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Polymorphism of the MHC class II *Eb* gene determines the protection against collagen-induced arthritis

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Abstract Collagen-induced arthritis (CIA) is an animal model of auto immune polyarthritis, sharing similarities with rheumatoid arthritis (RA). Paradoxally, susceptibility to mouse CIA is controlled by the *H2A* loci (*DQ* homologous) while RA is linked to *HLA.DR* genes (*H2E* homologous). We recently showed that the Eβ^d molecule prevents CIA development in susceptible *H2^q* mice. We addressed the question of whether *H2Eb* polymorphism will influence CIA incidence as *HLA.DRB1* polymorphism does in RA. In F_1 mice, only H2Eb^d and H2Eb^s molecules showed protection. Using recombinant B10.RDD (*Eb$^{d/b}$*) mice, we found that CIA protection was mediated by the first domain of the Eβ^d molecule. Using peptides covering the third hypervariable region of the Eβ chain, we found a perfect correlation between presentation of Eβ peptides by the H2A^q molecule and protection on CIA. Therefore, the mechanism by which *H2Eb* protects against CIA seems to rely on the affinity of Eβ peptides for the H2A^q molecule.

Introduction

Collagen-induced arthritis (CIA) is an animal model of autoimmune inflammatory polyarthritis induced by injection of heterologous type II collagen (CII) emulsified in complete Freund's adjuvant [(CFA) (Trentham 1982)]. CIA bears many similarities with rheumatoid arthritis [(RA) (Trentham 1982; Holmdahl et al. 1989)]. Like RA, susceptibility to arthritis in mice is influenced by genes within the major histocompatibility complex (MHC), *H2*, and it is restricted to haplotypes *H2^q* and *H2^r* (Holmdahl et al. 1989; Wooley et al. 1985). Using H2 recombinant strains, susceptibility in the *H2^q* haplotype was narrowed down to the class II molecule encoded by *H2A* loci (Holmdahl et al. 1989; Gustafsson et al. 1990; Wooley et al. 1981). Brunsberg and co-workers (1994) have elegantly demonstrated that introduction of an *H2Ab^q* transgene in *H2^p* mice is sufficient to confer CIA susceptibility.

The mouse class II molecules are encoded by *Aa* and *Ab* genes for the heavy and light chains of the A molecule, and *Ea* and *Eb* for the heavy and light chains of the E molecule (Hood et al. 1983). While the A molecule is expressed in all mouse haplotypes studied, the E molecule is not (Begovich et al. 1990; Donovan et al. 1989). Four haplotypes of inbred mouse strains b, s, q, and f, as well as 19 of 33 mouse strains carrying wildtype MHC haplotypes do not express E molecules on the surface. Both Eα and Eβ chains are not synthesized in mice of H2^f and H2^q haplotypes. Mice of the H2^b and *H2^s* haplotypes do not make Eα chain but produce Eβ molecules which remain in the cytoplasm as partially glycosilated precursors (Jones et al. 1981; Mathis et al. 1983; Nizetic et al. 1984).

Recently, we showed that the introduction of an *H2Eb^d* transgene into CIA-susceptible B10.RQB3 (*H2A^q*) mice re-established functional expression of the H2E molecule and caused a dramatic reduction in the incidence and severity of arthritis (Gonzalez-Gay et al. 1994). The *Eb* genes are homologous to human *DRB1* genes which are associated with RA susceptibility (Ollier and Thomson 1992). Since *DRB1* polymorphism is thought to be important in RA susceptibility, we generated F_1 mice between CIA susceptible B10.RQB3 and strains carrying *H2$^{b, d, k, p, s}$* haplotypes to investigate the role of the *EB* polymorphism in protecting CIA. The role of the third hypervariable (HV3) region of the H2Eβ molecule in CIA protection was examined using synthetic peptides in a T-cell proliferation assay.

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Table 1 MHC class II haplotypes of the mice used in this study

Mice	Haplotypes			
B10.RQB3	$Ab^{q/q}$	$Aa^{q/q}$	$Eb^{o/o}$	$Ea^{k/k}$
B10.RQB3.Eb^{d+}	$Ab^{q/q}$	$Aa^{q/q}$	$Eb^{d/o}$	$Ea^{k/k}$
B10.RQB3 × B10	$Ab^{q/b}$	$Aa^{q/b}$	$Eb^{o/b}$	$Ea^{k/o}$
B10.RQB3 × B10.P	$Ab^{q/p}$	$Aa^{q/p}$	$Eb^{o/p}$	$Ea^{k/p}$
B10.RQB3 × B10.A	$Ab^{q/k}$	$Aa^{q/k}$	$Eb^{o/k}$	$Ea^{k/k}$
B10.RQB3 × B10.S	$Ab^{q/s}$	$Aa^{q/s}$	$Eb^{o/s}$	$Ea^{k/o}$
B10.RQB3 × B10.D2	$Ab^{q/d}$	$Aq^{q/d}$	$Eb^{o/d}$	$Ea^{k/d}$
B10.RQB3 × B10.RDD	$Ab^{q/d}$	$Aa^{q/d}$	$Eb^{o/d/b}$	$Ea^{k/o}$

Materials and methods

Mice

All the mice used in this study were bred and maintained in our pathogen-free mouse colony.

Generation of B10.RQB3-F1 mice

CIA-susceptible B10.RQB3 ($H2^q$) mice were mated with the following strains of mice: B10.RDD ($H2^{d3}$), B10.D2 ($H2^d$), B10.S ($H2^s$), B10.P ($H2^p$), B10.A ($H2^a$), and B10 ($H2^b$; Table 1). B10.RQB3-Eb^d transgenic mice were generated as previously described (Gonzalez-Gay et al. 1994).

Flow cytometry

Analysis of the Eβ expression in peripheral blood lymphocytes (PBL) in the F_1 mice was performed as previously described (Gonzalez-Gay et al. 1994), using H2E^d-specific monoclonal antibody (mAb) 34-1-4S (Ozato et al. 1982) or H2E$^{b,\ k,\ r,\ s}$-specific mAb Y-17 (Lerner et al. 1980). Comparative expression of the H2E molecule in B10.RQB3 and F_1 mice was analyzed by Eα-specific 14-4-4S mAb [(Ozato et al. 1980) (Figure 1)].

Collagen

Purified cattle CII (BII) was prepared as previously described (Wooley et al. 1985).

Induction and quantification of arthritis

BII was dissolved in 0.1 M acetic acid at a concentration of 2 mg/ml and then emulsified with an equal volume of CFA (Mycobacterium Tuberculosis, strain H37 Ra; Difco Laboratories, Detroit, MI). One hundred μg of cold emulsion was injected intradermally into the base of the tail of 8-to-12 week-old animals. Mice were monitored three times a week from the third to the twelfth week postimmunization for the onset and development of CIA. The percent incidence of CIA remained unchanged after 12 weeks. The arthritic severity of all four limbs was determined as previously described (Wooley et al. 1981) using a grading system for each paw based on a scale of 0 to 3 as follows: 0 = normal, 1 = redness and swelling in paws or toes, 2 = deformity in paw, and 3 = ankylosis in the affected joint. The clinical score from each limb was summed, thus giving a severity range of 0–12 per mouse and the mean arthritic severity was determined using arthritic animals only.

Measurement of serum CII-specific antibody

Sera from experimental mice were tested at 1 : 100 and 1 :400 dilutions using a standard ELISA for CII-specific antibody as previously described (Gonzalez-Gay et al. 1994).

Peptide synthesis

Peptides covering the sequences 65–79 of the third hypervariable (HV3) region of H2Eβ^d, H2Eβ^s, H2Eβ^d, H2Eβ^k, and H2Eβ^p chains were synthesized at the Peptide Core Facility, Mayo Medical School, using methods as previously described (Krco et al. 1992).

The sequences of the synthesized peptides are:

Eβ$^{b,\ k}$ 65–79:
Pro-Glu-Phe-Leu-Glu-Gln-Lys-Arg-Ala-Glu-Val-Asp-Thr-Val-Cys
Eβ^d 65–79:
Pro-Glu-Ile-Leu-Glu-Asp-Ala-Arg-Ala-Ser-Val-Asp-Thr-Tyr-Cys
Eβ^s 65–79:
Pro-Glu-Phe-Leu-Glu-Gln-Arg-Arg-Ala-Ala-Val-Asp-Thr-Tyr-Cys
Eβ^p 65–79:
Pro-Glu-Leu-Leu-Glu-Arg-Arg-Arg-Ala-Glu-Val-Asp-Thr-Val-Cys

T-cell proliferation assay

T-cell proliferation assays using lymph node cells (LNC) from B10.RQB3 ($H2^{qb3}$) mice were performed as described previously (Krco et al. 1992). Incorporation of [^{3}H]thymidine was determined

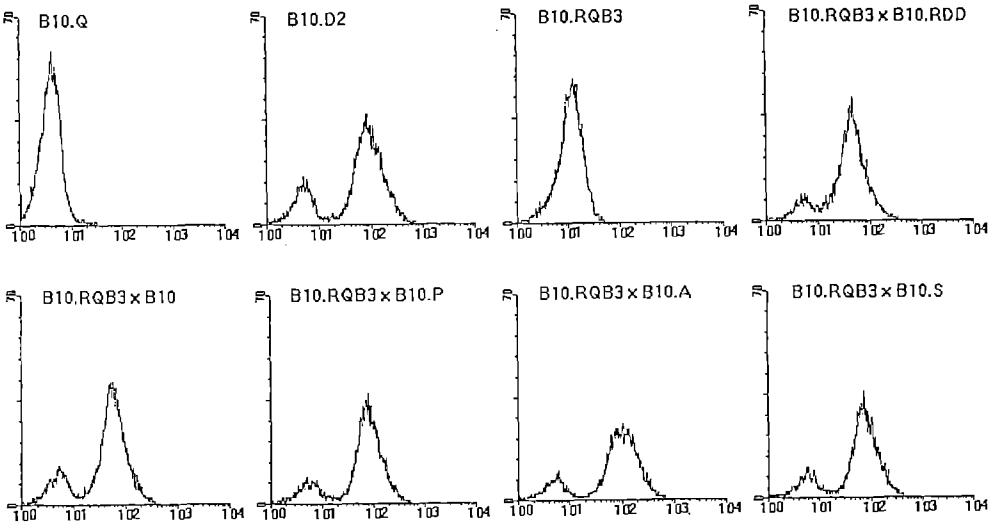

Fig. 1 Restored expression of H2E molecules in B10.RQB3-F_1 mice measured by Eα-specific 14-4-4S mAb

Table 2 Effect of *Eb* polymorphism on CIA in F_1 mice

Mice[a]	Incidence[b]	%	Onset (day) mean ± SD	Score[c] mean ± SD
B10.RQB3	26/33[d, e]	79	45±15	4.5±2.4
B10.RQB3 × B10	20/32	63	42±14	3.6±1.7
B10.RQB3 × B10.P	8/14	57	46±13	2.0±1.1
B10.RQB3 × B10.A	7/13	54	45±2	2.3±1.1
B10.RQB3 × B10.S	13/35[d]	37	53±11	2.7±2.0
B10.RQB3 × B10.D2	4/15[d]	27	47±13	1.8±1.0
B10.RQB3 × B10.RDD	7/32[e]	22	52±15	1.3±0.5
B10.RQB3-Eb^{d+}	2/12[e]	17	52±5	1.5±0.7

[a] Mice were immunized with 100 μg. BII in CFA on day 0 and monitored regularly for the onset and development of arthritis
[b] Final score determined at 12 weeks post-immunization
[c] The mean severity of the arthritis was calculated using arthritic animals only
[d] $P < 0.005$
[e] $P < 0.001$

by liquid scintillation counting. Results are expressed in Δcpm (mean cpm in experimental wells – mean cpm in the control wells).

Statistical analysis

Arthritis incidence between groups was analyzed using χ^2 test with Yates' correction. Antibody levels were compared using the Student's t-test.

Results

Production of mice expressing functional Eβd,b,p,k,s molecules

Unlike mice carrying the standard *H2^q* haplotype (B10.Q, DBA/1), recombinant B10.RQB3 mice carry *K^{q}Aa^qAb^q-Eb^{o}Ea^{k}D^b* genes. Thus, these animals express the CIA-susceptible *A^q* loci but, because of a nonfunctional *Eb^o* gene, do not express an intact E molecule on the cell surface despite synthesis of an Eα molecule. When B10.RQB3 mice are mated to any strain with a viable *Eb* gene, an intact and functional E molecule is expressed on the cell surface. We mated B10.RQB3 mice with B10.D2, B10, B10.P, B10.A, and B10.S animals to generate offspring which express Eβd,b,p,k,s molecules (Table 1). As seen in Figure 1, all F_1 animals expressed a viable E molecule on the cell surface.

H2Eb polymorphism determines protection in collagen-induced arthritis

The F_1 mice were monitored for the development of CIA and at 12 weeks post immunization the incidence of arthritis was determined. Table 2 shows that expression of the Eβ^d molecule significantly inhibited the incidence of CIA in (B10.RQB3 × B10.D2)F_1 mice ($P < 0.005$). Also, F_1 animals expressing the Eβ^s molecule developed a lower incidence of CIA versus parental B10.RQB3 mice ($P < 0.005$), while arthritis incidence in F_1 mice expressing Eβ^p, Eβ^k, or Eβ^b molecules were similar to the B10.RQB3 controls (Table 2). In addition to the decrease in frequency of CIA, arthritic Eβ^d-positive mice showed a marked reduction in the severity of the disease. Moreover, despite a reduced incidence of CIA, the mean arthritis score in Eβ^s mice was essentially similar to mice bearing the Eβ^p or Eβ^k molecules. Analysis of sera at day 35 post immunization showed a 36% reduction in mean O.D. of BII reactive antibodies in (B10.RQB3 × B10.D2)F_1 mice compared with B10.RQB3 mice, as well as a significant decrease in reactivity against homologous mouse CII [(MII) ($P < 0.02$, Table 3)]. In *Eb^s* mice, decreased titers were only observed for MII antibodies. These results show that the Eβ^d molecule confers the greatest protection against CIA, followed by Eβ^s with intermediate effect, and the remaining Eβ molecules with negligible effect. Previous studies have shown that H2E-negative F_1 animals generated between B10.Q and B10 (*H2A^b*), B10.S (*H2A^s*), B10.A(4R) (*H2A^k*), and B10.GD (*H2A^d*) mice remain CIA-susceptible, indicating that the non-H2A^q molecules or other *H2* genes from these strains do not protect *H2A^q* mice against CIA (C. S. David, unpublished data). As expected, the Vβ T-cell repertoire in these mice reflected the presence of a functional H2E molecule in all the F_1 mice [(Bill et al. 1989; Woodland et al. 1990) (data not shown)]. Therefore, deletion of specific Vβ-bearing T cells is not involved in this protection.

Table 3 Measurement of serum CII-specific antibodies in F_1 mice

Mice[a]	Anti-MII mean±SD	Anti-BII mean ± SD
B10.RQB3	0.15±0.06[b]	0.25±0.13
B10.RQB3 × B10	0.17±0.06	0.23±0.08
B10.RQB3 × B10.P	0.12±0.04	0.25±0.09
B10.RQB3 × B10.A	0.14±0.06	0.22±0.10
B10.RQB3 × B10.S	0.09±0.04[b]	0.24±0.07
B10.RQB3 × B10.D2	0.08±0.04[b]	0.16±0.10
B10.RQB3 × B10.RDD	0.10±0.06[b]	0.17±0.09
B10.RQB3-Eb^{d+}	0.09±0.03[b]	0.18±0.07

[a] Mice were bled at 5 weeks post immunization and the level of antibody against BII and MII determined by an ELISA. Data is presented as the mean OD at a 1:100 dilution
[b] $P < 0.02$

Mapping of CIA protection to the first domain of the Eβ molecule

The B10.RDD strain is a recombinant mouse carrying the *H2* haplotype *K^{d}Aa^dAb^dEb$^{d/b}$Ea^{o}D^d*. The class II region in B10.RDD was derived from the B10.GD mouse which bears a recombination between the second and the third exon of the *Eb* gene (Kobori et al. 1986). Thus, B10.RDD mice possess a hybrid *Eb$^{d/b}$* gene in which the first domain is derived from the *d* haplotype. When (B10.RQB3 × B10.RDD)F_1 mice were immunized with BII, the incidence of CIA was extremely low (7/32, $P < 0.001$) versus parental B10.RQB3 and (B10.RQB3 × B10)F_1 mice

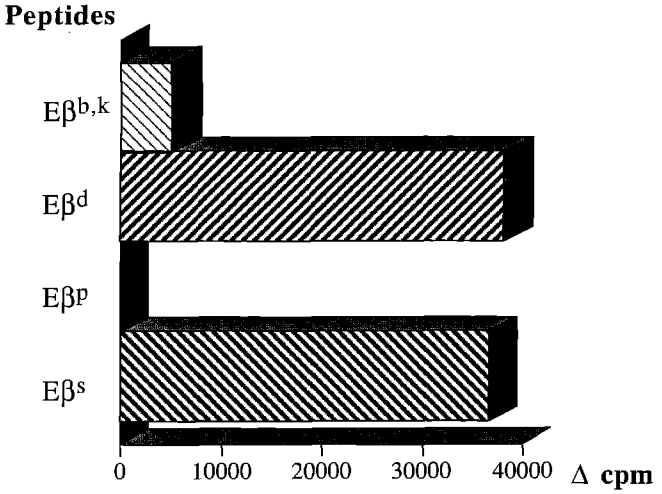

Fig. 2 B10.RQB3 mice were responsive to Eβ^{d} and Eβ^{s} peptides (65–79) but failed to mount a proliferative response to Eβ^{p} and Eβ^{b,k} peptides (65–79). For each peptide, two animals were immunized with 200 μg emulsified in CFA. LNC were cultured in vitro without peptide (negative control: mean cpm <5000), with Concavalin A (mean cpm >100000) or with peptide (100 μg/ml). Results are expressed in Δcpm (mean cpm in experimental wells – mean cpm in control wells). Values shown in the Figure are Δcpm of one representative experiment

(P <0.005; Table 2). The severity of CIA in the seven (B10.RQB3 × B10.RDD)F$_1$ mice which developed arthritis was considerably reduced (arthritic scores ranging from 1 to 2). Furthermore, a 32% reduction of BII-reactive antibodies and a 33% reduction of MII-reactive antibodies was detected compared with control B10.RQB3 sera (P <0.02). These results demonstrate that *H2* genes distal to the *Eb* locus play no role in CIA protection and strongly suggest that the protective effect on CIA is mediated by determinants within the first domain of the Eβ^{d} molecule.

Correlation between presentation of HV3 peptides and protection against arthritis

Our results stressed the importance of the polymorphism of the first domain of the Eβ chain in CIA protection. This is strongly reminiscent of the situation encountered in humans where the HV3 region of *DRB1* alleles confer susceptibility to RA (Winchester et al. 1992). Moreover, it is already known that, at least in mice, this HV3 region can constitute an antigenic determinant, as Roudier and co-workers (1991) have shown for the Eβ^{s} peptide (65–79) in *H2A^{d}*, H2A^{k}, and *H2A^{s}* animals. We looked at the proliferative response of T cells from B10.RQB3 animals against HV3 peptides (65–79) from Eβ^{b,k}, Eβ^{d}, Eβ^{p}, and Eβ^{s} chains. Interestingly, LNC from B10.RQB3 mice mounted a strong proliferative response to Eβ^{d} and Eβ^{s} (65–79) peptides (38182 and 36451 Δcpm, respectively), while Eβ^{p} and Eβ^{b,k} (65–79) peptides were unable to induce proliferation [(0 and 5147 Δcpm, respectively) (Fig. 2)]. Thus, in vitro proliferation to HV3 region peptides correlates with protection to CIA in mice.

Discussion

In this paper, using F$_1$ offspring from crosses between B10.RQB3 and *H2*-congenic B10 strains bearing various *H2Eb* genes, we showed that the protective role of the Eβ molecule is modulated by the polymorphic sequences of the *Eb* genes. Indeed, the *H2Eb* polymorphism defined a hierarchy in the level of CIA protection where the Eβ^{d} molecule confers the greatest protection, followed by Eβ^{s} with a clear effect on incidence, but not severity, and Eβ^{b,k,p} molecules with minimal influence. Also, through the use of B10.RDD mice bearing a recombination within the *Eb* gene, we eliminated any contribution of genes distal to *Eb* in the experimental results. Previous studies involving F$_1$'s lack of expression of H2E molecules ruled out contribution of other *H2*-linked genes. Therefore, unlike other experimental models of autoimmune diseases (Lund et al. 1990; Merino et al. 1992, 1993; Nishimoto et al. 1987; Podolin et al. 1993; Uehira et al. 1989), we clearly showed that one copy of the *Eb* gene is sufficient to confer protection (Table 2). To date, only in the lupus model of (NZB × NZW)F$_1$ mice have similar results been described (Hirose et al. 1994).

In RA, the disease-conferring element has been narrowed to the stretch of amino acids 67–74 of several *HLA-DRB1* alleles (Gregersen et al. 1987; Winchester et al. 1992). Differences among DR4 subtypes such as *Dw4* and *Dw14*, with respect to *Dw10*, are located at positions 67, 70, and 71 of the third hypervariable region (HV3) of the *DRB1* gene, which are of major importance in T-cell recognition (Nepom and Erlich 1991; Reinsmoen and Batch 1990). This finding has led to the generally admitted "shared epitope" hypothesis (Winchester et al. 1992). According to this hypothesis, a large proportion of RA patients carry *Dw1, Dw4, Dw14, Dw15, Dw16*, or *DR10* haplotypes which bear similar sequences in the HV3 region of the *HLA-DRB1* gene (Ollier and Thomson 1992). However, the biological significance of the shared epitope remains unknown.

Because the shared epitope is carried by different HLA-DR molecules, it is very unlikely that this motif is simply modulating the binding of an autoantigenic determinant in the groove of HLA-DR molecules. On the other hand, it is known that the HV3 Eβ^{s} peptide (65–79) is an antigenic determinant that binds to H2A^{d}, H2A^{k}, and H2A^{s} molecules (Roudier et al. 1991). On the basis of these findings, we speculated that the CIA protection mediated by Eβ^{d} and Eβ^{s} molecules might be due to the presentation of HV3 peptides from the H2Eβ chain by the H2A^{q} molecule. To investigate this possibility, T cells from B10.RQB3 animals were tested by T-cell proliferation assay using HV3 peptides (65–79) of H2Eβ^{b,d,k,p,s} molecules. As shown in Figure 2, it is striking that a perfect correlation exists between the binding and presentation of an HV3 Eβ peptide (65–79) to H2A^{q} molecule and the low incidence of arthritis mediated by its corresponding Eβ chain.

It has been a paradox as to why the susceptibility to CIA in mice maps to the *H2Ab* locus, which is homologous to the human *HLA-DQ* genes, while RA is associated with the

DRB1 alleles in humans, which are analogous to the mouse *Eb* genes. The observation that certain Eβ molecules can play a protective role in CIA suggests that the role of the HLA-DRβ1 molecules may also be one of protection in human RA. We hypothesize that while most DRβ1 molecules protect RA in humans, certain alleles such as *DR4.Dw4, DR4.Dw14*, and *DR1.Dw1* fail to do so. Since protection is dominant, it is understandable why homozygosity for the above non-protective alleles increases the incidence of RA in humans (Weyand et al. 1992). Thus, the human class II molecule which may be involved in presentation of an arthritogenic epitope could possibly be HLA-DQ, which is similar to the mouse H2A molecule. For instance, in *Dw4* haplotypes, *DQw7* or *DQw8* are prime candidates due to the linkage disequilibrum in the human MHC class II region. Because protection by DR molecules could be dominant over susceptibility, linkage studies would show association with nonprotective *DR* alleles and not with susceptible *DQ* alleles. In agreement with our hypothesis, Salvat and co-workers (1994) have recently showed that, while T cells from normal individuals carrying the *HLA-Dw2* subtype mount a proliferative response against Dw4 HV3 peptide, T cells from individuals bearing *Dw4/Dw2* haplotypes did not proliferate in response to the same peptide. Therefore, in both mice and humans, predisposition to arthritis seem to correlate with the inefficiency of HV3 peptides from H2Eβ or HLA-DR chains to bind to H2A or HLA-DQ molecules, respectively. At this point, two possible mechanisms may explain this protective effect. One is that self-MHC peptides present in the groove of H2A or HLA-DQ molecules are competing for binding of potentially "arthritogenic" peptide(s). The second is that H2Eβ- or DRβ1 peptides-specific autoreactive Th2-type T cells constitute a new population of "regulatory" T cells which modulate the T-cell response against self-peptides through the release of interleukins 4 and 10. Understanding this mechanism could open new avenues in the immunomodulation of RA susceptibility and generation of peptide-based vaccines.

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