Children's Healthcare of Atlanta

EMORY UNIVERSITY

2020 Pediatric Research and Career Development Symposium

Tuesday, August 4, 2020

9:00 am – 4:15 pm

Abstracts

BASIC SCIENCE POSTERS ONLY

Sponsored by the NICHD supported
Atlanta Pediatric Scholars Program, K12HD072245

TABLE OF CONTENTS

Basic Science POSTERS ONLY

Poster Presentations: B5 - B10 (In Order by Presenter Last Name)

B5. A Patient-Specific 3D Bioprinted Platform for *In Vitro* Disease Modeling and Treatment Planning in Pulmonary Vein Stenosis

Authors: Serpooshan, Vahid; Tomov, Martin L.; Jing, Bowen; Kumar, Akaash; Panoskaltsis, Nicki; Mantalaris, Athanasios; Slesnick, Timothy C.; Lindsey, Brooks; and Bauser-Heaton, Holly

Presenting Author:	**Holly Bauser-Heaton, MD, PhD**; basuerh@kidsheart.com
Type:	Basic - Poster
Poster Session Zoom Room Link:	**Visit Bauser-Heaton Zoom Room**

Pulmonary vein stenosis (PVS) is an acute pediatric cardiovascular disease that is always lethal if not treated early. While current clinical interventions (stenting and angioplasties) have shown promising results in treating PVS, they require multiple re-interventions that can lead to re-stenosis and diminished long-term efficacy. Thus, there is an unmet need to develop functional *in vitro* models of PVS that can serve as a platform to study clinical interventions. Patient-inspired 3D bioprinted tissue models provide a unique model to recapitulate and analyze the complex tissue microenvironment impacted by PVS. Here, we developed perfusable *in vitro* models of healthy and stenotic pulmonary vein by 3D reconstruction and bioprinting of patient CT data (Figure 1). Models were seeded with human endothelial (ECs) and smooth muscle cells (SMCs) to form a bilayer structure and perfused using a bioreactor to study cell response to stenotic geometry, and to the stent-based treatment. Flow hemodynamics through printed veins were quantified via CFD modeling, 4D MRI and 3D ultrasound imaging. Cell growth and endothelialization were analyzed. Our work demonstrates the feasibility of bioprinting various cardiovascular cells, to create perfusable, patient-specific vascular constructs that mimic complex *in vivo* geometries. Deeper understanding of EC-SMC crosstalk mechanisms in *in vitro* biomimetic models that incorporate tissue-like geometrical, chemical, and biomechanical ques could offer substantial insights for prevention and treatment of PVS, as well as other cardiovascular disease.

B6. Epigenetic and Transcriptional Dynamics in Acute and Chronic Human Myeloid Inflammation

Authors: Cammarata-Mouchtouris, Alexandre; Moncada, Diego; Giacalone, Vincent; Dobosh, Brian; Prahalad, Sampath; and Tirouvanziam, Rabindra

Presenting Author:	**Alexandre Cammarata-Mouchtouris, PhD**; acammar@emory.edu
Type:	Basic - Poster
Poster Session Zoom Room Link:	**Visit Cammarata-Mouchtouris Zoom Room**

RATIONALE: Beyond receptors and signaling proteins, genetic, epigenetic and transcriptional regulators are important in the unfolding of immuno-inflammatory responses. Epigenetic and transcriptional regulators in particular allow for rapid adaptation of responses in different types of cells and pathophysiological contexts. For example, our laboratory demonstrated in patients with Cystic Fibrosis (CF) that neutrophils recruited from blood into the airway lumen undergo reprogramming that causes them to promote a complex reorganization of the local tissue, in coordination with macrophages and epithelial cells. Such reprogramming of human neutrophils runs counter to the conventional assumption that the fate of myeloid cells is pre-programmed and establishes these fast-acting subsets as adaptable coordinators of tissue responses.

APPROACH: In ongoing studies, we are expanding on our past investigations in CF to characterize epigenetic and transcriptional dynamics in other neutrophil-dominated diseases of peripheral tissues including Juvenile Idiopathic Arthritis, and late stage COVID-19. Our pipeline combines the Cut & Run epigenetic assay method with shotgun Illumina sequencing and long-read Oxford Nanopore MinION sequencing to understand the regulatory landscape of neutrophils and monocytes recruited to diseased airways and joints.

RESULTS: Output from Cut & Run assays conducted in tissue neutrophils and macrophages in CF and JIA will be compared to illustrate the scope and specificity of epigenetic and transcriptional adaptations occurring over time in these fast-acting subsets. Rapid epigenetic changes can sometimes remain in effect even after the initial stimulus has waned, leading to transcriptional memory (a component of trained innate immunity). Therefore, the potential impact on long-term immune polarization / dysregulation will also be outlined.

CONCLUSIONS: A better understanding of dynamic adaptations in myeloid subsets associated with human peripheral inflammation using this and other approaches will help identify novel epigenetic and transcriptional targets for therapeutic modulation of various intractable diseases.

ACKNOWLEDGMENTS: CTID Pilot and Cystic Fibrosis Foundation.

B7. Complement-Mediated Acute Lung Injury in Sickle Cell Disease: Novel Mechanisms and Therapies

Authors: Chonat, Satheesh; Patel, Seema; Jeffers, Lauren; Cisneros de la rosa, Eduardo; Fields, Earl; Archer, David; Koval, Michael; Joiner, Clinton, and Stowell, Sean

Presenting Author:	**Satheesh Chonat, MD**; Satheesh.chonat@emory.edu
Type:	Basic - Poster
Poster Session Zoom Room Link:	**Visit Chonat Zoom Room**

Sickle cell disease (SCD) is a common and life-threatening autosomal recessive hematological disorder that affects millions worldwide. Amongst acute complications in SCD, acute chest syndrome (ACS) is a leading cause of hospitalization and the most common cause of death due to SCD. Unfortunately, supportive care remains the primary approach to alleviate these complications. This in part reflects an incomplete understanding of the pathophysiology and accompanying pharmacological targets that could specifically mitigate acute disease complications. Retrospective analysis of stored plasma samples from our SCD patients with ACS revealed acute hemolysis, and significantly increased levels of anaphylatoxins (C3a and C5a) and markers of the alternative complement pathway (Ba, Bb) during episodes of ACS compared to their baseline values. To examine the underlying mechanism of the role of complement in ACS, we developed a pre-clinical model of acute lung injury (ALI) in humanized sickle cell (SS) mice. Injection of cobra venom factor (CVF) to SS mice, a commonly used approach to induce complement activation, resulted in rapid deoxygenation, hypopnea and bradycardia (all hallmarks of ALI in mice), followed by death. In contrast, CVF treated littermate control (AA) mice did not develop detectable hemolysis, pulmonary compromise or increase in mortality. The SS mice had markedly increased levels of plasma anaphylatoxin C5a and increased complement component (C3) deposition in kidneys and lungs by immunofluorescence when compared to their controls and those treated with vehicle. While erythrocytes in these SS mice had elevated levels of C3b/iC3b/C3c deposition when compared to AA mice, no difference was noted in the total plasma C3, suggesting sickle erythrocytes are prone to complement-mediated hemolysis.

We then developed a humanized SS x C3 knock-out mice, and preliminary data suggests that these mice are protected from CVF mediated ALI and death. Our data thus far suggest that complement activation in SS mice results in hemolysis, release of free heme and production of C5a, which is a potent pro-inflammatory mediator, all of which possibly play a role in ALI. These results demonstrate that inhibition of C3a or C5a production may represent pharmacological targets to treat ACS in patients with SCD.

Poster Presentations: B5 - B10 (In Order by Presenter Last Name)

B8. Bortezomib Significantly Enhances Gamma Delta T Cell-Mediated Lysis of Acute Myeloid Leukemia and T-cell Acute Lymphoblastic Leukemia

Authors: Story, Jamie; Zoine, Jaquelyn; Burnham, Rebecca; Hamilton, Jamie; Spencer, H. Trent; Doering, Christopher; and Raikar, Sunil

Presenting Author:	**Jamie Story, BS**; jamie.y.story@emory.edu
Type:	Basic - Poster
Poster Available:	Yes - P10
Poster Session Zoom Room Link:	**Visit Story Zoom Room**

Previous studies have shown bortezomib, a proteasome inhibitor, increases surface expression of NKG2D ligands on cancer cells and enhances their sensitivity to innate immune cell-mediated cytotoxicity. Here, we investigate the combination of bortezomib and γδ T cells as a novel therapy for acute myeloid leukemia (AML), a hematologic malignancy in which CAR-based cellular immunotherapy has been challenging. Our results showed 24 hour treatment with bortezomib significantly increased ULBP2/5/6 expression in Kasumi-1 cells and Nomo-1 cells ($n = 3$, $p < 0.05$). Day 12 *ex vivo* expanded γδ T cells were incubated with target cells at the following effector to target (E:T) cell ratios: 0:1, 1:4, 1:2, 1:1, and 2.5:1. Total target cell death of Kasumi-1 cells was significantly increased with bortezomib treatment compared to vehicle control from 16.8% to 52.1% at a 1:4 E:T ratio, 30.0 % to 64.0% at 1:2, and 48.0% to 77.8% at the 1:1 ($n = 3$, $p < 0.05$). Nomo-1 cell death was significantly increased with bortezomib and γδ T cell combination treatment, compared to vehicle control, from 8.8% to 33.7%, 13.9% to 43.6%, 23.1% to 56.5%, and 45.8% to 71.3% at the 1:4, 1:2, 1:1, and 2.5:1 E:T ratios, respectively ($n = 3$, $p < 0.05$). We further validated this combination approach in two T-ALL cell lines, Jurkat and MOLT4, which also had significantly increased surface expression of ULBP2/5/6 with 5 nM bortezomib treatment ($n = 3$, $p < 0.05$). The total cytotoxicity against bortezomib treated Jurkat cells significantly increased from 39.9% to 64.0% at the 1:4 E:T ratio and from to 58.1% to 76.4% 1:2 ratio compared to vehicle control treated cells ($n = 3$, $p < 0.05$). The total cytotoxicity of MOLT-4 cells was also significantly increased with bortezomib treatment compared to vehicle treated cells from 19.6% to 35.5% at the 1:4 E:T ratio, 36.7% to 48.4% at the 1:2 E:T ratio, and 57.4% to 68.3% at the 1:1 E:T ratio ($n = 3$, $p < 0.05$). These results provides proof-of-concept for developing a platform for effective combination therapy of γδ T cells with stress ligand inducing drugs for high-risk leukemias.

B9. Postexposure Prophylaxis to Mitigate the Neurodevelopmental Consequences of Postnatal Zika Virus Infection in Infant Rhesus Macaques

Authors: Raper, Jessica; Schoof, Nils; Richardson, Rebecca; Medina, Alejandra; Rusnak, Rebecca; Kovacs-Balint, Zsofia; Sanchez, Mar; and Chahroudi, Ann

Presenting Author:	**Jessica Raper, PhD**; jraper@emory.edu
Type:	Basic - Poster
Poster Session Zoom Room Link:	**Visit Raper Zoom Room**

Although Zika virus (ZIKV) typically causes mild or no symptoms in adults, infection during pregnancy can result in a spectrum of disease in infants, including birth defects and neurodevelopmental disorders identified in childhood. While intense research has focused on prenatal ZIKV infection, the consequences of postnatal infection in early life are understudied. Using a highly clinically relevant rhesus macaque (RM) model, we have shown that ZIKV infection during infancy negatively impacted brain development resulting in long-term behavioral, cognitive and motor impairments. Considering that ZIKV has infected individuals in 92 countries and is endemic in many areas, it is important to investigate whether a postexposure prophylaxis could mitigate the negative neurodevelopmental consequences of postnatal ZIKV exposure. Using our established postnatal ZIKV RM model, we investigated whether an antiviral treatment could limit viral dissemination into the CNS and alleviate the impact of ZIKV on the developing brain. Three infant RMs received 14-day Sofosbuvir (SOF, 15mg/kg p.o.) treatment starting at 3 days post-infection (dpi). ZIKV+SOF infant RMs were monitored longitudinally for their immune response to ZIKV and SOF treatment, as well as assessing their behavioral, cognitive, motor, and brain development. ZIKV+SOF RMs cleared the virus at a similar rate to ZIKV-infected infant RMs, such that ZIKV was below detection by 7 dpi. Despite similar viral clearance, ZIKV+SOF RMs exhibited social behavior more similar to age- and rearing-matched uninfected controls. Similar to controls, ZIKV+SOF infant RMs exhibited caregiver attachment and prosocial behaviors. However, emotional assessments and neuroimaging suggest an intermittent phenotype. For example, ZIKV+SOF infant RMs exhibited the species-typical response of freezing during the profile condition of the human intruder task, but their level was lower than controls. At 3 months of age, ZIKV+SOF RMs had normal lateral ventricle volumes, but exhibited smaller amygdalae, hippocampi, and total white mater volume compared to controls. The current data suggests that antiviral treatment may help ameliorate some, but not all, of the neurodevelopmental consequences associated with early postnatal ZIKV infection. Further assessments are needed to determine degree that postexposure treatment can alleviate the cognitive and motor impacts of postnatal ZIKV infection.

B10. FVIII-Specific CD4 T Cell Proliferation Requires Multiple Previous Exposures to FVIII

Authors: Zerra, Patricia; Patel, Seema; Baldwin, W. Hunter; Cox, Courtney; Lollar, Pete; Stowell, Sean; and Meeks, Shannon

Presenting Author:	**Patricia Zerra, MD**; pzerra@emory.edu
Type:	Basic - Poster
Poster Available:	Yes - P11
Poster Session Zoom Room Link:	**Visit Zerra Zoom Room**

Factor VIII (FVIII) replacement in hemophilia A can be complicated by neutralizing anti-FVIII IgG alloantibodies that can actively block FVIII activity and prevent optimal replacement efficacy. Currently, no prophylactic therapy prevents inhibitor development, likely due to poor understanding of key immune regulators governing inhibitor formation.

In contrast to other model antigens, inhibitor formation occurs only following multiple FVIII exposures both in humans and mouse models. This suggests that early exposure events may prime subsequent development of long-lasting antibodies. Despite previous studies suggesting that CD4 T cells play an important role in inhibitor development, their timing and overall role in this key immune response remains incompletely understood. Thus, defining the role of CD4 T cells in inhibitor development is important if novel therapeutics for inhibitor prevention are to ever be realized.

As no tools exist to study FVIII specific CD4 T cells, we engineered the model antigen, OVA, into the B domain site of B-domain-deleted FVIII (HOVA) for tracking antigen specific CD4 T cells. HOVA had equivalent FVIII activity and immunogenicity to recombinant FVIII. Additionally, the OVA CD4 T cell epitope in HOVA was functional, as proliferation of CD4 T cells from OTII mice was observed following culture with HOVA *in vitro*. Surprisingly, no proliferation or activation of OTII CD4 T cells was detected in hemophilia A mice immunized with 1-2 HOVA injections. However, in previously highly immunized mice, 1 HOVA injection induced significant proliferation and activation of OTII CD4 T cells.

Understanding initiating immune events in the CD4 T cell-dependent process of FVIII inhibitor development is paramount to the development of novel therapies to prevent inhibitor formation in hemophilia A. HOVA is a unique immunologic tool for examining the FVIII specific CD4 T cell response following FVIII exposure. Using this tool, we found that FVIII specific CD4 T cell proliferation requires more than 2 prior exposures to HOVA, consistent with the observation that multiple FVIII exposures are required prior to inhibitor development. These findings provide an important clue to early steps in the development of FVIII inhibitors, with further studies needed to elucidate the mechanisms underlying this phenomenon.

Posters

All basic science posters provided by abstract authors are available in the following pages. Available posters are labeled P10 - P11.

Bortezomib Significantly Enhances γδ T Cell-Mediated Lysis of Acute Myeloid Leukemia and T-Cell Acute Lymphoblastic Leukemia

Jamie Y. Story, B.A.[1,3], Jaquelyn T. Zoine, B.S.[2,3],, Rebecca E. Ryan, B.S.[1,3], Jamie A. Hamilton[2,3], H. Trent Spencer, Ph.D.[1,3], Christopher B. Doering, Ph.D.[1,3] and Sunil S. Raikar, M.D.[3]

[1]Graduate Program in Molecular and Systems Pharmacology, Emory University, Atlanta, GA, [2]Graduate Program in Cancer Biology, Emory University, Atlanta, GA, [3]Aflac Cancer and Blood Disorders Center, Department of Pediatrics, Emory University, Atlanta, GA

Contact information: jystory@emory.edu

Background

- Relapse still remains a clinical barrier in the field of childhood leukemia
  - Allogeneic hematopoietic stem cell transplantion (HSCT) is only realistic chance at a cure for relapsed AML and T-ALL, but requires patients to be in remission
  - CAR T cell therapy has been difficult to adapt in both leukemias due to on-target, off-tumor effects
  - Other immunotherapies need to be explored for AML and T-ALL
- γδ T cells are an attractive candidate for cancer immunotherapy
  - Can induce cell lysis through engagement with stress antigens on tumor cells, such as natural-killer group 2, member D (NKG2D) ligands
  - Our lab previously developed a serum free GMP method for *ex vivo* expansions of γδ T cells (Vγ9Vδ2)
- Certain drugs can increase stress antigens on cancer cells
  - Bortezomib previously shown to increase NKG2D ligands on AML cells
  - Limited benefit from addition of bortezomib to standard chemotherapy in clinical setting for AML

Hypothesis

We hypothesize a more effective approach is a combination of bortezomib with *ex vivo* expanded γδ T cells, potentially as a bridge to transplant by inducing remission

Research Design

- Incubated Kasumi-1, Nomo-1, Jurkat, and MOLT-4 cells with 2.5, 5, and 10 nM of bortezomib over 48 hours to determine optimal drug treatment for increase in NKG2D ligands' surface expression
- Treated Kasumi-1, Jurkat and MOLT-4 cells with 5 nM bortezomib and Nomo-1 cells with 10 nM bortezomib or vehicle control for 24 hours prior to a 4 hour *in vitro* cytotoxicity assay with *ex vivo* expanded γδ T cells at various effector to target (E:T) ratios
  - Measured target cell death by flow cytometry via 7-AAD and Annexin V staining
- Assessed whether bortezomib treatment had negative effects on γδ T cells
  - Treated γδ T cells with 1-5 nM bortezomib for 24 hours, which is range of expected plasma concentration 24 hours after bortezomib injection in adults
- Incubated GFP$^+$ target cells with γδ T cells at lower E:T ratios over 48 hours to assess if bortezomib treatment accelerated target cell death over a longer time period

Results

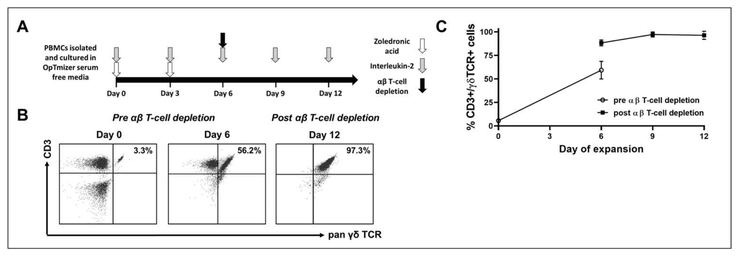


Figure 1. Expansion of γδ T cells from healthy donor PBMCs. (A) Healthy donor PBMCs were cultured in serum free media over a day 12 period. Cultures were supplemented with 500 IU IL-2 and 5 μM zoledronic acid on days 0 and 3, and 1000 IU IL-2 on days 6, 9, and 12. On day 6, cultures were depleted of αβ T cells using positive αβ T cell selection. B. (n = 6 different donors). (B) Representative flow of the percentage of γδ T cells over the course of expansion for one donor. Depleting cultures of αβ T cells yielded a highly pure γδ T cell product by day 12 of expansion. (C) Flow cytometry was performed every 3 days to monitor the percentage of γδ T cells over the expansion. Live cells were gated on and γδ T cell percentage was determined by CD3$^+$/γδ TCR$^+$. Post-αβ depleted cultures were 88.3 ± 3.1% γδ T cells, compared to 59.3 ± 9.4% prior to depletion on day 6 (n = 6 separate donors). By day 12 of expansion, all cultures had an average of 96.4 ± 4.3% γδ T cells (n = 6 separate donors).

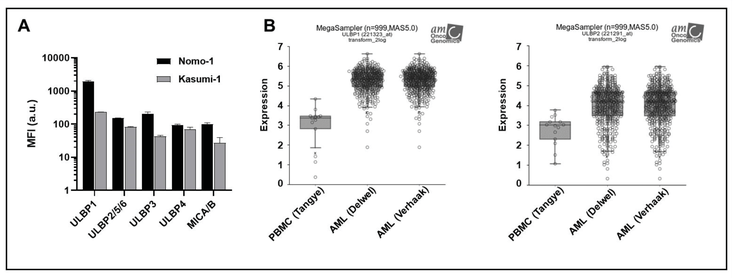


Figure 2. Expression of NKG2D ligands in AML cell lines and primary AML patient samples. (A) Baseline protein expression of NKG2D ligands on AML cell lines was assessed by gating on Annexin V and 7-AAD double negative cells and analyzing surface marker expression via flow cytometry. Both AML cell lines showed highest expression of ULBP1 (n = 3, 1934.0 ± 126.2 MFI arbitrary units for Nomo-1 and 230.0 ± 3.5 MFI arbitrary units for Kasumi-1). (B) RNA sequencing data from primary AML patient samples showed elevated gene expression in ULBP1 and ULBP2 compared to gene expression in normal PBMCs.

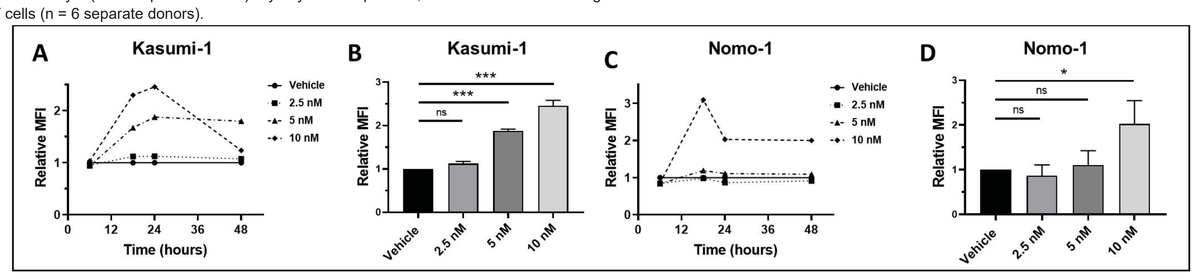


Figure 3. Bortezomib significantly increases expression of ULBP2/5/6 in AML cell lines after 24 hours of treatment. (A and C) AML cell lines were treated with vehicle control or increasing doses of bortezomib for 48 hours. Live cells were gated on (7-AAD$^-$/Annexin V$^-$) and analyzed for changes in NKG2D ligand surface expression at the various time points and concentrations (n = 3, average relative MFI). Peak expression of ULBP 2/5/6 was seen between hour 18 and 24 in both cell lines. (B) Surface expression of ULBP2/5/6 on Kasumi-1 cells significantly increased with 5 nM and 10 nM bortezomib treatment (n = 3, p < 0.0001 for both groups). (D) Nomo-1 cells had a significant increase in ULBP2/5/6 at the 10 nM dose (n = 3, p = 0.0124). Data are displayed as an average +/- SD of relative MFI of bortezomib treated cells compared to vehicle control treated cells. Significance was determined using one-way ANOVA with Dunnett's multiple comparisons test.

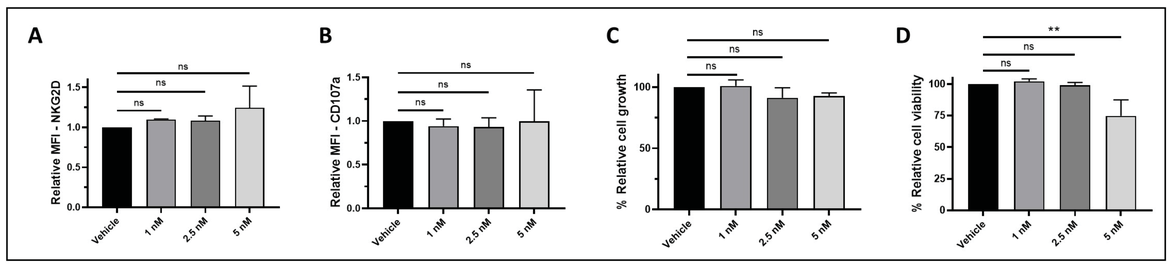


Figure 4. Bortezomib has no major negative effects on γδ T cells. γδ T cells were treated with 1, 2.5, or 5 nM bortezomib for 24 hours at day 10 of the expansion. There was no significant decrease in surface expression of NKG2D receptor (A) and CD107a (B) on live γδ T cells (7-AAD$^-$/Annexin V$^-$) at any bortezomib dose (n = 3 separate donors, p > 0.05). (C) Bortezomib treatment had no negative effect on cell growth (n = 3 separate donors, p > 0.05). (D) There was a significant decrease in cell viability at 5 nM, from 98.9% to 74.8% (p = 0.004, n = 3 separate donors). Data are displayed as an average +/- SD of relative MFI of bortezomib treated cells compared to vehicle control treated cells. Significance was determined using one-way ANOVA with Dunnett's multiple comparisons test.

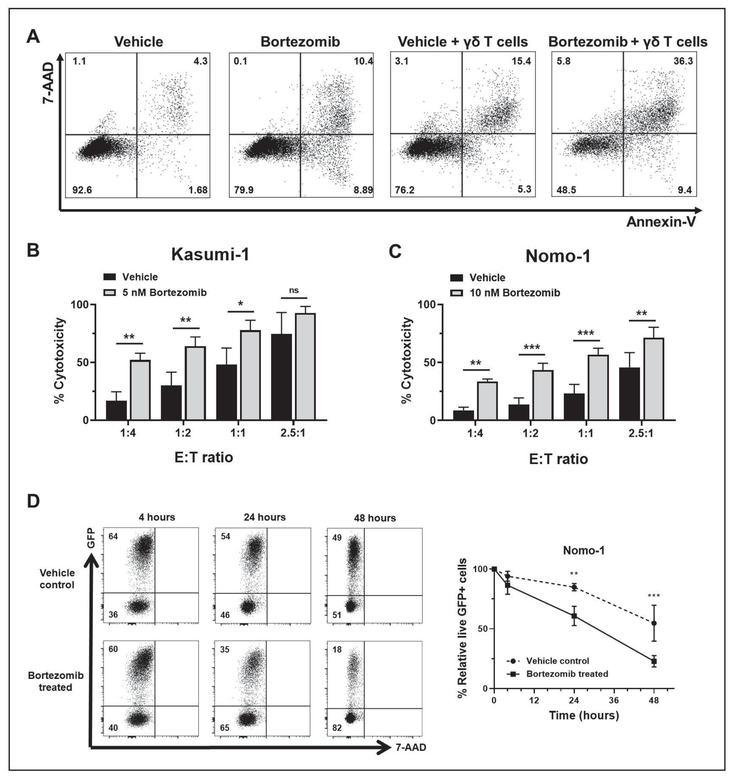


Figure 5. Bortezomib enhances cytotoxicity of γδ T cells against AML cell lines. (A) Representative flow plots from a 4 hour cytotoxicity assay with γδ T cells and Nomo-1 cells at a 1:1 effector to target (E:T) ratio. After 24 hour treatment with bortezomib or vehicle, AML cells were incubated with day 12 ex vivo expanded γδ T cells at 1:4, 1:2, 1:1, and 2.5:1 E:T ratios for 4 hours. The difference in cytotoxicity of γδ T cells against bortezomib-treated target cells and vehicle-treated target cells was analyzed at each E:T ratio. Replicates represent γδ T cells expanded from 3 different donors. (B) Treating Kasumi-1 cells with bortezomib significantly increased target cell death at the 1:4, 1:2, and 1:1 E:T ratios (n = 3, p = 0.0044, 0.0058, and 0.016, respectively). (C) Cytotoxicity of Nomo-1 cells significantly increased in bortezomib-treated cells compared to vehicle-treated cells at all E:T ratios (n= 3, p = 0.0025, 0.0005, 0.0001, and 0.0021, respectively). (D) Representative flow plots from a longer time course cytotoxicity assay with γδ T cells and Nomo-1 cells at a 1:2 E:T ratio. After 24 hours of treatment with bortezomib or vehicle, GFP$^+$ Nomo-1 cells were incubated with day 11 ex vivo expanded γδ T cells for 4, 24, and 48 hours. The percentage of remaining live GFP$^+$ cells (GFP$^+$/7-AAD$^-$) was determined at each time point to determine whether bortezomib treatment increased the cytotoxicity of GFP$^+$ Nomo-1 cells compared to vehicle-treated cells. Bortezomib-treated Nomo-1 cells incubated with γδ T cells had a significantly lower percentage of live cells after 24 hours and 48 hours compared with vehicle-treated cells (n = 3, p = 0.003 and 0.0002, respectively). Significance was determined using two-way ANOVA with Bonferroni's multiple comparisons test.

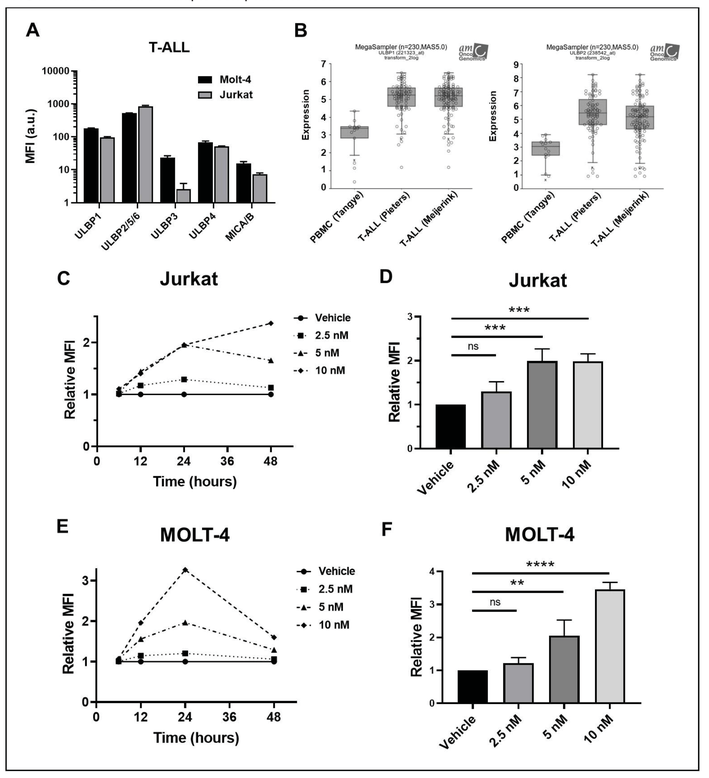


Figure 6. Bortezomib significantly increases expression of ULBP2/5/6 in T-ALL cell lines after 24 hours of treatment. (A) Baseline protein expression of NKG2D ligands on T-ALL was assessed by gating on Annexin V and 7-AAD double negative cells and analyzing surface marker expression via flow cytometry. Both T-ALL cell lines showed highest expression of ULBP2/5/6 (n = 3, 853.667 ± 51.287 MFI arbitrary units for Jurkat and 532.667 ± 4.041 MFI arbitrary units for MOLT-4). (B) RNA sequencing data from primary T-ALL patient samples showed elevated gene expression in ULBP1 and ULBP2 compared to gene expression in normal PBMCs . (C and E) T-ALL cell lines were treated with vehicle control or increasing doses of bortezomib for 48 hours. Live cells were gated on (7-AAD-/Annexin V-) and analyzed for changes in NKG2D ligand surface expression at the various time points and concentrations (average relative MFI, n= 3). (C) Surface expression of ULBP2/5/6 in Jurkat cells peaked between 24 and 48 hours at all doses. (D) There was a significant increase in ULBP2/5/6 with 5 nM and 10 nM bortezomib treatment at the 24 hour time point (n = 3, p = 0.007). (E) Surface expression of ULBP2/5/6 in MOLT4 cells peaked between 12 and 24 hours at all doses. (F) ULBP2/5/6 expression was also significantly increased on MOLT-4 cells at 5 nM and 10 nM bortezomib (n = 3, p = 0.0037 and p < 0.0001, respectively). Significance was determined using one-way ANOVA with Dunnett's multiple comparisons test.

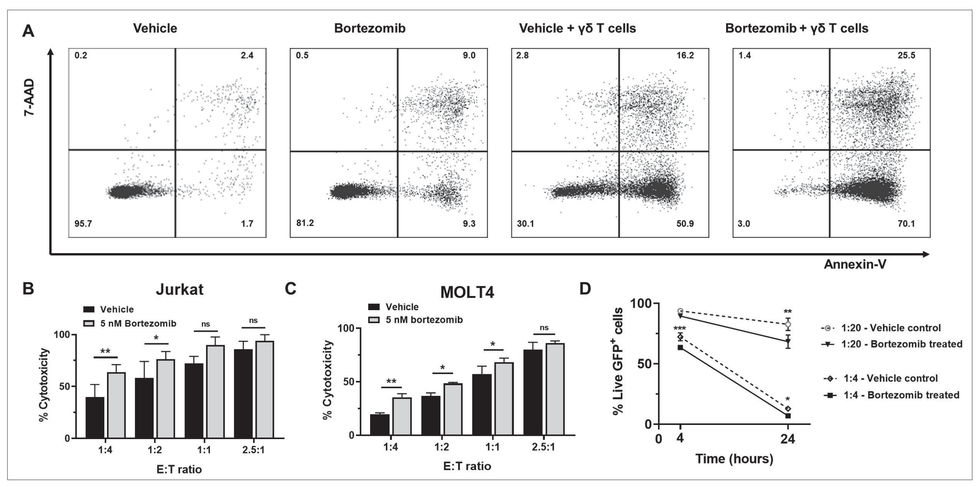


Figure 7. Bortezomib increases cytotoxicity of γδ T cells against T-ALL cell lines. (A) Representative flow plots from a 4 hour cytotoxicity assay with γδ T cells and Jurkat cells at a 1:1 effector to target (E:T) ratio. (B) Vehicle or bortezomib treated cells Jurkat and MOLT-4 cells were incubated for 4 hours with day 12 ex vivo expanded γδ T cells at 1:4, 1:2, 1:1, and 2.5:1 E:T ratios. The difference in target cell death between bortezomib-treated cells and vehicle-treated cells was compared at each E:T ratio. Replicates represent γδ T cells expanded from 3 different donors. Bortezomib-treated Jurkat cells had a significantly higher cytotoxicity (% dead target cells) compared to vehicle-treated Jurkat cells at the 1:4 and 1:2 E:T ratios (n = 3, p = 0.0054 and 0.0448, respectively). (C) MOLT-4 cells treated with bortezomib also had a significantly higher percentage of dead target cells at the 1:4, 1:2, and 1:1 E:T ratios (n = 3, p = 0.001, 0.014, and 0.023, respectively). (D) After 24 hours of treatment with bortezomib or vehicle, GFP+ Jurkat cells were incubated with day 11 ex vivo expanded γδ T cells for 4 and 24 hours at a 1:4 or 1:20 E:T ratio. The percentage of remaining live GFP+ cells (GFP+/7-AAD-) was determined at each time point to determine whether bortezomib treatment increased the cytotoxicity of GFP+ Jurkat cells compared to vehicle-treated cells. Bortezomib-treated Jurkat cells had a significantly lower percentage of live cells after 24 hours compared with vehicle-treated cells at both E:T ratios (n = 3, p = 0.011 and p = 0.003, respectively). Significance was determined using two-way ANOVA with Bonferroni's multiple comparisons test.

Conclusions

- ULBP2/5/6 surface expression significantly increases with 24 hour bortezomib treatment at 5 or 10 nM in AML and T-ALL cell lines
- Administering γδ T cells 24 hours after bortezomib treatment in humans should not negatively affect their *in vivo* cytotoxicity against cancer cells
- Bortezomib treatment significantly increased cytotoxicity of *ex vivo* expanded γδ T cells at low E:T ratios
  - Target cell death >30% was seen at 1:4 E:T ratio in all cell lines
- Bortezomib treated cells were killed faster than vehicle control treated cells in a 24-48 hour cytotoxicity assay
- Demonstrates feasibility of γδ T immunotherapy with stress antigen inducing drugs, such as bortezomib, in the AML and T-ALL setting without having to develop antigen specific immunotherapies

Future Directions

- Investigate whether bortezomib treatment increases NKG2D ligand surface expression on primary cells from AML and T-ALL patients
- Determine if bortezomib treatment enhances susceptibility of primary AML and T-ALL cells to γδ T cell-mediated cytotoxicity in an *in vitro* based cytotoxicity assay
- Perform *in vivo* experiments to investigate whether bortezomib and γδ T cells combination decreases tumor burden and/or increases survival in mice
  - Inject luciferase-expression Nomo-1 and Jurkat cells into NSG mice and treat with vehicle control, bortezomib alone, γδ T cells alone, or bortezomib + γδ T cells
  - Measure tumor burden via bioluminescence imaging and overall survival

Acknowledgements

This research was supported by:

- Hyundai Hope on Wheels
- Curing Kids Cancer
- Atlanta Pediatric Scholars Program – K12HD072245 through NICHD

FVIII-SPECIFIC CD4 T CELL PROLIFERATION REQUIRES MULTIPLE PREVIOUS EXPOSURES TO FVIII

Patricia E. Zerra[1,2], MD, Seema R. Patel, PhD[2], W. Hunter Baldwin[2], John Healey[2], Ernest Parker[2], Courtney Cox[2], Pete Lollar, MD[2], Sean R. Stowell, MD, PhD[1] and Shannon L. Meeks, MD[2]

[1]Center for Transfusion and Cellular Therapies, Department of Pathology and Laboratory Medicine, Emory University School of Medicine, Atlanta, GA

[2]Aflac Cancer and Blood Disorders Center, Children's Healthcare of Atlanta/Emory University School of Medicine, Atlanta, GA

INTRODUCTION

FVIII replacement in hemophilia A can be complicated by the development of neutralizing anti-FVIII IgG alloantibodies (inhibitors)[1,2]. These inhibitors can actively block FVIII activity and prevent optimal replacement efficacy[3]. Despite their significant clinical implications, there are currently no prophylactic therapies to prevent inhibitor development. This is likely in part due to a poor understanding of the key immune regulators governing inhibitor formation.

In contrast to other model antigens, inhibitor formation occurs only following multiple FVIII exposures both in humans and mouse models. This suggests that early exposure events may prime subsequent development of long-lasting antibodies. In particular, as CD4 T cells facilitate IgG formation, it is possible that each FVIII exposure event may propagate a CD4 T cell response to a threshold necessary to generate an optimal IgG response. Despite previous studies suggesting that CD4 T cells play an important role in inhibitor development[4,5], their timing and overall role in this key immune response remains incompletely understood. Thus, defining the role of CD4 T cells in inhibitor development is important if novel therapeutics for inhibitor prevention are to ever be realized.

MATERIALS AND METHODS

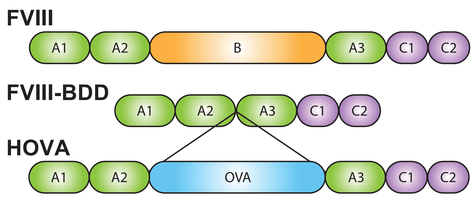


Figure 1. HOVA development. As no tools exist to study FVIII specific CD4 T cells, we engineered the model antigen, ovalbumin (OVA), into the B domain site of B-domain-deleted (BDD) FVIII for tracking antigen specific CD4 T cells. We utilized human FVIII containing an AvrII site at the end of the A2 domain and a BsiWI site in the activation peptide. Annealing of forward and reverse ultramers (containing AvrII and BsiWI sites flanking the ova consensus sequence), followed by cutting with AvrII and BsiWI which enabled us to ligate the product into human BDD FVIII.

Testing HOVA FVIII activity, immunogenicity, and function. FVIII activity of HOVA was assessed by a clotting assay. Immunogenicity was assessed by administering either HOVA or FVIII to hemophilia A mice, followed by anti-FVIII IgG measurement in plasma via ELISA. To determine OVA CD4 T cell epitope function in HOVA, CD4 T cells from OTII mice that express a T cell receptor specific to $OVA_{323-339}$ were CFSE-labeled and cultured with HOVA *in vitro* for 7 days. Proliferation was evaluated by flow cytometry.

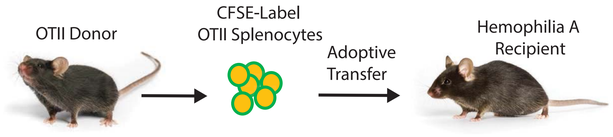


Figure 2. Adoptive Transfer of CFSE-labeled OTII splenocytes. To test whether HOVA exposure results in OTII activation *in vivo*, CFSE-labeled splenocytes from OTII transgenic mice with CD4 T cells specific for HOVA were adoptively transferred into naïve or highly immunized hemophilia A mice, followed by HOVA administration. Flow cytometric analysis assessed OTII CD4 T cell proliferation and activation.

RESULTS

HOVA is immunogenic in mice with Hemophilia A

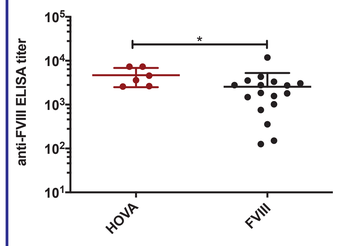


Figure 3. Anti-FVIII Antibody Titers. After 4 weekly doses of 2 µg HOVA or recombinant FVIII, plasma was evaluated for anti-FVIII IgG by ELISA. Error bars indicate SD. Horizontal lines represent mean of each group. *P=0.03, Mann-Whitney test. (HOVA also had equivalent FVIII activity via measurement of clotting time.)

HOVA CD4 T cell epitope is functional *in vitro*

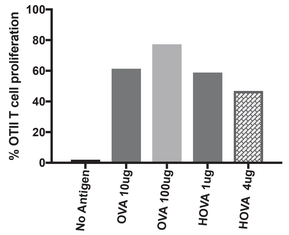


Figure 4. Splenocytes from OTII transgenic mice were CFSE-labeled, then cultured for 7 days either with no antigen, with 10 or 100 µg OVA or with 1 or 4 µg HOVA *in vitro*. OTII CD4 T cell proliferation was assessed by flow cytometry.

No FVIII-specific CD4 T cell proliferation or activation after 1-2 exposures to HOVA *in vivo*

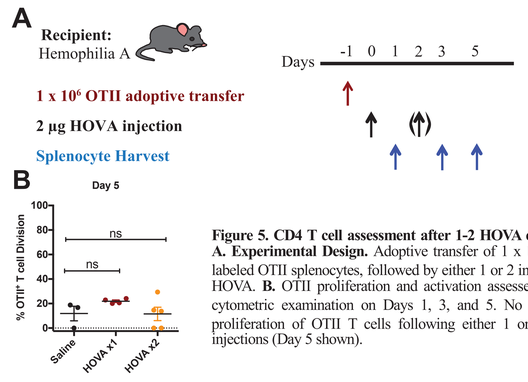


Figure 5. CD4 T cell assessment after 1-2 HOVA exposures. A. Experimental Design. Adoptive transfer of 1 x 10^6 CFSE-labeled OTII splenocytes, followed by either 1 or 2 injections of HOVA. **B.** OTII proliferation and activation assessed by flow cytometric examination on Days 1, 3, and 5. No significant proliferation of OTII T cells following either 1 or 2 HOVA injections (Day 5 shown).

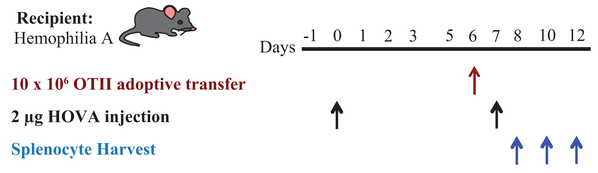


Figure 6. CD4 T cell assessment after 2 HOVA exposures - Experimental Design. Mice received 1 injection of HOVA, followed by adoptive transfer of 10 x 10^6 CFSE-labeled OTII splenocytes one week later. Mice then received a second HOVA injection on Day 7. No significant OTII T cell activation or proliferation upon analysis.

FVIII-specific CD4 T cells show proliferation and activation in highly immunized mice

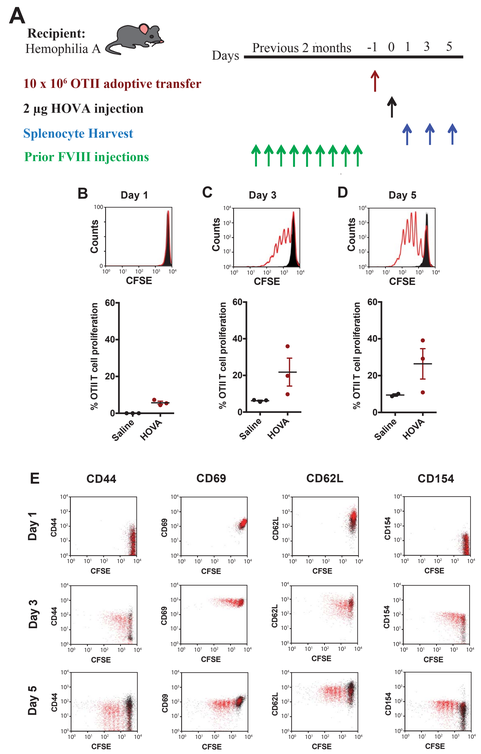
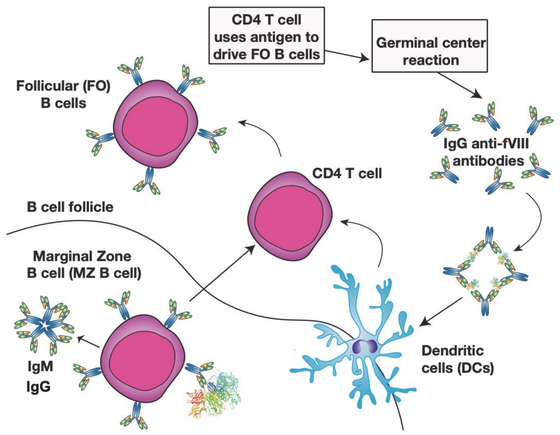


Figure 7. CD4 T cell assessment in highly immunized mice. A. Experimental Design. Mice with multiple previous exposures to FVIII were adoptively transferred with 10 million OTII splenocytes, followed by one 2 µg injection of HOVA. Representative histograms and % OTII CD4 T cell proliferation assessed on Days 1 **(B)** 3 **(C)** and 5 **(D)** by flow cytometric analysis. **E.** Representative dot plots of OTII CD4 T cell activation in mice receiving saline (black) or HOVA (red).

CONCLUSIONS



- **HOVA allows examination of the FVIII-specific CD4 T cell response**
- **FVIII-specific CD4 T cells do not proliferate in response to 1-2 doses of FVIII**
- **FVIII-specific CD4 T cell proliferation and activation requires >2 prior exposures to FVIII, consistent with the observation that multiple FVIII exposures are required prior to inhibitor development**
- **Further studies needed to elucidate the underlying mechanisms: ? Immune complex formation**

REFERENCES

[1] Ehrenforth S, Kreuz W, Scharrer I, Linde R, Funk M, Gungor T, Krackhardt B, Kornhuber B. Incidence of development of factor VIII and factor IX inhibitors in haemophiliacs. *Lancet.*1992;339(8793):594-8.

[2] Lollar P. Pathogenic antibodies to coagulation factors. Part one: factor VIII and factor IX. *J Thromb Haemost.* 2004;2(7):1082-95.

[3] Meeks SL, Healey JF, Parker ET, Barrow RT, Lollar P. Antihuman factor VIII C2 domain antibodies in hemophilia A mice recognize a functionally complex continuous spectrum of epitopes dominated by inhibitors of factor VIII activation. *Blood.* 2007;110(13):4234-42.

[4] Bray GL, Kroner BL, Arkin S, Aledort LW, Hilgartner MW, Eyster ME, Goedert JJ. Loss of high-responder inhibitors in patients with severe hemophilia A and human immunodeficiency virus type 1 infection: a report from the Multi-Center Hemophilia Cohort Study. *Am J Hematol.* 1993;42(4):375-9.

[5] Reding MT, Wu H, Krampf M, Okita DK, Diethelm-Okita BM, Christie BA, Key NS, Conti-Fine BM. Sensitization of CD4+ T cells to coagulation factor VIII: response in congenital and acquired hemophilia patients and in healthy subjects. *Thromb Haemost.* 2000;84(4):643-52.

Patricia E. Zerra, MD, received a **2018 HTRS/Novo Nordisk Mentored Research Award** in Hemophilia and Rare Bleeding Disorders from the Hemostasis and Thrombosis Research Society (HTRS), which was supported by an educational grant from Novo Nordisk Inc.