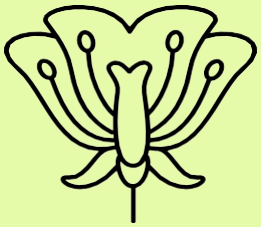
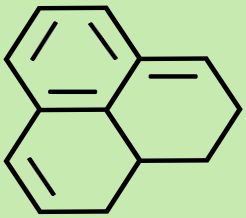
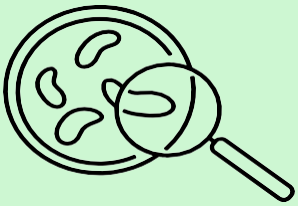
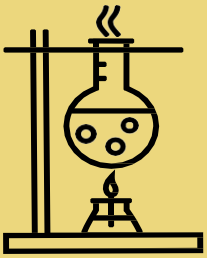
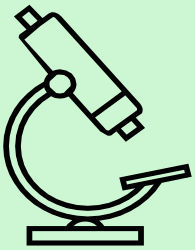
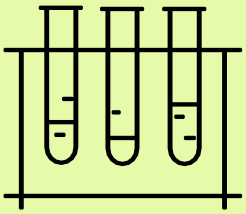
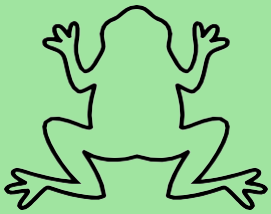
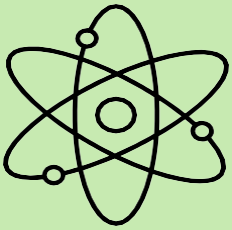
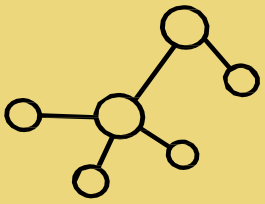
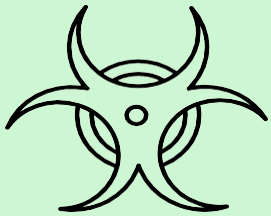
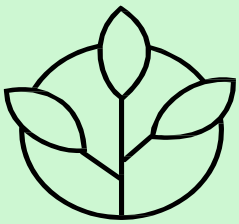
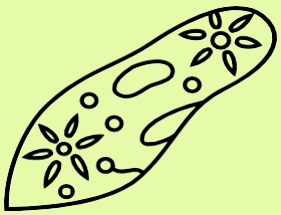
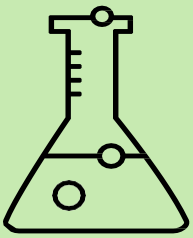
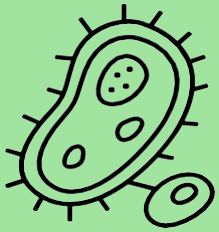



CBER Science Symposium

Poster Abstracts

September 17-18, 2024

Flash Talks & In-Person Poster Sessions (open to the public)

FDA White Oak Great Room - 12:00 PM – 2:00 PM

Topic Area	Presenting Author Last Name	Presenting Author First Name	Abstract Title	Page Number	Poster Number	Presenting Date
Cell, Tissue & Gene Therapy	Afroj	Sayma	Evaluation of RNA extraction and Illumina NGS library preparation methods to detect viral RNA from different sample matrices	7	3	9/17/24
	Bajgain	Pratima	Identification of Cas-derived peptides on MHC-I proteins	8	15	9/17/24
	Bing	Sojin	Low cross reactivity between wild type and deamidated AAV can lead to false negative results in immune monitoring T-cell assays	9	7	9/17/24
	Colon-Moran	Winston	Identification of a novel strategy to modify AAV vector capsid to reduce immunogenicity ***FLASH TALK***	10	19	9/17/24
	Hodges	Shelby	Rhesus Broadly Neutralizing SIV Antibodies Mirror Developmental Features of Human HIV-1 Antibodies	11	5	9/17/24
	Khanal	Supreet	Identification of cellular factors that contribute to peripheral blood-derived CAR-NK cell mediated inflammatory toxicities	12	1	9/17/24
	Kotian	Shweta	Multi-omics Analysis of Adult Human Stem Cell-Derived Retinal Pigment Epithelium (RPE) Cells that Influence Transplant Efficacy for Vision Rescue	13	21	9/17/24
	Le	Tommy	Culture Methods Affect Expression of Molecular Markers Associated with Neural Stem Cell Differentiation	14	11	9/17/24
	Li	Xiaoqing	Evaluation of in vitro human placental barrier models for assessing drug toxicity on placenta immunity (maternal IgG transfer)	15	17	9/17/24
	Najera	Susana	Evaluation of 3D Spheroids in AAV Transduction Studies	16	9	9/17/24
	Shi	Bo	Specific Mutations in Blood Coagulation Factor VIII Have a Cumulative Effect for Increasing its Secretion	17	13	9/17/24
Advanced Manufacturing and Analytics, including New and Emerging Technologies	Baer	Alan	Developing a novel manufacturing platform for adeno-associated virus (AAV)-based vectors for gene therapy ***FLASH TALK***	18	35	9/17/24
	Dorosky	Robert	Use of Bacteriocins to Improve Live Biotherapeutic Product Purity Assays ***FLASH TALK***	19	39	9/17/24
	Jackson	Joseph	405 nm violet-blue light inactivates hepatitis C cell culture virus (HCVcc) in ex vivo human platelet concentrates and plasma	20	23	9/17/24
	Khoshi	Amir	Effect of Concurrent Pathogen Reduction (Amotosalen/UVA) and Gamma Irradiation on Biochemical Characteristics of Apheresis Platelets in Additive Solution	21	29	9/17/24
	Kim	Byung Woo	Efficient and robust generation of functional hematopoietic cells from human pluripotent stem cells in albumin-free conditions ***FLASH TALK***	22	41	9/17/24
	Neupane	Dharmendra	Glycoengineered outer membrane vesicles from Escherichia coli as a platform for developing a vaccine against Streptococcus pyogenes	23	27	9/17/24
	Panthi	Santosh	Development of an assay to perform high-throughput drug screen in HEK293 cells to improve AAV vector production	24	25	9/17/24
	Sharma	Kavita	Identifying Immunodominant Major Urinary Proteins (MUPs) In Mouse Urine	25	31	9/17/24
	Surov	Stepan	Investigation of the reversal potential of engineered Factor Xa agents in plasma treated with Factor Xa inhibitors ***FLASH TALK***	26	37	9/17/24
	Vertrees	Devin	Placenta Model for Investigating Viral Infection and Antibody Protection	27	33	9/17/24

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Advances in Computational Science Supporting Biologics'	Jankowska	Katarzyna	Revealing the role of micro-RNAs as post-transcriptional regulators of ADAMTS13 through binding to its coding region and understanding the effects of synonymous mutations on ADAMTS13 functionality ***FLASH TALK***	28	4	9/18/24
	Katagiri	Nobuko	The evolutional codon context of coagulation factors: positional codon and codon pair usage biases in ADAMTS13, Von Willebrand Factor, Factor 8, and Factor 9 ***FLASH TALK***	29	6	9/18/24
	Katneni	Upendra	CoCoPUTs, TissueCoCoPUTs and CancerCoCoPUTs: Databases for codon, codon pair and dinucleotide usage information	30	2	9/18/24
	Oakley	Miranda	Heterogeneity in pathogenic brain sequestered CD8+ T cells during experimental cerebral malaria revealed by single cell sequencing	31	8	9/18/24
Emerging and Re-emerging Diseases	Acharyya	Nirmallya	Development of isothermal and CRISPR-based diagnostics for the detection of Babesia parasites	32	54	9/18/24
	Cervenak	Juraj	Transmission of variant Creutzfeld-Jakob disease by blood transfusion in macaques.	33	58	9/18/24
	Cho	Jung-Sun	Double negative (CD4-CD8-) T cells persist in the gut of mice chronically infected with Trypanosoma cruzi and inhibit parasite growth in myoblasts in vitro	34	48	9/18/24
	Hashemi	Fatema	Innovative Nylon Affinity Networks for Enhanced Bacterial Reduction in Blood Transfusions	35	46	9/18/24
	Hastie	Jessica	Siderophore Utilization in Clostridioides difficile	36	50	9/18/24
	Hayashi	Clifford	High antibody response and resolution of parasitemia in the absence of CD4+ T cells during blood stage Plasmodium yoelii malaria infection	37	62	9/18/24
	Kanai	Tapan	Analyzing interactions of Influenza HA vaccine-elicited monoclonal antibodies using cryo-electron microscopy ***FLASH TALK***	38	53	9/17/24
	Kendra	Joseph	Probing the minimum required sequence constraints for stable incorporation of chimeric segments into rotaviruses	39	66	9/18/24
	Landivar	Michael	Highly Variable Antigenic Site Located at the Apex of GII.4 Norovirus Capsid Protein Can Induce Cross-reactive Neutralizing Antibodies	40	64	9/18/24
	Madere	Ferralita	Development of Specific and Sensitive Devices for Differential Diagnosis of Dengue, West Nile and Zika Viruses	41	43	9/17/24
	Neerukonda	Sabarinath	Comparison and characterization of myeloid cell line models for Anaplasma phagocytophilum infection ***FLASH TALK***	42	51	9/17/24
	Okeze	Efemena	Antibody Cross-Reactivity Among Dengue Virus (DENV), West Nile Virus (WNV) and Zika Virus (ZIKV) in Asymptomatic Infections	43	45	9/17/24
	Otubu	Temisan	Detection of transfusion-relevant bacteria spiked in human plasma using real-time polymerase chain reaction	44	60	9/18/24
	Ragupathy	Viswanath	Unbiassed metagenomic exploration of Transfusion-Transmitted infections with Nanopore sequencing. ***FLASH TALK***	45	55	9/17/24
	Rawal	Atul	A Machine Learning Driven Investigation of the Association between HLA and Asymptomatic SARS-CoV-2 Infection ***FLASH TALK***	46	61	9/17/24
	Scholl	Aaron	Understanding the host regulatory circuits behind host defense against flavivirus infection	47	52	9/18/24

Topic Area	Presenting	Presenting	Abstract Title	Page Numb	Poster Num	Presenting
Emerging and Re-emerging Diseases	Silberstein	Erica	Single-cell transcriptomics reveals the immune landscape of the mouse colon during chronic Trypanosoma cruzi infection ***FLASH TALK***	48	49	9/17/2024
	Smith	Emily M.	Investigation of novel bacteriophage resistance mechanisms in vancomycin-resistant Enterococcus (VRE) ***FLASH TALK***	49	47	9/17/2024
	Subramani	Sakthivel	Host transcriptional responses against hepatitis E virus infection in gerbils ***FLASH TALK***	50	57	9/17/2024
	Visser	Bryan	Characterization of hepatitis B virus (HBV) from deferred potential blood donors and infectivity in primary human hepatocytes cultures ***FLASH TALK***	51	59	9/17/2024
	Yakovleva	Oksana	Evaluation of antimicrobial 405 nm violet-blue visible light inactivation potential of Treponema pallidum spiked in human platelets in a rabbit infectivity model.	52	56	9/18/2024
Methods and Biomarker Discovery for Product Safety and Quality	D'Agnillo	Felice	Exposure to Low Steady State Hydrogen Peroxide Induces Red Blood Cell Vesiculation and Loss of Deformability	53	63	9/18/2024
	Fatima	Tahira	Metabolite profiles distinguish exposure to Zika and Dengue flaviviruses in human induced pluripotent stem cells (hiPSCs) ***FLASH TALK***	54	38	9/18/2024
	Jean	Catherine	High-throughput APTT and one-stage APTT-based FVIII potency assays in low volume of mouse plasma ***FLASH TALK***	55	44	9/18/2024
	Klenow	Laura	Evaluation of the Leishmanin Skin Test antigen as a biomarker of vaccine immunogenicity and disease surveillance ***FLASH TALK***	56	40	9/18/2024
	Konduru	Krishnamu	Exploring host microRNAs in plasma as non-immune biomarkers for differential diagnosis between Dengue and Zika ***FLASH TALK***	57	42	9/18/2024
	Kumar	Supriya	Aptamer-based detection of Trypanosoma cruzi-derived biomarkers in blood of Chagas disease patients from Brazil and Bolivia	58	24	9/18/2024
	Olivares	Philip	Development of a simple purification scheme with a high yield for laboratory-scale production of recombinant blood coagulation Factor VIII	59	34	9/18/2024
	Park	Bu	Investigating Brain Structure in PP2A-B56e KO Mouse Model using 7T MRI	60	32	9/18/2024
	Simakova	Olga	Long-term physicochemical and microbiological stability and in vitro prothrombin-converting activity neutralization potency of outdated antivenom products indicated for exotic snake species envenomation	61	36	9/18/2024
	Singh	Olga	Development of a qmosRT-PCR assay for simple detection and identification of contamination of novel oral-poliovirus vaccines with Sabin viruses	62	30	9/18/2024
	Tirrell	Nikki	NLP-based biological cell type annotator for single cell transcriptomics	63	26	9/18/2024
	Zhao	Jiangqin	Preparation and Validation of CBER Diverse HIV-1 Variants NAT Reference Panel (v18.51)	64	28	9/18/2024

Topic Area	Presenting Author Last Name	Presenting Author First Name	Abstract Title	Page Number	Poster Number	Presenting Date
Immune Responses to Vaccination	Kim	Jaekwan	Combined immunization with SARS-CoV-2 spike and SARS-CoV nucleocapsid results in protection but increased lung pathology	65	16	9/18/24
	liu	lunhua	Sustained antigen delivery improves germinal center reaction and increases antibody responses in neonatal mice	66	14	9/18/24
	Pacheco-Fernandez	Thalia	A Live-Attenuated Leishmania Vaccine Shapes the Cellular Response in The Bone Marrow ***FLASH TALK***	67	18	9/18/24
	Parvathaneni	Swetha	Establishment of a robust experimental system for efficient generation of functional TFH cells in vitro in both adults and neonates	68	10	9/18/24
	Pilewski	Kelsey	Defining Humoral Immunity and Antibody Immunodominance Following Human Norovirus Challenge ***FLASH TALK***	69	20	9/18/24
	Rajasagi	Naveen	Qualitative analysis of immune responses to hepatitis C virus E2 protein induced with viral vector versus recombinant protein ***FLASH TALK***	70	22	9/18/24
	Toney	Mykia	Evaluating the In Vitro Immune Responses against Francisella tularensis Schus4-ΔclpB in Fischer 344 Rats	71	12	9/18/24

Evaluation of RNA Extraction and Illumina NGS library preparation methods to detect viral RNA from difference sample matrices

Sayma Afroj[1], Aaron Scholl[1], Binsheng Gong[2], Bingjie Li[1], Joshua Xu[2] and Sandip De[1]*
[1]Tumor Vaccine & Biotechnology Branch, Division of Cellular Therapy 2, Office of Cell Therapy and Human Tissues, Office of Therapeutic Products, US Food and Drug Administration, 10903 New Hampshire Avenue, Silver Spring, MD, 20993; [2]Division of Bioinformatics and Biostatistics, National Center for Toxicological Research, Food and Drug Administration 3900 NCTR Rd Jefferson, AR 72079.

Objective: Current pathogen and adventitious agent detection assays face limitations, such as specificity to certain pathogens and reduced sensitivity, which challenge the safe use of HCT/Ps. This project aims to evaluate various RNA extraction and NGS library preparation methods to achieve rapid, broad, sensitive, and specific detection of pathogens and adventitious agents.

Methods: We used three study designs to assess RNA extraction methods: spiking Zika virus (ZIKV) into naïve cells, using a cell line persistently infected with ZIKV, and spiking persistently infected ZIKV cells into buffy coat samples. RNA was extracted using five methods and ZIKV quantification was performed using qRT-PCR. To evaluate RNA library preparation methods, we spiked virus panels HEV RR.1 and BEI- NR-59622 (containing PCV1, Reo, FeLV, RSV, EBV) into U937 cells, extracted total RNA, and constructed libraries with various commercial kits and combinations. The libraries were sequenced on the Illumina NovaSeq 6000 platform. NGS data is being analyzed to determine virus genome mapping, genome coverage, and percentage of mapped reads.

Results and Conclusion: Overall, TRIzol extraction with silica column purification proved to be the best method for extracting viral RNA. As anticipated, we observed significant variability between different NGS library preparation methods. rRNA depletion effectively reduced human rRNA in the NGS libraries. Further data analysis is underway to study the impact of various library preparation methods on the total number of NGS reads generated from reference viral RNAs.

Plain Language Summary:
Efficient viral nucleic acids extraction and library preparation methods are crucial for adventitious virus detection from cell and tissue using NGS technique and for safety of cell and tissue therapy. Our study evaluated several RNA extraction methods and observed variations in viral RNA extraction efficiency among the tested methods. Overall, TRIzol with silica column purification was most sensitive method for virus detection across tested sample matrices. As anticipated, we observed significant variability between different NGS library preparation methods. Further data analysis is underway to study the impact of various library preparation methods on the total number of NGS reads generated from reference viral RNAs.

Identification of Cas-derived peptides on MHC-I proteins

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Hemostasis Branch 1, Division of Hemostasis, Office of Plasma Protein Therapeutics, Center for Biologics Evaluation and Research, Food and Drug Administration, Silver Spring, MD, USA.

Objective: The CRISPR-Cas systems are efficient gene editing tools with the potential to treat numerous human diseases. Despite the immense clinical promise, potential immune responses to Cas proteins present an obstacle to the widespread use of CRISPR-Cas editors. Antigen presenting cells (APCs) process foreign proteins which are then presented by Major Histocompatibility Complex (MHC) molecules. There are two classes of MHC molecules: Class I (MHC-I) and Class II (MHC-II) which present peptides from endogenously expressed and exogenous proteins respectively. Here, we focus on the MHC-I mediated immune response to Cas proteins. This is because in most protocols, the Cas molecules are delivered as RNA using viral vectors and are endogenously expressed in the target cells.

Methods: We used 9 monoallelic cell lines (each cell line expresses a unique MHC-I variant) and carried out lentivirus transduction using SaCas9 and Casφ lentiviral vector with Internal Ribosome Entry Site2-enhanced Green Fluorescent Protein (IRES2-eGFP). The transduced cells were sorted twice for cells that were positive for GFP and HLA-A, B, C (MHC-I subtypes). The expression of Cas proteins was also monitored on an immunoblot. We used the MHC- Associated Peptide Proteomics (MAPPs) assay to identify Cas-derived peptides on MHC-I proteins using mass spectrometry.

Results: Our results demonstrate successful transduction of SaCas9 and Casφ in all the 9 monoallelic cell lines. Using the MAPPS assay, we identified 45 unique peptides from SaCas9 and 14 unique peptides from Casφ.

Conclusions: Identification of Cas-derived peptides is an important first step in the comprehensive immunological characterization of Cas proteins. These results have significant implications in the choice of Cas proteins to be used in clinical, in vivo gene editing. Moreover, the identification of T cell epitopes also provides targets for de-immunization of Cas proteins.

Plain Language Summary:
The CRISPR-Cas system is a powerful gene editing tool with promising clinical applications. However, immune responses to Cas proteins limit the clinical potential of this technology. Peptides from a protein are loaded on Major Histocompatibility Complex (MHC) molecules found on antigen presenting cells. This is a necessary condition for eliciting an immune response to a protein. We used a mass spectrometry-based technique to identify Cas-derived peptides from two Cas proteins (SaCas9, and Casφ derived from Staphylococcus aureus and Biggiephage respectively) presented by 9 different MHC Class-I molecules. This study thus identifies the immunogenic regions on Cas proteins.

Low cross reactivity between wild type and deamidated AAV can lead to false negative results in immune monitoring T-cell assays

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During gene therapy trials, the immune response against adeno-associated virus (AAV) vectors is monitored by antibody assays that detect the humoral response and T-cell mediated cellular responses to AAV vectors. These assays commonly utilize the collection of patients' peripheral blood mononuclear cells (PBMCs) and stimulation with AAV-derived overlapping peptides. We recently described that spontaneous deamidation coincides with T cell epitopes in AAV capsids and that spontaneous deamidation may enhance or decrease immunogenicity in some individuals. This raised concerns of false negative results in antibody detection and PBMC immune monitoring assays because these assays use wild-type (WT) AAV or WT peptides for T cell re-stimulation and these peptides may not re-activate T cells that were stimulated with deamidated AAV capsid. To investigate this concern, we modeled the scenario by expanding T cells with deamidated peptides and evaluated the cross-reactivity of expanded T cells to WT peptides. In the majority of samples, cells that were expanded with deamidated peptides and restimulated with WT peptide had significantly lowered IL-2 and IFN-g production. Spiking the four deamidated peptides to the WT peptide pool used for restimulation restored the signal and corrected the performance of the assay. We also evaluated the impact of deamidation on anti AAV binding antibodies and did not observe a major impact on seroprevalence detection. These data indicate that a high level of deamidation of AAV therapy may result in underestimation or even failure to detect immune responses against these WT peptides during cellular immune monitoring.

Plain Language Summary:
In gene therapy trials using AAV vectors, we monitor immune responses with antibody and T-cell assays using patient blood cells and AAV-derived peptides. Spontaneous changes in AAV capsids, called deamidation, can affect immune reactions, potentially causing false negatives in monitoring assays. This underscores the impact of deamidation on immune response detection.

Identification of a novel strategy to modify AAV vector capsid to reduce immunogenicity

Winston Colon-Moran, Alan Baer, Santosh Panthi, Nirjal Bhattarai
Tumor Vaccine and Biotechnology Branch, Division of Cellular Therapy 2, Office of Cellular Therapy and Human Tissue, Office of Therapeutic Products, CBER, US Food and Drug Administration, Silver Spring, MD

AAV vectors are commonly used for vector-based gene therapies; however, host immune responses against AAV and/or transgene present a safety risk to subjects and can reduce therapeutic efficacy. To address this, we hypothesized that incorporating immunomodulatory factor(s) derived from naturally occurring human viruses into the AAV capsid may reduce AAV immunogenicity. To this end, we studied two immunomodulatory proteins expressed by the hepatitis B virus, e antigen (HBeAg) and s antigen (HBsAg), and its effect on human T cell activation (TCA). We found that HBeAg, but not HBsAg, inhibited T cell receptor-mediated TCA. Activation of human T cells with PMA and Ionomycin, which bypasses TCR and activates distal T cell signaling was not affected by HBeAg, suggesting that HBeAg specifically inhibits proximal TCR signaling. Deletion mutagenesis studies identified a short 11 amino acid peptide (11mer) derived from HBeAg that is sufficient to inhibit TCA. Mechanistically, we found that 11mer peptide inhibited activation of Interferon regulatory factor 3 (IRF3), potentially contributing to HBeAg-mediated inhibition of TCA. The 11-mer peptide was inserted into variable region (VR) III of the common VP3 region, which is exposed on the surface of the capsid. Importantly, *in silico* analysis showed that the insertion of the 11mer peptide did not affect the AAV capsid structure. Current studies are underway to assess quality, potency, and immunogenicity of the modified AAV vector and delineate mechanisms for the inhibition of T cell activation.

Plain Language Summary:
Gene therapy using viral vectors holds promise for treating various human diseases. Yet, the immune system's potential rejection of these vectors poses a challenge. Inspired by nature, where some human viruses have evolved to elude immune responses, we identified a specific peptide in the hepatitis B virus e protein that inhibits the human immune response. Leveraging this discovery, we are actively engineering a novel viral vector that incorporates this peptide. The goal is to create a vector capable of evading the human immune response, paving the way for safer and more effective gene therapy to treat diverse diseases.

Rhesus broadly neutralizing SIV antibodies mirror developmental features of human HIV-1 antibodies

Hodges, Shelby; Mason, Rosemarie; Roderer, Mario; Kwong, Peter; Gorman, Jason
Food and Drug Administration, Silver Spring, MD; National Institute of Health, Bethesda, MD

The study of antibodies targeting Simian Immunodeficiency Virus (SIV) has significant but underexplored potential in virology given the similarities between the SIV envelope (Env) and that of HIV-2, affecting ~1-2 million people globally, and the more distant HIV-1, impacting ~40 million. Additionally, at least a dozen zoonotic SIV crossover events resulting in HIV-1 and HIV-2 have been identified, but the actual total is unknown, implying more that didn't persist, and the risk of future crossovers remains. Lastly, while the SIV-NHP animal model is considered the standard, details about the development of SIV antibodies in NHPs have lagged compared to their human counterparts. This study defines structural characteristics of SIV and HIV-2 neutralizing rhesus antibodies, ITS102.03 and ITS103.01, aiming to bridge gaps in understanding both SIV and HIV.

Plain Language Summary:
This study structurally characterizes antibodies targeting Simian Immunodeficiency Virus (SIV) and explores the implications for HIV-2 and HIV-1. Using cryo-electron microscopy, we defined the structures of two rhesus antibodies in complex with the SIV viral envelope. These structures revealed interactions shared with both host receptors and human broadly neutralizing antibodies. The results suggest convergent evolution in antibody responses between humans and rhesus models. Overall, this research enhances our understanding of SIV and HIV-2 immune responses, their relationship to HIV-1, and underscores the value of the SIV-NHP model in evaluating HIV therapeutic and vaccination strategies.

Identification of cellular factors that contribute to peripheral blood-derived CAR-NK cell mediated inflammatory toxicities

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Background and Objective: Chimeric antigen receptor-expressing NK (CAR-NK) cells are increasingly being developed to treat a wide variety of human diseases. While CAR-T cells are known to activate bystander myeloid cells, contributing to inflammatory toxicities such as cytokine release syndrome (CRS) and immune effector cell-associated neurotoxicity syndrome (ICANS), the potential of CAR-NK cells to induce similar effects remains uncertain due to limited clinical data. Here we studied human peripheral blood-derived CAR-NK cells and assessed their ability to cause inflammatory toxicities.

Methods: We used PMA/Ionomycin and coculture to obtain activated NK cells and T cells. Activated supernatants were subjected to cytokine arrays to detect the levels of inflammatory cytokines. Antibody neutralizations and siRNA were used to deplete the candidate factors, and luciferase expressing Nalm6 was used to measure cytotoxicity.

Results: We found that following activation, NK cells secrete fewer inflammatory cytokines at lower levels compared to T cells obtained from the same donor, resulting in lower levels of myeloid cell activation and secretion of inflammatory cytokines IL-6 and IL-1β by myeloid cells. Nevertheless, activated NK cells demonstrated notable myeloid cell activation across all donors studied compared to resting NK cells. To assess the mechanism by which CAR-NK cells activate bystander myeloid cells, we analyzed the inflammatory cytokine profile of activated NK cells. Comparative analysis of secreted cytokines from activated and resting primary NK cells and NK cell lines identified key inflammatory factors with potential to cause myeloid cell activation. Antibody-mediated neutralization studies identified four NK cell factors, which, upon neutralization, significantly reduced NK-cell-mediated myeloid cell activation. This observation was confirmed through siRNA-mediated knockdown studies as knockdown of these four factors in peripheral blood-derived CAR-NK cells significantly reduced myeloid cell activation. Importantly, neutralization or knockdown of these four inflammatory factors in NK cells did not affect potency of CAR-NK cells.

Conclusions: Together, these data suggest that peripheral blood-derived CAR-NK cells can activate bystander myeloid cells and contribute to inflammatory toxicities during CAR-NK cell therapy. Furthermore, this study identified four major inflammatory factors secreted by activated NK cells, which, upon inactivation, can significantly reduce CAR-NK cell-mediated inflammatory toxicities without affecting their potency. The inactivation of these factors during CAR-NK cell manufacture may help improve product safety.

Plain Language Summary:
Cell-based gene therapies such as CAR-T and CAR-NK cells hold great potential for treating many human diseases. However, challenges such as inflammatory toxicities associated can impede their development and widespread use. In this study we identified some of the major inflammatory soluble factors from activated NK cells that can contribute to inflammation and toxicity during their use. Depletion of these soluble factors using antibody neutralization and siRNA decreased the activation of bystander myeloid cells, thus enhancing the safety profile of NK and CAR-NK cells.

Multi-omics Analysis of Adult Human Stem Cell-Derived Retinal Pigment Epithelium (RPE) Cells that Influence Transplant Efficacy for Vision Rescue

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It has been demonstrated previously that iPSCs can be produced from cadaveric retinas and differentiated into retinal pigment epithelium (RPE) cells. Using a multi-omics approach, we analyzed a population of these cells which has been shown to restore visual acuity significantly in a rodent model of human retinal dystrophy, with the aim of identifying the therapeutic sub-population. RPE cells at differentiation time-intervals of 2, 4, and 8 weeks were analyzed using the following assays by 10X Genomics: scRNA-seq, single cell ATAC sequencing (scATAC-seq) and a combined single cell multiome scRNA-seq+ATAC-seq (multiome-seq). Bioinformatics analyses were performed using Cell Ranger, Seurat and Signac. Initial exploration shows that the number of unique transcripts decreased with increasing time intervals. Clustering analysis showed that 2-, 4-, and 8-week cell clusters overlap, however, transcriptomic analysis showed subtle changes in the expression levels of genes relevant to the development and differentiation of RPE cells between 2-, 4-, and 8-weeks. Notably, the chromatin accessibility profiles of 2-, 4-, and 8-week cells were different, suggesting that chromatin accessibility analysis provides better discriminatory power between these differentiation states. We are currently parsing this data, as well as data from the multiomic analysis, in an attempt to connect the transcriptomic and epigenomic information and identify a subpopulation responsible for the therapeutic benefit. Any genes identified will be validated biologically by single cell PCR and by transplantation into the in vivo rat model. We believe these cells may meet an urgent need for therapy of age-related macular degeneration, the leading cause of blindness in the elderly, and other degenerative eye diseases.

Plain Language Summary:
We used a multi-omics approach by sequencing both mRNA and chromatin, to determine the therapeutic subset within a population of retinal pigmented epithelial (RPE) cells differentiated from adult human stem cells that have been shown to restore vision in a rat model of retinal dystrophy. We observed subtle transcriptomic changes but distinct epigenomic differences between RPE cells at various stages of differentiation. We are parsing the data to determine the biological significance of this finding.

Culture Methods Affect Expression of Molecular Markers Associated with Neural Stem Cell Differentiation

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Objective: To study how cell culture affects Neural Stem Cell (NSC) characteristics that determine their safety and effectiveness. We want to identify molecular markers that correlate with the differentiation state of NSCs.

Methods: Transgenic human iPSCs cell lines containing Sox2-GFP and CBF-Venus (Notch activity) reporters were used for these studies. The iPSCs were cultured under differentiating conditions to form neurospheres which were dissociated into single cells to propagate as adherent NSCs (AdNSCs). Forward and Side Scatter analysis were used to determine morphology and size characteristics of adNSCs. The cells were also analyzed for molecular markers via flow cytometry. To analyze NOTCH2+ cells specifically, adNSCs were dissociated into single cells and sorted via FACS into NOTCH2+ and NOTCH2– populations. Neurosphere formation assay was performed on NOTCH2+ and NOTCH2– populations and statistical analysis was carried out using Student t test. Immunohistochemistry was done on cells seeded on pre-coated chambered slides, stained with antibodies, and imaged using a confocal microscope.

Results: We have that found that adNSCs have a relatively homogenous morphology based on FSC and SSC when analyzed with flow cytometry. Flow cytometry analysis also found that Notch activation and Notch2 expression was highest shortly after the adNSCs were formed and decreased with passage number. Conversely, SOX2 expression increases with passage number. AdNSCs have the potential to differentiate into neuronal cell types, such as GABA and TH expressing cells. However, NOTCH2+ cells can differentiate more efficiently into these types of cells suggesting that they are more primitive stem cells. Beta-Tubulin III is highly expressed in adNSCs and only few GFAP expressing cells were present.

Conclusions: Culture conditions influence the potential therapeutic cell types that NSCs can differentiate into. Our results identify molecular markers that correlate with the differential potential of NSCs which is important information for manufacturing a NSC based cellular therapy.

Plain Language Summary:
This study examines how cell culture conditions affect Neural Stem Cell (NSC) characteristics potential to form therapeutic cell types.

Evaluation of in vitro human placental barrier models for assessing drug toxicity on placenta immunity (maternal IgG transfer)

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Objective: We developed two *in vitro* human placental barrier models, specifically a transwell model and a microfluidic device, to investigate drug toxicity on placental immunity by evaluating IgG transport.

Methods: We utilized human trophoblasts and endothelial cells to establish *in vitro* human placental barrier models. The characterization of the barriers includes: (1) immunocytochemistry staining, (2) ultrastructure visualized using TEM and SEM, (3) the transendothelial electrical resistance measurement, (4) permeability to various molecular weights substances including glucose, FITC-dextran and heparin. We pretreated the barrier with 10 μM dexamethasone (DEX) and 1 μM remdesivir (RDV) and proceeded with by IgG transfer detection. We also evaluated the IgG transport under drug treatment using a humanized mouse model, that expresses human *FCGRT* gene. Animals were dosed daily from gestational day (GD) 10-14 with 5 mg/kg DEX, 25 mg/kg RDV or vehicle control article via intravenous injection, followed by 2 mg/kg human IgG injection on GD 14.

Results: The transwell model was optimized by evaluating different culture parameters to obtain stable high resistance, intact trophoblast and endothelial cell layers, with spontaneous formation of syncytiotrophoblasts. This barrier was glucose permeable but heparin and dextran impermeable. After DEX or RDV pretreatment, our results demonstrated that DEX (10 μM) reduced the IgG transport, compromising placental immunity, in contrast to RDV (1 μM) which did not decrease human IgG transfer. Notably, the drug treatment didn't change expression of IgG binding receptor, FCGRT, and HCG. Furthermore, we enhanced the microfluidic model to demonstrate the formation of syncytiotrophoblast layers for further study of placental immunity. *In vivo*, IgG transfer was reduced in the DEX group, but not in the RDV group. The *in vivo* IgG transfer data were consistent with the results observed *in vitro*.

Conclusion: Our *in vitro* models are potentially useful tools that mimic human microenvironments for assessing drug toxicity on human placental immunity (placental IgG transfer).

Plain Language Summary:
We developed and studied two in vitro human placental barrier models. The purpose of these models is to better simulate the effects of drugs on the human placenta and the fetus. We showed the models can simulate immune function between the mother and baby, and showed that dexamethasone, a steroid, compromised it.

Evaluation of 3D Spheroids in AAV transduction studies

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Adeno-associated viruses (AAVs) are non-enveloped single-strand DNA viruses which are nonpathogenic in nature. AAVs can infect both dividing and non-dividing (quiescent) cells while expressing episomal DNA without integrating into the host cells genome. Therefore, AAV vectors are the leading candidate vectors for human gene therapy. Six FDA-approved gene therapy drugs utilizing AAV vectors, and many other products are currently under clinical trial and development. Screening for AAV gene therapy activity is usually performed using conventional 2D cell culture models. However, such a rudimentary approach and culture model does not reflect the physiological cell environment accurately, specifically lacking the role of the extracellular matrix (ECM) and cell-cell 3D contact.

Objective: Therefore, it is important to develop more physiologically representative culture models with high throughput screening capabilities to characterize the potency of AAV gene therapy candidates for successful clinical therapy.

Methods: Here, we evaluate 3D cultures by manufacturing 3 different AAV serotypes with self-complimentary and single-stranded GFP genes, representative of highly potent and medium potent genes, respectively, and evaluate their activity in different MOIs using 2D and 3D cultures in 2 different cell lines. RESULTS: We found that 3D cultures worked well for AAV transduction, required fewer cells and less AAV (10-fold less) with a lower limit of detection. This observation was true in different serotypes, different cell lines, and utilizing different reporter genes.

Conclusions: Thus, this work highlights the value of 3D cultures for activity evaluation of gene therapy vectors. We plan to use this system to evaluate the activity of clinically relevant genes, as well as to evaluate the effect of transduction boosters in these 2 culture systems, and/or use 3D culture for neutralization and immune cell killing assays.

Plain Language Summary:
In this project, we aim to evaluate the performance of 3D cultures for AAV transduction studies utilizing 3 different AAV constructs with different reporter genes. Currently, the existing methods for AAV transduction tend to underrepresent cells with low or transient gene expression, thus in this project we designed AAVs with low and high gene expression potency and evaluated their expression in 3D cultures. Here, we demonstrate that 3D cultures worked well for AAV transduction, required fewer cells and less AAV (10-fold less) with a lower limit of detection. This observation was true in different serotypes, cell lines, and utilizing different reporter genes.

Specific Mutations in Blood Coagulation Factor VIII Have a Cumulative Effect for Increasing its Secretion

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Background: Production of recombinant blood coagulation factor VIII, used for the therapy of its functional disorder (Hemophilia A), is known to be limited by low level of protein secretion. Previously, the mutation Phe309Ser was described to increase protein expression (*Swaroop et al, 1997*), as well as replacement of five specific amino acid residues (Ile86, Ala108, Gly132, Met147, and Leu152) within the same domain (A1) of FVIII into corresponding porcine residues (*Cao et al, 2020*). We aimed to investigate an effect of combining these mutations on FVIII expression.

Objective: To characterize protein expression for the five FVIII human to porcine replacements (x5) and the additional Phe309Ser mutation (x6) along with testing the transgene dose-dependence.

Methods: Lentivirus-based plasmid vectors with the x5 and x6 mutations within a codon optimized B domain-deleted FVIII (WT) gene were generated. Using a FVIII expression platform described previously (*Shestopal et al, 2017*), respective lentiviruses were transduced into Chinese Hamster Ovary cells with multiplicity of infection (MOI) 2, 4, or 8. Transgene copy number was tested by real-time (q) PCR, and media was tested for secreted FVIII by electro chemiluminescence assay (ECLA) using specific anti-FVIII antibodies.

Results: We observed a general positive correlation between increasing the number of introduced gene mutations, transgene copy number per cell, and protein secretion levels. For MOI=2, the difference in protein secretion levels between the x5 and x6 mutants was more pronounced; along with the MOI/transgene doubling, the secretion level doubled for each mutant, whereas increased only by ~60% for WT.

Conclusion: The six mutations of FVIII had cumulative effect on increasing protein secretion and were beneficial in converting the transgene number increase into additional protein secretion compared to non-mutated FVIII. The results can be useful for FVIII production for various applications.

Plain Language Summary:
Recombinant blood coagulation factor VIII (rFVIII), used for treatment of Hemophilia A, has low expression level in cell culture. Studies indicated that specific rFVIII mutations, including replacements into homologous porcine residues, increase protein yield. We tested effects of combining all these mutations on rFVIII expression at increasing transgene dosage per cell using a lentivirus expression platform, real-time PCR, and an electrochemiluminescence assay, and found a positive correlation between increasing the number of mutations, transgene copy per cell, and protein secretion in cell culture. The results demonstrated beneficial effect of combining these mutations, indicating usability of this approach for rFVIII production.

Developing a novel manufacturing platform for adeno-associated virus (AAV)-based vectors for gene therapy

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AAV vector-based gene therapy has the potential to treat many human diseases. Currently, there are six AAV vector-based gene therapy products approved by the U.S. FDA. While AAV vectors have been increasingly used in clinical studies, the vector manufacturing process is complex, resulting in poor yield and high costs. Since large quantities of AAV vectors are needed for preclinical and clinical studies, this presents a significant bottleneck and has negatively affected the availability and costs of these therapies.
AAV vectors are commonly manufactured using HEK293 cells in Erlenmeyer flask for research and large bioreactors for preclinical and clinical studies. This process is complex and requires product handling at multiple steps, which increases risks to the product. Furthermore, this contributes to lot-to-lot variability and increases manufacturing cost. To address these challenges, we are developing a novel, simplified and cost-effective manufacturing platform using a Gas Permeable Rapid Expansion (GREX) system, which is traditionally used for manufacturing cell therapy products. Our preliminary data identified that while the GREX system has the potential to produce AAV vectors in HEK293 cells, cell aggregation and clumping were found to be major challenges. To resolve these issues, we assessed various strategies known to reduce cell aggregation and clumping and identified a strategy to culture HEK293 cells in the GREX system without any cellular aggregation. We are currently manufacturing AAV vectors in GREX using a strategy that does not result in cell aggregation and comparing its quality and potency with AAV produced using the Erlenmeyer flask method. Furthermore, our current assessment suggests that the GREX system will significantly reduce the cost of AAV production compared to the current system.

Plain Language Summary:
Development of a novel method to improve adeno-associated viral (AAV) vector production for cell and gene therapy.

Use of Bacteriocins to Improve Live Biotherapeutic Product Purity Assays

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Objective: Live Biotherapeutic Products (LBPs) are biological products that contain live organisms and are intended for use as a drug. LBP microbial purity testing should demonstrate that the product is free of microbial contaminants. Product organism growth can obscure the results of culture-based microbial purity methods often used. Our approach is to use bacteriocins to selectively inhibit product strain growth thereby improving detection of contaminants.

Methods: Activities of four Class IIb bacteriocins were assessed against 32 anaerobic and facultatively anaerobic bacteria, including lactobacilli and non-lactobacilli, using a soft agar dilution assay. Contaminants were spiked into a mock LBP containing *L. delbrueckii* and the preparation was plated onto Plantaricin S (PS)-supplemented media. A commercially available *L. delbrueckii* containing probiotic was plated onto PS supplemented media.

Results: PS inhibited 14/17 lactobacilli with variable sensitivity. NC8 inhibited 5 lactobacilli strains and Lactocin 705 (L-705) and Plantaricin JK (Pln JK) inhibited a single lactobacilli strain. L-705 and PlnJK did not inhibit any of the non-lactobacilli tested. PS and NC8 inhibited 13/15 and 10/15 non-lactobacilli tested, respectively. Notably, lactobacilli were typically more sensitive to PS compared to the non-lactobacilli strains. For example, *L. delbrueckii* F1 and *L. crispatus* 33197 were inhibited by 0.17 and 1.7 µM PS, respectively, while *Enterococcus faecalis* 51299 inhibition required 28.8 µM PS. PS media-supplementation enabled unambiguous detection and enumeration of spiked anaerobic contaminants in a mock LBP and anaerobic contaminants in a commercial probiotic.

Conclusions: PS exhibited potent killing activity against some of the lactobacilli strains tested and the non-lactobacilli strains tested were typically less sensitive to PS compared to the lactobacilli tested. Media supplementation with PS significantly reduced the product viable counts and improved detection of product contaminants. These data suggest that PS is a promising candidate for LBP purity reagent development.

Plain Language Summary:
Gene therapy using viral vectors holds promise for treating various human diseases. Yet, the immune system's potential rejection of these vectors poses a challenge. Inspired by nature, where some human viruses have evolved to elude immune responses, we identified a specific peptide in the hepatitis B virus e protein that inhibits the human immune response. Leveraging this discovery, we are actively engineering a novel viral vector that incorporates this peptide. The goal is to create a vector capable of evading the human immune response, paving the way for safer and more effective gene therapy to treat diverse diseases.

405 nm violet-blue light inactivates hepatitis C cell culture virus (HCVcc) in *ex vivo* human platelet concentrates and plasma

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Background: Added safety measures coupled with the development and use of pathogen reduction technologies (PRT) significantly reduce the risk of transfusion-transmitted infections (TTIs) from blood products. Current PRTs utilize chemical and UV-light based inactivation methods. While the effectiveness of these PRTs in reducing pathogens are well documented, these can cause tolerable yet unintended consequences on the quality and efficacy of the transfusion products. As an alternative to UV-based approaches, we have previously demonstrated that 405 nm violet-blue light exposure successfully inactivates a variety of pathogens, including bacteria, parasites, and viruses, in both platelet concentrates (PCs) and plasma.

Objective: To assess the inactivation of HCVcc by 405 nm light exposure.

Methods: We assessed HCVcc titers after exposure to 405 nm blue light at indicated time points. We also assessed reactive oxygen species (ROS) production after exposure.

Results: We show that 405 nm light treatment effectively inactivates hepatitis C cell culture virus (HCVcc) by up to 4log10 in a variety of matrices, such as cell culture media, phosphate buffered saline, plasma, and PCs with 27 J/cm^2 of light exposure, and total inactivation of HCVcc after 162 J/cm^2 light exposure. Furthermore, we demonstrate that carry-over of media supplemented with fetal bovine serum enhances the production of ROS, providing mechanistic insights to 405 nm light-mediated viral inactivation.

Conclusion: Overall, 405 nm light successfully inactivates HCVcc, further strengthening this method as a novel PRT for platelets and plasma.

Plain Language Summary:
Current pathogen reduction technologies (PRTs) utilize chemical and UV-light based methods to successfully inactivate a variety of pathogens in blood products; however, these can cause tolerable yet unintended consequences on the quality and efficacy of the transfusion products. We have previously developed a novel, alternative PRT using 405 nm visible blue light, and have demonstrated inactivation of bacteria, viruses, and parasite. Herein this work, we've demonstrated that exposure to 405 nm visible blue light inactivation of hepatitis C cell culture virus (HCVcc) in both plasma and platelet concentrates.

Effect of Concurrent Pathogen Reduction (Amotosalen/UVA) and Gamma Irradiation on Biochemical Characteristics of Apheresis Platelets in Additive Solution

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Background: Pathogen reduction (PR) may be used as an alternative to gamma irradiation (GI) to prevent transfusion associated graft versus host disease (TA-GVHD) if the pathogen reduction technology has been shown to inactivate residual lymphocytes. However, as GI is considered the gold standard for reducing the risk of TA-GVHD, some centers continue to perform GI in addition to PR. This study investigated the effect of concurrent pathogen reduction and gamma irradiation (PR/GI) on the biochemical characteristics of apheresis platelets at day 1, 5 and 7 of storage at room temperature.

Methods: We compared in-vitro characteristics of apheresis platelets (PLTs), PR PLTs, GI PLTs and PR/GI PLTs at storage days of 1,5 and 7. PLTs suspended in 65% PAS-3/35% plasma were from six healthy volunteers prior to splitting and treatment with PR, GI, or PR/GI. Parameters measured were: PLT concentration, mean PLT volume (MPV), pH, glucose, CD62P, annexin V binding, aggregation induced by thrombin receptor activating peptide 6 (TRAP), mitochondrial membrane potential (MMP), and reactive oxygen species (ROS).

Results: PR/GI PLTs did not show significant changes in measured parameters when compared to PR PLTs. However, when compared to untreated control PLTs, PR and PR/GI PLTs showed significant declines in PLT content, pH, MMP, aggregation and significant increases in MPV, CD62P, annexin V binding, and ROS production, mostly on day 7 of storage.

Conclusions/Summary: While PR impacts PLT concentration, metabolic profile, activation status, and aggregation response of apheresis platelets, gamma irradiation of PR PLTs did not cause any additional significant changes of platelet in-vitro parameters.

Plain Language Summary:
Pathogen reduction (PR) may be used as an alternative to gamma irradiation (GI) to prevent transfusion associated graft versus host disease (TA-GVHD) if it has been shown to inactivate residual lymphocytes. However, as GI is considered the gold standard for reducing the risk of TA-GVHD, some centers continue to perform GI in addition to PR. we address an important question which we frequently receive from blood banks about the effects of concurrent pathogen reduction and gamma irradiation on platelet quality when stored up to 7 days. We show that additional gamma irradiation of pathogen reduced platelets does not significantly change the quality of platelets.

Efficient and robust generation of functional hematopoietic cells from human pluripotent stem cells in albumin-free conditions

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Objective: Generation of human hematopoietic stem and progenitor cells (HSPCs) from pluripotent stem cells (PSCs) holds significant promise in disease modeling, drug screening, and the development of cell and gene therapies for various hematologic and non-hematologic disorders. However, the efficient and consistent derivation of functional HSPCs remains a major challenge hindering their practical application in biomedical research and applications. In addition, human serum albumin (HSA) has been widely regarded as essential for supporting ex vivo maintenance and expansion of HSPCs, thereby contributing to high manufacturing costs. In this study, we address the manufacturing and regulatory challenges associated with using HSA in HSPC differentiation and propose a novel approach using a caprolactam-based polymer as an alternative.

Methods: Quantitative real-time PCR and flow cytometry were performed on PSC-derived HSPCs, erythrocytes, and NK cells to confirm the expression levels of HSPC markers at mRNA and protein levels. Colony forming unit (CFU) assay and Leishman stain were used to characterize differentiated cells in the myeloid lineage. Cytotoxicity assay and cytokine production assay were conducted to examine the functionality of differentiated NK cells in the lymphoid lineage.

Results: Our differentiation method demonstrates robust HSPC generation across various embryonic and induced PSC lines and culture conditions, highlighting its adaptability and reliability. Importantly, PSC-derived HSPCs exhibit functional versatility in differentiating into myeloid and lymphoid lineages, validated through colony forming assays, red blood cell and NK cell differentiations.

Conclusions: These findings suggest that HSA may be dispensable in HSPC differentiation and replacing it with synthetic polymers could mitigate lot-to-lot variation, reduce manufacturing costs, and expedite clinical applications.

Plain Language Summary:
Creating hematopoietic stem and progenitor cells (HSPCs) from pluripotent stem cells (PSCs) can help model diseases, test drugs, and develop treatments for blood disorders. However, generating these cells efficiently has been challenging. Usually, human serum albumin (HSA) is used in this process, but it's expensive. Our study introduces a new method that uses a caprolactam-based polymer instead of HSA. This new approach works well with different PSCs and culture conditions, demonstrating its adaptability. The HSPCs produced using this method can develop into various blood cell types, including erythrocytes and NK cells, suggesting that the caprolactam-based polymer can replace HSA. This substitution reduces costs and enhances the reliability of cell production.

Glycoengineered outer membrane vesicles from Escherichia coli as a platform for developing a vaccine against Streptococcus pyogenes

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Streptococcus pyogenes, also known as Group A *Streptococcus* (GAS), is a widespread human specific pathogen responsible for a wide range of infections such as sore throats and impetigo. GAS causes more than 500 million cases per annum, with diseases ranging from pharyngitis to life-threatening invasive infections, including streptococcal toxic syndrome, necrotizing fasciitis, septic arthritis, and meningitis. Due to the increase of antibiotic-resistant GAS infections, the World Health Organization has made development of a vaccine against GAS a priority. Group A carbohydrate (GAC) is a major virulence determinant and is conserved across all GAS strains. GAC consists of a polyrhamnose (pRha) backbone with an immunodominant N-acetylglucosamine (GlcNAc) side-chain. Due to the potential role of antibodies toward the GlcNAc side-chain in rheumatic fever, the polyRha backbone is a promising vaccine candidate as does not trigger cross-reactivity with human tissue. However, vaccines consisting solely of polysaccharides fail to generate immunological memory across all age groups. Outer membrane vesicles (OMVs) secreted by Gram-negative bacteria are an attractive vaccine technology. OMVs are spherical, secreted membranous structures generated from the outer membrane of the bacterium, and are composed of proteins, lipids, and lipopolysaccharide (LPS). Non-pathogenic *Escherichia coli* OMVs can be bioengineered to display heterologous glycans from other organisms as LPS on their surface. In this study, we have reconstituted the GAC/polyRha biosynthesis pathways in *E. coli*. We used multiple techniques for the physiochemical characterization of these vesicles before investigating their immunogenicity. We found that our glycoengineered OMVs induced a mixed anti-inflammatory and inflammatory immune responses *in vitro* and *in vivo*.

Development of an assay to perform high-throughput drug screen in HEK293 cells to improve AAV vector production

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Adeno-associated vector (AAV)-based gene therapy holds great promise for treating a range of human diseases. Despite the significant potential of AAV vectors in gene therapy, challenges persist in producing high-quality vectors on a large scale. Therefore, innovative strategies are needed to improve the AAV vector manufacturing process. Currently, AAV vectors are commonly produced using HEK293 cells, but during production, the activation of various cellular responses has been observed. The impact of these cellular responses on vector production and quality remains unknown. To explore this, we sought to perform a high-throughput drug screen in HEK293 cells, utilizing commercially available drug libraries that target antiviral, inflammatory, cell signaling, and checkpoint pathways to identify restriction factors that affect AAV quality and yield. To perform such a drug screen, first, we needed a high-throughput screening assay. Here, we report the development of a high-throughput drug screen assay that can be employed to identify small molecule compounds to improve AAV vector production in HEK293 cells. This 96-well plate assay uses an AAV-luciferase vector in adherent HEK293 cells, and the assay is reproducible, robust, and exhibits a high signal-to-noise ratio. Current studies are underway to identify compounds that improve AAV production using this high-throughput assay. Once chemical compounds are identified and their cellular targets in HEK293 are validated, we aim to develop a novel HEK293 producer cell line with knockouts of inhibitory cellular factors using CRISPR/Cas9. This novel HEK293 producer cell line may help to improve AAV vector quality and yield during manufacture.

Plain Language Summary:
AAV-based gene therapy holds a great promise for treating many human diseases. Despite the promise, challenges exist in producing high-quality AAV vectors on a large scale. HEK293 cells are commonly used for vector production, but the effect of the host factors on vector production and quality remains unexplored. Here, we developed a high-throughput drug screening assay to identify compounds that can enhance AAV vector production by inhibiting host restriction factors. The overall goal is to improve AAV quality and yield.

Identifying Immunodominant Major Urinary Proteins (MUPs) In Mouse Urine

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Objective: Mouse allergens trigger respiratory allergies in urban households and laboratory workers. Mouse allergen Mus m 1 consists of several major urinary proteins (MUPs) present in urine and pelt. The purpose of this study is to identify and quantify the immunodominant MUPs. Previously, we expressed two central (MUP7 and MUP10) and 3 peripheral (MUP4, MUP11 and MUP20) MUPs. To determine IgE reactivity to these rMUPs, we employed ImmunoCAP assays using clinical human sera.

Methods: We used ImmunoCAP allergens E72 (urine proteins) and E88 (epithelial proteins). Sera were preincubated with rMUPs, as well as mouse IgG and mouse albumin, and the reduction in IgE binding gives a relative concentration of sIgE to putative allergen. Fourteen sera were diluted to 25 kUA/L of sIgE and premixed with each allergen at final concentrations of 1.28×10^{-5} to 3.0×10^{-3} g/L. After initial experiments, extensive evaluations were done on MUP4, MUP7 and MUP20. Inhibition curves were calculated by linear regression of semilog inhibition data with at least 2 data points above and below 50% inhibition, and 50% inhibition (IC50) was estimated by linear interpolation for each combination.

Results: We observed no inhibition of binding with albumin or mouse IgG. All 5 recombinant MUPs inhibit the binding of sIgE to mouse sorbents. Using serum V9533 and sorbent E72, IC50 for MUP4 is 127.6g/L, MUP7 is 1.67g/L and MUP20 is 54.5g/L. Using V9533 and E88, IC50 for MUP4 is 14.9g/L, MUP7 is 0.0426g/L and MUP20 is 7.19g/L. Results with other sera were consistent with these results.

Conclusion: In this experiment, MUP7 (a central MUP) inhibits IgE at 1-2 logs lower concentrations than peripheral MUPs. In next step, we will be expanding our analyses and correlation of MUPs in environmental samples, wild mouse urines, and commercial allergen extracts by employing Parallel reaction monitoring Mass spectrometry (PRM-MS) approach.

Plain Language Summary:
Mouse allergens trigger respiratory allergies in urban households and laboratory workers. Mouse allergen Mus m 1 consists of several major urinary proteins (MUPs) present in urine and pelt. The purpose of this study is to identify and quantify the immunodominant MUPs. Previously, we expressed two central (MUP7 and MUP10) and 3 peripheral (MUP4, MUP11 and MUP20) MUPs. To determine IgE reactivity to these rMUPs, we employed ImmunoCAP assays using clinical human sera. We observed no inhibition of binding with albumin or mouse IgG. All 5 recombinant MUPs inhibit the binding of sIgE to mouse sorbents. Using serum V9533 and sorbent E72, IC50 for MUP4 is 127.6g/L, MUP7 is 1.67g/L and MUP20 is 54.5g/L. Using V9533 and E88, IC50 for MUP4 is 14.9g/L, MUP7 is 0.0426g/L and MUP20 is 7.19g/L. Results with other sera were consistent with these results. In this experiment, MUP7 (a central MUP) inhibits IgE at 1-2 logs lower concentrations than peripheral MUPs. We will be expanding our analyses and correlation of MUPs in environmental samples, wild mouse urines, and commercial allergen extracts by employing Parallel reaction monitoring Mass spectrometry (PRM-MS) approach. The current study will shed light on the complexity of immune response to mouse urine allergens, specifically in identifying the immunodominant allergens in mouse urine.

Investigation of the reversal potential of engineered Factor Xa agents in plasma treated with Factor Xa inhibitors.

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Background: Direct oral anticoagulants (DOACs) targeting Factor(F)Xa are crucial in prevention and treatment of thromboembolic disorders but can lead to anticoagulant-associated bleeding. The only approved reversal agent for FXa inhibitors, andexanet alfa, binds and temporarily sequesters DOACs, but requires high doses and associated with life threatening thrombosis. We previously reported the engineering and evaluation of novel FXa variants retaining enzymatic function in the presence of a DOAC (apixaban) and reducing apixaban-associated bleeding in mice. Here, we explore the potential of these FXa variants to reverse bleeding induced by other FXa DOACs.

Aims: Evaluate potential of two novel FXa variants to reverse low (0.1 μM) and high (2 μM) doses of DOACs that target FXa (apixaban, rivaroxaban and edoxaban) in normal pooled plasma (NPP) using the Thrombin Generation Assay (TGA). The reversal of DOAC bleeding by the FXa variants designed by use will be compared to a currently approved product (andexanet alfa).

Methods: FXa variants were computationally designed to retain enzymatic function in the presence of DOACs and reduce DOAC-associated bleeding. The procoagulant activity of FXa variants and andexanet alfa was evaluated in the TGA in NPP in the presence of FXa inhibitors. Samples were serially diluted and mixed with recalcified plasma, FXa inhibitors, phospholipid vesicles, Tissue Factor, and thrombin fluorogenic substrate. Thrombin Peak Height (TPH) from fluorescence curves was analyzed; TPH range for NPP was obtained without inhibitors and FXa variants.

Results: In TGA studies, FXa activity could be restored in a dose-dependent manner by either of the two FXa variants (RDR2_2 and HI_8) in the presence of FXa inhibitors, achieving reversal at notably lower doses than andexanet alfa.

Conclusion: Engineered FXa variants evade the effect of FXa inhibitors more effectively than the currently approved drug, andexanet alfa.

Plain Language Summary:
Direct oral anticoagulants (DOACs) targeting Factor Xa (FXa) that are used to prevent or treat thromboembolic disorders carry the risk of uncontrolled bleeding. Here, we present the design and evaluation of FXa variants which can be used to reduce DOAC-associated bleeding more efficiently than andexanet alfa which is the only specific DOACs reversal therapy currently approved.

Placenta Model for Investigating Viral Infection and Antibody Protection

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Objective: Human placenta has evolved to be a barrier to infection, but the mechanisms of this function are not fully elucidated. Using reporter-associated Zika virus (ZIKV), we aimed to characterize the susceptibility to infection of human placental cells. We also aimed to assess ZIKV infection in differentiated versus un-differentiated placental cells, and investigate molecular pathways involved in the differences we observed in infection levels.

Methods: Vero and human placental cell lines were infected with two reporter ZIKVs and infection levels were assessed with qRT-PCR and bioluminescence readout to show that infectivity was retained, and bioluminescence was reliably measurable. Forskolin-induced syncytialization of BeWo cells was used as a model of placental differentiation and markers of differentiation, chorionic gonadotropin and E-Cadherin, were assessed. Subsequently, the differentiated and un-differentiated cells were infected with reporter-ZIKV and infection levels measured. We also measured RNA and protein expression levels of putative ZIKV receptors, including FcRn, Axl, and Tim1, and a placental metalloprotease, ADAM12.

Results: We demonstrated that reporter-associated virus maintains infectivity using bioluminescence and qRT-PCR. We showed that Forskolin-differentiated BeWo cells are less susceptible to ZIKV infection. Differentiated BeWo cells exhibited lower Tim1 and FcRn mRNA levels and higher expression of metalloprotease ADAM12. Inhibition of metalloprotease function resulted in lower proteolysis of E-cadherin.

Conclusion: Our research demonstrated that reporter ZIKVs are suitable for use in research and product development assays. These experiments are necessary for preclinical analysis of potential ZIKV treatments and prophylaxis. Our research also provided new insights on the mechanisms underlying viral infections during pregnancy. The changes in expression of Tim1 could play a role in the observed decrease in viral susceptibility of differentiated BeWo cells. The increased expression of ADAM12 may contribute to functional decrease of this and putative viral receptors via the cleavage of extracellular domains that recognize and mediate viral entry.

Plain Language Summary:
We aim to understand the mechanisms used by the human placenta to prevent viral infection, as well as the methods used by viruses to establish infection across this barrier. Using reporter-associated virus, we demonstrated that differentiated placental cells are less susceptible to infection by Zika virus. Furthermore, we showed that the expression of metalloprotease and putative viral receptors changes due to placental cell differentiation. These findings may elucidate how virus susceptibility changes over the course of pregnancy.

Revealing the role of micro-RNAs as post-transcriptional regulators of ADAMTS13 through binding to its coding region and understanding the effects of synonymous mutations on ADAMTS13 functionality

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Background: The central premise for incorporating synonymous nucleotide variants (SNV) into genetic sequences for codon optimizing biopharmaceuticals and gene therapies is that these changes are benign alterations to the primary protein sequence. However, current studies show that even single synonymous variations may alter miRNA binding, mRNA expression, protein conformation and activity, which may lead to protein deficiency and disease manifestations. We reported a comprehensive series of in-silico analyses assessing the impact of sSNVs on A Disintegrin-like and Metalloprotease with Thrombospondin type-1 repeats, member-13 (ADAMTS13) and highlighted numerous variants that may affect protein functionality.
Deficiency of ADAMTS13 alters VWF thrombogenic potential and may cause microvascular thrombosis, such as thrombotic thrombocytopenic purpura (TTP).

Aim: To understand if sSNVs and codon optimization can affect the protein on transcriptional and translational levels and change ADAMTS13 properties *in-vitro*.

Methods: The binding of selected miRNAs to the ADAMTS13 coding sequence was validated in cellular systems using luciferase reporter vectors. MiRNAs were introduced into the cells by transient transfection of mimic miRNAs or through AAV-mediated delivery.

Results: Our results demonstrate that ADAMT13 synonymous variants disturb miRNA binding sites and significantly affect gene and protein expression in-vitro. Dual luciferase assay results revealed that Huh7 cells co-transfected with luciferase vectors that carry the ADAMTS13 variant sequence (c.972C>T) and miR-221 mimic sequence effects/decreases Luciferase signal compared to control RNAs. Studies in Huh7 cells co-transfected with ADAMTS13 expressing vectors (WT or variant c.972C>T) and miR221 revealed that miRNA221 differentially affects WT's and variant's gene and protein extracellular expressions compared to control. Finally, codon optimized ADAMTS13 variants are differentially affected on gene and proteins' intra- and extracellular- levels due to miRNA-221 overexpression (after AAV-miR221 transduction).

Conclusion: Our findings highlight the importance of considering sSNVs when assessing the complex effects of ADAMTS13 alleles and show the potential impact of sSNVs in modifying protein characteristics and disease severity.

Plain Language Summary:
Our results demonstrate that ADAMT13 synonymous variants disturb miRNA binding sites and significantly affect gene and protein expression in-vitro.

The evolutional codon context of coagulation factors: positional codon and codon pair usage biases in ADAMTS13, Von Willebrand Factor, Factor 8, and Factor 9

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Objective: To investigate positional variations of codon and codon-pair usage biases within the coding sequences of four blood clotting factors: *ADAMTS13*, *Von Willebrand factor*, *Factor 8*, and *Factor 9*. These genes were chosen for their different characteristics and important roles in human blood coagulation disorders. Understanding their biases and position-specific variations could be used to optimize translation and folding of their gene-encoded products.

Methods: We assessed sequence-position-specific codon and codon pair usage biases by aligning codons from mRNA homologs of human *ADAMTS13* (NM_139025.5), *Von Willebrand factor* (NM_000552.5), *Factor 8* (NM_000132.4), and *Factor 9* (NM_000133.4) using Discontiguous Megablast and MACSE. 157 sequences were analyzed for *ADAMTS13*, 148 for *F8*, 96 for *F9*, and 202 for *VWF*. Species ranged from *Even-toed Ungulates* to *Testudines*. A sliding-window divided the codon alignments to examine their biases (three or six nucleotides, respectively). Statistical significance was quantified with permutation tests modified for codon alignments.

Results: Our findings revealed positional variations in both codon and codon pair usage biases within each studied gene. Frequently observed codons and codon pairs tended to be the most biased positionally, with many sequence-position-specific, domain-independent deviations. Despite the poor correlation of codon and codon pair usage frequencies within sequences, they were closely associated positionally. These relationships were statistically significant and conserved in randomly sampled codon alignments.

Conclusions: This study provides new insights into the positional dynamics of codon and codon-pair usage biases within four critical coagulation genes. The distinct biases observed at different positions in coding sequences highlight the importance of considering positional effects in codon optimization strategies. These findings have significant implications for genetic engineering and synthetic biology, where tailored gene sequence optimization can enhance protein expression and functionality. Future research should focus on experimentally validating these predictions and developing positional bias models for more effective gene design.

Plain Language Summary:
This study investigates how codon and codon pair usage biases change depending on their position in four coagulation-related genes: *ADAMTS13*, *Von Willebrand factor*, *Factor 8*, and *Factor 9*. By analyzing these patterns, we identified significant variations at various positions within the genes. Understanding the factors influencing these positional biases can help improve gene engineering strategies for enhancing protein production and for developing novel genetic therapeutics. Future work will aim to validate these results experimentally.

CoCoPUTs, TissueCoCoPUTs and CancerCoCoPUTs: *Databases for codon, codon pair and dinucleotide usage information*

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Objective: Knowledge of up-to-date and accurate codon and codon-pair usage frequencies is essential for common biological techniques and a number of fields of study including evolutionary biology and drug design. Availability of codon usage frequency databases that are either outdated or limited in scope prompted us to develop HIVE-CUTS (High-performance Integrated Virtual Environment-Codon Usage Tables).

Methods: In partnership with CBER HIVE (High-PerformanceIntegratedVirtualEnvironment) at the FDA, we developed HIVE-CUTS(CodonUsageTables) comprising of CoCoPUTs, TissueCoCoPUTs and CancerCoCoPUTs containing codon, codon-pair and dinucleotide usage information at the level of genome, human tissue-specific transcriptome and primary tumor-specific transcriptome respectively. These data are calculated on HIVE's server and is accessible on HIVE's public portal (https://dnahive.fda.gov/dna.cgi?cmd=cuts_main).

Results: HIVE-CUTS is a publicly available database that includes codon usage tables for every organism with publicly available sequencing data and it is being routinely updated to keep up with the continuous flow of new data in GenBank and RefSeq. HIVE-CUTS was later updated to CoCoPUTs (Codon and Codon-pair Usage Tables) to include codon-pair, dinucleotide and junction-nucleotide usage frequency information. Compared to existing databases, the CoCoPUTs database is more comprehensive and provides several new functionalities, such as the ability to view and compare codon usage between individual organisms and across taxonomical clades, through graphical representation or through commonly used indices. Subsequently, databases containing similar information in healthy human tissues (TissueCoCoPUTs) and human cancer tissues (CancerCoCoPUTs) were developed. EmbryoCoCoPUTs and SARSCoCoPUTs with information related mouse embryonic tissue and SARS-CoV-2 virus respectively are currently in development.

Conclusions: Overall, the CoCoPUTs databases will aid in many fields of research including general and/or tissue-targeted gene optimization/therapy, viral vaccine development and genetic disease prediction tools.

Plain Language Summary:
The availability of accurate and up-to-date metrics of coding sequence usage for proteins of interest is crucial for common biological techniques and a number of fields of study including evolutionary biology, viral vaccine development and drug design. In partnership with CBER HIVE (High-Performance Integrated Virtual Environment) at the FDA, we developed HIVE-CUTS (Codon Usage Tables) comprising of CoCoPUTs, TissueCoCoPUTs and CancerCoCoPUTs containing coding sequence usage metrics including codon, codon-pair and dinucleotide usage information at the level of genome, human tissue-specific transcriptome and primary tumor-specific transcriptome respectively. These data are accessible on HIVE's public portal (https://dnahive.fda.gov/dna.cgi?cmd=cuts_main).

Heterogeneity in pathogenic brain sequestered CD8$^+$ T cells during experimental cerebral malaria revealed by single cell sequencing

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Brain sequestered CD8$^+$ T cells play a prominent role in the pathogenesis of experimental cerebral malaria (ECM) in mice and are found in the cerebrovasculature of young children with cerebral malaria. In mice, CD8$^+$ T cells accumulate in the brain during the effector phase of a *Plasmodium berghei* ANKA (*Pb-A*) infection and promote pathogenesis by inducing apoptosis of endothelial cells of the blood brain barrier. However, the molecular events associated with this CD8$^+$ T cell-mediated pathogenesis remain poorly understood. We performed single cell sequencing and bioinformatic analysis of brain sequestered CD8$^+$ T cells isolated from perfused tissue of *Pb-A* infected moribund and non-moribund mice and uninfected mice. We find that 42 genes associate with disease symptoms and 17 genes associate with *Pb-A* infection. Importantly, cluster analysis revealed that the brain sequestered CD8$^+$ T cells consists of two large clusters and ten small but distinct clusters indicating a large degree of heterogenicity in these cell populations during ECM. Furthermore, one of the large clusters is enriched in moribund mice and therefore associates with the symptoms of disease and the other large cluster preferentially expresses IFN-γ, a known biomarker of ECM pathogenesis. Lastly, we have created a transcriptional atlas of brain sequestered CD8$^+$ T cells by plotting the average expression and percentage of CD8$^+$ T cells that express various cytokines, chemokines, transcription factors, cell surface molecules such as checkpoint inhibitors, and other relevant molecules. The results of this study are being used to identify and test promising targets for adjunctive therapy to reduce the high mortality of human CM.

Plain Language Summary:
We performed single cell sequencing on pathogenic brain-sequestered leukocytes during experimental cerebral malaria in *Plasmodium berghei* ANKA infected mice. We measured differential expression in immune subsets that sequester within the brain in mice with experimental cerebral malaria compared to mice without cerebral malaria. We performed cluster analysis on brain-sequestered T cells and created an atlas of genes altered in pathogenic T cells during cerebral malaria. This research is being used to select novel targets for adjunctive therapy to lower the high mortality due to human cerebral malaria.

Development of isothermal and CRISPR-based diagnostics for the detection of *Babesia* parasites

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Objective: Human babesiosis is caused by infection of the red blood cells with *Babesia* species parasites and can result in severe disease and even death, especially in immunocompromised or asplenic individuals. In the USA there are ~2,000 cases of human babesiosis annually, primarily caused by *Babesia microti*, however, due to underdiagnosis the true number of infections has been estimated to be up to 10-fold higher. The number of cases of babesiosis is likely to continue increasing due to the expanding range of the ticks that transmit infection. Further, babesiosis can be transmitted by blood transfusion, although testing is now in place in endemic states. Diagnosis is currently based on the presence of parasites in a blood smear or by PCR. The objective of this study is to evaluate the utility of recombinase polymerase amplification (RPA) combined with Cas12 or Cas13-based CRISPR assays as simple but sensitive and specific point-of-care diagnostic tool for detecting Babesia nucleic acid.

Methods: We will optimize and compare the performance of RPA alone and two RPA-CRISPR based methods for the detection of *Babesia* spp. Optimization will include iterative primer, probe and guide design, as well as temperature and magnesium concentration. Initial optimization was performed with plasmid DNA and will further include *in vitro* cultured organisms and patient samples purified by various methods.

Results: The RPA assay was able to specifically detect two regions of the *Babesia* 18S rRNA from plasmid and not the human 18S negative control. We will present the sensitivity of the RPA, RPA-Cas12 and RPA-Cas13 assays for the detection of *Babesia* spp.

Conclusions: These methods offer the potential of a simple, rapid and scalable assay with the sensitivity and specificity of PCR and the ability to multiplex for the detection of other tickborne pathogens in the future.

Plain Language Summary:
Human babesiosis affects ~2,000 individuals annually in the USA, with actual cases potentially ten times higher. The disease is transmitted by ticks and blood transfusion, often undetected in early stage of infection. This study aims to evaluate new technologies for detecting pathogens, including RPA and Cas12/Cas13-based CRISPR assays. These technologies offer a simple and rapid method to detect multiple pathogens simultaneously while maintaining high sensitivity and specificity of more complex assays. We have demonstrated that our assays can specifically detect *Babesia* targeting highly conserved 18S rRNA, offering the ability to detect multiple tickborne pathogens simultaneously in the future.

Transmission of variant Creutzfeldt-Jakob disease by blood transfusion in macaques

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Background: Variant Creutzfeldt-Jakob disease (vCJD) and sporadic CJD (sCJD) are rare fatal diseases of the family of transmissible spongiform encephalopathies (TSEs) or prion diseases. vCJD is transmissible by blood transfusion (TTvCJD). There is no blood test for any form of human TSEs.
Objective: To demonstrate that blood from macaques experimentally infected with vCJD is transfusion-transmissible to naïve macaques and confirm appropriateness of the macaque vCJD model to reproduce TTvCJD in humans.

Methods: We transfused four adult macaques with approximately 100 ml each of blood from macaques clinical infected with vCJD. We monitored transfused macaques for clinical signs and collected blood and epithelial cells in nasal swab extracts every 3 months to detect abnormal prion protein, PrP^{TSE}, the biomarker of vCJD infection using in vitro PrP^{TSE} amplification assays. We also examined brain by histopathology and immunohistochemistry.

Results: Two macaques were euthanized for health reasons unrelated to vCJD 58- and 103-months post transfusion (mpt). These two macaques were vCJD negative by neuropathologic analysis of brain and in vitro PrP^{TSE} amplification assays.
Third macaque developed signs of neurological disease 103 mpt and was euthanized 4 months later. Brain analysis of this macaque confirmed vCJD infection which was also supported by detection of PrP^{TSE} in blood, post-mortem cerebral spinal fluid (CSF) and nasal swab extracts as early as 6 months before clinical onset.
Fourth macaque was asymptomatic and euthanized 117 mpt. In vitro PrP^{TSE} amplification assays of blood, nasal swab extracts and CSF were negative. Brain analysis is ongoing.

Conclusions: We demonstrated one transfusion-transmitted case of macaque vCJD. These results confirmed that blood from infected macaque can be used in assay validations as a surrogate of human blood. We also showed detection of PrP^{TSE} in nasal swab extracts that predicted infection.

Plain Language Summary:
Variant Creutzfeldt-Jakob disease (vCJD) is prion disease transmissible by blood transfusion. We transfused four macaques with blood from macaques infected with vCJD, monitored for clinical signs, and collected blood and nasal swabs. Post-mortem examination included immunohistology of brain and organs. Two macaques were euthanized for reasons unrelated to vCJD. Brain analysis was negative for vCJD in these animals. Third macaque developed neurological signs. Brain analysis confirmed vCJD diagnosis. Fourth macaque was euthanized asymptomatic. We demonstrated blood from macaques infected with vCJD is transmissible by transfusion. However, further investigation is needed to complete the study.

Double negative (CD4-CD8-) T cells persist in the gut of mice chronically infected with *Trypanosoma cruzi* and inhibit parasite growth in myoblasts *in vitro*

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Objective: Chagas disease (CD) is caused by the blood-borne parasite *Trypanosoma cruzi*, affecting 6-8 million individuals mostly in Latin America. Chronic CD involves progressive damage in main organs including the heart and gut often associated with the persistence of *T. cruzi* parasites. The gut is one of the main parasite reservoir tissues in mice models of chronic CD. Investigating the immune responses in the gut is critical to understand chronic pathogenesis and mechanisms of parasite persistence and could lead to improved therapies.

Methods: To that end, we collected gut tissues from *T. cruzi* Colombiana infected C57BL/6 mice during the acute (~30 days) and chronic phase (~100 days) of the disease. We isolated laminar propria immune cells from the colon using enzymatic and mechanical treatments and analyzed single cell suspensions by high parameter spectral flow cytometry targeting cell surface markers. Purified immune cells were also co-cultured with the *T. cruzi* infected C2C12 muscle cells *in vitro*.

Results: Flow cytometry analysis showed that double negative (CD4-CD8-) T cells (DNT cells) were increased > 2 folds in acutely infected tissues and remained significantly higher in chronically infected colons compared to controls. This pattern was not observed in other immune cells isolated from either whole blood, spleen, thymus, or bone marrow. Further, when *T. cruzi* infected C2C12 myoblasts were co-cultured with DNT cells isolated from infected mice spleen, parasite multiplication and release were inhibited.

Conclusions: These results suggest that DNT cells play a role in colon tissue immunity against *T. cruzi* infection and are capable of controlling parasite multiplication when in proximity to infected cells. Additional studies using DNT cells purified from chronically infected mice colon should further support the critical role of DNT cells in gut immunity and their possible contribution to parasite persistence and immune evasion in this chronic infection mouse model.

Plain Language Summary:
Little is known about the immune response in the gut during chronic Chagas disease. This tissue remains infected with *Trypanosoma cruzi* parasite causing this disease. We analyzed the immune cells isolated from the colon of infected mice and showed that a subset of T-lymphocytes (not expressing surface markers CD4 and CD8, called DNT cells) remained in high number (> 2 folds) during a chronic infection. They also inhibited the multiplication of parasites when added to infected cell cultures in vitro. These results suggest that DNT cells play a role in the gut immune response to *T. cruzi* infection.

Innovative Nylon Affinity Networks for Enhanced Bacterial Reduction in Stored Whole Blood and Blood Components

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Objectives: Ensuring safety in blood transfusions is crucial. Our research introduces a technique using ligands on nylon filaments to form affinity networks capable of capturing bacteria in plasma, platelets, and whole blood while preserving blood component integrity. We hypothesize that treating bacterially contaminated ex vivo whole blood and blood components with these affinity networks will significantly reduce the bacterial burden. Here, we tested this hypothesis in human plasma to develop proof-of-concept with two different bacteria.

Methods: Affinity networks were systematically dyed with Alcian blue and Reactive blue 21 using industry-level protocols. An unwoven nylon sheet format was selected to capture *Escherichia coli* and *Staphylococcus epidermidis* using agar plate cultures. Parameters for affinity net sterilization, wettability, mixing methods, spiked phosphate-buffered saline (PBS), and human plasma were tested.

Results: The incorporation rate of the affinity ligands in the network was high, with consistent inter-batch reproducibility. The affinity networks demonstrated bacteria reduction rates exceeding 90% for *E. coli* and 80% for *S. epidermidis* in both PBS and a 1:1 v/v mixture of human citrated plasma. These results were observed in 1 mL volumes, with ongoing evaluations for 50 mL volumes. Enhancements in the mixing of affinity networks with spiked plasma significantly improved capture efficiency. The highest performance was observed with the Alcian blue dye.

Conclusion: Affinity networks show great promise as a bacterial reduction method. The outstanding performance of the phthalocyanine dye warrants further research with other bacteria and blood components. These findings highlight the potential of affinity networks to enhance the safety of ex vivo stored blood components by effectively removing bacteria.

Plain Language Summary:
Innovative Nylon Affinity Networks for Enhanced Bacterial Reduction in Blood Transfusions Our research presents a novel method to enhance blood transfusion safety by utilizing nylon filaments treated with dyes (affinity networks) to capture bacteria in plasma, platelets, and whole blood. We used two dyes and tested the efficacy of these affinity networks in removing bacteria such as *Escherichia coli* and *Staphylococcus epidermidis* under various conditions. One affinity network consistently achieved over 90% reduction with *E. coli* and 80% with *S. epidermidis* in plasma. These findings indicate significant potential for improving transfusion safety by effectively removing bacteria from blood. Further research will explore various bacteria and blood products.

Siderophore Utilization in *Clostridioides difficile*

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Objective: *Clostridioides difficile* (*Cd*) is the leading cause of antibiotic associated diarrhea. During colonization, *Cd* must obtain essential nutrients for growth, including iron, which is used both by host cells and bacteria for many cell processes. Bacterial pathogens have evolved numerous mechanisms for acquiring iron, including small, high-affinity molecules called siderophores. Here, we first demonstrate that *Cd* can utilize a variety of siderophores as a sole iron source. Accordingly, two putative siderophore specific ABC transporters (FhuDBGC and YclNOPQ) are induced in iron depleted conditions. While many bacteria utilize siderophores produced by neighboring bacteria (xenosiderophores), some bacteria gain a competitive advantage by producing a local pool of siderophore. Although *Cd*630 does not encode siderophore biosynthesis genes, a bioinformatics analysis identified siderophore genes in approximately 5% of *Cd* strains. This work focuses on determining siderophore/transporter specificity and how siderophore production may correlate with disease severity and/or enhanced colonization.

Methods: We knocked out the genes predicted to be responsible for siderophore uptake and synthesis. We evaluated these mutants for siderophore utilization (growth in iron limited media, IDM) and siderophore production (Chrome Azurol S (CAS) assay).

Results: To assess the role of the transporter FhuDBGC in siderophore utilization, we made the *fhuDBGC* gene deletion in *Cd*630. This mutant was unable to utilize ferrichome efficiently in IDM, while complemented *fhuDBGC* restores ferrichrome utilization. It is unclear how *Cd* imports other siderophores and if YclNOPQ is involved. We knocked out the siderophore biosynthesis genes in strain VPI 10463. Compared to WT, this mutant reduces the rate that CAS dye changes color, suggesting siderophore production is altered.

Conclusions: Based on our observations, *Cd* utilizes siderophores as an iron source and FhuDBGC is responsible for ferrichrome uptake. Additionally, some *Cd* strains can produce a siderophore. Future work will examine the potential role of siderophore production and uptake on colonization.

Plain Language Summary:
Clostridioides difficile (*Cd*) is the leading cause of antibiotic associated diarrhea. During colonization *Cd* must obtain essential nutrients for growth, including iron, which is used both by host cells and bacteria for many cell processes. Bacterial pathogens have evolved numerous mechanisms for acquiring iron, including small molecules called siderophores that tightly bind iron. These siderophores are likely available to *Cd* from other commensals in the colon, but a subset of *Cd* strains produce a siderophore. We are currently working to determine if strains that produce a siderophore have an advantage during colonization and which transporters *Cd* uses to uptake siderophores.

High antibody response and resolution of parasitemia in the absence of $CD4^+$ T cells during blood stage *Plasmodium yoelii* malaria infection

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Objective: $CD4^+$ cells contribute to immunity against intraerythrocytic *Plasmodium* parasites by helping B cells generate an effective antibody response and by secreting cytokines that facilitate an effective cellular response. Utilizing C57BL/6 mice homozygous for *Cd4*^{tm1Mak} targeted mutation ($CD4^{-/-}$), we examined the requirement of $CD4^+$ T cells in parasite clearance and the generation of parasite specific antibodies.

Methods: C57Bl/6 and $CD4^{-/-}$ mice were infected with 1×10^6 *Plasmodium yoelii* (Py) infected red blood cells. On days 0, 4, 11, 17 and 35 post infection (PI), flow cytometric immune cell profiling of the spleen was conducted. Total IgG titers were measured via Immunofluorescence assay (IFA).

Results: $CD4^{-/-}$ mice had a compromised but effective ability to control Py malaria; $CD4^{-/-}$ mice had a delayed and higher peak parasitemia and a prolonged clearance of infection compared to WT mice. Analysis of the innate arm of immunity revealed expansion of macrophages and neutrophils but contraction of NK cells in the absence of CD4. Although there was no significant difference in the number of $CD19^+$ B cells, there was a three-fold expansion of $CD19^-CD138^+$ plasma cells (PC) on day 17 PI indicating the presence of a potent compensatory mechanism of antigen-specific expansion of PCs independent of $CD4^+$ T cells. Additionally, $CD4^{-/-}$ mice had comparable levels of *Py* specific IgG determined by IFA. One compensatory mechanism was significant expansion of $CXCR5^+PD1^+$ follicular $CD8^+$ T cells on days 11, 17 and 35 PI due to the absence of conventional follicular $CD4^+$ T cells in $CD4^{-/-}$ mice.

Conclusions: We present evidence of $CD4^+$ T cell independent mechanisms that control malaria infection. The identification of a novel immunological pathway that induces potent antibody responses in the absence of $CD4^+$ T cell help is under investigation and may lead to new strategies for the design of the next generation of malaria vaccines.

Plain Language Summary:
$CD4^+$ T cells provide help to B cells to generate an effective antibody response against invading pathogens. We provide for evidence for novel $CD4^+$ T cell independent immunological pathways that induced high antibody responses and protective immunity against the murine malaria, *Plasmodium yoelli*. $CD4^+$ T deficient mice that self-resolved their infection had antibody responses comparable to the wildtype mice but displayed an expansion of a unique $CD8^+$ T cell population, which may be compensating for the absence of $CD4^+$ T cells. These results have implications in designing malaria vaccines that induce superior immune responses by targeting novel immune cell populations.

Analyzing interactions of Influenza HA vaccine-elicited monoclonal antibodies using cryo-electron microscopy

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Objective: Influenza is responsible for significant hospitalizations and deaths across the globe, even in regions with available vaccines. Current vaccines are essentially strain-specific, and the immune response is often biased towards the variable head region of hemagglutinin (HA). We sought to define the epitopes of potent HA antibodies directed to the more conserved stem region and evaluate the potential for improving HA stem antigens to elicit a broader immune response.

Methods: We structurally assessed antibodies elicited during a phase I human clinical trial that targeted stem binding by using a stabilized stem on a nanoparticle as the immunogen. Using cryo-electron microscopy (cryo-EM), we determined the structure of mAb 1D01, a potently neutralizing stem-directed antibody, in complex with HA trimer and compared it to recently published structures from the same trial to evaluate the epitopes and form hypotheses for improving the next generation of antigens.

Results: Cryo-EM analysis showed the unique binding of 1D01 to the HA stem below the central epitope, recognized by more prominent classes of mAbs. The structure of 1D01 revealed substantial overlap with the epitope of a less potent mAb, 1B06, but a distinct binding orientation. Our analysis showed the buried surface area of 1D01 to be moderately higher than 1B06. However, docking 1D01 and 1B06 to an HA trimer on a virion membrane showed drastically different approach angles with potential hindrance of 1B06 caused by the viral membrane, with the FC region of the Fab only ~5-9 Å from the membrane surface.

Conclusions: The 1D01-HA structure allowed us to determine fine details in the immune response and compare the mAb to other antibodies isolated from vaccinated donors to identify features that enable 1D01's potent neutralization. The structure suggests potential adjustments to the antigen to block regions that elicit less effective antibodies and focus the immune response to where the most potent or broad stem antibodies are binding.

Plain Language Summary:
Atomic structures of antibodies, isolated from donors vaccinated against influenza virus, revealed details related to their specific epitopes and neutralizing potential. Our investigation used structural analysis to show that the potent human antibody, 1D01, is less sterically hindered than other antibodies binding the same region on the influenza HA stem near the viral membrane surface based primarily on its angle of approach. Understanding the details of antibodies elicited from vaccination informs the effectiveness of current immunogens and enables us to improve the next generation of candidate antigens.

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Probing the minimum required sequence constraints for stable incorporation of chimeric segments into rotaviruses

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Objective: Rotaviruses present an 11 segment, double-stranded RNA genome that has been adapted into a plasmid-based reverse-genetics system. Of interest is the potential to modify these segments to generate chimeric viruses as novel vaccine models. However, chimeric segments exceeding wild-type length degrade over successive passages, highlighting the need for improved cloning strategies. This work seeks to identify the minimum required viral sequence needed for the stable incorporation of foreign genes into permissible segments.

Methods: BHK-T7 cells were transfected with SA11 rotavirus plasmids that tested the following modifications to the NSP1 segment: enhanced green fluorescent protein (eGFP) inserted 81 nt into the open reading frame (ORF) (NSP1gfp); excision of the viral ORF sequence 3' of the eGFP insert (ouNSP1gfp); the eGFP sequence flanked by the NSP1 untranslated regions (UTRs) (uuNSP1gfp); the absence of NSP1 (ΔNSP1). Rescued rotaviruses were passaged up to 10 times and analyzed for infectious viral titers, fluorescence, and chimeric segment integrity.

Results: Rotaviruses containing uuNSP1gfp or ΔNSP1 segments could not be rescued, suggesting that all segments and some percentage of the NSP1 5' ORF are required for mature virions. NSP1gfp rotaviruses were passaged up to 10 times with high viral titers but a marked degradation of the chimeric NSP1 segment was observed in the latter passages, ablating eGFP expression. The truncated ouNSP1gfp segment preserved segment stability and fluorescence, with minimal signs of insert degradation in the final passages. Systematic truncations made to the remaining 5' ORF sequence of ouNSP1gfp have further demonstrated that as little as 27 nt of the original ORF sequences allows for robust eGFP incorporation and expression across 10 passages.

Conclusions: These results suggest that only a small initial region of the NSP1 ORF is required for self-recognition, encapsidation, and virion maturation, allowing for a near-total replacement of the remaining sequence with an outside gene.

Plain Language Summary:
The adaptation of the rotavirus segmented genome into a plasmid-based reverse-genetics system has garnered interest for the potential of developing chimeric viruses as novel vaccine candidates. However, segments with long insert sequences degrade across serial passages, necessitating more nuanced cloning strategies. This work seeks to identify the minimum required viral sequences needed for segment recognition and encapsidation via systematic truncations to the NSP1 segment with an Enhanced Green Fluorescent Protein insert. Results indicate that only a small initial region of the NSP1 sequence is needed for segment recognition and virion maturation, allowing for near-total replacement with a foreign gene.

Highly Variable Antigenic Site Located at the Apex of GII.4 Norovirus Capsid Protein Can Induce Cross-reactive Neutralizing Antibodies

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Objective: The predominant GII.4 human norovirus genotype is responsible for most norovirus outbreaks worldwide. The extreme antigenic and genetic diversity and incomplete understanding of humoral responses have made the development of a vaccine challenging. Humoral responses target the five major variable antigenic sites, site A being immunodominant. The factors influencing cross-reactive responses are poorly understood but gaining this knowledge may aid vaccine development.

Methods: We conducted bioinformatic analyses with amino acid patterns of >3000 GII.4 viruses circulating over the last 25 years. We then tested the cross-reactivity potential of mouse monoclonal and polyclonal antibodies mapping to site A. Mouse polyclonal responses were tested with a histo-blood group antigen (HBGA)-blocking antigen competition assay (HACA), which allows us to delineate observed blocking epitopes. Finally, we used structural information and mAb binding patterns to separate the site A residues into three clusters (I-III). We then designed mutant, cluster-swapped virus-like particles (VLPs) for use in binding assays to determine whether the antibody cross-reactivity is related to single or multiple epitopes.

Results: Large scale bioinformatics analyses demonstrated antigenic site A is the most variable antigenic site. Cross-reactive responses at the monoclonal and polyclonal level with the use of HACA attributed to antigenic site A demonstrated to be extensive and virus dependent. While investigating the epitopes that elicited cross-reactive antibodies, we found that most cross-reacting mAbs bind to different epitopes and such epitopes present varying levels of cross-reactivity.

Conclusions: These findings demonstrate the complexity of the antibody:virus interaction and that antigenic sites thought to be extremely variable are still able to induce cross-reactive responses. This is relevant for norovirus vaccine development as epitopes described to be conserved are poorly immunogenic. While further studies are required to better understand how antibodies can overcome antigenic diversification, this information provides new information that could guide norovirus vaccine design.

Plain Language Summary:
GII.4 human norovirus has extreme antigenic diversity and is responsible for the majority of outbreaks worldwide. With the absence of a vaccine, humoral responses is the best protection available but our understanding of it is incomplete. Bioinformatics analyses described antigenic site A to be the most variable antigenic site and the mutational patterns correlate strongest with variant fluctuation patterns. Monoclonal and polyclonal responses depicted varying degrees of cross-reactivity with multiple epitopes that are affected differentially by mutations elicited from antigenic site A. Understanding antigenic variability and the effects on humoral responses may aid vaccine development.

Development of Specific and Sensitive Devices for Differential Diagnosis of Dengue, West Nile and Zika Viruses

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Objective: Dengue (DENV), West Nile (WNV) and Zika (ZIKV) viruses, are emergent flaviviruses causing global outbreaks. Accurate diagnosis of these viruses presents significant challenges yet is critical to assist medical directives due to the disparate manifestations of disease in symptomatic infections (~20%). DENV, WNV and ZIKV are primarily transmitted through mosquito bites, but also through blood transfusion and organ transplant. Uniquely, ZIKV transmission can occur transplacentally from a pregnant mother to a fetus, and through sexual intercourse. DENV, WNV and ZIKV mostly cause indistinguishable mild flu-like diseases that can quickly progress to severe cases unique for each virus making early and accurate diagnosis essential. DENV can cause hemorrhagic fever, while WNV can lead to neuroinvasive diseases. ZIKV infection in pregnant women is critical to identify as fetuses are at risk for miscarriage and birth defects (i.e., microcephaly, mental retardation, and learning disabilities). ZIKV can also cause Gillian Barre syndrome in adults. There are no specific treatments or effective vaccines for these viruses. Existing diagnostic tools for DENV, WNV and ZIKV are limited (e.g., nucleic acid amplification tests due to short-term viremia and serological assays due to antibody cross-reactivity among DENV, WNV and ZIKV).

Methods: We used a peptide microarray to identify unique flavivirus-specific (DENV, WNV and ZIKV) epitopes that do not cross-react, allowing accurate serological differential diagnosis. In this work, a bead-based fluorometric peptide immunoassay analyzed using flow cytometry was employed to allow discrimination of flaviviral-specific antibodies.

Results: Using a fluorometric peptide bead immunoassay, we identified unique ZIKV peptides that do not show any cross-reactivity between DENV and ZIKV, but some cross-reactivity has been observed between ZIKV and WNV.

Conclusions: Completion of this work will allow for differentiation between infections and eliminate misdiagnosis resulting from IgG cross-reactivity and interference in IgM assays, which are observed between DENV, WNV and ZIKV.

Plain Language Summary:
Dengue (DENV), West Nile (WNV), and Zika (ZIKV) are related mosquito-spread viruses. Symptoms develop in 20% of infections with these viruses, being mostly mild and indistinguishable. However, they can progress to differing severe diseases, requiring accurate diagnoses to direct medical care. ZIKV is associated with birth defects in pregnant women requiring prenatal monitoring. DENV and WNV can cause hemorrhagic fever and encephalitis, respectively, leading to hospitalization. Current diagnostic tests use antibodies against these viruses but are unable to distinguish between them. Here, we identified proteins specific for theses viruses that can be potentially used for accurate differential diagnosis among them.

Comparison and characterization of myeloid cell line models for *Anaplasma phagocytophilum* infection

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Objective: *Anaplasma phagocytophilum* (*Ap*) is an obligate intracellular bacterium that is transmitted via *Ixodes* tick species to cause granulocytic anaplasmosis in humans and domestic animals. In humans, *Ap* infects neutrophils, endothelial cells and bone marrow (BM) progenitors of myeloid origin. Therefore, *in vitro* investigation of *Ap*-induced cellular subversion mechanisms have been predominantly studied in HL60 neutrophil-like cell line. However, genome wide manipulation and identification of host factors involved in *Ap* infection require studies in additional tractable cell models that model wider cellular tropism of *Ap* infection. The main aim of the current study is to identify whether myeloid cell lines U937, K562 and THP1 can sufficiently support *Ap* infection and therefore, might model *Ap* infection of neutrophils or BM myeloid progenitors.

Methods: We infected HL60, K562, U937 and THP1 cell lines with cell-free *Ap* to test their ability to support *Ap* replication. Cell-free *Ap* stock was generated by syringe lysis of *Ap*-infected HL60 cells. Post infection, cells were serially harvested on 3-, 5- and 7-days post infection. Cells were spun onto microscope slides and Hema-3 stained for enumerating *Ap*-vacuole (ApV) containing cells. Cellular DNA was isolated for the absolute quantification of *Ap* genome copies per cell using qPCR.

Results and Conclusions: We identified K562, a chronic myeloid leukemia-derived cell line, to support *Ap* propagation. Furthermore, as described for HL60 cells, *Ap* entry in K562 cells is sialic acid dependent. K562 is an erythroleukemia cell line that can undergo spontaneous differentiation into early precursors of the monocytic, granulocytic, and erythroid series and therefore, may serve as an additional model to study *Ap*-infection of myeloid progenitors. HL60 as a positive control also supported *Ap* propagation where as U937 and THP1 cell lines failed to support *Ap* propag

Plain Language Summary:
Anaplasma phagocytophilum (*Ap*) causes granulocytic anaplasmosis in humans and domestic animals. *Ap* infection of neutrophils in humans is challenging to study *ex vivo*, due to their short *ex vivo* lifespan and inability to manipulate its genome or gene programs by transfection and transduction procedures. Therefore, there is a need for additional cell types that model *Ap*-infection of neutrophils and bone marrow progenitors. We identified K562 cell line to support *Ap* propagation and therefore, can serve as a tractable cell type to model wider cellular tropism of *Ap* in human.

Antibody Cross-Reactivity Among Dengue Virus (DENV), West Nile Virus (WNV) and Zika Virus (ZIKV) in Asymptomatic Infections

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Objective: Global epidemics of flaviviruses cause over 400 million infections annually leading to major public health concerns. Flaviviruses are transmitted by mosquitos and/or ticks, and outbreaks of Dengue (DENV), West Nile (WNV), and Zika (ZIKV) viruses are on the rise. Most (80%) DENV, WNV and ZIKV infections are asymptomatic, and the majority of disease cases are mild, flu-like, self-limiting, and indistinguishable among them. However, they can quickly progress to severe distinct cases for each virus that have serious long-term consequences, including fatality. DENV fever may progress to hemorrhagic fever and WNV fever to encephalitis. ZIKV leads to Guillain-Barré Syndrome, and during pregnancy can cause miscarriage or birth defects even in asymptomatic mothers. There are no specific therapeutics or effective vaccines for these viruses. Hospitalization is needed for supportive care and survival in severe cases of DENV and WNV. Therefore, differential diagnosis is critical to guide medical directives. Although nucleic acid testing (NAT) can accurately identify the virus, it is restricted by short viremia (7-days of symptom onset). Serological tests can result in misdiagnosis due to the high degree of antibody cross-reactivity mainly between DENV and ZIKV, but cross-reactivity of WNV with DENV and ZIKV has not been established. Serological assays are largely used for diagnostics and in vaccine development to evaluate immune responses.

Methods: Commercial ELISAs were used to evaluate antibody cross-reactivity between DENV, WNV and ZIKV in 382 blood donor samples that tested NAT-positive for DENV (n= 88), WNV (n=179) and ZIKV (n=115).

Results and Conclusions: ZIKV cross-reactivity in asymptomatic infections was 17% (15 /88) with DENV and 4.0% (7/179) with WNV in samples collected prior to the U.S. ZIKV outbreak. WNVNAT-positive samples (n=68) collected prior to the 2009-2012 U.S. DENV outbreak, showed 51.5% (35/68) reactivity for DENV and 3.0% (2/68) for ZIKV documenting WNV cross-reactivity with DENV and ZIKV.

Plain Language Summary:
Dengue (DENV), West Nile (WNV), and Zika (ZIKV) viruses are transmitted to humans by mosquitos, with 80% of infections being asymptomatic. Disease is commonly flu-like and indistinguishable but can become severe with different clinical manifestations. DENV leads to hemorrhagic fever and WNV to encephalitis, both requiring hospitalization and differential diagnostics for medical care. ZIKV results in Guillain Bare syndrome, and in pregnant women, leads to miscarriage and neurological birth defects. Serological tests for diagnosis detect antibodies produced after infection and cross-reactivity is a setback for accurate diagnosis. Here, we evaluated the level of cross-reactivity among these viruses in asymptomatic infections.

Detection of transfusion-relevant bacteria spiked in human plasma using real-time polymerase chain reaction

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Background: Platelet units are currently screened for bacterial contamination using culture-based methods. These tests are sensitive and effective but more rapid methods applicable to platelets and whole blood are desirable. In this project, we focused on nucleic acid testing (NAT) of bacteria in plasma. In the future, we will expand the investigation to include whole blood.
Objective: To develop a rapid and sensitive NAT-assay to detect bacteria in blood components.

Methods: A total of eight primer sets, targeting conserved regions of the bacterial 16srRNA gene, were designed from the alignment of fourteen bacterial strains from the WHO international repository of platelet-relevant bacteria reference strains (WHO/BS/2015.2269). Bacterial DNA was extracted from seven selected bacterial strains and amplified using polymerase chain reaction (PCR). PCR product quality was assessed, and PCR conditions were optimized.
A pellet of 10^8 CFU from seven strains of transfusion- relevant bacteria was prepared and spiked, with a 10-fold serial dilution, into human plasma. Following the extraction of microbial DNA from spiked plasma, we used the two best primer sets and real-time PCR to detect the presence of bacteria.

Results: Primer standardization steps reduced the number of primers from eight to two. Real-time PCR conditions were optimized based on primer melting temperature (Tm), primer and microbial DNA concentrations, and the melt curve analysis. Early results showed the limit of detection (LOD) was 10 to 10^4 colony forming units [CFU]/mL and 10^2 to 10^4 for Gram-negative and Gram-positive bacteria, respectively.

Conclusions: Real-time PCR could serve as a rapid alternative method to conventional culture methods to screen bacterial contamination of blood components. To improve the sensitivity, we are investigating new methods to concentrate bacteria prior to detection.

Plain Language Summary:
Platelet units are currently screened for bacterial contamination using culture-based methods. These tests are sensitive and effective but more rapid methods applicable to platelets and whole blood are desirable. Our goal is to develop a nucleic acid amplification technique (NAT)-based assay to detect very small amounts of bacterial DNA in blood components. Early results from the assay showed that for some bacteria the minimum number of organisms that can be detected is 10 colony forming units [CFU]/ml. Overall, a NAT-based assay could serve as a rapid alternative method to conventional culture methods to screen bacterial contamination of blood components.

Unbiassed metagenomic exploration of Transfusion-Transmitted infections with Nanopore sequencing

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Objectives: 1. To evaluate the capability of nanopore metagenomic sequencing in detecting and characterizing novel and emerging microbial pathogens in blood donor samples. 2. To determine analytical sensitivity and Limit of detection of nanopore metagenomic sequencing using representative transfusion transmitted infections.

Methods: We evaluated plasma samples from individuals with natural infections of HBV, HCV, HIV, and WNV. Additionally, we determined the limit of detection (LoD) and precision of our metagenomic sequencing approach using representative viruses. Briefly, sample preparation and analysis process include DNA/RNA extraction, cDNA synthesis, barcoded library preparation for Nanopore sequencing, followed by use of core bioinformatics tools, algorithms, and microbial databases were employed for data analysis. Targeted sequence analysis was conducted using CLC Genomic Workbench v21.0.

Results: This study uncovered a diverse microbial landscape, with a majority of reads being host or run control, leaving about 3% for virus-specific analysis. WNV, HIV, and HCV were identified, and modified sample preparation enabled HBV detection. High viral loads provided extensive genome coverage, while low viral loads yielded limited hits. Notable findings included HCV genotype 4a, HIV genotype B, and high similarity in WNV sequences to Lineage 1A. Co-infections such as human pegivirus 1 (HPgV-1) in HCV and Torque Teno Virus (TTV) in HBV cases were also observed. The virus panel showed hits at 10^3 copies, but consistent hits with full genome coverage were seen at 10^4 copies. These results demonstrate the effectiveness of the methods in detecting and characterizing various viruses and co-infections.

Conclusions: The application of metagenomic characterization studies for agnostic viral detection opens a new opportunity towards identification of both known and unknown viruses for blood safety. The LoD for nanopore metagenomic sequencing approach is 10^4 copies/ml for the representative viruses tested. This approach enhances public health security by offering a comprehensive method for pathogen detection.
Disclaimer: My contributions are an informal communication and represent my own best judgement. These comments do not bind or obligate FDA.

Plain Language Summary:
In recent years blood safety has been dramatically improved but emergence of infectious agents that may be transmitted by blood products remains a challenge. Current assays used in blood establishments for pathogen detection are target specific, and ineffective for unknown pathogens. Pathogen reduction technologies are promising but not equally effective for all pathogens. Metagenomic sequencing enables unbiassed characterization of infectious disease agents. We developed a metagenomic nanopore sequencing approach to characterize pathogens transmitted through blood and blood products. Our goal is to apply this agnostic approach to detect pathogens before an outbreak and mitigate risk of transfusion transmitted infections.

A Machine Learning Driven Investigation of the Association between HLA and Asymptomatic SARS-CoV-2 Infection

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SARS-CoV-2 disease severity and symptoms can vary drastically within infected patients. Even though most studies investigating COVID-19 have focused on patients with severe form of the disease, investigations of asymptomatic infection have provided new insights into patient-specific immunological features that protect patients from COVID-19 symptoms. A recent study has shown an association between common leukocyte antigen (HLA) alleles such HLA-B*15:01 and asymptomatic COVID-19 infections [1]. Here we utilize machine learning in conjunction with explainable AI (XAI) to identify alleles in five HLA loci that can be either "protective" or "at-risk" for symptomatic COVID-19. Data from the public online HLA-COVID database (composed of 3238 samples) was used for training and validation of multiple ML classification models to identify the top performing model, which was chosen for further processing with XAI via SHAP (SHapley Additive exPlanations) to identify the "protective" and "symptomatic" HLA alleles. We determined that HLA-B *07:02, *40:01, and *15:01 are associated with asymptomatic disease, i.e., are protective and HLA-B* 08*01, 39*06, and 51*01 are associated with symptomatic disease i.e., at risk.

1. Augusto, D.G., Murdolo, L.D., Chatzileontiadou, D.S.M. et al. A common allele of HLA is associated with asymptomatic SARS-CoV-2 infection. Nature 620, 128–136 (2023).

Plain Language Summary:
SARS-CoV-2 disease severity and symptoms can vary drastically within infected patients. Even though most studies investigating COVID-19 have focused on patients with severe form of the disease, investigations of asymptomatic infection have provided new insights into patient-specific immunological features that protect patients from COVID-19 symptoms. A recent study has shown an association between common leukocyte antigen (HLA) alleles such HLA-B*15:01 and asymptomatic COVID-19 infections. Here we utilize machine learning in conjunction with explainable AI (XAI) to identify alleles in five HLA loci that can be either "protective" or "at-risk" for symptomatic COVID-19.

Understanding the host regulatory circuits behind host defense against flavivirus infection

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Background: Rising global temperatures are boosting vector (ticks, mosquitoes) populations, leading to more vector-borne flavivirus cases worldwide. It is well documented that human bodily fluids, tissues, and cells can transmit flaviviruses. Flaviviruses such as Zika (ZIKV) and Dengue (DENV) virus can remain in infected tissues for many months even when the individuals are no longer viremic. Herein, we investigate the molecular basis of high tissue tropism exhibited by flaviviruses.

Methods: Vero (monkey) and SK-N-SH (human) cells exhibit cytopathy in response to ZIKV, but not DENV, infection. Cells were infected with ZIKV-MR766 and DENV3 strains at an MOI of 1. Post-infection, cells were collected at various time points to investigate transcriptomic and protein level changes. RNA-seq was performed on the NovaSeq 6000, and differential gene expression was analyzed using DESeq2. Normalized RNA-seq data was visualized with IGV.

Results: Pathway analysis revealed differential expression of RNA processing factors following flavivirus infection. Transcript heatmaps indicated that genes involved in nonsense-mediated decay (NMD), RNA degradation, and the nuclear pore complex (NPC) pathways are upregulated in a time-dependent manner. Notably, our protein study found that ZIKV degrades specific NMD factors in host cells, a mechanism not observed with DENV. These findings demonstrate that flaviviruses employ a multifaceted strategy to hijack host defenses, impacting both transcript and protein levels.

Conclusions: We believe ZIKV, but not DENV, drives host cell cytopathy through targeted protein degradation. We've identified potential biomarkers of flavivirus infection and plan to test them via qRT-PCR on donor PBMCs infected with flaviviruses.

Plain Language Summary:
Flaviviruses like Zika and Dengue can persist in tissues long after viremia has resolved. Using next-generation sequencing, we compared samples from uninfected cells, short-term infections, and long-term infections of Zika and Dengue in monkey and human cell lines. We identified host regulatory circuits that are deregulated both at transcript and protein level upon flavivirus infection. We plan to test certain transcripts associated with these circuits as biomarkers for flavivirus infection in future studies. This study highlights the multifaceted approach flaviviruses use to hijack and maintain control over the host cell's machinery for replication.

Single-cell transcriptomics reveals the immune landscape of the mouse colon during chronic *Trypanosoma cruzi* infection

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Objective: Chagas disease (CD), caused by the blood-borne protozoan *Trypanosoma cruzi* (*T. cruzi*), affects nearly 6 million people worldwide. An estimated 300,000 infected individuals live in the United States, where transmission occurs mainly by local triatomine bugs and from mother-to-baby. Chronic CD can lead to life-threatening cardiac and gastrointestinal complications. Using bioluminescent parasites and ex-vivo imaging, we have shown that the colon is a major site of *T. cruzi* persistence in a chronically infected mice disease model. However, little is known about gastrointestinal CD and how the parasite evades the local host immune response to establish a lifelong infection. This study aims to evaluate the immune cell landscape of the chronically infected mouse colon using a single cell transcriptomic approach.

Methods: The large intestines of naïve and *T. cruzi* Colombiana chronically infected C57BL/6 mice were collected 110 days post-infection. Colon tissues were enzymatically and mechanically dissociated into single-cell suspensions. We performed single-cell separation and library preparation using the microwell-based BD Rhapsody platform combined with the whole transcriptome analysis kit.

Results: Single cell RNA sequencing analysis identified 18 cell clusters that were assigned to specific cell types by comparing highly expressed genes with canonical cell type-specific markers from online databases. We found that immune cells such as CD4+ and CD8+ T cells, NK cells, B cells and macrophages, were recruited to the colon of chronically infected mice. Ingenuity Pathway Analysis of differentially expressed genes predicted the activation of canonical pathways associated with the mouse immune response to *T. cruzi* such as S100 protein family, pathogen induced cytokine storm and phagosome formation in macrophages, T-cytotoxic and T-helper cells.

Conclusions: Our results revealed the cellular heterogeneity and immune cell enrichment of the mouse colon during chronic *T. cruzi* infection and identified several immune cell-related genes and pathways that could possibly influence long-term parasite persistence.

Plain Language Summary:
Trypanosoma cruzi is a blood-borne parasite that causes chronic Chagas gastrointestinal disease. The parasite infects the colon and can persist life-long in the host triggering tissue damage. To investigate defense and persistence mechanisms, we studied the immune cell populations isolated from the colon of infected mice. Using methodologies to isolate and analyze sequence information from individual cells, we found significantly more T cells, NK cells, B cells and macrophages in infected mice compared to non-infected animals. We also determined their gene expression patterns. Our results suggest that these immune cells are involved in the gut tissue response to *T. cruzi*.

Investigation of novel bacteriophage resistance mechanisms in vancomycin-resistant Enterococcus (VRE)

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Antimicrobial resistance (AMR) is a major health issue globally. Multidrug resistant organisms (MRDOs), including vancomycin-resistant *Enterococcus* (VRE), are of particular concern. Although commensal *Enterococcus* species colonize the gastrointestinal tract without causing disease, *E. faecalis and E. faecium* can spread to distal sites, including the urinary tract, and cause severe infection. Due to its antibiotic resistance, there are limited treatment options for those infected with VRE. Bacteriophage (phage) therapy is a potential alternative; however, it remains experimental with no products receiving FDA approval to date. Further experimentation is required to fully understand phage biology, including mechanisms responsible for bacterial resistance against phage and methods developed to overcome this challenge.

Objective: We aim to identify the genetic determinants of VRE resistance to phage killing through *in vitro* and *in vivo* phage infection experiments using transposon mutagenesis and transposon sequencing. We hypothesize that we will identify novel mechanisms of phage resistance by *E. faecium and E. faecalis*, which can help inform the future development of phage products.

Methods: Transposon libraries were developed using a plasmid-based system with a nisin-inducible transposase and mariner transposon. Subsequent experiments include transposon sequencing of these libraries before and after *in vitro* phage infection to identify isolates resistant to phage and to use these data to design and test an "optimal" phage cocktail with the goal of preventing the emergence of resistant VRE mutants.

Results: Transposon libraries in *E. faecium* P3-1 and UAA714 are in progress using pGPA1 and transposon libraries in *E. faecalis* VRE27 and OG1RF are in progress using a newly designed one plasmid system based on pCJK55 and pCJK72.

Conclusions: With the design of a phage cocktail to combat phage resistance by VRE, this work will increase our understanding of the advantages and limitations of phage therapy, which may reduce the burden of AMR infections globally.

Plain Language Summary:
Antibiotic resistance is a major health concern globally. Some bacteria are resistant to multiple antibiotics, such as vancomycin-resistant Enterococcus (VRE), and there is limited treatment available. Bacteriophage (phage) therapy, one in which phages are used to kill bacteria, is a possible alternative, but we need to understand how bacteria resist killing by phages. We aim to use new methods in VRE called transposon mutagenesis and transposon sequencing to identify new ways that VRE can resist phage killing using two different species E. faecium and E. faecalis. These discoveries will inform effective phage therapy to combat phage resistance by VRE.

Host transcriptional responses against hepatitis E virus infection in gerbils

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Objective: Hepatitis E virus (HEV) causes acute and chronic hepatitis E in immunocompetent and immunosuppressed humans respectively. HEV genotype 3 (gt3) is predominant in Europe and North America. HEV induced pathogenesis is mostly host driven. The objective of this study is to characterize the differentially expressed host genes in response to HEV gt3 infection in immunocompetent and immunosuppressed gerbils.

Methods: Adult male gerbils with or without immunosuppression were mock- or HEV-infected. Immunosuppression was achieved by subcutaneous implantation of controlled release tacrolimus pellets at ~5 mg/kg. Two weeks later, gerbils were inoculated intraperitoneally with HEV gt3 or PBS. Viral RNA and antigen were monitored in serum by RT-qPCR and ELISA. Tissue viral load was assessed by RT-qPCR. Liver disease was assessed by serum alanine transaminase (ALT) activity. Bulk mRNA sequencing and analysis was performed on liver tissues. The host gene expression in liver tissue was confirmed by RT-qPCR and examined by in situ hybridization on liver tissue sections.

Results and Conclusions: HEV RNAemia and antigenemia peaked at 2 weeks post-infection (wpi) in immunocompetent gerbils but they were found persistently at high levels in immunosuppressed gerbils until 6 weeks. Increased serum ALT was detected in some immunocompetent gerbils at peak viremia. A large set of differentially expressed genes (DEGs) were identified in HEV-infected livers under immunocompetent conditions compared with those under immunosuppressive conditions at 2 wpi. Several interferon-stimulated genes (ISGs) were found mostly upregulated. The mRNA levels of selected ISGs (Ifit1, Irf7, Rsad2, Gbps) were found increased in liver tissue infected with HEV. The profibrogenic host factor Chi3L1 was upregulated in HEV-infected livers and was detected by in situ hybridization in clusters of bystander cells adjacent to HEV infected cells. Overall, our results suggest a robust host transcriptional response against HEV infection in immunocompetent gerbils, but not immunosuppressed gerbils.

Plain Language Summary:
Hepatitis E virus (HEV) causes acute and chronic liver disease in humans. Mechanisms of disease during HEV infection are difficult to study in humans. Gerbils are promising model to study HEV induced liver disease. In our study, gerbils showed characteristics of HEV infection seen in human patients and robust gene expression only in the presence of a functional immune system. There are no FDA-approved diagnostic tests, vaccines, or antiviral therapies for HEV. The gerbil model will be a useful tool for identifying host factors contributing to hepatitis E pathogenesis, and preclinical evaluation of new products to detect, prevent, and treat HEV.

Characterization of hepatitis B virus (HBV) from deferred potential blood donors and infectivity in primary human hepatocytes cultures

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Objectives: Blood donations for transfusion are tested for relevant transfusion-transmitted infections, including HBV. We aim to characterize HBV variants from deferred donors identified through routine donor screening using virological, serological, and biochemical methods, and to evaluate variant infectivity in human hepatocyte (HH) cultures.

Methods: Hepatitis B surface antigen (HBsAg) -positive plasma samples were purchased from the American Red Cross biospecimen repository. These included 34 reactive and 14 non-reactive for anti-HBV core antibodies (anti-HBc). Samples were further characterized by HBeAg ELISA, and virus genotype determined by sequencing of the P gene. To evaluate sample infectivity, plasma was used to inoculate HH cultures derived from humanized mice with chimeric livers and HBV DNA and HBsAg were measured in culture media over time. Sedimentation profiles of HBsAg, HBeAg, and capsid-associated HBV DNA were analyzed in HH culture media.

Results: HBV-positive deferred donor samples included genotypes A, B, C, and D. Most samples with initial viral loads $>10^8$ copies/ml resulted in HH infection, judged by HBsAg production in culture media at 14 days post infection, even when diluted 100- to 1000-fold. Samples with higher infectivity were more likely to be genotypes B or C, be anti-HBc positive, have higher HBeAg levels, and be from younger donors. Sedimentation profiles of HBV biomarkers showed that infectious samples contained higher levels of free HBeAg and HBsAg, than non-infectious samples.

Discussion: We have established methods for the propagation and study of HBV isolates in HH from deferred potential donors. HBV genotypes reflect those seen in high-risk populations in the US. As expected, samples with higher viral load tended to result in HBV infection in HH. Although no information is available on infection stage (acute vs chronic), our data suggest samples from persons infected for longer (anti-HBc+) or with chronic infection were associated with higher infectivity.

Plain Language Summary:
Blood donations in the US are routinely tested for transfusion-transmissible infections, including hepatitis B virus (HBV). HBV can lead to chronic infection and increase the risk of liver failure and cancer. We studied HBV from deferred potential blood donors who were otherwise healthy and unaware of their infection but tested positive during screening. We characterized the viruses genetically and established a cell culture model based on human liver cells to propagate and study HBV infectivity and biology. We used this model to identify blood donation characteristics that might predict robust HBV infectivity.

Evaluation of antimicrobial 405 nm violet-blue visible light inactivation potential of *Treponema pallidum* spiked in human platelets in a rabbit infectivity model

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Background: Currently, safety of ex vivo platelet concentrates (PCs) stored in plasma is achieved using two methods: testing PCs for pathogens, and pathogen inactivation using chemical and/UV light. However, UV light can be harmful to the products. In this study, we tested antimicrobial 405 nm violet-blue light in the visible spectrum, against *Treponema pallidum in PCs,* the bacterium that causes syphilis.

Objective: To assess the level of inactivation of *T. pallidum* spiked in ex vivo human platelets achieved by exposure to 405 nm visible light.

Methods: We performed three studies using platelets from different donors. We spiked platelets with treponemes, removed one aliquot (T0) and injected into rabbits intradermally. We separated the remainder into two parts, one sample was exposed to the violet-blue light while the other was unexposed to the light as a control. After 5 hours, we removed an aliquot from both samples (T5 and T5-control) and injected into rabbits intradermally. Rabbits were tested weekly serologically, and syphilitic lesions were monitored for two months. Also, we collected lesion biopsies and popliteal lymph nodes for *T. pallidum* DNA detection to detect bacteria. At the end of the study, we compared bacterial titers after inactivation to those of control samples.

Results: Three *T. pallidum* inactivation studies showed similar results. Animals injected with inactivated treponemes (T5) developed no lesions and were serologically negative. Rabbits injected with T0 and T5 controls developed syphilis as demonstrated by the appearance of lesions at the site of inoculation and the presence of antibodies against treponemes in serum (seropositivity). The studies demonstrated >2 $\log_{10}$ of *T. pallidum* infectivity reduction.

Conclusions: Exposure of platelets containing treponemes to the violet-blue visible light inactivated *T. pallidum* to the limit of detection of the bioassay. A fourth study with a higher titer of *T. pallidum* stock is in progress.

Plain Language Summary:
We investigated the antimicrobial effect of 405 nm violet-blue light against Treponema pallidum, the bacterium that causes syphilis, spiked in human platelets. We performed three independent experiments. T. pallidum activity was determined before and after treatment using rabbit bioassay. We tested rabbit sera for antibodies against treponemes and monitored the animals for syphilitic lesions. Rabbits injected with irradiated T. pallidum remained serologically negative and developed no syphilitic lesions. Rabbits injected with non-irradiated controls developed syphilitic lesions at inoculation sites and seropositivity. This study demonstrated that 405 nm violet-blue light killed more than 99% of Treponema pallidum bacteria in human platelets.

Exposure to Low Steady State Hydrogen Peroxide Induces Red Blood Cell Vesiculation and Loss of Deformability

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Background: Methods to determine the impact of blood processing technologies or storage conditions on the quality of red blood cell (RBC) products are not fully established. Processed or stored RBCs may be particularly vulnerable to oxidative stress-mediated reversible and irreversible damage. Understanding the mechanisms underlying these adverse changes using relevant in vitro oxidative stress models may facilitate the identification of early predictive biomarkers of RBC quality. Objective: Using an in vitro model of low enzymatic hydrogen peroxide (H2O2) production, we evaluated the structural and functional integrity of human RBCs using ektacytometry, flow cytometry, membrane zeta potential, and high-resolution image analyses.

Methods: Washed human RBCs were incubated in Dulbecco's phosphate-buffered saline containing different concentrations of glucose oxidase to generate low micromolar levels of H2O2. RBC deformability was measured by ektacytometry using the Laser-assisted Rotational Red Cell Analyzer (Lorrca). Membrane integrity was measured by spectrophotometric detection of hemolysis and flow cytometric analysis of externalized phosphatidylserine (PS) and calcein-AM fluorescence. Plasma membrane vesiculation was assessed by high-resolution differential interference microscopy combined with immunofluorescence labeling of glycophorin and band 3-expressing microvesicles. Membrane zeta potential was assessed by light scattering (M3-PALS) technology.

Results: RBCs exposed to low steady state levels of H2O2 over the course of 24 hours showed a concentration- and time-dependent loss of membrane deformability that correlated with increased microvesicle release. These changes were also accompanied by a gradual loss of membrane integrity characterized by low but detectable hemolysis and loss of calcein fluorescence without PS externalization. Reduced membrane surface charge was also detected at late incubation times.

Conclusions: This in vitro enzymatic model of low and sustained oxidative stress exposure can serve as a relevant approach to study the temporal changes in the structural and functional integrity of RBCs and may be useful for identifying early biomarkers of RBC quality.

Plain Language Summary:
Development of in vitro experimental models to assess RBC integrity for the purposes of identifying relevant early predictive biomarkers of RBC quality.

Metabolite profiles distinguish exposure to Zika and Dengue flaviviruses in human induced pluripotent stem cells (hiPSCs)

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Background: Previous studies suggest that flaviviruses can be transmitted through cells and tissues threatening safe use of human cells, tissues and cellular and tissue-based products (HCT/Ps). Therefore, developing sensitive and specific tools to detect flavivirus contamination is critical to ensure safety of HCT/Ps. Here, we studied metabolite profiles following flavivirus infection of hiPSCs with goals to develop metabolite markers based-detection assay.

Methods: hiPSCs were infected with Zika-MR766 or Dengue-3 viruses, and cell viability, cytopathic effect (CPE), and viral load were measured using immunofluorescence assay (IFA) and qRT-PCR respectively. We performed mass spectrometry (FIA-MS and LC-MS) of hiPSCs at 0, 8, 24, 48 and 96 hours post infection to identify virus-specific shift in metabolome. LC-MS raw files were converted to mzML using msConvert and peak extraction was performed using MZmine. Features with a signal-to-noise ratio below three in pooled quality control (QC) samples compared to blank, missing in more than 20% of study samples, or with a coefficient of variance greater that 20% in pooled QC samples were removed.

Results: We did not observe significant differences in cell viability and CPE in hiPSCs following infection with Zika or Dengue compared to mock infection. However, IFA and qRT-PCR results indicated higher infectivity of ZIKV compared to DENV. FIA-MS results demonstrated that 0.2 million cells were sufficient to detect a maximum number of metabolites. The positive ionization mode in LC-MS yielded 10,077 features. After filtration, 1,052 features remained. Currently data from LC-MS is further evaluated via various machine learning techniques and statistical methods to identify a panel of biomarker specific for Zika and Dengue virus.

Conclusions: Preliminary data demonstrate that metabolomic profile can distinguish between Dengue or Zika virus infected and uninfected hiPSCs. This suggest that metabolite-based biomarkers have potential to detect flavivirus infection of hiPSCs.

Plain Language Summary:
It is well established that flaviviruses can transmit through cells and tissues from donors who is no longer viremic. So, creating sensitive and specific flavivirus detection tools is critical for the safety of cell and tissue therapy. Our study using hiPSCs infected with DENV and ZIKV viruses demonstrates that 'Metabolomics' is highly sensitive compared to standard NAT and immunogenic assays. We've discovered potential flavivirus detection metabolite markers in hiPSCs, currently validating their specificity. This novel and comprehensive study on identifying host biomarkers specific to flaviviruses using metabolomics in cells used for therapy has significant importance for Public Health.

High-throughput APTT and one-stage APTT-based FVIII potency assays in low volume of mouse plasma

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Background: Activated Partial Thromboplastin Time (APTT) assay is a conventional coagulation test that is used in clinical laboratories for diagnostics of functionality of intrinsic coagulation pathway. APTT assays and instruments for them are developed to work with minimum 200 µL of human plasma. However, research and preclinical studies with small animals are limited by low volume of blood that could be collected. Some clinical trials may also have difficulty re-testing patient samples if insufficient sample volume remained.
Aim: To develop a high throughput APTT assay to test small volumes of mouse plasma.

Methods: We used robotic arms for reagent distribution, a modern 96 channel pipettor for simultaneous sample activation, and fast microplate readers to achieve parallel testing of samples on a single microplate.

Results: Based on SynthASil commercial reagent for APTT assays as well as potency assays, we developed APTT assay which uses about 1000 µL of plasma per sample. The APTT assay can analyze 24 samples simultaneously. Our developed one-staged APTT-based potency assay for assessing activity of coagulation factor VIII in mouse plasma takes about 10 minutes to run. The potency assay can test up to 5 samples in four dilutions in parallel with a calibrator. Clotting time in both assays is determined as the time of a half-height of normalized optical density. APTT and potency assays show linear (linear-log scale) dose-response to FVIII in the range 0.00015% - 100.0% IU/mL. Our potency assay showed 80%, 77%, and 97% recovery of FVIII for 5, 20, and 50 IU/mL of FVIII, respectively, Our potency APTT assay demonstrated about a 20% shift in FVIII activity when compared to commercial SynthASil assay on ACL TOP coagulometer.

Conclusions: The proposed experimental design achieves high throughput APTT and one-stage potency assay for FVIII in low volume of mouse plasma.

Plain Language Summary:
Activated Partial Thromboplastin Time (APTT) assay is a conventional coagulation test that is used in clinical laboratories for diagnostics of functionality of intrinsic coagulation pathway. APTT assays and instruments for them are developed to work with minimum 200 µL of human plasma. However, research and preclinical studies with small animals are limited by low volume of blood that could be collected. Some clinical trials may also have difficulty re-testing patient samples if insufficient sample volume remained. Here we developed a high throughput APTT assay to test small volumes of mouse plasma.

Evaluation of the Leishmanin Skin Test antigen as a biomarker of vaccine immunogenicity and disease surveillance

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Objective: Leishmaniasis is a bloodborne pathogen transmitted through sand fly vectors carrying protozoan parasites from the genus *Leishmania*. Currently, there is no vaccine or donor screening assay available for human use to prevent or detect the spread of leishmaniasis. This study characterizes leishmanin skin test (LST) antigens produced under Good Laboratory Practice (GLP) for use as a biomarker of vaccine immunogenicity and *Leishmania* surveillance in endemic and non-endemic areas.

Methods: The GLP-LST specificity and immunogenicity was evaluated in murine models of infections: leishmanization with wildtype *Leishmania major*, and mice immunized with the live-attenuated vaccine *L. major* lacking the *centrin* gene *(LmCen$^{-/-}$).* Following these infections, the GLP-LST antigen was administered and its ability to induce a delayed-type hypersensitivity (DTH) response was monitored for 48 hours. The resulting DTH site was harvested, immune responses were analyzed via flow cytometry to assess the potency of the GLP-LST antigens.

Results: High-dimensional flow cytometry analysis of the cell populations isolated from the DTH sites showed an enrichment of CD69^{+}CD4^{+} skin resident memory T-cells. Additionally, the presence of activated macrophages and Langerhans cells at the DTH site was detected, consistent with previous studies.

Conclusions: The studies described here demonstrate the utility of the animal models and methods to assess the potency of the GLP-LST antigen. In the future, cGMP-LST can be used in vaccine clinical trials as a marker for immunity, and in active surveillance studies of endemic and emerging areas of *Leishmania* infection.

Plain Language Summary:
Leishmaniasis is a bloodborne pathogen transmitted through sand fly vectors carrying protozoan parasites from the genus Leishmania. Currently, there is no vaccine available for human use to prevent the spread of leishmaniasis, or surveillance tool to monitor cases of Leishmaniasis in endemic and emerging areas of transmission. In this study, a GLP-LST antigen has been evaluated for its ability to determine prior exposure to both wildtype Leishmania infections, as well as a live-attenuated LmCen-/- vaccine. Using the described animal models and methods we are able to effectively assess the potency of this antigen.

Exploring host microRNAs in plasma as non-immune biomarkers for differential diagnosis between Dengue and Zika viruses

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Objective: Dengue (DENV) and Zika (ZIKV) have caused extensive outbreaks. Approximately 80% of infections are asymptomatic and most symptomatic cases are flu-like, but they can quickly become serious disease of high fatality rates with significantly different manifestations, requiring differential diagnosis for supportive care. However, NAT assay sensitivity is challenged by low viral loads during early infection and short-lived viremia (~7 days), and serological assays are inadequate for differential diagnosis due to antibody cross-reactivity among these viruses. This study aims to evaluate the use of microRNAs (miRNAs) as biomarkers for detection of infection and discrimination between DENV and ZIKV infections.

Methods: NGS sequencing was used to establish miRNAs composition in plasma samples from subjects infected with DENV (n=10), ZIKV (n=10), and non-infected controls (n=10). A total of 1945 miRNA were identified and analyzed using the selection criteria of log2 fold-change ≥ 1.5, $p<0.05$, resulting in 427 differentially expressed miRNAs between ZIKV- and DENV-infected subjects compared to the controls.

Results: We selected 80 miRNAs with high levels of differential magnitude (40 up- and 40-down regulated) between ZIKV- and DENV-infected and control. qRT-PCR for all 80 miRNA was performed using additional samples from 86 subjects:24 ZIKV-infected, 24 DENV-infected, and 38 non-infected-controls. Twenty-two miRNAs showed significant levels of differential expression between DENV, ZIKV and controls. Nine of them exhibited the highest differential expression, of which 2 (miR-454-5p and miR-3195) allows to detect infection and discriminate DENV from ZIKV. Additionally, 4 miRNAs (miR-4707-3p, miR-9-3p, miR-320a-3p, 2, miR-20a-3p) are DENV-specific only, and 2 (miR-369-3p, miR-4485-3p) are ZIKV-specific only.

Conclusions: Our data shows that miRNA are promising non-immune biomarkers suitable for detection and differential diagnosis between DENV and ZIKV infections. Further logistic regression analysis and additional studies are needed to validate findings and better understand the potential use of the selected miRNA as a panel for DENV and ZIKV differential diagnosis.

Plain Language Summary:
Mosquito-borne viruses such as Dengue (DENV) and Zika (ZIKV) outbreaks are increasing, with most infections (~80%) being asymptomatic, but symptomatic cases can progress to severe disease. DENV can cause hemorrhagic fever, and ZIKV can cause birth defects. Current diagnostics rely on viral RNA detection by NAT and antibody detection by serology assays. However, both assays have limitations NAT due to short-time viremia and low viral loads, and serological assays due to antibody cross reactivity. We are evaluating the suitability of host microRNAs as non-immune biomarkers to differentiate DENV and ZIKV infections.

Aptamer-based detection of *Trypanosoma cruzi*-derived biomarkers in blood of Chagas disease patients from Brazil and Bolivia

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Objective: Chagas disease (CD) is a lifelong illness caused by the blood-borne parasite, *Trypanosoma cruzi*. CD can lead to cardiac and digestive complications if untreated. Current drug treatments may cause serious adverse events and are not fully effective for chronic infections. CD is typically diagnosed using serological (e.g., ELISA) and parasitological (e.g., PCR) assays during the acute stage of infection when parasitemia is elevated. However, these methods are inadequate for evaluating chemotherapy outcomes in chronically infected patients as they often exhibit very low parasitemia, and antibody levels decrease slowly over the years following treatment.

Methods: We developed aptamers (short RNA molecules) targeting *T. cruzi* Excreted Secreted Antigens (TESA). Using an Enzyme-Linked Aptamer (ELA) assay, we previously demonstrated that TESA biomarkers (BMK) levels rapidly declining in blood of drug-treated mice. To assess the clinical application of the ELA assay using Aptamer-29, we tested sera from different cohorts in endemic regions of Brazil and Bolivia at various stages of CD, including pre- and post-treatment.

Results: Brazilian samples had significantly higher TESA BMK levels in individuals with acute or reactivated disease and in patients with cardiac symptoms compared to endemic healthy controls. However, results were inconsistent in seropositive individuals at the asymptomatic (indeterminate) stage. In one Brazilian cohort, BMK levels were significantly higher than endemic controls and returned to non-endemic control levels following benznidazole treatment. Conversely, no significant differences in BMK levels were observed between infected and endemic controls in samples from Bolivia and an additional Brazilian cohort. Sample collection site and presence of cardiac symptoms did not impact BMK levels between seropositive and seronegative individuals in the Bolivian samples.

Conclusions: Results indicate that TESA BMKs can be detected in individuals with CD. Nevertheless, further testing is necessary to determine the ELA assay's utility in assessing treatment efficacy and cure in clinical trials.

Plain Language Summary:
Caused by the *Trypanosoma cruzi* parasite, Chagas disease (CD) can lead to heart and digestive complications if untreated. Current drugs can have serious side effects and are mostly ineffective for chronic infections. Blood tests diagnosing acute CD are inadequate for checking if treatments work in chronic cases. We developed RNA aptamers targeting *T. cruzi* antigens detectable through an Enzyme Linked Aptamer assay. Tests on mice samples confirmed its effectiveness. With clinical samples, biomarker levels were higher in acute cases and patients with cardiac symptoms. Results varied in infected individuals without symptoms. Further testing is needed to assess clinical utility.

Development of a simple purification scheme with a high yield for laboratory-scale production of recombinant blood coagulation Factor VIII

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Objective: Hemophilia A affects people lacking functional Factor VIII (FVIII), resulting in life-threatening bleeding generally treated with recombinant (rFVIII) or plasma-derived FVIII (pdFVIII) infusions. Purification from either source involves difficulties, as a low protein expression levels both physiologically (~ 1 nM in blood) and in mammalian cell culture (of rFVIII), complex purification schemes in both commercial and research-scale FVIII production, and attachment of FVIII secreted in cell culture to cell membranes results in its depletion from media typically used for rFVIII isolation in standard lab conditions. In rFVIII production, one approach is to stabilize secreted FVIII in culture media and simplify the purification scheme previously described. In that study, we used bovine von Willebrand Factor (VWF), homologous to human VWF stabilizing plasma FVIII, supplemented to media by addition of fetal bovine serum, and a two-column procedure for protein purification.

Methods: Numerous purification schemes involving (i) adjusting pH and salt addition (NaCl, $CaCl_2$) to culture media and respective buffers, testing (ii) ion-exchange and (iii) FVIII-affinity chromatography using various wash/elution buffer compositions, FVIII eluate concentration was measured via electrochemiluminescence assay (ECLA), purity was measured vie densitometry on stained gels. Cell culture media with expressed rFVIII is adjusted for salt concentration by addition of NaCl before loading onto an VIIISelect column (Cytiva). Using an updated VIIISelect buffer system with changed buffering agent, increased NaCl and $CaCl_2$ concentrations, and decreased pH, the column is washed and FVIII is eluted.

Results: The procedure first utilized metal-affinity chromatography resulted to the low yield (7.0±.16%), most likely because interactive rFVIII areas are masked by bound bovine VWF. The optimized scheme results with 65% yield and 95% purity in a single-column procedure.

Conclusions: The developed expression and purification scheme allows simple high yield rFVIII production with acceptable purity, sufficient for the use in majority of research applications.

Plain Language Summary:
Hemophilia A is a life-threatening disease in which people lack functional endogenous Factor VIII (FVIII). Treatment generally includes infusion of purifies FVIII from either recombinant or plasma-derived sources. FVIII purification is historically difficult, requiring multiple many steps starting with low concentrations expressed by cells. The combination of low FVIII expression and low-yield purification schemes necessitate development of improved methods for more efficient use of laboratory time and materials in regards of generating useful amounts of FVIII for research. Here, a more efficient purification scheme was developed.

Investigating Brain Structure in PP2A-B56e KO Mouse Model using 7T MRI

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Objective: Evaluate the hypothesis that PP2A dysregulation can cause structural, and tissue changes in the brain. Specifically, evaluate the effects of inactivating the PP2A B56e regulatory subunit in mice to assess brain structural changes over time using 7T MRI.

Methods: 14 PP2A-B56ε homozygous knockout (KO) mice at timepoint 1 (6 mice; 125-225 days old) and timepoint 2 (8 mice; 320-430 days old) were prepared. 13 and 11 matched wild-type littermates (+/+, +/-) were also included as controls at timepoint 1 and 2, respectively. Multi-echo gradient echo (GRE) acquisition was performed on a 7T MR scanner (Agilent Technologies, Santa Clara, CA, USA) to acquire T2* map and quantitative susceptibility mapping (QSM) using the following parameters: FOV = 30×30 mm², TR/TE = 1500/3.57 ms, eight echoes = 3.57 (start):4.00 (deviation):31.57 (end) ms, image resolution = 0.12×0.12 mm², flip angle = 67º, 40 slices, slice thickness = 0.25 mm, and total scan time ~19 min. The lateral and 3rd ventricular volumes were measured and normalized by the whole brain volume. QSM was acquired using MEDI software. For region-of-interest (ROI) analysis, 3 ROIs (hippocampus, thalamus, hypothalamus) were manually drawn and mean values of measures (T2*, QSM) were calculated within the ROIs. Comparison within the groups was performed using an analysis of variance (ANOVA) with age and sex as covariates using SPSS. A p-value of less than 0.05 ($p < 0.05$) was considered significant.

Results: Significant enlargement of the 3rd ventricle at both timepoints ($p < 0.001$) and of the lateral ventricles at timepoint 2 was observed in KO mice compared with matched control mice. However, no significant differences were found in weight, T2* and QSM values in ROIs at both timepoints except QSM in hippocampus at timepoint 1.

Conclusions: This study found significant ventricle enlargement in PP2A-B56e KO mouse model suggesting its link with neurodegeneration.

Plain Language Summary:
Protein phosphatase 2A (PP2A) controls the phosphorylation state of many key proteins in the brain known to be related to neurodegeneration. In this study, we investigate the effects of inactivating the PP2A B56epsilon regulatory subunit to assess structural changes to the brain using 7T MRI. Our findings show significantly increased ventricle volume, particularly in the 3rd ventricle, in the KO mice compared with controls. This result shows that dysregulation of PP2A may play a role in hydrocephalus and other neurodegenerative diseases.

Long-term physicochemical and microbiological stability and *in vitro* prothrombin-converting activity neutralization potency of outdated antivenom products indicated for exotic snake species envenomation

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Objective: Evaluation of Antivenom (AV) products stability, including physiochemical and microbiological properties and neutralizing potency, is important for their potential emergency use if in-date product is unavailable.

Methods: Six AVs (25 lots/50 vials) were characterized by: *In vitro* potency assessed by neutralization of Echis carinatus venom prothrombin-converting activity (PCA), appearance evaluated by visual inspection (opalescence/color, particulates), AV immunoglobulin characteristics - molecular size distribution and purity (SE-HPLC), protein concentration, Dynamic Light Scattering (DLS), and endotoxin concentration (LAL).

Results: Overall, 42% AV vials passed pre-screening based on evaluation of physicochemical and microbiological parameters. Protein concentration and pH were uniform in all AV products. Failures were due to endotoxin level > 10 EU/mL (24%), high opalescence (24%), out-of-range color (8%), presence of foreign particulates (22%), or intrinsic particulates (10%). SE-HPLC demonstrated decreased major AV product content {F(ab')2} and increased dimer/multimer populations in vials that failed visual inspection/endotoxin pre-screening, suggesting protein denaturation and agglomeration which has been associated with adverse effects caused by IgG products. DLS showed elevated polydispersity indices ($p<0.0001$) and aggregate content in failed pre-screened AV vials. Potency of expired vials of E. carinatus AVs to neutralize E. carinatus venom PCA was detected in all tested vials, but those which passed pre-screening had significantly higher PCA neutralization potency when compared to failed vials ($p<0.017$).

Conclusions: AV visual inspection results correlate with protein degradation and particle analysis, and may be useful for pre-screening under emergent conditions. Comprehensive physicochemical and microbiological evaluation of expired AVs may predict their potency. AV neutralization of venom PCA may serve as expired AV potency assay for several epidemiologically significant snake species. Based on our physicochemical and microbiological evaluation of AVs for their qualification for emergency clinical use, some AV products have potential for long-term stability even decades after their expiration.

Plain Language Summary:
To consider emergency use of expired antivenoms (AV) in life-threatening situations when no in-date product is available, continuing stability of AVs beyond their expiry needs to be evaluated. We assessed stability of physicochemical and microbiological parameters and *in vitro* potency of 6 non-FDA licensed investigational AVs indicated for envenomation by epidemiologically important snakes nonnative in the USA. 42% of 50 tested AV vials passed appearance (opalescence/color, particulates), protein content, pH, and microbiological screening. Visual inspection results correlated with protein degradation, particle analysis, microbiological, and neutralization of venom, suggesting that some outdated AVs might qualify for emergency use.

Development of a qmosRT-PCR assay for simple detection and identification of contamination of novel oral-poliovirus vaccines with Sabin viruses

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The live oral poliovirus vaccines (OPVs) are effective against poliomyelitis. They are produced from three attenuated poliovirus Sabin strains that can revert to a neurovirulent phenotype during replication in cell cultures or in vaccinees, resulting in rare cases of vaccine-associated paralytic poliomyelitis in vaccinees and/or their contacts.

Recently, a novel OPV2 vaccine (nOPV2) with enhanced genetic stability was developed using Sabin 2 genome as backbone. The vaccine included a domain V which was genetically modified. As a result, the stability of domain V is improved, reducing the chance of mutations associated with neurovirulence, and improving the attenuation of the virus. In addition, the nOPV2 cis-acting replicative element was relocated from the middle of the genome to the 5′ UTR, preventing the replacement of the modified domain V through single recombination events. nOPV2 also includes D53N and K38R amino acid changes in the RNA polymerase (3D protein) to improve replication fidelity and decrease the recombination degree, respectively. nOPV strains of type 1 and type 3 were developed using the genome of the nOPV2 as a backbone in which the capsid precursor (P1) was replaced with the P1 of Sabin strains type 1 and 3, respectively. It is crucial to eliminate the possibility that nOPV lots are contaminated with Sabin viruses.

Here we describe the development of the Quantitative Multiplex One-Step Reverse-Transcriptase Polymerase Chain Reaction (qmosRT-PCR) assay for simple detection and identification of Sabin viruses in the presence of overwhelming amounts of nOPV strains. The assay is very specific and sensitive, able to detect 0.00001% of Sabin viruses in nOPV and generate reproducible results. Also, 12 BioFarma trivalent nOPV lots were analyzed by the method, the results showed that the nOPV lots were not contaminated with Sabin virus.
This assay can be used for quality control and lot release of nOPV vaccines.

Plain Language Summary:
Development of a multiplex RT-PCR assay for simple detection and identification of Sabin viruses in the presence of overwhelming amounts of nOPV strains. The assay is very sensitive and specific, it can be used for quality control and lot release of nOPV vaccines.

NLP-based biological cell type annotator for single cell transcriptomics

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Objective: Cell type annotation is a critical step in single-cell transcriptomics analyses. Currently available options and published software rely on resources heavily populated with immune cell annotations. This leads to skewing the results and overpredicting computationally computed cell clusters as immune cells (and sub-populations), which is inaccurate. This is a common problem with biological datasets, referred to as "imbalanced datasets."
With the increasing use of single-cell transcriptomics in clinical trials, there is a need for robust tools that best predict cell types from marker genes for the purpose of identifying safety/efficacy signals. Such tools should consider the diverse types of cells found in different tissues sampled without bias. Our proposed solution uses advanced Natural Language Processing (NLP) techniques to annotate cell types.

Methods: The proposed tool uses key-word matching to predict cell types from a set of marker genes that capture the top quantile of genes expressed as highly expressed markers. Cell type gene markers were accumulated and filtered from all publicly available databases (CellMarker, CellMarker 2.0, Human Protein Atlas, PanglaoDB as well as entire CellxGene human dataset). We then curated a single database to host all normalized data which serves as the main resource for developing our NLP-based solution.

Results: We have prepared and assembled all sources to minimize bias. Pilot studies demonstrated that an NLP-based QA pipeline could effectively parse this database.

Conclusions: The proposed approach will enable 1) Accurate prediction of cell type(s) for a cluster of cells given as input tissue type and a list of gene markers. 2) Identification of novel/specific gene markers for known cell types (like summarization for regular text).

Plain Language Summary:
Cell type annotation is a critical step in single-cell transcriptomics analyses. Due to the increasing use of single-cell transcriptomics in clinical trials, there is a need for robust tools that can best predict cell types from marker genes that should consider the diverse types of cells found in different tissues sampled without bias. Our proposed tool uses advanced NLP techniques to annotate cell types. Cell type gene markers were accumulated and filtered from all publicly available databases. We then curated a single database to host all normalized data and that serves as the main resource for developing our NLP-based solution.

Preparation and Validation of CBER Diverse HIV-1 Variants NAT Reference Panel (v18.51)

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Objective: The HIV virus remains highly diverse, presenting ongoing challenges for diagnostics and treatment. Continuous monitoring of emerging HIV variants is crucial, as is the assessment of their impact on diagnostic tools. To address this, a new CBER Diverse HIV-1 Variants NAT Reference Panel (v18.51) is now publicly available.

Methods: This new HIV reference panel consists of five subtypes, five circulating recombinant forms (CRFs), and eight unique recombinant forms (URFs). There are three dilutions of the heat-inactivated viruses in the scale of low, medium, and high viral load (VL) for each strain. All members of the variant panel have been prepared by diluting the starting material with pooled defibrinated normal human plasma tested to be negative for HIV-1 and the major blood borne viruses. The validation studies were performed in four labs using different VL platforms.

Results: The VL panel for each strain representing low, medium, and high-end range of VL were prepared and assigned the titer (log 10) between 2.4-2.82(±0.39), 3.39-3.82(±0.34), and 4.40-4.87(±0.47) respectively after analyzing validation studies performed in 3 labs. The difference between the scale is 0.979(±0.65). Only three strains (CRF 06_cpx, CRF 18_cpx, and CRF 02_A1) missed a high titer of 50.000 copies/mL due to lower titers of the original virus stock. The panel materials were assembled in 1.25 mL per aliquots. Each set of HIV reference panel consisted of maximum 51 panel members representing 18 diverse HIV viruses.

Conclusions: The v18.51 panel is designed to be used as q reference reagent to evaluate assays for HIV diagnosis, viral load monitoring, and donor screening NAT assays to assess test sensitivity for diverse strains. The panel may be used for the qualitative and semi-quantitative evaluation of in vitro tests to detect HIV-1 RNA in human serum or plasma. It is not intended to be used for routine lot release testing of lots of HIV-1 detection kits of nucleic acid tests licensed for donor testing. The panel will be distributed to manufacturers of HIV NAT tests and other testing laboratories by the NIAID contract facility, the Duke External Quality Assurance and Oversight Proficiency Laboratory (EQAPOL).

Plain Language Summary:
The HIV virus remains highly diverse, presenting ongoing challenges for diagnostics and treatment. Continuous monitoring of emerging HIV variants is crucial. A new HIV panel is designed to be used as reference reagent to evaluate assays for HIV diagnosis, viral load monitoring, and donor screening NAT assays to assess test sensitivity for diverse strains, the panel may be used for the qualitative and semi-quantitative evaluation of in vitro tests to detect HIV-1 RNA in human serum or plasma, however, it is not intended to be used for routine release testing of lots of HIV-1 detection kits produced by licensed manufacturers of nucleic acid tests.

Combined immunization with SARS-CoV-2 spike and SARS-CoV nucleocapsid results in protection but increased lung pathology

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Objective: With the emergence of SARS-CoV-2 variants of concern (VOCs), vaccines that are cross-protective have become important. As the nucleocapsid (N) protein of SARS-CoV-2 is relatively conserved, it has been considered as a variant-stable vaccine target through the induction of T-cells. However, previous studies on SARS-CoV suggested that the induction of immune response to N could result in enhanced disease upon infection. We investigated immunization with a variant nucleocapsid, from SARS CoV, in combination with spike from SARS-CoV-2, and compared this to nucleocapsid from SARS-CoV-2.

Methods: Adenovirus-based vaccines encoding the N protein (N) from either SARS-CoV (Ad-N1) or SARS-CoV-2 (Ad-N2) were administered to K18-hACE2 transgenic mice either alone or in combination with an Adenovirus vector expressing the SARS-CoV-2 spike protein (S) (Ad-S) (WA strain) and immunogenicity, protection and lung pathology were assessed.

Results: Immunization with Ad-N1 or Ad-N2 alone induced robust T and B cell immune responses and provided low-level protection against SARS-CoV-2 challenge. Ad-N1+Ad-S or Ad-N2+Ad-S resulted in higher CD8+ T-cell responses to S and increased S-binding antibodies compared to Ad-S alone. However, neutralizing antibody titers were significantly lower in the combined immunized groups, suggesting possible impact of N-antigen on S-mediated immunogenicity. All S-containing immunization provided 100% survival and significantly lower lung viral titers upon challenge compared to controls. However, the addition of Ad-N1 to Ad-S resulted in increased lung pathology compared to Ad-N2+Ad-S or Ad-S alone, albeit less severe than in controls. Increased pathology was associated with higher T and B cell immune responses in nucleocapsid-immunized mice prior to challenge.

Conclusions: These findings suggest that spike-nucleocapsid-based vaccines are safe and effective, even with variant nucleocapsid sequences, but that viral control may be associated with higher lung pathology, compared to spike immunization alone, due to the immunogenic qualities of the nucleocapsid antigen.

Plain Language Summary:
With the emergence of SARS-CoV-2 variants of concern, vaccines that are cross-protective have become important. As the nucleocapsid (N) protein of SARS-CoV-2 is relatively conserved, it has been considered as a variant-stable vaccine target. However, previous studies on SARS-CoV suggested that the induction of immune response to N could result in enhanced disease upon infection. We investigated immunization with a variant nucleocapsid in combination with spike from SARS-CoV-2, and and found that spike-nucleocapsid-based vaccines are safe and effective, but viral control may be associated with higher lung pathology due to the immunogenic qualities of the nucleocapsid antigen.

Sustained antigen delivery improves germinal center reaction and increases antibody responses in neonatal mice

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Objective: Neonates and young infants are known to have limited responses to pediatric vaccines due to reduced germinal center formation. Prolonging vaccine antigen presentation has shown to expand germinal center formation and improve humoral responses in adult mice. However, it is unknow whether the sequential dosing strategy could overcome the weak germinal center development in neonatal mice.

Methods: Neonatal mice were immunized with tetanus conjugated pneumococcal type 14 (PPS14-TT) vaccine in three different dosing regimens: a single 0.2 μg bolus dose on Day 0; four constant doses of 0.05 μg on Days 0, 2, 4, and 6; or four escalating doses of 0.02, 0.04, 0.06, and 0.08 μg on Days 0, 2, 4, and 6 respectively. Humoral responses after vaccination were measured by ELISA, ELISpot, and flow cytometry.

Results: Compared to the single bolus dose PPS14-TT vaccinated neonatal mice, neonatal mice receiving four sequential doses of PPS14-TT vaccine have increased PPS14-specific IgG1 antibody titers, and significantly more PPS14-specific antibody secreting cells. Moreover, significant increases of Foxp3- T follicular helper cells and germinal center B cells were also observed in neonatal mice administrated with constant and escalating doses of the vaccine.

Conclusion: Sustained antigen delivery through sequential dosing overcomes the neonatal limitations to form germinal center reaction and improves humoral immunity. Thus, vaccine strategies that extend the release of vaccine antigen may reduce the number of doses, and time needed, to achieve protective immunity in neonates and young infants.

Plain Language Summary:
Sustained antigen delivery may reduce the number of doses to achieve protective immunity in neonates and young infants.

A Live-Attenuated *Leishmania* Vaccine Shapes the Cellular Response in The Bone Marrow

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Objective: *Leishmania* is a blood borne pathogen that causes leishmaniasis for which no vaccine is available. We developed a live-attenuated parasite vaccine lacking the *centrin* gene (*LmCen*$^{-/-}$). Immunization with *LmCen*$^{-/-}$ induced durable protection in murine models. It remains to be confirmed if the protective immunity requires the presence of a persistent infection. Our objective is to identify the possible persistence of *LmCen*$^{-/-}$ and its role in protection.

Methods: C57BL/6J mice were intradermally infected using virulent *L. major* (*Lm*WT) and *LmCen*$^{-/-}$ parasites. Using dual scRNA-seq, parasitized cell populations in the bone marrow (BM) were detected at 28 days post infection (dpi). Persistent presence of parasites was verified by flow cytometry at 14, 28, and 42 dpi using *Lm*WT and *LmCen*$^{-/-}$ fluorescent (mCherry+) parasites. Cell populations in the BM were identified via flow cytometry. Functional characteristics of BM monocytes was determined using virulent *Lm*WT luciferase-reporter parasites by measuring bioluminescence.

Results: About 1% of BM cells from mice infected with virulent *LmWT* or vaccinated with *LmCen*$^{-/-}$ harbored leishmania transcripts 28dpi. Identification of different cell populations via scRNAseq within the BM showed that *LmCen*$^{-/-}$ infection led to differential expansion of myeloid populations, compared to *Lm*WT infection. Validation via flow cytometry confirmed that vaccination with *LmCen*$^{-/-}$ leads to the expansion of neutrophils and myeloid progenitors giving rise to monocytes and macrophages overtime. Monocytes obtained from the BM of *Lm*WT or *LmCen*$^{-/-}$ infected mice at 28dpi were challenged ex-vivo with virulent parasites and were able to eliminate such parasites more efficiently than the BM monocytes which had not been previously exposed to the parasite.

Conclusions: *Lm*WT and *LmCen*$^{-/-}$parasites can reach the BM in very low numbers and alter the immune landscape in the BM.

Plain Language Summary:
Leishmaniasis, now declared endemic to the US, is a parasitic disease for which no vaccine has been approved by FDA. Vaccination is possible since prior infections can protect against re-infection, and the persistence of low numbers of parasites in the host is suspected to favor protection. We have developed a live-attenuated vaccine candidate, which shows excellent protection against leishmaniasis. We demonstrated that low number of parasites are found in the bone marrow of vaccinated mice, which generates changes in the monocyte populations of the bone marrow allowing them to resolve a second encounter with the parasite more effectively.

Establishment of a robust experimental system for efficient generation of functional TFH cells *in vitro* in both adults and neonates

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Objective: The inability of neonates to develop CD4+Foxp3-CXCR5+PD-1+ T follicular helper (TFH) cells contributes to their weak vaccine responses. Co-injection of IL6 with a pneumococcal conjugate vaccine (PCV) suppresses TFH cell expansion in neonatal mice. This is in sharp contrast to adults, where IL6 improves vaccine responses by downregulating the expression of IL2Rβ on TFH cells, thereby protecting them from the inhibitory effect of IL2. Recently, we showed that unlike in adults, IL6 enhances the activity of IL2 and its receptors on neonatal TFH cells, thereby suppresses immune response to vaccines. Here, we developed an in vitro system to differentiate TFH cells from adult and neonatal CD4+ T cells to study differential role of IL6.

Methods: For this study, we adapted established protocols for the in vitro generation of TFH -like cells using plate-bound anti-CD3/CD28 in combination with blocking antibodies and cytokines. TFH cell differentiation was analyzed by flow cytometry.

Results: Our data shows that recapitulating the in vivo results, addition of IL6 robustly induces the differentiation of murine adult CD4 T cells to TFH cells under TFH polarizing conditions. In contrast to adults, in neonates, removal of IL6 improves TFH differentiation. Our data also suggests that the effect of IL6 on TFH differentiation is mediated via regulation of CXCR5 expression. IL6 improves TFH in adults by downregulating the expression of IL2 receptors, IL2Rα and IL2Rβ; In neonates, removal of IL6 suppressed IL2 receptor expression, thereby leading to improved TFH differentiation. Additionally, confirming the suppressive activity of IL2, addition of exogenous IL2 resulted in decreased TFH differentiation in adults.

Conclusions: The *in vitro* system we developed allows the generation of TFH cells that captures *in vivo* attributes of TFH cells in both adults and neonates. This method will facilitate further research to study TFH cells, especially those that require a large amount of TFH cel

Plain Language Summary:
In adults, conjugate polysaccharide vaccines can elicit protective immune response after one dose. In infants, a 3 to 4 dose regimen is required to elicit adult-like immune responses. Children remain vulnerable to infections until they complete the four-dose regimen. IL6 that is required for improved immune response in adults, suppresses vaccine response in neonatal mice. The development of tools to study the totally opposite effect of IL6 in different age groups is important in gaining knowledge that will enable age-specific vaccine design based on the immunobiological differences between the two age groups.

Defining Humoral Immunity and Antibody Immunodominance Following Human Norovirus Challenge

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Introduction: Human norovirus is a leading agent of acute non-bacterial gastroenteritis in all age groups. Norovirus has diversified into ~40 different genotypes capable of infecting humans, each comprised of multiple distinct strains. This extreme diversity, in the context of immunity, has contributed to the lack of approved norovirus vaccines or specific therapeutics. Further, in the absence of traditional cell culture and small animal models, experimental human challenge models provide critical insights into human norovirus immunity for vaccine design.

Methods: Here, we profiled the timing, breadth, immunodominance and functionality of the humoral immune response in 14 participants following experimental human norovirus challenge with GII.2 Snow Mountain virus. Using a diverse panel of virus-like particles (VLPs) and soluble capsid domain antigens, we measured the serological antibody response before and after infection (days: 15, 30 and 45).

Results: Virus challenge induced strong homotypic IgG and IgA antibody responses, with variable titers directed to Protruding and Shell domains. Similar cross-genotype antibody titers were observed before and after challenge. Infection elicited strong HBGA-blocking titers, a surrogate for virus neutralization, to the challenge agent in 78.6% of people (11/14) and although cross-blockade of diverse noroviruses genotypes was limited, 50% of people (7/14) demonstrated blockade against the most prevalent circulating strain, GII.4_Sydney. Using a competitive neutralization assay we recently developed, we determined the contribution of cross-reactive and specific epitopes to the observed functional response over time. We found that antibodies recognizing the Protruding domain, and highly variable antigenic sites A and G on the GII.4 Protruding domain, greatly contribute to viral HBGA-blockade. Interestingly, contribution of more variable sites peaks before day 45, while cross-neutralizing antibodies increase to low levels over time.

Conclusions: Beyond providing critical insights into norovirus immunity, this study illustrates the use of human challenge models for the evaluation of norovirus vaccines and therapeutics.

Plain Language Summary:
Human norovirus is a leading agent of acute non-bacterial gastroenteritis in all age groups, costing the U.S. more than $10 billion every year. Norovirus is incredibly diverse, which has proven a barrier to vaccine and antiviral design. Here, we studied the antibody response to human norovirus infection in 14 adults over a 45-day period. We found that most individuals generated strong protective antibody responses following infection, but these responses start to decrease by 30 days. Beyond providing critical insights into norovirus immunity, this study illustrates the use of human challenge models for the evaluation of norovirus vaccines and therapeutics.

Qualitative analysis of immune responses to hepatitis C virus E2 protein induced with viral vector versus recombinant protein

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Background: Effective prophylactic vaccines to prevent hepatitis C virus (HCV) spread are still lacking. Successful vaccines targeting the HCV envelope should induce memory B cells, germinal center cells and T cells in addition to neutralizing antibodies. The main objective of this study is to compare qualitative and quantitative cellular and antibody responses in mice using different vaccine platforms (viral vector and recombinant protein formulated with different adjuvants).

Methods: BALB/c mice were immunized intramuscularly at days 0 and 21 with Adenovirus expressing HCV envelope glycoprotein E2 (Ad5-E2) or HCV E2 protein (rE2) adjuvanted with CPG ODN1826 and Alum or rE2 adjuvanted with AddaS03. Lymph nodes, spleens and serum samples were collected post-boost to evaluate cellular and antibody responses using flow cytometry, ELISA, and neutralization assays.

Results: Ad5-E2 immunization induced IFN-gamma, TNF-alpha and IL-2-producing CD8+ and CD4+ T cells detectable at day 14 post-boost. HCV rE2 combined with CpG/alum or AddaS03 induced only a weak or undetectable cytokine response in T cells at the same time point. In addition, Ad5-E2 induced higher germinal center B cell and T follicular helper cell responses compared with HCV rE2-CpG/Alum or AddaS03 groups. HCV E2-specific antibody responses were detected in all three immunized groups. ELISA antibody titers were higher in the E2/CpG/Alum and E2/AddaS03 groups compared to the Ad5-E2 treated group at day 14 post boost, although neutralizing antibody titers were similar.

Conclusions: The intracellular expression of E2 using the Ad5-E2 prime-boost immunization induced a superior cellular immune response, with respect to CD4+, CD8+, GC and Tfh cells compared to the rE2 adjuvant strategy. ELISA antibody titers were higher in the E2/CpG/Alum and E2/AddaS03 groups compared to the Ad5-E2 treated group, although neutralizing antibody titers were similar at these time points. Long-term follow-up is ongoing to assess the quality of long-term memory responses in all groups.

Plain Language Summary:
Qualitative analysis of immune responses to hepatitis C virus E2 protein induced with viral vector versus recombinant protein.

Evaluating the In Vitro Immune Responses against *Francisella tularensis Schus4-ΔclpB* in Fischer 344 Rats

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Francisella tularensis, like many intracellular bacteria, have evolved to successfully evade immune responses mounted by their hosts. Understanding the immune system's defenses and how to effectively harness and enhance them can be an important step in protecting humans from such evasive pathogens. To accomplish this, an animal model that closely reflects the infection and immune responses of humans is important. The Fischer 344 rat is a valuable research model used to evaluate immune responses against *Francisella tularensis* due to similarities between outcomes in rats and human outcomes. However, much remains unknown regarding how exactly adaptive immune responses defend against this intracellular pathogen. To validate previous studies evaluating control of bacterial growth and predictors of protection against *Francisella tularensis*, rats were treated with PBS, vaccinated with *F. tularensis* LVS, or vaccinated with *F. tularensis* Schus4-ΔclpB and restimulated in vitro 6 weeks after vaccination. To evaluate immune responses to restimulation in a co-culture assay, vaccinated animals were euthanized, and different concentrations of spleen and peripheral blood lymphocyte (PBL) preparations were overlayed over *F. tularensis* Schus4-ΔclpB-infected macrophages from naïve rats. Spleens and PBLs were stained with a 24-color flow cytometric panel before and after 48 hours of culture. Bacterial growth control was determined by measuring recovered bacterial CFU (plated directly from cultures). Supernatants from cultures were evaluated for nitric oxide and IFN-γ, both mediators that have been shown to be important in control of bacterial growth. Each concentration of splenocytes and PBLs (especially *F. tularensis* Schus4-ΔclpB vaccinated) effected similar levels of bacterial growth control, suggesting that control is neither organ specific nor concentration dependent. Therefore, vaccinated leukocytes at any of the tested concentrations are sufficient for control of bacterial growth. Future studies would serve to confirm these findings, as well as to further define the roles of nitric oxide and IFN-γ in controlling growth of *F. tularensis* Schus4-ΔclpB in rat macrophages.

Plain Language Summary:
We treated Fischer 344 rats with PBS, vaccinated with *F. tularensis* LVS, or vaccinated with *F. tularensis* Schus4-ΔclpB and restimulated in vitro 6 weeks after vaccination to validate previous studies evaluating control of bacterial growth and predictors of protection against *Francisella tularensis*. Different concentrations of spleen and peripheral blood lymphocyte (PBL) preparations were overlayed over *F. tularensis* Schus4-ΔclpB-infected macrophages from naïve rats to assess immune responses to restimulation in a co-culture assay. Each concentration of splenocytes and PBLs (especially *F. tularensis* Schus4-ΔclpB vaccinated) effected similar levels of bacterial growth control, suggesting that vaccinated leukocytes at any of the tested concentrations are sufficient for control of bacterial growth.