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TITLE: Precision Combinatorial Immunotherapeutic Targeting of Cytokine Receptor Kinase Signaling in CRLF2-Rearranged ALL

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CONTRACTING ORGANIZATION: University of Colorado, Aurora, CO

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14. ABSTRACT: Ph-like ALL is a high-risk subset of B-ALL defined by an activated kinase gene expression profile similar to that of BCR-ABL1-rearranged (Ph+) ALL and driven by a diverse range of genetic alterations that activate cytokine receptor signaling pathways. Children, AYAs, and older adults with Ph-like ALL have >60% relapse risk and experience significant leukemia-associated mortality. Approximately 50% of Ph-like ALL cases harbor rearrangements in CRLF2 (CRLF2-R) and frequent concomitant JAK2 point mutations. In addition to patients with Ph-like ALL, CRLF2 rearrangements (usually P2RY8-CRLF2 fusions) with JAK2 point mutations occur in approximately 60% of children and AYAs with trisomy 21/Down Syndrome-associated ALL (DS-ALL) and also induce hyperactive JAK/STAT signaling. Children with DS-ALL have substantial toxicity with chemotherapy and inferior clinical outcomes. CD19CART immunotherapy has proven highly successful at inducing remissions in 80-90% of patients with relapsed/refractory ALL. However, emerging data indicates that up to 50% of children and AYAs will relapse, most within a year. As an alternative strategy, the Fry laboratory developed CAR constructs targeting the TSLPR (encoded by CRLF2) and demonstrated potent in vivo activity of T cells transduced with anti-TSLPR CAR constructs (TSLPRCART) in CRLF2-R Ph-like ALL PDX models generated by the Tasian laboratory. Based on our promising preclinical data, a phase 1 clinical trial of TSLPRCART for children and AYAs with relapsed CRLF2/TSLPR-overexpressing ALL will soon open at the NIH. TKIs and CART immunotherapies have the potential to act synergistically in acute leukemias via co-targeting of oncogenic pathways using two distinct approaches: one (CART) targeting a cell surface cytokine receptor protein and the other (TKI) targeting critical receptor-mediated and intracellular kinase signaling pathways. Furthermore, combining multi-targeted CAR T cells with TKIs is strategically analogous to the paradigm of non-cross-resistant cytotoxic chemotherapy regimens that is required to achieve cure in children with ALL.					
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TABLE OF CONTENTS

Page

1. Introduction
2. Keywords
3. Accomplishments
4. Impact
5. Changes/Problems
6. Products
7. Participants & Other Collaborating Organizations
8. Special Reporting Requirements
9. Appendices

1. INTRODUCTION: *Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.*

Ph-like ALL is a high-risk subset of B-ALL defined by an activated kinase gene expression profile similar to that of *BCR-ABL1*-rearranged (Ph+) ALL and driven by a diverse range of genetic alterations that activate cytokine receptor signaling pathways. Children, AYAs, and older adults with Ph-like ALL have >60% relapse risk and experience significant leukemia-associated mortality. Approximately 50% of Ph-like ALL cases harbor rearrangements in *CRLF2* (*CRLF2-R*) and frequent concomitant *JAK2* point mutations. In addition to patients with Ph-like ALL, *CRLF2* rearrangements (usually *P2RY8-CRLF2* fusions) with *JAK2* point mutations occur in approximately 60% of children and AYAs with trisomy 21/Down Syndrome-associated ALL (DS-ALL) and also induce hyperactive JAK/STAT signaling. Children with DS-ALL have substantial toxicity with chemotherapy and inferior clinical outcomes. CD19CAR immunotherapy has proven highly successful at inducing remissions in 80-90% of patients with relapsed/refractory ALL. However, emerging data indicates that up to 50% of children and AYAs will relapse, most within a year. As an alternative strategy, the Fry laboratory developed CAR constructs targeting the TSLPR (encoded by *CRLF2*) and demonstrated potent *in vivo* activity of T cells transduced with anti-TSLPR CAR constructs (TSLPRCART) in *CRLF2-R* Ph-like ALL PDX models generated by the Tasian laboratory. Based on our promising preclinical data, a phase 1 clinical trial of TSLPRCART for children and AYAs with relapsed *CRLF2*/TSLPR-overexpressing ALL will soon open at the NIH. TKIs and CART immunotherapies have the potential to act synergistically in acute leukemias via co-targeting of oncogenic pathways using two distinct approaches: one (CART) targeting a cell surface cytokine receptor protein and the other (TKI) targeting critical receptor-mediated and intracellular kinase signaling pathways. Furthermore, combining multi-targeted CAR T cells with TKIs is strategically analogous to the paradigm of non-cross-resistant cytotoxic chemotherapy regimens that is required to achieve cure in children with ALL. This application is directly relevant to FY18 PRCRP Topic Areas of (1) Blood Cancer, (2) Immunotherapy, and (3) Cancer in Children, Adolescents, and Young Adults. *The primary hypothesis of this proposal is that durable remissions in patients with CRLF2-R Ph-like ALL or DS-ALL can be achieved using rationally combined immune and molecular kinase therapies that target critical and necessary signaling pathways in malignant cells.* The project is divided into the following Aims:

Aim 1. Develop combinatorial CAR constructs targeting TSLPR plus CD19 and/or CD22 and test the anti-leukemia efficacy of multi-targeted CARTs against *CRLF2-R* ALL in children, adults, and DS patients.

Aim 2. Determine the preclinical efficacy of multi-specific CARTs and kinase inhibitors against *CRLF2-R* ALL.

Aim 3. To delineate the impact of DS-associated immunodeficiency and aging on the potency of CART generated from patients with DS-ALL and to determine functionality of autologous T cell transduction for clinical immunotherapy with CARTs.

Study Design: These complementary and imminently clinically-translatable studies will test and validate the potential treatment efficacy of multi-targeted CART immunotherapy and/or kinase inhibition in multiple subtypes of *CRLF2-R* ALL characterized by TSLPR overexpression and hyperactivation of cytokine signaling, as well as increase our understanding of T cell functionality and therapeutic potential in patients with trisomy 21. In this work, we will develop synergistic, non-

overlapping treatment strategies to improve leukemia remission durability and to mitigate development of immunotherapeutic resistance in three very high-risk subtypes of B-ALL.

2. KEYWORDS: *Provide a brief list of keywords (limit to 20 words).*

Acute lymphoblastic leukemia (ALL), chimeric antigen receptor T cell, cytokine receptor-like factor 2 (CRLF2), Down syndrome, immunotherapy, kinase, Philadelphia chromosome-like (Ph-like), thymic stromal lymphopoietin (TSLPR)

3. ACCOMPLISHMENTS: *The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction.*

What were the major goals of the project?

List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

Specific Aim 1: Develop combinatorial CAR constructs targeting TSLPR plus CD19 and/or CD22 and test the antileukemia efficacy of multi-targeted CARTs against CRLF2-R ALL.

Major task 1: design of bispecific TSLPR x 19/22 CAR constructs & T cell manufacturing

Major task 2: new CRLF2+ ALL PDX model creation

- expand previously established childhood/adolescent CRLF2-R Ph-like ALL for subsequent experimental therapeutic studies
- establish 2-4 primary xenograft models of adult CRLF2-R Ph-like ALL and expand in secondary recipients for subsequent experimental therapeutic studies
- establish 2-4 primary xenograft models of DS-ALL and expand in secondary recipients for subsequent experimental studies

Major task 3: *in vitro* testing of TSLPRCARTs with CRLF2-R ALL cell lines MUTZ5 and MHH-CALL-4

- cytokine production assays (IL-2, IFN- γ)
- cytotoxicity assays (Cell Titer Glo) with ALL cell lines + CAR T cells +/- TKI

Major task 4: *in vivo* testing of TSLPRCARTs

- preclinical testing of TSLPRCART and ruxolitinib monotherapies in bioluminescent CRLF2-R cell line MUTZ5 xenograft models
- preclinical testing of TSLPRCART + ruxolitinib in bioluminescent CRLF2-R cell line MUTZ5 xenograft models
- preclinical testing of TSLPRCARTxCD19 in bioluminescent CRLF2-R cell line MUTZ5 xenograft models
- preclinical testing of TSLPRCARTxCD22 in bioluminescent CRLF2-R cell line MUTZ5 xenograft models

Major task 5: TSLPR surface density quantification of phase 1 clinical trial ALL patient samples

- NIH TSLPRCART trial anticipated to open sometime in 2024 (delayed due to scientific and administrative holds during viral pandemic, IND submitted to FDA and placed on clinical hold pending resolution of specific FDA comments); estimated patient accrual over 2-3 years
- Flow cytometric immunophenotyping of bone marrow TSLPR surface antigen density at study entry (and relapse if applicable)

Milestones achieved: identification of optimal combinatorial TSLPRCART(s) *in vivo* in CRLF2+ ALLs, final data analysis. Awaiting clinical trial status update from NIH colleagues.

Progress to date:

1. Task 1: TSLPRxCD19 and TSLPRxCD22 construct engineering initially completed with *in vitro* and some *in vivo* optimization. Based on emerging data with an ongoing CD19xCD22 CAR T cell product at the University of Colorado, we generated modified versions of the CD19xTSLPR which will likely be amongst the lead constructs. In the next several months, we will pursue more detailed *in vivo* testing in CRLF2-rearranged ALL PDX models.
2. Task 2: Childhood and adult ALL PDX models expansion complete. Ten DS-ALL (some CRLF2-R and some CRLF2-wild-type) PDX models established and now s/p secondary expansion. Extensive genetic characterization of PDX models performed to confirm CRLF2 rearrangement status and other genetic alterations (see Table below).

DS-ALL PDX model	Disease status	Cytogenetics/alterations	RAS mutation	JAK mutation
TCH839	diagnosis	47,XX,+21, P2RY8-CRLF2	none	JAK2 Ile682Phe
TCHK150	diagnosis	47,XX,+21, IGH-CRLF2	none	None
DSALL47	diagnosis	47,XY,+21, P2RY8-CRLF2	none	JAK2L730F, K926R, I951L
DSALL515	relapse	47,XY,+21, P2RY8-CRLF2	NRAS G12D	JAK2 I682insGA
DSALL1846	diagnosis	47,XX,+21	NRAS G12V	JAK2 I951L
DSALL2068	relapse	47, X jdic(Y)(q1122), +21c	NRAS G12D	none
DSALL2407	relapse	47, X jdic(Y)(q1122), +21c	NRAS G12D	none
DSALL2945	relapse	47, X jdic(Y)(q1122), +21c	NRAS G12D	none
DSALL2116	diagnosis	47,XX,+21	NRAS G12D	copy loss
DSALL2297	second relapse	47,XX,+21	KRAS Ala146V	none

* serial relapses from same child s/p CD19 & CD22 immunotherapies

3. Task 3: MUTZ5 *in vitro* cytotoxicity and cytokine production studies completed. Methods optimized also for *in vivo* cytokine measurement assays from TSLPRCART-treated ALL xenograft mice.
4. Task 4: Detailed TSLPRCART and ruxolitinib *in vivo* testing in MUTZ5 xenograft models for dose and timing optimization completed. *In vivo* testing of TSLPRCART +/- ruxolitinib completed in 3 DS-ALL models with plans for bispecific CAR T cell testing with optimized constructs. Additional *in vivo* ruxolitinib + CD19CART studies were completed in cell line and PDX models for additional CAR T cell control experiments needed for a manuscript planned for submission in spring 2024. Dr Tasian also presented data about our Ph-like and DS-ALL preclinical testing to date at a small Down Syndrome leukemia-focused scientific meeting hosted by the Lejeune Foundation in October 2022 in Paris, France and the second annual meeting in September 2023 at the St Jude Children's Research Hospital hosted by Dr John Crispino.
5. Task 5: Phase 1 TSLPR CAR T cell clinical trial was delayed due to need for TSLPR clinical CAR construct re-optimization and some pandemic-related administrative delays at the NIH. The

IND was submitted to the FDA and was placed on clinical hold pending resolution of comments related to non-clinical safety and biodistribution studies which are now being completed with plans to submit amended IND to FDA by June 2024. We have optimized and quantified TSLPRCART site density on *CRLF2*-rearranged and non-rearranged DS-ALL PDX models (n=10) and multiple Ph-like ALL models (n=25) in preparation for evaluation of clinical trial samples. Additional grant applications submitted for potential alternative phase 1 clinical trial planning. The Fry and Tasian team is planning a successor two-site academic phase 1 trial of bispecific TSLPRxCD19CART immunotherapy at the University of Colorado/Children's Hospital Colorado and Children's Hospital of Philadelphia based in part upon results of these DoD-supported preclinical studies.

Specific Aim 2: Determine the preclinical efficacy of multi-specific CARTs and JAKi against *CRLF2*-R ALL

Major task 1: *in vivo* testing of TSLPRCARTs/TKIs peds ALL PDXs

- preclinical testing and efficacy comparison of TSLPRCART, CD19CART, CD22CART, TSLPRxCD19CART, TSLPRxCD22CART in 2-4 childhood *CRLF2*-R ALL PDX models (see tables below for cohort and total mouse numbers)
- preclinical testing and efficacy comparison of TSLPRCART + ruxolitinib in 2-4 childhood *CRLF2*-R ALL PDX models

Major task 2: *in vivo* testing of TSLPRCARTs/TKIs adult ALL PDXs

- preclinical testing and efficacy comparison of TSLPRCART, CD19CART, CD22CART, TSLPRxCD19CART, TSLPRxCD22CART in 2-4 adult *CRLF2*-R ALL PDX models
- preclinical testing and efficacy comparison of TSLPRCART + ruxolitinib in 2-4 adult ALL *CRLF2*-R ALL PDX models

Major task 3: *in vivo* testing of TSLPRCARTs/TKIs DS-ALL PDXs

- preclinical testing and efficacy comparison of TSLPRCART, CD19CART, CD22CART, TSLPRxCD19CART, TSLPRxCD22CART in 2-4 *CRLF2*-R DS-ALL PDX models
- preclinical testing and efficacy comparison of TSLPRCART + ruxolitinib in 2-4 DS-ALL *CRLF2*-R ALL PDX models

Major task 4: ALL TKI effects *in vitro* and *in vivo*

- Normal T cell donor flow cytometric immunophenotyping (pediatric Ph-like, adult Ph-like, children with trisomy 21/DS)
- T cell and ruxolitinib coincubation studies: T cell subset flow immunophenotyping, cell death (Annexin-V/PI flow cytometry, cell counting), cell proliferation (CSFE assays)
- TSLPRCART and ruxolitinib coincubation studies: cytokine quantification, effects on proliferation

Milestone(s) Achieved: selection of optimal TSLPRCART/TKI therapy *in vivo* in each *CRLF2*+ ALL subtype, final data analysis

Progress to date:

1. Task 1: Bispecific TSLPRxCD19 and TSLPRxCD22 CAR constructs made as above with *in vitro* and *in vivo* experiments in antigen-engineered Nalm-6 cell lines (CRISPR/Cas9) completed. More detailed testing in *CRLF2*-R ALL cell lines and PDX models planned in near future, but has been delayed due to timing of collaboration with biotech partner to make vector. PDX model studies have with TSLPR CAR T cells and ruxolitinib been completed. Ongoing discussions with industry colleagues regarding expanded bispecific constructs work and potential for phase 1 clinical trial development in pediatric patients.
2. Task 2: Adult PDX models established with *in vivo* activity of ruxolitinib assessed and compared to pediatric model data.

Task 3: Detailed TSLPRCART and ruxolitinib *in vivo* testing in MUTZ5 xenograft models for dose and timing optimization completed. Five large-scale *CRLF2*-R ALL PDX model studies now completed (3 Ph-like ALL, 2 DS-ALL). New studies of ruxolitinib withdrawal and/or ALL rechallenge (relapse) completed. Detailed correlative biology assessments of *in vivo* cytokine production and T cell activation/exhaustion markers also completed for some models. Data update presented at ASH 2021 (poster presentation by Dr Bagashev: <https://ashpublications.org/blood/article/138/Supplement%201/1705/480789/Precision-Co-Targeting-of-the-Thymic-Stromal> and EHA 2022 (https://journals.lww.com/hemasphere/Fulltext/2022/06003/P1421_BIMODAL_TARGETING_OF_CYTOKINE_RECEPTOR_LIKE.1305.aspx). Manuscript draft has been written with edits by coauthors ongoing and final control experiments as above now completed by new postdoctoral fellows and technicians in the Tasian laboratory (planned submission spring 2024). We experienced delays in animal studies in 2023 due to major infectious issues (*C. bovis*) that requiring culling of our entire immunocompromised NSG mouse colony at CHOP and re-derivation and repeat of several studies.

Task 4: Normal young adult T cell studies with ruxolitinib completed (n=4 healthy donors) *in vitro*. T cell functionality and immunophenotyping studies performed to elucidate potential effects of ruxolitinib. Also obtained normal T cells from DS patients at Colorado (unable to access easily at CHOP after much investigation, access in Colorado initially delayed during research pandemic) with studies in process.

Specific Aim 3: To delineate the impact of immunodeficiency associated with Down Syndrome and aging upon T transduction efficiency and functionality of CARTs

Major task 1: B-ALL adult patient T cell functionality for CAR engineering

- Identification of patient samples at Penn (n=20-30)
- T cell flow immunophenotyping, analysis of senescence biomarkers, pre/post-treatment Treg suppression
- RNAseq to measure TCR repertoire diversity before and after T cell expansion and CAR construct transduction

Major task 2: DS-ALL T cell functionality for CAR engineering

- Identification of patient samples at CHOP and Colorado (n=6-12)

- T cell flow immunophenotyping, analysis of senescence biomarkers, pre/post-treatment Treg suppression
- RNAseq to measure TCR repertoire diversity before and after T cell expansion and CAR construct transduction

Milestone(s) Achieved: elucidation of T cell biologic features that contribute to CAR T cell success and failure, final data analysis

Progress to date:

1. Task 1: Identified normal T cell donor sources at CHCO and CHOP/Penn. T cell activation and exhaustion biomarker panels designed with flow cytometry assays completed on first sample set (n=3 healthy donors). Single-cell/RNAseq studies of sorted CAR T cells done from above xenograft mice studies to assess T cell expression changes in vivo over time and will be included in main manuscript.
2. We have completed work on the assessment of CAR T cell function manufactured from DS vs non-DS samples. The results have been interesting and informative. Overall, there was no decrease in short-term effector function in CAR T cell generated from individuals with DS. In fact, there was a trend towards increased cytokine production that may be related to the recently identified type 1 interferon inflammatory state associated with DS (identified by Dr. Joaquin Espinosa from the University of Colorado (<https://pubmed.ncbi.nlm.nih.gov/31722205/>)). Preliminary data in repetitive stimulation assays has suggested increase susceptibility to T cell exhaustion. In collaboration with Dr. Espinosa we will continue to dissect the mechanism for these observation that will potentially contribute to rationale designs of CAR T cell constructs or combination therapies.
3. Task 2: Normal DS T cell specimens obtained at CHCO. CD19CART and TSLPRCART products made with preclinical in vitro optimization performed. Planning for *in vivo* evaluation in ALL cell line xenograft models and PDX models compared to non-DS T cell sources to assess and to compare anti-leukemia functionality and other characteristics. Dr Tasian asked to join DS leukemia scientific working group organised by the Jérôme Lejeune Foundation in Paris (first annual meeting rescheduled for October 2022) and also participated in first NCI ImpactT21 meeting in May 2022: <https://ncifrederick.cancer.gov/events/conferences/impact21>

Human and animal subjects: Coded human ALL specimens (n=12-18 pediatric *CRLF2*-R, 10 adult *CRLF2*-R, 6 DS-ALL; Site 2) have been created for use in PDX model experiments. Human T cell samples (n=20 adult, 10 DS patients) will be used (Sites 1 & 2). A total of 800 NSG mice are needed for Aims 1 and 2 ALL PDX studies (Sites 1 & 2). Use of coded patient specimens without PHI is not considered human subjects research by the NIH. The human *CRLF2*-R ALL cell lines MUTZ5 and MHH-CALL-4 will be used for some studies and were commercially purchased from the DSMZ cell line biorepository in Braunschweig, Germany.

Clinical trial subjects: Correlative TSLPR antigen density quantification studies of patients' ALL cells pre- and post-TSLPRCART treated on the TSLPRCART phase 1 clinical trial will be assessed in this proposal. The trial itself will be conducted outside of the DoD TTSA studies and is planned to open now hopefully in Q3 2021. Up to 33 patients with relapsed/refractory *CRLF2*+ ALL are estimated to enroll over 1.5-3 years.

	Year 1				Year 2			
Target quarterly enrollment	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4
Total patients	6	6-9	6-9	6-9	3-9	3-9	3-9	3-9

What was accomplished under these goals?

ACURO approvals for CHCO and CHOP were previously established. ACURO renewal/re-approval at CHOP was obtained this quarter.

Describe the Regulatory Protocol and Activity Status (if applicable).

Describe the Protocol and Activity Status for sections a-c, as applicable, using the format described for each section. If there is nothing significant to report during this reporting period, state “Nothing to Report.”

(a) Human Use Regulatory Protocols

Nothing to report to report for this quarter. No human subjects protocols are involved. Use of coded leukemia specimens from our CHOP biorepository is considered non-human subjects research by our Institutional Review Board.

This project does not include a clinical trial, but we propose to assess TSLPR site density in a subset of patients enrolled at the NIH on a TSLPR CAR trial. This trial is not yet open, and no specimens from clinical trial subjects have thus been collected.

This research project involves use of previously obtained, coded, banked human leukemia specimens or normal T cells from adults and children following obtainment of written informed consent. All samples in the leukemia biorepositories are annotated with genetic mutation data obtained from routine diagnostic clinical testing, but have been disassociated from patient health information (PHI) and coded with a unique specimen identifier (USI) number. Patient samples are banked routinely by the biorepository personnel by a third party data management repository, which may be accessed upon appropriate Institutional Review Board (IRB) approval. Leukemia specimens have been or will be used to create patient-derived xenograft (PDX) models for preclinical experimental testing in these DoD studies.

What opportunities for training and professional development has the project provided?

If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. “Training” activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-

on-one work with a mentor. “Professional development” activities result in increased knowledge or skill in one’s area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.

Five post-doctoral fellows and one junior faculty member have been involved and/or are actively leading hands-on work for this award in our respective laboratories at Children’s Hospital Colorado and Children’s Hospital of Philadelphia. Six young research technicians (who ultimately plan to attend medical or graduate school and one master’s-level senior research assistant have assisted or are assisting with CAR construct engineering, *in vitro* experiments, and/or *in vivo* animal studies in our labs. Trainees are encouraged to present their work formally at lab meetings and scientific symposia, as well as to submit abstracts to and attend national hematology meetings. Trainees meet regularly one-on-one with their mentors Drs Fry and Tasian and regularly attend scientific seminars/workshops at their institutions to increase their knowledge and continue their career development. Our trainees presented two abstracts (one poster, one oral presentation) at the American Society of Hematology 2021 annual meeting and one poster presentation at the European Hematology Association 2022 annual meeting for work related to this project, which is wonderful visibility for our trainees and their career development.

How were the results disseminated to communities of interest?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.

Research-in-progress and results have been shared locally with other scientific researchers at our institutions. During the past year, updates about this work were shared via oral presentation at the American Society of Hematology annual meeting in December 2021 and European Hematology Association annual meeting in June 2022.

Updates about this DoD TTSA project have also been shared via the NCI Pediatric Immunotherapy Discovery and Development Network (PI-DDN) meetings given shared scientific interests (Fry, Tasian) with virtual talks by Fry and Tasian lab trainees in January 2022 at our monthly PI-DDN meeting and an in-person oral presentation by Dr Tasian in October 2022 at the PI-DDN annual meeting in North Bethesda, Maryland. Related studies from our separate NCI PI-DDN work were also presented at our American Society of Hematology 2021 annual meeting.

- <https://www.sciencedirect.com/science/article/pii/S0006497121023983>
- https://journals.lww.com/hemasphere/Fulltext/2022/06003/P1421_BIMODAL_TARGETING_OF_CYTOKINE_RECEPTOR_LIKE.1305.aspx

We also shared updates about the DS-ALL-specific component of this project at two annual ‘think tank’ meetings of Down syndrome leukemia-focused scientists in October 2022 and September 2023.

What do you plan to do during the next reporting period to accomplish the goals?

If this is the final report, state “Nothing to Report.”

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

We will continue to make experimental progress to achieve the aims of the award according to the proposal timeline. We will continue to submit abstracts of our work and will plan research manuscript writing of initial results in the next year of this award. A major manuscript of our above TSLPRCART + ruxolitinib combinatorial preclinical studies is being written with anticipated submission in early 2024 (Balestra, Niswander, Bagashev ... Fry/Tasian).

4. **IMPACT:** *Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:*

What was the impact on the development of the principal discipline(s) of the project?

If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

We have made important discoveries about the impact of the JAK inhibitor ruxolitinib upon normal T cell and TSLPR CAR T cell function *in vitro* and *in vivo* in our leukemia studies. These preclinical results will definitively impact our design of and planning for clinical trials testing new precision medicine treatments for patients with *CRLF2*-rearranged ALL. In addition to our established Ph-like ALL models, we have also successfully developed important new mouse models of pediatric Down Syndrome-associated ALL and tested our combinatorial CAR T cell + TKI strategy, validating earlier observations and data in our Ph-like ALL preclinical models. These models collectively provide a critical resource for this project and can also be shared with the greater scientific community in the future. Importantly, our DS-ALL PDX models are of major interest to a newly-formed Down Syndrome leukemia scientific group recently established with support of the Lejeune Foundation in Paris with some of our DoD TTSA data shared by Dr Tasian at meetings in October 2022 and September 2023.

What was the impact on other disciplines?

If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

Results from our work have broad implications for studies of other cellular immunotherapies and tyrosine kinase inhibitors in human cancer models and clinical trial development. We have learned that ruxolitinib suppresses the functionality of, but does not kill, CAR T cells. We anticipate that this inhibitor could be broadly used to mitigate cytokine release syndrome induced by CAR T cells and non-cellular immunotherapies beyond TSLPRCART studied in this project. This could be a particularly useful adjunctive approach while tocilizumab and other medications are on (inter)national shortage during the viral pandemic and beyond.

What was the impact on technology transfer?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:

- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*
- *adoption of new practices.*

Nothing to report in this reporting period. Our prior TSLPR monovalent CAR construct was licensed by Miltenyi/Lentigen. Some of multispecific TSLPR CAR constructs (TSLPRxCD19 and TSLPRxC22) were developed in collaboration with Miltenyi. Miltenyi has initiated multiple CAR trials worldwide, including trials using CAR formats targeting multiple antigens. As such, there is the potential for future engagement of an experienced biotech company to facilitate translation of these results from these preclinical studies to clinical trials. Drs Fry and Tasian are also planning academic bench-to-bedphase 1 clinical trials based upon their

What was the impact on society beyond science and technology?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:

- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

Nothing to report at this time.

5. **CHANGES/PROBLEMS:** *The PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, “Nothing to Report,” if applicable:*

Changes in approach and reasons for change

Describe any changes in approach during the reporting period and reasons for these changes. Remember that significant changes in objectives and scope require prior approval of the agency.

Nothing to report at this time. Like many others, our laboratories experienced pandemic-related delays in 2020-2021 with particular ‘ramp down’ of animal studies and some supply chain shortages. Despite these issues, we have remained on track with needed research progress accomplished.

Actual or anticipated problems or delays and actions or plans to resolve them

Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

Please note that our research laboratories were closed from March to July 2020 due to institutional research restrictions during the COVID-19 pandemic. Both Fry and Tasian laboratories were now fully operational, although some intermittent supply chain issues have persisted and needed extra timing to ‘ramp up’ animal breeding and *in vivo* experiments have occurred this year. As aforementioned, we had major infectious issues with the Tasian laboratory’s mouse facility at CHOP that required culling of the entire immunocompromised colony and re-establishment of NSG mice over a period of several months in 2023, which led to experimental delays and markedly increased costs of replacement mouse purchasing. All needed *in vivo* experiments required for our major milestones and planned manuscript submission have now been completed or are back on track for continued studies needed for this project.

Changes that had a significant impact on expenditures

Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

Due to laboratory shut-downs from March to July 2020 resulting from the COVID-19 pandemic, personnel effort, experimental expenditures, and animal costs were lower than anticipated. The Tasian laboratory cost-shared some laboratory personnel efforts during this time with hospital-sourced funding for COVID-19-related clinical support and research while our laboratories were shut down, which resulted in some personnel support carry-over. During the past year, we have been working at full-speed in both Tasian and Fry laboratories to make continued progress on-track for this project.

The laboratory animal facility at CHOP unfortunately had a major *C bovis* infection in spring/summer 2022, resulting in the Tasian Laboratory and others needing to cull their entire animal colonies of NSG mice for *in vivo* studies in August 2022 and again in May 2023. This has resulted in 3 month delays in animal studies and significant expense to re-derive our NSG breeding colony and to purchase experimental animals. No *in vivo* studies for this project were able to be performed for three months as a result of this major issue and needed time to re-establish a safe and ‘clean’ NSG colony for experimental studies. Work resumed in October 2022, then was again halted in May-July 2023 due to recurrent infectious issues and need to re-establish a major animal colony and to repeat experiments in summer/autumn 2023.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

Significant changes in use or care of human subjects

Nothing to report.

Significant changes in use or care of vertebrate animals

Nothing to report.

Significant changes in use of biohazards and/or select agents

Nothing to report.

6. PRODUCTS: *List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state “Nothing to Report.”*

- **Publications, conference papers, and presentations**

Report only the major publication(s) resulting from the work under this award.

Journal publications. *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume; year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Nothing to Report

Books or other non-periodical, one-time publications. *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Several abstracts of our work have been submitted to date:

1. Bagashev *et al.* Precision Co-Targeting of TSLPR in CRLF2-Rearranged Ph-like ALL. European Hematology Association annual meeting (June 2022; Vienna, Austria - poster presentation by Dr Bagashev)
https://library.ehaweb.org/eha/2022/eha2022-congress/358279/asen.bagashev.bimodal.targeting.of.cytokine.receptor-like.factor.2.28crlf229.html?f=menu%3D6%2Abrowseby%3D8%2Asortby%3D2%2Amedia%3D3%2Ace_id%3D2233%2Aot_id%3D26860%2Amarker%3D1769%2Afeatured%3D17676

2. Bagashev *et al.* Precision Co-Targeting of TSLPR in *CRLF2*-Rearranged Ph-like ALL. American Society of Hematology annual meeting (December 2021; Atlanta, Georgia - poster presentation by Dr Bagashev)
<https://www.sciencedirect.com/science/article/pii/S0006497121036697>
3. Bagashev *et al.* Precision Co-Targeting of the Thymic Stromal Lymphopoietin Receptor in Childhood *CRLF2*-Rearranged Acute Lymphoblastic Leukemia. International Society of Pediatric Oncology annual meeting (October 2021; Honolulu, Hawaii - oral presentation by Dr Tasian; meeting held virtually due to viral pandemic)
<https://onlinelibrary.wiley.com/doi/10.1002/pbc.29349?mi=49buyt&af=R&AllField=seminars+dialysis&content=articlesChapters&target=default>
4. Bagashev *et al.* Co-Targeting of Thymic Stromal Lymphopoietin Receptor Signaling to Decrease Immunotherapeutic Resistance in *CRLF2*-Rearranged ALL. 12th Biennial Childhood Leukemia and Lymphoma Symposium abstract CLLS20-0053 (May 2020; Valencia, Spain - oral presentation by Dr Tasian; meeting delayed to March 2021 and held virtually due to viral pandemic) <https://www.clls2021.org/programme>
5. Ross *et al.* Multi-Antigen Targeting of CD19, CD22 and TSLPR to Prevent Ph-like ALL Resistance. AACR 2020 annual meeting abstract #3234 (Chicago, Illinois; June 2020 - poster presentation by Dr Ross; meeting held virtually due to viral pandemic)
https://cancerres.aacrjournals.org/content/80/16_Supplement/3234

Other publications, conference papers and presentations. *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.*

Nothing to report.

- **Website(s) or other Internet site(s)**

List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

Nothing to report.

- **Technologies or techniques**

Identify technologies or techniques that resulted from the research activities. Describe the technologies or techniques were shared.

During the course of this project, we have successfully developed several important new patient-derived xenograft (PDX) models of *CRLF2*-overexpressing Ph-like ALL and Down Syndrome-associated ALL. These PDX models are a critical resource for this project for *in*

vivo investigation and could also be shared with the greater scientific community in the future and were offered for sharing amongst the newly-formed Down Syndrome leukemia consortium sponsored by the Lejeune Foundation as above.

- **Inventions, patent applications, and/or licenses**

Identify inventions, patent applications with date, and/or licenses that have resulted from the research. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

Nothing to report.

- **Other Products**

Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment and /or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:

- *data or databases;*
- *physical collections;*
- *audio or video products;*
- *software;*
- *models;*
- *educational aids or curricula;*
- *instruments or equipment;*
- *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
- *clinical interventions;*
- *new business creation; and*
- *other.*

Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate “no change”.

Name: Terry Fry, MD (CHCO)

Project Role: PI

Researcher Identifier (e.g. ORCID ID): 0000-0001-8044-5226

Nearest person month worked: 2.4 Calendar

Contribution to Project: Dr Fry prepared documentation necessary for initiation of research to be conducted on the proposal and oversees the entire project with Dr. Tasian.

Name: Lillie Leach, BS (CHCO)

Project Role: research technician

Researcher Identifier (e.g. ORCID ID): none

Nearest person month worked: 1.2 Calendar

Contribution to Project: Ms Leach has assisted with TSLPR CAR T cell manufacturing in the Fry laboratory following departure of Dr Ross from CHCO at completion of her post-doctoral fellowship. All animal studies at CHCO commenced after obtainment of ACURO approval.

Name: Sarah Tasian, MD (CHOP)

Project Role: Co-PI

Researcher Identifier (e.g. ORCID ID): 0000-0003-1327-1662

Nearest person month worked: 2.4 Calendar

Contribution to Project: Dr Tasian prepared documentation necessary for initiation of research to be conducted on the proposal and oversees the entire project with Dr. Fry.

Name: Asen Bagashev, PhD (CHOP)

Project Role: Scientist

Researcher Identifier (e.g. ORCID ID): 0000-0003-9900-8106

Nearest person month worked: 3 Calendar

Contribution to Project: Dr Bagashev was responsible in this project for *in vitro* and *in vivo* studies performed at CHOP in the Tasian laboratory. He completed his training in the Tasian lab in August 2022 and has moved to a principal scientist position in industry. All animal studies at CHCO commenced after obtainment of ACURO approval.

Name: Tommaso Balestra, PhD (CHOP)
Project Role: Post-doctoral fellow
Researcher Identifier (e.g. ORCID ID): none
Nearest person month worked: 3 Calendar
Contribution to Project: Dr Balestra joined the Tasian laboratory in July 2022 and is directing additional studies *in vitro* and *in vivo* of CD19CART + ruxolitinib in ALL cell lines and our DS-ALL PDX models. All animal studies at CHCO commenced after obtainment of ACURO approval.

Name: Robert Chen, BS (CHOP)
Project Role: Research technician
Researcher Identifier (e.g. ORCID ID): none
Nearest person month worked: 6 Calendar
Contribution to Project: Mr Chen joined the Tasian laboratory in June 2022. He is responsible for ALL PDX model maintenance and experimental *in vivo* studies at CHOP in the Tasian laboratory. Mr Chen is working on Ph-like and DS-ALL PDX model studies of ruxolitinib and CD19CART in collaboration with Dr Balestra. All animal studies at CHCO commenced after obtainment of ACURO approval.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

Nothing to report.

What other organizations were involved as partners?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.

Provide the following information for each partnership:

Organization Name: Stand Up to Cancer co-funded by the Emily F Whitehead Foundation

Location of Organization: New York/California and Pennsylvania

Partner's contribution to the project (identify one or more): We received a two-year Phillip A Sharp Award for Innovation in Collaboration for an extension of these Department of Defense Studies (funded June 2019 to May 2021 with NCE until May 2022 due to pandemic delays). This additional funding has primarily supported development and serial transplantation of our Down Syndrome ALL PDX models in costly immunocompromised mice with detailed genetic characterization. There is no budgetary overlap with the Department of Defense award.

We also applied for extended support for TSLPRxCD19CART bench to bedside development to phase 1 clinical trial investigation to the CHOP Center for Cell and Gene Therapy Seed Grant mechanism in March 2022 (declined for funding) and the V Foundation for Cancer Research in October 2022 (pending) based upon the successful results of this DoD TTSA-supported project.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: *For collaborative awards, independent reports are required from BOTH the Initiating Principal Investigator (PI) and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to <https://ers.amedd.army.mil> for each unique award.*

Please see detailed reports and allocation of research efforts for Drs Fry and Tasian detailed in our quarterly progress reports and most recent update in October 2020.

QUAD CHARTS: *If applicable, the Quad Chart (available on <https://www.usamraa.army.mil>) should be updated and submitted with attachments.*

9. **APPENDICES:** *Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.*

The following publications acknowledge support from this DoD TTSA mechanism:

1. Hurtz *et al* JCI 2020: <https://pubmed.ncbi.nlm.nih.gov/32191635/>
2. Loftus *et al* Haematologica 2021: <https://pubmed.ncbi.nlm.nih.gov/32414848/>
3. Thomas *et al* Leukemia 2021: <https://pubmed.ncbi.nlm.nih.gov/34193976/>
4. Niswander *et al* Haematologica 2021: <https://pubmed.ncbi.nlm.nih.gov/34196168/>
5. Ding *et al* Clinical Cancer Research 2021: <https://pubmed.ncbi.nlm.nih.gov/34210682/>
6. Qin *et al* JITC 2021: <https://pubmed.ncbi.nlm.nih.gov/34531250/>
7. Niswander *et al* Haematologica 2022: <https://pubmed.ncbi.nlm.nih.gov/35950535/>

FIGURES:

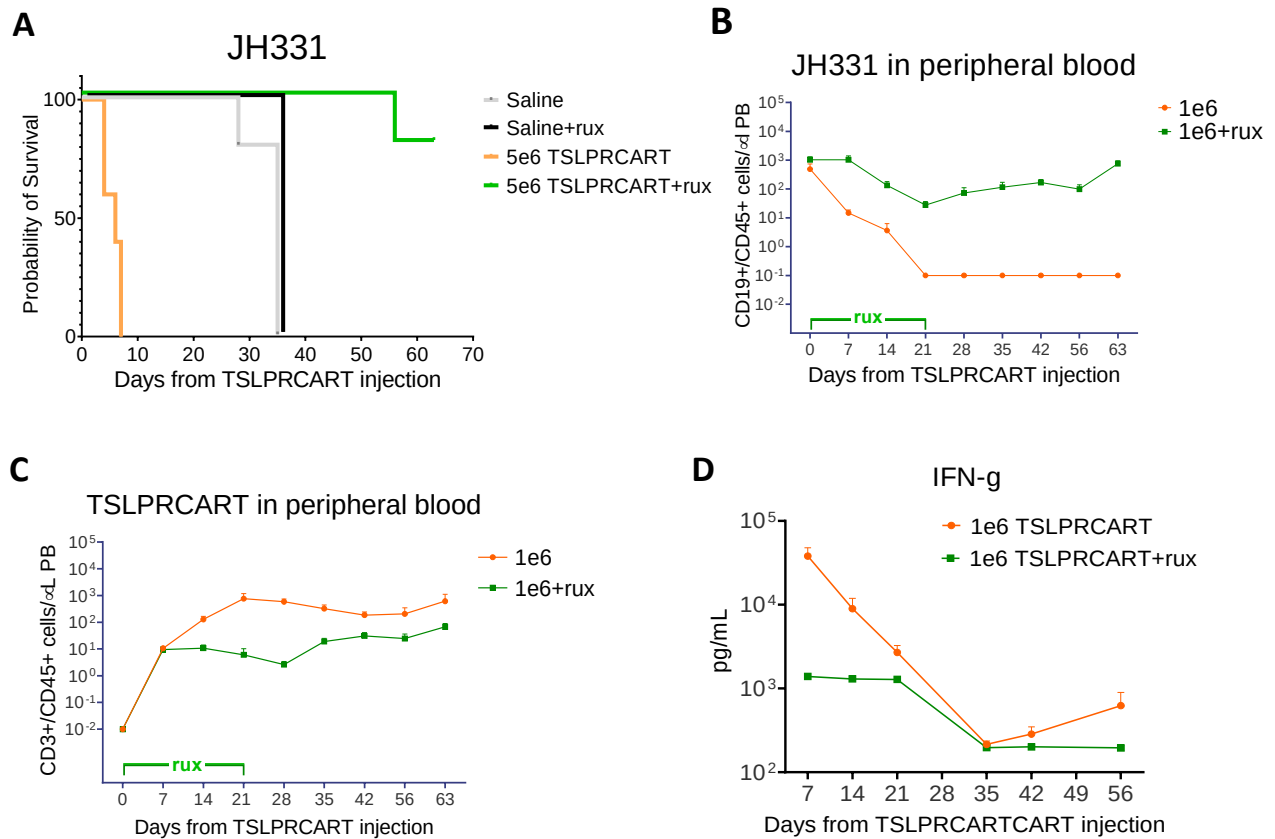


Figure 1. Simultaneous JAK inhibition ameliorates *in vivo* CART-associated mortality and suppresses TSLPRCART activity in a ruxolitinib-insensitive *IGH::CRLF2* Ph-like ALL patient-derived xenograft model. (A) Kaplan–Meier Curves demonstrating survival of mice engrafted with JH331 (*IGH::CRLF2*) PDX mouse model after treatment with intravenous 5e6 TSLPRCART or saline control with or without simultaneous exposure to ruxolitinib chow beginning on day 0 through day 21. (B) Human CD19+/CD45+ ALL cells and (C) human CD3+/CD45+ T cells were quantified weekly via flow cytometric analysis of sampled peripheral blood from mice engrafted with JH331 (*IGH::CRLF2*) PDX and treated with lower-dose 1e6 TSLPRCART with or without simultaneous exposure to ruxolitinib chow beginning on day 0 through day 21 (treatment exposure indicated by green bar). (D) IFN- γ levels in plasma isolated from JH331 mice treated as in B and C with 1e6 TSLPRCART are lower in mice treated with simultaneous ruxolitinib.

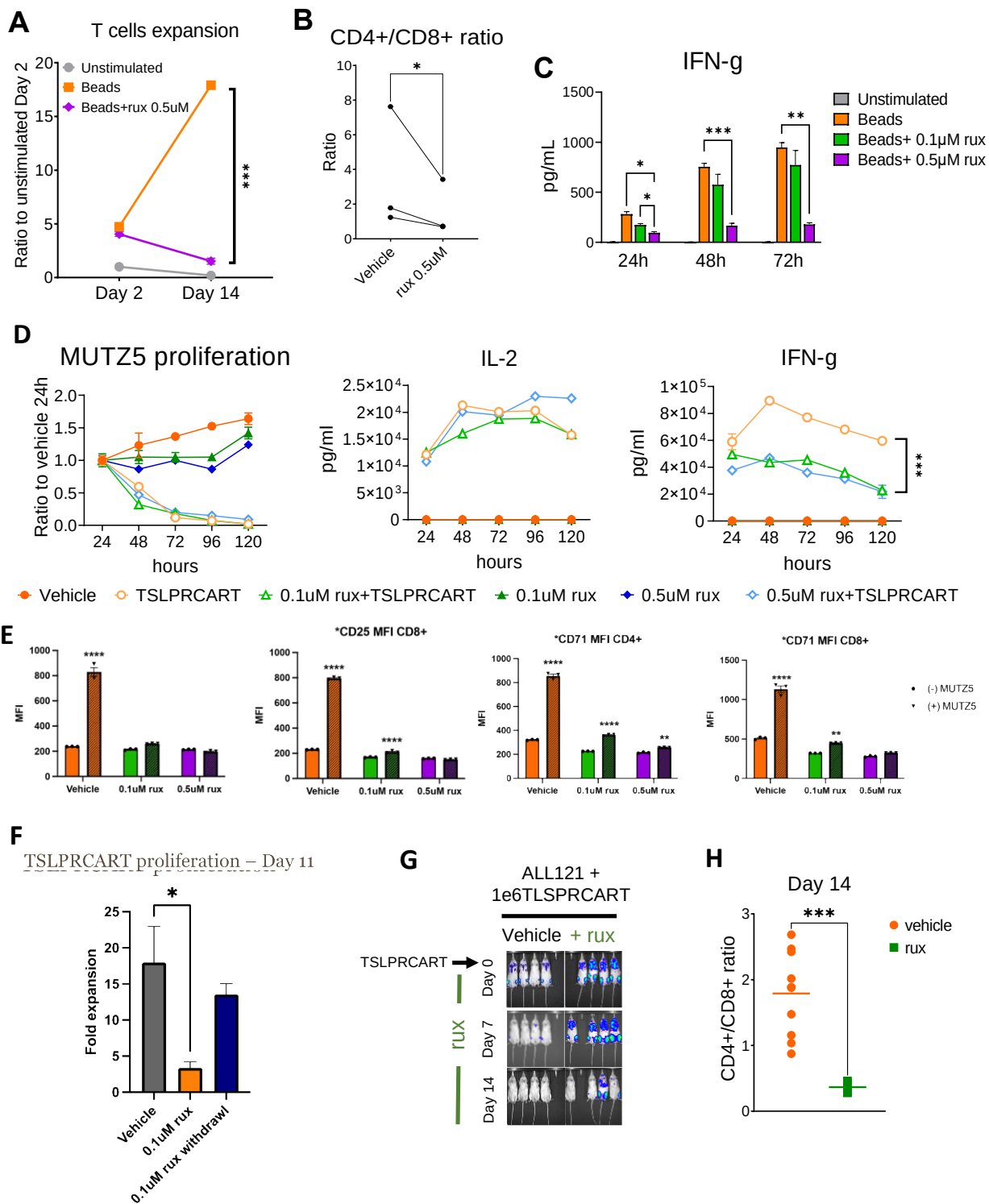


Figure 2. Ruxolitinib co-administration inhibits T cell expansion and cytokine production and alters CD4/CD8 T cell ratio. (A) Normal T cells from healthy donors (n=3 donors) were cultured with CD3/CD28 beads with or without ruxolitinib (0.5 μ M) *in vitro* for two weeks, with unstimulated T cells cultured without CD3/CD28 beads as a control. T cell expansion was assessed via Cell Titer Glo viability assay. Depicted data represent the mean of 3 independent T cell donors plated in technical triplicates $\pm$ standard error of the mean (SEM) are shown. **(B)** The ratio of CD4/CD8⁺ T cells was determined by flow cytometric immunophenotyping in normal healthy donor T cells exposed to 0.5 μ M ruxolitinib for 72 hours. **(C)** IFN γ in culture supernatant of CD3/CD28 bead activated T cells treated with or without ruxolitinib was quantified by ELISA at the indicated timepoints. **(D)** Luciferase-transduced *CRLF2*-rearranged ALL cell line MUTZ5 was co-incubated *in vitro* with TSLPRCART T cells at 1:15 E (effector):T (target) ratio and either vehicle or ruxolitinib (0.1 and 0.5 μ M). ALL cytotoxicity via luciferase reporter assays (left) and production of IL-2 (middle) and IFN γ (right) via ELISA at the indicated time point. **(E)** TSLPRCART were incubated in the absence (solid bars) or presence (lined bars) of MUTZ5 B-ALL cells in 1:1 E:T ratio for 24 hours with or without ruxolitinib. Induction of CD25 (left two panels) or CD71 (right two panels) surface expression on CD4⁺ and CD8⁺ T cells was evaluated by flow cytometry analysis. Quantification of median fluorescent intensity for CD4⁺ and CD8⁺ T cell subsets with n=3 technical replicates for each condition is displayed $\pm$ standard deviation. Ruxolitinib blunts upregulation of cell surface T cell activation markers CD25 and CD71 on both CD4⁺ and CD8⁺ TSLPRCART T cells when co-incubated with target ALL cells. **(F)** TSLPRCART T cells were co-incubated 1:1 with target MUTZ5 with or without ruxolitinib. Every 3-4 days, TSLPRCART were sampled for enumeration by flow cytometry to determine expansion from prior plating and the remaining cells re-stimulated 1:1 with MUTZ5 and fresh ruxolitinib containing media. On day 7, a subset of ruxolitinib-exposed TSLPRCART were replated in the absence of ruxolitinib (withdrawl) for 4 days. Ruxolitinib-induced decrease in TSLPRCART expansion is reversible with ruxolitinib withdrawal. **(G)** Representative bioluminescent imaging demonstrating the effect of simultaneous ruxolitinib on the anti-leukemia activity of TSLPRCART against patient-derived xenograft ALL121 (*IGH::CRLF2*, JAK2 R683G). Luciferase-expressing ALL121 cells were injected (1e6) intravenously (IV) into NSG mice. Once engraftment was confirmed by bioluminescent imaging, mice were treated with 1e6 TSLPRCART cells IV and simultaneously exposed to ruxolitinib or control chow. **(H)** At day 14 of treatment peripheral blood CD4⁺ and CD8⁺ T cells were quantified by flow cytometry. Statistical analysis in A and C was performed with two-way ANOVA with Tukey post-test for multiple comparisons and in D, E, and F with one-way ANOVA with Tukey post-test for multiple comparisons. A paired T test was performed in B. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

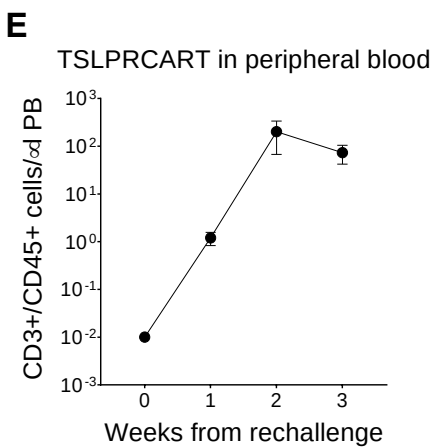
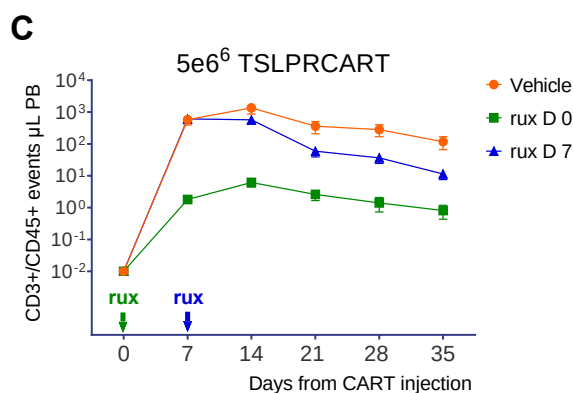
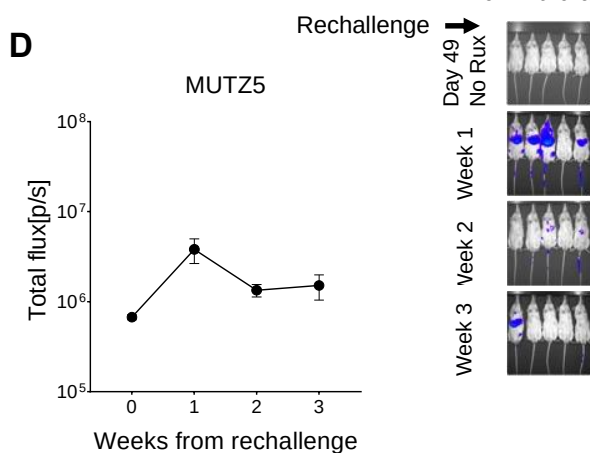
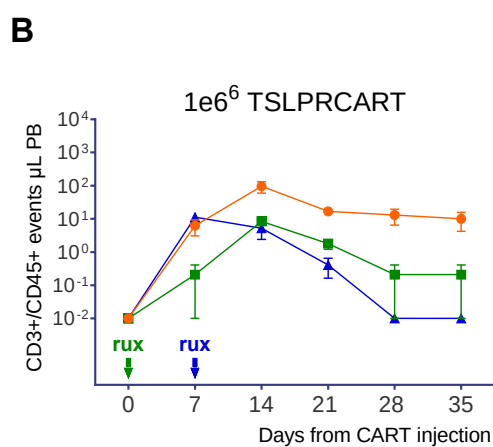
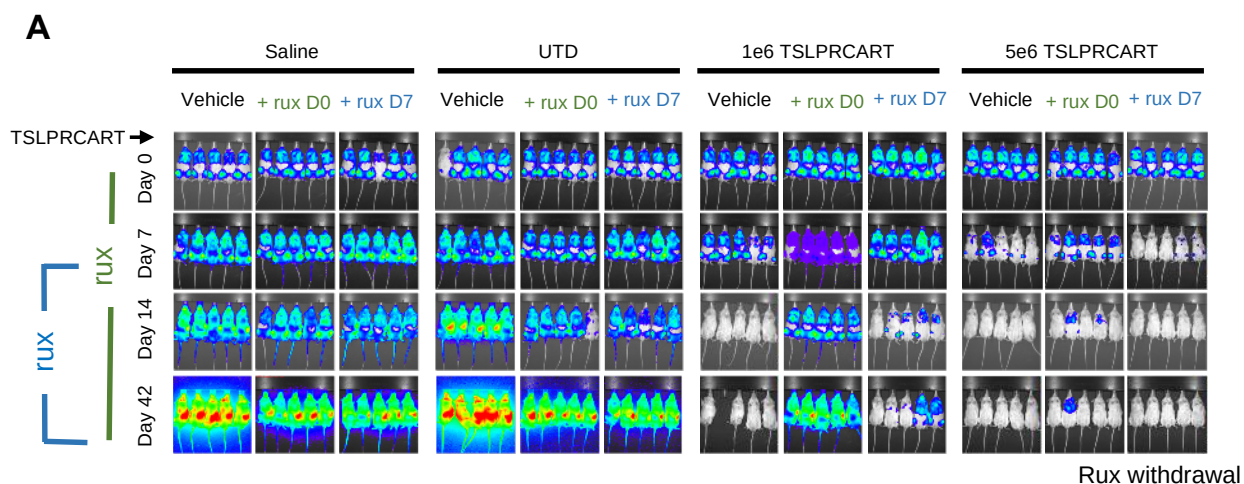
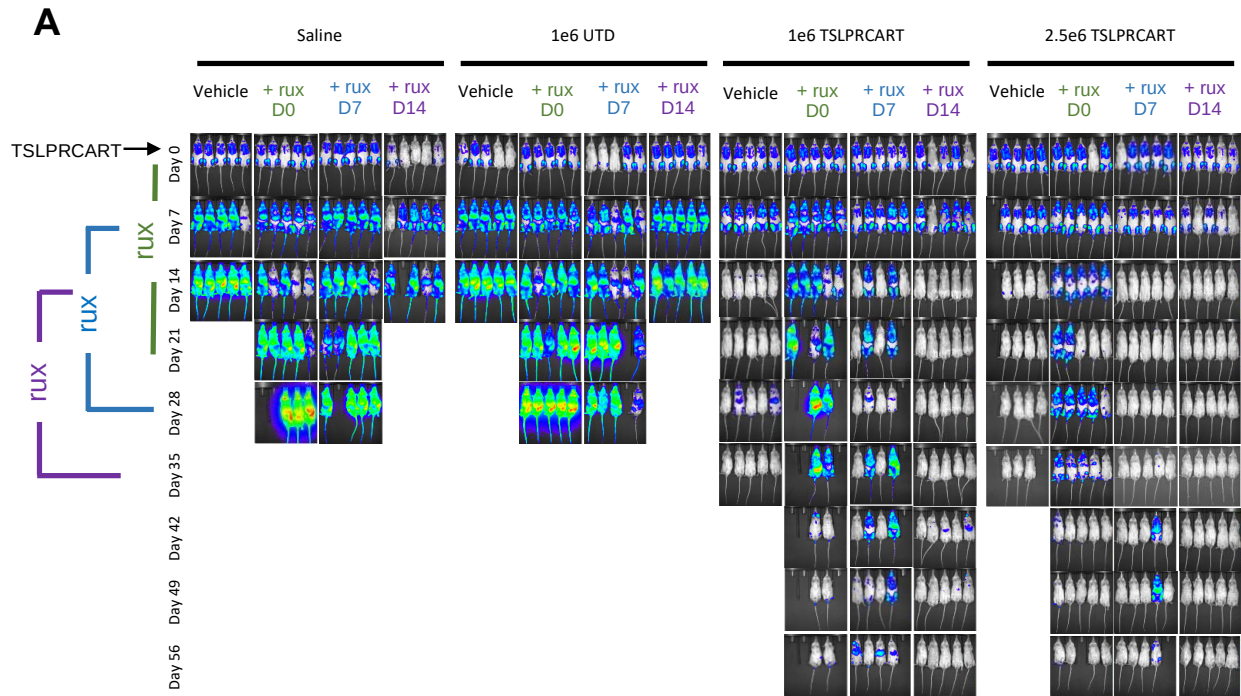
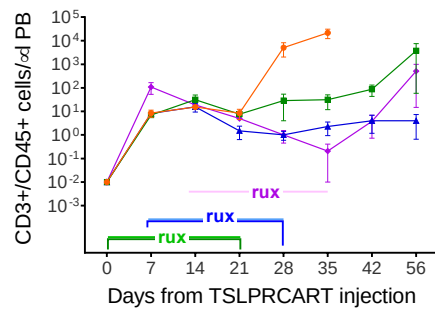


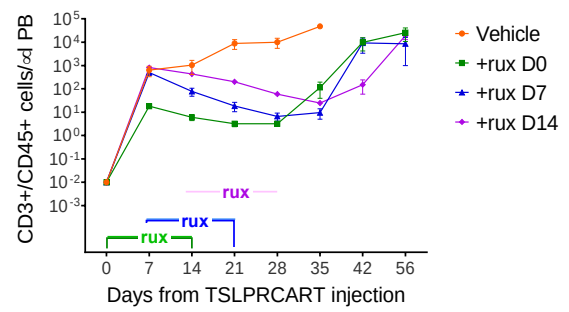
Figure 3. Ruxolitinib-associated inhibition of TSLPRCART is reversible and TSLPRCART functionality is improved with delayed co-administration. (A) Luciferase-transduced MUTZ5 cells (c) were injected IV in NSG mice. Once engraftment was documented by BLI, cohorts of 5 mice were randomized to IV treatment with saline, 1×10^6 untransduced T cells (UTD), or high (5×10^6) or low (1×10^6) dose TSLPRCART. As indicated, ruxolitinib (rux) chow was added at day 0 (green) contemporaneously with T cell treatments or delayed on day 7 (blue). Leukemia progression was monitored weekly by BLI. Peripheral blood was sampled weekly and human CD3+/CD45+ T cells were quantified by flow cytometry in mice receiving **(B)** 1×10^6 low dose TSLPRCART and **(C)** 5×10^6 high dose TSLPRCART. **(D)** Ruxolitinib treatment was discontinued on day 42 for the mice who had received high dose TSLPRCART and delayed ruxolitinib on day 7 (lower panel of **A**). After 49 days without ruxolitinib exposure, mice received 10×10^6 luciferase-expressing MUTZ5 cells IV. **(D)** Graphical representation of summary BLI radiance data following leukemia rechallenge is displayed. **(E)** Peripheral blood was sampled weekly following MUTZ5 rechallenge and human CD3+/CD45+ T cells were quantified by flow cytometry. TSLPRCART with previous delayed-ruxolitinib exposure expanded following leukemia rechallenge and low leukemia burden was maintained.



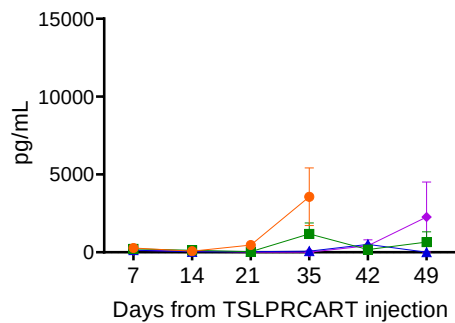
B 1e6 TSLPRCART in peripheral blood



2.5e6 TSLPRCART in peripheral blood



C 1e6 TSLPRCART- INF γ



2.5e6 TSLPRCART- INF γ

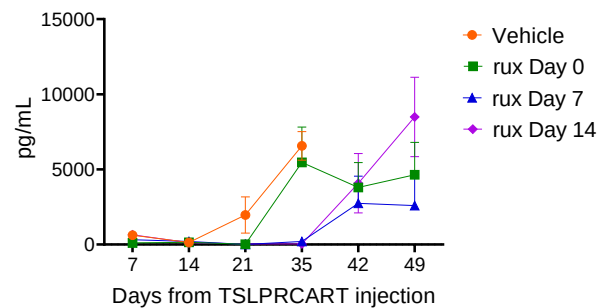


Figure 4. Delayed JAK inhibitor co-treatment improves *in vivo* TSLPRCART efficacy against a ruxolitinib-sensitive *IGH::CRLF2* Ph-like ALL PDX model. (A) Luciferase-transduced *IGH::CRLF2*, JAK2-mutant PDX model ALL121 cells (1×10^6) were injected IV in NSG mice. Once engraftment was documented by BLI, cohorts of 5 mice were randomized to IV treatment with saline, 1×10^6 untransduced T cells (UTD), or high (5×10^6) or low (1×10^6) dose TSLPRCART. As indicated, ruxolitinib (rux) chow was added at day 0 (green) contemporaneously with T cell treatments or delayed on day 7 (blue) or day 14 (purple). Ruxolitinib treatment window was 21 days. Leukemia burden was monitored weekly with BLI. (B) Peripheral blood was sampled weekly and human CD3+/CD45+ T cells were quantified by flow cytometry in mice receiving 1×10^6 low dose TSLPRCART (left panel) and 5×10^6 high dose TSLPRCART (right panel). (C) IFN- γ was measured by ELISA in plasma prepared from weekly peripheral venous blood from mice receiving 1×10^6 low dose TSLPRCART (left panel) and 5×10^6 high dose TSLPRCART (right panel). A sequenced three-week course of ruxolitinib co-therapy beginning after the initial peak of TSLPRCART expansion (day 14) improves long-term clearance of Ph-like ALL.

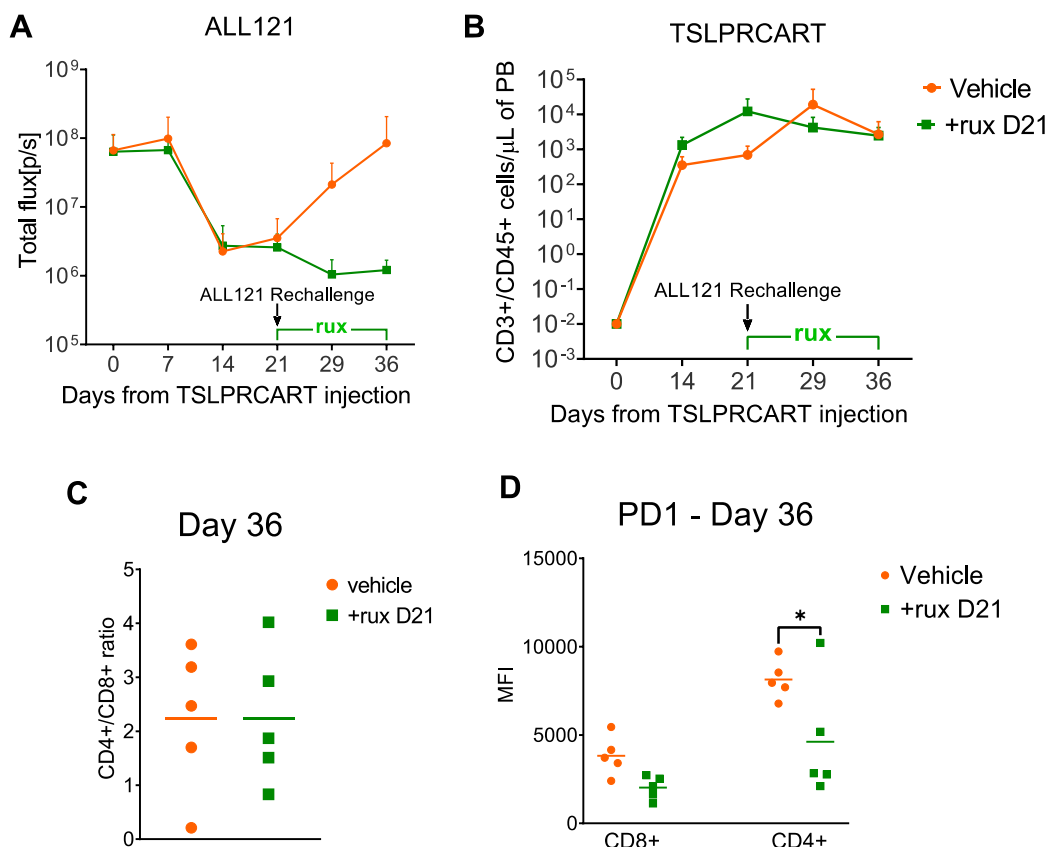


Figure 5. Maintenance ruxolitinib following TSLPRCART-induced leukemia clearance prevents ALL relapse *in vivo*. (A) Luciferase-transduced IGH::CRLF2, JAK2-mutant ALL121 PDX cells (1×10^6) were injected IV in NSG mice. Once engraftment was documented by BLI, all mice were treated IV with 2.5×10^6 TSLPRCART cells. Weekly BLI measurements are displayed graphically by total flux. On day 21, once leukemia was cleared by TSLPRCART below the level of initial engraftment by BLI, mice were rechallenged with 1×10^7 luciferase-expressing ALL121 cells IV and randomized to continue receiving control chow (orange) or transitioned to ruxolitinib-infused chow (green) for 2 weeks. Mice co-treated with ruxolitinib at ALL rechallenge remained in remission while control mice experienced leukemia progression (B) Flow cytometric quantification of CD3+ T cells in the peripheral blood revealed the addition of ruxolitinib did not affect TSLPRCART number. (C) At end of study, the ratio of CD4/CD8+ T cells isolated from spleens was determined by flow cytometry and was not altered by late ruxolitinib exposure. (D) Surface expression of exhaustion marker PD-1 in CD4+ and CD8+ T cell subsets is reported as median fluorescence intensity measured by flow cytometric analyses. PD-1 expression on TSLPRCART is decreased with late ruxolitinib co-treatment. Statistical analyses in D were performed by Two-way ANOVA with Tukey post-test for multiple comparisons * $p < 0.05$.

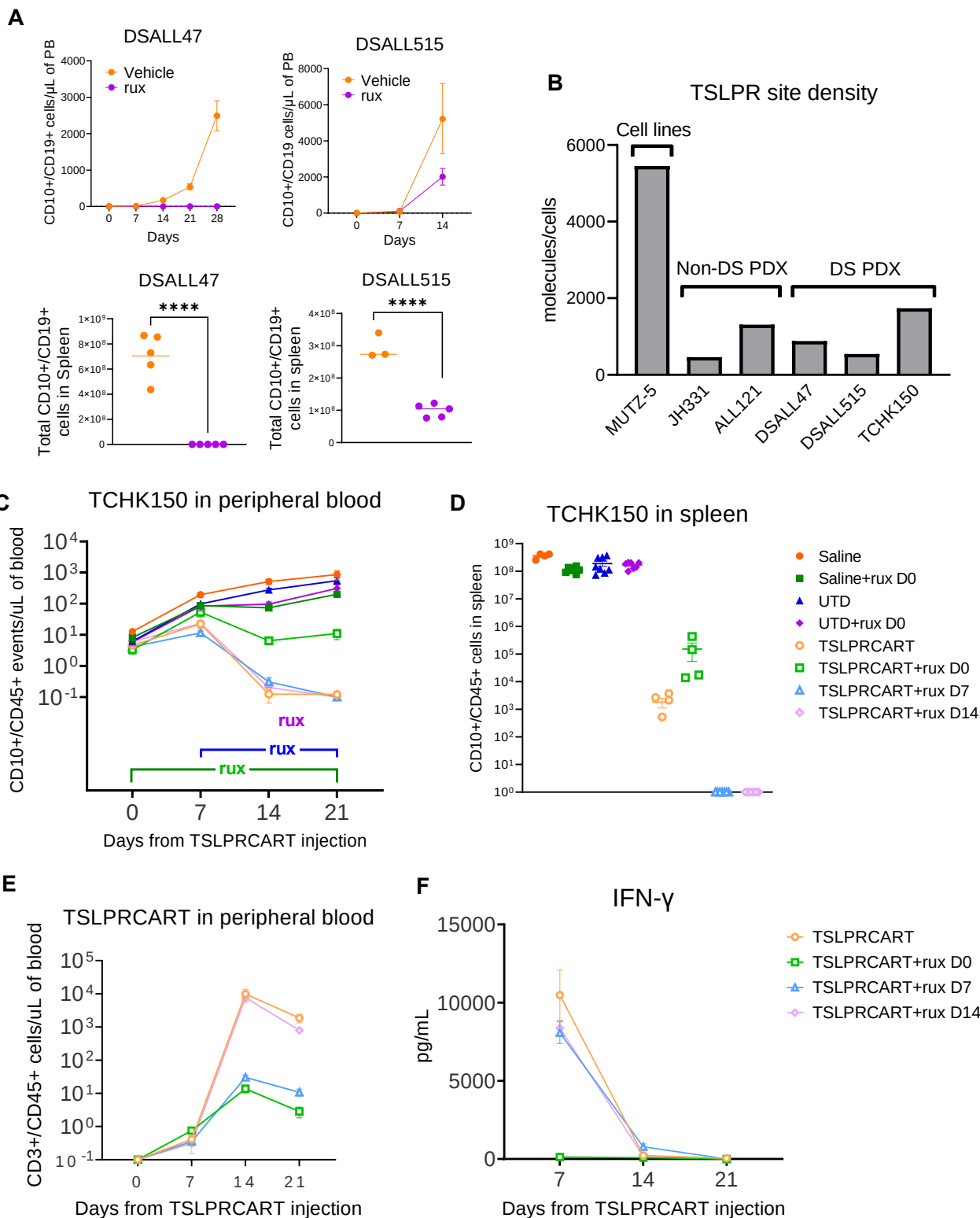


Figure 6. Ruxolitinib and TSLPRCART are highly effective against P2RY8/CRLF2 Down Syndrome ALL models *in vivo*. (A) NSG mice were engrafted with 5×10^6 DSALL515 (left) or DSALL47 (right) PDX cells. Once $>1\%$ CD10 $^+$ /CD19 $^+$ human ALL cells were detectable in the peripheral blood, mice (n=3-5/group) were randomized to treatment with control chow (orange) or ruxolitinib- infused chow (purple). ALL progression was monitored by weekly flow cytometric analysis of human CD10 $^+$ /CD19 $^+$ cells in peripheral blood (top panels) and quantified in spleens at end of study (bottom panels), which was determined by rate of leukemia progression in control mice for each model (14 days for DSALL515 and 28 days for DSALL47). (B) Flow cytometric quantification of TSLPR surface antigen density on *CRLF2*-R ALL models used in this study demonstrates similar levels in DS and non-DS PDX models. (C) TSLPRCART and ruxolitinib co-treatment was tested in mice engrafted with DS-ALL PDX model TCHK150. NSG mice were engrafted with 1×10^6 TCHK150 PDX cells. Once $>1\%$ CD10 $^+$ /CD19 $^+$ human ALL cells were detectable in the peripheral blood, mice were randomized to IV treatment with saline, 2.5×10^6 control UTD T cells, or 2.5×10^6 TSLPRCART cells. Ruxolitinib- infused chow was added for simultaneous treatment on day 0 (green) or for delayed co-treatment on day 7 (blue) or day 14 (purple). ALL progression was monitored by weekly flow cytometric analysis of human CD10 $^+$ /CD19 $^+$ cells in peripheral blood (D) and at study endpoint after 3 weeks of treatment in spleens. Delayed ruxolitinib co-treatment with TSLPRCART improved leukemia clearance compared to monotherapy or simultaneous co-treatment in this DS-ALL model. (E) Human CD3 $^+$ /CD45 $^+$ T cells quantification in peripheral blood demonstrated no change in TSLPRCART number with 14 day delayed ruxolitinib. (F) IFN- γ was measured by ELISA in plasma prepared from weekly peripheral venous blood.

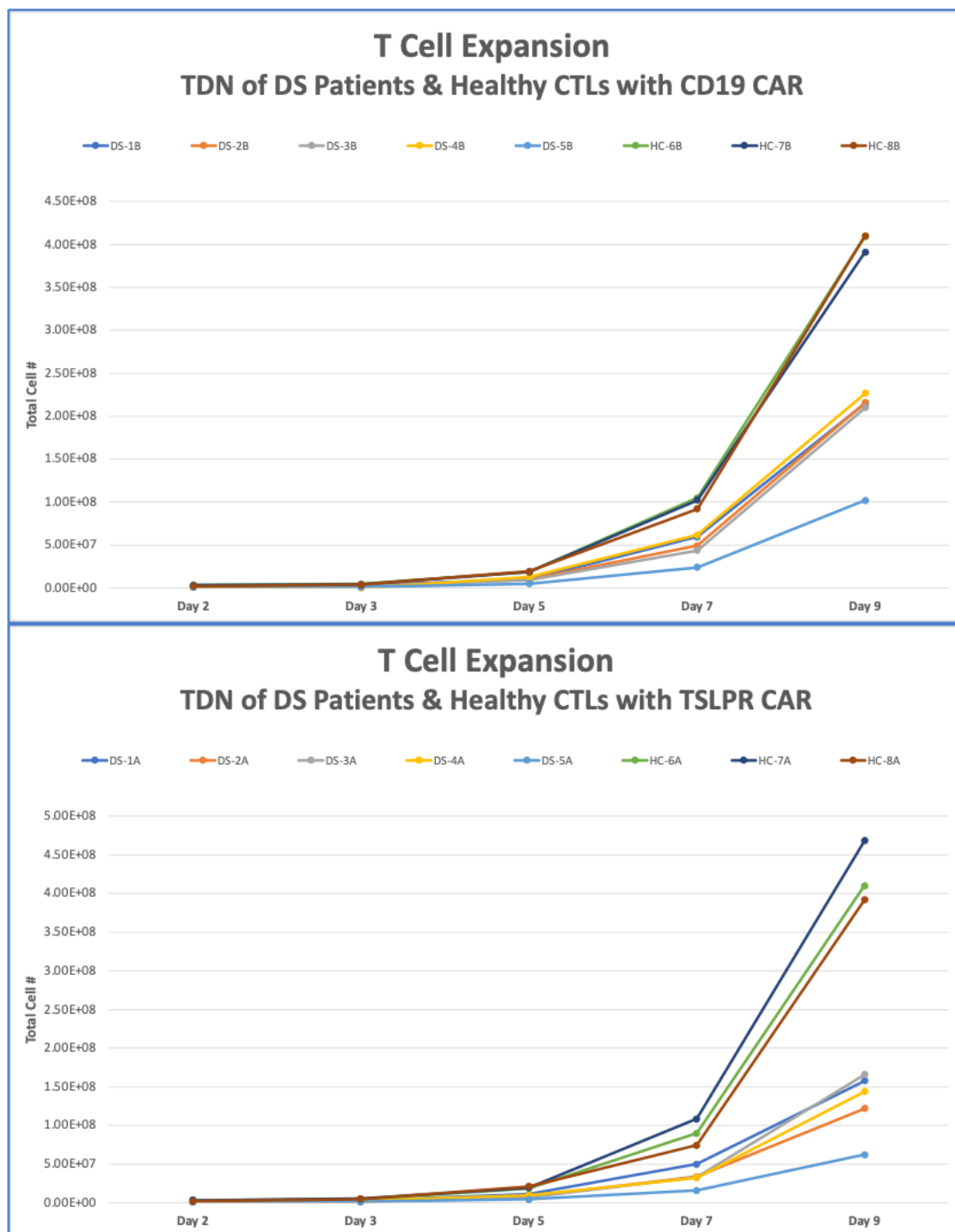


Figure 7. CAR T cells generated from individuals with Downs Syndrome may proliferate less at late timepoints.

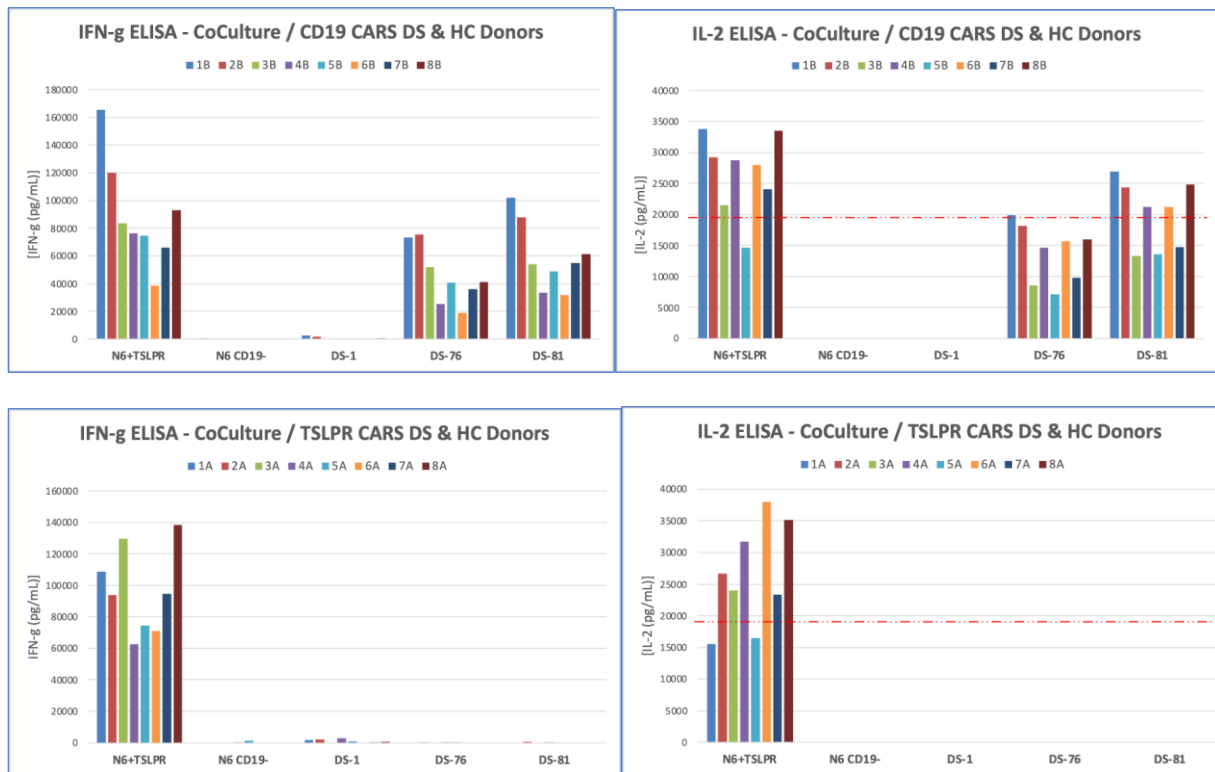


Figure 7. CART T cells generated from individuals with Downs Syndrome produce comparable levels of inflammatory cytokines upon Car stimulation in comparison to healthy donors.