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Immune responses to peptides containing homocitrulline or citrulline in the DR4-transgenic mouse model of rheumatoid arthritis

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ABSTRACT

Antibodies to proteins/peptides containing citrulline are hallmarks of Rheumatoid Arthritis (RA). These antibodies are strongly associated with the expression of the Shared Epitope (SE). RA patients also generate antibodies to homocitrulline-containing proteins/peptides (also referred to as anti-carbamylated protein antibodies (Anti-CarP)). This study was undertaken to investigate the relationship between homocitrulline and citrulline immune responses using an established mouse model of RA: DR4-transgenic (DR4tg) mice that express the human SE. C57BL/6 (B6) and DR4tg (on a B6 background) mice were immunized subcutaneously with a homocitrullinated peptide (HomoCitJED). Splenic T cell proliferation was evaluated by ^{3}H-thymidine incorporation assay. Antibodies to homocitrullinated and citrullinated antigens were screened by enzyme-linked immunosorbent assay (ELISA). Antibody cross-reactivity was examined by inhibition with HomoCitJED and its citrullinated counterpart peptide, CitJED (the number of homocitrullines in HomoCitJED is equal to the number of citrullines in CitJED). HomoCitJED-immunized DR4tg mice developed early T and B cell responses to HomoCitJED and late responses to CitJED. These mice also developed anti-CCP2 antibodies. In some mice, antibodies to HomoCitJED were also reactive to CitJED. B6 mice immunized with HomoCitJED developed late T and B cell responses to HomoCitJED, but did not generate responses to citrullinated antigens. Unlike DR4tg mice, anti-HomoCitJED antibodies from B6 mice did not react to CitJED. In conclusion, DR4tg mice immunized with HomoCitJED developed immune responses to CitJED, indicating cross-reactivity. CitJED immune responses were dependent on the SE. HomoCitJED responses occurred in the absence of the SE (B6 mice); however, they developed earlier in DR4tg SE-expressing mice.

1. Introduction

RA is characterized by the production of autoantibodies that target citrullinated proteins/peptides (ACPA). Clinically, the most commonly measured ACPA is anti-cyclic citrullinated peptide antibodies (anti-CCP2), which are highly specific for RA [1,2] and predictive of erosive joint disease [3,4]. The susceptibility to ACPA-positive RA is significantly higher in individuals expressing HLA alleles [5,6] that encode a consensus sequence called the Shared Epitope (SE) [7]. The SE binds to peptides containing citrulline with high affinity initiating citrulline-specific T and B cell responses [8,9]. Target antigens for these immune responses are present in the joint, including citrullinated fibrinogen [10], α-enolase [11,12], vimentin [13,14] and collagen II [15]. Evidence suggests that ACPA bind to these targets in the joint leading to complement activation and chronic inflammation [16]. Experiments done in animal models further support the notion that T and B cell citrulline-specific immune responses are pathogenic [17–20].

It is known that some ACPA also bind peptides or proteins containing homocitrulline [21–25], an amino acid that is structurally related to citrulline and is also present in RA synovium [26,27]. Antibodies to homocitrullinated antigens (also referred to as anti-carbamylated antibodies (anti-CarP)) have been detected in the sera of RA patients [21,28–31] and are associated with more severe disease [28,32]. *In silico* evidence suggests that homocitrullinated peptides can be accommodated within the binding

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groove of the SE [21]. However, the relationship between the SE and homocitrullinated peptides remains unclear. When immunized with homocitrullinated filaggrin-derived peptides and given an intra-articular injection of citrullinated peptide, different strains of mice developed arthritis with varying frequency, suggesting that the MHC-H2 haplotype influences the development of arthritis [33]. The objective of this study was to examine the role of the SE in the development of immune responses to a peptide containing homocitrulline using a humanized SE-expressing DR4-transgenic mouse model of RA.

2. Materials and methods

2.1. Antigens

The antigens used in this study were Citrullinated JED (CitJED) [21,25,34,35], Homocitrullinated JED (HomoCitJED), Arginine JED (ArgJED), and Lysine JED (LysJED) which are synthetic cyclic peptides containing 18 amino acids with 9 residues of citrulline, homocitrulline, arginine, and lysine respectively on the same peptide backbone [25]. CitJED and HomoCitJED were synthesized by Creative Peptides (Shirley, NY, USA) and ArgJED and LysJED were synthesized by Toronto Sick Kids Hospital (Toronto, ON, Canada). Unmodified human fibrinogen (VWR), citrullinated fibrinogen, and homocitrullinated fibrinogen were also used for the antibody assays. Citrullination of fibrinogen was previously described by Hill et al. [36] and homocitrullination was performed as per the method of Scinocca et al. [21].

2.2. Mice and immunizations

DR4-IE transgenic mice deficient in endogenous MHC class II on the C57Bl/6 background (referred to as DR4tg) [17,37] were bred in house. C57Bl/6 mice (B6) mice were purchased from The Jackson Laboratory (Maine, USA). Both strains of mice were housed in pathogen-free conditions at the Animal Care and Veterinary Services barrier facility at the University of Western Ontario as per the Canadian Council on Animal Care guidelines. The study was approved by the Animal Care and Use Committee (The University of Western Ontario, London ON, Canada). Female and male DR4tg and B6 mice were immunized and boosted 21 days later with 100 μg of HomoCitJED suspended in PBS or PBS alone using the method of Hill et al. [17].

2.3. Splenocyte proliferation

The splenocyte proliferation assay was adopted from Hill et al. [17]. In brief, splenocytes were harvested from mice sacrificed at various time points and cultured in complete RPMI media (Gibco) at a concentration of 4×10^5 splenocytes/well. Splenocytes were treated with 100 μg/mL of CitJED, HomoCitJED, or with controls (ArgJED, LysJED or no peptide) and incubated for 54 h at 37 °C, 5% CO_2. One μCi of ^{3}H-thymidine was added to each well and incubated for an additional 18 h. Splenocytes were harvested using a Harvester96 (Tomtec) and radioactivity was measured with a MicroBeta JET (Perkin Elmer). Proliferation experiments were conducted at least in quadruplicate and replicate counts per minute (cpm) were averaged (<20% intra-assay variation). Proliferative responses are reported as a Stimulation Index (SI; cpm of samples with peptide/cpm of samples with media alone) +/− standard error of the mean. A cut-off value of 2.0 was considered a positive proliferative response.

2.4. Antibody assays

Sera from mice at various time points were screened for IgG anti-HomoCitJED, anti-CitJED, anti-citrullinated fibrinogen (anti-CitFib), anti-homocitrullinated fibrinogen (anti-HomoCitFib), anti-fibrinogen (anti-Fib) and anti-cyclic citrullinated peptide (anti-CCP2) antibodies by direct antibody-binding ELISA. HomoCitJED and CitJED were dissolved in 15% HCl in sterile distilled water. The 15% HCl did not alter the structure of CitJED or its binding properties (data available upon request). The protocol for anti-HomoCitJED and anti-CitJED direct antibody-binding ELISA was similar to Hill et al. [36] with the following modifications: wells were coated with 20 μg/mL of HomoCitJED, 40 μg/mL of CitJED, or their controls (ArgJED and LysJED) at 20 μg/mL in carbonate coating buffer, and 100 μL/well of biotin-conjugated goat anti-mouse IgG (1:5000; Jackson) with streptavidin horseradish peroxidase polymer (1:4000; Abcam) were used for antibody detection. The ELISA protocol for anti-CitFib, anti-HomoCitFib and anti-Fib was also adapted from Hill et al. [36] as described above, but 10 μg/mL of CitFib, HomoCitFib and Fib in carbonate coating buffer were used instead of the peptides. These ELISAs were performed at least twice for the majority of serum samples with the average optical density (OD) being reported (<20% inter-assay variation). The detection of anti-CCP2 antibodies in mouse sera was performed using an anti-CCP2 ELISA kit (Euroimmun). The manufacturer's protocol was followed with the exception that peroxidase-conjugated rabbit anti-mouse IgG (1:5000, Dako) was used. The OD was determined at 450 nm. The cut-off value for anti-CCP2 was 0.44 OD, equal to two standard deviations above the mean value for PBS-immunized B6 mice. The cut-off values for the other antibodies were 0.1 OD, which is the lower detection limit of the ELISA (the mean values for these antibodies in PBS immunized mice were below 0.1).

The cross-reactivity of IgG anti-HomoCitJED, anti-CitJED, and anti-CCP2 antibodies was examined by inhibition ELISA. The protocol was adapted from Scinocca et al. [21] with the following plate-bound antigens: CitJED, HomoCitJED and CCP2. Inhibitions were performed with soluble CitJED and HomoCitJED at various concentrations (2−100 μg/mL) or with ArgJED and LysJED at 100 μg/mL. The average percent inhibition of two repeat determinations is reported, except for CCP2 that was performed once due to insufficient quantity of sera. A minimum OD of 0.5 by direct antibody-binding ELISA was required to obtain a dose-dependent, reproducible inhibition.

2.5. Statistical analysis

Graphpad Prism 6.0 software was used for data analysis. The Mann-Whitney *U* test was used to compare SI or OD between DR4tg and B6 mice at the indicated time points ($p < 0.05$ was considered significant). Two-way ANOVA with Bonferonni correction was used for statistical analysis of inhibition experiments ($p < 0.01$ was considered significant).

3. Results

3.1. Splenic T cell proliferative responses

Splenocytes from DR4tg and B6 mice were assessed for proliferative responses to HomoCitJED and CitJED peptide by ^{3}H-thymidine incorporation assay. In DR4tg mice, HomoCitJED-specific splenocyte proliferative responses were observed as early as day 10 in 3/10 (30%) of mice post-immunization with HomoCitJED. These responses increased at later time points with 9/11 (82%) of mice having a significant proliferative response to HomoCitJED at day 100 (Fig. 1A). We also monitored DR4tg mice for

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