





## Research Article

## PAD2 immunization induces ACPA in wild-type and HLA-DR4 humanized mice

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Rheumatoid arthritis (RA) is associated with HLA-DRB1 alleles expressing the "shared epitope." RA is usually preceded by the emergence of anti-citrullinated protein autoantibodies (ACPAs). ACPAs recognize citrulline residues on numerous proteins. Conversion of arginine into citrulline is performed by enzymes called peptidyl arginine deiminases (PADs). We have previously demonstrated that C3H mice immunized with PADs can produce ACPAs by a hapten-carrier mechanism. Here, we address the influence of HLA-DR alleles in this model in mice expressing RA-associated HLA-DRB1\*04:01 (KO/KI\*04:01), HLA-DRB1\*04:04 (KO/KI\*04:04), or non-RA-associated HLA-DRB1\*04:02 (KO/KI\*04:02) after murine PAD2 immunization. Immunization with mPAD2 triggers production of ACPAs in wild-type (WT) and HLA-DR4 C57BL/6 mice. Both I-A<sup>b</sup> and HLA-DR are involved in the activation of mPAD2-specific T lymphocytes. Among HLA-DR4 mice, mice expressing RA-associated HLA-DRB1\*04:01 are the best responders to mPAD2 and the best anti-citrullinated peptide antibody producers.

**Keywords:** ACPAs · HLA-DR4 · Humanized mice · PADs · Rheumatoid arthritis

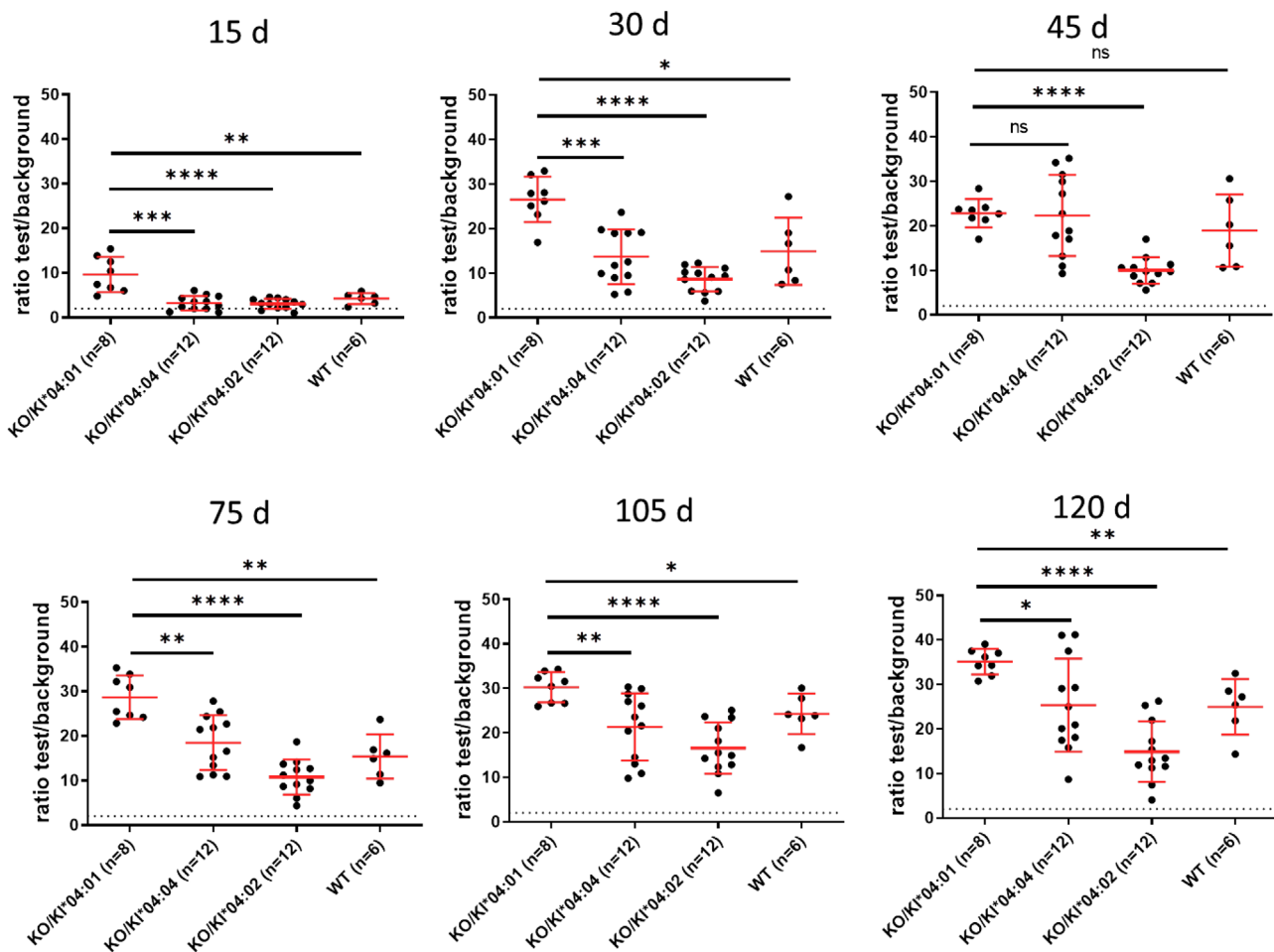
## Introduction

Rheumatoid arthritis (RA) is a chronic destructive joint disease with a prevalence of 0.5% worldwide. HLA-DR genes are the major RA genetic susceptibility factors. Most patients with RA express particular HLA-DRB1 alleles, like HLA-DRB1\*04:01, \*04:04, \*04:05, \*04:08, \*01:01, \*01:02, \*10:01, and \*14:02. RA-associated HLA-DR alleles share a highly conserved amino acid motif expressed in the third hypervariable region of their DRB1 chain. This motif is called the shared epitope (SE) [1]. Inheriting two SE-positive HLA-DRB1 alleles results in very high risk of developing RA [2].

The sera of patients with RA contain disease-specific anti-citrullinated protein antibodies (ACPAs) which recognize

citrulline-centered epitopes on numerous proteins, such as filaggrin, vimentin, and fibrin [3–7]. Citrulline is a modified form of arginine obtained after action of enzymes called peptidyl arginine deiminases (PAD) [8]. As of today, the mechanisms leading to the production of ACPAs are unknown. We suggested that T-cell autoimmunity to PAD triggers production of autoantibodies to citrullinated proteins by a hapten-carrier mechanism. This model was first demonstrated in normal mice. Indeed, C3H mice immunized with PADs develop antibodies and T cells to PADs and IgG antibodies to citrullinated fibrinogen peptides, in the absence of any T-cell response to native or citrullinated fibrinogen [9]. In this model, we observed the importance of the major histocompatibility complex (MHC) background. Indeed, C3H mice express an I-E beta<sup>k</sup> chain homologous to the beta 1 chain encoded by RA-associated HLA-DRB1\*04:01. DBA/2 mice, which fail to develop antibodies to citrullinated fibrinogen peptides, express an I-E beta<sup>d</sup> chain homologous to the beta 1 chain encoded by non-RA-associated HLA-DRB1\*04:02.

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**Figure 1.** IgG response to mPAD2 in C57BL/6 mice immunized with mPAD2. Sera diluted to 1/40 from mice immunized with mPAD2 were tested by ELISA for IgG response to mPAD2 at 15, 30, 45, 75, 105, and 120 days (d) after first immunization. OD was read at 405 nm. A ratio test OD/background OD (see Material and Methods) equal or higher than 2 defined positive sera (dotted line). Data are combined from two experiments with 38 mice per experiment. Each black dot represents result from a mouse. Means and SD (in red) were calculated for each group of mice. Asterisks represent significant Mann–Whitney  $p$  values (\* $p = 0.01$ , \*\* $p = 0.001$ , \*\*\* $p = 0.0001$ , \*\*\*\* $p < 0.0001$ ). NS: nonsignificant.

To further address the influence of HLA-DR alleles in the development of ACPA after murine PAD2 (mPAD2) immunization, we used HLA-DR humanized C57BL/6 mice expressing HLA-DRB1\*04:01 (SE+), HLA-DRB1\*04:04 (SE+) or HLA-DRB1\*04:02 (SE-).

## Results

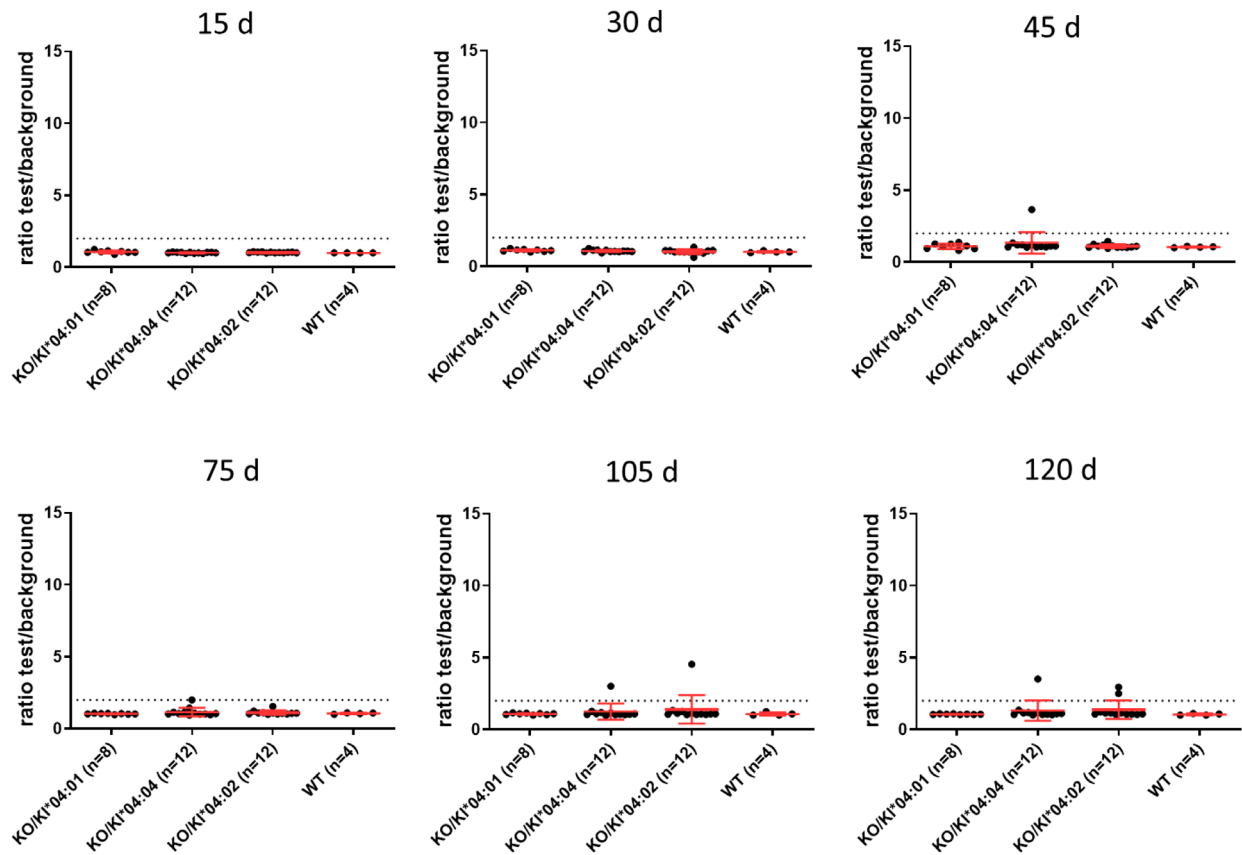
### Autoantibodies to mPAD2 in mice immunized with mPAD2

WT and HLA-DR humanized C57BL/6 mice were immunized subcutaneously with mPAD2 or PBS in CFA. Three booster injections with mPAD2 or PBS in IFA were given subcutaneously 15, 30, and 45 days later. Sera from mice obtained at 15, 30, 45, 75, 105, and 120 days after first immunization were tested for IgG responses to mPAD2 by ELISA.

All the mice immunized with mPAD2 developed anti-mPAD2 IgG antibodies after two immunizations (Fig. 1) and this was different from mice immunized with PBS (Fig. 2) (Fisher's exact test,  $p = 6 \times 10^{-22}$ ). Anti-mPAD2 IgG antibodies persisted over time. The anti-mPAD2 IgG response was significantly higher in the 8 KO/KI\*04:01 (SE+) mice than in the other 30 mice at 15, 30, 75, 105, and 120 days after first immunization (Mann–Whitney test, see Fig. 1).

### T-cell proliferation to mPAD2 in mice immunized with mPAD2

T-cell proliferation to mPAD2, native or citrullinated fibrinogen, or phytohemagglutinin (PHA) was evaluated by BrdU incorporation 120 days after the first immunization in spleen and LN cell cultures. Only experiments with positive proliferation to PHA were considered.



**Figure 2.** IgG response to mPAD2 in C57BL/6 mice immunized with PBS. Sera diluted to 1/40 from mice immunized with PBS were tested by ELISA for IgG response to mPAD2 at 15, 30, 45, 75, 105, and 120 days (d) after first immunization. OD was read at 405 nm. A ratio test OD/background OD (see Materials and Methods) equal or higher than 2 defined positive sera (dotted line). Each black dot represents result from a mouse. Data are combined from two experiments with 36 mice per experiment. Means and SD (in red) were calculated for each group of mice.

No T-cell proliferation was detected to native or citrullinated fibrinogen in mice immunized with mPAD2 or PBS (Fig. 3).

T-cell proliferation to mPAD2 was detected in 17 of 37 (46%) mice immunized with mPAD2 (Fig. 3A) versus zero of 35 mice immunized with PBS (Fig. 3B) (Fisher's exact test,  $p = 1.5 \times 10^{-6}$ ). T-cell proliferation to mPAD2 was observed in six of eight (75%) KO/KI\*04:01 (SE+) mice, five of 11 (45%) KO/KI\*04:04 (SE+) mice, five of 12 (42%) KO/KI\*04:02 (SE-) mice, and one of six (17%) WT mice. T-cell proliferation to mPAD2 was more frequent in the eight KO/KI\*04:01 mice than in the other 29 mice (Fisher's exact test,  $p = 0.1$ ).

In mice expressing both HLA-DR and I-A<sup>b</sup> (any KO/KI), inhibition tests showed that both I-A<sup>b</sup> and HLA-DR are involved in mPAD2-specific lymphocyte activation (Fig. 4).

### Anti-citrullinated fibrinogen peptide antibodies in mice immunized with mPAD2

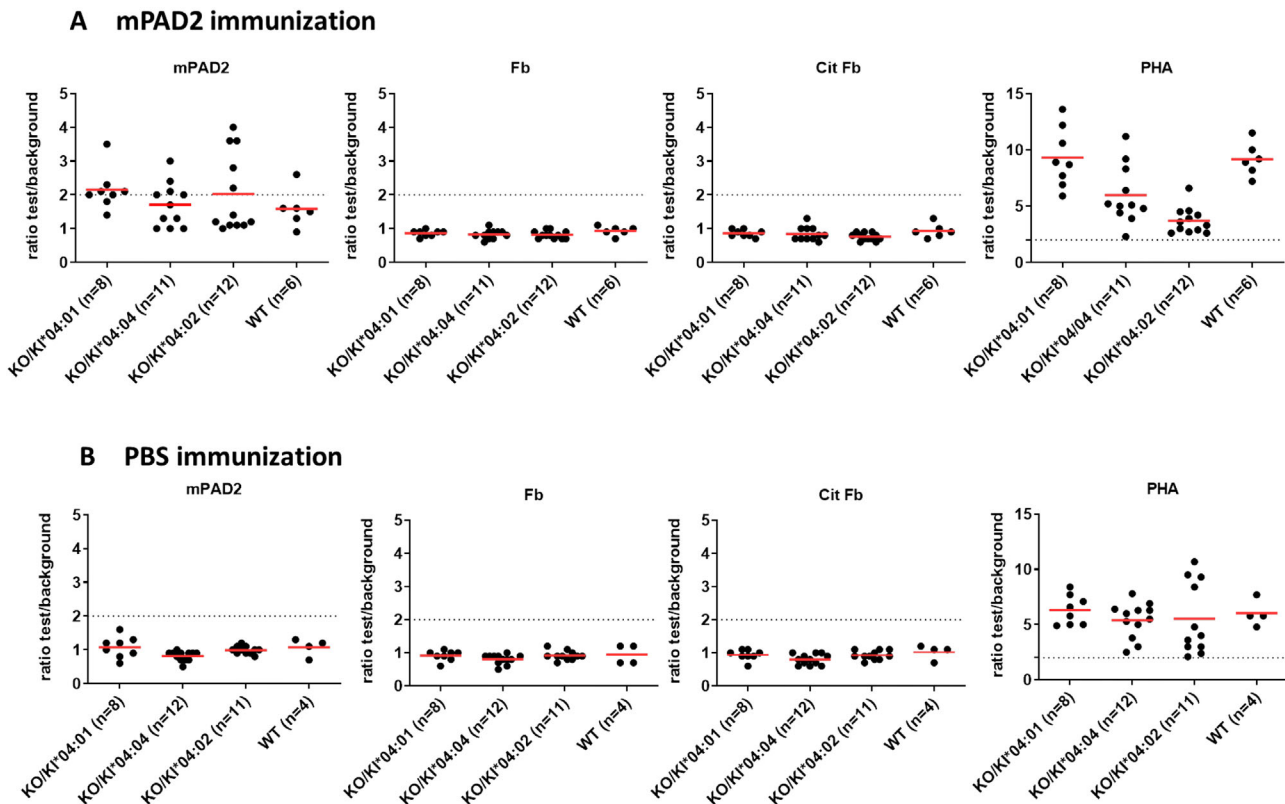
To test whether anti-citrullinated fibrinogen peptide antibodies were produced in mice after mPAD2 immunization, we screened sera from mice on 10 peptides from the beta chain of fibrinogen in their citrulline (C) or arginine (R) forms (Fig. 5).

Citrullinated fibrinogen peptide-specific IgG responses were detected in 12 of 38 (32%) mice immunized with mPAD2 (Fig. 5A) versus one of 36 (3%) mice immunized with PBS (Fisher's exact test,  $p = 0.001$ ) (Fig. 5B). Citrullinated fibrinogen peptide-specific IgG responses were detected in four of eight (50%) KO/KI\*04:01 (SE+) mice, two of 12 (17%) KO/KI\*04:04 (SE+) mice, one of 12 (8%) KO/KI\*04:02 (SE-) mice, and five of six (83%) WT mice. No IgG responses were detected to native fibrinogen peptides (Fig. 5).

Peptide 8C was the most often recognized citrullinated epitope. It was recognized by eight of 38 (21%) mice immunized with mPAD2 versus zero of 36 mice immunized with PBS (Fisher's exact test,  $p = 0.005$ ). In mice immunized with mPAD2, IgG response to peptide 8C was detected in two of eight (25%) of KO/KI\*04:01 (SE+) mice, two of 12 (17%) of KO/KI\*04:04 (SE+) mice, one of 12 (8%) of KO/KI\*04:02 (SE-) mice, and three of six (50%) of WT mice.

### Discussion

In most patients with RA, onset of disease is preceded by the development of autoantibodies to citrullinated proteins



**Figure 3.** T cell response to mPAD2 in C57BL/6 mice. Spleen and lymph nodes were obtained at 120 days after first immunization. Cells were extracted and cultured at a density of  $5 \times 10^6$  cells with  $2 \mu\text{g}$  of mPAD2, native fibrinogen (Fb), citrullinated fibrinogen (Cit Fb), or PHA. T-cell responses were evaluated by BrdU incorporation after mPAD2 immunization (A) or PBS immunization (B). Data are combined from three experiments with 72 mice per experiment. Positive T-cell responses were defined by test OD/ background OD ratio higher than 2 (dotted line). Means (red line) were calculated for each group of mice.

(ACPAs). Citrullination is the conversion of arginine into citrulline catalyzed by peptidyl arginine deiminases (PADs). ACPAs appear years before the onset of RA, which suggests they influence disease development. Both RA and ACPAs develop on the same genetical background characterized by HLA-DR molecules expressing the SE, a five-amino acid stretch on their DR beta 1 chain. RA- and ACPA-associated alleles are HLA-DRB1\*04:01, which encodes QKRAA, HLA-DRB1\*01:01, \*04:04, \*04:05, \*04:08, which encode QRRAA, and HLA-DRB1\*10:01, which encodes RRRRAA.

How SE-positive HLA-DR molecules contribute to the production of ACPA remains to be explained and each allele may not contribute in the same way. The QKRAA motif of HLA-DRB1\*04:01 is a binding motif for the human 70 kD heat shock protein hsp73. Interaction with hsp73 causes original trafficking of HLA-DRB1\*04:01 in B cells [10], which makes processing/presentation of antigen most efficient in cells that express HLA-DRB1\*04:01 [11]. HLA-DRB1\*04:04, which encodes a QRRAA motif, has been associated with high autoantibody responses [12–14].

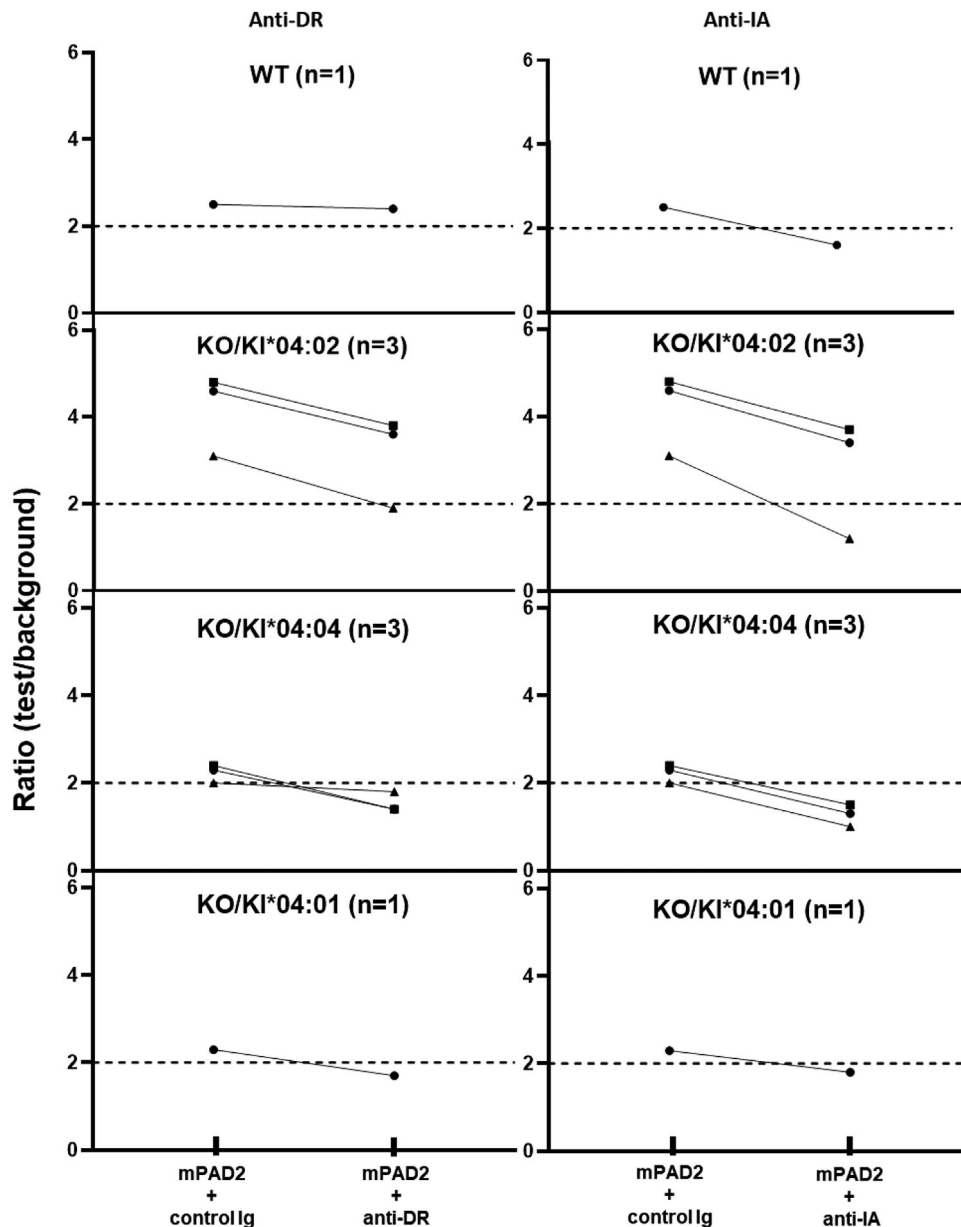
The association between RA and HLA-DRB1 alleles suggests that HLA-DR restricted T cells help antibody responses to the very numerous citrullinated proteins known to be recognized by

ACPAs. The identity of the peptides whose presentation by RA-associated HLA-DR molecules and recognition by helper T cells provides help to the B cells specific for the many citrullinated antigens recognized by RA patients' ACPAs is still unknown. Indeed, approximately 100,000 distinct citrullinated peptides are recognized by RA-specific autoantibodies, suggesting a requirement for many helper T lymphocytes with different specificities [15] even if cross-reactivity between citrullinated epitopes can somewhat diminish the number of required citrullinated peptide specific helper T cells.

Moreover, RA-associated HLA-DR alleles are associated with production of ACPAs, but they do not bind citrullinated peptides better than native peptides [12, 16]. A strong correlation between PAD4 peptide binding and HLA-DRB1 genotypic risk for RA has been observed [17].

We have suggested that PADs might be T cell targets whose recognition provides help for the production of autoantibodies to citrullinated proteins by a classical hapten-carrier model.

In this model, proteins under citrullination by PADs are processed with PADs in B cells specific for citrullinated epitopes. This model applies to any protein containing at least two arginine residues, one arginine residue bound by the PAD enzyme and a citrulline residue bound by the surface ACPA B-cell receptor. This



**Figure 4.** T-cell proliferation to mPAD2 is inhibited by anti-HLA-DR and/or anti-I-A<sup>b</sup>. Cells from spleen and lymph nodes obtained at 120 days after first immunization were cultured at a density of  $5 \times 10^6$  cells with 2  $\mu$ g of mPAD2, with anti-HLA-DR or anti-I-A<sup>b</sup> antibodies or control IgG. T-cell responses were evaluated by BrdU incorporation. Data are combined from three experiments with eight mice per experiment. Positive T-cell responses were defined by test OD/background OD ratio higher than 2 (dotted line).

is the case, for instance, of the alpha and beta chains of fibrinogen, a major autoantigen in RA, which contains 39 and 26 arginine residues, respectively. Thus, citrullinated epitope-specific B cells, because they present PAD peptides, can benefit from the help of PAD-specific T helper cells.

We have observed that RA patients have both antibodies and T-cell responses to PAD4 [18].

Moreover, C3H mice, expressing an I-E beta k molecule with an amino acid sequence very similar to the RA-associated HLA-DR $\beta$ 1\*04:01, develop IgG anti-citrullinated fibrinogen antibodies after PAD2 immunization [9]. In contrast, DBA/2 mice, express-

ing an I-E beta d chain with an amino acid sequence very similar to the “non-RA associated” HLA-DR $\beta$ 1\*04:02, failed to develop antibodies to citrullinated fibrinogen peptides.

Here, to test the contribution of HLA-DR alleles to the hapten-carrier model, we performed PAD2 immunization in C57BL/6 mice expressing:

- human HLA-DRA and HLA-DRB1\*04:01 and I-A<sup>b</sup> (KO/KI\*04:01);
- human HLA-DRA and HLA-DRB1\*04:04 and I-A<sup>b</sup> (KO/KI\*04:04);



- human HLA-DRA and HLA-DRB1\*04:02 and I-A<sup>b</sup> (KO/KI\*04:02);
- I-A<sup>b</sup> (C57BL/6).

The choice of mPAD2 rather than murine PAD4 immunization is trivial: in our early experiments in nontransgenic mice, we had trouble producing murine PAD4 and therefore used mPAD2 that we found easier to produce and efficient at triggering T-cell proliferation.

After immunization with mPAD2 in WT and HLA-DR humanized C57BL/6 mice, we obtained IgG and T-cell responses to mPAD2. While all mice were immunized against mPAD2, mPAD2-specific T cells were only observed in 46% of them. This could be explained by the fact that we analyzed T response at 120 days after first immunization, the time required for mice to produce antibodies to citrullinated fibrinogen peptides. Analysis of T response is often done after the first immunizations, when the T-cell response is strongest. This late analysis may explain weaker T-cell proliferations.

Remarkably, KO/KI\*04:01 humanized mice show better antibody response to mPAD2 than all other mice. Indeed, antibody response to mPAD2 is significantly higher in the KO/KI\*04:01 mice than in the other mice at 15, 30, 75, 105, and 120 days after first immunization. They also show better T-cell proliferative response to mPAD2 than other mice with 75% versus 38%, although results did not reach statistical significance.

To detect whether anti-citrullinated protein antibodies are produced in mice after mPAD2 immunization, we used previously identified B-cell epitopes from the beta chain of fibrinogen [9] to screen the sera from WT and HLA-DR humanized C57BL/6 mice immunized with mPAD2 or PBS. After mPAD2 immunization, citrullinated fibrinogen peptide-specific IgG responses were detected in 32% of mice.

The citrullin specificity of the antibodies was checked by comparison with the arginine version of the same peptides.

Peptide 8C was identified as a strong citrullinated fibrinogen B-cell epitope. We had already shown that it was recognized by the sera from 15% of C3H mice immunized with PADs [9]. Here, it was recognized by the sera from 21% of any C57BL/6 mice (KO/KI or not) immunized with mPAD2. Because peptide 8C (VWWMNWKGSWYSMR\*K) is a good citrullinated epitope and adjacent peptide 167C (WYSMR\*KMSMKIR\*PFF) is not, it is possible that amino acids present on the COOH side of the first R\* (citrulline) residue on 167C might impair either the binding of the peptide to the ELISA well or the recognition of the citrulline residue in the context of these amino acids. For instance, the Proline residue following the second Citrullin residue may alter the shape of the peptide chain.

IgG responses to citrullinated fibrinogen peptides were detected in 50% KO/KI\*04:01 (SE+) mice, 17% KO/KI\*04:04 (SE+) mice, 8% KO/KI\*04:02 (SE-) mice, and 83% WT mice. While KO/KI\*04:01 humanized mice show better antibody and T-cell proliferative response to mPAD2, WT mice are the best anti-citrullinated fibrinogen antibody producers. This may reflect the fact that both I-A<sup>b</sup> and HLA-DR proteins are involved in

mPAD2 peptide presentation, as suggested by anti-HLA-DR and anti-mouse I-A<sup>b</sup> antibody inhibition studies. Indeed, C57BL/6 mice (H-2<sup>b</sup>) do not express I-E, because the I-E alpha chain is not expressed on the H-2<sup>b</sup> haplotype. Thus, I-A<sup>b</sup> is the only class II MHC molecule available to C57BL/6 mice to develop their immune system. It is already known that peptides from PADs presented by I-A<sup>b</sup> are involved in the selection of the T-cell repertoire in C57BL/6 mice [19]. The amino acid sequence of I-A<sup>b</sup> has 64% homology with the sequence of the HLA-DRB1\*04 alleles and that the I-A<sup>b</sup> molecule could play the role of a DR4-like molecule. Further experiments aimed at suppressing the expression of the I-A<sup>b</sup> molecule must be done to evaluate the own effect of the HLA alleles without the dominant effect of I-A<sup>b</sup>.

Here, we confirm the "haptent-carrier" model for ACPA production, in which T cells specific for PADs help the development of IgG autoantibodies specific for citrullinated antigens. Indeed, immunization with mPAD2 induces IgG anti-citrullinated peptide autoantibodies in WT and HLA-DR4 humanized C57BL/6 mice.

An alternative haptent-carrier model could define PAD as the haptent and exogenous peptides as carriers. However, an exogenous source of peptides was excluded by confirming the purity of recombinant mPAD2 and the absence autocitrullination of mPAD2 or citrullinated peptides/mPAD2 complex. Obviously, when two proteins are bound to each other, they can be processed together and T cells specific for peptides from one protein can help B cells specific for the other. Here, we believe that the carrier effect is that of the PAD4 protein, because it is recognized by T cells in more than half of patients with RA [18].

Unexpectedly, WT mice whose only expressed MHC class II molecule is I-A<sup>b</sup> are the best anti-citrullinated fibrinogen autoantibody producers, further pointing to the privileged presentation of PAD peptides by I-A<sup>b</sup> [19]. Among the HLA-DR humanized mice, KO/KI\*04:01 mice are the best responders to mPAD2 and the best anti-citrullinated peptide autoantibody producers.

## Materials and methods

### Mice

C57BL/6NRj mice were purchased from Janvier Labs, Le Genest-Saint-Isle, France. We tested wild type (WT) and knock-out/knock-in (KO/KI) C57BL/6NRj mice. KO/KI mice were made by replacing I-E<sup>b</sup> genomic DNA (KO) with gene fragments containing HLA-DRA and HLA-DRB1\*04 (KI) (CIPHE, Marseille, France). All mice were genotyped by quantitative PCR assays. KO/KI mice express RA-associated HLA-DRB1\*04:01 (KO/KI\*04:01) or RA-associated HLA-DRB1\*04:04 (KO/KI\*04:04) or non-RA-associated HLA-DRB1\*04:02 (KO/KI\*04:02). KO/KI C57BL/6 mice also express I-A<sup>b</sup>. MHC class II expression in each mouse strain is summarized in Table 1 and has been verified by flow cytometry (data not shown). All mice were 7- to 9-week-old females, weighing 20–30 g, randomly divided into two groups (immunized by mPAD2 or PBS) (see Supporting Information).

**Table 1.** MHC class II expression of mice (X: no expression)

Mice	Name	MHC class II	
		I-A	I-E
C57BL/6	Wild type	b	X
C57BL/6	KO/KI*04:01	b	DRB1*04:01
C57BL/6	KO/KI*04:02	b	DRB1*04:02
C57BL/6	KO/KI*04:04	b	DRB1*04:04

Mice were housed at the Luminy INSERM animal facility, Marseille (A1301303).

## Proteins

mPAD2 was purchased from Proteogenix, Schiltigheim, France. Purity of recombinant mPAD2 protein was higher than 90%. Purity evaluation was made on SDS-PAGE gel using the GelAnalyzer software by Proteogenix. The absence autocitrullination of mPAD2 was checked by (i) using sera from ACPA-positive RA patients in ELISA assays and (ii) using the anti-modified citrulline Western blot detection kit, Millipore, France. Citrullinated fibrinogen, a target antigen of ACPA expressed in the synovial tissue of RA patients, allows ACPA detection with the same efficiency as anti-cyclic citrullinated peptide version 2 (CCP2) [20]. Citrullinated fibrinogen was obtained after incubation of native fibrinogen in 0.1 M Tris HCl (pH 7.4), 10 mM CaCl<sub>2</sub>, and 5 mM dithiothreitol buffer at a concentration of 1 mg/ml with rabbit PAD2 [21]. Noncitrullinated proteins were treated identically, except that water was added instead of rabbit PAD2.

## Immunization protocols

Mice were immunized subcutaneously with 100 µg of mPAD2 or, as a control with phosphate-buffered saline (PBS) in Freund's complete adjuvant (CFA). Three booster injections with 100 µg of mPAD2 or PBS in Freund's incomplete adjuvant (IFA) were given subcutaneously 15, 30, and 45 days later.

## Detection of anti-mPAD2 antibodies by ELISA

Plates were coated with 0.5 µg of mPAD2 and blocked with bovine serum albumin (BSA). Sera from mice obtained at 15, 30, 45, 75, 105, and 120 days after first immunization were diluted to 1/40 and incubated with mPAD2. After washing with PBS, anti-mouse IgG-peroxidase was added. After washing with PBS, tetramethyl benzidine (TMB) was added. Optical density (OD) was read at 405 nm. Background OD was obtained by adding each serum to a well without mPAD2. Positive sera were defined by test OD/background OD ratios higher than 2 (OD ratio).

**Table 2.** Amino acid sequence of beta fibrinogen peptides (R\*: citrulline)

Peptides	Sequences
163R	VIQNRQDGSVDFGRK
163C	VIQNR*QDGSVDFGR*K
110R	LMGENRTMTIHNGMF
110C	LMGENR*TMTIHNGMF
111R	FSTYDRDNDGWLTSD
111C	FSTYDR*DNDGWLTSD
165R	LTSDPRKQCSKEDGG
165C	LTSDPR*KQCSKEDGG
4R	PRKQCSKEDGGGWY
4C	PR*KQCSKEDGGGWY
5R	WYNRCHAANPNGRYY
5C	WYNR*CHAANPNGR*YY
6R	NRCHAANPNGRYYWG
6C	NR*CHAANPNGR*YYWG
8R	VVWMNWKGSWYSMRK
8C	VVWMNWKGSWYSMR*K
167R	WYSMRKMSMKIRPFF
167C	WYSMR*KMSMKIR*PFF
115R	MSMKIRPFFPQQ
115C	MSMKIR*PFFPQQ

## T-cell proliferation assays

Proliferation was evaluated using the colorimetric bromodeoxyuridine (BrdU) kit (Roche Diagnostics, Meylan, France). Mice were euthanized at day 120 after first immunization, cells from spleen and lymph nodes were obtained and cultured with mPAD2, native (arginine) or citrullinated fibrinogen or phytohemagglutinin (PHA). Inhibition of proliferation to mPAD2 was tested by monoclonal anti-HLA-DR (L243; Sigma Aldrich, Saint Quentin Fallavier, France) or purified anti-I-A<sup>b</sup> (AF61201) or purified mouse IgG2a isotype control (G155178; BD Biosciences, Le Pont de Claix, France). After 3 days, BrdU was added to the cells for 18 h. Cells were lysed and fixed. Anti-BrdU-peroxidase was added. After washing, TMB was added. OD was read at 405 nm. Background OD was obtained for cells cultured without protein. Positive T-cell responses were defined by test OD/background OD ratios higher than 2.

## Synthetic peptides

We used the same peptides from fibrinogen as in previous experiments [9]. Peptides were synthesized using a solid-phase system and purified (>60%) (Proteogenix, Schiltigheim, France). We tested 10 15-mers from the beta chain of human fibrinogen (locus NP\_005132) with 70–100% identity with their murine counterparts (Table 2). The arginine version of these peptides was used to test citrullinated peptide specificity of anti-citrullinated fibrinogen antibodies.



## Detection of anti-citrullinated fibrinogen peptide antibodies by ELISA

Sera from mice obtained at 120 days after first immunization were tested by ELISA. Plates were coated with 5 µg of fibrinogen peptides under citrullinated or arginine form as previously described [9]. After blocking, sera diluted to 1/80 were incubated with peptides. After washing with PBS, anti-mouse IgG-peroxidase was added. After washing with PBS, TMB was added. OD was read at 405 nm. Background OD was obtained by adding each serum to a well without peptide. Positive sera were defined by test OD/background OD ratios higher than 2.

## Statistical analysis

Comparisons between groups were performed using Fisher's exact and Mann–Whitney tests. GraphPad Prism 9.1.2 (GraphPad Software) was used for all statistical analyses.

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**Conflict of interest:** The authors declare no commercial or financial conflict of interest

**Ethics approval:** All animal care and experimental procedures were performed in agreement with the Animal Ethics Committee of Marseille and the Ministère de l'Enseignement Supérieur et de la Recherche, France (APAFIS No. 0300703 and No. 10065).

**Author contributions:** JR and IA designed research. MH, NL, and IA performed research. MH, NL, IA, and JR analyzed data. JR and IA wrote the paper.

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**Data availability statement:** The data that support the findings of this study are available from the corresponding author upon reasonable request.

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**Abbreviations:** **ACPA:** anti-citrullinated protein autoantibody · **mPAD2:** murine PAD2 · **PAD:** peptidyl arginine deiminase · **RA:** rheumatoid arthritis · **SE:** shared epitope · **TMB:** tetramethyl benzidine

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