

Project Title: Study of immune phenotypes in long-term childhood cancer survivors

Working group and investigators:

Primary working group: Chronic disease

Secondary working group: Second malignancies

Investigators

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BACKGROUND AND RATIONALE

Burden of chronic disease in childhood cancer survivors: The outcome of children with cancer has improved considerably in the past 3 decades.(1) However, these improvements come at a significant cost, including premature/accelerated aging, cardiotoxicity, endocrine dysfunction, reproductive health issues, subsequent cancers, financial toxicity, neurocognitive deficits, and psychosocial issues.(2-6) Of particular interest, cardiotoxicity and the potential mediators such as metabolic syndrome and obesity(2-6), develop prematurely, and are one of the leading causes of premature mortality in childhood cancer survivors. While the clinical epidemiology of these comorbidities in has been extensively studied, the biologic and actionable mechanisms that underlie the development of these comorbidities in survivors remain poorly understood.

Immune system and chronic health conditions: While the immune system likely evolved in the context of mediating protection from pathogens and external dangers, it is now well appreciated that the immune system plays a major role in the pathogenesis of diverse chronic health conditions, including heart disease, cancer and obesity.(2-6) For example, chronic inflammation is thought to underlie the pathogenesis of atherosclerosis, metabolic syndrome and obesity.(7) In preclinical models, the immune system provides surveillance against the development of cancer.(8) Recent preclinical studies have also implicated a major role for the immune system in preventing accumulation of senescent cells within tissues, implicated in tissue aging.(9) Several studies have begun to identify patterns associated with aging in cancer survivors(10). Immune-based approaches to treat cancer have already begun to transform cancer therapeutics. Strategies to modulate underlying inflammation are under active evaluation in the setting of heart disease, metabolic syndrome and obesity. The immune system may therefore be a potentially actionable target to alter the risk and trajectory of chronic diseases and subsequent malignancies in survivors of childhood hematologic malignancies.

Immune system in children with leukemia and survivors: Given that pediatric cancers are characterized by relatively few oncogenic mutations, it was initially believed that these tumors are unlikely to elicit strong immune responses due to paucity of neoantigens. Most early studies utilized a limited set of markers. However, in recent studies, we and others have utilized several high-dimensional single cell approaches to study the immune system in children with newly diagnosed leukemia.(11) These studies have shown that not only is there evidence of immune activation, but that this leads to emergence of immune dysfunction with accumulation of terminal effector T cells and attrition of naïve T cells.(11) Importantly, these features correlate with disease risk and outcome. In other studies, we have also utilized high dimensional mass cytometry to identify distinct race/ethnicity dependent changes in the tumor microenvironment in leukemia.(12)

Management of cancer involves myelosuppressive chemotherapy/ radiation. These therapies lead to cytopenias and immune suppression in all children receiving these therapies. Several studies have evaluated the early immune reconstitution that occurs within the first few years after completion of anti-leukemic therapy.(13) Most of these early studies utilized a limited set of markers and functional assays to characterize the immune system. We have recently utilized several complementary high-dimensional approaches(1, 2) to study the immune system in children with B-ALL at early recovery (<3 years) following completion of therapy (unpublished). Our data demonstrate wide variability in phenotypes and trajectories of immune recovery in these children. At present, nearly all the data relating to the immune system in survivors has focused on early stages of immune reconstitution post chemotherapy.(13) Data relating to variability in the immune system in adult survivors of childhood cancer are extremely limited and lack the level of depth in studies focused on early survivors. We therefore propose to characterize the immune phenotype and function of adult survivors of childhood hematologic malignancies with a combination of high-dimensional assays to address this unmet need.

SPECIFIC AIMS / OBJECTIVES / RESEARCH HYPOTHESES

As discussed above, emerging data from our labs demonstrate marked variability in terms of immune phenotypes and function in early survivors of B-ALL (<3 years post completion of therapy). We **hypothesize** that adult survivors of childhood hematologic malignancies will exhibit similar (or even greater) degrees of variability in terms of immune phenotypes, and these differences will be associated with key chronic health conditions (cardiometabolic diseases) and certain second malignancies that have not been treated with chemotherapy/radiation (thyroid cancer, meningioma and basal cell carcinoma [BCC]). Understanding this biology may pave the way for targeted interventions to mitigate the risk and severity of these chronic health conditions. The **specific aims** are:

Aim 1. To identify broad “immune-types” in adult survivors of childhood hematologic malignancies with unbiased approaches and determine demographic, clinical characteristics and therapeutic exposures associated with these phenotypes. We hypothesize that childhood cancer survivors can be classified into distinct groups based on immune phenotypes and signaling patterns in specific immune cells.

Aim 2. Compare immune phenotypes identified in adult survivors of childhood hematologic malignancies (Aim1) versus matched healthy controls. We hypothesize that immune cells in survivors will have distinct immunophenotypic and phosphoproteomic characteristics compared to healthy controls indicative of potentially targetable inflammatory signaling pathways and immune dysfunction.

Aim 3. Describe immune signatures in adult survivors of childhood hematologic malignancies with and without cardiometabolic chronic health conditions, physiologic frailty, and key second cancers (thyroid, meningioma, BCC). We hypothesize that survivors with adverse health outcomes such as cardiometabolic syndrome, second malignancies, and physiologic frailty will be characterized by distinct phenotypes and signaling alterations.

Overall Analytic framework

OUTCOME(S) OF INTEREST

AIM 1: Broad “immune-types” in childhood hematologic malignancy survivors using an agnostic approach

AIM 2: Prevalence of immune-types in survivors c/w health controls

AIM 3: Chronic health conditions and second cancers listed below.

Chronic health conditions:

- Grade ≥ 3 heart failure
- Grade ≥ 3 coronary heart disease
- Grade ≥ 2 diabetes
- Grade ≥ 2 dyslipidemia
- Grade ≥ 2 hypertension
- Obesity (By self-reported BMI ≥ 30 at any time-point)

Second cancers:

- Meningioma
- Thyroid cancer
- BCC

Planned research population:

Our studies will leverage previously collected blood samples from patients in the CCSS registry who are survivors of hematologic malignancies (leukemia/lymphoma). We will include patients from the initial cohort as well as the expansion CCSS cohort for these samples.

Aim 1 and 2: A sample of 200 adult survivors of childhood hematologic cancers with available whole blood banked with CCSS (≥ 18 y at sample donation) will be selected to represent the underlying CCSS hematologic malignancy population. We have procured and banked over 100 healthy donor blood samples. We obtain these both through our local blood bank as well as through blood draws using our institutional protocol. We have analyzed a cohort of healthy donors with the proposed assays and these donors will provide a source of age/sex/race/ethnicity-matched controls. The healthy donor blood is obtained via our local blood bank and through blood draws using our institutional protocols. We will expand the pool of healthy donors to match the demographics of the CCSS samples obtained for our studies. Based on our prior experience and a review of the literature we feel that the proposed sample size should be sufficient for identifying the clusters of immune phenotypes and conducting downstream analysis.

Aim 3: Adult survivors of childhood hematologic malignancies (not chosen as part of Aim 2) and with available blood sample (≥ 18 y at sample donation) will form the framework for selection of matched cases and controls:

- Chronic health conditions
 - presence of any of the following conditions that would fall under the category of cardiometabolic syndrome (grade 3+ heart failure; Grade 3+ coronary heart disease; Grade 2+ diabetes; Grade 2+ dyslipidemia; Grade 2+ hypertension; obesity (n=100)
 - Matched controls: matching criteria: primary cancer diagnosis, age at sample draw, race/ethnicity, length of follow-up for the controls to exceed that of the corresponding case (n=100). These are controls are childhood hematologic malignancy survivors and are distinct from the healthy controls in aim 2
- Subsequent cancers
 - BCC (n=40), thyroid cancer (n=40), meningioma (n=40). These cancers were chosen as they are unlikely to be treated with chemotherapy.
 - Matched controls: matching criteria: primary cancer diagnosis, age at sample draw, race/ethnicity, length of follow-up for the controls to exceed that of the corresponding case (n=120). These are controls are childhood hematologic malignancy survivors and are distinct from the healthy controls in aim 2

Exploratory variables:

- i) Age at primary cancer diagnosis
- ii) Age at survey completion
- iii) Age at sample procurement (not for Aim 3 but may examine to see if matching is good)
- iv) Sex
- v) Race/ethnicity (not for Aim 3, but may include as a variable if not perfectly matched)
- vi) Education
- vii) Income
- viii) Primary cancer diagnosis (not for Aim 3, but may include as a variable if not perfectly matched)
- ix) Age at BCC diagnosis
- x) Age at thyroid cancer diagnosis
- xi) Age at meningioma diagnosis
- xii) Age heart failure, coronary heart disease, hypertension, dyslipidemia and diabetes diagnosis
- xiii) Radiation (site, dose)
- xiv) Chemotherapy (y/n)

- xv) Cumulative doses of anthracyclines, alkylators, platinum
- xvi) Smoking (never, current former)
- xvii) Physical activity status and frailty (closest to the time of sample procurement)

Samples Requested

1. 10 million viably frozen PBMCs/patient
2. 500 mcl plasma/patient
3. 10-15mcg gDNA from PBMCs or whole blood/patient

Tests performed on samples

PBMCs.

Single cell mass cytometry. PBMCs from all patients will be used to characterize immune cell phenotype using single cell mass cytometry as described in our prior studies(11, 14-18).

Single cell phosphoproteomics. We will also use the PBMCs from all patients for single cell phosphoproteomics to determine the functional/activation status of the immune cells.

T cell proliferation assay. We plan to perform T cell proliferation assay on all samples. However, it is possible that we will obtain sufficient PBMCs after thawing for all assays, in this case we will prioritize single cell mass cytometry and single cell phosphoproteomic studies over T cell proliferation assays

Single cell transcriptomics and single cell proteomics (CiTE-seq) will be performed on selected patients using the 10X genomics platform, as described in our prior studies(16). We will target 20 samples each from patients who develop cardiometabolic syndrome, 20 patients who do not develop cardiometabolic syndrome, 20 patients who develop second malignancy and 20 who do not develop second malignancy.

Plasma.

All samples will be used to detect plasma cytokine levels

gDNA.

T cell receptor sequencing and TREC analysis will be performed on all gDNA samples received.

In recent studies we have successfully utilized these assays to characterize the immune landscape in childhood leukemia with similar samples.(19) We have also extensively utilized these tools to study the immune system in cancer tissues in adult cancer, as detailed in prior publications.(14-17, 20)

Summary of Samples and Assays for each aim

	Aim 1	Aim 2	Aim 3
Single cell mass cytometry	N= 200*	N= 200*	N= 440
Single cell phosphoproteomics	N= 200*	N= 200*	N= 440
T cell proliferation assay	N= 20*	N= 20*	N= 80
Single cell transcriptomics/proteomics (CiTE-seq)	N= 20*	N= 20*	N= 40
Plasma cytokines	N= 200*	N= 200*	N= 440
T cell receptor sequencing and TREC analysis	N= 20*	N= 20*	N= 40

*Same samples for aims 1 and 2

Immune Assays:

Mass cytometry: Immune profiles of mononuclear cells will be analyzed with a 53-marker custom mass cytometry panel. Mass cytometry data will be analyzed using CYTOBANK software as previously described.(19) We will also perform unbiased UMAP and flowSOM analysis using all mass cytometry markers to identify additional markers that may correlate with inferior immune recovery. All comparisons will be corrected for multiple comparisons utilizing Bonferroni correction.

CITEseq: CITEseq will be performed in a subset of patients with or without cardiometabolic health condition, second malignancy and matched controls. These samples will be selected based on availability of adequate PBMCs from samples and matched controls. We anticipate sequencing of at least 5000 cells per patient from at least 20 patients in each cohort, leading to sequencing of at least 100,000 cells in each cohort which will allow for adequate power to detect differences in genomic profiles that correlate with improvement versus not. We will use 5' sequencing libraries from 10X genomics (drop seq platform). This has the advantage of allowing us to perform T cell receptor sequencing on these samples as well which may be helpful in understanding the T cell repertoire and may serve as another indicator of thymic recovery along with assessment of T cell receptor excision circles (TRECs) described below. Data for single cell transcriptomic data will be analyzed utilizing Seurat V3 package in R as described in our prior studies.(19, 21) For visualization of results, uniform manifold approximation and projection (UMAP) will be performed and cluster identity determined using single R as well as antibody data from CITEseq. Significantly differentially expressed genes will be identified by the Wilcoxon rank-sum test with a Bonferroni correction ($P < 0.05$). Pathway analysis of significantly differentially expressed genes (Wilcoxon rank-sum Bonferroni adjusted $P < 0.05$) between clusters of interest will be performed using the pre-ranked workflow with gene set enrichment analysis (GSEA) software and the Molecular Signature Database (MSigDB) from the Broad Institute, as described in our prior studies.(21)

Analysis of thymic function: Prior studies have indicated an important role of age and thymic function in terms of recovery of lymphoid cells following intensive chemotherapy.(22, 23) Loss of naïve cells as a marker for immune dysfunction in our studies suggests that these patients have altered thymic function at initial diagnosis.(19) We will track their T cell receptor excision circles (TRECs) as a marker of thymic recovery following completion of therapy.(24) For some patients we will also obtain information on TCR diversity through CITEseq analysis, coupled to TCR sequencing.

Functional Assays: Important aspects of T cell function include their ability to proliferate in response to antigen stimulation as well as their ability to secrete cytokines that help clear pathogens or abnormal cells. We will assess these functions through the following assays:

T cell Proliferation Assay: We will assess the ability of the T cells to undergo expansion using the CFSE assay.(25) In this assay, T cells are labeled with a cell tracer dye, which halves every division. Number of cell divisions can be quantified by dilution of the cell surface dye. T cells will be stimulated using CD3/28 beads or PHA (phytohemagglutinin) or left unstimulated as negative control. T cell proliferation following stimulation with Candida antigen will be determined to understand their ability to recognize antigen.

Detection of cytokine secretion: Cell supernatants will be collected 48 hours following stimulation as above. Secreted cytokines will be measured using multiplex Luminex which allows detection of up to 48 different cytokines in 50 µl of cell supernatant. This will determine the quality of the T cells (i.e., whether they are able to secrete Th1 cytokines [IFN γ , TNF] or whether they are producing greater amounts of Th2 cytokines [IL4, IL10, IL13])(25).

Single cell phospho-proteomics: Mitogen activated protein (MAP) kinase and JAK-STAT pathways are important for several aspects of T and myeloid biology. We have recently developed a flow and mass cytometry based panel to systematically analyze phosphorylation of key target proteins within these pathways. This includes analysis of phosphorylated STAT1 (p-STAT1), p-STAT3, p-STAT5 (JAK-STAT pathway) as well as p-ERK and p-38 (MAPK pathway) coupled to specific cell surface markers to evaluate these changes at a single cell level. In our preliminary studies, these studies were particularly informative when looking for race/ethnicity-based differences in phospho-proteomics of immune cells. It will also provide direct information relating to inflammatory phenotype in target cellular populations, as has been implicated in the setting of chronic inflammation and aging(7).

Overall analytic strategy:

Aim 1: We will integrate data from mass cytometry and phosphoproteomics into hierarchical clustering algorithms as described in our recent studies (11), to identify distinct immune phenotypes and signaling states that cluster together(11). Appropriate statistical validation of selecting K clusters of immune phenotypes will be provided based on 'prediction strength' from 5-fold cross-validation as proposed by Tibshirani and Walther (2005).(26) This would aid in measuring the confidence of finding the K number of immune phenotypes/groups as opposed to any ad-hoc choice of clusters. We will also focus on each major immune cell type (e.g. B, T, myeloid) and utilize self-organizing maps (e.g., with FlowSOM) to identify distinct immune meta-clusters as described our recent publications

The downstream analysis will be undertaken with the number of clusters that biologically makes more sense. We anticipate that these analyses will yield distinct cohorts (for example cohorts with T cell dysfunction, exhaustion or myeloid inflammation). These studies will be complemented with T cell repertoire analysis and proliferation assays. Plasma cytokines will be analyzed to correlate with inflammatory phenotypes.

Aim 2: We will perform hierarchical clustering as in aim 1 using samples from both healthy donors and survivors to identify distinct groups as in aim 1. We will focus on each major immune cell type (e.g. B, T, myeloid) and utilize self-organizing maps (e.g., with FlowSOM) to identify distinct immune meta-clusters as described our recent publications(11, 14, 16, 17, 27). We will compare the immune meta-clusters between cancer survivors and matched healthy controls to identify major immunophenotypic and functional differences in survivors. Data from single cell transcriptomic studies on a subset of patients will be utilized to further understand transcriptomic underpinnings of these differences. The analytical approach would be similar to the one described for Aim 1.

Aim 3: we will utilize mass cytometry and phosphoproteomics as in Aims 1 and 2 above, to identify immune profiles enriched in cohorts with chronic health conditions or subsequent cancers, versus matched cohorts that do not develop these conditions. For functional studies, we will focus in particular on potentially actionable signaling pathways, that could serve as future biomarkers for and targets for specific preventive strategies in the future.

Anticipated results / potential pitfalls / alternatives

We have extensive experience with all of the proposed assays utilizing <10 million PBMCs obtained from other comparable biorepositories (such as COG or Aflac leukemia biorepository). We also have extensive experience with downstream bioinformatic tools. Therefore, we do not anticipate any technical issues with these studies. We also have support from our institutional funds to be able to begin these studies. We do however expect to apply for NIH support as well. We expect to successfully identify broad "immune types" based on integration of our immune data from several assays (aim1). These immune types may for example be biologically characterized by dominant alterations in phenotype or signaling in specific immune cells, such as myeloid or T cells. We also expect to find evidence of immune dysfunction as well as greater variability in immune function in survivors of childhood cancer compared to matched healthy controls (aim2). Finally, survivors who develop chronic health conditions may have distinct immune signatures compared to those who do not. This is also expected to be true for the cohort that developed second cancers (aim3). Our data may therefore not only provide insights into immune alterations in adult survivors of childhood cancer, but also may lead to future prospective studies specifically targeting aberrant pathways to prevent long-term complications.

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