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Targeting the Canonical Nuclear Factor-κB Pathway with a High-Potency IKK2 Inhibitor Improves Outcomes in a Mouse Model of Idiopathic Pneumonia Syndrome

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Idiopathic pneumonia syndrome (IPS) is a noninfectious inflammatory disorder of the lungs that occurs most often after fully myeloablative allogeneic hematopoietic stem cell transplantation (HSCT). IPS can be severe and is associated with high 1-year mortality rates despite existing therapies. The canonical nuclear factor-(NF) κB signaling pathway has previously been linked to several inflammatory disorders of the lung, including asthma and lung allograft rejection. It has never been specifically targeted as a novel IPS treatment approach, however. Here, we report that the IκB kinase 2 (IKK2) antagonist BAY 65-5811 or "compound A," a highly potent and specific inhibitor of the NF-κB pathway, was able to improve median survival times and recipient oxygenation in a well-described mouse model of IPS. Compound A impaired the production of the proinflammatory chemokines CCL2 and CCL5 within the host lung after transplantation. This resulted in significantly lower numbers of donor lung infiltrating $CD4^+$ and $CD8^+$ T cells and reduced pulmonary inflammatory cytokine production after allograft. Compound A's beneficial effects appeared to be specific for limiting pulmonary injury, as the drug was unable to improve outcomes in a B6 into B6D2 haplotype-matched murine HSCT model in which recipient mice succumb to lethal acute graft-versus-host disease of the gastrointestinal tract. Collectively, our data suggest that the targeting of the canonical NF-κB pathway with a small molecule IKK2 antagonist may represent an effective and novel therapy for the specific management of acute lung injury that can occur after allogeneic HSCT.

INTRODUCTION

Idiopathic pneumonia syndrome (IPS) refers to generalized, noninfectious, inflammatory lung injury occurring after hematopoietic stem cell transplantation (HSCT). The incidence of IPS has been estimated to range between 3% to 15%, is more common after myeloablative allogeneic transplantations than after reduced-intensity or autologous HSCTs, and has been associated with the use of total body irradiation (TBI) and cyclophosphamide-based conditioning regimens and concurrent acute graft-versus-host disease (GVHD) [1]. IPS after allogeneic transplantation is often resistant to therapy, with mortality rates of 60% to 80% being reported in some series, and new treatment approaches are needed.

The canonical nuclear factor (NF)-κB pathway is an important signaling cascade that is involved in multiple inflammatory pathways and has been implicated in the pathogenesis of several pulmonary disorders, including asthma and lung allograft rejection [2,3]. NF-κB itself is a dimer composed most commonly of a p50 and a RelA subunit. P50/RelA NF-κB is present in all cell types and is ordinarily sequestered in the cytoplasm in an inactive state by members of the IκB family of proteins [4,5]. Multiple proinflammatory signals, including TNF, CD40L, CD3/CD28, lipopolysaccharide (LPS), and IL-1 are able to induce the phosphorylation of IκB by activating IκB kinase (IKK), a heterotrimer composed of IKK1 and IKK2 catalytic subunits and a regulatory NEMO subunit. When this occurs, phosphorylated IκB is marked for ubiquination and ultimately degraded by the proteasome. This, in turn, liberates NF-κB and allows for its translocation to the nucleus where it induces the transcription of numerous inflammatory mediators [6-9].

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Although specific inhibition of the canonical NF-κB signaling pathway has not been previously explored as a treatment for IPS, there has been considerable interest in antagonizing NF-κB as a therapy for GVHD. Bortezomib is a reversible antagonist of the proteasome with inhibitory properties against NF-κB and has been evaluated for the management of GVHD in both preclinical models and human clinical trials [10-15]. In mouse studies, bortezomib was found to be effective in preventing lethal acute GVHD. Its therapeutic index, however, was small. Repeated bortezomib administration during the early transplantation period after an irradiation-based conditioning regimen accelerated acute GVHD and exacerbated gastrointestinal pathology [10,11]. Nevertheless, several therapy trials in humans have suggested a benefit for bortezomib in the management of chronic GVHD [12,14].

Although it is active against NF-κB, bortezomib demonstrates numerous additional effects via its action on the proteasome. As a result, there has been interest in examining other compounds that more specifically block the canonical NF-κB pathway. In work by Vodanovic-Jankovic et al., the authors evaluated the use of PS-1145, a direct inhibitor of IKK2, as a means for preventing acute GVHD in a mouse model system. There, PS-1145 appeared to be well tolerated and repeated doses given over the first post-transplantation week did not reproduce the gastrointestinal pathology seen with bortezomib [11].

Subsequent to this work, a structurally distinct inhibitor of IKK2 termed BAY 65-5811 or "compound A" became available, which had previously demonstrated both anti-inflammatory and antitumor activity in vivo [16,17]. Compound A is considerably more potent than PS-1145 against IKK2 with an IC_{50} of only 4 nM, and it exhibits minimal off-target effects against a variety of intracellular protein kinases and phosphatases [17]. As a result, we set out to determine if this structure could improve upon the results previously obtained with bortezomib and PS-1145. Here, we report that compound A was unable to prevent acute GVHD lethality in a B6 into B6D2 model where recipient death is primarily driven by gastrointestinal injury [18]. Subsequent in vivo trafficking studies, however, indicated that compound A was very effective at attenuating donor T cell accumulation within pulmonary tissues. As a result, we examined the effects of compound A in a well-described mouse model of IPS and found that the drug significantly improved median survival times and reduced hypoxemia. To our knowledge, our data are the first to demonstrate that an IKK2 antagonist can improve IPS outcomes in a preclinical model and suggest that the specific targeting of the canonical NF-κB pathway might represent a new therapeutic approach for the management of acute lung injury after HSCT.

METHODS

Mice

C57BL/6 ("B6"), B6xDBA/2 F1 ("B6D2"), and B10.BR mice were purchased from The Jackson Laboratory. Enhanced green fluorescent protein (eGFP)–expressing B6 mice were generated as previously prescribed [19]. All experiments were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee at The University of North Carolina.

Compound A

Compound A (BAY 65-5811, Bayer Pharmaceuticals) was supplied by the laboratory of Dr. Albert Baldwin. For all in vitro and in vivo work, compound A was suspended in 100% dimethylsulfoxide.

Mixed Lymphocyte Reaction

Mixed lymphocyte reactions (MLRs) using [3H] thymidine were performed as described previously [20].

Transplantation Systems

Donor T cell–depleted (TCD) bone marrow (BM) cells and whole CD25-depleted splenic conventional T cells (T_{cons}) were prepared as previously described [21,22]. Natural killer cell–depleted splenocytes were prepared by subjecting freshly isolated murine splenocytes to a negative-selection process using anti-NK1.1 antibodies coupled to ferromagnetic beads and a magnetic-activated cell sorter column (Miltenyi Biotec).

Stereomicroscopy

Recipient mice were imaged with a Zeiss Stereolumar V12 microscope with eGFP band-pass filter at room temperature. eGFP intensities were determined with Axiovision (Carl Zeiss) software. Specific exposure times and magnifications were as follows:

Day + 7 $eGFP^+$ donor T_{con} imaging:

Colon, exposure = 5 seconds, magnification = 12×;
liver, exposure = 6 seconds, magnification = 40×;
lung, exposure = 10 seconds, magnification = 17×

Organ eGFP Quantification

Recipient organs were homogenized and absolute eGFP levels determined with an ELISA kit as has been described previously (Cell Biolabs) [20,21].

Immunohistochemistry

Recipient lungs were perfused through with saline and then fixed in formalin. Paraffin-imbedded tissue sections were then stained with a mouse monoclonal antiphospho-IkBa-Ser32/36 antibody (Cell Signaling, Danvers MA) diluted 1:100 using a mouse on mouse peroxidase kit [16].

Isolation of Leukocytes from Tissues

Mice were killed and perfused through the heart with saline. Organs were then digested with a collagenase solution and donor $eGFP^+$ leukocytes isolated by centrifugation through a Percoll gradient, as described previously [22]. Leukocytes were then stained and quantified using flow cytometry.

Organ Chemokine/Cytokine Analysis

Recipient mice were perfused through with PBS and their organs then removed and homogenized. Total cytokine levels were then determined by ELISA (eBioscience).

Quantitative Reverse Transcription PCR

$CD4^+CD25^-$ B6 T_{cons} were isolated using column purification. The cells were then activated in vitro with plate-bound anti-CD3 and anti-CD28 antibody in the presence of supplemental murine IL-2 at 100 IU/mL. After 48 hours, their total RNA was isolated for quantitative reverse transcription PCR, as previously described [21].

Mouse Pulse Oximetry

Blood arterial oxygen saturation was tested in each mouse using a MouseOx Plus pulse oximeter (Starr Life Sciences; Oakmont, PA). Mice were anesthetized using isoflurane during the testing. A thigh sensor recorded data for a minimum of 22 seconds, and an average oxygen saturation level for that recorded time was calculated as a single data point in each mouse.

Antibodies for Cell Purifications and Flow Cytometry

The following antibodies were purchased from eBioscience: anti-CD4 (RM 4.5), CD8 (53-6-7), B220 (RA3-6B2), CD25 (PC61), NK1,1 (PK136), CD19 (1D3), CD11b (M1/70), Ly6C (HK1.4), annexin V (VAA-33).

Statistical Analysis

Survival curves were constructed using the method of Kaplan and Meier and median survival times compared using the log-rank test. Continuous variables were compared using the 2-tailed Student *t*-test. *P* values less than .05 were considered significant. Unless otherwise indicated, error bars represent SEM.