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TITLE: In-Depth Analysis of Citrulline-Specific CD4 T-Cells in Rheumatoid Arthritis

PRINCIPAL INVESTIGATOR: Bernard Ng, MD

CONTRACTING ORGANIZATION: Seattle Institute for Biomedical and Clinical Research
Seattle, WA 98108

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14. ABSTRACT
The goal of this project is to test the hypothesis that cit-specific CD4 T cells present in rheumatoid arthritis (RA) patients exhibit a distinct cell surface phenotype and transcriptional signature that could be used to predict disease, response to therapy and identify novel therapeutic targets for the treatment of RA. In Year 2, we have made significant progress on all our goals. For Aim 1, we have completed patient recruitment for the cross-sectional study, and begun analysis of the frequency and phenotype of cit-specific T cells in this cohort. In addition, we have developed and validated new tools for characterizing of cit-specific T cells. This work was presented at the 2016 American College of Rheumatology (ACR) Annual Meeting (Milestone #2) and a manuscript based on this work is in preparation (Milestone #3). We are on track to complete Aim 1 by the end of the first quarter of 2017. For Aim 2, we have completed whole blood RNAseq on the cross-sectional cohort. Although data analysis is ongoing, we have already identified novel RA specific transcript markers that will be used for the transcript profiling of the tetramer sorted cit-specific T cells. For Aim 3, recruitment to the longitudinal study is on track with phenotypic analysis scheduled to start at in the second quarter of 2017. Given these accomplishments in Year 2, we do not anticipate any problems achieving Milestones 3 and 4 by the end of Year 3.

15. SUBJECT TERMS
Rheumatoid arthritis; CD4 T cells; citrulline; HLA class II tetramers; RNAseq; Transcriptional profiling

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1. INTRODUCTION

Rheumatoid Arthritis (RA) affects over 1.3 million Americans. It is a chronic disease, which if untreated results in pain and permanent disability. Our current approaches to treatment are expensive, lead to systemic immune suppression and do not cure the disease. It is now known that joint-associated proteins are biochemically altered by inflammation and that these alterations provoke cellular immune responses against joint tissue. In particular, T cell responses directed against the joints drive development of RA, but are not well understood. Our research group has developed the ability to identify and isolate joint specific T cells from the blood of RA patients using a tool called HLA class II tetramers. In this proposal our objective is to use this tool to better understand the unique features of joint specific T cells and how these features change with disease activity and with therapy. The information will be useful to diagnose RA earlier – which could allow for earlier intervention, decreasing the morbidity of disease. Further it may be a means to predict response to therapy very soon after the initiation of a new therapy, which would decrease the expense and exposure to drugs that are unhelpful or potentially harmful. Findings from our DoD funded work will not only enhance our scientific knowledge related to the causes of RA, but also identify new determinants which can be therapeutically targeted while protecting the remaining immune cells needed for the patient's health.

2. KEYWORDS

Rheumatoid arthritis; CD4 T cells; citrulline; HLA class II tetramers; RNAseq; Transcriptional profiling

3. ACCOMPLISHMENTS

What were the major goals of the project?

The major goal of this project is to test the hypothesis that cit-specific CD4 T cells present in RA patients exhibit a distinct cell surface phenotype and transcriptional signature that could be used to predict disease, response to therapy and identify novel therapeutic targets for the treatment of RA. In Specific Aim 1, we will utilize HLA class II tetramers to visualize and characterize T cells that recognize citrullinated epitopes in RA patients with respect to time from diagnosis and disease activity. In Specific Aim 2, we will utilize C1 Fluidigm technology combined with RNAseq to examine the transcriptional profiles of tetramer sorted cit-specific T cells at the single cell level using samples from healthy controls and RA subjects. In Specific Aim 3, we will utilize informative cell surface markers and transcript profiles to determine the impact of biologic therapy on the immune phenotype of cit-specific T cells isolated from RA patients.

Table 1 lists the Major Tasks and Milestones associated with each Specific Aim as outlined in the approved Statement of Work (SOW). It includes both a projected timeline and actual completion dates or percent complete. In Year 2, our major goals were to complete patient recruitment for the disease activity cross-sectional study, analyze these samples and present at a national meeting.

TABLE 1: MAJOR GOALS, MILESTONES, TIMELINE AND COMPLETION DATES

Specific Aim 1: Utilize HLA class II tetramers to visualize and characterize T cells that recognize citrullinated epitopes in RA patients with respect to time from diagnosis and disease activity.	**Projected Timeline**	**Year 2 Progress**
Major Task 1: Recruit patients and conduct studies to characterize T cells that recognize citrullinated epitopes by direct ex vivo tetramer staining.	**Months**	**Completion Dates (or % Complete)**
Subtask 1: Submit documents for local IRB review.	1-2	**100% Complete** BRI IRB approved: 07/28/2014 VA IRB approved: 09/24/2014
Subtask 2: Submit IRB approval and necessary documents for HRPO review.	3-4	**100% Complete**
Milestone #1: HRPO approval received	4	**100% Complete** BRI HRPO approved: 12/24/2014 VA HRPO approved: 03/27/2015
Subtask 3: Recruit at least 20 RA subjects in each of the four disease activity groups as defined by RAPID3 score as well as 20 healthy control subjects.	4-15	**100% Complete**
Subtask 4: Ex vivo tetramer analysis of citrulline reactive T cells.	4-15	**75% Complete**
Milestone #2: Successful comparison of the frequency and phenotype of cit-specific T cells in RA subjects based on RAPID3 score categories. Submission of these data as an abstract at a national meeting.	15-16	**75% Complete**
Specific Aim 2: Utilize C1 Fluidigm technology combined with RNAseq to examine the transcriptional profiles of tetramer sorted cit-specific T cells at the single cell level using samples from healthy controls and RA subjects.		
Major Task 2: Sorting of tetramer sorted cit-specific T cells and transcript profiling.	**Months**	**Completion Dates (or % Complete)**
Subtask 1: Preliminary Fluidigm C1 analysis of citrulline specific CD4 T cells in 2-4 RA subjects and healthy controls known to have high T cell frequency.	6-15	Whole Blood RNAseq: 90% Cit-specific T cell RNAseq: 20%
Subtask 2: Confirmation of RNA seq transcript signatures using qPCR of the same amplified cDNA samples.	16	20% complete
Subtask 3: Further validate C1 findings on new or frozen PBMC samples using 96 well PCR analysis and/or flow cytometry.	17	0% complete
Subtask 4: Select and re-sample (if needed) previously identified Tmr+ RA and healthy control subjects for transcript analysis.	17-20	0% complete
Subtask 5: Transcript analysis and flow cytometric assessment of the significance of RA specific transcript markers in cit-specific T cells in population identified in Subtask 4.	20-24	0% complete

Milestone #3: Co-author manuscript on the frequency, phenotype, and transcript profile of cit-specific T cells in RA subjects.	**24**	25% complete
Major Task 3: Longitudinal study of the immune phenotype of cit-specific T cells in RA patients following first administration of biologic or non-biologic therapy.	**Months**	**Completion Dates (or % Complete)**
Subtask 1: Recruitment of patients for longitudinal studies.	16-26	70% Complete
Subtask 2: Selection of informative panel of markers for longitudinal studies.	24	80% complete
Subtask 3: Longitudinal study of the immune phenotype of cit-specific T cells in RA patients.	24-32	10% complete
Subtask 4: Data analysis / correlation of informative phenotypic markers with response to therapy	32-34	0% complete
Milestone #4: Co-author manuscript on the impact of biologic therapy on the immune phenotype of cit-specific T cells isolated from RA patients	34-36	0% complete

What was accomplished under these goals?

Specific Aim 1: Utilize HLA class II tetramers to visualize and characterize T cells that recognize citrullinated epitopes in RA patients with respect to time from diagnosis and disease activity.

We have completed recruitment for the cross-sectional study proposed in Specific Aim 1. For this cohort, we consented a total of 162 subjects. Of these 162 subjects, 112 were eligible for enrollment based on required HLA-DR genotype and presence of anti-cyclic citrullinated peptide (CCP) antibodies; 150 subjects were from BRI and 12 subjects from the Puget Sound VA. Table 2 shows recruitment numbers for each of the four disease activity groups in the cross-sectional study. We have completed ex vivo tetramer assays for 52 of the 81 subjects that were HLA DRB1*04:01 (**Appendix II: Figure 1**). The median frequency of the RA epitope antigen-specific T cells was 9.23 cells per million, and preliminary phenotypic characterization suggests these cells have a Th1 like phenotype (**Appendix II: Figure 1**). Data analysis is ongoing. We anticipate completing the remaining subjects in this cross-sectional cohort (DRB1*04:01 and DRB1*04:04) by the end of the first quarter of 2017.

TABLE 2: PATIENT ENROLMENT FOR CROSS-SECTIONAL STUDY'S DISEASE ACTIVITY GROUPS

		DISEASE ACTIVITY GROUP (RECRUITMENT GOAL)				
HLA genotype	**TOTAL ENROLLED 12/12/2016**	**REMISSION (20 SUBJECTS)**	**LOW SEVERITY (20 SUBJECTS)**	**MEDIUM SEVERITY (20 SUBJECTS)**	**HIGH SEVERITY (20 SUBJECTS)**	**RAPID 3 not available**
DRB1*04:01	81	19	14	27	17	4
DRB1*04:04	31	6	9	6	9	1
TOTAL	**112**	**25**	**23**	**33**	**26**	**5**

We have also extended our HLA-DRB1*04:01 tetramer panel to include tetramers that detect citrullinated aggrecan. Citrullinated aggrecan epitopes were predicted for their binding to DRB1*04:01, and candidates confirmed using binding assays with recombinant protein (**Appendix II: Table 1**). The immunogenicity of confirmed binders was then assessed using 14-day *in vitro* peptide stimulation cultures followed by tetramer staining and single-cell cloning from selected subjects (**Appendix II: Figures 2 and 3**). Epitopes eliciting a significant response were then used for *ex vivo* tetramer staining of PBMC from healthy controls and from CCP+ RA patients in our cross-sectional cohort. *Ex vivo* tetramer analysis of PBMC revealed that a subset of RA patients had significantly increased frequencies of cit-aggrecan specific T cells in comparison to healthy controls (**Appendix II: Figure 4**). Ongoing studies are determining the effect of both disease activity and time

since diagnosis. We are also determining whether cit-aggrecan specific T cells have a distinct functional profile in RA subjects compared to healthy controls. We presented this study at the 2016 American College of Rheumatology (ACR) Annual Meeting in Washington DC in November, and are currently preparing a manuscript based on this work (**Appendix II: Abstract**)). Furthermore, we have developed HLADRB1*04:01 tetramers recognizing citrullinated tenascin C. Validation of these tetramers is ongoing.

Specific Aim 2: Utilize C1 Fluidigm technology combined with RNAseq to examine the transcriptional profiles of tetramer sorted cit-specific T cells at the single cell level using samples from healthy controls and RA subjects

As outlined in our report dated January 9, 2016, proposed RNAseq will be done on bulk instead of single tetramer positive cells due to challenges with single cell capture using C1 Fluidigm technology.

In the past year, we have completed whole blood RNAseq on a total of 228 subjects, which includes the entire cross-sectional cohort, additional individuals with known RA disease activity scores, and healthy control subjects with no history of autoimmune disease. The additional RA subjects were already in our registry and were included in order to increase the statistical power of the study. The healthy control subjects were also in our registry and were included as controls for the effect of disease. Although data analysis is ongoing, we have already made two novel observations with this dataset. First, neutrophil count appears to correlate with disease activity with a significant increase in the number of neutrophils in subjects with medium and high disease severity compared to either subjects in near remission or subjects with low disease severity (**Appendix II: Figure 5**). Our second novel observation was that T cell exhaustion appears to be dysregulated in subjects carrying a high risk HLA allele as low risk HLA alleles are associated with an exhaustion-like signature (**Appendix II: Figure 6**). This second finding is directly relevant to our study of cit-specific CD4 T cells and suggests a set of transcripts and cell surface markers that will be assessed on tetramer positive CD4 T cells. Ultimately, these findings have the potential to be novel biomarkers and new therapeutic targets. We are on schedule to complete data analysis for the whole blood RNAseq dataset by the end of the first quarter of 2017.

Specific Aim 3: Utilize informative cell surface markers and transcript profiles to determine the impact of biologic therapy on the immune phenotype of cit-specific T cells isolated from RA patients.
Patient recruitment for this longitudinal study is ongoing. As of 12/12/2016, we have consented a total of 84 subjects, of which 47 have qualified for enrolment based on HLA genotype and presence of CCP antibodies. Table 3 lists the number of enrolled subjects per treatment group for each time point as of 12/12/2016.

TABLE 3: PATIENT ENROLMENT FOR LONGITUDINAL STUDY'S TREATMENT GROUPS as of 12/12/2016

TREATMENT GROUP (RECRUITMENT GOAL)	TIME POINT 1 (PRE-Tx)	TIME POINT 2 (2-6 MONTHS POST Tx)	TIME POINT 3 (7-10 MONTHS POST Tx)
NON-BIOLOGIC MTX[1] (12 SUBJECTS)	8	7	1
NON-BIOLOGIC DMARDS[2] (12 SUBJECTS)	6	1	0
BIOLOGIC ANTI-TNF[3] (12 SUBJECTS)	21	17	10
BIOLOGIC ORENCIA[4] (12 SUBJECTS)	11	7	5
BIOLOGIC ACTEMRA[5] (12 SUBJECTS)	3	3	0
BIOLOGIC RITUXAN[6] (12 SUBJECTS)	4	4	0
BIOLOGIC XELJANZ[7] (12 SUBJECTS)	1	1	0

1. Methotrexate; 2. DMARDs: Leflunomide, Azathioprine, Sulfasalazine, and Hydroxychloroquine; 3. Anti-TNF: Adalimumab (Humira); Etanercept (Enbrel); Golimumab (Simponi); and Infliximab (Remicade); 4. CTLA-4Ig: Abatacept; 5. Anti-IL6R: Tocilizumab; 6. Anti-CD20: Rituximab; 7. JAK inhibitor: Tofacitinib.

Based on our current recruitment numbers, we propose three minor changes to our approach for the longitudinal study. Note, we believe these changes are minor as they do not alter either the objectives or scope of the study. First, the MTX and DMARDS treatment groups will be combined into a single non-biologic treatment group; there are a total of 8 subjects with 2 time points collected in this combined group as of

12/12/26. Second, the primary study will focus on time point 1 (pretreatment) and time point 2 (2-6 months post treatment) and only include time point 3 when available. Third, due to slow recruitment of the Actemra treatment group, we will no longer include this group in the primary study. Instead this treatment group will be combined with individuals treated with Rituxan and Xeljanz to form a separate group that will be used to determine if the immune phenotypes found in the both the anti-TNF and the Orencia (CTLA-4Ig) treatment groups are also seen in subjects treated with biologics of comparable efficacy that target distinct immune pathways.

For the primary study, we anticipate completing recruitment for the all three treatment groups (anti-TNF; Orencia; and Non-Biologic MTX & DMARDS) by the end of the first quarter of 2017. *Ex vivo* tetramer analysis and RNAseq will begin at the start of the second quarter. Note, selection of the panel of informative phenotypic markers for this study is ongoing and will be completed by the end of the first quarter.

What opportunities for training and professional development has the project provided?
This project has provided opportunities for training and professional development for the following postdoctoral fellows: Hannes Uchtenhagen, Jing Song and Virginia Muir. Dr. Uchtenhagen has been actively engaged in the ex vivo tetramer analysis of citrulline reactive T cells include analyzing cross-sectional cohort samples and developing both reagents and combinatorial assays. He has regularly presented his work at BRI and had the opportunity to present his work at the 2016 ACR Annual Meeting in Washington DC. He is currently preparing a manuscript based on this work. These opportunities allowed him to grow as a scientist and he recently accepted a position as Senior Scientist at Anocca AB in Sodertalje, Sweden.

Drs. Song and Muir joined the BRI in 2016. Dr. Song has been actively involved in both the ex vivo tetramer analysis of the cross-sectional cohort and the expansion of our tetramer panels. She is currently preparing a manuscript describing citrullinated Tenascin C peptides as targets of autoreactive CD4+ T cells in rheumatoid arthritis. Dr. Muir is currently using bioinformatics and systems immunology to analyze the whole blood RNAseq data. She will also be responsible for the cit-specific T cell RNAseq analysis. Both Drs. Song and Muir have opportunities to regularly present at BRI and hope to present their work at either ACR or FOCIS in 2017. They will also be lead authors on any manuscripts based on their work.

How were the results disseminated to communities of interest?
In the past year, we have presented our findings at the ACR Annual Meeting in Washington DC and at the “Be The Cure” Tolerance Workshop in Sigtuna, Sweden. We have continued to share our findings and technology with our collaborators at both the Karolinska University in Stockholm, Vivianne Malmstrom and Lars Klareskog, and the University of Colorado in Denver, Michael Holers. We have also worked with the Accelerated Medical Partnership RA project group, using the tetramers to evaluate synovial T cells isolated from joint biopsies that are being obtained by this research group. In addition, I have presented our findings at Rheumatology Grand Rounds at the following universities: University of Washington, Seattle; University of Pittsburgh, Pittsburgh; and University of Alabama, Birmingham. Collectively, these presentations demonstrate that we are regularly disseminating our results to the national and international Rheumatology Community. Further the tools generated in this project will be made available to the Rheumatology Community for the application to questions and sample sets beyond the scope of our DOD project.

What do you plan to do during the next reporting period to accomplish the goals?
We plan to complete Milestones #3 and #4 by the end of the next reporting period (Year 3). For Milestone #3, we will submit a manuscript describing the effect of disease activity on the frequency, phenotype and transcript profile of cit-specific T cells in RA subjects. For Milestone #4, we will submit a manuscript on the impact of biologic therapy on the immune phenotype of cit-specific T cells isolated from RA patients.

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
We do not believe that any significant changes have been made in our study design or in its application. However, we are proposing to make three minor changes in order to complete our patient recruitment for the longitudinal study on schedule. Importantly, these changes do not alter the objectives or scope of the study.

Changes in approach and reasons for change
We are proposing to make the following three minor changes to our approach for the longitudinal study. First, the MTX and DMARDS treatment groups will be combined into a single non-biologic treatment group. This change is necessary due to the limited number of patients that are only treated with either MTX or DMARDS. This group will include individuals treated with hydroxychloroquine, methotrexate, leflunamide, sulfasalazine and azathioprine, each of which has a different mechanisms of action, but can have comparable efficacy. Second, we propose to make the primary endpoint of our longitudinal study a comparison between time point 1 (pretreatment) and time point 2 (2 to 6 months post treatment), we will add an analysis of time point 3 (7 to 10 months post treatment) when available. Our rationale for this change is that we find that decisions related to response to therapy are typically made within 3 to 6 months of initiating a treatment, and if the response is poor the therapeutic intervention is changed so time point 3 is available only for subjects that respond positively to a therapy. Third, we will focus the primary study on a comparison of subjects treated with DMARDS, anti-TNF biologics and Orencia. We will continue to recruit individuals treated with Actemra as well as Rituxan and Xeljanz, however these subjects, for whom we have fewer subjects, will be studied in a more targeted way to evaluate how results found in the primary longitudinal cohorts are reflected in these other therapies. This was proposed in the original application but was Actemra upgraded to the primary study in the Year 1 progress report as we appeared to be on track to reaching our recruitment goal of 12 for this biologic. This has not occurred so we will now combine this group with subjects treated with Rituxan or Xeljanz. We will use this group to help us assess the phenotypic changes in response to therapy that are specific to the type of biologic intervention or common across all biologics. Note, we currently have 8 subjects recruited to this treatment group; 3 subjects on Actemra, 4 subjects on Rituxan and 1 subject on Xeljanz.

Actual or anticipated problems or delays and actions or plans to resolve them
Nothing to Report

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

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

American College of Rheumatology 2016 Annual Meeting Abstract Number: 3108
Title: Citrullinated Aggrecan Peptides Are Targets of Auto-Reactive CD4+ T-Cells in Rheumatoid Arthritis
Authors: Hannes Uchtenhagen, Cliff Rims, Eddie James and Jane H. Buckner
Session Title: T Cell Biology and Targets in Autoimmune Disease – Oral Session
Presented on Tuesday, November 15, 2016

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

Biospecimen collections: In the past year, through recruitment of RA subjects for this study, we have made a significant contribution to the BRI Immune Mediated Disease Registry and Repository (BRI-IMDR). Specifically the number of RA subjects in the BRI-IMDR has increased from 626 to 738. The samples collected from these subjects will be first used to address questions related to the DOD project, but remaining samples will be available to other scientists for their investigation into the causes of immune-mediated disease.

Research material: We have also developed a panel of HLA-DRB*04:04 tetramers. This new tool allows us to characterize T cell responses in patients with RA and healthy subjects with the DDRB1*04:04 haploytpes.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	**Jane Buckner, MD**
Project Role:	Principal Investigator
Research Identifier (e.g. ORCID):	JBUCKNER
Nearest person month worked:	1
Contribution to project:	Dr. Buckner will direct the research, supervise the postdoctoral fellow and research technicians in this project. She will meet with all investigators on a monthly basis and be responsible for preparation of publications.

Name:	**Bernard Ng, MD**
Project Role:	Principal Investigator
Research Identifier (e.g. ORCID):	BERNARDNG

Nearest person month worked:	1
Contribution to project:	Dr. Ng will supervise recruitment of study participants at the Seattle VA.

Name:	**Eddie James, PhD**
Project Role:	Co-Investigator
Research Identifier (e.g. ORCID):	EJAMES2
Nearest person month worked:	1
Contribution to project:	Dr. James will work closely with Dr. Buckner and her team on Aim 1 applying the myc–tagged tetramer technology and multiparameter flow cytometry to RA samples. Dr. James will assist in analysis of these data and preparation of publications.

Name:	**Peter Linsley, PhD**
Project Role:	Co-Investigator
Research Identifier (e.g. ORCID):	PLINSLEY
Nearest person month worked:	1
Contribution to project:	Dr. Linsley will direct the RNAseq studies, and oversee the work of biostatisticians analyzing the data. He will also assist with data interpretation and preparation of publications.

Name:	**Hannes Uchtenhagen**
Project Role:	Visiting Postdoctoral Fellow
Research Identifier (e.g. ORCID):	
Nearest person month worked:	6
Contribution to project:	Dr. Uchtenhagen worked with Drs. James and Linsley for the tetramer analyses and RNAseq studies. He left the BRI in October 2016, and Dr. Song will continue his studies.
Funding Support:	National Institute of Arthritis and Musculoskeletal and Skin Diseases, National Institute of Allergy and Infectious Diseases, Benaroya Research Institute at Virginia Mason

Name:	**Jing Song**
Project Role:	Postdoctoral Fellow
Research Identifier (e.g. ORCID):	
Nearest person month worked:	6
Contribution to project:	Dr. Song will work with Drs. James and Linsley for the tetramer analyses and RNAseq studies.
Funding Support:	National Institute of Arthritis and Musculoskeletal and Skin Diseases, National Institute of Allergy and Infectious Diseases, Benaroya Research Institute at Virginia Mason

Name:	**Virginia Muir**
Project Role:	Postdoctoral Fellow
Research Identifier (e.g. ORCID):	

Nearest person month worked:	6
Contribution to project:	Dr. Muir will work with Dr. Linsley on the RNAseq studies.
Funding Support:	National Institute of Arthritis and Musculoskeletal and Skin Diseases, National Institute of Allergy and Infectious Diseases, Benaroya Research Institute at Virginia Mason

Name:	**Cliff Rims**
Project Role:	Research Technician
Research Identifier (e.g. ORCID):	
Nearest person month worked:	11
Contribution to project:	Mr. Rims will assist Dr. James and Dr. Buckner in handling blood samples, FACs staining and tetramer analysis.

Name:	**Jeffrey Carlin, MD**
Project Role:	Director of the BRI Rheumatic Disease Registry
Research Identifier (e.g. ORCID):	JSCARLIN
Nearest person month worked:	1
Contribution to project:	Dr. Carlin is the director of the rheumatic disease registry at BRI. He oversees patient recruitment at BRI-Virginia Mason Medical Center.
Funding Support:	Virginia Mason Medical Center

Name:	**Sylvia Posso**
Project Role:	Clinical Research Coordinator
Research Identifier (e.g. ORCID):	
Nearest person month worked:	2
Contribution to project:	Ms. Posso is the clinical research coordinator responsible for patient recruitment, maintaining IRB approval and clinical data management at BRI-Virginia Mason Medical Center.
Funding Support:	Benaroya Research Institute at Virginia Mason internal funding

Name:	**Kevin Criste**
Project Role:	Research Assistant
Research Identifier (e.g. ORCID):	
Nearest person month worked:	1
Contribution to project:	Mr. Criste was responsible for patient recruitment at BRI-Virginia Mason Medical Center. He has since left the BRI and was replaced by Mohammad Pourmandi.

Name:	**Mohammad Pourmandi**
Project Role:	Research Assistant
Research Identifier (e.g. ORCID):	
Nearest person month worked:	2
Contribution to project:	Mr. Pourmandi is responsible for patient recruitment at BRI-Virginia Mason Medical Center. He has since left the BRI and was replaced by Kaytlyn Ly.

Name:	Kaytlyn Ly
Project Role:	Research Assistant
Research Identifier (e.g. ORCID):	
Nearest person month worked:	2
Contribution to project:	Ms. Ly is responsible for patient recruitment at BRI-Virginia Mason Medical Center.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
See Appendix I, Senior/Key Personnel Other Support

What other organizations were involved as partners?

Organization Name: Seattle Institute for Biomedical & Clinical Research
Location of Organization: 1100 Olive Way, Seattle WA 98101
Partner's contribution to the project: Collaboration

The Seattle Institute for Biomedical & Clinical Research receives and administers non-VA appropriated funds in support of research performed at the VA Puget Sound Health Care System. The collaborating organization is the Seattle Institute for Biomedical & Clinical Research. The performance site is the VA Puget Sound Health Care System.

Partnering Organization Performance Site:
Department of Veterans Affairs
Puget Sound Health Care System
1660 S. Columbian Way
Seattle, WA 98108-1597

8. SPECIAL REPORTING REQUIREMENTS

The collaborating Principal Investigator's (Bernard Ng) technical report is a duplicate that is separately submitted.

9. APPENDICES

Appendix I. Senior/Key Changes in Other Support
Appendix II. Table, Figures and Abstract

OTHER SUPPORT

BUCKNER, Jane H

ACTIVE

1 DP3 DK097672-01 (Buckner) 09/15/2012 – 03/14/2017 1.20 calendar
NIH/NIDDK $739,964
Defining the functional impact of T1D genes in mouse and man: A Unified Strategy
This project proposes to define *how* alterations in immune function mediated by T1D genes contribute to disease pathology. To achieve this goal we propose a collaborative and integrated strategy in which we will identify and study causative genetic variants which are: 1) likely to impact important immune pathways, 2) amenable to dissection through the use of targeted homologous mutations in mice, and 3) common enough to allow robust studies in adequate numbers of healthy control and T1D subjects. We propose 3 specific areas of concentration: 1) Human Genetic Studies, 2) Murine models of human coding variants, and 3) Human Immune Phenotyping Studies, to be performed in tandem with information sharing between all aims to keep colleagues informed of the progress.

5 U01 AI101990-04 (Buckner) 07/01/2012 – 06/30/2017 2.04 calendar
NIH/NIAID $2,008,657
Defining the role of altered cytokine signaling pathways on autoimmunity
We pose the hypothesis that in autoimmune individuals, enhanced phosphorylation of STAT3 and diminished phosphorylation of STAT5 establish a functional program biasing immune responses towards a skewed, pro-autoimmune profile. Award includes an administrative supplement in support of the Cooperative Study Group for Autoimmune Disease Prevention (CSGADP). The CSGADP goals is to halt the development of autoimmune disease prior to clinical onset by means other than global immunosuppression, the CSGADP will support collaborative projects, innovative pilot and feasibility projects, and development of reagents and resources. The Infrastructure and Opportunities Fund (IOF) of the CSGADP will facilitate the mission.

1 DP3 DK104466-01 (Buckner) 09/19/2014 – 08/31/2017 0.18 calendar
NIH/NIDDK $145,683
Investigating the role of IL-6 signaling in Teff resistance and T1D development
The focus of this project is enhanced responsiveness to IL-6 and or IL-21 predates the development of T1D and contributes to the development of Teff resistance. Additionally, we will examine the hypothesis that the combination of enhanced IL-6(pSTAT3) and blunted IL-2(pSTAT5) responses result in an increase in pathogenic CD4 T cells and impaired Treg development and function resulting in the progression to T1D.

W81XWH-15-1-003 (Buckner) 12/10/2014 – 12/09/2017 0.60 calendar
Department of Defense/USAMRAA $247,619
In Depth Analysis of Citrulline-specific CD4 T Cells in Rheumatoid Arthritis
The focus of this project is to test the hypothesis that cit-specific CD4 T cells present in RA patients exhibit a distinct cell surface phenotype and transcriptional signature that could be used to predict disease, response to therapy and identify novel therapeutic targets for the treatment of RA.

1 R01AR065952-01A1 (Hawkins) 07/01/2015 – 06/30/2020 0.24 calendar
NIH/NIAMS $4,109
Functional validation of Rheumatoid Arthritis-associated distal related regulatory SNPs
This proposal is to functionally validate distal regulatory SNPs associated with rheumatoid arthritis.

1 UC4 DK097835-01 (Krischer) 05/01/2015 – 02/28/2017 0.20 calendar
NIH/NIDDK $16,680
NIDDK T1D TrialNet Data Coordinating Center | TrialNet Core Biomarkers and Mechanisms Panel (BMP)
Members of the TrialNet Core BMP are charged with development and execution of a strategic plan for mechanistic and biomarkers research within TrialNet towards its mission of Type 1 Diabetes prevention.

5 UM1 AI109565-03 (Nepom) 08/01/2014 – 1/31/2018 0.60 calendar

Immune Tolerance Network $18,717
Dr. Buckner is Co-Protocol Chair of this trial with Dr. Carla Greenbaum. ITN Clinical Trial Protocol Chairs have overall responsibility for the conduct of the study, including oversight of all scientific, reporting, and financial matters. They will oversee recruitment activities, patient care management, and participate in monthly study teleconferences. Under the guidance of the ITN members, Protocol Chairs also interact with regulatory agencies, as needed, for matters regarding this clinical trial.

5 U01 AI101981-04 (Holers) 07/05/12 – 06/30/2017 0.60 calendar
NIH/NIAID $389,375
Prevention Center U01: Early targets for antigen-specific tolerance induction in Preclinical Rheumatoid Arthritis
The focus of this project is to identify novel synovial T cell epitopes in Rheumatoid Arthritis (RA), and develop HLA class II tetramers to detect autoreactive T cells. The data generated from these studies will be vital in the development of tools to predict the development of RA and will also yield information that will assist in the development of treatments to prevent the development of RA.

2016PG-T1D039 (Rawlings) 01/01/2016-11/30/2017 0.24 calendar
Helmsley Foundation $177,000
Durable regulatory cell therapy of TID using gene editing
Based upon our parallel existing expertise in T cell engineering and isolation of islet-specific T cells, and our preliminary dataset showing the capacity to efficiently edit the human FOXP3 locus, we predict that we will establish scalable methods to produce regulatory T-cell products directly amenable to clinical application.

PENDING

2 U01 AI101981 (Holers) 07/01/2017-06/30/2022 0.60 calendar
NIH/NIAID $125,000
Early Targets for Antigen Specific Tolerance Induction in Preclinical Rheumatoid Arthritis
This study will provide novel insight into the role of microbial antigens in the development of RA and be vital in the development of prognostic biomarkers for RA and will also generate new tools for interrogating the mechanism of disease pathogenesis.

Number not yet assigned (Buckner) 07/01/2017-06/30/2022 1.80 calendar
NIH/NIAID $490,997
Mechanisms of IL-6 mediated T cell pathogenesis in autoimmunity
The goal of this study is to test the hypothesis that elevated membrane bound IL-6 receptor expression leads to altered T cell fate and function resulting in pathogenic autoreactive T cells due to changes in the magnitude and balance of STAT1 and STAT3 phosphorylation.

Number not yet assigned (Buckner) 07/01/2017-06/30/2022 2.40 calendar
Department of Defense/USAMRAA $1,898,536
Understanding the dysregulation and interplay of immune programs implicated in SLE
The goal of this study is to determine how four different immune programs, alone and in combination, contribute to the development and progression of lupus. The four "Immune Programs" are the Type 1 Interferon Program (Project 1), the TLR Endosomal Signaling program (Project 2), the Germinal Center Program (Project 3), and the Plasmablast Differentiation Program (Project 4).

OVERLAP

None

OTHER SUPPORT

Ng, Bernard

No changes in other support

ACTIVE
NONE

COMPLETED RESEARCH SUPPORT

Grant # VA.SCV.1010./000-00.B_N 10/01/2010 – 09/30/2012
South Central VA Health Care Network Research Grants Program
Evaluating the Optimal Use of Traditional Disease-Modifying Drugs of Rheumatic Diseases in the Biologic Era
Brief summary – Since the advent of biological DMARDs, there has been reduced interest in the use of traditional DMARDs. Most recent literature, especially those funded by large pharmaceuticals, focuses on new and expensive drugs. There is little funding and interest to look at optimizing MTX use and the use of triple traditional DMARDs RA therapy. Knowing the trends of prescription behavior of DMARDs will be important to make cost-effective recommendations regarding traditional and biological DMARDs.
Role: Principal Investigator

Grant # CF.HQU.LFP.0110.000-00.B_N10/01/2009 09/30/2010
Houston VA Health Services Research and Development Center of Excellence
Is low or moderate alcohol consumption safe in patients with Rheumatoid Arthritis (RA) on Methotrexate
Brief summary- Methotrexate (MTX) has been used extensively in the treatment of RA since the early 1980s. It is a drug with a high benefit-to-risk ratio compared to many traditional disease-modifying anti-rheumatic drugs (DMARDs). In addition, low cost, convenient weekly dosing and wide availability make MTX a preferred drug over several other DMARDs. It is usually recommended that one should abstain from alcohol while taking MTX because of the fear of liver toxicities. This is a conservative recommendation that is based solely on expert opinions because there is no clinical data about the quantity of alcohol that can be safely consumed with MTX. A study commented that more patients refused Leflunomide, another DMARD which abstinence from alcohol is required, when told that they needed to abstain from alcohol. Though it is not unreasonable to assume that alcohol will adversely affect MTX compliance, there are no well-established studies in this area unlike the treatment of HIV and diabetes. If low or moderate alcohol consumption is found to be safe when taking MTX, the compliance for MTX may be improved when patients are told that complete abstinence from alcohol is not required.
Role: Principal Investigator

Grant # (Huston, D.P.) 07/01/2002 – 06/30/2003
GlaxoSmithKline/American Academy of Allergy Asthma and Immunology
Atomic Delineation of the βc Receptor Binding Domain of IL-5
Brief summary –The grant funded a study that tested the hypothesis that the IL-5 domain that engages and/or activates the βc subunit of the IL-5 receptor involves a specific charge field around the Glu13 residue. The corollary hypothesis is that a charge alteration at this site will result in an IL-5 molecule still capable of binding the IL-5Rα subunit but unable to transduce and/or recruit an agonistic signal through the βc subunit. Such molecules should have the potential to be a therapeutic molecular antagonist of IL-5-mediated eosinophilic inflammation.
Role: Research/Clinical Fellow

OVERLAP
There is no scientific or budget overlap between these projects. If pending grants are awarded, the percent/calendar month effort will be adjusted accordingly.

OTHER SUPPORT

JAMES, Eddie A.

ACTIVE

1-SRA-2017-344-S-B (James, E) 08/01/2016 - 07/31/2019 1:2 calendar months
JDRF $225,000
Developing a biomarker assay for T cells that recognize modified antigens
The goal of this project is to develop and validate an assay to broadly characterize CD4+ T cells that recognize PTM epitopes, thereby demonstrating that monitoring these responses can provide a meaningful biomarker.

2 R01 DK081166 (Haskins, K) 04/01/2016 – 03/31/2021 0.60 calendar
NIH $110,822
Hybrid Peptides as Antigens for Diabetogenic CD4 T Cells
The goal of this project is to test the hypothesis that CD4+ T cell responses to hybrid insulin peptides are relevant in human T1D

1 DP3 DK106909-01 (Kwok, W) 09/01/2015–08/31/2018 0.24 calendar
NIH/NIDDK $1,290,209
Phenotypic analysis of islet antigen-specific effector T cells in pre-diabetic subjects
The aims are to test the following hypotheses: 1. that an increase in the frequency of recently activated auto-reactive CD4+ and CD8+ T cells precedes the onset of clinical diabetes; 2. that prediabetic subjects that progress to T1D acquire an expanded TCR repertoire of islet antigen specific T cells and that those T cells exhibit a distinct transcript signature; and 3. that progression toward T1D is accompanied by an imbalance between Treg function and effector T cell responsiveness and by periods of active beta cell destruction.

2-SRA-2015-107-Q-R (James, E) 08/01/2015 – 07/31/2017 1.44 calendar
Juvenile Diabetes Research Foundation $107,496
Validation of an improved HLA class I Combinatorial Multimer Assay
The goal of this project is to validate an improved combinatorial T cell assay as an effective biomarker to predict risk of imminent loss of residual insulin secretion in subjects with type 1 diabetes.

W81XWH-15-1-0003 (Buckner, J) 12/10/2014 – 12/09/2017 1.08 calendar
Department of Defense $243,841
In-Depth Analysis of Citrulline-Specific CD4T Cell in Rheumatoid Arthritis
The goal of this project is to test the hypothesis that cit-specific CD4 T cells present in RA patients exhibit a distinct cell surface phenotype and transcriptional signature that could be used to predict disease, response to therapy and identity novel therapeutic targets for the treatment of RA.

2-SRA-2014-297-Q-R (James, E) 10/01/2014 – 1/31/2017 1.2 calendar
Juvenile Diabetes Research Foundation $259,065
T cell recognition of modified epitopes as a mechanistic contributor and biomarker of progression in type 1 diabetes
We hypothesize that CD4+ T cells that recognize PTM epitopes are an important component of autoimmune responses that target pancreatic beta cells and that monitoring these responses can target a useful biomarker. With recently developed panels of epitopes within beta cell antigens, such as GAD65, IA2, and IAPP, we will utilize established ex vivo assays to measure the frequency of CD4+ T cells that recognize modified epitopes from beta cell antigens.

HHSN272201400049C (Koelle, D) 09/30/2014 – 09/29/2017 0.24 calendar
NIH/BAA/NIAID/DAIT $365,646
Large Scale T Cell Epitope Discovery
The goal is to map T cell epitopes in a HLA restricted fashion for Vaccinia virus and T cell epitopes in a HLA restricted fashion for Varicella virus. All the epitopes identified will be verified by ex vivo tetramer staining and

examined for the frequency and phenotype of T cells of recent both Vaccinia vaccinees and subjects that received small pox vaccines 20 years ago. Lastly, we will examine the cross reactivity between varicella and HSV-1/2 reactive T cells.

3-SRA-2014-315-M-R (James, E) 09/01/2014 – 08/31/2017 1.20 calendar
Juvenile Diabetes Research Foundation $416,773
Immune effector and regulatory balance as a predictor for preserved beta cell function in subjects with established T1D
This project will investigate the central hypothesis that T cell effector and regulatory balance represents a key mechanism that determines the persistence of C-peptide in established T1D.

1 DP3 DK097653-01 (Kwok, W) 09/15/2012 – 09/14/2017 2.40 calendar
NIH-NIDDK $2,216,781
Mechanisms for HLA-DQ mediated disease protection and susceptibility
This project will examine the role of DQ-restricted T cells in T1D pathogenesis and protection from disease. This work will apply unique reagents and new technologies and extensive collections of DQ-restricted T cell clones and DQ-transfected cell lines.

5 U01 AI101981-04 (Holers, VM) 07/05/2012 – 06/30/2017 0.60 calendar
NIH-NIAID $98,103
Prevention Center: Early targets for antigen-specific tolerance induction in preclinical rheumatoid arthritis
The goal is to identify novel synovial T cell epitopes in RA, and develop HLA class II tetramers to detect autoreactive T cells. The data generated from these studies will be vital in the development of tools to predict the risk of RA and will also yield information that will assist in the development of treatments to prevent RA.

5 U19 AI100275-04 (Sette, A) 04/01/2012 – 03/31/2017 0.36 calendar
NIH-NIAID $27,238
LIAI Epitope Validation Center: Characterization of Allergen Specific T cells
Core B: Reagent Development
Benaroya Research Institute (BRI) will construct expression vectors for expression of different DP molecules in S2 cells. BRI will also purify DP molecules from transfected S2 cells and assemble allergen-specific DP tetramers.

PENDING

Number not yet assigned (Mathieu, C) 07/01/2017-06/30/2019 0.6 calendar months
JDRF $68,182
Citrullinated GRP78 in human type 1 diabetes: staging, pathogenesis and therapeutic utility
The objective of this project is to improve our understanding of the events that trigger loss of self-tolerance in T1D. We will utilize tetramer reagents to visualize cells that recognize modified epitopes in peripheral blood samples from newly diagnosed T1D patients and AutoAb positive first degree relatives.

OVERLAP
There is no scientific or budgetary overlap at present. If any overlap in calendar month effort occurs as pending grants are awarded, PI effort will be adjusted in accordance with sponsor regulation and institutional policy.

OTHER SUPPORT

LINSLEY, Peter

ACTIVE

1 DP3 DK104465-01 (Linsley) 09/25/2014 – 08/31/2017 3.6 calendar
NIH/NIDDK $640,925
Determining the molecular basis for different rates of T1D progression
Our goal is to identify molecular and/or cellular signatures in whole blood that characterize non-progressor responses to different therapies and during natural history, and to determine whether these signatures are unique or treatment-specific. From these signatures, we anticipate discovering unique, data-driven insights into immunological aspects of T1D progression.

1 DP3 DK110867-01 (Linsley) 08/01/2016-7/31/2017 1.25 calendar
NIH/NIDDK $297,376
Single Cell Transcriptome Analysis of Islet Antigen Reactive Memory CD4+ T Cells in Established TID
We are using cutting edge now cytometry and systems biology approaches to identify single cell transcriptome signatures in islet antigen reactive memory CD4+ (IARM-CD4) T cells from subjects with established TID and healthy controls. We anticipate discovering unique, data-driven insights into immunological aspects of T1D progression. This study will also provide information regarding the feasibility, lime, cost, effect size and variability needed to design appropriate follow on studies to rigorously evaluate IARM-CD4 T cells as new biomarkers and therapeutic targets.

5 R01 AI108839-03 (Wambre) 07/01/2014 – 06/30/2019 .075 calendar
NIH/NIAID $250,000
Induction and signature of pathogenic T cells in allergy
The purpose of this study is to identify a CD4+T cell signature for allergic diseases resulting from a comprehensive understanding of the mechanisms associated with the pathogenesis of allergic disease and peripheral tolerance to allergens.

5 P01 DE021954-05 (Rose) 05/01/2013 – 04/30/2017 0.87 calendar
NIH/NIDCR $55,793
Oral Pathogenesis and Host Interactions of KSHV Infection - *Supplement*
Dr. Linsley will be responsible for overseeing the RNAseq analysis of KSHV-infected patient samples in his laboratory. Dr. Linsley will participate in the analysis of RNAseq experiments and help direct the activities of Bioinformaticians needed for advanced statistical analysis, and in systems analysis.

3-SRA-2014-315-M-R (James, E) 09/01/2014 – 08/31/2017 .9 calendar
JDRF $416,773
Immune effector and regulatory balance as a predictor for preserved beta cell function in subjects with established T1D
This project will investigate the central hypothesis that T cell effector and regulatory balance represents a key mechanism that determines the persistence of C-peptide in established T1D.

W81XWH-15-1-003 (Buckner, JH) 12/10/2014 – 12/09/2017 1.2 calendar
Department of Defense – USAMRAA $243,841
In Depth Analysis of Citrulline-specific CD4 T Cells in Rheumatoid Arthritis
The focus of this project is to test the hypothesis that cit-specific CD4 T cells present in RA patients exhibit a distinct cell surface phenotype and transcriptional signature that could be used to predict disease, response to therapy and identify novel therapeutic targets for the treatment of RA.

2-PAR-2015-123-Q-R (Nepom) 04/28/2015 – 03/31/2017 1.2 calendar
JDRF-ITN Partnership $434,067
Profiling immune subsets for biomarker assessment
There is a critical need to predict, at the time of diagnosis of T1D, the rate of decline in beta cell function that will occur in the first few years of disease. The goal of this study to define blood transcriptome signatures from

blood samples collected within six months of enrollment in recent onset T1D clinical trials that will predict the change in MMTT-stimulated c-peptide production at 2 years compared to baseline. Our primary aim is to define which sets of transcripts, either by their baseline level or as they change over time, correlate with rate of c-peptide decline.

1 DP3 DK106909-01 (Kwok) 09/01/2015–08/31/2018 0.48 calendar
NIH/NIDDK $1,290,209
Phenotypic Analysis of Islet Antigen-specific Effector T cells in Pre-diabetic Subjects
The aims are to test the following hypotheses: 1. that an increase in the frequency of recently activated auto-reactive CD4+ and CD8+ T cells precedes the onset of clinical diabetes; 2. that prediabetic subjects that progress to T1D acquire an expanded TCR repertoire of islet antigen specific T cells and that those T cells exhibit a distinct transcript signature; and 3. that progression toward T1D is accompanied by an imbalance between Treg function and effector T cell responsiveness and by periods of active beta cell destruction.

5 UM1 AI109565-03 (Nepom) 02/01/2016 – 01/31/2017 0.60 calendar
NIH/NIAID $193,487
Single Cell Transcriptome Analysis of Islet Antigen Reactive Memory CD4+ Cells
The aims are to test the following hypotheses: 1) Determine the frequency, stability and specificity of expanded TCR clonotypes in IARM-CD4 T cells. 2) Validate the specificity of expanded TCR pairs. 3) Characterize the transcript phenotypes of IARM-CD4 T cells and determine the generality, stability and clonotype specificity of these signatures.

5 UM 1AI109565-03 (Nepom) 05/01/2016 – 01/31/2017 0.48 calendar
NIH/NIAID $109,691
Exploring the Role of T Cell Exhaustion in Preserving C-Peptide Levels After Alefacept Treatment
The aims are to test the following hypotheses: We aim to test the hypotheses that delayed T1D progression is the result of increased T cell exhaustion following alefacept treatment, and that a T cell exhaustion signature pre-treatment can predict C-peptide level maintenance or decline.1) Examine evidence of T cell exhaustion during and after alefacept treatment. 2) Determine whether a T cell exhaustion signature predicts C-peptide stability.

2-SRA-2016-307-S-B (Linsley) 09/01/2016 – 08/31/2018 1.2 calendar
JDRF $225,910
Triggering EFFECTOR T cell exhaustion to enhance tolerance to autoantigens induced by TCR-targeted agents in T1D
We aim to further characterize the distribution of MCD8TEK cells, their T cell exhaustion phenotype(s), and their relationship to islet autoreactive T cells. Our goals are to overcome obstacles to successful therapy with teplizumab; identify new biomarkers for successful therapy; and to exploit T cell exhaustion as a novel mechanism for treatment of T1D.

PENDING

None

OVERLAP

There is no scientific or budgetary overlap at present. If any overlap in calendar month effort occurs as pending grants are awarded, PI effort will be adjusted in accordance with sponsor regulation and institutional policy.

AWARD NUMBER: W81XWH-15-1-003
TITLE: In-Depth Analysis of Citrulline-Specific CD4 T Cells in Rheumatoid Arthritis

PRINCIPAL INVESTIGATORS:
Jane Buckner, MD, Benaroya Research Institute and Bernard Ng, MD, Puget Sound Veterans Administration

APPENDIX II

1. Figures and Tables:
    - Table 1. Binding of predicted cit-aggrecan epitopes to DRB1*04:01
    - Figure 1. Ex vivo tetramer analysis of antigen specific T cells from RA subjects in DoD cross-sectional cohort
    - Figure 2. Immunogenicity of citrullinated aggrecan epitopes that bind DRB1*04:01
    - Figure 3. T cell clones specific for citrullinated aggrecan epitopes
    - Figure 4. Cit-aggrecan specific T cells and aggrecan specific antibodies are elevated in RA subjects
    - Figure 5. Neutrophil CBC counts correlate with RA disease severity
    - Figure 6. Low risk HLA alleles are associated with an exhaustion-like signature in whole blood

2. American College of Rheumatology 2016 Annual Meeting Abstract
    - American College of Rheumatology 2016 Annual Meeting Abstract Number: 3108 Citrullinated Aggrecan Peptides Are Targets of Auto-Reactive CD4+ T-Cells in Rheumatoid Arthritis Authors: Hannes Uchtenhagen, Cliff Rims, Eddie James and Jane H. Buckner

TABLE 1. BINDING OF PREDICTED CIT-AGGRECAN EPITOPES TO DRB1*04:01[1]

Peptide Source	Sequence	Cit (μM)	Arg (μM)
agg 3	**TLLWVFVTL(CIT)VITAAVTV**	**35.30**	**36.06**
agg 70	AP(CIT)IKWS(CIT)VSKEKEVVLLVA	>100	>100
agg 82	KEVVLLVATEG(CIT)V(CIT)VN	>100	>100
agg 91	EG(CIT)V(CIT)VNSAYQDKVS	>100	>100
agg 116	ATLEVQSL(CIT)SNDSGVY(CIT)CE	>100	>100
agg 128	SGVY(CIT)CEVMHGIEDS	>100	>100
agg 153	**IVFHY(CIT)AIST(CIT)YTLDF**	**0.98**	**50.96**
agg 161	**ST(CIT)YTLDFD(CIT)AQ(CIT)ACLQ**	**0.99**	**1.02**
agg 200	**DAGWLADQTV(CIT)YPIHT**	**2.95**	**2.53**
agg 208	TV(CIT)YPIHTP(CIT)EGCYG	>100	>100
agg 225	**DEFPGV(CIT)TYGI(CIT)DTNETYDV**	**2.18**	**>100**
agg 274	(CIT)(CIT)LGA(CIT)LATTGQLYL	>100	>100
agg 298	**SAGWLAD(CIT)SV(CIT)YPISK**	**1.90**	**2.94**
agg 306	SV(CIT)YPISKA(CIT)PNCGGNL	>100	>100
agg 319	GGNLLGV(CIT)TVYVHANQ	>100	>100
agg 385	P(CIT)NITEGEA(CIT)GSVIL	>100	>100
agg 477	**GVVFHY(CIT)PGPT(CIT)YSLTF**	**14.69**	**>100**
agg 490	SLTFEEAQQACL(CIT)TG	>100	>100
agg 498	QACL(CIT)TGAVIASPEQ	>100	>100
agg 520	**GYEQCDAGWL(CIT)DQTV(CIT)YPIV**	**12.62**	**15.61**
agg 553	**PGV(CIT)TYGV(CIT)PSTETYDVY**	**7.96**	**>100**
agg 568	**DVYCFVD(CIT)LEGEVFFA**	**23.42**	**3.06**
agg 579	**EVFFAT(CIT)LEQFTFQE**	**78.43**	**15.33**
agg 608	TGQLYAAWS(CIT)GLDKCYAG	>100	>100
agg 621	**KCYAGWLADGSL(CIT)YPIV**	**6.90**	**4.66**
agg 631	SL(CIT)YPIVTP(CIT)PACGG	>100	>100
agg 670	HAFCF(CIT)GMQPHFGSNS	>100	>100
agg 684	**NSPFCLE(CIT)TPLGSPDPA**	**0.12**	**0.13**

[1.] 28 citrullinated aggrecan epitopes that consist of G1 and G2 domains were predicted to bind DRB1*04:01. Epitope number corresponds to amino acid location in full length aggrecan. Of the 28 epitopes, 13 (indicated in red) were positive for DR04:01 binding. Epitopes whose IC_{50} was greater than 100 μM were considered non-binders. Corresponding native epitopes were assessed for binding whose citrullinated peptides were IC_{50} was less than 100 μM.

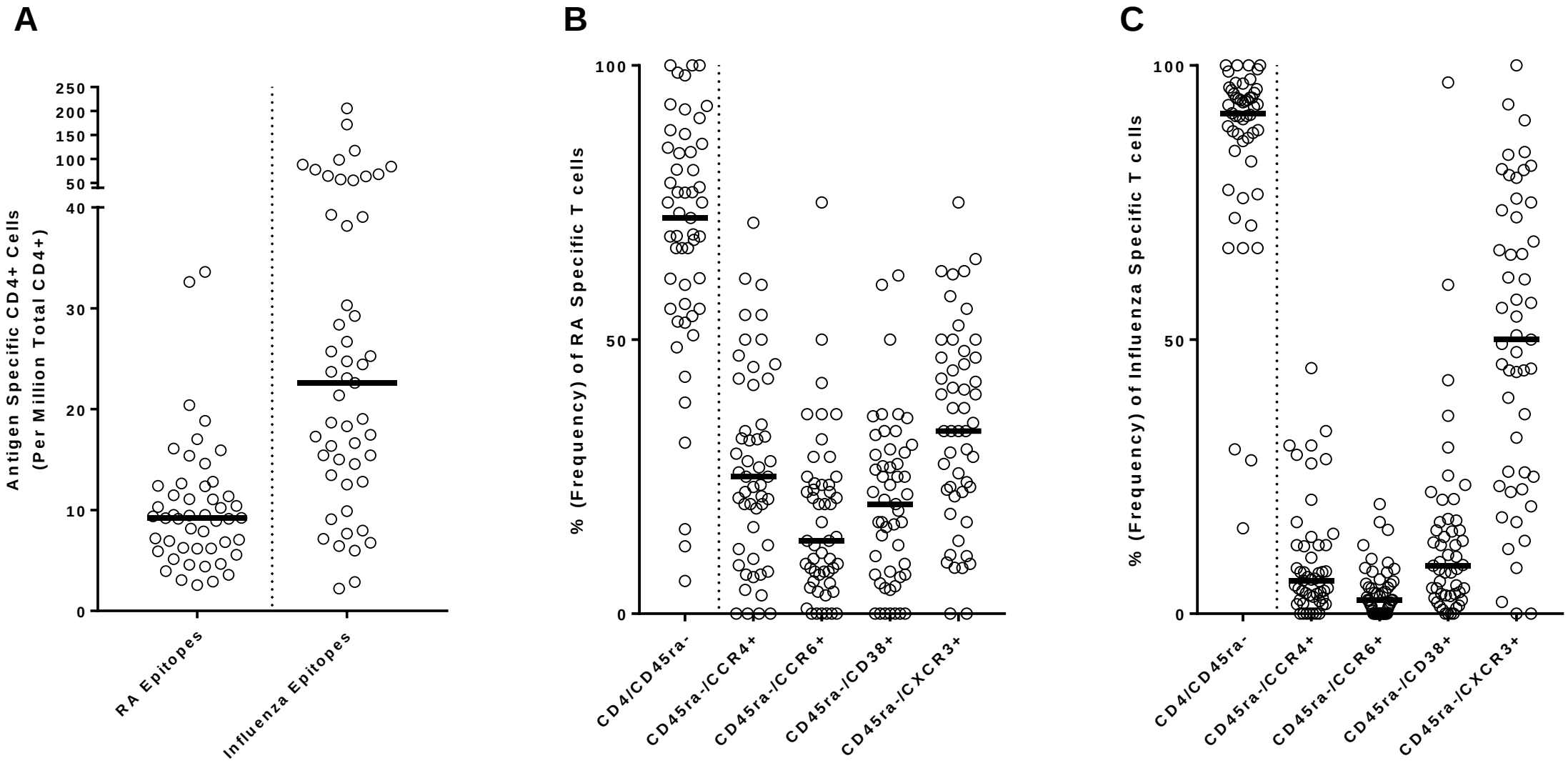


Figure 1. Ex vivo tetramer analysis of antigen specific T cells from RA subjects in DoD cross-sectional cohort Ex vivo tetramer analysis has been completed for 52 of the 81 HLA DRB1*04:01 subjects in the DoD cross-sectional cohort. Antigen specific T cell frequencies were determined directly ex vivo. The tetramer panel included epitopes for enolase, CILP, vimentin, fibrinogen, aggrecan and influenza, which was included as a positive control. All frequencies are expressed as number of antigen-specific cells/million total CD4+ T cells. (**A**) Frequency of RA epitope antigen-specific CD4 T cells and influenza epitope antigen specific CD4 T cells in 52 subjects in the cross-sectional cohort; median RA epitope antigen-specific CD4 T cell frequency = 9.23 cells per million. (**B**) Phenotypic analysis of RA epitope antigen-specific T cells and (**C**) Phenotypic analysis of influenza epitope antigen-specific T cells. For phenotypic analysis, T cell lineage markers were assessed using flow cytometry. Elevated expression of CXCR3 suggests that the RA epitope antigen-specific T cells have a Th1 like phenotype.

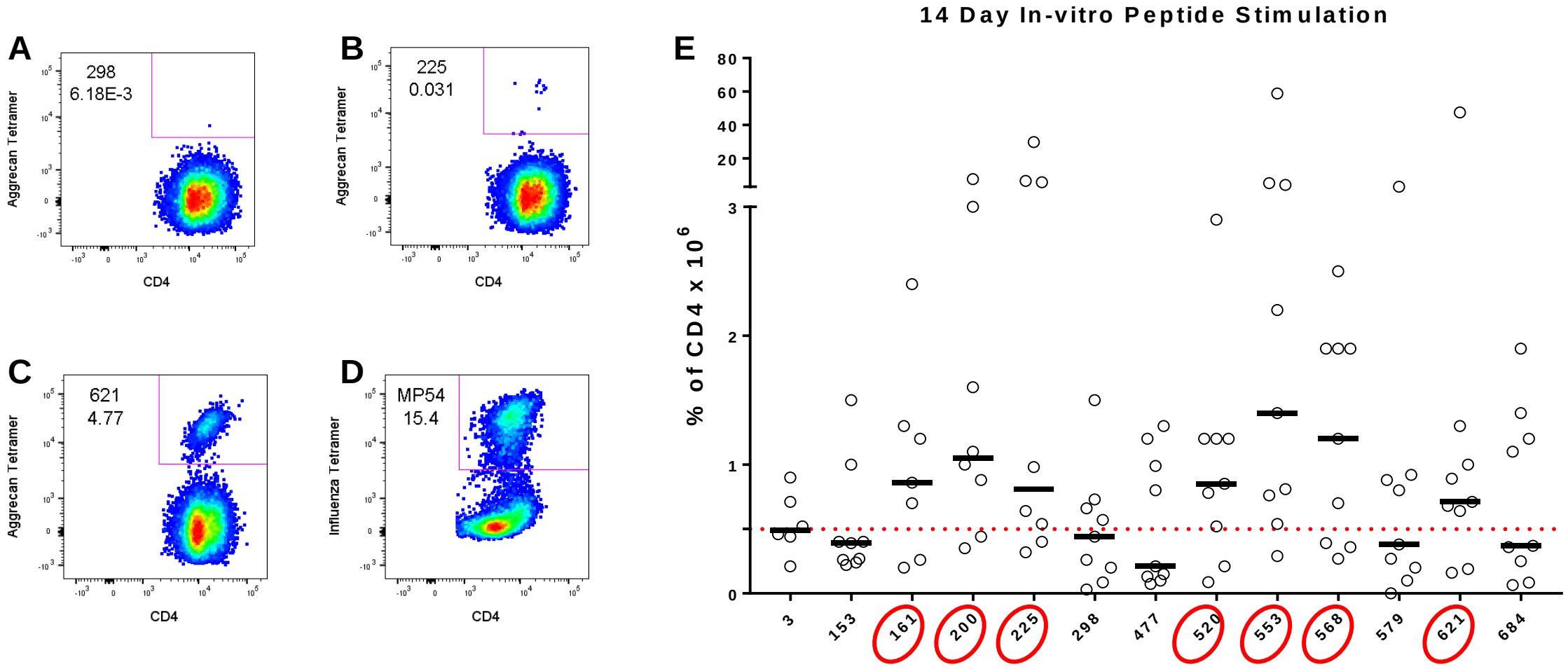


Figure 2. Immunogenicity of citrullinated aggrecan epitopes that bind DRB1*04:01. Immunogenicity was assessed using 14-day in vitro peptide stimulation cultures followed by tetramer staining and single cell cloning. Peptides elicited a clonal expansion that ranged from (**A**) zero expansion, (**B**) moderate, to (**C**) high. (**D**) Influenza MP54 used as reference. Representative FACS plots shown. (E) Summary of PBMC in-vitro peptide responses. 7 out of 13 citrullinated aggrecan epitopes were considered immunogenic (circled in red). Epitopes that elicited a response median ≤ 0.05% of total CD4 were considered weak epitopes and were eliminated from the study.

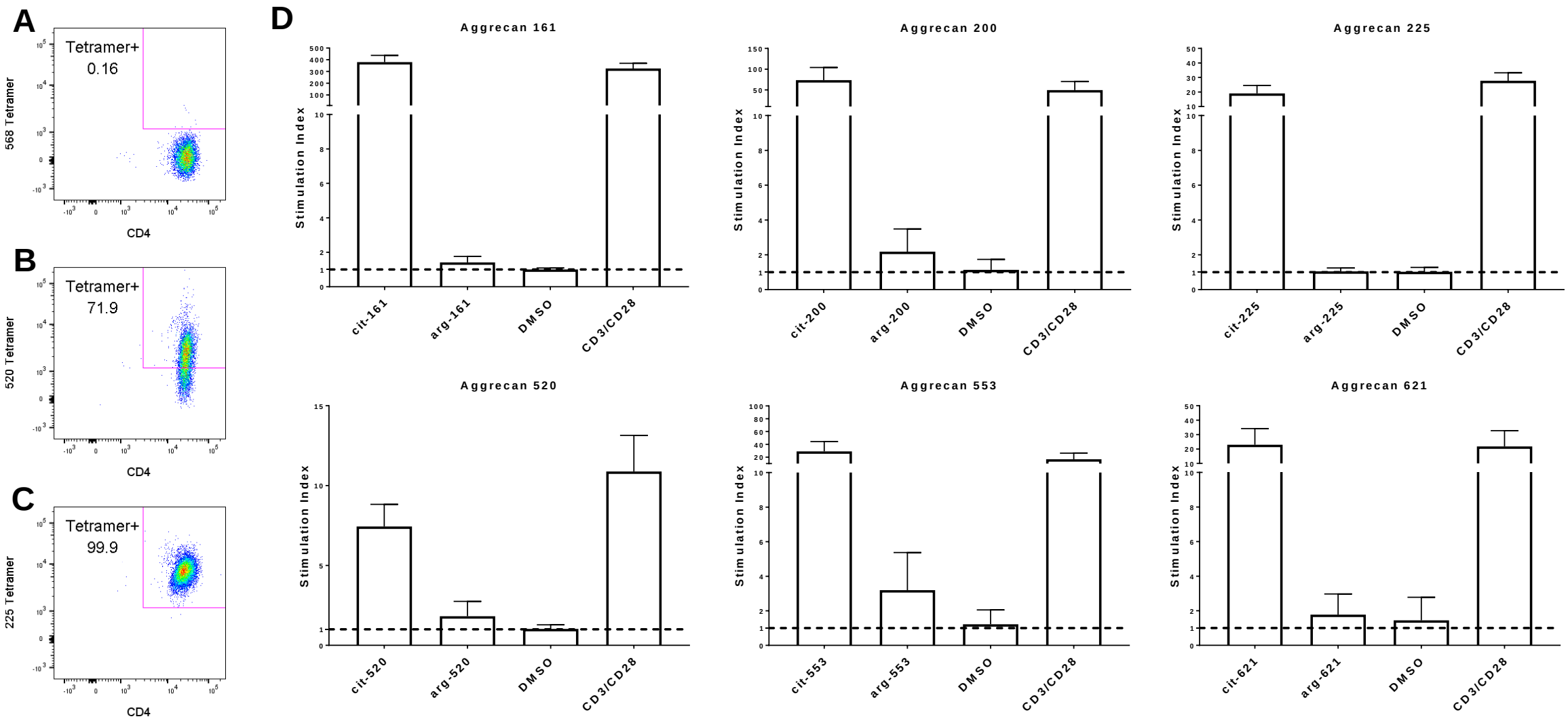


Figure 3. T cell clones specific for citrullinated aggrecan epitopes. Six citrullinated aggrecan specific T cell clones were isolated from *in vitro* PBMC peptide stimulation. Clones screened by tetramer stain were assessed as (**A**) negative, (**B**) positive low-expressing mono-clonal, and (**C**) positive high-expressing mono-clonal. (**D**) Positive clones were then screened for epitope specificity through proliferation by thymidine incorporation after 96 hours in the presence of DRB1*04:01 irradiated feeders and either citrullinated aggrecan peptide, native aggrecan peptide, DMSO, or CD3/CD28 antibodies. Stimulation index equals the ratio of peptide condition to DMSO no-peptide control.

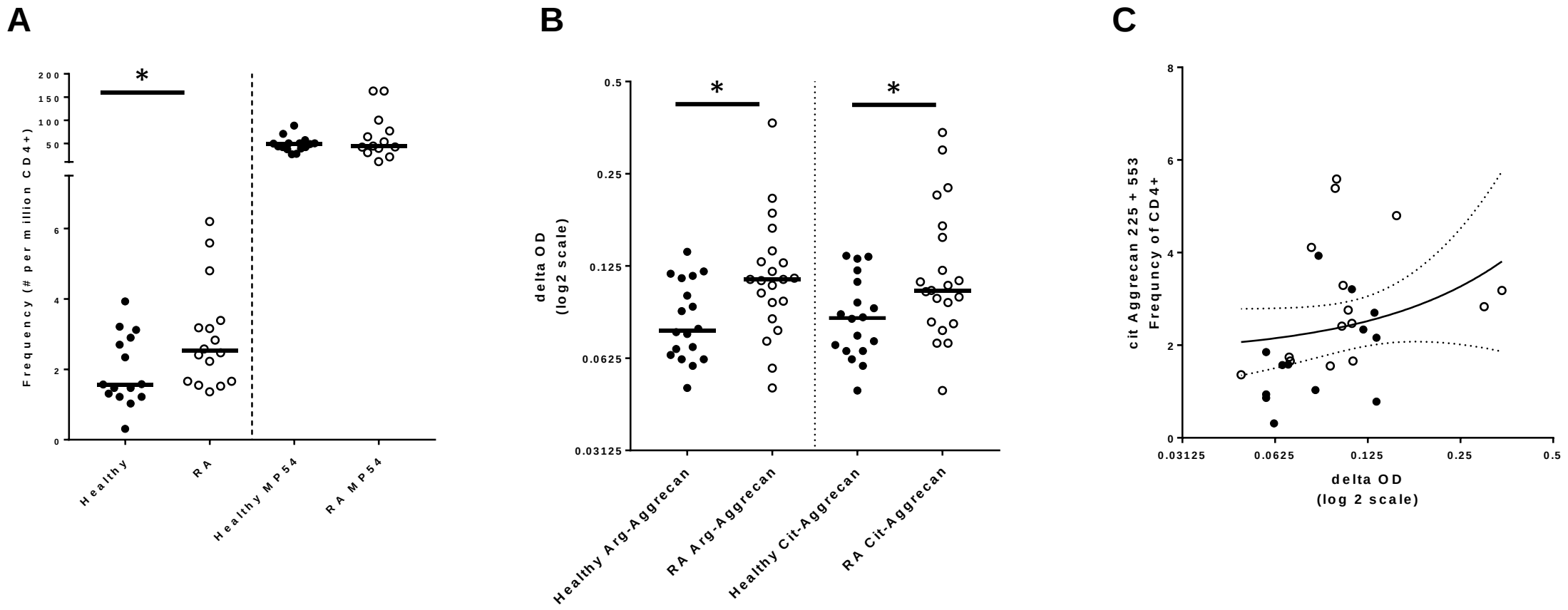


Figure 4. cit-Aggrecan specific T cells and aggrecan specific antibodies are elevated in RA subjects (**A**) Ex-vivo analysis of cit-Aggrecan 225 and 553 specific T cells in PBMC showed that the frequency of these cells are significantly elevated in RA subjects compared to healthy controls (Mann-Whitney test P=0.0395; two-tailed P value; Healthy n=15, RA n=16). (**B**) Subjects were assessed for antibodies against aggrecan-G1 domain fragments in serum. Compared to healthy subjects, those with RA show significant elevated native (Mann-Whitney test P=0.0208; two tailed P value) and citrullinated (Mann-Whitney test P=0.0341; two tailed P value) aggrecan-G1 domain antibodies (HC n=18, RA n=21) Influenza MP54 used as assay control. (**C**) Subjects that were assessed for both aggrecan 225/553 T cell frequency and aggrecan-G1 domain antibodies show correlation by Spearman r 0.4808, approximate P value ** 0.0096 between G1/G2 T cells and G1 antibody levels; best-fit curves obtained through linear regression with band at 95% confidence; n=28 combined healthy and RA subjects (closed circle healthy, open circle RA).

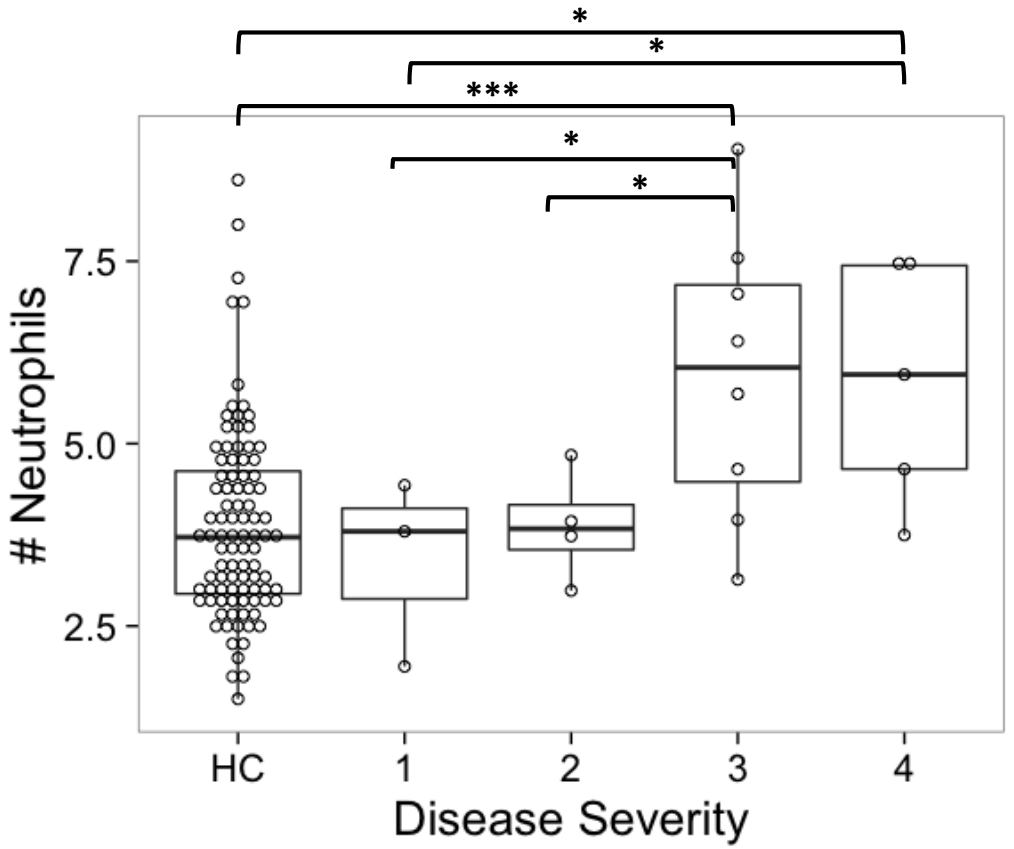
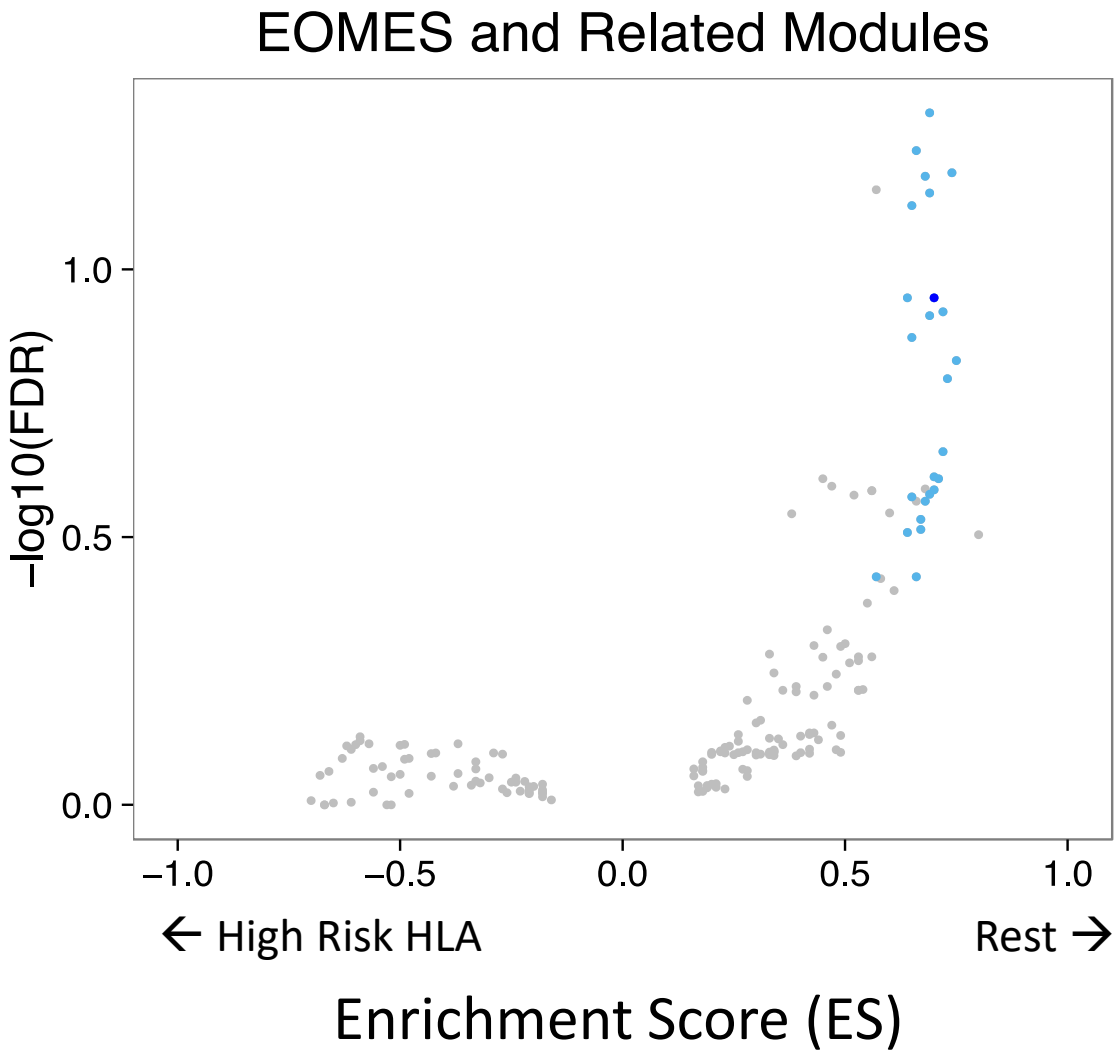


Figure 5. Neutrophil CBC counts correlate with RA disease status. 56 RA subjects were binned on weighted Rapid 3 scores as follows: High Severity (4): 4.3 -10.0, Moderate Severity (3): 2.3 – 4.0, Low Severity (2): 1.3 – 2.0, Near Remission (1): 0 - 1.0. Individuals with more severe RA had increased neutrophil counts compared to individuals with lower disease severity or the 79 healthy controls (HC). One asterisk indicates FDR-corrected t-test < 0.05, three asterisks indicates FDR-corrected t-test < 0.001.



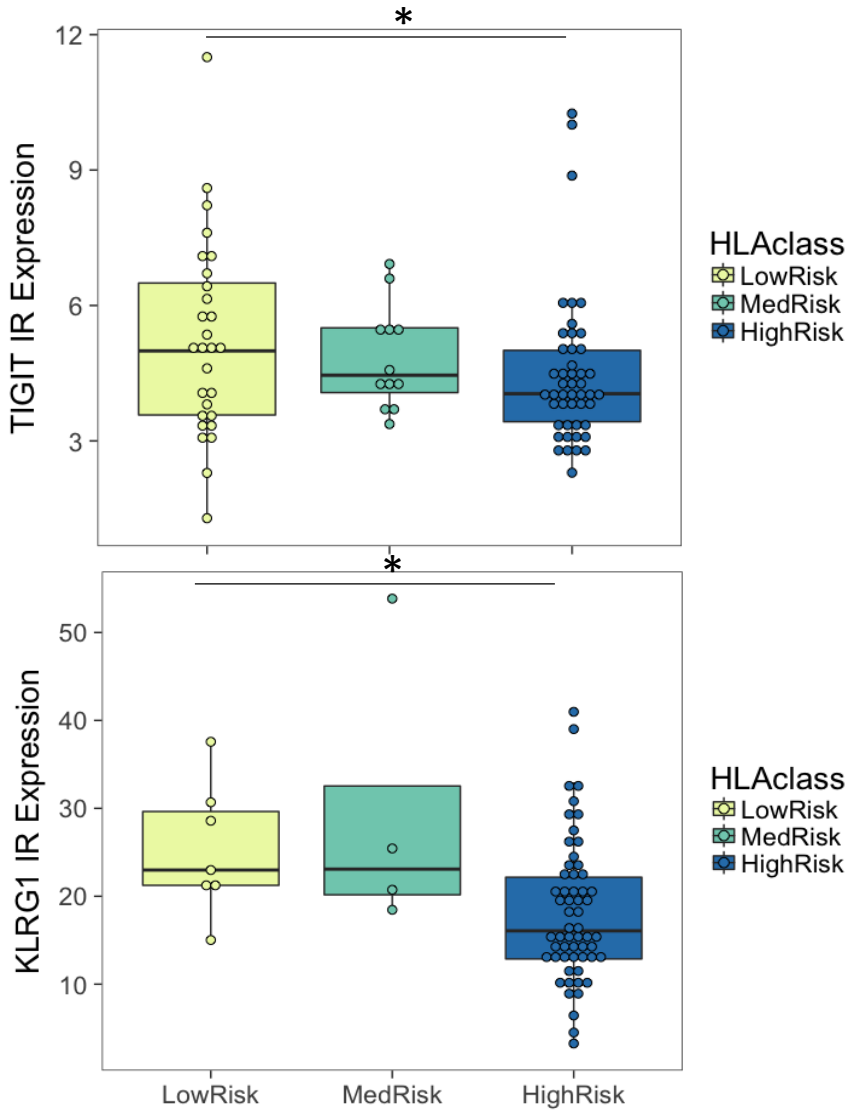


Figure 6. Low risk HLA alleles are associated with exhaustion-like signature in whole blood. (A) Gene set enrichment analysis of whole blood RNAseq indicates that the module of genes that are co-expressed with EOMES (dark blue) and highly similar gene modules (light blue) exhibit expression, which is well correlated with lower risk HLA alleles. **(B)** Whole blood RNA-seq samples derived from healthy control individuals with at least one HLA-DRB *0404, *0401, *0408, or *1001 allele (classified as high risk alleles) exhibited decreased inhibitory receptor expression for TIGIT compared to samples from individuals with either moderate-risk HLA-DRB alleles (*0101 and *0102) or non-risk HLA-DRB alleles. Mann-Whitney test p = 0.028. **(C)** Whole blood RNA-seq samples derived from RA individuals with at least one high risk HLA-DRB allele exhibited decreased inhibitory receptor expression for KLRG1 compared to samples from individuals with non-risk HLA-DRB alleles. Mann-Whitney test p = 0.022.

ABSTRACT NUMBER: 3108

Citrullinated Aggrecan Peptides Are Targets of Auto-Reactive CD4+ T-Cells in Rheumatoid Arthritis

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SESSION INFORMATION

Date: Tuesday, November 15, 2016

Session Title: T Cell Biology and Targets in Autoimmune Disease - Oral Session

Session Type: ACR Concurrent Abstract Session

Session Time: 4:30PM-6:00PM

Background/Purpose: T-cell frequencies against citrullinated epitopes derived from major auto-antibody targets (vimentin, fibrinogen, α-enolase) are increased in rheumatoid arthritis (RA). Emerging serologic data suggests antibody reactivity against additional citrullinated proteins, including histones and aggrecan, in RA. Among these potential targets, previous studies found T-cell reactivity against aggrecan peptides in models of RA and to some extent in clinical samples (Boots AM *et al.,* 1997; Law SC *et al.,* 2012; Aggarwal A *et al.,* 2013). Here, we undertook a systematic approach to verify the relevance of aggrecan specific CD4+ T-cell responses in RA.

Methods: Citrullinated aggrecan epitopes were predicted for their binding to DRB1*04:01 and candidates confirmed using binding assays with recombinant protein. The immunogenicity of confirmed binders was then assessed using 14-day *in vitro* peptide stimulation cultures followed by tetramer staining and single-cell cloning from selected subjects. Epitopes eliciting a significant response were then used for *ex vivo* tetramer staining of PBMC from healthy controls and from CCP+ RA patients across a range of disease activities (based on RAPID3 scores).

Results: Starting with 28 cit-aggrecan peptides predicted to bind DR04:01¸ we identified 6 epitopes that activated and expanded CD4+ T cells with unusual strength for (modified) self-antigens. Using the corresponding tetramers we isolated cit-aggrecan specific T-cell clones specific for these peptides from RA patients. These clones selectively recognized citrullinated peptide and exhibited a Th1-like functional phenotype. *Ex vivo* tetramer analysis of PBMC revealed that a subset of RA patients had significantly increased frequencies of cit-aggrecan specific T-cells in comparison to healthy controls. Ongoing studies will determine whether cit-aggrecan specific T-cells arise early or late in disease, whether they have a distinct functional profile compared with healthy controls, and whether they exhibit expanded TCR clonotypes in established disease. Additionally, we are investigating the degree to which the serum from the blood or synovium contains antigens that specifically activate cit-aggrecan specific T-cell clones.

Conclusion: Citrullinated aggrecan, a protein associated with autoantibodies in a subset of RA

patients, have the potential to elicit very strong CD4+ T cell responses. In a subset of patients these are apparent as strong *ex vivo* response and show a disease associated Th1-like phenotype and some signs of clonal expansion. So far the clinical correlations of these high frequencies remain unclear but are an intriguing target of our ongoing studies.

Disclosure: H. Uchtenhagen, None; **C. Rims**, None; **E. James**, None; **J. H. Buckner**, None.

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