

Humanized Mice, a New Model To Study the Influence of Drug Treatment on Neonatal Sepsis

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Bacterial infection with group B *Streptococcus* (GBS) represents a prominent threat to neonates and fetuses in the Western world, causing severe organ damage and even death. To improve current therapeutic strategies and to investigate new approaches, an appropriate *in vivo* model to study the immune response of a human immune system is needed. Therefore, we introduced humanized mice as a new model for GBS-induced sepsis. Humanized mice feature deficiencies similar to those found in neonates, such as lower immunoglobulin levels and myeloid cell dysfunction. Due to the husbandry in specific-pathogen-free (SPF) facilities, the human immune cells in these mice also exhibit a naive phenotype which mimics the conditions in fetuses/neonates. Following infection, cytokine release and leukocyte trafficking from the bone marrow to the lymphoid organ (spleen) and into the peritoneum (site of infection) as well as bacterial spreading and clearance were traceable in the humanized mice. Furthermore, we investigated the effects of betamethasone and indomethacin treatment using this novel sepsis model. Although both drugs are commonly used in perinatal care, little is known about their effects on the neonatal immune system. Treatment of infected humanized mice not only induced the reduction of human leukocytes in the spleen but also increased the bacterial load in all analyzed organs, including the brain, which did not show infiltration of live GBS in untreated controls. These studies demonstrate the utility of the humanized mice as a new model to study an immature human immune response during bacterial infection and allow the investigation of side effects induced by various treatments.

Streptococcus agalactiae (group B streptococcus [GBS]) is a leading cause of invasive neonatal infection, inducing sepsis, pneumonia, and meningitis (1–3). This problem is aggravated by the emergence of strains with antibiotic resistance against clindamycin, erythromycin, and penicillin which are routinely used in the clinic for intrapartum antibiotic prophylaxis (4, 5).

While GBS-colonized healthy adults do not show any signs of infection, this pathogen can cause severe invasive bacterial infections in immunocompromised hosts, in elderly patients, and especially in neonates. Primarily responsible for the rapid disease progression in neonates is the inexperienced and immature immune system, which features various deficiencies. To mimic the naive and immature human immune system *in vivo*, we introduced the humanized mouse model in our studies: transplantation of CD34⁺ hematopoietic stem cells (e.g., isolated from human cord blood) into immunodeficient mice (NOD.Cg-Prkdc^{scid}Il2rg^{tm1Wjl}/SzJ [NSG]) induces the development of a human immune system (6, 7) and allows the investigation of human diseases in a small animal model (8–11). However, some human immune cell populations are weakly present or exhibit functional deficiencies in these mice which mimic the impaired neonatal immune system in many cases. For example, the low number of human granulocytes in humanized mice is comparable to the situation in neonates with a limited neutrophil storage pool in the bone marrow (12) and multiple dysfunctions of neutrophils such as reduced chemotaxis, phagocytosis, and bactericidal activity (13).

Likewise, monocytes from humanized NSG mice and human cord blood-derived neonatal monocytes displayed a similar phenotype concerning their phagocytic activity and expression of costimulatory molecules (14).

Furthermore, the protective environment of the amniotic cavity of neonates and humanized mouse husbandry in a specific-pathogen-free (SPF) facility induces a similar naive phenotype of

the adaptive immune cells in neonates (15–18) and humanized mice (11). The maturation of human T cells in the mouse thymus is dependent on the murine major histocompatibility complex (MHC) (lack of positive selection on human MHC). As a result, the induction of T cell-dependent B cell activation, the production of human immunoglobulins (Ig), and the class switch toward antigen-specific IgG are generally poor in humanized mice (19). Similar findings are observed in neonates, including reduced MHC II and CD86 expression (20, 21) and low Ig levels (16, 18, 22). Neonatal B cells also exhibit a reduced class switch capacity toward antigen-specific IgG because of lower interleukin-4 (IL-4), IL-5 receptor, and IL-2 receptor γ chain (IL-2RG) expression (23–25).

Another example of immunological similarities found in both neonates and humanized mice is complement deficiency. Several studies described reduced amounts of complement factors with weak activity in newborns (26–29), impairing the clearance of bacterial infections. Similarly, there is a lack of

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human complement as well as dysfunctional mouse complement in humanized mice (30–32).

On the basis of these criteria, we selected the humanized mouse model for our studies using NSG newborns transplanted with cord blood-derived hematopoietic stem cells. These mice developed a naive human immune system due to the SPF husbandry. Subsequently, mice were inoculated with GBS and the effects of two different agents which are already applied in the clinic in situations of threatened preterm delivery were investigated: betamethasone, a glucocorticoid which is used to prevent neonatal respiratory distress syndrome and decreases neonatal mortality in infants born at 24 to 34 weeks of gestation (33), and indomethacin, a nonsteroidal anti-inflammatory drug which is applied in the clinic as a second-line tocolytic agent (33, 34). Since more than 40% of preterm births are associated with infection (35, 36), both drugs sometimes counteract an ongoing bacterial infection in the fetus. Studies using neonatal rats and piglets investigated the effect of indomethacin and glucocorticoids during GBS infection, giving valuable insights into inflammation alteration in a neonatal immune system (37–40). However, the immune responses in infection and after treatment sometimes exhibit severe differences between species (41, 42). Hence, the objective of this study was to establish a new neonatal humanized sepsis model to investigate the effects of treatment on a human immune system *in vivo* in detail.

MATERIALS AND METHODS

Animals. NOD.Cg-Prkdc^{scid}Il2rg^{tm1Wjl/SzJ} (NSG) mice were obtained from Jackson Laboratories (Bar Harbor, ME) and kept and bred in a specific-pathogen-free animal facility at the University of Regensburg. In order to generate humanized mice, newborn NSG mice received sublethal irradiation (1 Gy) and were subsequently injected intrahepatically with 2×10^5 CD34⁺ stem cells, isolated from human cord blood using the MACS cell separation system according to the manufacturer's protocol (Miltenyi Biotec, Bergisch Gladbach, Germany).

Ethics statements. All animal work was approved by the local veterinary authorities from the district government based on the international European guidelines and national regulations of the German animal protection act (permission no. 54-2532.1-18/10).

Cord blood samples were taken with approval from the Ethics Committee of the University of Regensburg (permission no. 11-101-0231). All patients included in the experiments provided written informed consent. All experiments were performed in accordance with relevant institutional and national guidelines, regulations, and approvals.

Bacterial strain. The group B streptococcus (GBS) reference strain ATCC 13813 (generously provided by the Department of Medical Microbiology and Hygiene, University Hospital of Regensburg) was grown from frozen stocks on 8% sheep blood agar plates. Subsequently, bacteria were adjusted to an optical density of 0.4 corresponding to 2×10^8 CFU/ml. For injection, appropriate numbers of CFU were adjusted to 1 ml in phosphate-buffered saline (PBS) (Pan Biotech, Aidenbach, Germany).

Infection model. Mice were infected with GBS at the age of 3 to 5 months. In order to determine appropriate infectious doses for humanized mice, 10^5 to 10^8 CFU was injected intraperitoneally (i.p.) ($n = 56$ humanized mice, $n = 11$ irradiated control mice).

For the majority of experiments, two doses were defined. A moderate dose of infection with GBS (10^6 CFU; animals sacrificed after 3 [$n = 10$] and 7 [$n = 7$] days, respectively) was chosen to study the human immune response with high rates of survival. In order to analyze more pronounced short-time effects of GBS infection, a high dose of GBS (10^7 CFU) was inoculated i.p. and animals were sacrificed and analyzed after 24 h postinfection ($n = 10$).

For the treatment studies, infected animals received either betametha-

none (5 mg/kg; $n = 28$), indomethacin (3 mg/kg; $n = 18$), or vehicle (PBS; $n = 42$) alone. High-dose-infected animals were treated 3 h postinfection. In the moderate-dose model, animals received two treatments i.p.: the first one after 24 h, the second one 48 h postinfection.

Preparation of mononuclear cells from various tissues and peripheral blood. Mononuclear cells (MNC) were isolated from peripheral blood (pb), spleen, mesenteric lymph node (mLN), liver, lung, kidney, brain, bone marrow (bm), and peritoneum. For preparation of MNC from pb, mice were anesthetized and bled retrobulbarly. Blood (100 μ l) was mixed with 20 μ l 0.5 M EDTA to prevent clotting and stained for flow cytometric analysis. Subsequently, animals were killed by cervical dislocation and cells from various tissues were extracted. The peritoneal cavity of each mouse was rinsed with 10 ml PBS plus 2 mM EDTA plus 2% fetal calf serum to isolate peritoneal exudate cells (PEC). To obtain MNC of bm, femurs were removed and the ends were clipped off and rinsed with 20 ml PBS plus 2 mM EDTA using a syringe with a 27-gauge needle (BD Bioscience, Franklin Lakes, NJ). The spleen, mLN, liver, and lung were weighted, passed through a 40- μ m-pore-size cell strainer (BD), and rinsed with 20 ml PBS plus 2 mM EDTA. The resulting MNC suspensions were either used directly to determine the number of CFU in the corresponding organ or analyzed by flow cytometry.

Cells from lung and liver were resuspended using 5 ml 40% Percoll (GE Healthcare, Uppsala, Sweden) diluted with RPMI medium (Pan Biotech, Aidenbach, Germany) and underlayered using 5 ml 70% Percoll/RPMI. Gradients were centrifuged for 20 min at 800 relative centrifugal force (RCF) at room temperature, and the interphase was collected and washed with PBS. Liver and lung stromal cells from the upper layer and erythrocytes from the bottom layer were discarded. Subsequently, MNC from the interphase were counted and 10^6 cells were stained for flow cytometric analysis.

CFU in various organs. Organs (spleen, liver, lung, kidneys, brain, and bm) were homogenized and serially diluted 1:10 down to 10^{-8} using PBS, plated on 8% sheep blood agar plates, and grown overnight at 37°C. Colonies were counted, and the number of CFU per g of organ was calculated (for bm and peritoneal lavage, the number of CFU per ml was determined).

Flow cytometric analysis of MNC. Reconstitution of humanized mice with human leukocytes and leukocyte subpopulations were analyzed with an LSR-II flow cytometer (BD Bioscience, San Jose, CA). Cells from pb, spleen, liver, lung, peritoneum, mLN, and bm were stained using the following human-specific monoclonal antibodies: anti-CD45-APC (clone HI30), anti-CD19-Pe (clone HIB19), anti-CD4-FITC (clone SK3), anti-CD66-Pe (clone B1.1/CD66), and anti-CD33-PerCP-Cy5.5 (clone P67.6) from BD Bioscience, anti-HLA-DR-Pe-Cy7 (clone LN3), anti-CD80-Biotin (clone 2D10.4), anti-CD3-PerCP-Cy5.5 (clone OKT3), anti-CD45RA-Pe-Cy7 (clone HI100), and anti-CD27-Biotin (clone 0323) from eBioscience (San Diego, CA), and anti-CD8a-APC (clone HIT8a) from Biologend (San Diego, CA). For isotype control stainings, the appropriate monoclonal mouse antibodies were used.

Cytokine detection. To determine cytokine production of human leukocytes, 100 μ l blood was drawn retrobulbarly from humanized mice. Blood was allowed to clot at 4°C for 20 min, and serum was collected after two succeeding centrifugations (3,000 RCF and 10,000 RCF, 10 min at 4°C each). The resulting serum was stored at -80°C. Human cytokine concentrations (pg/ml) of serum samples were analyzed by Multimatrix GmbH (Regensburg, Germany) using the cytokine human 6 Plex Panel (interleukin [IL]-1 β , IL-6, IL-8, IL-10, gamma interferon [IFN- γ], and tumor necrosis factor [TNF]) (Life Technologies GmbH, Darmstadt, Germany). For acquisition of the mean fluorescence intensity, the Luminex xMAP 100 system (Luminex Corp., Austin, TX) was used and the data were analyzed using the Liquichip Analyzer software (Qiagen, Hilden, Germany).

Histology. Tissue specimens (spleen, liver, brain, thymus, lymph nodes, and lung) were fixed with 4% formalin, embedded in paraffin, and stained as previously described (11). Briefly, specimens were deparaffinized and pretreated by microwave heating for 30 min at 320 W in 0.1 M

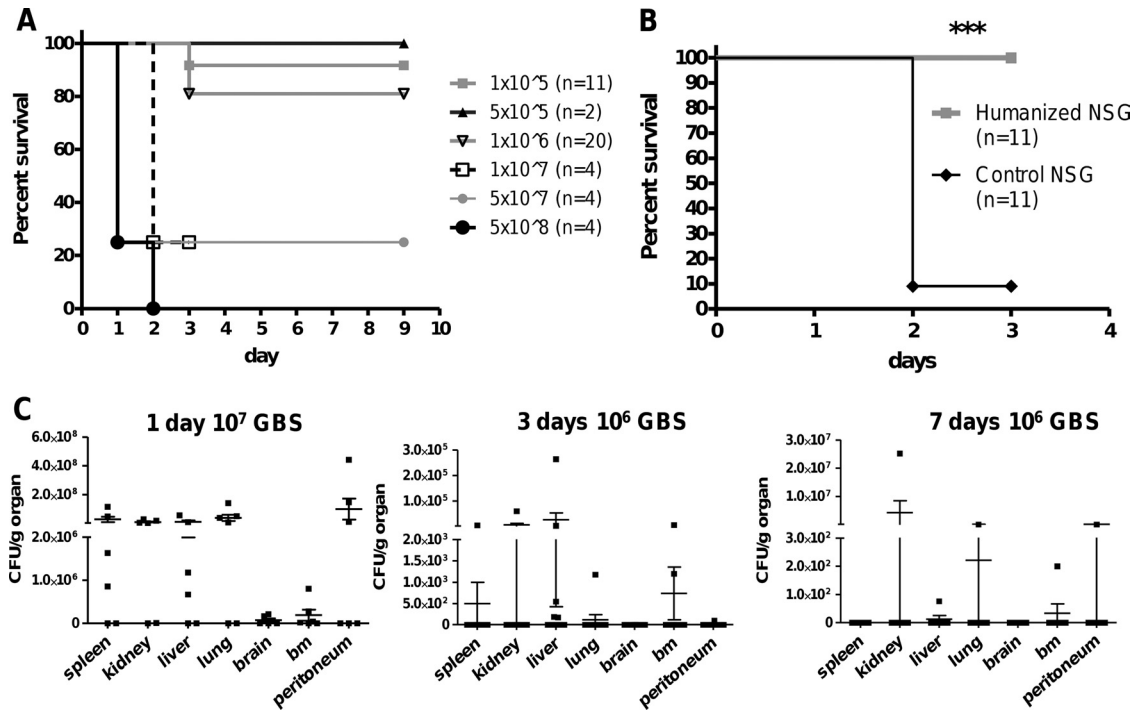


FIG 1 Dose-dependent survival of infected humanized mice and controls. (A) Survival curves of *Streptococcus agalactiae* (GBS)-infected humanized mice. (B) Survival curves of 1×10^6 GBS-infected humanized mice and conditioned control mice (irradiated but not reconstituted with human CD34⁺ cells). Numbers of animals are indicated in parentheses. Statistical differences were calculated using Mantel-Cox (log rank) test (***, $P < 0.001$). (C) CFU count for each organ was determined after 1 day of high-dose infection (left graph, 10^7 CFU GBS; $n = 10$) and 3 days (middle graph, $n = 10$) and 7 days (right graph, $n = 6$) of moderate-dose (1×10^6) infection.

citrate buffer adjusted to pH 7.3. The immunostaining was automatically performed on a Ventana Nexes autostainer (Ventana, Tucson, AZ) by using the streptavidin-biotin-peroxidase complex method with 3,3'-diaminobenzidine as a chromogen. The following antibodies were used: anti-CD3 clone SP (Thermo Fisher Scientific Inc., Fremont, CA) and anti-CD79a clone JCB117 and anti-CD45/LCA clone 2B11-PD7/26 (Dako, Glostrup, Denmark).

Statistics. Statistical analysis was performed using GraphPadPrism (GraphPad Software, La Jolla, CA). All data are represented as mean \pm standard error of the mean (SEM) and were tested for statistical significance using Student's *t* test, analysis of variance (ANOVA), Bonferroni posttest, log rank, or Tukey's multiple comparison test, as indicated in the figure legends.

RESULTS

Dose-dependent disease progression in GBS-infected humanized mice. The transplantation of CD34⁺ stem cells resulted in high levels of human immune cell reconstitution (percentage of human CD45/isolated cells) in various organs, including the spleen, liver, bone marrow, and lymph nodes of humanized mice 3 to 5 months posttransplantation (see Fig. S1A in the supplemental material). Lower levels of reconstitution (percentage of human CD45/isolated cells) were measured in the lung and peritoneal exudate cells (PEC). Of note, lung and liver stromal cells and erythrocytes were removed before the reconstitution levels were calculated. To monitor the development of human lymphocytes, the mice were bled and analyzed by flow cytometry at different time points (see Fig. S1B). Human T cell differentiation in the thymus of these mice requires a longer period of time than human B cell maturation in the bone marrow, and therefore T cell num-

bers increase steadily in the peripheral blood (see Fig. S1B). To ensure a reliable T cell population during infection, we performed all experiments in humanized mice older than 15 weeks. The presence of human immune cells in the different organs obtained from flow cytometric analysis (see Fig. S1) was confirmed using immunohistochemistry (see Fig. S2 in the supplemental material).

To identify the optimal dose of infection, different doses of bacteria were injected intraperitoneally. Lower doses of GBS (1×10^5 to 5×10^5) led to the survival of 90 to 100% of infected animals (Fig. 1A), whereas injection of 10^7 GBS resulted in the death of 80% of the animals 3 days postinfection. Higher doses led to a more rapid and pronounced disease progression with $\sim 75\%$ (5×10^7) to 100% (5×10^8) lethality. In order to ensure that the human and not the residual mouse leukocytes play a prominent role in the immune response to the GBS infection, conditioned NSG mice (without human immune cells) as well as humanized littermates were infected with 10^6 CFU. After 2 days, 10 of 11 infected radiated NSG mice died, whereas all (11/11) humanized littermates survived (Fig. 1B). CFU concentration for each organ was determined for humanized mice infected with high and moderate doses of GBS (Fig. 1C).

For the following experiments, two doses were defined. A moderate dose of infection (10^6 CFU; animals sacrificed after 3 and 7 days) was chosen to study the human immune response with high rates of survival. In order to analyze more pronounced short-term effects of GBS infection, a high-dose infection (10^7 CFU) was inoculated and animals were sacrificed after 24 h.

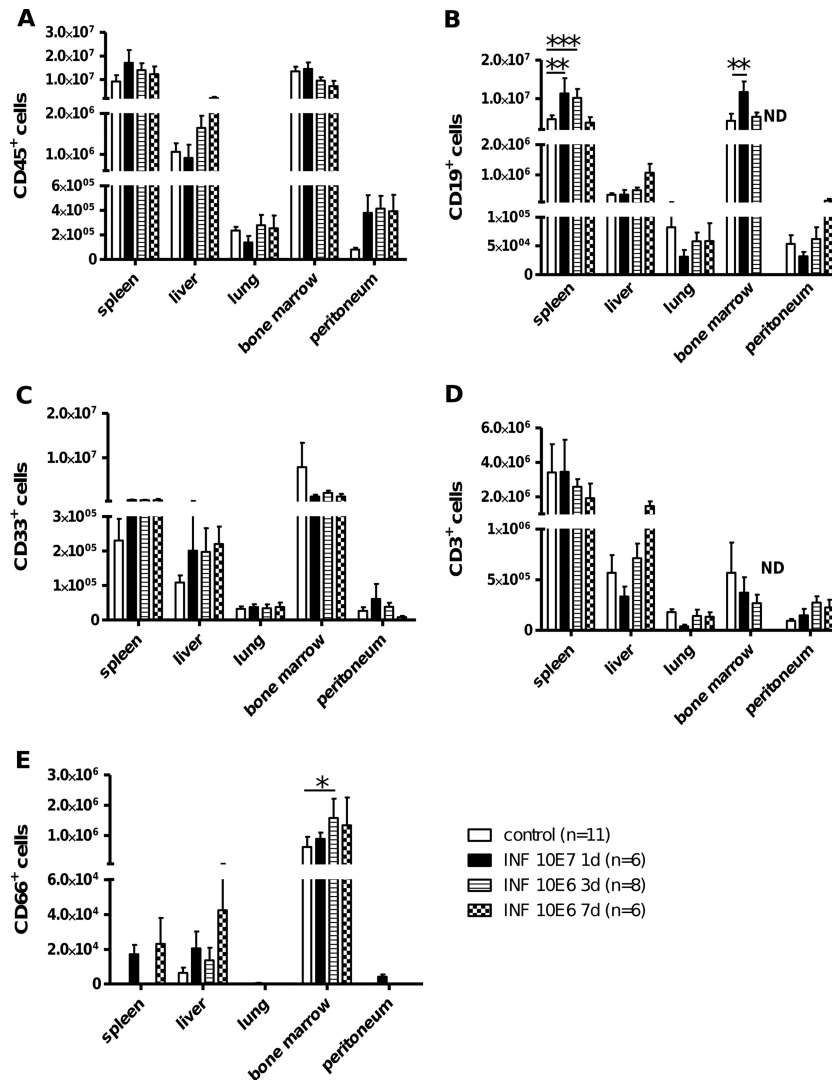


FIG 2 Human immune cell trafficking in GBS-infected humanized mice. Humanized mice were infected with a moderate dose of GBS (1×10^6 CFU) for 3 days or 7 days or a high dose (1×10^7 CFU) for 1 day. The total numbers of human immune cells (CD45⁺) (A), human B cells (CD19⁺) (B), human myeloid cells (CD33⁺) (C), human T cells (CD3⁺) (D), and human granulocytes (CD66⁺) (E) were calculated from different organs, including peritoneal exudate cells (PEC). Significances between groups were analyzed using the Bonferroni posttest: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. ND, not done.

Although intraperitoneal (i.p.) injection of 10^6 CFU led to a rapid systemic spread of GBS (data not shown), some humanized mice were able to clear the infection completely after 3 (4/10) and 7 (4/6) days. These animals were free of live bacteria in all tested organs (lung, liver, peritoneum, bm, kidney, and spleen), were in generally good health, and gained weight (data not shown). Infection with 10^7 CFU led to rapid systemic dissemination of live bacteria. While the highest concentration of CFU was at the site of injection (peritoneum), high numbers of bacteria were found in all organs investigated, even in the brain of 5/10 animals (Fig. 1C).

Migration of immune cells after *Streptococcus agalactiae* infection in humanized mice. To analyze the effect of GBS infection on migration of cells in humanized mice after bacterial infection, the total numbers of cells from different organs (lung, liver, spleen, peritoneum, and bm) were determined. Most organs did not show significant changes in the total leukocyte count (without differentiation of human or mouse origin) after GBS infection

(data not shown). However, there was a marked decrease in the total number of cells in the bm 3 days postinfection, which vanished 4 days later. A trend toward higher numbers of splenocytes could be detected 1 and 3 days postinfection, which became significant on day 7. Further investigation of the total numbers of different human immune cell subsets in various organs after infection revealed no significant changes in the total amount of human leukocytes (CD45), myeloid cells (CD33), or T cells (CD3) (Fig. 2A, C, and D). No significant changes were detectable in the CD4-CD8 distribution or their phenotype (naive versus memory) (data not shown). Only human B cells (CD19) significantly increased in the bm (3 days postinfection) and in the spleen (3 and 7 days postinfection) after a moderate dose of GBS inoculation (Fig. 2B). CD66⁺ granulocyte levels were low in all organs but were significantly increased after 3 days of a moderate dose of infection in the bm (Fig. 2E).

Further, we investigated whether leukocyte trafficking in pe-

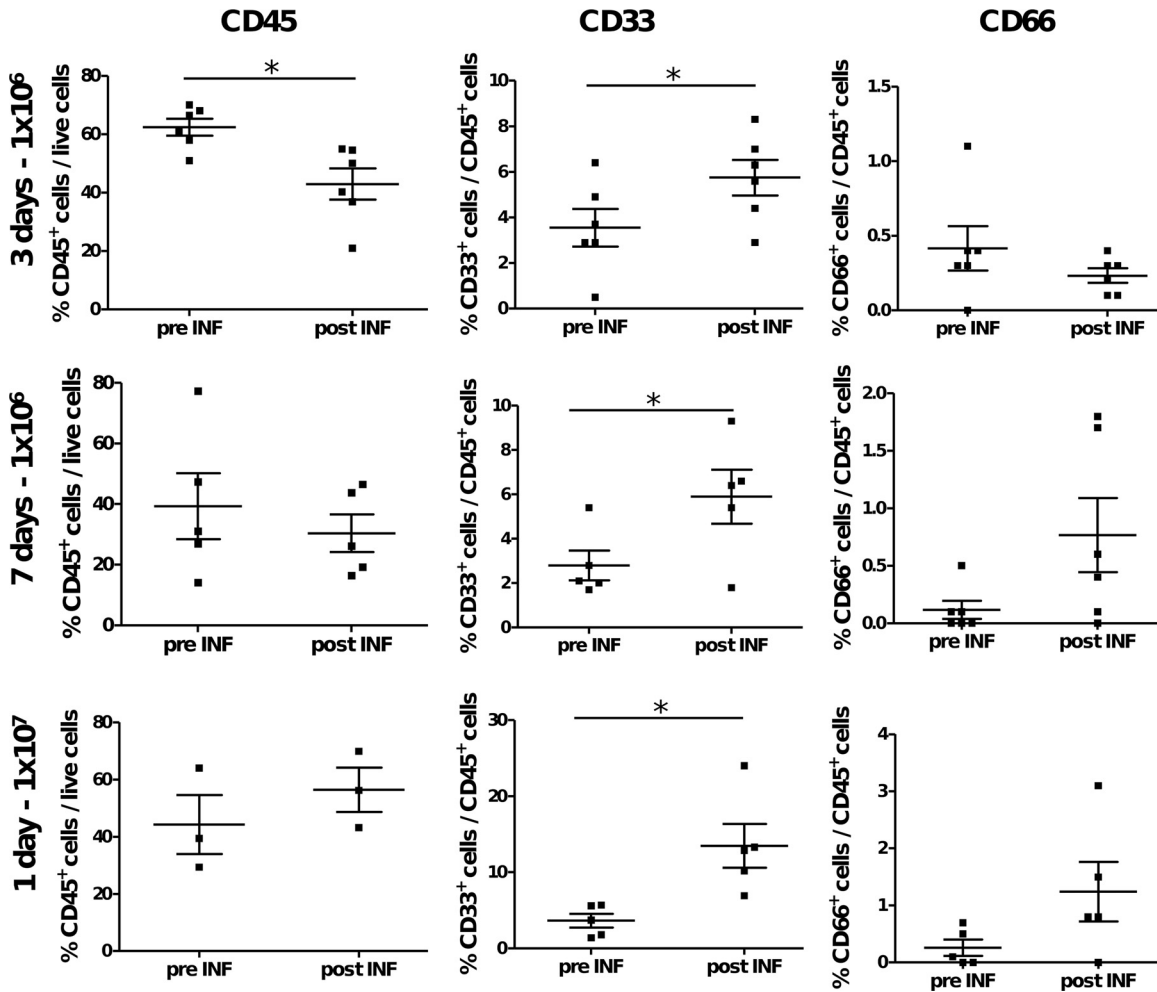


FIG 3 Influence of GBS infection on peripheral blood leukocyte populations in humanized mice. Humanized mice were infected with a moderate dose of GBS (1×10^6 CFU) for 3 days and 7 days as well as 1 day with a high dose of GBS (1×10^7 CFU). The percentage of human leukocytes (CD45⁺), human myeloid cells (CD33⁺), and human granulocytes (CD66⁺) in pb before (pre INF) and after (post INF) the infection was determined using flow cytometry. Values were analyzed using Student's *t* test; *, $P < 0.05$.

peripheral blood occurred. On day 3, the percentages of human CD45⁺ cells (Fig. 3) and human B cells (data not shown) were significantly reduced whereas the percentage of myeloid cells (CD33) was significantly elevated on day 3 and on day 7 (10^6 CFU) and also after 24 h of high-dose infection (10^7 CFU) (Fig. 3). The percentage of human T cells (CD3) was significantly increased after high-dose infection but not after moderate infection (data not shown).

To investigate the migration capacity of human immune cells to the site of infection, PEC were further analyzed and revealed a significant increase in the percentage of human leukocytes at each time point and dose of infection compared to those of uninfected controls (Fig. 4A). On day 3, a marked increase of T cells was observed (Fig. 4B). An increase of the myeloid cell population was obvious after high dose and after 3 days of a moderate dose of infection but was reduced again on day 7. Granulocytes were only present 24 h after high-dose inoculation with GBS.

Increased serum cytokine levels in GBS-infected humanized mice. Serum was analyzed during the acute inflammation after high-dose infection (10^7 CFU; 1 day) and revealed the induction

of human proinflammatory cytokines (IFN- γ , TNF, IL-1 β , and IL-6), chemokines (IL-8), and counterregulatory (anti-inflammatory) cytokines (IL-10) (Fig. 5). A trend of increased cytokine levels (except IL-1 β) was also found in humanized mice 2 and 3 days postinfection with moderate doses of GBS (10^6) (data not shown).

Indomethacin and betamethasone influence bacterial load and immune cell distribution. To study the influence of both treatments on the infection in humanized mice, animals were injected with GBS (10^7 or 10^6 CFU) and treated with either betamethasone or indomethacin in the high-dose (1 day) or moderate-dose (3 or 7 days) infection model.

In order to successfully fight off bacterial infections, the ability to inhibit the systemic spreading of bacteria is crucial. Therefore, the effect of treatment on systemic bacterial clearance (spleen, kidney, liver, lung, brain, bm, and peritoneum) was analyzed in all experiments. In the moderate-dose model (3 days postinfection), betamethasone significantly reduced systemic clearance compared to vehicle (PBS)-treated animals (Fig. 6A). Although indomethacin-treated animals also displayed a trend toward reduced

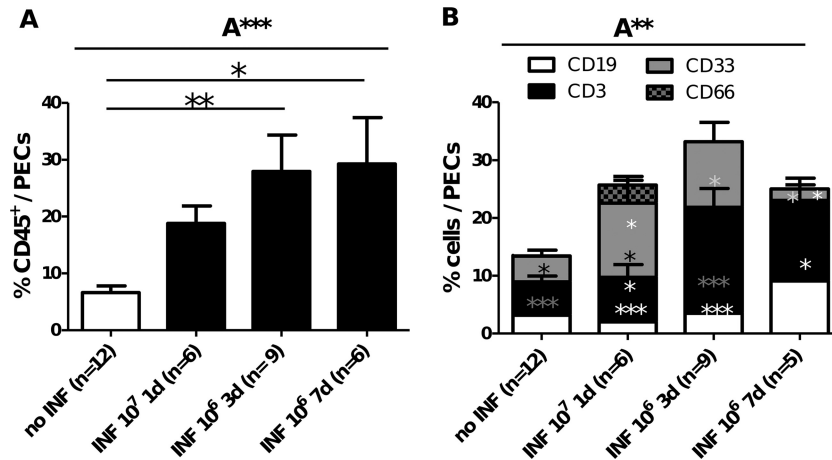


FIG 4 Influx of human immune cells into the peritoneal cavity of GBS-infected humanized mice increased over time. Humanized mice were infected (INF) for 1 day with a high dose (1×10^7 CFU) as well as 3 or 7 days with a moderate dose (1×10^6 CFU) of GBS. The percentage of human leukocytes (CD45⁺) (A) as well as that of human B cells (CD19⁺), T cells (CD3⁺), myeloid cells (CD33⁺), and granulocytes (CD66⁺) (B) was analyzed and compared to levels in noninfected humanized mice. Numbers of animals are indicated in parentheses. Significances between groups were analyzed with 1-way (A) or 2-way (B) ANOVA: A***, $P < 0.001$; A**, $P < 0.01$. Additionally, significances between groups were analyzed with Tukey's multiple comparison test (1-way ANOVA) (*, $P < 0.05$; **, $P < 0.01$) and the Bonferroni posttest (2-way ANOVA; significances are marked within the columns in the same shading style for corresponding comparisons) (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

clearance in nearly all organs, the differences were not significant (Fig. 6B). Importantly, the presence of live bacteria in the brain after infection with 10^6 CFU was detected only in animals receiving treatment and never observed in untreated controls (Fig. 6C).

After 7 days of moderate-dose infection, a trend toward larger amounts of CFU in betamethasone- as well as indomethacin-injected mice was detectable, indicating a lower rate of clearance in treated animals at this time point as well (data not shown).

Characterizing the influence of treatment on cytokine production during acute infection (high dose) revealed a reduction in levels of the proinflammatory cytokines TNF, IFN- γ , and IL-6 after betamethasone treatment (Fig. 6D). Indomethacin treatment of a moderate 3-day infection increased IL-8 significantly but did not alter other cytokine levels (data not shown).

To determine the effect of betamethasone and indomethacin on the migration of leukocytes during GBS infection, the percentage of the cell populations in the pb (Fig. 7A) and the total number of immune cell populations in the organs (Fig. 7B) were analyzed in moderate-dose-infected humanized mice 3 days postinfection. In pb, a reduction of the percentage of CD45⁺ cells occurred after infection but was not changed by either treatment whereas both treatments significantly decreased the percentage of B cells (CD19) (Fig. 7A). Betamethasone induced an increase in the CD33⁺ myeloid cell population after infection. This effect was even more pronounced when animals were treated with indomethacin (Fig. 7A).

In the spleen, betamethasone treatment reduced the total number of human CD45⁺ cells, human B cells (CD19), and human T cells (CD3) (Fig. 7B, panels I, II, and III). In the bm, glucocorticoid treatment significantly increased the number of human B cells (Fig. 7B, panel II) and decreased the number of human myeloid cells (Fig. 7B, panel IV) compared to PBS-treated mice.

Indomethacin significantly reduced the levels of human leukocytes (Fig. 7B, panel I), human B cells (Fig. 7B, panel II), and human T cells in the spleen (Fig. 7B, panel III) and significantly decreased the amount of human CD8⁺ T cells (Fig. 8) compared

to PBS-treated mice. No differences in immune cell distribution were found after 7 days of infection (data not shown).

DISCUSSION

Bacterial infections, especially GBS infections, continue to present a major threat to neonates and fetuses, and an appropriate model to study the effect of new or already existing treatments in the context of an immature human immune system is still missing.

In this study, we introduced humanized mice as a new animal model for neonatal GBS infection. The human immune system in the animals was generated by transplanting CD34⁺ hematopoietic stem cells from human cord blood into newborn NSG mice which developed a naive human immune system due to SPF husbandry. The ongoing human immune response against GBS infection was characterized, and the feasibility of testing side effects of treatments in this humanized sepsis model was investigated exemplarily using betamethasone and indomethacin.

During the last decades, studies in mice have helped to elucidate the role of the immature neonatal immune system during bacterial and parasitic infection (43–48). Nevertheless, many significant differences between the mouse and human immune systems have been described (41), including differential expression of TLR, different immunoglobulin isotypes and receptors, and various proportions of leukocyte populations, as well as effects of certain proteins and factors (e.g., nitric oxide production). These differences between mice and humans might explain why virtually none of the treatments that had a significant impact in murine models of sepsis worked in the following 25 clinical trials in patients (49, 50). In comparison with humans, rodents are generally highly resistant to most types of induced inflammation. For example, the sensitivity to lipopolysaccharide (LPS) is 1,000 to 10,000 times higher in humans (51, 52) than in mice (53). These distinctly different immune responses are most likely due to diverse reactions in humans and mice, such as, e.g., cytokine release. This aspect became clearly recognizable during the first clinical tri-

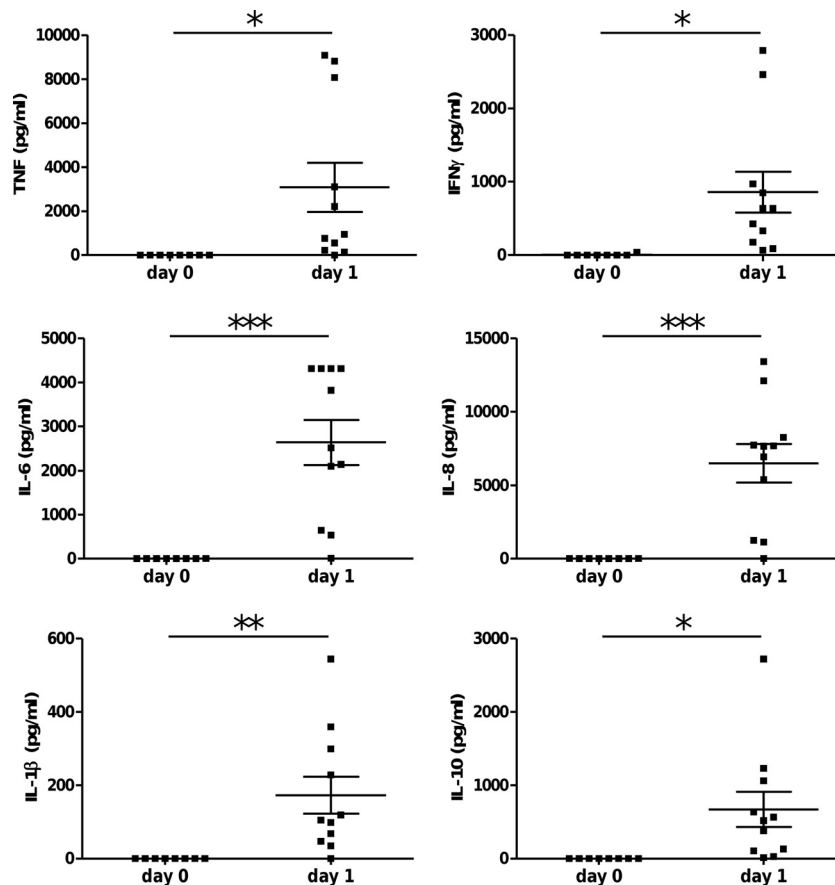


FIG 5 Human cytokine response in GBS-infected humanized mice. Humanized mice were infected with a high dose (1×10^7 CFU; $n = 11$) of GBS, and 24 h later the serum levels of human TNF, IFN- γ , IL-1 β , IL-6, IL-8, and IL-10 were determined. Humanized mice without infection served as controls ($n = 8$). Significant differences were calculated using the Student *t* test: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

als in which TNF was injected for cancer treatment and did not induce symptoms in mice (54) but triggered severe side effects in humans (55). Similarly, the manipulation of immune responses using monoclonal antibodies can result in a completely different outcome in both species. The latest example is the agonistic monoclonal antibody TGN1412, which did not show critical side effects in mice (56, 57), whereas human volunteers receiving this antibody displayed respiratory distress, pulmonary infiltrates, cytokine storm, and lymphopenia (58). In addition, mice often clear infections more rapidly without systemic spreading, inflammation, or kidney, lung, and liver damage, which are an important part of the sepsis syndrome in humans leading to the death of the patient.

Unsinger and colleagues used humanized mice to study apoptosis induction in human immune cells following cecal ligation and puncture (CLP). The study revealed that the immune response (e.g., cytokine release and lymphocyte apoptosis induction) after bacterial challenge in these animals is comparable to that in humans (59).

We extended this study and investigated human immune cell trafficking in GBS-infected humanized mice. Despite certain deficiencies, the human immune cells displayed migration capacity, as shown by the emigration out of the bm (reduced cell count after infection) into the peritoneal cavity (site of infection) and the lymphoid organs (spleen) passing the transport medium (pb). In

addition, the induction of proliferation and maturation was reflected by a significantly increased population of CD66⁺ granulocytes in the bm at 3 and 7 days postinfection. One day after high-dose infection, human CD66⁺ granulocytes were able to migrate into the peritoneum but were unable to control the infection or prevent systemic spreading.

The influence of treatment with betamethasone or indomethacin on migration and/or proliferation in various organs was visible in infected humanized mice as observed in the overall reduction of CD45⁺ leukocytes in the spleen (caused mainly by a reduction of human T cells). In the bone marrow, the number of myeloid cells decreased but the number of human B cells increased after both treatments.

Induction of human proinflammatory as well as counterregulating anti-inflammatory cytokines and chemokines in the serum proved the functional activation of human immune cells in these mice. The plasma cytokine levels in infected humanized mice are comparable with those of septic neonatal mice (60–63). Septic human adults and neonates also display increased plasma levels of the cytokines measured in this study; however, they do not reach the plasma concentrations of existing animal models for sepsis or infected humanized mice (64, 65). This might be due to a lower bacterial burden in human patients. Glucocorticoid treatment had no prominent effect on the cytokine response but resulted in systemic elevation of CFU counts after 3 days of infection. An

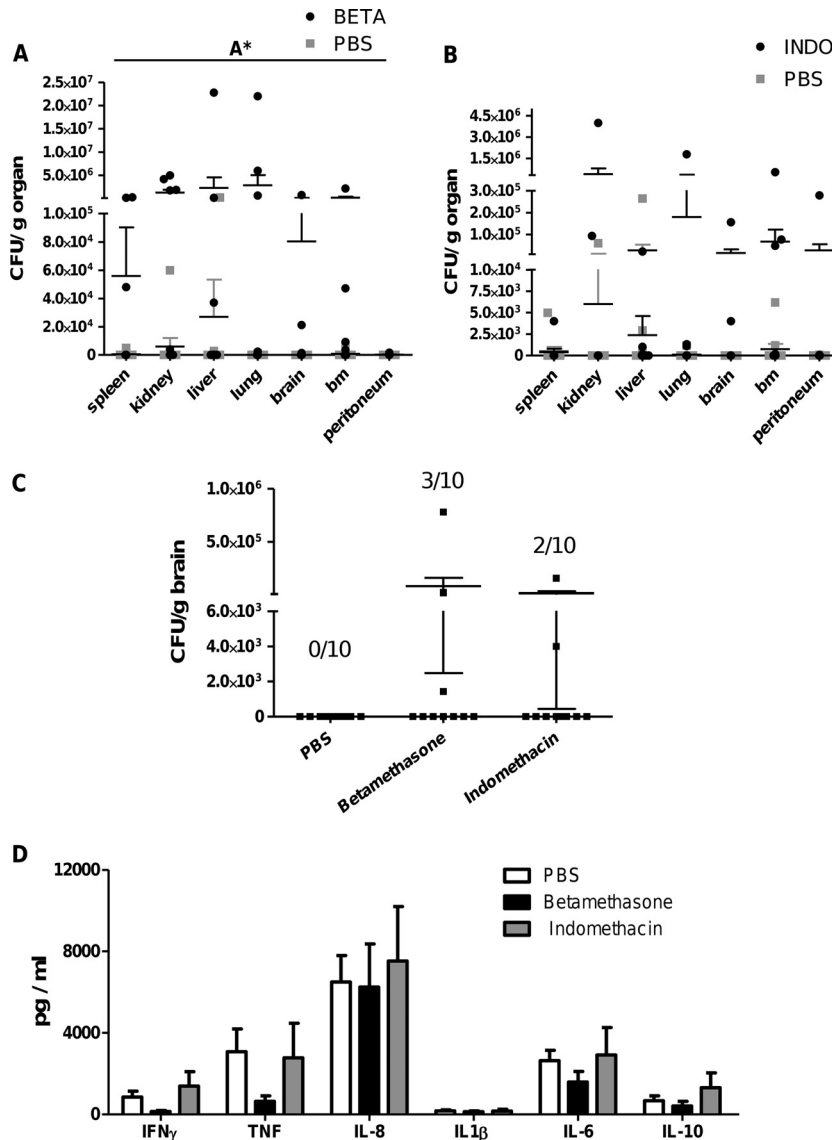


FIG 6 Influence of betamethasone (BETA) and indomethacin (INDO) treatment on bacterial load and cytokine release in GBS-infected humanized mice. Humanized mice were infected with a moderate dose (1×10^6 CFU) of GBS and treated twice (24 and 48 h p.i.) with betamethasone (A) ($n = 10$), indomethacin (B) ($n = 10$), or vehicle alone (PBS) ($n = 10$). On day 3, mice were sacrificed and CFU per organ were analyzed for each animal. (C) CFU/g brain after 3 days of infection with or without indomethacin or betamethasone treatment. Numbers indicate CFU⁺ mice/total number of analyzed animals. (D) Humanized mice were infected with a high dose of GBS (1×10^7 CFU). Three hours postinfection, animals received betamethasone ($n = 8$), indomethacin ($n = 4$), or vehicle (PBS) alone ($n = 11$). After 24 h of infection, the serum cytokine levels were analyzed. Significant differences between groups were calculated using 2-way ANOVA (A*, $P < 0.05$).

increased bacterial load following glucocorticoid treatment was also described by Tran and Weisman after infecting newborn rats with GBS (39).

Animals receiving indomethacin only displayed a tendency toward reduced bacterial clearance but showed treatment-induced changes in leukocyte populations similar to those in glucocorticoid-treated humanized mice. Importantly, only in indomethacin (2/10) and betamethasone (3/10)-treated animals infected with a moderate dose was GBS able to translocate into the brain tissue. Our data suggest that indomethacin treatment might have detrimental effects on human neonates suffering from GBS infection. However, studies using neonatal animal models revealed that treatment with this anti-inflammatory drug also has beneficial

effects during GBS infection. Indomethacin reduces sepsis-related pulmonary hypertension, increases arterial partial pressure of oxygen as well as cardiac output, prevents metabolic acidosis, and augments hemodynamics, improving the outcome in GBS-induced sepsis (37, 38, 40, 66).

Our results indicate that in the situation of imminent preterm delivery induced by GBS infection, treatment with betamethasone or indomethacin may have the potential to trigger the course of disease. However, since both therapeutic options (prevention of preterm birth by indomethacin and induction of lung maturation by betamethasone) are necessary for neonatal survival, antibiotic treatment should always be combined with both treatments in the case of suspected bacterial infection to prevent the increase of

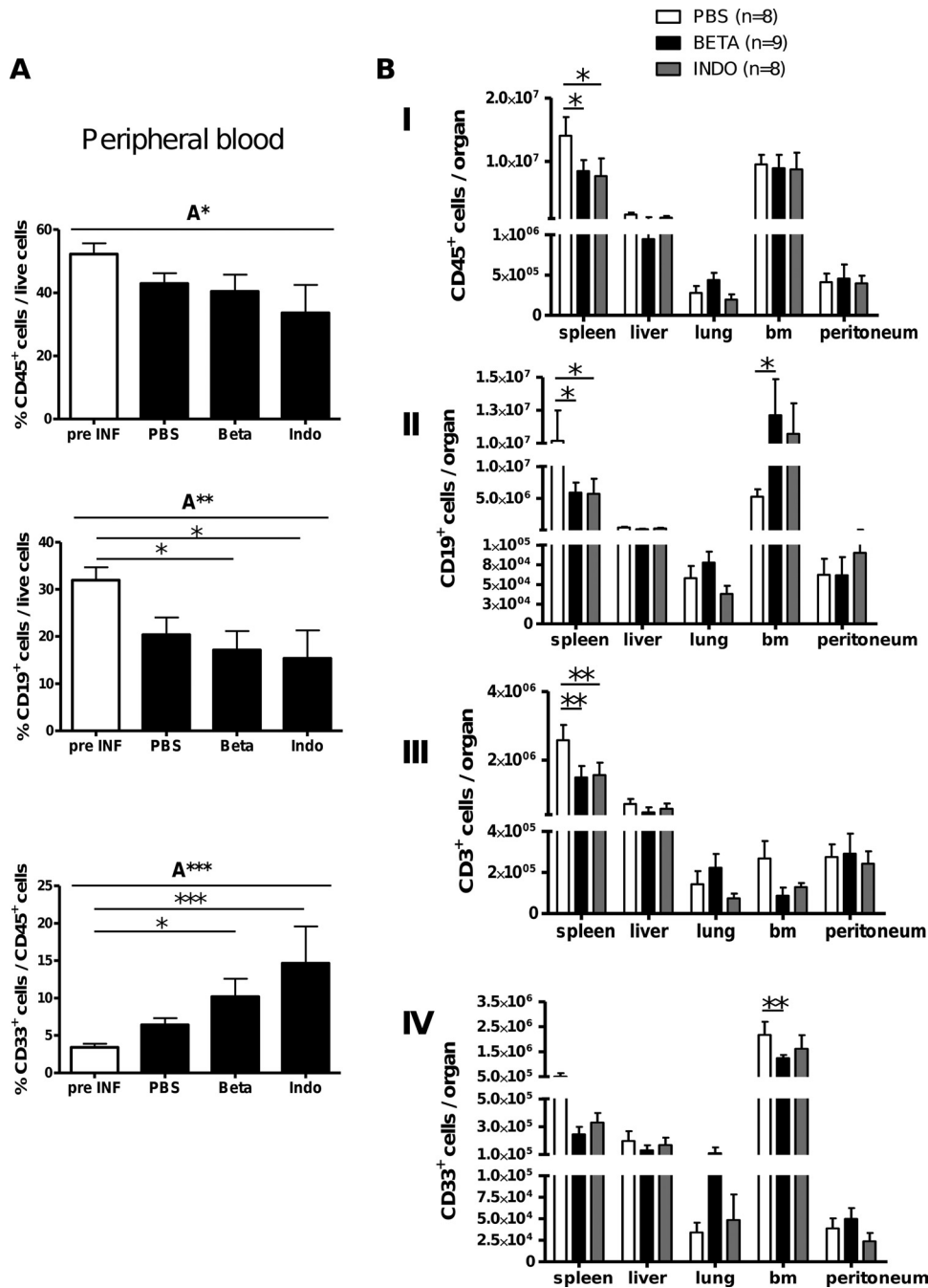


FIG 7 Influence of indomethacin (INDO) and betamethasone (BETA) treatment on human leukocyte populations in GBS-infected humanized mice. Humanized mice were infected with a moderate dose (1×10^6 CFU) of GBS and treated twice (24 and 48 h) with betamethasone ($n = 9$), indomethacin ($n = 6$), or vehicle alone (PBS; $n = 8$). On day 3, mice were sacrificed and leukocyte populations in pb (A) and organs (spleen, liver, lung, bm, and peritoneal cavity) (B) were analyzed. Significant differences were calculated using 1-way (A) or 2-way (B) ANOVA: A*, $P < 0.05$; A**, $P < 0.01$; A***, $P < 0.001$. In addition, significant differences between different treatment groups were calculated using Tukey's multiple comparison test (1-way ANOVA) and the Bonferroni posttest (2-way ANOVA): *, $P < 0.05$; **, $P < 0.01$.

bacterial load and therefore avoid the possible drug-induced side effects described above.

As seen in the individual differences in bacterial load and immune cell distribution, heterogeneity (induced by different stem cell donors) sometimes interfered with the pooling of data and impeded significant differences between groups. Nevertheless,

this model corresponds to the situation in humans more accurately than that of inbred mice with their identical genetic background. To assess the effects of new treatment strategies, a higher number of humanized mice (compared to inbred mice) might be necessary.

Of note, humanized NOD/*scid* and NSG mice still possess

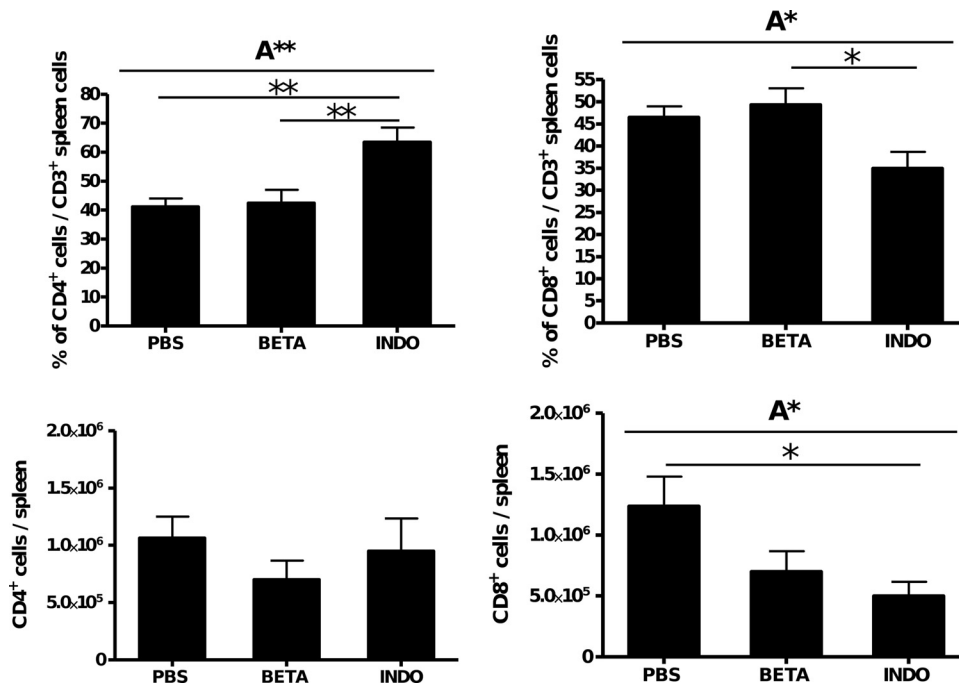


FIG 8 Influence of indomethacin and betamethasone treatment on human T cell population in GBS-infected humanized mice. Humanized mice were infected with a moderate dose (1×10^6 CFU) of GBS and treated twice (24 and 48 h) with betamethasone ($n = 9$), indomethacin ($n = 6$), or vehicle alone (PBS; $n = 8$). On day 3, mice were sacrificed and CD4 and CD8 T cell populations in the spleen were analyzed. Significant differences were calculated using 1-way ANOVA: A*, $P < 0.05$; A**, $P < 0.01$. In addition, significant differences between different treatment groups were calculated using Tukey's multiple comparison test: *, $P < 0.05$; **, $P < 0.01$.

mouse macrophages and granulocytes, but these cells exhibit dysfunctions in maturation and activation (7, 32). These deficient myeloid cells do not seem to play a prominent role in fighting off bacterial infections, since we found that only 1/11 conditioned NSG mice survive GBS infection compared to 11/11 humanized littermates. Nevertheless, mouse cell activity most likely contributes to the immunological processes to a certain degree.

In conclusion, humanized mice feature deficiencies in their immune system similar to those in human neonates. Therefore, they offer a new opportunity to investigate the effects of GBS infection in combination with treatment on a human immune system in a small animal model. Although an animal model will never be able to mimic the situation in human neonates entirely, humanized mice offer a new—and in certain aspects improved—approach to investigate sepsis in the context of human immune responses.

Mouse models remain to be the first choice to test the efficiency of new therapies in high-throughput studies under standardized conditions. However, humanized mice might help to bridge the gap between *in vitro* as well as *in vivo* mouse models and clinical trials to elucidate possible side effects before they enter the stage of clinical testing.

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