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Preventive Azithromycin Treatment Reduces Noninfectious Lung Injury and Acute Graft-versus-Host Disease in a Murine Model of Allogeneic Hematopoietic Cell Transplantation

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Noninfectious lung injury and acute graft-versus-host disease (GVHD) after allogeneic hematopoietic cell transplantation (allo-HCT) are associated with significant morbidity and mortality. Azithromycin is widely used in allogeneic HCT recipients for pulmonary chronic GVHD, although current data appear controversial. We induced GVHD and noninfectious lung injury in lethally irradiated B6D2F1 mice by transplanting bone marrow and splenic T cells from allogeneic C57BL/6 mice. Experimental groups were treated with oral azithromycin starting on day 14 until the end of week 6 or week 14 after transplantation. Azithromycin treatment resulted in improved survival and decreased lung injury; the latter characterized by improved pulmonary function, reduced peribronchial and perivascular inflammatory cell infiltrates along with diminished collagen deposition, and a decrease in lung cytokine and chemokine expression. Azithromycin also improved intestinal GVHD but did not affect liver GVHD at week 6 early after transplantation. At week 14, azithromycin decreased liver GVHD but had no effect on intestinal GVHD. In vitro, allogeneic antigen-presenting cell (APC)– dependent T cell proliferation and cytokine production were suppressed by azithromycin and inversely correlated with relative regulatory T cell (Treg) expansion, whereas no effect was seen when T cell proliferation occurred APC independently through CD3/CD28-stimulation. Further, azithromycin reduced alloreactive T cell expansion but increased Treg expansion in vivo with corresponding downregulation of MHC II on $CD11c^+$ dendritic cells. These results demonstrate that preventive administration of azithromycin can reduce the severity of acute GVHD and noninfectious lung injury after allo-HCT, supporting further investigation in clinical trials.

INTRODUCTION

Noninfectious lung injury and acute graft-versus-host disease (GVHD) are major causes of morbidity and mortality after allogeneic hematopoietic cell transplantation (allo-HCT). In 2011, the American Thoracic Society issued a consensus statement on noninfectious lung injury summarized under the term *Idiopathic Pneumonia Syndrome*, encompassing various clinical forms, which differ based on the time of onset, course of disease, and treatment response [1]. Various models of rodents have been established to study noninfectious lung injury after allo-HCT, and 2 major histopathologic features similar to that seen in patients receiving allo-HCT are commonly observed across models: peribronchial and perivascular lymphocytic infiltration and acute pneumonitis of the alveolar spaces and the interstitium [1,2]. Although the exact pathophysiology is not completely understood, donor $CD4^+$ T cells activated by antigen presenting cells (APCs) [3-5] and shifting towards Th1 or Th17 phenotype [6,7], cytotoxic $CD8^+$ T cells [8], and macrophages [5]

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contribute to the tissue injury. Cytokines and chemokines such as TNF-α, IL-1β, IFN-γ, and CCL2, CCL5, CXCL9-11, and CXCL1 [9-11], respectively may also be contributive to the disease process. Acute GVHD itself, sharing similar pathophysiological characteristics, may be a predisposing and driving factor of disease development.

Azithromycin is a macrolide antibiotic with established anti-inflammatory properties and favorable toxicity profile, which attains very high and durable lung concentrations [12]. Azithromycin treatment has been found to decrease pulmonary exacerbations and improve lung function in patients with cystic fibrosis (CF) [13,14], chronic obstructive pulmonary disease, and non-CF bronchiectasis [15,16]. Azithromycin has also been shown to be beneficial in lung transplantation for the prevention and treatment of chronic allograft rejection [17,18]. Accordingly, azithromycin has been studied in the setting of allo-HCT to treat bronchiolitis obliterans syndrome (BOS), but studies were limited by small patient numbers and demonstrated conflicting evidence of therapeutic efficacy [19,20]. In none of these studies, azithromycin was given prophylactically with the intention to prevent lung injury.

In this study, we use a well-established mouse model of noninfectious lung injury after allo-HCT to determine whether azithromycin started early and given over a prolonged time period is beneficial in preventing pulmonary damage, thereby improving pulmonary function and overall outcome.

MATERIALS AND METHODS

Animal experiments were done at 3 different institutions: early GVHD and pulmonary function testing experiments were done at the University of Regensburg Medical Center (UKR), Regensburg, Germany, and the splenic T cell expansion study was done at the University of Utah (UofU), Salt Lake City, Utah. All other data presented are from experiments performed at Louisiana State University Health Sciences Center Shreveport (LSUHSC-S), Louisiana. All experiments were done with the approval of the local institutional animal committees.

Induction of GVHD and lung Injury

Female B6D2F1 (H-2^{bxd}) and C57BL/6 (H-2^{b}) mice were purchased from the Jackson Laboratory (Bar Harbor, ME) or Charles River Laboratories (Sulzbach, Germany) and acclimatized in the respective animal facility for at least 1 week before starting the experiments. Animals were between 10 and 20 weeks old at the time of HCT or when used for in vitro studies.

Mice underwent bone marrow transplantation according to standard protocol as previously described [9,21]. B6D2F1 mice were conditioned using total body irradiation. Irradiation was done using either a linear accelerator, 150 cGy/minute (UKR) or using a Cesium source irradiator (LSUHSC-S and UofU), at a total dose of 12 Gy. Radiation was delivered in 2 fractions, 3 hours apart to reduce gastrointestinal toxicity. Animals then received a cell mixture of 4 to 5 $\times$ 10^6 bone marrow cells and 6 $\times$ 10^6 splenocytes from either syngeneic B6D2F1 or allogeneic C57BL/6 donors via tail vein injection. Mice were housed in sterilized microisolator cages and received autoclaved (UKR) or normal (LSUHSC-S and UofU) chow and autoclaved water for the first 2 weeks.

At day 14 after transplantation, syngeneic and allogeneic recipients were equally stratified based on weight loss into 2 subgroups: 1 group received autoclaved water only whereas the other group received autoclaved water supplemented with azithromycin dihydrate (Pfizer, Germany) at a concentration of 1 mg/mL from day +14 until day +17 and .5 mg/mL from day +18 until end of week +14 (UKR). At LSUHSC-S and UofU, azithromycin (Sagent Pharmaceuticals, Schaumburg, IL) dosing was increased to 3 mg/mL to attain a dose of 450 to 600 mg/kg/day to optimize study set up [22]. Survival was monitored daily and GVHD clinical scores were assessed weekly using 5 clinical parameters: weight loss, posture, mobility, fur texture, and skin integrity, as previously described [2]. Animals were sacrificed for analysis at end of week +6 and +14.

Histopathology

Lung injury was assessed by examination of lung histopathology. Hematoxylin and eosin–stained lung sections were independently evaluated by the pathologist for the severity of periluminal infiltrates (around airways and vessels) or parenchymal pneumonitis (involving the alveoli or interstitium), using a previously described semiquantitative scoring system [2]. Final scores for periluminal and parenchymal infiltrates were obtained by multiplication of the respective severity and extent. Liver and gut pathology were also assessed by hematoxylin and eosin staining of the tissues and independently evaluated by the pathologist, using previously described scoring systems [23,24]. Trichrome staining for collagen (NovaUltra Masson Trichrome Stain kit, IW-3006, IHC World, Woodstock, MD) was performed as per manufacturer's protocol. For immunostaining, 4 μm of organ slices were dewaxed and rehydrated, followed by antigen unmasking using citrate buffer pH 7.2 and microwave at 300W for 30 minutes. Next, peroxidase blocking solution (S2023, Dako) was added for 5 minutes to quench endogenous peroxidase activity. $CD3^+$ cells were then detected by rabbit IgG anti-mouse CD3 antibody (NeoMarkers, RM-9107-S [Thermo Fisher Scientific GMBH, Dreieich, Germany]) in 1:50 dilution in antibody diluent (S2022, Dako). Simple Stain Mouse MAX PO anti rabbit (414141F, Histofine, NICHIREI BIOSCIENCES INC, Tokyo, Japan) was used as the secondary antibody, visualization using DAB (K3467, Dako) and counterstained using hematoxylin. $CD3^+$ staining was scored as cells per high power field.

Bronchoalveolar Lavage

Bronchoalveolar lavage (BAL) and cell surface phenotyping were performed as previously described and cells were analyzed by flow cytometry [2].

Pulmonary Function Testing

Assessment of pulmonary function was performed using a Buxco lung function analysis system (Buxco Electronics, Troy, NY) consisting of a pulmonary function test/forced maneuvers analyzer (SFT3840, Buxco) plus pressure panel for mouse maneuvers (AUT6100, Buxco), and an anesthetized mouse–pulmonary function test (PFT) plethysmograph (PLY3112, Buxco) as previously described [25]. Data acquisition and analysis were done using BioSystem XA software (SFT3850).

Tissue Cytokine and Chemokine Expression

At time of analysis, tissues were harvested and snap frozen. Supernatant of homogenized tissue was obtained as described before [26]. Total protein concentration in the supernatant was determined using Coomassie Bradford protein assay (Thermo Scientific, Rockford, IL) to allow for cytokine and chemokine concentration normalization to picograms per milligram of total protein.

Cytokines and chemokines, such as TNF-α, IL-1β, CXCL1, CXCL9, CXCL10, IL-15, CCL5, CCL3, and others were assayed by Millipore's MILLIPLEX MAG Mouse Cytokine/Chemokine kit (Billerica, MA) using Luminex xMAP technology according to manufacturer's protocol. Cytokine/chemokine analysis was also performed on tissues using ELISA for CXCL9, IFN-γ,TNF-α, CCL2, CXCL1, MIP-2, IL-6, and CXCL10 (Duoset, R&D Systems GmbH, Germany).

T cell Activation and Proliferation Assays

Mixed lymphocytic reaction (MLR) was done using irradiated (40 Gy) BALB/c or B6D2F1splenocytes (8 $\times$ 10^5 cells per well) as stimulators. C57BL/6 effector T cells were purified from splenocytes using MACS CD90·2 beads and QuadroMACS system using LS columns (Miltenyi Biotec, Auburn, CA) according to manufacturer's protocol and cocultured in complete RPMI medium in 2:1 stimulator to effector ratio at 4 $\times$ 10^5 cells per well in a 96-well flat bottom plate. CD3/CD28 T cell activation assay was performed using C57BL/6 splenocytes at a concentration of 2 $\times$ 10^5 cells per well in complete RPMI using antiCD3ε (Clone 145-2C11) and anti-CD28 (Clone 37·51, eBioscience, San Jose, CA) antibody stimulation as per manufacturer's protocol. Cultures were incubated at 37°C and 5% CO_2, pulsed at 72 hours with ^{3}H thymidine (1 μCi) for MLR and at 48 hours for CD3/CD28 T cell activation assay and harvested 24 hours later using PHD cell harvester (Brandel, MD). Proliferation was quantified by using Wallac 1409 liquid scintillation counter. Azithromycin was added to respective groups at the concentrations 5 μg/mL, 10 μg/mL, and 20 μg/mL versus controls for the duration of the assay. Average counts per minute were obtained from triplicates, and experiments were done in duplicates. Supernatants were obtained before the addition of ^{3}H thymidine and stored at −80° C for cytokine analysis.

MLR and CD3/CD28 T cell activation assay supernatant were analyzed for IFN-γ, TNF-α, IL-10, IL-17, IL-2, IL-4, and IL-6 cytokine levels using Cytometric Bead Array Mouse Th1/Th2/Th17 Cytokine Kit (BD Biosciences Pharmingen, San Diego, CA) per manufacturer's protocol.

T cell Migration Assay

Migration assay was performed using murine recombinant CXCL9 (DevaTal Inc., Trenton, NJ) as chemotactic stimulant at 100 nM/well using a 24-well transwell migration plate. Purified T cells at 5 $\times$ 10^5 cells per well