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Autoantibodies to citrullinated proteins (ACPAs) are present in two-thirds of patients with rheumatoid arthritis (RA). ACPAs are produced in the absence of identified T cell responses for each citrullinated protein. Peptidyl arginine deiminase 4 (PAD4), which binds proteins and citrullinates them, is the target of autoantibodies in early RA. This suggests a model for the emergence of ACPAs in the absence of detectable T cells specific for citrullinated antigens: ACPAs could arise because PADs are recognized by T cells, which help the production of autoantibodies to proteins bound by PADs, according to a "hapten/carrier" model. Here, we tested this model in normal mice. C3H are healthy mice whose IEβk chain is highly homologous to the β1 chain HLA-DRB1*04:01, the allele most strongly associated with RA in humans. C3H mice immunized with PADs developed antibodies and T cells to PAD and IgG antibodies to citrullinated fibrinogen peptides, in the absence of a T cell response to fibrinogen. To analyze the MHC background effect on hapten/carrier immunization, we immunized DBA/2 mice (whose IEβd chain is similar to that of HLA-DRB1*04:02, an HLA-DR4 subtype not associated with RA). DBA/2 mice failed to develop antibodies to citrullinated fibrinogen peptides. Thus, T cell immunization to PAD proteins may trigger ACPAs through a hapten/carrier mechanism. This may constitute the basis for a new mouse model of ACPA-positive RA.

rheumatoid arthritis | PAD protein | anticitrullinated protein antibody | mouse model | MHC

Rheumatoid arthritis (RA), a chronic inflammatory joint disease, is associated with HLA-DRB1 alleles expressing the "shared epitope"-like HLA-DRB1*04:01 and HLA-DRB1*01:01 (1–4). RA is usually preceded by the development of anticitrullinated protein autoantibodies (ACPAs) (5, 6). ACPAs recognize citrullins [citrullin is an amino acid obtained by posttranslational modification of arginin by enzymes called Peptidyl Arginyl Deiminases (PADs)] on many different proteins like fibrin, vimentin, enolase, collagen, and so forth (7–10).

The function of HLA-DR molecules is to present peptides to helper T cells to help the production of IgG antibodies by B cells. Therefore, an obvious explanation for the association of RA with HLA-DR and ACPAs could be that HLA-DR–restricted T cells might help antibody responses to the many different citrullinated proteins known to be recognized by ACPAs. However, T cells specific for citrullinated proteins have not been identified so far. Furthermore, this explanation implies that RA-associated HLA-DR alleles bind citrullinated peptides to allow their presentation to T cells. This "citrullinated peptide binding" hypothesis is controversial, supported by very limited binding data (11) and contradicted by extensive binding data on fibrinogen, vimentin, collagen, and Epstein–Barr virus peptides under native and citrullinated forms (12–14).

While trying to identify new autoantibodies in patients with early or late RA by using human protein chips, we found that IgG antibodies to PAD4, one of the five isotypes of PAD, are present in 20% of patients with early and 40% of patients with late RA (15). This finding led us to propose the hypothesis that PAD4 is the T cell target whose recognition provides help for the production of autoantibodies to citrullinated proteins (ACPAs), according to a classical hapten/carrier model. Here, we demonstrate this model in normal mice.

Results

Anti-PAD Antibodies in C3H Mice Immunized with PADs. C3H mice were immunized with murine or human PAD2, or murine or human PAD4, or PBS in Freund's complete adjuvant (CFA). Three booster injections with PAD or PBS in Freund's incomplete adjuvant (IFA) were given s.c. 15, 35, and 55 d later (Fig. 1). IgG responses to PADs were analyzed by ELISA. Sera were diluted at 1/40. Positive sera were defined by an OD value higher than twice the background OD (obtained by adding each serum to a well without PAD).

Only murine PAD2, human PAD2, and PAD4 were immunogenic.

C3H mice immunized with PBS did not develop anti-PAD antibodies (Fig. 2*A*). The average background OD for wells without PAD was 0.06, and the average OD for wells with PADs was 0.07.

In C3H mice immunized with murine PAD2 or human PAD2 or human PAD4, anti-PAD antibodies were detected after first immunization and persisted over time in 28/28 mice immunized with either murine PAD2 or human PAD2 or human PAD4 versus 0/20 mice immunized with PBS (Fisher's test, $P = 6 \times 10^{-14}$) (Fig. 2).

Significance

The presence and development of autoantibodies to citrullinated proteins (ACPAs) are highly associated with rheumatoid arthritis (RA). The mechanisms leading to the production of ACPAs are unknown. Here, we propose a model to explain the emergence of anticitrullinated protein autoantibodies in RA. Indeed, we could trigger the development of anticitrullinated fibrinogen autoantibodies in normal mice by immunization with peptidyl arginine deiminase (PAD), most likely through a hapten/carrier mechanism in which the carrier is the PAD enzyme that performs citrullination and the hapten is any protein being citrullinated (hence bound) by PAD. Our results allow us to understand the birth of anticitrullin autoimmunity.

Author contributions: J.R. and I.A. designed research; F.A., C.M., E.P., and I.A. performed research; N.C.L., N.B., J.R., and I.A. analyzed data; and J.R. and I.A. wrote the paper.

The authors declare no conflict of interest.

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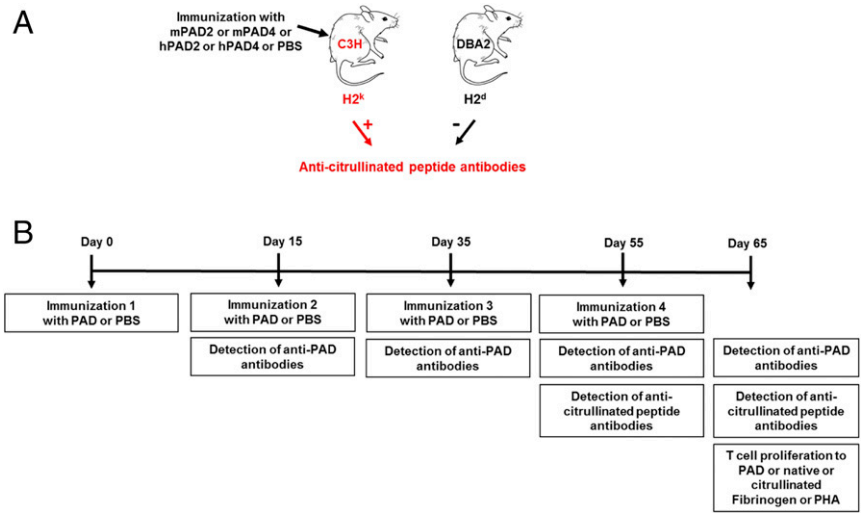

Fig. 1. Model and methods. Shown are the immunization protocol (*A*) and antibody and T cell analysis kinetic (*B*).

In C3H mice immunized with murine PAD2, the average background OD was 0.06, and the average test OD was 1 after the first immunization, 1.4 after the second immunization, 1.8 after the third immunization, and 1.7 after the fourth immunization (Fig. 2*B*).

In C3H mice immunized with human PAD2, the average background OD was 0.06, and the average test OD was 0.8 after the first immunization, 0.9 after the second immunization, 1.5 after the third immunization, and 1.6 after the fourth immunization (Fig. 2*B*).

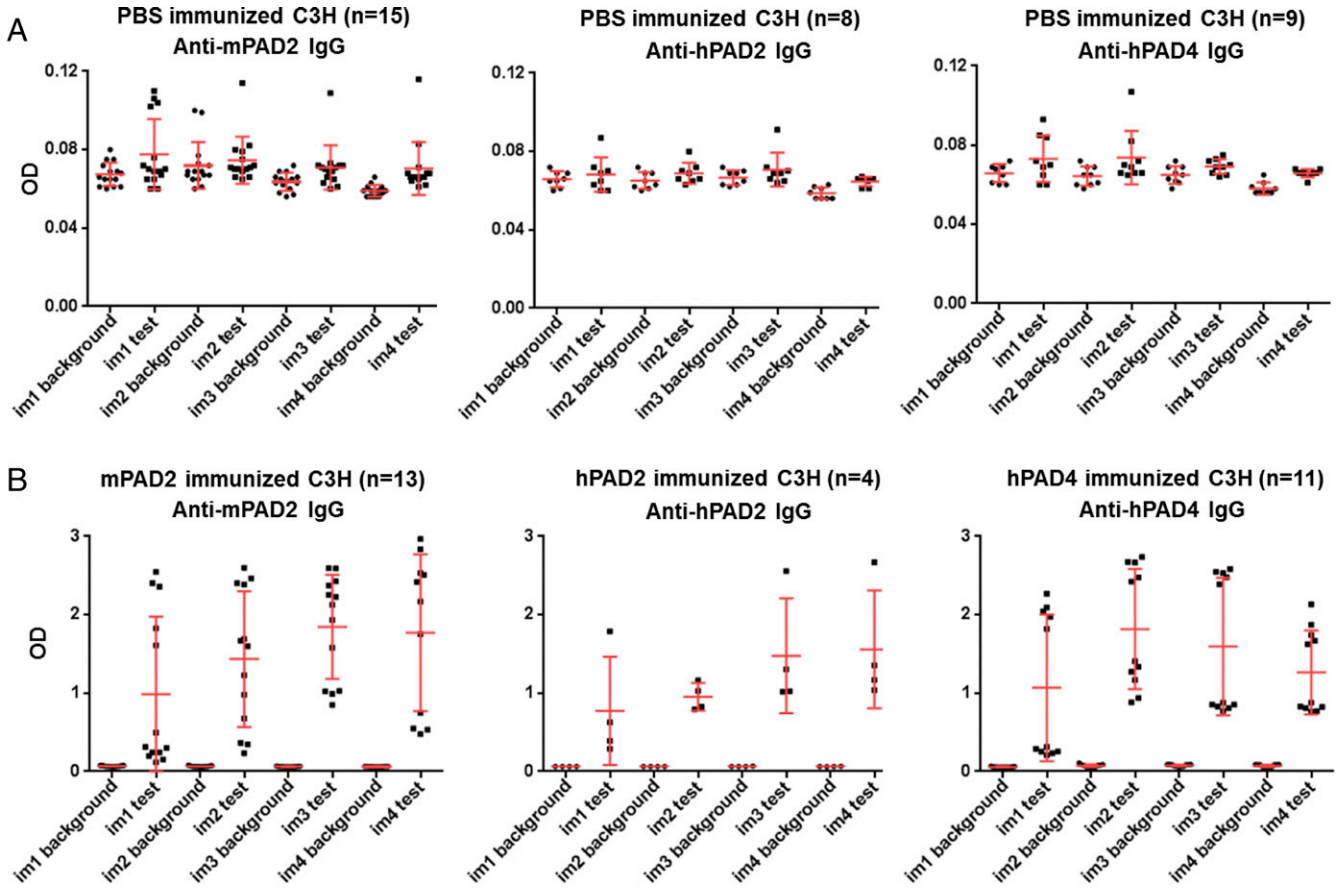

Fig. 2. IgG responses to PADs in C3H mice. IgG responses to murine PAD2 or human PAD2 or human PAD4 were analyzed by ELISAs. Plates were coated with PADs and blocked with BSA. Sera from primed mice were obtained at 15, 35, 55, and 65 d postimmunization and were diluted at 1/40. (*A*) For PBS-immunized mice, 15 sera were tested for murine PAD2, 8 sera were tested for human PAD2, and 9 sera were tested for human PAD4. (*B*) For PAD-immunized mice, each serum was tested against the same PAD used for each immunization. After washing, peroxidase-conjugated antimurine IgG was added. The OD was read at 405 nm. The background OD was obtained by adding each serum to a well without PAD (negative). Positive sera were defined as an OD value higher than twice the background OD.

In C3H mice immunized with human PAD4, the average background OD was 0.07, and the average test OD was 1.1 after the first immunization, 1.8 after the second immunization, 1.6 after the third immunization, and 1.3 after the fourth immunization (Fig. 2*B*).

The presence of anti-PAD antibodies was confirmed by titration assays for 12 C3H mice immunized with PADs (Fig. S1).

T Cell Responses to PADs in C3H Mice Immunized with PADs. Spleen and lymph nodes were obtained 65 d after immunizations with murine PAD2 or human PAD2 or human PAD4 or PBS. T cell proliferation to PADs, native or citrullinated fibrinogen, or phytohemagglutinin (PHA) was evaluated by bromodeoxyruridine incorporation. Positive responses were defined by OD values higher than twice the background OD obtained for cells cultured without antigen.

T cell responses to PADs were detected in 24/28 C3H mice immunized with PADs versus 4/20 C3H mice immunized with PBS (Fisher's test, $P = 6 \times 10^{-6}$) (Fig. 3).

Indeed, 11/13 C3H mice immunized with murine PAD2, 4/4 C3H mice immunized with human PAD2, and 9/11 C3H mice immunized with human PAD4 developed immunizing PAD-specific T cell responses (Fig. 3*B*). No T cell response was detected for native or citrullinated fibrinogen in mice immunized with PADs or PBS. Among the four mice immunized with PBS who responded to PADs, two recognized murine PAD2, one recognized human PAD2, and one recognized murine PAD2, human PAD2, and human PAD4 (Fig. 3*A*).

In C3H mice immunized with PBS, the average background OD was 0.17 for cells cultured without antigen, and the average test OD was 0.26 for cells cultured with murine PAD2 or human PAD2 or human PAD4, 0.16 for cells cultured with native or citrullinated fibrinogen, and 1.1 for cells cultured with PHA (Fig. 3*A*).

In C3H mice immunized with murine PAD2, the average background OD was 0.18 for cells cultured without antigen, and the average test OD was 0.48 for cells cultured with murine PAD2, 0.17 for cells cultured with native or citrullinated fibrinogen, and 1.1 for cells cultured with PHA (Fig. 3*B*).

In C3H mice immunized with human PAD2, the average background OD was 0.19 for cells cultured without antigen, and the average test OD was 0.82 for cells cultured with human PAD2, 0.19 for cells cultured with native or citrullinated fibrinogen, and 1.8 for cells cultured with PHA (Fig. 3*B*).

In C3H mice immunized with human PAD4, the average background OD was 0.25 for cells cultured without antigen, and the average test OD was 0.6 for cells cultured with human PAD4, 0.22 for cells cultured with native or citrullinated fibrinogen, and 1.3 for cells cultured with PHA (Fig. 3*B*).

Antibodies to Citrullinated Peptides from Human Fibrinogen in C3H Mice Immunized with PADs. To test whether anticitrullinated peptide antibodies were produced in C3H mice after PAD immunization, we screened sera from 28 mice immunized with murine PAD2 or human PAD2 or human PAD4 and sera from 20 mice immunized with PBS with 46 citrullinated peptides from the alpha, beta, and gamma chain of human fibrinogen. These

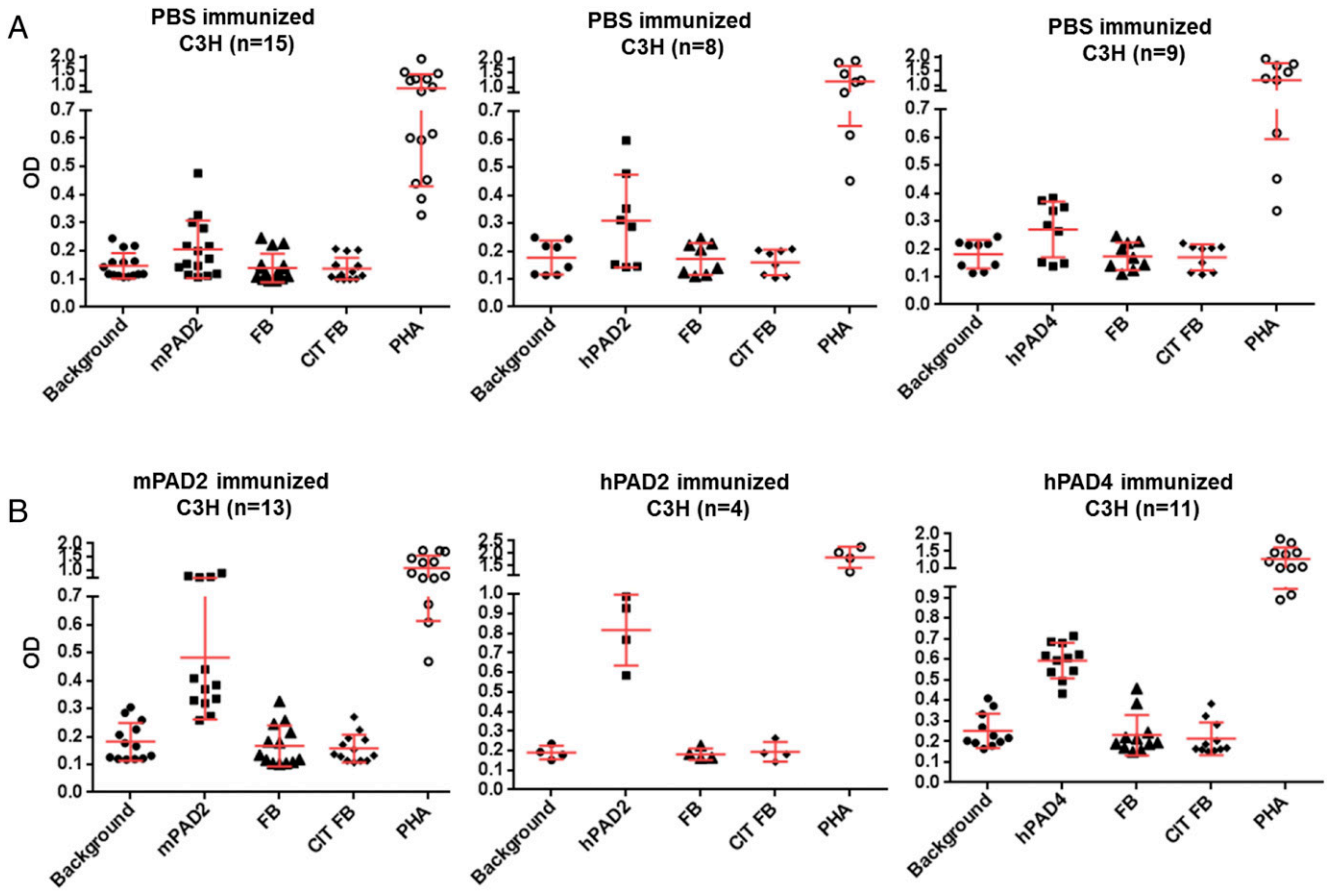

Fig. 3. T cell responses to PADs in C3H mice. Spleen and lymph nodes were obtained at 65 d postimmunization for PBS-immunized mice (*A*) or PAD-immunized mice (*B*). Cells were extracted and cultured at 5×10^6 cells per well with 2 μg of proteins or PHA. T cell responses were evaluated by bromodeoxyruridine incorporation. Positive responses were defined as an OD value higher than twice the OD for cells cultured without protein.

peptides were 17 15-mers from the alpha chain of human fibrinogen (locus NP_000499) with 50–100% identity with their murine counterparts, 28 15-mers from the beta chain of human fibrinogen (locus NP_005132) with 70–100% identity with their murine counterparts, and one 15-mer from the gamma chain of human fibrinogen (locus NP_068656.2) with 100% identity with its murine counterpart.

We identified four major citrullinated epitopes: peptides 4C, 5C, 6C, and 8C (Fig. 4). These peptides encompassing residues 420–479 of the beta chain of human fibrinogen were recognized by 8/28 sera from mice immunized with PADs versus 0/20 sera from mice immunized by PBS (Fisher's test, $P = 0.014$).

The presence of anticitrullinated peptide antibodies was confirmed by titration assays for six C3H mice immunized with PADs (Figs. S2–S4).

To check for the presence of citrullin residue-specific antibodies, we then screened the same sera from 28 mice immunized with murine PAD2 or human PAD2 or human PAD4 and from 20 mice immunized with PBS, with peptides 4, 5, 6, and 8 under their native and citrullinated form (Fig. 5).

IgG responses to native peptides were detected in 10/28 mice immunized with PADs versus 2/20 mice immunized with PBS (Fisher's test, $P = 0.05$) (Fig. 5).

Citrullinated peptide-specific IgG responses were detected in 6/28 mice immunized with PADs versus 0/20 mice immunized with PBS (Fisher's test, $P = 0.03$) (Fig. 5).

Peptide 8C was the most interesting because it was only recognized under its citrullinated form by the sera from 4/28 mice immunized with murine PAD2 or human PAD2 or human PAD4 (Fig. 5).

Influence of MHC Background on Anticitrullinated Peptide Immunization. To test whether polymorphism of the IEβ chain influences the development of T cell responses to PAD and antibody responses to citrullinated peptides from fibrinogen, we immunized mice expressing an IEβ allele other than IEβk with PAD.

We chose DBA2 mice whose IEβd chain is similar to that of non-RA–associated HLA-DRB1*0402 (16) (Fig. S5). IgG responses to PADs were analyzed by ELISA. Sera were diluted at 1/40. DBA/2 mice were immunized with murine PAD2 or human PAD4 proteins, which had given the highest anti-PAD antibody titers in C3H mice after PAD immunization.

We detected IgG responses to PADs in 10/10 DBA/2 mice immunized with murine PAD2 and human PAD4 and 0/9 DBA/2 mice immunized with PBS (Fisher's test, $P = 1 \times 10^{-5}$) (Fig. 6).

In DBA/2 mice immunized with PBS, the average background OD was 0.06, and the average test OD was 0.06 when adding each serum to a well with murine PAD2 or human PAD4 (Fig. 6*A*).

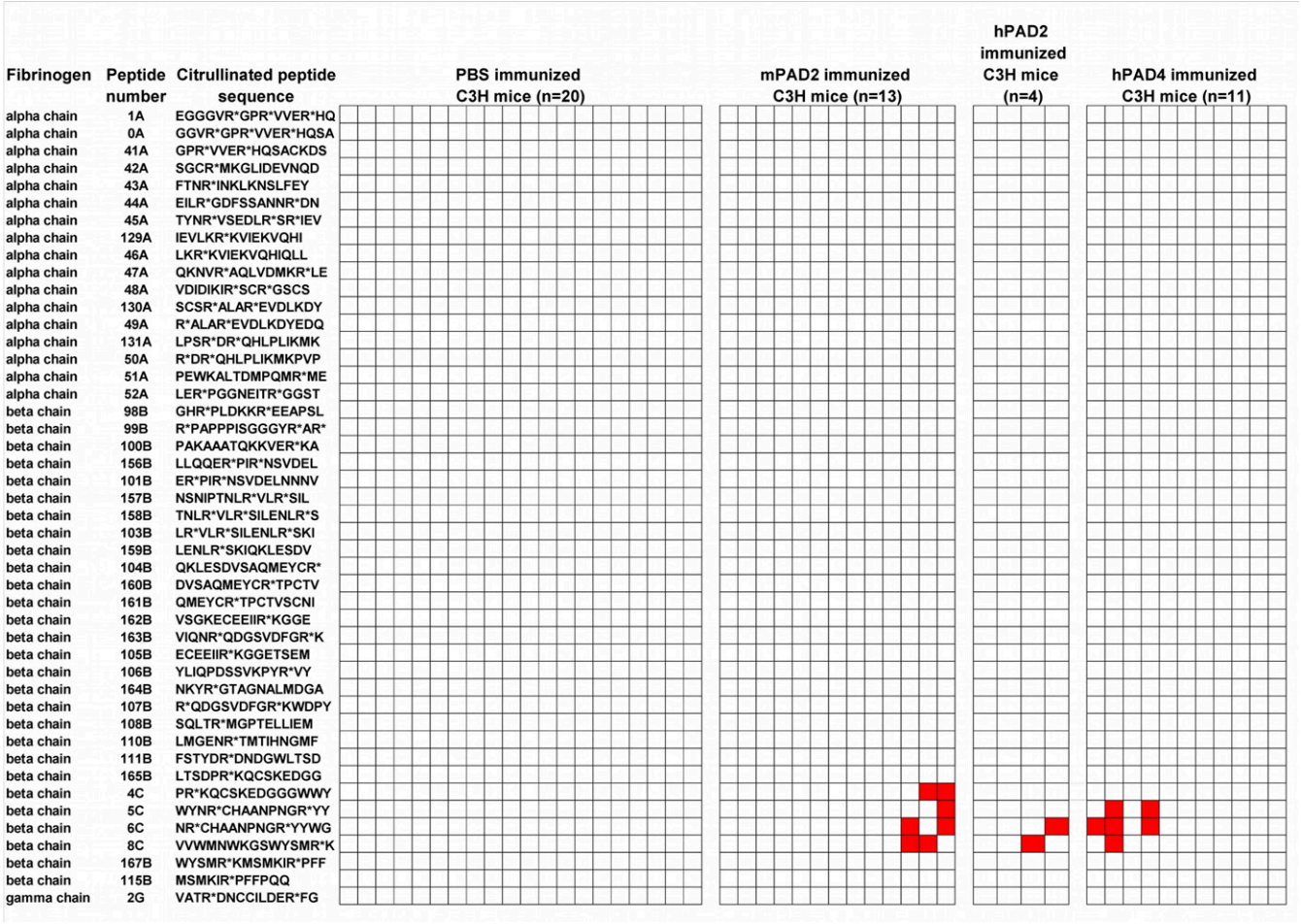

Fig. 4. Citrullinated peptides recognized by the sera of C3H mice immunized with PADs. ELISA plates were coated with 46 citrullinated peptides and blocked with BSA. Sera from primed mice obtained at 55 and 65 d postimmunization were diluted at 1/80. After washing, peroxidase-conjugated antimurine IgG was added. The OD was read at 405 nm. The background OD was obtained by adding each serum to a well without peptide. Positive sera were defined as an OD value higher than twice the background OD. A column corresponded to one mouse. IgG to citrullinated peptide is indicated in red.

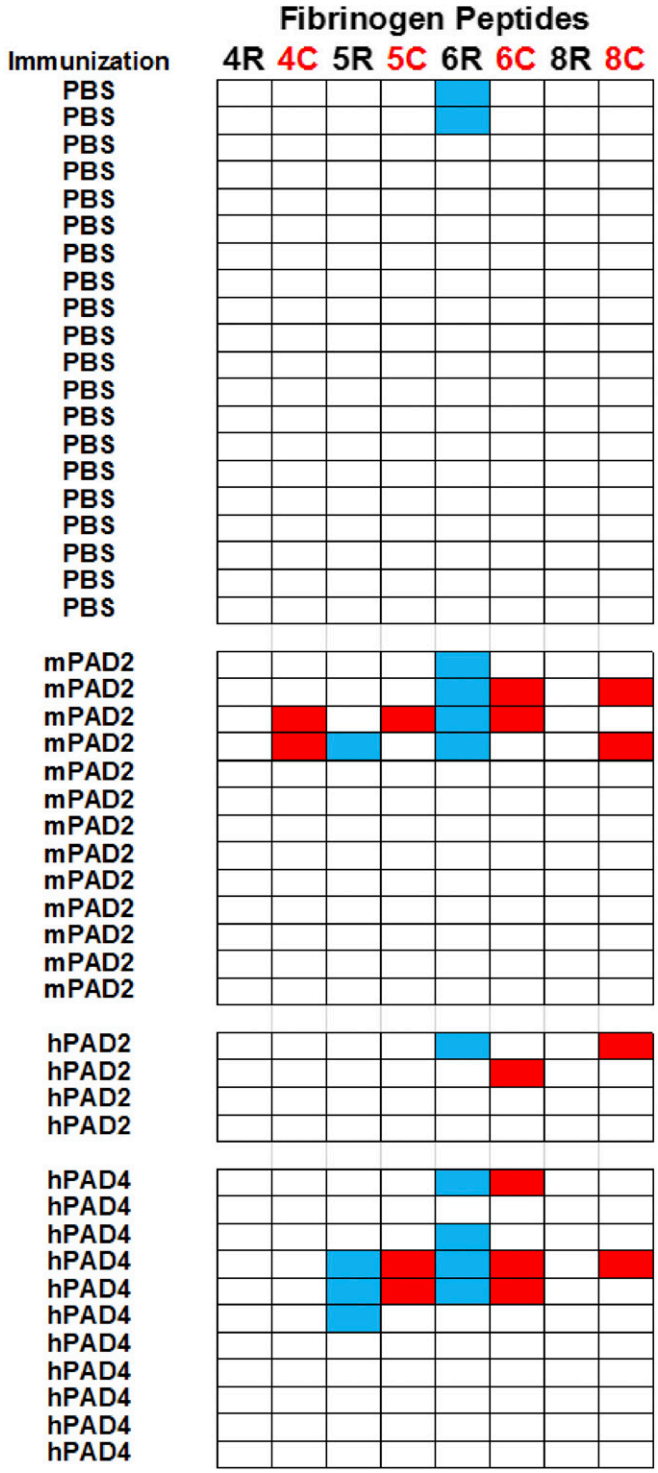

Fig. 5. PAD immunization in C3H mice triggers production of anticitrullinated fibrinogen peptide antibodies. Plates were coated with peptides in unmodified forms (4R, 5R, 6R, and 8R) or citrullinated forms (4C, 5C, 6C, and 8C). After blocking, sera from primed mice obtained at 55 or 65 d postimmunization were diluted at 1/80. After washing, peroxidase-conjugated antimurine IgG was added. The OD was read at 405 nm. The background OD was obtained by adding each serum to a well without peptide. Positive sera were defined as an OD value higher than twice the background OD. A line corresponded to one mouse. IgG to native peptide is indicated in blue, and IgG to citrullinated peptide is indicated in red.

In DBA/2 mice immunized with murine PAD2, the average background OD was 0.05, and the average test OD was 0.27 after the first immunization, 0.77 after the second immunization, 0.7 after the third immunization, and 0.8 after the fourth immunization (Fig. 6*B*).

In DBA/2 mice immunized with human PAD4, the background OD was 0.06, and the average test OD was 0.44 after the first immunization, 0.47 after the second immunization, 0.88 after the third immunization, and 0.93 after the fourth immunization (Fig. 6*B*).

Anti-PAD antibody levels (as indicated by test ODs) were lower in DBA/2 than in C3H mice after immunization with PADs.

The presence of anti-PAD antibodies was confirmed by titration assays for 10 DBA/2 mice immunized with PADs (Fig. S6).

T cell responses to PADs were detected in 8/10 DBA/2 mice immunized with murine PAD2 and human PAD4 and 3/9 DBA/2 mice immunized with PBS (Fisher's test, $P = 0.07$) (Fig. 7).

Indeed, in DBA/2 mice immunized with PBS, the average background OD was 0.15 in cells cultured without antigen, and the average test ODs were 0.2 in cells cultured with murine PAD2 or human PAD4, 0.14 in cells cultured with native or citrullinated fibrinogen, and 0.35 in cells cultured with PHA (Fig. 7).

In DBA/2 mice immunized with murine PAD2, the average background OD was 0.18 in cells cultured without antigen, and the average test OD was 0.37 in cells cultured with murine PAD2, 0.17 in cells cultured with native or citrullinated fibrinogen, and 0.6 in cells cultured with PHA (Fig. 7).

In DBA/2 mice immunized with human PAD4, the average background OD was 0.18 in cells cultured without antigen, and the test ODs were 0.53 obtained in cells cultured with human PAD4, 0.18 in cells cultured with native or citrullinated fibrinogen, and 0.61 in cells cultured with PHA (Fig. 7).

T cell responses (as indicated by test ODs) to murine PAD2 were lower in DBA/2 mice immunized with murine PAD2 than in C3H mice immunized with murine PAD2. T cell responses (as indicated by test ODs) to human PAD4 were similar in DBA/2 and C3H mice immunized with human PAD4. No T cell responses were detected in native or citrullinated fibrinogen in DBA/2 mice immunized with PADs or PBS. T cell responses to PHA (as indicated by test ODs) were lower in DBA/2 than in C3H mice. DBA/2 mice are known to have the lowest PHA responsiveness of all classical mouse strains, whereas C3H are among the low responders (17).

Finally, we screened sera from mice immunized with PBS or murine PAD2 or human PAD4 on the 46 citrullinated peptides from the alpha, beta, and gamma chain of human fibrinogen with 50–100% homology with their murine counterparts. No IgG response to citrullinated fibrinogen peptides was detected in any of the DBA/2 mice immunized with PADs or PBS (Fig. 8). The lack of anticitrullinated peptide antibodies was confirmed by titration assays for seven DBA/2 mice immunized with PADs (Figs. S2–S4).

Discussion

In this study, we tested whether immunization to PAD proteins triggers ACPAs through a hapten/carrier mechanism in which the carrier is PAD, which performs citrullination, and the hapten is any protein being citrullinated by PAD.

Indeed, the sera of two-thirds of patients with RA contain anticitrullinated peptide autoantibodies. These IgG antibodies recognize citrullin residues on different proteins. ACPAs are central to the development of RA. However, the mechanisms leading to the production of ACPAs are unknown.

A tentative model proposes that cigarette smoking may induce citrullination of proteins in the lungs. HLA-DRB1 alleles associated with RA would then bind citrullinated peptides and present them to helper T cells. This mechanism would explain anticitrullinated protein immunization in patients whose HLA-DRB1 molecules express the shared epitope. However, this model has serious limitations:

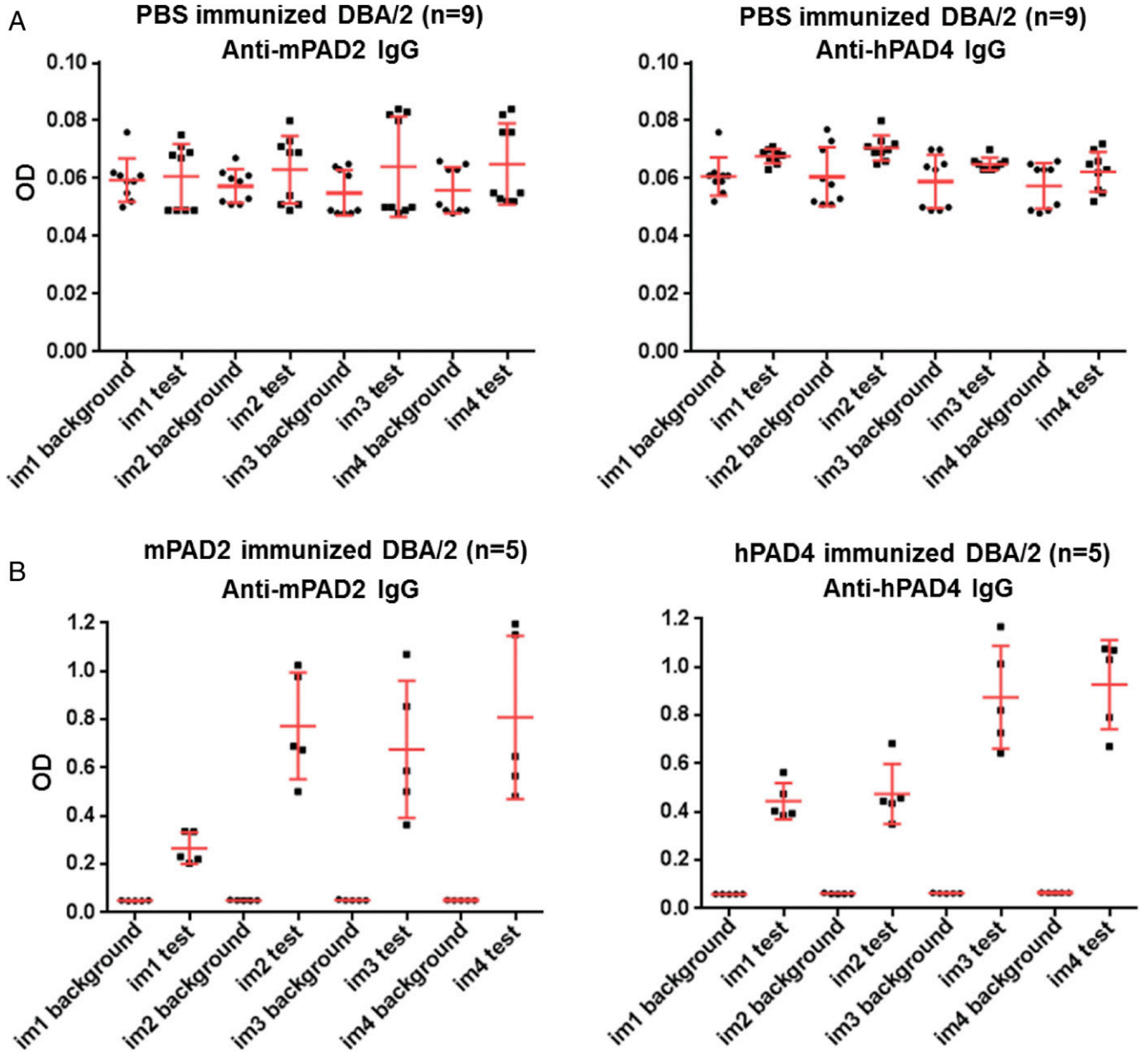

Fig. 6. IgG responses to PADs in DBA/2 mice. Plates were coated with PADs and blocked with BSA. Sera from primed mice were obtained at 15, 35, 55, and 65 d postimmunization and were diluted at 1/40. (*A*) For PBS-immunized mice, nine sera were tested for murine PAD2 and human PAD4. (*B*) For PAD-immunized mice, each serum was tested against the same PAD used for each immunization. After washing, peroxidase-conjugated antimurine IgG was added. The OD was read at 405 nm. The background OD was obtained by adding each serum to a well without PAD (negative). Positive sera were defined as an OD value higher than twice the background OD.

i) RA may occur in nonsmokers.
ii) IgG antibodies against citrullinated proteins usually develop in the absence of identified helper T cell response.
iii) The preferential binding of citrullinated peptides to shared epitope-positive HLA-DRB1 alleles has never been properly demonstrated. Indeed, the article that was supposed to have demonstrated the point studied the binding of one unique citrullinated vimentin peptide to three shared epitope-positive and five shared epitope-negative HLA-DRB1 alleles (11). Conversely, thorough studies of the binding of hundreds of peptides to shared epitope-positive or -negative HLA-DRB1 alleles did not show preferential binding of citrullinated peptides to shared epitope-positive alleles, compared with their native counterparts (12–14).

Thus, the identity of the antigen(s) whose recognition by T cells can help the development of IgG autoantibodies directed at citrullinated proteins is still unknown.

We focused on PAD2 and PAD4 as candidate antigens in anticitrullinated protein immunization for several reasons:

i) Anti-PAD4 IgG autoantibodies are detectable during the preclinical phase of RA in one-third of patients and are associated with ACPA positivity (18–24).
ii) PAD2 and PAD4 expressed in the RA synovium (25) are involved in the citrullination of fibrin, the major synovial autoantigen in RA (10).
iii) PADI4, the gene encoding PAD4, is a susceptibility locus for RA in Asians and in some, but not every, Western populations

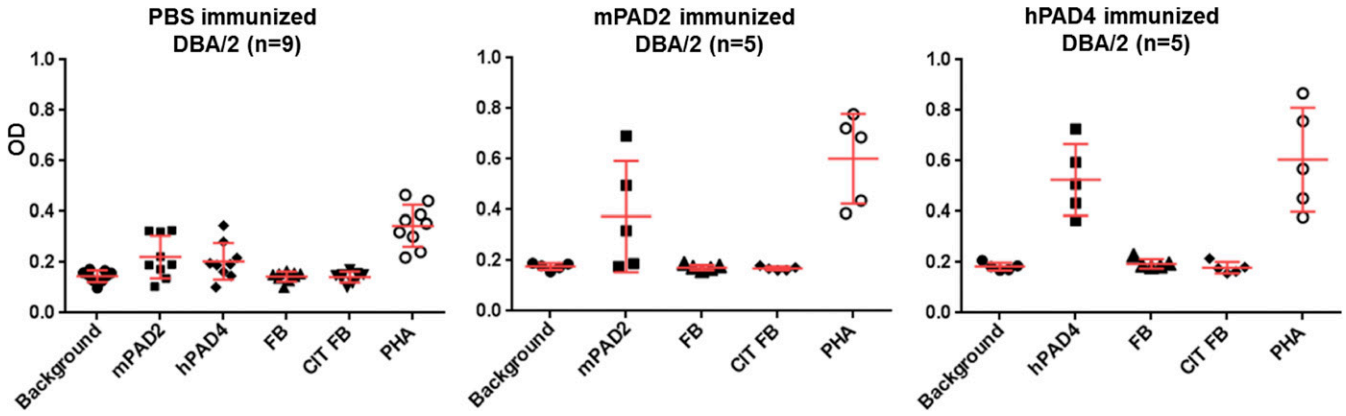

Fig. 7. T cell responses to PADs in DBA/2 mice. Spleen and lymph nodes were obtained at 65 d postimmunization. Cells were extracted and cultured at 5×10^6 cells per well with 2 μg of proteins or PHA. T cell responses were evaluated by bromodeoxyruridine incorporation. Positive responses were defined as an OD value higher than twice the OD for cells cultured without protein.

(26). Increased susceptibility to develop RA might be due by higher stability of PAD4 transcripts and thus higher expression of PAD4 (27).

To test whether we could induce ACPAs by immunizing normal mice with PADs, we used C3H mice whose IEβk chain is similar to that of RA-associated HLA-DRB1*04:01. We immunized C3H mice with four PAD proteins (murine and human PAD2 and PAD4) and obtained IgG and T cell responses to PAD after immunization with murine and human PAD2 or with human PAD4.

No T cell response was observed to native or citrullinated fibrinogen in C3H mice immunized with PADs.

To detect anticitrullinated protein antibodies, we used 46 citrullinated peptides from human fibrinogen with 50–100% homology with their murine counterparts. Among these 46 peptides, four were preferentially recognized by sera from primed mice. These peptides are located in the C-terminal domain of human beta fibrinogen (amino acids 420–479). IgG responses to citrullinated fibrinogen peptides were only detected in C3H mice immunized with murine or human PAD2 or human PAD4.

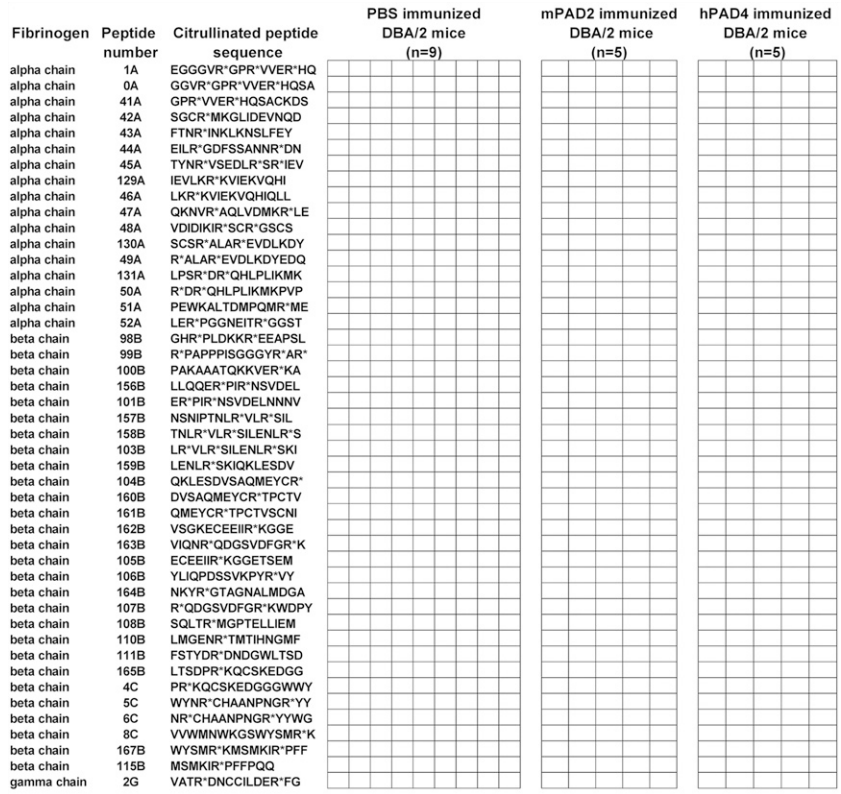

Fig. 8. Citrullinated peptide detection in the sera of DBA/2 mice immunized with PADs or PBS. ELISA plates were coated with 46 citrullinated peptides and blocked with BSA. Sera from primed mice obtained at 55 and 65 d postimmunization were diluted at 1/80. After washing, peroxidase-conjugated antimurine IgG was added. The OD was read at 405 nm. The background OD was obtained by adding each serum to a well without peptide. Positive sera were defined as an OD value higher than twice the background OD. A column corresponded to one mouse.

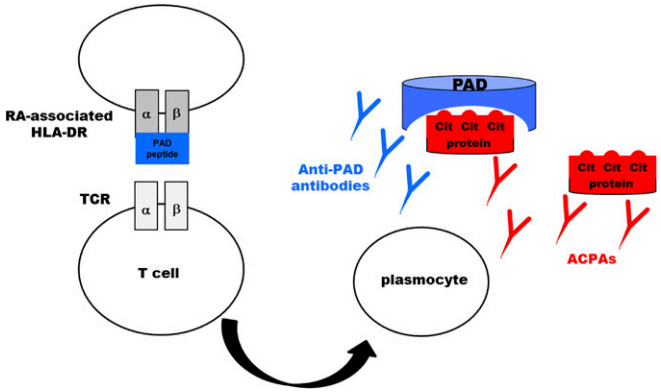

Fig. 9. Hapten (citrullinated proteins)/carrier (PAD) model. PAD may be the carrier seen by the helper T cells, which help production of antibodies to any protein being citrullinated.

We also used DBA/2 mice whose IEβd chain is similar to that of non-RA–associated HLA-DRB1*04:02. No IgG responses to citrullinated fibrinogen peptides were detected in DBA/2 mice immunized with PADs.

Our data suggest a model for the HLA and T cell contribution to the production of IgG anticitrullinated proteins in RA. In this model, HLA-DR alleles bind PAD peptides. T lymphocytes recognize PAD peptides and help production of antibodies to any antigen being citrullinated by PAD (Fig. 9).

A corollary of this model is that the molecular basis for the association between HLA-DRB1 alleles like HLA-DRB1*04:01 and HLA-DRB1*01:01 and RA is the capability of these alleles to bind peptides derived from PAD.

It must be noted that any protein (even bacterial) with PAD activity may be the target of helper T cells that may help production of IgG antibodies to any protein under citrullination. In this respect, the aggravating (but not mandatory) role of tobacco smoking on RA susceptibility might occur through increased periodontal disease. Indeed, porphyromonas gingivalis, the bacterium most associated with periodontal disease, has a PAD protein of its own. Peptides from this bacterial PAD might bind HLA-DR and be recognized by helper T cells. This is consistent with the fact that residues 11 and 13, located on the floor of the HLA-DRB1 molecule and influencing peptide binding, contribute a major effect in RA susceptibility, which is enhanced by tobacco smoking (28, 29).

It remains to be determined whether the mere production of ACPAs is enough to induce arthritis. Of interest, in vitro studies in humans suggest that ACPA/rheumatoid factor immune complexes activate macrophages and induce production of inflammatory cytokines including TNF alpha (30).

However, the goal of this article was only to demonstrate the production of ACPA after PAD immunization in mice through a hapten/carrier mechanism.

Whether this may trigger arthritis will be the subject of future studies.

Materials and Methods

Mice. C3H/HeNHsd and DBA/2 mice were purchased from Envigo Laboratories. Mice were housed at the Luminy INSERM Institute, Marseille (A13 01303). All experiments were approved by the animal ethics committee (no. 03007.03). 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.

Proteins. Murine PAD2 and PAD4 and human PAD2 and PAD4 proteins were purchased from Proteogenix. They were produced in baculovirus and purified. Their activity and autocitrullination status were tested before immunization.

Citrullinated Proteins. Citrullinated fibrinogen, the most likely target antigen of ACPA, expressed in the synovial tissue of RA patients, allows ACPA detection with the same efficiency as the anticyclic peptide antigen kits (anti-CCP2) in RA. Citrullinated human fibrinogen (Calbiochem and Oxford Biomedical Research) was obtained after incubation in 1 M Tris·HCl (pH7.4), 100 mM CaCl2, 50 mM DTT buffer at a concentration of 1 mg/mL with rabbit PAD2 (Sigma Aldrich), for 2 h at 37 °C. Noncitrullinated proteins were treated identically, except that water was added instead of rabbit PAD2.

Immunization Protocols. C3H or DBA/2 mice were immunized s.c. with 100 μg of murine or human PAD2, or murine or human PAD4, or PBS in CFA. Three booster injections with 100 μg of PADs or PBS in IFA were given s.c. 15, 35, and 55 d later.

Detection of Anti-PAD Antibodies. Sera from primed mice were obtained at days 15, 35, 55, and 65 and tested by ELISA. Plates were coated overnight with PADs. Plates were blocked with 2% BSA. Sera were incubated. After washing with Tween 20, peroxidase-conjugated antimurine IgG was added. The OD was read at 405 nm. Background OD was obtained by adding each serum to a well without protein. Positive sera were defined as an OD value higher than twice the background OD.

Synthetic Peptides. Each peptide was synthesized using a solid-phase system (Neosystem) and purified. We started with 46 citrullinated peptides from the alpha, beta, and gamma chain of human fibrinogen and used them for ELISAs in 67 mice. We tested 17 15-mers from the alpha chain of human fibrinogen (locus NP_000499) with 50–100% identity with their murine counterparts, 28 15-mers from the beta chain of human fibrinogen (locus NP_005132) with 70–100% identity with their murine counterparts, and one 15-mer from the gamma chain of human fibrinogen (locus NP_068656.2) with 100% identity with its murine counterpart. Peptides 4C, 5C, 6C, and 8C from the beta chain of human fibrinogen were the targets of all of the anticitrullinated fibrinogen peptide antibody responses. They are 93–100% identical to their murine counterpart. They encompass residues 420–479 of the beta chain of human fibrinogen (locus NP_005132). The arginine version of these four peptides was used to confirm citrullinated peptide specificity of anticitrullinated fibrinogen antibodies.

Detection of Anticitrullinated Peptide Antibodies. Sera from primed mice were obtained at days 55 and 65 and tested for anticitrullinated peptide antibodies by ELISA. Plates were coated with human fibrinogen peptides under citrullinated and native forms. After blocking, sera from primed mice were incubated with peptides. After washing, peroxidase-conjugated antimurine IgG was added. The OD was read at 405 nm. Background OD was obtained by adding each serum to a well without peptide. Positive sera were defined as an OD value higher than twice the background OD.

T Cell Proliferation Assays. Spleen and lymph nodes were obtained at day 65 postimmunization and used in antigen-specific proliferation assays. T cell responses to PADs, native or citrullinated fibrinogen, or PHA were evaluated using the colorimetric bromodeoxyruridine kit (Roche Diagnostics) (31). Background OD was obtained for cells cultured without antigen. Positive responses were defined as an OD value higher than twice the background OD.

Statistical Analysis. Comparisons between groups were performed using Fisher's exact test.

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