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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 arginin free peptides from the alpha chain and 12 arginin 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

	PAD4 peptides	HLA-DRB1* 01:01	04:01	04:04	04:02	07:01
1	MAQGTLIRVTPEQPTHAVCV					
2	PEQPTHAVCVLGTLTQLDIC			1		
3	LGTLTQLDICSSAPEDCTSF					
4	SSAPEDCTSFSINASPGVVV					
5	SINASPGVVVDIAHSPPAKK					
6	DIAHSPPAKKKSTGSSTWPL					
7	KSTGSSTWPLDPGVEVTLTM					
8	DPGVEVTLTMKAASGSTGDQ	2	3	4	3	4
9	KAASGSTGDQKVQISYYGPK					
10	KVQISYYGPKTPPVKALLYL					
11	TPPVKALLYLTAVEISLCAD					
12	TAVEISLCADITRTGKVKPT		1			
13	ITRTGKVKPTRAVKDQRTWT		1	1		1
14	RAVKDQRTWTWGPCGQGAIL					
15	WGPCGQGAILLVNCDRDNLE					
16	LVNCDRDNLESSAMDCEDDE		1	1	1	
17	SSAMDCEDDEVLDSEDLQDM					
18	VLDSEDLQDMSLMTLSTKTP					
19	SLMTLSTKTPKDFFTNHTLV					
20	KDFFTNHTLVLHVARSEMDK					
21	LHVARSEMDKVRVFQATRGK			1		
22	VRVFQATRGKLSSKCSVVLG		1			
23	LSSKCSVVLGPKWPSHYLMV		1			
24	PKWPSHYLMVPGGKHNMDFY					
25	PGGKHNMDFYVEALAFPDTD					
26	VEALAFPDTDFPGLITLTIS					
27	FPGLITLTISLLDTSNLELP					
28	LLDTSNLELPEAVVFQDSVV					
29	EAVVFQDSVVFRVAPWIMTP					
30	FRVAPWIMTPNTQPPQEVYA					
31	NTQPPQEVYACSIFENEDFL					
32	CSIFENEDFLKSVTTLAMKA					
33	KSVTTLAMKAKCKLTICPEE					
34	KCKLTICPEEENMDDQWMQD					
35	ENMDDQWMQDEMEIGYIQAP					
36	EMEIGYIQAPHKTLPVVFDS					
37	HKTLPVVFDSPRNRGLKEFP					
38	PRNRGLKEFPIKRVMGPDFG					
39	IKRVMGPDFGYVTRGPQTGG					
40	YVTRGPQTGGISGLDSFGNL					
41	ISGLDSFGNLEVSPPVTVRG					
42	EVSPPVTVRGKEYPLGRILF					
43	KEYPLGRILFGDSCYPSNDS					
44	GDSCYPSNDSRQMHQALQDF					
45	RQMHQALQDFLSAQQVQAPV					
46	LSAQQVQAPVKLYSDWLSVG					
47	KLYSDWLSVGHVDEFLSFVP					
48	HVDEFLSFVPAPDRKGFRLL					
49	APDRKGFRLLLASPRSCYKL		2			
50	LASPRSCYKLFQEQQNEGHG					
51	FQEQQNEGHGEALLFEGIKK					
52	EALLFEGIKKKKQQKIKNIL					
53	KKQQKIKNILSNKTLREHNS					
54	SNKTLREHNSFVERCIDWNR					
55	FVERCIDWNRELLKRELGLA					
56	ELLKRELGLAESDIIDIPQL					
57	ESDIIDIPQLFKLKEFSKAE					
58	FKLKEFSKAEAFFPNMVNML					
59	AFFPNMVNMLVLGKHLGIPK					
60	VLGKHLGIPKPFGPVINGRC					
61	PFGPVINGRCCLEEKVCSLL	1	2	1	2	2
62	CLEEKVCSLLEPLGLQCTFI					
63	EPLGLQCTFINDFFTYHIRH					
64	NDFFTYHIRHGEVHCGTNVR					
65	GEVHCGTNVRRKPFSFKWWNMVP		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.

		Fibrinogen peptides	HLA-DRB1* 01:01	04:01	04:04	04:02	07:01
1	alpha	GPRVVERHQSACKDS					
2	alpha	DWPFCSDEDWNYKCP	1	1	1	1	1
3	alpha	SGCRMKGLIDEVNQD					
4	alpha	FTNRINKLKNSLFEY				1	
5	alpha	QKNNKDSHSLTTNIM					
6	alpha	EILRGDFSSANNRDN					
7	alpha	TYNRVSEDLRSRIEV					
8	alpha	IEVLKRKVIEKVQHI					
9	alpha	LKRKVIEKVQHIQLL					
10	alpha	QKNVRAQLVDMKRLE					
11	alpha	VDIDIKIRSCRGSCS	2	6	6	6	2
12	alpha	SCSRALAREVDLKDY	1		1		
13	alpha	RALAREVDLKDYEDQ					
14	alpha	QKQLEQVIAKDLLPS				1	
15	alpha	LPSRDRQHLPLIKMK			1		
16	alpha	RDRQHLPLIKMKPVP					
17	alpha	DLVPGNFKSQLQKVP					
18	alpha	PEWKALTDMPQMRME	2	4	3	6	11
19	alpha	MELERPGGNEITRGG					
20	alpha	LERPGGNEITRGGST					
21	alpha	SYGTGSETESPRNPS					
22	alpha	SAGSWNSGSSGPGST					
23	alpha	SGPGSTGNRNPGSSG					
24	alpha	GNRNPGSSGTGGTAT					
25	alpha	WKPGSSGPGSTGSWN					
26	alpha	SGSSGTGSTGNQNPG					
27	alpha	QNPGSPRPGSTGTWN			1		
28	alpha	SPRPGSTGTWNPGSS					
29	alpha	GSSERGSAGHWTSES					
30	alpha	ERGSAGHWTSESSVS					
31	alpha	GSTGQWHSESGSFRP					
32	alpha	DSPGSGNARPNNPDW					
33	alpha	GTFEEVSGNVSPGTR					
34	alpha	VSPGTRREYHTEKLV					
35	alpha	REYHTEKLVTSKGDK					
36	alpha	GDKELRTGKEKVTSG			1		
37	alpha	ELRTGKEKVTSGSTT					
38	alpha	TTTRRSCSKTVTKTV					
39	alpha	TTRRSCSKTVTKTVI					1
40	alpha	PDGHKEVTKEVVTS					
41	alpha	EDGSDCPEAMDLGTL					
42	alpha	SGIGTLDGFRHRHPD					
43	alpha	EAAFFDTASTGKTFP					
44	alpha	GFFSPMLGEFVSETE					
45	alpha	SETESRGSESGIFTN	1		1		
46	alpha	SRGSESGIFTNTKES					
47	alpha	SSHHPGIAEFPSRGK					
48	alpha	SSSYSKQFTSSTSYN		2			4
49	alpha	SYNRGDSTFESKSYK	1		2		
50	alpha	RGDSTFESKSYKMAD					
51	alpha	EAGSEADHEGTHSTK					
52	alpha	GTHSTKRGHAKSRPV					
53	alpha	RGHAKSRPV					
54	beta	GHRPLDKKREEAPSL					
55	beta	DKKREEAPSLRPAPP					
56	beta	RPAPPPISGGGYRAR					
57	beta	PAKAAATQKKVERKA					
58	beta	PDAGGCLHADPDLGV					
59	beta	LCPTGCQLQEALLQQ					
60	beta	LLQQERPIRNSVDEL			2		
61	beta	ERPIRNSVDELNNNV					
62	beta	EAVQSTSSSSQFYMY					1
63	beta	LLKDLWQKRQKQVKD					
64	beta	NENVVNEYSSELEKH					
65	beta	QLYIDETVNSNIPTN					
66	beta	NSNIPTNLRVLRSIL	3		2	1	
67	beta	TNLRVLRSILENLRS	5				
68	beta	LRVLRSILENLRSKI		1	1	1	
69	beta	LENLRSKIQKLESDV	2		2		
70	beta	QKLESDVSAQMEYCR	2		2	2	1
71	beta	DVSAQMEYCRTPCTV	4	1	2	2	
72	beta	QMEYCRTPCTVSCNI	7	2		3	3
73	beta	TPCTVSCNIPVVSGK	2	4		2	1
74	beta	VSGKECEEIIRKGGE	2		2	1	
75	beta	ECEEIIRKGGETSEM					
76	beta	YLIQPDSSVKPYRVY					
77	beta	CDMNTENGGWTVIQN	2				
78	beta	VIQNRQDGSVDFGRK			2		
79	beta	RQDGSVDFGRKWDPY					
80	beta	KQGFGNVATNTDGKN					
81	beta	YCGLPGEYWLGNDKI	2		1	1	1
82	beta	SQLTRMGPTELLIEM					
83	beta	EDWKGDKVKAHYGGF					
84	beta	TVQNEANKYQISVNK				1	
85	beta	NKYRGTAGNALMDGA					
86	beta	YRGTAGNALMDGASQ					
87	beta	LMGENRTMTIHNGMF					1
88	beta	FSTYDRDNDGWLTSD					
89	beta	LTSDPRKQCSKEDGG			2		
90	beta	PRKQCSKEDGGGWWY					
91	beta	WYNRCHAANPNGRYY	3	1	2	1	
92	beta	NRCHAANPNGRYYWG	2	4	3	9	5
93	beta	GQYTWDMAKHGTDDG					
94	beta	VVWMNWKGSWYSMRK	5	8	5	6	6
95	beta	WYSMRKMSMKIRPFF	11	10	2	5	4
96	beta	MSMKIRPFFPQQ					
		Number of bound peptides	20	12	23	18	14

		Citrullinated Fibrinogen peptides	HLA-DRB1* *01:01	*04:01	*04:04	*04:02	*07:01
1	alpha	GPR*VVER*HQSACKDS					
2	alpha	DWPFCSDEDWNYKCP	1	1	1	1	1
3	alpha	SGCR*MKGLIDEVNQD				1	
4	alpha	FTNR*INKLKNSLFEY					
5	alpha	QKNNKDSHSLTTNIM					
6	alpha	EILR*GDFSSANNR*DN					
7	alpha	TYNR*VSEDLR*SR*IEV					
8	alpha	IEVLKR*KVIEKVQHI	1				1
9	alpha	LKR*KVIEKVQHIQLL					
10	alpha	QKNVR*AQLVDMKR*LE					
11	alpha	VDIDIKIR*SCR*GSCS	1	3	1	4	7
12	alpha	SCSR*ALAR*EVDLKDY	1		2	1	1
13	alpha	R*ALAR*EVDLKDYEDQ					
14	alpha	QKQLEQVIAKDLLPS				1	
15	alpha	LPSR*DR*QHLPLIKMK	1		2	1	1
16	alpha	R*DR*QHLPLIKMKPVP					
17	alpha	DLVPGNFKSQLQKVP					
18	alpha	PEWKALTDMPQMR*ME					
19	alpha	MELER*PGGNEITR*GG	1				
20	alpha	LER*PGGNEITR*GGST					
21	alpha	SYGTGSETESPR*NPS					
22	alpha	SAGSWNSGSSGPGST					
23	alpha	SGPGSTGNR*NPGSSG			1		
24	alpha	GNR*NPGSSGTGGTAT					
25	alpha	WKPGSSGPGSTGSWN					
26	alpha	SGSSGTGSTGNQNPG					
27	alpha	QNPGSPR*PGSTGTWN			2		
28	alpha	SPR*PGSTGTWNPGSS					
29	alpha	GSSER*GSAGHWTSES	1				
30	alpha	ER*GSAGHWTSESSVS					
31	alpha	GSTGQWHSESGSFR*P					
32	alpha	DSPGSGNAR*PNNPDW					
33	alpha	GTFEEVSGNVSPGTR*					
34	alpha	VSPGTR*R*EYHTEKLV	1				
35	alpha	R*EYHTEKLVTSKGDK					
36	alpha	GDKELR*TGKEKVTSG			2	1	
37	alpha	ELR*TGKEKVTSGSTT					
38	alpha	TTTR*R*SCSKTVTKTV					
39	alpha	TTR*R*SCSKTVTKTVI					
40	alpha	GPDGHKEVTKEVVTS					
41	alpha	EDGSDCPEAMDLGTL					
42	alpha	SGIGTLDGFR*HR*HPD					
43	alpha	EAAFFDTASTGKTFP					
44	alpha	GFFSPMLGEFVSETE					
45	alpha	SETESR*GSESGIFTN			2		
46	alpha	SR*GSESGIFTNTKES					
47	alpha	SSHHPGIAEFPSR*GK					
48	alpha	SSSYSKQFTSSTSYN		2			4
49	alpha	SYNR*GDSTFESKSYK	1		2		
50	alpha	R*GDSTFESKSYKMAD					
51	alpha	EAGSEADHEGTHSTK					
52	alpha	GTHSTKR*GHAKSR*PV			1		
53	alpha	R*GHAKSR*PV					
54	beta	GHR*PLDKKR*EEAPSL					
55	beta	DKKR*EEAPSLR*PAPP			2		
56	beta	R*PAPPPISGGGYR*AR*			1	1	
57	beta	PAKAAATQKKVER*KA					
58	beta	PDAGGCLHADPDLGV					
59	beta	LCPTGCQLQEALLQQ					
60	beta	LLQQER*PIR*NSVDEL			1		
61	beta	ER*PIR*NSVDELNNNV					
62	beta	EAVQSTSSSSQFYMY					1
63	beta	LLKDLWQKR*QKQVKD					
64	beta	NENVVNEYSSELEKH					
65	beta	QLYIDETVNSNIPTN					
66	beta	NSNIPTNLR*VLR*SIL	3	1	3	1	
67	beta	TNLR*VLR*SILENLR*S	10	3		4	4
68	beta	LR*VLR*SILENLR*SKI	3	1	1	2	1
69	beta	LENLR*SKIQKLESDV	2		2		
70	beta	QKLESDVSAQMEYCR*					
71	beta	DVSAQMEYCR*TPCTV	5	1		3	2
72	beta	QMEYCR*TPCTVSCNI	7	1		3	3
73	beta	TPCTVSCNIPVVSGK	2	4		2	1
74	beta	VSGKECEEIIR*KGGE			2		
75	beta	ECEEIIR*KGGETSEM					
76	beta	YLIQPDSSVKPYR*VY					
77	beta	CDMNTENGGWTVIQN	2				
78	beta	VIQNR*QDGSVDFGR*K	3		2	1	
79	beta	R*QDGSVDFGR*KWDPY					
80	beta	KQGFGNVATNTDGKN					
81	beta	YCGLPGEYWLGNDKI	2		1	1	1
82	beta	SQLTR*MGPTELLIEM					
83	beta	EDWKGDKVKAHYGGF					
84	beta	TVQNEANKYQISVNK				1	
85	beta	NKYR*GTAGNALMDGA					
86	beta	YR*GTAGNALMDGASQ					
87	beta	LMGENR*TMTIHNGMF					
88	beta	FSTYDR*DNDGWLTSD					
89	beta	LTSDPR*KQCSKEDGG	3		2		
90	beta	PR*KQCSKEDGGGWWY				1	1
91	beta	WYNR*CHAANPNGR*YY	3		2	1	1
92	beta	NR*CHAANPNGR*YYWG					2
93	beta	GQYTWDMAKHGTDDG					
94	beta	VVWMNWKGSWYSMR*K	4	7	4	6	11
95	beta	WYSMR*KMSMKIR*PFF	12	5	9	6	6
96	beta	MSMKIR*PFFPQQ					
		R*= Citrullin					
		Number of bound peptides	23	11	23	21	18

Fig. 2. Binding of native and citrullinated fibrinogen 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 the HA peptide. Numbers indicate the ratios peptide signal OD divided by HA peptide OD. Thus, a number higher than 1 indicates positive binding.

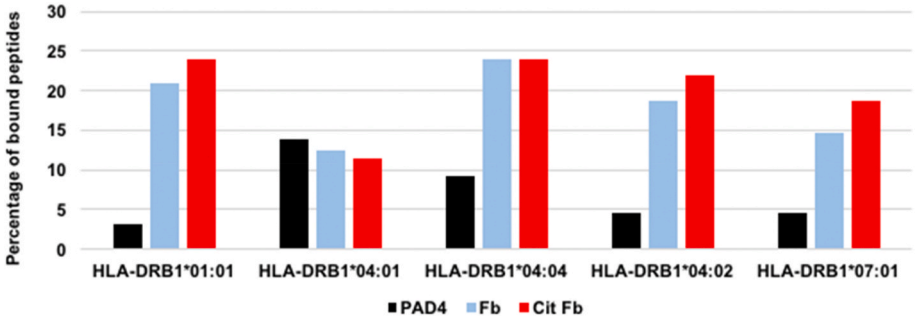

Fig. 3. Percentages of peptides bound by purified HLA-DR molecules encoded by HLA-DRB1*01:01,*04:01, *04:04, *04:02, *07:01. The individual binding of each of 65 peptides from PAD4, each of 96 native or citrullinated peptides from Fibrinogen to purified HLA-DR molecules encoded by HLA-DRB1*01:01, *04:01, *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 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

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

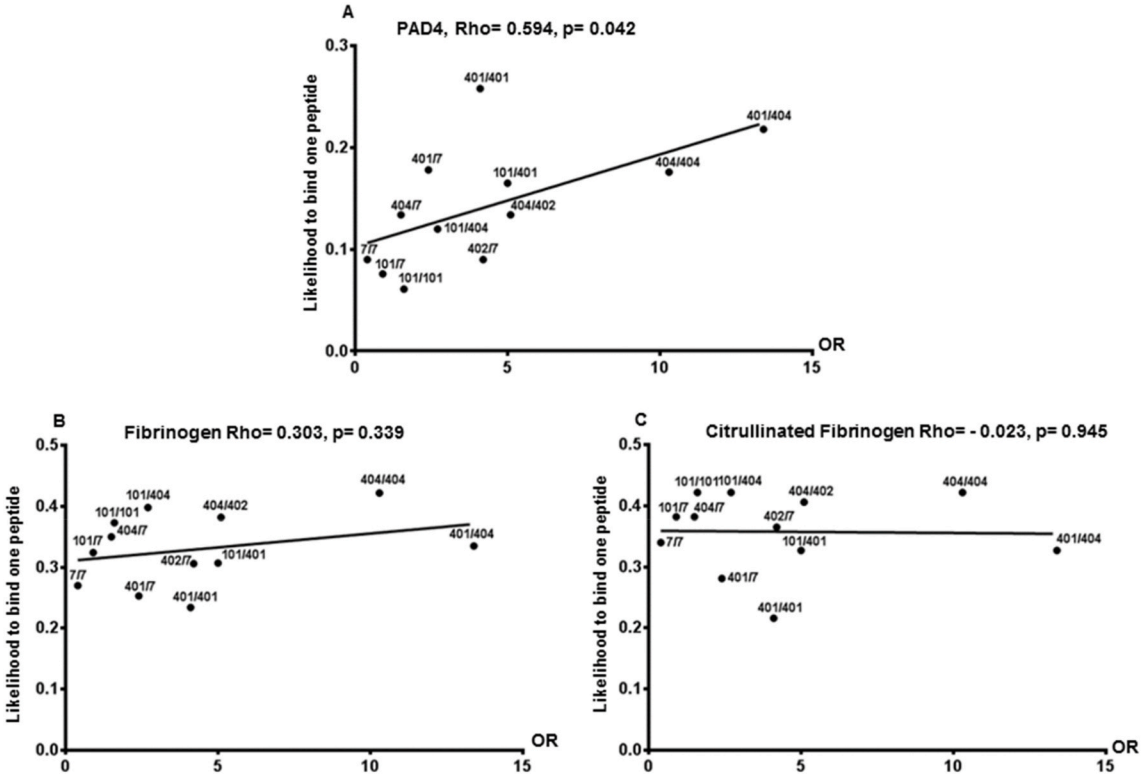

Fig. 4. Correlation between OR to develop RA and likelihood to bind at least one peptide from PAD4 or native or citrullinated Fibrinogen. Correlation between HLA-DRB1 genotypic risks (OR) for RA and likelihood of binding PAD4 (A), Fibrinogen (B) or citrullinated Fibrinogen (C) peptides for a given genotype 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_{FH} 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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