

AWARD NUMBER: W81XWH-18-1-0239

TITLE: Breaking B-Cell Tolerance to Produce Antibodies that Eradicate Leukemias and Lymphomas

PRINCIPAL INVESTIGATOR: Stefanie Sarantopoulos

CONTRACTING ORGANIZATION: Duke University, Durham, NC

REPORT DATE: January 2022

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
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1. REPORT DATE January 2022		2. REPORT TYPE Final		3. DATES COVERED 30Sep2018-29Sep2021	
4. TITLE AND SUBTITLE Breaking B-Cell Tolerance to Produce Antibodies that Eradicate Leukemias and Lymphomas				5a. CONTRACT NUMBER W81XWH-18-1-0239	
				5b. GRANT NUMBER	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Stefanie Sarantopoulos, PhD E-Mail: stefanie.sarantopoulos@duke.edu				5d. PROJECT NUMBER 0011172009	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Duke University 2200 W Main Street, Suite 800 Durham, NC 27708-4677				8. PERFORMING ORGANIZATION REPORT NUMBER 1	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Development Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION STATEMENT: Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT Targeted immunotherapies are urgently needed for the treatment of life-threatening leukemias and lymphomas. Tumor specific antigens are not universally present on cells and the antigen profiles of tumors change when single antigens are targeted by monoclonal antibodies. Thus, while monoclonal antibodies have proven capacity to eradicate hematolymphoid and solid tumors, tumor specific and tumor associated antigen (TAA) targets are variably immunogenic and animal vaccinations with these antigens do not readily produce therapeutically viable antibodies. Polyreactive anti-tumor antibodies are not produced when peripheral B cell tolerance mechanisms are intact. Our work studying allo-HCT patient samples has now led to improved understanding about how B cell tolerance mechanisms dampen recognition of host/self. Cancer relapse after allo-HCT in patients who never develop chronic graft versus host disease (cGVHD) reveals how host-protective mechanisms dampen responsiveness to tumor. Our primary objective is to develop urgently needed B-cell immunotherapies for the treatment of life-threatening hematolymphoid malignancies.					
15. SUBJECT TERMS Immunotherapy, anti-tumor antibody, B cell, allogeneic stem cell transplantation					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRDC
Unclassified	Unclassified	Unclassified	Unclassified	33	19b. TELEPHONE NUMBER (include area code)

TABLE OF CONTENTS

	<u>Page</u>
1. Introduction	4
2. Keywords	4
3. Accomplishments	4
4. Impact	10
5. Changes/Problems	10
6. Products	11
7. Participants & Other Collaborating Organizations	11
8. Special Reporting Requirements	12
9. Appendices	13

1. INTRODUCTION:

Our major objective is to develop targeted immunotherapies that are urgently needed for the treatment of life-threatening leukemias and lymphomas. Tumor specific antigens are not universally present on cells and the antigen profiles of tumors change when single antigens are targeted by monoclonal antibodies. Thus, while monoclonal antibodies have proven capacity to eradicate hemato-lymphoid and solid tumors, tumor specific and tumor associated antigen (TAA) targets are variably immunogenic and animal vaccinations with these antigens do not readily produce therapeutically viable antibodies. We have previously demonstrated that constitutive IgG production by aberrantly activated B-cells after hematopoietic allogeneic stem cell transplantation (HCT) potentially produce antibodies that might target tumor antigens. We have also demonstrated that these B-cells are promoted by RNA-receptor (TLR7) responsive. We focus on a high order RNA-packaged vaccine vector called VRP that we showed promotes B-cell responses to pursue--our aims 1) to augment B-cell responses and 2) to harness anti-tumor antibody producing B-cells.

2. KEYWORDS:

Leukemia, Lymphoma, anti-tumor antibodies, stem cell transplantation, B cell

3. ACCOMPLISHMENTS:

What were the major goals of the project?

Specific Aim 1: Devise a strategy for safe B cell adoptive therapy that eradicates hematolymphoid tumors.

Major Task 1: Induce anti-tumor B cell responses in mice.

We have systematically approached this hypothesis as outlined in our proposal and we have accomplished our major task as detailed below in the subsections under “What was accomplished under these goals?” below (major and specific objectives).

Subtask 1: Submit and obtain documents for ACURO approvals (1-3)

Task completed 100%

Subtask 2: Test antibody and anti-tumor response of adoptively transferred syngeneic B cell products (3-9)

Task completed 100%

Subtask 3: Test whether B cell response after VRP-Flt3 is TLR9 or TLR7 mediated, using TLR9 -/- donor cell (10-12)

Task is 60% completed. We have made VRP vector needed for experiments and experiments and we are prepared to perform additional tumor studies pending review of our manuscript.

Subtask 4: Elucidate molecular mechanisms induced by TLR9 agonist CpG in human B cells after auto-HCT.

Task is 75% completed. We have tested CpG and TLR7 responses of B cells from patients and determined relative diminished TLR9 responses in patients who have achieved cancer remission.

What was accomplished under these goals?

Major Task 1: Induce anti-tumor B cell responses in mice.

This is our final report. Major Accomplishments

De novo immune responses to myeloid and other blood-borne tumors are notably limited and ineffective, making our ability to promote immune responses with vaccines a major challenge. While focus has been largely on cytotoxic cell-mediated tumor eradication, B-cells and the antibodies they produce also have roles in anti-

tumor responses. Indeed, therapeutic antibody-mediated tumor cell killing is routinely employed in patients with hematolymphoid cancers, but whether endogenous antibody responses can be incited to blood-born tumors remains poorly studied. A major limitation of immunoglobulin therapies is that cell surface expression of single tumor-associated antigen (TAA) targets is dynamic and varied, making promotion of polyclonal, endogenous B cell responses appealing. Since many TAA are self-antigens, strategies for tumor vaccines that lead antibodies to non-polymorphic antigen targets remains a challenge. Thus, we asked whether IgG were produced that recognize non-polymorphic TAA targets on hematolymphoid tumors. Since B cell responses to RNA vaccines are known to occur, we employed the Viral Replicon Particle (VRP). VRP constructed to encode mouse FLT3 provoked a rapid IgG B-cell response to this self-antigen and model TAA in leukemia and lymphoma mouse models. In addition, IgG to other TAA were also produced. Our data suggest that vaccination with RNA viral particle vectors incites a loss of B-cell tolerance that enables production of anti-tumor antibodies. The work provides impetus to employ such strategies that lead to a break in B-cell tolerance and enable production of broadly reactive anti-TAA antibodies as potential future therapeutic agents for patients with hematolymphoid cancers. **(Our submitted manuscript is appended at the end of this report).**

Major accomplishment: We have demonstrated that anti-tumor responses can be mounted by breaking B cell tolerance in syngeneic model system.

Subtask 3 and 4: We did not complete these subtasks largely because of technical challenges with VRP vaccine production efficiency and because global TLR 9 and TLR7 knockouts would unlikely give us an answer to whether B cells relied on TLR7 activation for rapid antibody production found after VRP vaccination. Given logistical challenges faced (in part due to the pandemic), we focused efforts on Major Task 2 and production of human anti-tumor antibodies. In a separate study in the laboratory we chronic graft versus host disease patients (NIH R01 HL129061-06). By studying patients who have lost B cell tolerance after allogeneic stem cell transplantation, we found that TLR9 responses were not increased in cGVHD B cells. By contrast when we used the TLR7 agonist, we found that TLR7 responsiveness was significantly higher in cGVHD B cells that we previously published are hyperresponsive to antigen. This demonstration of increased TLR7 responsiveness corroborates our hypothesis that RNA vaccines like the VRP tumor vaccine promote rapid IgG production by B cells.

Summary of Specific Aim 1, Major Task 1. Our data show that a rapid specific and broad antibody response to circulating tumor cells is elicited with this RNA virus system. We found that after VRP-Flt3 vaccination, antibodies to leukemia cells that over express FLT-3 are produced. Importantly, antibodies from these mice also bind to other unknown antigens since responses to VRP-mock leukemia cells that do not overexpress FLT3 were also targeted by antibodies from these mice. Thus, our data suggest that after vaccination with a same species self antigen using the VRP vector can ‘break B-cell tolerance’ and result in anti-self antigen and anti-leukemia antigen targeting by B-cells. While B cells from patients who were post syngeneic (autologous) did not show increased TLR 7 or 9 responsiveness, we found that B cells from patients with cGVHD who have ongoing anti-tumor immune responses (no cancer relapse) after transplant, we have now also shown that B cells have increased responsiveness through TLR7. These data (funded through NIH R01 HL129061 and manuscript in final editing stages for submission to Blood) support the hypothesis that RNA/TLR7 ligands like the VRP incite rapid responses from antigen-activated B cells and the pursuit of VRP-like vector therapies. The challenge with the VRP vector system (low efficiency of viral particle packaging and expression) are now also being overcome through development of synthetic vector systems by other investigators, including investigators at Duke. With the capacity to produce RNA viral cancer vaccine in a more efficient manner, we have plans to continue this work and to apply for funding for this tumor vaccine project with our Duke collaborators.

Milestone(s) Achieved in Major Task 1: We have demonstrate feasibility and safety of inducing anti-tumor B cell responses in preclinical models. We submitted a manuscript to Frontiers in Immunology on January 28, 2022.

Specific Aim 2: Use activated B cells from patients after stem cell transplantation to produce broadly reactive antitumor antibodies.

Major Task 2: Produce human antibodies

This task is 85% completed. We have produced IgG from cloned B cells from patients after transplantation and we have devised an antigen capture system for screening. We are now cloning B cells from CLL patients for production of full length anti-tumor antibodies.

Subtask 1: Obtain local IRB and DoD HRPO approval for the use of human anatomical substances (1-2)

Grant started September 2018.

This task is 100% complete. The approval for this work was August 12, 2019

Subtask 2: Screen for patient-specific anti-tumor antibodies (2-18)

This subtask is 85% completed. We have developed a work flow for antibody production and screening as detailed below. We have dissected and worked on each piece of the flowchart. Having identified tumor specific IgG from subcloned B cells from patients, we have used pooled primary tumor cells to identify antibodies that bind to tumor but not to normal cells. We sequenced the antigen binding portion of these antibodies and we are producing full length antibodies for further testing. We will complete in vitro assays to show that these antibodies mediate antibody dependent cytotoxicity (the Sarantopoulos lab discretionary funds will be used to cover cost of this remaining work).

What was accomplished under these goals?

1) Major activities – This is our final report. Thus, we focus here on major accomplishments.

Our major accomplishment is that we have now devised and tested our antibody production strategy using patient samples (**Fig 5**). While this work was stalled because of the COVID-19 pandemic (please see our request for no-cost extension), we have now subcloned and produce antibodies that we have shown using our flow cytometry based assay bind preferentially to tumor, but not healthy cells (**Fig 6**).

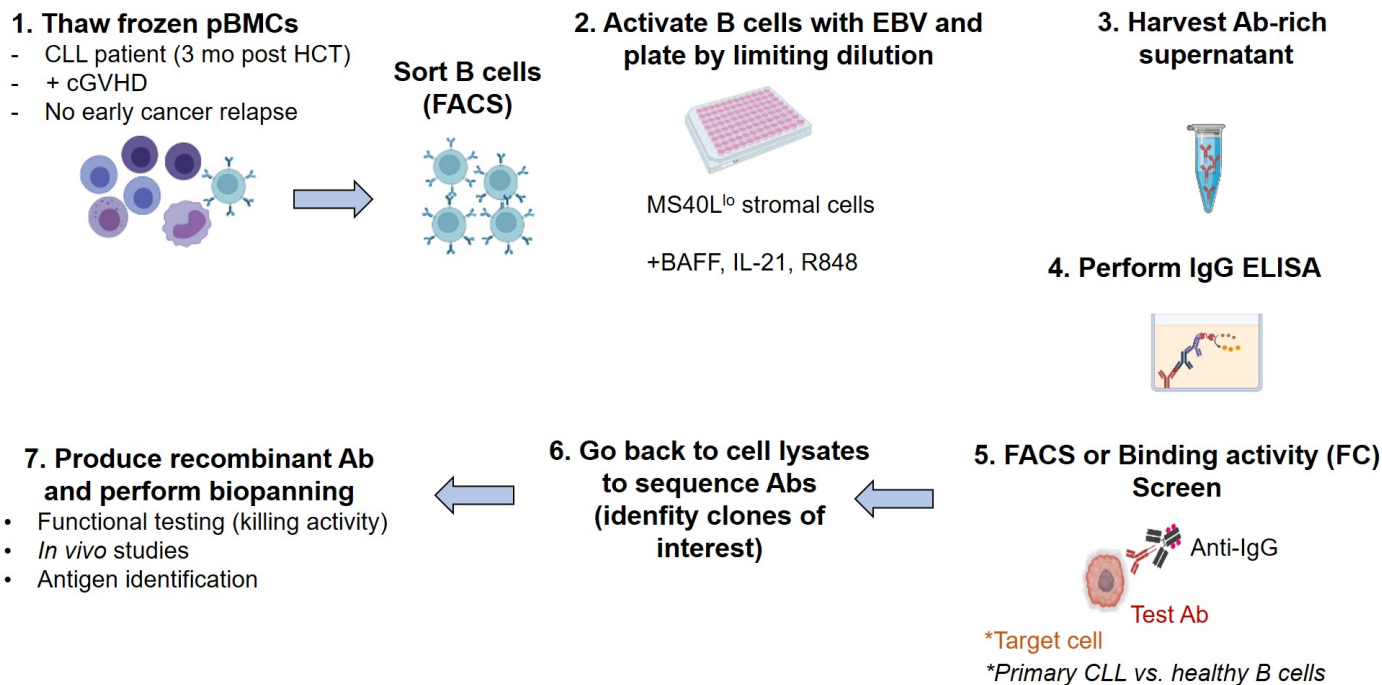
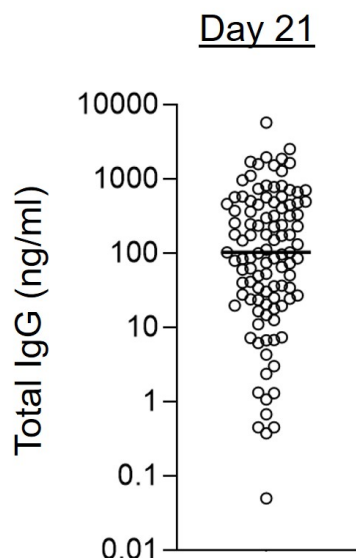


Fig 5. Method for cloning human anti-tumor (chronic lymphocytic leukemia, CLL) antibodies from patients. B cell subsets known to be activated and able to constitutively produce IgG will be single cell cloned and activated either with ebstein-barr virus (EBV) or cytokine cocktails. IgG from the supernatants (SN) will be tested for IgG production and used in functional antibody-dependent cellular cytotoxicity screening assays that identify anti-tumor antibodies. The full length antibodies will then be produced and using these antibodies, tumors can be eradicated and novel antigen targets can be identified.

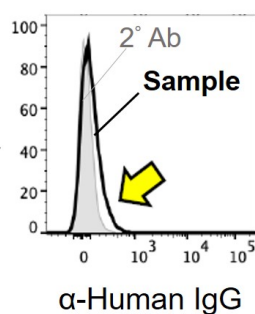
Culture efficiency: $105/302 = 34.77\%$



Binding Activity

10 IgG samples bound to CLL

Primary CLL



vs. healthy B cells - no/very low binding (not shown)

9/10 samples – Variable heavy (VH) and light (VL) chains sequenced

Produce recombinant Ab and perform biopanning

- Functional testing (killing activity)
- *In vivo* studies
- Antigen identification

Fig 6. IgG levels measured by sandwich ELISA from A patient whose cancer is in remission who has loss of B cell tolerance (cGVHD). 35% of the subcloned wells produced a median of 100ng/ml IgG. The IgG from the supernatant of these 105 wells was tested for binding capacity on pooled tumor CLL samples versus healthy B cell samples. RNA was then extracted from the cells from the 10 wells that produced antibodies to tumor, were subjected to RT-PCR and of the 10 subclones we have 9/10 of the VH-VL pairs sequenced (**Table 1 below**).

Table 1. Variable heavy (VH) and light (VH) chain gene families, mutation frequencies and complementary determining region (CDR3) lengths.

Sample	VH_LENGTH (# amino acid AA)	VH GENE	VH_D GENE	VH_J GENE	VH_ MUT FREQ	VH_ CDR3 LEN AA
5F5	340	IGHV3-48*03	IGHD1-26*01	IGHJ4*02	1.04%	6
6B10	346	IGHV3-11*01	IGHD6-6*01	IGHJ1*01	0.35%	8
1C6	402	IGHV1-18*04	IGHD3-3*01	IGHJ6*02	1.04%	27
1G8	388	IGHV3-23*04	IGHD6-6*01	IGHJ4*02	0.35%	22
2G10	352	IGHV3-30*18	IGHD5-12*01	IGHJ4*02	1.05%	10
3C10	367	IGHV4-59*01	IGHD6-13*01	IGHJ5*02	0.00%	16
3C11	364	IGHV3-23*04	IGHD4-17*01	IGHJ4*02	0.35%	14
3D2	367	IGHV3-30*18	IGHD1-26*01	IGHJ4*02	0.35%	15
3G5	366	IGHV3-30*04	IGHD4-17*01	IGHJ6*02	1.05%	15

Sample	L_LENGTH (# amino acid AA)	VL GENE	VL_JGENE	VL_MUTFREQ	VL_ CDR3 LEN AA
5F5	327	IGLV1-47*01	IGLJ3*02	1.12%	9
6B10	334	IGLV2-8*01	IGLJ2*01	1.12%	11
1C6	316	IGKV3-20*01	IGKJ1*01	0.00%	9
1G8	325	IGLV1-40*01	IGLJ3*02	30.04%	9
1G8	334	IGLV2-8*01	IGLJ2*01	1.11%	11
2G10	303	IGKV1-39*01	IGKJ5*01	0.82%	9
2G10	334	IGLV2-8*01	IGLJ2*01	0.76%	11
2G10	349	IGLV5-37*01	IGLJ2*01	1.04%	10
3C10	304	IGLV3-21*01	IGLJ2*01	1.71%	13
3C11	337	IGLV1-44*01	IGLJ7*01	3.19%	13
3D2	331	IGKV2D-29*01	IGKJ4*01	30.30%	12
3G5	337	IGKV2-28*01	IGKJ2*01	0.72%	9

In **Table 1**, we provide the variable heavy (VH, D, and J) gene families of the 9 clones along with VH (and VJ) family information. In two of the subcloned wells, we sequenced more than one VL-J gene set, making VH-VL

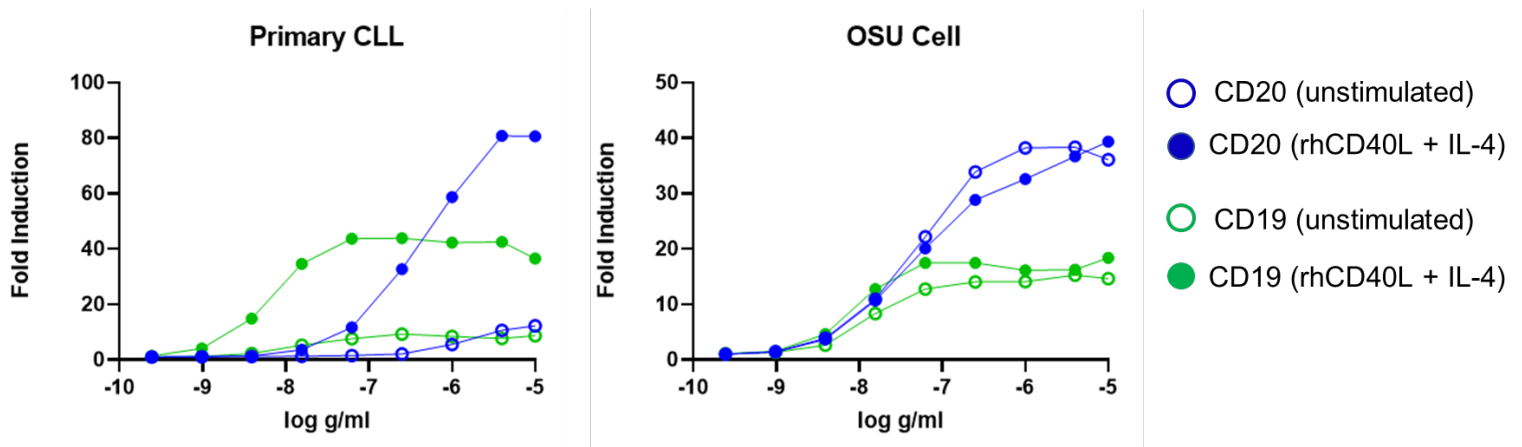


Fig 7. CD40 ligand/IL-4 co-stimulation enhanced the ADCC activity of anti-CD20 (Rituximab) and anti-CD19 (Inebilizumab) antibodies to the primary CLL tumor cells. Primary CLL cells and OSU-CLL line cells were stimulated with 100 ng/mL rhCD40L and 5 ng/mL rhIL-4 at 37°C for 24 hours. Cells were then harvested, washed, and incubated with various concentrations of anti-CD20 or anti-CD19 antibodies and with the effector cells for a 6-hr induction according to the instruction of Promega ADCC Reporter Bioassay. The effector: target cell ratio was optimized to 1:1 according to previous experiments. The ADCC activity was shown as fold of induction using the following formula: RLU (induced) / RLU (no antibody control).

fidelity in these 2 instances unclear. We are producing full-length antibody from our top tumor binding antibodies (and fortunately these are from clones with known VH-VL fidelity. As previously reported, we have developed a screening strategy that uses an ADCC Reporter Bioassay. This assay uses an engineered Jurkat cells stably expressing the FcγRIIIa receptor, V158 (high affinity) variant, and an NFAT response element driving expression of firefly luciferase as effector cells. Antibody biological activity in ADCC mechanism of action (MOA) is quantified through the luciferase produced as a result of NFAT pathway activation; luciferase activity in the effector cell is quantified with luminescence readout. During the funding period, we tested, then established and validated a method for screening patient-specific anti-tumor antibodies (Fig. 7).

Developing this assay for use on primary tumor cells required an activation step of the primary CLL cells. Our development of this screening assay is a major advancement that will allow us to screen for functional antibodies. We have also developed a flow cytometric based screening assay for detecting the CLL tumor targeting antibodies that will provide the first level screening of low prevalence antibodies that bind to cell surface antigens of interest. We will then expand B-cell clones of interest and purify the IgG prior to use in our ADCC assay.

Major Accomplishment: We have now developed a method for B cell cloning and IgG production as well as a functional assay to screen for antibodies that bind to tumor cells. Importantly, using this strategy, we have now harnessed monoclonal antibodies that bind to tumor but not normal cells. We are in the process of large scale production of these tumor specific antibodies in order to verify their tumor-eradicating capacity.

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

A primary goal of my academic medicine laboratory is to train promising young physician-scientists. This grant is not intended to train investigators, but the work proposed in this innovative project have afforded opportunities for training. During this funding period, I was fortunately able to recruit Sonali Bracken MD PhD. Dr Bracken is a trainee in the laboratory who had salary coverage by a training grant from an R38 NIH grant. I provide 1:1 mentoring regarding pursuit of experiments on this DoD expansion grant, and I also implemented a professional development plan with this trainee.

Another major goal in my lab that is enabled by funded projects like this one is the professional development of laboratory scientists. Hsuan Su, MS is a research associate who has implemented and helped refine our VRP vaccine work in our mouse models. Because of this D0D funded project, she was able to advance her professional development by completing a first authored manuscript. My senior lab scientists (Jon Poe PhD, and Wei Jia, MD directly mentored Hsuan Su regarding development and composition of her manuscript.

How were the results disseminated to communities of interest?

Nothing to report.

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

Nothing to report.

4. IMPACT:

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

Nothing to report.

What was the impact on other disciplines?

The work has implications in the solid tumor oncology field. It also has implications for understanding loss of B-cell immune tolerance that is relevant in bone marrow transplantation/Graft versus Host disease and in de novo rheumatological disorders (in the Rheumatology field).

This work pertains to the field (oncology and hematopoietic stem cell transplantation). The work also has potential implications for other fields. If we are able to identify anti-tumor antibodies, we may also be able to identify antibodies important in chronic graft versus host disease. Thus, our work impacts the immunology and rheumatology fields.

What was the impact on technology transfer?

Nothing to Report

What was the impact on society beyond science and technology?

Nothing to report

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

Nothing to report

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

Nothing to report

Changes that had a significant impact on expenditures

The COVID-19 pandemic impacted our ability to complete work within the time-constraints of the grant as planned. Thus, work was completed via a no-cost extension.

Update: We are now actively cloning B cells in the lab and testing for IgG production.

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

none

Significant changes in use or care of vertebrate animals

none

Significant changes in use of biohazards and/or select agents

none

6. PRODUCTS:

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

Manuscript submitted to Frontiers in Immunology.

Hsuan Su^{1,2†}, Kazuhiro Imai^{3†}, Wei Jia^{1,2}, Zhiguo Li⁴, Rachel A. DiCioccio^{1,2}, Jonathan S. Serody^{5,6,7}, Jonathan C. Poe^{1,2}, Benny J. Chen^{1,8}, Phuong L. Doan^{1,8}, Stefanie Sarantopoulos^{1,2,8}, Alphavirus Replicon Particle Vaccine Breaks B cell Tolerance and Rapidly Induces IgG to Murine Hematolymphoid Tumor Associated Antigens. *Submitted to Frontiers in Immunology on January 28, 2022.*

Journal publications.

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

Nothing to report

Other publications, conference papers and presentations.

R38 executive committee presentation by Dr. Sonali Bracken February 24, 2020 “Harnessing B-cells to Eradicate Tumors”

Duke Department of Medicine Research Retreat. Invited oral presentation by Sonali Bracken, entitled “Exploiting the lack of B cell tolerance in chronic graft-versus-host disease (cGVHD) to develop antibody-based therapies for cancer” February 3, 2022.

- **Website(s) or other Internet site(s)**
none
- **Technologies or techniques**
none
- **Inventions, patent applications, and/or licenses**
none
- **Other Products**

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name: Stefanie Sarantopoulos
Project Role: Primary Investigator
Researcher Identifier (e.g. ORCID ID): 0000-0003-4551-6687
Nearest person month worked: 5 (calendar months 3.0)- 20% effort

Contribution to Project: Dr. Sarantopoulos is the PI of the grant and is directly supervising all work performed.

Name: Sonali Bracken
Project Role: Post-doctoral fellow, MD/PhD R38 trainee

Researcher Identifier (e.g. ORCID ID): 0000-0003-1814-4099

Nearest person month worked: 5 (calendar months 6.0) – 50% effort

Contribution to Project:

Dr. Bracken has performed work on developing an assay to identify tumor-specific antibodies and she has successfully started sorting and cloning B cells for high-throughput antibody-production and screening

Dr. Bracken is completing a manuscript that demonstrates the role of TLR7 in chronic GVHD—while not part of the SOW, this work is important related work and the paper is going to be submitted to Blood in February 2022 (it is in final editing stage)

Funding Support:

R38 NIH physician scientist training grant;

We received a grant for 25K from the R38 parent to support part of Hsuan Su's effort.

Name:

Hsuan Su

Project Role:

research technician, MS

Researcher Identifier (e.g. ORCID ID): n/a

Nearest person month worked: 6 (calendar months 6.0) – 50% effort

Contribution to Project:

Hsuan Su, MS has performed mouse studies, compiled data, produced comprehensive figures and helped write a manuscript that is currently being submitted to PLoS ONE by the end of August.

Funding Support:

This CMDRP grant and Sarantopoulos lab funds from Duke

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?

Nothing to report

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:

QUAD CHARTS:

9. APPENDICES:

Alphavirus Replicon Particle Vaccine Breaks B cell Tolerance and Rapidly Induces IgG to Murine Hematolymphoid Tumor Associated Antigens

Hsuan Su^{1†}, Kazuhiro Imai^{1,3†}, Wei Jia¹, Zhiguo Li^{4,8}, Rachel A. DiCioccio¹, Jonathan S. Serody^{5,6,7}, Jonathan C. Poe¹, Benny J. Chen^{1,8}, Phuong L. Doan^{1,8}, Stefanie Sarantopoulos^{1,2,8*}

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†These authors have contributed equally to this work and share first authorship

Keywords: Alphavirus Replicon Particle, hematolymphoid tumor, B cell tolerance, autoantigen, antitumor antibody, VRP, cancer vaccine.

1. Abstract

De novo immune responses to myeloid and other blood-borne tumors are notably limited and ineffective, making our ability to promote immune responses with vaccines a major challenge. While focus has been largely on cytotoxic cell-mediated tumor eradication, B-cells and the antibodies they produce also have roles in anti-tumor responses. Indeed, therapeutic antibody-mediated tumor cell killing is routinely employed in patients with hematolymphoid cancers, but whether endogenous antibody responses can be incited to blood-born tumors remains poorly studied. A major limitation of immunoglobulin therapies is that cell surface expression of single tumor-associated antigen (TAA) targets is dynamic and varied, making promotion of polyclonal, endogenous B cell responses appealing. Since many TAA are self-antigens, strategies for tumor vaccines that lead antibodies to non-polymorphic antigen targets remains a challenge. Thus, we asked whether IgG were produced that recognize non-polymorphic TAA targets on hematolymphoid tumors. Since B cell responses to RNA vaccines are known to occur, we employed the Viral Replicon Particle (VRP). VRP constructed to encode mouse FLT3 provoked a rapid IgG B-cell response to this self-antigen and model TAA in leukemia and lymphoma mouse models. In addition, IgG to other TAA were also produced. Our data suggest that vaccination with RNA viral particle vectors incites a loss of B-cell tolerance that enables production of anti-tumor antibodies. The work provides impetus to employ such strategies that lead to a break in B-cell tolerance and enable production of broadly reactive anti-TAA antibodies as potential future therapeutic agents for patients with hematolymphoid cancers.

2. Introduction

Monoclonal antibodies can be utilized to manipulate the immune response to tumors or to directly lead to tumor cell killing, especially when combined with chemotherapy by covalently linking the cytotoxic compounds to antibodies. Importantly, rapidly emerging chimeric antigen receptor (CAR) T cell and CAR NK cell therapies are also extended applications of tumor antigen-specific antibodies (1-6). Thus, strategies that induce the active humoral immune response against tumor-associated antigens (TAAs) in the host have also received considerable attention for their therapeutic promise, whereby tumor cell killing is mediated primarily by antibody-dependent cellular cytotoxicity (ADCC). TAAs may emerge through mutations that lead to new epitopes in self-antigen (7-9). More commonly, TAAs are self-antigens that are either overexpressed or aberrantly expressed normal intracellular molecules. Because normal self-proteins are typically unrecognized by the host immune system without a breach in immune tolerance, producing antibodies to TAA remains a major challenge, especially in tumors that circulate and potentially induce ongoing immune tolerance to TAA. During the development of B cells, remarkable polyreactive repertoire potential arises (10). Autoimmune disease can arise unless the autoreactive antibodies are silenced by ongoing active mechanisms of tolerance that are operational in healthy individuals. In cancer patients, however, immune responses against self-antigens have been found (8). In addition, although many TAAs are self-antigens that are typically poorly immunogenic, antibodies against TAAs have been found in leukemia patients both before and after stem cell transplantation and in a variety of cancers (8, 9, 11-14). The tumor-specific antibodies found in cancer patients have been reported to be associated with better prognosis (9). Studies have also shown that B cells undergo clonal expansion, isotype switching, and affinity maturation within tumor-associated tertiary lymphoid structures (9). These findings further strengthen the role of the B cell response in antitumor immunity and suggest that the antitumor effects of B cells could be augmented through breaking the constraints of immune tolerance toward TAAs (15).

Promoting endogenous immune responses in the host that afford effective tumor eradication is of potential high impact. Venezuelan equine encephalitis virus (VEE) is a positive, single-stranded RNA alphavirus. Packaged replicon particles derived from an attenuated strain of VEE (16, 17) have been used for the development of vaccines for infectious diseases and cancer (18, 19). These VEE replicon particles (VRP) are propagation-defective, but can infect lymphoid cells and have a tropism for dendritic cells (17, 20). They can only undergo one round of infection and are not able to replicate in the host. VRP encoding an inserted gene not only serve as efficient vectors that express the gene of interest in infected cells at high levels (16, 17, 21, 22), they have also been shown to activate both cellular and humoral immune responses against the encoded tumor antigens in murine models of breast tumor (23-26), melanoma (27, 28) and prostate cancer (29, 30). Clinical trials have shown that the VRP vaccines are well-tolerated and are safe to use in patients (31-33). Subsequent trials testing VRP-HER2 vaccines in breast cancer, and VRP-CEA vaccines in colon cancer patients are ongoing (34, 35). Without an inserted gene, VRP have been demonstrated to elicit strong adjuvant activity (20, 36-38). Given what is now known about B-cell activation and autoantibody production that is driven by nucleic acid receptor activation (39, 40), VRP utilization is considered an ideal approach that may provide signals through nucleic acid receptors and facilitate production of antibodies to the TAAs.

Eradication of melanoma utilizing a TAA-encoding VRP was in part mediated via ADCC in an Fc dependent manner (28). These responses do not appear to be impeded by host anti-vector immunity, and notably, repetitive vaccinations of VRP augments the anti-tumor responses (27, 30, 33). Given the efficacy of VRP in solid tumor models, we sought to evaluate the potential of VRP as an approach to vaccination against hematolymphoid malignancies. We chose the *fms*-like tyrosine kinase-3 (FLT3), a receptor tyrosine kinase that is involved in the proliferation and survival of hematopoietic stem cells and dendritic cells as a model TAA target in our studies. This is in part because studies have shown that FLT3 is highly expressed in most acute leukemias, and the internal tandem duplications (ITD) mutation of this gene that occurs in approximately 25% of AML cases, and increase FLT3 expression on malignant cells has been associated with an adverse prognosis (41, 42). Given the high rate of FLT3 mutations and prognostic impact, FLT3 has been recognized as an attractive target for immunotherapy.

Here we investigated the potency of VRP encoding FLT3 as a therapeutic vaccine in mouse hematolymphoid tumor models. We found that B cell responses against self-antigen FLT3 and other surface antigens expressed on tumor cells in mice with established disease were induced. We demonstrate the potential of using VRP vaccines in patients with leukemias or lymphomas and that immune responses incited by single-antigen vectors may lead to TAA epitope spreading. These findings also have promising intimations for the design of novel antibody-based immunotherapies for hematolymphoid malignancies. Our results warrant further study of VRP vaccines in patients with leukemias and lymphomas.

3. Materials and Methods

3.1. Animal and cell lines

B6.SJL and BALB/c mice were purchased from Taconic Biosciences Inc. (Germantown, NY) and Charles River (Wilmington, MA), respectively. 8- to 12-week-old female mice were used for all experiments. All experiments were performed under a protocol approved by Duke University Institutional Animal Care and Use Committee. C1498 and A20 were purchased from American Type Culture Collection (Manassas, VA). The pcDNA 3.1/ myc-His vectors (Invitrogen) harboring a gene encoding the extracellular and transmembrane domains and a short portion of the intracellular domain of mouse FLT3 (AA 1 to 584) was transfected into C1498 and A20 cells using Amaxa® Cell Line Nucleofector® Kit V (Lonza). The transfected cells were selected and maintained in RPMI medium containing 10% FBS, 10mM HEPES, 2 mM L-Glutamine, 1 mM sodium pyruvate, MEM non-essential amino acid, 0.055 mM 2-mercaptoethanol, and 400 µg/mL Geneticin™ (Gibco) at 37°C under 5% CO₂. The FLT3 expression level on the transfected cells was determined by flow cytometry using fluorochrome-conjugated antibody against mouse FLT3/ CD135 (Clone A2F10, BioLegend).

3.2. Preparation of VRP

The extracellular-transmembrane domain of mouse FLT3 (AA 1 to 584) was cloned into the pVKE plasmid which was provided by Dr. Jonathan S. Serody (University of North Carolina at Chapel Hill, NC). Plasmid with the FLT3 transgene, pVKE-FLT3, or plasmid without the transgene, pVKE-mock, alone with other two helper plasmids encoding the VEE capsid and glycoprotein genes, respectively, were linearized by NotI (NEB) digestion, and T7 mMESSAGE mMACHINE® Kit (Ambion) were used for *in vitro* transcription. Capped VRP transcripts were electroporated into Baby hamster kidney (BHK) cells (ATCC) under a condition of 1000V/ 100ohms/ 25uF/ 3 pulses using ECM® 630 Exponential Decay Wave Electroporation System (BTX) for VRP packaging. The transfected cells were cultured with MEM- α medium (Gibco) containing 10% FBS, 2 mM L-Glutamine, 1 mM sodium pyruvate, pen/strep and 10% Tryptose Phosphate Broth (Sigma) at 37°C under 5% CO₂ for 24-26 hours. Culture supernatants containing the VRP were harvested and clarified by centrifugation at 800 x g/ 4°C for 10 minutes. VRP were further concentrated by ultracentrifugation at 24,000 rpm/ 4°C for 3 hours through a 5 mL cushion of 20% (w/v) sucrose dissolved in PBS and were resuspended in PBS.

The titer (IU/mL) of VRP encoding the FLT3 gene (VRP-FLT3) or VRP lacking an inserted transgene (VRP-Ctrl) was determined by flow cytometry. BHK cell monolayers were infected with serial diluted VRP for 16-18 hours at 37°C under 5% CO₂. Cells were harvested and counted with a hemacytometer. Cells were then fixed with Fixation/Permeabilization Solution Kit (BD Bioscience), followed by an incubation with 1:1000 diluted anti-VEE NSP mouse serum (Global Vaccine) at 4°C for 30 min. After being washed, cells were stained with 1:500 diluted Alexa Fluor® 488-conjugated goat anti-mouse IgG antibodies (Clone Poly4053, BioLegend) at 4°C for 30 min. Stained cells were washed, suspended in FACS buffer, and analyzed by flow cytometry. The fraction of infected cells and the volume of VRP stock used for infection was plotted, and data points in the linear range were used for titer determination. The titer of VRP was calculated by the following equation: Titer (IU/mL) = (fraction of infected cell x number of cells at time of infection) / (volume of the viral stock used for infection).

3.3. Tumor challenge and VRP vaccination

For C1498-FLT3 tumor model, CD45.1 congenic B6.SJL mice were inoculated with 1 x 10⁶ C1498-FLT3 cells intravenously via the lateral tail vein. For A20-FLT3 tumor model, BALB/c mice were subcutaneously inoculated with 1 x 10⁶ A20-FLT3 cells on the back. Tumor-bearing mice were vaccinated subcutaneously on

the rear footpad with $1 - 2.5 \times 10^6$ IU VRP in a volume of 20 μ L PBS on days 4 and 18 post tumor inoculation. A third vaccine was given on day 32 post tumor inoculation in one repeat of the C1498-FLT3 tumor model. Mice in the vehicle control group were received PBS only (**Figure 1**).

The expansion of C1498-FLT3 tumor in the blood of B6.SJL mice was detected using antibodies against mouse CD45.1 (Clone A20, BioLegend) and CD45.2 (Clone 104, BioLegend) by flow cytometry. Mice were monitored and were euthanized when hind limb paralysis or other signs of morbidity were observed to minimize the pain and discomfort. The growth of A20-FLT3 tumor was monitored by measuring two perpendicular diameters using a caliper. Tumor area was calculated according to the following formula: area (mm^2) = length (mm) x width (mm). Mice were monitored and were euthanized when tumors reached an area of approximately 500 mm^2 , before reaching any further clinical signs and symptoms, in order to avoid unnecessary pain and discomfort. Maximum tumor size was always maintained within Duke IACUC guidelines.

3.4. Flow cytometric analysis

For analysis of cell frequency and number in the blood, an equivalent volume of whole blood sample was drawn from the retro-orbital sinus of each mouse, and the blood was transferred immediately to tubes containing heparin (25 U/mL). After lysis of red blood cells with RBC Lysis Buffer (eBioscience), cells were then washed, and non-specific binding to Fc receptors was blocked with TruStain FcX (BioLegend) prior to the surface marker staining. Fluorochrome-conjugated antibodies against CD19 (Clone 6D5, BioLegend), CD4 (Clone GK1.5, BioLegend), CD8a (Clone 53-6.7, BioLegend), CD44 (Clone IM7, BioLegend) and CD62L (Clone MEL-14, BioLegend) were used for B or T cell population analysis. 7-AAD (BioLegend) was used for exclusion of dead cells, and a fixed volume of CountBright Absolute Counting Beads (ThermoFisher Scientific) were used for cell number calculation. Lymphocytes were gated based on the forward and side scatter parameters.

For detection of tumor-binding plasma IgG, 2.5×10^5 tumor cells in each test were used. Cells were first stained with LIVE/DEAD™ Fixable Green Dead Cell Stain Kit (ThermoFisher Scientific). After washing, cells were blocked with TruStain FcX™ (BioLegend). Plasma samples from tumor-bearing mice were diluted to adjust the total IgG amount to 1 μ g per test according to the result of total IgG ELISA, and were incubated with tumor cells at 4°C for 90 minutes. Cells were washed and followed by staining with 1:200 diluted fluorochrome-conjugated goat anti-mouse IgG antibodies (Clone Poly4053, BioLegend) and anti-mouse CD135 antibody (A2F10, BioLegend) at 4°C for 30 minutes. Stained cells were analyzed on a 3-laser BD FACSCanto II flow cytometer (BD), and the data were further analyzed using the FlowJo software (FlowJo, LLC).

3.5. ELISA

Plasma samples were separated from blood by centrifugation at 600 x g/ 4°C for 10 minutes and were stored at -80°C until used. For plasma IgG determination, goat anti-mouse kappa (SouthernBiotech, cat# 1050-01) and goat anti-mouse lambda (SouthernBiotech, cat# 1060-01) antibodies were coated on the ELISA plate (Corning) at a total concentration of 2 μ g/mL in coating buffer (pH 9.5, BioLegend) overnight at 4°C. Wells were washed with PBS containing 0.1% Tween-20 (PBST) and were blocked with PBST containing 0.5% (w/v) BSA (blocking buffer) at room temperature (RT) for 1 hour. Blocking buffer was also used as dilution buffer. After wells were washed, purified mouse IgG (BIO-RAD, cat# PMP01) was used as a standard in the assay and the plasma samples were serially diluted, and applied to the wells for a 2-hour incubation at RT. Wells were washed and were incubated with 1:5000 diluted HRP-conjugated goat anti-mouse IgG secondary antibody (SouthernBiotech, cat# 1030-05) at RT for 1 hour. Wells were washed again, and TMB substrate (ThermoFisher Scientific) was used according to the manufacturer's instruction for color development. Reaction was stopped with 2M H_2SO_4 solution and the absorbance at 450nm was measured with the correction wavelength set at 570 nm. A standard curve with four-parameter logistic curve-fit was generated with GraphPad Prism software, and the IgG concentration of plasma samples was calculated according to the standard curve.

For the anti-FLT3 IgG ELISA, recombinant mouse FLT3 protein (rFLT3, Asn28 – Ser544, Sino Biological Inc.) diluted in coating buffer at a concentration of 2 μ g/mL was coated on the ELISA plates overnight at 4°C.

Wells were blocked with PBS containing 0.5% casein at RT for 1 hour. Plasma samples were serially diluted and applied to wells for a 2-hour incubation at RT. 1:5000 diluted HRP-conjugated goat anti-mouse IgG antibody (SouthernBiotech, cat# 1030-05) was added to wells and incubated at RT for 1 hour. Between each incubation step, wells were washed with PBST. TMB substrate was used for color development as described above. The titration curves of anti-FLT3 plasma IgG were generated with four-parameter logistic curve-fit.

For the tumor-binding IgG ELISA, membrane protein of C1498-mock cells was extracted using Mem-PER™ Plus Membrane Protein Extraction Kit (ThermoFisher Scientific), and was coated on plate at a concentration of 10 µg/mL in coating buffer (pH 9.5, BioLegend) overnight at 4°C. Wells were blocked with PBS containing 0.5% casein (BIO-RAD) at RT for 1 hour. Plasma samples were serially diluted and applied to the wells for a 2-hours incubation at RT. Wells were incubated with 1:5000 diluted HRP-conjugated goat anti-mouse IgG antibody (SouthernBiotech, cat# 1030-05) at RT for 1 hour. Wells were washed with PBST between each incubation step. TMB substrate was used for color development as described above. The titration curves were generated with four-parameter logistic curve-fit.

3.6 Immunoblotting

For the western blotting, rFLT3 protein (Sino Biological Inc.) was separated on NuPAGE Bis-Tris gel (ThermoFisher Scientific) under reducing conditions and transferred to nitrocellulose membranes using the iBlot system (ThermoFisher Scientific). Membranes were blocked with 1% casein protein in PBS (BIO-RAD) at RT for 1 hour, and were then cut into strips. Strips were incubated with rabbit anti-mouse FLT3 polyclonal IgG (LifeSpan BioSciences, Inc., cat# LS-C6858) or 1:250 diluted plasma samples overnight at 4°C. Strips were washed with PBST and were then probed with 1:15,000 diluted fluorochrome-conjugated antibody against rabbit IgG (H+L) (LI-COR, cat# 926-68073) or antibody against mouse IgG Fcγ fragment (Jackson ImmunoResearch, cat# 115-655-071) at RT for 1 hour. Strips were washed, rinsed with PBS and imaged by Odyssey infrared imaging system (LI-COR).

For dot blotting, 1 µL of rFLT3 protein diluted to a concentration of 50 ng/µL was spotted on the nitrocellulose membranes in duplicate and was dried on bench top for 6 min. Membranes were then blocked with 1% casein protein in PBS at RT for 1 hour, and were then cut into strips. Strips were incubated with 1:500 diluted plasma samples overnight at 4°C. Strips were washed with PBST and were then probed with fluorochrome-conjugated antibody against mouse IgG Fcγ fragment at RT for 1 hour. Strips were washed, rinsed with PBS and imaged by Odyssey infrared imaging system (LI-COR).

3.7. Statistical analysis

Significant differences for cell numbers or frequencies in blood, total IgG concentration, level of tumor antigen-specific IgG and MFI of tumor-binding IgG among experimental groups were determined by ordinary one-way ANOVA or Kruskal-Wallis test (non-parametric one-way ANOVA) as detailed in each figure legend.

Significant differences in survival were determined by log-rank test. All statistical analyses were performed with GraphPad Prism software (Version 9.3.1). P-values less than 0.05 were considered significant.

4. Result

4.1. VRP-FLT3 vaccination induces robust FLT3-specific IgG production in mouse lymphoma model.

To investigate the efficacy of VRP vaccines on inducing immune response toward a self-antigen, a mouse B cell lymphoma model was employed in our study. A20 cells were transfected with plasmid vectors harboring the mouse *Flt3* gene. The FLT3 expression was confirmed by flow cytometry (**Supplementary Figure 1**) before tumor cells were subcutaneously inoculated on the back of BALB/c mice. FLT3-harboring VRP (VRP-FLT3) vs control VRP (VRP-Ctrl, vector alone) were administered by footpad injection as therapeutic vaccines on days 4 and 18 after tumor challenge. Blood was withdrawn on day 14 and 28 for analyzing the lymphocyte compartment of the tumor-bearing mice. With VRP-FLT3 vaccination, the frequency of lymphocytes was significantly increased (**Figure 2A**). Among the lymphocytes, an increase of B cell frequency on day 28 was only observed in VRP-FLT3 vaccinated mice (**Figure 2B**). Both VRP-FLT3 and VRP-Ctrl vaccinations

induced a T cell response and resulted in a significant increase of activated CD44⁺ CD62L⁻ CD8⁺ T cell frequency (**Supplementary Figure 2A**).

The increase in B cell number suggested a B cell response might be mounted with VRP-FLT3 vaccination in our mouse model. Therefore, we further investigated antibody production in the tumor-bearing mice. Plasma samples collected on day 28 were analyzed for total IgG concentration, and we found that both VRP-FLT3 and VRP-Ctrl vaccinations significantly increased the total IgG level (**Figure 2C**). To further evaluate the specificity of antibody to FLT3, we performed western blot and dot blot with the rFLT3 protein. Plasma collected on days 35-42 was tested in western blot, and the result showed that only samples from VRP-FLT3 vaccinated mice contained IgG that recognized the recombinant FLT3 protein (**Figure 2D**). The dot blot, where the rFLT3 protein remained in a native form, showed a consistent result. It also indicated that the FLT3-specific IgG developed rapidly in tumor-bearing mice as early as 10 days after the first VRP-FLT3 vaccination. Furthermore, the titer of FLT3-specific IgG increased by a second vaccination and the response was sustained over time (**Figure 2E**). These findings suggest that the VRP-FLT3 vaccine induces a rapid IgG antibody response to the model TAA, a self-antigen, and the response can be boosted with repetitive vaccination.

4.2. VRP-FLT3 vaccination alters the composition of circulating B cells in a mouse lymphoma model.

To investigate whether VRP vaccination affected the composition of the peripheral B cell compartment in tumor-bearing mice that received control vs VRP-FLT3 vaccination, we examined different subsets of B cells in the blood after vaccination. It has been shown that the GL7⁺ B cell subset from secondary lymphoid organs has a higher capacity for antibody production compared to the GL7⁻ subset after reactivating with the same antigen used for immunization (43). We previously showed that the circulating GL7⁺ B cells were proportionally increased after alloantigen exposure when B-cell tolerance was lost in mice with chronic graft versus host disease, and production of host tissue-reacting antibodies was promoted (44, 45). In the current mouse lymphoma model, we also found that the GL7⁺ B cell frequency was significantly increased in VRP-FLT3 vaccinated mice on day 14 after tumor inoculation (**Figure 3A**).

In addition to the increase of GL7⁺ B cells, we found a significant elevation of CD93⁺ transitional-type B cells in the circulation after VRP-FLT3 vaccination by day 14 (**Figure 3B**). Moreover, within the CD93⁺ population, the frequency of IgM⁺CD23⁻ T1 transitional B cells also raised (**Figure 3C**). The overexpression of TLR7 was reported to promote the expansion of transitional-type B cells and further resulted in autoantibody production (46). These data suggest breaking tolerance to self-antigen and production of FLT3-specific IgG induced by the VRP-FLT3 vaccine may reside in the expanded GL7⁺ or transitional B cell populations.

4.3. VRP-FLT3 vaccination induces robust FLT3-specific IgG production in mouse leukemia model.

To investigate the potential use of VRP vaccines to break B cell tolerance in leukemia, a C1498 mouse leukemia model was employed. C1498 cells were transfected with plasmid vectors harboring the mouse *Flt3* gene. FLT3 expression on C1498-FLT3 cells was verified (**Supplementary Figure 1**) before the tumor cells were intravenously injected into B6.SJL mice. VRP-FLT3 or VRP-Ctrl were administered by footpad injection as therapeutic vaccines on days 4 and 18 after inoculation of C1498-FLT3 tumor cells. Peripheral lymphocyte compartments of these mice were then investigated for the immune responses elicited by VRP vaccines. With VRP-FLT3 vaccination, the number of lymphocytes in blood on day 28 was significantly increased (**Figure 4A**). Within the lymphocyte population, the B cell number in the peripheral blood of VRP-FLT3 vaccinated tumor-bearing mice increased significantly compared to that of the mice receiving PBS or VRP-Ctrl vaccines (**Fig 4B**). Besides the enrichment of B cells, the frequency of activated CD44⁺ CD62L⁻ CD4⁺ T cells and CD44⁺ CD62L⁻ CD8⁺ T cells were significantly higher in both VRP-FLT3 and VRP-Ctrl vaccination groups compared to the control mice that received PBS (**Supplementary Figure 2B**).

The concentration of total IgG in plasma samples collected on day 28 after tumor inoculation was determined by ELISA. Both VRP-FLT3 and VRP-Ctrl vaccines increased the total IgG level in tumor-bearing mice (**Figure 4C**). We then employed an ELISA using a recombinant mouse FLT3 (rFLT3) protein to investigate the production of antibody specific to FLT3 in tumor-bearing mice. A significant level of plasma IgG that

recognized rFLT3 was detected in mice vaccinated with VRP-FLT3, while this was not observed in VRP-Ctrl vaccinated group (**Figure 4D, E**). Thus, we demonstrated that VRP-FLT3 vaccine is capable of inducing a prompt antibody response to FLT3 in both our lymphoma and leukemia mouse models. These data suggest that vaccination of VRP-FLT3 can overcome the intrinsic B cell tolerance to the model self-antigen.

4.4. Antibodies against tumor membrane antigens are induced by VRP-FLT3 vaccination in mouse leukemia model.

The production of FLT3-specific IgG induced by VRP-FLT3 vaccine was confirmed by an ELISA using rFLT3. We then applied a flow cytometric approach to investigate the reactivity of plasma antibody to the tumor cells. C1498-FLT3 cells that were used to inoculate the mice or non-FLT3 expressing C1498-mock cells were used as targets in these assays. The FLT3 expression level of these target cells was confirmed (**Supplementary Figure 3**). Plasma samples collected on days 35-42 after tumor challenge were diluted according to the total IgG concentration to tailor the IgG amount in each test to 1 ug. Plasma IgG that reacted with tumor cells was detected by a fluorophore-conjugated secondary antibody. Although the difference did not reach statistical significance, the heatmap of MFI showed that plasma samples from VRP-FLT3 vaccinated mice tend to have overall higher reactivity to the C1498-FLT3 tumor cells than plasma samples from mice vaccinated with PBS or VRP-Ctrl (**Figure 5A**). Since FLT3 is only one of numerous antigens expressed on tumor cells and epitope spreading is known to occur when B-cell tolerance is lost (15, 47), we also wanted to test whether VRP vaccines could elicit antibody responses against TAAs other than FLT3. To do so, we performed the same flow cytometric binding test with the target cells changed to C1498-mock cells. Plasma samples from mice that received the VRP-FLT3 vaccination had higher levels of IgG bound to the C1498-mock tumor cells than plasma samples from PBS control mice (**Figure 5B**).

To verify this finding, we performed an ELISA with the membrane protein extracts of C1498-mock cells. Membrane protein extracts were coated on the plates, and plasma samples collected on days 35-42 from tumor-bearing mice were titrated and tested. The result showed that mice vaccinated with VRP-FLT3 developed a significantly higher level of IgG that reacted to tumor membrane proteins other than FLT3 compared to the PBS control mice (**Figure 5C, D**). These results indicate that vaccination of VRP-FLT3 not only elicits an antibody response to FLT3 (**Figure 4D, E**) but also elicits antibody responses to other TAAs in mice inoculated with FLT3-expressing tumor.

4.5. VRP-FLT3 vaccination attenuates growth of tumor in both mouse lymphoma model and leukemia model.

Since we observed the induction of antibody responses to both FLT3 and other tumor membrane antigens, along with the activation of T cells, we assessed whether our VRP vaccine strategy had a therapeutic effect against lymphoma and leukemia. Remarkably, a therapeutic potency of VRP vaccine was observed in the A20-FLT3 tumor model. This was evident in that the size of tumor inoculated on the back of mice significantly decreased in mice vaccinated with VRP-FLT3 compared to the mice receiving PBS or VRP-Ctrl vaccines (**Figure 6A**). The VRP-FLT3 vaccine was unable to completely eliminate the tumor, however, and there was no survival benefit observed with either VRP-FLT3 or VRP-Ctrl vaccination for this particular model (**Supplementary Figure 4A**). Attenuation of tumor growth was also observed in the C1498-FLT3 tumor model. We found an initial decrease in tumor cell number on day 28 after tumor challenge with both VRP-FLT3 and VRP-Ctrl vaccines. Importantly, tumor decrease was maintained at day 35 only in the VRP-FLT3 group although a statistical analysis was not possible since there were only 2 PBS-treated mice alive at day 35 (**Figure 6B**). Strikingly, there was also a significant improvement in survival in mice that received the VRP-FLT3 vaccine compared with the PBS control group (**Supplementary Figure 4B**). These data indicate that VRP vaccination attenuates the expansion of tumor cells *in vivo*, and the efficacy of VRP-FLT3 vaccine was superior to VRP-Ctrl vaccine, although complete tumor regression was not achieved.

5. Discussion

Previous studies have demonstrated that VRP encoding a TAA was a potential vaccination strategy in different solid tumor models. In this study, we described the efficacy of VRP to induce broadly reactive antibodies to self TAAs in two different hematolymphoid cancer mouse models. VRP encoding the TAA, FLT3, which is a self-antigen known to be overexpressed in acute leukemias, was given to mice bearing FLT3-expressing tumors. Although a complete tumor regression was not achieved in this pilot study, the growth of tumors was attenuated by the VRP-FLT3 vaccination in both mouse models, and a prolongment of survival was seen in the leukemia mouse model, suggesting that efficacious tumor reduction was associated with the immune response elicited by VRP-FLT3 vaccine. The more solid tumor-like A20-FLT3 lymphoma model may have very different tumor microenvironment from that of the C1498-FLT3 leukemia model. This could explain why a survival benefit was not seen in our lymphoma model. Both tumor cells used in our lymphoma or leukemia models were aggressive and fast-growing. Further optimization of the VRP vaccination strategy may be investigated to strengthen the anti-tumor immunity induced by this VRP system. It has been shown that the anti-vector immunity to VRP did not hinder the efficacy of repetitive vaccination with VRP, and development of immunity to the encoded antigen increased as more VRP vaccinations were administered (27, 30, 32). Additional doses of VRP-FLT3 vaccine could potentially further boost and/or maintain the protection against the tumors in these mouse models.

Induction of T-cell responses with VRP has been reported in both animal and human studies of different solid tumors (26-30, 32-35), and this was also seen in our study, as the frequency of activated T cells increased in the blood of tumor-bearing mice with the VRP vaccinations. In addition to the cellular immune response, VRP is also able to elicit a potent humoral response as a vector system for delivering the antigen of interest or simply as an adjuvant co-administered with antigens (23, 25, 27, 28, 30, 33, 36, 38, 48). Breaking tolerance to self-antigens is challenging but essential for vaccine approaches targeting TAAs that are recognized as self-antigens by the immune system in the cancer immunotherapy. It has been shown that VRP can induce specific antibodies to TAAs in studies of melanoma, breast cancer and colon cancer (23, 26-28, 33). Here we clearly demonstrated that the VRP-FLT3 vaccination overcame the intrinsic self-tolerance mechanisms to induce a strong antibody response to FLT3 in the tumor-bearing mice in both of our hematolymphoid tumor models. Our study, together with others, reveals the reproducible induction of immune responses to the self-antigen encoded by VRP.

FLT3 was chosen as the target antigen in our study for inciting antitumor activity due to the high correlation between FLT3 and leukemia; however, there are many other TAAs that have yet to be discovered in blood cancers. Antigen/epitope spreading induced by immunotherapy in cancer treatment has been reported (15, 49-51). In our study, in addition to the FLT3-specific antibody, we also found that vaccination of VRP-FLT3 can induce the production of antibodies reacting to the non-FLT3 expressing C1498-mock cells. The immunity to FLT3 elicited by VRP-FLT3 might serve as a trigger and raise a subsequent immune response against additional antigens on the tumor cells. Thus, here we demonstrate for the first time that VRP encoding a TAA can promote a polyclonal B cell response to not only the encoded TAA but also other TAAs expressed on tumor cells. Our result indicates that, with this VRP system, it is possible to activate the immune responses to currently unknown TAAs through the few that have been identified, including FLT3.

VRP lacking a transgene has been shown to possess an adjuvant activity that promoted the immune response to co-delivered antigen (36, 52, 53). This could account for the increase in frequency of activated T cells in the periphery, and for the increase in total IgG level in serum of mice that received the VRP-Ctrl vaccine in our study. VRP-Ctrl vaccine, which do not encode a transgene, was able to activate the immune system of tumor-bearing mice, yet it was unable to achieve TAA-specific responses. TAA-specific responses were only observed in tumor-bearing mice that received VRP-FLT3 vaccine, suggesting that a target antigen is needed to incite a desired immunity toward tumor cells. The critical parameters that mediate the immune activity of VRP have not yet been fully elucidated and the mechanisms of B cell responses induced by VRP remain unclear. UV inactivation abrogated the adjuvant effect of VRP, indicating that viral RNA and/or the RNA replication plays an important role in triggering the immune responses (36). Several cellular sentinel sensors could be responsible for recognizing the viral replicative molecules (36, 54), and Toll-like receptor 7 (TLR7) is one of the candidates. TLR7 signaling in B cells has been related to activation of autoreactive B cells and induction of

autoantibodies (39, 46, 55). Although a preferential infection of DCs and macrophages by VRP has been demonstrated, it has been shown that B cells can also be infected by VRP (20). Further study is needed to investigate whether VRP vaccine can directly or indirectly promote the activation of autoreactive B cells that have potential anti-tumor activity in the presence of a trigger TAA, without causing autoimmune disease comorbidity. This will help us to devise a better strategy for promoting the B cell response against tumor in the immunotherapy of hematolymphoid cancers.

While as stated above, VRP encoding TAA can provide potent tumor protection in prophylactic settings in studies of melanoma, breast cancer, and prostate cancer (23, 24, 26-29, 56). However, the tumor protecting effect was relatively modest when tumors were established prior to the administration of VRP (28, 56). In some instance, failure in inhibiting the growth of preexisting tumors by VRP vaccine can occur despite the ability of VRP to induce some degree of TAA-specific immune responses (25, 29). Nevertheless, employing other treatments in combination with VRP vaccine has been shown to yield a synergistic therapeutic activity (25, 56), indicating a possibility to shape the immune responses and enhance the antitumor efficacy of VRP vaccine when used alongside other therapies. In our study, activation of both cellular and humoral responses were observed, suggesting the possibility to utilize both arms of the immune response depending on the type of tumor and its aggressiveness.

Hematopoietic stem cell transplantation (HCT) is an important immunotherapy in hematolymphoid malignancies, especially for the refractory cases. Combining autologous HCT (auto-HCT) with a VRP-TAA vaccine strategy could be a future direction that is worth further study. For example, it is known that the B-cell activating factor (BAFF) is critical for development and survival of mature B cells, and elevation of BAFF levels can subvert the B cell tolerance and rescue the intermediate affinity, self-reactive B cells that are normally deleted during late maturation (10, 44, 57). Myeloablative treatment before HCT induces a lymphopenia condition in which the B cell compartment reconstitutes in a setting of a high BAFF-to-B cell ratio (58, 59). Applying a VRP vaccine strategy, which has proven to be an effective treatment to alter the self-tolerance, utilized at the right time point after auto-HCT may enable the rescue of those potentially tumor-reactive B cells and augment more efficient anti-tumor immunity without the immune toxicity found after allogeneic HCT.

6. Figure legends

Figure 1. The mouse lymphoma model and leukemia model, and the VRP vaccination strategy.

FLT3-expressing A20 tumor cells were inoculated subcutaneously on the back of BALB/c mice on day 0. Tumor-challenged mice were vaccinated on days 4 and 18 with 1×10^6 IU of VRP-Ctrl or VRP-FLT3 on the footpad. Control mice were received PBS ($n=5$ per group). FLT3-expressing C1498 tumor cells were inoculated intravenously through tail vein of B6.SJL mice on day 0. Mice were vaccinated on day 4 and 18, and day 32 in one repeat of the this model with $1-2.5 \times 10^6$ IU of VRP-Ctrl or VRP-FLT3 on the footpad. The control mice were received PBS ($n=5$ or 10 per group). Blood samples were harvested on day 14, 28, and 35-42.

Figure 2. VRP-FLT3 vaccination elicits a FLT3-specific antibody response in a mouse lymphoma model.

Mice challenged with A20-FLT3 tumor cells were vaccinated as described in Fig 1. (A-B) Blood samples harvested on days 14 and 28 were analyzed by flow cytometry for the frequencies of (A) lymphocytes and (B) CD19⁺ B cells. (C) Concentrations of total IgG in plasma on day 28 were determined by ELISA. Each symbol represents an individual mouse ($n=5$ per group). Bars represent the mean $\pm$ SD. Statistical significance was determined by one-way ANOVA, * $P<0.05$, ** $P<0.01$. (D) Western blot of recombinant mouse FLT3 protein. Plasma collected on days 35-42 was 1:250 diluted and used for probing. Arrow indicates the FLT3 protein. Two representative samples per group are shown. (E) Dot blot of recombinant mouse FLT3 protein. Protein was spotted in duplicate and probed with 1:500 diluted plasma collected on indicated time points. Two to three representative samples per group are shown. In both (D) and (E), fluorochrome-conjugated anti-mouse IgG secondary antibody was used for signal development.

Figure 3. VRP-FLT3 vaccination alters the composition of circulating B cells in a mouse lymphoma model.

Mice challenged with A20-FLT3 tumor cells were vaccinated as described in Fig 1. Blood samples harvested on days 14 were analyzed by flow cytometry for the frequency of (A) GL7⁺ B cells, (B) CD93⁺ mature B cells and CD93⁻ non-mature B cells, or (C) CD93⁺ IgM⁺ CD23⁻ T1 B cells (n=5 per group). A representative sample of each group is shown in contour plot on the right. Bars represent the mean $\pm$ SD. Statistical significance was determined by one-way ANOVA, **P<0.01, ***P<0.001, ****P<0.0001.

Figure 4. VRP-FLT3 vaccination elicits a FLT3-specific antibody response in a mouse leukemia model.

Mice challenged with C1498-FLT3 tumor cells were vaccinated as described in Fig 1. (A-B) Blood samples harvested on days 14 and 28 were analyzed by flow cytometry for the numbers of (A) lymphocytes and (B) CD19⁺ B cells. (C) Concentrations of total IgG in plasma on day 28 were determined by ELISA. Levels of FLT3-specific plasma IgG on day 28 were determined by ELISA, and the (D) titration curves and (E) O.D. value at 1:500 dilution are shown. Each symbol represents an individual mouse (n=5 per group). Bars represent the mean $\pm$ SD. Statistical significance were determined by one-way ANOVA, *P<0.05, **P<0.01, ****P<0.0001. Data represents one of two independent experiments.

Figure 5. VRP-FLT3 vaccination induces broad reactivity of antibody to mouse leukemia cell membrane antigens.

Mice challenged with C1498-FLT3 tumor cells were vaccinated as described in Fig 1. (A-B) Plasma samples collected on days 35-42 were diluted to a level of 1 ug total IgG and were incubated with (A) C1498-FLT3 cells or (B) C1498-mock cells. The tumor cell-bound IgG was detected by a fluorophore-conjugated secondary anti-mouse IgG antibody using flow cytometry. Heatmap of MFI of each sample are shown (n=8 or 10 per group). Statistical significance was determined by Kruskal-Wallis test (nonparametric) ANOVA, *P<0.05. A representative sample of each group is shown in histogram on the right (2nd antibody alone in shade grey and samples in blue line). (C-D) Plasma IgG bound to C1498-mock membrane extracts were determined by ELISA. Days 35-42 plasma samples were tested. (C) Titration curves (symbols represent the mean values in each group) and (D) O.D. values at 1:25 dilution (each symbol represents an individual mouse, n=7 per group) are shown. Bars represent the mean $\pm$ SD. Statistical significance was determined by one-way ANOVA, *P<0.05, **P<0.01.

Figure 6. VRP vaccination attenuates the growth of FLT3-expressing C1498 tumor cells and FLT3-expressing A20 tumor cells.

(A) Mice challenged with A20-FLT3 tumor cells were vaccinated as described in Fig 1. Tumor area of individual mice are depicted (n=5 per group). Lines represent the mean value of each groups. Statistical significance was determined by one-way ANOVA, *P<0.05, **P<0.01. (B) Inoculation of FLT3-expressing C1498 tumor cells and the vaccination schedule are as described in Fig 1. C1498-FLT3 tumor cells in peripheral blood were determined by Flow cytometry. Each symbol represents an individual mouse (n=5 per group). Bars represent the mean $\pm$ SD. Statistical significance was determined by one-way ANOVA, *P<0.05.

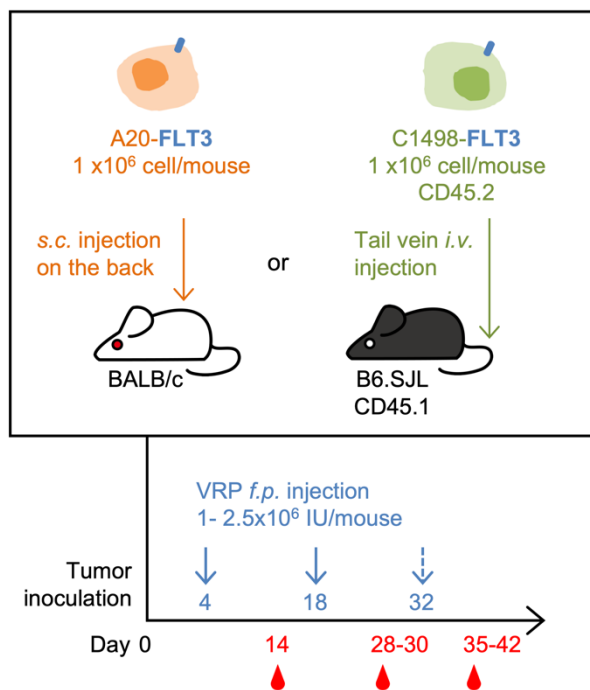


Figure 1.

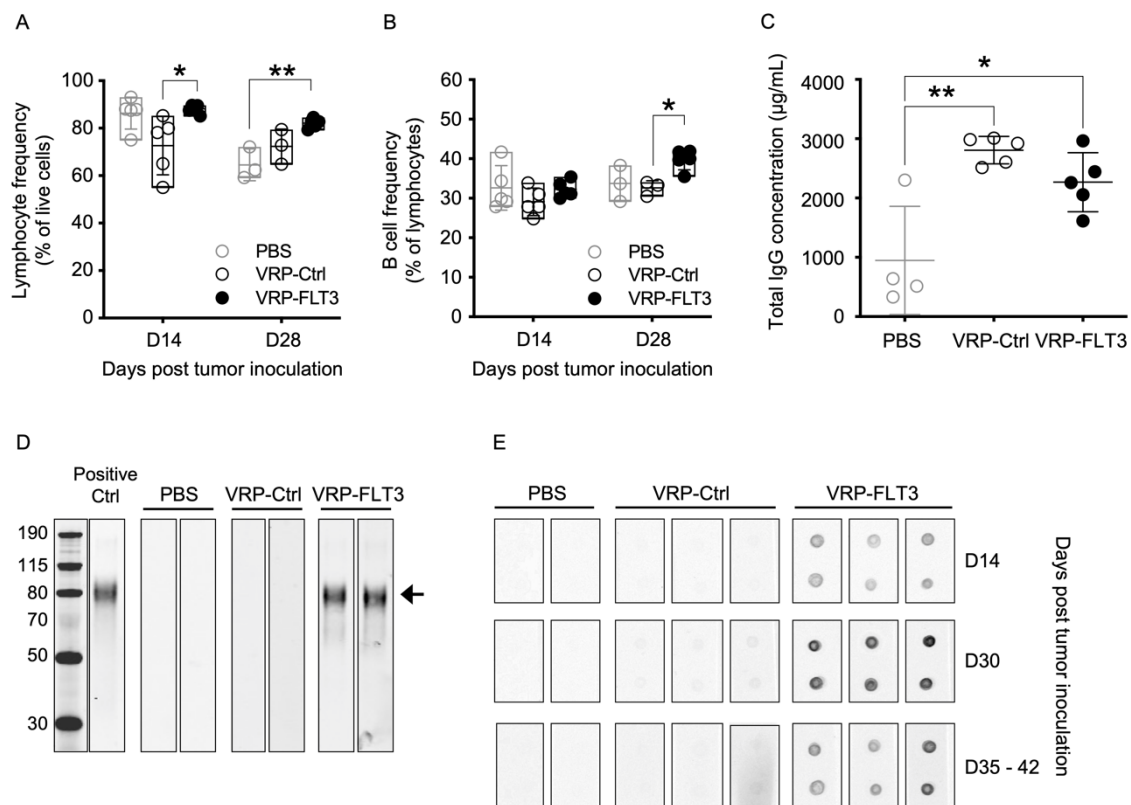


Figure 2.

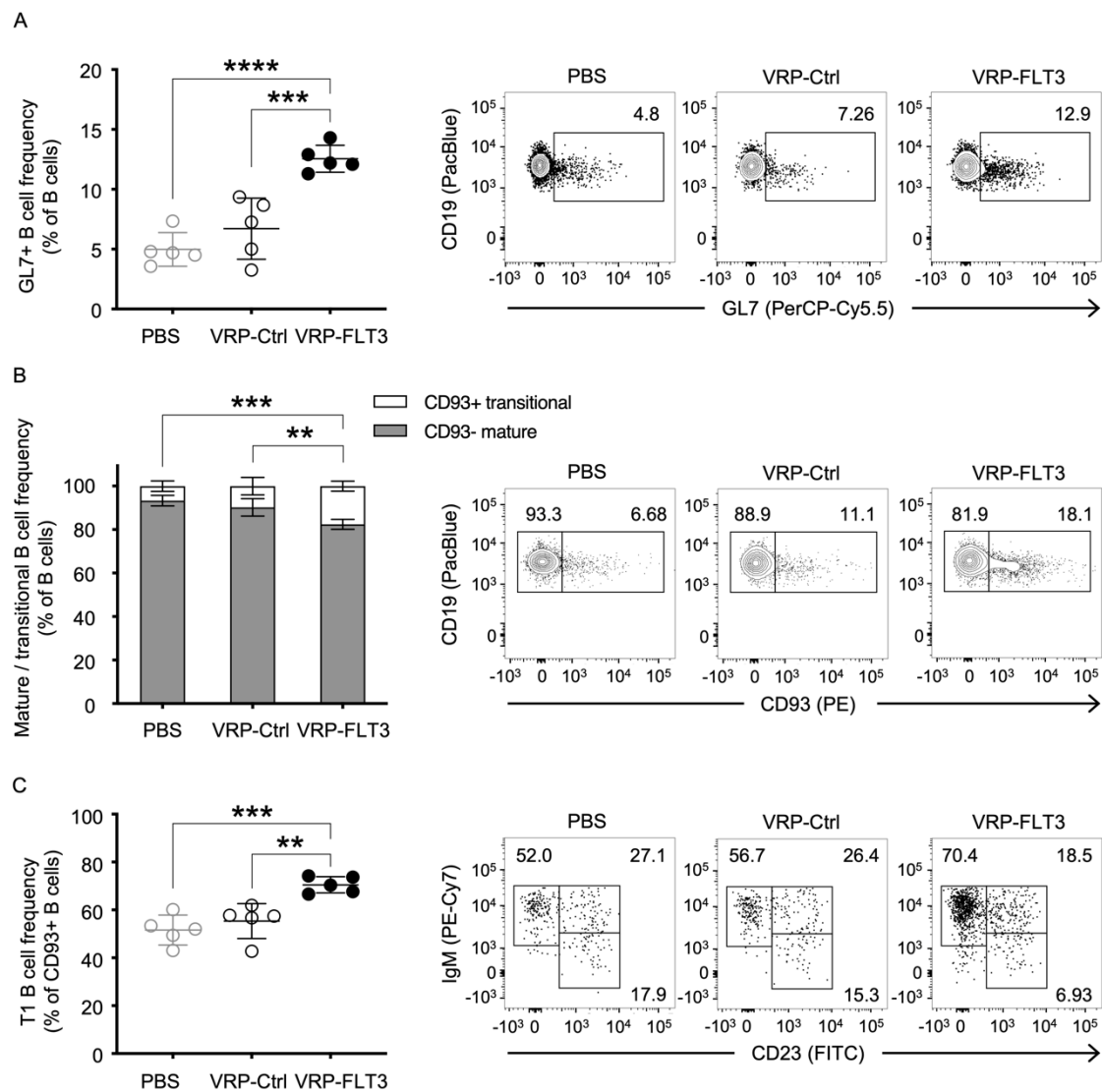


Figure 3.

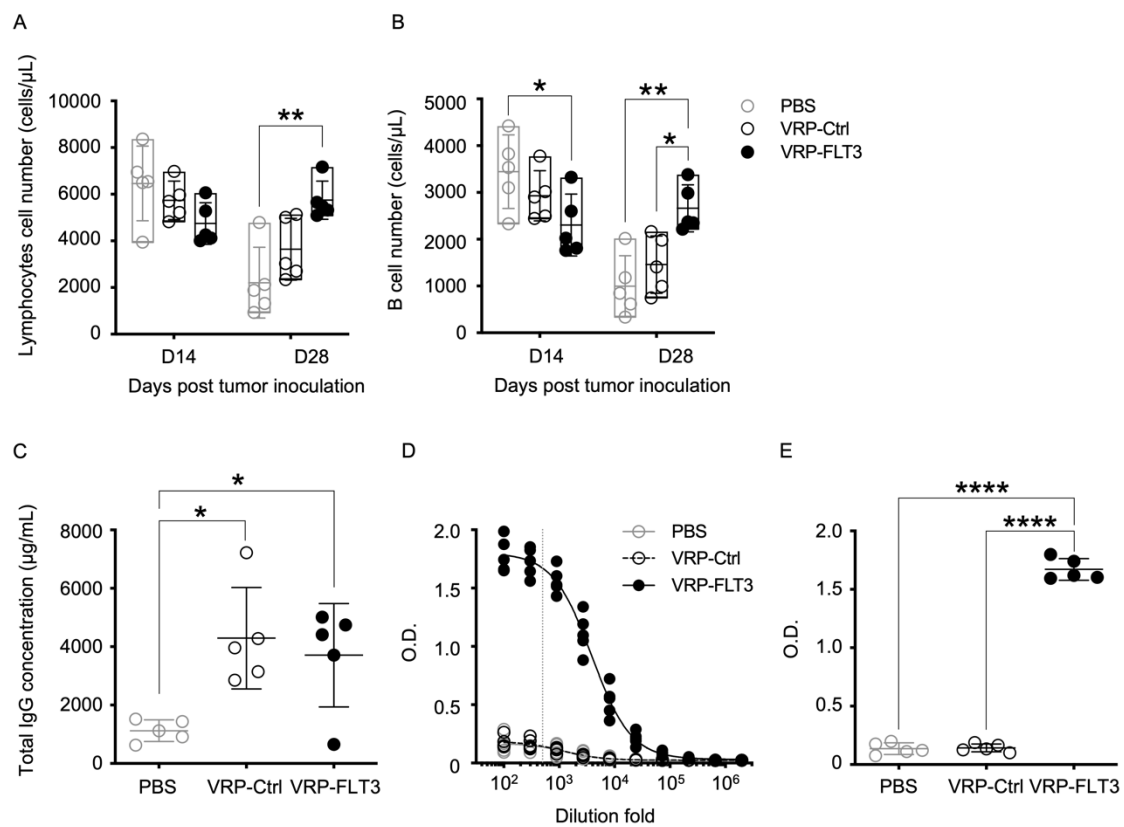


Figure 4.

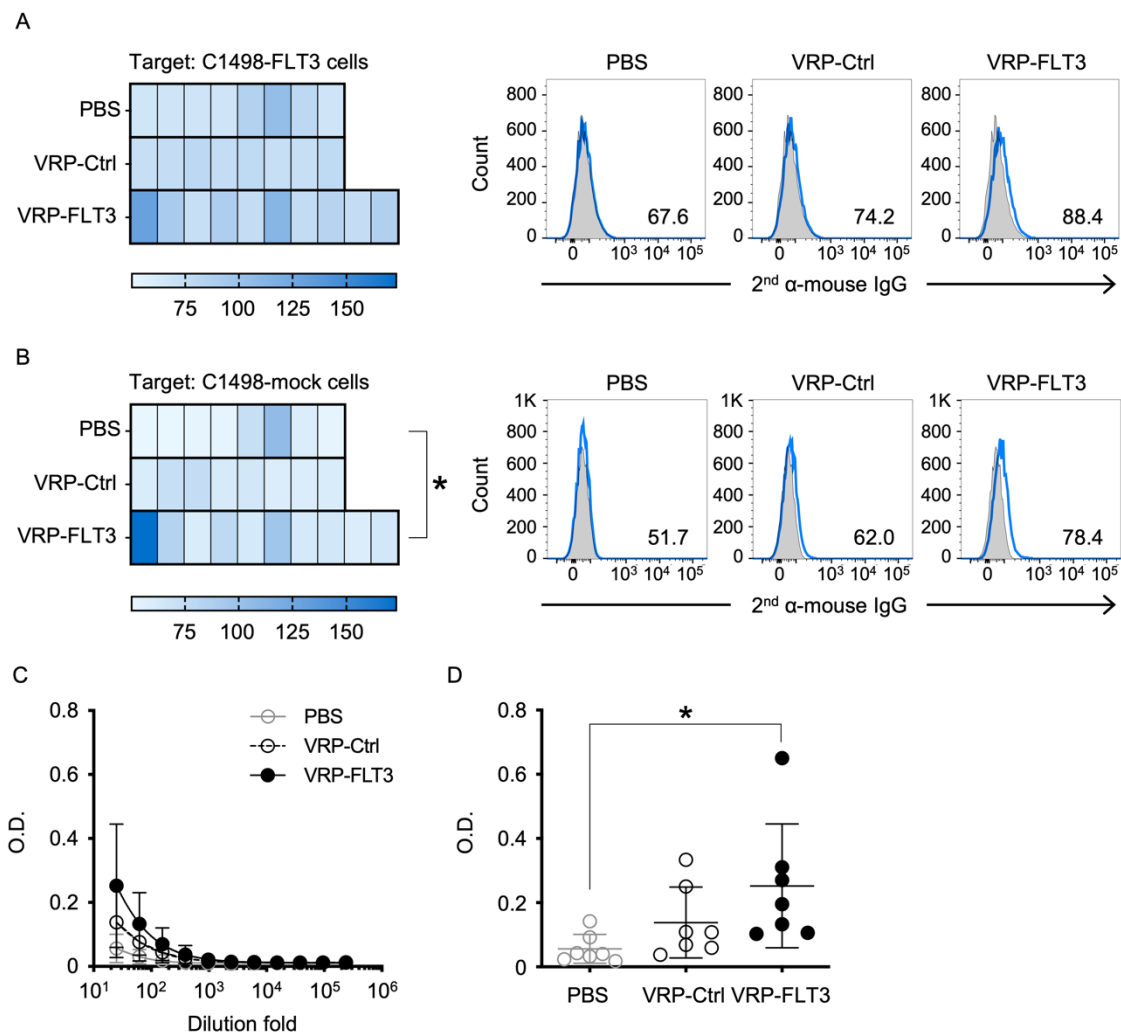


Figure 5.

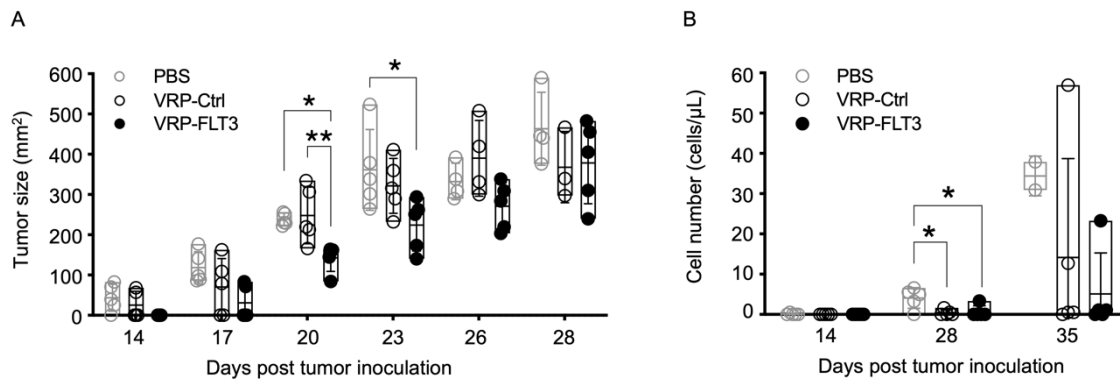


Figure 6.

7. Acknowledgments

This work was funded by the Department of Defense, Congressionally Mediated Research Program W81XWH-18-1-0239.

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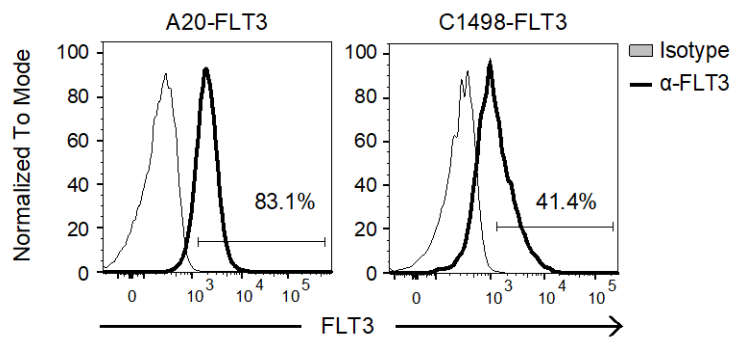
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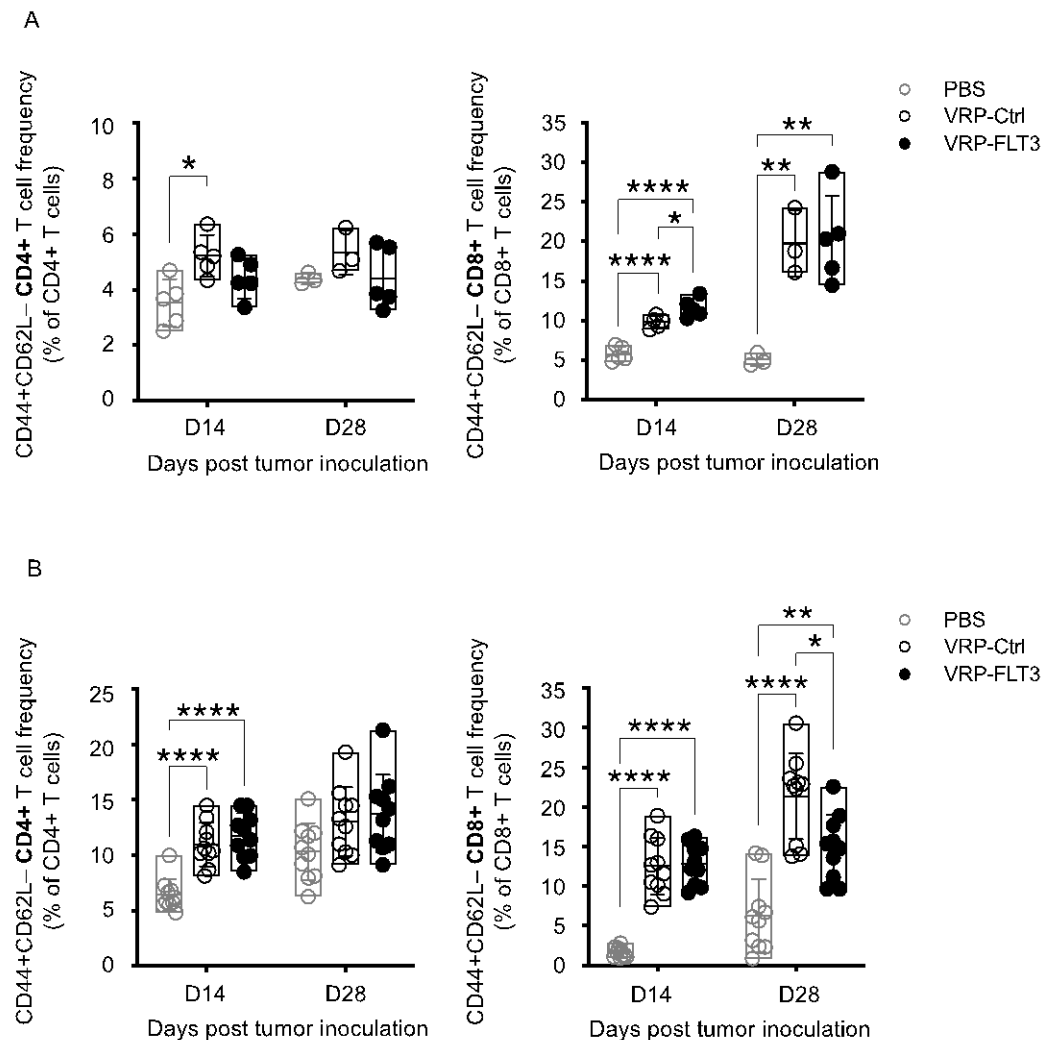
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Supplementary Figures



Supplementary Figure 1. FLT3 expression level on tumor cells.

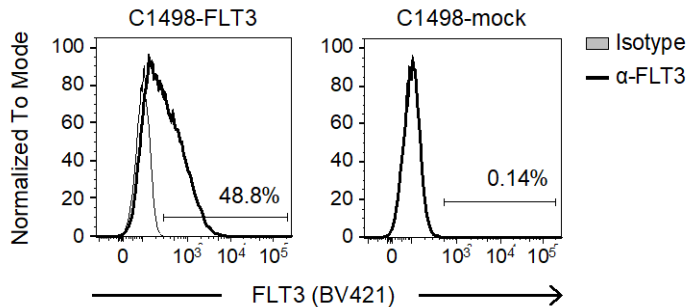
A20 and C1498 tumor cell lines were transfected with mouse FLT3. The FLT3 expression levels were determined by flow cytometry. Grey shade represents the isotype control. Solid line represents the sample.



Supplementary Figure 2. VRP vaccine induces T cell response in tumor-bearing mice.

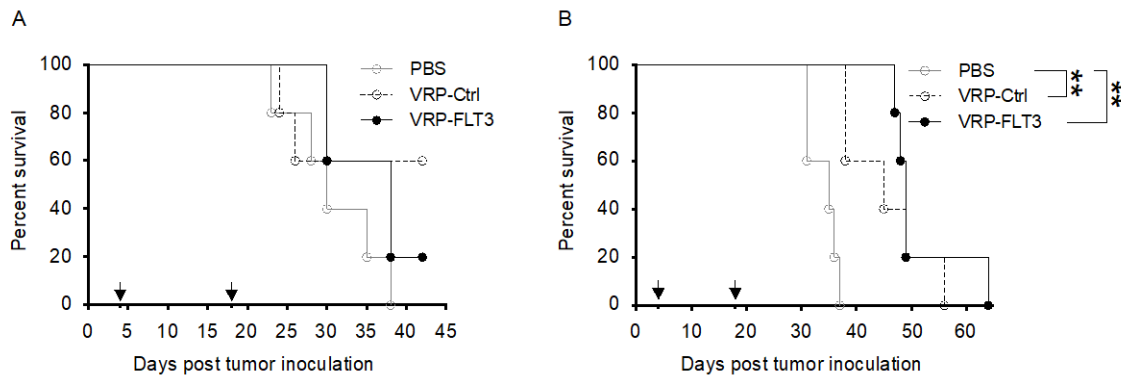
Tumor inoculation and VRP vaccination schedule are as described in Fig 1. Blood samples harvested on days 14 and 28 were analyzed by flow cytometry for the frequencies of activated CD4+ and CD8+ T cells (CD44+

CD64L-). (A) A20-FLT3 tumor model (n=5 per group). (B) C1498-FLT3 tumor model (n=10 per group). Each symbol represents an individual mouse. Bars represent the mean $\pm$ SD. Statistical significance were determined by one-way ANOVA, *P<0.05, **P<0.01, ****P<0.0001.



Supplementary Figure 3. FLT3 expression level of tumor cells that were used as the target cells in the flow cytometric binding assay.

FLT3-expressing C1498 cells (left) or the non-FLT3 expressing C1498-mock cells (right) were incubated with diluted plasma collected from C1498-FLT3 tumor bearing mice. Tumor cell-reacting plasma IgG were detected by a secondary antibody. Grey shade represents the isotype control. Solid line represents the sample.



Supplementary Figure 4. VRP vaccine improves the survival of tumor-bearing mice in the leukemia model but did not show a significant survival benefit in the lymphoma model.

Tumor inoculation and VRP vaccination schedule are as described in Fig 1. Kaplan-Meier survival curves of (A) A20-FLT3 tumor inoculated mice (n=5 per group) and (B) C1498-FLT3 tumor inoculated mice (n=5 per group). Arrows indicate the days of VRP vaccination. Statistical significance was determined by Log-rank (Mantel-Cox) test, **P<0.01. For the C1498-FLT3 tumor model, the best of two independent experiments is depicted. The repeat experiment of the C1498-FLT3 tumor model included 10 mice per group and was ended on day 40 after tumor inoculation.