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TITLE: Modeling Human Gamma Delta T Cells as Antitumor Agents In Vivo

PRINCIPAL INVESTIGATOR: Jenny Gumperz, PhD

CONTRACTING ORGANIZATION: University of Wisconsin, Madison
MADISON, WI 53715-1218

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14. ABSTRACT The central objective of this project is to provide mechanistic data that will support the development of human V γ 9V δ 2 T cells as an anti-tumor cellular immunotherapy for lymphomas driven by Epstein-Barr virus (EBV) infection. During this research period we have concluded our efforts on Major Task 1 and made progress on Major Tasks 2, 3, and 4. The most significant findings were: i) the observation that our $\gamma\delta$ T immunotherapy cells express the CD16 receptor and can therefore mediate antibody-dependent cell-mediated cytotoxicity (ADCC); ii) identification of a new live cell imaging methodology to perform screening and optimization experiments that will make our studies more efficient and reduce our animal usage; iii) preliminary experimental results demonstrating effector responses by the $\gamma\delta$ T immunotherapy cells against EBV-infected B lymphocytes, that suggest that the EBV-infected B cells have upregulated their production of key endogenous ligands that allow for TCR-mediated recognition of target cells by $\gamma\delta$ T cells without the need for added drugs.					
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1. **INTRODUCTION:** The central objective of this project is to provide mechanistic data that will support the development of human $\gamma\delta$ T cells as an anti-tumor cellular immunotherapy for lymphomas driven by Epstein-Barr virus (EBV) infection. The project employs a humanized mouse model, in which highly immunodeficient mice are injected with human umbilical cord blood cells that were briefly exposed *in vitro* to EBV. Over the ensuing three weeks, the human immune cells (including both T and B lymphocytes) expand *in vivo*, and human B cell lymphomas form in the fatty tissue surrounding peritoneal organs. The B cell lymphomas are infiltrated by autologous T cells, but these are suppressed by inhibitory ligands and fail to control the cancer. We have shown that administration of human $\gamma\delta$ T cells ($V\gamma9V\delta2^+$ T cells) that were expanded *in vitro* from peripheral blood of unrelated healthy adult donors is highly effective at reducing the tumor burden in this model. Thus, in contrast to the endogenous T lymphocytes, the immunotherapeutic $\gamma\delta$ T cells appear to avoid becoming suppressed by the existing inhibitory pathways. The two Specific Aims of this project focus on investigating the nature of the activation signals required for the immunotherapeutic effects of the $\gamma\delta$ T cells. Specifically, Aim 1 investigates the requirement for stimulation via T cell receptor (TCR)-mediated recognition of butyrophilin (BTN3A1) molecules that associate with lipid metabolites such as isopentenyl pyrophosphate (IPP). Aim 2 investigates the role of activation mediated by an "NK" receptor called NKG2D, that is expressed by $V\gamma9V\delta2^+$ T cells. The information gained from these studies will allow us to identify human lymphoma patients who are likely to fulfill the requirements for $V\gamma9V\delta2^+$ T cell activation, in that their lymphomas present the required activating signals. This will allow us to proceed with setting up a pilot clinical trial to test this therapeutic approach.
2. **KEYWORDS:** Epstein-Barr virus (EBV); B cell lymphoma; $\gamma\delta$ T cell; cellular immunotherapy; humanized mouse; umbilical cord blood.
3. **ACCOMPLISHMENTS:**
 - o **What were the major goals of the project?**

Specific Aim 1: Use human EBV-driven lymphomagenesis model to test the impact of TCR-mediated activation of $V\gamma9V\delta2^+$ T cells on their anti-tumor effects <i>in vivo</i> .	Completion status
Major Task 1: Perform <i>in vivo</i> blocking experiments to test requirement for recognition of BTN3A1 protein by adoptively transferred $\gamma\delta$ T cells	100%
Subtasks 1-3, 5 and 6: completed in previous reporting period	100%
Subtasks 4, and 7-18: Studies using anti-BTN3A1 blocking mAb as an approach to assess the importance of TCR-mediated activation of $V\gamma9V\delta2^+$ T cells for their anti-tumor effects <i>in vivo</i> .	See below for discussion of unexpected experimental results that compromise the interpretation of this approach and have thus led us to truncate the analyses for this task.

Major Task 2: Use pharmacological inhibitors (e.g. statins) to inhibit production of IPP	20%
Subtasks 1 and 3: completed in previous reporting period	100%
Subtasks 2 and 4-9: Studies using statin treatment as an approach to inhibit the production of IPP by EBV-infected B cells, to test for the importance of this ligand for the anti-tumor effects of V γ 9V δ 2 ⁺ T cells <i>in vivo</i> .	See below for discussion of alternative experimental approaches that we propose to use to improve our efficiency and allow us to accomplish this goal
Milestone(s) Achieved: Information on whether IPP production is required.	20%

Major Task 3: Test effects of compounds (e.g. bisphosphonates, synthetic ligands) that increase Vγ9Vδ2⁺ T cell activation	20%
Subtask 1: Expand human V γ 9V δ 2 ⁺ T cells from adult peripheral blood samples and make sterile frozen aliquots	100%
Subtasks 2-10: Studies to test whether drugs that increase the availability of V γ 9V δ 2 ⁺ T cell TCR ligands leads to improved anti-tumor efficacy.	See below for discussion of alternative experimental approaches that we propose to use to improve our efficiency and allow us to accomplish this goal
Milestone(s) Achieved: Determination of whether enhancing mevalonate pathway activation results in more efficient anti-tumor effects.	20%

Specific Aim 2: Test impact of NKG2D-mediated activation on anti-tumor effects of Vγ9Vδ2⁺ T cells <i>in vivo</i>.	
Major Task 4: Test effect of blocking the NKG2D-mediated activation pathway.	20%
Subtask 1: Generate viral stock	100%
Subtask 2:	100%

Perform preliminary experiment to titrate viral dose required for visible tumor formation within 30 days	
Subtask 3: Expand human V γ 9V δ 2 ⁺ T cells from adult peripheral blood samples and make sterile frozen aliquots	100%
Subtask 4: Test different anti-NKG2D mAb clones to identify those that provide the best blocking of V γ 9V δ 2 ⁺ T cell responses <i>in vitro</i> . If isotype is different than anti-BTN3A1 mAb, identify and test appropriate isotype control mAb.	50%
Subtasks 5-12: Perform experiments in EBV lymphomagenesis model system to assess the importance of NKG2D-mediated activation for the anti-tumor effects of V γ 9V δ 2 ⁺ T cells.	0%
Milestone(s) Achieved: Information on importance of NKG2D-mediated activation.	20%

Major Task 5: Test whether Vγ9Vδ2⁺ T cells control tumors caused by LMP1- or LMP2A-deficient EBV	5%
Subtask 1: Generate LMP1- and LMP2A-deficient viral stocks for use in studies	100%
Subtasks 2-18: Analysis	0%
Milestone(s) Achieved: Information on whether anti-tumor effects of Vγ9Vδ2⁺ T cells depends on specific viral genes that are thought to upregulate host cell expression of ligands for NKG2D	5%

○ **What was accomplished under these goals?**

1. *Re-establishment of our humanized mouse model of EBV-driven lymphomagenesis.* As noted in the last progress report, we experienced a number of technical difficulties with our humanized mouse model system that were apparently associated with the human cord blood mononuclear cell (CBMC) samples used to generate human immune engrafted mice for our experiments. By the end of the last reporting period we had resolved the problem of the CBMCs lysing upon exposure to EBV, and were able to generate engrafted mice infected with EBV. However, during the current reporting period we found that even when we purified CBMCs ourselves, samples obtained from certain blood banks (e.g. Medical College of Wisconsin) often yielded experimental results that were not satisfactory for testing experimental interventions. Specifically, in many experiments using cord samples obtained from MCW, the peritoneal tumor masses were very small (Fig. 1A). Thus, although the $\gamma\delta$ T cell immunotherapy probably had worked in that compared to the control mice there were more mice showing no detectable tumor

tissue in the immunotherapy treatment conditions (e.g. in the experiment shown in Fig. 1A, 4 out of 9 immunotherapy mice had no visible tumors, while all 3 control mice had tumors), the small tumor sizes meant we would not have enough statistical power to determine whether our experimental interventions had a significant effect. We were not able to determine the reason that many of the cord blood samples from MCW yielded only small sized tumors in our EBV-lymphomagenesis model. However, we did discover that this problem did not occur when we use cord blood samples from a different source. Cord blood samples obtained from a clinical laboratory that is accredited and FDA-approved for transfusion medicine (the Clinical Laboratory of the University of Colorado Hospital) have consistently yield acceptable results, such that the EBV-driven tumors of our control mice are quite large, and we observe a dose-dependent reduction in tumor size after administration of the $\gamma\delta$ T cell immunotherapy (Figure 1B). Thus, we feel confident that it is feasible for us to perform *in vivo* experiments to test pathways of activation of the $\gamma\delta$ T cell immunotherapy, albeit with somewhat reduced availability of cord samples to support our experiments (UCH only supplies leftover samples that are not used for clinical transfusion, and we can usually only obtain samples from them about 2 times per month). Because of this lower than expected availability of cord blood samples, we put effort into identifying an alternative approach (see #3 below) that will allow us to be more efficient in carrying out our proposed experiments.

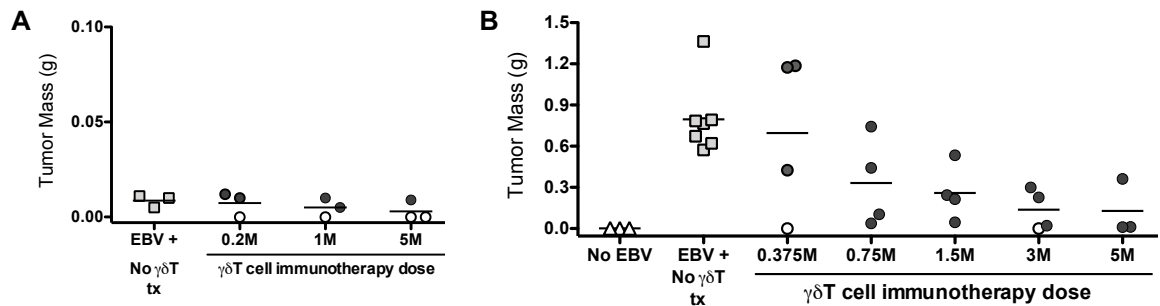
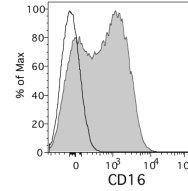


Figure 1. Re-establishment of *in vivo* EBV-lymphomagenesis model. NSG mice were administered EBV-infected CBMCs and 23 days later were either mock-treated with PBS (square symbols) or given the indicated doses (in millions) of $\gamma\delta$ T cell immunotherapy (circles). Each symbol represents the tumor mass obtained from an individual mouse. White symbols represent cases where no tumor tissue was detected. **A)** Results from an experiment performed with a cord blood sample obtained from the Medical College of Wisconsin. Note that the immunotherapy in this experiment may have been effective, since a total of 4 out of 9 mice given the highest dose showed no detectable tumors and all three of the control mice did have detectable tumors. However, the extremely small tumor size (less than 0.05g) in the positive control mice posed a problem for interpreting the impact of any experimental manipulations. **B)** Results from an experiment performed using a cord blood sample obtained from the University of Colorado Hospital. Using these cord samples, tumor sizes from mock-treated mice have consistently been much larger, and a dose-dependent effect of the $\gamma\delta$ T cell immunotherapy is apparent. Thus, by cord samples of this type, we can determine whether experimental manipulations to block $\gamma\delta$ T cell activation pathways have reduced the effectiveness of the immunotherapy.

2. *Results impacting Major Task 1.* While carrying out experiments for Major Task 1 to identify an appropriate anti-BTN3A1 blocking mAb, we determined that our $\gamma\delta$ T immunotherapy cells express the CD16 receptor (see Figure 2). This is an activating Fc receptor that mediates antibody-dependent cell-mediated cytotoxicity (ADCC) against antibody-coated target cells. The ability of the $\gamma\delta$ T immunotherapy cells to mediate ADCC will most likely significantly complicate our ability to interpret the results of experiments where we had proposed to administer an anti-BTN3A1 mAb to block $\gamma\delta$ T immunotherapy cell activation by BTN3A1 molecules expressed on potential target cells (i.e. in addition to blocking recognition of BTN3A1 by the TCR, the presence of the antibody can activate ADCC). For this reason, we have decided not to pursue the *in vivo* blocking studies originally proposed as Major Task 1. It is important to note, however, that this CD16 observation also provides a new direction for analysis that has very high potential clinical significance, since administration of immunotherapeutic $\gamma\delta$ T cells in

the presence of an FDA-approved therapeutic mAb that binds B-lymphoma cells (e.g. Rituximab) may provide a synergistic anti-tumor effect that may lead to substantially improved efficiency of the immunotherapy. Therefore, as discussed below, if our results suggest that the original plan for Aim 2 (analysis of the importance of the NKG2D activation pathway *in vivo*) is unlikely to yield a positive finding, we propose instead to focus this Aim on using the *in vivo* EBV-lymphomagenesis model to explore the impact of co-administering an antibody to engage the CD16-activation along with $\gamma\delta$ T cell immunotherapy.

Figure 2. Results impacting Major Task 1 - Expression of CD16 by $\gamma\delta$ T immunotherapy cells. Flow cytometric analysis of immunotherapy $\gamma\delta$ T cells expanded from adult human PBMCs, showing cell surface staining for CD16 (shaded histogram) compared to an isotype-matched control mAb (dashed line).



- 3. Identification of a new approach that will facilitate our efficiency in carrying out the proposed studies.** Our department recently purchased a live cell imaging instrument (an IncuCyte system, made by Sartorius corp.) This instrument is housed in a tissue culture CO₂ incubator and generates repeated phase-contrast and 2-color fluorescent microscopy images over time of cells cultured in 96-well tissue culture plates. It can be used to assess interactions between effector cells (e.g. our $\gamma\delta$ T immunotherapy cells) and tumor cells. Of particular interest, the instrument can be used to analyze tumor spheroids, which are thought to closely mimic tumor tissues *in vivo* because they include a microenvironment that effector cells must negotiate in order to carry out anti-tumor cytotoxicity. Since it would be more efficient for us to use live cell imaging to optimize our experimental manipulations before assessing physiological outcomes in our *in vivo* model, we performed a series of pilot experiments to establish feasibility. Figure 3 shows an example of an analysis we performed of colony-forming unit cells that were grown into spheroids in matrigel, and monitored over time in the IncuCyte instrument in the presence or absence of lymphokine-activated killer (LAK) cells. Based on this and other preliminary analyses, we believe it will be feasible not only to analyze $\gamma\delta$ T immunotherapy cell responses to cultured EBV-infected B lymphoblastoid cells, but also to harvest nascent EBV-lymphomas from our *in vivo* model and to assess the immunotherapy cell responses to these as spheroids growing in matrigel.

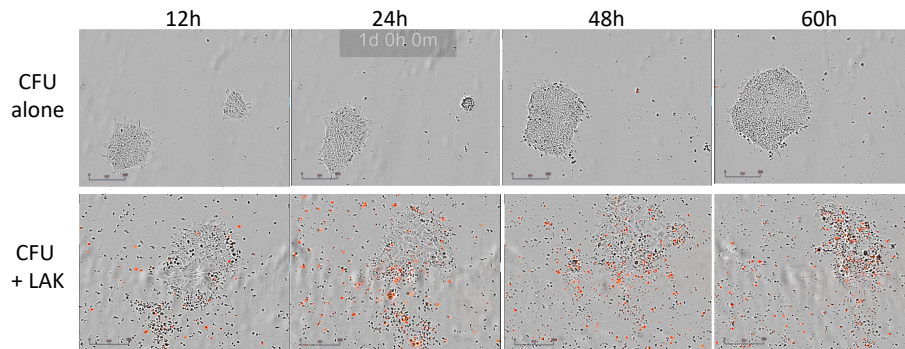


Figure 3. Real-time live cell imaging analysis of spheroid cell clusters. IncuCyte Live Cell Imager analysis showing the disruption and killing of a spheroid cluster by human peripheral blood lymphokine-activated killer (LAK) cells. Images are shown in phase contrast with red fluorescent signal representing cells that have lost membrane integrity (i.e. dying cells).

- 4. Progress on Major Tasks 2 and 3.** We have carried out a series of initial studies to investigate the role of TCR-mediated activation in the responses of our $\gamma\delta$ T immunotherapy cells to EBV-infected B cells. Our preliminary findings show that the $\gamma\delta$ T cells mediate both cytolytic degranulation and cytokine release in response to EBV-infected B cells, but show significantly lower responses to B-lymphoma cells that are not infected with EBV (Fig. 4A). The observation

of cell surface CD107a expression (an indicator of cytolytic degranulation) is particularly important, since our prior studies have shown that cytolysis by $\gamma\delta$ T cells is activated primarily by TCR-stimulation and not NKG2D, whereas IFN- γ secretion can be activated by either one (Zumwalde et al., *J. Clin. Invest.* 2017). Preliminary results from additional experiments showed increases in $\gamma\delta$ T cell CD107a expression in some cases after BP-treatment of the EBV-infected cells, but little or no effect of this treatment on IFN- γ production by the $\gamma\delta$ T cells (Fig. 4B). These preliminary results suggest that it is not necessary to treat EBV-infected cells with compounds (such as BP) that increase the availability of cellular BTN3A1 ligands in order for the $\gamma\delta$ T cells to mediate anti-tumor responses. (The impact of activators such as BP on the anti-tumor effects of the $\gamma\delta$ T immunotherapy cells is the subject of Major Task 3, and will be further investigated in the upcoming grant period). We also carried out preliminary experiments to determine whether responses by our $\gamma\delta$ T immunotherapy cells are blocked by treating the EBV-infected B cells with a statin drug to inhibit the endogenous production of BTN3A1 ligands (the subject of the experiments in Major Task 2). The results of these experiments showed modest $\gamma\delta$ T cell inhibition after statin treatment (Fig. 4C). However, these studies are still in the optimization stage to determine the statin dose and treatment time necessary to eliminate relevant endogenous metabolites made by the EBV-infected cells.

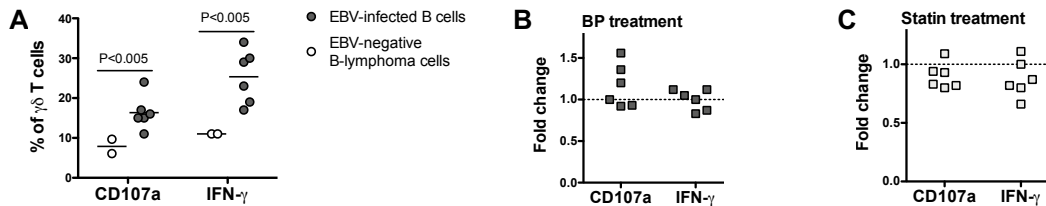


Figure 4. Progress on Major Tasks 2 and 3 - initial studies on TCR-activation pathway of $\gamma\delta$ T immunotherapy cells. **A)** Aggregated results from a series of preliminary analyses to assess cytolytic degranulation (cell surface CD107a expression) and intracellular IFN- γ production by our $\gamma\delta$ T immunotherapy cells in response to B-lymphoma cells that are either infected with EBV (dark symbols) or negative for EBV (light symbols). **B)** Fold-change in the amount of cell surface CD107a or intracellular IFN- γ produced by $\gamma\delta$ T immunotherapy cells in response to B-lymphoma cells that were treated with bisphosphonate (BP) compared to mock-treated. **C)** Fold change in CD107a or IFN- γ in response to statin treated compared to mock-treated B-lymphoma cells.

- **What opportunities for training and professional development has the project provided?**
 - Dr. Jenny Gumperz Ph.D. and Dr. Dana Baiu Ph.D. each provided training to undergraduate students in association with this project: Ms. Isabel Monti and Mr. Matthew Dandan. Both of these undergraduate students independently wrote a project proposal for the major undergraduate scientific research award offered here at the University of Wisconsin (the Hilldale award), and both presented posters about their research at UW's Undergraduate Research Symposium.
- **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?**
 - For Aim 1, we will not further pursue the experiments originally proposed for Major Task 1 (BTN3A1 blocking *in vivo*), since it will probably not be possible to interpret the results given the potential for CD16-mediated ADCC by the $\gamma\delta$ T immunotherapy cells. We will move forward with Major Tasks 2 and 3, but as discussed below, we will slightly alter the approach for these studies by using *in vitro* analyses to perform optimization experiments, and only using the *in vivo* model for later experiments. These proposed changes to the approach are

thus expected to make our studies more efficient (and will also reduce our animal usage), but they do not substantially change the scope of this aim, since we still expect to assess the importance of TCR-mediated activation in our *in vivo* model system.

- For Aim 2, our preliminary findings have suggested that the NKG2D-mediated activation pathway may not play a major role, since we do not observe noticeable upregulation of the most potent NKG2D ligands on B-lymphoma cells from EBV-infected mice (Zumwalde et al., *J. Clin. Invest.* 2017). We therefore propose to use our *in vitro* assays to further assess this question, and will only perform the originally proposed *in vivo* blocking studies if our *in vitro* analyses do suggest an important role for the NKG2D-activation pathway. If our studies do not support a role for the NKG2D-activation pathway, we will also not perform Major Task 5 (analysis of the effects of V γ 9V δ 2⁺ T cells immunotherapy on lymphomas caused by LMP1- and LMP2A-deficient EBV), since the main purpose of this part of Aim 2 was to provide additional support for the role of the NKG2D-activation pathway. Thus, we anticipate the possibility that negative results may limit the scope of the work on Aim 2.
- However, if we do determine that it is unlikely to be productive to pursue Aim 2 according to the original plan, we will revise Aim 2 to instead pursue studies on whether CD16-mediated ADCC can enhance the anti-tumor effects of V γ 9V δ 2⁺ T cell immunotherapy in our *in vivo* EBV-lymphomagenesis model. Briefly, we will test whether administering FDA-approved therapeutic antibodies (e.g. an anti-CD20 antibody such as Rituximab) that bind to the EBV-lymphoma cells, in combination with titrated doses of V γ 9V δ 2⁺ T cells immunotherapy produces a synergistic effect where lower numbers of immunotherapy cells are required to achieve the same amount of reduction in tumor burden (see titration curve in Fig. 1B for an example). We feel strongly that this new direction is completely consistent with the overarching objective of this project ("to provide mechanistic data that will support the development of human $\gamma\delta$ T cells as an anti-tumor cellular immunotherapy for lymphomas driven by EBV infection"), since the demonstration of a synergistic anti-tumor effect of therapeutic antibodies and V γ 9V δ 2⁺ T cell immunotherapy would be very significant for advancing these studies towards clinical translation.

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?**
 - Nothing to Report.
- **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**
 1. In place of some of the proposed *in vivo* experiments (e.g. initial investigations of $\gamma\delta$ T immunotherapy responses and optimization of antibody blocking and drug stimulation

protocols), we will carry out live cell imaging experiments using the IncuCyte imager. The *in vitro* imaging approach will be more efficient because it will allow us to test more different conditions in parallel than it would be possible for us to test in our *in vivo* model, and will also reduce our animal usage.

2. For Aim 1, we will not complete the studies initially proposed as Major Task 1, because we have found that our $\gamma\delta$ T immunotherapy cells express the CD16 receptor. Since this receptor mediates ADCC of antibody-coated target cells, it will not be possible to accurately interpret the results of blocking assays in which an anti-BTN3A1 mAb is bound to potential target cells. (Note that this complication is less of a concern for experiments where the blocking antibody binds to a receptor on the $\gamma\delta$ T immunotherapy cells, such as the experiments proposed for the first part of Aim 2).
3. For Aim 2, we will perform the proposed anti-NKG2D antibody blocking studies using the IncuCyte live cell imaging system (i.e. analysis of EBV-infected B cells or EBV-lymphoma spheroids co-cultured with $\gamma\delta$ T immunotherapy cells, in the presence or absence of a mAb that blocks the NKG2D receptor on the $\gamma\delta$ T cells) to investigate the importance of the NKG2D-activation pathway. If the results from these experiments suggest little or no role for the NKG2D, we will shift our focus for this Aim to investigating whether adding a therapeutic mAb that binds to B lymphoma cells (Rituximab) synergistically enhances the anti-tumor efficiency of our $\gamma\delta$ T immunotherapy cells. We believe this change is justified because of our earlier observation that ligands for NKG2D are not upregulated on B lymphoma cells from our EBV-infected mice (suggesting the NKG2D-activation pathway may not be critical), and because the demonstration of a synergistic anti-tumor effect mediated via the CD16-activation pathway would have strong pre-clinical significance that would clearly advance the stated overarching goal of these studies.

- **Actual or anticipated problems or delays and actions or plans to resolve them**
 - See above sections.
- **Changes that had a significant impact on expenditures**
 - Nothing to Report.
- **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**
 - Nothing to Report.

6. PRODUCTS:

- **Publications, conference papers, and presentations**
 - **Journal publications.** Nothing to Report.
 - **Books or other non-periodical, one-time publications.** Nothing to Report.
 - **Other publications, conference papers, and presentations.** Nothing to Report.
- **Website(s) or other Internet site(s).** Nothing to Report.
- **Technologies or techniques.** Nothing to Report.
- **Inventions, patent applications, and/or licenses.** Nothing to Report.

- **Other Products.** Nothing to Report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

- **What individuals have worked on the project?**

Name:	Jenny E. Gumperz, Ph.D.
Project Role:	Principal Investigator
Researcher Identifier (e.g. ORCID ID):	0000-0003-1852-2192
Nearest person month worked:	4
Contribution to Project:	Dr. Gumperz oversees all aspects of the project to ensure that the proposed goals are accomplished. These activities include the design of experiments, analysis and interpretation of the data, budget oversight, and manuscript and progress report preparation.
Funding Support:	National Institutes of Health.
<hr/>	
Name:	Dana C. Baiu, Ph.D.
Project Role:	Associate Scientist
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	8
Contribution to Project:	Conducts experiments and analyses; interprets, analyzes, and summarizes data.
Funding Support:	
<hr/>	
Name:	Nicholas A. Zumwalde, Ph.D.
Project Role:	Assistant Scientist
Researcher Identifier (e.g. ORCID ID):	0000-0003-3786-7735
Nearest person month worked:	1
Contribution to Project:	Prepared reagents, conducted experiments
Funding Support:	American Cancer Society

Name:	Shannon C. Kenney
Project Role:	Collaborator
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	1
Contribution to Project:	Generates virus stocks and provides expertise on aspects of experimental analyses and interpretation.
Funding Support:	National Institutes of Health.

- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**
 -
- **What other organizations were involved as partners?**
 - Nothing to Report.

8. SPECIAL REPORTING REQUIREMENTS

- **COLLABORATIVE AWARDS:** Not applicable.
- **QUAD CHARTS:** Not applicable.

9. APPENDICES: Not applicable.