Journal of Innate Immunity

Research Article

J Innate Immun 2023;15:1–15
DOI: 10.1159/000524583

Received: July 23, 2021
Accepted: March 17, 2022
Published online: May 10, 2022

Bruton's Tyrosine Kinase in Neutrophils Is Crucial for Host Defense against *Klebsiella pneumoniae*

Zhe Liu[a, b] Alexander P.N.A. De Porto[a, b] Regina De Beer[a, b] Joris J.T.H. Roelofs[b, c] Onno J. De Boer[c] Sandrine Florquin[c] Cornelis Van't Veer[a, b] Rudi W. Hendriks[d] Tom Van der Poll[a, b, e] Alex F. De Vos[a, b]

[a]Center for Experimental and Molecular Medicine, Amsterdam UMC, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands; [b]Amsterdam Infection and Immunity Institute (AI&II), Amsterdam UMC, Amsterdam, The Netherlands; [c]Department of Pathology, Amsterdam UMC, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands; [d]Department of Pulmonary Medicine, Erasmus MC, University Medical Center, Rotterdam, The Netherlands; [e]Division of Infectious Diseases, Amsterdam UMC, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands

Keywords
Bruton's tyrosine kinase · *Klebsiella pneumoniae* · Innate immunity · Neutrophils · Reactive oxygen species

Abstract
Humans with dysfunctional Bruton's tyrosine kinase (Btk) are highly susceptible to bacterial infections. Compelling evidence indicates that Btk is essential for B cell-mediated immunity, whereas its role in myeloid cell-mediated immunity against infections is controversial. In this study, we determined the contribution of Btk in B cells and neutrophils to host defense against the extracellular bacterial pathogen *Klebsiella pneumoniae*, a common cause of pulmonary infections and sepsis. *Btk*$^{-/-}$ mice were highly susceptible to *Klebsiella* infection, which was not reversed by Btk re-expression in B cells and restoration of natural antibody levels. Neutrophil-specific Btk deficiency impaired host defense against *Klebsiella* to a similar extent as complete Btk deficiency. Neutrophil-specific Btk deficiency abolished extracellular reactive oxygen species production in response to *Klebsiella*. These data indicate that expression of Btk in neutrophils is crucial, while in B cells, it is dispensable for in vivo host defense against *K. pneumoniae*.

Introduction

Bruton's tyrosine kinase (Btk) is a member of the family of nonreceptor tyrosine kinases designated "tyrosine kinase expressed in hepatocellular carcinoma". Btk is expressed in all cells of the hematopoietic lineage, except in T and plasma cells [1]. Btk is vital for B-cell development due to its role in signaling of the B-cell receptor and its

Zhe Liu and Alexander P.N.A. De Porto contributed equally to this study.

Karger@karger.com
www.karger.com/jin

Correspondence to:
Alex F. De Vos, a.f.devos@amsterdamumc.nl

immature form, the pre-B-cell receptor [2]. In humans, defects in Btk result in the primary immunodeficiency X-linked agammaglobulinemia (XLA). XLA is characterized by an almost complete block of B-cell development at the pre-B-cell stage, and as a result, circulating B cells and antibodies are very low [3]. The phenotype of Btk-deficient (*Btk*$^{-/-}$) mice, also known as X-linked immune deficiency mice, partially mimics XLA. In *Btk*$^{-/-}$ mice, pre-B-cell differentiation is only mildly impaired, follicular B-cell numbers are reduced, and B-1 cells are absent, and as a result, levels of natural IgM and IgG3 antibodies are low [2]. Both XLA patients and *Btk*$^{-/-}$ mice are highly susceptible to infections [3–5]. Since treatment of XLA patients and *Btk*$^{-/-}$ mice with antibody supplementation causes dramatic reductions in infections and greatly improves survival rates [4, 6], the increased susceptibility for infection due to functional Btk deficiency is thought to result predominantly from impaired B cell-mediated immunity.

Btk, however, mediates several myeloid cell effector functions [1]. In monocytes and macrophages, Btk regulates complement receptor-, Fcγ receptor (FcγR)- and dectin-1-mediated phagocytosis [7–9], although conflicting findings have also been reported [10–12]. Moreover, Btk controls specific bactericidal responses by macrophages [10, 13, 14] and contributes to Toll-like receptor (TLR), triggering receptors expressed on myeloid cells 1 and NLR family pyrin domain containing 3 as well as FcγR-mediated production of inflammatory cytokines by macrophages [8, 13, 15–20]. In neutrophils, Btk has been implicated in integrin-mediated recruitment and activation [21, 22], and in generation of TLR4-mediated reactive oxygen species (ROS) and nitric oxide production [10]. Targeting of Btk in neutrophils protected mice from FcγR/TLR4-mediated acute lung injury [23]. In addition, Btk is required for neutrophil development and expression of granule proteins [24]. Together, these studies led us to hypothesize that the enhanced susceptibility of XLA patients and *Btk*$^{-/-}$ mice for bacterial infections could also be attributed to impaired effector functions of myeloid cells, including neutrophils.

In addition to *Haemophilus influenzae* and *Streptococcus pneumoniae*, *Klebsiella pneumoniae* is among the most frequent pathogens isolated in XLA patients [25–27]. The Gram-negative bacterium *K. pneumoniae* is a common cause of nosocomial pneumonia and sepsis [28]. Severe systemic infections can be caused by hypervirulent *Klebsiella* strains that generate thick mucoviscous polysaccharide capsules [29]. Neutrophils are the most abundant circulating leukocytes, being the first line of host defense against bacterial infection [30]. Previous studies have shown that neutrophils are essential in host defense against *K. pneumoniae*-evoked pneumosepsis [31, 32]. In the present study, we determined the role of Btk in B cells and neutrophils in the immune response against *Klebsiella*. For this purpose, we studied *Btk*$^{-/-}$ mice, *Btk*$^{-/-}$ mice with re-enforced expression of Btk specifically in B cells, μMT mice lacking peripheral B cells and antibodies, and neutrophil-specific *Btk*$^{-/-}$ mice in a well-established model of hypervirulent *Klebsiella*-induced pneumonia and sepsis [31–34].

Materials and Methods

Mice

Wild-type (WT) mice were either purchased from Charles River (Maastricht, Netherlands) or derived from heterozygous breeding of *Btk*$^{-/-}$ mice. *Btk*$^{-/-}$ mice and *Cd19-BTK*$^{+}$ mice were generated as previously described [35, 36], and the latter strain was maintained on a *Btk*$^{-/-}$ background. *Btk*$^{fl/fl}$ mice were generated from *Btk*^{tm1a} embryos (EUCOMM, Institute Clinique de la Souris, Illkirch, France) as described previously [37]. Female *Btk*$^{fl/fl}$ mice were bred to male *Mrp8cre* transgenic mice (Jackson Laboratory) [38] to generate male *Mrp8cre.Btk*^{fl}*/Y* mice with neutrophil cell-specific Btk deletion and littermate controls (*Btk*^{fl}*/Y*) [39]. μMT mice were purchased from Jackson Laboratory. All mice used in these studies were backcrossed at least 8 times to C57BL/6 background and used at 8–12 weeks of age.

Pneumonia and Sepsis Models

Pneumonia was induced using *K. pneumoniae* K2:O1 (ATCC43816) by intranasal inoculation with approximately 1 × 10^4 colony-forming unit (CFU) *K. pneumoniae*, as previously described [32, 33]. Ibrutinib (Selleckchem; 25 mg/kg in water, 5% mannitol, 0.5% gelatin) or vehicle was administered orally 27 and 3 h prior to inoculation as previously described [40]. Mice were sacrificed at 6, 24, or 30–42 h after infection or followed for 9–11 days. Sepsis was induced by intravenous administration of approximately 1–5 x 10^4 CFU *K. pneumoniae* [41], and mice were sacrificed 6 or 24 h later. To determine bacterial loads, samples were processed exactly as previously described [32, 33].

Cell Isolation and Characterization

Mouse bone marrow neutrophils were isolated as previously described [42], using a single layer (62.5%) of Percoll (Sigma). Isolated neutrophils were washed with Hank's balanced salt solution (HBSS) and rested in HBSS with 1.26 mM $CaCl_2$ and 0.49 mM $MgCl_2$ ($HBSS^{+/+}$) for 30 min at room temperature prior to the experiments. Purity of isolated neutrophils was verified by flow cytometry after staining with anti-Ly6G mAb (see below). Single-cell suspensions of the lungs were generated by incubation with 1 mg/mL collagenase D (Roche) for 30 min at 37°C followed by passage through a 19 G needle. Single-cell suspensions of the spleen were generated either by passage through a mesh or by incubation with 1 WU/mL Liberase TL (Roche) and 4 mg/mL lidocaine for 15 min at 37°C. Concentration of cell suspensions and blood leukocytes

were determined using a Z1 counter (Beckman Coulter). Flow cytometric analysis was performed as previously described [40]. Cells were stained using the following labeled antibodies: CD19-PE/eF610 (1D3), CD23-PE/Cy7 (B3B4), CD43-PE (R2/60), CD45-PE/eF610 (30-F11), CD115-PE (AFS98), CD169-eF660 (SER4), F4/80-PE (BM8), MHCII-AF700 (M5/114.15.2) (all eBioscience), CD3-PerCP/Cy5.5 (17A2), CD4-FITC (RM4-5), CD11b-PE/Cy7 (M1/70), CD11c-PerCP/Cy5.5 (HL3), CD21/35-APC (7G6), B220-FITC (RA3-6B2), Ly6C-AF700 (AL-21), Ly6G-APC (1A8), and SiglecF-A647 (E50-2440) (all BD Biosciences). Alveolar macrophages (AMs) were identified as $CD45^+/CD11c^+/SiglecF^+$ cells and lung dendritic cells as $CD45^+/CD11c^+/SiglecF^-/MHCII^+$ cells. In blood, monocytes were identified either as $CD45^+/CD11b^+/Ly6G^-/Ly6C^+$ cells or as $CD45^+/CD11b^+/Ly6G^-/Ly6C^-$ cells, granulocytes as $CD45^+/CD11b^+/Ly6G^+$ cells, and B lymphocytes as $CD45^+/B220^+$ cells. In spleen, granulocytes were identified as $CD45^+/Ly6G^+$ cells and B lymphocytes as $CD45^+/B220^+$ cells. Flow cytometry was performed using a FACSCanto II. Flow cytometry results were analyzed using FlowJo.

ELISA

Cytokine ELISA (TNF, IL-6, IL-1β, IL-10, CXCL1, CXCL2, CCL2, myeloperoxidase [MPO], elastase, MRP8/14: R&D Systems) and bead arrays (TNF, IL-6, MCP-1: BD Biosciences) were performed according to the manufacturer's instructions. For measurement of natural antibodies, plates were coated with 10^8 CFU heat-killed *K. pneumoniae* or 10 μg/ml *K. pneumoniae* lipopolysaccharide (LPS) (Sigma-Aldrich) and bound antibodies were detected using biotinylated anti-mouse IgM or IgG3 antibodies (Southern Biotechnology Associates) and streptavidin-HRP.

Lung Histology and Immunohistochemistry

Lung sections for histological analysis were prepared as described earlier [43]. Sections were analyzed for bronchitis, edema, interstitial inflammation, intra-alveolar inflammation, pleuritis, and endothelialitis. All parameters were graded on a scale of 0–4 with 0 as "absent" and 4 as "severe." The total "lung inflammation score" was expressed as the sum of the scores for each parameter, the maximum score being 24. Granulocyte staining using FITC-labeled rat anti-mouse Gr1 mAb (RB6-8C5; BD Biosciences) or Ly6G mAb (1A8; BioLegend) and quantification was done exactly as described [41]. Staining for citrullinated histone 3 (CitH3; NB100-57135; Novus Biologicals) [44] and phosphorylated histone H2AX (γH2AX; clone 20E3; Cell Signaling Technology) [45] was performed as previously described. The presence of neutrophil extracellular traps (NETs) in inflammatory foci in the lung was scored semiquantitatively in a blinded manner as 0: negative; 1: <10% of inflamed area positive for CitH3; 2: 10–50% of inflamed area positive for CitH3; and 3: >50% of inflamed area positive for CitH3. Cell death was scored in a blinded manner by counting γH2AX-positive cells in inflammatory foci in the lung in 4 microscope fields at ×40 magnification and calculating the average number of γH2AX-positive cells per infiltrate.

Neutrophil Phagocytosis

K. pneumoniae (ATCC43816) was cultured as previously described [33]. Phagocytosis assays were performed as described previously [34] with minor modifications. In summary, 1×10^5 bone marrow-derived neutrophils were plated in 96-well plates precoated with 10% FCS and 1×10^6 bacteria were added to each well (MOI10). Plates were centrifuged at 500 × g for 3 min to synchronize phagocytosis and incubated at 37°C for 30 min. Gentamicin (100 μg/mL) was added to specific wells and incubated for 1 h at 37°C to kill extracellular bacteria. Control wells with bacteria only were not treated with gentamicin. After incubation, neutrophils were washed extensively and treated with 0.1% Triton X-100 for 10 min at 4°C. Samples were plated on bacterial culture plates to quantify surviving bacteria.

Neutrophil ROS Assay

Neutrophil ROS production was measured using isoluminol-amplified chemiluminescence as reported previously [46] with minor modifications. 1×10^5 bone marrow-derived neutrophils in $HBSS^{++}$, isoluminol (50 μM; Sigma-Aldrich), and horse radish peroxidase (15U/mL; Sigma-Aldrich) were plated in FCS pre-coated 96-well plates. *K. pneumoniae* was added to the cells at a MOI of 10 or 20. As positive control, phorbol myristate acetate (Sigma) was added to some wells to a final concentration of 100 nM. As negative control, cells were treated with HBSS++. Plates were centrifuged to load bacteria onto the cells as described above. Thereafter, chemiluminescence was measured immediately every 3 min for up to 2 h using a Synergy HT plate reader (BioTek).

Statistical Analysis

Data are expressed as scattered dot plot plus median line. Data were analyzed using GraphPad Prism. The Kruskal-Wallis test was used for comparison of multiple groups, and Mann-Whitney U test was used for comparison between groups. Survival curves are depicted as Kaplan-Meier plots and compared using the log-rank test. A p value below 0.05 was considered statistically significant.

Results

$Btk^{-/-}$ Mice Are Highly Susceptible to K. pneumoniae-Evoked Pneumosepsis

To determine whether Btk is involved in the immune response during *K. pneumoniae*-evoked pneumosepsis, WT and $Btk^{-/-}$ mice were infected via the nostrils with a gradually growing hypervirulent strain of *K. pneumoniae* after which survival and bacterial loads in organs were monitored. $Btk^{-/-}$ mice displayed accelerated and increased mortality after *K. pneumoniae* infection, as all mice died within 2 days (Fig. 1a), whereas 50% of the WT mice died by the end of day 10. Numbers of bacteria in the lung did not differ between WT and $Btk^{-/-}$ mice at 6 h after inoculation, but were significantly increased in $Btk^{-/-}$ mice at 24 and 35 h (Fig. 1b). At these later time points, $Btk^{-/-}$ mice also had more bacterial dissemination than WT mice, as revealed by markedly increased CFUs in blood and spleen (Fig. 1c, d). To exclude that the difference in antibacterial defense of $Btk^{-/-}$ and WT mice resulted from differences in the microbiome between in house bred $Btk^{-/-}$ mice and commercial WT mice, we repeated the experiment with WT and $Btk^{-/-}$ littermates and

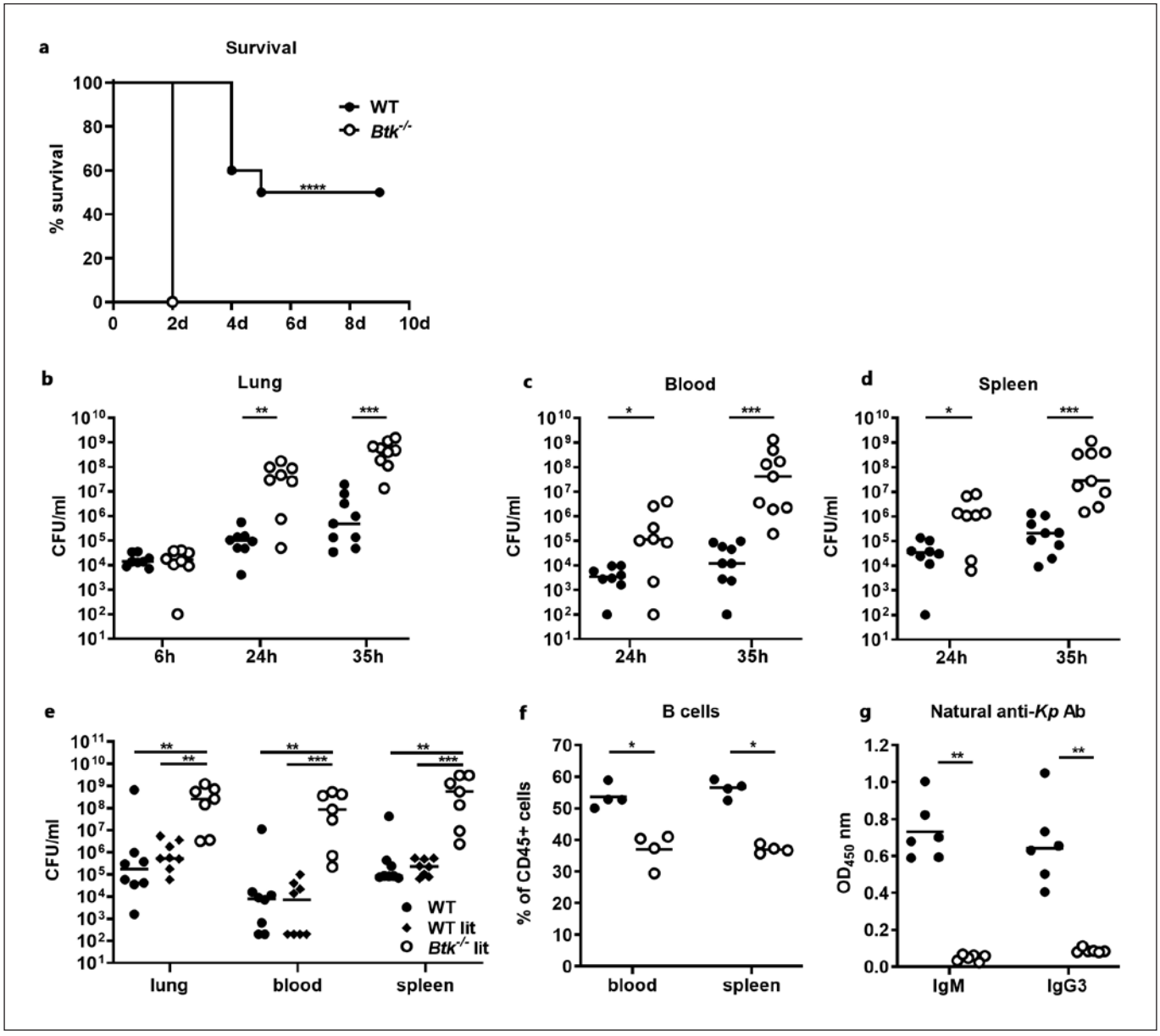


Fig. 1. *Btk*$^{-/-}$ mice are highly susceptible to *K. pneumoniae*-evoked pneumosepsis; WT and *Btk*$^{-/-}$ mice were intranasally inoculated with *K. pneumoniae*. Survival of WT and *Btk*$^{-/-}$ mice (n = 10 per group), bacterial counts of WT (**a**) and *Btk*$^{-/-}$ mice (n = 8–9 per group) in the lung (**b**), blood (**c**), and spleen (**d**). **e** Bacterial counts in the lung, blood, and spleen of purchased WT, WT littermate, and *Btk*$^{-/-}$ littermate 36 h after inoculation (n = 7–8 per group). **f** Percentage of B cells in the blood and spleen of naive WT and *Btk*$^{-/-}$ mice (n = 4 per group). **g** Relative concentrations of anti-*K. pneumoniae* IgM and IgG_3 in serum of naive WT and *Btk*$^{-/-}$ mice (n = 6 per group). Survival is expressed as the Kaplan-Meier plot, and all other graphs are dot plots with individual observations plus median. Survival was compared with the log-rank test, and bacterial loads and cell counts were compared to control mice with the Mann-Whitney U test (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$).

obtained similar findings (Fig. 1e). To obtain insight in the enhanced susceptibility of *Btk*$^{-/-}$ mice for *K. pneumoniae* infection, we analyzed B-cell numbers and natural antibodies against *Klebsiella* in naïve *Btk*$^{-/-}$ and WT mice. As expected [35], naïve *Btk*$^{-/-}$ mice had significantly lower B-cell numbers than WT mice in blood and spleen (Fig. 1f). Moreover, natural IgM and IgG3 antibodies against *K. pneumoniae* were detectable in the plasma of naïve WT

mice, but not in *Btk*$^{-/-}$ mice (Fig. 1g). Analysis of myeloid cells revealed similar numbers of AMs, lung dendritic cells, and blood monocytes in WT and *Btk*$^{-/-}$ mice (online suppl. Fig. S1a, b; for all online suppl. material , see www.karger.com/doi/10.1159/000524583). In line with previous findings [24], *Btk*$^{-/-}$ mice had increased neutrophil numbers in blood and spleen when compared with WT mice (online suppl. Fig. S1c).

Natural Antibodies and Btk Expression in B Cells Play a Limited Role in Host Defense against K. pneumoniae

To establish whether the enhanced susceptibility of *Btk*$^{-/-}$ mice for *K. pneumoniae* resulted from decreased numbers of B cells and lack of natural antibodies, we next performed infection experiments in *Btk*$^{-/-}$ mice with enforced transgenic expression of human BTK specifically in B cells (designated *Cd19-BTK*$^{+}$) [36], in B cell-deficient (μMT) mice [47], and in WT mice treated with the irreversible Btk inhibitor ibrutinib [48]. Expression of the *Cd19-BTK* transgene is restricted to the B-cell lineage and rescues the B-cell defects in *Btk*$^{-/-}$ mice [36]. Plasma levels of anti-*K. pneumoniae* IgM and IgG3 in *Cd19-BTK*$^{+}$ mice were increased compared to *Btk*$^{-/-}$ mice and compared to WT mice (Fig. 2a). *Cd19-BTK*$^{+}$ mice however were equally susceptible to *K. pneumoniae* as *Btk*$^{-/-}$ mice (Fig. 2b). All *Cd19-BTK*$^{+}$ and *Btk*$^{-/-}$ mice succumbed within 3 days after infection and at a similar rate, whereas 50% of the WT mice died by the end of day 10 and at a much slower rate (Fig. 2b). Bacterial counts in the lung and blood did not differ between *Btk*$^{-/-}$ and *Cd19-BTK*$^{+}$ mice (Fig. 2c, d). *Cd19-BTK*$^{+}$ mice had reduced bacterial counts in the spleen 24 h after infection as compared to *Btk*$^{-/-}$ mice, but this phenotype faded by 32 h (Fig. 2e). To confirm that natural antibodies did not provide protection against *K. pneumoniae* in *Cd19-BTK*$^{+}$ mice, we inoculated mice directly into the blood stream and assessed bacterial loads in blood, spleen, and liver 6 h later. *Btk*$^{-/-}$ mice had increased CFUs in blood, spleen, and liver, as compared to WT mice and a similar increase in CFU was found in the spleen and liver of *Cd19-BTK*$^{+}$ mice (Fig. 2f). To further establish the role of B cells and natural antibodies in host defense against *K. pneumoniae*, we next studied B cell-deficient μMT mice [47]. In μMT mice, natural anti-*K. pneumoniae* antibodies were absent (Fig. 2g). After *K. pneumoniae* inoculation, however, CFUs in lung, blood, and spleen of μMT mice were significantly reduced as compared to *Btk*$^{-/-}$ mice and compared to WT mice (Fig. 2h–j). To further distinguish the contribution of Btk and natural antibodies to host defense against *K. pneumoniae*, we treated WT mice with the irreversible inhibitor ibrutinib. Previously, we have established that short-term oral treatment of mice with ibrutinib abrogated kinase activity of Btk and reduced lung inflammation during antibiotic-treated pneumococcal pneumonia [40]. Ibrutinib treatment did not alter plasma levels of natural anti-*K. pneumoniae* IgM and IgG3 (Fig. 2k). Bacterial counts in lung and spleen of Ibrutinib-treated mice were significantly increased as compared to vehicle-treated mice 40 h after inoculation (Fig. 2l). Together, these findings indicate that natural antibodies and B cell-mediated immunity play a limited role in innate host defense against *K. pneumoniae* and suggest that the enhanced susceptibility of *Btk*$^{-/-}$ mice for *K. pneumoniae* results from a critical role of Btk in cells other than B cells.

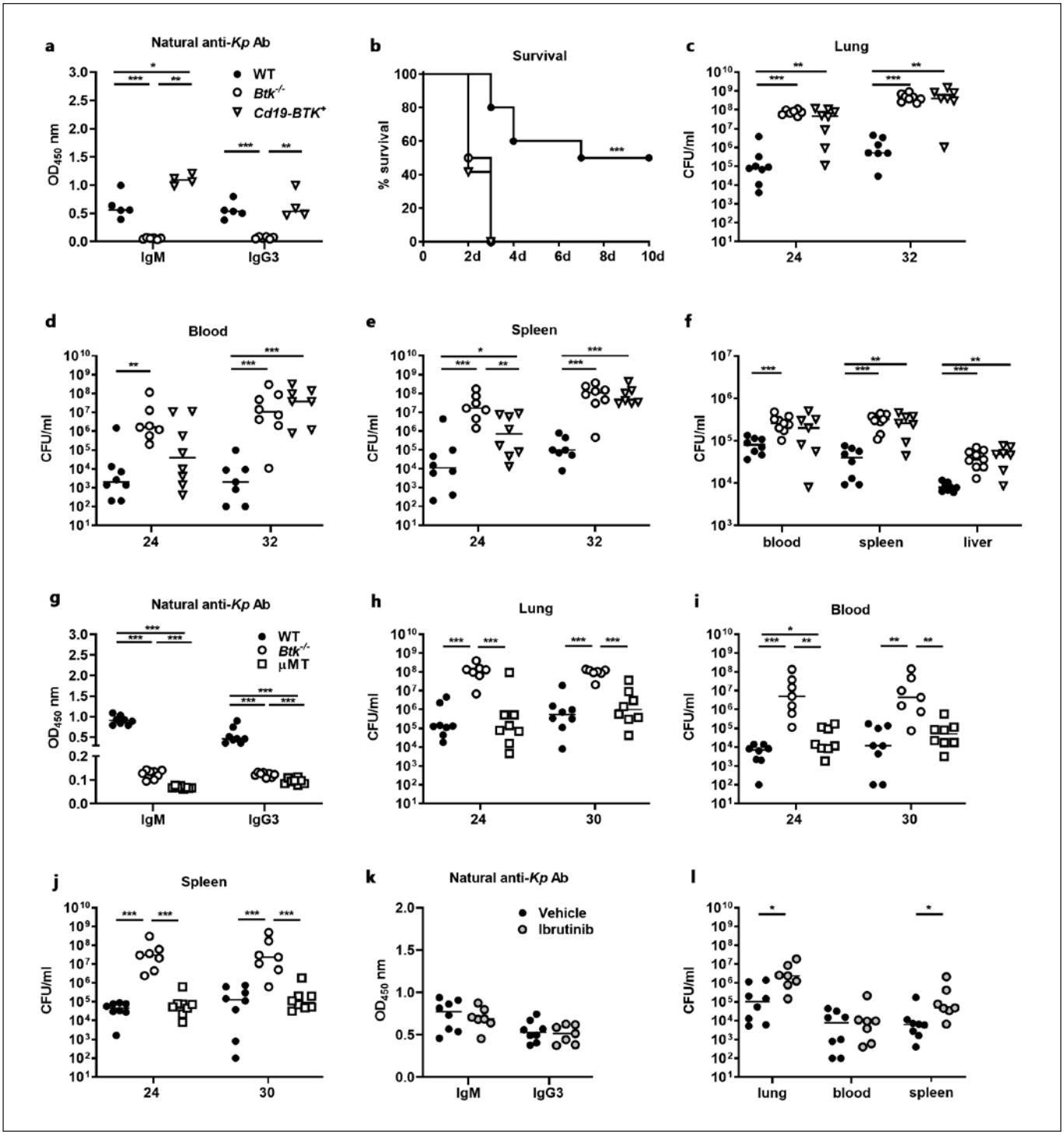
Btk Expression in Neutrophils Is Required for Host Defense against K. pneumoniae

Previously, it has been shown that neutrophils are eminent for host defense against *K. pneumoniae* [31, 32]. To assess whether Btk in neutrophils is important for host defense against *K. pneumoniae*, we generated conditional knockout mice by breeding *Mrp8cre* mice [38] with *Btk*$^{fl/fl}$ mice [37]. In line with the known expression of Cre recombinase in the bulk of neutrophils of *Mrp8cre* mice [49, 50], *Mrp8cre.Btk*^{fl}*/Y* mice displayed strongly reduced Btk protein levels in neutrophils, whereas Btk protein levels in B cells, monocytes, and alveolar macrophages were unaltered as compared to *Btk*^{fl} controls [39]. Intranasal inoculation with *K. pneumoniae* resulted in increased bacterial numbers in lung, blood, spleen, and liver of *Mrp8cre.Btk*^{fl}*/Y* mice 24 and 42 h after infection (Fig. 3a–d), comparable to *Btk*$^{-/-}$ mice (Fig. 1b–d). To determine whether the increased bacterial loads in blood and spleen resulted from impaired systemic host defense, we intravenously injected *Mrp8cre.Btk*^{fl}*/Y* mice and WT littermates with *K. pneumoniae* and assessed bacterial loads 24 h later. *Mrp8cre.Btk*^{fl}*/Y* mice had similar bacterial counts as WT mice after intravenous *K. pneumoniae* infection (Fig. 3e). These results indicate that Btk in neutrophils is required to control *K. pneumoniae* infection in the lungs during pneumosepsis and suggest that enhanced susceptibility of *Btk*$^{-/-}$ mice for *K. pneumoniae* pneumonia results from a paucity of Btk in neutrophils.

Btk Deficiency in Neutrophils Does Not Impair Pulmonary Inflammatory Response during K. pneumoniae Infection

To further evaluate the impact of Btk deficiency in neutrophils after *K. pneumoniae* infection, we assessed the inflammatory responses in infected *Mrp8cre.Btk*^{fl}*/Y*

and WT mice. *Mrp8cre.Btk*^{fl}*/Y* mice had similar lung pathology (Fig. 4a, b) and confluent lung inflammation (online suppl. Table S1) as WT after *K. pneumoniae* infection. Moreover, levels of TNF, IL-1β, and IL-6 in lung and BALF (Fig. 4c; online suppl. Table S2) were largely similar between groups, except for IL-1β levels in lung at 42 h (higher in *Mrp8cre.Btk*^{fl}*/Y* mice). Likewise, *Btk*$^{-/-}$ and WT mice displayed similar lung pathology after intranasal *K. pneumoniae* infection (online suppl. Fig. S2a), while *Btk*$^{-/-}$ mice had higher lung IL-1β and IL-6 levels relative



(For legend see next page.)

to WT mice at 35 h, paralleling the much higher bacterial loads at this time point (online suppl. Table S3). Furthermore, Btk deficiency in neutrophils did not impact on plasma cytokine and chemokine levels after intranasal *K. pneumoniae* infection (online suppl. Table S4), except for IL-6 (higher in *Mrp8cre.Btk^{fl}/Y* mice at 42 h after intranasal infection). These data indicate that Btk deficiency in neutrophils does not significantly alter inflammatory responses in the lung during *K. pneumoniae* infection.

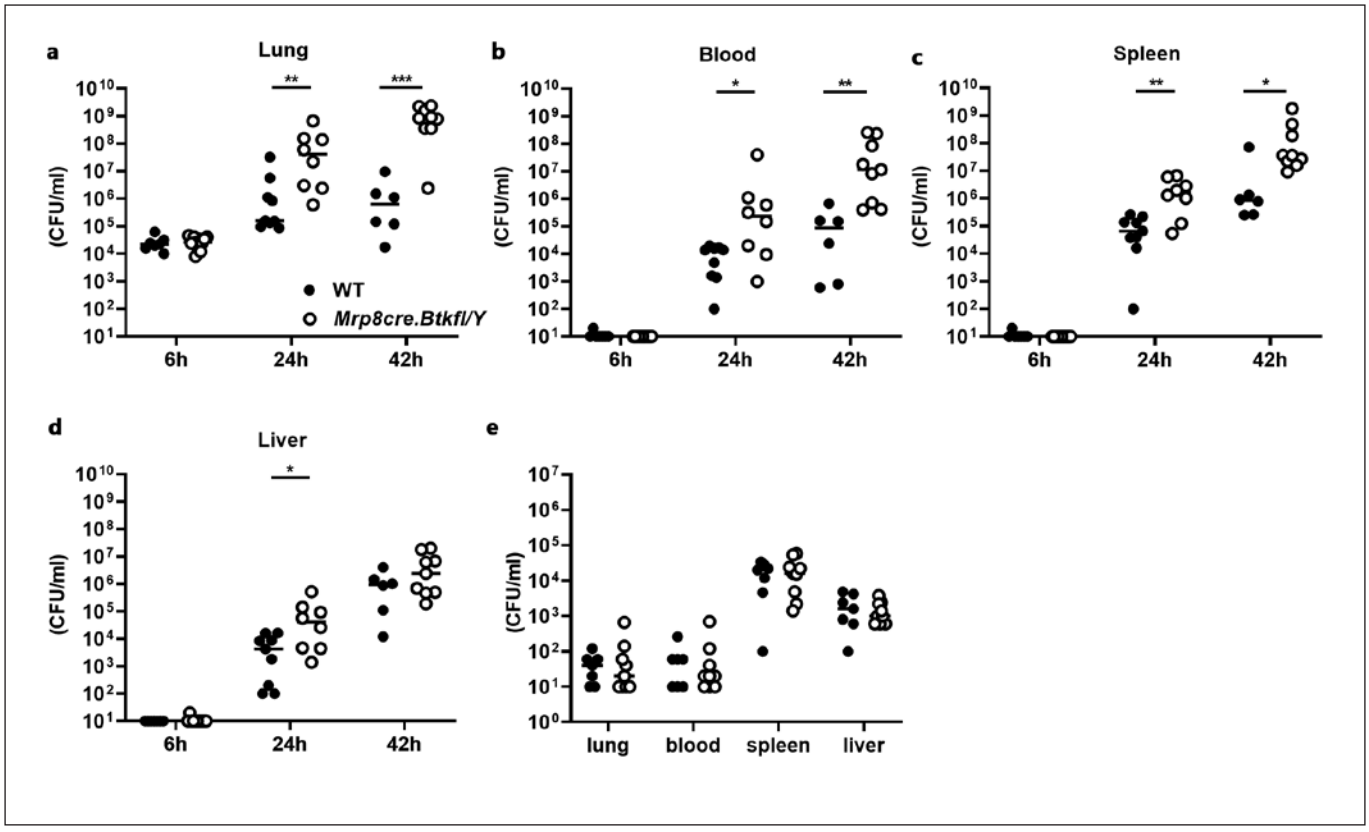
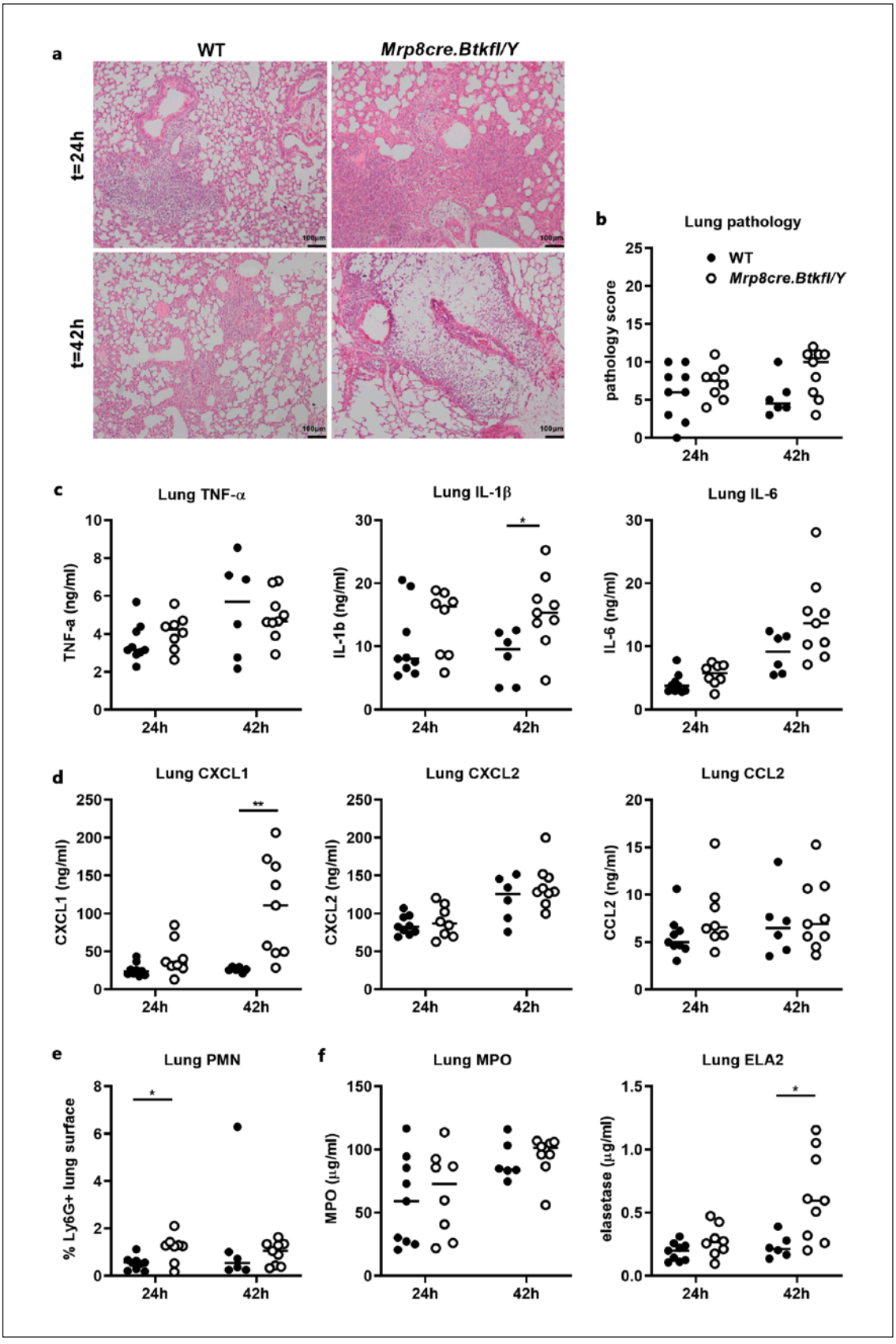


Fig. 3. Btk expression in neutrophils is required for host defense against K. *pneumoniae Mrp8cre.Btk^{fl}/Y*, and littermate control (WT) mice were infected intranasally with *K. pneumoniae* and euthanized 6, 24, or 42 h later (n = 6–11 per group). Bacterial loads in lung (**a**), blood (**b**), spleen (**c**), and liver (**d**). *Mrp8cre.Btk^{fl}/Y* and WT mice were infected intravenously with ~$\times 1 \times 10^4$ CFU *K pneumoniae* euthanized at 24 h (n = 8 per group). **e** Bacterial loads were determined in blood, lung, spleen, and liver. Data are represented as dot plot with individual observations plus median (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

Fig. 2. Natural antibodies and Btk expression in B cells play a limited role in host defense against *K. pneumoniae*. **a** Relative concentrations of anti-*K. pneumoniae* IgM and IgG_3 in serum of naive WT, *Btk*$^{-/-}$, and *Cd19-BTK*$^+$ mice (n = 4–6 per group). WT, *Btk*$^{-/-}$, and *Cd19-BTK*$^+$ mice were intranasally inoculated with *K. pneumoniae*. Survival of WT, *Btk*$^{-/-}$, and *Cd19-BTK*$^+$ mice (n = 10–12 per group) (**b**); bacterial counts of WT, *Btk*$^{-/-}$, and *Cd19-BTK*$^+$ mice (n = 7–8 per group) in the lung (**c**), blood (**d**), and spleen (**e**). **f** WT, *Btk*$^{-/-}$, and *Cd19-BTK*$^+$ mice were intravenously inoculated with ~$\times 5 \times 10^4$ CFU *K. pneumoniae* (n = 7–9 per group); bacterial counts in the blood, spleen, and liver were determined 6 h after inoculation. WT, *Btk*$^{-/-}$, and μMT mice were intranasally inoculated with *K. pneumoniae*. **g** Relative concentrations of anti-*K. pneumoniae* IgM and IgG_3 in serum of infected WT, *Btk*$^{-/-}$, and μMT mice (n = 8 per group). Bacterial counts of WT, *Btk*$^{-/-}$, and μMT mice (n = 7–8 per group) in the lung (**h**), blood (**i**), and spleen (**j**). WT mice were treated orally with vehicle or ibrutinib (25 mg/kg) 27 and 3 h prior to intranasal inoculation with *K. pneumoniae* (n = 7–8 per group). **k** Relative concentrations of anti-*K. pneumoniae* IgM and IgG_3 in serum of infected vehicle-treated or ibrutinib-treated WT mice (n = 7–8 per group). **l** Bacterial counts in the lung, blood, and spleen were determined 40 h after inoculation (L). Survival is expressed as the Kaplan-Meier plot, and all other graphs are dot plots with individual observations plus median. Survival was compared with the log-rank test; bacterial loads, and cell counts were compared to control mice with the Mann-Whitney U test (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).



4

(For legend see next page.)

Btk Deficiency in Neutrophils Does Not Alter Neutrophil Influx into the Lung during K. pneumoniae Pneumonia

To determine whether the higher bacterial burden in *Mrp8cre.Btk*^{fl}*/Y* mice resulted from reduced neutrophil migration into lung, we determined neutrophil chemoattractant levels and neutrophil numbers in the lung after intranasal infection with *K. pneumoniae*. Levels of CXCL1, CXCL2, and CCL2 in lung (Fig. 4d) and BALF (online suppl. Table S5) were largely similar between groups, but lung CXCL1 levels at 42 h were higher in *Mrp8cre.Btkfl/Y* mice compared to WT mice. Ly6G staining of lung sections indicated that *Mrp8cre.Btk*^{fl}*/Y* mice had more neutrophil influx at 24 h (Fig. 4e and S2d). Neutrophil numbers in the alveolar space however were not different between *Mrp8cre.Btk*^{fl}*/Y* and WT mice (online suppl. Fig. S2e). In line with these results, lung MPO and elastase (ELA2) levels (indicators of total neutrophil content in tissue homogenates) were similar in WT and *Mrp8cre.Btk*^{fl}*/Y* mice, except that *Mrp8cre.Btk*^{fl}*/Y* mice had higher elastase level at 42 h (Fig. 4f). Intranasal *Klebsiella* infection produced comparable results in WT and *Btk*$^{-/-}$ mice (online suppl. Fig. S2b, c). Together, these data indicate the increased bacterial outgrowth in *Mrp8cre.Btk*^{fl}*/Y* mice after infection with *K. pneumoniae* via the airways is not due to impaired neutrophil migration into the lung, but rather results from deficient neutrophil functions.

Btk Deficiency in Neutrophils Does Not Affect Neutrophil Activation or Degranulation in the Lung during K. pneumoniae Pneumonia

Since neutrophil activation is critical in innate immunity and neutrophil degranulation is implicated in host defense against *K. pneumoniae* [51, 52], we assessed neutrophil activation and degranulation after *K. pneumoniae* infection. Analysis of CD11b surface expression on neutrophils in BALF and blood showed similar results in *Mrp8cre.Btk*^{fl}*/Y* and WT mice (online suppl. Fig. S3a, b). Moreover, *Mrp8cre.Btk*^{fl}*/Y* mice had MPO, elastase, and MRP8/14 protein levels in BALF that were equivalent to those in WT mice (online suppl. Fig. S3c). Plasma MPO and elastase levels paralleled blood CFUs in *Mrp8cre.Btkfl/Y* and WT mice after intranasal *K. pneumoniae* infection (online suppl. Fig. S3d), while no difference was found after intravenous *Klebsiella* infection (online suppl. Fig. S3e). Formation of NETs has been implicated to contribute to host defense against *K. pneumoniae* [53]. To determine whether Btk is required for NET formation, we analyzed the presence of CitH3, a surrogate marker for NETs [44], in the lungs of infected *Mrp8cre.Btk*^{fl}*/Y* and littermate control mice. CitH3 was detectable in inflammatory foci in the lungs of both groups (online suppl. Fig. S3f, g), indicating that Btk in neutrophils is not required for formation of NETs. To assess whether Btk deficiency impacted on cell death of neutrophils, we analyzed the presence of γH2AX, a marker for double-stranded DNA breaks during various forms of cell death [45], in the lungs. In both *Mrp8cre.Btk*^{fl}*/Y* mice and littermate controls, *K. pneumoniae* infection was associated with γH2AX expression at the site of cell infiltration (online suppl. Fig. S3h, i). Together, these data suggest that the impaired host defense against *Klebsiella* of *Mrp8cre.Btk*^{fl}*/Y* mice is not due to defects in neutrophil activation or degranulation, nor by inability to form NETs or trigger cell death.

Btk in Neutrophils Is Essential for K. pneumoniae-Induced Extracellular ROS Production

Although most *Klebsiella* strains are known to be resistant to uptake by phagocytic cells [29], phagocytosis and intracellular killing have been implied as neutrophil antimicrobial mechanisms involved in host defense against *K. pneumoniae* infection [54]. To determine the role of Btk in phagocytosis of *K. pneumoniae*, we utilized a gentamicin protection assay [34]. Both WT and Btk-deficient neutrophils phagocytosed less than 0.01% of the added *K. pneumoniae* bacteria at a multiplicity of infection of 10 (Fig. 5a; online suppl. Fig. S4a). These results indicate that the *K. pneumoniae* ATCC43816 strain used in our studies is resistant to neutrophil phagocytosis independent of Btk expression.

Fig. 4. Btk deficiency in neutrophils does not impair pulmonary inflammatory responses nor neutrophil influx into the lung during *K. pneumoniae* pneumonia *Mrp8cre.Btk*^{fl}*/Y*, and littermate control (WT) mice were infected intranasally with *K. pneumoniae* and euthanized at 24 or 42 h (n = 6–9 per group). Representative pictures of hematoxylin and eosin-stained lung sections (**a**) and lung pathology scores of individual mice (**b**). **c** Levels of TNF, IL-1β, and IL-6 in lung homogenates. **d** Levels of CXCL1, CXCL2, and CCL2 in lung homogenates. **e** Lung sections were stained with anti-Ly6G, and the percentage of Ly6G positive lung surface was quantified. **f** Levels of MPO and ELA2 in lung homogenates. Data are represented as scatter dot plots plus median (*$p < 0.05$, **$p < 0.01$).

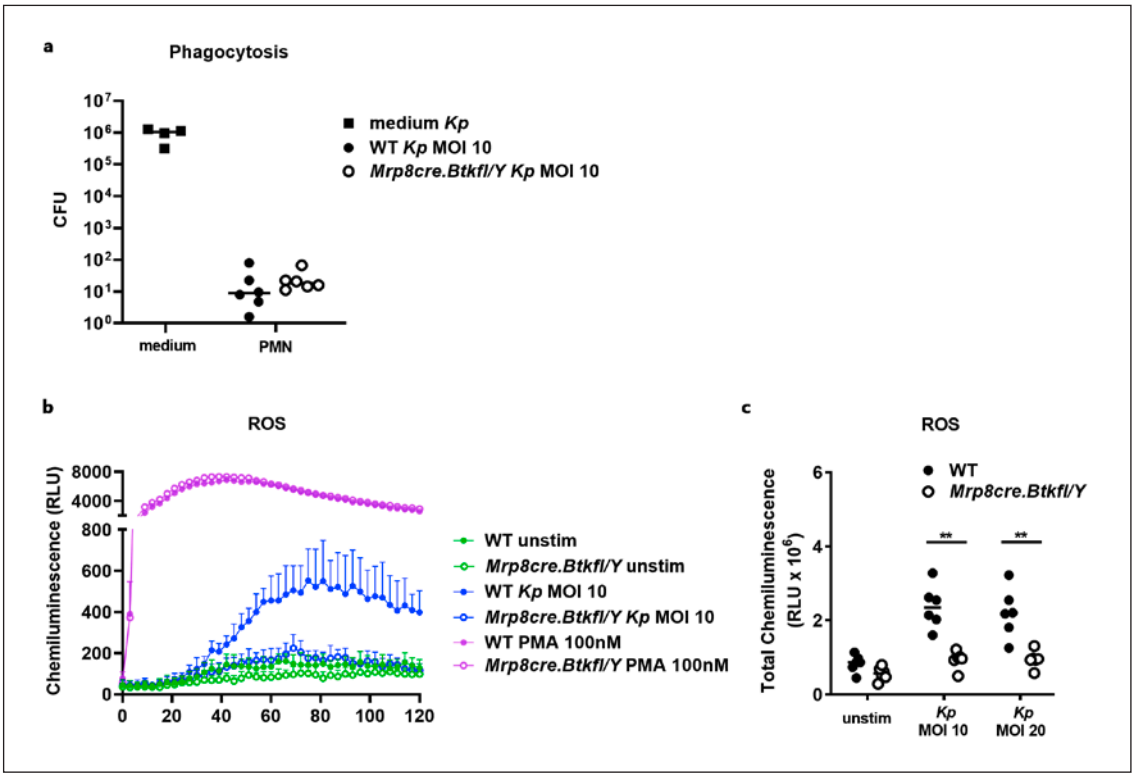


Fig. 5. Btk in neutrophils is essential for *K. pneumoniae*-induced extracellular ROS production *Mrp8cre.Btk*^{fl}*/Y*, and WT neutrophils were incubated with live *K. pneumoniae* at MOI10 ($n = 6$ per group). **a** *Klebsiella* bacteria in medium and internalized bacteria (phagocytosis) by PMN after gentamicin treatment are shown as CFU. Analysis of respiratory burst of neutrophils from *Mrp8cre.Btk*^{fl}*/Y* and WT mice using isoluminol chemiluminescence ($n = 6$ per group). Neutrophils were treated with HBSS$^{+/+}$, live *K. pneumoniae* (MOI10 or 20), or 100 nM PMA. **b** Representative graph of 6 experiments. **c** Total ROS production during 120-min stimulation was calculated as the total area under the curve. Data are represented as dot plots with individual observations plus median (*$p < 0.05$, **$p < 0.01$). PMA, phorbol myristate acetate.

Extracellular ROS is an important mechanism for host defense against *K. pneumoniae* [34, 55]. To investigate whether Btk deficiency resulted in a defect in neutrophil ROS production after *K. pneumoniae* infection, we employed an assay with isoluminol to detect extracellular O_2^- [56]. In contrast to neutrophils from WT mice, neutrophils from *Mrp8cre.Btk*^{fl}*/Y* mice were severely hampered in extracellular ROS production following exposure to *K. pneumoniae* (Fig. 5b, c). Similar results were obtained with neutrophils from *Btk*$^{-/-}$ mice (online suppl. Fig. S4b). Lack of ROS production of Btk-deficient neutrophils was not caused by a defective NADPH oxidase system as after challenge with phorbol myristate acetate, which activates protein kinase C directly and independent of receptor-mediated signaling pathways [34], releases ROS robustly in Btk-deficient neutrophils (Fig. 5b). All together, these results indicate that Btk is essential for *K. pneumoniae*-induced neutrophil ROS production.

Discussion

Since susceptibility of XLA patients and *Btk*$^{-/-}$ mice to bacterial infections [4, 5] is markedly reversed by antibody supplementation treatment [4, 6], it is generally accepted that Btk in myeloid cells is redundant for innate immunity against bacteria. In the present study, we challenged this precept and revealed that Btk in neutrophils is crucial for innate host defense against the common human Gram-negative bacterial pathogen *K. pneumoniae*. Using *Btk*$^{-/-}$ mice rescued for Btk expression in B cells, mice devoid of B cells, and mice treated for a short term with ibrutinib, we demonstrated that natural antibodies and B cell-mediated immunity contribute little to host immunity against *K. pneumoniae*. In contrast, neutrophil-specific Btk-deficient *Mrp8cre.Btk*^{fl}*/Y* mice displayed severely impaired antibacterial defense during *Klebsiella* pneumonia as compared to littermate controls.

Btk was not required for neutrophil migration or activation in the lung in response to *Klebsiella*. Btk however was essential for extracellular ROS production by neutrophils induced by *Klebsiella*. Taken together, the results of the current study indicate that Btk in neutrophils is crucial to regulate innate host defense against *K. pneumoniae* in mice.

One of the most important functions of B-1 cells is the generation of natural antibodies [57]. Besides, B-1 cells are involved in the regulation of immune responses by cytokine secretion [57]. Previous studies have implicated B-1 cells and natural antibodies in host defense during Gram-negative infections. *Btk*$^{-/-}$ mice, lacking B-1 cells, display increased mortality after challenge with a lethal dose of LPS [58, 59], a component of the outer membrane of Gram-negative bacteria, which has been attributed to a paucity in LPS clearing natural antibodies [58] and to reduced expression of the anti-inflammatory cytokine IL-10 [59]. Natural IgM and IgG antibodies against LPS and capsular polysaccharides of *K. pneumoniae* are present in human serum, and anti-capsular polysaccharide antibodies promote phagocytosis of *Klebsiella* [60]. Moreover, serum transfer from conventional mice to germ-free mice was able to render germ-free mice resistant to *K. pneumoniae* infection [61], suggesting that natural antibodies present in serum are protective against this pathogen. The results of the current study with *Cd19-BTK*$^{+}$ and μMT mice however indicate that natural antibodies against *K. pneumoniae* do not confer protection to this bacterium in mice. The discrepancy between the previous studies [60, 61] and our current findings may result from different serotypes used. In the present study, we infected mice with a highly virulent *Klebsiella* strain, which is resistant to opsonophagocytosis [62] and complement-mediated killing [29].

Several studies with chemokine-deficient mice, transgenic chemokine overexpressing mice, as well as with mice depleted of neutrophils [31, 63–65], have demonstrated that neutrophils are essential to restrain *K. pneumoniae* infection. Various degranulation products of neutrophils, including MPO and elastase, have been shown to contribute to host defense against *K. pneumoniae* [66, 67]. Our findings with *Mrp8cre.Btk*^{fl}*/Y* mice demonstrate that Btk in neutrophils contributes to control of *K. pneumoniae* growth in the lungs and dissemination to other organs after infection via the airways. Although Btk deficiency in neutrophils has been described to result in inefficient development of granules and reduced expression of MPO and elastase [24], we have not been able to reproduce these findings [39]. Moreover, in contrast to a prior investigation that examined the role of Btk in neutrophil migration during sterile inflammation [22], our study argues against a role for Btk in neutrophil migration, activation, and degranulation during *Klebsiella* pneumonia. Strikingly, we recently reported that *Nbeal2*-deficient mice, which have neutrophils devoid of primary, secondary, tertiary, and secretory granules, were not impaired in innate host defense against *Klebsiella* [68]. These findings suggest that antibacterial components of neutrophils other than degranulation products are required to protect against this bacterium. In addition, our study does not reveal a critical role for Btk in formation of NETs during *Klebsiella*-induced pneumonia, opposite to what was reported for influenza-induced lung inflammation [69]. Furthermore, the results of our study also argue against a pivotal role for Btk in the induction of lung inflammation. Strikingly, pulmonary levels of particular inflammatory markers (IL-1β, CXCL1, and ELA2) were increased in neutrophil-specific Btk-deficient mice rather than reduced, consistent with higher bacterial numbers. Further studies are required to establish the cellular processes causing these specific differences in the inflammatory response.

Hypermucoviscous *K. pneumoniae* is considered an extracellular pathogen due to its thick polysaccharide capsule, which prevents uptake by neutrophils [29, 62]. Consistent with previous studies [34, 70], we here demonstrate the hypervirulent *K. pneumoniae* ATCC43816 strain is resistant to phagocytosis. Few studies however have demonstrated that macrophages are able to phagocytose hypervirulent *Klebsiella* in vivo [71, 72], but the uptake did not trigger intracellular killing and was suggested to benefit persistent infection. Since Btk is involved in regulation of phagocytosis by neutrophils [22], further in vivo experiments are required to establish whether Btk-deficient neutrophils are hampered in bacterial phagocytosis during *Klebsiella*-evoked pneumosepsis and whether this contributes to impaired host defense. In addition to resistance to phagocytosis, we found that incubation of *K. pneumoniae* for 2 h with isolated neutrophils from either WT or *Btk*$^{-/-}$ mice resulted in bacterial outgrowth rather than killing (data not shown), suggesting that this strain is able to resist neutrophil bactericidal activity in vitro. Recently, it was reported that *Cybb*$^{-/-}$ mice (with deficient ROS production) were susceptible to hypervirulent *K. pneumoniae* infection [34], indicating that ROS are important for host defense against this pathogen. In line with these findings, humans with chronic granulomatous disease due to mutations in NADPH oxidase components are frequently in-

fected by *Klebsiella* species [73]. In the current study, we found that Btk-deficient neutrophils produced less extracellular ROS after *K. pneumoniae* stimulation. Results from experiments with WT neutrophils treated with ibrutinib and *K. pneumoniae* [34] corroborate our findings. Taken together, these results indicate that Btk in neutrophils is essential for *Klebsiella*-induced extracellular ROS production and thereby crucial for host defense against this pathogen.

The ROS generating NADPH oxidase complex in neutrophils can be primed through the activation of several surface receptors, including G-protein-coupled receptors, integrin receptors, TNF receptors, FcγR, and TLRs [74, 75]. Btk-deficient neutrophils failed to produce superoxide in response to LPS and when plated on poly-RGD-coated surface, indicating that Btk is involved in TLR4 and integrin receptor-mediated neutrophil activation [10, 22]. In the current study, the signaling pathways and pattern recognition receptors served by Btk and that mediate antibacterial responses are unknown. Since the impaired host defense, accelerated mortality, and modestly altered pulmonary cytokine response of *Klebsiella* infected $Btk^{-/-}$ mice closely mimic the outcome of these parameters in myeloid cell-specific MyD88-deficient mice [76] and chimeric mice lacking MyD88 in hematopoietic cells [77], it is tempting to speculate that hampered TLR receptor signaling is responsible for the hypersusceptibility of $Btk^{-/-}$ mice for *Klebsiella*. Btk is involved in signaling of TLR2, TLR4, and TLR9 [16, 19, 78], and these TLRs have been shown to contribute to host defense against *K. pneumoniae* [33, 43, 79]. Further studies are required to identify the pattern recognition receptors which depend on Btk to generate ROS production and control *Klebsiella* infection.

Although our study unequivocally demonstrates that the innate host response against *Klebsiella* in *Mrp8cre.* Btk^{fl}/Y mice phenocopies that of complete $Btk^{-/-}$ mice after intranasal infection, the antibacterial response in these mouse strains differed after intravenous inoculation. Although bacterial numbers in $Btk^{-/-}$ mice were significantly increased in organs as compared to WT mice, *Mrp8cre.* Btk^{fl}/Y mice did not show enhanced susceptibility to *Klebsiella* after intravenous inoculation. These findings suggest that Btk in other myeloid cells, such as spleen and liver macrophages, may contribute to host defense against *Klebsiella*. Further studies with specific macrophage Cre recombinase expressing mice in conjunction with Btk^{fl} mice may reveal whether Btk in spleen and liver macrophages restrains systemic *K. pneumoniae* infection.

In summary, the results of this study indicate that natural antibodies and B cell-mediated immunity are dispensable for host defense against *K. pneumoniae* and reveal that Btk expression in neutrophils is crucial for *Klebsiella*-induced extracellular ROS production and innate immunity against this pathogen. We speculate that impaired Btk signaling in myeloid cells may explain why some patients treated with ibrutinib [80] and a proportion of XLA patients treated with antibodies [3] develop bacterial infections.

Acknowledgments

The authors would like to thank J.M. Pater, S. Terpstra, M.S. ten Brink, and J.B. Daalhuisen for their excellent technical assistance.

Statement of Ethics

The animal experiments were reviewed and approved by the Central Authority for Scientific Procedures on Animals (CCD) and the Animal Ethical Committee (DEC) and the Animal Welfare Body (IvD) of the Academic Medical Center Amsterdam; approval numbers DIX21 and DIX17-4125-1. The animal care and use protocol adhered to the Dutch Experiments on Animals Act (WOD) and European Directive of September 22, 2010 (Directive 2010/63/EU), in addition to the Directive of May 6, 2009 (Directive 2009/41/EC).

Conflict of Interest Statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Funding Sources

Zhe Liu was funded by the Chinese Scholarship Council (CSC No. 201706170060). Alexander P.N.A. De Porto was funded by a PhD scholarship from the Academic Medical Center, Amsterdam, the Netherlands.

Author Contributions

Conceptualization: Zhe Liu, Alexander P.N.A. De Porto, Cornelis Van't Veer, Rudi W. Hendriks, Tom Van der Poll, and Alex F. De Vos. Data curation: Zhe Liu, Alexander P.N.A. De Porto, and Alex F. De Vos. Formal analysis: Zhe Liu, Alexander P.N.A. De Porto, Joris J.T.H. Roelofs, Onno J. De Boer, Sandrine Florquin, and Alex F. De Vos. Investigation: Zhe Liu, Alexander P.N.A. De

Porto, Regina De Beer, and Alex F. De Vos. Supervision: Cornelis Van't Veer, Tom Van der Poll, and Alex F. De Vos. Resources: Rudi W. Hendriks. Writing – original draft: Zhe Liu, Alexander P.N.A. De Porto, and Alex F. De Vos. Writing – review and editing: Zhe Liu, Alexander P.N.A. De Porto, Cornelis Van't Veer, Rudi W. Hendriks, Tom Van der Poll, and Alex F. De Vos.

Data Availability Statement

All data generated and analyzed during this study are included in this article and its supplementary material files. Further inquiries can be directed to the corresponding author.

References

1 Rip J, Van Der Ploeg EK, Hendriks RW, Corneth OBJ. The role of Bruton's tyrosine kinase in immune cell signaling and systemic autoimmunity. Crit Rev Immunol. 2018;38(1):17–62.
2 Corneth OBJ, Klein Wolterink RGJ, Hendriks RW. BTK Signaling in B cell differentiation and autoimmunity. Curr Top Microbiol Immunol. 2016;393:67–105.
3 Shillitoe B, Gennery A. X-linked agammaglobulinaemia: outcomes in the modern era. Clin Immunol. 2017;183:54–62.
4 Briles DE, Nahm M, Schroer K, Davie J, Baker P, Kearney J, et al. Antiphosphocholine antibodies found in normal mouse serum are protective against intravenous infection with type 3 streptococcus pneumoniae. J Exp Med. 1981;153(3):694–705.
5 Winkelstein JA, Marino MC, Lederman HM, Jones SM, Sullivan K, Burks AW, et al. X-linked agammaglobulinemia: Report on a United States Registry of 201 Patients. Medicine. 2006;85(4):193–202.
6 Maarschalk-Ellerbroek LJ, Hoepelman IM, Ellerbroek PM. Immunoglobulin treatment in primary antibody deficiency. Int J Antimicrob Agents. 2011;37(5):396–404.
7 Amoras AL, Kanegane H, Miyawaki T, Vilela MM. Defective Fc-, CR1- and CR3-mediated monocyte phagocytosis and chemotaxis in common variable immunodeficiency and X-linked agammaglobulinemia patients. J Investig Allergol Clin Immunol. 2003;13(3):181–8.
8 Jongstra-Bilen J, Puig Cano A, Hasija M, Xiao H, Smith CI, Cybulsky MI. Dual functions of Bruton's tyrosine kinase and Tec kinase during Fcgamma receptor-induced signaling and phagocytosis. J Immunol. 2008;181(1):288–98.
9 Strijbis K, Tafesse FG, Fairn GD, Witte MD, Dougan SK, Watson N, et al. Bruton's tyrosine kinase (BTK) and Vav1 contribute to Dectin1-dependent phagocytosis of Candida albicans in macrophages. PLoS Pathog. 2013;9(6):e1003446.
10 Mangla A, Khare A, Vineeth V, Panday NN, Mukhopadhyay A, Ravindran B, et al. Pleiotropic consequences of Bruton tyrosine kinase deficiency in myeloid lineages lead to poor inflammatory responses. Blood. 2004;104(4):1191–7.
11 Ren L, Campbell A, Fang H, Gautam S, Elavazhagan S, Fatehchand K, et al. Analysis of the effects of the Bruton's tyrosine kinase (Btk) inhibitor ibrutinib on monocyte Fcγ receptor (FcγR) function. J Biol Chem. 2016;291(6):3043–52.
12 O'Riordan CE, Purvis GSD, Collotta D, Krieg N, Wissuwa B, Sheikh MH, et al. X-linked immunodeficient mice with no functional Bruton's tyrosine kinase are protected from sepsis-induced multiple organ failure. Front Immunol. 2020;11:581758.
13 Mukhopadhyay S, George A, Bal V, Ravindran B, Rath S. Bruton's tyrosine kinase deficiency in macrophages inhibits nitric oxide generation leading to enhancement of IL-12 induction. J Immunol. 1999;163(4):1786–92.
14 Mukhopadhyay S, Mohanty M, Mangla A, George A, Bal V, Rath S, et al. Macrophage effector functions controlled by Bruton's tyrosine kinase are more crucial than the cytokine balance of T cell responses for microfilarial clearance. J Immunol. 2002;168(6):2914–21.
15 Horwood NJ, Mahon T, McDaid JP, Campbell J, Mano H, Brennan FM, et al. Bruton's tyrosine kinase is required for lipopolysaccharide-induced tumor necrosis factor alpha production. J Exp Med. 2003;197(12):1603–11.
16 Horwood NJ, Page TH, McDaid JP, Palmer CD, Campbell J, Mahon T, et al. Bruton's tyrosine kinase is required for TLR2 and TLR4-induced TNF, but not IL-6, production. J Immunol. 2006;176(6):3635–41.
17 Ormsby T, Schlecker E, Ferdin J, Tessarz AS, Angelisova P, Koprulu AD, et al. Btk is a positive regulator in the TREM-1/DAP12 signaling pathway. Blood. 2011;118(4):936–45.
18 Lee KG, Xu S, Kang ZH, Huo J, Huang M, Liu D, et al. Bruton's tyrosine kinase phosphorylates toll-like receptor 3 to initiate antiviral response. Proc Natl Acad Sci U S A. 2012;109(15):5791–6.
19 Herbst S, Shah A, Mazon Moya M, Marzola V, Jensen B, Reed A, et al. Phagocytosis-dependent activation of a TLR9-BTK-calcineurin-NFAT pathway co-ordinates innate immunity to aspergillus fumigatus. EMBO Mol Med. 2015;7(3):240–58.
20 Liu X, Pichulik T, Wolz OO, Dang TM, Stutz A, Dillen C, et al. Human NACHT, LRR, and PYD domain-containing protein 3 (NLRP3) inflammasome activity is regulated by and potentially targetable through Bruton tyrosine kinase. J Allergy Clin Immunol. 2017;140(4):1054–67.
21 Mueller H, Stadtmann A, Van Aken H, Hirsch E, Wang D, Ley K, et al. Tyrosine kinase Btk regulates E-selectin-mediated integrin activation and neutrophil recruitment by controlling phospholipase C (PLC) gamma2 and PI3Kgamma pathways. Blood. 2010;115(15):3118–27.
22 Volmering S, Block H, Boras M, Lowell Clifford A, Zarbock A. The neutrophil Btk signalosome regulates integrin activation during sterile inflammation. Immunity. 2016;44(1):73–87.
23 Krupa A, Fol M, Rahman M, Stokes KY, Florence JM, Leskov IL, et al. Silencing Bruton's tyrosine kinase in alveolar neutrophils protects mice from LPS/immune complex-induced acute lung injury. Am J Physiol Lung Cell Mol Physiol. 2014;307(6):L435–48.
24 Fiedler K, Sindrilaru A, Terszowski G, Kokai E, Feyerabend TB, Bullinger L, et al. Neutrophil development and function critically depend on Bruton tyrosine kinase in a mouse model of X-linked agammaglobulinemia. Blood. 2011;117(4):1329–39.
25 Zhu Z, Kang Y, Lin Z, Huang Y, Lv H, Li Y. X-linked agammaglobulinemia combined with juvenile idiopathic arthritis and invasive Klebsiella pneumoniae polyarticular septic arthritis. Clin Rheumatol. 2015;34(2):397–401.
26 Lougaris V, Soresina A, Baronio M, Montin D, Martino S, Signa S, et al. Long-term follow-up of 168 patients with X-linked agammaglobulinemia reveals increased morbidity and mortality. J Allergy Clin Immunol. 2020;146(2):429–37.
27 Rawat A, Jindal AK, Suri D, Vignesh P, Gupta A, Saikia B, et al. Clinical and genetic profile of X-linked agammaglobulinemia: a multicenter experience from India. Front Immunol. 2020;11:612323.
28 Van der Poll T, Opal SM. Host-pathogen interactions in sepsis. Lancet Infect Dis. 2008;8(1):32–43.
29 Paczosa MK, Mecsas J. Klebsiella pneumoniae: going on the offense with a strong defense. Microbiol Mol Biol Rev. 2016;80(3):629–61.
30 Németh T, Sperandio M, Mócsai A. Neutrophils as emerging therapeutic targets. Nat Rev Drug Discov. 2020;19(4):253–75.
31 Xiong H, Carter RA, Leiner IM, Tang YW, Chen L, Kreiswirth BN, et al. Distinct contributions of neutrophils and CCR2+ monocytes to pulmonary clearance of different Klebsiella pneumoniae strains. Infect Immun. 2015;83(9):3418–27.

32 Ramirez-Moral I, Blok DC, Bernink JH, Garcia-Laorden MI, Florquin S, Boon L, et al. Interleukin-33 improves local immunity during Gram-negative pneumonia by a combined effect on neutrophils and inflammatory monocytes. J Pathol. 2021;253(4):374–83.
33 Wieland CW, Van Lieshout MH, Hoogendijk AJ, Van der Poll T. Host defence during Klebsiella pneumonia relies on haematopoietic-expressed toll-like receptors 4 and 2. Eur Respir J. 2011;37(4):848–57.
34 Nguyen GT, Shaban L, Mack M, Swanson KD, Bunnell SC, Sykes DB, et al. SKAP2 is required for defense against *K. pneumoniae* infection and neutrophil respiratory burst. eLife. 2020;9:e56656.
35 Hendriks RW, De Bruijn MF, Maas A, Dingjan GM, Karis A, Grosveld F. Inactivation of Btk by insertion of lacZ reveals defects in B cell development only past the pre-B cell stage. Embo J. 1996;15(18):4862–72.
36 Maas A, Dingjan GM, Grosveld F, Hendriks RW. Early arrest in B cell development in transgenic mice that express the E41K Bruton's tyrosine kinase mutant under the control of the CD19 promoter region. J Immunol. 1999;162(11):6526–33.
37 De Porto A, Claushuis TAM, Van der Donk LEH, De Beer R, De Boer OJ, Florquin S, et al. Platelet Btk is required for maintaining lung vascular integrity during murine pneumococcal pneumosepsis. Thromb Haemost. 2019;119(6):930–40.
38 Passegué E, Wagner EF, Weissman IL. JunB deficiency leads to a myeloproliferative disorder arising from hematopoietic stem cells. Cell. 2004;119(3):431–43.
39 De Porto AP, Liu Z, De Beer R, Florquin S, Roelofs J, De Boer OJ, et al. Bruton's tyrosine kinase-mediated signaling in myeloid cells is required for protective innate immunity during pneumococcal pneumonia. Front Immunol. 2021;12:723967.
40 De Porto AP, Liu Z, De Beer R, Florquin S, De Boer OJ, Hendriks RW, et al. Btk inhibitor ibrutinib reduces inflammatory myeloid cell responses in the lung during murine pneumococcal pneumonia. Mol Med. 2019;25(1):3.
41 De Stoppelaar SF, Claushuis TA, Jansen MP, Hou B, Roelofs JJ, Van't Veer C, et al. The role of platelet MyD88 in host response during gram-negative sepsis. J Thromb Haemost. 2015;13(9):1709–20.
42 Mócsai A, Zhang H, Jakus Z, Kitaura J, Kawakami T, Lowell CA. G-protein-coupled receptor signaling in Syk-deficient neutrophils and mast cells. Blood. 2003;101(10):4155–63.
43 Branger J, Knapp S, Weijer S, Leemans JC, Pater JM, Speelman P, et al. Role of toll-like receptor 4 in gram-positive and gram-negative pneumonia in mice. Infect Immun. 2004;72(2):788–94.
44 Claushuis TAM, Van der Donk LEH, Luitse AL, Van Veen HA, Van der Wel NN, Van Vught LA, et al. Role of peptidylarginine deiminase 4 in neutrophil extracellular trap formation and host defense during *Klebsiella pneumoniae*-induced pneumonia-derived sepsis. J Immunol. 2018;201(4):1241–52.
45 Perlee D, de Beer R, Florquin S, Van der Poll T, Van't Veer C, de Vos AF. Caspase-11 contributes to pulmonary host defense against *Klebsiella pneumoniae* and local activation of coagulation. Am J Physiol Lung Cell Mol Physiol. 2020;319(1):L105–14.
46 Dahlgren C, Karlsson A, Bylund J. Measurement of respiratory burst products generated by professional phagocytes. Methods Mol Biol. 2007;412:349–63.
47 Kitamura D, Roes J, Kühn R, Rajewsky K. A B cell-deficient mouse by targeted disruption of the membrane exon of the immunoglobulin mu chain gene. Nature. 1991;350(6317):423–6.
48 Honigberg LA, Smith AM, Sirisawad M, Verner E, Loury D, Chang B, et al. The Bruton tyrosine kinase inhibitor PCI-32765 blocks B-cell activation and is efficacious in models of autoimmune disease and B-cell malignancy. Proc Natl Acad Sci U S A. 2010;107(29):13075–80.
49 Van Ziffle JA, Lowell CA. Neutrophil-specific deletion of Syk kinase results in reduced host defense to bacterial infection. Blood. 2009;114(23):4871–82.
50 Abram CL, Roberge GL, Hu Y, Lowell CA. Comparative analysis of the efficiency and specificity of myeloid-Cre deleting strains using ROSA-EYFP reporter mice. J Immunol Methods. 2014;408:89–100.
51 Mócsai A. Diverse novel functions of neutrophils in immunity, inflammation, and beyond. J Exp Med. 2013;210(7):1283–99.
52 Hirche TO, Gaut JP, Heinecke JW, Belaaouaj A. Myeloperoxidase plays critical roles in killing *Klebsiella pneumoniae* and inactivating neutrophil elastase: effects on host defense. J Immunol. 2005;174(3):1557–65.
53 Papayannopoulos V, Metzler KD, Hakkim A, Zychlinsky A. Neutrophil elastase and myeloperoxidase regulate the formation of neutrophil extracellular traps. J Cell Biol. 2010;191(3):677–91.
54 Bengoechea JA, Sa Pessoa J. Klebsiella pneumoniae infection biology: living to counteract host defences. FEMS Microbiol Rev. 2018;43(2):123–44.
55 Paczosa MK, Silver RJ, McCabe AL, Tai AK, McLeish CH, Lazinski DW, et al. Transposon mutagenesis screen of *Klebsiella pneumoniae* identifies multiple genes important for resisting antimicrobial activities of neutrophils in mice. Infect Immun. 2020;88(4):e00034–20.
56 Lundqvist H, Dahlgren C. Isoluminol-enhanced chemiluminescence: a sensitive method to study the release of superoxide anion from human neutrophils. Free Radic Biol Med. 1996;20(6):785–92.
57 Baumgarth N. A hard(y) look at B-1 cell development and function. J Immunol. 2017;199(10):3387–94.
58 Reid RR, Prodeus AP, Khan W, Hsu T, Rosen FS, Carroll MC. Endotoxin shock in antibody-deficient mice: unraveling the role of natural antibody and complement in the clearance of lipopolysaccharide. J Immunol. 1997;159(2):970–5.
59 Barbeiro DF, Barbeiro HV, Faintuch J, Ariga SK, Mariano M, Popi AF, et al. B-1 cells temper endotoxemic inflammatory responses. Immunobiology. 2011;216(3):302–8.
60 Lepper PM, Möricke A, Held TK, Schneider EM, Trautmann M. K-antigen-specific, but not O-antigen-specific natural human serum antibodies promote phagocytosis of Klebsiella pneumoniae. FEMS Immunol Med Microbiol. 2003;35(2):93–8.
61 Cisalpino D, Fagundes CT, Brito CB, Ascencao FR, Queiroz-Junior CM, Vieira AT, et al. Microbiota-induced antibodies are essential for host inflammatory responsiveness to sterile and infectious stimuli. J Immunol. 2017;198(10):4096–106.
62 Domenico P, Salo RJ, Cross AS, Cunha BA. Polysaccharide capsule-mediated resistance to opsonophagocytosis in *Klebsiella pneumoniae*. Infect Immun. 1994;62(10):4495–9.
63 Tsai WC, Strieter RM, Wilkowski JM, Bucknell KA, Burdick MD, Lira SA, et al. Lung-specific transgenic expression of KC enhances resistance to *Klebsiella pneumoniae* in mice. J Immunol. 1998;161(5):2435–40.
64 Cai S, Batra S, Lira SA, Kolls JK, Jeyaseelan S. CXCL1 regulates pulmonary host defense to Klebsiella Infection via CXCL2, CXCL5, NF-kappaB, and MAPKs. J Immunol. 2010;185(10):6214–25.
65 Batra S, Cai S, Balamayooran G, Jeyaseelan S. Intrapulmonary administration of leukotriene B(4) augments neutrophil accumulation and responses in the lung to Klebsiella infection in CXCL1 knockout mice. J Immunol. 2012;188(7):3458–68.
66 Belaaouaj A, McCarthy R, Baumann M, Gao Z, Ley TJ, Abraham SN, et al. Mice lacking neutrophil elastase reveal impaired host defense against gram negative bacterial sepsis. Nat Med. 1998;4(5):615–8.
67 Hirche TO, Gaut JP, Heinecke JW, Belaaouaj A. Myeloperoxidase plays critical roles in killing *Klebsiella pneumoniae* and inactivating neutrophil elastase: effects on host defense. J Immunol. 2005;174(3):1557–65.
68 Claushuis TAM, De Stoppelaar SF, De Vos AF, Grootemaat AE, Van der Wel NN, Roelofs J, et al. Nbeal2 deficiency increases organ damage but does not affect host defense during Gram-negative pneumonia-derived sepsis. Arterioscler Thromb Vasc Biol. 2018;38(8):1772–84.

69 Florence JM, Krupa A, Booshehri LM, Davis SA, Matthay MA, Kurdowska AK. Inhibiting Bruton's tyrosine kinase rescues mice from lethal Influenza induced acute lung injury. Am J Physiol Lung Cell Mol Physiol. 2018;315(1): L52–8.
70 March C, Cano V, Moranta D, Llobet E, Pérez-Gutiérrez C, Tomás JM, et al. Role of bacterial surface structures on the interaction of *Klebsiella pneumoniae* with phagocytes. PLoS One. 2013;8(2):e56847.
71 Cano V, March C, Insua JL, Aguilo N, Llobet E, Moranta D, et al. *Klebsiella pneumoniae* survives within macrophages by avoiding delivery to lysosomes. Cell Microbiol. 2015; 17(11):1537–60.
72 Wanford JJ, Hames RG, Carreno D, Jasiunaite Z, Chung WY, Arena F, et al. Interaction of *Klebsiella pneumoniae* with tissue macrophages in a mouse infection model and ex-vivo pig organ perfusions: an exploratory investigation. Lancet Microbe. 2021;2(12): e695–703.
73 Roos D. Chronic granulomatous disease. Br Med Bull. 2016;118(1):50–63.
74 Kahn ML, Koretzky GA. Integrins and ITAMs: more than just good neighbors. Nat Immunol. 2006;7(12):1286–8.
75 El-Benna J, Hurtado-Nedelec M, Marzaioli V, Marie JC, Gougerot-Pocidalo MA, Dang PM. Priming of the neutrophil respiratory burst: role in host defense and inflammation. Immunol Rev. 2016;273(1):180–93.
76 Van Lieshout MH, Anas AA, Florquin S, Hou B, Van't Veer C, De Vos AF, et al. Hematopoietic but not endothelial cell MyD88 contributes to host defense during gram-negative pneumonia derived sepsis. PLoS Pathog. 2014;10(9):e1004368.
77 Van Lieshout MH, Blok DC, Wieland CW, De Vos AF, Van't Veer C, Van der Poll T. Differential roles of MyD88 and TRIF in hematopoietic and resident cells during murine gram-negative pneumonia. J Infect Dis. 2012; 206(9):1415–23.
78 Liljeroos M, Vuolteenaho R, Morath S, Hartung T, Hallman M, Ojaniemi M. Bruton's tyrosine kinase together with PI 3-kinase are part of Toll-like receptor 2 multiprotein complex and mediate LTA induced Toll-like receptor 2 responses in macrophages. Cell Signal. 2007;19(3):625–33.
79 Bhan U, Lukacs NW, Osterholzer JJ, Newstead MW, Zeng X, Moore TA, et al. TLR9 is required for protective innate immunity in Gram-negative bacterial pneumonia: role of dendritic cells. J Immunol. 2007;179(6): 3937–46.
80 Varughese T, Taur Y, Cohen N, Palomba ML, Seo SK, Hohl TM, et al. Serious infections in patients receiving ibrutinib for treatment of lymphoid cancer. Clin Infect Dis. 2018;67(5): 687–92.