Rheumatoid Arthritis, *how it happens*

University of Washington
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Disclosures

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Consulting agreements with :

Eli Lilly, Janssen

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Overview of todays talk

- What do we know about the development of RA?
  - Defining pre-clinical disease
  - Role of Genetics
  - Role of autoantibodies
  - Environmental factors

- Defining the role of CD4 T cells in RA
  - Identification of synovial autoantigens implicated in the pathogenic CD4 T cells response in RA
  - Utilization of HLA Class II tetramers to define frequency and phenotype of citrulline specific CD4 T cells in RA

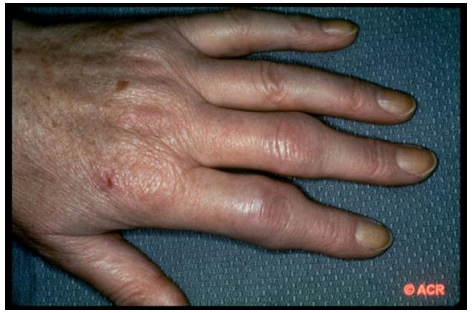
EULAR Recommendations for Terminology and Research in Rheumatoid Arthritis Development

Genetic Risk → Environmental Risk Factors → Systemic Autoimmunity → Arthralgia → Unclassified Arthritis → Rheumatoid Arthritis

HLA
PTPN22
CTLA-4,
STAT4
TRAF-1,
PAD4

Smoking
Hormones
Infections/
Stress?

ACPA / RF
Inflammatory
Markers



Gerlag et al, Ann Rheum Dis 71:638-641

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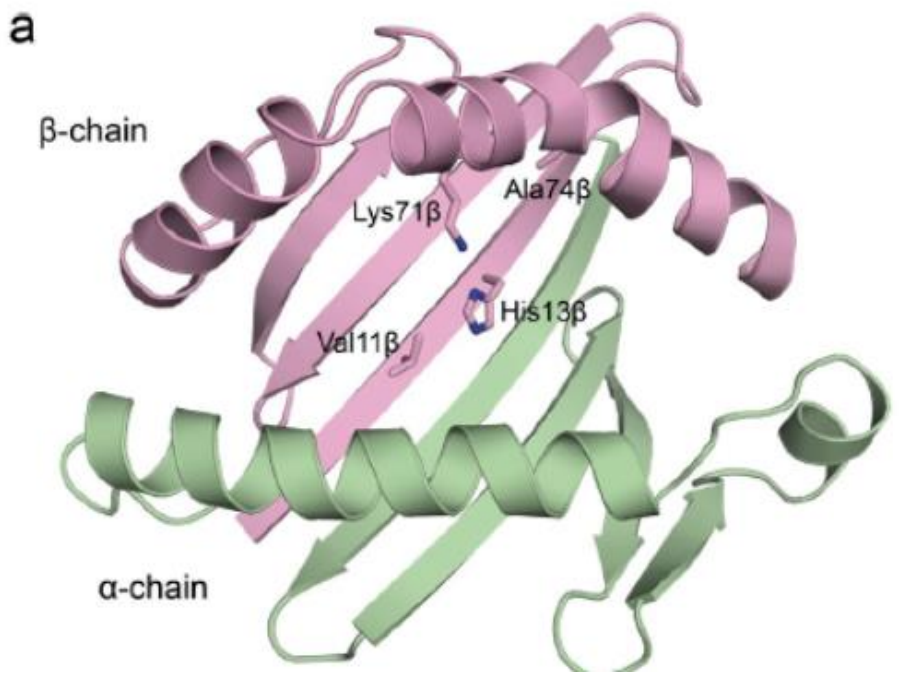
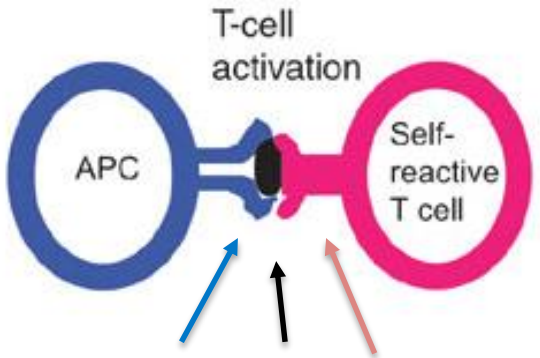
Factors that drive the development of RA

Genetic Risk
HLA Class II

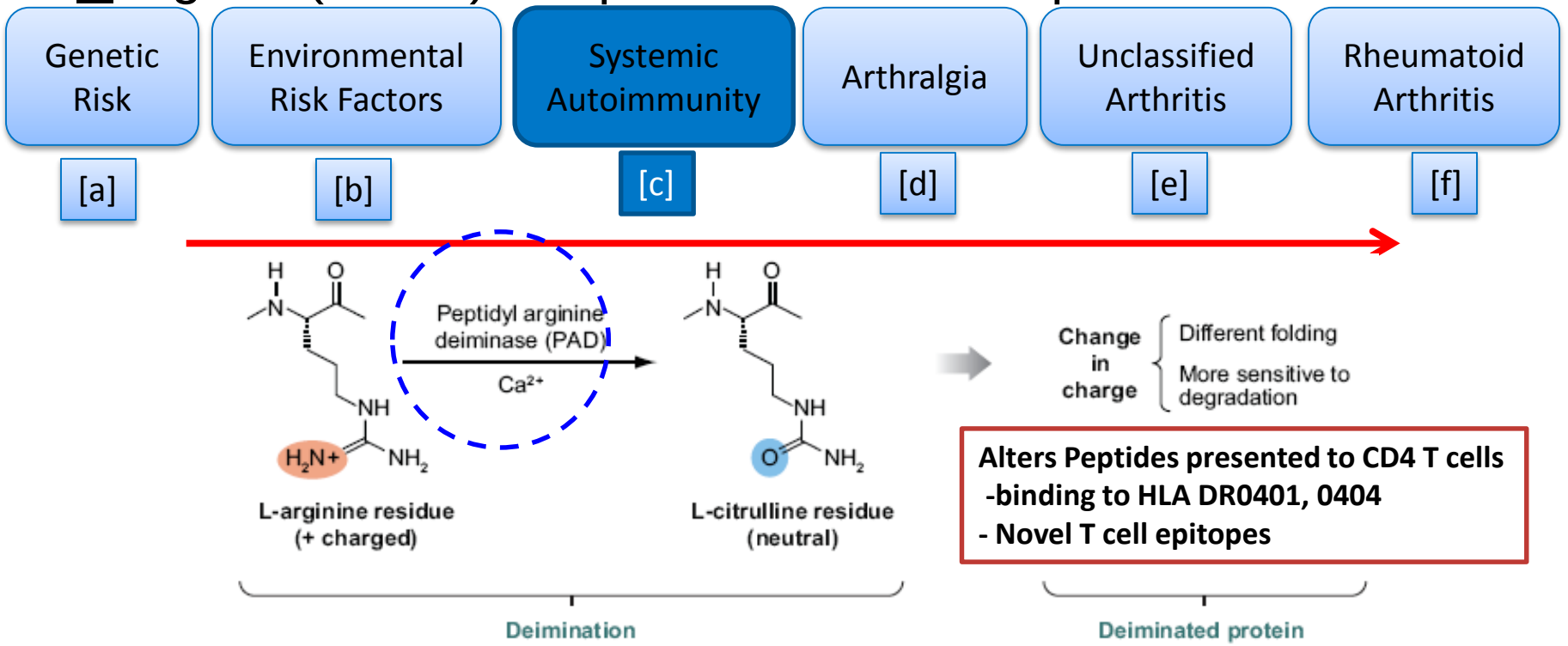
Amino acid sequence	SE motif	Coding *HLA-DRB1* alleles
QKRAA	+	**0401;*0409;*0413;*0416; *0421;*1419;*1421*
DERAA	–	**0402;*0414;*0103;*1102;*1116;*1120;*1121;*1301;*1302;*1304;*1308;*1315;*1315;*1317;*1319;*1322;*1416*
QRRAE	–	**0403;*0406;*0407;*0417;*0420*
QRRAA	+	**0101;*0102;*0105;*0404;*0405;*0408;*0410;*0419; *1402;*1406;*1409;*1413;*1417;*1420*
RRRAA	+	**1001*
RRRAE	–	**09;*1401;*1404;*1405;*1407;*1408;*1410;*1411;*1414;*1418*
DRRAA	–	**0415;*0805;*11011;*11012;*11041;*11042;*1105;*1106;*11081;*11082*1109;*1110;*1112;*1115;*1118;*1119;*1122;*1201;*12021;*12022;*12031;*12032;*1305;*1306;*1307;*1311;*1312;*1314;*1321;*1601;*1602;*1605*
QARAA	–	**15;*1309*
QKRGR	–	**03; *0422;*1107*





Class II- peptide- TCR

Antibodies to Citrullinated Protein Antigens (ACPA) are present in RA and precede disease



- Post-translational modification by PADs 1-4,6
- Citrullinated proteins found ubiquitously at sites of inflammation
- Biologic roles:
  - Protein folding and cleavage (filaggrin & vimentin)
  - Gene transcription (histone modification)
  - Protein activity (chemokine citrullination)
  - Neutrophil NET Formation

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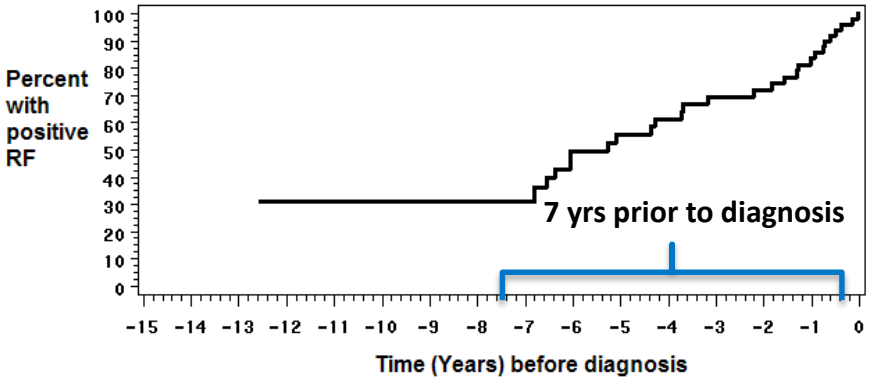
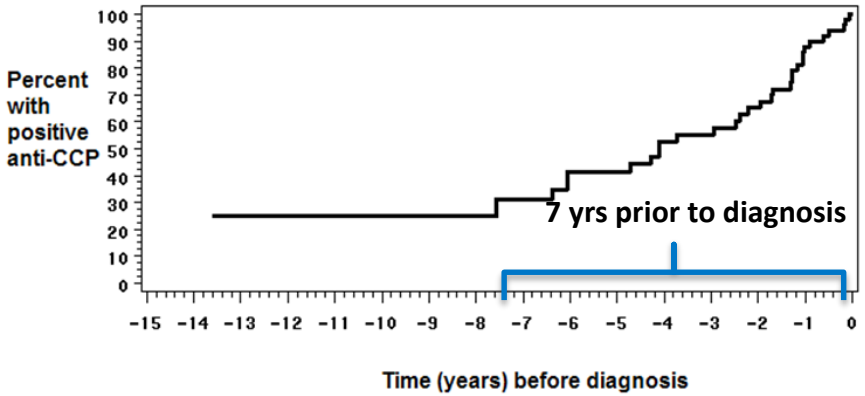
Autoimmunity precedes development of RA

Systemic Autoimmunity

- Rheumatoid Factor and Antibodies to Citrullinated Protein Antigens (ACPA) are present in RA and precede disease
- ACPA are highly specific for RA, and 70% sensitive
- ACPA presence and levels correlate with disease severity and poor outcomes





Majka, Deane et al, Ann Rheum Dis 2007

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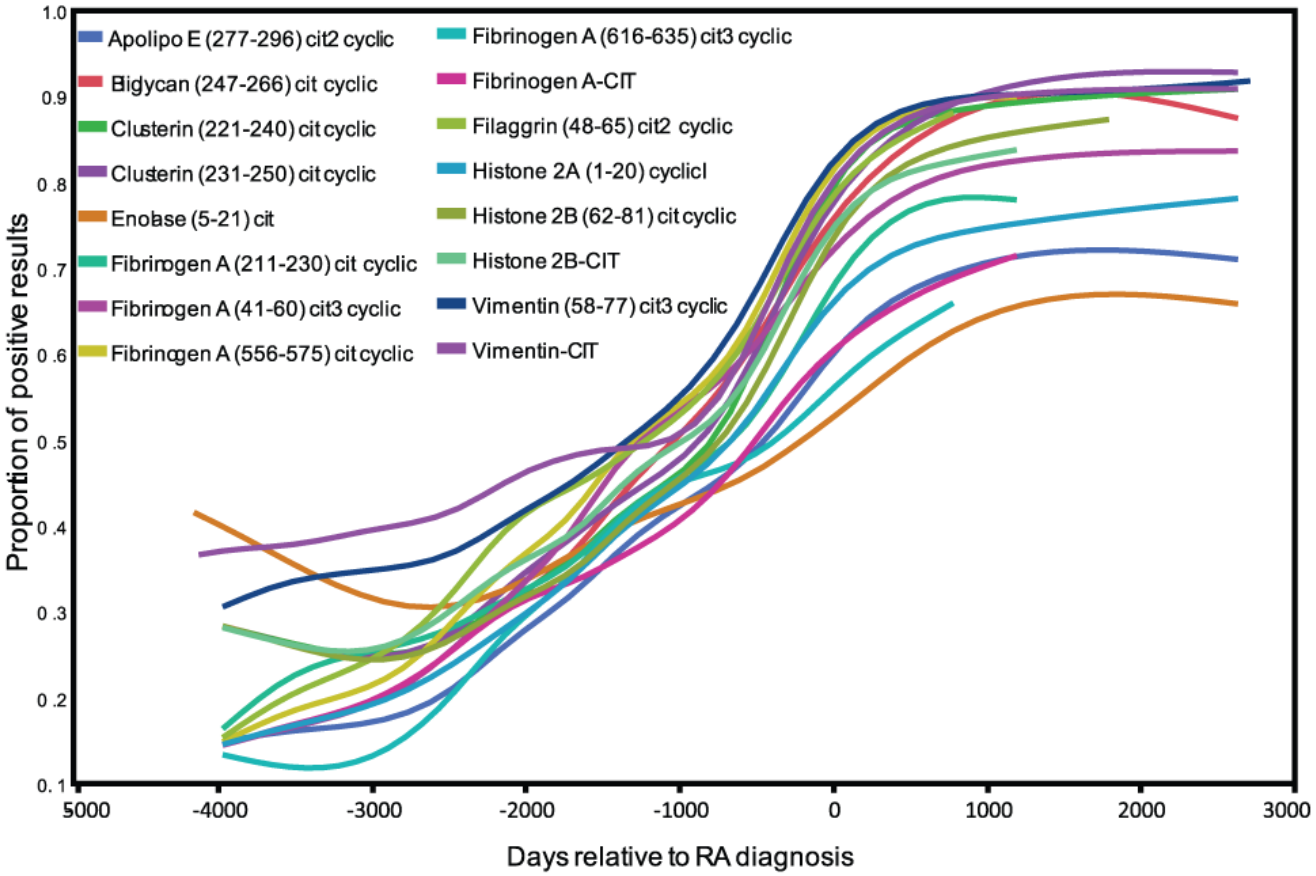
Individual ACPA Responses Rapidly Expand Prior to the Development of Clinically Apparent Disease

Genetic Risk [a] → Environmental Risk Factors [b] → Systemic Autoimmunity [c] → Arthralgia [d] → Unclassified Arthritis [e] → Rheumatoid Arthritis [f]



Sokolove et al, PLoS One 2012

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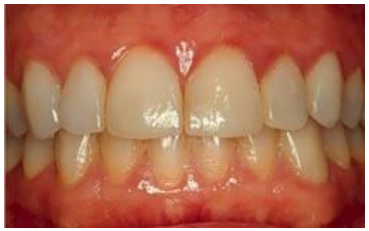
Does RA originate in the Lung and other mucosal sites?

Genetic Risk | **Environmental Risk Factors** | Systemic Autoimmunity | Arthralgia | Unclassified Arthritis | Rheumatoid Arthritis

Smoking, silica…..

Environmental factors (i.e. smoking, dysbiosis)



Periodontitis

1) Lung disease can be associated with elevations of RA-related autoantibodies in absence of inflammatory arthritis
2) Lung disease precedes arthritis in some cases
3) Known local generation of RF and anti-citFib Abs in the lung in subjects with established RA

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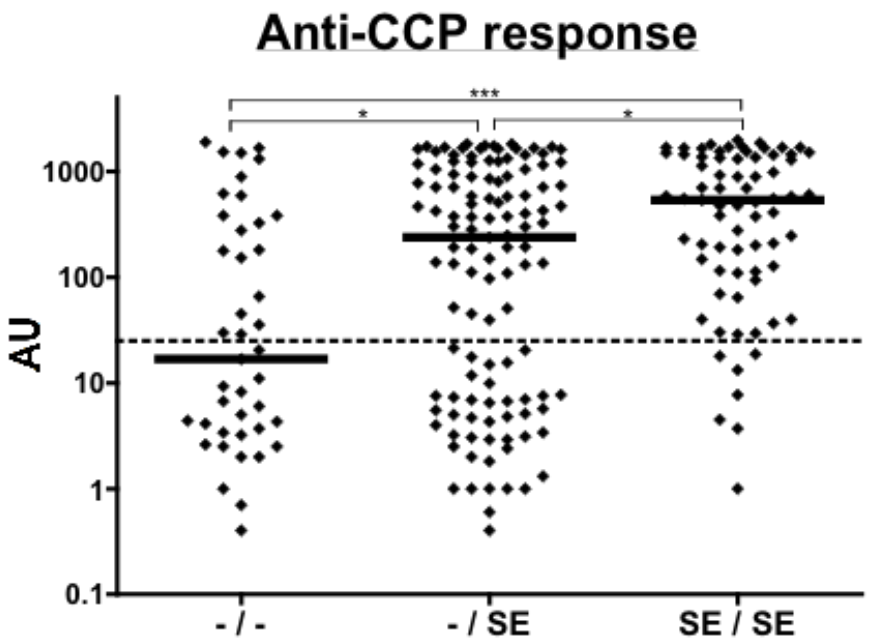
4) *p.gingivalis* has abundant endogenous PAD enzymes and drive self and host protein citrullination

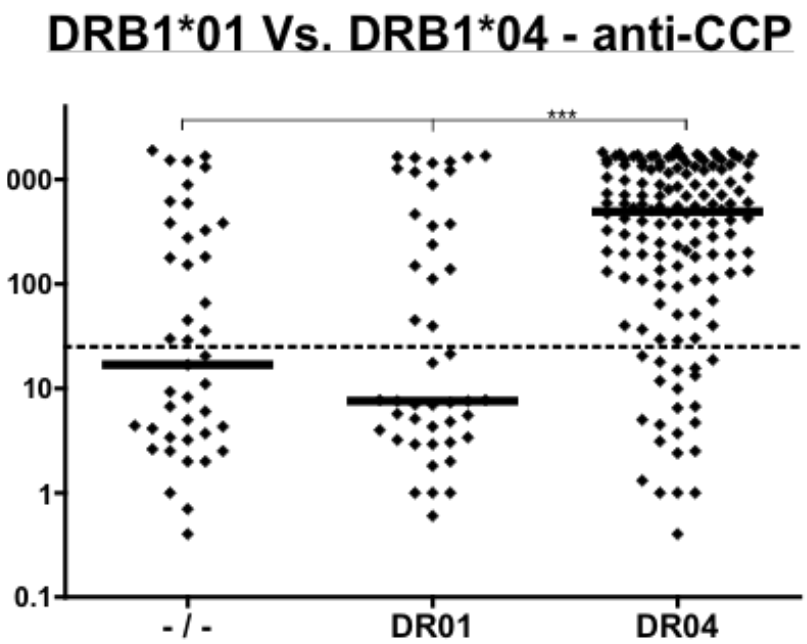
5) ACPA found to be cross reactive with *p. gingivalis* proteins (*Li S et al Arth Rheum 2016)*

Klareskog et al Ann Rheum Dis 2010; Hart et al Environ Health Perpect 2009,; Gizinski et al Clin Rheum 2009; Demoruelle et al Arthritis Rheum 2012; Fischer et al Respiratory Med 2012; Rangel-Moreno et al J Clin Invest 2006. Li S et al Arth Rheum 2016

Evidence that Genetic Risk + Environment lead to RA

Genetic Risk → Environmental Risk Factors → Systemic Autoimmunity → Arthralgia → Unclassified Arthritis → Rheumatoid Arthritis





Strongest association is with the HLA-DR4

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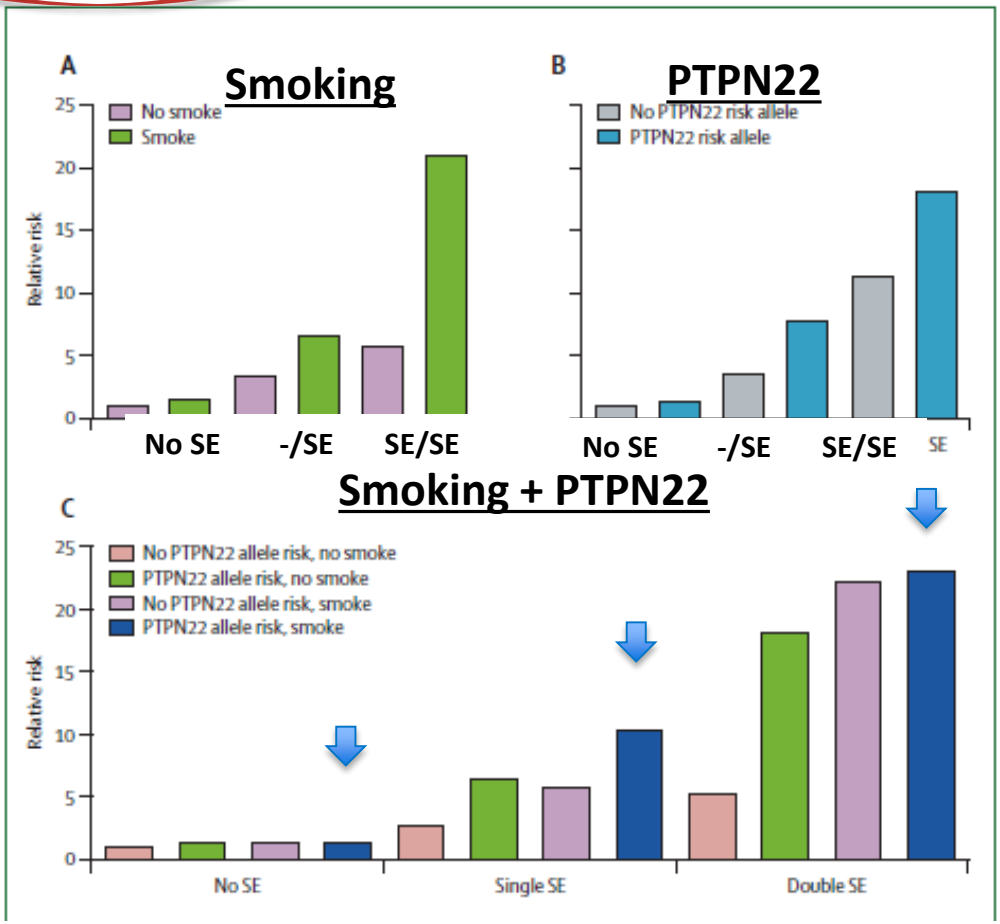
Evidence that Genetic Risk + Environment lead to RA

Genetic Risk → Environmental Risk Factors → Systemic Autoimmunity → Arthralgia → Unclassified Arthritis → Rheumatoid Arthritis

ACPA+ RA



Klareskog Lancet 2009

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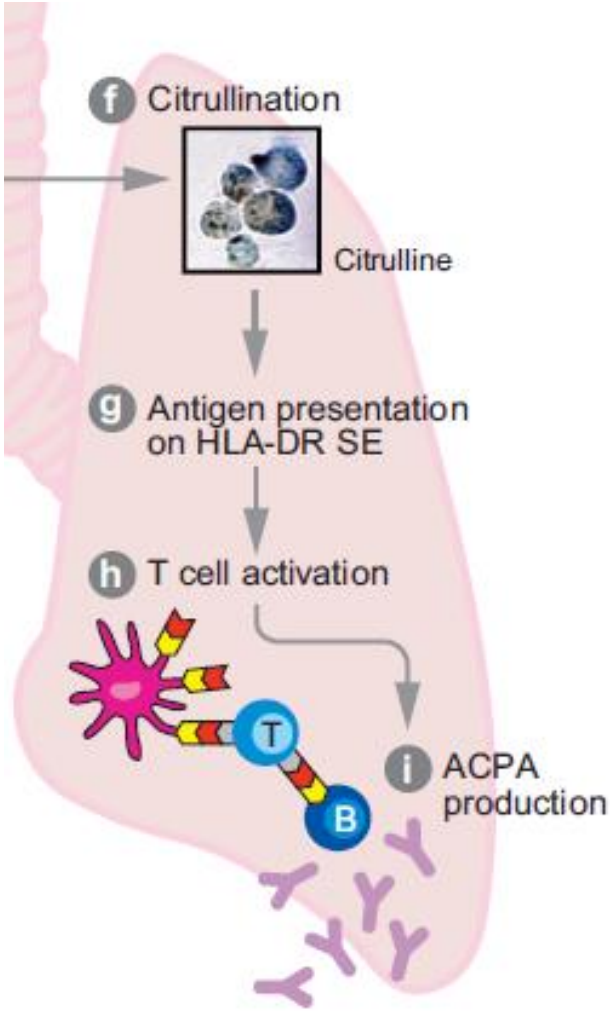
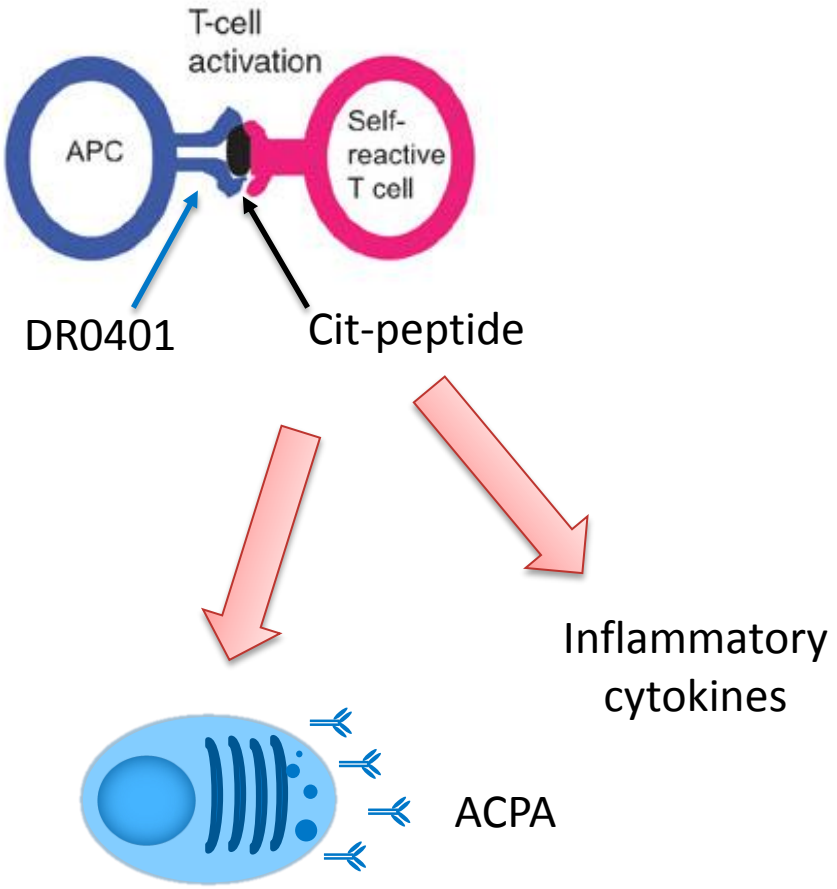
Implications of link between HLA Class II and ACPA





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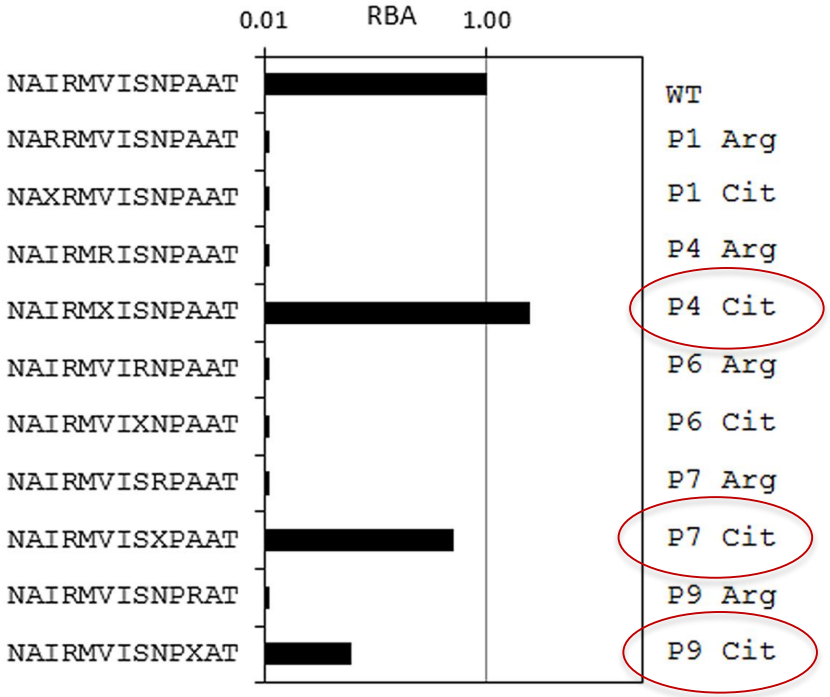
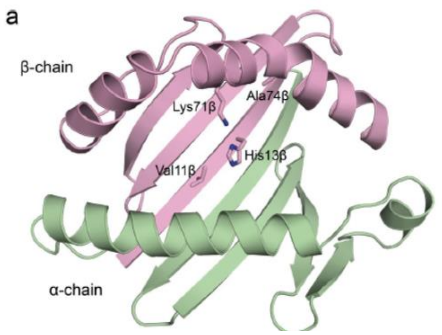
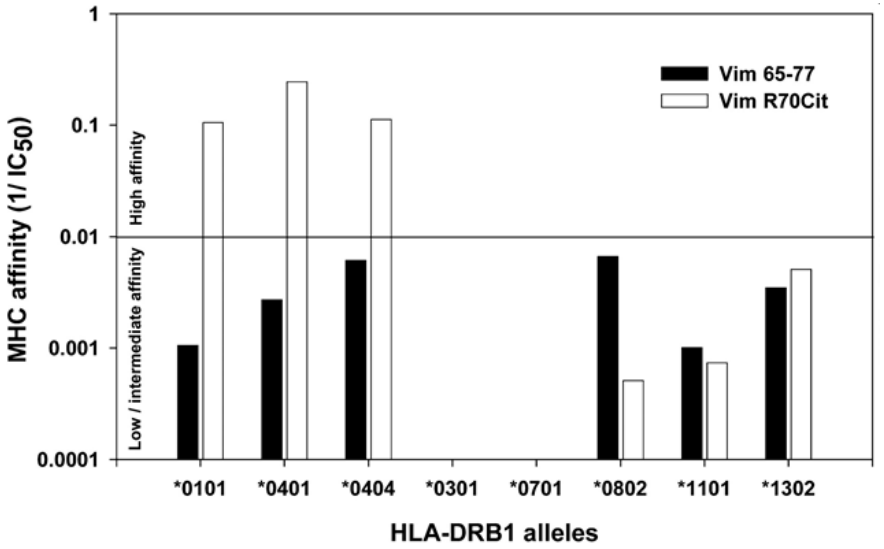
Citrullinated peptides preferentially bind shared epitope Class II alleles

Define Citrulline Binding to pockets of DRB1*0401





Pocket 4 of DRB1*0401 favors binding of citrulline and does not bind R



Hill et al, JI 2003

Hypothesis:

CD4 T cells that recognize and respond to citrullinated antigens are present in individuals with CCP+ RA and contribute to development of high affinity antibodies and to RA pathology.

Objectives:

To visualize T cells specific for citrullinated epitopes in RA patients in order to characterize these cells

- Which proteins and epitopes do they recognize?
  - These could be targets for antigen specific tolerance
- What is their frequency and function?
  - This could help us target pathogenic cells specifically
- Can they be detected prior to disease onset?
  - Aiding in disease prediction/ prevention
- Do number or function change with treatment or disease activity?
  - Will this help us understand response to therapy

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Experimental Approach

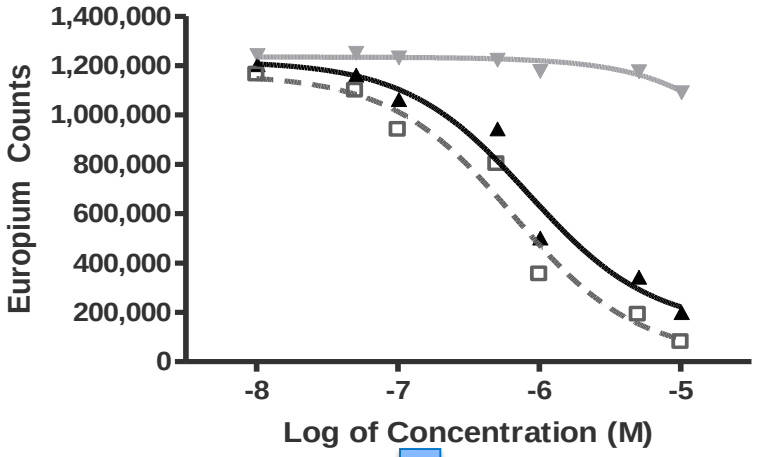
1. Development of a relevant panel of tetramers
   - Prediction of putative citrullinated epitopes
   - Assessment of binding for putative epitopes
   - *in vivo validation in immunized DR4 Tg mice*
   - *In vitro* validation using human PBMCs

2. *Ex vivo* characterization of cit-specific CD4+ T cells
   - Frequency and phenotype in RA patients
   - Frequency and phenotype in HLA matched controls
   - Variation based on disease duration and therapy

A Pipeline for Epitope Discovery

Test ability of predicted peptides to bind DR0401

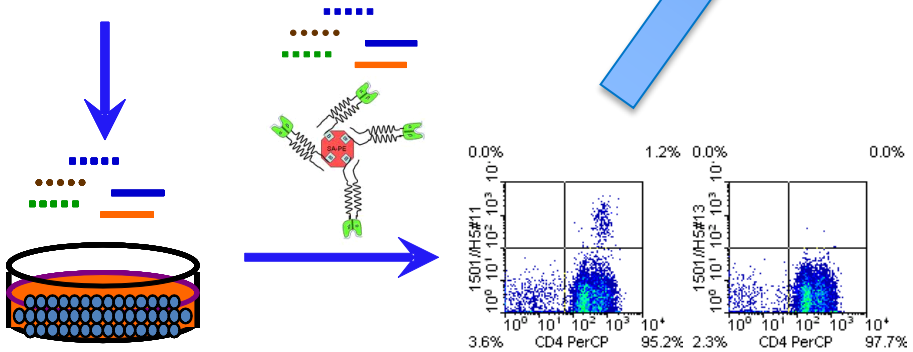
Competition binding assay



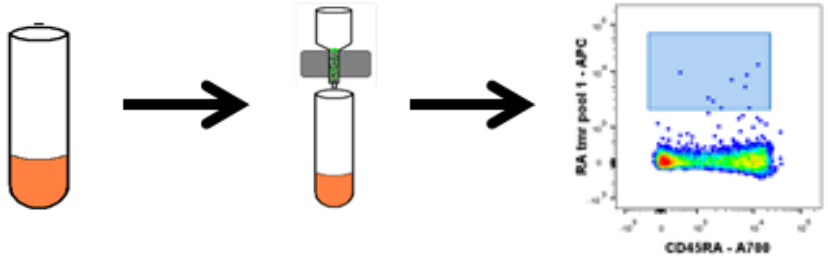
Sorting T Cell Clones

In vitro antigenicity screening

Selected Peptides



Ex vivo tetramer enrichment



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Predicting potential citrullinated epitopes

DRB1*04:01 Matrix

P1		P4		P6		P7		P9	
Residue	Value	Residue	Value	Residue	Value	Residue	Value	Residue	Value
G	0.00	G	0.31	G	0.43	G	1.80	G	1.00
A	0.08	A	0.86	A	0.93	A	1.82	A	1.00
V	0.24	V	0.67	V	1.56	V	0.60	V	0.50
L	0.24	L	1.32	L	0.65	L	0.42	L	0.40
I	0.30	I	1.16	I	0.91	I	0.42	I	0.35
M	0.18	M	2.08	M	0.54	M	1.08	M	1.12
P	0.00	P	0.07	P	0.90	P	0.10	P	0.17
F	1.50	F	1.97	F	0.30	F	0.51	F	0.10
W	0.93	W	0.47	W	0.39	W	0.60	W	0.10
S	0.00	S	1.14	S	1.36	S	0.57	S	3.48
T	0.00	T	0.91	T	1.83	T	0.57	T	0.85
N	0.00	N	3.65	N	1.21	N	1.50	N	0.72
Q	0.00	Q	1.04	Q	0.32	Q	0.90	Q	0.60
Y	0.97	Y	0.89	Y	0.27	Y	0.56	Y	0.18
C	0.00	C	1.36	C	0.72	C	0.12	C	0.40
K	0.00	K	0.06	K	0.02	K	0.03	K	0.05
R	0.00	R	0.03	R	0.02	R	0.09	R	0.07
H	0.00	H	1.27	H	0.30	H	0.39	H	2.64
D	0.00	D	1.17	D	0.84	D	0.12	D	0.70
E	0.00	E	1.93	E	0.87	E	0.12	E	0.40
X	0.0001	X	1.66	X	0.0005	X	0.77	X	0.07

$$RBA = C_{p1} \times C_{p4} \times C_{p6} \times C_{p7} \times C_{p9}$$

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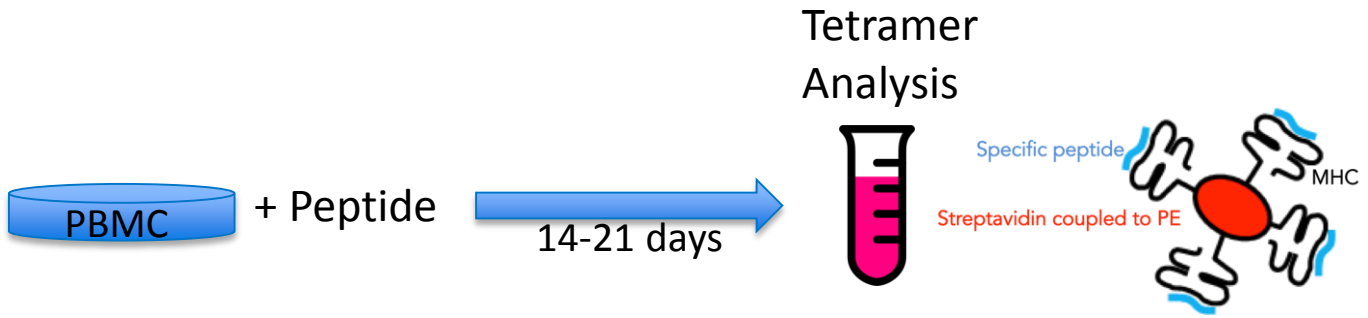
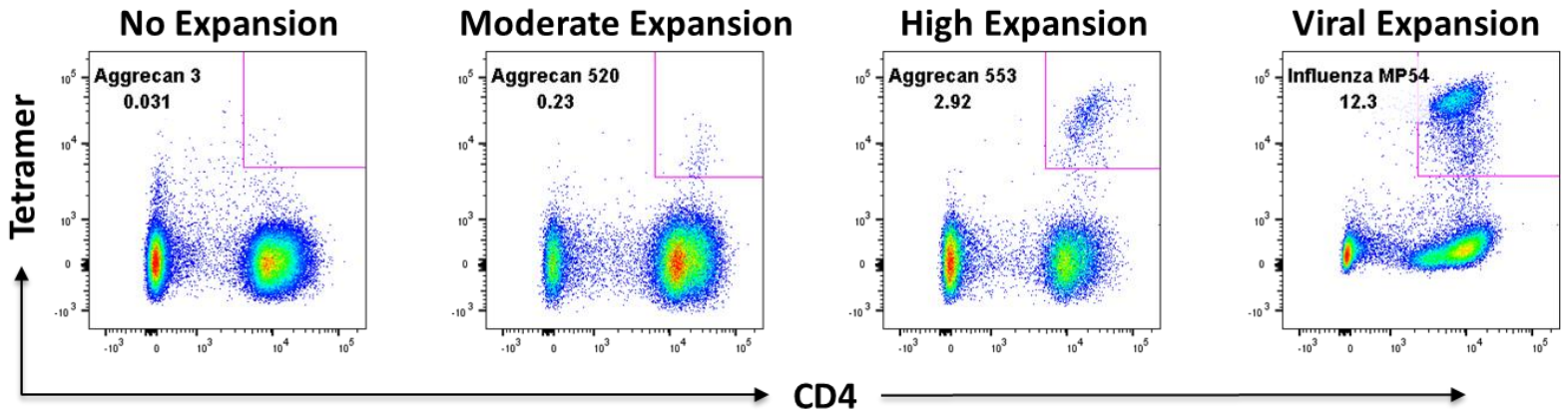
In-vitro Expansion of Aggrecan Specific CD4 T cells





6/13 peptides resulted in CD4 T cell expansion in vitro which are detectable by tetramer

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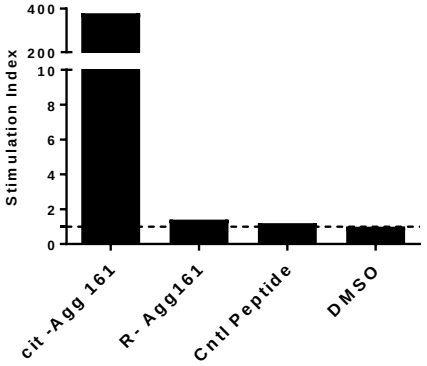
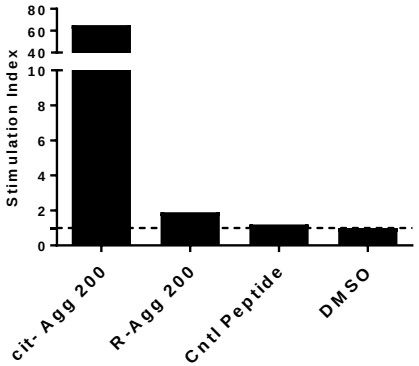
Confirmation of aggrecan epitopes by cloning

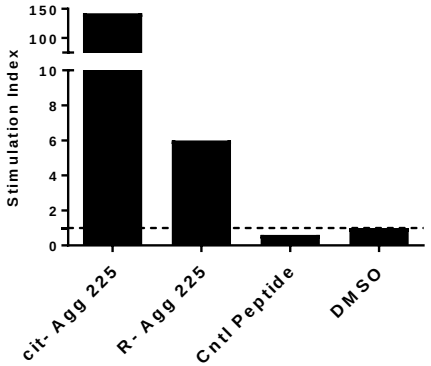
ST(Cit)YTLDFD(Cit)AQ(Cit)ACLQ



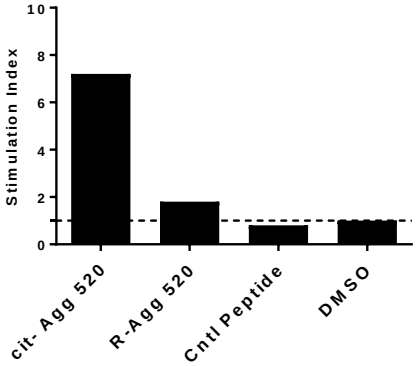
DAGWLADQTV(Cit)YPIHT



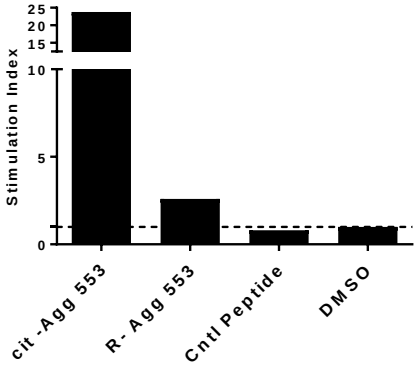
DEFPGV(Cit)TYGI(Cit)DTNETYDV



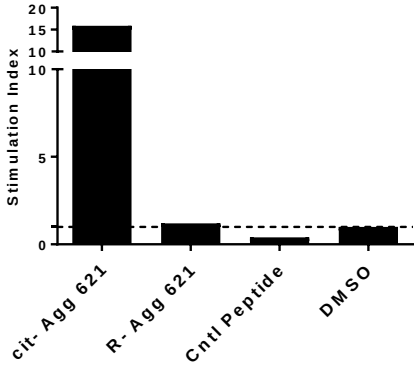
GYEQCDAGWL(Cit)DQTV(186)YPIV



PGV(Cit)TYGV(Cit)PSTETYDVY



KCYAGWLADGSL(Cit)YPIV



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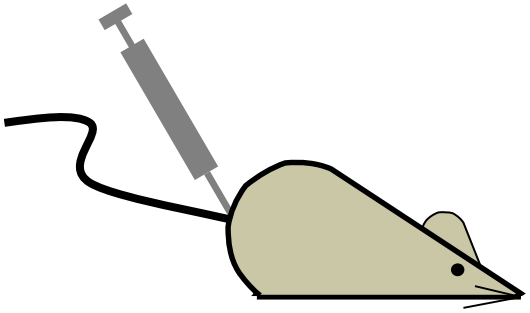
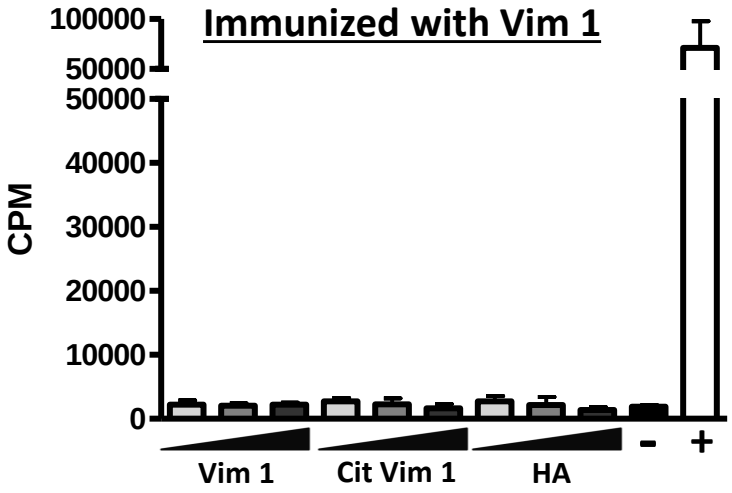
Validation of novel epitopes using DR4 Tg mice

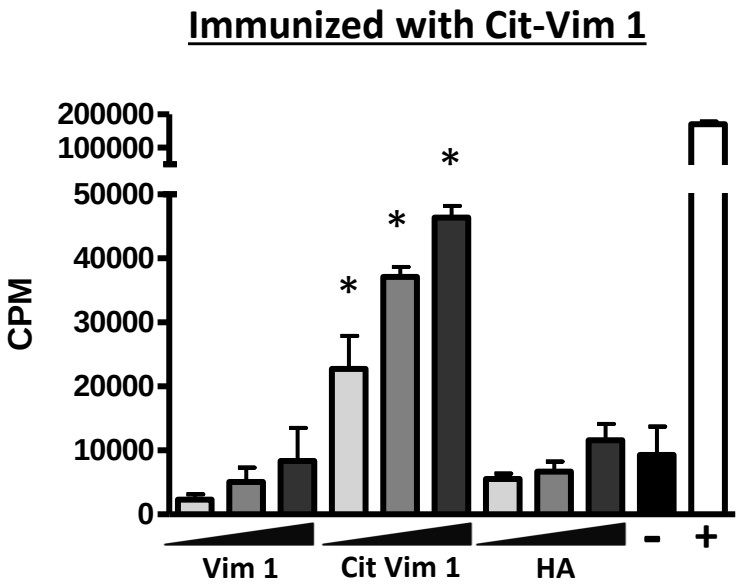


DR0401 mice

→

Day 14 Harvest
spleen and draining LN
(ILN & ParaLN)





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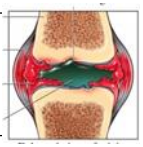
Predicting Citrullinated Epitopes

cannot bind

able to bind

Predict peptides from antigens of high interest



β-chain Fibrinogen

Vimentin
Alpha-enolase
Fibrinogen (alpha, beta, & gamma)
Cartilage Intermediate Layer Protein
Aggrecan

Alleles of high interest:

DR0401, DR0404, DR0101 and DRB4

DRB1_0401:

MKRMVSWSFHKLKTMKHLLLLLLCVFLVKSQGVNDNEEG
FFSARGHRPLDKKREEAPSLRPAPPPISGGGYRARPAKA
AATQKKVERKAPDAGGCLHADPDLGVLCPTGCQLQEALL
QQERPIRNSVDELNNNVEAVSQTSSSSFQYMYLLKDLWQ
KRQKQVKDNENVVNEYSSELEKHQLYIDETVNSNIPTNL
RVLRSILENLRSKIQKLESDVSAQMEYCRTPCTVSCNIP
VVSGKECEEIIRKGGETSEMYLIQPDSSVKPYRVYCDMN
TENGGWTVIQNRQDGSVDFGRKWDPYKQGFGNVATNTDG
KNYCGLPGEYWLGNDKISQLTRMGPTELLIEMEDWKGDK
VKAHYGGFTVQNEANKYQISVNKYRGTAGNALMDGASQL
MGENRTMTIHNGMFFSTYDRDNDGWLTSDPRKQCSKEDG
GGWWYNRCHAANPNGRYYWGGQYTWDMAKHGTDDGVVWM
NWKGSWYSMRKMSMKIRPFFPQQ

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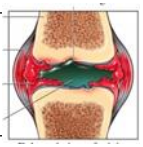
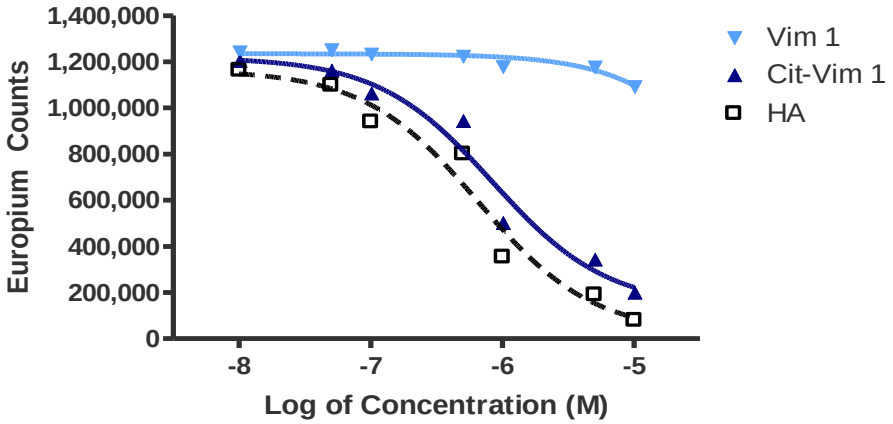
Creating a panel of potential arthritogenic peptides in RA

Predict peptides from antigens of high interest





Vimentin
Alpha-enolase
Fibrinogen (alpha, beta, & gamma)
Cartilage Intermediate Layer Protein
Aggrecan

Alleles of high interest: **DR0401**, DR0404, DR0101 and DRB4

DRB1*0401 binding results (to date)

- 164 peptides of interest were tested for binding, 30 peptides bound
- 17 peptide pairs where both the R & cit versions bound
- 13 peptide pairs where the cit version bound and the R version did not

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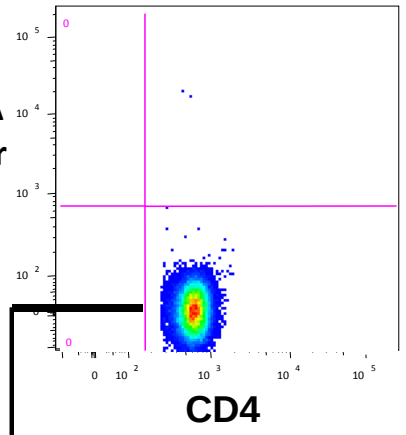
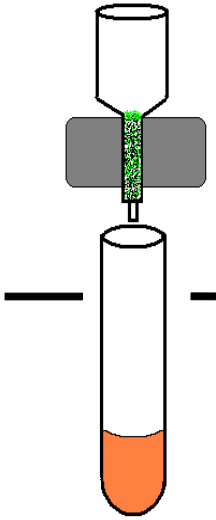
Ex vivo characterization of CD4+ T cells

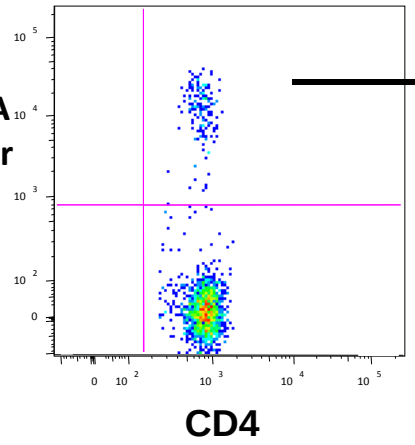
Magnetic bead enrichment

1% of the sample before enrichment





All the sample after enrichment



# of CD4+ cells in 1% of the pre-enriched sample = N

# of CD4+ tetramer+ cells eluted from the column = n

$$\text{Frequency} = \frac{n}{100N}$$ = 62 antigen specific T cells per million

Kwok et al, JACI 2010

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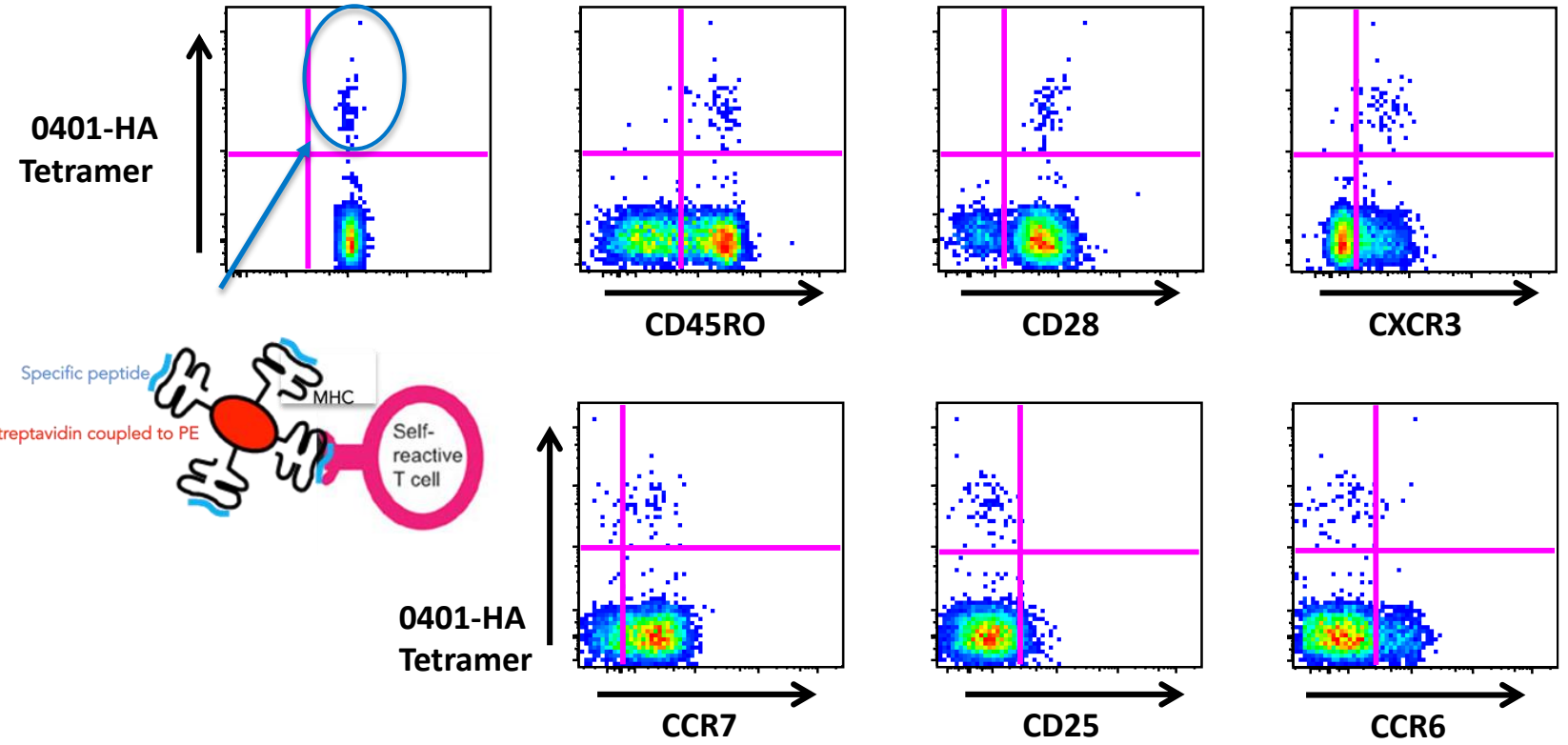
Ex vivo characterization of CD4+ T cells

Influenza specific



Frequency:
62/million CD4+ T cells

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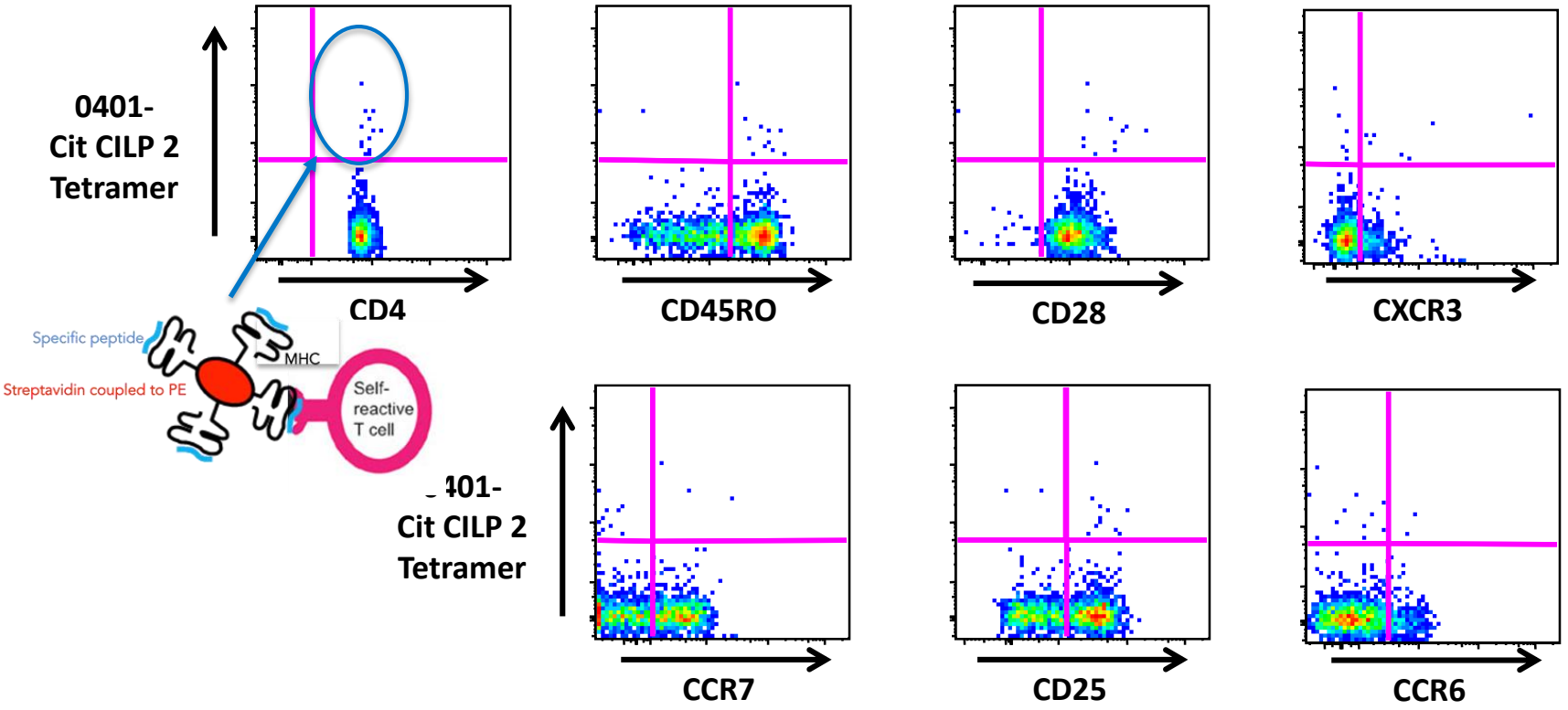
Ex vivo characterization of CD4+ T cells

Cit-CILP specific



Frequency:
4/million CD4+ T cells

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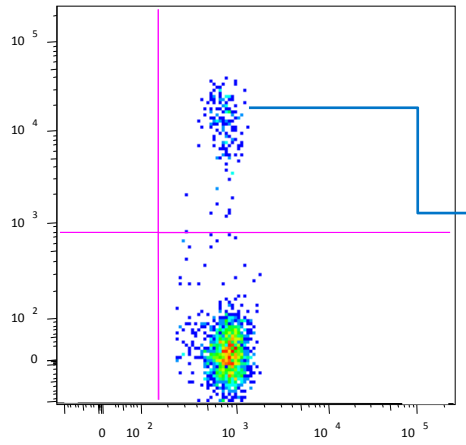
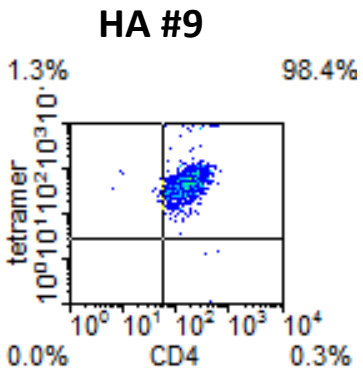
Validating the specificity of ex vivo staining

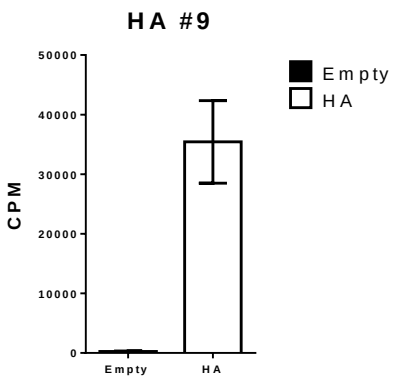


Single cell sort & clone

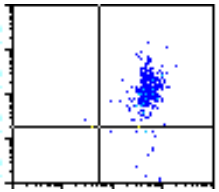
Test for specificity

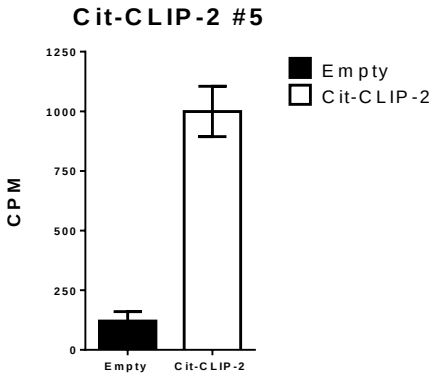
- Tmr Staining
- Proliferation assay





Cit-CILP-2 #5





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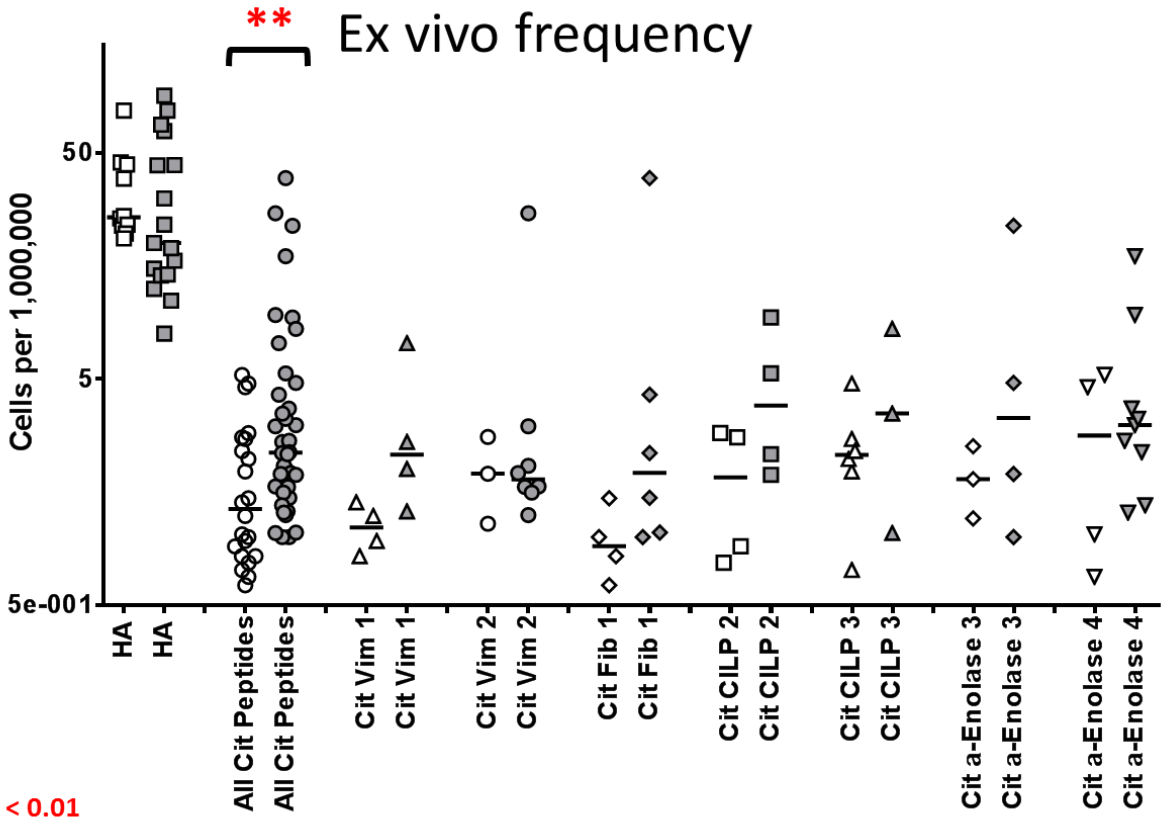
Cit-specific T cells are more frequent in patients than in controls

- 19 RA subjects had an age range of 25-81 (mean, 52+/- 13.5)
- 15 Healthy subjects had an age range of 25-82 (mean, 44+/- 17.5)
- All subjects carry at least one copy of the HLA-DRB1*0401 allele.



Jame E et al A&R 2014

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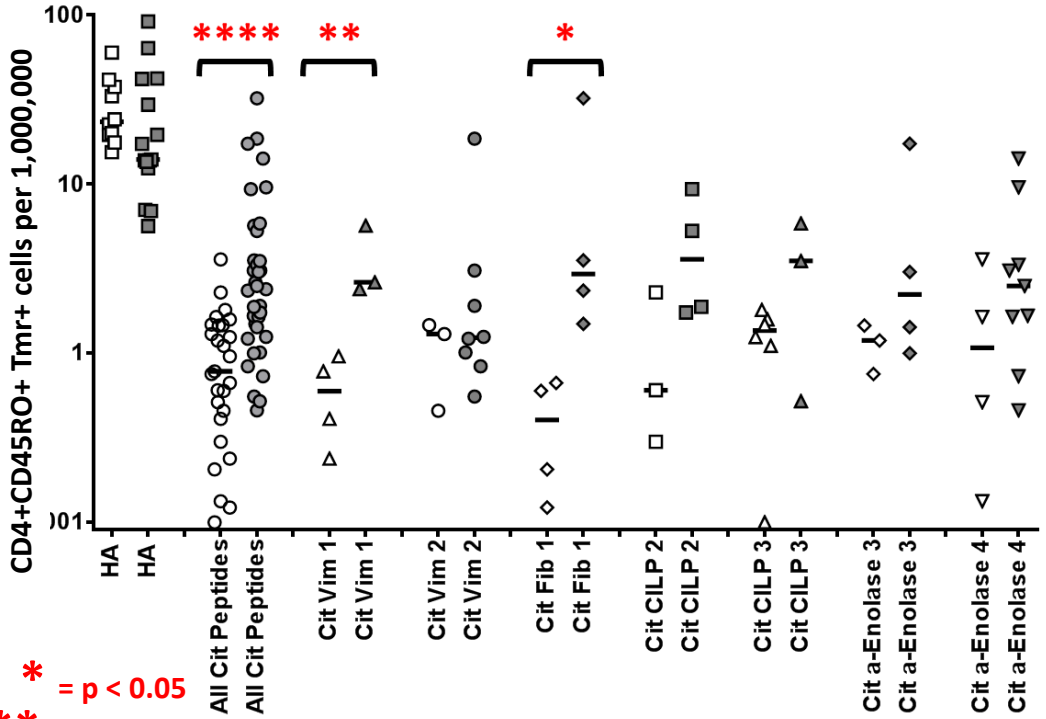
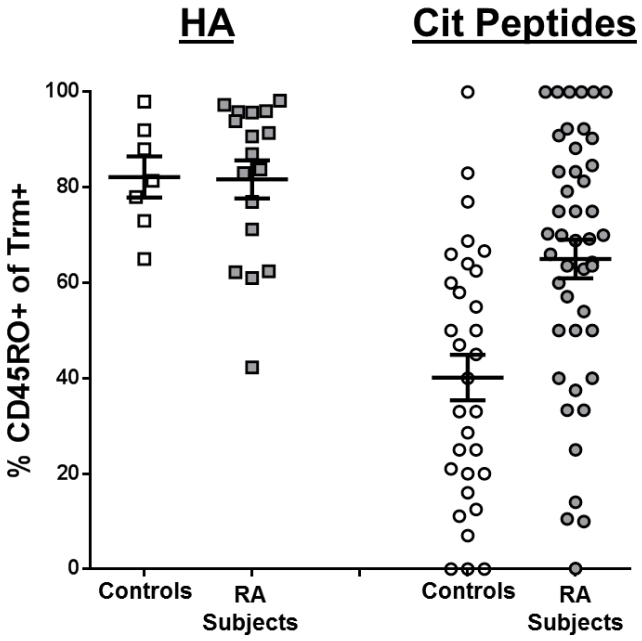
Cit-specific memory T cells are much more frequent in patients than in controls



* = p < 0.05

** = p < 0.01

**** = p < 0.0001



The frequencies of individual Tmr specificities become significant

The % of CD45RO+ Tmr+ T cells is increased in RA.

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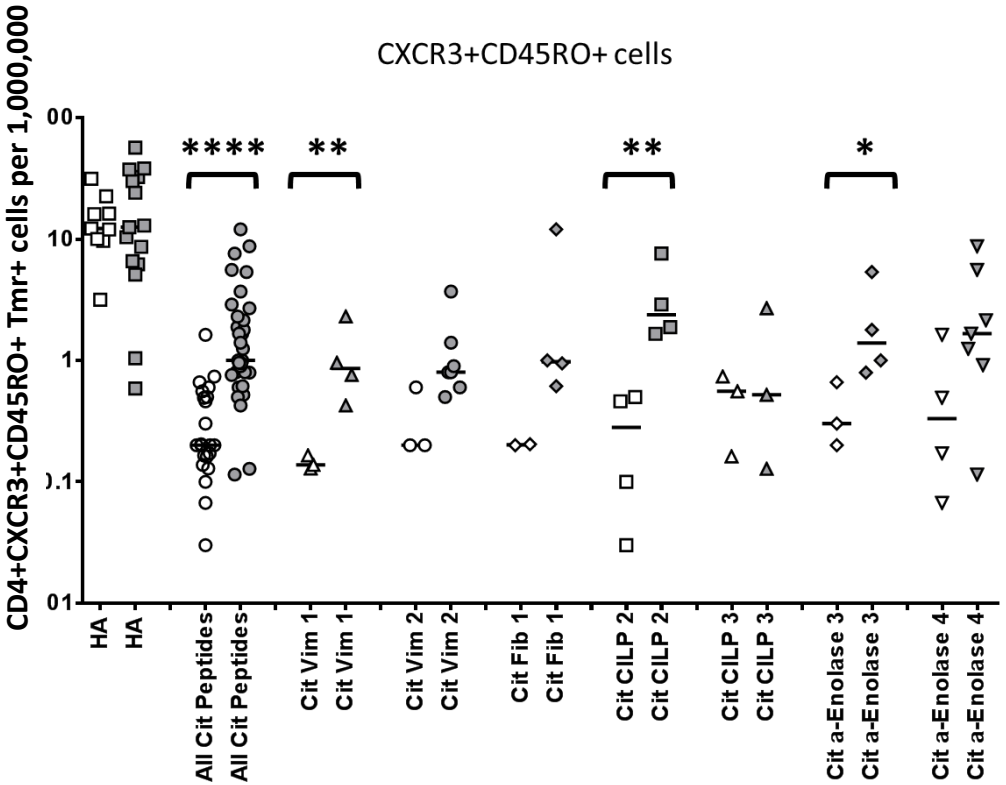
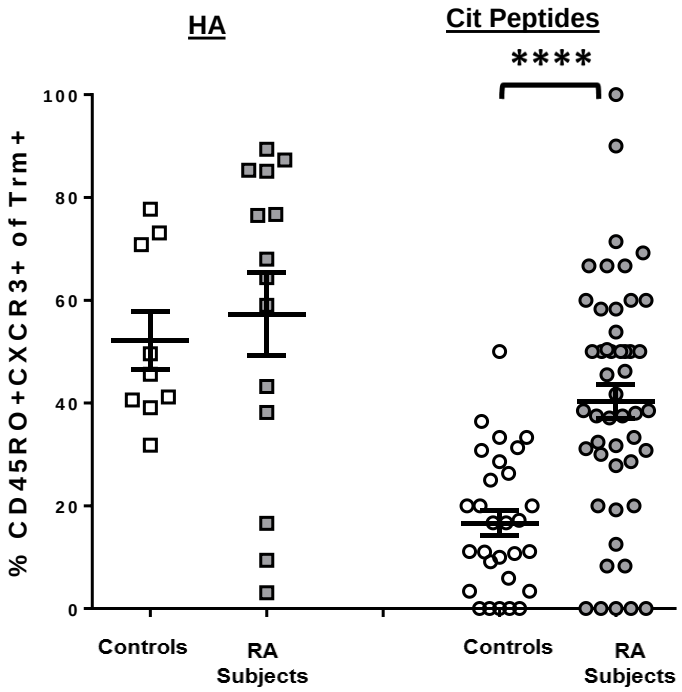
Cit-specific Th1 memory T cells are much more frequent in patients than in controls





The frequencies of individual TH1 Tmr specificities become more significant

The % of CXCR3+CD45RO+ Tmr+ T cells is increased in RA.

* = p < 0.05

** = p < 0.01

**** = p < 0.0001

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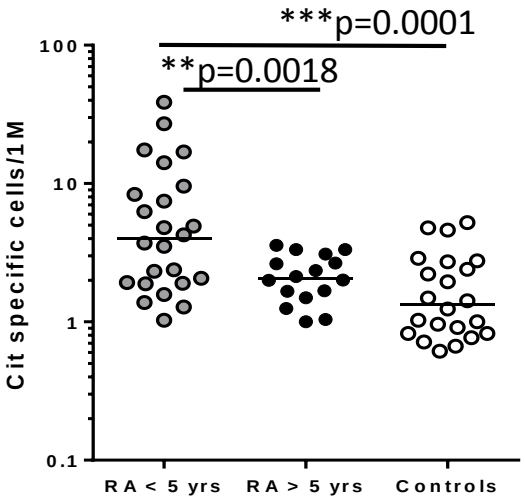
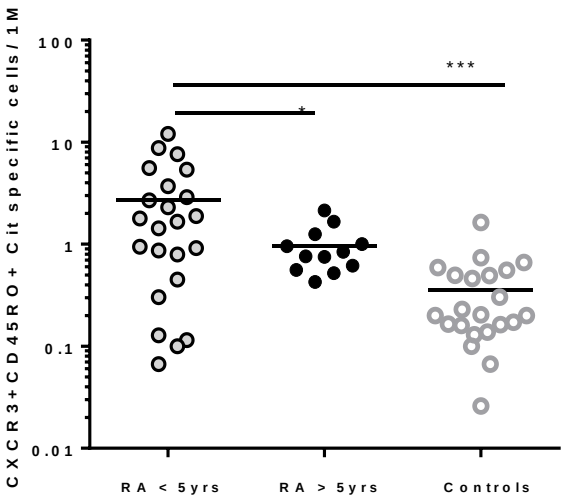
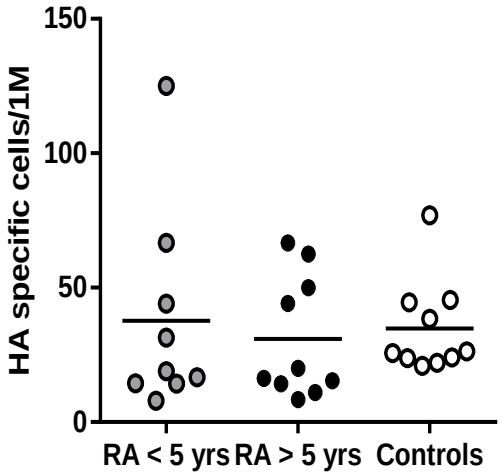
A greater frequency of Cit-Tmr+ cells are present in early in RA

Citrulline Specific CD4 T cells





HA Specific CD4 T cells



The number of HA specific cells does not change with time from disease, suggesting that this finding is specific for the pathogenic CD4 T cells in RA.

Control n=11 with Tmr+ of 15 total
RA<5 yrs n=9 with Tmr+ cells (of 9 total)
RA> 5yrs n= 7 with Tmr+ cells (of 10)

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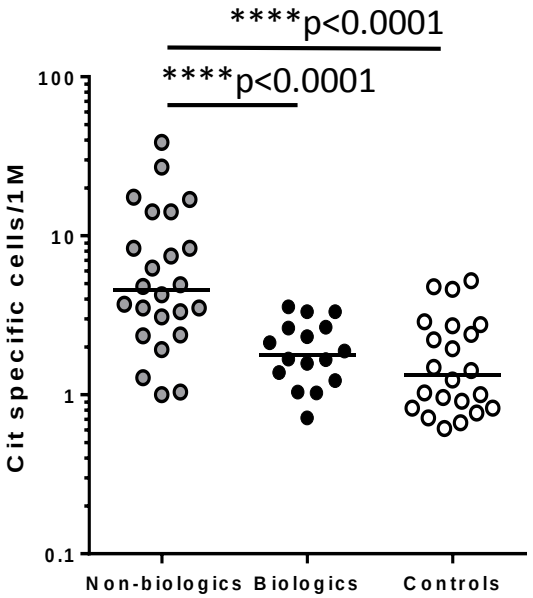
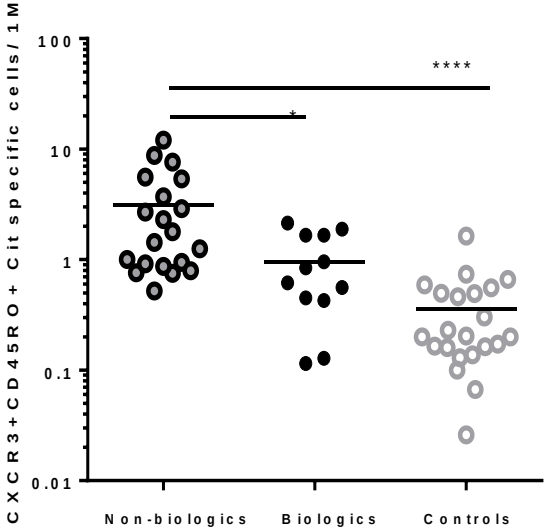
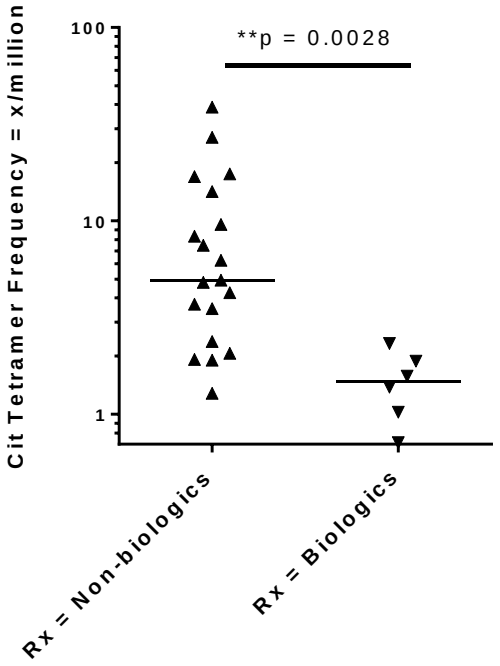
Cit-Tmr+ T cell frequency is lower in individuals treated with biologics irrespective of time from diagnosis

Citrulline Specific CD4 T cells





RA subjects <5ys of dx



The number of Citrulline specific cells is lower in individuals treated with a biologic agent- irrespective of time from diagnosis

Biologics n=7 with Tmr+ cells (of 11 total)
non-biologic n= 8 with Tmr+ cells (of 9)
One subject is studied twice and is in both groups

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Conclusions: Technical

- Citrullinated HLA DR0401 binding epitopes from synovial antigens can be predicted based on known binding motifs
- Confirmation of peptide epitopes can be performed through:
  - Binding studies/Immunization of HLADR4 Tg mice
  - In vitro expansion of human CD4 T cells in response to the epitope
- CD4 T cells specific for these epitopes can be identified directly ex vivo using HLA Class II tetramers.
  - Frequency can be determined
  - Phenotype- including cell surface markers, cytokine production can be determined using this approach.

Conclusions: Findings and Implications

- RA patients have CD4 T cells to multiple citrullinated epitopes
  - Establishes a link between Abs and T cell responses
  - Different epitope patterns seen in different subjects
  - No single epitope was positive in all subjects
- RA patients demonstrate an increase in the frequency of citrulline specific CD4 T cells
  - Implies disease relevance for cit-specific T cells
  - predominantly memory cells
  - predominantly Th1
- Frequency of these cells is linked to disease duration and therapy in these individuals
  - Suggests Cit-specific CD4+ T cells could be a useful biomarker

Remaining questions

- Are other epitope specificities important?
  - Expand study to include additional antigens
  - Investigate other HLA restrictions (DR 0404, 1001, 0101)
- Does the number of epitopes recognized expand over time and/or does fine specificity impact disease outcome?
  - Utilize a multiplex strategy to examine multiple specificities in each blood sample
  - Examine longitudinal samples
- Are other phenotypic attributes important?
  - Examine the transcriptome of citrulline specific T cells from the peripheral blood and synovial fluid using RNA seq
- Does T cell frequency or phenotype change with disease activity and treatment?
  - Cross sectional analysis to confirm and expand current findings
  - Longitudinal analysis
  - What would we see prior to disease diagnosis?
- Are Cit-specific CD4 T cells in the joint of similar specificity and phenotype to those in the periphery?

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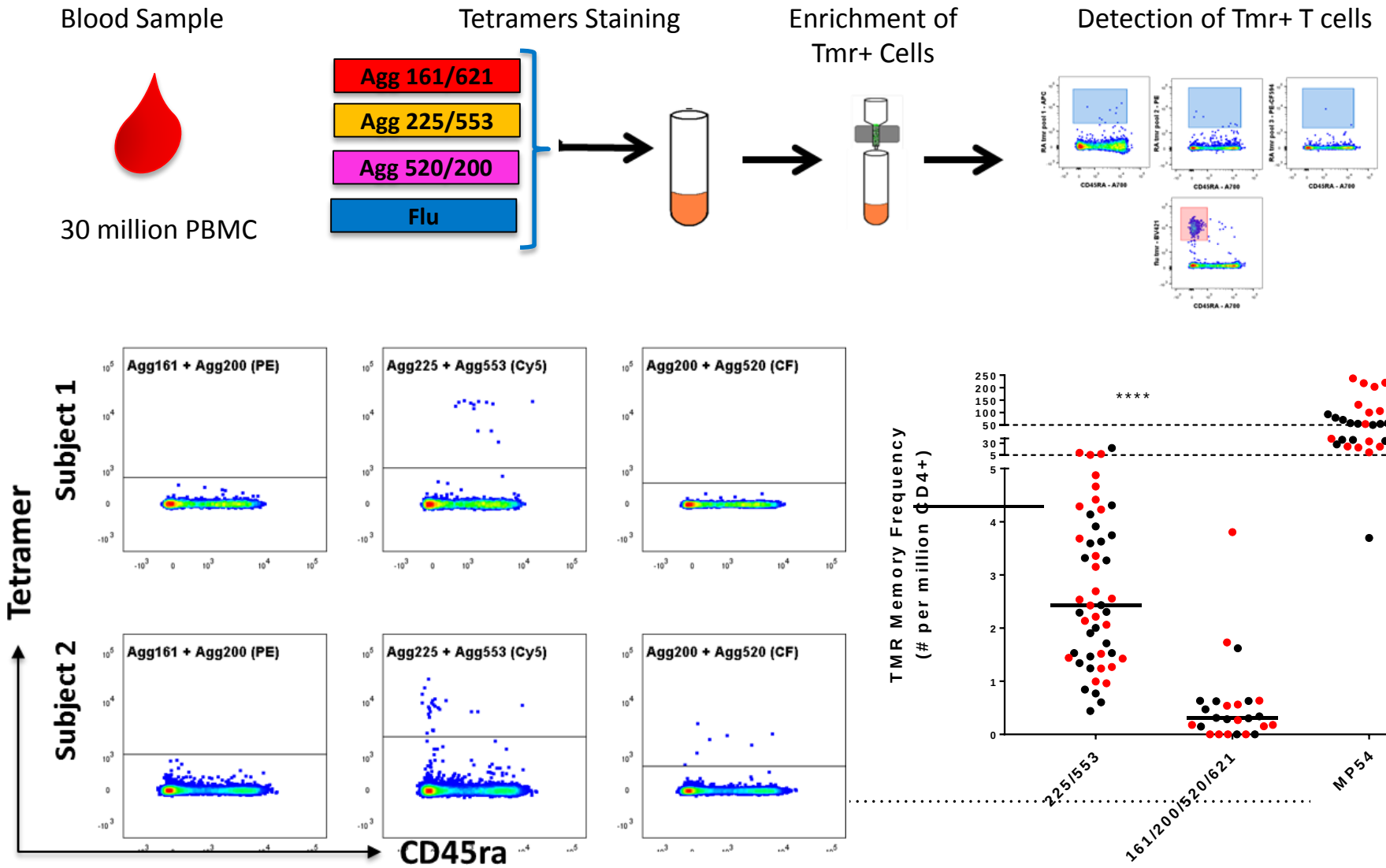
Direct *ex vivo* evaluation of aggrecan epitopes



Red = RA Subjects

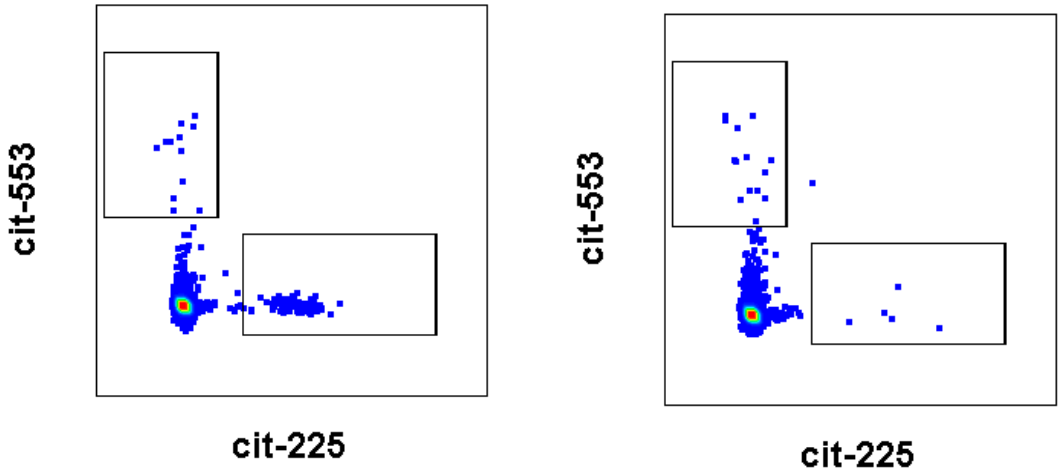
Defining minimal aggrecan epitopes

Red font indicates amino acids that can be truncated without loss of recognition
Arrows indicates Cit residues that are required for recognition
Underline indicates predicted minimal core 9mer

Agg 225 DEFPGVXTYGIXDTNETYDV

Agg 553 PGVXTYGVXPSTETYDVY

In spite of considerable homology, T cells do not appear to cross-stain with Agg 225 and Agg 553 *ex vivo*



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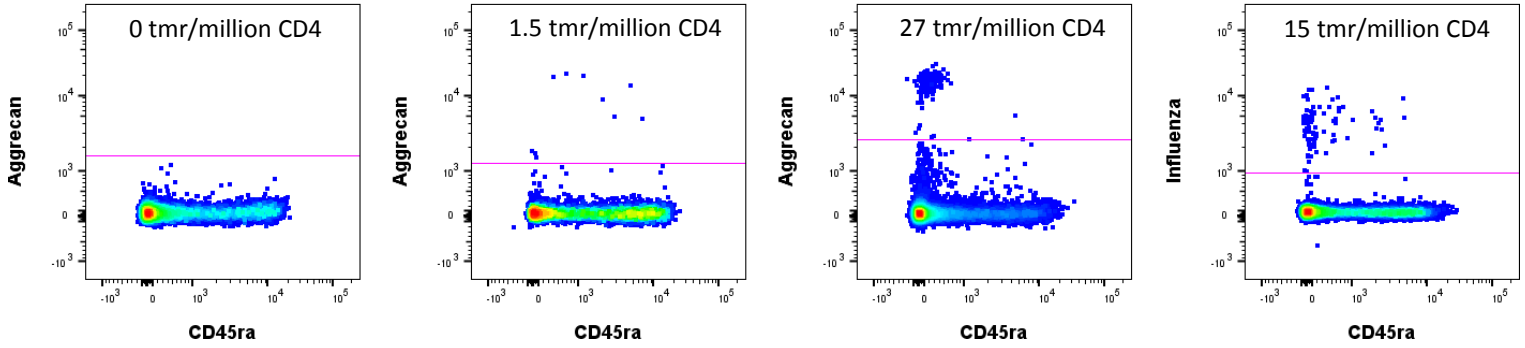
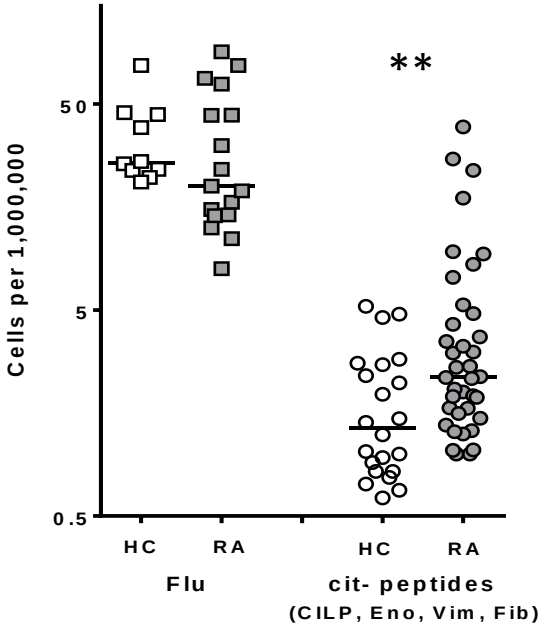
Evaluating cit- specific T cell frequency in RA



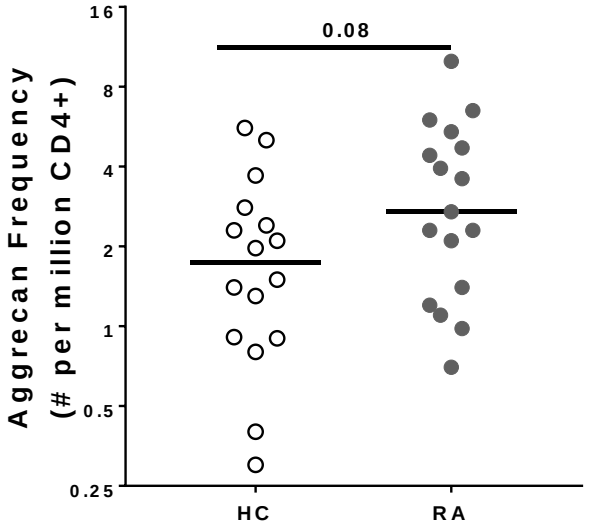
Evaluating cit-specific T cells
CILP, Enolase, Vimentin, Fibrinogen



** = p < 0.01

James et al A&R 2014

Aggrecan Frequency HC vs RA



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Remaining questions

- Are other epitope specificities important?
  - Expand study to include additional antigens
  - Investigate other HLA restrictions (DR 0404, 1001, 0101)
- Does the number of epitopes recognized expand over time and/or does fine specificity impact disease outcome?
  - Utilize a multiplex strategy to examine multiple specificities in each blood sample
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- Are other phenotypic attributes important?
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- Are Cit-specific CD4 T cells in the joint of similar specificity and phenotype to those in the periphery?

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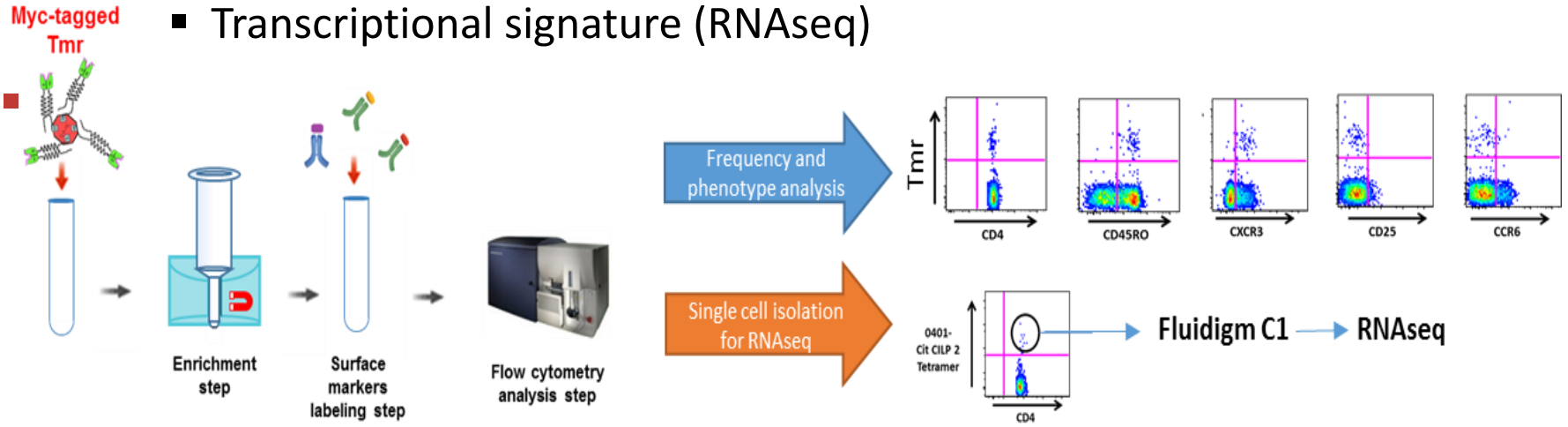
Summary and Conclusions

- A comprehensive screening of cit-aggrecan epitopes in context of HLA DRB1*0401 identifies 13 peptides.
- 6 cit-aggrecan epitopes are consistently immunogenic when tested in peripheral blood.
- Several cit-Aggrecan peptides elicit unusually strong *in vitro* response in some individual
- Two epitopes are dominant targets in the periphery
  - These epitopes are not cross-reactive
- Frequencies cit-Agg specific CD4 T cells are slightly elevated in RA
  - Memory frequencies appear elevated in a subset of patients
  - Isolated clones reveal cit-specificity and Th1 phenotype
- Frequency in the periphery increases with biologic therapy and when disease is well controlled.

Technical Challenges

- Goal: Direct ex vivo identification and isolation of CD4 T cells specific for synovial cit-epitopes to determine:
  - Frequency
  - Phenotype-
    - cell surface markers/cytokine production (FACS)
    - Transcriptional signature (RNAseq)



- Obstacle: Large number of potential CD4 T cell epitopes among synovial antigens require high cell numbers to test individually

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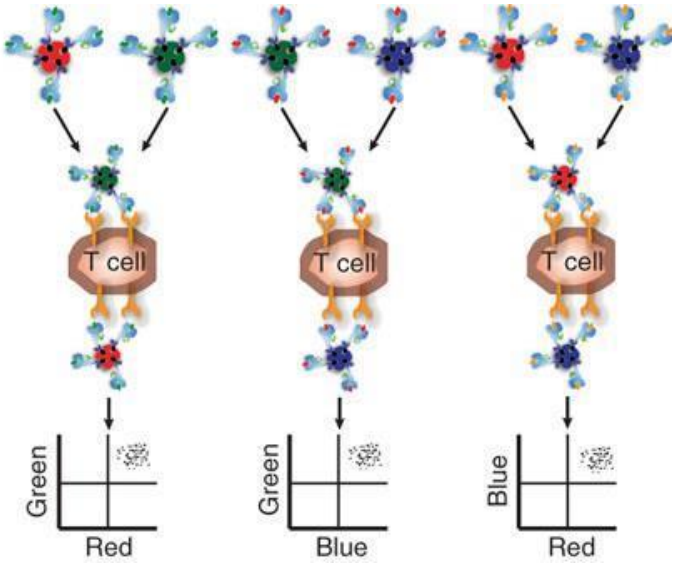
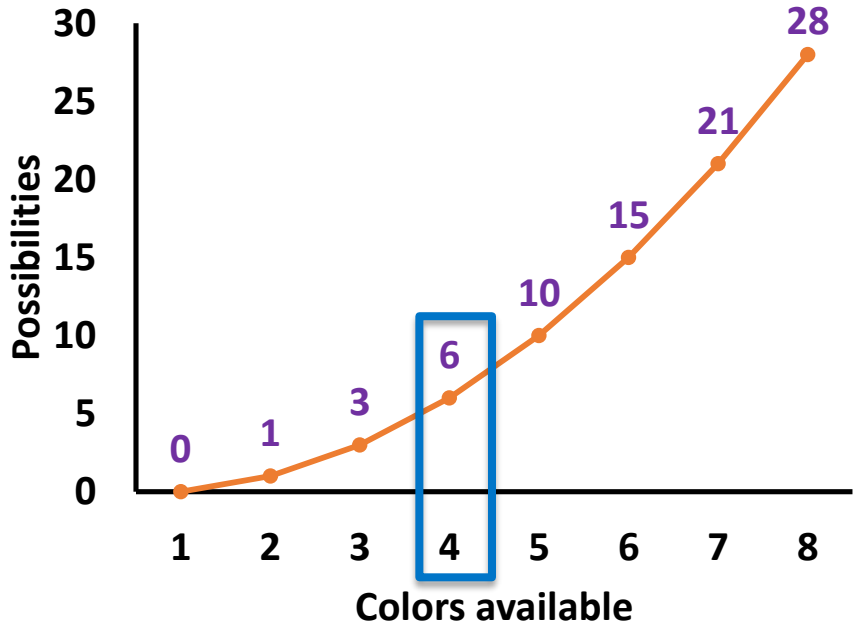
Miniaturize the tetramer assay for small volumes and frozen cells

Develop multiplexing with tetramers so we can do it all in 1 tube!





Make more fluorophores available for HLA II tetramer staining
Develop a robust combinatorial HLA II tetramer staining protocol.

Characterize multiple responses from the seasonal flu vaccine using moderate amounts of frozen PBMC

For MHC class I
2009 Nature Methods
Davis MM *et al.*,
Schumacher TN *et al.*

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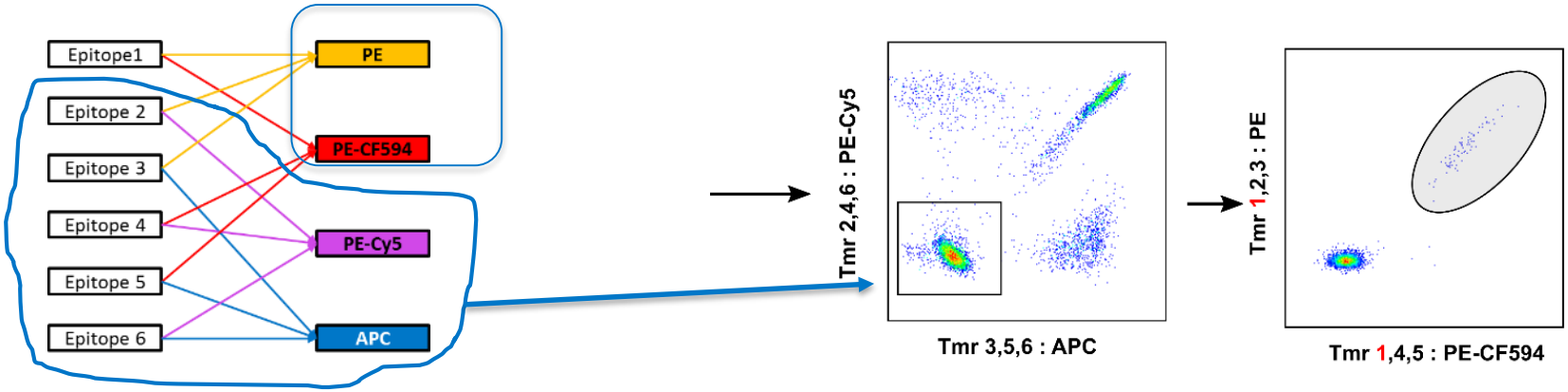
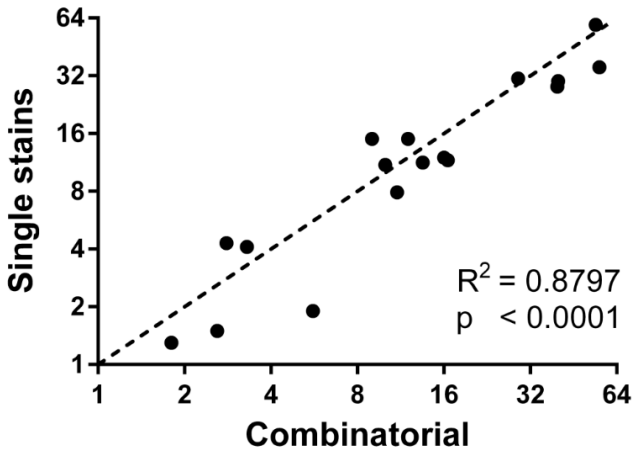
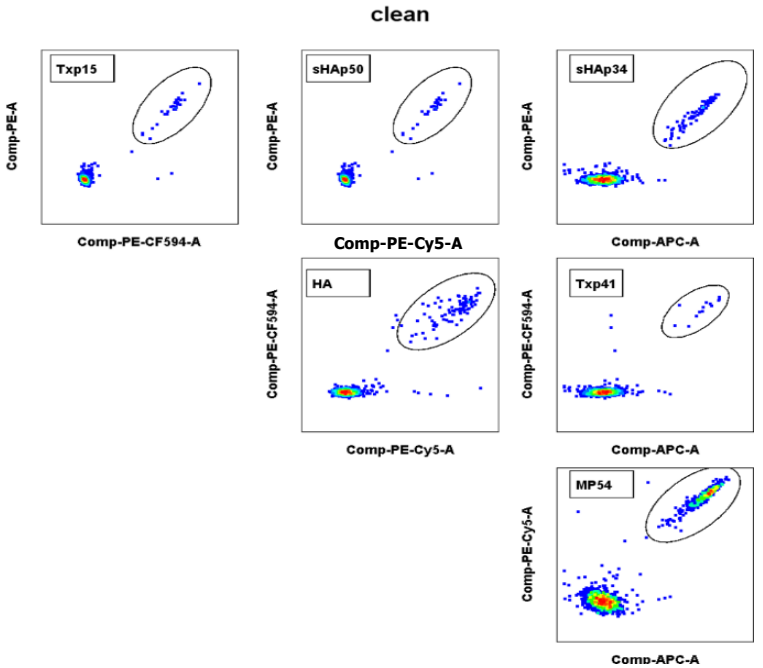
Combinatorial staining – removes background and confirms specificity through use of two Tmr



Frequency unchanged by use of multiplex approach





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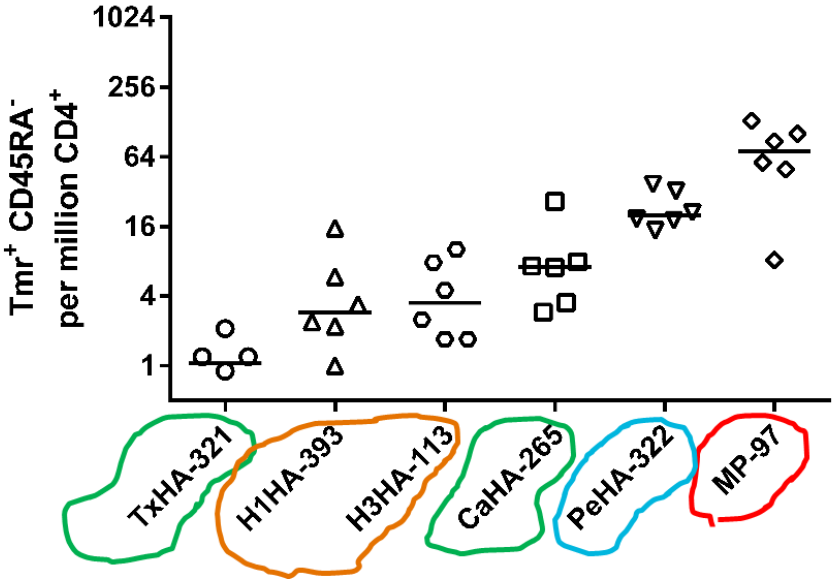
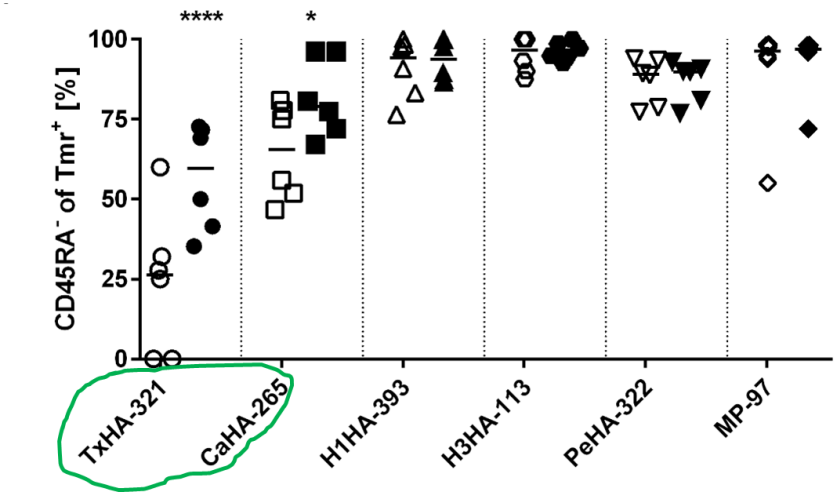
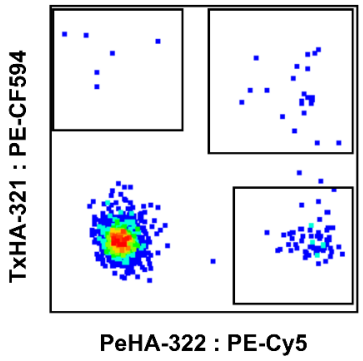
1: Frequency Hierarchy

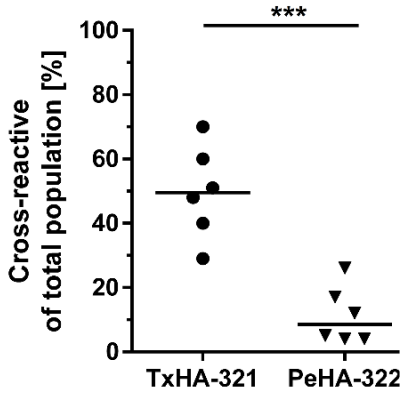


2: Memory Phenotype



3: Cross-Reactivity





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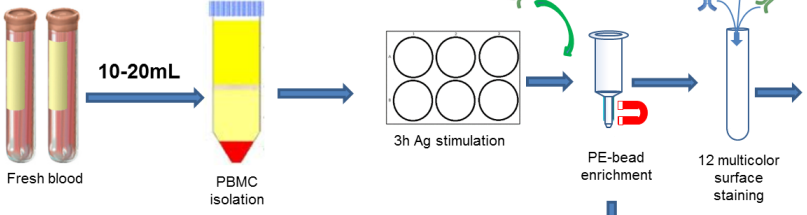
Summary

- Expanded useable fluorophores for HLA II tetramer staining
- Developed a robust protocol for the combined detection and characterization of multiple antigen-specific CD4 T-cell responses *ex vivo*
  - From modest amounts of frozen PBMC
- Window into prominent responses to the seasonal flu vaccine
  - Frequency hierarchy
  - Activation and memory phenotype
  - Cross-reactivity
  - Lineage composition
  - Cytokine expression
- Feed into single-cell RNAseq (TCR repertoire analysis)
- Technique with broad relevance for human immunology

Antigen-Specific T cell Profiling Pipeline:

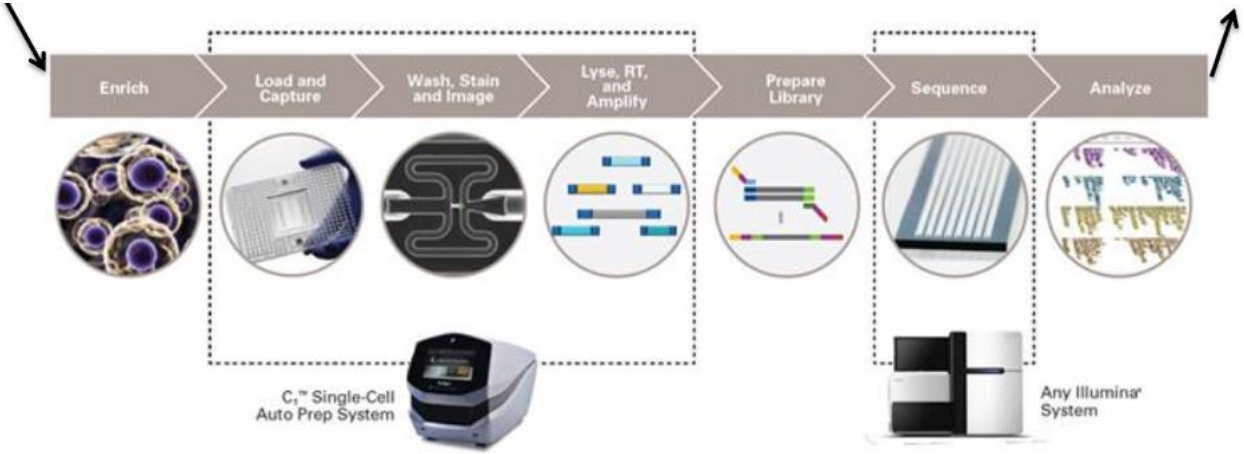
Tracking and dissecting antigen-specific CD4+ T cell responses *ex vivo*



12C Surface markers analysis + Frequency

Color	Panel
FITC	CD161
PE	CD154-Ara h
ECD	CD45RO
PC5.5	Dump
PC7	CCR4
BV421	CXCR3
APC	CRTH2
APC7	CD27
BV711	CD38
BV650	CD3
BV605	CD4
BV510	CCR6

RNASeq
C1 single-cell profiling



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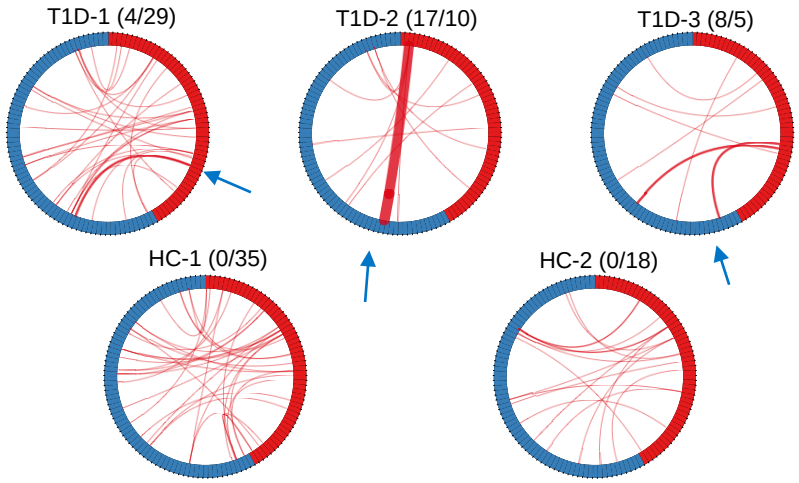
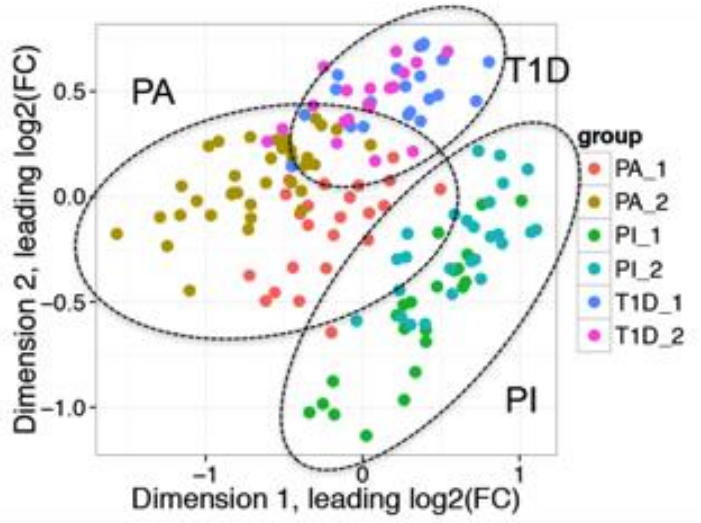
Single cell analysis of antigen-specific CD4 provides unique information on TCR and transcriptional profiles that are distinct with each disease

- Autoantigen- and allergen-specific CD4+ T cell single cell profiles cluster by donor rather than by batch



Parentheses indicate numbers of TCRs Expanded (E) >2/Not-Expanded > 2 (NE). E versus NE TCRs in T1D subjects were significantly higher than in HC subjects (p-value = 9.1e-9). Arrows, most expanded clonotypes.



T1D (n=2) subjects → islet specific T cells

PA, peanut allergen skin test + IgE+; PI, peanut allergen skin test + IgE- → peanut specific T cells

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Conclusions

- RA continues to be a disease that is challenging to treat and does not yet have a cure.
- Genetic and environmental factors are involved and understanding these is leading to an better understanding of disease pathogenesis
- The role of CD4 T cells in disease is clear.
- New tools that include HLA Class II tetramers and RNAseq will allow us to defining the character and specificity of these cells
- This has the potential to improve diagnosis, follow response to therapy and ultimately allow us to use antigen specific therapy.

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At BRI:
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