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

PRINCIPAL INVESTIGATOR: Terry Fry, MD

CONTRACTING ORGANIZATION: University of Colorado, Aurora, CO

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E-Mail:

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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

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1. INTRODUCTION:

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 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 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:

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:

What were the major goals of the project?

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-gamma)
- 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 to open in Q2 2021; 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

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 in pediatric ALL PDX models
• 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 in adult ALL PDX models
• 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 in DS-ALL PDX models
• 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

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.

What was accomplished under these goals?

Specific Aim 1 Progress to date:

1. Task 1: TSLPRxCD19 and TSLPRxCD22 construct engineering initially completed with *in vitro* and some *in vivo* optimization.
2. Task 2: Childhood and adult ALL PDX models expansion complete. Nine DS-ALL (some *CRLF2*-R and some *CRLF2*-wild-type) PDX models established and now s/p secondary expansion. *In vivo* testing of TSLPRCART +/- ruxolitinib completed in 3 DS-ALL models with plans for bispecific CAR T cell testing with optimized constructs.
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. 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/in process for others.
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. Trial now planned to open in Q2 2022. We have optimized and quantified TSLPRCART site

density on *CRLF2*-rearranged and non-rearranged DS-ALL PDX models (n=9) and multiple Ph-like ALL models (n=18) in preparation for clinical trial samples.

Specific Aim 2 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. Ongoing construct optimization ongoing prior to more detailed testing in *CRLF2*-R ALL cell lines and PDX models planned in near future. PDX model studies ongoing with TSLPR CAR T cells and ruxolitinib.
2. Task 2: Adult PDX models established with *in vivo* activity of ruxolitinib assessed and compared to pediatric model data.
3. Task 3: Detailed TSLPRCART and ruxolitinib *in vivo* testing in MUTZ5 xenograft models for dose and timing optimization completed. Four large-scale *CRLF2*-R ALL PDX model studies now completed (2 Ph-like ALL, 2 DS-ALL). 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.
4. 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 Progress to date:

1. Task 1: Identified normal T cell donor sources at CHCO and CHOP. Initial T cell activation and exhaustion biomarker panels designed with flow cytometry assays optimized. New single-cell/RNAseq studies pending in sorted CAR T cells from above xenograft mice studies to assess T cell expression changes in vivo over time.
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 soon 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.

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

Three post-doctoral fellows and one junior faculty member 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.

How were the results disseminated to communities of interest?

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 12th Biennial Childhood Leukemia & Lymphoma Symposium (March 2021, Tasian; rescheduled from 2020 meeting that was delayed due to viral pandemic) and via oral presentation at the International Society of Pediatric Oncology annual meeting (October 2021, Tasian). An abstract of additional studies was submitted and will be presented as a poster at the American Society of Hematology annual meeting (December 2021, trainee Bagashev in Tasian laboratory). Updates about this DoD TTSA project have also been shared via the NCI Pediatric Immunotherapy Discovery and Development Network monthly and annual meetings given shared scientific interests (Fry, Tasian).

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

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 2022 (Bagashev … Fry/Tasian).

4. IMPACT:

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

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. These models collectively provide a critical resource for this project and can also be shared with the greater scientific community in the future.

What was the 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?

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?

Nothing to report at this time.

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

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

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

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 needed to re-purchase breeder mice to build back up our animal colony to full efficiency.

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

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:

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

  Journal publications. Nothing to Report

  Books or other non-periodical, one-time publications.

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. American Society of Hematology annual meeting (December 2021; Atlanta, Georgia - poster presentation by Dr Bagashev)

2. 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

3. 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

4. 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.

Nothing to report.

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

Nothing to report.

- **Technologies or techniques**

  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.

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

  Nothing to report.

- **Other Products**

Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

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: 0.6 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 is responsible for *in vitro* and *in vivo* studies performed at CHOP in the Tasian laboratory. All animal studies commenced after obtainment of ACURO approval.

Name: Joseph Loftus, BS (CHOP)
Project Role: Research technician
Researcher Identifier (e.g. ORCID ID): none
Nearest person month worked: 3 Calendar
Contribution to Project: Mr Loftus was responsible creation and maintenance of the needed ALL PDX models at CHOP in the Tasian laboratory through July 2021. All animal studies commenced after obtainment of ACURO approval.

Name: Catherine Falkenstein, BS (CHOP)
Project Role: Research technician
Researcher Identifier (e.g. ORCID ID): none
Nearest person month worked: 3 Calendar
Contribution to Project: Ms Falkenstein joined the Tasian laboratory in June 2021 and replaced Mr Loftus in July 2021. She is now responsible for all ALL PDX model maintenance and experimental *in vivo* studies at CHOP in the Tasian laboratory.

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

Nothing to report.

What other organizations were involved as partners?

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). 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.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: 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:

9. APPENDICES:

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/

FIGURES:

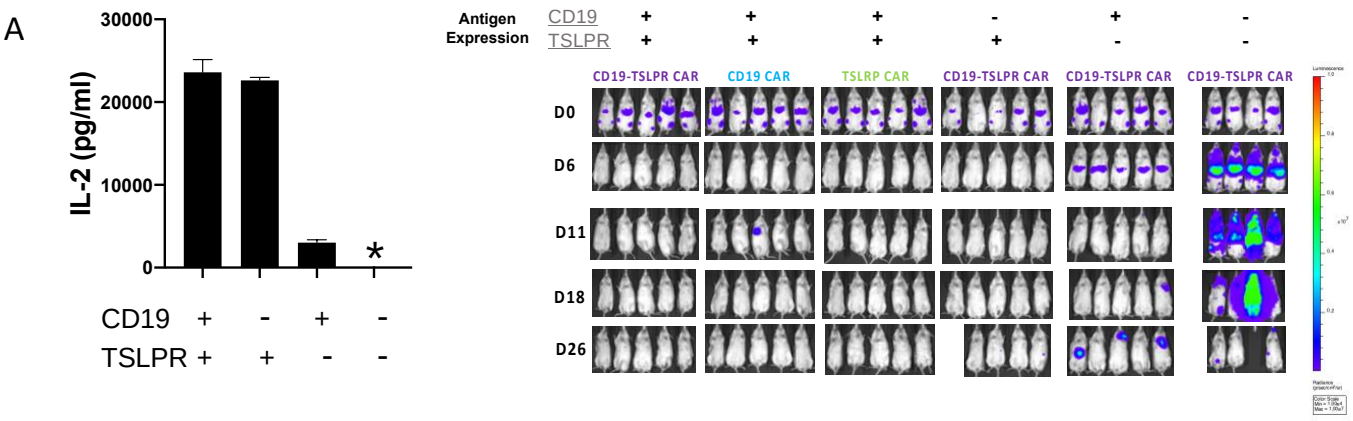

Figure 1. Development of a bivalent CD19xTSLPR CAR construct. Multiple bivalent formats were generated and screened in vitro against Nalm6 ALL cell lines engineered to express different combinations of TSLPR and CD19 on the surface. Panel A shows IL-2 production after 12 hours of co-

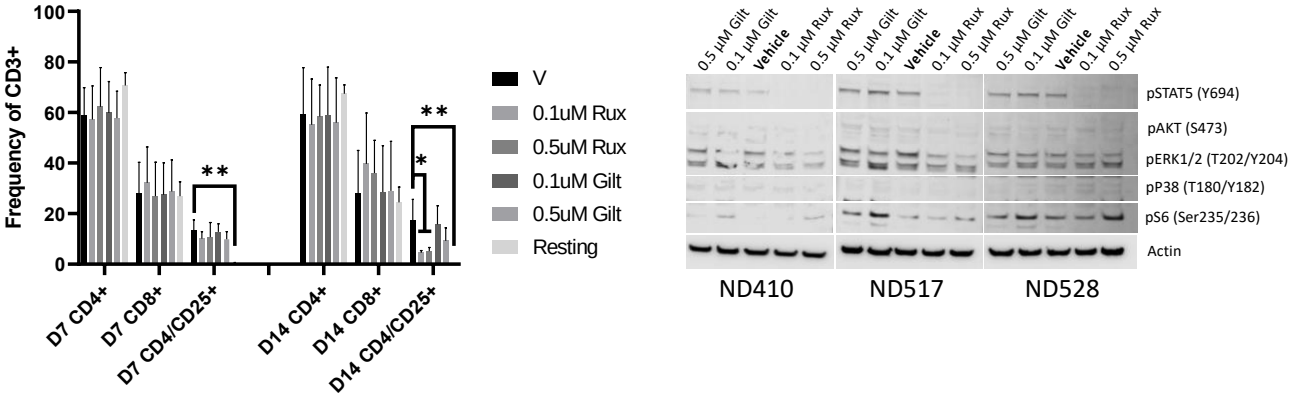

Figure 2. Effects of tyrosine kinase inhibitors (TKIs) upon normal CD3/CD28 bead-activated human T cells. **(A)** Immunophenotype of normal T cells treated with JAKi (ruxolitinib) or FLT3i (gilteritinib) demonstrates ruxolitinib-induced decreased CD4+ CD25+ Treg populations, but minimal effects of TKIs upon CD4 and CD8 cells. Gilteritinib had minimal effects upon normal T cell. **(B)** *In vitro* signaling effects of JAKi or FLT3i on normal T cells (incubated x 1h) are shown by Western blotting analysis.

ALL121 PDX model (*IGH-CRLF2*, *JAK2* R683G): relatively ruxolitinib-resistant

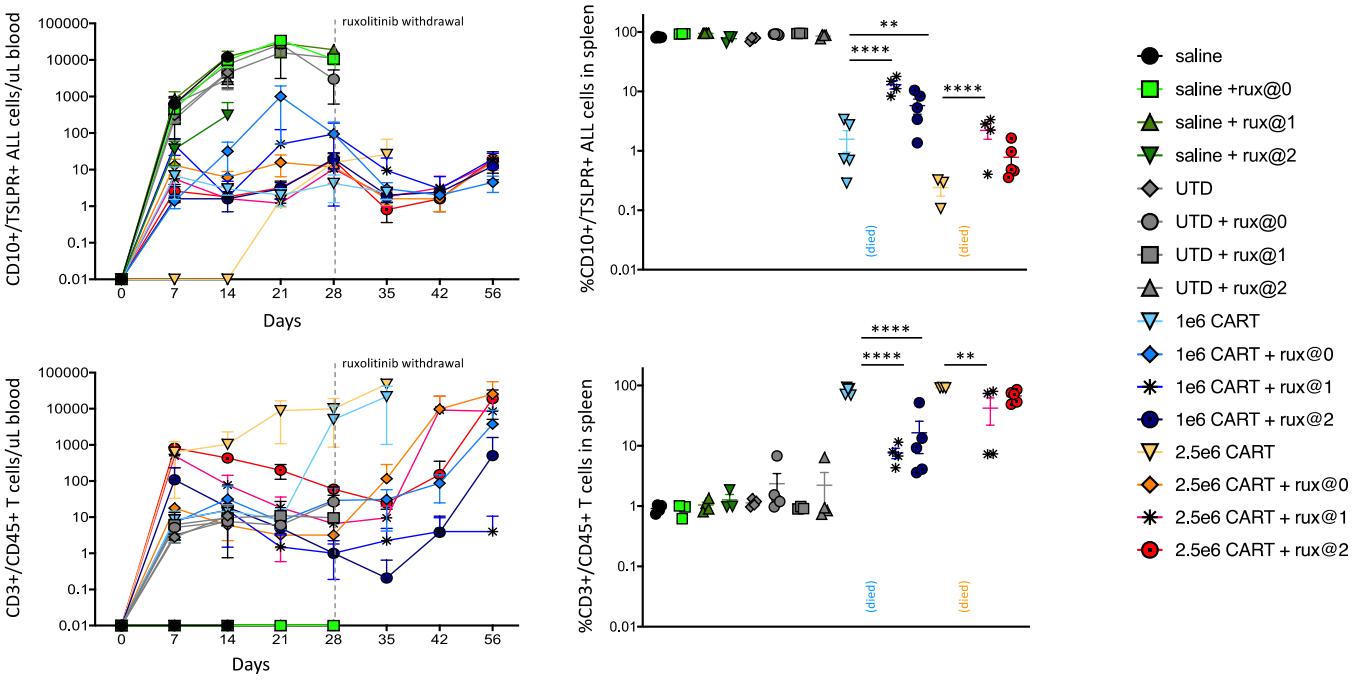

Figure 3. Ruxolitinib co-treatment hinders *in vivo* anti-leukemia functionality of TSLPRCART, but also protects mice from life-threatening cytokine release syndrome (CRS). *CRLF2*-rearranged Ph-ALL PDX mice (model ALL121) were treated with saline, untransduced T cells (UTD), ruxolitinib (rux), or TSLPRCART (CART) at the indicated cell doses and staggered timing of ruxolitinib (@0 = simultaneous with CART, @1 = 1 week after CART, and @2 = 2 weeks after CART). Mice were bled weekly for flow cytometric quantification of human CD10+/TSLPR+ ALL cells and CD3+ CART cells (left panels) and in end-study spleens (right panels). Ruxolitinib given @0 and @1 hindered desired anti-ALL activity of TSLPRCART, while administration @2 did not impair inhibition of leukemia proliferation. Co-administration of ruxolitinib was protective against grade 5 CRS in mice treated with 2.5x10e6 TSLPRCART. Ruxolitinib withdrawal further allowed eventual TSLPCART recovery and regained expansion and anti-ALL functionality.

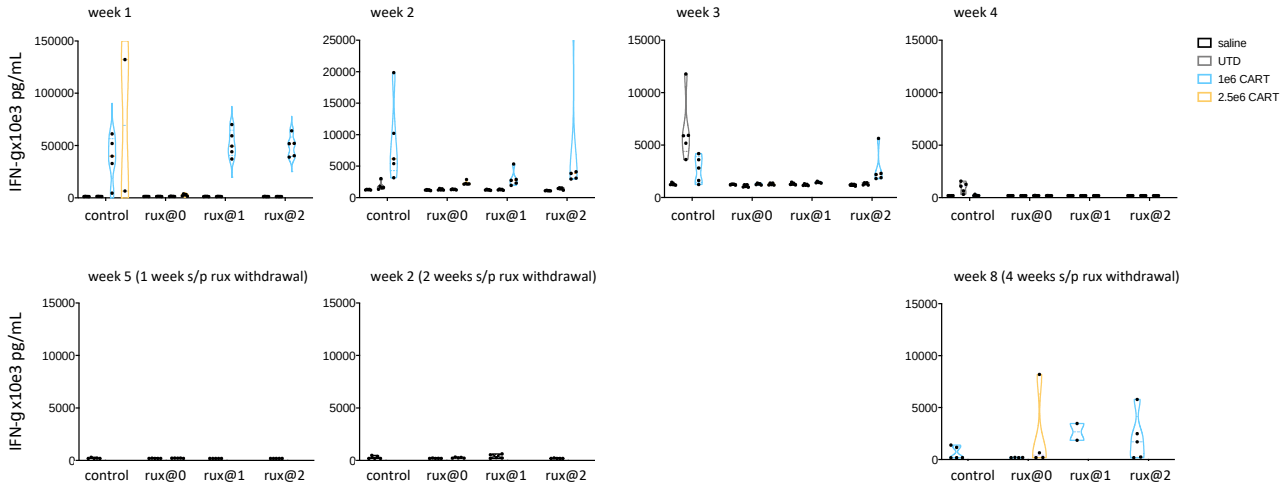

Figure 4. Ruxolitinib co-administration impairs TSLPRCART-induced inflammatory cytokine production. *CRLF2*-rearranged Ph-like ALL PDX mice from the experiment shown in Figure 3 were treated with saline, UTD, or TSLPRCART (CART) at the designated cell doses with ruxolitinib co-administration at week 0, 1, or 2. Mice were bled weekly after/during therapy, and IFN-γ levels were quantified in murine plasma over time. IFN-γ is suppressed during ruxolitinib co-treatment and rebounds after ruxolitinib withdrawal

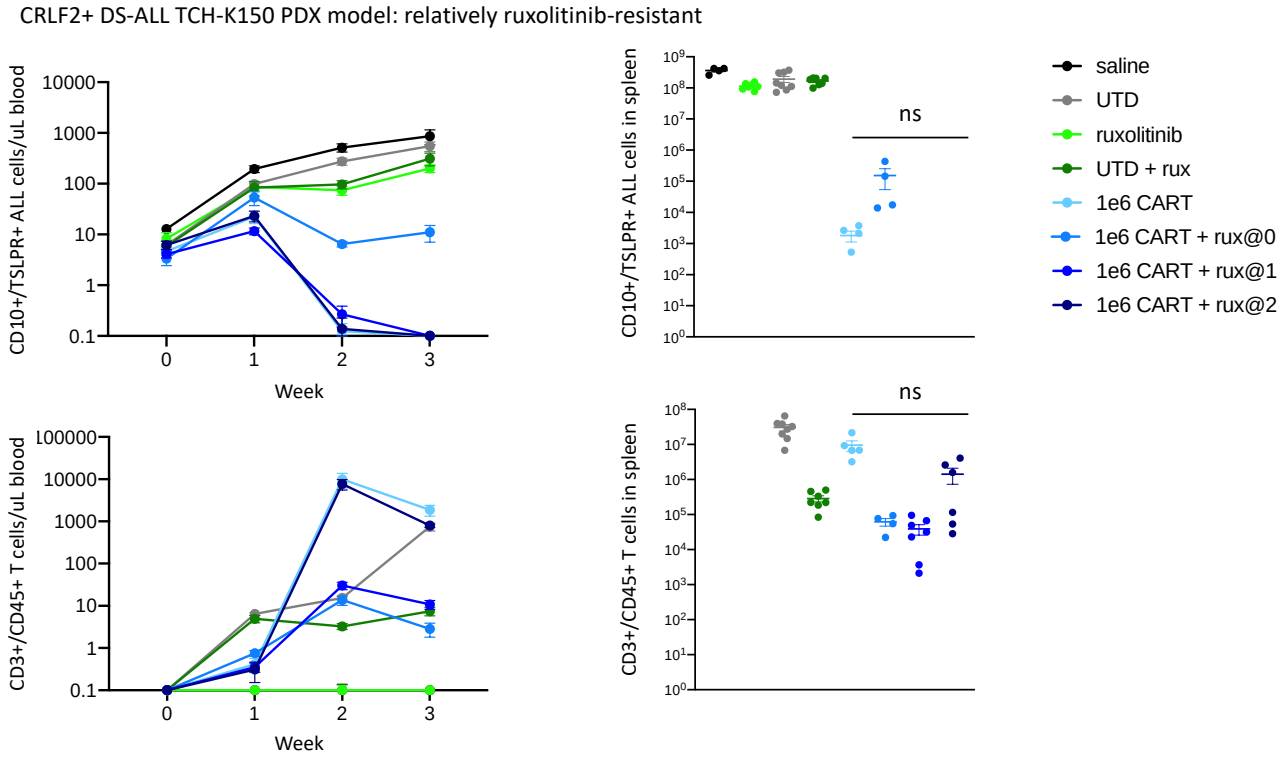

Figure 5. Ruxolitinib impairs TSLPCART functionality in *CRLF2*-rearranged Down Syndrome-associated ALL. DS-ALL PDX models were treated as in Figure 3 for Ph-like ALL PDX models with weekly quantification of human ALL and CD3+ TSLPRCART cells in murine blood (left panels) and in end-study spleens (right panels).

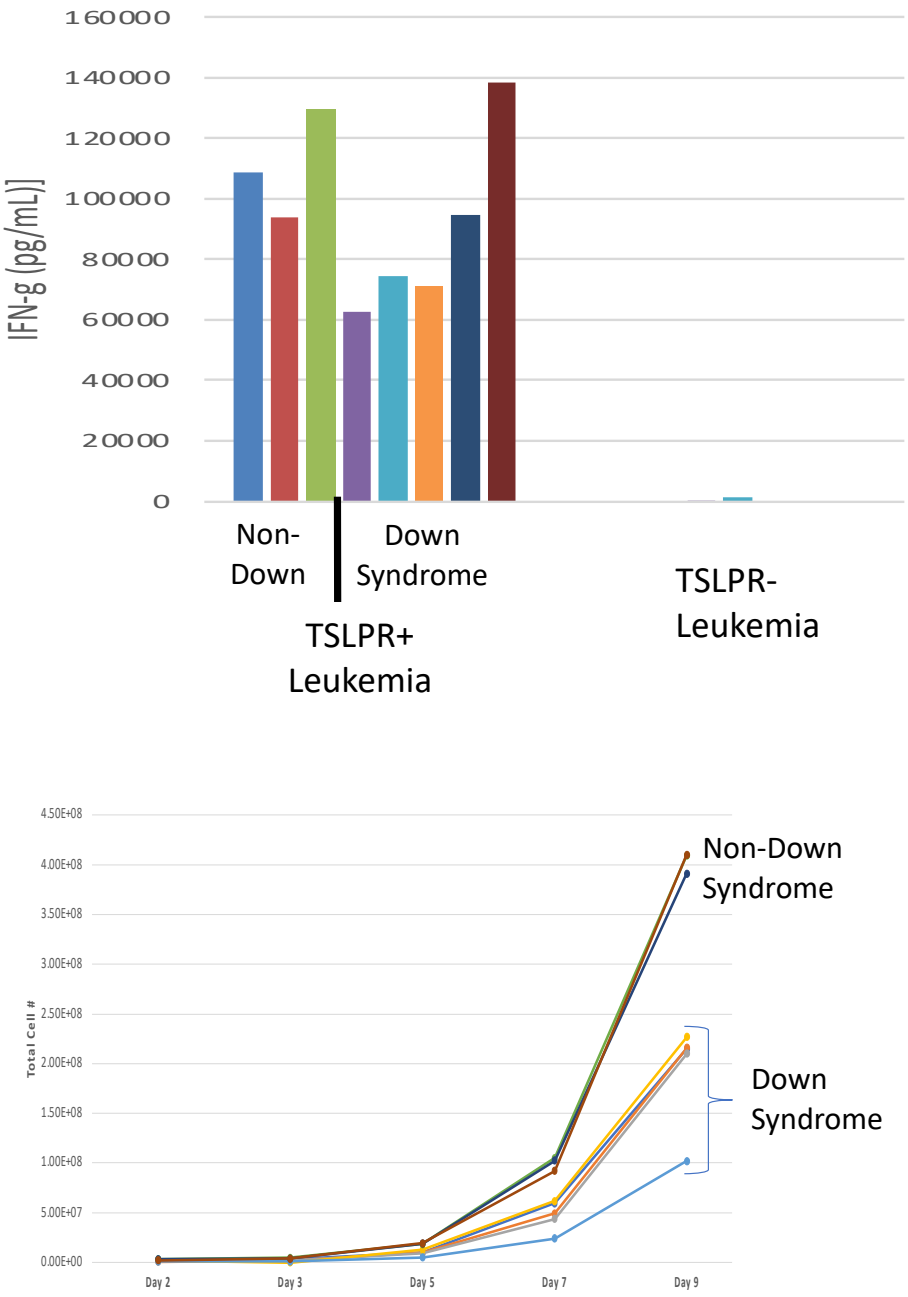

Figure 6. TSLPR CAR T cells from Down Syndrome Patients are functional but proliferate less well than non-Down CAR T cells. T cells from DS donors and non-DS donors demonstrated comparable transduction (data not shown) and produced comparable IFNg upon co-culture with leukemia expressing TSLPR (top panel). DS CAR T cells proliferate less well than CAR T cells from non-DS donors (bottom panel).