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Do RA associated HLA-DR molecules bind citrullinated peptides or peptides from PAD4 to help the development of RA specific antibodies to citrullinated proteins?

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ABSTRACT

Purpose: Rheumatoid arthritis (RA) is associated with HLA-DRB1 genes encoding a five amino acid basic motive, the shared epitope (SE). Each HLA-DRB1 genotype defines a genotype specific risk of developing RA.

RA is preceded by the emergence of anti citrullinated protein antibodies (ACPAs). Citrullin is a neutral version of arginin, a basic amino acid, formed after post translational modification by Peptidyl Arginyl Deiminases (PADs). HLA-DRB1 genes associated with RA are also associated with ACPAs.

Two models might explain this association.

Here we tested both models for prediction of HLA-DRB1 genotypic risks of developing RA.

Methods: We calculated the likelihoods for the 2 HLA-DR molecules encoded by 12 common HLA-DRB1 genotypes to bind at least one randomly chosen peptide from PAD4 or fibrinogen(native or citrullinatd) and compared them with the 12 respective HLA-DRB1genotypic risks of developing RA.

Results: HLA-DRB1 Genotypic risks of developing RA correlate with likelihoods of binding PAD4 peptides, not citrullinated Fibrinogen peptides. Thus, the molecular basis for the association of HLA-DR and ACPA positive RA is most likely the capability for RA associated HLA-DR molecules to bind peptides(s) from PAD4.

- RA associated HLA-DR molecules might bind citrullinated peptides better than non RA associated HLA-DR molecules.
- RA associated HLA-DR molecules might bind PAD4 peptide(s) better than non RA associated HLA-DR molecules.

1. Introduction

Rheumatoid arthritis (RA) is a chronic destructive autoimmune joint disease of unknown origin.

HLA-DRB1 genes are the major genetical component of RA susceptibility. Indeed, HLA-DR molecules with the so called "shared epitope" (SE), a basic five amino acid motive in the third hypervariable region of their HLA-DRB1 chain are associated with RA [1]. Both HLA-DR molecules expressed by an individual influence his/her risk to develop RA with Odds Ratios (ORs) ranging from 30 for HLA-DRB1 genotypes

encoding 2 RA associated HLA-DR molecules to 0.2 for HLA-DR genotypes encoding no shared epitope positive HLA-DR molecule [2].

The development of RA is preceded by the emergence of anti citrullinated protein antibodies (ACPAs). Citrullin is a neutral version of arginin, a basic amino acid formed after post translational modification of arginin by enzymes called Peptidyl Arginyl Deiminases (PADs).

HLA-DRB1 genes associated with RA are also associated with the presence of ACPAs in patients with RA [2]. This suggests that RA associated HLA-DR molecules predispose to RA because they contribute to the development of ACPAs.

Indeed, the function of HLA-DR molecules is to present peptides to Follicular B Helper T cells (T_{FH}) to allow the development of IgG antibodies, suggesting that RA associated HLA-DR molecules might present peptides to the T_{FH} cells which help the development of ACPAs. However, the identity of the peptide(s) presented by RA associated HLA-DR molecules to the T_{FH} cells which help the development of IgG ACPAs is

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unknown.

It was first proposed that RA associated HLA-DR molecules bound and presented citrullinated peptides to T_{FH} cells allowing them to help the development of IgG antibodies to citrullinated proteins. This was demonstrated by showing that one peptide from Vimentin: Vim 65-77, when citrullinated, bound RA associated HLA-DR molecules better than non RA associated HLA-DR molecules. This suggested that RA associated HLA-DR molecules were, in general, better than non RA associated HLA-DR molecules at binding citrullinated peptides, explaining why they were associated with ACPAs and RA [3].

However, thorough HLA-DR peptide binding studies did not confirm the preferential binding of citrullinated peptides to RA associated HLA-DR molecules [4,5].

We have proposed an alternative hypothesis, suggesting that T_{FH} cells recognize PAD4, the citrullinating enzyme and help the development of ACPAs of multiple specificities by a hapten carrier mechanism in which PAD4 is the carrier and proteins bound by PAD4 and being citrullinated are the haptens [6,7]. We found evidence for this hypothesis in mice and in humans.

In short, two models may explain the RA/ACPA/HLA-DR association. One states that RA associated HLA-DR molecules bind citrullinated peptides better, the second that RA associated HLA-DR molecules bind PAD4 peptide(s) better.

Here, we decided to evaluate how each of the two models is consistent with the risk to develop ACPA positive RA associated with 12 common HLA-DRB1 genotypes.

To do so, we used previously published binding data of 65 peptides from PAD4, 96 peptides from Fibrinogen (under their native or citrullinated form) to HLA-DR molecules encoded by HLA-DRB1*04:01, *04:04, *01:01, *04:02, *07:01 [4,7]. We evaluated the likelihoods of binding at least one randomly chosen peptide from PAD4 or native or citrullinated fibrinogen for the 2 HLA-DR molecules encoded by 12 different HLA-DRB1 genotypes and compared them with HLA-DRB1 genotypic risks of developing RA (2).

We found that the risk of developing RA associated with each HLA-DRB1 genotype matches the likelihood of binding at least one peptide from PAD4, but not the likelihood of binding a peptide from native or citrullinated Fibrinogen.

2. Methods

2.1. What are the relevant citrullinated antigens to test for HLA-DRB1 binding?

There is no evidence that any particular citrullinated antigen is at the origin of anti citrullinated protein immunization. We chose to test the binding of peptides from Fibrinogen because Fibrinogen is strongly expressed in the rheumatoid joint and because positivity of antibodies to citrullinated Fibrinogen matches closely that of the anti CCP2 test [8].

2.2. How to characterize the peptide binding capabilities conferred to each individual by the two HLA-DRB1 alleles he/she expresses?

To evaluate the capability for an individual with a given HLA-DRB1 genotype, to mount an efficient T cell response to a relevant, unknown, peptide from PAD4 or Fibrinogen, we calculated, for the two HLA-DR molecules expressed by each HLA-DRB1 genotype, the likelihood to bind at least one peptide from PAD4 or Fibrinogen or citrullinated Fibrinogen. We counted how many peptides tested from 65 overlapping peptides from PAD4 or 96 peptides from Fibrinogen or citrullinated Fibrinogen could be bound by HLA-DRB1*0401, *0404, *0101, *0402, *0101 molecules, with a signal as strong or higher than that obtained with HA, a peptide from the Haemophilus Influenzae hemagglutinin known to be a pan HLA-DR binder [5].

2.3. Binding of peptides from PAD4 and fibrinogen to different HLA-DR molecules

We previously published the results of a direct binding assay of peptides from PAD4 and native and citrullinated Fibrinogen to HLA-DR molecules [4,7]. Homozygous cell lines expressing SE positive HLA-DRB1*01:01 (JESTHOM), *04:01 (SAVC), and *04:04 (PEYSSON), or SE negative HLA-DRB1*04:02 (YAR) and DRB1*07:01 (MOU) were cultured in RPMI 1640 supplemented with 10% fetal calf serum. After cell lysis (in 10 mM Tris pH8, 10 mM NaCl, 10 mM MgCl₂, 1% Triton X100, 0.05 mg/ml Dnase and protease inhibitors), total protein extracts were immunoprecipitated by anti-HLA-DR LB3.1 antibody covalently coupled on CNBr-activated Sepharose 4B (Sigma Aldrich, St. Quentin-Fallavier, France). After washing, HLA-DR molecules were eluted in phosphate buffered saline (pH 2) with 0.5% octyl glucoside, neutralized in 1 M Tris, and quantified.

The binding of each purified HLA-DR allele was tested on ELISA plates coated with 10 µg of peptide from PAD4 or native and citrullinated fibrinogen. Peptides were synthesized using the solid-phase system and purified (>60%) (Neosystem, Strasbourg, France). Each peptide was tested in duplicate wells and, as controls, 2 empty wells (not coated with any peptide) and 2 wells coated with a positive binder, influenza hemagglutinin (HA) peptide (amino acid sequence: PKYVKQNTLKLAT) [5]. After peptide coating, plates were blocked with 1% bovine serum albumin. One microgram of each purified HLA-DR molecule was added to each well. After washing, bound HLA-DR was detected by biotinylated anti-HLA-DR antibody (Immunotech, Marseille, France) followed by peroxidase-conjugated avidin (Sigma Aldrich, St. Quentin-Fallavier, France). After tetramethyl benzidine incubation, optical density (OD) was read at 405 nm. Positive binding was defined as an OD value equal or higher than to the OD for the HA peptide.

2.4. Peptides from PAD4 tested for HLA-DR binding

65 overlapping peptides covering the entire PAD4, a 663 aa protein (locus NM_012387) were tested for HLA-DR binding as described above.

Relevant peptides may be 10-20 amino acid long. For each peptide length, there are about 660 possible peptides on PAD4. Thus, a maximum number of 6600 PAD4 peptides may exist, and 65 peptides represent about 1% of this maximum number of possible peptides. Sequences and HLA-DR binding properties of the 65 tested peptides from PAD4 have been published recently and are shown in Fig. 1 [7].

2.5. Peptides from fibrinogen and citrullinated fibrinogen tested for HLA-DR binding

96 overlapping peptides covering the entire alpha (631 aa) (locus NP_000499) and beta (447 aa) (locus NP_005132) chains of Fibrinogen were synthesized. A native set included 40 peptides from the alpha chain, 31 peptides from the beta chain, 13 arginine free peptides from the alpha chain and 12 arginine free peptides from the beta chain. A citrullinated set included the same peptides, with all arginine residues replaced by citrullins. Sequences and HLA-DR binding properties of these 96 citrullinated or native peptides from Fibrinogen have been published previously and are shown in Fig. 2 [4].

The alpha chain and beta chains of Fibrinogen encompass a total 1078 amino acids, thus may theoretically yield up to 10.000 possible peptides in the 10-20 amino acid range. Thus, 96 peptides from the alpha and beta chains of Fibrinogen represent about 1% of the maximum number of possible peptides, a coverage very similar to that of PAD4.

2.6. Likelihood for an HLA-DR molecule of binding a random peptide from a given protein

To evaluate the capability of HLA-DR molecules encoded by different HLA-DRB1 alleles of binding an unknown relevant peptide from a given

		HLA-DRB1*				
		01:01	04:01	04:04	04:02	07:01
PAD4 peptides						
1	MAQGLTRVTPQPHAVCV			1		
2	PEQPTHAVCVLGLTLQDIC					
3	LGLTLQDICSSAPEDCTSF					
4	SSAPEDCTSFSSINASPGVV					
5	SINASPGVVDAHSPPAKK					
6	DAHSPPAKKSTGSSTWPL					
7	KSTGSSTWPLDPGVEVLTLM					
8	DPGVEVLTLMKAASGSTGDQ	2	3	4	3	4
9	KAASGSTGDQKVQISYGP					
10	KVQISYGPKTTPPVKALLYL					
11	TPPVKALLYLTAVEISLCA					
12	TAVEISLCAIDTRTGKVKPT		1			
13	ITRTGKVKPTRAVKDQRTWT		1	1		1
14	RAVKDQRTWTWGPGCGGAIL					
15	WGPGCGGAILLVNCDRDLN					
16	LVNCDRDLNLESSAMDCEDDE		1	1	1	
17	SSAMDCEDDEVLDSEDLQDM					
18	VLDSEDLQDMSLMTLSTKTP					
19	SLMTLSTKTPKDFFTNHTLV					
20	KDFFTNHTLVHVARSEMDK					
21	LHVARSEMDKVRVQATRGK			1		
22	VRVQATRGKLSKCSVVLG		1			
23	LSSKCSVVLGPKWPSHYLMV		1			
24	PKWPSHYLMVPGGKHMDFY					
25	PGGKHMDFYVEALAFPD					
26	VEALAFPDTPFGLITLITIS					
27	FPGLITLITLSDNLELP					
28	LLDTSNLELPEAVFQDSVV					
29	EAVFQDSVFRVAPWIMTP					
30	FRVAPWIMTPNTQPPQEVYA					
31	NTQPPQEVYACSFENEDFL					
32	CSFENEDFLKSVTTLAMKA					
33	KSVTTLAMKAKCKLTICPEE					
34	KCKLTICPEEENMDQWMQD					
35	ENMDQWMQDEMEIGYQAP					
36	EMEIGYQAPHTLPPVFD					
37	HKTLPPVFDSPRNRGLKEFP					
38	PRNRGLKEFPKRVMPDFG					
39	IKRVMPDFGYVTRGPQTGG					
40	YVTRGPQTGGISGLDSFGN					
41	ISGLDSFGNLEVPVTVRG					
42	EVSPVTVRGKEYPLGRILF					
43	KEYPLGRILFGDSCYPSNDS					
44	GDSCYPSNDSRQMHQALQDF					
45	RQMHQALQDFLSAQVQAPV					
46	LSAQVQAPVKLYSDWLSVG					
47	KLYSDWLSVGHVDFLSFVP					
48	HVDFLSFVPAPDRKGFRLL					
49	APDRKGFRLLASPRSCYKL		2			
50	LASPRSCYKLFEQQNEGHG					
51	FEEQQNEGHGALLFEGIKK					
52	EALLFEGIKKKQKIKNIL					
53	KKQKIKNILSNKTLREHNS					
54	SNKTLREHNSFVERCIDWNR					
55	FVERCIDWNRLLKRELGLA					
56	ELLKRELGLAESDIIDIPQL					
57	ESDIIDIPQLFKLKEFSKAE					
58	FKLKEFSKAFAFFPNMVMNL					
59	AFPPNMVMNLVLGKHLGIPK					
60	VLGKHLGIPKPGPVINGRC					
61	PFGPVINGRCCLLEKVCSSL	1	2	1	2	2
62	CLEEKVCSSLLEPLGLQCTFI					
63	EPLGLQCTFINDFFTYHIRH					
64	NDDFFTYHIRHGEVHCCTNVR					
65	GEVHCCTNVRKPFSSKWWNMV		2			
Number of bound peptides		2	9	6	3	3

Fig. 1. Binding of PAD4 peptides to HLA-DRB1*01:01, *04:01, *04:04, *04:02, *07:01 molecules. Ten micrograms of peptide were coated to each well. One microgram of purified HLA-DR molecule was added. After washing, bound HLA-DR was detected by biotinylated anti-HLA-DR antibody followed by peroxidase-conjugated avidin and tetramethyl benzidine incubation. OD was read at 405 nm. Positive binding was defined by an OD value equal or higher than the OD for HA peptide. Numbers indicate the ratios tested peptide OD divided by HA peptide OD. Thus, a number higher than 1 indicates positive binding.

protein, we used a surrogate marker: we evaluated the likelihood of binding an unknown, randomly chosen peptide from this protein.

This can be done by studying the binding of a limited number of overlapping peptides, covering the entire protein by the HLA-DR molecule. The likelihood of binding is the ratio of the number of bound peptides divided by the total number of tested peptides.

2.7. Likelihood for one (at least) of the 2 HLA-DR molecules encoded by an HLA-DRB1 genotype of binding a random peptide from a given protein

The likelihood for none of the two HLA-DR molecules encoded by an HLA-DRB1 genotype to bind a peptide is the product of the likelihood for each HLA-DR molecule of not binding it.

The likelihood for one (at least) of the 2 HLA-DR molecules encoded by an HLA-DRB1 genotype of binding a random peptide from a protein is 1 minus the likelihood for none of the two HLA-DR molecules to bind it.

2.8. HLA-DRB1 genotypic risk of developing RA

We previously genotyped for HLA-DRB1 857 patients with ACPA positive RA and 2178 controls from South Eastern and Eastern France and calculated Odds Ratios (OR) for developing RA for 106 of 132 possible genotypes accounting for 97% of subjects. We found that HLA-DRB1 genotypic ORs for developing ACPA positive RA range from 30 to 0.2 [2].

2.9. Statistics

Correlation between the HLA-DRB1 genotypic Odds Ratios to develop RA and the likelihood to bind at least one peptide from PAD4 or Fibrinogen for this genotype was evaluated by Pearson's correlation test. Comparison between numbers of peptides bound by each HLA-DR molecule was done by chi-square test. GraphPad Prism 5.02 (GraphPad Software) was used for all statistical analyses.

3. Results

3.1. Likelihood of binding one randomly chosen peptide from PAD4, native or citrullinated fibrinogen for individual HLA-DR molecules encoded by HLA-DRB1*01:01, *04:01, *04:02, *04:04, *07:01

The results of the direct binding assay of 65 peptides from PAD4, 96 citrullinated peptides from Fibrinogen and their 96 native counterparts to 5 purified HLA-DR molecules, HLA-DRB1*01:01, *04:01, *04:04, *04:02, *07:01, previously published [4,7], are presented in Figs. 1 and 2.

Each purified HLA-DR molecule bound 2 to 9 of 65 PAD4 peptides, 12 to 23 of 96 fibrinogen peptides, 11 to 23 of 96 citrullinated fibrinogen peptides.

For each HLA-DR molecule, the number of bound citrullinated Fibrinogen peptides was not different from the number of bound native Fibrinogen peptides (Chi2, N.S.).

For each HLA-DR molecule, we calculated a likelihood of binding at least a peptide from PAD4, native Fibrinogen and citrullinated Fibrinogen. This likelihood was defined as the ratio of the number of bound peptides divided by the number of tested peptides. Likelihoods of binding ranged from 3% to 24% (Fig. 3).

Our binding data did not show differential HLA-DR binding between citrullinated or native Fibrinogen peptides, and allowed us calculate for each of 5 HLA-DR molecules, a likelihood of binding a peptide from PAD4 or native and citrullinated Fibrinogen.

		HLA-DRB1*							HLA-DRB1*								
		01:01	04:01	04:04	04:02	07:01			01:01	04:01	04:04	04:02	07:01				
1	Fibrinogen peptides						1	1	1	1	1	1	1				
2	alpha GPRVVERHQSACKDS	1	1	1	1	1											
3	alpha DWPFCSDEEDWNYKCP																
4	alpha SGCRMKGIDEVNDQ																
5	alpha FTNRINKLNSLFY				1												
6	alpha QKNNKDSHSLTTNIM																
7	alpha EILRGDFSSANNRDN																
8	alpha TYNRVSEDLRSRIEV																
9	alpha IEVLKRKVKIEKVQHI																
10	alpha LKRKVKIEKVQHIQLL																
11	alpha QKNVRAQLVDMKRLE																
12	alpha VIDIKIRSCRSGSCS	2	6	6	6	2											
13	alpha SCSRALAREVDLKDY	1		1													
14	alpha RALAREVDLKDYEDQ																
15	alpha QKQLEQVIAKDLLPS				1												
16	alpha LPSRDRQHLPLIKMK			1													
17	alpha RDRQHLPLIKMKPVP																
18	alpha DLVPGNFKSQKQKVP																
19	alpha PEWKALTDMPQMRME	2	4	3	6	11											
20	alpha MELERPGGNEITRG																
21	alpha LERPGGNEITRGGST																
22	alpha SYGTGSETSESPRNP																
23	alpha SAGSWNSGSSGPGST																
24	alpha SGPGSTGNRNPSSG																
25	alpha GNRNPSSGSGTGTAT																
26	alpha WKPSSGSGPGSTGSWN																
27	alpha SGSSGTGSTGNQNP																
28	alpha QNPSPRPGSTGTWN			1													
29	alpha SPRPGSTGTWNPGSS																
30	alpha GSSERGSAGHWTSES																
31	alpha ERGSAGHWTSESVS																
32	alpha GSTGQWHSSESGFRP																
33	alpha DSPGSGNARNPNPDW																
34	alpha GTFEEVSGNVSPGTR																
35	alpha VSPGTRREYHTEKLV																
36	alpha REYHTEKLVTSKGDK																
37	alpha GDKELRTGKEKVTSG			1													
38	alpha ELRTGKEKVTSGSTT																
39	alpha TTTTRRSCKTVTKTV																
40	alpha TTRRSCKTVTKTV					1											
41	alpha PDGHKEVTEKEVTS																
42	alpha EDGSDCPEAMDGLT																
43	alpha SGIGTLDGFRHRHPD																
44	alpha EAAFFDTASTGKTFP																
45	alpha GFFSPMLGEFVSETE																
46	alpha SETESRGSESGIFTN	1		1													
47	alpha SRGSESGIFTNTKES																
48	alpha SSHHPGIAEFPSRGK																
49	alpha SSSYSKQFTSSTSYN		2			4											
50	alpha SYNRGDSFESKSYK	1		2													
51	alpha RGDSTFESKSYKMD																
52	alpha EAGSEADHEGTHSTK																
53	alpha GTHSTKRGHAKSRPV																
54	alpha RGHAKSRPV																
55	beta GHRPLDKKREEAPSL																
56	beta DKKREEAPSLRPAPP																
57	beta RPAPPISGGGYRAR																
58	beta PAKAAATQKKVERKA																
59	beta PDAGGCLHADPDLGV																
60	beta LCPTGCQLQEALLQQ																
61	beta LLQQRPIRNSVDEL			2													
62	beta ERPIRNSVDELNNV																
63	beta EAVQSTSSSQFYMY					1											
64	beta LLKDLWQKQKQKQKQ																
65	beta NENNVNYSSELEKH																
66	beta QLYIDETVNSNIPTN																
67	beta NSNIPTNLRVLRSL	3		2	1												
68	beta TNLRVLRSLLENLRS	5															
69	beta LRVLRSLLENLRSKI		1	1	1												
70	beta LENLRSKIQKLESDV	2		2													
71	beta QKLESDVSAQMEYCR	2		2	2	1											
72	beta DVSAQMEYCRTPCTV	4	1	2	2												
73	beta QMEYCRTPCTVSCNI	7	2		3	3											
74	beta TPCTVSCNIPVSGK	2	4		2	1											
75	beta VSGKECEEIRKGGGE	2		2	1												
76	beta ECEEIRKGGGETSEM																
77	beta YLIQPDSSVKPYRYV																
78	beta CDMNTENGWTVIQN	2															
79	beta VIQNRQDGSVDFGRK			2													
80	beta RQDGSVDFGRKWDPY																
81	beta KQGFNVATNTDGNK																
82	beta YCGLPGEYWLNDGKI	2		1	1	1											
83	beta SQLTRMGPTLLIEM																
84	beta EDWKGDVKVKAHYGGF																
85	beta TVQNEANKYQISVKN				1												
86	beta NKYRGTAGNALMDGA																
87	beta YRGTAGNALMDGASQ																
88	beta LMGENRTMTIHNGMF					1											
89	beta FSTYDRDNDGWLTS																
90	beta LTSDRPKQCSKEDGG			2													
91	beta PRKQCSKEDGGGWY																
92	beta WYNRCHAANPNRGYY	3	1	2	1												
93	beta NRCHAANPNRGYYW	2	4	3	9	5											
94	beta GQYTWDMAKHGTDG																
95	beta VVMNWKGSWYSMRK	5	8	5	6	6											
96	beta WYSMRKMSMKIRPFF	11	10	2	5	4											
	MSMKIRPFFPQQ																
Number of bound peptides		20	12	23	18	14	Number of bound peptides		23		11			23	21	18	
		HLA-DRB1*							HLA-DRB1*								
		01:01	04:01	04:04	04:02	07:01			01:01	04:01	04:04	04:02	07:01				
1	Citrullinated Fibrinogen peptides	1	1	1	1	1	1	1	1	1	1	1	1				
	2	alpha GPRVVERHQSACKDS															
	3	alpha DWPFCSDEEDWNYKCP															
	4	alpha SGCRMKGIDEVNDQ															
	5	alpha FTNRINKLNSLFY															
	6	alpha QKNNKDSHSLTTNIM															
	7	alpha EILRGDFSSANNRDN															
	8	alpha TYNRVSEDLRSRIEV															
	9	alpha IEVLKRKVKIEKVQHI															
	10	alpha LKRKVKIEKVQHIQLL															
	11	alpha QKNVRAQLVDMKRLE															
	12	alpha VIDIKIRSCRSGSCS	1	3	1	4								7			
	13	alpha SCSRALAREVDLKDY	1		2	1	1										
14	alpha R'ALAR'EVDLKDYEDQ																
15	alpha QKQLEQVIAKDLLPS					1											
16	alpha LPSR'DR'QHLPLIKMK			2	1	1											
17	alpha R'DR'QHLPLIKMKPVP																
18	alpha DLVPGNFKSQKQKVP																
19	alpha PEWKALTDMPQMR'ME																
20	alpha MELER'PGGNEITR'GG	1															
21	alpha LER'PGGNEITR'GGST																
22	alpha SYGTGSETSESPR'NPS																
23	alpha SAGSWNSGSSGPGST																
24	alpha SGPGSTGNR'NPSSG			1													
25	alpha GNR'NPSSGSGTGTAT																
26	alpha WKPSSGSGPGSTGSWN																
27	alpha SGSSGTGSTGNQNP																
28	alpha QNPSPR'PGSTGTWN			2													
29	alpha SPR'PGSTGTWNPGSS																
30	alpha GSSER'GSAGHWTSES																
31	alpha ER'GSAGHWTSESVS	1															
32	alpha GSTGQWHSSESGFR'P																
33	alpha DSPSGGNAR'PNPDW																
34	alpha GTFEEVSGNVSPGTR																
35	alpha VSPGTR'R'EYHTEKLV	1															
36	alpha R'EYHTEKLVTSKGDK																
37	alpha GDKELR'TGKEKVTSG			2	1												
38	alpha ELR'TGKEKVTSGSTT																
39	alpha TTTTR'R'SCKSTVTKTV																
40	alpha TTR'R'SCKSTVTKTV																
41	alpha GPDGHKEVTKEVTS																
42	alpha EDGSDCPEAMDGLT																
43	alpha SGIGTLDGFR'HR'HPD																
44	alpha EAAFFDTASTGKTFP																
45	alpha GFFSPMLGEFVSETE																
46	alpha SETESR'GSESGIFTN			2													
47	alpha SR'GSESGIFTNTKES																
48	alpha SSHHPGIAEFPSR'GK																
49	alpha SSSYSKQFTSSTSYN		2			4											
50	alpha SYNR'GDSFESKSYK	1		2													
51	alpha R'GDSFESKSYKMD																
52	alpha EAGSEADHEGTHSTK																
53	alpha GTHSTKR'GHAKSR'PV			1													
54	alpha R'GHAKSR'PV																
55	beta GHR'PLDKKR'EAPSL																
56	beta DKKR'EAPSLR'PAPP			2													
57	beta R'PAPPISGGGYR'AR			1	1												
58	beta PAKAAATQKKVER'KA																
59	beta PDAGGCLHADPDLGV																
60	beta LCPTGCQLQEALLQQ																
61	beta LLQQR'PIR'NSVDEL			1													
62	beta ER'PIR'NSVDELNNV																
63	beta EAVQSTSSSQFYMY					1											
64	beta LLKDLWQKR'QKQVMD																
65	beta NENNVNYSSELEKH																
66	beta QLYIDETVNSNIPTN																
67	beta NSNIPTNLR'VLR'SIL	3	1	3	1												
68	beta TNLR'VLR'SILENLR'SI	10	3		4	4											
69	beta LR'VLR'SILENLR'SKI	3	1	1	2	1											
70	beta LENLR'SKIQKLESDV	2		2													
71	beta QKLESDVSAQMEYCR																
72	beta DVSAQMEYCR'TPCTV	5	1		3	2											
73	beta QMEYCR'TPCTVSCNI	7	1		3	3											
74	beta TPCTVSCNIPVSGK	2	4		2	1											
75	beta VSGKECEEIR'KGGE			2													
76	beta ECEEIR'KGGETSEM																
77	beta YLIQPDSSVKPYRYV																
78	beta CDMNTENGWTVIQN	2															
79	beta VIQNR'QDGSVDFGR'K	3															
80	beta R'QDGSVDFGR'KWDPY			2	1												
81	beta KQGFNVATNTDGNK																
82	beta YCGLPGEYWLNDGKI	2		1	1	1											
83	beta SQLTR'MGPTLLIEM																
84	beta EDWKGDVKVKAHYGGF																
85	beta TVQNEANKYQISVKN				1												
86	beta NKYR'GTAGNALMDGA																
87	beta YR'GTAGNALMDGASQ																
88	beta LMGENR'TMTIHNGMF																
89	beta FSTYDRDNDGWLTS																
90	beta LTSDRPK'QKCSKEDGG	3		2													
91	beta PR'KQCSKEDGGGWY				1	1											
92	beta WYNR'CHAANPNGR'YY	3															
93	beta NR'CHAANPNGR'YYWG					2											
94	beta GQYTWDMAKHGTDG																
95	beta VVMNWKGSWYSMR'K	4	7	4	6	11											
96	beta WYSMR'KMSMKIR'PFF	12	5	9	6	6											
	MSMKIR'PFFPQQ																
R= Citrullin																	
Number of bound peptides		23	11	23	21	18											

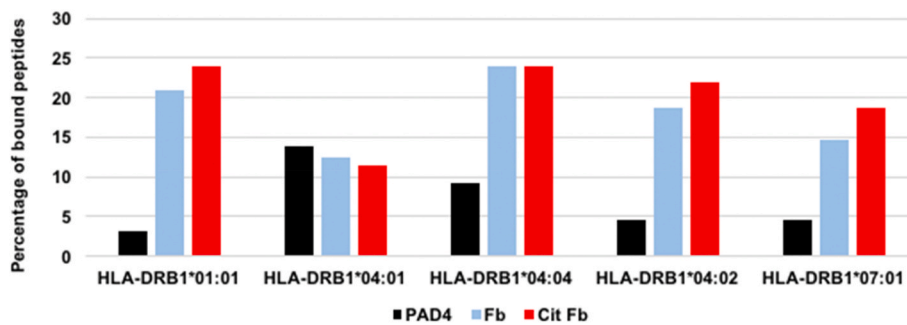


Fig. 3. Percentages of peptides bound by purified HLA-DR molecules encoded by HLA-DRB1*01:01, *04:01, *04:04, *04:02, *07:01. The individual binding of each of 65 peptides from PAD4, each of 96 native or citrullinated peptides from Fibrinogen to purified HLA-DR molecules encoded by HLA-DRB1*01:01, *04:01, *04:04, *04:02, *07:01 was tested by direct binding to purified HLA-DR. The ratios number of bound peptides/number of tested peptides define a likelihood of binding specific for a protein and an HLA-DR molecule. PAD4: Peptidyl Arginyl Deiminase 4, Fb: fibrinogen, Cit Fb: citrullinated fibrinogen.

3.2. Correlation between Odds ratios to develop ACPA positive RA and peptide binding likelihoods for one (at least) of the two HLA-DR molecules encoded by 12 different HLA-DRB1 genotypes

We calculated the likelihood for one (at least) of the 2 HLA-DR molecules encoded by each of 12 HLA-DRB1 genotypes containing HLA-DRB1*04:01, *04:04, *01:01, *04:02, *07:01 to bind at least one peptide from PAD4 or native or citrullinated Fibrinogen, as described in the Methods section (Table 1).

We then compared the likelihood for each of the 12 sets of HLA-DR molecules of binding at least one peptide from PAD4 or native or citrullinated Fibrinogen with each of the 12 respective genotypic risks of developing RA, using Pearson's rank correlation.

We found that genotypic risks of developing RA correlate with likelihoods of binding PAD4 peptide, not native or citrullinated Fibrinogen peptide (Pearson's, $p = 0.042$) (Fig. 4).

4. Discussion

HLA-DRB1 genes predispose to both rheumatoid arthritis and the development of antibodies which precede and are specific of RA: anti citrullinated protein antibodies (ACPAs). ACPAs recognize citrullin residues on many different proteins. Citrullins are arginin residues which have been modified after translation by enzymes called Peptidyl Arginyl Deiminases (PAD). Citrullins are neutral while Arginins are charged and basic.

How RA associated HLA-DRB1 alleles help the development of RA is

Table 1

HLA-DRB1 genotypic risk for RA and likelihood to bind PAD4 or Fibrinogen peptides for each of 12 different HLA-DRB1 genotypes.

HLA-DRB1* Genotypes	Risk to develop RA (Odds Ratio)	Likelihood to bind at least one peptide from		
		Native		Citrullinated
		PAD4	Fibrinogen	Fibrinogen
DRB1*07:07	0.4	0.090	0.270	0.340
DRB1*01:01/07	0.9	0.076	0.324	0.382
DRB1*04:04/07	1.5	0.134	0.350	0.382
DRB1*01:01/01:01	1.6	0.061	0.373	0.422
DRB1*04:01/07	2.4	0.178	0.253	0.281
DRB1*01:01/04:04	2.7	0.120	0.398	0.422
DRB1*04:01/04:01	4.1	0.258	0.234	0.216
DRB1*04:02/07	4.2	0.090	0.306	0.365
DRB1*01:01/04:01	5	0.165	0.307	0.327
DRB1*04:04/04:02	5.1	0.134	0.382	0.406
DRB1*04:04/04:04	10.3	0.176	0.422	0.422
DRB1*04:01/04:04	13.4	0.218	0.335	0.327

unknown. However, since RA is preceded by ACPAs, antibodies believed to cause RA, it is assumed that HLA-DRB1 genes contribute to the development of RA by allowing the development of ACPAs. This is expected, because the function of HLA-DR molecules is to present peptides to CD4 T cells, among which T_{FH} cells which help the development of IgG antibody responses. Still, the identity of the peptides presented by HLA-DR molecules to the T_{FH} cells that will help the development of ACPAs is unknown.

An early, classical hypothesis, proposed that HLA-DR molecules associated with RA were capable of binding citrullinated peptides with higher affinity than non RA associated HLA-DR molecules. The demonstration of this hypothesis relied on a classical study of the binding of one peptide from Vimentin: Vim 65-77 under its native (arginine) or citrullinated form, called Vim R70Cit, to 8 HLA-DR molecules, of which 3 (HLA-DRB1*04:01, *04:04, *01:01) contained the shared epitope. VimR70Cit was found to bind shared epitope positive HLA-DR molecules with higher affinity than Vim 65-77 [3]. This study was confirmed in 2018 by binding and structural studies performed with a few selected peptides including Vim 65-77 and its citrullinated variant [9].

However these findings have been challenged by many thorough peptide binding studies. The binding of 167 peptides from the alpha and beta chains of human Fibrinogen to 5 HLA-DR molecules did not give any indication of preferential binding of citrullinated fibrinogen peptides to SE positive HLA-DR molecules [4]. More recently, Sette et al. studied the binding of 200 peptides from Collagen, Fibrinogen, Aggrecan and Vimentin (including Vim 65-77) to 28 different HLA-DR molecules and failed to demonstrate a higher binding affinity of citrullinated peptides to SE positive HLA-DR molecules [5].

We have developed an alternative hypothesis. Indeed, the emergence of ACPAs is often preceded by the development of anti PAD4 IgG antibodies [10]. In normal mice, immunization with PADs can trigger the development of ACPAs by a hapten carrier mechanism [6]. Similarly, RA patients have both antibodies and T cell responses to PAD4, suggesting that the target for the helper T cells involved in the development of ACPAs may be not citrullinated antigens but PADs [7].

To sort between the two hypotheses, we evaluated which one matches HLA-DRB1 genotypic risks for the development of ACPA positive RA better. Indeed, the risk to develop ACPA positive RA can be calculated based on an individual's HLA-DRB1 genotype. In 2013, we calculated these risks for 106 of the 136 most common HLA-DRB1 genotypes in France [2]. Here, we compared the risks (Odds Ratios ORs) of developing ACPA positive RA for 12 common HLA-DRB1 genotypes with the likelihood for one at least of the two HLA-DR molecules encoded by each genotype to bind one randomly chosen peptide from PAD4, native and citrullinated Fibrinogen. We found that HLA-DRB1 genotypic ORs to develop RA correlate with the likelihood for at least one of the encoded HLA-DR molecules to bind one randomly chosen peptide from PAD4 but not from citrullinated or native Fibrinogen.

This result suggests that HLA-DRB1 genes act on the triggering of ACPA immunization and the development of RA by allowing presentation of peptide(s) from PAD4, according to a classical hapten carrier

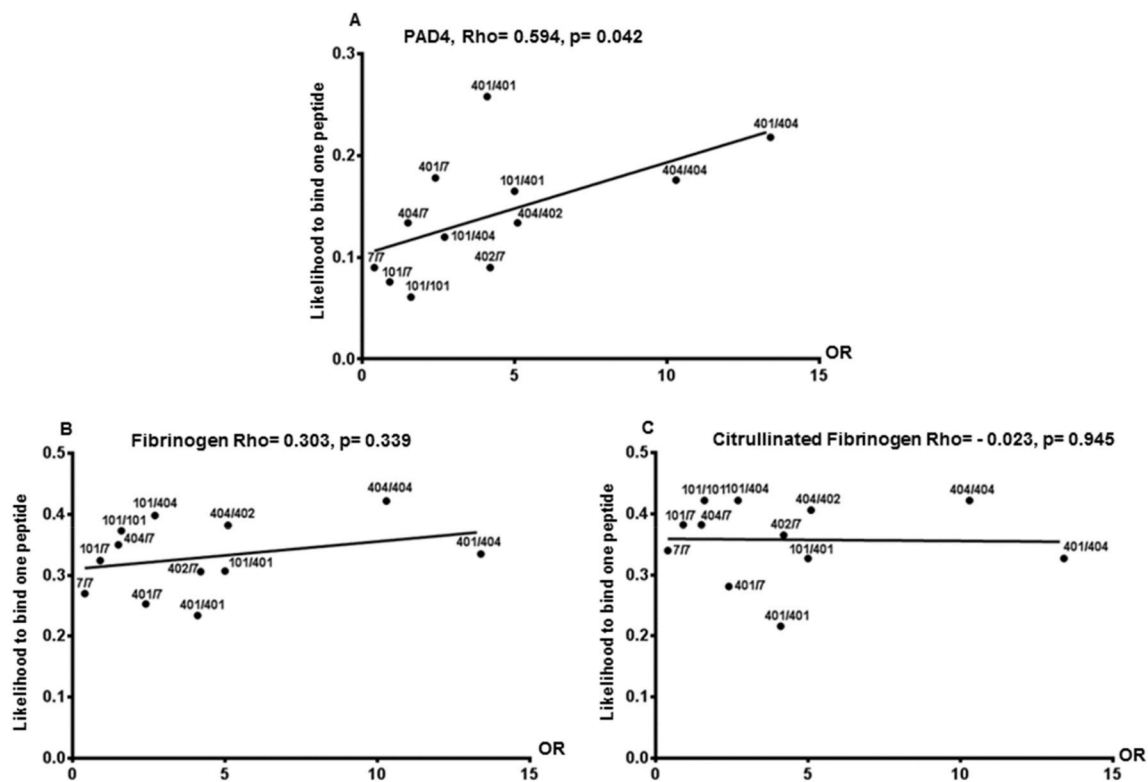


Fig. 4. Correlation between OR to develop RA and likelihood to bind at least one peptide from PAD4 or native or citrullinated Fibrinogen. Correlation between HLA-DRB1 genotypic risks (OR) for RA and likelihood of binding PAD4 (A), Fibrinogen (B) or citrullinated Fibrinogen (C) peptides for a given genotype was evaluated by Pearson's rank correlation. Each HLA-DRB1 genotype is indicated by a black dot.

model in which PAD4, the citrullinating enzyme, is the carrier. Thus, B lymphocytes recognizing citrullinated epitope(s) on any protein being citrullinated by PAD4 may process the PAD4/citrullinated protein complex, present PAD4 peptide on HLA-DR and benefit from the help of PAD4 peptide specific T_H cells (7).

We compared the capabilities of the HLA-DR molecules encoded by 12 different genotypes to bind peptides from PAD4, native or citrullinated Fibrinogen. We found that the risk to develop RA, associated with each pair of HLA-DR molecules, matches the pair's capability to bind a peptide from PAD4, not a peptide from citrullinated or native Fibrinogen. This finding is consistent with the T cell proliferation and activation data in patients with RA. Indeed, we found that T cell response to the PAD4 protein, but not to citrullinated or native Fibrinogen is common and associated with antibodies to PAD4 and the shared epitope of HLA-DR, in RA patients, not controls. Furthermore, p8, the peptide from PAD4 which binds HLA-DR best according to our binding data is recognized by T cells in almost half of RA patients, not controls and this is associated with shared epitope positive HLA-DR alleles (7).

Thus, far from solving a purely theoretical issue, our findings identify PAD4 as an important triggering antigen in the development of RA. Their practical implication is the prevention of RA by PAD4 tolerization in high risk individuals identified by their high risk HLA-DRB1 genotypes (2).

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Declaration of competing interest

None.

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