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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.

15. SUBJECT TERMS

NONE LISTED

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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. 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. 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 <u>hypothesis</u> 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 of 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 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-g)
• 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 2022 (delayed due to scientific and administrative holds during viral pandemic); 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*-R 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. 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	*JAK2*L730F, 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 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 now ongoing in cell line and PDX models for additional CAR T cell control experiments. Dr Tasian 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.
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. Protocol has been written with anticipated IRB submission soon. Not certain of planned trial opening at this time due to NIH POB delays (estimated early 2023). 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.

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 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. Manuscript draft has been written with edits by coauthors ongoing and final control experiments as above in process (planned submission January/Februar 2023).

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. 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 ImpacT21 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				Year			
Target quarterly	**Q 1**	**Q2**	**Q 3**	**Q4**	**Q 1**	**Q 2**	**Q3**	**Q4**
Total patients	6	6-9	6-	6-9	3-	3-	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.

Three 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. Four 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 Hemtatology 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 Hemtatology 2021 annual meeting.

- https://www.sciencedirect.com/science/article/pii/S0006497121023983

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 2023 (Bagashev … Fry/Tasian).

4. **IMPACT:** D*escribe 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 a meeting in October 2022.

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 wordwide, 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.

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 are 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.

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 facilty 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. This has resulted in a 3 month delay 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 has resumed in October 2022.

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. He 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

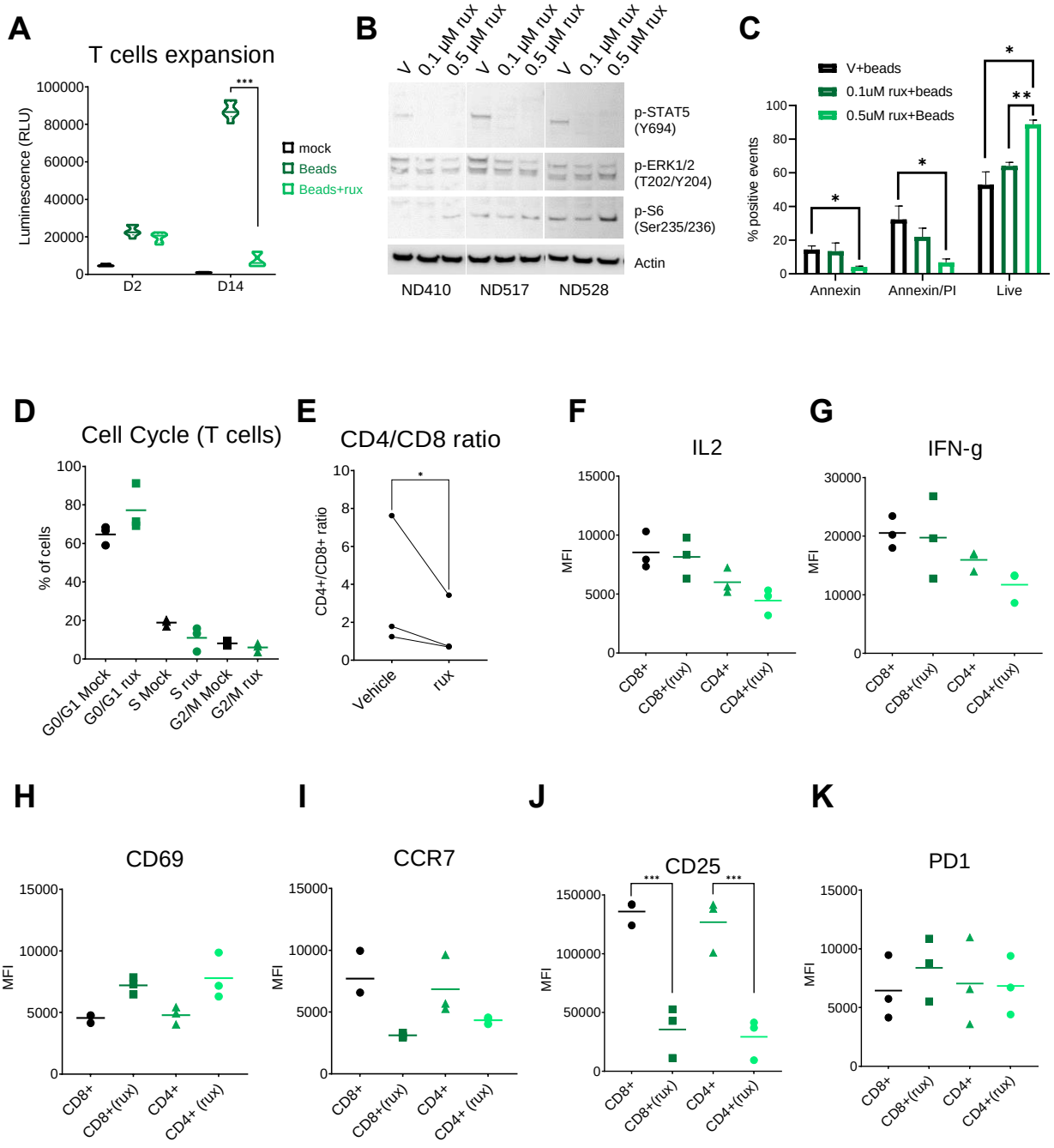

Figure 1. Ruxolitinib inhibits normal T cell expansion *in vitro* without inducing cell death.
(A) Three number of normal healthy donor T cells were unstimulated (mock) or stimulated with CD3/CD28 activation beads in the absence or presence of the JAK inhibitor Ruxolitinib (0.5 μM) *in vitro* in cell culture medium for up two weeks in triplicate in 96 well-plates. T cell expansion was assessed via Cell-Glo assay. Ruxolitinib blunted bead-stimulated T cell expansion in co-culture at 14 days. Summary data from 3 independent T cell donors plated in triplicate are shown. *** $p<0.001$ by 2way ANOVA with Tukey post-test for multiple comparisons . **(B)** Western blotting analysis of protein lysates from normal healthy donor T cells (n=3 donors; ND410, ND517, ND528) were incubated *in vitro* with vehicle (V) the designated concentrations of ruxolitinib (0.1 uM or 0.5 uM rux) for 72 hours demonstrates potent inhibition of phosphorylated (p) STAT5 with minimal effects upon pERK and pS6. **(C)** Flow cytometric cell death assays with annexin-V/propidium iodide staining of bead-stimulated normal healthy donor T cells without and with ruxolitinib exposure *in vitro* for 72 hours demonstrated dose-dependent prevention of apoptosis, suggesting that ruxolitinib does not kill T cells despite suppression of functionality, as shown in (A). **(D)** Cell cycle Flow cytometry analysis of normal healthy donor T cells incubated with beads an 0.5 uM ruxolitinib for 72 hours showed modest G0/G1 arrest with minimal S-phase or G2/M transition effects and **(E)** a reduction in the CD4/CD8 ratio via flow cytometric immunophenotyping. **(F)** and **(G)** Modest effects of ruxolitinib exposure upon normal T cell activation markers IL2 and IFNγ were also detected via flow cytometry of permeabilized cells in both CD4+ T cell subsets. **(H)** and **(I)** Live cell flow cytometry of cell surface activation markers and cytokines CD69 and CCR7. **(J)** and **(K)** potent reduction of CD25 but not PD1 was detected in both CD4+ and CD8+ T cells with ruxolitinib exposure. Statistical analyses were performed by one-way ANOVA with Dunnett's post-test for multiple comparisons, *** $p<0.001$
.

Figure 2

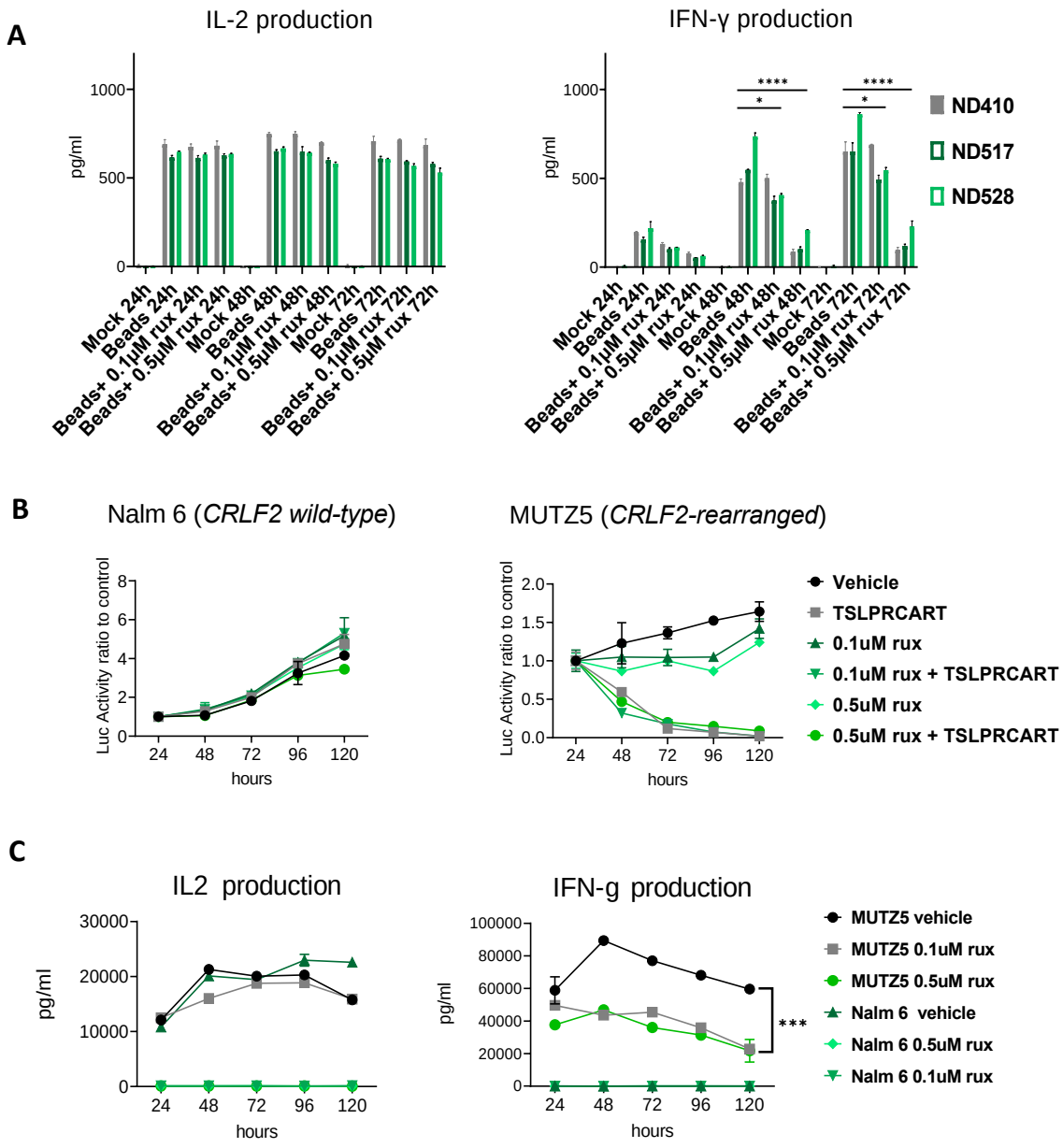

Figure 2. Ruxolitinib does not affect TSLPRCART efficacy in vitro.
(A) Elisa assay showing IL-2 and IFN-ɣ levels in culture medium produced by CD3/CD28 beads activated normal T cells treated with ruxolitinib or vehicle for the indicated timepoints. **(B)** Functional killing assay of TSLPRCART cells with luciferase+ NALM-6 or MUTZ5 cells in vitro. 20000 cells/ well were seeded in biological triplicates at 1:15 E (effector):T (target) cells ratio. Viability analysis was performed via luciferase reporter assays in technical triplicates. **(C)** Elisa assay cell culture with supernatants from (B) showing IL-2 and IFN-ɣ produced by TSLPRCART cells treated with ruxolitinib or vehicle. Statistical analyses were performed by one-way ANOVA with Tukey post-test for multiple comparisons *** $p<0.001$.

Figure 3

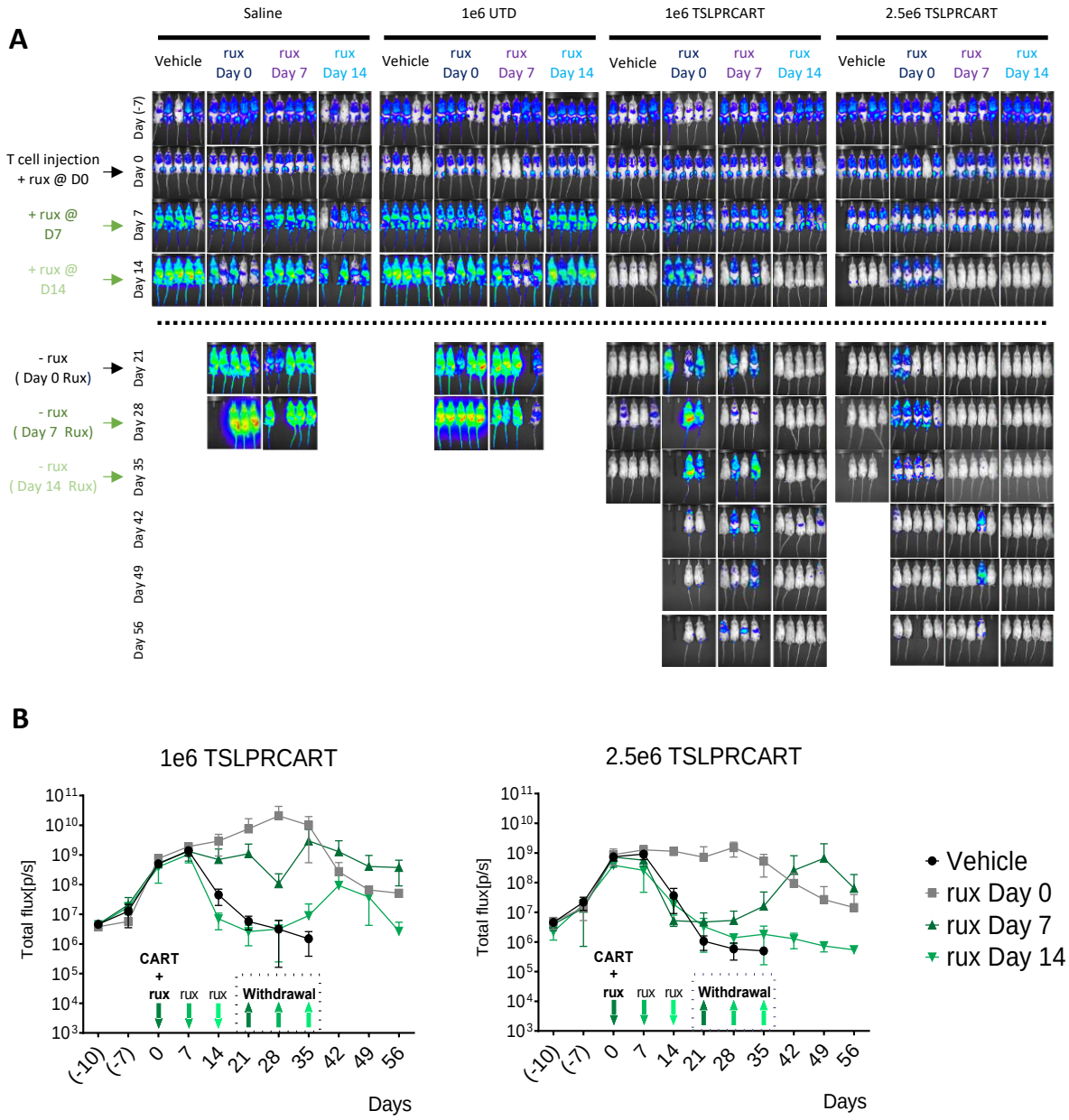

Figure 3. Early ruxolitinib co-treatment impairs TSLPRCART efficacy against Ph-like ALL. **(A)** Representative images demonstrating the effect of TSLPRCART and ruxolitinib treatments on the leukemia progression in MUTZ5-CBG (IGH-CRLF2, JAK2 R683G) mouse model. MUTZ5-CBG cells were injected ($1e^6$) in NSG mice and allowed to engraft until 10^6 Total Flux [p/s]. Mice were then injected either with Saline, $1e^6$ UTD (untransduced T cells) as controls or with $1e^6$ (low dose) and $2.5e^6$ (high dose) TSLPRCART cells. At the indicated times (arrows) normal chow was replaced with ruxolitinib infused chow. Leukemia progression was monitored by injecting the mice with Luciferin reagent (15g/ml) and imaging 5 minuets later. **(B)** Graphs representing the quantification of the images from (B) for the corresponding TSLPRCART treatment groups (1e6 and 2.5e6 TSLPRCART). Color-matched arrows indicate the time of CART injection or ruxolitinib chow addition and withdrawal.

Figure 4

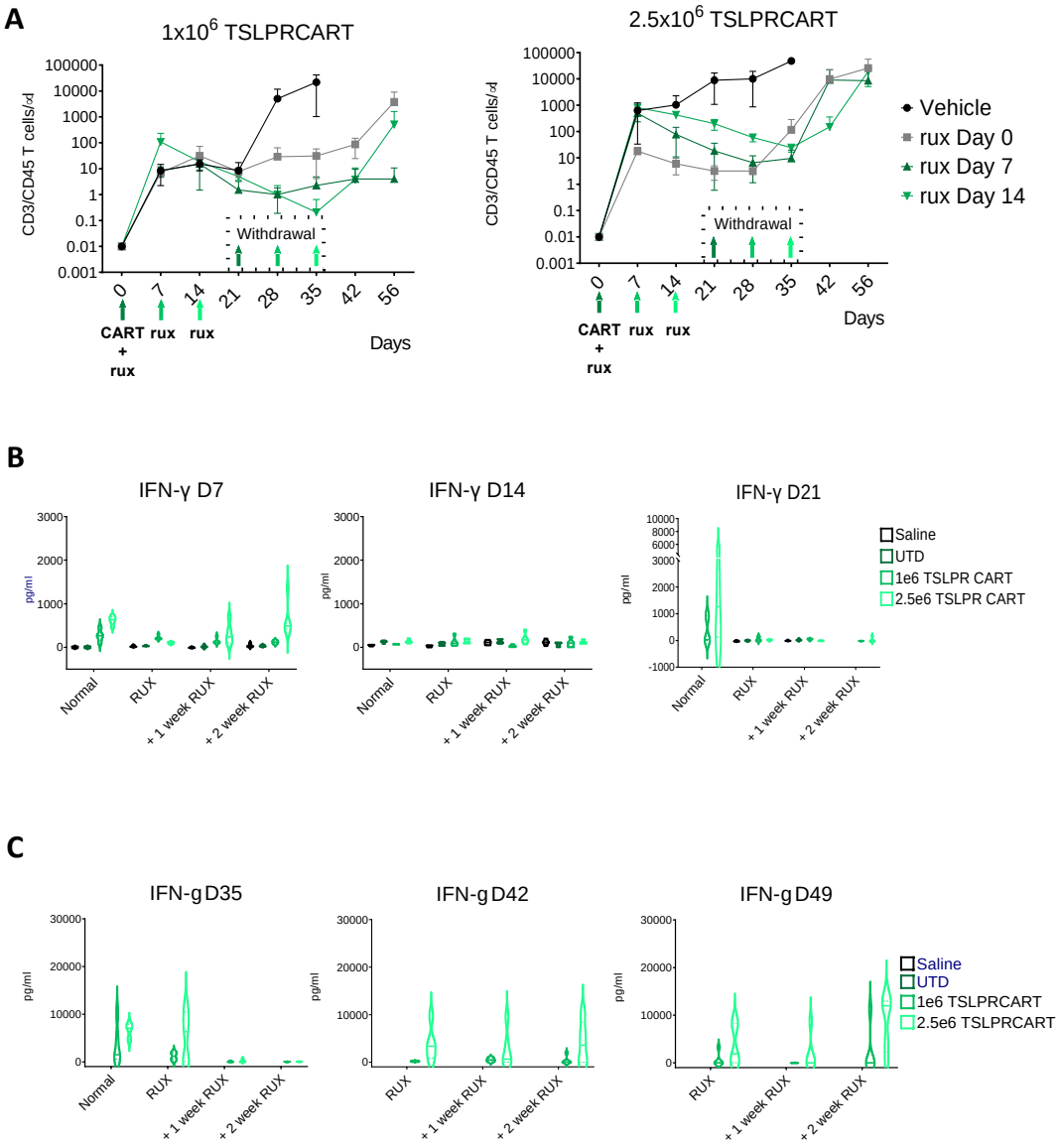

Figure 4. TSLPRCART regains functionality with ruxolitinib withdrawal.
(A) Flow cytometry measurements of human CD45/CD3 positive T cells in ALL121-CBG (IGH-CRLF2) PDX mouse model showing peripheral blood CART cells number in both low (1e6) and high (2.5e6) TSLPRCART dose cohorts after addition of ruxolitinib at Day 0, Day 7 or Day 14. Color-coded arrows indicate the time of CART cells injection or ruxolitinib chow addition and withdrawal. **(B)** ELISA assay with plasma from ALL121-CBG (IGH-CRLF2) PDX mouse model showing IFN-γ plasma levels in after ruxolitinib treatment (Days 7, 14 and 21). **(C)** ELISA assay with plasma from ALL121-CBG (IGH-CRLF2) PDX mouse model (Day 35, 42 and 49) demonstrating IFN-γ levels in mice treated with TSLPRCART and after ruxolitinib withdrawal for at least 2 weeks.

Figure 5

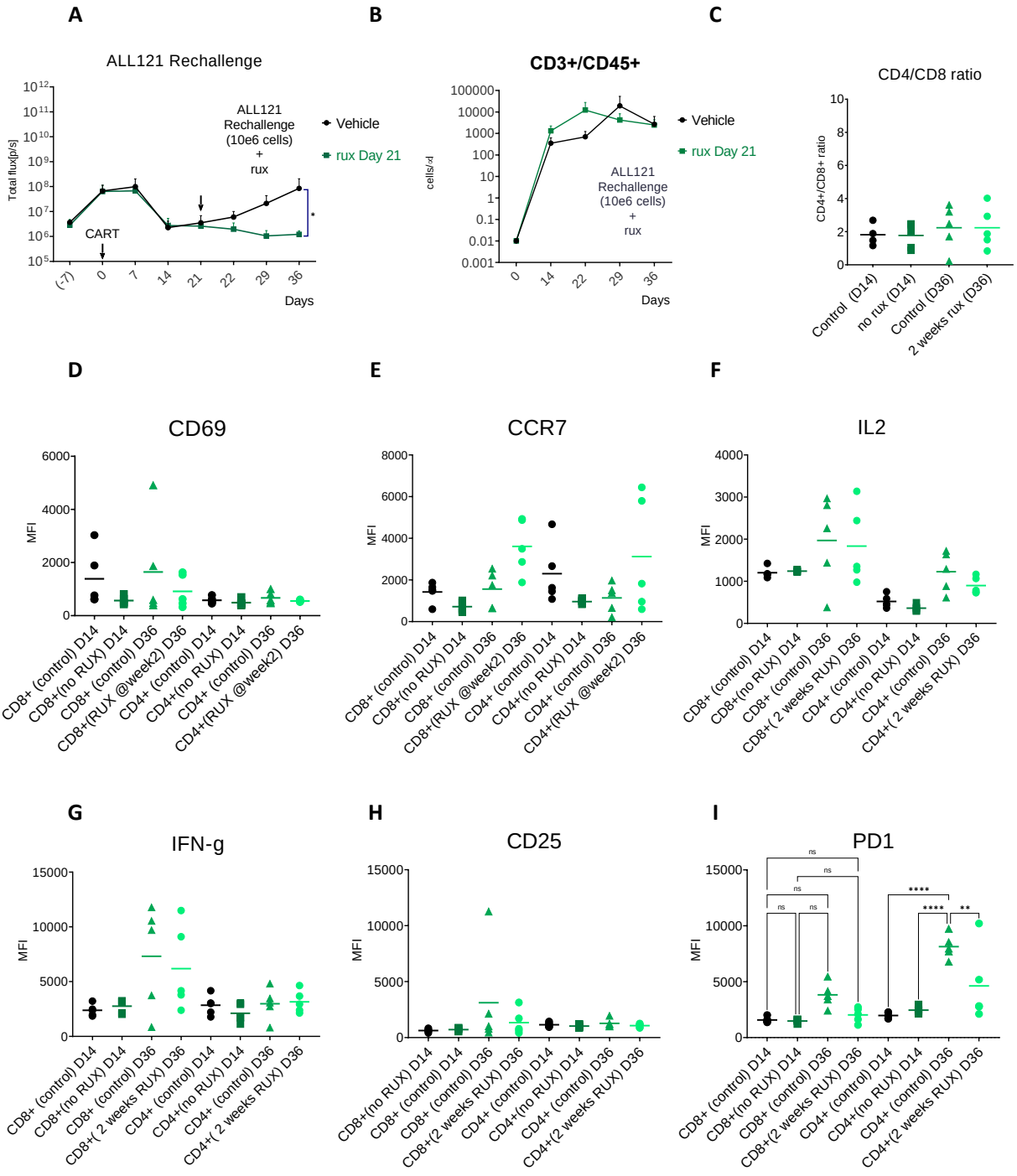
A
ALL121 Rechallenge
Total flux[p/s]
ALL121 Rechallenge (10e6 cells) + rux
CART
Vehicle
rux Day 21
Days
B
CD3+/CD45+
cells/ml
ALL121 Rechallenge (10e6 cells) + rux
Vehicle
rux Day 21
Days
C
CD4/CD8 ratio
CD4+/CD8+ ratio
Control (D14)
no rux (D14)
Control (D36)
2 weeks rux (D36)
D
CD69
MFI
CD8+ (control) D14
CD8+(no RUX) D14
CD8+ (control) D36
CD8+(RUX @week2) D36
CD4+ (control) D14
CD4+(no RUX) D14
CD4+ (control) D36
CD4+(RUX @week2) D36
E
CCR7
MFI
CD8+ (control) D14
CD8+(no RUX) D14
CD8+ (control) D36
CD8+(RUX @week2) D36
CD4+ (control) D14
CD4+(no RUX) D14
CD4+ (control) D36
CD4+(RUX @week2) D36
F
IL2
MFI
CD8+ (control) D14
CD8+(no RUX) D14
CD8+ (control) D36
CD8+(2 weeks RUX) D36
CD4+ (control) D14
CD4+(no RUX) D14
CD4+ (control) D36
CD4+(2 weeks RUX) D36
G
IFN-g
MFI
CD8+ (control) D14
CD8+(no RUX) D14
CD8+ (control) D36
CD8+(2 weeks RUX) D36
CD4+ (control) D14
CD4+(no RUX) D14
CD4+ (control) D36
CD4+(2 weeks RUX) D36
H
CD25
MFI
CD8+(no RUX) D14
CD8+ (control) D14
CD8+ (control) D36
CD8+(2 weeks RUX) D36
CD4+ (control) D14
CD4+(no RUX) D14
CD4+ (control) D36
CD4+(2 weeks RUX) D36
I
PD1
MFI
ns
****
**
CD8+ (control) D14
CD8+(no RUX) D14
CD8+ (control) D36
CD8+(2 weeks RUX) D36
CD4+ (control) D14
CD4+(no RUX) D14
CD4+ (control) D36
CD4+(2 weeks RUX) D36

Figure 5. Delayed ruxolitinib treatment improves TSLPR CART therapy persistence in leukemia re-challenge mouse model. (A) Graphs representing Total Flux [p/s] quantification of luciferin injected ALL121-CBG (IGH-CRLF2) rechallenge PDX mouse model. Arrows indicate the time of ALL121-CBG or CART cells injection and ruxolitinib chow addition. **(B)** Flow cytometry measurements of human CD45/CD3 positive T cells in ALL121-CBG (IGH-CRLF2) rechallenge PDX mouse model showing peripheral blood CART cells numbers. **(C)** Graph representing the quantification of the CD4 to CD8 ratios at Day 36 from (B). **(D-I)** Mean Florescent intensity (MFI) graphs form flow cytometry analyses of peripheral blood from ALL121-CBG (IGH-CRLF2) rechallenge PDX mouse model for day 14 and 36 demonstrating the surface levels of CD69, CCR7, CD25 and PD1 as well as intracellular levels of IL-2 and IFN-γ. Statistical analyses were performed by one-way ANOVA with Tukey post-test for multiple comparisons *** $p<0.001$.

Figure 6

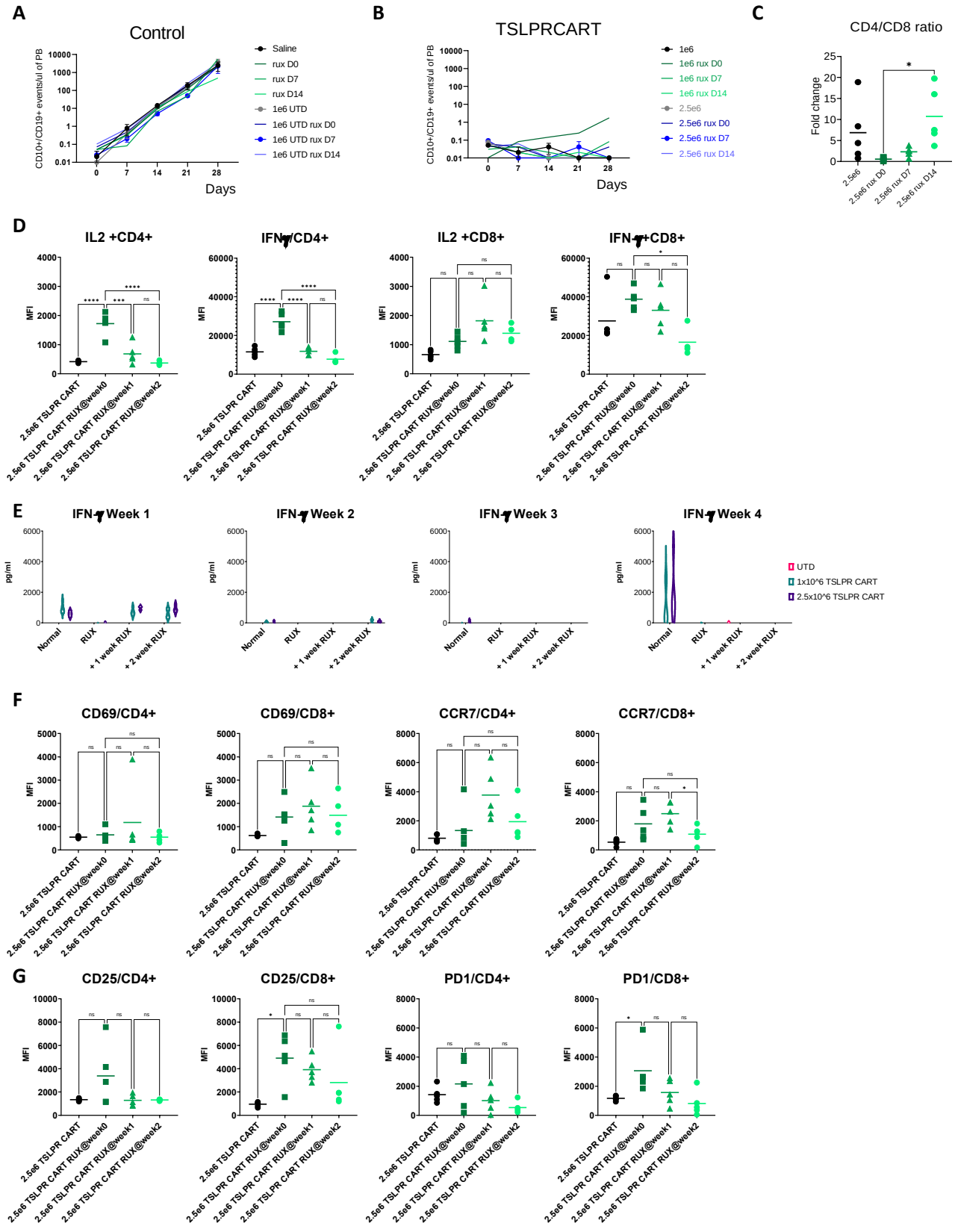
A
Control
CD10+/CD19+ events/ul of PB
Days
Saline
rux D0
rux D7
rux D14
1e6 UTD
1e6 UTD rux D0
1e6 UTD rux D7
1e6 UTD rux D14
B
TSLPRCART
CD10+/CD19+ events/ul of PB
Days
1e6
1e6 rux D0
1e6 rux D7
1e6 rux D14
2.5e6
2.5e6 rux D0
2.5e6 rux D7
2.5e6 rux D14
C
CD4/CD8 ratio
Fold change
2.5e6
2.5e6 rux D0
2.5e6 rux D7
2.5e6 rux D14
D
IL2 +CD4+
IFN-γ+CD4+
IL2 +CD8+
IFN-γ+CD8+
MFI
2.5e6 TSLPR CART
2.5e6 TSLPR CART RUX@week0
2.5e6 TSLPR CART RUX@week1
2.5e6 TSLPR CART RUX@week2
E
IFN-γ Week 1
IFN-γ Week 2
IFN-γ Week 3
IFN-γ Week 4
pg/ml
Normal
RUX
+ 1 week RUX
+ 2 week RUX
UTD
1x10^6 TSLPR CART
2.5x10^6 TSLPR CART
F
CD69/CD4+
CD69/CD8+
CCR7/CD4+
CCR7/CD8+
MFI
G
CD25/CD4+
CD25/CD8+
PD1/CD4+
PD1/CD8+
MFI

Figure 6. Ruxolitinib/TSLPR CART treatment in Ruxolitinib resistant P2RY8/CRLF2 Down Syndrome PDX model recapitulates IGH@-CRLF2 non-Down Syndrome PDX results.
(A) and **(B)** Graphs representing human CD10+/CD19+ cells quantification in peripheral blood from DSALL515 (P2RY8-CRLF2) PDX mouse model with Saline , UTD or TSLPRCART injected mice. **(C)** Graph representing the Flow Cytometry quantification of CD4 to CD8 TSLPRCAR T cell ratios in peripheral blood from DSALL515 (P2RY8-CRLF2) PDX mouse model. **(D)** Mean Florescent intensity (MFI) graphs from flow cytometry analyses of peripheral blood from DSALL515 (P2RY8-CRLF2) PDX mouse model demonstrating the intracellular levels of IL-2 and IFN-γ in CD4 and CD8 TSLPRCART cells subset. **(E)** ELISA assay with plasma DSALL515 (P2RY8-CRLF2) PDX mouse model (Day 7, 14, 21 and 28) demonstrating IFN-γ levels after ruxolitinib exposure. **(F)** and **(G)** Mean Florescent intensity (MFI) graphs form flow cytometry analyses of peripheral blood from DSALL515 (P2RY8-CRLF2) PDX mouse model demonstrating the surface levels of CD69, CCR7, CD25 and PD1 in CD4 and CD8 T cells subset. Statistical analyses were performed by one-way ANOVA with Tukey post-test for multiple comparisons *** $p<0.001$.

Figure 7

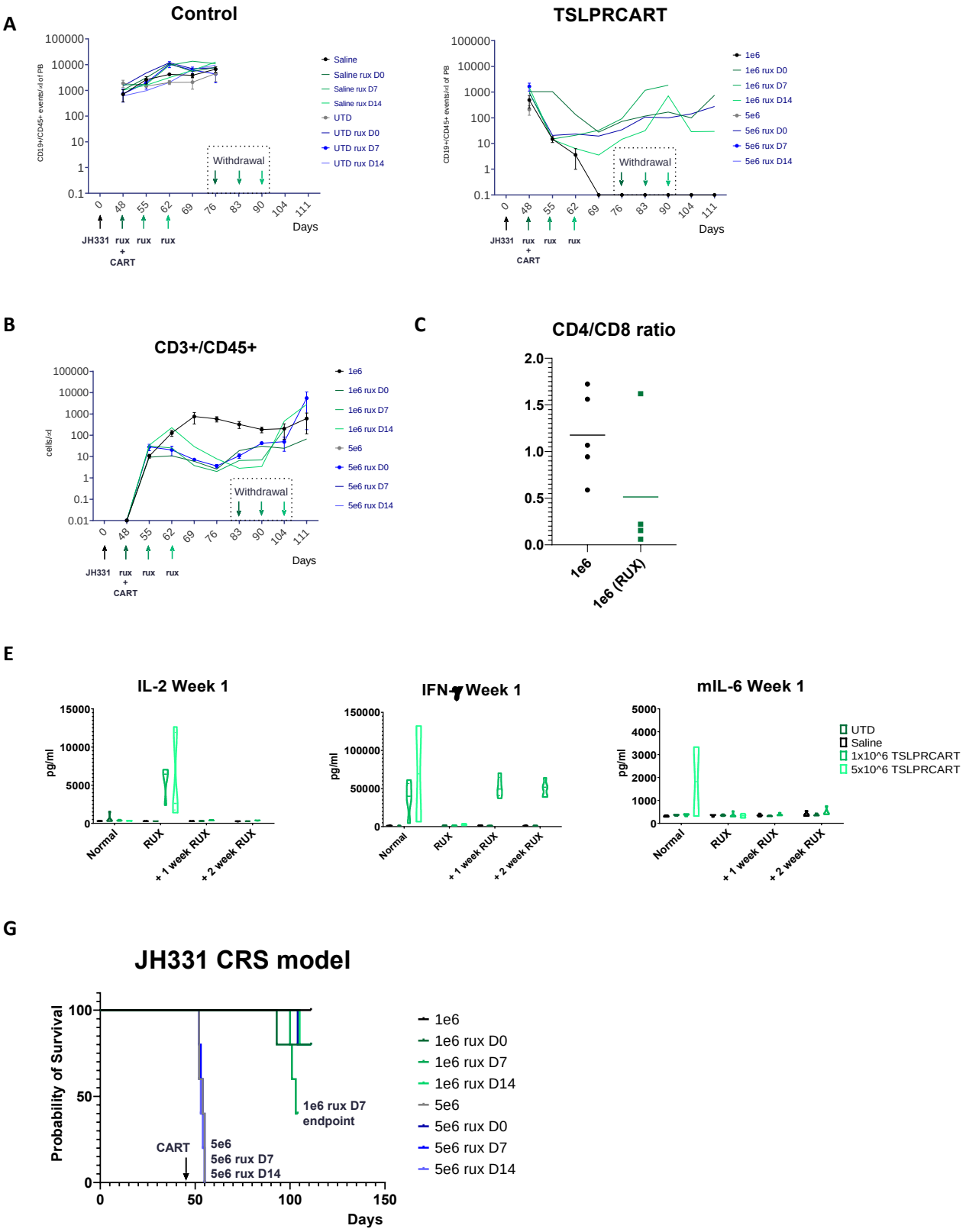
A
Control
TSLPRCART
CD19+/CD45+ events/-d of PB
Withdrawal
Days
JH331
rux
+
CART
Saline
Saline rux D0
Saline rux D7
Saline rux D14
UTD
UTD rux D0
UTD rux D7
UTD rux D14
1e6
1e6 rux D0
1e6 rux D7
1e6 rux D14
5e6
5e6 rux D0
5e6 rux D7
5e6 rux D14
B
CD3+/CD45+
cells/-d
C
CD4/CD8 ratio
1e6
1e6 (RUX)
E
IL-2 Week 1
IFN-γ Week 1
mIL-6 Week 1
pg/ml
Normal
RUX
+ 1 week RUX
+ 2 week RUX
UTD
Saline
1x10^6 TSLPRCART
5x10^6 TSLPRCART
G
JH331 CRS model
Probability of Survival
CART
5e6
5e6 rux D7
5e6 rux D14
1e6 rux D7
endpoint
Days

Figure 7. Simultaneous ruxolitinib administration prevents TSLPRCART-induced CRS.
(A) and **(B)** Graphs representing human CD19+/CD45+ cells quantification in peripheral blood from JH331 (IGH-CRLF2) CRS PDX mouse model in Saline , UTD or TSLPRCART injected mice. Color-matched arrows indicate the time of CART injection or ruxolitinib chow addition and withdrawal. **(B)** Flow cytometry measurements of human CD45/CD3 positive T cells in ALL121-CBG (IGH-CRLF2) rechallenge PDX mouse model showing peripheral blood CART cells numbers. **(C)** Graph representing the quantification of the CD4 to CD8 ratios at Day 36 from (B). **(E)** ELISA assay with plasma from JH331 (IGH-CRLF2) CRS PDX mouse model (Day 7, 14, 21 and 28) demonstrating human IL-2, human IFN-γ or mouse IL-6 levels after ruxolitinib exposure. **(G)** Kaplan–Meier Curves for JH331 (IGH-CRLF2) CRS PDX mouse model.