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14. ABSTRACT The goal of this project was 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. Our studies resulted in novel findings, unique resources and new capabilities for the field of RA research. Our findings indicate that the dominant autoantigen driving disease may differ between individuals and that the character of the inflammatory response in RA may be linked to the antigen during the CD4 T cell response. Furthermore, we provide evidence that cit-antigen-specific phenotypes may stratify patients based on disease activity and disease duration. In addition, our whole blood RNA sequencing dataset suggest that T cell exhaustion is dysregulated in subjects carrying the HLA-DRB1 alleles associated with increased risk of RA. The project also generated in two unique cohorts of RA subjects that included Veterans: a cross-sectional cohort and a longitudinal cohort with samples collected before and after initiation of therapy. There are banked samples with linked clinical data for each subject. Lastly, we also developed three new capabilities: 1) a computational analysis tool for analyzing rare cell populations; 2) a method for combining index sorting and single cell RNA sequencing of rare cell populations; and 3) a panel of HLADRB1*04:01 tetramers. We will share these resources and capabilities both within the RA community, and the field of autoimmune disease.

15. SUBJECT TERMS
Rheumatoid arthritis; CD4 T cells; citrulline; HLA class II tetramers; RNA-seq; 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 was 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; RNA-seq; Transcriptional profiling

3. ACCOMPLISHMENTS

What were the major goals of the project?

The major goal of this project was 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 *Specific Aim 1*, we utilized our multiplex HLA class II tetramer assay 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 proposed to utilize C1 Fluidigm technology combined with RNA-seq 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. However, this Specific Aim was modified due to technical challenges with tetramer positive (Tmr+) cell capture using the C1 Fluidigm technology. Instead, we developed a novel activation and capture approach in order to perform single cell RNA-seq (scRNA-seq) on Tmr+ CD4 T cells. We also added a whole blood RNA-seq study comparing the transcriptional profile of whole blood from individuals with RA and healthy control subjects. In *Specific Aim 3*, we used the multiplex HLA class II tetramer assay to determine the impact of biologic therapy on the immune phenotype of cit-specific T cells isolated from RA patients

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.	**Progress**
Major Task 1: Recruit patients and conduct studies to characterize T cells that recognize citrullinated epitopes by direct ex vivo tetramer staining.	**Completion Dates (or % Complete)**
Subtask 1: Submit documents for local IRB review.	**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.	**100% Complete**
Milestone #1: HRPO approval received	**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.	**100% Complete**
Subtask 4: Ex vivo tetramer analysis of citrulline reactive T cells.	**100% 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.	**100% Complete** Presentations included in Appendix II.
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.	**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.	Whole Blood RNAseq: 100%
Subtask 2: Confirmation of RNA seq transcript signatures using qPCR of the same amplified cDNA samples.	100% Complete
Subtask 3: Further validate C1 findings on new or frozen PBMC samples using 96 well PCR analysis and/or flow cytometry.	Not performed due to inability to perform C1 analysis. Whole blood RNA-seq was performed instead as noted above
Subtask 4: Select and re-sample (if needed) previously identified Tmr+ RA and healthy control subjects for transcript analysis.	100% 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.	80% Complete

Milestone #3: Co-author manuscript on the frequency, phenotype, and transcript profile of cit-specific T cells in RA subjects.	75% 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.	**Completion Dates (or % Complete)**
Subtask 1: Recruitment of patients for longitudinal studies.	100% Complete
Subtask 2: Selection of informative panel of markers for longitudinal studies.	100% Complete
Subtask 3: Longitudinal study of the immune phenotype of cit-specific T cells in RA patients.	100% Complete
Subtask 4: Data analysis / correlation of informative phenotypic markers with response to therapy	50% 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	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.

Using a multiplex HLA class II tetramer staining assay, we characterized cit-specific CD4 T cells in a cross-sectional cohort of 80 RA subjects and 30 healthy control (HC) subjects matched for HLA, age, sex, and race (**Table 1**). All subjects were HLA DRB1*0401, and all RA subjects were positive for anti-citrullinated protein antibodies (ACPA). The cohort was cross-sectional with respect to disease activity, disease duration and therapy (**Table 1**). We assayed the frequency and phenotype of CD4 T cells specific for five citrullinated autoantigens expressed in the joint: alpha-enolase, aggrecan, cartilage intermediate layer protein (CILP), fibrinogen and vimentin. Influenza was included to allow comparison to a known foreign antigen response. Notably, our multiplex tetramer assay allows simultaneous detection of multiple antigen specificities and cell surface proteins in a single blood sample (*Uchtenhagen et al., Nat Commun. 2016 37:12614*). Consistent with our previous published study analyzing single antigen specificities (*James et al., Arthritis Rheumatol. 2014 66:1712-22*), the frequency of cit-specific CD4 T cells was significantly increased in RA subjects compared to healthy control (HC) subjects, whereas the frequency of influenza-specific CD4 T cells was not significantly different between RA and HC subjects (**Figure 1A**). While the overall frequency of cit-specific CD4 T cells was increased in RA subjects compared to HC subjects, antigen-specific differences were observed. Aggrecan- and vimentin/fibrinogen-specific cells were increased in RA whereas alpha-enolase-specific cells were decreased in RA (**Figure 1B**).

Table 1. Cross-sectional cohort characteristics

	RA (n=80)	HC (n=30)
Number of Females	57	20
Number of Males	23	10
Age (Years)	57.4±12.8	45.6±15.2
Under 35	5	6
35-45	8	6
46-55	18	7
56-65	27	8
Over 65	22	3
Disease Duration (Years)	12.5±10.9	
0-5 (Early)	24	
>5 (Established)	55	
Not Reported	1	
Disease Activity (RAPID3)		
Near Remission (0-1.0)	19	
Low Severity (1.3-2.0)	14	
Moderate Severity (2.3-4.0)	28	
High Severity (4.3-10.0)	16	
Not Reported	3	
Therapeutic*		
Steroids	28	
DMARDs	65	
Biologics	46	
Biologics: TNFi vs non-TNFi		
TNFi	35	
non-TNFi	11	

*Patients may be on more than one therapeutic

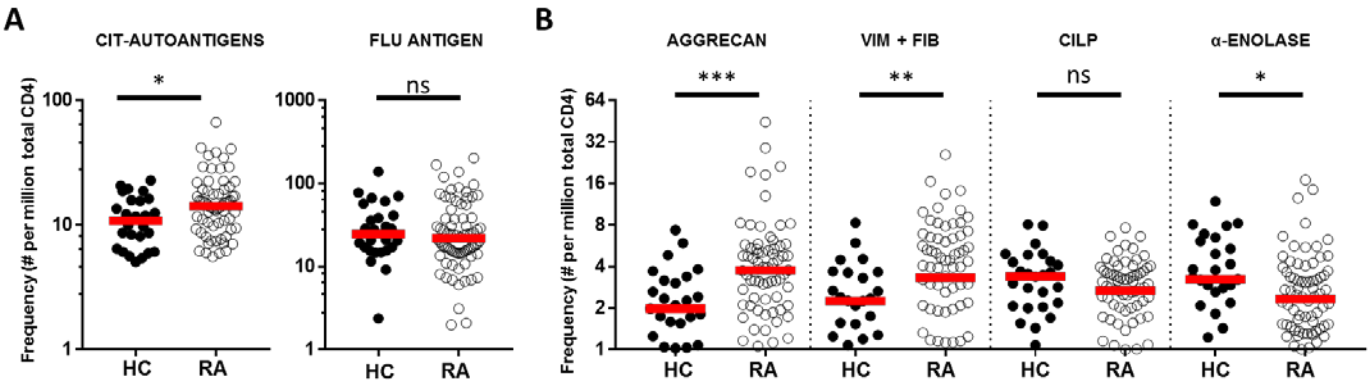


Figure 1. Frequency of CD4 T cells specific for citrullinated antigens derived from synovial proteins in RA and HC subjects. (**A**) Frequency of cit-specific CD4 T cells (left panel) and flu-specific CD4 T cells (right panel) in RA and HC subjects. (**B**) Frequency of cit-specific CD4 T cells by antigen specificity in RA and HC subjects. Data for cit-specific CD4 T cells in are from tetramers targeting cit-aggrecan, cit-alpha- enolase, cit-CILP, cit-vimentin and cit-fibrinogen in multiplex tetramer assay. Statistics: Unpaired non-parametric Mann-Whitney *p-value<0.05

We also investigated the influence of antigen specificity on the immunophenotype of cit-specific CD4 T cells by assessing expression of cell surface markers including chemokine receptors (CXCR3, CCR4 and CCR6), as well as maturation and activation markers CD45RA, CD38 and CCR7) (**Figure 2**). In the tmr+ population, the influenza-specific CD4 T cells expressed predominantly CXCR3 consistent with a Th1 phenotype. In contrast, the autoantigen-specific CD4 T cells exhibited broad heterogeneity across immunophenotype between individuals and within an individual. We also found antigen-based differences in the phenotype of cit-specific CD4 T cells with broad heterogeneity among the RA subjects with respect to the specificities of cit-Tmr+ cells as well as the phenotype of these cells. To better understand this heterogeneity in the context of multiple parameters, we worked with our co-investigator, Dr. Peter Linsley to develop a novel metacluster analysis algorithm to analyze this dataset. This algorithm which we call DISCOV-R (**DIS**tribution analysis across **C**lusters of a parent population **OV**erlaid with a **R**are subpopulation) is described in **Figure 3**. Importantly, DISCOV-R facilitates direct comparisons of complex phenotypes between subjects while minimizing: 1) skew introduced by disparate sample sizes, 2) sensitivity to outliers and 3) homogenization resulting from pooling cells or subjects, which in turn enables unbiased assessment of the distribution of rare, autoreactive cells both within and across subjects without masking individual heterogeneity.

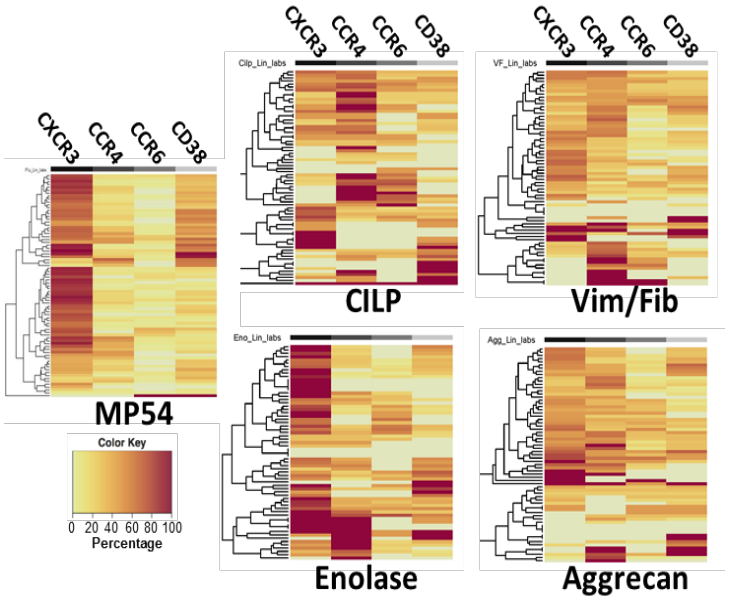


Figure 2. CD4 T cells for citrullinated synovial antigens have heterogeneous phenotypes. Heat maps of percentage of memory Tmr+ cells for specific citrullinated antigens showing expression of cell surface chemokine receptors, CXCR3, CCR4, and CCR6, and the activation marker CD38 from 55 RA subjects. MP54 is an influenza epitope used as a control. A dendrogram indicating the unsupervised hierarchical clustering relationships is shown to the left of each heat map.

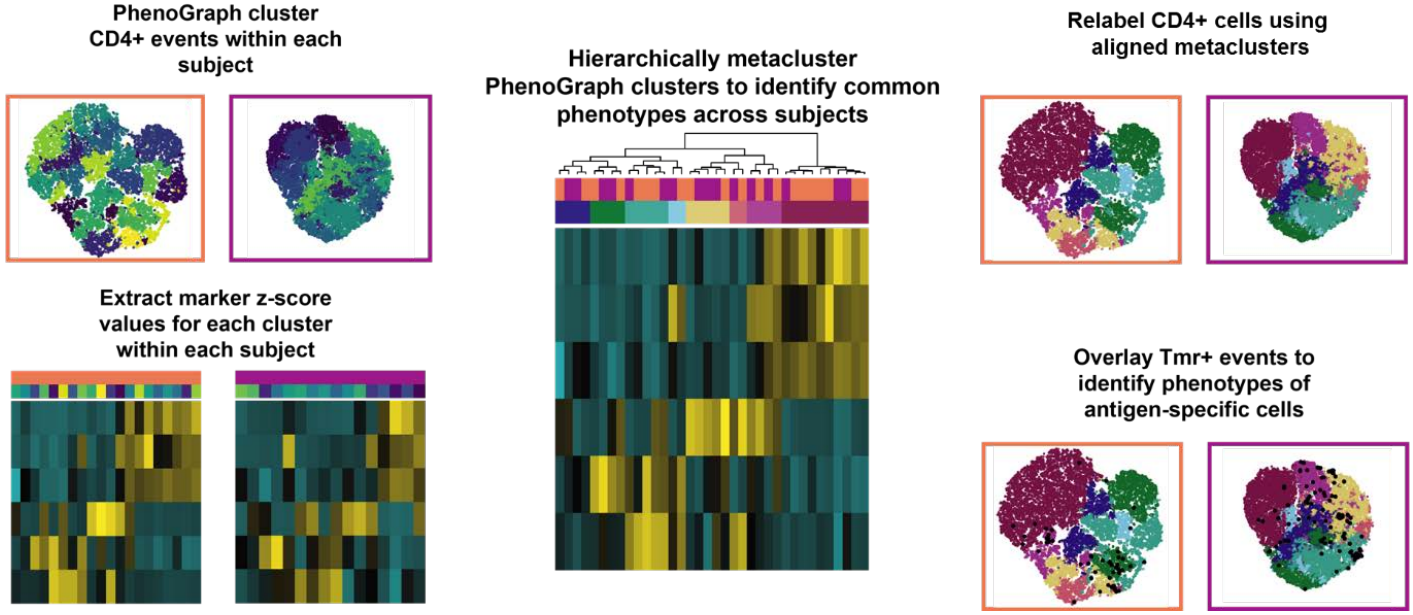
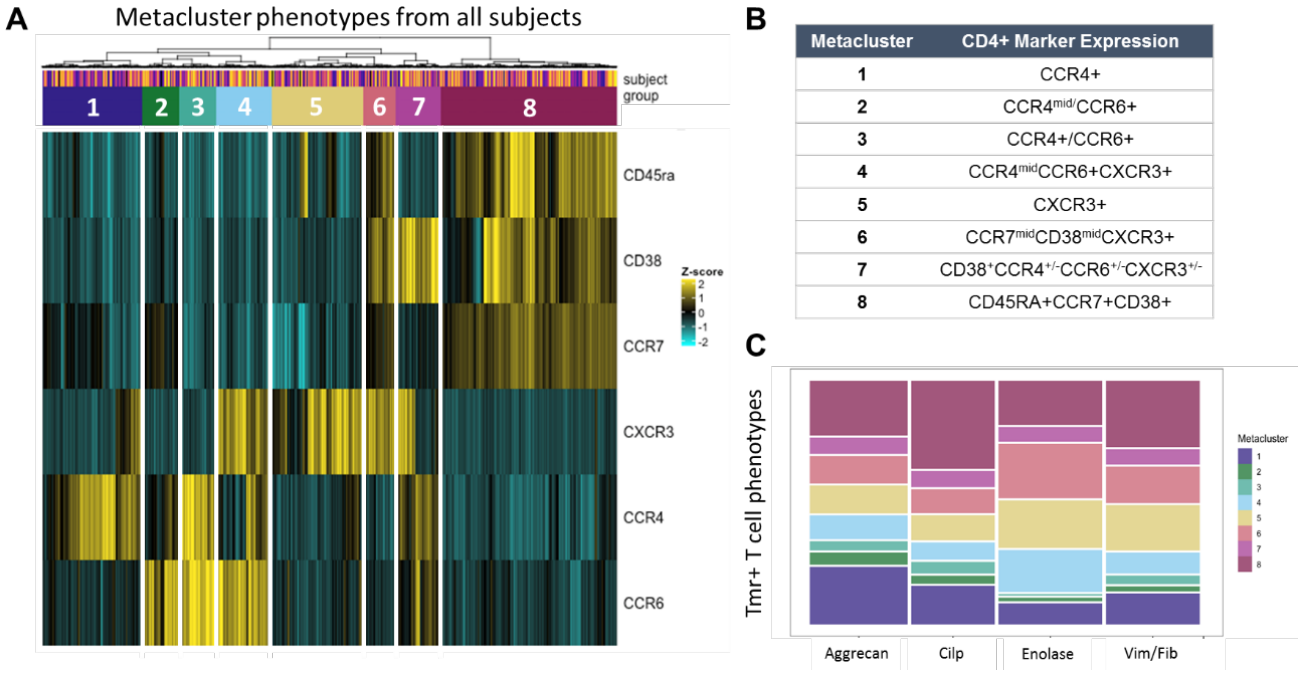


Figure 3. DISCOV-R Workflow
DIStribution analysis across **C**lusters of a parent population **OV**erlaid with a **R**are subpopulation**)**

Using DISCOV-R, we confirmed the significant phenotypic heterogeneity among cit-specific T cells and defined eight CD4 T cell immunophenotypes (metaclusters (MC)) shared across all subjects (**Figures 4A and 4B**). These immunophenotypes include Th1 (MC-5), Th2 (MC-1), Th17 (MC-2 and MC-3), Th17.1 (MC-4), T_{SCM} (MC-5), activated memory (MC-7) and naïve T cells (MC-8) (**Figures 4A and 4B**). Furthermore, DISCOV-R revealed that antigen-specificity influenced the predominant immunophenotype of cit-specific CD4 T cells. Cit-aggrecan-specific cells were primarily Th2-like (MC-1); cit-alpha-enolase-specific cells were principally Th1 (MC-5); and CILP-specific cells were mostly naïve (MC-8) (**Figure 4C**).



Metacluster	CD4+ Marker Expression
1	CCR4+
2	$CCR4^{mid/}CCR6+$
3	CCR4+/CCR6+
4	$CCR4^{mid}CCR6+CXCR3+$
5	CXCR3+
6	$CCR7^{mid}CD38^{mid}CXCR3+$
7	$CD38^{+}CCR4^{+/-}CCR6^{+/-}CXCR3^{+/-}$
8	CD45RA+CCR7+CD38+

Figure 4. DISCOV-R analysis of cit-specific CD4 T cells in RA and HC subjects. (A) Eight metaclusters that define CD4 T cell phenotypes shared across all subjects**. (B)** Cell surface markers that define metaclusters. **(C)** Mosaic plots showing percentage of antigen-specific cells in each metacluster

Using these metaclusters we were then able to perform analysis that demonstrate how specific characteristics of cit-specific CD4 T cell were associated with disease characteristics. For example, the frequency of cit-aggrecan-specific CD4 T cell with a Th1 phenotype (MC-5) was significantly higher in subjects with more than 5 years disease duration compared to subjects with less than 5 years duration (**Figure 5A**). In addition, cit-vimentin/fibrinogen-specific CD4 T cells with a stem cell memory phenotype (MC-6) were more frequent in subjects with moderate disease activity compared to those subjects near remission (**Figure 5B**). Together, these data reveal broad heterogeneity of autoantigen-specific CD4 T cells across individuals and within an individual and suggest that specific antigens may drive distinct immune responses. Future work may help stratify RA subjects based on the specificity and dominant

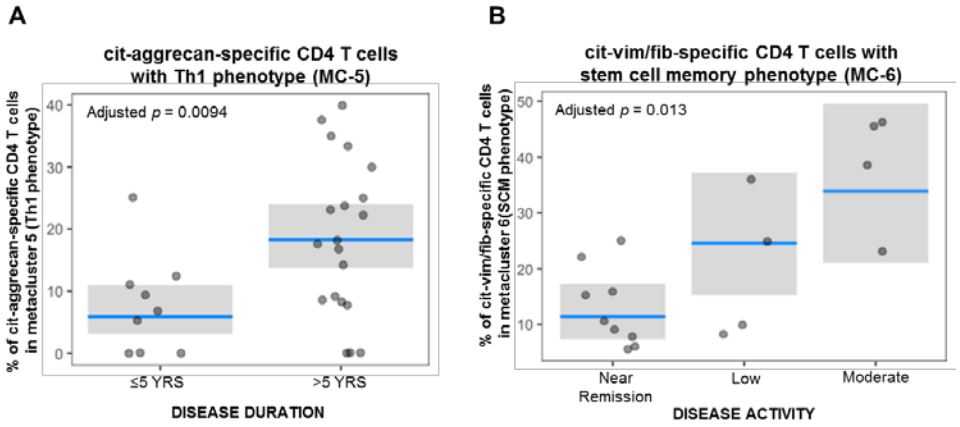


Figure 5. Frequency of cit-antigen-specific CD4 T cell phenotypes are influenced by disease duration and disease activity. (A) Increased frequency of cit-aggrecan-specific CD-4 T cells with Th1 phenotype (metacluster-5 (MC-5)) in longstanding disease (> 5 yrs) compared to recent onset disease (≤ 5 yrs). (B) Increased frequency of cit-vim/fib-specific CD4 T cells with stem cell memory (SCM) phenotype with increased disease activity.

phenotype of their T cell response, leading to targeted therapies.

As per Milestone 2, we have presented this work at 2017 American College of Rheumatology (ACR) Annual Meeting and the 2017 International Forum for RA (IFRA). We have also recently submitted an abstract based on this work to the upcoming annual meeting for the Federation of Clinical Immunology Societies, FOCIS2019. Appendix 1 includes the ACR abstract, and the FOCIS2019 abstract. We are currently preparing a manuscript based on this work with the goal to submit by April 2019.

An important part of this Specific Aim was our continued development and validation of HLADRB1*04:01 tetramers recognizing both citrullinated aggrecan and citrullinated tenascin C. This work has resulted in the following publications and meeting presentations. The aggrecan study has resulted in a recent publication in the journal Arthritis and Rheumatology (*Rims et al., Arthritis Rheumatol. 2018 Nov 2 [Epub ahead of print] PMID: 30390384*). In addition, the aggrecan tetramers developed under the auspices of this DoD grant were also used in a study investigating synovial fibroblast-neutrophil interactions in RA that was published in the journal Science Immunology (*Carmona-Rivera et al., Sci Immunol 2017 Apr;2(10). Pii eaag3358 PMID: 28649674*). The tenascin C study was presented at FOCIS2017 (see Appendix 1) and is currently a manuscript in preparation.

Specific Aim 2: Utilize C1 Fluidigm technology combined with RNA-seq 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 indicated in the major goals section above, this Specific Aim was modified due to technical challenges with capturing tmr+ cells using C1 Fluidigm technology. With Department of Defense approval, we continued to work on developing a method for capturing tmr+ cells for single cell RNA-seq but also added a whole blood transcriptome profiling study comparing RA and healthy control subjects. The workflow for our capture method includes plate bound DRB401401 monomer stimulation, tetramer staining, dual enrichment of CD154+tmr+ cells and index sorting (**Figure 6A**). We are currently applying this approach to cit-specific T cells targeting aggrecan based on our observations in Specific Aim 1 that these antigen-specific T cell populations are unique with regard to phenotype and appear to be associated with disease duration. Our preliminary data demonstrates our ability to detect productive TCRs (**Figure 6B**), and also provides evidence of expanded TCR clonotypes over time (**Figure 6C**).

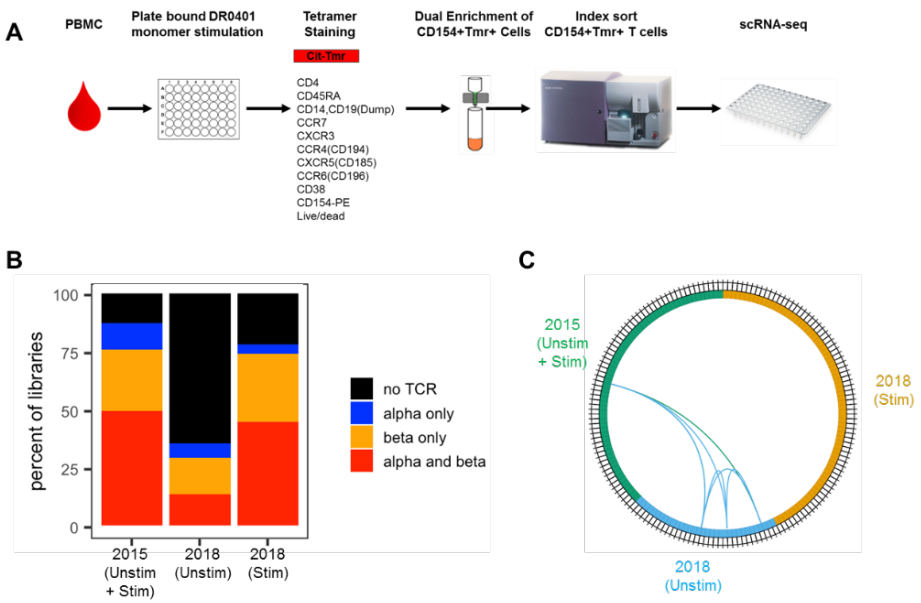


Figure 6. Index sorting and single cell RNA-seq of Tmr+ cit-specific CD4 T cells from a single RA subject at two different time points (**A**) Schematic of workflow. (**B**) TCR identification in cit-specific CD4+ T cells. T cell receptor (TCR) sequences were assembled from single cell profiles from Tmr+ cit-specific CD4+ T cells using MiXCR. Shown is the percentage of total individual antigen-specific CD4+ T cells yielding TCR chains. (C) TCR sharing over time in single RA subject. The circos plot depicts each sample from (B) in a different color. Lines connect cells TCR clonotypes shared within a sample and between different time points. The four single cells that share a TCR clonotype are all CD4+CCR4+Tmr+, which is consistent our finding that a Th2-like phenotype is predominant for cit-specific tmr+ cells

In the transcriptome profiling study, we identified differences in whole blood gene expression profiles based on HLA alleles. Specifically, modular gene expression analysis identified differential expression of several T cell exhaustion-like molecules, including EOMES, TIGIT and KLRG1, between subjects that were HLA DRB1*0401, *0404, *0405, *0408 and *1001 (RA high risk) and all other subjects tested (low risk) (**Figure 7**). Flow cytometric analysis confirmed this finding and determined that it was specific to CD8 T cells and to a lesser degree, CD4 T cells (**Figure 8**). Notably, both the transcript and flow cytometric findings were present in RA subjects and healthy individuals based on HLA haplotype. This novel finding formed the basis of a recently awarded NIH Exploratory Research Grant (R21) testing the hypothesis that the HLA DRB1*04 and *1001 alleles associated with increased risk of RA promote alterations in T cell fate and function that contribute to the development and progression of RA, independent of antigen specificity.

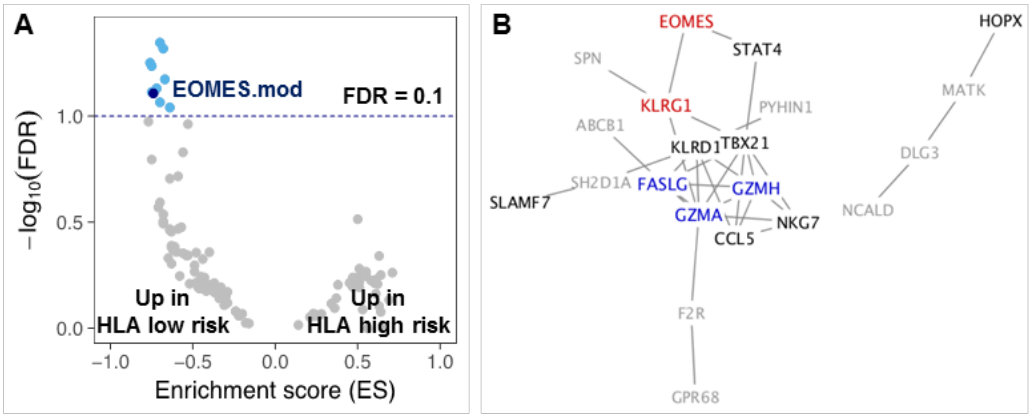


Figure 7. A gene signature associated with T cells exhaustion was detected in whole blood of low risk HLA-DRB1 individuals. (A) Gene module enrichment within a gene list rank ordered by ratio in HLA low risk versus high risk individuals using GSEA analysis. Dark blue dot, EOMES.mod; light blue dots, enriched gene sets having significant gen overlap (5% overlap, hypergeometric p-value 2e-5) with EOMES.mod (SMAD7.mod, NFATC3.mod, RUNX3.mod, GZMM.mod, E2F1.mod, STAT4.mod, TOX.mod, TIGIT.mod and CD8A.mod, with descending significance); gray dotes, all other modules. Horizontal dashed line, FDR + 0.1. (B) Protein-protein interaction network of 21 interconnected genes in the leading edge metagene from EOME.mod enrichment in (A) (enrichment for protein-protein interactions, FDR + 9.72e-10; for the GO Biological process term, “Immune Response”, FDR = 0.019). Network nodes are colored by functional classification: red text, exhaustion; blue text, effector molecules; black text, differentiation markers; grey test, other classifications.

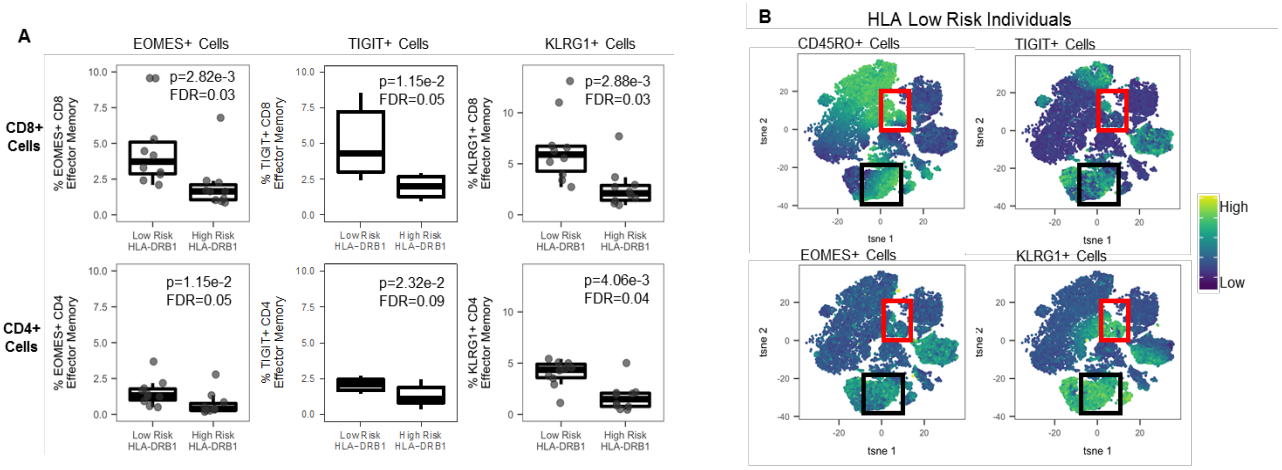


Figure 8. Accumulation of memory subsets in HLA low risk individuals marked by inhibitory receptors, EOMES, TIGIT, and KLRG1 protein expression. (A) Boxplots demonstrating higher frequency of effector memory CD8+ and CD4+ T cells expressing exhaustion markers and effector molecules via flow cytometry in both low risk HLA DRB1 and high risk HLA DRB1 individuals. P-values form uncorrected Mann- Whitney tests and multiple test corrected FDRS shown. (**B**) Exhaustion markers are co-expressed on memory CD4+ and CD8+ T cells. Flow cytometry analysis was performed on PBMCs from ten individuals with HLA DRBR1 low risk alleles. 2,500 flow cytometry events were randomly sampled form each donor, combined and subjected to unsupervised clustering using t-SNE. Boxes highlight regions of the t–SNE plot where exhaustion markers are co-expressed on memory CD4+ (red box) and CD8+ (Black box) T cells.

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.

For this Specific Aim, we have completed the following: subject recruitment, sample acquisition, and ex vivo tetramer assays. Data analysis is ongoing. The longitudinal cohort included 36 RA subjects with samples collected at three time points: baseline immediately prior to initiation of therapy, 3 months, and 9 months after initiation of therapy. We also recruited 9 healthy control subjects with two samples collected three months apart in order to account for the effect of time. Cohort characteristics are summarized in **Table 2**. As in Specific Aim 1, we used a multiplex tetramer assay with the selected tetramers and cell surface proteins based on our findings in Specific Aims 1 and 2. The tetramer staining panel was similar to the one used for the cross-sectional cohort including aggrecan, alpha-enolase, fibrinogen and vimentin with influenza as a foreign antigen but with the addition of tenascin C based on our recent work identifying T cells recognizing citrullinated tenascin C epitopes in RA subjects. We have also added T cell exhaustion markers to the immunophenotyping panel based on our results from the whole blood RNA-seq study in Specific Aim 2. Although data analysis is ongoing, our preliminary analysis suggests that time has no significant effect on the frequency of cit-specific CD4 T cells in either RA or healthy control subjects (**data not shown**). We are currently applying the DISCOV-R algorithm to this dataset to determine how therapy influences the phenotype of cit-specific CD4 T cells and are on track to complete this by the end of June 2019. Once our analysis of the flow cytometric data is complete, we will select several subjects for scRNA-seq of Tmr+ cells from two points in time pre and post therapy. We will utilize the approach that we have developed in Specific Aim 2 to assess both sharing of TCR over time and transcriptional profiles of these cells on a single cell level. We will then present our findings at the next ACR Annual Meeting, and submit a manuscript based on this work by the end of 2019.

Table 2. Longitudinal cohort characteristics

	RA (n=37)	HC (n=10)
Number of Females	23	5
Number of Males	14	5
Age at First Draw (Years)	59.9±13.6	43.8±16.5
Under 35	2	4
35-45	3	1
46-55	9	2
56-65	8	2
Over 65	15	1
Starting Therapeutic*		
DMARDs	14	
Biologics	28	
Biologics: TNFi vs non-TNFi**		
TNFi	13	
non-TNFi	15	

*Patients may be on more than one therapeutic
** non-TNFi includes rituximab (n=7), abatacept (n=5), tocilizumab (n=2), and tofacitinib (n=1)

Other Achievements:

- Development of two unique seropositive RA cohorts of HLA DRB1*0401 or 0404 individuals
  - Cross-sectional cohort of 115 individuals with banked samples and linked clinical data
    - Note, in addition to the Aim 1 study, we have also used samples from the cross-sectional cohort in an unrelated study investigating the enhanced IL-21 response in B cells from RA subjects. This work was recently published in Frontiers in Immunology citing DoD funding (*Dam et al., Front Immunol. 2018 Sep 5; 9:1978. PMID: 30233580*.)
  - Longitudinal cohort of 74 individuals with banked samples collected pre and post therapy and linked clinical data

- Development of HLA DRB1*0404 tetramer panel

- Development of new computational algorithm (DISCOV-R) for analyzing rare subpopulations
  Importantly, we will share these unique resources and capabilities with the wider scientific community. In addition to the Aim 1 study, we also used samples from this cohort to investigate the enhanced B cell response to IL-21 in RA. This work was recently published in Frontiers in Immunology

What opportunities for training and professional development has the project provided?
This project has provided opportunities for training and professional development for the following personnel: Jing Song, Virginia Muir and Cliff Rims. Dr. Song, a postdoctoral fellow in Dr. Buckner's lab, has been actively involved in the expansion of our tetramer panels. Her work has shown that T cells that recognize citrullinated tenascin-C peptides are present in HLA-DRB1*0401 patients with rheumatoid arthritis. She has regularly presented her work at BRI and had the opportunity to give an oral presentation at the 2017 FOCIS Annual Meeting in Chicago. She is currently preparing a manuscript based on the Tenascin C study. Dr. Muir in Dr. Linsley's group, is using bioinformatics and systems immunology to analyze both the tetramer data and the whole blood RNA-seq data from the cross-sectional cohort. She was the lead scientist for the development of the DISCOV-R algorithm. She has recently submitted an abstract describing the results from the cross-sectional cohort study to FOCIS2019. Based on her work for the DoD project, Dr. Muir has been promoted to Bioinformatics Project Lead at BRI. Cliff Rims, a research technician in Dr. Buckner's lab, has been actively involved in the tetramer analysis of the cross-sectional cohort. He had the opportunity to give an oral presentation based on this work at the 2017 ACR Annual Meeting. Drs. Muir and Rims are co-first authors on the manuscript in preparation for the Specific Aim 1 study. The RA cohorts developed in this study have also be used to support studies of two additional post-doctoral fellows, Elizabeth Dam and Britta Jones. Dr. Dam used the DoD funded RA cohort for her study investigating the enhanced B cell response to IL-21 in RA. She recently published this work in Frontiers in Immunology (*Dam et al., Front Immunol. 2018 Sep 5; 9:1978. PMID: 30233580*) and presented it at the 2017 ACR Annual Meeting. Dr. Jones is using the DoD funded RA cohort to investigate the role of LAG-3 in T cell function in RA. She recently gave a poster presentation based on this work at the Keystone Symposia on Transcription and RNA Regulation in Inflammation and Immunity.

How were the results disseminated to communities of interest?
We have presented our findings at a number of national and international meetings including FOCIS2017, the 2017 ACR Annual Meeting, and the International Forum for RA in 2017. Dr. Buckner also presented this work at University of Pittsburgh, University of Alabama, Birmingham and NYU Langone Health. Presentations are described in more detail in the Products section. 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. Collectively, these presentations demonstrate that we are regularly disseminating our results to the national and international rheumatology community. Furthermore, 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?
Nothing to Report

4. IMPACT
What was the impact on the development of the principal discipline(s) of the project?
The work performed has influenced the assessment of antigen specific CD4 T cells in human disease, in particular those involved in autoimmunity, although the findings could extend to Allergy, Transplant, cancer immunotherapy and infectious disease as well. We have demonstrated the ability to probe the frequency and phenotype of antigen specific T cells using a multiplex Tmr based approach. This is an advance in the field of tetramer analysis. Our analysis of this data through the development of DISCOV-R also extends the capacity to understand this type of data. Further our development of a scRNA-seq approach with pre-activation of Tmr+ T cells will assist in studies of TCR repertoire, understanding questions of clonal expansion and cellular heterogeneity of antigen specific T cells in disease. Our goal is with the publication and presentations related to this work to assist the scientific community in applying these approaches more broadly.

What was the impact on other disciplines?
As noted above the impact of the approach that we have develop can extend to the study of rare antigen specific T cell populations across disciplines in immunology. We believe this can be helpful in the area of allergy, organ transplantation, cancer as well as other autoimmune diseases.

What was the impact on technology transfer?
None at this time

What was the impact on society beyond science and technology?
RA is a debilitating disease that leads to disability and to early death. This in addition to the current therapeutic approaches is of high societal cost both financially and with respect to human health. Our hope is that the findings from these studies will help drive better and more targeted therapy for RA. This approach may be used in the future to predict and ultimately prevent RA.

5. CHANGES/PROBLEMS

Changes in approach and reasons for change
There were two challenges that needed to be resolved in this project. The first challenge was technical and related to the scRNA-seq for Specific Aim 2. In our original proposal, we anticipated that capturing single Tmr+ cells would be challenging due to their rarity in the T cell population and poor capture of lymphocytes by the C1 technology. This was indeed the case and the small number of single Tmr+ cells captured (typically 20-40 individuals cells from 100cc blood) lead to challenges related to the breadth of genes that could be analyzed in addition to problems with data analysis for such a small sample size. We resolved this challenge by developing a new approach to activate and capture Tmr+ CD4 T cells. In brief, we activate T cells with tetramer for 6 hours, stain and sort the Tmr+ cells directly into 96 well plates using index sorting so that the surface phenotype of each cell can be determined. Using this approach we are able to isolate 50-100 Tmr+ cells from 40-50cc of blood, which allows us to more fully characterize specificity, cell surface phenotype and obtain transcriptional and TCR alpha and beta chain sequences (see Figure 6 above). We also addressed this challenge by adding a whole blood RNA-seq study to Aim 2, which has already resulted in a novel finding about T cell exhaustion in individuals carrying high-risk HLA alleles for RA (see Accomplishments). The second challenge was related to patient recruitment for the longitudinal cohort with unexpectedly slow recruitment to the DMARD (non-biologic) treatment group. However, with a no-cost extension we were able to complete both recruitment assessment of cit-specific CD4 T cells using the multiplex tetramer assay.

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

Journal publications see Appendix I

1. Rims C, Uchtenhagen H, Kaplan MJ, Carmona-Rivera C, Carlucci P, Mikecz K, Markovics A, Carlin J, **Buckner JH**, James EA. Citrullinated Aggrecan Epitopes as Targets of Auto-reactive CD4+ T cells in Patients with Rheumatoid Arthritis. Arthritis Rheumatol. 2018 Nov 2. doi: 10.1002/art.40768. [Epub ahead of print]

2. Dam EM, Maier AC, Hocking AM, Carlin J, **Buckner JH**. Increased Binding of Specificity Protein 1 to the IL21R Promoter in B Cells Results in Enhanced B Cell Responses in Rheumatoid Arthritis. Front Immunol. 2018 Sep 5;9:1978. doi: 10.3389/fimmu.2018.01978. eCollection 2018. PMID: 30233580; PMCID: PMC6134023.

3. Carmona-Rivera C, Carlucci PM, Moore E, Lingampalli N, Uchtenhagen H, James E, Liu Y, Bicker KL, Wahamaa H, Hoffmann V, Catrina AI, Thompson P, **Buckner JH**, Robinson WH, Fox DA, Kaplan MJ. Synovial fibroblast-neutrophil interactions promote pathogenic adaptive immunity in rheumatoid arthritis. Sci Immunol. 2017 Apr;2(10). pii: eaag3358. doi: 10.1126/sciimmunol.aag3358. Epub 2017 Apr 14. PMID: 28649674; PMCID: PMC5479641.

Books or other non-periodical, one-time publications.
Nothing to report

Other publications, conference papers, and presentations – see Appendix II for meeting abstracts

1. **2016 University of Pittsburgh Rheumatology Grand Rounds, Pittsburgh, PA – Jane Buckner (Invited Speaker)**

2. **2016 University of Alabama Rheumatology Grand Rounds, Birmingham, AL – Jane Buckner (Invited Speaker)**

3. **2017 FOCIS Annual Meeting Abstract Number: T.18.**
   Identification and Functional Characterization of T cell Reactive to Citrullinated Tenascin-C in HLADRB1 0401-Positive Rheumatoid Arthritis Patients*
   Jing Song, Cliff Rims, David Arribas-Layton, Eddie James and Jane Buckner
   Benaroya Research Institute at Virginia Mason, Seattle, WA

4. **2017 ACR/ARHP Annual Meeting Abstract Number: 951**
   Cross Sectional Analysis of Citrullinated-Synovial Antigen-Specific CD4+ T Cells in an RA Cohort Demonstrates Antigen Based Differences in T Cell Frequency, Phenotype and the Influence of Immunotherapy
   Cliff Rims[1], Sylvia Posso[1], Bernard Ng[2], Jeffrey Carlin[3], Eddie James[4] and Jane H. Buckner[4], [1]Translational Research, Benaroya Research Institute at Virginia Mason, Seattle, WA, [2]Rheumatology, VA Puget Sound Healthcare System, Seattle, WA, [3]Rheumatology, Virginia Mason Medical Center, Seattle, WA, [4]Benaroya Research Institute at Virginia Mason, Seattle, WA

5. **2017 ACR/ARHP Annual Meeting Abstract Number: 19**
   The Transcription Factor Specificity Protein 1 Up-Regulates IL-21 Receptor Expression on B Cells in Rheumatoid Arthritis Leading to Altered Cytokine Production and Maturation.
   Elizabeth Dam[1], Alison Maier[1], Anne Hocking[1], Jeffrey Carlin[2], and Jane H. Buckner[1], [1]Translational Research, Benaroya Research Institute at Virginia Mason, Seattle, WA, [2]Rheumatology, Virginia Mason Medical Center, Seattle, WA, [4]Benaroya Research Institute at Virginia Mason, Seattle, WA

6. **2017 International Forum for RA (IFRA), Sweden – Jane Buckner (Invited Speaker)**
   Session 7: Adaptive immunity vs. innate immunity and mesenchymal functions in RA
   Genetics, T cell specificity and T cell regulation in RA

7. **2018 Colton Center Symposium Advances in Autoimmunity, NYU Langone Health, New York City, NY – Jane Buckner (Invited Speaker)**

8. **2019 Keystone Symposium on Transcription and RNA Regulation in Inflammation and Immunity**
   Regulation of LAG-3 and other co-inhibitory receptors in autoimmunity
   Britta E. Jones[1], Megan D. Maerz[1], Jane H. Buckner[1]
   Translational Research Program, Benaroya Research Institute at Virginia Mason, 1201 9th Avenue Seattle, WA 98101, USA

9. **2019 FOCIS Annual Meeting – Submitted**
   Computational Analysis of Citrulline-Specific CD4+ T Cell Frequency and Phenotype Reveals Differences that are Driven by Antigen Specificity and Disease Characteristics among Rheumatoid Arthritis Patients.
   Virginia Muir, Cliff Rims, Hannes Uchtenhagen, Anne M. Hocking, Sylvia Posso, Heather Bukiri, Jeffrey Carlin, Bernard Ng, Eddie James, Peter S. Linsley, and Jane H. Buckner

Website(s) or other Internet site(s)
Nothing to Report

Technologies or techniques
1. Development of DISCOV-R computational algorithm for analysis of rare cell populations

2. Development of novel approach for activation and capture of Tmr+ CD4 T cells for scRNA-seq.

Inventions, patent applications, and/or licenses
Nothing to Report

Other Products
Biospecimen collections: Repository of human samples including DNA, RNA, serum, plasma, and peripheral blood mononuclear cells with linked clinical data from 303 individuals with RA including 31 Veterans. Of these 303 subjects, 188 qualified for enrolment in either the cross-sectional cohort or the longitudinal cohort based on their HLA genotype and presence of anti-citrullinated protein antibodies. 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? – Final period of the award 12/10/2016-12/09/2018:

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:	2
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:	**Virginia Muir**
Project Role:	Postdoctoral Fellow
Research Identifier (e.g. ORCID):	
Nearest person month worked:	1
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:	3
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:	Kaytlyn Ly
Project Role:	Research Assistant
Research Identifier (e.g. ORCID):	
Nearest person month worked:	12
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 III, Senior/Key Personnel Other Support

What other organizations were involved as partners?

Organization Name: Benaroya Research Institute at Virginia Mason
Location of Organization: 1201 Ninth Avenue, Seattle WA 98101-2579
Partner's contribution to the project: Collaboration

Partnering Organization Performance Site:
Benaroya Research Institute at Virginia Mason
1201 Ninth Avenue
Seattle, WA 98101-2579

8. SPECIAL REPORTING REQUIREMENTS
The collaborating Principal Investigator's (Jane Buckner) technical report is a duplicate that is separately submitted.

9. APPENDICES

Appendix I: Publications

1. Rims C, Uchtenhagen H, Kaplan MJ, Carmona-Rivera C, Carlucci P, Mikecz K, Markovics A, Carlin J, **Buckner JH**, James EA. Citrullinated Aggrecan Epitopes as Targets of Auto-reactive CD4+ T cells in Patients with Rheumatoid Arthritis. Arthritis Rheumatol. 2018 Nov 2. doi: 10.1002/art.40768. [Epub ahead of print]

2. Dam EM, Maier AC, Hocking AM, Carlin J, **Buckner JH**. Increased Binding of Specificity Protein 1 to the IL21R Promoter in B Cells Results in Enhanced B Cell Responses in Rheumatoid Arthritis. Front Immunol. 2018 Sep 5;9:1978. doi: 10.3389/fimmu.2018.01978. eCollection 2018. PMID: 30233580; PMCID: PMC6134023.

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RHEUMATOID ARTHRITIS

Synovial fibroblast-neutrophil interactions promote pathogenic adaptive immunity in rheumatoid arthritis

Carmelo Carmona-Rivera,[1] Philip M. Carlucci,[1] Erica Moore,[1] Nithya Lingampalli,[2,3] Hannes Uchtenhagen,[4] Eddie James,[4] Yudong Liu,[1] Kevin L. Bicker,[5] Heidi Wahamaa,[6] Victoria Hoffmann,[7] Anca Irinel Catrina,[6] Paul R. Thompson,[8] Jane H. Buckner,[4] William H. Robinson,[2,3] David A. Fox,[9] Mariana J. Kaplan[1]*

Rheumatoid arthritis (RA) is characterized by synovial joint inflammation and by development of pathogenic humoral and cellular autoimmunity to citrullinated proteins. Neutrophil extracellular traps (NETs) are a source of citrullinated autoantigens and activate RA synovial fibroblast-like synoviocytes (FLS), cells crucial in joint damage. We investigated the molecular mechanisms by which NETs promote proinflammatory phenotypes in FLS and whether these interactions generate pathogenic anti-citrulline adaptive immune responses. NETs containing citrullinated peptides are internalized by FLS through a RAGE-TLR9 pathway, promoting FLS inflammatory phenotype and their up-regulation of major histocompatibility complex (MHC) class II. Once internalized, arthritogenic NET peptides are loaded into FLS MHC class II and presented to antigen-specific T cells. HLA-DRB1*04:01 transgenic mice immunized with mouse FLS loaded with NETs develop antibodies specific to citrullinated forms of relevant autoantigens implicated in RA pathogenesis as well as cartilage damage. These results implicate FLS as notable mediators in RA pathogenesis, through the internalization and presentation of NET citrullinated peptides to the adaptive immune system, leading to pathogenic autoimmunity and cartilage damage.

INTRODUCTION

Rheumatoid arthritis (RA) is the second most prevalent autoimmune condition, affecting 1% of the world population. It is a chronic, systemic inflammatory disease that affects the peripheral synovial joints and is associated to high morbidity and enhanced mortality. A significant proportion of RA patients exhibit RA-related autoantibodies, which include rheumatoid factor and antibodies to citrullinated protein antigens (ACPAs) (*1*). RA is characterized by a prolonged (3 to 5 years) subclinical phase where ACPAs are detected before the onset of clinically apparent disease (*2*–*5*). ACPA reactivity is directed against various citrullinated intracellular and extracellular antigens, including vimentin, histones, fibrinogen, and enolase. T cell responses to citrullinated peptides also develop in RA. Reactivity to citrullinated antigens correlates with the presence of the HLA-DRB1*04:01 shared epitope, which includes HLA-DRB1*04:01, HLA-DRB1*04:04, and HLA-DRB1*01:01, haplotypes associated with risk of developing RA (*6*, *7*). Citrullination of specific anchor residues enhances the ability of peptides to bind and be presented by the major histocompatibility complex class II (MHC II)–shared epitope alleles, allowing the activation and expansion of citrulline-specific $CD4^+$ T cells, and the subsequent promotion of ACPA generation (*8*–*12*).

In early stages of RA, neutrophils are abundant in both synovial tissue and fluid, supporting an important role for these cells in the initial events contributing to the pathogenesis of this disease (*13*). Recent work from our group and others indicates that RA synovial and peripheral blood neutrophils display an enhanced capacity to form neutrophil extracellular traps (NETs) (*14*, *15*). During NET formation, there is intracellular activation of peptidylarginine deiminase-4 (PAD4), a myeloid-specific PAD involved in citrullination, and neutrophils extrude a meshwork of nuclear material coupled to cytoplasmic and granular proteins. Because of PAD activation, proteins externalized in NETs become citrullinated, and several of them have been characterized as important RA autoantigens (*14*). Hence, NET formation may represent an important process leading to the citrullination of autoantigens that, in a genetically predisposed host, could promote activation of innate and adaptive immune responses and contribute to RA development.

One important cellular participant in RA is the fibroblast-like synoviocyte (FLS). These cells are major effectors in cartilage damage and participate in synovial inflammation in the rheumatoid joint. FLS express a variety of Toll-like receptors (TLRs) and have the capacity to act as antigen-presenting cells (APCs) in the synovium (*16*–*19*). Recent evidence demonstrates that FLS are activated by NETs, leading to up-regulation of inflammatory cytokine and adhesion molecule synthesis (*14*). However, the mechanisms by which NETs activate FLS remain to be fully characterized. We hypothesized that specific citrullinated autoantigens contained in NETs can be taken up by FLS and presented to T cells in an MHC II–dependent manner, leading to antigen-specific enhanced T and B cell responses relevant to disease pathogenesis.

RESULTS

ACPAs induce NETosis and recognize multiple citrullinated autoantigens exposed in NETs

Putative arthritogenic peptides such as histones H3 and H4 and vimentin have been reported to be citrullinated in NETs (*14*, *20*, *21*). Because

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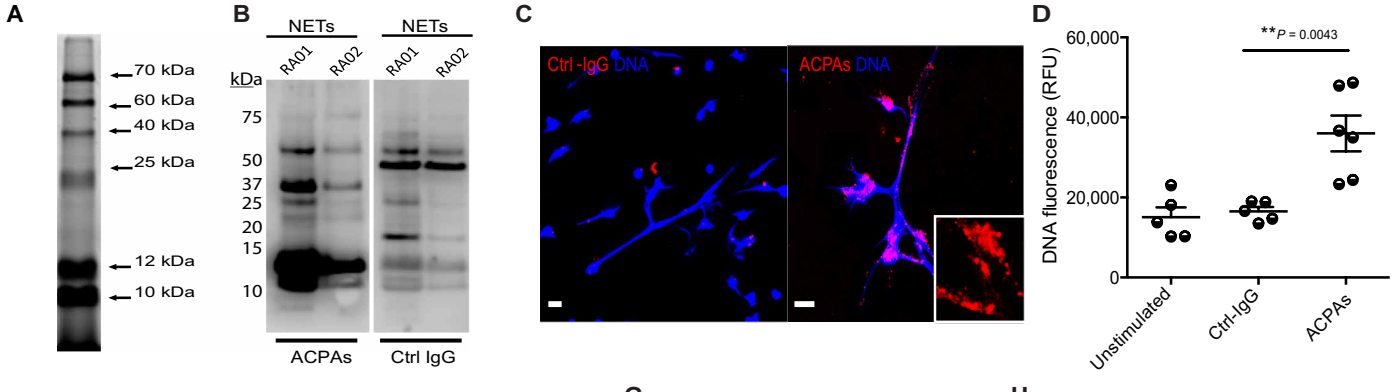
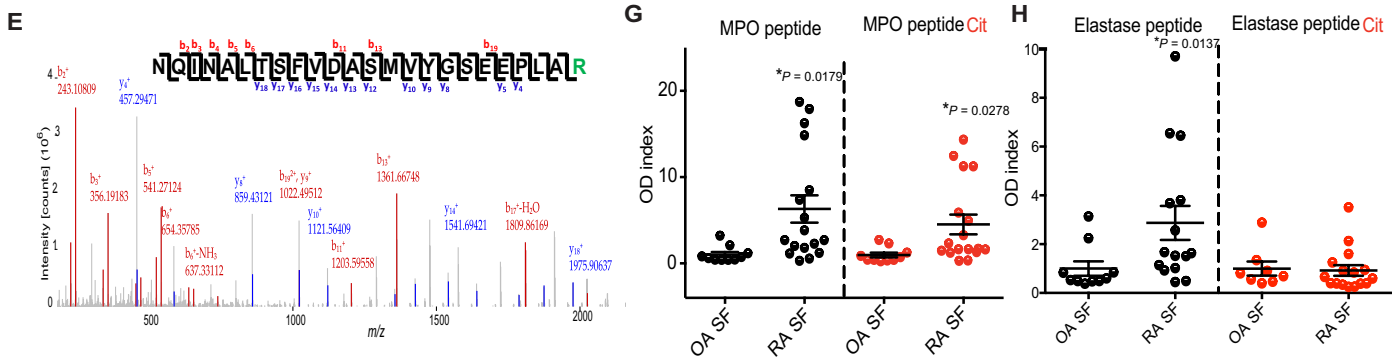
*Corresponding author. Email: mariana.kaplan@nih.gov

citrullination and response to citrullinated antigens are considered key in RA pathogenesis, we investigated whether other peptides are citrullinated in NETs and could function as autoantigens in this disease. Using a rhodamine phenylglyoxal (Rh-PG)–based probe to quantify citrullination, multiple citrullinated proteins were detected in NETs that were induced in control neutrophils by rheumatoid factor stimulation, a known inducer of NETosis (*14*) (Fig. 1A). Western blot and immunofluorescence analyses demonstrated that ACPAs isolated from RA patients differentially recognized multiple citrullinated proteins in spontaneously generated RA-NETs when compared with control immunoglobulin Gs (IgGs), suggesting that RA-specific autoantigens are contained within these structures (Fig. 1, B and C, and fig. S1). In addition, purified RA ACPAs enhanced NETosis in control neutrophils when compared with control IgG (Fig. 1D). These results confirm the hypothesis that RA-NETs or NETs induced by RA-relevant stimuli externalize multiple citrullinated autoantigens; in turn, RA autoantibodies enhance NET formation.

To identify in more detail the proteins that are citrullinated in NETs, we performed proteomic analysis in NETs similarly induced





F

NET protein	Citrullinated peptide
Azurocidin 1 (AZU1)	QFPFLASIQNQGCit QTFSISSMSENGYDPQQNLNDLMLLQLDCit
Catalase	GPLLVQDVVFTDEMAHFDCit
Histone H2A type 2-C	VGAGAPVYMAAVLEYLTAEILELAGNAACit
Histone H2B	AMGIMNSFVNDIFECit
Myeloperoxidase (MPO)	NQINALTSFVDASMVYGSEEPLACit SLMFMQWGQLLDHDLDFTPEPAACit IANVFTNAFCit
Neutrophil elastase (ELA)	QAGVCFGDSGSPLVCNGLIHGIASFVCit CitLGNGVQCLAMGWGLLGR
Profilaggrin	TENTCitLGDNR
Protein S100-A12	LEEHLEGIVNIFHQYSVCit
Protein S100-A9	VIEHIMEDLDTNADKQLSFEEFIMLMACit

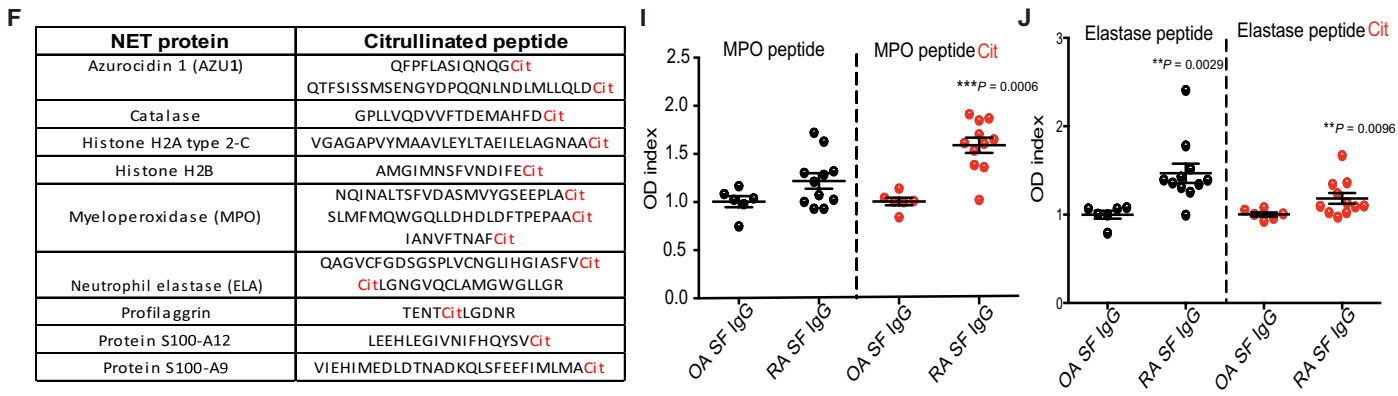


Fig. 1. ACPAs recognize multiple citrullinated peptides in NETs and induce NETosis. (**A**) Rh-PG probe against citrulline was used to detect specific citrullinated proteins in purified NETs generated by stimulating control neutrophils with IgM rheumatoid factor. (**B**) ACPAs differentially recognize citrullinated autoantigens in NETs when compared with control IgG. Spontaneously generated NETs from peripheral blood neutrophils from two RA patients (RA01 and RA02) were isolated and resolved in SDS-PAGE. Western blot was performed using ACPAs or control IgG. (**C**) ACPAs bind to NETs and (**D**) enhance NETosis. Red, control IgG (ctrl-IgG) or ACPAs; blue, Hoechst stain. (**E**) Representative histogram of citrullinated peptides detected in NETs using PEAKS' software (Thermo-Fisher Scientific). (**F**) Mass spectrometry analysis demonstrates multiple citrullinated peptides in NETs. (**G** and **H**) ELISA analysis of synovial fluid from OA ($n = 10$) and RA ($n = 17$) patients to detect autoantibodies recognizing citrullinated or noncitrullinated forms of MPO and neutrophil elastase, respectively. (**I** and **J**) ELISA analysis of IgGs isolated from synovial fluid (SF) of OA ($n = 6$) and RA ($n = 11$) patients showing recognition of native or cit-MPO or elastase. (A) to (C) are representative of three independent experiments. Scale bars, 10 μm. Results are the means ± SEM of $n = 5$ to 6. For statistical analyses, Mann-Whitney *U* test was used. *m/z*, mass/charge ratio; OD, optical density.

in peripheral blood control neutrophils by rheumatoid factor stimulation [control IgM does not induce NETs (*14*)]. This analysis detected several citrullinated proteins in these structures (Fig. 1, E and F, and table S1), including azurocidin, catalase, histone H2B, myeloperoxidase (MPO), neutrophil elastase, profilaggrin, S100-A12, and S100-A9. Because MPO and neutrophil elastase play important roles in NET formation (*22*) and are abundant and citrullinated in NETs, we investigated whether RA patients develop autoantibodies against citrullinated forms of these proteins. We selected two epitopes, IANVFTNAF**R** (citrullinated in MPO) and RLGNGVQCLAMGWGLLG**R** (citrullinated in neutrophil elastase), and generated synthetic peptides with or without citrullination sites. We identified the presence of autoantibodies against the citrullinated MPO peptide (cit-MPO) and against the native-form elastase peptide in RA synovial fluid but not in the synovial fluid from patients with osteoarthritis (OA) (Fig. 1, G and H). Confirming these findings, IgGs purified from RA synovial fluid recognized cit-MPO and cit-elastase (Fig. 1, I and J). These observations confirm and expand the repertoire of citrullinated molecules present in NETs to which RA patients develop autoantibody responses. These results also support previous observations that, in RA, antibody responses to both native and citrullinated version of various proteins are detected (*23*).

Internalization of NET components by FLS promotes their inflammatory phenotype

We previously showed that both RA and OA FLS exposed to NETs become proinflammatory and synthesize significantly increased levels of interleukin-6 (IL-6) (*14*). We then assessed the putative mechanisms involved in NET-induced FLS activation. OA and RA FLS and control dermal fibroblasts were incubated in the presence or absence of spontaneously formed peripheral blood RA-NETs for 2 hours. By confocal microscopy, we demonstrated that NETs are internalized by both OA and RA FLS, whereas skin fibroblasts showed minimal internalization (Fig. 2, A and B). To determine whether the activation status of the cell has an influence in NETs' internalization, we also incubated skin fibroblasts harvested from psoriasis patients with NETs from peripheral blood RA neutrophils. Psoriatic skin fibroblasts were also capable of internalizing NETs, suggesting that the activation status of the fibroblast has an impact on its ability to internalize NETs (fig. S2). Internalized NET components colocalized with the early endosome antigen-1 (EEA1) (*24*), implicating the endocytic pathway in NET internalization by FLS (Fig. 2A). To characterize the intracellular trafficking of NETs, we preincubated FLS with chloroquine (CQ), a lysosomotropic agent that prevents endosomal acidification, or with cytochalasin D (cyto D), an inhibitor of actin polymerization. FLS internalized NETs when exposed to cyto D but not when cultured with CQ (Fig. 2, C and D). By fluorescence microscopy visualization, we demonstrated significant decreases in the number of FLS positive for intracellular MPO, and Western blot analysis showed that MPO is not internalized by FLS treated with CQ (Fig. 2, C and E). These observations indicate that NET internalization was impaired by antimalarials. Moreover, the levels of IL-6 synthesized by FLS significantly associate with the capacity to internalize NETs (Fig. 2F). These findings suggest that the internalization of NETs by FLS is independent of actin filaments and uses the endocytic pathway, most likely clathrin-coated vesicles. Furthermore, the release of proinflammatory cytokines by FLS triggered by NETs requires the internalization of these structures.

The RAGE-TLR9 signaling pathway mediates NET internalization by FLS

NETs contain DNA and nuclear and granule proteins (*25*). Given that unmethylated CpG sequences are recognized by TLR9, we tested whether this receptor was involved in the internalization of NETs by FLS. Western blot analysis demonstrated that OA and RA FLS preincubated with a TLR9 antagonist and then exposed to RA-NETs are impaired in their ability to internalize MPO, a molecule present in NETs (Fig. 3A). In addition, IL-6 and IL-8 synthesis by FLS was significantly reduced in the presence of a TLR9 antagonist, with the effect being more predominant on IL-8 expression (Fig. 3, B and C). The expression levels of TLR9 were significantly higher in OA and RA FLS than in control skin fibroblasts. Furthermore, TLR9 expression was significantly up-regulated when FLS were incubated with NETs, suggesting a positive regulatory feedback loop (Fig. 3D). Given the role of TLR9 in the internalization of NETs and the fact that this molecule is involved in type I interferon (IFN) synthesis in certain cell types, we quantified type I IFN genes in FLS after incubation with NETs for 24 hours. IFN-α was significantly up-regulated after NETs incubation (fig. S3). Because TLR9 resides in endosomes, we hypothesized that a receptor present on the plasma membrane of FLS would mediate internalization of NETs. The receptor for advanced glycation end products (RAGE) was previously reported to recognize HMGB1 (a molecule present in NETs) (*26*, *27*), to promote DNA uptake into endosomes, and to lower the immune recognition threshold for TLR9 activation (*28*). FLS were incubated with 2 to 4 μM RAGE peptide inhibitor for 30 min, followed by exposure to NETs for 1 hour. Coimmunoprecipitation analysis demonstrated that RAGE interacts with the active form of TLR9 (cleaved TLR9) to mediate NET internalization, and this interaction was abolished in the presence of a RAGE inhibitor (Fig. 3E and fig. S4). We also detected a constitutive interaction of RAGE with the inactive form of TLR9, but this interaction was independent of NETs (Fig. 3E). We hypothesized that RAGE and the inactive form of TLR9 interact in the plasma membrane of FLS and that the functional interaction with the active form of TLR9 occurs intracellularly. Immunofluorescence of nonpermeabilized cells demonstrated interaction of RAGE with TLR9 in the plasma membrane of FLS, and this interaction was not perturbed by the presence of a RAGE inhibitor (Fig. 3F). Intracellularly, however, RAGE and TLR9 interactions were blocked with a RAGE inhibitor (Fig. 3F). These results suggest that NETs are internalized by FLS via a RAGE-TLR9 axis and that the proinflammatory profile induced in FLS is dependent on NET internalization.

NET internalization induces MHC II up-regulation in FLS in an IL-17B–dependent manner

FLS have the ability to acquire APC capabilities in the inflamed synovium (*18*), including MHC II up-regulation. We tested the hypothesis that the uptake of NETs by FLS would lead to presentation of NET citrullinated peptides to the adaptive immune system in an MHC II–dependent manner. We observed that, upon NET internalization, MHC II was up-regulated in FLS (Fig. 4A and fig. S5) when compared with cells not exposed to NETs or isotype control. Flow cytometry analysis confirmed up-regulation of MHC II intracellularly and in the plasma membrane of FLS incubated with NETs (Fig. 4B). This suggests that a molecule present in NETs may promote up-regulation of FLS MHC II. IFN-γ is a known inducer of MHC II up-regulation in APCs, including FLS (*19*). However, NETs showed no detectable levels of this cytokine by Western blot and enzyme-linked immunosorbent

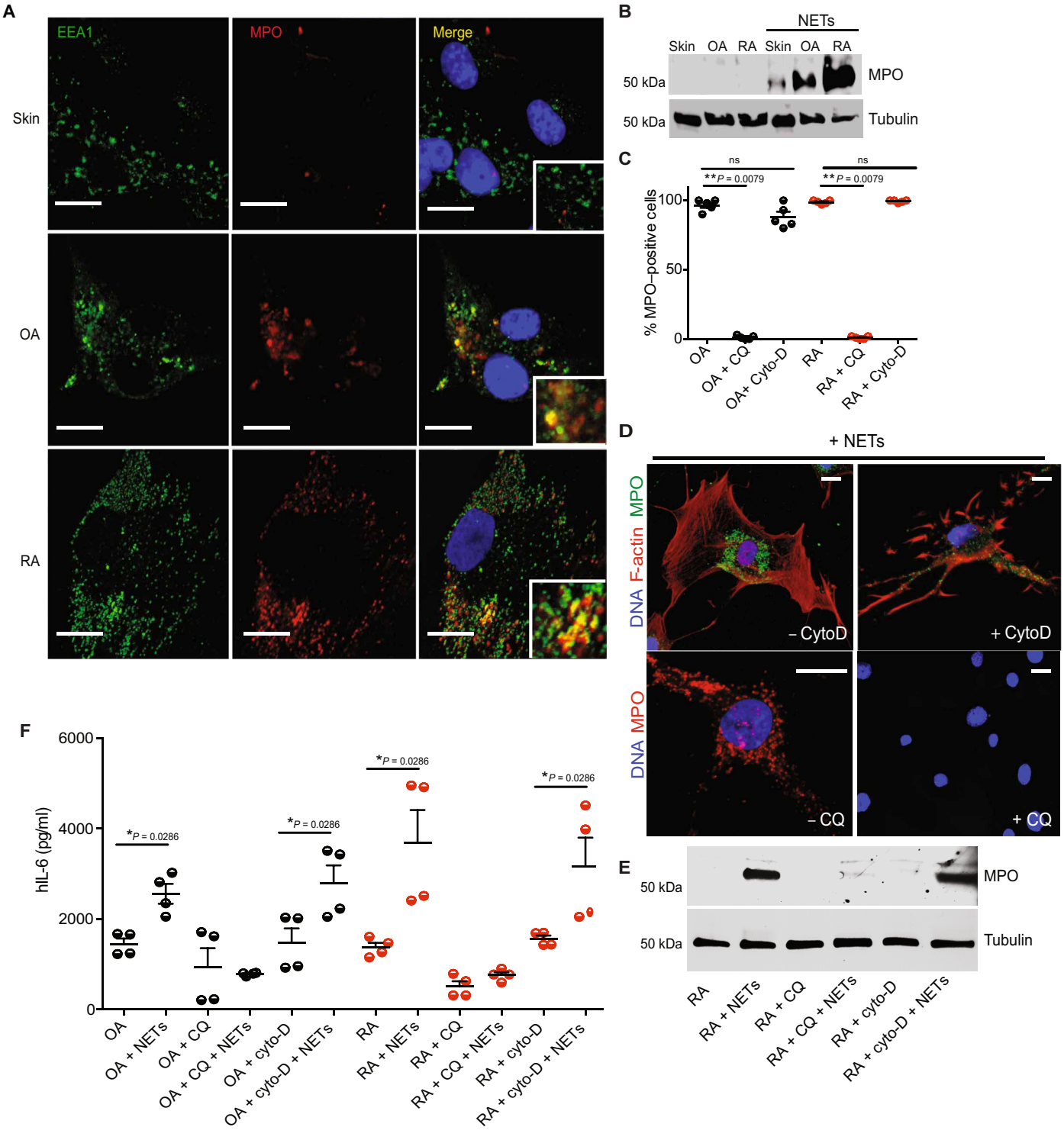


Fig. 2. NETs are internalized by FLS into EEA1-positive compartments. RA-NETs were incubated with OA or RA FLS or skin fibroblasts for 2 hours. (**A**) Internalized NETs colocalize with EEA1 compartments in FLS. Red, MPO; green, EEA1; blue, DNA. Results are representative of three independent experiments performed with a confocal microscope, Scale bars, 10 μm. (**B**) Western blot analysis of fibroblasts incubated in the presence or absence of RA-NETs shows that MPO bound to NETs is internalized by OA and RA FLS but minimally by healthy control skin fibroblasts. Results are representative of three independent experiments. (**C**) Percentage of MPO-positive cells (FLS that internalized NETs) decreased after CQ but not cyto D exposure. (**D**) Representative confocal images after treatment with cyto D and CQ. Red, MPO or F-actin; green, MPO; blue, DNA. Scale bars, 10 μm. (**E**) Western blot analysis confirms that MPO internalization is impaired in FLS preincubated with CQ but not in FLS preincubated with cyto D. (**F**) IL-6 release by FLS is dependent on NET internalization. Results are the means ± SEM of $n = 4$. For statistical analyses, Mann-Whitney U test was used. ns, not significant; hIL, human IL.

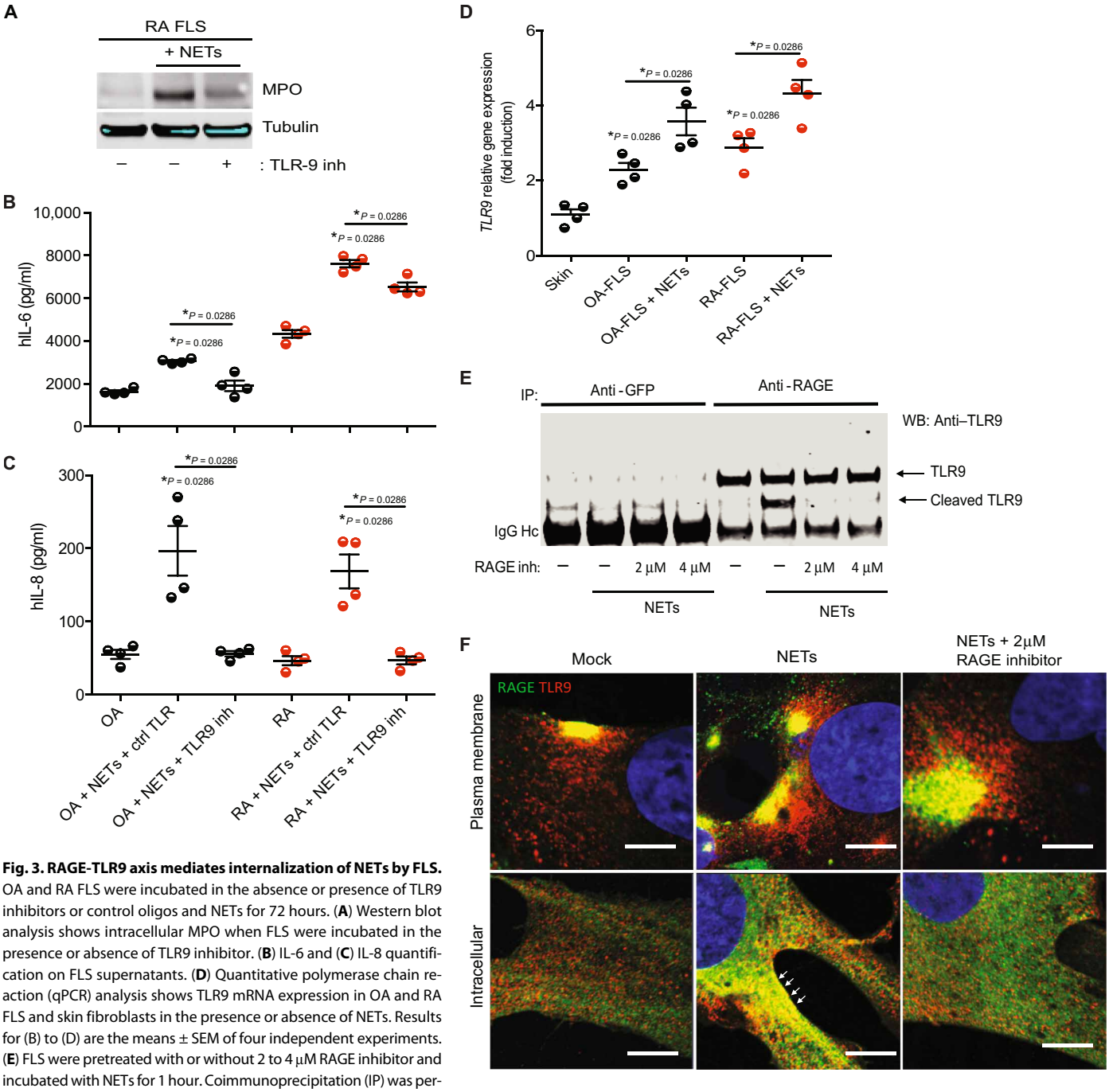


Fig. 3. RAGE-TLR9 axis mediates internalization of NETs by FLS. OA and RA FLS were incubated in the absence or presence of TLR9 inhibitors or control oligos and NETs for 72 hours. (**A**) Western blot analysis shows intracellular MPO when FLS were incubated in the presence or absence of TLR9 inhibitor. (**B**) IL-6 and (**C**) IL-8 quantification on FLS supernatants. (**D**) Quantitative polymerase chain reaction (qPCR) analysis shows TLR9 mRNA expression in OA and RA FLS and skin fibroblasts in the presence or absence of NETs. Results for (B) to (D) are the means ± SEM of four independent experiments. (**E**) FLS were pretreated with or without 2 to 4 μM RAGE inhibitor and incubated with NETs for 1 hour. Coimmunoprecipitation (IP) was performed against RAGE, and TLR9 was detected by Western blot. Anti–green fluorescent protein (GFP) was used as negative control. (**F**) Plasma membrane (top) and intracellular (bottom) detection of RAGE and TLR9 were performed on FLS pretreated with or without RAGE inhibitor. Red, TLR9; green, RAGE; blue, DNA of three independent experiments; white arrows highlight areas of colocalization of RAGE and TLR9. Scale bars, 5 μm. Mann-Whitney *U* test was used.

assay (ELISA) (fig. S6, A and B). IFN-γ was also quantified by ELISA in supernatants of FLS incubated for up to 12 days with RA-NETs and was found to be undetectable (fig. S6C). This ruled out the possibility that FLS were induced to produce IFN-γ in response to NETs. Members of the IL-17 family play important roles in inflammatory responses in RA (*29*). Although most studies have focused on IL-17A, IL-17B synthesized by neutrophils was recently described as the most abundant IL-17 isoform in the RA synovium (*30*). Immunofluorescence and Western blot analyses demonstrated that RA-NETs were decorated with significantly increased amounts of IL-17B but not IL-17A (Fig. 4, C and D, and fig. S7). Neutralization of IL-17B in spontaneously generated RA-NETs induced significant decreases in expression of HLA-DRA and HLA-DRB MHC II molecules (Fig. 4E). Furthermore, incubation of RA FLS with IL-17B or IL-17A led to up-regulation of HLA-DRA and HLA-DRB mRNA (Fig. 4F). Mature MHC II compartments were detected in FLS incubated with recombinant IL-17B (Fig. 4F). Inhibition of

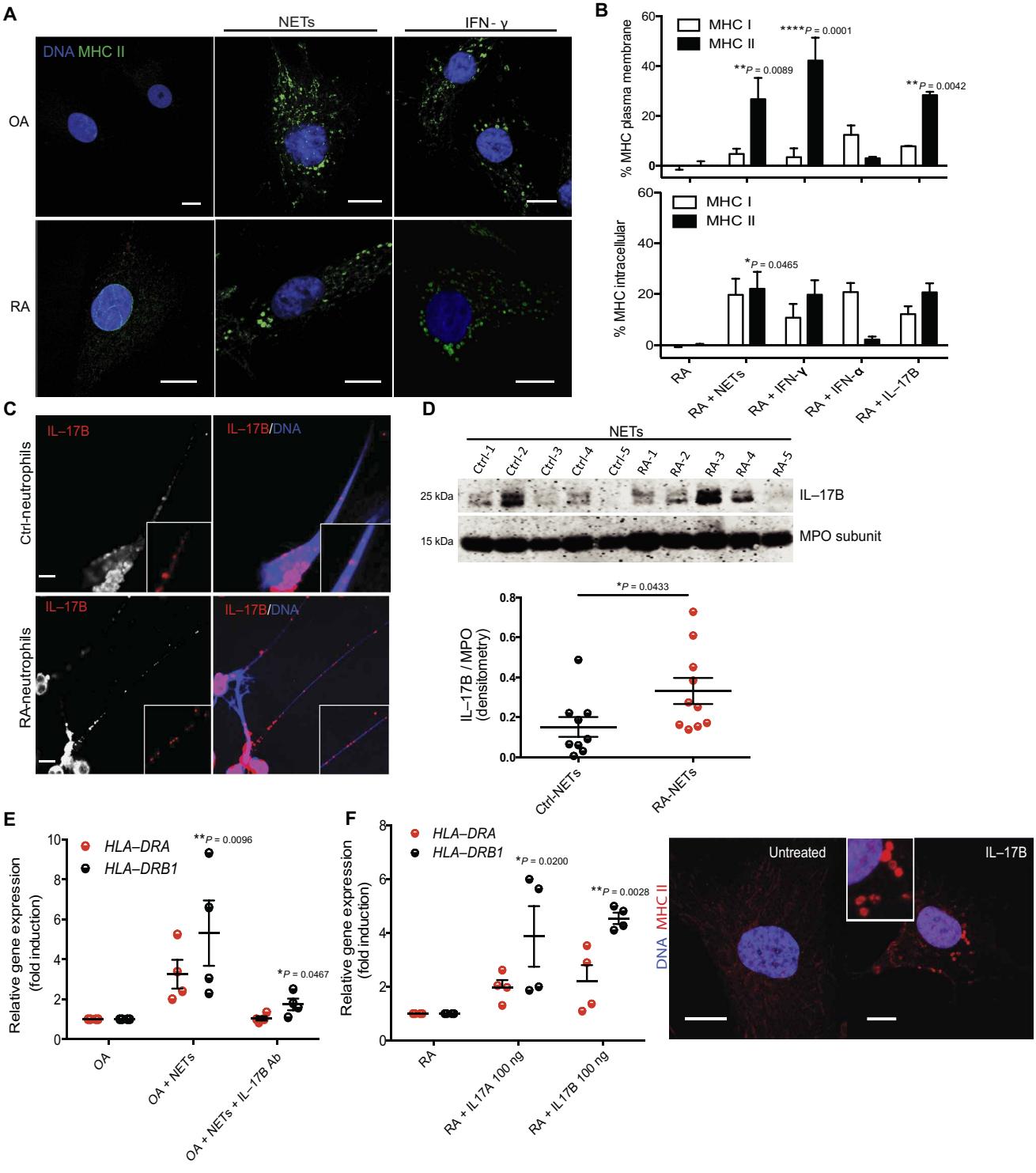


Fig. 4. IL-17B present in NETs up-regulates MHC II in OA and RA FLS. OA and RA FLS were incubated in the presence or absence of spontaneously generated RA-NETs or IFN-γ (1000 U/ml). (**A**) Detection of MHC II in FLS by immunofluorescence. Green, MHC II; blue, DNA. Results are representative of three independent experiments. Scale bars, 10 μm. (**B**) Plasma membrane and intracellular MHC I and II were quantified by flow cytometry in RA FLS treated with NETs, IFN-γ, or IFN-α for 5 days. (**C**) IL-17B (red) is externalized in control (Ctrl) NETs generated with LPS (1 μg/ml) and in spontaneously generated RA-NETs. (**D**) IL-17B is detected in isolated NETs by Western blot analysis. Each lane depicts independent NET isolation per group (Ctrl and RA). Mann-Whitney *U* test was used. Results are the means ± SEM of 10 independent experiments,*$P < 0.05$. (**E**) OA FLS were incubated with RA-NETs in the presence or absence of IL-17B (1 μg/ml) neutralizing antibodies for 48 hours. Quantification of HLA-DRA and HLA-DRB mRNA was performed by real-time PCR. Bars are the means ± SEM of four independent experiments. (**F**) RA FLS were incubated with 100 ng of human recombinant IL-17A or IL-17B for 72 hours. qPCR and immunofluorescence analyses assessed MHC II mRNA expression and protein localization, respectively. ANOVA with Bonferroni's test was performed for (B), (E), and (F). Results are the means ± SEM of four independent experiments.

IL-17R using a neutralizing antibody did not impair NET internalization (fig. S8), suggesting that RAGE-TLR9 is the main pathway of NET internalization and that internalization is independent of IL-17R. Overall, these results indicate that IL-17B externalized in NETs can induce up-regulation of MHC II in FLS.

Arthritogenic NET peptides internalized by FLS colocalize with MHC II and are presented to antigen-specific CD4$^+$ T cells

Because FLS internalize molecules present in NETs and up-regulate MHC II after this internalization, we tested whether these NET peptides were loaded onto the FLS MHC II compartment. After a 5-day incubation of FLS in the presence or absence of NETs, immunofluorescence confocal microscopy analysis demonstrated that MPO colocalizes with MHC II in both OA and RA FLS, suggesting that peptides derived from NETs are loaded onto MHC II compartments intracellularly (Fig. 5A). In addition, colocalization of NET peptides and MHC II was detected in the plasma membrane of nonpermeabilized FLS (Fig. 5B and fig. S9), suggesting that the complex traffics to the plasma membrane to expose the peptide.

In addition to ACPAs, autoantibodies targeting native proteins have been described in RA. In particular, a subset of RA patients develops autoantibodies to human cartilage–glycoprotein 39 (HC-gp39) (*31*). To investigate whether noncitrullinated arthritogenic peptides are also contained within the NETs, we performed Western blot analysis, which revealed that HC-gp39 is synthesized by neutrophils and externalized in NETs (fig. S6A). To determine the functionality of the peptide–MHC II complex with regard to the ability to activate antigen-specific T cells, we incubated FLS loaded with NETs with murine T cell hybridomas specific for immunodominant portions of HC-gp39 263 to 275 (RSFTLASSETGVG) and quantified IL-2 synthesis by the T cells. HC-gp39 T cell hybridomas synthesized significantly higher levels of IL-2 when incubated with FLS loaded with NETs than when exposed to FLS alone (Fig. 5C).

Because NETs contain citrullinated vimentin (cit-vimentin), among other citrullinated peptides, and to corroborate that FLS can present citrullinated peptides to antigen-specific T cells, DRB1*04:04 RA FLS loaded with NETs were incubated with DRB1*04:04 cit-vimentin–specific CD4$^+$ T cells isolated and expanded from RA patients (fig. S10). After 5 days of incubation, cit-vimentin–specific CD4$^+$ T cells displayed significant increases in secretion of IFN-γ, TNF-α (tumor necrosis factor–α), IL-10, and IL-1ra (Fig. 5D) when compared with T cells exposed to FLS alone. The release of these cytokines was reduced when cells were incubated with neutralizing antibodies against MHC II or against CD28 (Fig. 5D), indicating that costimulation is required. To test specificity of FLS toward citrullinated peptides, we cocultured CD4$^+$ T cells against citrullinated Aggrecan 225 with FLS. FLS were unable to activate Aggrecan 225 CD4$^+$ T cells (fig. S11), suggesting that specific citrullinated peptides are presented by FLS. Results indicate that FLS that internalize NET components can present arthritogenic peptides to antigen-specific T cells and activate adaptive immunity.

Humanized HLA-DRB1*04:01 transgenic mice develop ACPAs in response to immunization with FLS loaded with NETs

To confirm that NETs are an important source of citrullinated peptides and that FLS that internalize NETs can induce adaptive immune responses characteristic of RA in vivo, we used the humanized HLA-DRB1*04:01 transgenic mouse model (*32*). These mice are DRB1*04:01.AEo; therefore, they lack endogenous class II molecules (both I-A and I-E chains). In this model, transgenic expression of this DR1 allele confers susceptibility to inflammatory arthritis induced by immunization with various stimuli including citrullinated fibrinogen (cit-fibrinogen) (*33*). Synovial tissue was harvested from healthy HLA-DRB1*04:01 mice to isolate and expand FLS in culture (Fig. 6A). Mouse FLS were incubated for 3 days in the presence or absence of NETs isolated from peripheral blood RA neutrophils. Internalization of NETs by mouse FLS was confirmed by intracellular immunofluorescence against human MPO (Fig. 6A). A total of 100,000 FLS with or without internalized NETs were injected in one knee of each HLA-DRB1*04:01 mouse (Fig. 6A). After seven rounds of injections performed every other week, serum ACPA levels were quantified using a commercial assay [anti–cyclic citrullinated peptide (CCP), Wuhan Huamei Biotech Co.]. High titers of anti-CCP antibodies were detected in the sera of mice that received intra-articular injections of FLS loaded with NETs when compared with animals that received FLS alone (Fig. 6B). Dot blot analysis demonstrated that sera from animals immunized with FLS loaded with NETs developed antibodies recognizing cit–histone H3 (cit-H3), cit–histone H4 (cit-H4), and cit-MPO as well as antibodies recognizing NET proteins (Fig. 6C). In addition, serum samples from mice immunized with FLS loaded with NETs differentially recognized proteins contained in RA-NETs, when compared with serum from animals that were immunized with FLS alone (Fig. 6D). Splenocytes from mice injected with FLS loaded with NETs displayed a significant response to citrullinated peptides (a cocktail of cit-H3, cit-H4, cit-MPO, and cit-vimentin) when compared with a cocktail of native peptides, as assessed by IL-2 synthesis (Fig. 6E). ACPA generation in these animals was dependent on CD4$^+$ T cells because CD4$^+$ T cell–depleted animals (using an antibody approach) injected with FLS loaded with NETs displayed significantly decreased levels of ACPAs (Fig. 6F and fig. S12).

Given that we detected systemic ACPAs in animals immunized with FLS loaded with NETs, we hypothesized that FLS can migrate to the spleen and/or lymph nodes to interact with T cells. Labeled FLS loaded with NETs were detected in spleen and lymph nodes of animals immunized with FLS when compared with naïve mice (Fig. 6G). These results indicate that induction of antigen-specific T cell responses and ACPA synthesis by FLS loaded with NETs likely occurs both intra-articularly and outside the joint.

An antigen array was used to identify the repertoire of autoantibodies generated after immunization. Animals immunized with FLS loaded with NETs displayed elevated levels of antibodies recognizing citrullinated histones cit-H2A, cit-H2B, and cit-H3 (Fig. 6H). Supporting our observation that NETs activate HC-gp39 T cell hybridomas, mice immunized with FLS-NETs also displayed elevated levels of antibodies against HC-gp39 (HC-gp39 154 to 169, HC-gp39 258 to 279, HC-gp39 322 to 337, and HC-gp39 344 to 363 epitopes) (Figs. 5C and 6H). Antibodies recognizing α-enolase (α-enolase 414 to 433), cit-fibrinogen, and cit-vimentin (vim 58 to 77 cit3) were also present in animals immunized with FLS-NET, when compared with those immunized with FLS alone (Fig. 6H and fig. S13). Other antibodies identified included those recognizing cartilage components, such as biglycan (epitopes 238 to 257, 247 to 266, and 247 to 266 cit3), suggesting that NETs may also modify FLS behavior, potentially promoting cartilage damage. Although animals immunized with FLS alone or FLS loaded with NETs did not develop overt arthritis, safranin O staining of murine synovial cartilage showed disruption in cartilage integrity, significantly increased cartilage loss and cartilage irregularity, and higher prevalence of pannus in those animals immunized with FLS-NETs

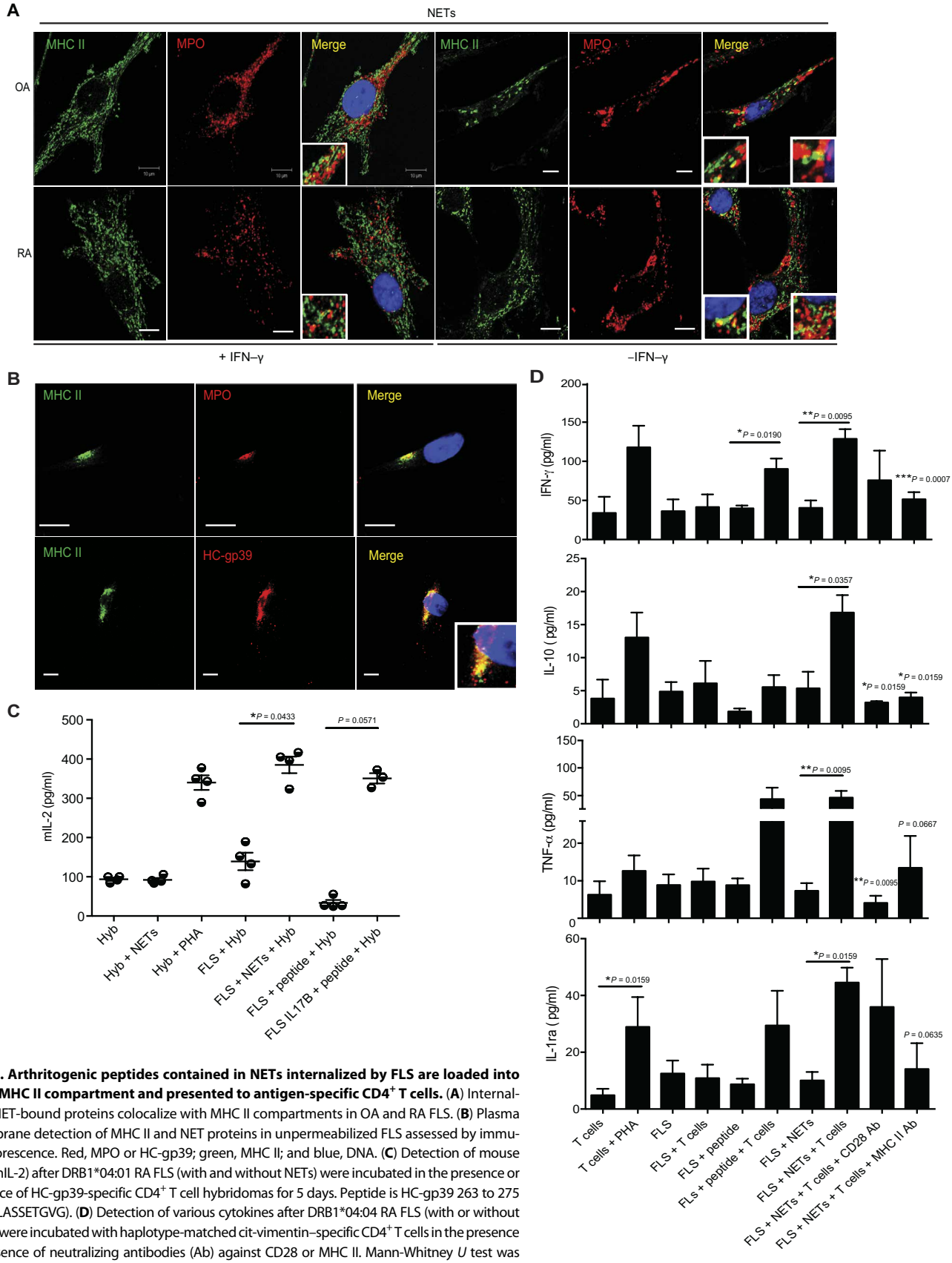


Fig. 5. Arthritogenic peptides contained in NETs internalized by FLS are loaded into their MHC II compartment and presented to antigen-specific CD4$^+$ T cells. (**A**) Internalized NET-bound proteins colocalize with MHC II compartments in OA and RA FLS. (**B**) Plasma membrane detection of MHC II and NET proteins in unpermeabilized FLS assessed by immunofluorescence. Red, MPO or HC-gp39; green, MHC II; and blue, DNA. (**C**) Detection of mouse IL-2 (mIL-2) after DRB1*04:01 RA FLS (with and without NETs) were incubated in the presence or absence of HC-gp39-specific CD4$^+$ T cell hybridomas for 5 days. Peptide is HC-gp39 263 to 275 (RSFTLASSETGVG). (**D**) Detection of various cytokines after DRB1*04:04 RA FLS (with or without NETs) were incubated with haplotype-matched cit-vimentin–specific CD4$^+$ T cells in the presence or absence of neutralizing antibodies (Ab) against CD28 or MHC II. Mann-Whitney *U* test was used. Results are the means ± SEM of four to six independent experiments. Scale bars, 10 μm.

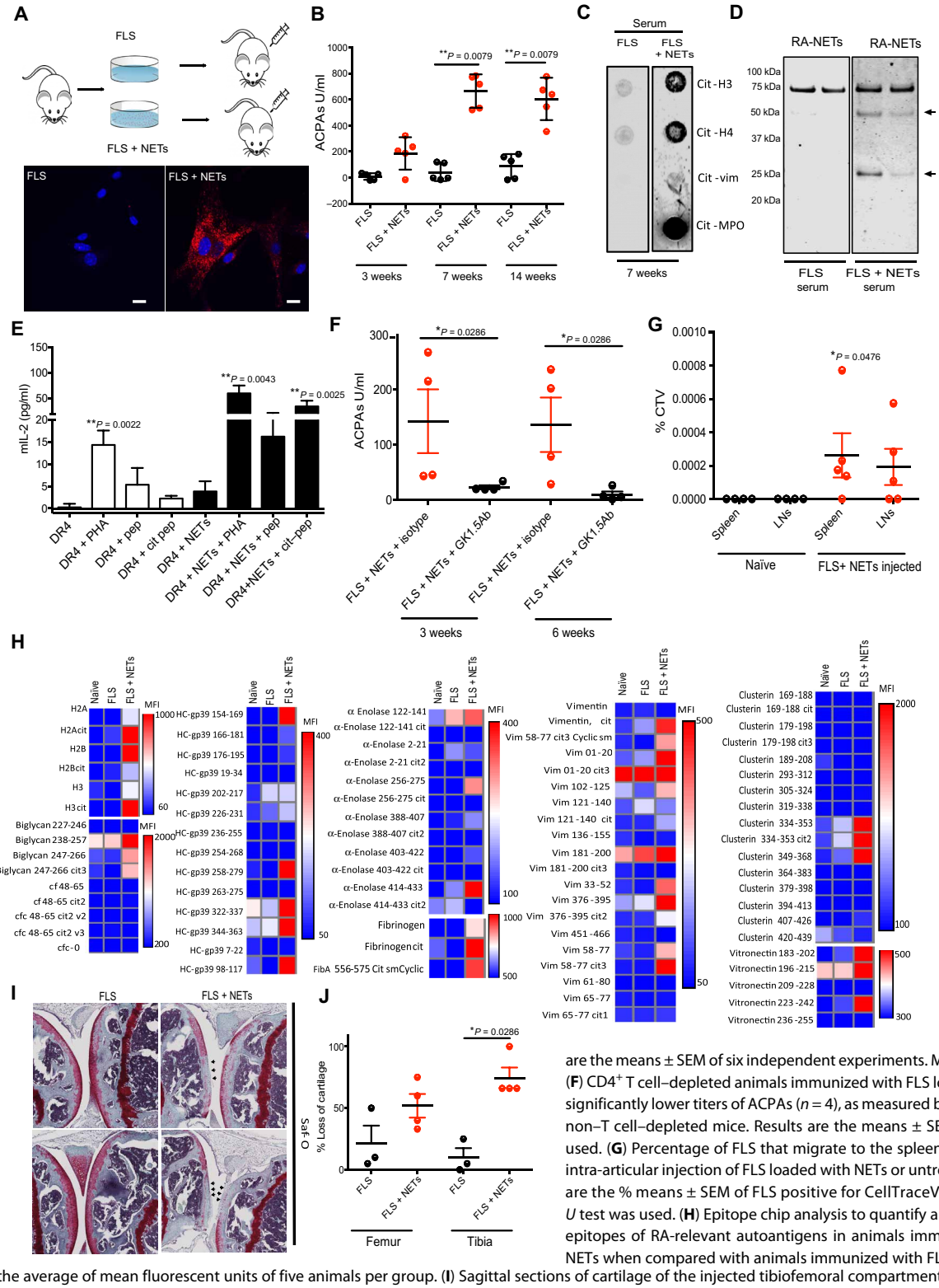


Fig. 6. DRB1*04:01 humanized mice that receive intra-articular injections of mouse FLS loaded with RA-NETs develop ACPAs and cartilage damage. DRB1*04:01 FLS were isolated and incubated with human RA-NETs for 3 days before intra-articular injection. (**A**) Internalization of NETs by FLS was assessed by immunofluorescence using an antibody against MPO (red). (**B**) Serum ACPA levels at various time points in animals immunized with FLS alone or FLS loaded with NETs ($n = 5$ per group). Results are the means ± SEM. Mann-Whitney *U* test was used. (**C**) Sera from three mice immunized for 7 weeks with FLS or with FLS loaded with NETs were analyzed by dot blot against citrullinated proteins. (**D**) Western blot analysis to detect serum autoantibodies recognizing human NET proteins (arrows) in DRB1*04:01 animals that received FLS alone or FLS-NETs; each lane depicts two independent NET isolations. (**E**) IL-2 synthesis by DRB1*04:01 mouse splenocytes incubated with a cocktail of native (pep; H3, H4, MPO, and vimentin) or citrullinated peptides (cit-pep; a cocktail of cit-H3, cit-H4, cit-MPO, and cit-vimentin), when comparing animals immunized with FLS loaded with NETs (DR4 + NETs) with animals immunized with FLS alone (DR4). PHA is used as positive control. Results are the means ± SEM of six independent experiments. Mann-Whitney *U* test was used. (**F**) $CD4^+$ T cell–depleted animals immunized with FLS loaded with NETs demonstrate significantly lower titers of ACPAs ($n = 4$), as measured by ELISA, when compared with non–T cell–depleted mice. Results are the means ± SEM. Mann-Whitney *U* test was used. (**G**) Percentage of FLS that migrate to the spleen and lymph nodes (LNs) after intra-articular injection of FLS loaded with NETs or untreated mice ($n = 4$ to 5). Results are the % means ± SEM of FLS positive for CellTraceViolet (% CTV). Mann-Whitney *U* test was used. (**H**) Epitope chip analysis to quantify antibodies recognizing specific epitopes of RA-relevant autoantigens in animals immunized with FLS loaded with NETs when compared with animals immunized with FLS alone. Heat map represents the average of mean fluorescent units of five animals per group. (**I**) Sagittal sections of cartilage of the injected tibiofemoral compartment were stained with safranin O demonstrating impaired cartilage integrity (arrowheads) in animals immunized with FLS loaded with NETs when compared with animals immunized with FLS alone. (**J**) Percentage of cartilage loss of the femurs and tibias of animals immunized with FLS or FLS loaded with NETs. Results are the means ± SEM of two to three independent experiments. Mann-Whitney *U* test was used.

when compared with those immunized with FLS alone (Fig. 6, I and J). Overall, these results suggest that, in the presence of the shared epitope, FLS that have internalized citrullinated peptides present in NETs can induce adaptive immunity in vivo, effectively activating citrullinated antigen–specific T cells, which could, in turn, promote ACPA generation, as well as the promotion of synovial cartilage degradation.

DISCUSSION

Increasing experimental evidence suggests that FLS in the RA synovium can act as immune sentinels. Their interaction with various leukocytes may promote intra-articular and peripheral inflammation and immune responses characteristic of this disease (*34*, *35*). However, how the presentation of citrullinated autoantigens (key targets of the immune system in RA) to the adaptive immune system occurs in the synovium and the role of FLS in this process have been unclear. We now describe how neutrophil-FLS interactions in the RA synovium may play crucial roles in the promotion of joint damage and in the development of systemic dysregulated innate and adaptive immunity against citrullinated intracellular autoantigens. We found that NETs containing citrullinated and arthritogenic peptides are internalized by FLS through a RAGE-TLR9 endocytic pathway, leading to a proinflammatory phenotype in these cells. NET internalization promotes up-regulation of MHC II in the FLS, loading of NET peptides into the MHC II, trafficking to the FLS cell membrane, and presentation to antigen-specific T cells. This promotes T cell activation and modulation of B cell responses, leading to the generation of ACPAs and the propagation of inflammatory responses and cartilage damage.

A key histologic finding of the RA joint is a hyperplastic synovial lining and an invasive, inflammatory pannus across the surface of synovial joints. In addition to FLS, a distinct structural cell, the RA synovium contains macrophages, lymphocytes, and neutrophils and appears to function as a tertiary lymphoid structure (*36*). FLS display many proinflammatory properties that contribute to RA pathogenesis, including their ability to function as APCs through up-regulation of MHC II after exposure to inflammatory stimuli and their expression of costimulatory molecules that can provide second signals leading to T cell activation (*18*). During active phases of disease and in early disease, large numbers of activated neutrophils are found in the synovial fluid of RA patients as well as at the cartilage pannus interface, where they may interact with FLS (*37*, *38*). In addition, T cell–FLS interactions are readily observed in the inflamed synovium and promote T cell recruitment and T helper cell 1 (T_H1) and T_H17 differentiation (*39*).

We previously showed that the RA synovium, characterized by ACPA and rheumatoid factor generation and increased proinflammatory cytokines, is highly conducive to NET formation. In turn, NETs may provide the immune system with access to enhanced sources of citrullinated proteins and thereby may represent an early event preceding epitope spreading. We now add additional evidence on the role of NETs as a source of immunogenic peptides by identifying several specific citrullinated antigens present in these structures that are recognized by ACPAs. In turn, ACPAs induce NET production, potentially creating a vicious inflammatory cycle in the synovium and in the periphery. This loop may promote disease by allowing an expansion of the citrulline specificities in RA. Our observations could help explain the broad array of citrullinated antigens seen by the antibody and T cell repertoire of RA patients. Whether citrullination of these proteins involved in NET formation alters not only immunogenicity but also their physiologic function remains to be determined and was not explored in this manuscript.

NETs externalize substantial amounts of DNA bound to granule proteins, and this could be a mechanism by which FLS internalize NET components. RAGE promotes uptake of alarmin/DNA complexes into endosomes and lowers immune recognition threshold for TLR9 activation in other cell types (*28*). This pathway is operational in FLS and mediates NET internalization. This is also in accordance with recent observations that some NET proteins (e.g., LL37 and HMGB1) promote APC activation by facilitating antigen uptake, interaction with endosomal TLRs, and inflammatory cytokine release (*40*). Beyond nucleic acids, it is possible that other molecules present in NETs could bind to RAGE, but this was not explored in this study.

In the case of the RA synovium, we propose that NET internalization augments cytokine synthesis by FLS and leads to their up-regulation of MHC II, thereby enhancing APC capabilities. Specifically, we showed that IL-17B is externalized by NETs and promotes MHC II up-regulation in the FLS and that this phenomenon is IFN-γ–independent. Our results support and expand previous observations that IL-17B induces neutrophilia, is expressed by synovial neutrophils, and is the predominant IL-17 cytokine in the RA synovium (*41*, *42*), and that FLS express the IL-17RB receptor (*30*). Overall, our observations suggest that proteins present in NETs can significantly alter the FLS phenotype and endow these cells with APC capabilities.

Citrullinated peptides are preferentially recognized by the HLA-DRB1*04:01/04 alleles, leading to their presentation to autoreactive T cells, which then have the ability to promote ACPA generation, a feature of severe erosive RA (*10*, *43*, *44*). In this context, we found that HLA-DRB1*04:01/04–positive RA FLS that internalized NETs efficiently stimulated haplotype-matched antigen-specific T cells in vitro, and that HLA-DRB1*04:01 transgenic mice developed ACPAs and enhanced T cell responses and cartilage damage when immunized with syngeneic FLS loaded with RA-NETs. These findings support a crucial interplay between genetic susceptibility factors and environmental stimuli (e.g., microbes known to promote NETosis) in promoting and amplifying local and systemic inflammatory responses. Although the administration of NET-loaded FLS to these mice did not lead to overt arthritis, they displayed disruptions in cartilage integrity that suggest a pathogenic local effect in the joint. It is likely that additional inflammatory triggers may be needed to promote full-blown disease, and this is supported not only by other animal models of arthritis (*44*) but also by the observation that ACPA development and immune dysregulation precede overt RA by many years (*4*).

B cells isolated from RA synovial tissue produce antibodies to NET targets and selectively recognize NETs synthesized by RA neutrophils (*45*). It is then conceivable that the activation of antigen-specific T cells by FLS loaded with NETs promotes ACPA production by B cells present in the synovium and in the periphery, and this is supported by our observation that in vivo T cell depletion abrogates ACPA production induced by NET-loaded FLS transfer. Our results support the concept that T_Hs with T cell receptors specific for processed NET peptides (including citrullinated and noncitrullinated peptides derived from proarthritogenic proteins) are present in the RA synovium and respond to local APCs, such as FLS, loaded with citrullinated NET antigens. Our findings are also consistent with previous evidence that NET products can be taken up by professional APCs. Myeloid dendritic cells that internalize NET components induce autoimmunity when injected into naïve mice (*46*).

Neutrophils may play additional roles in the resolution of inflammation in RA (*47*). Although FLS that internalized NETs activated T cells and induced proinflammatory cytokine synthesis, they also induced higher synthesis of the anti-inflammatory IL-1 receptor antagonist, and it will be important to assess whether these interactions also mediate resolution of inflammatory responses in RA. Furthermore, it is important to emphasize that other mechanisms inducing citrullination likely play important roles in subsets of RA patients (*48*).

Our observations highlight a novel mechanism that promotes immune dysregulation and pathogenic autoimmunity in RA and further supports the rationale for testing NETosis inhibitors and strategies that disrupt specific cell-cell interactions in the synovial joint in future clinical trials in RA and, potentially, other chronic inflammatory conditions. Antimalarials have been widely used in RA for many years, although their exact mechanism of action to explain efficacy remains to be determined. Because the internalization of NETs is decreased in the presence of antimalarials, our observations may provide an additional mechanism of action for this group of drugs and suggest that further exploring strategies that limit the interactions of NETs with FLS and other target cells may have potential therapeutic benefit.

MATERIALS AND METHODS

Study design

The study investigated the interactions between RA FLS and NETs and their role in driving adaptive immunity in this disease. Observations using human cells in vitro were also corroborated in animal models that recapitulate the genetic predisposition found in humans. Mice sample size and the number of in vitro experiments using murine and human samples were chosen on the basis of previous publications, and no randomization was performed to select groups (*14*, *26*). The number of samples used per experiment is explained in each figure legend.

Patient recruitment, cell isolation, and culture and generation of T cell hybridomas and antigen-specific T cells

Patient selection

Patients recruited fulfilled the 1987 American College of Rheumatology criteria for RA or were diagnosed with OA based on clinical and radiographic features and confirmed by pathological findings at joint surgery (*49*). Healthy controls were recruited by advertisement. All individuals gave written informed consent and enrolled in a protocol approved by the National Institute of Arthritis and Musculoskeletal and Skin Diseases/National Institute of Diabetes and Digestive and Kidney Diseases (NIAMS/NIDDK) Institutional Review Board (IRB; 01-AR-0227), the University of Michigan IRB (HUM00043667 or HUM00045058), the Benaroya Research Institute IRB (07109-139), or the Karolinska Institute IRB (2003-138, 2010/935-31/3, and 2011/583-32). Peripheral blood was obtained by venipuncture, collected in EDTA-containing tubes, and fractionated via Ficoll Paque Plus (GE Healthcare) gradient. Neutrophils were isolated by dextran sedimentation and hypotonic salt solution, as previously described (*14*).

Isolation of FLS and dermal fibroblasts

Human OA and RA FLS were obtained, as previously described (*18*). In brief, FLS were obtained by collagenase (Worthington Biochemical) digestion of human synovial tissue obtained at arthroplasty or synovectomy from RA or OA joints. Cells were maintained in CMRL medium (Invitrogen Life Technologies) and used after passage 4 from primary cultures. Healthy control human dermal fibroblasts [a gift from E. Romm, National Institutes of Health (NIH)] and psoriasis human dermal fibroblasts (a gift from J. T. Elder, University of Michigan) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS; Invitrogen), 2 mM glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml) and grown in a humidified incubator with 5% CO_2 at 37°C.

Generation of HC-gp39 T cell hybridomas

The generation and characterization of murine MHC II–restricted T cell hybridomas specific for a 13–amino acid peptide of the arthritogenic HC-gp39 were previously described (*50*). These cells were cultured in RPMI 1640 medium (Invitrogen) with 0.6 mM sodium pyruvate, 1 mM Hepes, and 0.055 mM β-mercaptoethanol. All cell cultures were supplemented with 10% FBS, 2 mM glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml) and grown in a humidified incubator with 5% CO_2 at 37°C.

Cloning of citrulline-specific T cells

T cell clones specific for cit-vimentin restricted by HLA-DRB1*04:04 were generated by staining peripheral blood mononuclear cell (PBMC) samples directly ex vivo, as previously described (*51*). Briefly, tetramer-positive $CD4^+$ cells were sorted on a FACSAria II at single-cell purity. Clones were expanded in 96-well plates in the presence of 1.0×10^5 irradiated PBMCs and phytohemagglutinin (PHA) (2 μg/ml; Remel Inc.) and screened by restaining with tetramers. Antigen-specific T cell proliferation was assessed by stimulating 1.0×10^4 T cells in wells with 1×10^5 irradiated allogenic PBMCs in the presence of peptide (10 μg/ml). After 72 hours, cells were pulsed with H3-thymidine for an additional 24 hours and thymidine uptake measured by liquid scintillation counting. Figure S3 shows the specificity of the response to citrullinated versus native forms of vimentin, validation of immunogenicity, and the proliferative response of the clone. Aggrecan-specific T cell clones were used as negative control because aggrecan is not expressed in NETs.

Quantification of NET formation by plate assay

NET quantification was performed, as previously described (*14*). Sytox green is a nonpermeable DNA dye that will bind to DNA present in NETs generated during neutrophil culture. Briefly, neutrophils were resuspended in RPMI without phenol red containing 0.2 μM Sytox green (Invitrogen) for 2 to 3 hours. Neutrophils (2×10^5) were incubated in the presence or absence of control IgG or ACPAs in 96-well black plates for 1 hour at 37°C. Fluorescence was measured in a BioTek Synergy H1 Hybrid Reader. Results were reported as DNA relative fluorescence units.

Isolation and purification of ACPAs

Plasma samples were obtained from RA patients ($n = 38$) with high anti-CCP2 antibody levels (more than 3× the cutoff levels). IgGs were purified from plasma samples on Protein G columns followed by anti-CCP2 IgG affinity purification on CCP2 columns, as previously described (*52*). IgGs from OA and from ACPA-seropositive RA synovial fluids were purified using the Melon Gel IgG purification kit (Thermo Fisher) according to the manufacturer's protocol.

NET isolation

NETs were isolated, as previously described (*14*). Briefly, RA neutrophils were purified and seeded in 24-well tissue culture plates in RPMI without phenol or stimuli and incubated for 2 hours at 37°C. Supernatants were harvested, and NETs were digested with micrococcal nuclease (10 U/ml; Thermo Fisher) for 15 min at 37°C. Supernatants were

collected and centrifuged at 300*g* for 5 min at 4°C. NET supernatants were transferred to a fresh tube and stored at −80°C until used.

Detection of citrullinated proteins using Rh-PG probe

Isolated NETs from control neutrophils stimulated with rheumatoid factor (*14*, *53*) were analyzed using the Rh-PG probe, as previously described (*54*). Briefly, a 30-μl aliquot of each sample was prepared in 50 mM Hepes and treated with 20% trichloroacetic acid and 0.1 mM Rh-PG for 30 min at 37°C. Samples were quenched with citrulline (Sigma), cooled on ice for 30 min, and centrifuged at 14,000 revolutions per minute for 15 min at 4°C. The supernatant was removed, and samples were washed with cold acetone and dried at 100°C for 5 min. After resuspending in 50 mM Hepes, samples were separated by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) (12%; 170 V for 50 min) and imaged on a Typhoon Imager (excitation, 532 nm; emission, 580 nm).

Assessment of FLS–T cell interactions

FLS were cultured in the presence or absence of spontaneously generated RA-NETs for 3 days, washed twice with phosphate-buffered saline (PBS) to remove noninternalized NETs, and then plated at a density of 100,000 cells per well in T cell hybridoma medium or human T cell medium. HC-gp39–specific T cell hybridomas or human cit-vimentin–specific T cells were added to FLS cultures at a ratio of 1:2 (FLS/T cell) and incubated in the presence or absence of neutralizing antibodies (20 μg/ml) against CD28 or MHC II for 5 days. Supernatants were then collected and centrifuged at 300*g* for 5 min at room temperature. Mouse IL-2 was quantified using ELISA Ready-SET-Go! (eBioscience) for the hybridoma experiments, and the Bio-Plex pro-human cytokine assay (Bio-Rad) was performed for the human cit-vimentin T cell experiments, according to the manufacturers' recommendations. Aggrecan-specific T cell clones were used as negative control.

Generation of mouse FLS and in vivo administration of FLS with and without NETs

Breeding pairs of DR4 transgenic mice were a gift from C. David (Mayo Clinic) and were housed and bred at the NIH animal facility. These mice are DRB1*04:01.AEo and lack endogenous class II molecules (both I-A and I-E chains) (*33*). All animal studies were performed according to the guidelines established by the NIAMS Laboratory Animal Care and Use Section and following approved protocol (A013-08-05). Eight-week-old DRB1*04:01 female mice were euthanized, and synovium from the tibiofemoral compartment was isolated and dissected. A piece of synovial tissue was incubated in a 12-well plate with CMRL 1066 medium supplemented with 10% FBS, 1% L-glutamine, and 1% penicillin-streptomycin. DRB1*04:01 FLS were used at passage 4 to ensure purity of the cells. FLS were cultured in the presence or absence of 50 μg of human RA-NETs, isolated as above, for 3 days before injection. FLS were washed with PBS, detached with trypsin, centrifuged at 300*g* for 5 min at room temperature, washed, and resuspended in 1× Hanks' balanced salt solution. Uptake of NETs by FLS was confirmed by immunofluorescence microscopy. Twelve-week-old DRB1*04:01 female mice were anesthetized using isoflurane vaporization, and the left hindleg was shaved to expose the knee joint. A total of 1×10^5 DRB1*04:01 FLS with or without uploaded NETs were injected into the synovial space using a 27-gauge needle. This procedure was performed every 14 days for a total of 14 weeks (seven injections), and mice were sacrificed at 24 weeks of age.

Epitope mapping by antigen array

Antigens were diluted to PBS (0.2 mg/ml) or water and robotically spotted onto SuperEpoxy 2 Microarray Substrate Slides (ArrayIt), as previously described (*55*). A total of 330 RA-associated autoantigens were used, of which 52 are citrullinated, 263 are native, and 9 are control. Arrays were circumscribed using a hydrophobic Aqua-Hold Pap Pen 2 (Fisher Scientific). Chip was blocked overnight with PBS containing 3% fetal calf serum and 0.05% Tween 20. Arrays were probed with 1:300 diluted mouse sera, washed, and incubated in a 1:2000 dilution of Cy3-conjugated goat anti-mouse IgG + IgM secondary antibody (Jackson ImmunoResearch). Arrays were scanned using the GenePix 4000 scanner at a wavelength of 532 nm, and the median pixel intensities of the features and background values were determined using GenePix Pro version 3.0 software (Molecular Devices). Results were expressed as median fluorescence units, representing the median values from four to eight identical replicates of an antigen on each array after subtraction of the median values of both intraslide negative control bovine serum albumin and interslide negative control (blank well features). The investigator who performed the array experiments was blinded to the experimental conditions used for each sample.

Statistical analysis

Sample size for experiments using human samples was determined using similar patient numbers per experimental condition as in our previous publications assessing inhibition of NET responses (*14*). No samples, mice, or data points were excluded from the reported analysis once obtained. Data were analyzed using GraphPad Prism software. For samples with non-Gaussian distribution, we used Mann-Whitney *U* test. For multiple comparisons, we used analysis of variance (ANOVA) with Bonferroni's test. Results are presented as the means ± SEM.

See the Supplementary Materials for additional materials and methods.

SUPPLEMENTARY MATERIALS

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Materials and Methods
Fig. S1. ACPAs recognize NET peptides.
Fig. S2. Skin fibroblasts from patients with psoriasis internalize NETs.
Fig. S3. NETs up-regulate type I IFN-inducible genes in FLS.
Fig. S4. Endogenous expression of RAGE and TLR9 in FLS.
Fig. S5. Up-regulation of MHC II in OA FLS incubated with NETs.
Fig. S6. IFN-γ is not detected in NETs or FLS supernatants.
Fig. S7. Detection of IL-17A and IL-17B in neutrophil lysate and NETs.
Fig. S8. NETs are internalized by a TLR9-dependent and IL-17R–independent mechanism.
Fig. S9. FLS do not express HC-gp39 or MPO.
Fig. S10. Characterization of vimentin epitope binding to HLA-DRB1*04:04 and T cell clone specificity.
Fig. S11. Cytokine profile of cocultures of Aggrecan 225 $CD4^+$ T cells and haplotype-matched FLS.
Fig. S12. GK1.5 antibody depletes specifically CD4 T cells but not CD8 T cells.
Fig. S13. DRB1*04:01 transgenic mice immunized with FLS loaded with NETs develop in vivo antibody responses to citrullinated peptides.
Table S1. Mass spectrometry analysis demonstrates multiple citrullinated peptides in NETs induced with IgM rheumatoid factor.
Source data Excel file
Source data blots
Reference (*56*)

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Science Immunology

Synovial fibroblast-neutrophil interactions promote pathogenic adaptive immunity in rheumatoid arthritis

Carmelo Carmona-Rivera, Philip M. Carlucci, Erica Moore, Nithya Lingampalli, Hannes Uchtenhagen, Eddie James, Yudong Liu, Kevin L. Bicker, Heidi Wahamaa, Victoria Hoffmann, Anca Irinel Catrina, Paul R. Thompson, Jane H. Buckner, William H. Robinson, David A. Fox and Mariana J. Kaplan

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NETs Blow the Joint

Neutrophil extracellular traps (NETs) activate synovial fibroblast-like synoviocytes (FLS) in joints of individuals with rheumatoid arthritis. Now, Carmona-Rivera *et al*. investigate the mechanism behind this activation. They found that NETs containing citrullinated peptides could be internalized by FLS through the RAGE-TLR9 pathway and then loaded onto major histocompatibility complex class II and presented to antigen-specific T cells, which contribute to joint inflammation. NET-loaded FLS induced autoantibody production and joint disease in mice. These data suggest that cross-talk between NETs and FLS may contribute to rheumatoid arthritis.

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Increased Binding of Specificity Protein 1 to the *IL21R* Promoter in B Cells Results in Enhanced B Cell Responses in Rheumatoid Arthritis

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B cells are implicated in rheumatoid arthritis (RA) based on the presence of autoantibodies and the therapeutic response to B cell depletion. IL-21 has a significant role in B cell development and function. Here we assess B cell responses to IL-21 and the mechanisms responsible for altered IL-21R expression in RA. Flow cytometry of PBMC and cultured B cells was used to quantify protein and mRNA levels of IL-21R, IL-21 signaling through pSTAT3, specificity protein 1 (SP1) and to determine cytokine production (IL-6) and maturation status of B cells in RA and healthy control subjects. SP1 binding to the *IL21R* promoter region in B cells was assessed with ChIP-qPCR. We demonstrate an increase in IL-21R expression in total and memory B cells from RA subjects, which correlated with responsiveness to IL-21 stimulation. Stimulation of naïve RA B cells with IL-21 and CD40L resulted in an increase in differentiation into plasmablasts and an increase in IL-6 production in comparison to healthy controls, which was dose dependent on IL-21 stimulation. IL-21R expression on memory B cells in RA synovial fluid was comparable to peripheral blood making our study pertinent to understanding B cell responses in the joint and site of inflammation. We identified an increase in SP1 protein and mRNA in RA B cells and demonstrate an increase in binding of SP1 to the *IL21R* promoter region, which suggests a mechanism by which IL-21R expression is enhanced on B cells in RA. Taken together, our results indicate a mechanism by which IL-21 enhances B cell development and function in RA through an SP1 mediated increase in IL-21R expression on B cells.

Keywords: rheumatoid arthritis, B cells, IL-21R, specificity protein 1, IL-6

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INTRODUCTION

Rheumatoid arthritis (RA) is a chronic inflammatory and destructive joint disease characterized by the presence of rheumatoid factor and anti-cyclic citrullinated peptide autoantibodies (ACPA) (1, 2). Although there has been advances in the diagnosis and treatment of RA, there is a gap in knowledge regarding molecular and cellular mechanisms that cause RA. B cells contribute via their capacity to produce autoantibodies, proinflammatory cytokines and as antigen presenting cells. Early tolerance is altered in RA resulting in an increase in autoreactive B cells (3). The

development of ACPA and rheumatoid factor autoantibodies precede disease development and expansion of ACPA and rheumatoid factor specific plasmablasts in RA patients with significant levels of somatic mutation, further indicating dysregulation in B cell maturation (4). Notably, B cell depletion with rituximab is efficacious in RA (5) and response to therapy with rituximab and abatacept (6) correlate with decreases in the level of ACPA. This highlights the need to better understand how B cells escape tolerance in RA and their role in propagating disease.

The role of IL-21 in B cell development and function has been well described. IL-21 induces B cell activation, expansion and the induction of plasma cells following stimulation and directly contributes to the generation of antibodies (7). IL-21 signals through the IL-21R and the common gamma chain (γc) and via the STAT signaling pathway. Phosphorylated STAT3 homodimerizes and translocates to the nucleus where it activates IL-21 target genes such as *PRDM1,* a critical transcription factor for plasma cell development (8, 9). It also has a direct role in the germinal center where it contributes to antibody production, including isotype switching and affinity maturation (10, 11).

Growing evidence suggests that IL-21 has a role in B cell dysfunction in RA. IL-21 levels in the serum and SF are increased in RA (12) and correlate to 28-joint count disease activity scores (13). A recent study demonstrated an increase in the frequency of $CD19^{+}IL\text{-}21R^{+}$ cells in RA compared to controls and observed enhanced proliferation and differentiation of B cells from RA patients compared to controls (13).

The importance of IL-21 in has also been demonstrated in murine models of rheumatoid arthritis. In the K/BxN model of spontaneous autoantibody mediated arthritis, K/BxN $IL\text{-}21R^{-/-}$ mice are protected from initiation of arthritis or autoantibody development (14). Further, the treatment of K/BxN mice with an IL-21R-Fc fusion protein combined with an adjuvant delayed disease onset and decreased the severity of disease (14). The collagen-induced arthritis (CIA) IL-21R knockout murine model are resistant to CIA induction, demonstrate impaired antibody production and showed that IL-21 signaling in B cells was indispensable for the development of CIA (15). DBA/1 treated with bovine type II collagen mice were also used to investigate whether IL-21 contributed to disease development (16). Treating these mice with an IL-21R.Fc fusion protein reduced the clinical signs of collagen-induced arthritis, decreased antibody levels and decreased IL-6 levels thereby demonstrating a pathogenic role for IL-21 in mouse models of RA (16).

The goal of this study was to determine the mechanism that leads to increased IL-21R, extend our understanding of how it influences the function of RA B cells and more fully understand it in the context of disease. We demonstrate an increase in the expression of IL-21R on total and memory B cells in RA compared to controls, which positively correlated with pSTAT3 levels following IL-21 stimulation. Functionally, this increase in IL-21R on B cells was associated with increased expression of IL-21 target genes, plasmablast development and IL-6 production in response to IL-21. We link SP1 to the source of increased IL-21R based on increased SP1 expression in RA B cells and increased binding to the *IL21R* promoter region in RA. Together these findings suggest that increased expression of SP1 drives an increase in IL-21R, which potentiates the expansion of pathogenic B cells and autoantibody production in RA.

MATERIALS AND METHODS

Patients

All samples used in this study were from participants in the Benaroya Research Institute Immune-Mediated Disease Registry. All patients gave written informed consent. Patient characteristics are summarized in **Tables 1–4**. RA subjects were drawn from a general rheumatology clinic and carry a diagnosis of RA based on the 2010 American College of Rheumatology criteria. There were two different cohorts of RA subjects. The first cohort ($N = 110$, **Table 1**) was cross-sectional with respect to disease duration, disease activity, antibody status and therapy although no one was on biologic DMARDs at the time of study. This cohort was compared to age-, gender- and race-matched healthy control subjects ($N = 93$, **Table 1**). The second RA cohort ($N = 52$, **Table 2**) was selected to determine whether therapy had an effect on IL-21R or signaling responses. Individuals with SLE ($N = 20$, **Table 3**) carried a diagnosis of SLE based on the 1997 American College of Rheumatology criteria (17) and were age-, gender-, and race-matched to healthy control subjects ($N = 21$, **Table 3**). All individuals with MS had relapsing-remitting MS ($N = 21$, **Table 4**) based on the Revised McDonald Diagnostic Criteria for MS (18) and were age-, gender- and race-matched to healthy control subjects ($N = 27$, **Table 4**). Healthy control subjects that were matched to the MS cohort are a subset of the healthy controls presented in **Figure 1**. Only samples that are matched are graphed together. Note all healthy control subjects had no history of autoimmune disease themselves or among their first-degree relatives. Disease status, gender, age, therapy and race was blinded until the conclusion of the study. All subjects were included in IL-21R expression studies, other assays were performed with selected subjects as defined in the figure legends. All PBMC samples were cryogenically frozen and thawed at the time of experiment except for synovial fluid/PBMC comparisons, which were fresh.

Synovial Fluid Processing

Synovial fluid was obtained from RA subjects undergoing therapeutic arthrocentesis. Synovial fluid samples were diluted 1:12 with 10% human serum RPMI 1640 (Gemini, GE). Diluted samples were treated with hyaluronidase (VWR) and benzonase (Sigma) for 30 minutes at 37°C, centrifuged and resuspended in 2 mL hemolytic buffer. Samples were quenched with 30 mL PBS, centrifuged, resuspended in 10% RPMI, filtered through a 100 μm cell strainer and washed with 10% RPMI media.

Flow Cytometry

PBMC were rested in XVIVO 15 (Lonza), stained with a viability dye (eBioscience) and blocked with Human TruStain FcX (Biolegend). PBMCs were incubated with CD19 (HIB19), CD20 (2H7), CD24 (ML5), CD10 (HI10a), IgM (MHM-88), CD3 (UCHT1), CD8 (RPA-T8), CD45RO (UCHL1), CD45RA (HI100), CD138 (MI15), IL-21R (17A12), from Biolegend; CD38

TABLE 1 | RA and healthy control cohort characteristics.

	RA subjects ($n = 110$)	Control subjects ($n = 93$)
Age at draw (mean ± SD years)	51.7 ± 14.9	46.3 ± 14.2
Male/Female	24/86	25/68
Race	94.6% Caucasian, 5.4% other	91.4% Caucasian, 8.6% other
Disease duration (mean ± SD years)	6.8 ± 8.4	
RAPID3 score (mean ± SD)	3.1 ± 2.2	
CCP^+	60.9%	
RF^+	57.3%	
Therapy	15% Steroid 50.9% DMARD 40% NSAID 10% Biologic	

RAPID3, the routine assessment of patient index data with 3 measures (0–9); CCP, cyclic citrullinated peptide; RF, rheumatoid factor. Steroid therapy includes: prednisone (5–20 mg/day); DMARD includes: methotrexate (10–25 mg/week or 10 mg/2X weekly), sulfasalazine, hydroxychloroquine (200 mg/2X or 1X daily), azathioprine, and leflunomide; NSAID includes: ibuprofen, naproxen, aspirin, meloxicam, and celecoxib; and biologics includes: etanercept, abatacept, infliximab, and adalimumab.

TABLE 2 | Second RA cohort disease distribution.

	RA subjects ($n = 52$)
Age at draw (mean ± SD years)	57.3 ± 14.3
Male/Female	14/38
Race	92.3% Caucasian, 7.7% other
Disease duration (mean ± SD years)	13.5 ± 10.8
RAPID3 score	2.6 ± 1.7
CCP^+	86.5%
RF^+	61.5%
Therapy	30% Steroid 75% DMARD 36.5% NSAID 55.8% Biologic

RAPID3, the routine assessment of patient index data with 3 measures (0–9); CCP, cyclic citrullinated peptide; RF, rheumatoid factor. Steroid therapy includes: prednisone (5–20 mg/day); DMARD includes: methotrexate (10–25 mg/week or 10 mg/2X weekly), sulfasalazine, hydroxychloroquine (200 mg/2X or 1X daily), NSAID includes: ibuprofen, naproxen, aspirin, meloxicam, and celecoxib; and biologics includes: golimumab, etanercept, abatacept, infliximab, tofacitinib, and adalimumab.

(HIT2), CD27 (L128), CD4 (SK3), CD27 (L128), Blimp-1 (5E7), γ_C (TUGh4), STAT3 (M59-50), from BD, SP1 (D4C3) from CST and IL-6 (MQ2-13AS) from eBioscience. IL-6 and IgM levels were determined after brefeldinA (Biolegend)/monensin (Biolegend) stimulation for 4 h, fixed with cytofix (BD), permeabilized with cytoperm (BD) followed by intracellular staining. Transcription factor staining was conducted according to the manufacturers protocol (BD). Where the mean fluorescent intensity (MFI) is analyzed we utilized the geometric mean fluorescent intensity. All flow cytometry experiments were acquired on a BD FACSCanto II (BD) and data were analyzed with FlowJo software (Tree Star).

TABLE 3 | SLE and healthy control cohort characteristics.

	SLE subjects ($n = 20$)	Control subjects ($n = 21$)
Age at draw (mean ± SD years)	45.0 ± 17.8	44.0 ± 16
Male/female	1/19	1/20
Race	60% Caucasian, 20% Asian, 10% African American, 10% Unknown	66.6% Caucasian, 19% Asian, 9.5% African American, 4.7% Pacific Islander
Disease duration (mean ± SD years)	10 ± 9.6	
SLEDAI score (mean ± SD)	4.6 ± 3.7	
ANA^+	100%	
Therapy	45% Prednisone (2.5–7 mg/day) 80% Hydroxychloroquine	

SLEDAI, Systemic lupus erythematosus disease activity index; ANA, antinuclear antibodies.

TABLE 4 | MS and healthy control cohort characteristics.

	MS subjects ($n = 21$)	Control subjects ($n = 27$)
Age at draw (mean ± SD years)	32.7 ± 8.6	33.9 ± 9.6
Male/female	8/13	13/14
Race	95% Caucasian, 4.7% Asian	88.9% Caucasian, 11.1% Asian
Disease duration (mean ± SD years)	4.4 ± 7.0	
Patient classifications	100% RRMS	
Therapy	100% untreated	

RRMS, Relapsing remitting multiple sclerosis.

RNA Flow Cytometry

Intracellular RNA flow cytometry was conducted using the manufacturer's protocol (PrimeFlow RNA, Affymetrix, Santa Clara, CA). *RPL13A* RNA probe was used as a positive control and a no probe negative control was included. The following Type I RNA probes were obtained from eBioscience: *IL21R, SP1, PRDM1, SOCS1, SOCS3*, and miR155.

Intracellular Signaling

PBMCs were rested in XVIVO15 (Lonza). Cells were stimulated with media or IL-21 (50 ng/mL) (Miltenyi) for 45 min. PBMC was fixed with methanol and labeled with surface antibodies, intracellular CD20 antibody (H1, BD), and pSTAT3 (pY705) (4/P-STAT3, BD). Cells were stimulated with IL-10 (10 ng/mL) (BD) for 45 min or IL-4 (50 ng/mL) (R&D Systems) for 15 min. Cells stimulated with IL-10 were analyzed for pSTAT3 fold change levels and those stimulated with IL-4 were analyzed for pSTAT6 (pY641) (18/P-Stat6, BD). PSTAT3 fold change was calculated by the geometric mean of the stimulated well divided by the geometric mean of the unstimulated well.

Gating B Cells in Flow Cytometry

Example gating for B cell populations and IL-21R gating of a bimodal population is visualized in **Figure 1A** and is applied throughout the paper. CD20 was used to gate total B cells to allow a direct correlation in our signaling assays: the CD19 epitope is stripped during methanol permeabilization, thus we used surface CD20 to delineate B cells in **Figure 1** and intracellular CD20 (clone: H1, BD) in **Figure 2**. For RNA flow cytometry, we used intracellular CD20 gating.

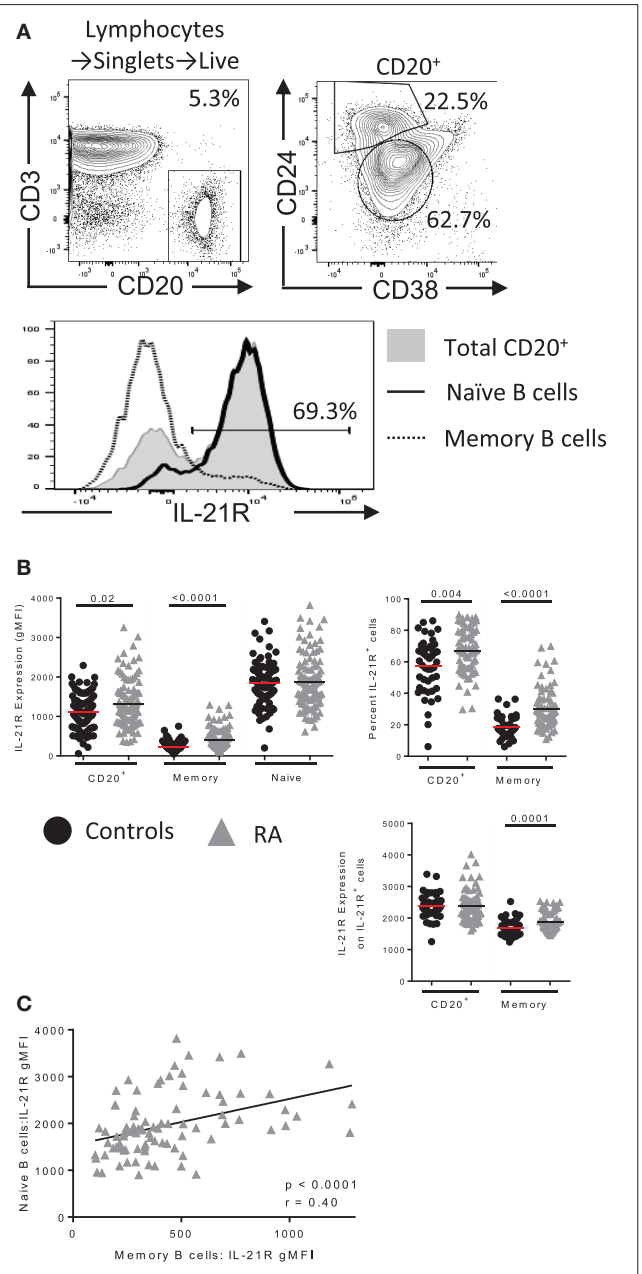

FIGURE 1 | IL-21R expression is increased in B cells in RA. **(A)** (top) Gating scheme for total B, memory and naïve B cells with IL-21R expression in each population (bottom) **(B)** (left) IL-21R levels on $CD20^+$, total memory ($CD20^+CD38^-CD24^{hi}$) and naïve ($CD20^+CD38^{int}CD24^{int}$) B cells in (black circles) healthy control (n=46) and (gray triangles) RA subjects (n=71) by flow cytometry. (Right) Percent of $IL\text{-}21R^+$ cells (center, left) and IL-21R expression was quantified on $IL\text{-}21R^+$ cells (bottom, right) in $CD20^+$ and memory B cells in RA and control subjects. **(C)** IL-21R levels in memory and naïve B cells were correlated in RA subjects. Significance was determined using a Mann Whitney *U* test and a Pearson correlation.

B Cells Cultures and Plasmablast Induction

For 24 or 48 h cultures, PBMC was thawed and stimulated with IL-21 (50 ng/mL) (Miltenyi) with and without CD40L (5 μg/mL) (Peprotech). Four hours prior to harvest, brefeldinA/monensin were added to the cultures. Cells were harvested and stained for flow cytometry.

For plasmablast cultures, naïve B cells were isolated using the human naïve B cell isolation kit II (Miltenyi) and purity was assessed following 12-day cultures. Samples with purity >90% were plated at two million cells per well and cultured with IL-21 (15 pg/mL or 50 ng/mL) (Miltenyi) and CD40L (5 μg/mL) (Peprotech).

Okadaic Inhibitor Assays

An aliquot of PBMC was removed to determine levels of SP1 and IL-21R prior to stimulation to obtain baseline levels. PBMC were plated with 0.1 μM Okadaic acid or DMSO control for 24 h.

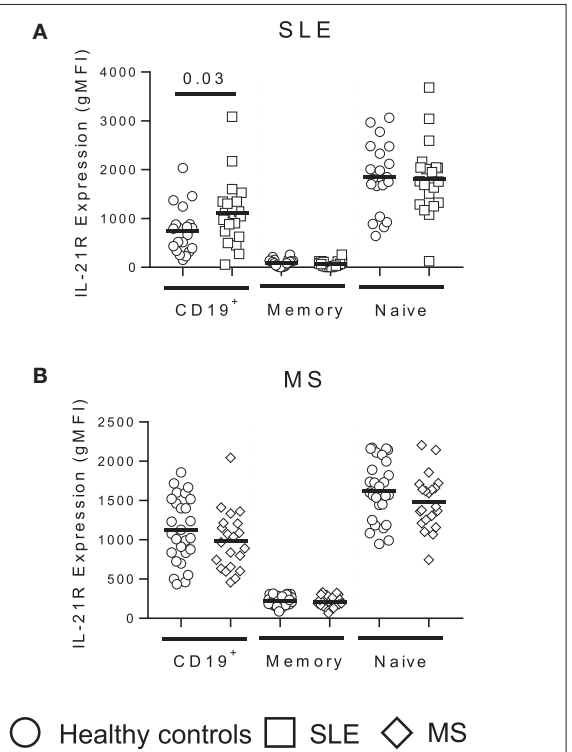

FIGURE 2 | IL-21R expression is enhanced in total B cells in SLE subjects but unchanged in MS. IL-21R expression levels in SLE (white squares) **(A)** and MS (white triangles) **(B)** subjects compared to matched healthy controls (white circles) in total $CD19^+$ B cells, total memory ($CD20^+CD38^-CD24^{hi}$) and naïve ($CD20^+CD38^{int}CD24^{int}$) B cells. Significance was determined using Mann Whitney *U* tests.

CCP3 IgG, Rheumatoid Factor IgM, IL-6 and IL-10 ELISAs

Serum from RA subjects was assessed for anti-cyclic citrullinated peptide 3 IgG (Inova Diagnostics), anti-rheumatoid factor IgM (Abcam), IL-6 (eBioscience), and IL-10 (eBioscience) levels according to the manufacturer's instructions.

Chromatin Immunoprecipitation

B cells were isolated by negative selection using the human B cell isolation kit II (Miltenyi). Cells were fixed with 4% paraformaldehyde for 10 minutes at 37°C. The cell pellet was frozen at −80°C until sample preparation. Magnetic beads were blocked (PBS/0.5% BSA) and incubated with either 10 μg SP1 antibody (07-645, Millipore) or control IgG overnight at 4°C. Nuclei were isolated the following day and sheared using a truChIP Chromatin Shearing Reagent Kit (Covaris), an AFA microTUBE (Covaris) and a M220 focused ultrasonicator (Covaris). Conditions for sonication: 20% duty cycle, 30 min. Sonicated samples were immunoprecipitated with magnetically labeled SP1 antibody or control IgG overnight at 4°C. The next day samples were reverse cross-linked. The Qiagen QIAquick PCR purification kit was used to purify the DNA.

Quantitative RT-PCR

Gene expression analysis was performed using SYBR green real-time quantitative PCR on an ABI7900 HT analyzer (Applied Biosystems). Primers used to detect SP1 binding in the IL-21R promoter region were: forward (5′-GGTCCC TAAGAGGGAAGTGTCA−3′) and reverse (5′-GCACCCACTG TCACCAAAGG−3′) as previously published by Wu et al (19). Primers to detect SP1 binding outside of the *IL21R* gene are: site 1: forward (5′-GTCCCACAGGTCCCTAAGGT−3′) and reverse (5′-CCAGCAACTTCCCTGAGATG−3′); site 2: forward (5′-AAGGTAAGGCTGGGCTCAAT−3′) and reverse (5′-GCAATCTCAGGTCACTGCAA−3′).

Statistics

Data was analyzed using GraphPad Prism. Statistical significance between controls and RA; or between RA-IL-21R^{high} compared to controls or IL-21R^{low}, was assessed with a two-sided Mann Whitney *U* or Student's *t* test of which a P value of < 0.05 was considered significantly different. A Mann Whitney *U* or Student's *t* test was selected after determining the normality of the groups with a D'Agostino-Pearson omnibus normality test. Correlations were assessed by calculating the Pearson's correlation coefficient, *r*.

The RA-IL-21R^{high} cohort was defined as subjects for whom the gMFI of IL-21R is two standard deviations (SD) above the mean of the control group. The RA-IL-21R^{low} cohort was defined by the IL-21R gMFI within two SD of the mean gMFI of the control group (see **Figure 1** for further details). Samples selected from the RA-IL-21R^{high} and IL-21R^{low} groups were selected randomly once the groups were designated.

RESULTS

IL-21R Expression on B Cells Is Enhanced in RA

Example gating for B cell populations and IL-21R gating of a bimodal population is visualized in **Figure 1A** and is applied throughout the paper. IL-21R expression was calculated on the entire B cell population as some subjects demonstrated a continuum of expression (**Figures 1A,B**). We observed increased IL-21R expression in total B cells as well as an increase in frequency of IL-21R$^+$ B cells in RA subjects compared to healthy controls (**Figure 1B**). Frequency of IL-21R$^+$ memory B cells was obtained by applying the total B cell IL-21R$^+$ gate to the memory B cell population. This finding was most significant in the memory B cell population, however IL-21R gMFI in memory B cells correlated with IL-21R gMFI in naïve B cells indicating that this may extend to naïve B cells as well (**Figure 1C**). Also of note, IL-21R expression is decreased on memory compared to naïve B cells, this is due to the reduced need for IL-21 in mature B cells (20). No differences in IL-21R surface expression was present in T cells from RA subjects (**Figure S1**) indicating that this is a B cell specific finding.

B cells are increased in the RA joint, and have been shown to produce ACPA (21). To determine whether enhanced expression of IL-21R observed in the periphery is also seen in the RA joint we obtained paired synovial fluid and peripheral blood samples from RA subjects and measured IL-21R expression. Synovial fluid samples had an increased frequency of memory B cells with an increase in the frequency of switched memory as compared to peripheral blood (**Figure S2A**). IL-21R levels on synovial fluid B cells were as high as that seen in peripheral blood (**Figures S2B–C**). This suggests that IL-21 signaling in peripheral blood B cells may reflect that seen in the synovium.

Autoimmune disease immune phenotypes often demonstrate similarities suggesting similar mechanisms of action or disease pathogenesis. To determine whether IL-21R expression is a RA specific finding, we analyzed the IL-21R in SLE and MS subjects compared to screened healthy controls that were specifically matched for age, race and gender (**Tables 2**,**3**). We detected enhanced IL-21R expression in total CD19$^+$ B cells in SLE subjects, though no differences were measured in memory or naïve B cell subsets (**Figure 2A**). Further, MS subjects demonstrated no differences in total CD19$^+$, memory or naïve B cell subsets compared to controls (**Figure 2B**). This suggests that these findings are specific to autoimmune diseases under the rheumatic disease umbrella.

In our cohort of RA subjects with established disease, we observed only modest correlations with disease duration, ESR, rheumatoid factor IgM, and IL-21R expression, driven in part by a single outlier, and no correlations with CRP, CCP3-IgG or Rapid 3 score (**Figure S3**). We were able to assess IL-21R expression levels at two time points for a subset of RA subjects and found that it was stable over time (**Figure S4**). New therapies are under development to treat RA by targeting the IL-21 signaling pathway (22–25). We questioned whether current therapies used to treat RA may influence enhanced IL-21R expression that we have observed in some RA subjects.

However, the increase in IL-21R on B cells was unrelated to therapeutic intervention (**Table 2**, **Figure S5**) and was modestly associated with disease duration. Together these data indicate that alterations in IL-21R expression we observe in RA are not likely to be secondary to either inflammation, longstanding RA and/or therapy.

Comparison of IL-21R Expression and Response to IL-21

To determine whether increased IL-21R influences IL-21 signaling we measured pSTAT3 in PBMC stimulated with IL-21 (**Figure S6A**). pSTAT3 fold change was calculated by the geometric mean of stimulated cells divided by the geometric mean of unstimulated cells. We observed a significant correlation between IL-21R and pSTAT3 fold change in total ($p < 0.0001$), memory ($p = 0.0087$), and naïve ($p < 0.0001$) B cells (**Figures 3A–C**). There was no difference in γ_C expression or signaling through IL-4 (which signals through the γ_C) suggesting that enhanced pSTAT3 response was not due to alterations in signaling via γ_C or a target downstream of it (**Figures S6B,C**). To determine whether all signaling through STAT3 was impacted, we examined baseline levels of STAT3 and pSTAT3 in response to IL-10, and found no significant differences between RA and controls (**Figures S6D,E**).

To determine whether factors other than IL-21R play a role in IL-21 signaling in RA, we examined the expression of *SOCS1* and *SOCS3*, which negatively regulate IL-21 signaling (26, 27). We observed a significant decrease in *SOCS3* levels in B cells from RA compared to controls (**Figure 4A**). We did not detect a difference in *SOCS1* levels, nor did we observe a difference in miR155 levels in B cells between control and RA (**Figure 4B**). MiR155 acts by suppressing *SOCS1* and is elevated in B cells from RA that are ACPA$^+$ (28). These data indicate IL-21R is the predominant factor implicated in the enhanced response to IL-21 in our RA cohort.

To determine whether IL-21 target genes in B cells were altered in RA, *PRDM1* levels were assessed following a stimulation with IL-21 (**Figure 4C**). Memory B cells from RA significantly induced *PRDM1* whereas there was no significant increase in *PRDM1* mRNA from controls (**Figure 2D**). These data suggest that increased IL-21R translates to increased IL-21 signaling resulting in enhancement of IL-21 target genes.

Increased IL-21-Mediated Plasmablast Differentiation Is Present in RA

IL-21 promotes the development of plasmablasts through the induction of *PRDM1,* which encodes Blimp-1, a transcription factor required for plasmablast development (29). To assess whether increased IL-21R expression enhances plasmablast development, we performed *in vitro* IL-21/CD40L stimulations, an approach successfully used by others to assess plasmablast differentiation (8, 30, 31). For these studies naïve B cells were used, as they would allow us to understand how enhanced IL-21 signaling influenced B cell development. The correlation between IL-21R on naïve and memory B cells among the subjects in our RA cohort support this approach. We observed a significant increase in the frequency of plasmablasts in RA as compared to control (**Figure 5A**), which was most pronounced at a low dose of IL-21. In addition, we observed an increase in expression of CD138 on RA plasmablasts (**Figure 5B**). The increase in frequency was among IgM$^+$ plasmablasts (**Figure 5C**), with a corresponding increase in surface IgM levels (**Figure 5C**) though no difference in IgG levels (data not shown). Together, these data suggest increased plasmablast development in response to increased IL-21R expression.

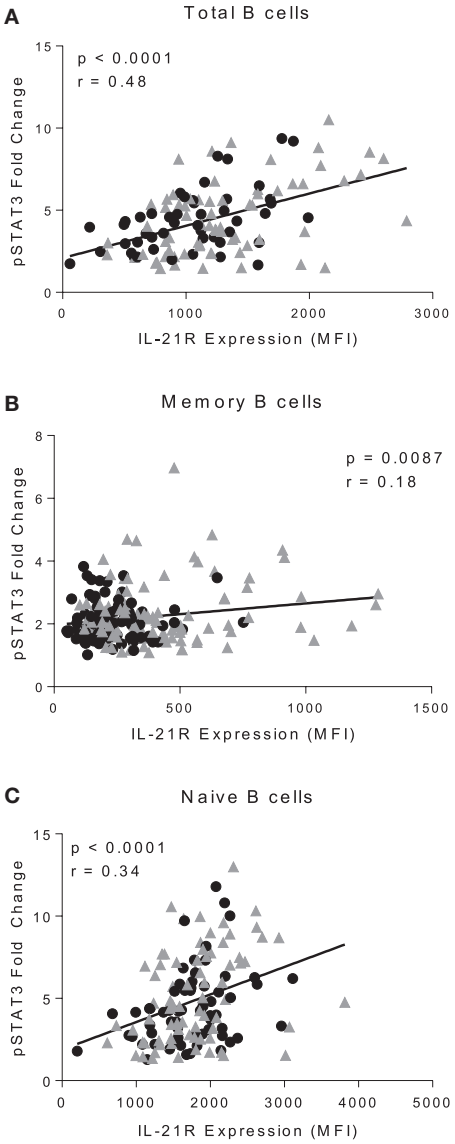

FIGURE 3 | IL-21R levels correlate with IL-21-mediated pSTAT3 signaling. Correlation of IL-21R expression and pSTAT3 fold change in CD20$^+$ **(A)**, total memory B cells **(B)**, and naïve B cells **(C)**. pSTAT3 levels were assessed by stimulating with IL-21 for 45 min and measuring pSTAT3 gMFI with no stimulation or with stimulation. Fold change was calculated by dividing IL-21 stimulation pSTAT3 gMFI by the no stimulation pSTAT3 gMFI. As there were no differences in slope between controls and RA patients, the correlation was conducted on both controls (black circles) and RA patients (gray triangles) ($n = 116$). Correlation was assessed with the Pearson correlation.

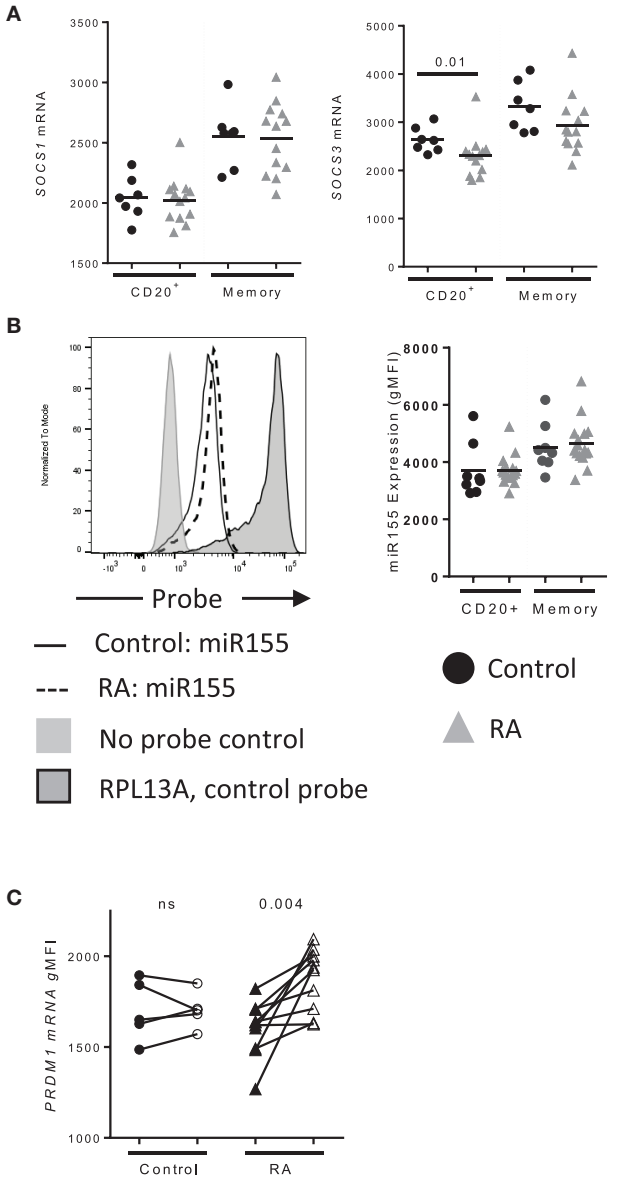

FIGURE 4 | *PRDM1* levels are enhanced in memory B cells following IL-21 stimulation. **(A)** *SOCS1* (left) and *SOCS3* (right) mRNA expression in $CD20^+$ and total memory B cells from (black circles) Control ($n = 7$), (gray triangles) RA ($n = 14$) subjects as assessed by mRNA flow cytometry. **(B)** miR155 levels in total and memory B cells (black circles) Control ($n = 7$), (gray triangles) RA ($n = 14$) subjects collected with miRNA flow cytometry. **(C)** *PRDM1* mRNA levels in memory B cells from control (circles, $n = 5$) and RA subjects (triangles, $n = 10$) at baseline (filled) and 48 h (unfilled) after stimulation with IL-21 (50 ng/mL) and measured by mRNA flow cytometry ($n = 15$). Significance was determined using Mann Whitney *U* tests.

IL-21R Expression and IL-6 Production Are Correlated in B Cells

Plasmablasts produce increased quantities of IL-6 as compared to naïve B cells (32). We observed an increase in IL-6 production by the RA plasmablasts in *in vitro* plasmablast cultures compared to controls (**Figure 5D**). Since IL-6 production was

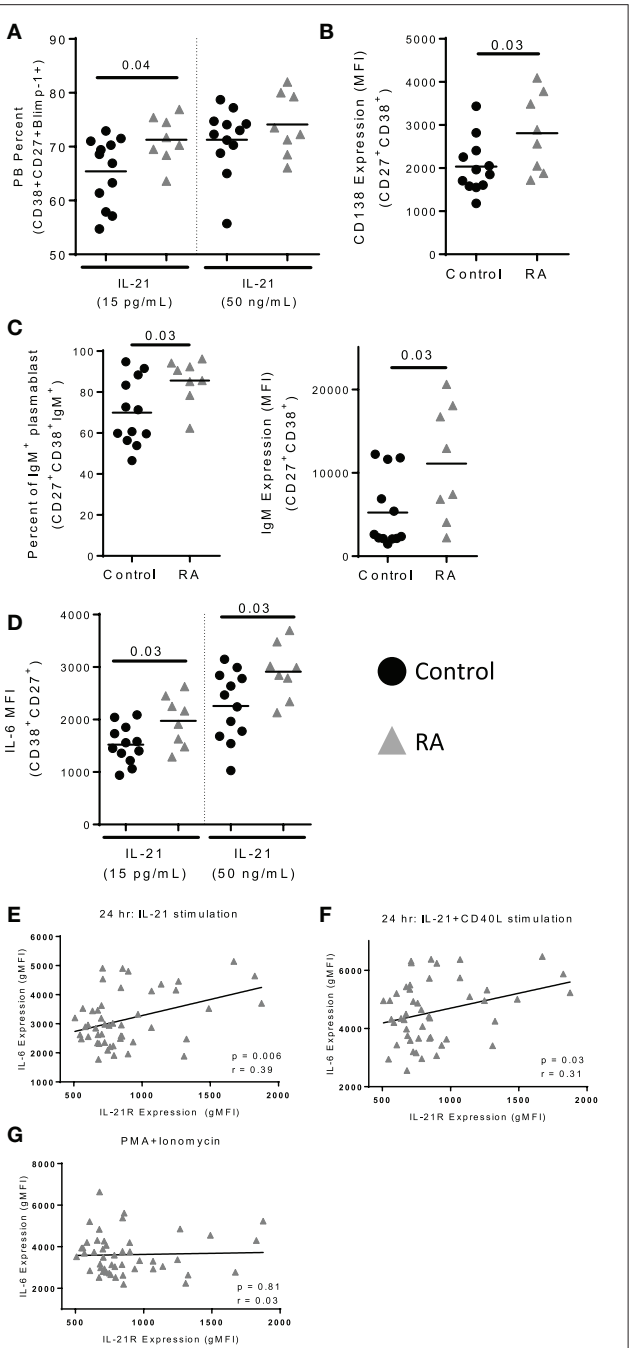

FIGURE 5 | IL-21 mediated plasmablast differentiation is enhanced in RA**.** Naïve B cells were isolated from PBMC of control ($n = 12$) and RA patients ($n = 8$) and cultured with IL-21 (15 pg/mL or 50 ng/mL) and CD40L for 12 days. **(A)** Plasmablast percent was measured by staining for $CD38^+CD27^+Blimp\text{-}1^+$ cells following 12 days of culture with either low (15 pg/ml) or high (50 ng/ml) levels of IL-21 in addition to CD40L. **(B)** CD138 expression was determined on plasmablasts ($CD38^+CD27^+$) from RA subjects and controls following high dose IL-21 and CD40L culture. **(C)** Frequency of IgM^+ plasmablasts ($CD38^+CD27^+$) were assessed following 12 days of culture with high dose IL-21 and CD40L (left). IgM gMFI was assessed in plasmablasts by intracellular staining (right). **(D)** IL-6 gMFI was assessed on plasmablasts in response to low or high dose IL-21 in addition to CD40L stimulation in controls and RA subjects. **(E)** IL-21 expression correlates with

(Continued)

FIGURE 5 | IL-6 production following IL-21 stimulation **(F)** and IL-21+CD40L stimulation but not **(G)** PMA+Ionomycin stimulation. PBMCs from RA subjects ($n = 52$) were stimulated with IL-21 **(E)** or IL-21+CD40L **(F)** for 24 h. In the last 4 h of incubation, brefeldin A and monensin were added to prevent export of IL-6. The level of IL-6 was measured by flow cytometry on total B cells. IL-6 production was measured by flow cytometry. IL-21R expression was measured at day 0 on memory B cells. Black circles are controls and gray triangles are RA patients. Significance was assessed using Mann Whitney *U* tests. Correlation was assessed with the Pearson correlation.

altered in cultures, we investigated the relationship between IL-21R and IL-6 production. In RA, we found that IL-21R on memory B cells correlated with IL-6 production following stimulation with IL-21 or IL-21+CD40L (**Figures 5E–F**). In contrast, there was no correlation between IL-21R and IL-6 production or intracellular IL-6 levels following stimulation with PMA/ionomycin (**Figure 5G**, **Figure S7A**), suggesting that the differences in IL-6 production are dependent on IL-21. Although not significant, in our cohort there was a trend suggesting a correlation between IL-21R and increased serum IL-6 levels in RA ($p = 0.07$) (**Figures S7B,C**). In addition, no correlation with levels of IL-10 was seen in these subjects (**Figures S7D,E**). Together, this suggests that IL-6 production by B cells is IL-21 signaling dependent.

SP1 Binding to the IL-21R Promoter

We next asked whether increased IL-21R levels were due to increased transcription. RNA flow was used for this purpose as it can be used to determine protein and mRNA levels simultaneously in rare cell types (representative staining, **Figure S8A**). Subjects were randomly selected from RA high IL-21R (RA-IL-21R^{high}) and RA low IL-21R (RA-IL-21R^{low}) groups. We defined RA-IL-21R^{high} as subjects for whom the gMFI of IL-21R is two standard deviations (SD) above the mean of the control group. RA-IL-21R^{low} were RA subjects with IL-21R gMFI within two SD of the mean gMFI of the control group (**Figure 6A**). *IL21R* mRNA levels were significantly increased in RA-IL-21R^{high} expressers compared to RA-IL-21R^{low} in total and memory B cells (**Figures 6B,C**) suggesting that enhanced IL-21R on RA B cells is transcriptionally regulated.

Specificity protein 1 (SP1) has been reported to regulate IL-21R expression in T cells (19). We assessed whether SP1 is responsible for enhanced IL-21R in B cells in RA and we found that SP1 levels were increased in B cells from RA-IL-21R^{high} compared to controls and RA-IL-21R^{low} subjects (**Figure 7A**). In addition, SP1 protein levels correlated significantly with IL-21R protein levels in B cells (**Figure 7A**). *SP1* mRNA levels were increased in memory B cells from RA-IL-21R^{high} expressers (**Figures 7B,C**) and this significantly correlated with *IL21R* mRNA levels (**Figure 7C**).

We next assessed the binding of SP1 to the *IL21R* promoter in B cells. As before, we analyzed B cells from controls, RA-IL-21R^{high} and IL-21R^{low} subjects and performed ChIP-qPCR on purified B cells; we analyzed the SP1 binding site that were identified in the *IL21R* promoter in T cells (19). We used total B cells as we could not obtain sufficient quantities of memory B cells from RA and we observed alterations in total B cells, as well as memory B cells, in IL-21R and SP1 expression. We observed enhanced binding of SP1 to the *IL21R* promoter in RA-IL-21R^{high} B cells compared to both controls and RA-IL-21^{low} subjects

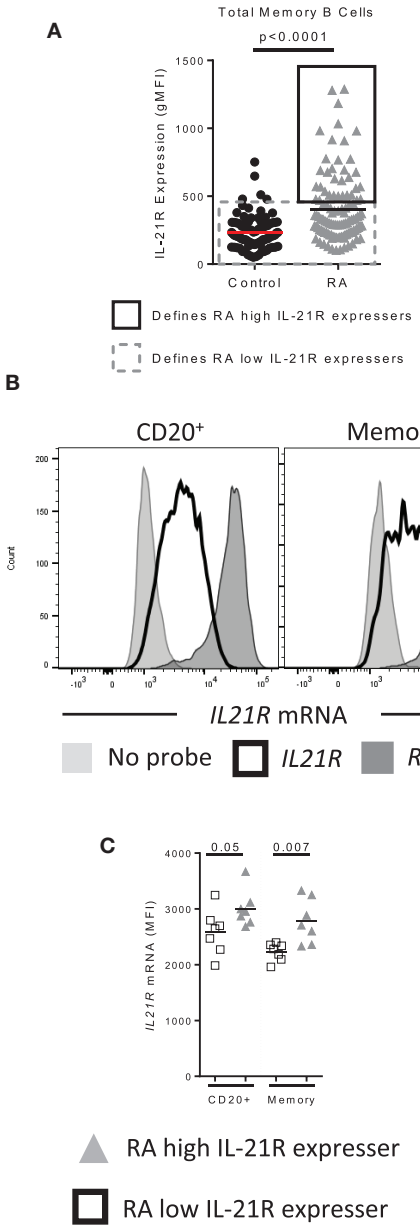

FIGURE 6 | IL-21R transcript levels are increased in RA subjects with high levels of IL-21R **(A)** Data from **Figure 1A** used to show how RA high IL-21R expressers and RA low IL-21R expressers are defined. RA high IL-21R expressers in the solid black box express IL-21R at levels two times the standard deviation of the mean of the control samples. RA low IL-21R expressers in the gray dashed box are less than two times the standard deviation of the mean of the control samples. **(B)** Representation of the positive control probe (*RPL13A)* (dark gray histogram), *IL21R* (black line histogram) and negative control (no probe) (light gray histogram) in CD20$^+$ or memory B cells in a control subject. **(C)** *IL21R* mRNA levels in total and memory B cells from RA high IL-21R expressers (gray triangles, $n = 7$) and RA low IL-21R expressers (white squares, $n = 7$) Significance was determined using a Mann Whitney *U* test.

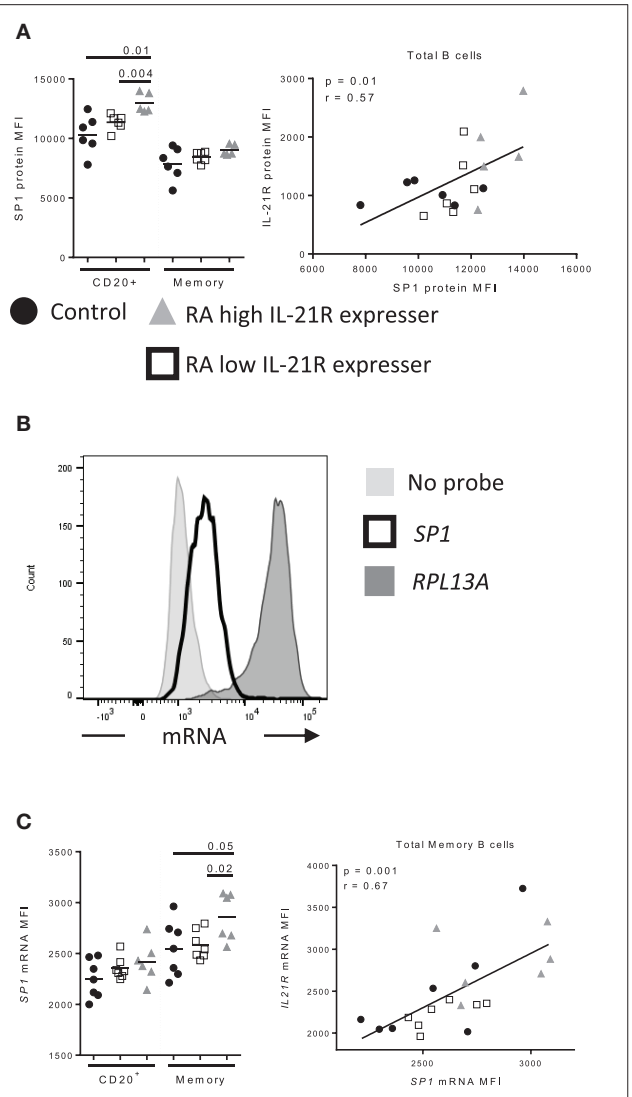

FIGURE 7 | Increased SP1 expression correlates with increased IL-21R expression in memory B cells in RA subjects **(A)** SP1 protein levels were assessed by flow cytometry in control ($n = 6$, black circles), RA low IL-21R expressers ($n = 6$; open squares) and RA high IL-21R expressers ($n = 5$; gray triangles) in $CD20^+$ and memory B cells ($CD20^+CD38^-CD24^+$) (left). SP1 protein levels from left were correlated with IL-21R protein expression in total B cells in RA and control subjects ($n = 17$) (right). **(B)** Representative histogram of mRNA levels from a no probe negative control, *SP1* and positive control probe, *RPL13A*. **(C)** (left) *SP1* mRNA levels were determined in total memory B cells in controls ($n = 7$, black circles), RA low IL-21R expressers ($n = 7$; open squares) and RA high IL-21R expressers ($n = 6$; gray triangles). (right) *SP1* mRNA levels from left were correlated with IL-21R protein expression in memory B cells in RA and control subjects combined ($n = 20$). Significance was determined using Mann Whitney U tests (to compare RA-IL-21^{high} to controls and RA-IL-21^{low}) and correlations were assessed with Pearson correlations.

(**Figure 8A**). There were no significant differences in SP1 binding when we analyzed sites outside the *IL21R* promoter region (**Figure S8**). Importantly, there was a strong and significant correlation between IL-21R and SP1 binding to the *IL21R* promoter (**Figure 8B**).

To explore this mechanism further, we used okadaic acid to determine whether inhibition of SP1 dephosphorylation would result in a decrease in IL-21R on B cells. Okadaic acid specifically blocks protein phosphatase 2A (PP2A) which has been shown to control the dephosphorylation of SP1 (33). Dephosphorylation of SP1 has been previously reported to be required for SP1 induced IL-21R in T cells (19). We observed a significant reduction in the levels of protein for both SP1 and IL-21R in controls, RA-IL-$21R^{low}$ and IL-$21R^{high}$ subjects following okadaic acid treatment which was normalized to the DMSO control (**Figure S9**). These findings suggest that enhanced binding of SP1 to the *IL21R* promoter may be responsible for increased IL-21R expression in B cells in RA.

DISCUSSION

We find that RA subjects with elevated IL-21R expression demonstrate hyper-responsiveness to IL-21, increased plasmablast differentiation and IL-6 production. These features could participate in early RA by promoting the development of autoantibodies and the persistent inflammation in late disease via the production of IL-6. These findings are consistent with the observation that circulating plasmablasts are increased in the setting of active RA disease and supports a role for the IL-21/IL-21R pathway in autoantibody formation in RA (34). A role for IL-21 signaling in autoantibody production is supported by prior work showing that serum IL-21 has a significant, albeit modest, correlation with CCP antibodies and a trend suggesting correlation with rheumatoid factor IgG antibodies in a previous study (13). Similarly, we found a modest correlation between IL-21R on RA memory B cells and levels of rheumatoid factor IgM in our study.

The correlation between memory and naïve B cell IL-21R expression in RA subjects suggests that IL-21R expression is typically downregulated during development/activation (20). In our study, IL-21R expression is maintained as naïve B cells progress to memory B cells, which appears to result in enhanced plasma cell differentiation. Enhanced IL-21 expression has also been shown to participate in the generation of autoreactive $CD11c^{hi}$t-bet^+ B cells as described in human SLE (35). Interestingly, these cells can be induced by IL-21 (36, 37) or IFN-γ (36, 38), and are present in healthy control subjects (39). Other studies have shown that $CD11c^{hi}$t-bet^+ B cells are expanded in SLE (35, 40) and MS (41). These cells have also been characterized in RA by their low expression of CD21 where we previously demonstrated an increased frequency of these cells (39). Given the involvement of IL-21 in the development of $CD11c^{hi}$t-bet^+ B cells, our study suggests that the enhanced IL-21R we observe may contribute to the development of these autoreactive cells. Further investigation is needed to determine how enhanced IL-21R signaling contributes to the development of these cells in RA.

Multiple factors may influence the enhanced plasmablast development and IL-6 production by B cells in RA patients. Our group previously observed an increase of approximately 2% in unconventional memory cells ($CD19^+CD27^-CD21^-$) in RA

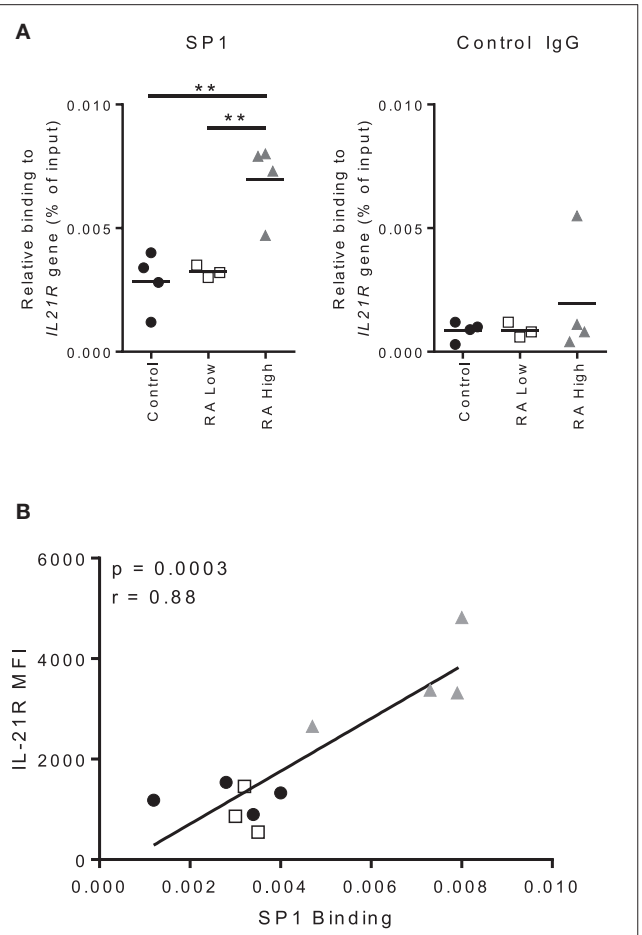

FIGURE 8 | Increased IL-21R expression correlates with increased SP1 binding to the IL-21R promoter in RA. **(A)** Total B cells were isolated from whole blood from RA patients and controls. SP1 binding to the IL21R promoter region and negative control IgG binding were determined using ChIP-qPCR analysis. The results are presented relative to input DNA. Significance was assessed using the student's *t*-test; $n = 4$ [Control (black circle), RA High (gray triangle)] $n = 3$ [RA Low (white square)]. $^{**}p < 0.01$. **(B)** Correlation between IL-21R expression in total B cells as assessed by flow cytometry and SP1 relative binding to the IL21R gene as assessed by ChIP-qPCR and presented in A. One subject from the RA low cohort was excluded due to low purity of the B cell population. Significance was determined using Mann Whitney U tests **(A)** and Pearson correlations **(B)**.

subjects compared to controls in the naïve B cell population (39). Unconventional memory cells have the capacity to rapidly mature into plasma cells (42). The frequency of unconventional memory cells in the maturation cultures, while not zero, is likely too small to make an impact. Further, *in vivo*, increased IL-6 production in response to IL-21, by both memory B cells and plasmablasts, may be a driving factor in the expansion of T follicular helper and Th17 cells in RA and ultimately promote B cell maturation and autoantibody production (12, 13, 32, 43, 44).

SLE subjects demonstrated enhanced IL-21R expression on total $CD19^+$ B cells compared to controls while MS subjects did not. This finding was modest, and not observed in memory or naïve B cell subsets. Notably, there are several papers in the literature that have assessed IL-21R expression in SLE subjects. These papers provide opposing views to whether SLE subjects express increased, decreased or no difference in expression compared to controls (45–47). Le Coz et al. only observed an increase on memory B cells in SLE subjects compared to controls (46). SLE is a heterogeneous disease and our cohort was drawn from our clinic population which was not controlled for disease activity at time of draw, disease duration or organ involvement. The analysis of SLE subjects is further complicated by the involvement of the *IL21R* SNP rs3093301 in SLE (48, 49), which is not associated with RA or MS. It is interesting to note that that about 44% of RA subjects will develop at least one SLE criteria in addition to arthritis (50). This suggests there may be similar pathogenic mechanisms between diseases (50) and our findings presented here suggest that the mechanisms of IL-21R regulation may be shared between SLE and RA.

In this study we determined the mechanism that leads to enhanced IL-21R in RA, show that IL-21R mRNA levels are increased in RA B cells and investigated the role of SP1 in the regulation of IL-21R expression. Augmented transcriptional activity of SP1 has been described in fibroblast-like synovial cells and in bone marrow mononuclear cells in RA (51–53). T cell receptor induced SP1 activity has been shown to mediate IL-21R expression in human T cells (19). Our finding that SP1 regulates the maintenance of baseline IL-21R in human B cells in RA is, to our knowledge, a novel insight as the role of SP1 in induction of IL-21R in B cells and its role in modulating IL-21R expression in RA has not been investigated. Chronic inflammation experienced by RA subjects may drive SP1 and IL-21R expression, promoting IL-6 production and the expansion of the autoantigen specific B cells ultimately contributing to the expanding specificities of ACPA and the maturation of high affinity responses seen in RA over time. Alternatively, genetic or epigenetic factors may lead to SP1 driven IL-21R expression early in disease in the absence of inflammation. In this study, the role of IL-21R expression in the development of RA was not investigated. In this setting, increased IL-21R expression on B cells could play a role in the development of autoantibodies, epitope spreading and the increased inflammatory mediators (54) seen in progression to disease.

Other mechanisms may contribute to increased IL-21R expression or IL-21 responsiveness in the setting of RA. We observed a significant decrease in *SOCS3* in B cells in RA. As *SOCS3* is a negative regulator of IL-21 signaling, it may contribute to the enhanced response to IL-21 seen in RA B cells. Prior studies have found *SOCS1* and *SOCS3* transcripts in PBMC to be increased in RA (55). Due to the relatively small percentage of B cells in PBMC, it is unlikely that the *SOCS1* and *SOCS3* expression in B cells would be reflected in this analysis. No differences in miR155 expression between RA and controls in our cohort were observed which is in contrast to a report from Alivernini's group that miR155 levels are elevated in B cells in RA (28). These differences may be due to cohort characteristics and/or the method of miR155 detection; we used miRNA flow cytometry whereas Alivernini's group used qPCR. In either case, a decrease in *SOCS3* expression in RA could contribute to enhanced IL-21/IL-21R signaling.

Defining the role of IL-21/IL-21R signaling in RA is important because of the interest in blocking this pathway therapeutically by targeting IL-21, IL-21R (56) and the Janus kinases (JAK) (57, 58). Two JAK inhibitors are approved for patients with RA (Tofacitinib and Baricitinib) and both demonstrate efficacy as defined by improvements in DAS28-CRP scores (59–62); however, these therapies influence multiple cytokine signaling pathways, increasing the importance of understanding the impact of alterations in the IL21/IL-21R signaling pathway on disease development and progression. Studies as we present here could shed light on patients most likely to respond to IL-21 targeted therapies, and the ideal time point for intervention. Our findings in a cross-sectional cohort of individuals with RA and controls suggest that IL-21 based therapies for RA may be more efficacious if targeted to individuals with increased IL-21R expression on memory B cells; studies that evaluate response to therapy, requiring a longitudinal cohort with samples collected before and after initiation of therapy would be of particular interest.

We have shown that IL-21R expression is increased on total and memory B cells in peripheral blood and synovial fluid from RA subjects. Increased IL-21R in peripheral blood correlates with enhanced response to IL-21 with respect to *PRDM1* expression, plasmablast differentiation and IL-6 production. Mechanistically we showed that the increased IL-21R was due to enhanced binding of the transcription factor, SP1. Together, these studies highlight the importance of IL-21 signaling in B cells and provide insight into how to target therapies to the IL-21 signaling pathway in RA.

ETHICS STATEMENT

All subjects gave written informed consent in accordance with the Declaration of Helsinki and according to Institutional Review Board-approved protocols at the Benaroya Research Institute.

AUTHOR CONTRIBUTIONS

ED designed the research studies, conducted experiments, acquired data, analyzed data, and wrote the manuscript. AM conducted experiments, acquired data, and analyzed data. AH wrote the manuscript. JC recruited subjects. JB recruited subjects, designed the research studies and wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2018.01978/full#supplementary-material

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Title: Citrullinated Aggrecan Epitopes as Targets of Auto-reactive CD4+ T cells in Patients with Rheumatoid Arthritis

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ABSTRACT

Objective. Recognition of citrullinated antigens such as vimentin, fibrinogen, and alpha-enolase is associated with rheumatoid arthritis (RA). Emerging data suggests that the matrix protein aggrecan is also recognized as a citrullinated antigen. The goal of this study was to directly visualize cit-aggrecan specific T cells and characterize them in subjects with RA.

Methods. Citrullinated aggrecan peptides with likely DRB1*04:01 binding motifs were predicted using a previously published scanning algorithm. Peptides with detectable binding were assessed for immunogenicity by HLA tetramer staining, followed by single cell cloning. Selectivity for citrullinated peptide was assessed through tetramer staining and proliferation assays. E*x vivo* tetramer staining was then employed to assess frequencies of aggrecan specific T cells in peripheral blood. Finally, disease association was assessed by comparing T cell frequencies in RA patients and controls and correlating aggrecan specific T cells with levels of aggrecan specific antibodies.

Results. We identified six immunogenic peptides, two of which were the predominant T cell targets in peripheral blood. These two epitopes were citrullinated at HLA binding residues and shared homologous sequences. RA patients had significantly higher frequencies of cit-aggrecan-specific T cells than healthy subjects. Furthermore, T cell frequencies were significantly correlated with antibodies against citrullinated aggrecan.

Conclusion. Our findings indicate that T cells that recognize citrullinated aggrecan are present in subjects with RA and correlate with antibodies that target this same antigen. Consequently, aggrecan-specific T cells and antibodies are potentially relevant markers that could be utilized to monitor patients with RA or at risk subjects.

Rheumatoid arthritis (RA) is a chronic disease in which joints are destroyed through inflammatory processes (1). Serological markers, including rheumatoid factor and anti-citrullinated protein antibodies (ACPA) and strong association with high risk alleles such as *HLA-DRB1*04:01* implicate autoreactive CD4+ T cells as an important facet of disease etiology (2, 3). Detailed studies of ACPA specificity establish that vimentin, fibrinogen, and α-enolase are recognized as citrullinated antigens (4-6). These proteins are also recognized by autoreactive CD4+ T cells, supporting the notion that T cells provide help for antibody responses in RA (7). Furthermore, T cell frequency has been shown to be influenced by disease duration and therapy, suggesting that changes in T cell number and function are reflective of changes in the overall disease process (8).

Emerging data suggests that the matrix protein proteoglycan aggrecan is recognized as a citrullinated antigen (7, 9). Aggrecan is an abundant component of extracellular matrix within the joints (along with proteins such as tenascin-C and type II collagen) and has been conclusively shown to be citrullinated in human articular cartilage (10). The presence of aggrecan fragments has been documented within synovial fluid and is reported to increase with age (11, 12). Furthermore, immunization with aggrecan was shown to induce arthritis in murine models (13). Autoantibodies to citrullinated epitopes within the G1 domain are elevated in RA but

not in Osteoarthritis, indicating that such antibodies are specifically associated with autoimmunity rather than merely accompanying joint damage (10). Citrullinated CD4+ T cell epitopes from aggrecan have been described and elevated responses to some were shown to be associated with RA (9, 14, 15). However, a systematic HLA-specific characterization of aggrecan derived T cell epitopes has yet to be performed and tools to directly visualize aggrecan specific T cells have yet to be developed. The goals of our study were to define citrullinated aggrecan epitopes in the context of DRB1*04:01, to visualize and characterize cit-aggrecan-specific T cells, and to investigate their relevance in subjects with RA.

MATERIALS AND METHODS

Epitope prediction and peptide synthesis. A previously described prediction method was utilized to identify citrullinated aggrecan peptides with motifs likely bind to HLA-DRB1*04:01 (henceforth DR0401) (8, 16) . Briefly, motif scores were calculated by multiplying coefficients corresponding to each anchor residue for all possible core 9-mers within the protein that included an internal or flanking arginine (R) or citrulline (Cit) residue. A total of 28 peptides with motif scores of 0.1 or higher were synthesized by Mimotopes. For tetramer production and further studies, selected citrullinated peptides and their corresponding native peptides were re-synthesized by Sigma. All peptides were dissolved in DMSO to a stock concentration of 20 mg/ml.

Peptide binding to DR0401. The binding capacity of citrullinated aggrecan peptides to DR0401 was assessed using a previously described assay (17). Briefly, candidate peptides were plated at increasing concentrations against a fixed

concentration of a biotinylated reference peptide, influenza $HA_{306-318}$ (PKYVKQNTLKLAT) in wells coated with anti-HLA-DR antibody (Clone L243, supplied by the Benaroya Research Institute (BRI) Tetramer Core). Europium conjugated streptavidin was used to label residual HLA-bound biotinylated peptide (PerkinElmer) and quantified using a Wallac Victor 2 Multi-label Counter. Binding curves were fitted by a sigmoidal regression model using Graphpad Prism 7.0 and EC50 values were calculated as the peptide concentration needed to displace 50% of the reference peptide.

Subject recruitment. Control and RA subjects were recruited with informed consent through the BRI healthy control and rheumatic disease registries. Sample use was approved by the BRI Institutional Review Board. All subjects had *HLA-DRB1*04:01* haplotypes. Control subjects had no autoimmune disease, no first degree relatives with RA, and ranged in age from 23 to 66 years (mean ± SD; 50.2 ± 10.9 years). All RA patients were ACPA positive, ranged in age from 25 to 71 years (mean ± SD; 52.5 ± 11.3 years), and met 1987 American College of Rheumatology criteria (1).

PBMC isolation and preparation. PBMC were separated from whole blood over Ficoll-Hypaque gradient, cryopreserved in heat inactivated fetal bovine serum supplemented with 10% DMSO, and stored in liquid nitrogen. PBMC were subsequently thawed at 37°C, washed with RPMI media supplemented with 10% FBS and 0.001% DNase/Benzonase (Sigma Aldrich), and re-suspended in RPMI media supplemented with 10% human serum (Gemini Bio-Products).

Tetramer production. Class-II tetramers were generated by the BRI Tetramer Core as previously described (18). Briefly, DR0401 monomer was purified from insect cell cultures and biotinylated at a sequence-specific site. Biotinylated monomer was loaded with 0.2 mg/ml of peptide, incubating at 37°C for 72 hours in the presence of 2.5 mg/ml n-octyl-β-D-glucopyranoside and 1 mM Pefabloc SC (Sigma-Aldrich). Loaded monomers were conjugated into tetramers with fluorescently labeled streptavidin for 6-18 hours at room temperature at a molar ratio of 8:1.

***In vitro* PBMC expansion, HLA class-II tetramer staining and T cell clone isolation.** PBMC were cultured at $5x10^6$ cells/well in 48-well plates in 1 ml RPMI supplemented with 10% human serum and 10µg/mL of peptide. On day 6, cells were transferred to fresh wells and 10 U/ml of rIL-2 (Roche) was added. After 14 days, cultures were screened by staining a 50 µl aliquot with 0.5 µl of tetramer (final concentration 5 ng/ml) at 37°C for 1 hour, co-stained with anti-CD4 APC (BD Biosciences) for 30 minutes at 4°C in the dark, washed, and analyzed by flow cytometry. To isolate T cell clones, staining was repeated and tetramer-labeled cells were single cell sorted using a BD FACS Aria II into 96-well round bottom plates containing 150 µl of human T cell media and expanded by adding 10^5 irradiated feeders, 10 U/ml IL-2 and 2 µg/ml phytohaemagglutinin (PHA, Thermo). After 10-14 days, expanded cells were transferred to 96-well flat bottom plates and split as needed for an additional 14 days. The resulting clones were re-screened by tetramer staining. Positive clones were further expanded by additional rounds of PHA stimulation and then cryopreserved.

Proliferation assays. To assess proliferation in response to citrullinated, partially citrullinated, or unmodified peptide, clones or cells were plated at $2.5x10^4$ cells/well in 96-well round bottom plates in human T cell media with 10^5 irradiated antigen presenting cells from a *HLA-DRB1*04:01* positive donor plus peptide and incubated at 37°C. After 72 hours, 1 µCi of [^{3}H]-thymidine was added and incubated for 24 hours. Cells were then washed, water lysed, and DNA was collected onto glass fiber filter membranes (PerkinElmer) using a plate harvester. Each filter mat was immersed in Betaplate Scint (PerkinElmer) and counts collected on a Wallac 1450 LSC and Luminescence Counter (PerkinElmer). Stimulation index was determined by calculating the ratio of counts of peptide-stimulated cells to counts of non-stimulated cells.

Intracellular cytokine staining of T cell clones. To assess their functional profiles, T cell clones were activated with 50 ng/mL phorbol myristic acid (PMA) and 1 µg/mL ionomycin for 30 min, treated with brefeldin A (eBioscience) and incubated for 3 h at 37°C. Cells were fixed using fixation/permeabilization buffer (eBioscience), washed in permeabilization buffer (eBioscience), stained using antibodies to GM-CSF (PerCP-Cy5.5), granzyme B (FITC), IFNγ (AF700), IL-4 (AF647), IL-21 (PE) (all from Biolegend), collected on an LSR II flow cytometer (BD Biosciences), and analyzed using FlowJo (TreeStar).

Activation of T cell clones by fibroblast-like synoviocytes (FLS). Human FLS were HLA typed and isolated as previously described (19), were co-cultured with T cell clones by plating 1 x 10^5 FLS (Passage 10 0401, passage 12 non-0401) with 2 x 10^5 T cells per well, and incubated in the presence or absence of anti-DR

antibody (L243, BRI Tetramer Core, 20 µg/ml) or corresponding peptide as a positive control for 5 days. Clones and FLS were also cultured individually to assess background. On day 4, 1 µCi of [^{3}H]-thymidine was added to each well. Cells were harvested after 24 hours and counts collected on day 5 as described above.

Gene expression of aggrecan by fibroblast-like synoviocytes (FLS)

Human FLS were plated on a single 100mm tissue culture dish (BD). Once 80% confluent, cells were treated with 1000 Units/ml interferon gamma (RnD #285-IF) for 72 hours upon which cells were treated with trypsin and RNA was extracted using a Qiagen RNeasy Mini Kit (Qiagen #74134) following manufacturer's protocol. RNA was quantified using a Nanodrop, cDNA was synthesized using ThermoFisher SuperScript IV reverse transcriptase kit (Thermo #18091050) as per manufacturer instructions in a PTC-225 Peltier thermal cycler. cDNA was probed using TaqMan Fast Advanced Master Mix (Applied Biosystems #4444557) in an Applied Biosystems 7500 Fast Real-Time PCR System cycler with the following Applied Biosystems Taqman primers: VIC-MGB GAPDH Hs02786624_g1, FAM-MGB PDPN Hs00366766_m1, FAM-MGB CD3E Hs01062241_m1, FAM-MGB ACAN Hs00153936_m1, FAM-MGB VCAN Hs00171642_m1. Gene expression were assessed by comparing mean Ct values to mean Ct of GAPDH. Ct values defined as "undetermined" by 7500 software v2.3 assigned a default value of "40" or above 35 were considered "undetectable".

***Ex vivo* detection of cit-Aggrecan-specific T cells.** *For ex vivo* detection of antigen-specific T cells, 3.5 x 10^7 PBMCs were thawed and rested for 2 hours at 37°C, re-suspended in 200 µl of T cell media and treated with Dasatanib for 10

minutes at 37°C to prevent internalization of T cell receptors. Cells were stained by adding 4.5 µl of each tetramer (final concentration 11 ng/ml) at room temperature for 90 minutes, with gentle manual shaking every 15 minutes. Cells were then labeled with anti-PE and anti-Myc magnetic beads (Miltenyi) for 20 minutes at 4°C, enriched on a magnetic column according to manufacturer's protocols (Miltenyi), reserving a 1% cell fraction before enrichment to estimate the total number of CD4+ T cells in the sample. Cells were surface-stained for 30 minutes at 4°C with CD14/CD19/Annexin V-FITC (all from Biolegend), CD4-V500 (BD), CD45RA-AF700 (BD), and CCR7-APC/Cy7 (Biolegend). Samples were collected to completion on a BD FACS Canto II. Flow cytometry data was analyzed using FlowJo v10 and Graphpad Prism 7.0. The frequency (F) of antigen specific T cells was calculated as: F = (1,000,000 x tetramer positive events from enriched sample)/(100 x number of CD4+ cells from the non-enriched fraction). Supplemental Figure 1 depicts the gating strategy.

IgM Depetion and Serum Antibody Detection. IgM from serum samples of RA patients and healthy control subjects (HC) were depleted via immuno-absorption. Briefly, diluted sera were incubated with goat anti-human IgM (µ chain-specific) antibody conjugated to agarose beads (Millipore Sigma) for 90 minutes at 4°C on a rotary wheel. IgM depletion was verified by anti-IgM Western blot. For ELISA, wells of 96-well ELISA plates (Nunc) were coated overnight with recombinant human (rh)-aggrecan G1 or citrullinated rh-aggrecan G1 domain (0.2 µg/well each) in 100 µl/well of 0.15 M sodium carbonate buffer (pH 9.6) at room temperature (10). Unbound antigen was removed by washing with HRP wash buffer (Inova Diagnostics). Wells were blocked with heat-inactivated normal goat serum (R & D Systems) diluted to

1:10 in HRP sample diluent (Inova Diagnostics) at for 2 hours at room temperature. IgM-depleted serum samples were diluted 1:100 in sample diluent and incubated with the antigen-coated wells (100 µl/well, duplicate wells) for 2 hours at room temperature. Bound IgG was detected by incubation with 100 µl/well of HRP-conjugated polyclonal goat anti-human IgG (Abcam) at 1:3,000 dilution for 1 hour at room temperature. Unbound material was removed with HRP wash buffer between each of these steps. The color reaction was developed by incubation with 100 µl/well 3,3',5,5'-tetramethylbenzidine (BD OptEIA TMB substrate set) for 10 minutes in the dark at room temperature and stopped with 25 µl/well stop solution (4N HCl). Absorbance at 450 nm was read in a Synergy 2 ELISA reader (BioTek Instruments). Net optical density (ΔOD) values were calculated by subtracting the OD of wells not containing samples (but coated with rhG1 or CitrhG1 and reacted with HRP secondary antibody) from the OD values of serum samples.

Anti-IgM Western Blot

Paired non-depleted and IgM-depleted serum samples (60 µg protein each) were run in a 7.5% SDS-PAGE gel under reducing conditions. The proteins were transferred to a PVDF membrane, probed with HRP-conjugated goat anti-human IgM µ chain-specific antibody (Abcam) and visualized by enhanced chemiluminescence (ECL, Amersham)

Statistical Methods. All statistical tests were performed using PRISM version 7 software (GraphPad). Tests that were used (as appropriate) included unpaired t tests, unpaired t tests with Welch's correction, Mann-Whitney tests, or ANOVA with Sidak's multiple comparisons test, and Spearman correlation. P values

< 0.05 were considered significant. Data are reported as the mean ± standard error of the mean.

RESULTS

Aggrecan peptides bind to DR0401 and are immunogenic. Following our previously described approach for identifying epitopes from joint-associated antigens (8, 16), we synthesized 28 citrullinated peptides corresponding to aggrecan sequences that contain DR0401 binding motifs (Supplemental Table 1). Detectable *in vitro* binding to recombinant DR0401 protein was confirmed for thirteen of these peptides; among these, three were preferentially bound in their citrullinated form (Table 1). To assess the immunogenicity of these arginine-containing (arg-Agg) or citrullinated aggrecan (cit-Agg) peptides, PBMC from DR0401 positive RA patients and healthy controls were stimulated *in vitro*. For cit-Agg or arg-Agg peptides with detectable HLA binding, expansion of epitope specific T cells was evaluated through HLA class II tetramer staining. Possible responses to arg-agg peptides that corresponded to cit-Agg peptides but lacked detectable binding were evaluated through proliferation assays, but no responses above background proliferation were observed (Supplemental Figure 2A). Tetramer staining indicated that aggrecan peptides display varying degrees of immunogenicity (Figure 1A). Two arg-Agg peptides occasionally elicited responses (Figure 1B), but all median responses were well below our established cutoff and consequently were not advanced for further study. In total, seven cit-Agg elicited median responses in subjects with RA that were above a previously established cutoff (Figure 1C). Among these, six were verified through isolation T cell clones (Supplemental Figure 2B) and were advanced for further study. Examining *in vitro* responses to cit-Agg peptides, responses were

also detectable in HLA matched controls (Figure 1D) but were more frequently seen in subjects with RA (Figure 1E).

In toto, our approach identified six citrullinated aggrecan peptides that bound to DR0401 and elicited *in-vitro* T cell responses. Two of these epitopes exhibited preferential binding to DR0401. For the remaining peptides, the citrulline residues within the predicted binding register are positioned as T cell contacts (Table 2). Some of these immunogenic cit-Agg peptides overlapped with previously described epitopes. In particular, our cit-Agg_{200}, cit-Agg_{298} and cit-Agg_{621} peptides correspond to epitopes described by Boots et al. (20). The cit-Agg_{298} peptide also overlaps with the p49 aggregan sequence reported by Markovics et al. (MDMCSAGWLAD(Cit)SVR) (9). Surprisingly, the cit-Agg_{84} peptide (VVLLVATEG(Cit)VRVNSAYQDK) described by Law et al bound with very low affinity. (21) (Not shown).

Aggrecan specific CD4+ T cells are citrulline selective and respond to FLS-derived antigens. To assess the selectivity of aggrecan specific T cells, clones corresponding to each specificity were tested by proliferation assay for their responsiveness to cit-Agg or the corresponding arg-Agg peptide. Each clone preferentially responded to citrullinated peptide (Figure 2A). To assess their function, we characterized the cytokine profiles of aggrecan-specific T cell clones by intracellular staining. Following activation with PMA/ionomycin, the clones predominantly secreted IFNγ and GM-CSF with lesser amounts of granzyme B and IL-4 (Supplemental Figure 2C). It is possible that a less robust antigen specific activation of the clones would have elicited a more focused cytokine response. To assess whether the cit-Agg epitopes that we identified are naturally processed and

presented, we utilized co-culture experiments with aggrecan-specific clones with human FLS cells. FLS lines isolated from DR0401+ and DR0401- donors had detectable levels of aggrecan mRNA (albeit lower than versican), expressed podoplanin, and (as expected) lacked CD3e expression (Figure 2B). Each clone showed increased proliferation when co-cultured with DR0401+ FLS cells alone or pulsed with peptide or with cognate peptide presented by irradiated DR0401+ feeder cells and this proliferation was blocked by an anti-HLA-DR antibody (Figure 2C and 2D). Clones with all six specificities (cit-Agg_{161}, cit-Agg_{200}, cit-Agg_{225}, cit-Agg_{520}, cit-Agg_{553}, and cit-Agg_{200}) had significantly higher levels of proliferation in response to DR0401+ FLS cells as compared with DR0401- FLS cells (Figure 2D). However, a negative control T cell clone that recognized an influenza epitope did not proliferate in response to FLS cells. These observations demonstrate that aggrecan is produced by FLS cells and that epitopes corresponding to cit-Agg peptides can be processed and presented by FLS cells.

T cells that recognize the dominant cit-aggrecan epitopes are more frequent in the peripheral blood of RA patients. To investigate the frequency of CD4+ T cells specific for citrullinated aggrecan epitopes in the peripheral blood of RA patients, *ex vivo* tetramer staining was performed. As an initial screen, we performed a grouped analysis, examining the combined T cell frequencies for groups of tetramers: cit-Agg_{225} and cit-Agg_{553} (both of which preferentially bound in their citrullinated form); cit-Agg_{161}, cit-Agg_{200}, cit-Agg_{520} and cit-Agg_{621} (which bound in both their citrullinated and unmodified forms), and cit-Agg_{84} (which bound with very low affinity to DR0401). Previously published work (22) and our preliminary assays (Supplemental Figure 3) demonstrate that this tetramer staining approach is reliable.

The observed frequencies demonstrated a hierarchy in which the cit-Agg_{225} and cit-Agg_{553} epitopes stood out as the dominant specificities. T cells specific for the other aggrecan epitopes were present, but at lower frequencies that in some cases was near the threshold of detection (Figure 3A). Additional *ex vivo* analysis of 12 *HLA-DRB1*04:01* positive RA subjects showed that cit-Agg_{553}-specific T cells were significantly higher in frequency than cit-Agg_{225}-specific cells suggesting that the cit-Agg_{553} epitope is the most prominent specificity (Figure 3B).

Previous studies have reported immunogenic aggrecan peptides and demonstrated responses to citrullinated aggrecan in samples from patients with RA (9, 10, 23). To corroborate the relevance of citrullinated aggrecan epitope-specific T cells, we compared the frequency of T cells specific for cit-Agg_{225} and cit-Agg_{553} in RA patients and healthy controls with *DRB1*04:01* haplotypes. We observed that patients had significantly higher T cell frequencies than controls (Figure 3C). These differences did not merely reflect global differences between patients and controls, as no difference was observed for T cells specific for the immunodominant influenza epitope.

The dominant citrullinated aggrecan epitopes have similar sequences but are recognized by distinct T cell clones. The cit-Agg_{225} and cit-Agg_{553} peptides are derived from the G1 and G2 domains respectively, but have homologous sequences, raising the possibility that these peptides could be cross-recognized by overlapping sets of T cells. Based on binding assays and proliferation assays with variants of these two sequences, we identified truncated peptides that met minimum requirements for binding to DR0401 and recognition by cit-Agg_{225} or cit-Agg_{553} specific T cell clones. These results (Supplemental Table 2) were

consistent with the predicted minimal motifs: YGI(Cit)DTNET for cit-Agg_{225} and YGV(Cit)PSTET for cit-Agg_{553}. Based on these results, both peptides bind to DR0401 in similar registers. These registers predict that a citrulline residue would be positioned within binding pocket 4. This supposition is further supported by the fact that citrullination of the corresponding arginine residue is required for binding. Despite sharing 10 of 14 residues within their minimal peptides (Supplemental Figure 4A), the cit-Agg_{225} and cit-Agg_{553} peptides were not cross recognized by T cell clones (Supplemental Figure 4B). Furthermore, *ex vivo* tetramer staining showed no significant signs of cross-reactivity (Supplemental Figure 4C). Therefore, these sequences appear to represent distinct epitopes, in spite of their considerable homology.

Citrullinated aggrecan-specific T cell frequency correlates with aggrecan-specific antibodies. A recent study demonstrated that, although ACPA+ RA patients had increased levels of antibodies against a citrullinated aggrecan epitope, there was no clear correlation between antibodies and T cell reactivity with the same epitope in these patients (9). Having identified two dominant cit-Agg T cell epitopes, we were curious to explore correlations between T cell and antibody responses against aggrecan. Therefore, we measured citrullinated aggrecan antibody levels in the serum and the frequency of T cells that recognize cit-Agg_{225} and cit-Agg_{553} in matched samples. IgM-depleted serum samples (Supplemental Figure 5A) from 18 healthy control and 21 ACPA+ RA subjects were screened for the presence of IgG antibodies against native and citrullinated G1 domain of aggrecan (an assay for G2 domain antibodies has yet to be developed). Based on either background adjusted OD (Figure 4A) or raw OD (Supplemental Figure 5B) RA

patients had significantly higher levels of antibodies against both the native and citrullinated G1 domain of aggrecan than HLA matched controls. Levels of antibody recognition of citrullinated and native aggrecan were not different. Notably, we observed a positive correlation between the frequency of aggrecan-specific memory T cells and antibody levels in subjects with RA (Figure 4B). This correlation was absent in controls, due at least in part to their decreased levels of antibody and lower T cell frequencies.

DISCUSSION

Previous studies have indicated that T cell and antibody responses against citrullinated aggrecan contribute to the loss of peripheral tolerance in patients with RA. Of note, Boots et al. assessed the HLA binding of native (non-citrullinated) aggrecan peptides to DR0401 (and also DRB1*04:04 and DRB1*01:01) and identified multiple aggrecan peptides as being immunogenic in the context of DR0401 (20). That study, which focused on unmodified (rather than citrullinated) peptides, observed proliferative responses that were lower in RA patients than in controls. Buzas et al. identified the $Agg_{84\text{-}103}$ peptide as an immunodominant epitope recognized by BALB/C mice immunized with human aggrecan, and observed that some aggrecan peptides were "conditionally immunogenic" in that responses were only elicited in arthritic animals (15). Subsequently, Law et al. examined responses to citrullinated $collagen_{1237\text{-}1249}$, $vimentin_{66\text{-}78}$, $aggrecan_{84\text{-}103}$ and $fibrinogen_{79\text{-}91}$ in RA patients and in a limited number of controls, observing that the citrullinated aggrecan peptide was the most immunogenic, eliciting IL-6 and TNF-alpha secretion that was not observed in response to the corresponding unmodified peptide (21). This result appears to imply that functional responses to aggregan are citrulline selective.

However, the T cell responses observed in the study by Law et al. were not exclusively restricted to DR0401; 10 of the 21 RA subjects sampled were not DR0401 positive and among those 4 were DR4 negative.

Our present study provides new evidence that CD4+ T cells selectively recognize citrullinated aggrecan epitopes in the context of the high risk *DRB1*04:01* haplotype. We identified six aggrecan peptides that are recognized by T cell clones from subjects with RA when crucial arginine residues are converted to citrulline. Citrullinated residues could increase recognition by enhancing HLA binding or alter peptide recognition by modulating TCR interactions (depending on the positioning of the residue within its HLA binding motif). Indeed, two of the aggrecan epitopes identified through our study (cit-Agg_{225} and cit-Agg_{553}) bound to DR0401 and elicited T cell responses only in their citrullinated form. The remaining aggrecan epitopes (cit-Agg_{161}, cit-Agg_{200}, cit-Agg_{520}, and cit-Agg_{621}) bound comparably in their citrullinated and unmodified forms and were citrullinated at predicted TCR contact residues. Among these Agg_{200} and Agg_{621} were able to elicit T cell responses in a limited number of subjects. Since cit-Agg_{225} and cit-Agg_{553} specific T cells were present at significantly higher frequencies in RA patients than other cit-Agg specificities, it could be suggested that the poor HLA binding of the unmodified Agg_{225} and Agg_{553} peptides may lead to impaired T cell tolerance to the corresponding cit-epitopes. In spite of sharing considerable sequence homology, the cit-Agg_{225} and cit-Agg_{553} peptides originate from different domains of aggrecan and T cells specific for these peptides showed no evidence of cross-reactivity.

Cit-Agg-specific T cells were present at elevated frequencies in RA patients in comparison with HLA-DR matched healthy controls. The mere presence of CD4+ T cells that recognize citrullinated aggrecan does not guarantee that these cells play a

role in disease. However, T cell clones that recognize citrullinated aggrecan epitopes exhibited a Th1-like phenotype that is consistent with the functional phenotype that we previously observed for more established citrullinated antigens such as vimentin, fibrinogen, and enolase (8). In addition, T cell clones specific for all six of the citrullinated aggrecan peptides proliferated in response to DR0401+ FLS cells, suggesting that these epitopes can be naturally processed and presented to T cells by synovial cells. Interestingly, these same clones failed to proliferate in response to the corresponding arg-Agg peptides (Figure 2A), suggesting that aggrecan is citrullinated by FLS.

A recent study documented the presence of citrullinated aggrecan in human cartilage extracts and established an ELISA assay to detect unmodified or citrullinated aggrecan G1 domain-specific serum autoantibodies (10). Applying this methodology to analyze serum autoantibodies in patients and controls from our cohort, aggrecan G1 domain specific antibodies were present at significantly higher levels in RA patients than in HLA matched controls, but cit-Agg- and arg-Agg were recognized at similar levels, suggesting either that G1 domain specific antibodies cross-recognize native and citrullinated aggrecan or that there are roughly equivalent levels of antibodies that uniquely recognize native and citrullinated aggrecan respectively. We observed a significant positive correlation between cit-Agg-specific memory T cell frequency and antibody levels in RA patients (but not controls). This correlation raises the possibility that antigen specific T cell help may contribute to antibody responses. Cumulatively, our results demonstrate that T cell responses to cit-Agg are associated with RA. Such responses are of interest because aggrecan is an extracellular matrix proteoglycan that interacts with cells and extracellular molecules through its globular domains. Given that aggrecan fragments are present

within synovial fluid, this protein is apparently subject to fragmentation and release in inflamed joints, where it can be presented to T cells as a citrullinated antigen by either FLS or professional APCs.

Our study does have limitations. Although we were able to demonstrate differences in the frequency of cit-Agg specific CD4+ T cells in RA patients and HLA matched controls, the characteristics of the RA patients selected for our study did not allow us to effectively examine variations with respect to disease activity or severity. In general, our patients had well controlled disease, which may explain in part the overlap in aggrecan-specific T cell frequencies observed between controls and some patients. In addition, although it would be of considerable interest to document the presence of cit-Agg specific T cells within inflamed joints, synovial fluid and tissue samples from HLA typed subjects were not available for our study. However our *ex vivo* tetramer staining approach should be applicable to address these questions in future studies that utilize such samples.

Although previous studies have documented T cell responses to citrullinated aggrecan, our study represents the first HLA-controlled study to define disease relevant citrullinated aggrecan epitopes and to characterize T cells that recognize these peptides in subjects with established RA. To our knowledge, this is also the first study to demonstrate a direct correlation between T cell frequency and antibody responses against the same joint-associated antigen. Together, these results suggest that cit-Agg specific CD4+ T cells could play a role in the etiology of RA and that future studies of the frequency, phenotype, and role of cit-Agg-specific T cells in various stages of disease are warranted.

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Table 1. Binding characteristics of aggrecan peptides chosen for further study

Peptide*	Sequence	Cit (μM)	Arg (μM)
agg 3-20	TLLWVFVTL(Cit)VITAAVTV	35.30	36.06
agg 153-168	**IVFHY(Cit)AIST(Cit)YTLDF**	**0.98**	**50.96**
agg 161-178	ST(Cit)YTLDFD(Cit)AQ(Cit)ACLQ	0.99	1.02
agg 200-215	DAGWLADQTV(Cit)YPIHT	2.95	2.53
agg 225-244	**DEFPGV(Cit)TYGI(Cit)DTNETYDV**	**2.18**	**>100**
agg 298-313	SAGWLAD(Cit)SV(Cit)YPISK	1.90	2.94
agg 477	**GVVFHY(Cit)PGPT(Cit)YSLTF**	**14.69**	**>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 621	KCYAGWLADGSL(Cit)YPIV	6.90	4.66
agg 684	NSPFCLE(Cit)TPLGSPDPA	0.12	0.13

* Sequences that bind better as citrullinated peptides are shown in boldface

Table 2. Predicted motifs for immunogenic aggrecan peptides

Peptide	Sequence*
agg 161-178	ST(CIT)**Y**TLDFD(Cit)AQ(Cit)ACLQ
agg 200-215	DAG**W**LADQTV(Cit)YPIHT
agg 225-244	DEFPGV(Cit)T**Y**GI(Cit)DTNETYDV
agg 520	G**Y**EQCDAGWL(Cit)DQTV(Cit)YPIV
agg 553	PGV(CIT)T**Y**GV(Cit)PSTETYDVY
agg 621	KCYAG**W**LADGSL(Cit)YPIV

* Predicted DR0401 binding motifs are underlined with the first anchor shown in boldface

Figure Legends

Figure 1. Assessing the immunogenicity of aggrecan peptides. Peptides with positive binding to DR0401 were evaluated for immunogenicity by tetramer staining after 14 days of peptide stimulation. As demonstrated by the representative FACS plots, peptides elicited diverse levels of T cell expansion (A) that ranged from negligible expansion (upper left), modest expansion (upper middle), moderate expansion (upper right and lower right), to robust expansion (lower left); expansion in response to an immunodominant influenza peptide was used as reference (lower middle). (B) The candidate arg-agg epitopes exhibited relative low immunogenicity, with median responses that were less than 0.05% of total CD4+ T cells. (C) Several candidate cit-agg epitopes were immunogenic, exhibiting median responses that were above 0.05% of total CD4+ T cells. (D) The six most promising cit-agg epitopes elicited *in vitro* responses in some HLA matched controls. (E) The percentage of subjects with responses above the 0.05% cutoff was always higher for RA patients. Differences between RA patients and controls were significant for cit-161 and cit-520 (p-values 0.028 and 0.021 respectively) and approached significance for cit-553 and cit-621 (p-values 0.08 and 0.01 respectively, Fisher Exact test). Dotted lines in panels B-D indicate a threshold value of 0.05% used to define positive responses.

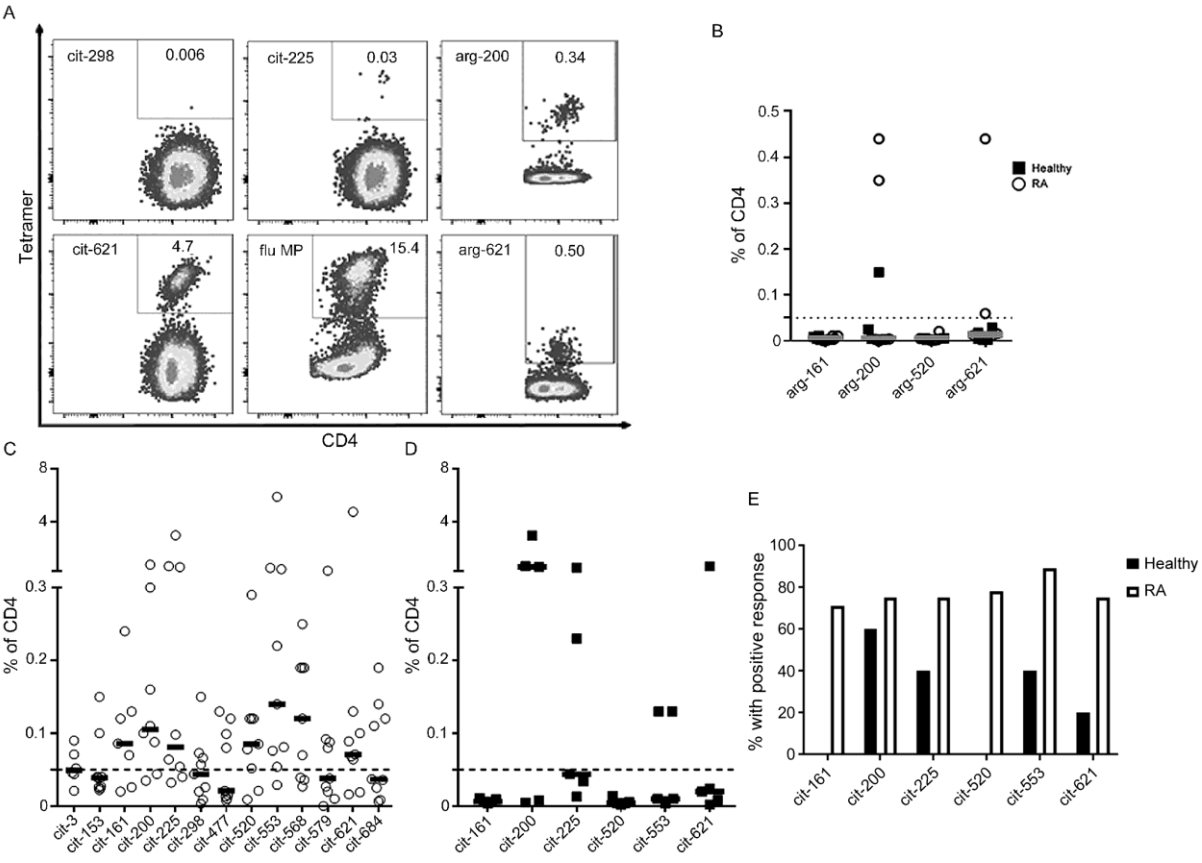
Figure 2. Aggrecan-specific T-cell clones respond when co-cultured with fibroblast-like-synoviocytes (FLS). (A) As a specificity control, each clone was stimulated with cit-agg or arg-agg peptide in the presence of HLADR0401+ APCs. (B) Aggrecan expression by DR0401 and Non-DR4 FLS lines was assessed by qPCR with GAPDH (reference), podoplanin (PDPN; positive control), CD3e

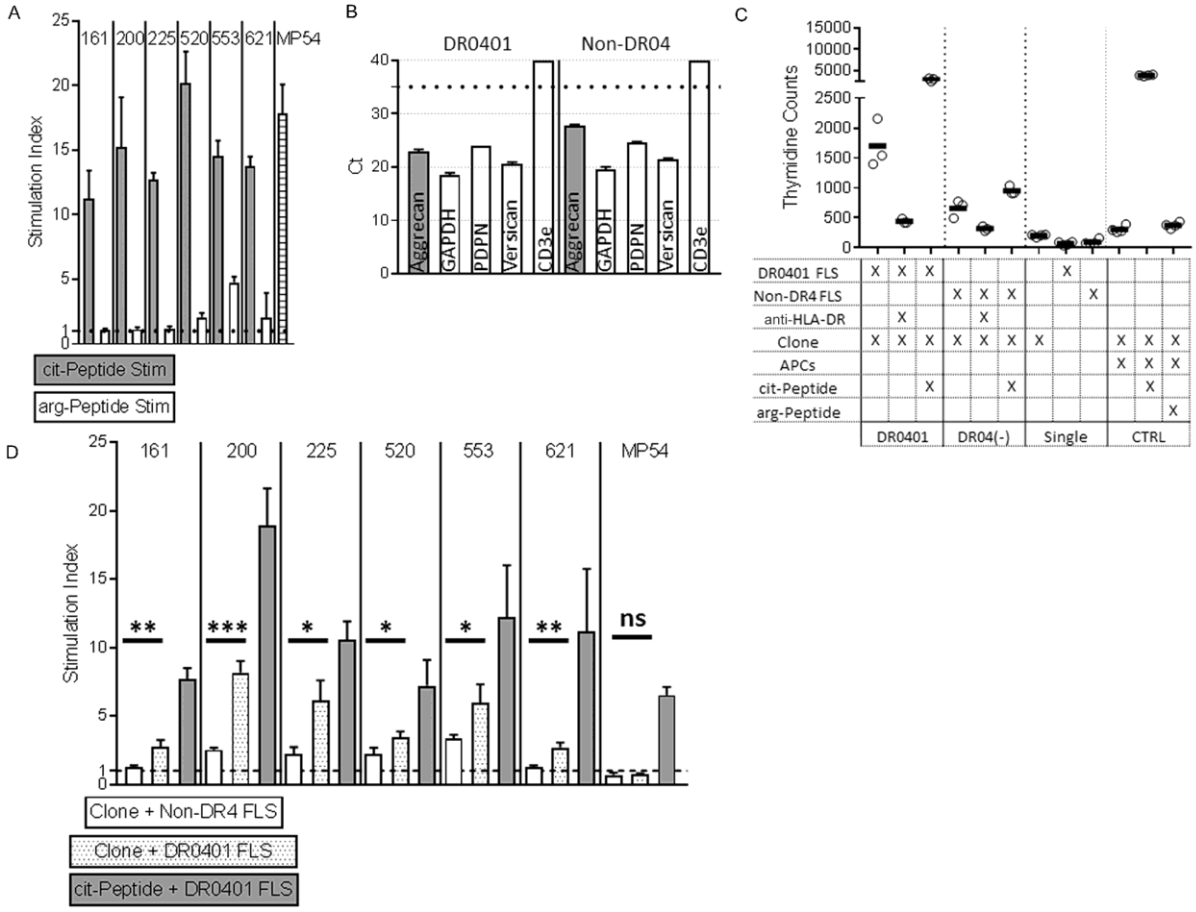
(negative control), and versican (related proteoglycan). Ct is inversely correlated with mRNA levels and values over 35 (dotted line) were considered undetectable. (C) Representative raw proliferation results for an aggrecan specific T cell clone co-cultured with an FLS line from a DR0401+ RA subject, a DR0401- subject, and each of these cell types individually, or the same T cell clone co-cultured with irradiated DR0401+ PBMC. Antigen presentation via HLA-DR0401 was required to elicit proliferation above background. (D) Stimulation indices for T cell clones co-cultured with DR0401- FLS(white bars), DR0401+ FLS (shaded bars), or DR0401+ FLS plus peptide(solid gray bars). Clones specific for cit-Agg 161, 200, 225, 520, 553, and 621 showed higher expansion when co-cultured with DR0401+ FLS as compared to DR0401- FLS (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, n=3). In contrast, the influenza (MP54) specific clone could not be activated by DR0401+ FLS cells in the absence of peptide.

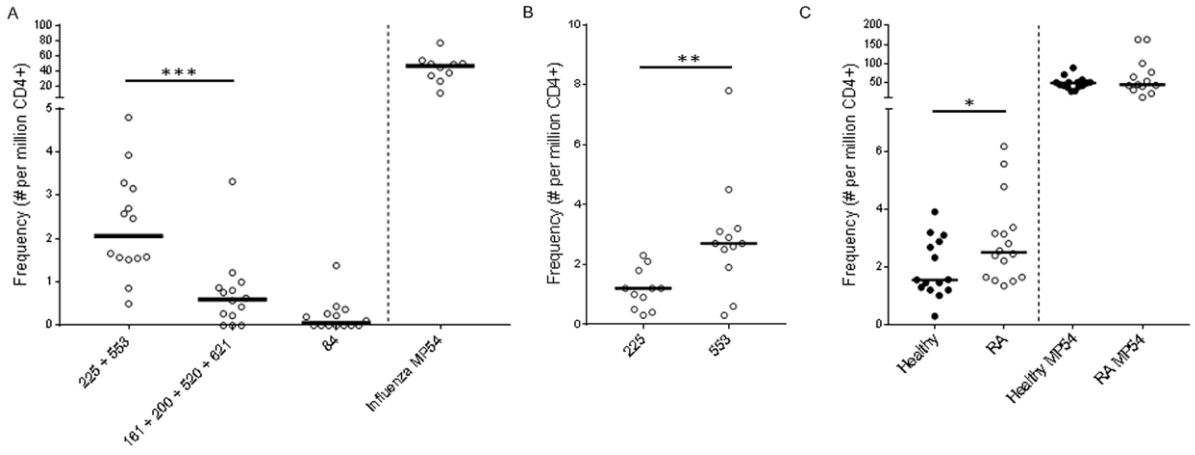
Figure 3. T cells that recognize dominant aggrecan epitopes are more prevalent in RA patients than in healthy subjects. (A) The ex-vivo frequencies of T cells specific for cit-Agg_{225} + cit-Agg_{553} were significantly higher in RA patients (n=14 DRB1*04:01 subjects) than cit-Agg_{161} + cit-Agg_{200} + cit-Agg_{520} + cit-Agg_{620} or cit-Agg_{84} (P=0.0002; two tailed Mann Whitney test). An influenza tetramer (MP54) was used as a positive control. (B) In a separate comparison, the frequencies of T cells specific for cit-Agg_{553} were significantly higher in RA patients (n=12 DRB1*04:01 subjects) than cit-Agg_{225} (p=0.0037; two tailed Mann Whitney test). (C) Ex-vivo frequencies of cit-Agg_{225} and cit-Agg_{553} specific T cells were significantly higher in RA patients (open circles, n=16 DRB1*04:01 subjects) than in healthy

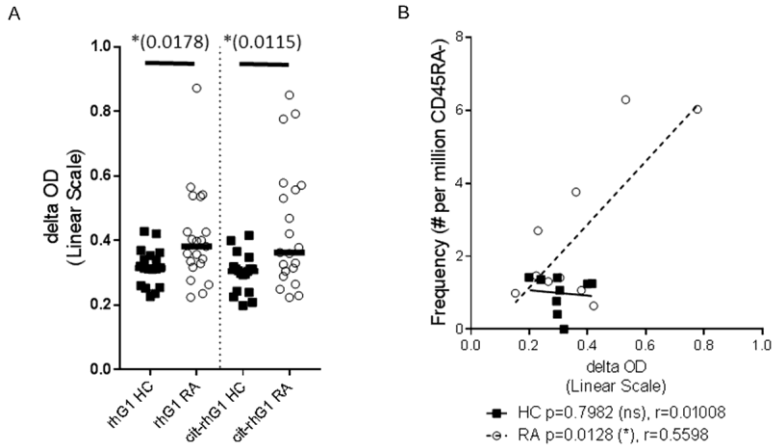
subjects (filled circles, n=15 DRB1*04:01 subjects) (p=0.0395; two-tailed Mann-Whitney test).

Figure 4. Aggrecan-specific antibodies are elevated in patients and positively correlate with T cell frequency. (A) Based on the background adjusted delta OD, the levels of antibodies against the native or citrullinated aggrecan-G1 domain in IgM-depleted serum were significantly higher in RA patients (open circles, n=21 subjects) than in healthy subjects (filled circles, n=18 subjects) (* indicates $p<0.05$; two tailed Mann-Whitney test). (B) Serum levels of aggrecan-G1 domain antibodies and combined frequencies of memory positive cit-Agg 225 + cit-Agg553 specific T cells showed a significant correlation ($r^2 = 0.7982$, $p=0.0128$) in RA subjects (N=10) when compared to healthy subjects ($r^2 = 0.01008$, $p=0.5598$, N=8). Correlation by two-tailed Pearson r, memory cells defined as CD45RA negative.









Appendix II: Meeting abstracts

1. **2017 FOCIS Annual Meeting Abstract Number: T.18.**
   Identification and Functional Characterization of T cell Reactive to Citrullinated Tenascin-C in HLADRB1 0401-Positive Rheumatoid Arthritis Patients*
   Jing Song, Cliff Rims, David Arribas-Layton, Eddie James and Jane Buckner
   Benaroya Research Institute at Virginia Mason, Seattle, WA

2. **2017 ACR/ARHP Annual Meeting Abstract Number: 951**
   Cross Sectional Analysis of Citrullinated-Synovial Antigen-Specific CD4+ T Cells in an RA Cohort Demonstrates Antigen Based Differences in T Cell Frequency, Phenotype and the Influence of Immunotherapy
   Cliff Rims[1], Sylvia Posso[1], Bernard Ng[2], Jeffrey Carlin[3], Eddie James[4] and Jane H. Buckner[4], [1]Translational Research, Benaroya Research Institute at Virginia Mason, Seattle, WA, [2]Rheumatology, VA Puget Sound Healthcare System, Seattle, WA, [3]Rheumatology, Virginia Mason Medical Center, Seattle, WA, [4]Benaroya Research Institute at Virginia Mason, Seattle, WA

3. **2017 ACR/ARHP Annual Meeting Abstract Number: 19**
   The Transcription Factor Specificity Protein 1 Up-Regulates IL-21 Receptor Expression on B Cells in Rheumatoid Arthritis Leading to Altered Cytokine Production and Maturation.
   Elizabeth Dam[1], Alison Maier[1], Anne Hocking[1], Jeffrey Carlin[2], and Jane H. Buckner[1], [1]Translational Research, Benaroya Research Institute at Virginia Mason, Seattle, WA, [2]Rheumatology, Virginia Mason Medical Center, Seattle, WA, [4]Benaroya Research Institute at Virginia Mason, Seattle, WA

4. **2019 Keystone Symposium on Transcription and RNA Regulation in Inflammation and Immunity**
   Regulation of LAG-3 and other co-inhibitory receptors in autoimmunity
   Britta E. Jones[1], Megan D. Maerz[1], Jane H. Buckner[1]
   Translational Research Program, Benaroya Research Institute at Virginia Mason, Seattle, WA

5. **2019 FOCIS Annual Meeting – Submitted**
   Computational Analysis of Citrulline-Specific CD4+ T Cell Frequency and Phenotype Reveals Differences that are Driven by Antigen Specificity and Disease Characteristics among Rheumatoid Arthritis Patients.
   Virginia Muir[1], Cliff Rims[2], Hannes Uchtenhagen[2], Anne M. Hocking[2], Sylvia Posso[2], Heather Bukiri[3], Jeffrey Carlin[2], Bernard Ng[4], Eddie James[2], Peter S. Linsley[1], and Jane H. Buckner[2]
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2017 FOCIS ANNUAL MEETING

T.18. Identification and Functional Characterization of T cell Reactive to Citrullinated Tenascin-C in HLADRB1* 0401-Positive Rheumatoid Arthritis Patients

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Anti-citrullinated protein antibodies (ACPAs) are a hallmark of rheumatoid arthritis (RA) and target a number of synovial and inflammation associated proteins. Antibodies against Tenascin-C, an extracellular matrix protein, have been observed in ACPA+ RA patients and a citrullinated peptide was recently identified as their major target. Our aim was to determine whether T cell responses against cit-tenascin-C are present in subjects with RA. We utilized an algorithm to predict 64 possible HLA-DRB1*0401 restricted epitopes within tenascin-C based on its binding motif. These peptides were tested in a binding assay, identifying 10 citrullinated peptides that bound with moderate to high affinity. We next performed *in vitro* assays, expanding PBMC obtained from HLA-DRB1*0401+ patients and staining with HLA class II tetramers, confirming 6 of these peptides as immunogenic. We utilized tetramers to directly stain cit-tenascin-C specific T cells and observed that tenascin-C specific cells were readily visualized in the peripheral blood of HLA-DR*0401+ patients. To further investigate the specificity of tenascin-C specific T cells, we isolated a T cell clone from an RA patient using *ex vivo* single cell sorting. The expanded clone remained tetramer positive and proliferated in response to a citrullinated tenascin-C peptide (1012-1026 modified at amino acids 1014 and 1016). These results demonstrate that T cells that recognize citrullinated tenascin-C peptides are present in HLA DRB1*0401+ RA patients. We expect that further characterization of cit-tenascin-C specific T cells will indicate unique functional characteristics and others that are shared by T cells that recognize conventional RA autoantigens

ABSTRACT NUMBER: 951

Cross Sectional Analysis of Citrullinated-Synovial Antigen-Specific CD4+ T Cells in an RA Cohort Demonstrates Antigen Based Differences in T Cell Frequency, Phenotype and the Influence of Immunotherapy

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Meeting: 2017 ACR/ARHP Annual Meeting

Date of first publication: September 18, 2017

Keywords: anti-citrullinated protein/peptide antibodies (ACPA), antigen RA, flow cytometry and therapeutic targeting, T cells

SESSION INFORMATION

Date: Sunday, November 5, 2017

Session Title: T Cell Biology and Targets in Autoimmune Disease

Session Type: ACR Concurrent Abstract Session

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

Background/Purpose:

The presence of ACPA in RA indicates that an immune response directed toward citrullinated synovial antigens participates in disease development or persistence. Research from our group have identified T cell targets derived from the auto-antigens aggrecan, vimentin, fibrinogen, alpha-enolase, and cartilage intermediate layer protein (CILP). In this study, we visualized peripheral antigen-specific CD4+ T cells using a multiplexed flow-cytometry based HLA class II tetramer assay in a cross-sectional cohort of 80 RA and 30 matched healthy control subjects to understand their relevance to RA disease progression and response to therapy.

Methods:

All subjects were DRB1*04:01. RA subjects were CCP positive and represented a range of characteristics including time from diagnosis, disease activity and treatment at the time of blood draw. Antigen-specific T cells were visualized by directly staining peripheral blood mononuclear cells (PBMC) with multiple tetramers corresponding to different antigens. Frequencies and phenotypic features of antigen-specific CD4+ T cells were assessed for correlation with clinical characteristics.

Results:

Ex-vivo analysis of PBMC revealed an increase in synovial targeted CD4 T cells when compared to matched healthy DRB1*04:01 subjects. When analyzed by individual antigen CD4 T cells,

aggrecan, vimentin and fibrinogen were increased in RA, and by contrast cartilage-intermediate-layer-protein (CILP) and enolase specific T cells were reduced in comparison to healthy subjects, suggesting that the characteristics of the CD4+ T cells response to synovial epitopes may be unique to antigen specificity.

Within this patient cohort we found a lower frequency of synovial specific T cells in individuals on TNF therapies sampled within 5 years of diagnosis. These differences were most pronounced in the CD4 T cells specific for aggrecan, vimentin and fibrinogen, and showed alterations in chemokine receptor and activation marker expression in the treated group.

Ongoing studies will determine if frequency, phenotype and specificity of synovial specific CD4 T cells correlate directly with disease duration, therapeutic duration, and clinical diagnostic values such as level of RF, CCP, CRP, and disease severity.

Conclusion:

We have shown that a multiplexed tetramer assay can define the breadth and character of the T cell response to synovial antigens. Characterizing a relatively large cohort of subjects, we demonstrate differences in the phenotype and frequency of the T cells that respond to a diverse set of synovial antigens thought to be important targets in RA. In particular, we show that synovial specific T cell frequency is influenced by therapeutic interventions. Better understanding the interplay of antigen specificities and phenotypes in RA is vital to understanding disease pathogenesis, response to therapy and ultimately developing antigen specific therapies.

Disclosure: C. Rims, None; **S. Posso**, None; **B. Ng**, None; **J. Carlin**, None; **E. James**, None; **J. H. Buckner**, None.

ABSTRACT NUMBER: 19

The Transcription Factor Specificity Protein 1 up-Regulates IL-21 Receptor Expression on B Cells in Rheumatoid Arthritis Leading to Altered Cytokine Production and Maturation

Elizabeth Dam[1], Alison Maier[1], Anne Hocking[1], Jeffrey Carlin[2] and Jane H. Buckner[1], [1]Benaroya Research Institute at Virginia Mason, Seattle, WA, [2]Rheumatology, Virginia Mason Medical Center, Seattle, WA

Meeting: 2017 ACR/ARHP Annual Meeting

Date of first publication: September 18, 2017

Keywords: B cells

SESSION INFORMATION

Date: Sunday, November 5, 2017

Session Type: ACR Poster Session A

Session Title: B Cell Biology and Targets in Autoimmune Disease Poster

Session Time: 9:00AM-11:00AM

Background/Purpose: Growing evidence suggests that IL-21 has a role in B cell dysfunction in rheumatoid arthritis (RA). Previous studies have reported that IL-21 levels are increased in the serum and synovial fluid of RA subjects and correlate with the 28-joint count disease activity score. While an increase in the percent of IL-21R positive B cells in RA has been described, the significance of this finding in B cells with respect to signaling, B cell differentiation and function has not been investigated. The goal of this study was to address this gap in knowledge and determine the mechanism that leads to increased IL-21R expression on B cells in RA.

Methods: We analyzed IL-21 receptor expression in B cells isolated from whole blood and synovial fluid from a cohort of RA subjects and healthy controls matched for age, gender and race. The RA subjects all met the ACR classification criteria and none of the subjects were on biologics at the time of the draw. Flow cytometry was used to quantify protein and mRNA levels of IL-21R, specificity protein 1 (SP1) and to determine cytokine production (IL-6) and maturation status of B cells. IL-21 signaling was assessed by measuring pSTAT3 levels following IL-21 stimulation. IL-21R levels were correlated to serum levels of rheumatoid factor (RF) IgM which was assessed by ELISA. SP1 binding to the *IL21R* promoter region in B cells was assessed with ChIP-qPCR.

Results: We demonstrate an increase in IL-21R expression in total and memory B cells from RA subjects, which correlated with responsiveness to IL-21 as measured by phosphorylation of STAT3. IL-21R expression on memory B cell also correlated with serum rheumatoid factor IgM levels. There was comparable levels of IL-21R expression between memory B cells from peripheral blood and those from synovial fluid in the same subject. In addition, stimulation of naïve B cells from RA subjects with IL-21 and CD40L resulted in an increase in differentiation into plasmablasts. Further investigation showed that IL-21R expression correlated with an increase in the level of the SP1 transcription factor. Mechanistic experiments showed increased binding of SP1 to the *IL21R* promoter region in B cells in RA.

Conclusion: Our results suggest a mechanism by which IL-21 enhances B cell development and function in RA through an SP1 mediated increase in IL-21R expression on B cells. This suggests that

therapies for RA may be more efficacious if targeted to memory B cells and/or target SP1.

Disclosure: E. Dam, None; **A. Maier**, None; **A. Hocking**, None; **J. Carlin**, None; **J. H. Buckner**, None.

2019 KEYSTONE SYMPOSIUM ON TRANSCRIPTION AND RNA REGULATION IN INFLAMMATION AND IMMUNITY (FEBRUARY 2-5, 2019)

Regulation of LAG-3 and other co-inhibitory receptors in autoimmunity

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Background: The expression of inhibitory receptors (IR) on T cells plays a key role in the regulation of effector immune responses. In cancer, over-expression of these receptors on the cell surface allows tumors to evade immune surveillance and blocking these receptors is an effective therapeutic approach. In autoimmunity, the use of IR agonists may prove useful, but the role of these receptors in autoimmunity is not well-defined. Our goal in this study is to understand how cell surface expression and the regulation of LAG-3 in tandem with other IR are altered in relapsing remitting multiple sclerosis (RRMS), type 1 diabetes (T1D) and rheumatoid arthritis (RA) as compared to healthy controls (HC).

Methods: We measured surface expression of LAG-3 (CD223) by flow cytometry in 121 RRMS, 105 T1D, 35 RA and 123 age and gender matched HC. In a subset of this cohort, we measured surface expression of additional inhibitory receptors PD-1 and TIGIT alongside qPCR for mRNA expression of the genes encoding the inhibitory receptor molecules: *Lag3*, *Pdcd1 (PD-1)*, *Havcr2* (TIM-3) and *Tigit* along with the transcription factor *Maf,* in a purified T cell population.

Results: We describe a significant decrease in both the frequency and mean fluorescence intensity of LAG-3 on the surface of CD4 and CD8 T cells in patients with RA, RRMS and T1D, as compared to HC. In the smaller sub-cohort of RRMS and RA, there is a decrease in PD-1 on the surface of T cells which is not seen in T1D. In RA, there is decreased expression of *Havcr2 (*1.7-fold less), *Pdcd1 (*2.1-fold less) and *Lag3 (*1.2-fold less) mRNA compared to HC. There is evidence that Maf regulates a module of IR, but preliminary data suggests this relationship is dysregulated in patients with RA.

Conclusion: Surface expression of LAG-3 is diminished across multiple autoimmune diseases and T cell subsets. In RA, a module of IR appears to be dysregulated both transcriptionally and on the surface. Increased understanding of the expression and role of inhibitory receptors on T cells in patients with autoimmune disease may further characterize the status of ongoing inflammation and disease progression in these individuals and ultimately guide therapeutic interventions targeting these pathways.

2019 FOCIS ANNUAL MEETING - Submitted

Computational Analysis of Citrulline-Specific CD4 T Cell Frequency and Phenotype Reveals Differences that are Driven by Antigen Specificity and Disease Characteristics among Rheumatoid Arthritis Patients

Virginia Muir[1], Cliff Rims[2], Hannes Uchtenhagen[2], Anne M. Hocking[2], Sylvia Posso[2], Heather Bukiri[3], Jeffrey Carlin[3], Bernard Ng[4], Eddie James[2], Peter S. Linsley[1], and Jane H. Buckner[2]

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The presence of anti-citrullinated protein antibodies in rheumatoid arthritis (RA) indicates that an immune response directed toward citrullinated antigens participates in disease development. Using a combinatorial HLA class II tetramer staining approach and applying a new computational algorithm for phenotyping rare cell populations, we characterized cit-specific CD4 T cells in a cross-sectional cohort of 80 RA subjects and 30 matched healthy control (HC) subjects. We assayed the frequency and phenotype of CD4 T cells specific for five citrullinated autoantigens expressed in the joint: alpha-enolase, aggrecan, cartilage intermediate layer protein (CILP), fibrinogen and vimentin. While the overall frequency of cit-specific CD4 T cells was increased in RA subjects compared to HC subjects, antigen-specific differences were observed. Aggrecan- and vimentin/fibrinogen-specific cells were increased in RA whereas alpha-enolase-specific cells were decreased. Further, antigen-specificity influenced the predominant immunophenotype of cit-specific CD4 T cells. Cit-aggrecan-specific cells were primarily Th2-like; cit-alpha-enolase-specific cells were principally Th1; and CILP-specific cells were mostly naïve. Lastly, certain cit-specific cell immunophenotypes were significantly associated with disease characteristics. Cit-aggrecan-specific and CILP-specific cells both exhibited phenotypic shifts between early and longstanding disease. Vimentin/fibrinogen-specific stem cell memory cells were more frequent than predicted from the CD4 landscape and were linked to disease activity. These data reveal the heterogeneity of autoantigen-specific CD4 T cells across individuals and within an individual and suggest that specific antigens may drive distinct immune responses. Future work may help classify RA subjects based on the specificity and dominant phenotype of their T cell response, leading to more targeted therapies.