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PRINCIPAL INVESTIGATOR: Dr. Klaus Frueh

CONTRACTING ORGANIZATION: Oregon Health & Science University

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Dr. Klaus Frueh

E-Mail:

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Oregon Health & Science University
3181 SW Sam Jackson Park Rd.
Portland, Oregon 97293-3011

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14. ABSTRACT
Enter a brief (approximately 200 words) unclassified summary of the most significant find during the research period.
We investigate a new paradigm for vaccines and immunotherapies of prostate cancer (PCa). We demonstrate that strain 68-1 rhesus cytomegalovirus (RhCMV)-expressing tumor antigens elicit broad CD8+ T cell responses to epitopes presented by non-polymorphic major histocompatibility complex (MHC)-E molecules instead of polymorphic, classical MHC-Ia. Due to the high conservation of MHC-E we were able to show that human PCa cell lines and human primary tumor cells stimulate MHC-E-restricted CD8+ T cells elicited in rhesus macaques (RM) by 68-1 RhCMV expressing prostate acid phosphatase (PAP). We began identifying T cell receptors (TCRs) recognizing PAP-derived peptides presented by MHC-E and characterizing such TCRs. Since MHC-E is non-polymorphic, unlike classical MHC type I molecules, TCRs expressed by MHC-E-restricted T cells are not limited to a given MHC-I but can be universally employed regardless of the MHC-type thus enabling the development of a universal T-cell based immunotherapy.

15. SUBJECT TERMS
Immunotherapy of prostate cancer, T cell therapy, major histocompatibility complex E, cancer vaccines using cytomegalovirus vectors

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1. INTRODUCTION:

We propose to investigate a new paradigm for vaccines and immunotherapies of prostate cancer (PCa) that was made possible by our discovery that strain 68-1 rhesus cytomegalovirus (RhCMV)-based vectors elicit broad CD8+ T cell responses to epitopes presented by non-polymorphic major histocompatibility complex (MHC)-E molecules instead of polymorphic, classical MHC-Ia. We will explore the targeting of PCa via prostatic acidic phosphatase (PAP)-specific, MHC-E-restricted CD8+ T cells (ETc) by 1) assessing the capacity of PCa cell lines and primary tumor cells to stimulate ETc, 2) identifying TCRs recognizing PAP-derived peptides presented by MHC-E, 3) transfecting human T cells with these TCRs to identify E-restricted PAP epitopes.

2. KEYWORDS:

Prostate cancer, immunotherapy, human leukocyte antigen E, T cell receptor, prostatic acidic phosphatase, epitope, rhesus macaque, cytomegalovirus, single cell sequencing

3. ACCOMPLISHMENTS:

What were the major goals of the project?

Specific Aim 1: To assess the capacity of PCa cell lines and primary tumor cells to stimulate PAP-specific, MHC-E-restricted CD8+ T cells.
Task 1 (Month 1): Identification of E-expressing PCa cell lines (Status: completed)
Task 2 (Months 2-3): Monitoring stimulation of ETc by PCa cell lines (Status: completed)
Milestone 1 (Month 3): PCa cell lines identified that stimulate PAP-specific CD8+ T cells via HLA-E (Status: achieved)
Task 3 (Months 1-18): Generating cell suspensions from PAP and E-positive PCa (Status: complete)
Task 4 (Months 1-18): Monitoring stimulation of ETc by primary PCa tumor cells (Status: complete)
Milestone 2 Month 18: HLA-E mediated targeting of PAP-positive PCa tumors demonstrated (Status: achieved)

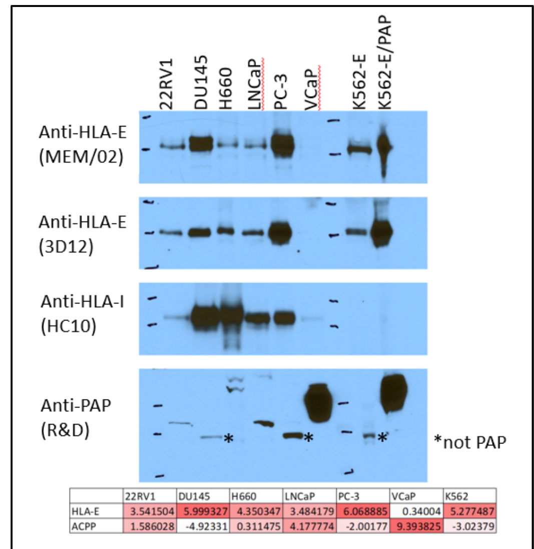
Specific Aim 2: To identify TCRs recognizing PAP-derived peptides presented by PCa cells via MHC-E
Task 5 (Month 4-10): Bulk identification of TCR sequences (Status: completed)
Milestone 3 (Month 10): Frequency of TCRα and TCRβ sequences in PCa reactive and PAP-peptide-specific T cells determined for each RM (Status: completed)
Task 6 (Months 11-18): Identification of individual PAP-specific, MHC-E restricted TCRs (Status: complete)
Milestone 4: Identification of sequences of TCR α/β pairs that were identified in both peptide and PCa-stimulated T cell clones (Status: achieved)

Specific Aim 3: To identify PAP-derived peptides presented by PCa cells via HLA-E
Task 7 (Month 19-36): Mapping of PAP-derived, MHC-E restricted peptides recognized by individual TCR α/β pairs (Status: modified task, partially completed)
Milestone 5 (Month 36): Identification of MHC-E restricted TCRs and PAP epitopes (Status: not completed)
Task 8 (Month 19-36): Stimulation of TCR clones by PCa cells (Status: in progress)
Milestone 6: Identification of TCRs recognizing cancer cell lines and primary cancer cells (Status: not completed)
Milestone 7: MHC-E restricted PAP epitopes shared by, or limited to, individual tumors (Status: not completed)

Major Activity 1: To assess the capacity of PCa cell lines and primary tumor cells to stimulate PAP-specific, MHC-E-restricted CD8+ T cells.

Specific Objective 1.1: Expression of HLA-E on established PCa tumor cell lines

To demonstrate that prostate cancer (PCa) cell lines are recognized by prostate acidic phosphatase (PAP)-specific MHC-E-restricted T cells (ETcs) in a HLA-E- and PAP-specific manner we selected PCa cell lines that, according to transcriptomic analysis by the Broad-institute (Ghandi et al. Nature 569:503, 2019), either express a) both HLA-E and PAP (LnCaP, 22RV1), b) PAP only (VCaP), c) HLA-E only (DU145). We also generated K562 cells (that lack endogenous HLA-ABC and express low levels of HLA-E) stably expressing HLA-E alone or together with full-length PAP. Expression of each of these proteins was monitored by immunoblot. As shown in **Fig. 1** there was overall a good correlation between reported mRNA levels and observed protein levels. However, the commercial anti-PAP antibody used in these experiments showed some cross-reactivity with another protein, most likely ACP2 (lysosomal acid phosphatase).



	22RV1	DU145	H660	LNCaP	PC-3	VCaP	K562
HLA-E	3.541504	5.999327	4.350347	3.484179	6.068885	0.34004	5.277487
ACPP	1.586028	-4.92331	0.311475	4.177774	-2.00177	9.393825	-3.02379

Figure 1: PAP and HLA-E expression in PCa cell lines.
The indicated cell lines were cultured according to published protocols. Cell lysates were subjected to SDS-PAGE and immunoblot with the indicated antibodies. Note that the PAP-specific antibody obtained from R&D systems recognizes a lower MW band in PAP-negative cell lines (indicated by *). The bottom Panel shows relative mRNA levels as published previously (1).

Specific Objective 1.2: Monitoring the stimulation of MHC-E-restricted, PAP-specific CD8+ T cells by prostate cancer cell lines.

We next determined whether PAP and HLA-E expression of prostate cancer cell lines correlates with their ability to stimulate PAP-specific, MHC-E restricted CD8+ T cells. We co-cultured representative cell lines for each of the three expression types (PAP+, HLA-E+), (PAP-, HLA-E+), (PAP+, HLA-E-) with CD8+ T cells isolated from peripheral blood mononuclear cells of rhesus macaques (RM) immunized with strain 68-1 RhCMV expressing rhesus PAP (RhPAP). After enrichment by magnetic bead sorting, CD8+ T cells were co-incubated with PCa cell lines and T cell stimulation was monitored by

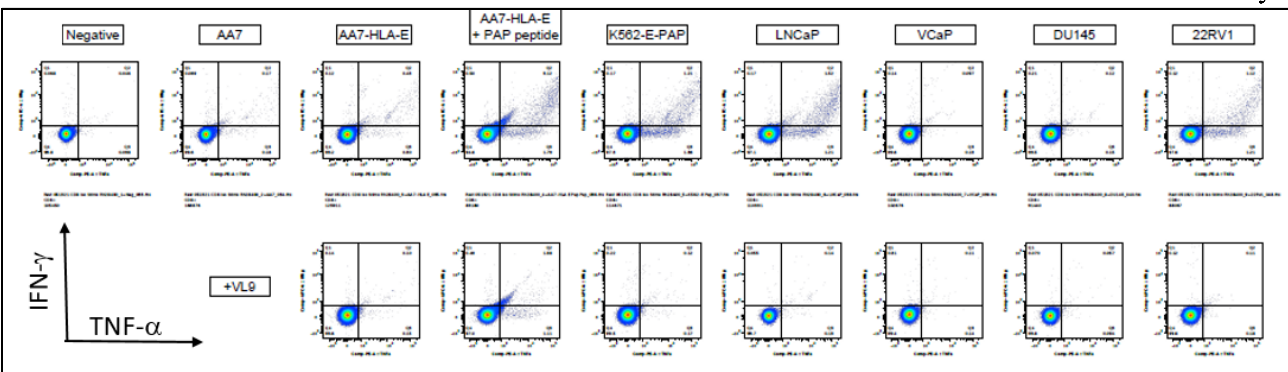


Figure 2: CD8+ T cells from RhCMV/RhPAP-immunized RM recognize PAP-expressing cancer cell lines. CD8+ T cells were isolated from RM previously immunized with 68-1 RhCMV/RhPAP and co-incubated with indicated cell lines. Dot plots showing the results of ICS for IFNγ and TNFα staining for each cell line. AA7 cells are derived from K562-cells in which the HLA-E gene was removed by CRISPR/Cas mutagenesis (kindly provided by Vir Biotechnology).

intracellular cytokine staining (ICS) for interferon-gamma (IFNγ) and tumor necrosis factor alpha (TNFα). To eliminate the possibility of MHC-II-dependent T cell stimulation these assays were performed in the presence of MHC-II blocking antibodies (anti-HLA-DR) and invariant chain derived peptide CLIP as described (Hansen et al. Science 351:714, 2016). In multiple experiments we observed robust stimulation with two PAP+ and HLA-E+ cell lines (LNCap and 22RV1) but not with PAP-negative (DU145) or HLA-E-negative (VCaP) cells (**Fig. 2** shows a representative experiment). In addition, these cells responded to K562-cells expressing HLA-E together with PAP or upon exogenous addition of PAP-peptides. Moreover, the blocking of these responses by VL9 shows that they are HLA-E restricted. These results unequivocally show that cancer cells present PAP-antigen via HLA-E.

Specific Objective 1.3: Generating cell suspensions from PAP and HLA-E-positive prostate cancer samples

Isolation of viable cells from radical prostatectomy (surgical resection) tissue specimens was described in the last progress report. One of the limitations was that previous explant cultures yielded very limited amounts of prostate cancer cells available for T cell assays (2×10^4 to 2×10^5 PCa cells per sample). Therefore, we recently increased the size of the tissue sample and shipped the sample from Mt. Sinai to OHSU after processing the tissue and generating cell suspensions. Primary prostate cancer tissue samples (Gleason score 6-10) were generated from prostate tissue of subjects consented to PPHS/IRB study. De-identified samples from 20 subjects in 29 cryovials were shipped to OHSU. Cell suspensions, prepared as described in previous reports, yielded between 5×10^4 and 1.5×10^6 cells. Suspension cells developed using this method consist of a heterogeneous mix of prostate epithelial cells, stromal cells and tissue resident immune cells and were frozen in Cyrostor solution. These cells will be used to demonstrate recognition of primary tumors by cloned TCRs.

Specific Objective 1.4: Monitoring the stimulation of MHC-E-restricted, PAP-specific CD8+ T cells by primary tumor cells

In the last progress report we showed the results of T cell assays obtained from 10 primary prostate cancer samples. Briefly, we observed that the CD8+ T cells from RhCMV/PAP immunized RM showed above-background staining for TNFα or IFNγ in the presence of prostate cancer cells from several patients. Since in most instances this staining was reduced in the presence of MHC-E binding peptide VL9, we concluded that prostate cancer cells present PAP antigen via HLA-E.

Conclusion for Major Activity 1: Our data suggest that prostate cancer cells are able to present peptides derived from PAP via the conserved HLA-I molecule HLA-E. These results strongly support the notion that prostate cancer can be targeted by HLA-E restricted CD8+ T cells.

Major Activity 2: To identify TCRs recognizing PAP-derived peptides presented by PCa cells via MHC-E

Objective 2.1 Bulk identification of TCR sequences

In the last progress report, we described the identification of individual T cell receptors expressed by MHC-E restricted, PAP-specific CD8+ T cells responding to K562-E cells expressing RhPAP. To identify TCRs that are present on T cells responding to PCa cell lines cells we stimulated CD8+ T cells from 68-1 RhCMV/RhPAP-immunized RM additionally with LNCaP cells. To allow for the sorting of responding CD8+ T cells we performed T cell stimulation in the presence of the compound TAPI-0, a metalloprotease inhibitor which prevents cleavage of TNFα and therefore traps it on the

surface of activated cells. As shown in **Fig.** 3, CD69 and TNFα positive, CD8+ T cells responding to LNCaP were sorted for single cell sequencing by 10x Genomics scRNA-seq system.

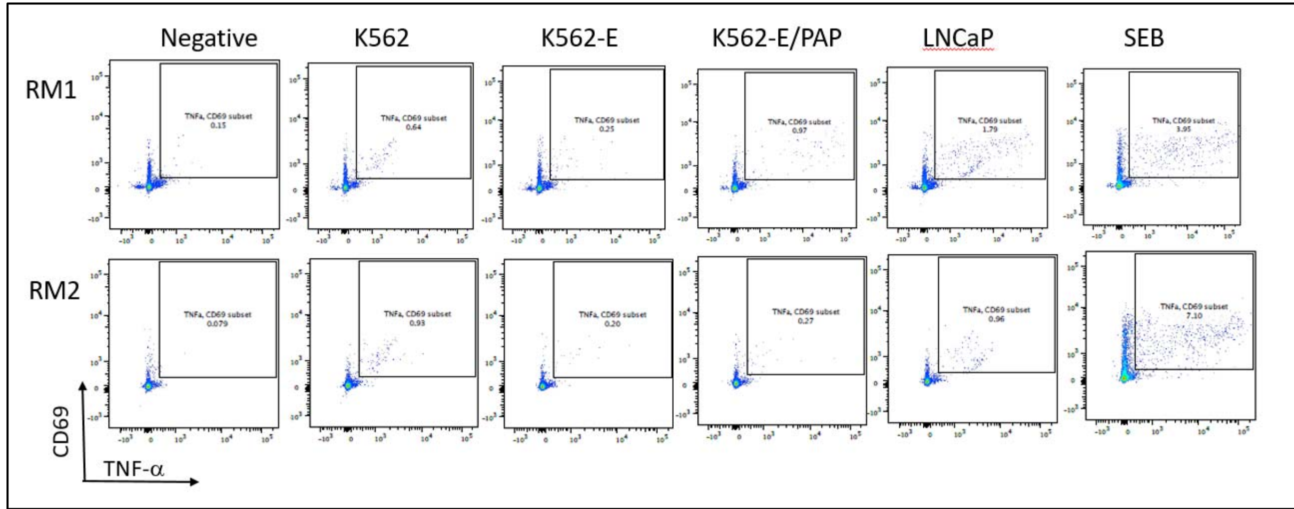


Figure 3: Sorting of CD8+ T cells from RhCMV/RhPAP-immunized RM responding to PAP-expressing cancer cell lines. CD8+ T cells were isolated from two RM previously immunized with 68-1 RhCMV/RhPAP and co-incubated with indicated cell lines. Dot plots showing the results of cell surface staining for the activation markers CD69 and TAP-I/0 trapped TNFα. The superantigen SEB (Streptococcal enterotoxin B) was included as positive control whereas the negative control only contained CD8+ T cells.

This produces Illumina-compatible sequencing libraries, which have been enriched by PCR for near full-length TCR molecules. Molecules from each individual cell were tagged with a molecular barcode unique to that cell. As shown in **Fig. 4** for the TCRβ-chain of one of the animals (RM1), this sequencing revealed the CDR3 clonotypic hierarchy for each TCR, with the same three clones identified in T cells responding to both K562-E/PAP and LNCaP. Only the TCRβ CDR3s are shown. However, the 10x genomic system captured the full length TCR sequence as well as the paired TCRα sequence for each clone. This information allows to generate synthetic TCR heterodimers.

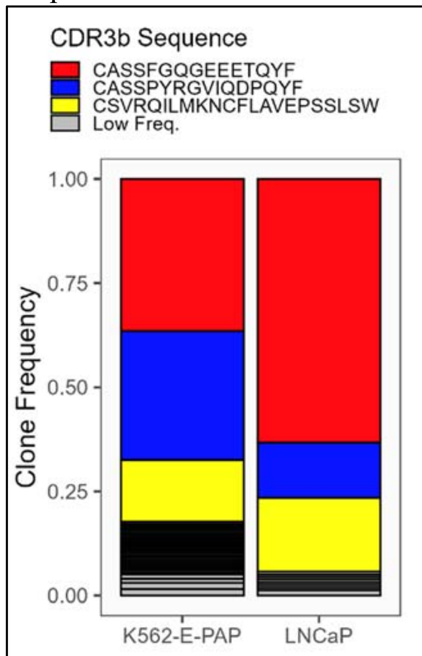


Figure 4: TCR clonotypic hierarchies of PAP-specific CD8+ T cells. RM2 had been previously immunized with 68-1 RhCMV/RhPAP. Shown are the relative frequencies of individual TCRβ sequences observed CD8+ T cells obtained stimulated with K562 cells expressing RhPAP or with the prostate cancer cell line LNCaP. The sequences of the complementarity determining region 3 (CDR3) of the most frequent TCRs are shown above.

Objective 2.2 Identification of individual PAP-specific, MHC-E restricted TCRs

As described in the last progress report, we selected 2 specific TCR αβ pairs for validation and further analysis. Synthetic genes encoding the respective TCRs fused to the murine constant region were inserted into the retroviral expressing vectors and transfected . As shown in **Fig. 5**, both TCRs were expressed on the surface of the human Jurkat T cell line. Experiments monitoring their ability to response to PAP-expressing cell lines are in progress.

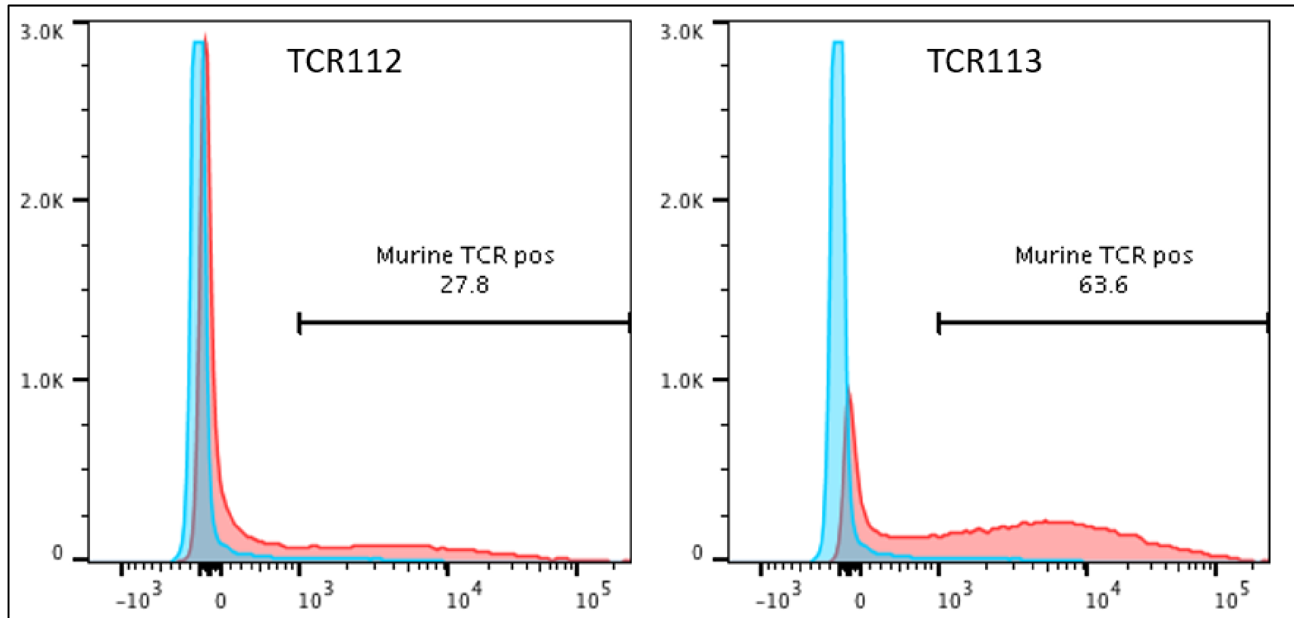


Figure 5: Expression of transduced PAP-reactive TCRs on human T cells. Human T cell lymphoblastic cell line Jurkat E6.1 48 hours after transduction with RD114 retroviral vectors encoding recombinant PAP TCRs 112 or 113 (red histograms) as compared to the non-transduced parental Jurkat-E6.1 cells (blue histogram). Cells were stained for their expression of murine TCR β constant region and quantified by flow cytometry.

Conclusion for Major Activity 2: We have identified TCRs in RM T cells responding to endogenously processed PAP by sorting and single cell sequencing. The resulting TCRs were successfully expressed in human T cells. Additional TCRs responding to PAP-expressing cancer cells will be similarly expressed. These TCRs will be used to determine responses to cancer cells and PAP-peptides.

Major Activity 3: To identify PAP-derived peptides presented by PCa cells via HLA-E

The original goal of this major activity was to identify some of the PAP-derived peptides presented on cancer cells by mapping the peptide specificity of PAP/MHC-E-specific TCRs. However, recent (as yet unpublished) observations in the SIV model suggest that MHC-E-restricted TCRs elicited by 68-1 RhCMV vectors are cross-reactive, i.e. they recognize multiple, often unrelated peptides within the same antigen presented by MHC-E. These new developments render it unlikely that PAP-specific TCRs can be used to identify individual peptides restricted by MHC-E. However, our studies in SIV also revealed that all of the MHC-E restricted TCRs react with at least one "supertope", i.e. epitopes shared among all animals. Supertopes thus seem to be the key epitopes that select for these cross-reactive TCRs. Revised objective 3.1 is therefore to identify MHC-E restricted supertopes in PAP and to determine which supertopes are recognized by the TCRs identified in Major Activity 2. Objective 3.2 is then to demonstrate that supertope-specific TCRs are able to recognize cancer cells.

Objective 3.1: Mapping of PAP-derived, MHC-E restricted peptides recognized by individual TCR α/β pairs

To identify the MHC-E restricted supertopes within the PAP sequence, we mapped the MHC-E restricted epitopes in multiple animals inoculated with 68-1 RhCMV/RhPAP (**Fig.6**). This study identified two MHC-E supertopes within the overlapping 15mer peptide pool covering Rhesus PAP: PAP24+25 (YKHEQVYIRST = shared amino acids) and PAP68 (NHMKRATQMPSYKKL). A comparison of these peptide sequences to human PAP as well as to Lysosomal Acid Phosphatase (LAP), the closest relative of PAP, reveals that these supertope peptides sequences are highly conserved between human and rhesus PAP, but not in other acid phosphatase sequences. These sequences are thus ideally suited as PCa-specific target.

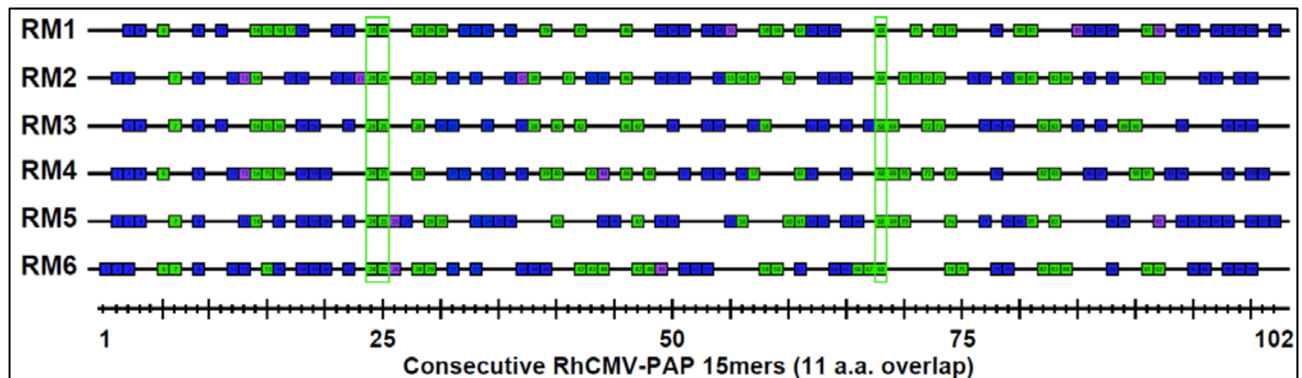


Figure 6: Mapping of MHC-E restricted supertopes. Intracellular cytokine staining for IFNγ and TNFα of PBMC from 6 RM previously inoculated with 68-1 RhCMV/RhPAP was conducted in the presence of single 15mer peptides from 102 overlapping peptides corresponding to the entire RhPAP protein sequence. Each square represents a peptide that resulted in above background staining for CD8+ T cells from indicated animals. The color of the square indicates whether the staining was blocked by antibody W6/32 and peptide VL9 (green=MHC-E block) or by anti-DR antibody and CLIP peptide (blue=MHC-II). Purple indicates indeterminate results.

Table 1: MHC-E-restricted Supertopes identified in RM immunized with 68-1 RhCMV/RhPAP

	PAP24+25 overlap	**PAP68**
Rhesus PAP	YKHEQVYIRST	NHMKRATQMPSYKKL
Human PAP	YKHEQVYIRST	NHMKRATQIPSYKKL
Human LAP	YHRQEVYVRST	TLMATTSQLP---KL

Since the 15mer peptides overlap by 11 amino-acids, the minimal 9mer supertope peptide is contained within the sequence overlap of PAP24 and PAP25. The minimal supertopes will be further defined using PAP-specific TCRs.

Conclusion for Major Activity 3: Two MHC-E restricted supertopes were identified in PAP. These supertopes will be used to determine peptide-specific stimulation of MHC-E restricted TCRs.

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

Nothing to report

How were the results disseminated to communities of interest?

Some of the results were presented at the Federation of Clinical Immunology Societies (FOCIS) conference, held virtually in October 2020.

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

We will clone the additional TCR sequences from PAP-specific, MHC-E restricted CD8+ T cells. We will transfect these TCRs into primary T cells. We will determine the stimulation of these cloned TCRs by PAP expressing cancer cells and supertope peptides. A manuscript describing some of the results is in preparation and we plan to publish this in the next reporting period

4. IMPACT:

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

We show for the first time that cancer cells not only express HLA-E, but can stimulate CD8+ T cells that recognize the tumor antigen PAP via HLA-E. This finding suggests that HLA-E presents tumor antigen-derived peptides on cancer cells thus enabling the targeting of HLA-E by immunotherapy. Since HLA-E is a non-polymorphic HLA molecule, this finding will enable the development of immunotherapies that are universal, i.e. they will work regardless of the HLA-type of a patient. We began to isolate and characterize T cell receptors (TCRs) specific for HLA-E presenting cancer antigens to enable immunotherapy by transgenic T cells expressing such TCRs.

What was the impact on other disciplines?

The techniques applied and optimized in this proposal will be generally applicable to isolate and characterize T cell receptors that recognize peptides in the context of HLA-E.

What was the impact on technology transfer?

The results of this work have been included in a patent application that was licensed to Vir Biotechnology.

What was the impact on society beyond science and technology?

Nothing to report

5. CHANGES/PROBLEMS:

As discussed above, recent results obtained in our SIV research suggests that MHC-E restricted TCRs recognize multiple peptides. TCRs can thus not be used to identify peptides. Instead we will now use supertope peptides to characterize the TCRs.

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

A limitation of studying primary tumor cells was the limited cell numbers available from resected prostate cancer. Thus problem has been solved.

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

Significant changes in use or care of human subjects

Nothing to report

Significant changes in use or care of vertebrate animals

Nothing to report

Significant changes in use of biohazards and/or select agents

Nothing to report

6. PRODUCTS

- **Publications, conference papers, and presentations**
  Journal publications.

  Oral presentation at the Federation of Clinical Immunology Societies (FOCIS) conference, held virtually in October 2020.

  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**

Patent application PCT/US2020/036481 entitled "Tumor Antigen-specific T cell responses" was submitted by OHSU based on provisional application No. 62/878,511, filed July 25, 2019, which claims the benefit of U.S. Provisional Application No. 62/858,756, filed June 7, 2019. Inventors are Klaus Frueh, Scott Hansen and Louis Picker. The patent was licensed exclusively to Vir Biotechnology as an appendix to a master licensing agreement between OHSU and Vir. The non-provisional PCT application was filed on June 5, 2020.

- **Other Products**

Sequences for TCRs will be provided to the public in publications if studies confirm their specificity for PAP-peptides presented by MHC-E

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

VACCINE & GENE THERAPY INSTITUTE/OHSU

Name: Klaus Frueh
Project Role: Principal Investigator
NIH Commons ID: kfrueh
Nearest person month worked: 1
Contribution to Project: As the Principal Investigator of the project, Dr. Früh supervised the experiments and interpreted the data together with other investigators on the project.

Name: Ben Bimber
Project Role: Co-Investigator
NIH Commons ID: bimberb
Nearest person month worked: 1
Contribution to Project: Dr. Bimber performed the bioinformatics analysis of sequencing results that identified the frequency MHC-E-restricted, PAP-specific TCRs. He helped selecting individual TCR constructs.

Name: Scott G. Hansen
Project Role: Co-Investigator
NIH Commons ID: hansensc
Nearest person month worked: 1
Contribution to Project: Dr. Hansen supervised the T cell assays with monkey-derived T cells using cancer cells or PAP-derived peptides.

Name: Louis Picker
Project Role: Co-Investigator
NIH Commons ID: pickerl
Nearest person month worked: 1
Contribution to Project: Dr. Picker advised Drs Frueh and Hansen with respect to experimental design, data analysis and trouble shooting.

Name: Kyle J. Taylor
Project Role: Research Assistant 2
Researcher Identifier (e.g. ORCID ID): not applicable
Nearest person month worked: 2
Contribution to Project: Ms. Taylor assisted Dr. Bimber with sequence analysis of PAP-specific TCRs.

Name: Abigail B. Ventura
Project Role: Research Associate
Researcher Identifier (e.g. ORCID ID): not applicable
Nearest person month worked: 2
Contribution to Project: Ms. Venture assisted Dr. Hansen with processing samples for T cell assays. She also assisted Dr. Bimber by FACS sorting of single MHC-E restricted T cells specific for PAP.

MT. SINAI SCHOOL OF MEDICINE

Name: Ashutosh Kumar Tewari
Project Role: Subaward Principal Investigator
NIH Commons ID: aktewari
Nearest person month worked: 1
Contribution to Project: Dr. Tewari provided prostate cancer samples for the project and coordinated the collaboration between Mt Sinai and OHSU. He arranged for regular meetings between the two teams and helped with data analysis in interpretatoin

Name: Nina Bhardwaj
Project Role: Subaward Co-Investigator
NIH Commons ID: bhardno2
Nearest person month worked: 1
Contribution to Project: Dr. Bhardwaj worked with Drs Tewari and Nair in designing the experiments using primary prostate cancer samples for immunological studies.

Name: Sujit Nair
Project Role: Subaward Co-Investigator
NIH Commons ID: nairs1
Nearest person month worked: 1
Contribution to Project: Dr. Nair isolated and cultured prostate cancer cells and provided them to OHSU for T cell analysis.

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?

Vir Biotechnology Inc. located in San Francisco provided funding for the animal cohorts used in this research. The Bill and Melinda Gates foundation, located in Seattle, and NIAID, located in Bethesda, provided funding for the development of tools such as TCR sequencing used in this project. Principal investigators at the Vaccine and Gene Therapy Institute (VGTI) of OHSU provided advice and input in experimental design and interpretation of the results.

8. SPECIAL REPORTING REQUIREMENTS

QUAD CHARTS: Attached

9. APPENDICES: N/A