape, extrusion of DNA/Histone-complexes (red), MPO (green) and/or nuclear material into the extracellular space, or having contact with neighboring NETs as previously shown.²³ The rate of NET formation was calculated as the percentage of NET-positive cells per all DAPI-stained nuclei as an average of five images per well.

Cytokine Analysis

The pro-inflammatory cytokines TNF-α and IL-6 were measured in the supernatants of cultured PMNs using bioassays as previously described.²⁴ TNF-α was measured based on its cytotoxic effect on a mouse fibrosarcoma cell line (WEHI 164

subclone 13), and the IL-6 level was determined based on its proliferative effect on a B9 hybridoma cell line. WEHI cells at a cell number of 50,000 cells/well and B9 cells at a cell count of 5,000 cells/well were cultured in a 96-well plate. The cells were maintained and incubated at 37 °C, 95% humidity, and 5% CO₂. Once the 96-well plates were prepared, the standards (National Institute for Biological Standards and Control) and samples were added in dilution series and incubated for 24 h (TNF- α) or 72 h (IL-6). Tetrazolium bromide (MTT, Sigma-Aldrich, Munich, Germany) was added for an additional 4 h, after which the reaction was stopped by adding an isopropanol-hydrochloric acid solution. The plates were left at RT for 12 h before photometric measurements to quantify the colorimetric effect of MTT conversion to formazan using an ELISA plate reader. The level of TNF- α present in supernatants was inversely proportional to the amount of living WEHI cells/detected formazan levels, while the level of IL-6 present was directly proportional to the amount of alive B9 cells/assessed formazan levels. Based on the known standards and linear regression analysis, the amounts of TNF- α and IL-6 were calculated with detection limits as follows: 3 I.U./mL of IL-6 and 6.0 pg/mL of TNF- α .

Flow Cytometry

After isolation, cells were stained with the following antibodies: FITC mouse anti-rat CD45 (OX-1), APC mouse anti-rat CD11b and PE mouse anti-rat granulocytes (RP-1) (BD Biosciences, Heidelberg, Germany) to quantify the purity of rat PMN cultures according to Francis et al (2019).²⁵ After fixation, cells were analyzed using a MACSQuant™ Analyzer 10 flow cytometer (Miltenyi Biotec, Bergisch Gladbach, Germany; [Supplementary Material](#)). Control samples included unstained samples, single-stained samples and fluorescence-minus-one controls. The data were analyzed using the FlowJo™ software (Tree Star, Ashland, OR, USA, v. 1.0.8.1). Populations were analyzed in triplicate samples from 4 independent experiments, each containing at least 5000 events. Some contamination with eosinophil granulocytes was determined ([Supplementary Figure 1](#)).

Statistical Analysis

To evaluate the effect of different treatments on the inflammatory response of PMNs, statistical analyses were conducted using the Prism GraphPad 9 software (GraphPad software, San Diego, CA, USA). Outliers were excluded using the ROUT method (Q=2%) and data were tested for normality using a Kolmogorov–Smirnov test. For normally distributed values, a one-way ANOVA followed by Dunnett's post-hoc test was performed. For non-normally distributed data, a Kruskal–Wallis' test followed by Dunn's post-hoc test was performed. To evaluate the effect of two parameters (treatment and incubation time), a two-way ANOVA and Tukey's post-hoc test were performed. $p < 0.05$ was applied for statistical significance and means $\pm$ SD are displayed on the graphs.

Results

GBS Increases NET Formation in Rat and Mouse PMNs

After treating the cells with GBS and LPS at different concentrations and time points, the percentage of NETs in relation to the total cell count was assessed. PBS was used as a negative control and CD (25 nM) as a positive control, inducing NETs in almost 100% of cells.²⁶ In rat PMN cultures, GBS induced NETosis in a dose-dependent manner. At MOI 10, GBS significantly increased the number of PMNs that form NETs. At MOI 100, the effect reached its maximum, resulting in around 10% of the PMNs undergoing NETosis, proving to be the most suitable concentration for further investigation ([Figure 1A](#)). Starting from 3h, GBS caused a significant induction of NETs when compared to the negative control. Moreover, the largest difference between the PBS negative and CD positive controls was also noted at the 3h time point and, thus, it proved to be the optimal time point for further investigation ([Figure 1B](#)).

Next, we compared NET formation in rat and mouse PMNs 3h after stimulation with different doses of LPS and GBS at a concentration of MOI 100. In both species, GBS significantly affected NET formation in PMN cultures, while LPS had no effect on NET induction. Only the highest dose of LPS (20 μ g/mL) triggered a significant increase in NET formation in rat PMNs ([Figure 1C](#) and [D](#) but only a tendency in B at 3h). No NET formation was noted in mouse PMNs stimulated with LPS.

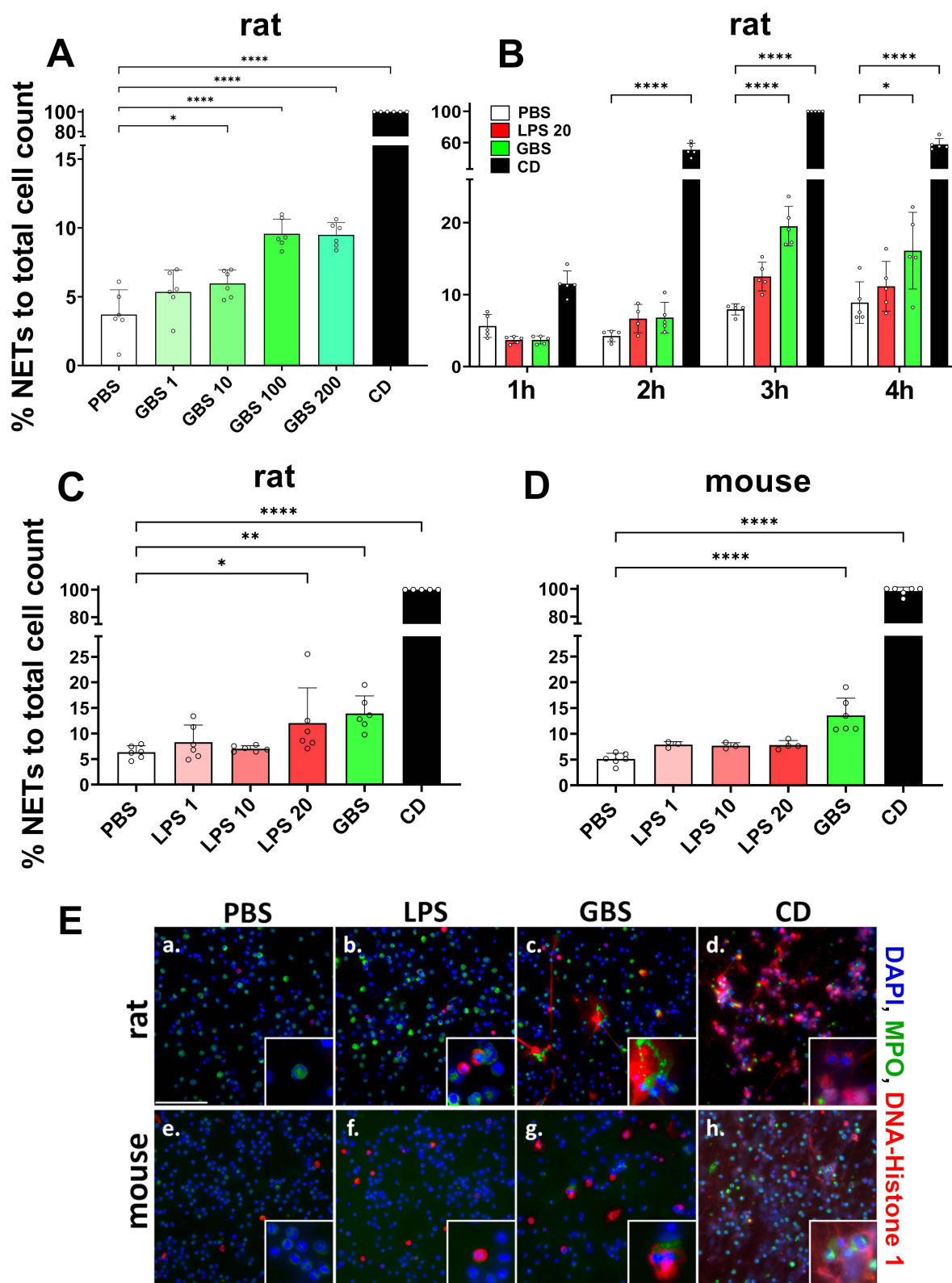


Figure 1 GBS significantly increased NET formation in rat and mouse PMNs, while LPS had minimal effects. **(A)** Percentage of PMNs forming NETs in relation to total cell count after treating rat PMN cultures with GBS lysates at MOI 1, 10, 100, 200 for 3h. **(B)** Percentage of PMNs forming NETs in relation to total cell count in rat cultures at different time points. Cell cultures were exposed to LPS (20 µg/mL) and GBS (MOI 100) and NET formation was assessed after 1, 2, 3 and 4h. **(C and D)** Percentage of PMNs forming NETs in relation to total cell count after exposing rat **(C)** or mouse **(D)** PMN cultures to LPS (1, 10, 20 µg/mL) or GBS (MOI 100) for 3h. **(E)** Representative microphotographs of NET formation stimulated for 3h by LPS (10 µg/mL) and GBS (100 MOI) compared to PBS controls. Cell cultures were stained with DNA-histone I (red) as a NET marker and myeloperoxidase (MPO, green) as marker for neutrophil granulocytes. Nuclei were stained with DAPI (blue). The chosen color for the image description on the right-hand side represents the respective color of the staining depicted in the microphotographs. High-magnification insets depict differences in the morphology of NETs between rat (a-d) and mouse (e-h). Scale bars in a. represent 100 µm, and 50 µm in insets and apply for a-h. The qualitative analysis of NET morphology was conducted in 6 independent experiments. Data are represented as bar graphs with mean data points and error bars showing mean $\pm$ SD for **(A, C and D)**. Each data point represents the mean of two to three technical replicates from one independent experiment, with each independent experiment corresponding to cells isolated from a single male or female animal (n= 3–6). Data demonstrate the percentage of NETs relative to the total cell count. Statistical analysis was performed by one-way ANOVA and Dunnett's post-hoc test. For **(B)** data are represented as bar graphs with individual data points and error bars showing mean $\pm$ SD out of 3 to 6 independent experiments. Statistical analysis was performed by two-way ANOVA and Tukey's post-hoc test.

Immunofluorescence imaging revealed a difference in NET morphology between mouse and rat PMNs. While NETs in mouse PMN cultures appeared to be more rounded and puffy, NETs in the rat PMN cultures formed more web-like structures as demonstrated in the exemplary microphotographs (Figure 1E).

GBS Increases the Secretion of Pro-Inflammatory Cytokines in Rat but Not in Mouse PMNs

In rat PMNs, TNF- α and IL-6 levels showed a significant increase after 3h under both gram-negative (LPS) and gram-positive (GBS) inflammatory conditions (Figure 2A and C).

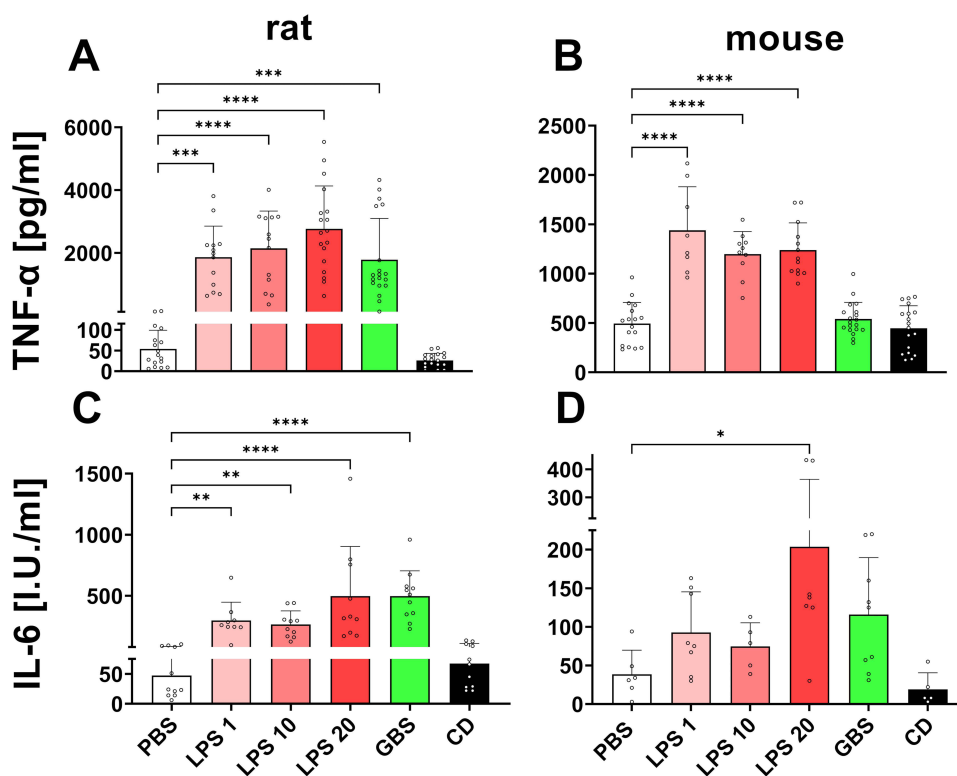


Figure 2 GBS significantly increased the secretion of TNF- α and IL-6 in rat PMNs, but not in mouse PMNs. **(A and B)** TNF- α levels (pg/mL) in the supernatants of rat PMN cultures **(A)** and mouse PMN cultures **(B)** after treating the cells with LPS (1, 10, 20 µg/mL) or GBS (MOI 100) for 3h. **(C and D)** IL-6 levels (I.U./mL) in the supernatants of rat **(C)** and mouse PMN cultures **(D)** after treating the cells with LPS (1, 10, 20 µg/mL) and GBS (MOI 100) for 3h. Data are represented as bar graphs with individual data points and error bars showing mean $\pm$ SD. Each point represents a technical replicate from 5 to 6 independent experiments. Statistical analysis was performed by Kruskal–Wallis' test and Dunn's post-hoc test for **(A, C and D)** Statistical analysis was performed by one-way ANOVA and Dunnett's post-hoc test for **(B)** P-values shown: *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

In mouse PMNs, TNF- α levels were significantly increased after stimulation with all LPS doses. IL-6 levels were only increased after treating the cells with the highest dose of LPS (20 μ g/mL). GBS had no significant impact on either TNF- α or IL-6 secretion by mouse PMNs (Figure 2B and D).

As expected, our positive control for NET induction (CD) was a very potent inducer of NETosis but did not alter the secretion of either cytokine in mice or rats (Figure 2A–D).

Discussion

Our data shows certain similarities and differences in the inflammatory response of rat and mouse PMNs, regarding NET formation and cytokine secretion.

A key limitation of studies investigating PMN cultures is purity. Sounbuli et al (2023) achieved a purity of $80.3 \pm 0.3\%$ using the EasySepTM Mouse Neutrophil Enrichment Kit to isolate PMNs from murine bone marrow.²⁷ Using the custom-made EasySepTM Rat Neutrophil Enrichment Kit, we obtained a purity of $65 \pm 8\%$ PMNs. Additionally, $10 \pm 3\%$ of the isolated cells were myeloid progenitor cells, likely containing immature PMNs (Supplementary Figure 1). By applying a similar FSC-SSC gating strategy as Sounbuli et al (2023) to rat samples, purity increases to $76 \pm 5\%$, comparable to the mouse level. The observed contamination by other cell types, particularly eosinophils ($13 \pm 5\%$), raises the question of whether the effects observed were exclusively attributable to direct stimulation of PMNs. Current data describing direct interactions between eosinophils and PMNs under physiological conditions are limited. In contrast, both cell types are known to contribute to inflammation in certain pathological conditions, such as chronic rhinosinusitis,²⁸ where eosinophil extracellular traps have also been described.²⁹ Although the contribution of eosinophils cannot be excluded, the observed responses most likely reflect the predominant PMN population. Further optimization of the isolation protocol in future studies may enable a more precise characterization of the contribution of eosinophils to GBS-stimulation.

Applying heat-inactivated GBS to rat PMNs induced NET formation in a dose-dependent manner with the strongest effect peaking at a MOI of 100. Previous studies performed with live GBS also showed a concentration dependent effect on the immune response of PMNs with a peak at MOI 20,³⁰ though studies elucidating the effects of GBS lysates on PMNs remain scarce.^{3,31}

The 3h time point proved to be optimal for our investigation since the observed effect of GBS on NET formation was the most pronounced compared to the negative control. After incubating the cells for more than 3h, the negative control showed an increase in NET formation. Previously, it was demonstrated that PMNs undergo spontaneous NETosis in vitro in a time-dependent manner.³² Thus, our findings suggest that incubation times longer than 3 hours should be considered a limitation for future studies investigating NET formation in rat PMNs.

LPS only increased NETosis minimally in our experiments, whereas it has previously been shown to be a potential NET inducer in vivo and in vitro.³³ The ability of LPS to induce NET formation in vitro has been shown to be dependent on different factors, such as the dose and serotype of LPS.³⁴ Interestingly, this study also demonstrated no NET induction after applying the same serotype of LPS (O111:B4) as in the present study.

The observed differences in NET morphology between mice and rats may either reflect species-specific variations³⁵ in NET composition or distinct mechanisms of NET formation. To gain further mechanistic insights, future studies should distinguish between vital and suicidal NET formation. To achieve this aim, different time points between 30 minutes and 3 hours of stimulation could be evaluated by establishing a cell death stain in addition to NET markers.^{36,37}

Cytokine analyses revealed that rat PMNs secrete greater amounts of TNF- α and IL-6 in response to LPS and GBS than murine PMNs. When investigating cytokine secretion, contamination by monocytes or eosinophils should be considered.^{38,39} Eosinophils accounted for $13 \pm 5\%$ of the rat PMN cultures, indicating a potential contribution to the observed cytokine release. A detectable contamination with monocytes could not be revealed (Supplementary Figure 1).

TNF- α and IL-6 belong to the canonical first-line innate immune response to infection and inflammation involved in immune-to-brain communication, acting as humoral mediators to transfer inflammation to the brain, inducing brain-controlled sickness-type responses such as fever.^{40–42} In our hands, GBS exposure did not induce TNF- α secretion in mouse PMNs. However, murine PMNs have already been shown to secrete TNF- α in response to heat-inactivated GBS.³⁰ The use of lyophilized bacteria, however, makes direct comparison difficult.

While few data are available on LPS-induced cytokine secretion of human PMNs,⁴³ we still do not know whether their secretion of IL-6 and TNF- α in response to GBS is more similar to murine or rat PMNs. However, studies employing human mononuclear cells⁴⁴ or whole blood,⁴⁵ have reported upregulation of cytokines.

Overall, rat PMNs could be a complementary model to investigate NET and cytokine induction by GBS.

Conclusion

These findings demonstrate species-specific differences between rat and mouse PMNs in their responses to inflammatory stimuli, particularly regarding NET formation and cytokine secretion. NET formation was induced in both rat and mouse PMNs after treatment with GBS. Interestingly, we observed different NET morphologies in mouse and rat NETs with immunofluorescent microscopy. These differences should be considered in the future when investigating NET formation using immunofluorescence.

Secretion of pro-inflammatory cytokines was more pronounced in rat PMNs with GBS significantly increasing TNF- α and IL-6 in rat, but not mouse PMNs. Overall, we demonstrate that both rat and mouse PMN cultures are suitable to study PMNs inflammatory response to pathogens in vitro, including GBS, complementing previous assessments in rat^{46–48} and mouse in vivo models.^{49,50}

Future research is warranted to gain more insights into potential sex- and age-dependent⁵¹ differences in the inflammatory response of rat and mouse PMNs, and to elucidate the signaling pathways and mechanisms underlying our observations.

Abbreviations

CD, β -methyl-cyclodextrine; FCS, fetal calf serum; GBS, Group B *Streptococcus*; iGBS, invasive GBS disease; LPS, lipopolysaccharide; MOI, multiplicity of infection; MPO, myeloperoxidase; NDI neurodevelopmental impairment; PMN, neutrophil granulocyte; NET, neutrophil extracellular trap; PBS, phosphate-buffered saline; PBS-T, PBS with TritonX-100; PFA, paraformaldehyde.

Data Sharing Statement

The data that supports the findings of this study are available from the corresponding author, CR, upon reasonable request.

Ethics Statement

All animal experiments were performed in accordance with the German animal welfare law and were declared to the Internal Animal Care and Use Committee of the Justus Liebig University Giessen (Animal Welfare Officers; registration no.: 679_M and 797_M). This study was reported in accordance with the Animal Research: Reporting of in vivo Experiments (ARRIVE) guidelines where relevant. They were consistent with the recommendations of the AVMA Guidelines for the Euthanasia of Animals.

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Author Contributions

Jan Stoll: Writing – original draft, Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Validation, Visualization; Sheila Bauch: Writing – original draft, Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Validation, Visualization; Blandine Lieubeau: Writing – review and editing, Data curation, Investigation, Methodology, Validation; Ivan Conejeros: Writing – review and editing, Data curation, Investigation, Methodology, Validation; Marita Meurer: Writing – review and editing, Conceptualization, Investigation, Methodology, Validation; Maren von Köckritz-Blickwede: Writing – review and editing, Conceptualization, Investigation, Methodology, Validation; Stephan Leisengang: Writing – review and editing, Validation; Lois May Harden: Writing – review and editing, Conceptualization, Validation. Jessica Kate Hernandez: Writing – review and editing, Conceptualization, Data curation,

Formal Analysis, Investigation, Methodology, Validation; Julie Hervé: Writing – review and editing, Funding Acquisition, Project Administration, Resources, Supervision, Methodology, Validation; Christoph Rummel: Writing – review and editing, Conceptualization, Funding Acquisition, Methodology, Project Administration, Resources, Supervision. All authors took part in drafting, revising, or critically reviewing the article, gave final approval to the version to be published, have agreed on the journal to which the article has been submitted, and agree to be accountable for all aspects of the work.

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Disclosure

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