Immunotherapy with Synergistic Pattern Recognition Receptor Agonists Improves Morbidity and Mortality in a Corticosteroid-Immunosuppressed Murine Model of Influenza-Associated Pulmonary Aspergillosis

Sebastian Wurster[1], **Jezreel Pantaleón García**[2], **Nathaniel D. Albert**[1], **Scott Evans**[2], **Dimitrios P. Kontoyiannis**[1]

[1] Department of Infectious Diseases, Infection Control and Employee Health, The University of Texas MD Anderson Cancer Center
[2] Department of Pulmonary Medicine, The University of Texas MD Anderson Cancer Center

THE UNIVERSITY OF TEXAS MD Anderson Cancer Center
Making Cancer History®

Background

Influenza-associated pulmonary aspergillosis (IAPA) is a feared complication in patients with severe influenza, especially those receiving corticosteroids. However, validated murine models of IAPA in a background of corticosteroid immunosuppression are lacking, compounding efforts to better characterize the immunopathology and treatment of this emerging entity. Furthermore, robust *in-vivo* data for immunotherapeutic interventions to improve the outcomes of IAPA are scarce. Therefore, we established a novel IAPA mouse model that allows us to compare the outcomes of IAPA in mice with and without corticosteroid immunosuppression and to study the effect of the synergistic pattern recognition receptor (PRR) agonists Pam-2 and ODN.

Methods

IAPA infection model: 8-week-old female BALB/c mice were infected with 1.5% or 7.5% of the 90% lethal dose (LD_{90}) of a mouse-adapted influenza A/Hong Kong/1968 (H3N2) strain (influenza A virus, IAV), delivered by aerosolization. Aerosolized saline was used as a control. Mice then received two intraperitoneal injections of 10 mg cortisone acetate (CA) or mock injections on days 5 and 8 after influenza infection. On day 9, mice were intranasally challenged with 50,000 *Aspergillus fumigatus* AF-293 conidia or mock-infected with saline.

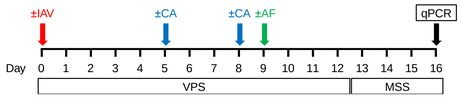

Figure 1: Experimental timelines for model establishment.

Anti-infective therapy: For selected experiments, mice received daily intravenous injections of liposomal amphotericin B (LAmB, 5 mg/kg/injection), starting either on the day of AF infection (day 9, "early") or on the day following AF infection (day 10, "late"). Empty liposomes (ELS) and 5% glucose (vehicle) were used as controls. For some experiments, some mice additionally received oseltamivir (OST, 10 mg/kg) or mock treatment with 5% glucose twice daily by oral gavage on days 3 through 6 after IAV infection.

Immunotherapy: For immunotherapeutic studies, mice received a 30-minute nebulization of the synergistic PRR agonists Pam2+ODN (4 µM/1 µM) or PBS (mock treatment) on day 8 (single-dose) or on days 8 and 12 (dual-dose).

Readouts: Survival and infection severity were monitored until day 16. Infection severity was scored using the viral pneumonia score (VPS, 0 = healthy to 12 = severely distressed/moribund)[1] and the modified murine sepsis score (MSS, 0 = healthy to 3 = moribund)[2]. Animals that died prior to the day of assessment received an MSS of 4. Fungal burden was determined in lung tissue homogenates on day 16 or upon death using an 18S qPCR assay[3].

Statistical analyses are described in the individual figure legends.

Results

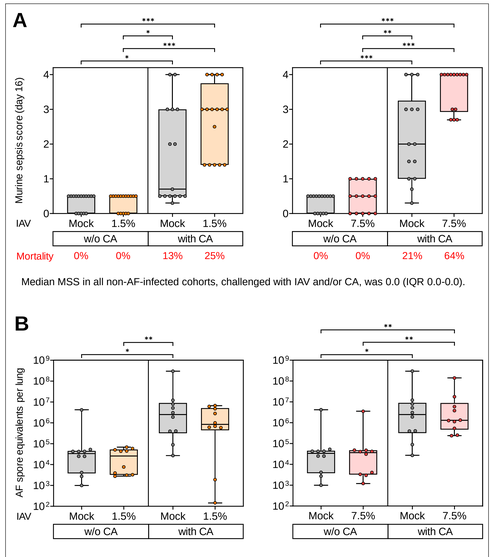

Figure 2: Glucocorticosteroid treatment predisposes mice to increased morbidity and mortality due to IAPA.
To optimize the IAPA model, mice were challenged with IAV (mock infection, 1.5% LD_{90}, or 7.5% LD_{90}) and treated or not with CA, as described in Materials & Methods. Mice were subsequently infected with AF and monitored until day 16 (7 days after AF infection). **(A)** Distributions of morbidity scores (MSS) on day 16 depending on IAV inoculum and CA treatment. N = 14-16 per group from 3 independent experiments. **(B)** Pulmonary fungal burden on day 16 or upon earlier natural death. N = 10 per group from 2 independent experiments. **(A-B)** Boxes indicate medians and inter-quartile ranges (IQRs). Whiskers indicate the spread (min/max). Kruskal-Wallis test with Dunn's post test. * p<0.05, ** p<0.01, *** p<0.001.

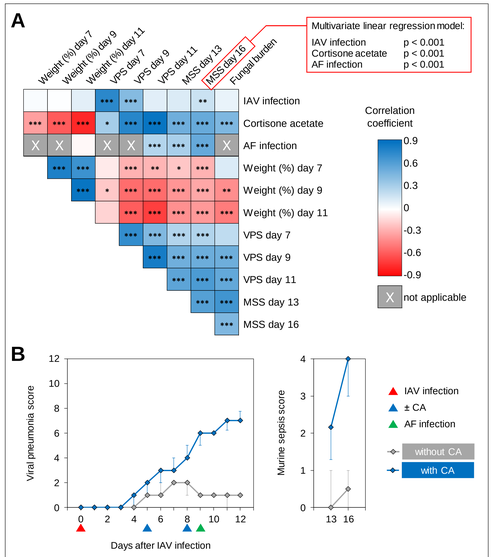

Figure 3: Cortisone acetate is an independent predictor of adverse outcomes in our IAPA infection model.
(A) Heat map comparing correlation coefficients between experimental challenges and outcomes (weight, VPS, MSS, and fungal burden). Spearman's rank correlation and rank-biserial correlation were used for comparisons of two continuous variables and comparisons of continuous and dichotomous variables, respectively. Additionally, a non-parametric general linear regression model was used to determine the independent impact of infectious challenges and CA therapy on morbidity/mortality outcomes. N = 14-15 per group from 3 independent experiments. * p<0.05, ** p<0.01, *** p<0.001. **(B)** Kinetics of daily VPS and MSS scores in IAV- (7.5% LD_{90}, ▲) and AF-infected (▲) mice depending on CA treatment (▲). Medians and IQRs are shown.

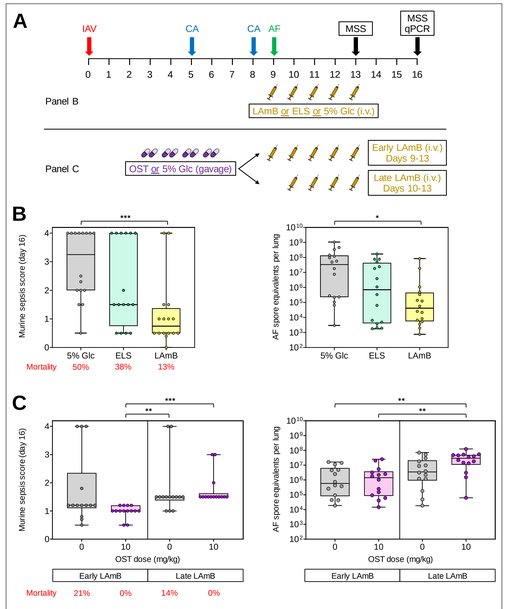

Figure 4: Timing of antifungal therapy determines the morbidity of corticosteroid-immunosuppressed mice with IAPA.
(A) Experimental procedures and timelines. **(B)** Day-16 MSS and pulmonary fungal burden of CA-immunosuppressed mice with IAPA depending on the antifungal treatment arm. N = 16 mice per treatment from 2 independent experiments. Kruskal-Wallis test with Dunn's post test. **(C)** Day-16 MSS and pulmonary fungal burden depending on OST treatment and timing of LAmB initiation. N = 14 mice per treatment from 2 independent experiments. **(B-C)** Boxes and whiskers denote inter-quartile range (with median bars) and minimum-to-maximum range. Asterisks indicate statistical significance: * p < 0.05, ** p < 0.01, *** p < 0.001.

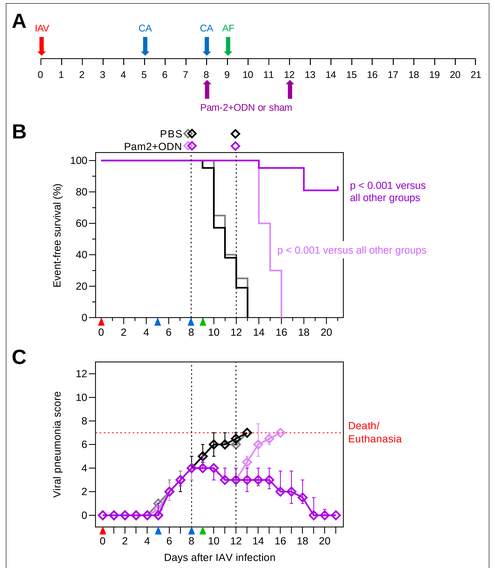

Figure 5: PRR agonists significantly improved mortality and morbidity of CA-immunosuppressed mice with IAPA.
(A) Timeline of experimental procedures. CA-immunosuppressed mice with IAPA were treated with aerosolized PRR agonists Pam2+ODN or PBS (sham), as described in the Methods section. A composite mortality/morbidity endpoint was used, with an event defined as death or VPS ≥7. N = 20-21 per group from 3 independent experiments. **(B)** Kaplan-Meier curves comparing event-free survival of mice with IAPA depending on their aerosolization treatments with single-dose (d8) or dual-dose (d8+d12) PBS or Pam2+ODN. **(C)** Kinetics of daily VPS scores of CA-immunosuppressed mice with IAPA depending on the treatment arm. Medians and IQRs are shown for all mice until the last day of event-free survival. ▲ = 7.5% LD_{90} IAV infection, ▲ = CA treatment, ▲ = AF infection.

Conclusions and Outlook

- We have established a unique murine IAPA model that allows us to compare the course and pathophysiology of IAPA in mice with and without CA immunosuppression.
- Our results underscore that corticosteroids are a major driver of IAPA-associated morbidity and mortality.
- Early antifungal treatment with liposomal amphotericin B was pivotal to improve IAPA outcomes in CA-immunosuppressed mice, even after prior antiviral therapy with oseltamivir.
- Immunomodulatory treatment of CA-immunosuppressed IAV-infected mice with PRR agonists significantly improved influenza- and IAPA-associated morbidity and mortality. Detailed studies of the mechanistic underpinnings of this effect are in progress.
- In the future, we will employ this novel *in-vivo* platform to study the impact of various antifungal, antiviral, and immunotherapeutic interventions on the natural history and immune pathogenesis of IAPA.
- Additionally, we will use this model to study the history and immune pathogenesis of other post-viral fungal superinfections such as coronavirus-associated pulmonary aspergillosis (CAPA).

References: [1] Rouxel et al., 2016, Plos One; [2] Mai et al., 2018, Intensive Care Med Exp.; [3] Ibrahim et al., 2005, Antimicrob Agents Chemother.

This study was supported by an investigator-initiated grant from Gilead Global Pharma (protocol IN-US-131-5756 to DPK). The sponsor had no influence on the research design.