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ORIGINAL RESEARCH ARTICLE

Preclinical and Toxicology Studies of BRD5529, a Selective Inhibitor of CARD9

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Abstract

Background The caspase recruitment domain-containing protein 9 (CARD9) inhibitor BRD5529 has been shown to be an effective in vitro inhibitor of *Pneumocystis* β-glucan-induced proinflammatory signaling, suggesting its viability as a candidate for preliminary anti-*Pneumocystis* drug testing in the rodent *Pneumocystis* pneumonia (PCP) model.

Methods Mice were injected intraperitoneally (IP) daily with either vehicle or BRD5529 at 0.1 or 1.0 mg/kg for 2 weeks. Mouse weights were taken daily. At day 14, mice were euthanized, weighed, and analyzed by flexiVent™ for lung stiffness. Lungs, liver, and kidney were then harvested for hematoxylin and eosin (H&E) staining and pathology scoring. Lung samples were further analyzed for proinflammatory cytokines via enzyme-linked immunosorbent assay (ELISA) and extracellular matrix generation via quantitative polymerase chain reaction (qPCR). Blood collection postmortem was performed for blood chemistry analysis. Furthermore, administration of BRD5529 prior to the intratracheal inoculation of fungal β-glucans, which are known proinflammatory mediators via the Dectin-1-CARD9 pathway, resulted in significant reductions in lung tissue interleukin-6 and tumor necrosis factor-α, suggesting the exciting possibility of the use of this CARD9 inhibitor as an additional therapeutic tool in fungal infections.

Results BRD5529 at both IP doses resulted in no significant changes in daily or final weight gain, and analysis of lung stiffness by flexiVent™ showed no significant differences between the groups. Furthermore, ELISA results of proinflammatory cytokines showed no major differences in the respective groups. qPCR analysis of extracellular matrix transcripts were statistically similar. Examination and pathology scoring of H&E slides from lung, liver, and kidney in all groups, as well as subsequent pathology scoring, showed no significant change. Blood chemistry analysis revealed similar, non-significant patterns.

Conclusions In our initial general safety and toxicology assessments, BRD5529 displayed no inherent safety concerns in the analyzed parameters. These data support broader in vivo testing of the inhibitor as a timed adjunct therapy to the deleterious proinflammatory host immune response often associated with anti-*Pneumocystis* therapy.

Key Points

An inhibitor of CARD9 termed BRD5529 was well tolerated when administered to mice via intraperitoneal injection.

Administration of BRD5529 prior to intratracheal administration of yeast β-glucans resulted in significant reductions in the host inflammatory response.

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1 Introduction

Caspase recruitment domain-containing protein 9 (CARD9) is a central mediator downstream of C-type lectin receptors (CLRs) that is vital for microbial pathogen proinflammatory host immune response and organism burden control [1]. CARD9 is highly expressed in myeloid cells and is shown to be particularly important in fungal infections [2]. Others have demonstrated CARD9 pathway intervention with the chemical CARD9 inhibitor BRD5529 can directly mimic a protective variant of the protein and may provide therapeutic benefit for those with inflammatory bowel disease [3]. We have recently shown that pre-incubation of BRD5529 with RAW macrophages prior to the application of proinflammatory β-glucans from the lung pathogen *Pneumocystis* spp. results in substantial reduction in downstream CARD9 proinflammatory signaling and subsequent tumor necrosis factor (TNF)-α release, suggesting that timed therapeutic intervention during or after anti-*Pneumocystis* treatment may greatly improve the deleterious effects on the host caused by organism killing and release of proinflammatory carbohydrates [4]. The purpose of this study was to evaluate the short-term administration of CARD9 inhibitor BRD5529 in mice via intraperitoneal (IP) administration and to address potential detrimental responses to the inhibitor via physiological, inflammatory, and toxicological analysis. Furthermore, we present evidence that BRD5529 can significantly dampen fungal β-glucan-induced inflammation in the lung. These data demonstrate the safety of BRD5529 and support broader clinical development of the CARD9 inhibitor for in vitro administration in the *Pneumocystis* pneumonia (PCP) mouse treatment model as a therapeutic tool to treat PCP host inflammation upon anti-*Pneumocystis* treatment.

2 Methods

2.1 Animals

Equal numbers of male and female C57BL/6 mice (Charles River Laboratories, Wilmington, MA, USA) aged 10–12 weeks were used for all experiments. Mice undergo a mandatory 2-day acclimation period before any experiments can be performed. The mice were separated by sex and were housed three to a cage. They were fed PicoLab® Mouse Diet 20 5058* ad libitum and were watered from an automatic watering valve. Included in the housing, the mice were provided 'standard enrichment material' for the facility of two Twist-n'Rich™ and a Bed-r'Nest® (The Andersons Plant Nutrient Group).

All animal procedures were performed in accordance with the Laboratory Animal Welfare Act, the Guide for the Care and Use of Laboratory Animals Welfare Act, and the Mayo Clinic Institutional Animal Care and Use Committee (IACUC; approval number A00005722-20).

2.2 Administration of BRD5529

BRD5529 was obtained for Sigma Aldrich. Dose selection (for 1 mg/kg) was based on that previously described [5] to achieve an in vivo half maximal inhibitory concentration (IC_{50}) value of approximately 10.7 μM, similar to that of a previously published IC_{50} in vitro value of 8.6 μM [3]. Due to the lack of solubility of the inhibitor in water or saline, the inhibitor was prepared with 1% Methocel™ [6]. IP treatment (100 μL) with 1% Methocel™ (vehicle, control mice group) or the indicated concentration (mg/kg) of the BRD5529 inhibitor in Methocel™ was initiated on day 0 and subsequently every day for 14 days. At day 14, the mice were sacrificed and subsequent analysis was performed as described below.

2.3 Flexivent™ Analysis

FlexiVent™ analysis was performed at described previously [7].

2.4 Enzyme-Linked Immunosorbent Assay (ELISA) Determination of Cytokine Release

Cytokines were analyzed from total lung homogenates. Enzyme-linked immunosobent assay (ELISA) kits to measure mouse interleukin (IL)-1β, IL-6, and TNFα were purchased from Thermo Fisher Scientific.

2.5 Quantitative Polymerase Chain Reaction Analysis

To extract RNA from mouse lung, tissue was lysed and homogenized with Buffer RLT Plus (supplied with the RNeasy® Plus Mini Kit; Qiagen). The lysate was passed through a genomic DNA eliminator spin column, ethanol was added, and the sample was applied to a RNeasy MinElute spin column according to the manufacturer's instructions. An iScript™ Select cDNA synthesis kit (Bio-Rad, Hercules, CA, USA) was used for reverse transcription using oligo (dT) primers and random hexamer primer mix. An SYBR green PCR kit (Bio-Rad) was used for quantitative real-time PCR and was performed and analyzed on a CF96 Touch™ Real-Time PCR Detection System (Bio-Rad). The sequences of the primer pairs are listed in electronic supplementary Table 1.

2.6 Biochemical Analysis

For blood chemistry analysis, serum was analyzed using the Piccolo Xpress™ Chemistry Analyzer according to the manufacturer's instructions.

2.7 Histology Analysis

For histological analysis, lung, liver, and kidney samples were fixed in 10% neutral formalin. Paraffin embedding and staining were performed at the Mayo Clinic Histology Core, Scottsdale, AZ, USA. Sections (5 μm) were stained with hematoxylin and eosin (H&E) and graded blindly for the extent of organ inflammation by a pathologist from the Mayo Clinic. The sections were scored as follows: 1+, mild perivascular aggregates; 2+, heavy perivascular aggregates; 3+, mild alveolar aggregates; 4+, alveolar exudate and heavy alveolar aggregates; and 0, normal. These scores were based on grading of the entire organ surface area present on the slide section.

2.8 In Vivo Inflammatory Analysis of BRD5529

To determine if administration of BR5529 via IP injection might result in systemic distribution, as well as the effects on the host lung inflammatory response, the following experiment was conducted. Briefly, 20 h prior to administering mice 100 ug/mL *Saccharomyces cerevisiae* β-glucans via intratracheal (IT) injection (similar to the study by Vassallo et al. [8]), mice were administered 1 mg/kg of BRD5529 or the vehicle control administered IP as noted above. The next day at 18 h post BRD5529 or vehicle treatment, mice were administered another 1 mg/kg of BRD5529 or vehicle as stated. After 2 h, mice were administered ±100 ug/mL *S. cerevisiae* β-glucans (Sigma Aldrich) administered IT. The following day mice were sacrificed and lung protein lysates were measured for IL-6 and TNFα as noted above.

2.9 Statistical Analysis

For multigroup data, initial analysis was first performed with analysis of variance (ANOVA) to determine the overall different differences. If ANOVA indicated overall differences, subsequent group analysis was then performed using the two-sample, unpaired Student's *t* test for normally distributed variables. The sample size of six animals per group was based on our preliminary and prior observations; with a sample size of six for each group, there is a power of at least 0.9 to detect a difference for a given parameter representing 1.5 times within the group standard deviation. The mouse groups ($n = 6$) contained equal numbers of male and female mice. Since there were no discernable differences in the data based on sex, the data from all mice were combined for the reported results. For the in vivo studies analyzing the effects of BRD5529 on β-glucan-induced lung inflammation, the sample size for each group was eight mice. Evaluation of data was conducted using Prism 9 for MacOS, version 9.3.1 (GraphPad, San Diego, CA, USA). P values < 0.05 were considered significant.

3 Results

3.1 BRD5529 Intraperitoneal (IP) Administration Resulted in no Significant Weight Loss

Administration of BRD5529 at 0.1 or 1.0 mg/kg IP resulted in no significant changes in daily and ending weight loss (Fig. 1).

3.2 Static Lung Compliance After BRD5529 IP Administration

To measure lung function in mice after 14 days of IP administration of vehicle or BRD5529 at 0.1 or 1.0 mg/kg, we used the flexiVent™ apparatus. Daily IP injections of all three conditions for 14 days resulted in no significant increases in lung static compliance between the three groups tested (Fig. 2).

3.3 Measurements of Lung Cytokines in BRD5529 IP Administration and Control Groups

Production of the inflammatory cytokines IL-1β, IL-6, and TNFα were measured in whole lung lysates, as shown in Fig. 3a–c. No significant alterations were noted from the vehicle control.

3.4 Analysis of mRNA Extracellular Matrix Generation

Quantitative polymerase chain reaction (qPCR) was implemented to determine the levels of messenger RNA (mRNA) expression of collagen type alpha-1 chain (Col1a1) and fibronectin (Fn), both extracellular matrix-related genes used as markers for profibrotic development [9]. β2 microglobulin (B2M) was used as a housekeeping gene. As shown in Fig. 4a–b, no significant differences were noted in the three groups at day 14 in the respective lung samples.

3.5 Serum Chemistry Data

Complete group mean serum chemistry data from data 14 are presented in Fig. 5a–n. There were no noteworthy changes in either of the BRD5529 dose groups compared with the vehicle control.

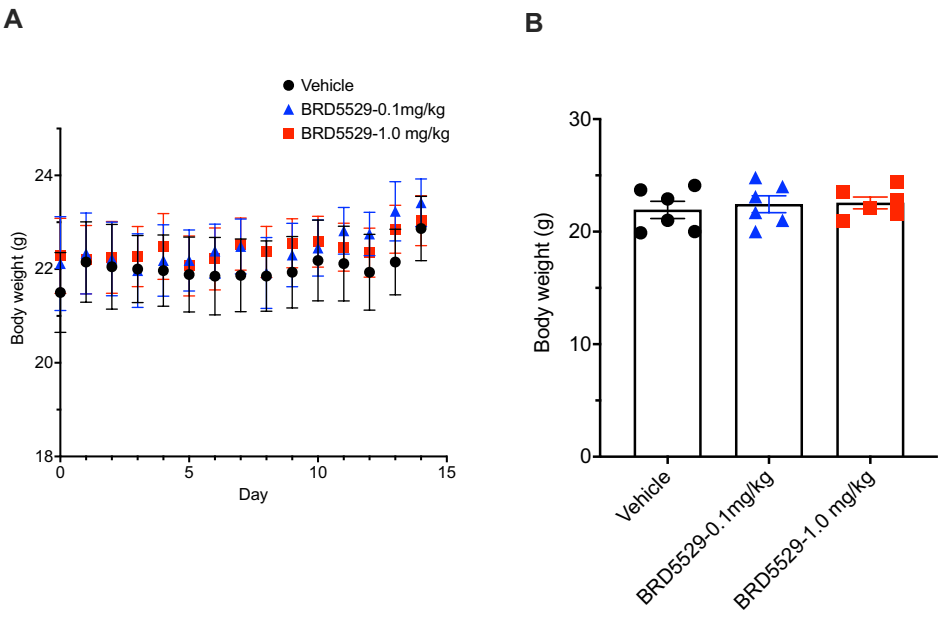

Fig. 1 Effects of mouse body weight with daily IP administration of BRD5529. **a** Daily weight changes in the vehicle control versus the 0.1 and 1.0 mg/kg doses of IP BRD5529 administered daily for 14 days. **b** Final weights for the three groups after 14 days ($n = 6$ mice/group). No significant weight changes were noted between the three groups. *IP* intraperitoneal

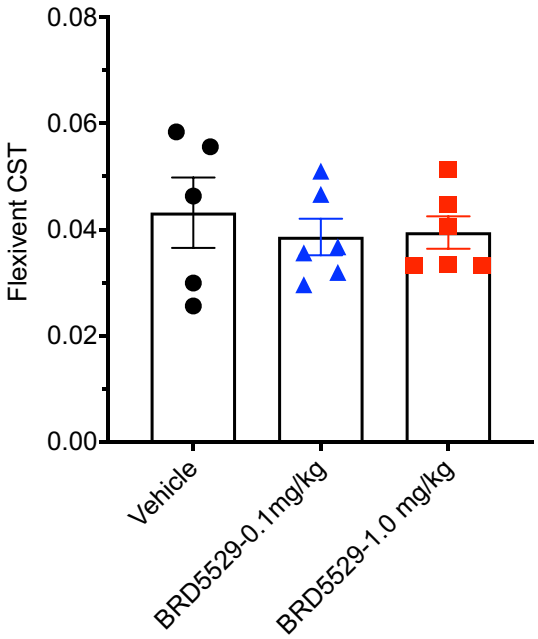

Fig. 2 CARD9 inhibitor BRD5529 effects on lung compliance. Lung quasi-static compliance (CST; reflects the intrinsic elastic properties of the lung and chest at rest) [$n = 6$ mice/group]. No significant changes in CST were noted between the three groups.

3.6 Histology Analysis

Histologic examination of all samples from lung, liver, and kidney from both the BRD5529-treated and vehicle groups did not reveal any abnormality and all organs appeared normal (score 0 for all parameters) (Fig. 6a–f).

3.7 In Vivo BRD5529 Anti-Inflammatory Analysis

Analysis of the effects of two doses of IP BRD5529 the day before (18 h) and 2 h before IT injection of yeast β-glucans demonstrated significant decreases in IL-6 and TNFα in lung protein lysates as measured by ELISA (Fig. 7a–b).

4 Discussion

Fungi are major contributors to opportunistic infections in those patients with advanced HIV. PCP, caused by *Pneumocystis jirovecii*, is the most common pathogen in AIDS populations across the globe. Implementation of highly active antiretroviral therapy (HAART) has decreased the overall incidence of PCP but the ability to receive this treatment is limited and the number of cases requiring hospitalization is still quite high [10].

The most current anti-*Pneumocystis* therapy, the use of trimethoprim-sulfamethoxazole (TMP-SMX), has proven to an effective antimicrobial combination to treat *Pneumocystis jirovecii* pneumonia (PJP). Although effective, the inflammatory response upon fungal cell death, via the exposure of exuberant newly exposed β-glucans, can prove highly detrimental to the host [11–13]. Indeed, when corticosteroids are utilized in HIV patients with moderate to severe PJP, a significant decrease in mortality and morbidity was noted [14]. In non-HIV patients, data on adjuvant corticosteroids is less clear and no consensus has been determined. Although corticosteroids may be beneficial to patients in these settings, there are still both short-term (co-infections, hyperglycemia) and long-term (myopathy and osteoporosis) factors that need to be considered [15].

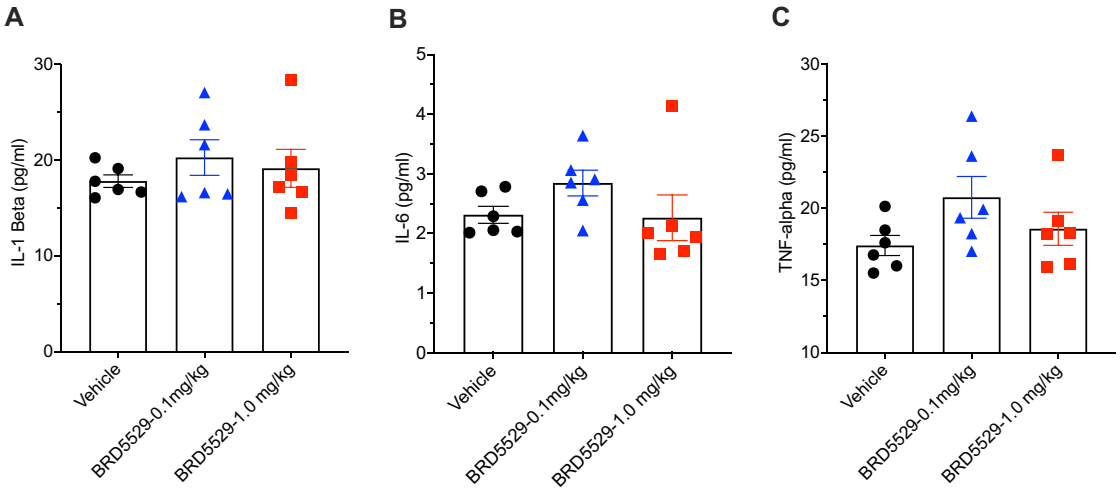

Fig. 3 CARD9 inhibitor BRD5529 effects on lung proinflammatory cytokine production. **a** IL-1β, **b** IL-6, and **c** TNFα production were measured from total lung lysates from day 14 of the experiment ($n = 6$ mice/group). No significant differences were noted between the groups. *IL* interleukin, *TNF* tumor necrosis factor

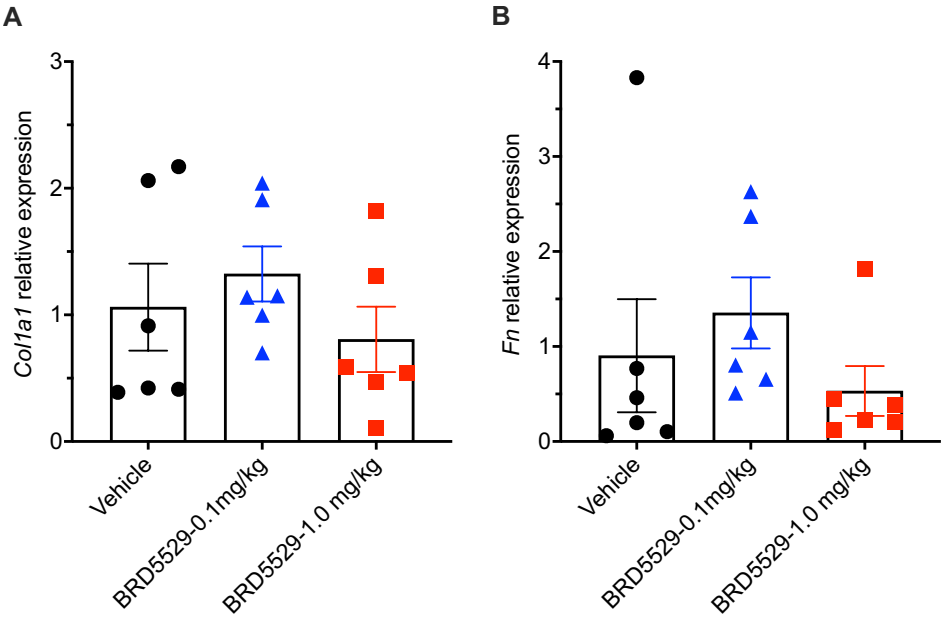

Fig. 4 Quantitation of *Col1a1* and *Fn* mRNA in total lung RNA after vehicle and BRD5529 administration for 14 days. Ratios of **a** *Col1a1* and **b** *Fn* to *B2M* in total lung RNA ($n = 6$ mice/group). No significant differences were noted between the groups. *mRNA* messenger RNA, *B2M* β2 microglobulin

Therefore, other adjunct therapies should be considered in PJP. We have recently demonstrated that in vivo, macrophages pre-incubated with the CARD9 inhibitor BRD5529 have significant reductions in their ability to generate proinflammatory signaling and downstream TNFα production upon stimulation with *Pneumocystis* β-glucans [4]. These results lead us to hypothesize that BRD5529 may be used in vivo as an adjunct therapy similar to corticosteroids [4]. As part of the development toward human clinical application, a thorough preclinical assessment must be conducted to evaluate the safety and potential toxicity of the BRD5529 CARD9 inhibitor. To the best of our knowledge, this is the first study conducted to examine this, and the preclinical profile presented here suggests that BRD5529 could meet these early criteria.

In this study, we administered BRD5529 to mice at either 0.1 or 1.0 mg/kg IP once daily for 14 days. In acute toxicity studies, administration of BRD5529 appeared to be well tolerated in mice at both doses. Parameters such as weight loss, lung function, lung-specific proinflammatory response, lung extracellular matrix mRNA generation, blood toxicology analysis, and H&E histological examination of lung, liver,

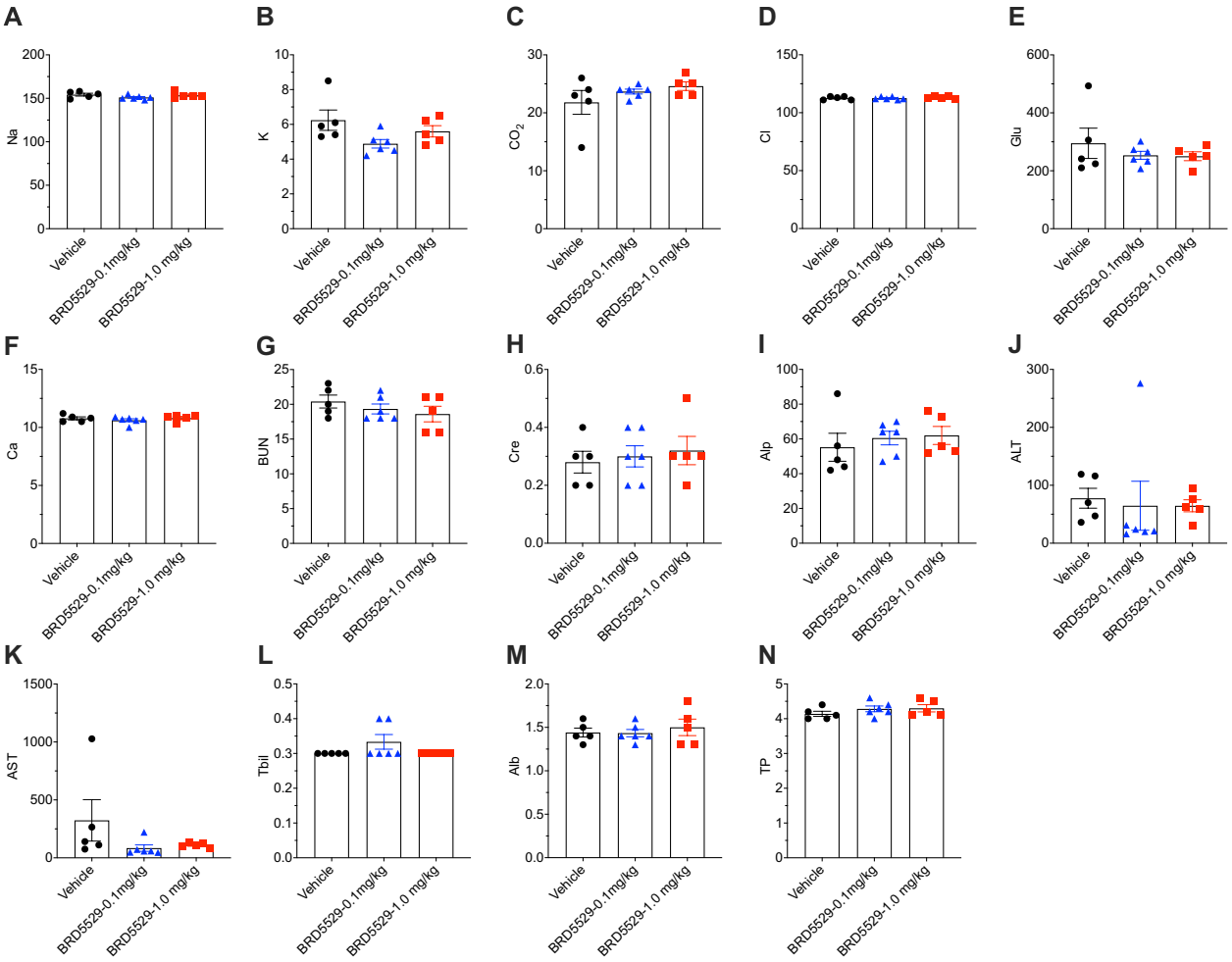

Fig. 5 Serum chemistry parameters (n = 5–6 mice/group). No significant differences were noted between the groups. *Na* sodium (mmol/L), *K* potassium (mmol/L), CO_2 carbon dioxide (mmol/L), *Cl* chloride (mmol/L), *Glu* glucose (mg/dL), *Ca* calcium (mg/dL), *BUN* blood urea nitrogen (mg/dL), *Cre* creatine (g/dL), *Alp* alkaline phosphatase (U/L), *ALT* alanine aminotransferase (U/L), *Tbil* total bilirubin (mg/dL), *Alb* albumin (g/dL), *TP* total protein (g/dL)

and kidney samples yielded no significant changes compared with the vehicle control. Furthermore, we demonstrated that pretreatment of mice with the CARD9 inhibitor BRD5529 prior to the addition of fungal β-glucans, a major cell wall proinflammatory constituent of pathogenic fungi, including *Pneumocystis*, can significantly reduce IL-6 and TNFα production in lung tissue.

5 Conclusions

Based on these preliminary findings, future use of BRD5529 in in vivo mouse studies to determine if the CARD9 inhibitor can be used to reduce the deleterious effects of the host proinflammatory response in the PCP model seems safe and feasible.

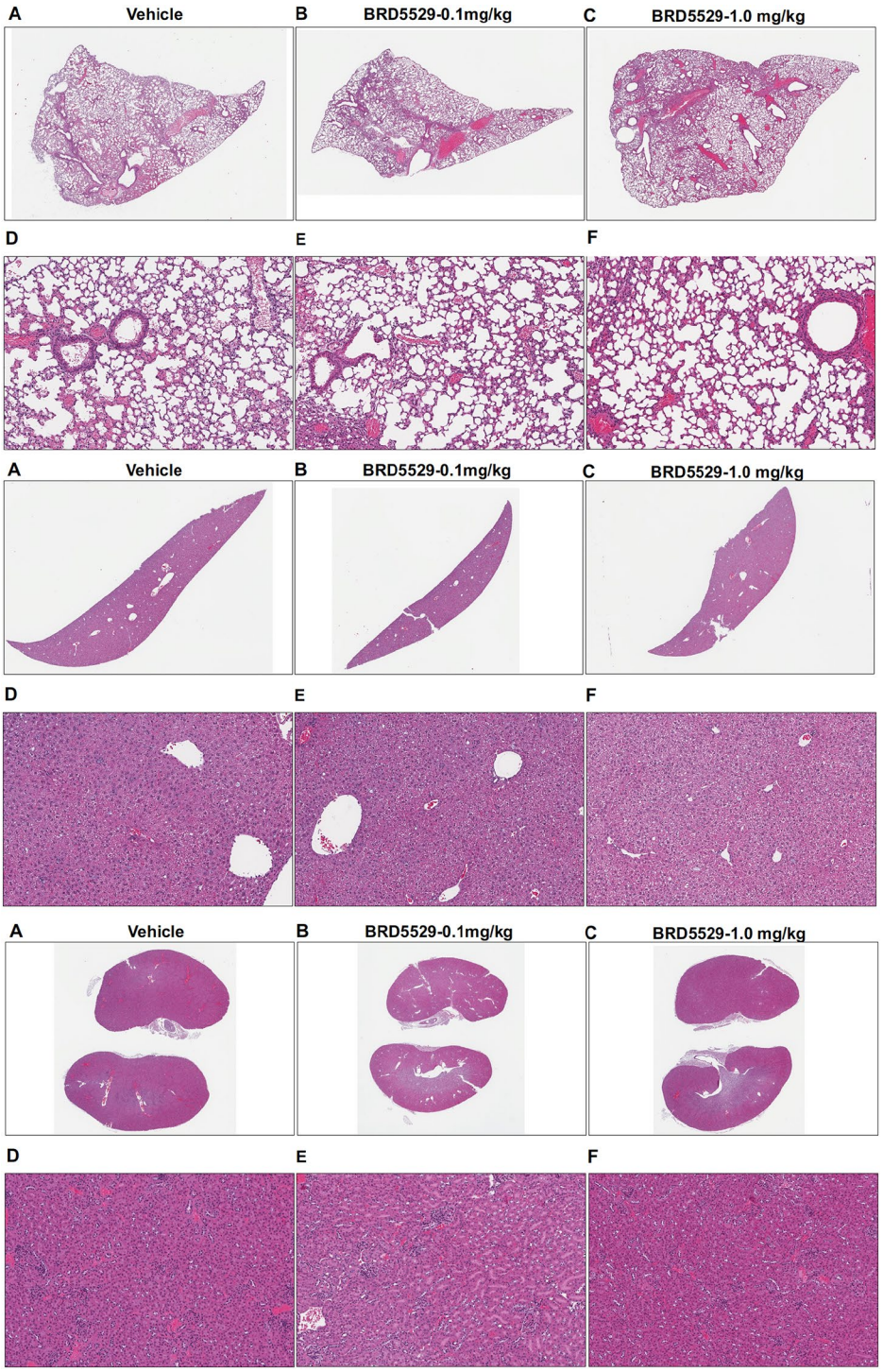

Fig. 6 Lung, liver, and kidney histopathology of 14-day IP-treated vehicle or BRD5529 CARD9 inhibitor. H&E staining was performed on sections of lung (top two panels), liver (middle two panels), and kidney (bottom two panels) from mice in all groups. **a** Vehicle control. **b** BRD5529 at 0.1 mg/kg. **c** BRD5529 at 1 mg/kg. **a–c** = ×1 magnification; **d–f** = ×10 magnification. No gross histological changes were present in lung, liver, and kidneys in the vehicle and BRD5529 doses tested. *IP* intraperitoneal, *H&E* hematoxylin and eosin

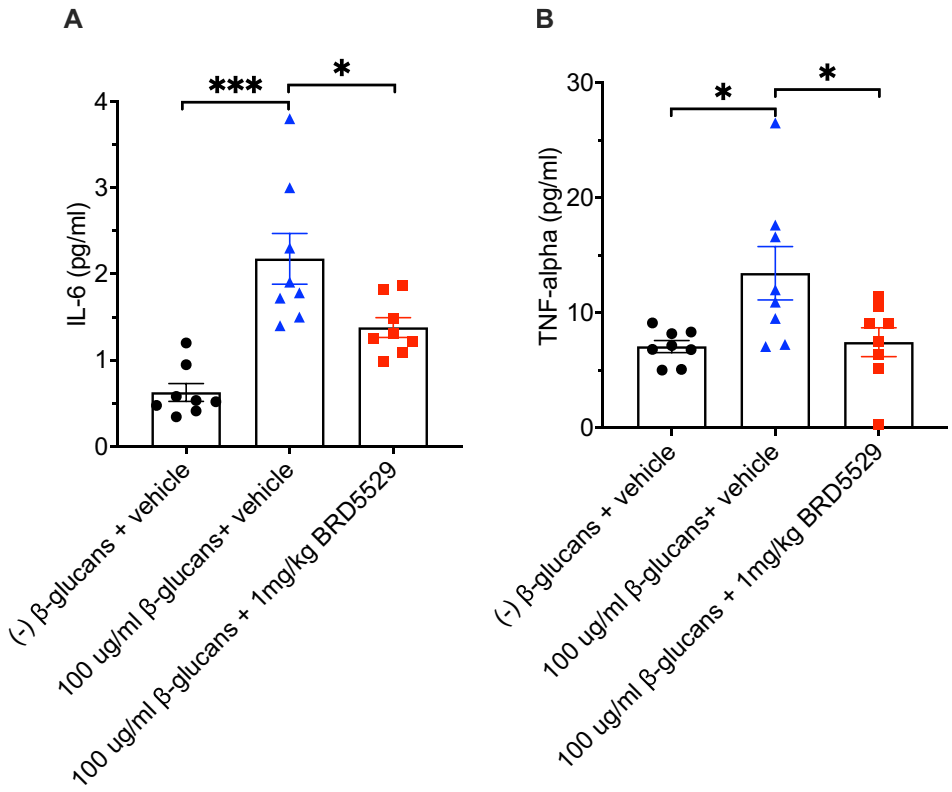

Fig. 7 Effects of IP injection of BRD5529 on yeast β-glucan-induced proinflammatory response. Twenty hours prior to administering mice 100 ug/mL *Saccharomyces cerevisiae* β-glucans via IT injection, mice were administered 1 mg/kg BRD5529 or the vehicle control via IP administration as noted above. The next day at 18 h post BRD5529 or vehicle treatment, mice were administered another 1 mg/kg of BRD5529, or vehicle as stated. After 2 h, mice were administered ± 100 ug/mL *S. cerevisiae* β-glucans via IT administration. The following day, mice were sacrificed and lung protein lysates (50 ug total) measured for **a** IL-6 and **b** TNFα. Bar graph represents the results from eight mice per group (±SEM; $*p < 0.05$, $***p < 0.001$). *IT* intratracheal, *IP* intraperitoneal, *IL* interleukin, *TNF* tumor necrosis factor, *SEM* standard error of the mean

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s40268-022-00389-0.

Declarations

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Conflict of interest Theodore J. Kottom, Kyle Schaefbauer, Eva M. Carmona, Eunhee S. Yi, and Andrew H. Limper declare no conflicts of interest.

Ethics approval This study was approved by the Mayo Clinic IACUC (approved protocol A00005722-20).

Consent to participate Not applicable.

Consent for publication Not applicable.

Availability of data and material The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Code availability Not applicable.

Author contributions TJK, KS, ESY, and AHL made substantial contributions to the conception or design of the work, and the acquisition, analysis, or interpretation of data. EMC revised the manuscript critically for important intellectual content and all authors approved the version to be published.

References

1. Tang J, Lin G, Langdon WY, Tao L, Zhang J. Regulation of C-type lectin receptor-mediated antifungal immunity. Front Immunol. 2018;9:123.

2. Drummond RA, Franco LM, Lionakis MS. Human CARD9: a critical molecule of fungal immune surveillance. Front Immunol. 2018;9:1836.
3. Leshchiner ES, Rush JS, Durney MA, Cao Z, Dancik V, Chittick B, et al. Small-molecule inhibitors directly target CARD9 and mimic its protective variant in inflammatory bowel disease. Proc Natl Acad Sci USA. 2017;114(43):11392–7.
4. Kottom TJ, Carmona EM, Limper AH. Targeting CARD9 with small-molecule therapeutics inhibits innate immune signaling and inflammatory response to *Pneumocystis carinii* beta-glucans. Antimicrob Agents Chemother. 2020;64(11):e01210-e1220.
5. Mansoor A, Mahabadi N. Volume of distribution. Treasure Island (FL): StatPearls; 2022.
6. Jung MY, Kang JH, Hernandez DM, Yin X, Andrianifahanana M, Wang Y, et al. Fatty acid synthase is required for profibrotic TGF-beta signaling. FASEB J. 2018;32(7):3803–15.
7. Yin X, Choudhury M, Kang JH, Schaefbauer KJ, Jung MY, Andrianifahanana M, et al. Hexokinase 2 couples glycolysis with the profibrotic actions of TGF-beta. Sci Signal. 2019;12(612):4067.
8. Vassallo R, Standing JE, Limper AH. Isolated Pneumocystis carinii cell wall glucan provokes lower respiratory tract inflammatory responses. J Immunol. 2000;164(7):3755–63.
9. Kang JH, Jung MY, Choudhury M, Leof EB. Transforming growth factor beta induces fibroblasts to express and release the immunomodulatory protein PD-L1 into extracellular vesicles. FASEB J. 2020;34(2):2213–26.
10. Thomas CF Jr, Limper AH. Current insights into the biology and pathogenesis of *Pneumocystis pneumonia*. Nat Rev Microbiol. 2007;5(4):298–308.
11. Evans HM, Simpson A, Shen S, Stromberg AJ, Pickett CL, Garvy BA. The trophic life cycle stage of the opportunistic fungal pathogen *Pneumocystis murina* hinders the ability of dendritic cells to stimulate CD4(+) T cell responses. Infect Immun. 2017;85(10):e00396-e417.
12. Kutty G, Davis AS, Ferreyra GA, Qiu J, Huang DW, Sassi M, et al. β-glucans are masked but contribute to pulmonary inflammation during pneumocystis pneumonia. J Infect Dis. 2016;214(5):782–91.
13. Linke MJ, Ashbaugh A, Collins MS, Lynch K, Cushion MT. Characterization of a distinct host response profile to *Pneumocystis murina* asci during clearance of pneumocystis pneumonia. Infect Immun. 2013;81(3):984–95.
14. Bozzette SA, Sattler FR, Chiu J, Wu AW, Gluckstein D, Kemper C, et al. A controlled trial of early adjunctive treatment with corticosteroids for *Pneumocystis carinii* pneumonia in the acquired immunodeficiency syndrome California Collaborative Treatment Group. N Engl J Med. 1990;323(21):1451–7.
15. Weyant RB, Kabbani D, Doucette K, Lau C, Cervera C. Pneumocystis jirovecii: a review with a focus on prevention and treatment. Expert Opin Pharmacother. 2021;22(12):179–1592.