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Towards a molecular basis for the association of HLA, Rheumatoid Arthritis and Autoantibodies

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CHAPTER 1

Introduction

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HLA and rheumatoid arthritis: How do they connect?
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THE ADAPTIVE IMMUNE SYSTEM

During life, every day, we encounter many different micro-organisms including potentially pathogenic bacteria, viruses, fungi and parasites. The immune system is critically important in the defence against these pathogens.

The immune system has many different components. These include a wide variety of immune cells, but also non-cellular effector mechanisms. The immune system can be roughly subdivided in two components: the innate and the adaptive immune system. Upon primary infection, the innate immune system is the first to act. The response is generally fast and unspecific. After a few days the adaptive immune system comes into play. This response is highly potent and extremely specific. In addition, after clearance of the pathogen, the adaptive immune system establishes memory resulting in a highly potent and fast specific immune response upon secondary infection.

The large specificity of the adaptive immune system is mainly established by two cell typed, the B and T cells. These lymphocytes express B or T cell receptors, highly specific receptors with a very large diversity. B and T cells differ in the way they recognize their antigen. B cells recognize their antigen in its native form. Antigen-recognition combined with different activation signals allows for B cell activation and their differentiation in antibody secreting plasma cells. T cells can provide these activation signals and are therefore critically important for the generation of potent antibody responses. In addition. T cells also have other effector functions including killing of infected cells and the production of inflammatory cytokines. In contrast to B cells that recognize native antigens, T cells only recognize processed antigens presented by antigen presenting molecules, predominantly by human leukocyte antigens (HLA) [1].

Due to the potency and specificity of adaptive immune response, B and T cells have to be strongly educated to distinguish self from non-self. Nevertheless, autoimmune diseases (AID), diseases characterized by a failure of the immune response to distinguish self from non-self, are highly prevalent in the western world. In the United States, the prevalence was estimated between 5-8% of the population [2]. B and T cells are implicated in the development of most of these AID.

HUMAN LEUKOCYTE ANTIGENS

HLA molecules present processed antigens to T lymphocytes. In the presence of foreign proteins, e.g. during infection, foreign peptides are presented by HLA molecules. Recognition of such HLA-peptide complexes by T cells results in T cell activation and its accompanying effector mechanisms. HLA molecules can be subdivided in two subclasses: HLA class I and class II molecules. HLA class I molecules present peptides derived from an intracellular source. T lymphocytes that are

specific for antigens presented by HLA class I molecules are distinguished by the expression of CD8, hence their name CD8+ T cells. HLA class II molecules present peptides derived from an extracellular source. T lymphocytes that are specific for HLA class II molecules express CD4, hence their name CD4+ T cells. The classical HLA class II molecules are named HLA-DQ, HLA-DR and HLA-DP and are composed of an alpha and a beta chain. Peptides are presented at a site of interaction between these two chains [3].

The genes encoding for HLA molecules are located in a region in chromosome 6 (Figure 1). The region has high linkage disequilibrium (LD) and the genes are highly polymorphic, resulting in a large variety of HLA molecules. Polymorphic residues are mainly located at sites important for antigen presentation resulting in large differences in peptide repertoire between different HLA molecules [4, 5].

Expression

HLA class I and HLA class II molecules differ in their expression pattern. HLA class I molecules are expressed by nearly all nucleated cells. In contrast, the expression of HLA class II molecules is more tightly regulated. Under physiological conditions, HLA class II molecules are predominantly expressed by professional antigen-presenting cells, including B lymphocytes, dendritic cells and macrophages. In inflammatory conditions, including in AID, other tissue cell types can also present HLA class II molecules [6].

Tolerance and autoimmunity

T cell are highly specific and potent. Therefore, it is very important to prevent their activation in response to processed self-antigens. In the thymus, precursor T cells are educated to distinguish self from non-self [7]. HLA molecules play a very important in role this process. Nevertheless, the HLA locus is the most important risk factor for the development of most AID [8]. Understanding the role of HLA molecules in the development of AID will likely offer very important insights in the AID pathogenesis.

RHEUMATOID ARTHRITIS

Rheumatoid arthritis (RA) is a chronic systemic AID characterized by extensive inflammation of the synovial joints. About 1% of the western population is affected by this disease resulting in a large disease burden. In the past decades seminal work was performed on the drivers of the inflammatory process, resulting in the development of a whole range of therapeutics that have greatly enhanced the quality of life of RA patients. Despite the identification of crucial pathways and the implementation of novel therapeutics, little is known about the factors driving the initiation of RA and about why the inflammation does not spontaneously resolve

Figure 1: Schematic representation of the HLA locus. Scheme shows the different genes located in the class-I and class-II region of the HLA locus. Genes that encode classical HLA class I and class II molecules are indicated in black.

[9].

RA is considered an autoimmune disease. Autoantibodies are an important hallmark of RA and several classes of autoantibodies have been described that precede the development of RA. These autoantibodies include rheumatoid factor, anti-citrullinated protein antibodies (ACPA) and the recently identified anti-carbamylated protein antibodies [10, 11].

ACPA are of particular interest as these autoantibodies are highly specific for RA and can be found in about 50% of early RA patients. This makes ACPA an important early clinical biomarker and ACPA-status was therefore recently added to the new criteria used for the classification of RA patients [12]. In addition, ACPA+ RA patients present with a faster rate of joint destruction. ACPA can therefore also be used as a biomarker for patients with a more severe disease phenotype and could be used to select those patients eligible for a more aggressive treatment [13].

Upon comparing ACPA+ with ACPA- RA patients, it was noted that these patient-groups do not only differ in clinical phenotype but also in genetic and environmental risk factors [14, 15]. It is therefore believed that these are two distinct disease subsets with a different underlying pathogenesis.

Citrullination

In 1964, it was first shown that RA patients harbour very specific antibodies, which were called anti-perinuclear factor or anti-keratin antibodies [16-18]. In a search for synovial targets to which these autoantibodies bind, fibrinogen and vimentin were identified [19, 20]. Proteins can undergo many types of post-translational modifications (PTMs). In 1998, it was demonstrated that the described autoantibodies only target citrullinated antigens, hence their name anti-citrullinated protein antibodies [21-23]. Citrullination is a physiological process catalysed by a family of enzymes called peptidylarginine deiminases (PADI1-4). These enzymes convert the positively charged amino acid (aa) arginine to a novel uncharged aa called citrulline. This process is linked to several aspects of cell biology including apoptosis, necrosis, netosis and histone modifications. PADI enzymes require relatively high concentrations of calcium for their activity allowing citrullination of the extracellular matrix when they are released from (dying) cells [24, 25].

Several data indicate an important role for citrullination in ACPA+ RA. Genome-wide

association studies identified single nucleotide polymorphisms (SNPs) in the region encoding the PADI4 gene that are associated with an increased risk to develop ACPA+ RA[26]. Also, smoking, the most important environmental risk factor, is described to associate with an increased PADI2 expression in bronchoalveolar lavage cells and bronchial mucosal biopsy sections [27]. Finally, it was recently shown that RA patients can carry autoantibodies specifically targeting PADI-enzymes, which can enhance the activity of these enzymes allowing them to function at lower calcium concentrations enabling enhanced citrullination of the extracellular matrix when they are released from (dying) cells [28].

Pathogenicity of ACPA

Because of the clear association between the rate of joint destruction and ACPA positivity, it has been suggested that ACPA may directly contribute to synovial inflammation. ACPA producing B cells are enriched in the synovial fluid, which suggests local production of ACPA in the synovial fluid and a direct role for ACPA and/or ACPA-producing B cells in synovial inflammation [29, 30]. The number of different ACPA isotypes is a predictor of radiographic damage [31]. As different isotypes can recruit different immune-effector mechanisms, this suggests that ACPA can contribute to damage using multiple different effector pathways. For example, stimulation of osteoclast precursors, cells involved in the degradation of bone, with ACPA has been reported to result in increased osteoclastogenesis and was suggested to explain the association between ACPA and more severe joint destruction [32, 33]. This was also confirmed in mice since injecting mice with experimental arthritis with ACPA aggravates arthritis severity [34].

Many antibody effector mechanisms are implicated in the recruitment and activation of inflammatory cells. For instance, ACPA can form immune complexes with citrullinated proteins, which can activate inflammatory cells and induce the production of TNF-alpha [35, 36]. Also, it was reported that ACPA are particularly good in activating the complement system and complement-mediated recruitment of inflammatory cells could therefore be an important effector mechanism of ACPA [37-39]. These data suggest that ACPA is not merely a biomarker for a more severe disease phenotype, but could be directly involved in the inflammatory process.

The antigens targeted by ACPA

Since the identification of the molecular nature of the antigens targeted, much work has been performed on identifying potential citrullinated proteins recognized by ACPA. Unfortunately, this is hampered by the characteristics of the ACPA response. The ACPA repertoire is highly diverse as it displays reactivity against many different citrullinated proteins. This is explained both by the polyclonality of the ACPA response and by several reports that showed that ACPA molecules can directly cross-react between different citrullinated antigens [30, 40-42]. An increasing list of

citrullinated proteins identified within the synovial compartment and recognized by ACPA is now known including proteins like collagen, vimentin, fibrinogen, enolase, fibronectin, vinculin and histones [19, 43-46]. It is therefore likely that a plethora of citrullinated proteins are recognized by ACPA in the synovial compartment that could all conceivably contribute to synovial inflammation. However, due to the high degree of ACPA cross-reactivity, it is difficult to identify those citrullinated antigens that play a critical role in the initiation or development of ACPA+ RA.

Characteristics of the ACPA response

The presence of ACPA can precede RA onset by up to ten years, without any clinical signs of arthritis [47, 48]. Interestingly, by comparing the characteristics of ACPA before and after disease onset, it has become clear that the ACPA response is, on average, not the same between these subjects. In RA patients, ACPA are increased in level, use more isotypes, display a different glycosylation pattern and are more cross-reactive towards different citrullinated epitopes. Interestingly, the "maturation" of the ACPA response takes place before disease onset. Prior to the start of clinical symptoms a sharp rise in ACPA levels, isotype-usage and epitope recognition-profile is observed [49].

These findings are in line with the notion that before disease onset the ACPA-response undergoes a "second hit", which suggests a role for a matured ACPA response in the onset and the pathogenesis of ACPA+ RA. As an "immature" ACPA response can be identified in serum up to ten years before disease onset, this provides a potentially interesting therapeutic window for interventions preventing maturation of the ACPA response. In a search for such interventions, it is important to understand the progression from ACPA positivity without disease in healthy subjects to ACPA+ RA.

HLA MOLECULES IN RA

Genetic risk

The most important genetic risk factor for RA is the HLA class II locus (Figure 1). Importantly, it was shown that this locus is strongly associated with ACPA+ RA, not with ACPA- RA. It was estimated that about 20% of the genetic variance in ACPA+ RA patients is explained by SNPs in the HLA locus. All the other known loci together account for less than 5% of the genetic variance (unpublished data).

Susceptibility

In 1976, it was first shown that the presence of HLA-DRB1*04 strongly predisposes to RA [50]. The HLA-DRB1 gene is in LD with genes encoding the alpha and the beta chain of HLA-DQ (HLA-DQA1 and HLA-DQB1) and these genes are inherited together
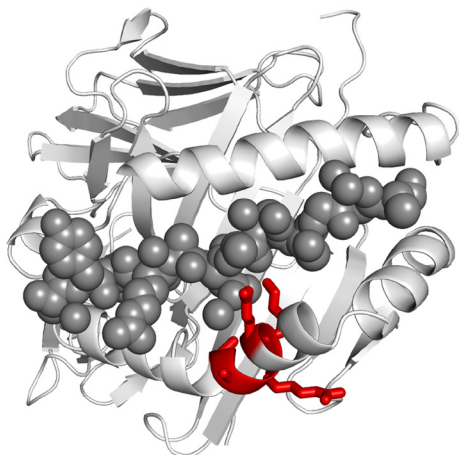
Figure 2: HLA-DR4 molecule with the SE sequence in positions 70-74. The crystal structure of HLA-DRB1*04:01/DRA1*01:01 complexed with a human collagen II-derived peptide (dark grey circles) shows the peptide binding groove created by the helical structures of the HLA-DR beta and alpha chain with the SE motive indicated (red) (Polyview-3D).

in haplotypes. In 1986, the shared epitope (SE) hypothesis was postulated [51]. This hypothesis assumes that the HLA association is explained by polymorphisms in the HLA-DRB1 chain. Upon comparing different HLA-DRB1*04 alleles it was observed that these alleles differ mostly in aa position 70-74 [52]. Haplotypes encoding predisposing HLA-DRB1 alleles share the sequences QRRAA, QKRAA and RRRAA at these positions and hence are collectively called HLA-SE alleles (Figure 2). It was postulated that this sequence directly influences peptide presentation or T cell recognition [51, 53]. Recently, the association of individual amino acids with ACPA+ RA was revisited by using a novel statistical approach leading to the formation of an "HLA-SE version 2.0" hypothesis. Variations encoding amino-acids 11 and 13 of the HLA-DRB1 chain explain, statistically, most of the genetic risk followed by "SE aa" 71 and 74. Based on these four amino acid positions, RA patients can be subdivided in 16 groups, each with their own genetic risk-profile [54]. The three groups with the highest odds ratios still harbor HLA-DR-DQ haplotypes encoding the SE alleles derived from HLA-DRB1*01, DRB1*04 and HLA-DRB1*10 alleles.

Notably, HLA-DRB1*04 positive RA patients are also HLA-DQ3 positive and it has also been proposed that the predisposing effect of HLA-DR4 can be explained by its LD with genes encoding for HLA-DQ3 [55]. The HLA-DR/HLA-DQ linkage makes it difficult to identify which of the two molecules is responsible for the association with RA. Therefore, it is still unclear whether the HLA-DQ-genes in linkage with the HLA-DR encoding SE genes can be excluded from the association with ACPA+ RA.

Protection

In addition to HLA-DRB1 alleles that contribute to RA susceptibility, other HLA-DRB1 alleles confer protection against the disease. HLA-DRB1*13 alleles are most strongly associated with protection, but only from ACPA+ RA. Interestingly, these alleles can also protect in the presence of SE alleles [56]. The dominant role of these protective alleles could suggest that these alleles work on a similar pathway. Likewise, protection can be transferred from a mother carrying a protective allele to a child lacking
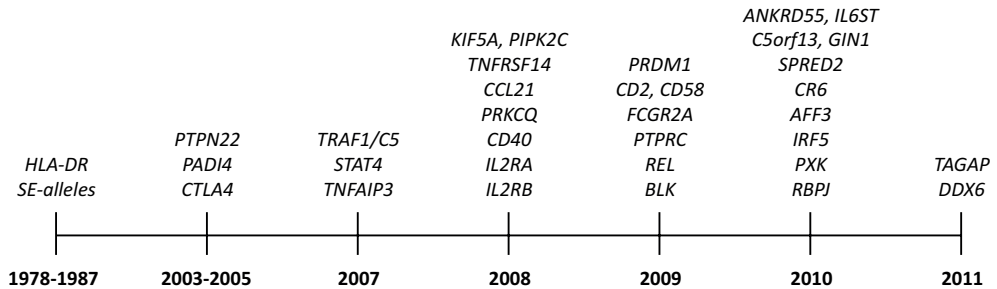
			KIF5A, PIPK2C		ANKRD55, IL6ST	
			TNFRSF14	PRDM1	C5orf13, GIN1	
			CCL21	CD2, CD58	SPRED2	
			PRKCQ	FCGR2A	CR6	
			CD40		AFF3	
	PTPN22	TRAF1/C5		PTPRC	IRF5	
HLA-DR	PADI4	STAT4	IL2RA	REL	PXK	TAGAP
SE-alleles	CTLA4	TNFAIP3	IL2RB	BLK	RBPJ	DDX6
1978-1987	**2003-2005**	**2007**	**2008**	**2009**	**2010**	**2011**

Figure 3: List of ACPA+ RA susceptibility genes

such allele, indicating that protection is an active and dominant process [57].

HLA involvement in ACPA positivity or in ACPA positive disease?

As mentioned above, the presence of ACPA can be found up to ten years before disease onset. This suggests that the progression to ACPA+ RA involves two "hits". Initially individuals are healthy and ACPA negative. After the "first hit" they become positive for ACPA. Then, presumably after a "second hit", individuals progress from ACPA positivity to ACPA+ RA, which is accompanied by a maturation of the ACPA response. The HLA class II locus is strongly associated with ACPA+ RA and understanding this association would provide important insights in disease pathogenesis. The association suggests an involvement of antigen-specific CD4+ T cells. This is strengthened by genome-wide association studies that identified many genes involved in the regulation of adaptive immune responses (Figure 3) [58]. In understanding the HLA class II association it is important to understand if this locus contributes to either the first or the second "hit" thought to accompany ACPA-positivity or ACPA-positive disease, respectively. As antigen-experienced CD4+ T cells are critically important for the generation of mature antibody responses, it seems likely that activated antigen-experienced (self-reactive) CD4+ T cells are involved in the maturation of the ACPA response. Importantly, recent evidence suggests that the HLA class II locus is not associated with the risk to become ACPA+, but with the risk to progress from ACPA+ to ACPA+ RA [59, 60]. This supports the hypothesis that the HLA class II locus is not directly involved in the formation of ACPA responses, but rather in its maturation, possibly via T cells providing help to ACPA producing B cells before the precipitation of disease (Figure 4).

THESIS OUTLINE

The general aim of this thesis was to provide a better understanding of the role of HLA class II dependent antigen presentation in both physiological conditions as well
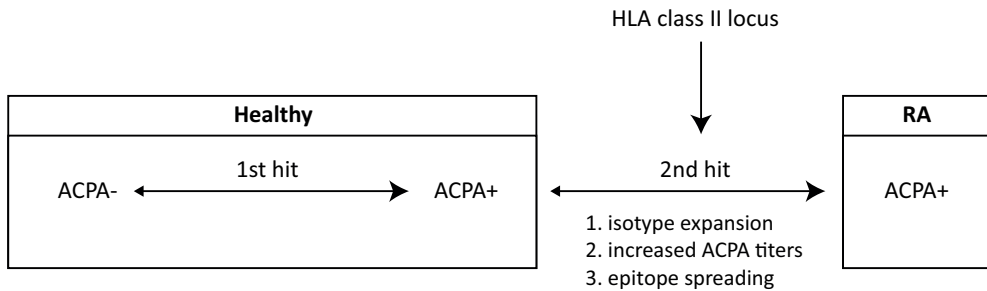
Figure 4: Schematic representation of the progression from healthy to ACPA+ RA. The HLA class II locus associates with the progression from ACPA positivity to ACPA+ RA. During this "2nd hit" the ACPA response matures as showed by isotype expansion, increased ACPA titers and epitope spreading. Together it supports a role for the HLA class II locus in the maturation of ACPA.

as in rheumatoid arthritis. To achieve this aim, our studies were separated in two parts.

In *PART I*, we focused on the expression of HLA class II molecules and the characteristics of the ligands presented by HLA class II molecules, starting with the observation that human mast cells can present HLA class II molecules on their surface (**Chapter 2**). Potentially this expression could play a role in rheumatoid arthritis as mast cells are frequent cells in the synovium [61]. The nature of peptides presented by HLA class I molecules have recently been extensively characterized [62]. In **Chapter 3**, we have now performed highly similar analyses in the context of HLA class II with a focus on HLA-DR3. HLA-DR3 is one of the most common HLA-DR molecules and associated with different autoimmune diseases including thyroid autoimmunity and ACPA negative RA [63, 64]. In **Chapter 4**, we have characterized peptides eluted from HLA-DQ6.3. This particular HLA-DQ molecule is in tight linkage disequilibrium with HLA-DRB1*13:01 and is strongly associated with protection from autoimmune diseases including RA and narcolepsy [56, 65].

In *PART II*, we studied the association between HLA class II, RA and citrullination. As discussed, the HLA locus is primarily a risk factor for ACPA+ RA. Several tests are now available to determine ACPA-positivity using model antigens (e.g. anti-CCP-2/3) [11]. In **Chapter 5**, we have examined a novel ACPA multiplex-test in comparison to the conventional tests [66]. In **Chapter 6**, we provide an opinion on recent genetic advances in identifying causal variants within the HLA locus associated with ACPA-positive rheumatoid arthritis. To study the contribution of protective and predisposing alleles in the ACPA+ RA it is important to determine in time where these alleles modulate risk. This has previously been performed for the predisposing alleles and in **Chapter 7** we have expanded this to protective HLA-DRB1*13 alleles. In **Chapter 8**, we provide a detailed molecular basis for the contribution of protective and predisposing HLA class II molecules in the HLA-RA connection. We have also studied an alternative hypothesis that assumes that

the HLA-RA connection is explained by the presentation of shared (citrullinated) antigens by HLA-SE alleles [67]. In **Chapter 9**, we studied the ability of different SE-positive HLA class II molecules to present citrullinated antigens. In **Chapter 10**, we expanded these analyses to SE-negative HLA-DR and HLA-DQ molecules. Finally, in **Chapter 11** we discuss the implications of the results and future directions.

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PART I

HLA class II expression and ligand-presentation

CHAPTER 2

Communication between human mast cells and CD4+ T cells through antigen-dependent interactions

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ABSTRACT

Mast cells are immune cells residing in tissues, where pathogens are first encountered. It has been indicated that mast cells might also be involved in setting the outcome of T cell responses. However, little is known about the capacity of human mast cells to express MHC class II and/or to capture and present antigens to CD4+ T cells.

To study the T cell-stimulatory potential of human mast cells, CD34+ stem cell-derived mast cells were generated. These cells expressed HLA-DR when stimulated with IFN-γ, and, importantly, presented peptide and protein for activation of antigen-specific CD4+ T cells. The interplay between mast cell and T cell led to increased HLA-DR-expression on mast cells. Mast cells were present in close proximity to T cells in tonsil and expressed HLA-DR and CD80, indicating their ability to present antigens to CD4+ T cells in T cell areas of human lymph nodes.

Our data show that mast cells can present native antigens to human CD4+ T cells and that HLA-DR expressing mast cells are present in tonsil tissue, indicating that human mast cells can directly activate T cells and providing a rationale to study the potential of mast cells to prime and/or skew human T cell responses.

INTRODUCTION

Mast cells are most well-known because of their role in IgE-mediated immune responses as they express the high affinity FcεRI. Mast cells are present at strategic locations of the environment/host interface where they can encounter pathogens. Therefore, they have been implicated in the regulation of adaptive immune responses as well as in the regulation of T cell immunity.

Secretion of TNF by skin mast cells was shown to be essential for lymph node hypertrophy and T cell recruitment during bacterial infection [1]. Furthermore, mast cells were shown to be capable of migrating from the skin to the draining lymph nodes in murine models of contact hypersensitivity and UV radiation [2, 3]. In the draining lymph nodes, the secretion of chemokines by mast cells has been implicated in the recruitment of T cells [2, 4]. These studies, therefore, indicate that mast cells can be involved in the regulation of T cell trafficking towards lymphoid organs, suggesting that mast cells may have a role in the induction of primary and/or local memory T cell responses. Indeed, anti-CD3-stimulated T cells displayed enhanced proliferation and cytokine secretion in the presence of mast cells, indicating that mast cells are capable of enhancing T cell responses [5-7].

A first direct demonstration that mast cells can also present antigens to T cells came from mouse studies showing that OVA-specific TCR-transgenic CD8+ T cells can be activated by mast cells via MHC class I [8, 9]. Likewise, using OVA-specific TCR-transgenic CD4+ T cells, it was demonstrated in vitro that mouse mast cells can also activate MHC class II-restricted T cells when loaded with OVA-peptide, but were less efficient in presenting OVA-protein [10, 11].

Thus far, little information is available on the antigen-presenting capacity of human mast cells. Human mast cells have also been shown to express MHC class II, especially after incubation with IFN-γ, which suggests their capacity to present antigen to CD4+ T cells [12-14]. Indeed, activation of T cell hybridomas with superantigens by these cells has been reported [14]. However, T cell activation by superantigen does not require the presentation of antigenic peptides by MHC-molecules or the processing of protein via endogenous pathways.

Human mast cells have been reported to be present in the interfollicular area in tonsil [7], indicating they may be involved in the activation of T cell responses in lymphoid organs. However, it is unknown whether these mast cells possess the required molecular make-up, such as HLA-DR and costimulatory molecules, to present antigen and activate T cells in lymphoid organs.

As it is unclear whether human mast cells are able to process and present native antigen to CD4+ T cells, we aimed to study the interaction between these cells. Our data show that human mast cells can present both peptide and entire protein antigens to CD4+ T cells, using two different antigen-specific CD4+ T cell responses as read-out. Interaction of CD4+ T cells with mast cells led to the enhanced expression of HLA class II molecules on mast cells via release of IFN-γ by T cells. In addition,

we show that human mast cells, analyzed ex vivo from tonsil, are present in T cell areas where they express HLA-DR and CD80. Together, these data indicate that antigen-specific mast cell-T cell interactions could contribute to the outcome of immune responses in the human.

MATERIALS AND METHODS

Mast cells

Buffy coats from healthy volunteers were obtained from the blood bank (Sanquin, The Netherlands). PBMCs were isolated using a standard Ficoll procedure, after which CD34+ hematopoietic stem cells were isolated with CD34 microbeads (Miltenyi Biotec). Isolated CD34+ stem cells were differentiated into mast cells using serum-free medium (StemPro 34 + supplement, Gibco) with 30 ng/mL IL-3, 100 ng/mL IL-6 and 100 ng/mL Stem Cell Factor (SCF) at 50.000 cells/mL as described [15, 16]. Half of the medium was replaced weekly with serum-free medium containing 100 ng/mL IL-6 and 100 ng/mL SCF. All recombinant cytokines were obtained from Peprotech. After 6-8 weeks, the purity of mast cells was determined by flow cyto-metric analysis of CD117 (c-kit), FcεRI and CD203c and intracellular tryptase. The purity of mast cells ranged from 90-99%.

CD4+ T cell clone

An adenovirus-specific CD4+ T cell clone (M2.11) was used to determine antigen presentation by mast cells [17]. This clone recognizes a peptide derived from hexon protein II, in the context of HLA-DRB1*03:01. Mast cells from blood of HLA-DRB1*03:01 positive donors were generated as described above. Mast cells were incubated with 5 µg/mL peptide for 24 hours, or 1-50 µg/mL protein (22.2 kDa polypeptide fragment D from hexon protein II [17]) for 6-72 hours, before coculture with the CD4+ T cells. Before coculture with the T cell clone, mast cells were thoroughly washed to remove all soluble protein or peptide. Subsequently, the mast cells were added to the CD4+ T cells in a 1:1 ratio in RPMI with 10% human serum and 100 ng/mL SCF at 200.000 cells/mL. After 1 hour, 5 µg/mL brefeldin A was added and incubated overnight. To determine the effects of activation of the T cell clone on the mast cells, the cells were cultured without brefeldin A for the indicated timepoints.

Blocking antibodies and isotype controls for HLA class II (BD Pharmingen, clone Tü39, 10 µg/mL) were added to the mast cells, and incubated for 1 hour at 37 °C before the mast cells were added to the T cells. Blocking antibodies remained present during the coculture. Blocking antibodies and isotype controls for IFN-γ (eBioscience, clone NIB42, 20 µg/mL) were added to the cocultures directly.

CD4 T cell bulk responses against recall antigens

Polyclonal CD4+ T cell bulks were generated by activation of PBMCs with the recall antigens tetanus toxoid at 0.75 Lf/mL (Netherlands Vaccine Institute), tuberculin purified protein derivative 0,5 µg/mL (Netherlands Vaccine Institute) and Candida albicans 0,005% (HAL allergy) in IMDM/5% human serum for 7 days, after which antigen presentation by autologous mast cells was evaluated. Mast cells or autologous PBMCs were incubated with the recall antigens mentioned above for 16 hours, before coculture with autologous bulk CD4+ T cells. After this incubation, the cells were thoroughly washed to remove all soluble protein. Autologous PBMCs were used as positive control and were allowed to adhere to the plate at 37°C for 2 hours, after which non-adherent cells were removed. Subsequently, the CD4+ T cells were added to the antigen presenting cells in IMDM/5% human serum at 2 x 10^6 cells/mL. After 1 hour, 10 µg/mL brefeldin A was added and incubated overnight.

Tonsils

Human tonsils were obtained after tonsillectomy. A portion of these tissues were fixed with 4% formaldehyde in PBS, stored in 70% ethanol and embedded in paraffin for immunofluorescent staining of sections. The remaining part of the tonsils was washed with RPMI with 10%FCS, and cut into small pieces. Subsequently, the tonsils were dispersed through a 70 um filter to get single-cell suspensions. Cells were frozen until flow cytometric staining and acquisition. This study was performed in accordance with the declaration of Helsinki and local ethical guidelines.

Immunofluorescence

Tonsil tissues were treated as previously described to deparaffinize and retrieve antigens with EDTA [18]. Slides were incubated with 8 µg/mL goat anti-human tryptase, 26.5 µg/mL rabbit anti-human CD117, 60 µg/mL rabbit anti-human CD3 or matching isotype controls for 1 hr at room temperature. After washing, slides were stained with 2 µg/mL donkey α-goat AF 568 and 2 µg/mL donkey α-rabbit AF 488 for 1 hr at room temperature. After washing, slides were covered with Vectashield containing DAPI (Vector laboratories) to stain nuclei. Slides were analysed on a Zeiss Axio ScopeA1 microscope.

Flow cytometry

For detection of HLA-DR and costimulatory molecules expression by cultured mast cells, cells were incubated with fluorochrome-conjugated antibodies recognizing CD117, CD203c, HLA-DR, CD80 and CD86 or matching isotype controls. Intracellular staining of tryptase was performed as described [19].

For detection of expression of HLA-DR and costimulatory molecules on tonsil mast cells, cells were incubated with fluorochrome-conjugated antibodies recognizing

CD117, CD203c, FcεRI, HLA-DR, CD86 and CD80 or matching isotype controls for each of these antibodies. Just prior to flow cytometric acquisition, 0.2 uM DAPI was added for exclusion of dead cells.

For intracellular cytokine staining by CD4+ T cells, the cells were incubated with fluorochrome-conjugated antibodies recognizing CD3 and CD4, after which they were permeabilized using CytoFix CytoPerm Kit (BD Biosciences). To gate out any remaining monocytes in the CD4+ T cell bulks, antibodies to CD14 were added as well. After washing, cells were incubated with PE-labeled anti-IFN-γ, TNF-alpha or matching isotype control. Cells were taken up in 1% paraformaldehyde until flow cytometric acquisition.

Flow cytometry was performed on FACS Calibur (BD) or LSR II (BD). Analysis was performed using FACS Diva (BD) and FlowJo software.

Statistical analysis

Results are expressed as mean + SEM. Statistical analysis was performed using SPSS PASW 17.0 and GraphPad Prism 4. For differences between two groups, student's T tests were performed. P values of <0.05 were considered statistically significant.

RESULTS

Human peripheral blood-derived mast cells express HLA-DR when stimulated with IFN-γ

Human mast cells were generated from CD34+ stem cells. Typical flow cytometry plots identifying mast cells by combined expression of CD117, CD203c (Ectoenzyme E-NPP3), FcεRI and intracellular tryptase are shown in Figure 1A, indicating that the six-week culture results in mature (CD117+ tryptase+) mast cells. No expression of the DC markers DC-SIGN and CD1c (BDCA-1) was found on our cultured mast cells (data not shown). Only low, but detectable, levels of HLA-DR were detected on resting mast cells (Figure 1B-C).

Because it was previously shown for mast cell-lines that IFN-γ could induce expression of HLA class II, we evaluated whether this could lead to upregulation of HLA-DR on our cultured mast cells as well. Incubation with 50 ng/mL IFN-γ for 24 h induced upregulation of HLA-DR (Figure 1B-C). No induction of expression of HLA class II was found after stimulation with TLR ligands, anti-IgE or recombinant IL-4 (data not shown). These results indicate that in the presence of IFN-γ, mast cells can acquire the expression of HLA class II, and possibly the ability to present antigen to CD4+ T cells. Of the costimulatory molecules tested (CD80, CD86, ICOSL, OX40L, CD40L, CD40), we found expression of CD80 but not CD86 or any of the other markers, as assessed by flow cytometry (Figure 1D-E).

Together, these data indicate that human mast cells can express molecules required for stimulation of CD4+ T cells.

Human mast cells can present peptide and protein to CD4+ T cells

To evaluate the potential of human mast cells to present antigen to CD4+ T cells, mast cells were pre-incubated with an adenovirus-derived peptide or hexon IID protein. Subsequently, mast cells were cocultured with an adenovirus-specific T cell clone recognizing the particular peptide. As shown by intracellular cytokine staining, mast cells pre-incubated with peptide, in contrast to control mast cells, were capable of inducing robust cytokine production by the T cells (Figure 2A-B). The capacity of mast cells to induce T cell responses with the peptide was comparable to that of PBMCs. Importantly, mast cells pre-incubated with the hexon IID protein were also capable of activating T cells, indicating the capacity of mast cells to take up, process and present native protein antigen to CD4+ T cells (Figure 2A-C).
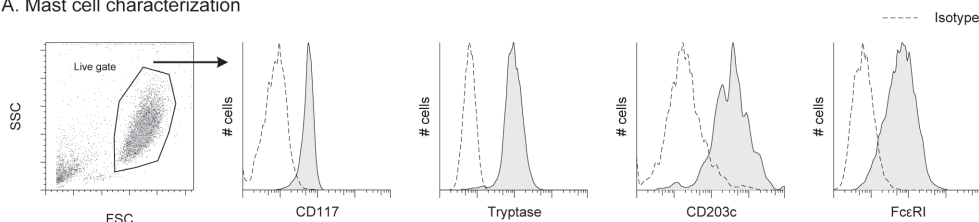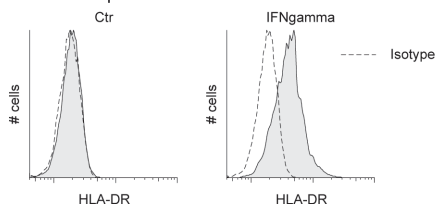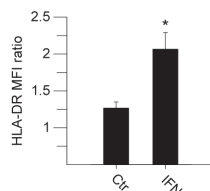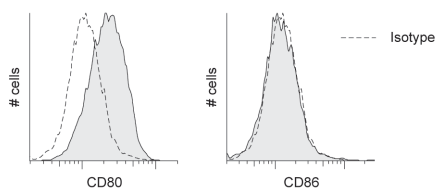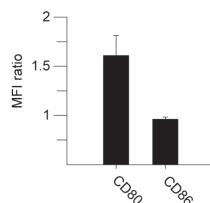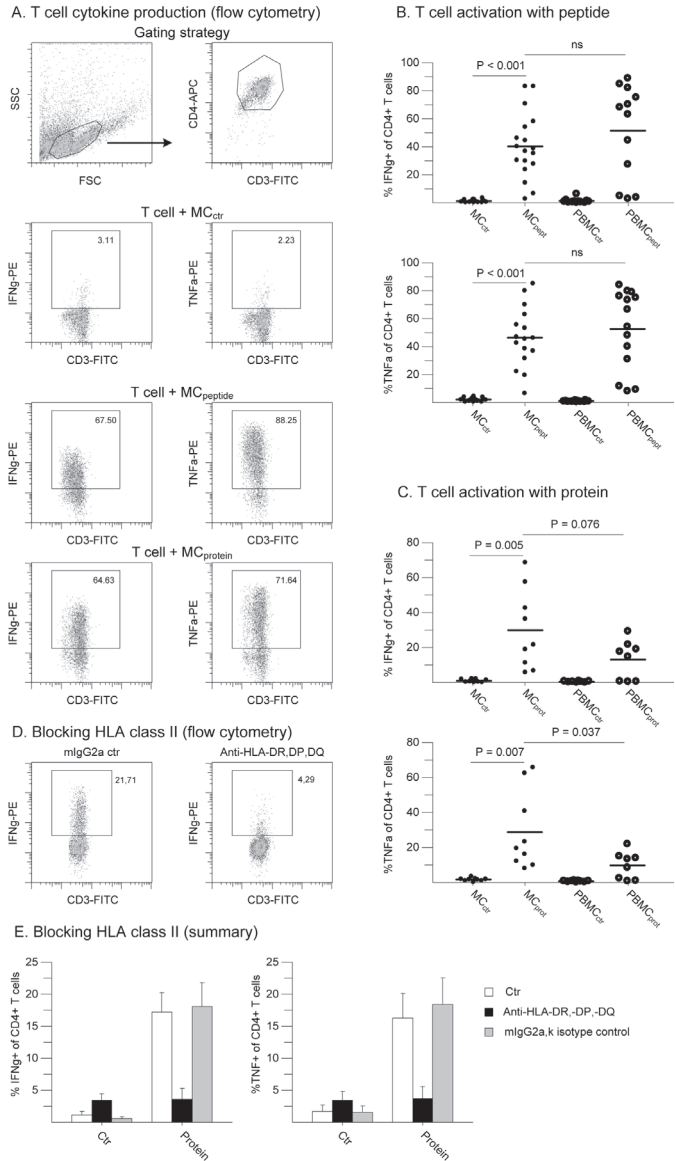

Figure 1. Expression of HLA-class II and costimulatory molecules by human peripheral blood-derived mast cells. (A) Representative flow cytometric plots of mast cell characterization after 6-8 week differentiation showing expression of CD117, intracellular tryptase, FcERI and CD203c. Histograms show gated live cells based on FSC/SSC characteristics as shown in the first panel. (B, C) Mast cells were cultured in the presence of 50 ng/mL IFN-γ for 24 hours, after which expression of HLA-DR was assessed. (D, E) Expression of CD80 and CD86 in resting mast cells. (B, D) Representative examples of mast cells gated as CD117+ CD203c+. (C, E) Summaries (mean ± SEM) of independent mast cell cultures (n=3). Expression (MFI ratio) is indicated as MFI of the marker of interest (e.g. HLA-DR) divided by the MFI of the matching isotype control. Asterix in (C) indicates significant (p<0.05) increase in IFN-γ treated cells compared with ctr mast cells, using student's T test.

Presentation of protein antigens by mast cells appeared rather efficient as 5 µg/mL protein, equivalent to 225 nM, was still able to activate adenovirus-specific T cells. Furthermore, although there was no difference between the T cell responses induced by mast cells compared with PBMCs when they were incubated with peptide, mast cells were more efficient than PBMCs in inducing T cell responses with the protein antigen (Figure 2C) Similar results were obtained when T cell-derived cytokine levels in supernatant were measured by ELISA (data not shown).

Figure 2. Antigen presentation by human mast cells leads to activation of adeno-virus-specific T cells. (A) Representative flow cytometric plots of TNF-alpha (TNFa) and IFN-γ (IFNg) production by gated CD3+CD4+ T cells after o/n coculture with mast cells preincubated with medium (ctr), adenovirus-specific peptide or protein, in the presence of brefeldin A. (B and C) Summary of T cell activation after preincubation of mast cells with peptide and protein. Each dot shows an independent mast cell culture or PBMCs donor (n = 18 and n = 9 for peptide and protein, respectively). P values comparing the indicated conditions were calculated using student's t-test. (D, E) Cytokine production by T cells after preincubation of mast cells with blocking HLA class II antibodies (n=3). (E) Data are shown as mean ± SEM pooled from (n=3) independent mast cell cultures

A. T cell cytokine production (flow cytometry)

B. T cell activation with peptide

C. T cell activation with protein

D. Blocking HLA class II (flow cytometry)

E. Blocking HLA class II (summary)

To confirm that these T cell responses were HLA class II-dependent, mast cells were next incubated with blocking HLA class II- or isotype control antibodies. CD4+ T cell responses induced by mast cells pre-incubated with hexon IID protein were almost completely inhibited by HLA class II-blocking antibodies, compared with the respective isotype control (Figure 2D-E), further confirming that the mast cells stimulated the T cells via HLA class II.
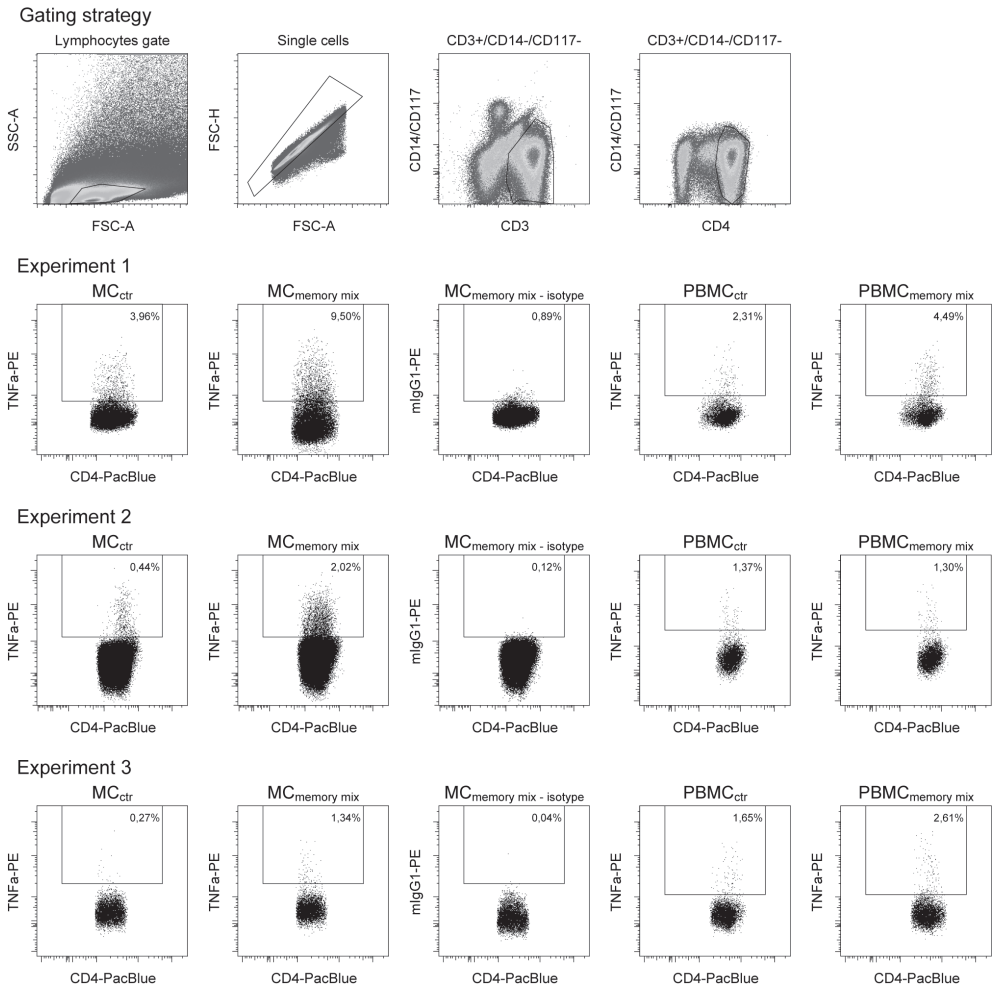

Figure 3. Antigen presentation by human mast cells to autologous bulk CD4+ T cells.
Polyclonal CD4+ T cell bulks were generated by activation of PBMCs with the recall antigens tetanus toxoid, tuberculin purified protein derivative and Candida albicans for 7 days. Autologous mast cells or adherent PBMCs preincubated with medium (ctr) or these recallantigens (memory mix) were cultured o/n with the bulk T cells in the presence of brefeldin A. Flow cytometric plots of TNF-alpha (TNFa) production by T cells in 3 independent experiments are shown. T cells were gated based on FSC/SSC characteristics, CD14/CD117-negativity, CD4+ and CD3+ as shown in top panel.

Although the data presented above indicate that human mast cells can present protein antigens to T cells, they do not show whether also polyclonal T cells can be activated by mast cells. To determine whether polyclonal T cells, rather than cloned T cells potentially harboring a high avidity TCR to the peptide-MHC complex, are also activated by mast cells, we next generated CD4+ bulk T cells expanded for one week with pooled tetanus toxoid, tuberculin and candida albicans. Subsequently, autologous mast cells were incubated with these recall antigens and used as APCs for polyclonal bulk T cells. Mast cells were capable of activating autologous polyclonal CD4+ T cells, confirming their T cell-activating capacity (Figure 3). The response by CD4+ T cells that was induced by mast cells was comparable to or better than our positive control, autologous adherent PBMCs.

Together, these results indicate that human mast cells are capable of taking up and processing native proteins for presentation to CD4+ T cells in the context of HLA class II.
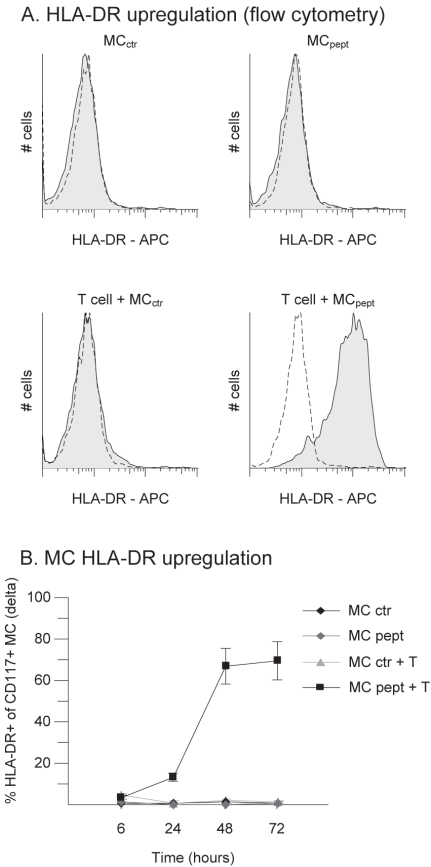
CD4+ T cells can modulate mast cells via IFN-γ

As the interaction between antigen-presenting cells and T cells can be bi-directional, the potential of CD4+ T cells to modulate MC phenotype was analysed. To this end, mast cells pre-incubated with peptide were cocultured with adenovirus-specific
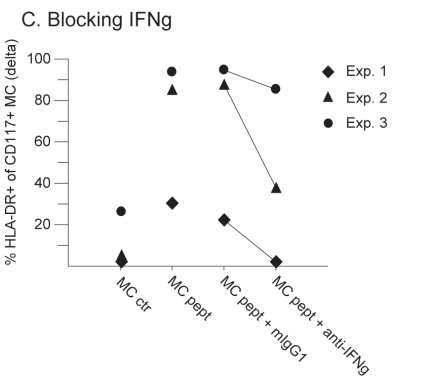

A. HLA-DR upregulation (flow cytometry)

MC$_{ctr}$ — MC$_{pept}$

T cell + MC$_{ctr}$ — T cell + MC$_{pept}$

cells — HLA-DR - APC

B. MC HLA-DR upregulation

% HLA-DR+ of CD117+ MC (delta) vs Time (hours)

- MC ctr
- MC pept
- MC ctr + T
- MC pept + T

C. Blocking IFNg

% HLA-DR+ of CD117+ MC (delta)

- Exp. 1
- Exp. 2
- Exp. 3

MC ctr — MC pept — MC pept + mIgG1 — MC pept + anti-IFNg

Figure 4. Induction of HLA class II on mast cells through IFN-γ production by CD4+ T cells. (A, B) Representative flow cytometric plots and summary of HLA-DR expression by mast cells preincubated with medium (ctr) or adenovirus-specific peptide (pept), after coculture with or without adenovirus-specific CD4+ T cells for the indicated time periods (n=2). (C, D) Representative flow cytometric plots and summary of HLA-DR expression by mast cells after coculture for 48 hrs with adenovirus-specific CD4+ T cells in the presence of isotype control antibodies (mIgG1) or blocking antibodies to IFN-γ (n=3). Mast cells are gated as CD203c+ cells as shown in Figure 1. Data are shown as mean ± SEM pooled from (n=3) independent mast cell cultures.

CD4+ T cells to evaluate potential effects of T cell activation on the mast cells. As shown in Figure 4A-B, the expression of HLA-DR by mast cells was enhanced when T cells were activated by mast cells pre-incubated with peptide. Mast cells cultured without T cells, or mast cells not pre-incubated with peptide did not show an increase in HLA-DR expression. Upregulation of HLA-DR was inhibited by blocking IFN-γ, ranging from 14-100% inhibition as compared with the isotype control (Figure 4C-D). These data indicate that the effect of activated T cells responsible for HLA class II-upregulation on mast cells was mainly mediated via IFN-γ. Together, these results show that mast cells cannot only activate T cells, but also that CD4+ T cells can modulate mast cells by upregulation of HLA class II, indicating a bidirectional interplay between mast cell and T cell.

Human tonsils contain HLA-DR+ mast cells

To evaluate whether HLA-DR+ mast cells can also be found in vivo in T cell areas of lymphoid organs, tonsil sections were stained for mast cells. Mast cells were identified as CD117+ Tryptase+ double-positive cells and were found in all (n=6) tissues analysed (Figure 5A). All tryptase+ cells expressed CD117, whereas also some CD117+ tryptase- cells could be identified, indicating that CD117 alone cannot uniquely define tonsillar mast cells. Tryptase+ mast cells were most often found in T cell areas characterized by abundant expression of CD3, and were commonly observed in close proximity to CD3+ T cells (Figure 5B-C). These results indicate that mast cells are present in T
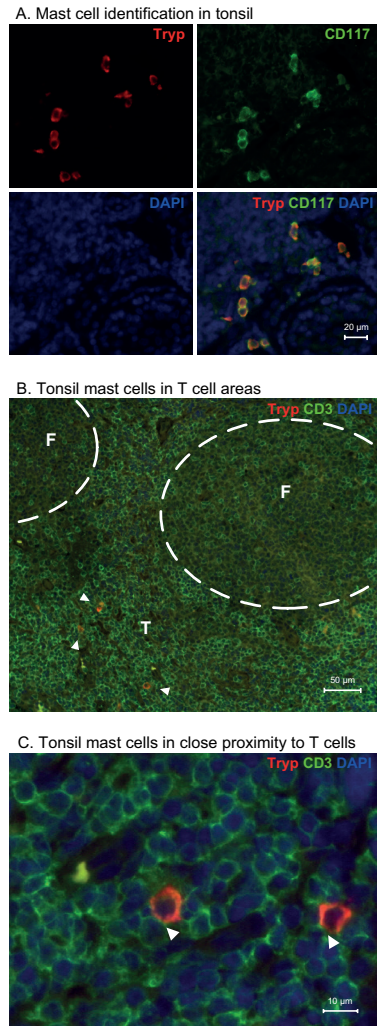

Figure 5. Mast cells are present in T cell areas of human tonsil. (A) Mast cells in tonsils are characterized as tryptase (tryp) + CD117+ double-positive cells. (B, C) Double-staining of tryptase and CD3. (B) Mast cells are mainly found in T cell areas of the tonsil. "F" indicates the B cell follicles; "T" indicates the T cell area, defined by expression of CD3. Arrowheads show Tryp+ mast cells present in the T cell area. (C) Mast cells are often found in close proximity to T cells. Arrowheads show examples of Tryp+ mast cells in close contact with CD3+ T cells. Original magnifications: 20x (A and B) and 40x (C). (A-C) Images are representative examples of fluorescent stainings of tonsils from 6 independent donors.

cell areas of lymphoid organs and at locations allowing T cell-mast cell interactions. Nonetheless, as evaluated by immunofluorescent stainings of tonsil sections, we could not detect HLA-DR on these tryptase+ mast cells (data not shown). As HLA-DR expression might be relatively low on tonsillar mast cells, the detection of its expression might be obscured by the high HLA expression of other APCs which are abundantly present in tonsil. Therefore, we also analyzed mast cells in tonsil cell suspensions using a more sensitive flow cytometric technique with antibodies recognizing CD117, CD203c, FcεRI, HLA-DR and costimulatory molecules (Figure 6). Mast cells were identified by their expression of high levels of CD117 and were further characterized by expression of the mast cell markers CD203c and FcεRI (Figure 6A-B), and absence of the DC markers DC-SIGN and CD1c (data not shown). HLA-DR expression was found on mast cells of all 6 donors analyzed, both when analyzed as percentage or mean fluorescence (Figure 6C-D). The relative immunofluorescence was approximately 10-fold lower than on other HLA-DR+ cells in the tonsil, which is in line with the difficulty to detect HLA-DR on mast cells in a convincing manner by staining of tonsil sections.
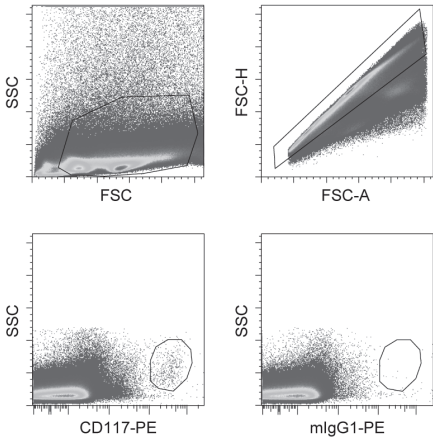
The expression of costimulatory molecules by tonsillar mast cells was also evaluated (Figure 6E-F). Although only low levels of CD86 were found, all four donors analyzed showed significant expression of CD80 compared with the isotype control, confirming the data obtained from mast cells cultured from blood (Figure 1C).

Together, these results show that mast cells expressing HLA-DR and costimulatory molecules are present in human tonsil, and that the majority of these mast cells are located in close proximity to T cells, arguing for mast cell-T cell interaction in human lymph nodes.

DISCUSSION

In this article we show for the first time that cultured human mast cells are capable of presenting native protein antigens to CD4+ T cells. The interaction between mast cells and CD4+ T cells modulated the mast cell phenotype, indicating that both cell types can influence each other in an antigen-dependent manner. Furthermore, the interaction between these cell types may be physiologically relevant as the presence of mast cells in proximity to T cells was also shown in lymphoid organs. A clear inherent limitation of our study is that we are unable to show the ability of mast cells to present antigen in vivo. Likewise, there were some differences between the cultured mast cells and their in vivo counterparts. Whereas the cultured mast cells readily expressed HLA-DR after coculture with the T cells, the tonsil-resident mast cells already expressed HLA-DR when evaluated directly ex vivo. This difference might be explained by the exposure of mast cells to IFN-γ either in the tonsil or in the periphery by either T cells or innate IFN-γ-producing cells such as NK-cells. Nonetheless, as the phenotype of mast cells for the other
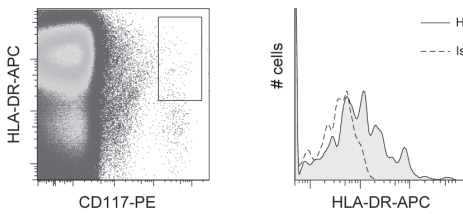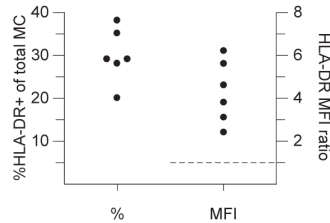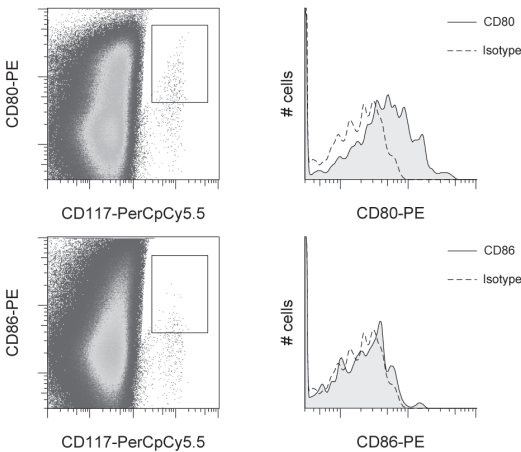
A. Mast cell characterization

B. MC frequency
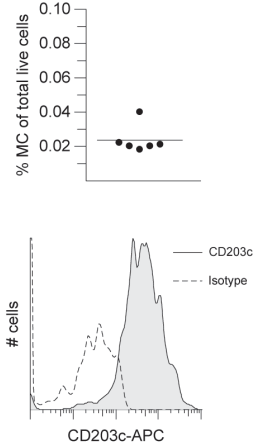
C. HLA-DR expression

D. Summary of HLA-DR

E. Costimulatory molecules
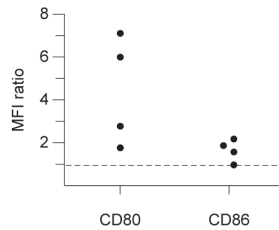
F. Summary of CD80/CD86

Figure 6. Mast cells in tonsil express HLA-DR and CD80. (A, B) Mast cell characterization in total tonsil cell suspensions by CD117, FcεRI and CD203c (n=6). Live cells were gated based on FSC/SSC characteristics and DAPI-negativity and their expression of CD117 or isotype control are shown in the dotplots. Histograms show expression of FcεRI and CD203c by gated CD117hi cells as shown in the dotplots. (C, D) Expression of HLA-DR by mast cells characterized as in A (n=6). (E, F) Expression of CD80 and CD86 by mast cells characterized as in (A) (n=4). Expression (MFI ratio) in (D) and (F) is indicated as MFI of the marker of interest (e.g. HLA-DR, CD80) divided by the MFI of the matching isotype control. Dotted lines in (D) and (F) indicate absence of expression of the indicated marker compared with the isotype control (MFI ratio = 1). Data shown are representative of tonsils from 4-6 independent donors.

markers evaluated (CD117, tryptase, CD80) was comparable, we believe that the cultured mast cells are a good representation of their counterparts in vivo, and that the biological effects observed in vitro are conceivably present in vivo as well.

The presentation of antigen to CD4+ T cells by human mast cells may serve to stimulate T cells in diverse locations, such as lymphoid organs and peripheral tissues. As mast cells can produce a different set of cytokines than other antigen-presenting cells [20-22], our findings could potentially have important implications for the skewing of T helper cells and maybe even for the outcome of naïve T cell-priming in lymph nodes. Since both cultured and tonsil-resident mast cells express HLA class II and CD80, their potential in stimulating naïve CD4+ T cells cannot be excluded. Skin mast cells have been shown to migrate from infected/allergic areas to draining lymph nodes in the mouse [2, 3]. Nonetheless, mouse mast cells, although able to activate memory T cells, were not able to prime naïve CD4+ T cells, probably due to the lack of costimulatory molecules [10, 11]. As mast cells only comprised approximately 0.02-0.04% of total tonsil cells as shown in figure 6, and as their expression of HLA class II molecules was much lower than that of other professional antigen presenting cells such as DCs, we do not consider it likely that mast cells play a crucial role in priming of T cells in primary immune responses. In contrast, we consider it more likely that they might be more important in activation of secondary T cells in either lymph node or peripheral tissue. Especially, the latter is an intriguing possibility as mast cells are present at several strategic locations where pathogens are first encountered. It could be very beneficial to the host to swiftly activate effector memory T cells that are known to reside in tissues [23], by mast cells that are well known for their immediate and strong actions upon activation. Furthermore, mast cells could produce several cytokines upon activation through activation via IgE and TLR, which may have an impact on T cell activation or skewing.

In addition, CD4+ T cells could also influence mast cells, for example by inhibiting or modulating their reactivity. As murine mast cells were shown to specifically enhance activation of CD4+ Tregs through MHC class II [10], such T cell-mast cell interactions could also function to inhibit mast cell responses, such as observed in a murine model of anaphylaxis [24]. The expression of MHC class II by mast cells depended on the production of IFN-γ by T cells in this study, showing the ability of T cells to influence mast cell phenotype. Therefore, interaction between mast cells and T cells might direct the responsiveness of both cell types, allowing for optimal control of both cell populations.

We have not shown how the mast cells have taken up antigen for presentation to T cells in our study. However, we observed no enhancement of antigen presentation when protein antigens were targeted to the FcεRI on the mast cells via hapten-specific IgE and hapten-coupled protein (data not shown). The lack of enhancement of presentation by Fc-receptor-targeted antigens by human mast cells contrasts findings obtained in mice, as two studies reported enhanced antigen presentation by Fc receptor-mediated uptake [11, 25]. Nonetheless, another study using murine

mast cells indicated decreased antigen presentation when antigen was routed to IgE/FcεRI, possibly because antigens were protected from proteolytic degradation and subsequent presentation as peptide in MHC [10]. Further research into this aspect is required, but our data indicate that Fc-receptor mediated uptake and presentation of Ig(E) bound antigen by human mast cells, if present, is likely to be less efficient as compared with the ability of DCs to take up and present immune complexed antigens [26].

In conclusion, our results show that cultured human mast cells can function as antigen presenting cells, by uptake and processing of protein, and subsequent presentation to CD4+ T cells via HLA class II. Furthermore, expression of HLA class II and CD80 is found on mast cells isolated from human tonsil, where they are found in close proximity to T cells. In addition, the interaction between mast cells and

CD4+ T cells led to changes in the mast cell phenotype in an antigen-specific manner, indicating an intimate interaction between these two distinguished cell types.

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CHAPTER 3

The HLA-class II ligandome of B cells is highly conserved on N- and C-terminal residues outside the peptide binding cleft.

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Manuscript in preparation

ABSTRACT

Presentation of peptide-ligands on HLA class II molecules to CD4+ T cells plays an important role in adaptive immunity. HLA class II molecules typically present ligands between 11 and 20 amino acid (aa) residues in length. The part of the peptide interacting with the HLA molecule is highly conserved at anchor-positions interacting with the peptide-binding pockets. As these peptides are generated by the action of specific proteases, we questioned if the N- and C-terminal side of the peptide, outside the peptide-binding cleft, is also conserved.

To study this we identified over 13 270 HLA-DRB1*03:01 ligands from EBV-transformed B cells, the largest data set of ligands isolated from a single HLA-class II molecule. We provide a detailed characterization of these ligands and studied N- and C-terminal conservations. To do this, we selected for peptides of at least 15 aa residues (n=7 593) and determined the frequency of aa residues at the N- and C-terminal side of the peptide relative to their frequency in the human proteome. Using this approach, we could demonstrate conservations of aa residues at both termini. Conserved residues are distinct from residues conserved at the p1 and p9 anchor residues. We identified similar conservations in a second set of ligands eluted from HLA-DQ2/DQ8 (n=480) and a set of HLA-DR ligands available through public databases (n=924).

Together, these data show that the HLA class II ligandome of B cells is conserved on the N- and the C-terminus. We propose that these conservations are the result of the action of specific proteases involved in antigen-processing.

INTRODUCTION

HLA class II molecules are heterodimers of an alpha and a beta chain and present peptide-ligands on the surface of professional antigen-presenting cells to CD4+ T cells[1, 2].

Activated CD4+ T cells play an essential role in the specific immune system and can have multiple effector mechanisms including the priming of CD8+ T cell responses and helping B cells in the production of antibodies. In addition these T cells can also produce effector molecules including many different cytokines that can shape the immune response. A proper CD4+ T cell response is crucial for the immune system to combat infections, but also plays an important role in anti-tumor responses[3-5]. CD4+ T cells can also play a detrimental role. In allergy CD4+ T cells targeting allergens are important for class-switching towards an IgE response[6]. In autoimmunity, CD4+ T cells are described to be important effector cells as they could cause tissue damage, can prime autoreactive CD8+ T cell responses and can be involved in the production of autoantibodies[3]. Likewise, the HLA class II locus is a very important risk factor for many different autoimmune diseases[7]. Finally, the HLA-system and

CD4+ T cells play an important role in allograft rejection[8].

Since the early 90s many groups have studied the peptide binding repertoire of MHC class II molecules[9-11]. From these studies it became clear that this repertoire strongly differs from HLA class I molecules. For instance, HLA-class II ligands are highly heterogeneous in size and are found in nested sets. Also, these ligands are the result of lysosome degradation of proteins[12]. The identification of HLA class II ligands was used to determine amino acid (aa) conservations in the different peptide-binding pockets of individual HLA class II molecules, which could be used to predict potential T cell epitopes.

Presented HLA class II ligands are generated upon antigen-processing by specific proteases (e.g. cathepsins) in the endosomal or lysomal compartments. Peptide-ligands are generally presented in a linear configuration with 9 amino acids interacting with the HLA molecule. Within, these 9 aa residues, p1, p4, p6, p7 and p9 residues are highly conserved as these anchor-residues interact with the peptide binding pockets. The characteristics of these pockets differ between HLA class II molecules, thereby allowing each HLA molecule to present a distinct peptide binding repertoire. As these ligands are generated by specific proteases, we questioned if this would be reflected in aa conservations on the N- and C-terminal side of the presented ligandome.

We now performed an in-depth analysis of the ligandome of HLA-DRB1*03:01[13], one of the most prevalent HLA-DR molecules in Caucasians[14]. The large number of identified ligands (n=13 270) allowed us to accurately determine the frequency of aa residues on the N- and C-terminus and to compare these frequencies with the human proteome.

METHODS

Data processing

The tandem mass spectra were matched against the IPI human database (version 3.87) using the Mascot search engine (version 2.2.04, Matrix Science, London, UK) with a precursor mass tolerance of 2 ppm, with methione oxidation as a variable modification and a product ion tolerance of 0.5 Da. Peptides were identified based on a false-discovery rate (FDR) of 1%. The GRAVY index of each peptide was calculated using the Protein GRAVY sequence manipulation suite. The pI values of the identified peptides were calculated using the ExPASy "Compute pI/Mw" tool. To generate motifs, the minimal core sequences found within nested sets were extracted and the resulting list of peptides were aligned using MEME where motif width was set to 9–15 and motif distribution set to 'one per sequence'. Icelogo (version 1.2) was used to generate the peptide binding motif and the presented heatmaps with human Swiss-Prot means as reference.

RESULTS

HLA-DRB1*03:01 derived ligands

To establish the HLA-DRB1*03:01 ligandome, we isolated HLA-DR3:peptide complexes from EBV-transformed B cell line DUCAF. This resulted in the identification of the largest set of HLA-class II derived ligands (13 270 unique ligands with a false discovery rate of 1%) ever described from a single HLA-class II molecule. This set of ligands allows us to perform a detailed characterization of the HLA-class II ligandome. To quantify the variety of identified ligands, we calculated for all identified ligands their theoretical isoelectric points (pI) and their gravy index, a measure of hydrophobicity (Figure S1A-B). These analyses showed us that we have identified a wide variety of ligands with large differences in hydrophobicity and in pI. Hydrophilic ligands are slightly disfavored as previously described for HLA-class I ligands [17].

Characteristics of the HLA-DRB1*03:01 ligandome

We next studied the identified ligands in more detail. These analyses were only performed on ligands with a best mascot ionscore above 60 (still leaving 4926 unique ligands), thereby further limiting false-positive results. In Figure 1A, we analyzed the length of the ligands and confirmed previous results showing MHC class II ligands are heterogeneous in size with 91% of the ligands between 12 and 20 aa in length [10, 11].

We next characterized the protein sources of the identified ligands. In Figure 1B, we studied the position in the protein of which the ligand was derived by looking at the start or stop position relative to the protein length. Interestingly, the ligands were derived from sequences throughout the length of the protein without an obvious bias. Ligands derived from the end of the protein are slightly underrepresented in the ligandome.

The 4926 ligands that we identified were derived from 1188 different proteins. When we plotted the number of ligands against the different proteins, we found up to 69 unique ligands derived from a single protein. In Figure 1C the number of ligands per protein are plotted. For 90% of the proteins, less than 10 unique ligands were found. As previously described, certain proteins, including different HLA proteins, were clearly overrepresented[10]. It was previously demonstrated for HLA-class I ligands, that the number of ligands per protein correlated with the length of the protein[17]. This is conceivable as longer proteins theoretically yield contain more different ligands. However for HLA-DRB1*03:01 ligands, we did not observe an association between protein length and the number of ligands that were identified (Figure 1D).

The HLA-DRB1*03:01 peptide-binding motif

In contrast to HLA-class I molecules, HLA class II molecules have an open confi-
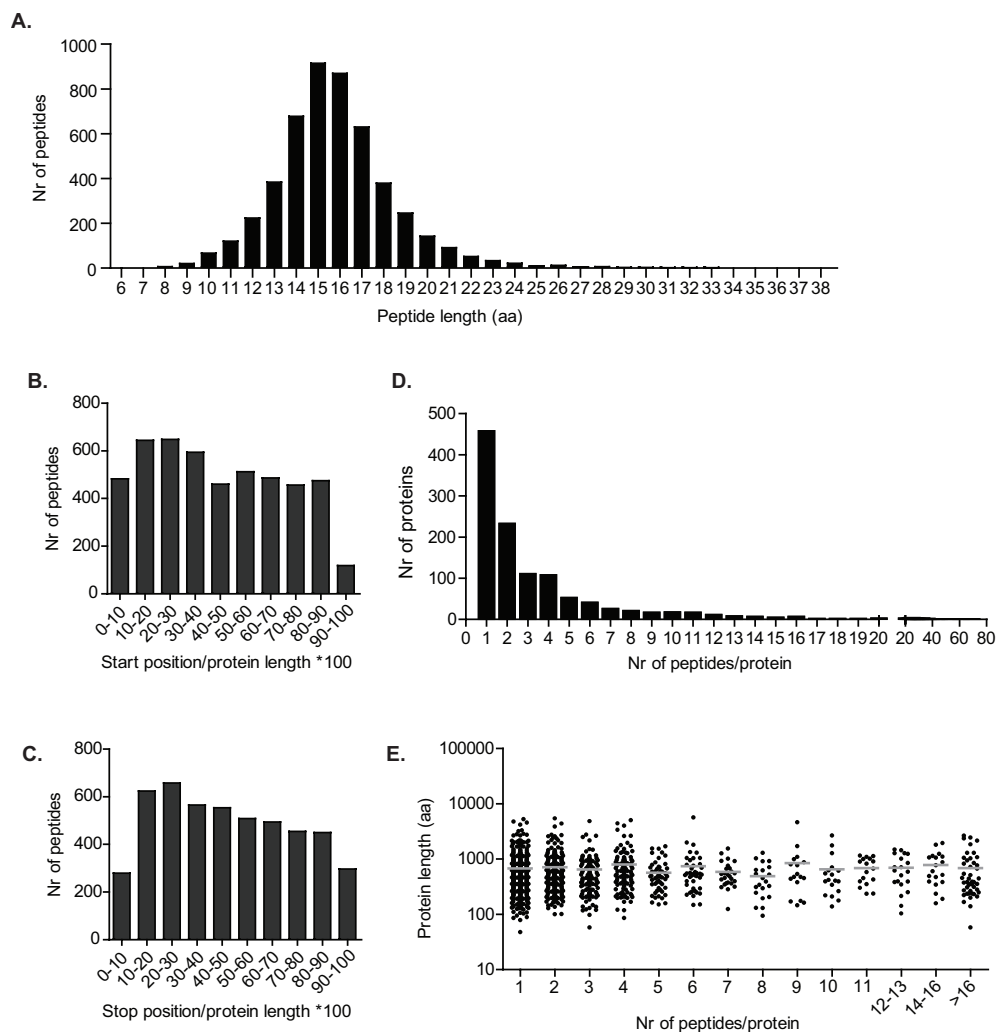
Figure 1: Characteristics of HLA-DRB1*03:01 ligands. (A) Distribution of the peptide-length of eluted DUCAF-DR3-ligands. Ligands were selected based on a BMI ≥ 60. (B-C) Plots depict the start (B) or stop position (C) of the ligands as a percentage of the total protein length versus the percentage of identified core-sequences. (D) Plot depicts the number of proteins as a function of the number of peptides identified per protein. (E) Plot depicts the protein length as function of the number of peptides by which the protein is represented.

guration allowing the accommodation of a wide variety of peptide lengths[2, 10, 11]. The part of the peptide that interacts with the HLA molecules is only 9 aa in length[2], but HLA-class II derived ligands usually present in nested sets of ligands of different length with the same 9 aa core sequence. An analysis of the association of the number of ligands versus protein length is therefore largely influenced by these nested sets. To correct for this bias, we determined the minimum core sequences by removing all length variants. This resulted in the identification of 1175 ligand-

A.

B.

C.

D. **P1 residue**

E. **P4 residue**

F. **P6 residue**

G. **P7 residue**

H. **P9 residue**
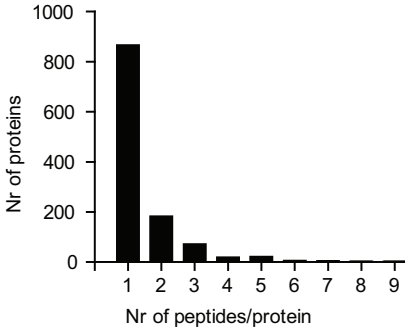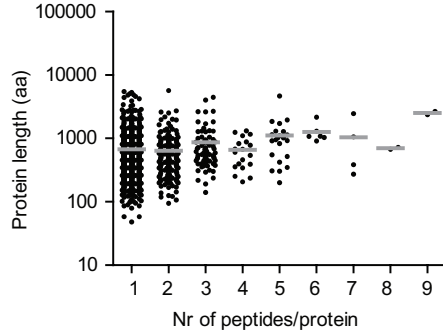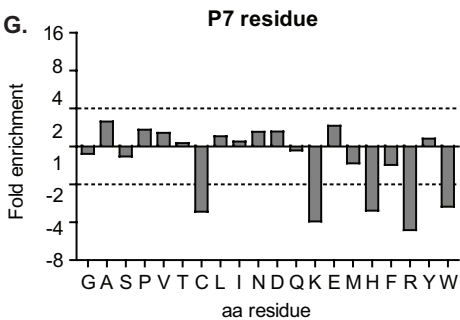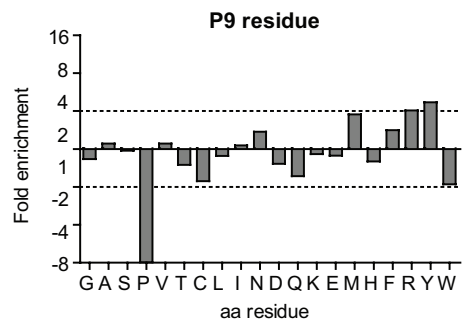
Figure 2: Characteristics of HLA-DRB1*03:01 core-ligands. (A) Figure depicts the number of proteins as a function of the number of core-peptides identified per protein. There is a gradual decrease in the number of core-peptides by which a protein is represented. (B) The protein length is depicted as function of the number of core-peptides by which the protein is represented. To determine the peptide-binding motif, identified cores were aligned using MEME to identify nonamers. The frequ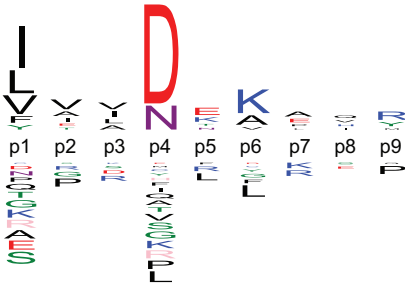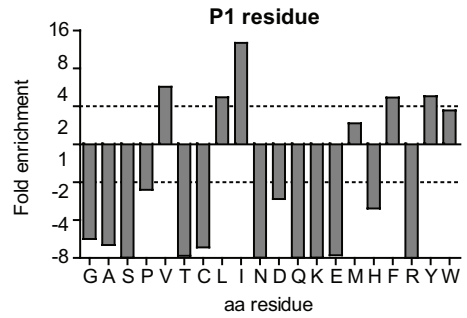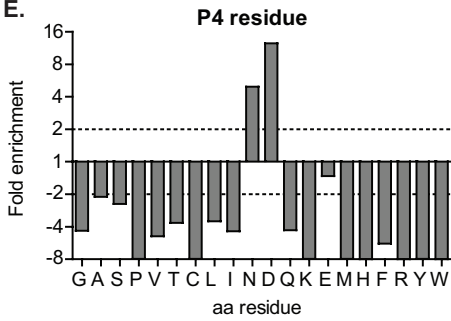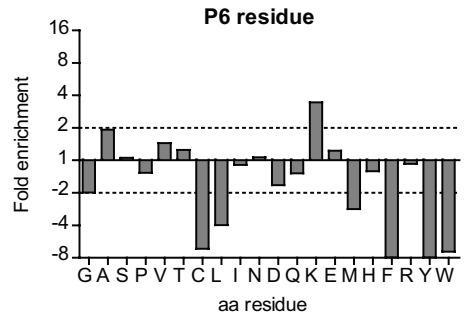ency of aa residues in the human proteome was used as a reference to study aa conservations. (C) Visualization of the peptide binding motif by ICELOGO. Positively associated residues (P>0.05 at each relative position) are shown above the X-axis and negatively associated residues are shown below. Residue height is proportionate to prevalence. (D-H) Plots depict enriched or deprived aa residues on the p1 (D), p4 (E), p6 (F), p7 (G) and p9 (H) residues of the nonamers.

core sequences for HLA-DRB1*03:01. For 74% of the proteins we identified a single ligand-core sequence (Figure 2A). Interestingly, again there was no obvious correlation between protein length and the number of ligand-cores/protein (Figure 2B). To determine the peptide binding motif of HLA-DRB1*03:01, we aligned all ligand-core sequences with MEME and subsequently compared the frequency of amino acids at different positions interacting with the peptide binding pockets with the frequency of these amino acids in the human proteome with Icelogo. This approach led to the identification of the peptide binding motif of HLA-DRB1*03:01 (Figure 2C). As previously shown, HLA-DR molecules have an hydrophobic anchor in p1 (Figure 2D). Likewise, we observed a clear preference of isoleucine, leucine, valine, phenylalanine and tyrosine residues in p1. In p4, we can observe a strong preference for aspartic acid and the highly related asparagine residue, likely caused by a combination of size and charge of this particular pocket (Figure 2E). In pocket 6 and 9, both electronegative pockets, we observed a preference for respectively lysine or arginine and tyrosine residues (Figure 2F-G).
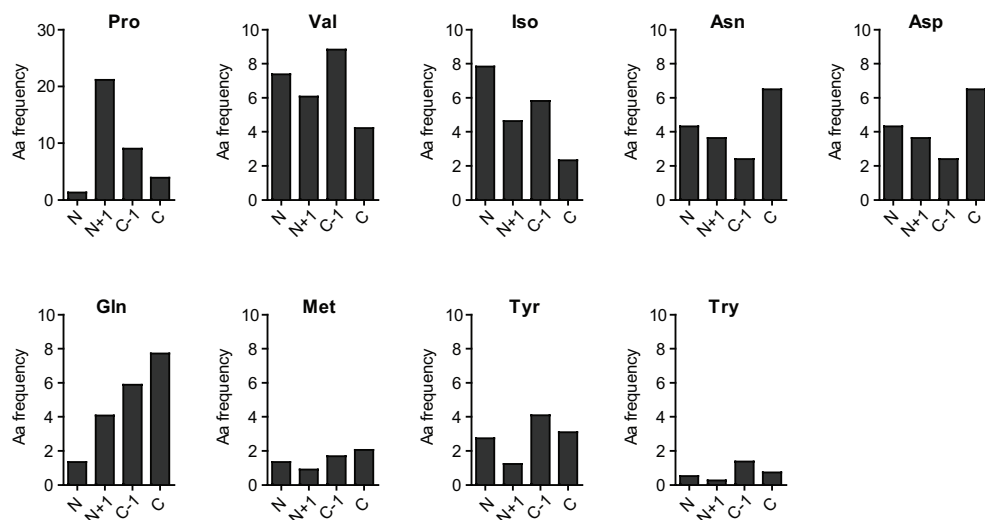
Figure 3: Frequency of aa residues on the N- and C-terminus of HLA-DRB1*03:01 ligands. Plots depict the frequency of the depicted aa residues at the ligands N- and C-terminal residue (designated "N" and "C") or the adjacent residues (designated "N+1" and "C-1") For this analysis HLA-DRB1*03:01-ligands with a length of >15 aa were selected.

A.

position N

Fold enrichment

aa residue: Gly, Ala, Pro, Val, Iso, Asn, Asp, Gln, Met, Tyr, Try

B.

position N+1

Fold enrichment

aa residue: Gly, Ala, Pro, Val, Iso, Asn, Asp, Gln, Met, Tyr, Try

C.

position C-1

Fold enrichment

aa residue: Gly, Ala, Pro, Val, Iso, Asn, Asp, Gln, Met, Tyr, Try

D.

position C

Fold enrichment

aa residue: Gly, Ala, Pro, Val, Iso, Asn, Asp, Gln, Met, Tyr, Try
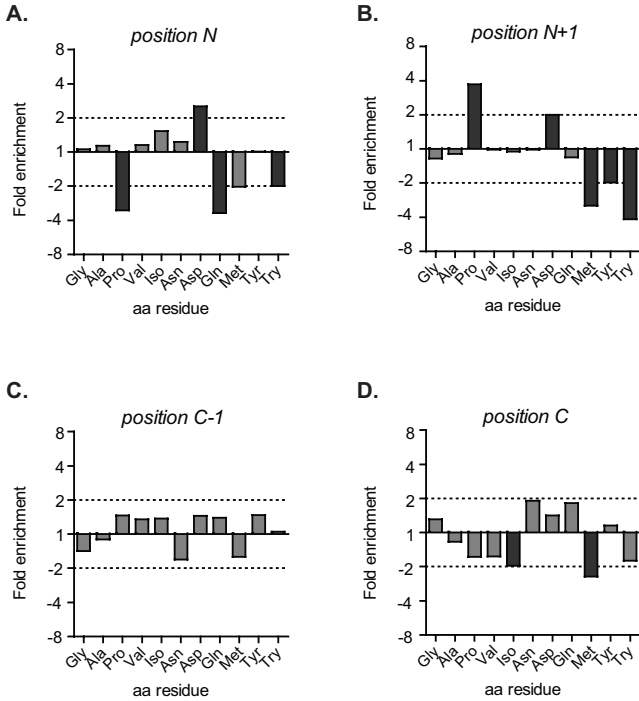
Figure 4: N- and C-terminal enrichment of aa residues in HLA-DRB1*03:01. Plots depict enriched or deprived aa residues on the N- (A and B) and C-terminal (C and D) residues of HLA-DRB1*03:01-ligands. For this analysis peptides with a length of >15 aa were selected. The frequency of aa residues in the human proteome (swiss-prot means) was used as a reference.

The HLA-DRB1*03:01 ligandome of B cells is conserved on the N- and C-terminus

HLA-class II ligands are generated upon lysosome degradation of proteins and are therefore the result of the action of specific proteases and peptidases[18]. We therefore proposed that the action of these proteases/peptidases would be reflected in conservations of particular aa residues in the N- and C-terminal peptide-positions. To analyze this, we selected ligands of ≥15 aa in length to make sure that we are not analyzing p1 and p9 residue conservations and we next determined the frequency of individual amino acid (aa) residues in the peptides N- and C-terminal residues in four different positions: the two N-terminal residues (designated N and N+1) and the two C-terminal residues (C-1 and C). Figure 3 shows aa residues that differ more than two-fold in frequency between the different positions. Importantly, these data provide a first indication that aa residues on the N- and C-terminus of HLA class II ligands are not randomly distributed.

To further study potential conservations, we next compared the frequency of the aa residues presented in Figure 3 with their frequency in the human proteome using IceLogo. Using this approach, we show a more than two-fold increase in aspartic acid and a more than two-fold decrease in proline and glutamine on the N-terminus (Figure 4A). Likewise, on the C-terminus, we observed a more than two-fold decrease in methionine and isoleucine (Figure 4B). We also focused on the peptide position adjacent to the N- or C-terminal position and could demonstrate a clear preference for proline in position 2 of the peptide (designated N+1, Figure 4C) and

A. *position N DR-ligands*

B. *position N +1 DR-ligands*

C. *position C-1 DR-ligands*

D. *position C DR-ligands*

E. *position N DQ-ligands*

F. *position N +1 DQ-ligands*

G. *position C-1 DQ-ligands*

H. *position C DQ-ligands*
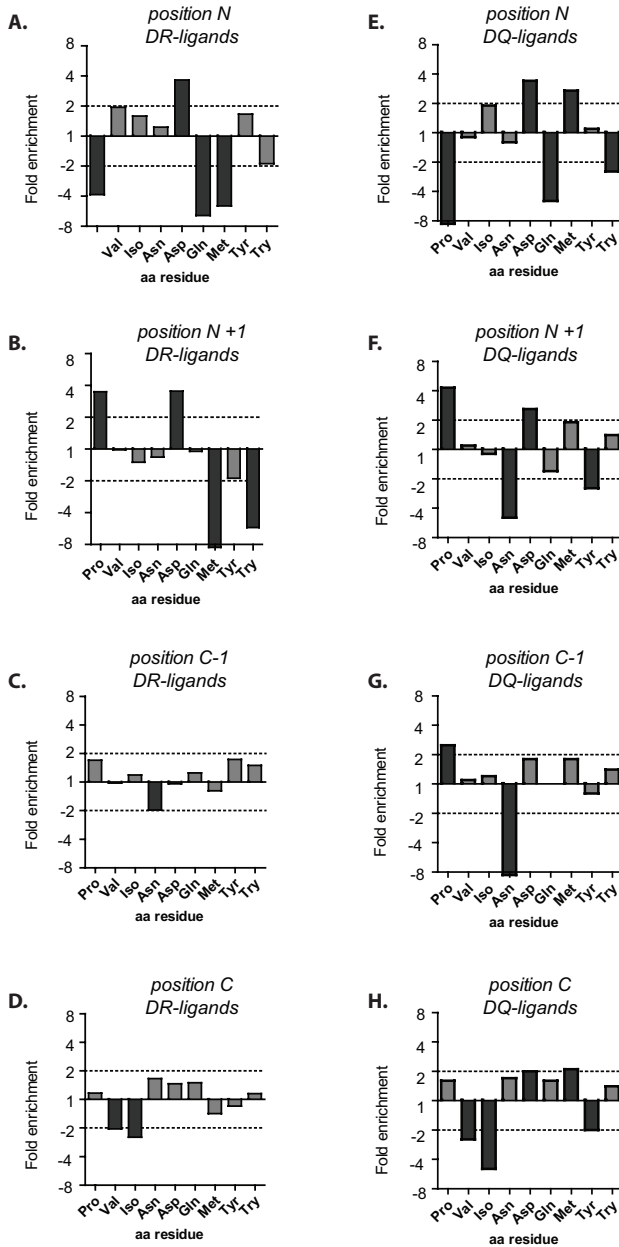
Figure 5: N- and C-terminal enrichment of aa residues in different HLA-DR and HLA-DQ ligandomes. Plots depict enriched or deprived aa residues on the N- (A, B, E and F) and C-terminal (C, D, G and H) residues of HLA-DR- (A-D) or HLA-DQ-ligands (E-H). For this analysis peptides with a length of >15 aa were selected. The frequency of aa residues in the human proteome (swiss-prot means) was used as a reference.

relatively low occurrence of methionine and tryptophan residues. In the second last peptide residues (designated C-1), no aa residues were clearly disfavored (Figure 4D). Importantly, the described conservations are different from the conservations at position p1 and p9 of the peptide binding motif (Figure 2), indicating that the conserved residues at position N, N+1, C, and C+1, are most likely projecting outside the HLA-molecule.

N- and C-terminal conservations are a general feature of HLA-class II B cell ligandomes.

The presented data indicate that the N- and C-terminal side of the studied ligandome is highly conserved. To investigate if this a general feature of HLA class II B cell ligandomes, we next focused on other HLA class II molecules isolated from EBV-transformed B cells. Figure 5 shows N- and C-terminal preferences in two additional sets of ligands (480 ligands ≥15 aa) derived from HLA-DQ (DQ2 and DQ8, in total 480 ligands ≥15 aa) and HLA-DR (DR4, DR8 and DR13, in total 924 ligands ≥15 aa) molecules. Importantly, we can again observe strong con-

servations on both the N- and C-terminus and these conservations largely overlap between different EBV-transformed B cell lines.

The applied reference set is based on Swiss-prot means of the frequency of aa residues in human proteins. To further validate our findings we made use of a second reference set of previously published HLA class I ligands (n=35 050 ligands of 9 aa). HLA class I ligands are conserved in positions p2 and p9. We therefore selected the p4 and p5 positions (at which no conservations are expected) and used the aa frequencies at these positions as a reference. Importantly, using this second set, we can again confirm the described aa conservations on the N- and C-terminus of the HLA class II ligandome (Figure S2).

Together these data indicate that the identified conservations are shared between cell lines, suggesting that these conservations are a general feature of HLA-class II-ligands derived from B cell lines. The observation that similar conservations are found in different HLA-DR/DQ molecules further supports that these conserved positions are not interacting with the polymorphic peptide-binding pockets.

DISCUSSION

We show that HLA-DRB1*03:01 can present a highly diverse ligandome. The presented ligands are mostly between 12 and 20 aa in length and both the core interacting with the HLA-molecule as well as the N-terminal and C-terminal part of these ligands are enriched for particular aa residues.

We show that the electronegative p6 and p9 pocket and the electropositive p4 pocket provide important constraints on the type of peptides that can be accommodated by HLA-DRB1*03:01. Previously it was attempted to elucidate the peptide binding motif of HLA-DRB1*03:01 using peptide substitutions or with small sets of eluted peptides[19-22]. We now constructed a detailed peptide binding motif with 1845 core sequences that could be used in the future to predict potential DR3:T cell epitopes.

Our analysis demonstrate new insights into the influence of antigen-processing on the ligandome. Firstly, The ligands that we identified are derived from sequences throughout the length of the protein, indicating that the enzymes used for processing don't bias the peptide repertoire towards epitopes from particular parts of the protein. Secondly, the number of different ligands or core-sequences derived from a particular protein is not related to protein length, which is in contrast to HLA class I molecules[17]. This observation could be explained by less stringent peptide binding motifs of HLA class II molecules. This is interesting as it suggests that smaller protein are equally represent in the HLA class II ligandome. How, this is achieved remains elusive. Thirdly, the N- and C-terminal ligand residues are conserved for particular aa residues. Our data suggests that this is a general phenomenon of B cell HLA class II ligandomes. We believe this could point to the action of specific pro-

teases/peptidases involved in antigen processing. For instance, we observe a clear conservation of a proline residue at p2. Similar preferences have been described for a member of the cathepsin-family, a family of proteases involved in antigen-processing[23]. These conservations can aid in better predicting potential T cell epitopes or in the design of long-peptide vaccines.

The 13 270 different ligands are now publicly available and can be used to answer a plethora of immunological questions regarding the role of HLA-DR3 in allergy, autoimmunity, infection and transplant rejection.

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SUPPLEMENTARY INFORMATION
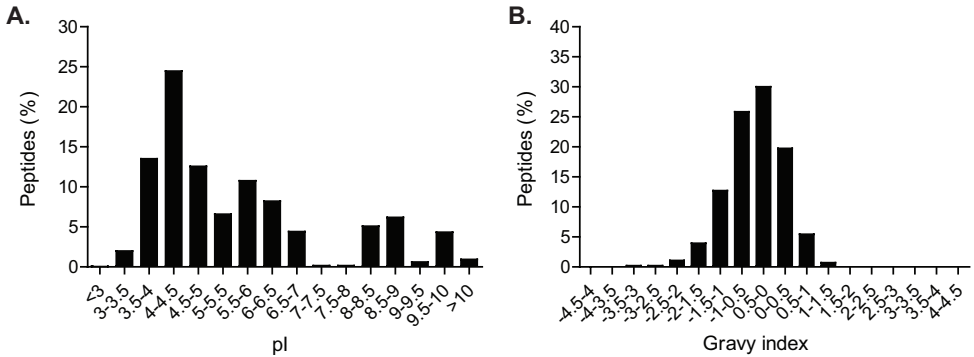
Figure S1: DUCAF-DR3-ligandome physiochemical properties. (A) Distribution of ligands over the pI range. (B) Distribution of ligands over the hydrophobicity range. Ligands were selected based on a BMI ≥ 60.
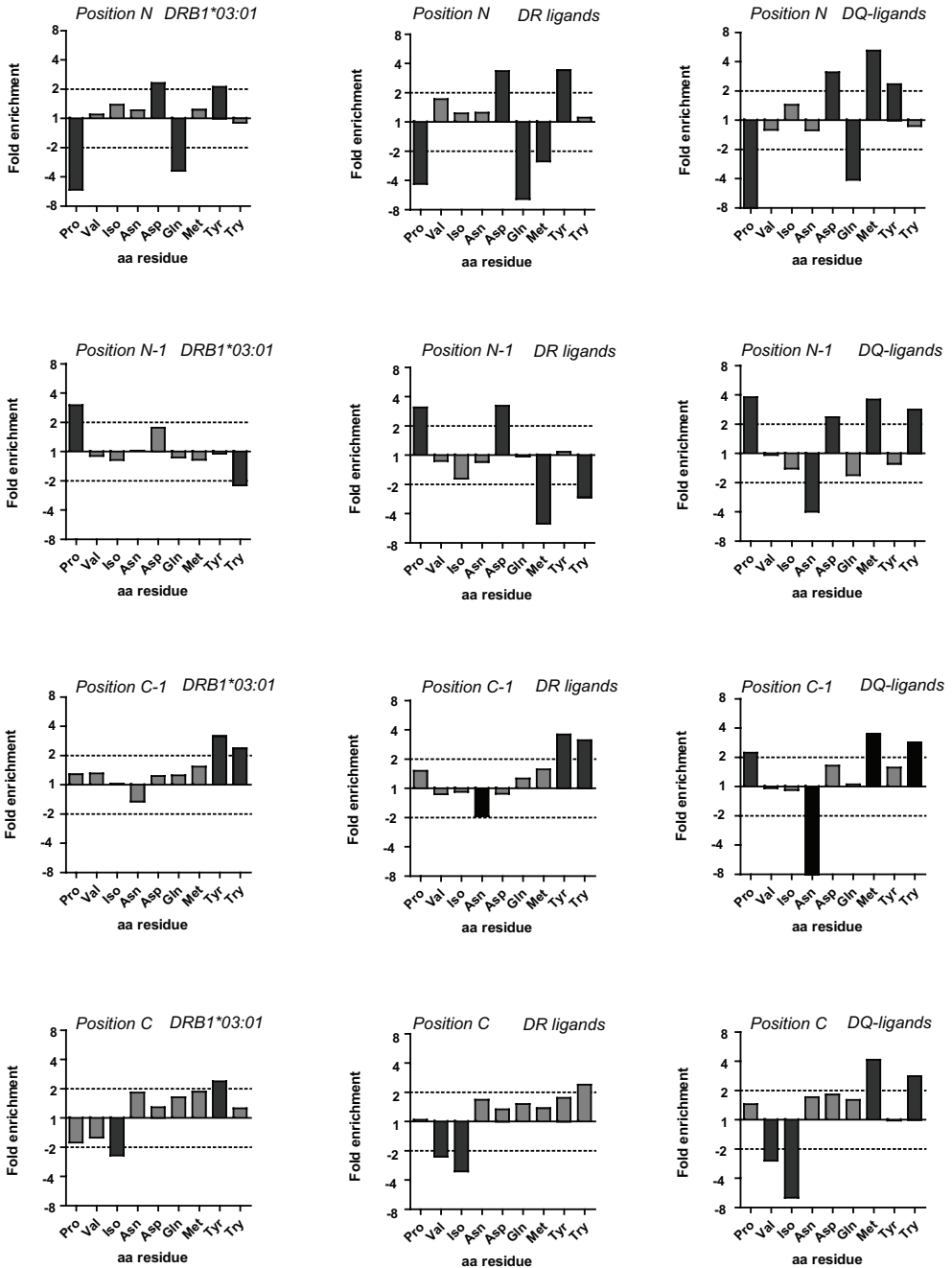
Figure S2: N- and C-terminal enrichment of aa residues in different HLA-DR and HLA-DQ ligandomes. Plots depict enriched or deprived aa residues on the N- (and C-terminal residues of HLA-DRB1*03:01-, HLA-DR- or HLA-DQ-ligands (E-H). For this analysis peptides with a length of >15 aa were selected. The frequency of aa residues in position 4 and 5 of 9mer HLA class I ligands was used as a references

CHAPTER 4

HLA-DQA1*01:03/DQB1*06:03, an HLA-DQ molecule linked to autoimmune resistance, presents a unique peptide repertoire.

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Manuscript in preparation

ABSTRACT

HLA-DQ0603 (DQA1*01:03;DQB1*06:03) is associated with resistance to multiple autoimmune diseases including rheumatoid arthritis, type 1 diabetes, narcolepsy and myasthenia gravis. We aimed to elucidate the peptide-binding repertoire of HLA-DQ0603 by isolating HLA-DQ0603 molecules from B-LCL lines and subsequent characterization of HLA-DQ0603-bound ligands. Characteristics of the identified ligands were comparable to what was previously shown for other HLA-class II molecules (e.g. ligand length and localization of protein-sources). After peptide-alignment we obtained the peptide-binding motif of HLA-DQ0603. We found that HLA-DQ0603 presented a unique peptide-binding repertoire that differed from previously identified peptide-binding motifs of closely related HLA-DQ6 molecules. Differences were mainly in pocket 1 and pocket 6 and can be translated to structural differences between different HLA-DQ molecules. This motif allows for more precision in the identification of disease-linked epitopes.

INTRODUCTION

The Human Leukocyte Antigen (HLA) class II locus is the primary risk factor for many different autoimmune diseases (AID), including multiple sclerosis (MS), rheumatoid arthritis (RA), type 1 diabetes (T1D), narcolepsy and Myasthenia Gravis (MG) [1-5]. These HLA associations with autoimmunity suggest an important role for antigen-specific T cells during pathogenesis. For most of these autoimmune diseases the antigens recognized by such autoimmunogenic T cells are unclear.

The HLA class II locus contains genes that are in strong linkage disequilibrium [6]. As a result, these genes inherit together in haplotypes making it difficult to genetically pinpoint the causative gene. In addition, this locus can play a dual role. Certain haplotypes can be associated with susceptibility, while others are neutral or even associated with resistance. Most haplotypes are differentially associated with multiple autoimmune diseases. For example, the HLA-DRB1*04-DQB1*03:01-DQA1*03:02 haplotype is strongly associated with risk for RA, yet protective in T1D [7-9]. However, other haplotypes display a similar effect throughout different autoimmune diseases. The haplotype HLA-DRB1*13:01-DQB1*06:03-DQA1*01:03 is of particular interest as it is strongly associated with resistance to different autoimmune diseases including RA [7, 10], T1D [9, 11-13], MG [14] and narcolepsy [15]. The underlying immunological pathways are currently elusive.

The ligandome of HLA-DRB1*13:01 was previously characterized [16], but the peptide-binding repertoire of HLA-DQ0603 (HLA-DQB1*06:03/DQA1*01:03) is unknown. We therefore aimed to elute naturally processed ligands and to characterize the peptide-binding repertoire for this HLA-DQ molecule.

MATERIALS AND METHODS

Ligand isolation from affinity-purified HLA class II molecules. HLA-DQ0603-molecules were isolated from DRB1*13:01-DQB1*06:03-DQA1*01:03 homozygous EBV-transformed B lymphoblastoid cell line APD. Approximately $2x10^9$ cells were grown in IMDM supplemented with L-glutamine and 10% FCS. Subsequently, the cells were harvested, washed with PBS, and the cell pellet was stored at -80°C. The cells were lysed with 50 ml of lysis buffer (50 mM Tris, 150 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40, and protease inhibitors (Complete inhibitor mix; Roche)) to remove the nuclei and insoluble material. The lysate was centrifuged for 60 min at 10,000g. It was subsequently precleared for 60 min with Sepharose beads and mixed with SPV-L3 coupled sepharose beads. After 60 min of gentle mixing, the beads were washed in 10 bead volumes of lysis buffer followed by washing with 20 mM Tris-HCl, 120 mM NaCl (pH 8.0), followed by washing with 20 mM Tris-HCl, 1 M NaCl (pH 8.0), 20 mM Tris-HCl (pH 8.0), and finally with 10 mM Tris-HCl (pH 8.0). Subsequently, the HLA-peptide complexes were eluted with 25 ml of 10% acetic

A

```
              1         11        21        31        41        51        61        71        81
DQB1*06:03 RDSPEDFVYQ FKGMCYFTNG TERVRLVTRH IYNREEYARF DSDVGVYRAV TPQGRPDAEY WNSQKEVLEG TRAELDTVCR HNYEVAFRGI
DQB1*06:02 --------F- ---------- ---------T ----Y----- ---------- ---------- ---------- ---------- ----------
DQB1*06:04 ---------- ---------- ---------- ---------- ---------- ------Y--- --------R- ---------- -----GY---
```

B

Figure 1: Characteristics of the HLA-DQ0603 molecule. (A) Sequences of HLA-DQB1*06:03 in comparison to HLA-DQB1*06:02 and *06:04. (B) Schematic representation of aa involved in shaping the different peptide-binding pockets of HLA-DQ0603 (DQB1*06:03/DQA1*01:03). Amino acids are colored according to their properties (white = hydrophilic, light-gray = hydrophobic, dark-gray = acidic, black = basic). Numbering below the amino-acids indicate their positions in the DQA1 (gray) or DQB1 (black) chain.

acid in water. All purification steps were performed at 4°C. High molecular mass material (HLA molecules) was removed by filtration through Centriprep filtration units with a cutoff value of 10 kDa. Ligands were identified as previously described. Briefly, ligands were fractionated on a C18 reverse phase HPLC system and analyzed by tandem mass spectrometry.

Peptide identification by mass spectrometry (MS) Ligands were characterized by an online nano-HPLC-MS system. The nano-HPLC column was connected to the needle of the electrospray source of the mass spectrometer. The mass spectrometer was run in data-dependent MS/MS mode during ligand elution. All tandem mass spectra produced in this way were searched against the human IPI database with the database search program MASCOT version 2.4 (Matrixscience). All reported hits were assessed manually, and ligands with best MASCOT ionscores <50 were discarded.

Peptide binding motif. The peptide binding motif of HLA-DQ0603 was constructed by aligning all the identified ligands and subsequent identification of the most likely nonamers. Aa frequency at each of the positions in the nonamer were compared to the frequency of these aa residues in the human proteome.

RESULTS

Characterization of the peptide-binding repertoire of HLA-DQ0603

The presence of HLA-DQ0603 is tightly associated with autoimmune resistance. Figure 1A shows the sequence of the HLA-DQB1*06:03 chain and a comparison with HLA-DQB1*06:02 and *06:04. Figure 1B shows a schematic representation

of the residues involved in shaping the various peptide-binding pockets of HLA-DQB1*06:03. Most notable is the β57D residue. The presence of β57D is strongly associated with T1D risk and it was described that this residue forms a salt-bridge with α76, thereby enhancing the stability of the peptide-MHC complex [17].

To examine the peptide-binding repertoire of HLA-DQ0603, we isolated HLA-DQ molecules from an EBV-transformed HLA-DQ0603 homozygous B cell line. Ligands derived from these HLA-DQ molecules were characterized. In total we identified 698 unique ligands. Most of the identified ligands were derived from HLA molecules (23%) as was previously described for ligands derived from other HLA class II molecules [18].

The ligands ranged in length between 7 and 20 amino acids. 94% of the ligands ranged between 12 and 18 aa in length as was previously shown for other HLA class II molecules (Figure 2A) [18, 19]. As HLA class II molecules are flexible in the size of ligands that can be accommodated, nested peptide-sets can be frequently found. These sets include different ligands with a shared core-sequence but of a different length. When we discard these length variants, 303 core sequences were identified. For 71% of the core sequences we found only a single length variant. For the remaining 29% we found up to 37 different length variants (Figure 2B). We also studied the localization of the proteins from which the identified ligands were derived. About 60% of the ligands were derived from the endocytic pathway, the remainder
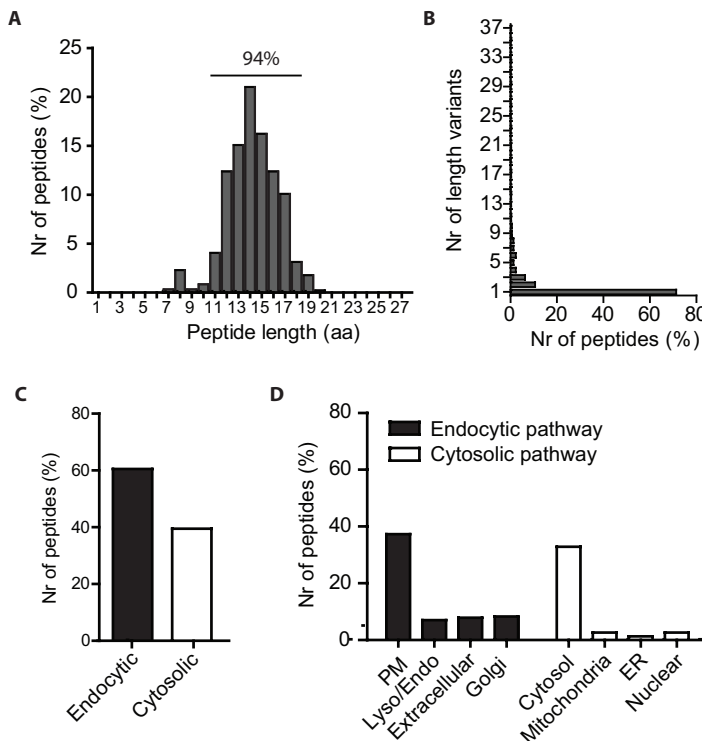
Figure 2: Characteristics of identified ligands. (A) Graph depicting the distribution of peptide length in the identified ligands. (B) Graph depicting the number of length variants identified for the different core ligand-sequences. (C) Graph depicting the percentage of peptides derived from a cytosolic or an endocytic source. (D) Graph depicting the different sources from which the identified peptides were derived. PM = plasma membrane, Lyso/Endo = lysosome or endosome, ER = endoplasmatic reticulum.
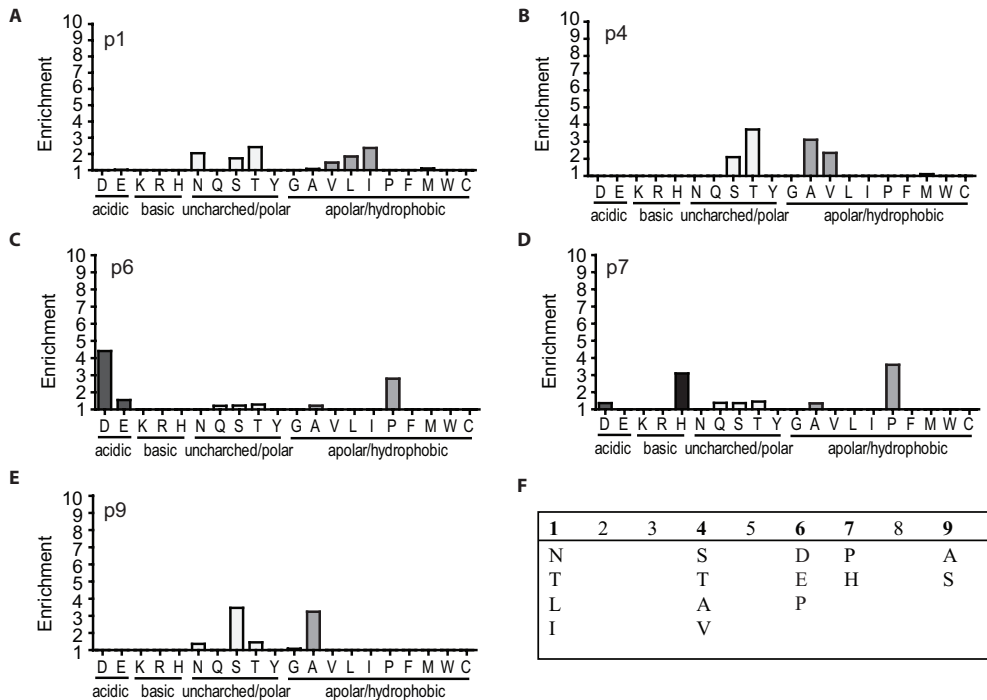
Figure 3: Peptide-binding motif of HLA-DQ0603. (A-E) Ligands derived from HLA-DQ0603 were aligned and the frequency of aa at positions interacting with pockets were compared to their distribution in the human proteome. Plots depict enrichment for amino acids at positions predicted to interact with pocket 1 (A), pocket 4 (B), pocket 6 (C), pocket 7 (D) and pocket 9 (F) of HLA-DQ0603 Aa color conventions are as in Figure 1. (F) Summary of the peptide-binding motif of HLA-DQ0603.

from the cytosolic pathway (Figure 2C). Most ligands were derived from the plasma membrane, which includes all the HLA-derived ligands. The second most abundant source is the cytosol (Figure 2D. Peptide-localizations were comparable to what was previously shown for other HLA class II molecules [20, 21].

The peptide-binding motif of HLA-DQ0603

To determine the peptide-binding motif of HLA-DQ0603 from the eluted ligands, we performed both manual and automated ligand alignment, both resulting in similar results. Figure 3 shows the results from automated ligands alignments. The presence of aa residues in positions interacting with the various pepti-de-binding pockets were compared to the frequency of that particular aa in the human proteome. In the aa residues predicted to interact with pocket 1, there are clear preferences for asparagine, serine, threonine, leucine and isoleucine residues, all small to medium sized amino acid residues (Figure 3A). In pocket 4, a serine, threonine, alanine and valine are enriched. These are all small amino acid residues (Figure 3B). Residues in p6 are enriched for acidic residues and

proline (Figure 3C). In pocket 7, ring-shaped aa histidine and proline are preferred (Figure 3D). Finally, pocket 9 prefers the small amino acid residues alanine and serine (Figure 3E). The corresponding binding motif is summarized in Figure 3F.

DISCUSSION

This is the first report characterizing the peptide-binding repertoire of HLA-DQ0603, an HLA-DQ molecule tightly associated with autoimmune resistance. Using naturally eluted ligand sequences we have also established its peptide-binding motif. This allows for a comparison with other previously characterized HLA-DQ molecules.

HLA-DQ0602 (DQB1*06:02-DQA1*01:02) is of particular interest. This HLA-DQ molecules is tightly linked with susceptibility for narcolepsy [15, 22]. This HLA-DQ molecule is highly related to the HLA-DQ0603 (Figure 1A). Both HLA-DQ molecules differ only in two amino acid positions: β9 that shapes peptide-binding pocket 9 and β30 that shapes peptide-binding pocket 6 [23]. The difference in β9 is relatively a minor difference (phenylalanine in DQB1*06:02 and tyrosine in DQB1*06:03). In contrast, the difference in β30 (tyrosine in DQB1*06:02 and a histidine in DQB1*06:03) would result in a more positively charged p6 pocket. Likewise, if we compare the peptide-binding motif of HLA-DQ0602(24;25) and HLA-DQ0603, we

Molecule	Name	Sequence
Hypocretin (O43612)	Hcrt 3-11	LPSTKVSWA
	Hcrt 6-14	TKVSWAAVT
	Hcrt 83-91	LLQASGNHA
	Hcrt 89-97	NHAAGILTL
Hypocretin receptor 1 (AAL47214)	HcrtR1 88-96	LSLADVLVT
	HcrtR1 125-133	LQAVSVSVA
	HcrtR1 300-308	LMVVLLVFA
Hypocretin receptor 2 (AAL47215)	HcrtR2 63-71	IVFVVALIG
	HcrtR2 70-78	IGNVLVCVA
	HcrtR2 96-104	LSLADVLVT
	HcrtR2 98-106	LADVLVTIT
	HcrtR2 111-119	TLVVDITET
	HcrtR2 135-142	TVSVSVSVL
	HcrtR2 427-435	TSISTLPAA
	HcrtR2 171-179	NSIVIIWIV

Table 1: List of predicted epitopes that bind to HLA-DQB1*06:02 but not to HLA-DQB1*06:03.

cannot identify differences in the p1, p4, and p9 residues, but we do find large differences in the p6 residues. HLA-DQ0602 is reported to prefer leucine, isoleucine and valine residues, all medium-sized hydrophobic residues [24, 25]. In contrast, we showed a preference for acidic residues in the p6 pocket of HLA-DQ0602, which can be directly explained by an β30Y→H substitution. An understanding of these differences allows for predicting candidate narcolepsy epitopes. In Table 1, we have summarized candidate epitopes from three narcolepsy candidate autoantigens (hypcretin and hypocretin receptor 1 and 2) that we predict to selectively bind to HLA-DQ0602 and not to DQ0603 and that can therefore be attractive candidate epitopes. Indeed, Hcrt3-11 was previously crystallized in the context of HLA-DQB1*06:02 and was presented in the predicted register [26].

The peptide-binding motif of HLA-DQ0604 (DQB1*06:04-DQA1*01:02) has also been established [25]. This allele is associated with T1D susceptibility. HLA-DQB1*06:03 differs from HLA-DQB1*06:04 in three different positions: β86 (A→G) that shapes the p1 pocket, β57 (D→V) that is involved in peptide-MHC stability and β70(G→R) that is involved in TCR recognition [17, 23, 27] (Figure 1A). β86 renders the p1 pocket more spacious allowing the accommodation of large residues [25]. Together, these comparison illustrates how small differences in the structure of HLA-DQ molecules are translated in large differences in the peptide-binding repertoire. This study shows that HLA-DQ0603 presents a unique peptide-binding repertoire, distinct from highly related HLA-DQ6 molecules, likely resulting in distinct T cell repertoires that could explain HLA-DQ0603-mediated autoimmune resistance.

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PART II

Towards a molecular basis for the connection between HLA, Rheumatoid Arthritis and Autoantibodies

CHAPTER 5

An investigation of the added value of an ACPA multiplex assay in an early arthritis setting.

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ABSTRACT

Introduction. Recently, arrays have become available that allow the simultaneous analysis of several anti-citrullinated protein antibody (ACPA) reactivities using distinct citrullinated peptides. Such assays are designed for exploratory studies. The interpretation of positive antibody reactivity's can best be made if the diagnostic and prognostic value of a multiplex array in an early-arthritis setting is known and if the multiplex-positive patients who are negative according to 3 commonly used commercial ACPA assays are characterized.

Methods. Using Thermo Scientifics' ImmunoCap ISAC system, a multiplexed array that determines reactivities to 11 citrullinated peptides, we analysed serum/plasma of 195 healthy controls and 1282 early arthritis patients from two independent cohorts: the Leiden Early Arthritis Clinic (n=1013) and the IMPROVED (n=269). Findings were compared to results primarily of the anti-CCP2 assay, but also to CCP3 and anti-MCV assays. The associations between ACPA reactivities and patient characteristics, risk factors (shared epitope, smoking) and disease outcomes (progression of undifferentiated arthritis to RA and severity of joint destruction) were assessed.

Results. 31% of anti-CCP-2-negative RA patients displayed reactivity towards citrullinated peptides in the multiplex assay. These patients had a positive signal towards a more restricted peptide repertoire than anti-CCP-2-positive RA patients (median = 1 versus 5). Within anti-CCP-2-negative patients, ACPA reactivity as detected by multiplex array was not significantly associated with known risk factors, clinical or prognostic parameters. The frequency of sera from anti-CCP-2-negative RA patients that were positive for the multiplexed peptides was comparable to the frequency in non-RA arthritic patients (27%).

Conclusions. Additive citrulline peptide reactivities detected by the current multiplex system did not reach significant power to be RA-specific. The presence of residual citrulline reactivities detected by the multiplex-system used in arthritis patients that are negative in commercial ACPA assays need to be interpreted with caution.

INTRODUCTION

Rheumatoid Arthritis (RA) is a systemic inflammatory disease characterized by extensive inflammation of synovial joints[1]. RA patients frequently present with autoantibodies, of which the anti-citrullinated protein antibodies (ACPA) are of particular interest. These autoantibodies are highly specific for RA and specifically target proteins that have undergone a post-translational modification that converts arginine to citrulline residues. ACPA can be used to predict the development of RA as these autoantibodies can be detected up to ten years prior to disease onset[2, 3]. Furthermore, ACPA can subdivide RA patients in distinct diseases subsets. These subsets differ substantially in clinical outcome and with regard to underlying genetic and environmental risk factors[4-6].

Several commercial tests are available that use specific citrullinated antigens to determine ACPA positivity. The most widely used tests are anti-citrullinated cyclic peptide 2 (CCP-2), anti-CCP-3 and anti-mutated citrullinated vimentin (anti-MCV)[7, 8]. Anti-CCP-2 has been used in most studies and has long been the standard test to determine reactivity to citrullinated peptides, although the sequence identity has not been disclosed [9]. It has been postulated that because commercial anti-CCP-tests employ peptide-sequences that are, most likely, not derived from human proteins, these tests do not detect part of the citrullinated-peptide reactivities[10]. Therefore, arrays have been developed to do exploratory studies on a large number of peptides. This could be important to enhance understanding of disease as well as identify antibody systems that are predictive of disease characteristics which can occur in a number of different diseases such as exocrine gland dysfunction which is associated with anti-SSA antibodies.

Recently, arrays have become available that can determine citrullinated peptide reactivities towards a wide variety of natural citrullinated antigens in a relatively high-throughput setting[10, 13]. Intriguingly, these arrays showed the presence of reactivities against citrullinated peptides within anti-CCP-2-negative RA patients. This raised the question what a positive signal in such an array means and to guide subsequent exploratory studies, we determined whether a positive signal in a multiplex assay has an additive value over commercial assays determining the presence of ACPA and whether these patients share the characteristics of RA patients positive in commercial ACPA assays.

We aimed to perform a detailed characterization of the value of multiplex ACPA-testing in an early-arthritis setting by studying the characteristics of anti-CCP-2-negative patients that are positive or negative for additional citrulline reactivities. We studied clinical parameters and the presence of genetic and environmental risk factors using a previously validated multiplex chip-based array, the ImmunoCAP ISAC system[13-16]. Using this array, we measured reactivity against 11 different citrullinated peptides derived from fillagrin, type II collagen, alpha-enolase, fibrinogen and vimentin in patients from two independent arthritis cohorts.

METHODS

Patients

Citrullinated peptide reactivities were assessed on baseline plasma samples of arthritis patients (n=1013) included in the Leiden Early Arthritis Clinic (EAC). This Dutch cohort was initiated in 1993 and includes patients with a recent onset of arthritis (symptom duration < 2 years) from the Leiden area[17, 18]. Definitive diagnoses were determined after 1 year of follow-up by an experienced rheumatologist. In the current analysis 564 patients fulfilled the ACR1987 criteria for RA. Patients not fulfilling the RA criteria, who had no other definite clinical diagnosis, were classified as undifferentiated/unclassified arthritis (UA, n=297). The remaining 149 patients were classified as non-UA/non-RA patients. 135 out of 149 patients were anti-CCP-2 negative and these presented with a wide variety of diagnoses (Table S1).

We also assessed citrullinated peptide reactivities in baseline serum samples of 269 patients that were included in the IMPROVED study and that fulfilled the ACR1987 criteria for RA at the time of inclusion [19]. The IMPROVED study (ISRCTN11916566) was a randomized controlled trial investigating the effect of different treatment regimens in early arthritis.

Genotyping of the HLA-DRB1 region in order to determine the number of RA-associated Shared Epitope (SE) alleles was available for 500 patients (EAC only)[20]. Within the EAC, radiographs were taken at baseline and at yearly follow-up visits for 7 years. In total 2340 sets of hands and feet radiographs of 463 RA patients were scored using the Sharp- van der Heijde scoring methods (SHS) by one experienced reader with known time-order (intra-class correlation coefficient 0.91) [21].

Patient samples were compared to serum samples of 195 healthy controls. These controls lived in the Leiden area and were matched to the patients for age and gender.

The study was performed in accordance with the declaration of Helsinki and protocols were approved by the Leiden University Medical Center ethics committee. Informed consent was obtained.

Citrullinated peptide assays

Anti-CCP-2 was measured on the Immunoscan RA Mark 2;Eurodiagnostica, Arnhem, The Netherlands with a cut-off of 25 arbitrary units in all patients. Anti-CCP-3 and anti-MCV were measured in 454 EAC RA patients by respectively Quanta lite CCP 3.1 IgG/IgA (INOVA Diagnostics Inc., San Diego, CA, USA) and Orgentec Diagnostika GmbH (Mainz, Germany) with a cut-off of 20 arbitrary units. Cut-offs were set using manufacturer's instructions. IgM-RF was determined by routine diagnostic laboratory screening in 614 RA patients (EAC and IMPROVED).

Antibody responses to citrullinated peptides were measured with the ImmunoCAP ISAC system (Thermo Scientific) that contains 11 different citrullinated peptides and their arginine counterparts: CCP1, CitC1 (CII355-378cit3), CEP1, Fibα36-50, Fibα621-

635, Fibβ36-52, Fibβ60-74, Fibβ563-583, Fibβ580-600, Vim2-17 and Vim60-75 as described elsewhere [13-16, 22]. We excluded 4% of the tested samples from the analyses as antibody responses could not be accurately determined due to high background signals or unspecific reactivity towards streptavidin.

For cut-off calculations, the citrulline-specific signal was used (difference in fluorescence intensity between the citrullinated peptide and the arginine-containing peptide). The cut-off was established as the mean plus two times the standard deviation (SD) of the citrulline-specific signals in 195 healthy control sera. For CitC1 (a peptide derived from collagen type II) we used the citrulline signal without subtracting the arginine signal as this is a non-linear peptide and have independent but exclusive reactivities to the arginine counterpart [23].

For reasons of sample availability, plasma of EAC patients and serum of IMPROVED patients and healthy controls was used for the multiplex assay. For several individuals (n=59) both serum and plasma with 92% reproducibility (data not shown).

Statistical analysis

Linear regression was performed to study the association between log-transformed median anti-CCP-2 levels and the number of peptides recognized in the multiplex array. Differences in anti-CCP-2 levels between groups were analysed with the Mann-Whitney test.

To determine the association between the risk factors HLA-SE, smoking and RA, unconditional logistic regression analysis was performed to calculate odds ratio's (ORs) and 95% confidence intervals (CIs). Multiplex-negative RA patients (either anti-CCP-2-negative or –positive) were used as the reference category and compared with multiplex-positive RA patients (again in both categories: anti-CCP-2-positive or anti-CCP-2-negative). Similarly the association between multiplex-positivity or anti-CCP-2-positivity and progression to RA was assessed. To study progression, we used selected patients that are classified as undifferentiated arthritis (UA) patients at baseline and we assessed the development of RA in the first year of follow-up using the ACR1987 criteria for RA.

To study biological interaction between smoking, HLA-SE and multiplex-positivity in anti-CCP2 negative RA patients was performed as described elsewhere[24]. To analyse the rate of joint destruction, a multivariate normal regression model for longitudinal data with log-transformed SHS as response variable was used as previously described[25]. This method analyses al repeated measures at once and takes advantage of the correlation between these measurements. Analyses were adjusted for age, gender and different inclusion periods as proxy for treatment strategy [17]. Anti-CCP-2-negative RA patients were used as reference and compared with anti-CCP-2-positive RA patients. We also used anti-CCP-2-negative multiplex-negative and anti-CCP-2-negative multiplex-positive RA patients as a reference and these were compared with respectively anti-CCP-2-negative multiplex-positive and anti-CCP-2-positive multiplex-positive RA patients. The beta (β) indicates the relative

fold increase in radiographic joint damage per year.

RESULTS

The presence of citrulline-reactivities by multiplex assay in RA patients.

Before embarking on determining the diagnostic value of a prototype multiplex array in an early arthritis setting, we first wished to validate the assay by comparing the results in the Leiden population of RA-patients to previously published data using other sets of RA-patients. We used a multiplex-array to measure reactivities against the arginine and citrulline variants of 11 different antigens. All citrullinated peptides were recognized by a proportion of RA patients in the EAC and the IMPROVED cohort (Figure 1A). In 8% of the tested healthy controls, citrulline-reactivities were present and 13/15 multiplex-positive controls recognized a single citrullinated peptide (Figure 1B). In RA patients, citrulline reactivities were observed in the majority of patients (64% in EAC, 75% in IMPROVED) and positive patients were generally positive for more than one peptide (79% in EAC and 84% in IMPROVED, Figure 1C). Notably, the frequency of anti-CCP-2-positive RA patients is higher in the IMPROVED than in the EAC (65% vs 51%), probably explained by the different inclusion criteria.

We next focused on anti-CCP-2-positive RA patients. The multiplex chip-based-array detected citrullinated peptide reactivities in 274/285 (96%) anti-CCP-2-positive EAC RA patients (Figure 2A). Percentages were comparable in the IMPROVED cohort (169/174, 97%) and with anti-MCV- or anti-CCP-3-positive EAC RA patients (241/273 (88%) and 234/261 (90%) respectively). The number of recognized citrullinated multiplex peptides associated with median anti-CCP-2 antibody levels (Figure 2B, R2 = 0.86, P < 0.0001) as has also been reported for ACPA fine specificities determined by ELISA[26]. Likewise, the 4% of anti-CCP-2-positive multiplex-negative RA patients displayed lower anti-CCP-2 levels. (Figure 2C, P=0.009). These data show that nearly all anti-CCP/MCV-positive RA patients are positive in the multiplex-array. The small multiplex-negative subgroup represents patients with lower anti-CCP-2 levels.

Most importantly, the test characteristics described above are comparable to previously published data, indicating that the multiplex array reproducibly detected reactivities against commonly used citrullinated peptide tests in RA patients.

Multiplex-positive anti-CCP-2 negative RA patients

Recent reports using multiplex-platforms with citrullinated peptides also showed positive response in anti-CCP-2-negative RA patients[10, 13, 26]. To determine whether this could also be found in the Leiden RA population, we next analysed the multiplex-array reactivity in the anti-CCP-2-negative RA population. In the EAC, 31% (86/279) of the anti-CCP-2-negative RA patients tested positive in the multiplex assay versus 34% (31/91) in the IMPROVED (Figure 3A). Similar percentages were
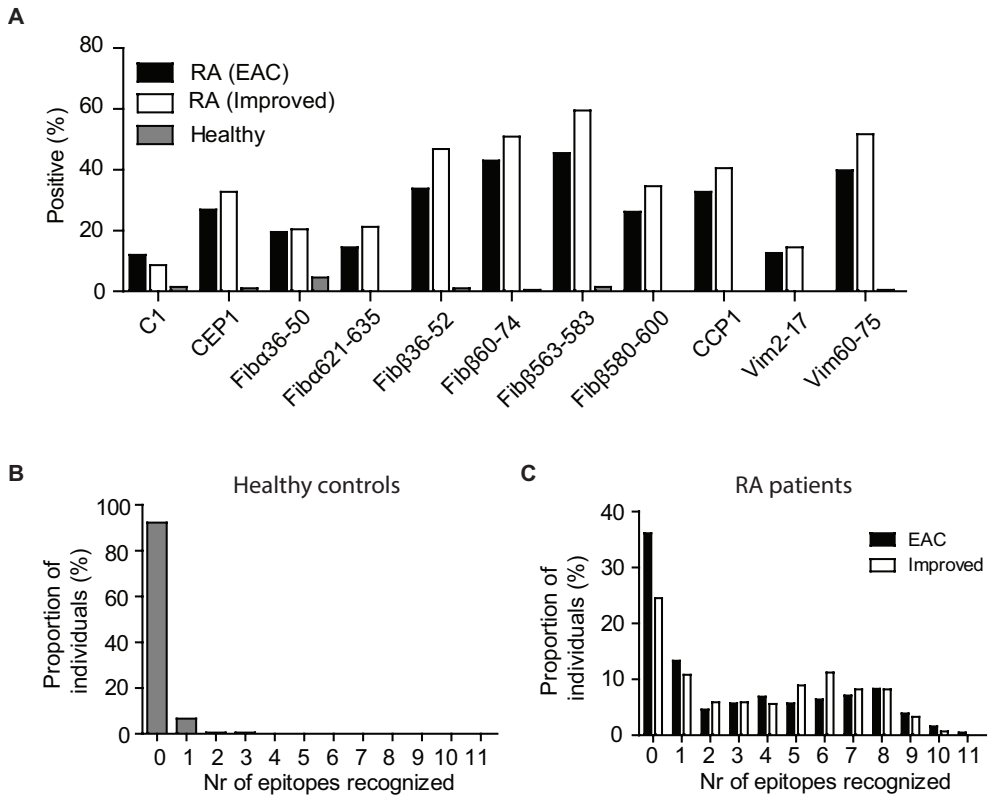
Figure 1: Citrulline-reactivities in RA patients and healthy controls. A. Plot depicts the frequency of positive RA patients in the EAC (n=564, in black) or the IMPROVED (n=269, in white) and healthy controls (n=195, in grey) for the different tested citrullinated peptides. B. Percentage of healthy controls plotted against the number of citrulline-reactivities. C. Percentage of RA patients (EAC in black, IMPROVED in white) plotted against the number of citrulline-reactivities.

found in anti-CCP-3-negative (29%, 56/193) or anti-MCV-negative (27%, 49/181) RA patient populations. These findings are also comparable to previously reported observations using similar assays in other sets of patients.

Diagnostic value of the multiplex-array in an early arthritis setting.

The finding that a substantial proportion of ACPA-negative RA patients, as determined by 3 commercial assays, shows reactivity in the multiplex assay is intriguing and prompted us to determine whether this assay has an additive value over "conventional" ACPA-testing in an "early arthritis setting". We next analysed multiplex-positivity in EAC patients with a form of arthritis other than UA or RA (so-called non-RA, n=149). 30% (45/149) of non-RA patients were multiplex-positive. In anti-CCP-2-negative (135/149) non-RA patients, 27% (37/135) was multiplex-positive, without a predilection for a particular diagnosis (Figure 3B). Similar findings were
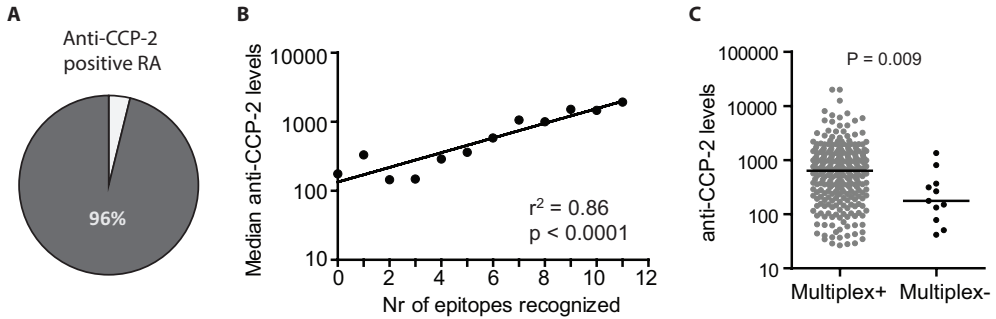
Figure 2: Multiplex-positivity correlates with anti-CCP-2 levels. A. Percentage of anti-CCP-2 positive RA patients (n=285) in the EAC that are positive or negative in the multiplex array. B. Median anti-CCP-2 levels plotted against the number of recognized peptides. A linear regression was used to determine the degree of correlation. C. Anti-CCP-2 levels in multiplex negative (n=11) and positive (n=274) RA patients. Each dot represent an EAC patient. Median anti-CCP-2 levels were compared using a Mann-Whitney test.

made when the anti-CCP-3 and anti-MCV-assays were used to determine the presence of ACPA.

The findings described above were reflected in the sensitivity and the specificity (in relation to the original ACR 1987 criteria) of the multiplex assay (63.8% and 63.7%) in comparison with the anti-CCP-2 ELISA (51% and 87%). To calculate the sensitivity and specificity, all 446 patients in the EAC without RA were taken into account. These patients had either UA or another form of arthritis. The positive likelihood-ratio (LR+) for the multiplex is lower (1.76 versus 4.02 for anti-CCP2), whereas the LR- is similar (0.57) (Figure 3C-D). These analyses were performed using the ACR1987 criteria for RA since the EULAR/ACR2010 criteria includes anti-CCP-2 status, thereby introducing potential circular reasoning.

The diagnosis of non-RA patients was initially assessed after 1 year by an experienced rheumatologist. To rule out the possibility that the multiplex-positive non-RA patients were misdiagnosed or that they developed RA later in time, we re-assessed their diagnosis, radiographs and medication use at their last visit. Two non-RA patients were now classified with RA. Taking these findings into consideration and assuming that these 2 patients were initially misdiagnosed, the sensitivity of the multiplex assay increased from 63.7% to 64.1%.

All analyses described above have been performed using the same cut-off (mean+2xSD of the citrulline-specific signal in healthy controls). However, using several different cut-offs did not change the outcome/conclusions of the studies (data not shown).

Together these data illustrate that a large subset of anti-CCP/MCV-negative arthritis patients is positive in the multiplex-array. However, the multiplex has a low RA-specificity.
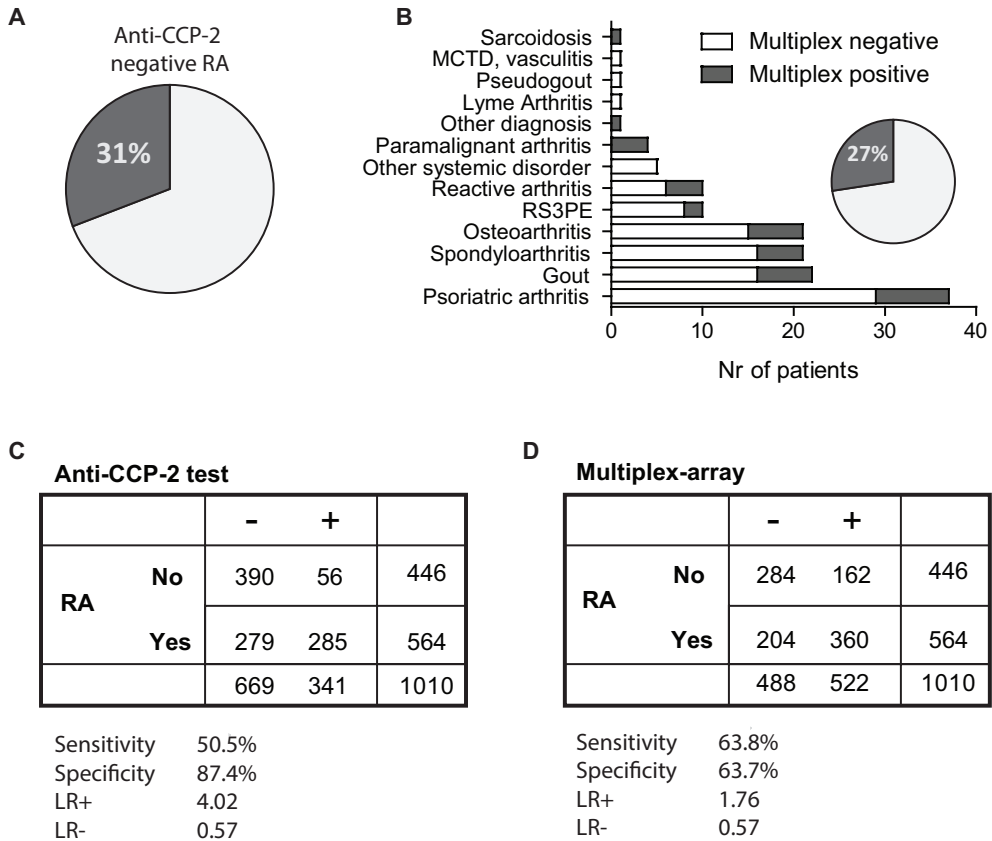
Figure 3: Multiplex-positivity in anti-CCP-2 negative RA patients is not RA specific. A. Percentage of anti-CCP-2 negative RA patients (n=279) in the EAC that are positive (n=86) or negative (n=193) in the multiplex array. B. Pie diagram summarizes the percentage of multiplex positive (n=37, grey) and negative (n=98, white) anti-CCP-2 negative non-RA/UA patients (n=135). Bar diagram plots the number of patients that are multiplex positive and negative with different diagnoses. C-D. The ACR1987 criteria were used to calculate the sensitivity and specificity of the anti-CCP-2 test (C, E) and of the multiplex-array (D, F).

Characteristics of anti-CCP-2-negative RA patients positive or negative for citrullinated peptide reactivities.

As the multiplex assay was designed to identify antibodies and antibody patterns to formulate new hypothesis regarding of value for understanding etiology, pathogenesis , prognosis, and prediction, we wished to investigate whether the multiplex assay could be useful to stratify anti-CCP-2-negative RA patients into different subsets using other outcome parameters. There were no statistical differences in baseline characteristics between multiplex-negative and –positive EAC patients, although female anti-CCP-2-negative patients were more often multiplex-positive (P = 0.04, Table S2). Taking into account the large number of comparisons performed, this is not significant.

	Shared epitope			
	No, n (%)	Yes, n (%)	OR (95% CI)	P-value
CCP2-	140 (57)	107 (43)	REFERENCE	
CCP2+	54 (21)	199 (79)	**4.82 (3.26-7.14)**	**P < 0.001**
CCP2-Multiplex-	93 (55)	78 (46)	REFERENCE	
CCP2-Multiplex+	47 (60)	29 (39)	0.74 (0.42-1.28)	P=0.28
CCP2-Multiplex+	47 (62)	29 (38)	REFERENCE	
CCP2+Multiplex+	51 (21)	192 (79)	**6.10 (3.50-10.64)**	**P < 0.001**
	Ever smoker			
	No, n (%)	Yes, n (%)	OR (95% CI)	P-value
CCP2-	274 (78)	78 (22)	REFERENCE	
CCP2+	301 (69)	138 (31)	**1.61 (1.17-2.22)**	**P = 0.004**
CCP2-Multiplex-	183 (79)	54 (21)	REFERENCE	
CCP2-Multiplex+	90 (79)	24 (21)	0.90 (0.53-1.56)	P = 0.71
CCP2-Multiplex+	90 (79)	24 (21)	REFERENCE	
CCP2+Multiplex+	281 (71)	125 (29)	**1.67 (1.01-2.74)**	**P = 0.004**

Table 1: Association of risk factors HLA-SE and smoking with multiplex-positivity in anti-CCP-2-negative RA patients. Statistical comparison of the frequency of smokers and HLA-SE alleles in anti-CCP2 positive vs anti-CCP2 negative RA patients and within multiplex-positive RA patients.

Citrullinated-peptide repertoire recognized by anti-CCP-2-negative, multiplex-positive RA-patients.

We next analysed in more detail the reactivity patterns of sera from multiplex-positive anti-CCP-2 negative sera. Multiplex-positive anti-CCP-2 negative subjects (either RA, healthy and non-RA) recognized mostly one peptide (Median=1 in anti-CCP2-negative vs. 5 in anti-CCP-2 positive) as previously reported[13].

85% of the anti-CCP-2-negative sera in the EAC that are positive for a single peptide are positive for Fibα36-50 or Fibβ563-583 (122/143), Table S3). As the fraction of anti-CCP-2-negative multiplex-positive patients largely relies on these two peptide-reactivities, we studied these in more detail (Figure S1). Anti-CCP2-negative patients with reactivities towards Fibβ563-583, were mostly positive just above the cut-off (Figure S1). This was not unique for this peptide as this was also observed for other citrullinated peptide reactivities. Fibα36-50 was the exception with a

substantial number of anti-CCP2-negative RA patients who tested clearly positive (Figure S1), although recognition of this peptide was not RA-specific. After removal of these two peptides from the analysis 15% of anti-CCP-2-negative RA patients were multiplex-positive versus 5% of non-RA patients. This also led to a decreased sensitivity (54%) but an increased specificity (81%) of the multiplex assay.

Risk factors and anti-CCP-2-negative RA patients positive or negative for citrullinated peptide reactivities

Previously, it was shown that the presence of anti-CCP2-antibodies associates with HLA-SE alleles and smoking[5, 6]. Indeed, we found an increased frequency of HLA-SE alleles (43% vs 79%, OR = 4.82, P < 0.001) and smoking (22% vs. 32%, OR = 1.57, P = 0.004) in anti-CCP-2-positive versus -negative RA patients (Table 1). In contrast, in the anti-CCP-2-negative subset, we found no difference in the frequency of HLA-SE alleles (46% vs. 39%, OR = 0.74, P = 0.28) and smokers (21% vs. 21%, OR = 0.90, P = 0.71, Table 1) between multiplex-negative and -positive RA patients. We did observe an increased frequency of HLA-SE alleles and smoking in multiplex-positive anti-CCP-2-positive RA patients compared to multiplex-positive anti-CCP-2-negative RA patients (79% vs. 38% and 29% vs. 21%, Table 1).

We also studied IgM-RF, which co-occurs with anti-CCP-2 (29% in anti-CCP-2-negative and 91% in anti-CCP-2-positive RA, P < 0.001), but, again, no increased frequency of IgM-RF was found in the subgroup of anti-CCP2-negative multiplex-positive RA patients compared to multiplex negative patients (25% versus 33%)[27]. These findings were replicated with anti-MCV- and anti-CCP-3 (data not shown).

Together, these data show that anti-CCP/MCV-negative multiplex-positive RA patients cannot be distinguished from anti-CCP/MCV negative multiplex-negative RA patients using the read-outs described.

Correlation of multiplex array positivity with clinical outcome in anti-CCP-2-negative patients.

Next, we wished to study whether the multiplex-assay aids in the prognosis of clinical outcome. In the EAC cohort, it is known that anti-CCP-2-positive UA patients have an increased risk to progress towards RA in the first year of follow-up as compared to anti-CCP-2-negative UA patients (30% versus 68%, Figure 4A). Within the anti-CCP-2-negative subset, positivity in the multiplex-array was not associated with progression (30% in multiplex negative vs 30% in multiplex positive). Accordingly, multiplex-positive UA patients that are anti-CCP-2-positive have a higher risk to progress than multiplex-positive anti-CCP-2-negative UA patients (71% versus 30%, Figure 4A).

Likewise, with regard to joint damage anti-CCP-2-positive RA patients had 11% more joint destruction per year (β = 1.11, CI = 1.08-1.14, P < 0.001), which accumulated over 7 years of follow-up (Figure 4B). There was no significant association

A

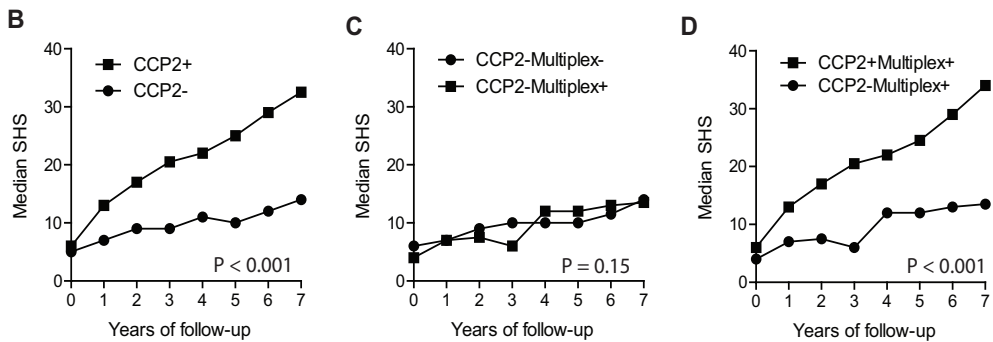|	Progression to RA			
	No n (%)	Yes n (%)	OR (95% CI)	P-value
CCP2-	311 (70)	132 (30)	REFERENCE	
CCP2+	45 (32)	97 (68)	5.08 (3.38-7.64)	**P < 0.001**
CCP2-Multiplex-	222 (70)	93 (30)	REFERENCE	
CCP2-Multiplex+	89 (70)	39 (30)	1.05 (0.67-1.64)	P = 0.84
CCP2-Multiplex+	89 (68)	39 (30)	REFERENCE	
CCP2+Multiplex+	37 (29)	91 (71)	5.61 (3.28-9.60)	**P < 0.001**

Figure 4: Multiplex positivity in anti-CCP-2 negative RA patients does not correlate with clinical outcome. A. Scheme depicts the number of patients that are diagnosed with UA at baseline. Patients were followed for one year to study their progression towards RA. B. Median SHS during 7 years of follow-up in anti-CCP-2 negative (N=1010) and positive (N=1330) RA patients. C. Median SHS during 7 years of follow-up in anti-CCP-2 negative RA patients that are multiplex negative (N=729) or positive (N=281). D. Median SHS during 7 years of follow-up in multiplex positive RA patients that are anti-CCP2 negative (N=281) or positive (N=1282). N depicts the number of radiographs.

with joint destruction over time when multiplex-negative anti-CCP-2-negative and multiplex-positive anti-CCP-2-negative patients were compared. (β = 1.02, CI = 0.99-1.06, P = 0.15, Figure 4C). In line with this observation, within the multiplex-positive RA subset, anti-CCP-2-positive patients have more joint destruction over time than anti-CCP-2-negative RA patients. (β = 1.09, CI = 1.05-1.13, P < 0.001, Figure 4D). Together, these data show no added clinical value of the multiplex-array, using 11 citrullinated peptides, over the existing anti-CCP-2-test with regard to clinical phenotype or outcome of disease.

DISCUSSION

In this report, we extensively analysed the first multiplex-array, consisting of 11 dif-

ferent citrullinated peptides for detection of ACPA, in an early arthritis setting. The multiplex-array can identify most RA patients that are positive in the conventional ACPA tests. A large subgroup of RA patients that are negative in commercial ACPA tests, including anti-CCP-2/3 and anti-MCV, are positive for one or more reactivities in the multiplex-array. These reactivities have potential to discover new autoantibody systems or lead to understanding of auto-immune reactions in RA. However on a group level, these "additional" identified reactivities does not contribute with more clinical information. Furthermore, patients that are multiplex positive but CCP2-negative differ from CCP-2-positive RA patients in ACPA risk factors (HLA-SE and smoking), the presence of IgM-RF and in clinical parameters (UA to RA progression and joint destruction), but do not differ from anti-CCP-2-negative patients.

The fact that the current analysis made use of a single platform is a limitation. Nonetheless, findings are in line with results obtained by other platforms[10, 26, 28]. Using a bead-based multiplex-platform containing 16 different citrullinated antigens Wagner et al. found that at least 10% of anti-CCP-2 negative RA patients were ACPA-positive. In this study ACPA-positive was much more stringently defined (positive for >2 antigen-reactivities with positive defined as mean + 3x the standard deviation of the signal in healthy controls). Consistent with our findings, they also described that anti-CCP-2-negative RA patients that are positive for citrulline-reactivities recognize a more restricted peptide repertoire[10]. Likewise, this observation was also confirmed in another study describing a surface plasmon resonance based multiplex platform[25].

Multiplex assays are designed to investigate the presence of antibodies to a large number of targets in a high throughput manner and with very small amounts of samples. The assay presently contains a large number of different peptides, which are derived from native proteins. It is not clear, however, whether antibodies to the peptides selected in the array in fact crossreact to native citrullinated proteins in vivo. It has been shown that antibodies to citrullinated type II collagen both bind joints in vivo, and induces arthritis in mice, but the presently included peptide contains two citrullines that block many antibodies reactive with single citrullines present on type II collagen in cartilage [29-30]. Thus, it is not clear, whether any of the selected peptides attract antibodies that have functional relevance in vivo. On the other hand this can also be said regarding the CCP-2 test as well, since in this case the sequence is not publically known and therefore no arginine-control can be used. Possibly the test contains several peptides that have been selected to give the highest specificity for RA. Thus, neither of the used test give any information on possible pathogenic or regulatory influence of the respective antibody reactivities. In this study we have focused on the clinical relevance of citrulline reactivities in anti-CCP-2-negative RA patients. To the best of our knowledge, only one other report has focused on the clinical relevance of citrulline reactivities in anti-CCP-2-negative patients. Pratt et al studied the predictive value of reactivity towards citrullinated antigens in an ELISA platform with regard to UA to RA progression and found that

none of the tested reactivities was as good as anti-CCP-2 in predicting progression. In line with our observations, reactivities to a set of citrullinated peptides in anti-CCP-2-negative UA patients were not associated with disease progression[28]. Although our findings are in line with results from other platforms, future work should focus on comparing citrulline reactivities in independent cohorts of anti-CCP-2 negative RA patients side-by-side on different platforms. To improve multiplex-platforms, this work should be directed towards identifying those citrullinated peptides that have an additive value and are specific for RA or a specific phenotypical aspect such as the anti-SSA antibodies. Most importantly, such specified clinical characteristics should be linked with functional knowledge of the detected antibodies. It is needed to both improve these platforms with more relevant peptides and to validate them using large cohorts of healthy controls, RA patients and disease controls. Future studies will also be useful to analyse the relationship of each reactivity with risk factors and prognosis, and to investigate the functional role of these autoantibody specificities in the disease process. This could facilitate the incorporation of the most meaningful reactivities in new future assays

Our data indicate that the positive signals found in anti-CCP-2 negative sera using the current multiplex-system, should be interpreted with caution. The detected signals are dependent on the particular assays of peptides bound to solidphase, known to twist the conformation and increase variability and false positive signals as compared with assaying protein-protein interactions. In addition, for most of the positive signals for detection of citrullinated peptides, the signal is just above the cut-off. False positive signals may therefore contribute to the observation that citrulline reactivities in anti-CCP/MCV-negative RA patients are not associated with any of the known risk factors for ACPA-positive disease. Another limitation is that the peptide composition of the array is limited and the truly relevant citrullinated peptides or proteins are not present. This is especially relevant since the number of non-fibrinogen peptide positive, anti-CCP-2 negative, multiplex-positive patients are low thereby making it impossible to analyse these reactivities. In fact, an increasing number of citrullinated peptides, not used in the present array, are reported to have potential pathogenic properties in the development of arthritis [29,31].

CONCLUSIONS

Our data indicate that residual reactivities to a wide range of citrullinated joint antigens as measured in the current multiplex-system are unable to subdivide anti-CCP-2-negative RA patients into clinically different patient subsets. Nonetheless, it is clear that anti-CCP-2-negative RA patients are clinically heterogeneous and there is a need for biomarkers for disease phenotypes within disease like RA but also across different diseases. This would require different studies in patient cohorts with detailed phenotypical characteristics which are selected in non-biased ways

[32]. An improved multiplex-assay could potentially accomplish this.

ACKNOWLEDGEMENTS

Chapter 5

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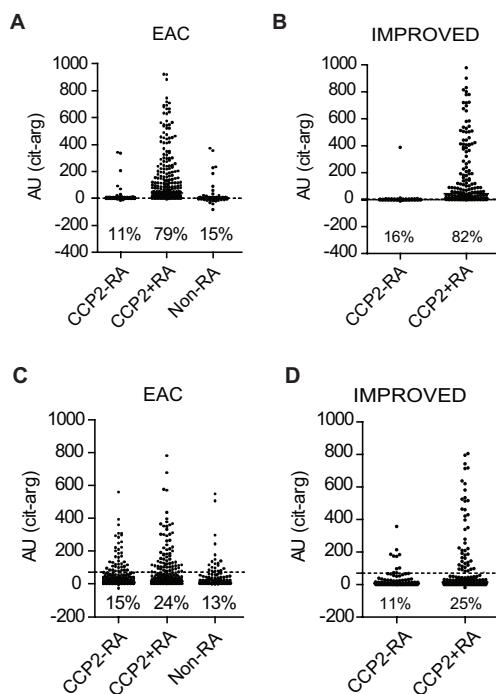
SUPPLEMENTARY INFORMATION

Chapter 5

Figure S1: Citrulline reactivities to Fibα36-50 and Fibβ563-583. Citrulline-reactivities towards Fibβ563-583 in anti-CCP-2 positive (n=285) and anti-CCP-2 negative (n=279) RA patients and non-RA patients (n=149) in the EAC. B. Citrulline-reactivities in arbitrary units (AU) towards Fibβ563-583 in anti-CCP-2 positive (n=174) and anti-CCP-2 negative (n=92) RA patients in the IMPROVED. C. Citrulline-reactivities towards Fibα36-50 in anti-CCP-2 positive (n=285) and anti-CCP-2 negative (n=279) RA patients and non-RA patients (n=149) in the EAC. D. Citrulline-reactivities towards Fibα36-50 in anti-CCP-2 positive (n=174) and anti-CCP-2 negative (n=92) RA patients in the IMPROVED. The dotted line indicates the cut-off. Reactivities are plotted in arbitrary units (AU). Each dot indicates a unique individual.

Group	Multiplex-negative	Multiplex-positive	Total
Reactive arthritis	6 (6%)	4 (11%)	10 (7%)
Gout	16 (16%)	6 (16%)	22 (16%)
Pseudogout	1 (1%)	0 (0%)	1 (1%)
Spondyloarthritis including psoriatric arthritis	45 (46%)	13 (35%)	58 (43%)
Osteoarthritis	15 (15%)	6 (16%	21 (16%)
Lyme arthritis	1 (1%)	0 (0%)	1 (1%)
Paramalignant arthritis	0 (0%)	4 (11%)	4 (3%)
Sarcoidosis	0 (0%)	1 (3%)	1 (1%)
Mixed connective tissue disease, vasculitis	1 (1%)	0 (0%)	1 (1%)
Other systemic autoimmune diseases	5 (5%)	0 (0%)	5 (4%)
Remitting seronegative symmetrical synovitis with pitting edema	8 (8%)	2 (8%)	10 (7%)
Rest	0 (0%)	1 (3%)	1 (1%)
Total	*98 (100%)*	*37 (100%)*	*135 (100%)*

Table S1: Diagnoses of anti-CCP2-negative non-RA patients (n=135) as assessed by an experienced rheumatologist after 1 year of follow-up stratified for multiplex-positivity.

Patient Characteristic	Multiplex-negative, n=193	Multiplex-positive, n=86	P-value
Anti-CCP2 titer in AU/ml, mean (SD)	13 (9)	12 (10)	0.53
median (IQR)	20 (3-21)	20 (1-20)	0.73
Age in yrs, mean (SD)	60 (17)	59 (17)	0.67
Female gender, n (%)	47 (55)	131 (68)	0.04
HAQ, median (IQR)	1.0 (0.6-1.6)	1.0 (0.6-1.6)	0.76
mean (SD)	1.1 (0.7)	1.1 (0.7)	0.92
CRP in mg/ml, mean (SD)	27 (30)	35 (42)	0.72
median (IQR)	15 (6-37)	23 (6-48)	0.46
SHS, mean (SD)	9 (11)	8 (9)	0.36
median (IQR)	6 (2-12)	4 (2-11)	0.19
ESR in mm/h, mean (SD)	35 (23)	33 (27)	0.54
Symptom duration at baseline in months, mean (SD)	6 (12)	5 (9)	0.58

Table S2: Baseline characteristics of anti-CCP-2-negative RA patients (n=279) that are multiplex-negative or –positive.

Group	Number	Percentage
CEP1	0	0%
Vim2-17	0	0%
Fibβ36-52	1	1%
Fibα621-635	0	0%
Fibβ680-600	0	0%
CCP1	1	1%
C1	4	3%
Vim60-75	6	4%
Fibβ60-74	9	6%
Fibβ563-583	49	34%
Fibα36-50	73	51%
Total	*143*	*100%*

Table S3: Peptides recognized by anti-CCP-2-negative EAC patients that are positive in the multiplex assay for a single epitope (n=143).

Chapter 5

CHAPTER 6

Fine-mapping the HLA locus in rheumatoid arthritis and other rheumatic diseases: Identifying causal amino acid variants.

Jurgen van Heemst, Tom W.J. Huizinga, Diane van der Woude and René E.M. Toes.

ABSTRACT

Purpose of review To provide an update on- and context of the recent findings obtained with novel statistical methods on the association of the Human leucocyte (HLA) locus with rheumatic diseases.

Recent findings Novel SNP fine-mapping data obtained for the HLA locus have indicated the strongest association with aa positions 11 and 13 of HLA-DRB1 molecule for several rheumatic diseases. On the basis of these data, a dominant role for position 11/13 in driving the association with these diseases is proposed and the identification of causal variants in the HLA-region in relation to disease-susceptibility implicated.

Summary The human leukocyte antigen (HLA) class II locus is the most important risk factor for several rheumatic diseases. Recently, new statistical approaches have identified previously unrecognized amino acid positions in the HLA-DR-molecule that associate with anti-citrullinated protein antibody negative and positive rheumatoid arthritis. Likewise, similar findings have been made for other rheumatic conditions such as giant cell arteritis and systemic lupus erythematosus. Interestingly all these studies point towards an association with the same amino acid positions: amino acid position 11 and 13 of the HLA-DR beta chain. As both these positions influence peptide binding by HLA-DR and have been implicated in antigen presentation, the novel fine-mapping approach is proposed to map causal variants in the HLA-region relevant to RA and several rheumatic diseases. If these interpretations are correct, they would direct the biological research aiming to address the explanation for the HLA-disease association. Here, we provide an overview of the recent findings and evidence from literature that, while relevant new insights have been obtained on HLA-disease associations, the interpretation of the biological role of these amino acids as causal variants explaining such associations should be taken with caution.

INTRODUCTION

Rheumatoid Arthritis (RA) is a chronic systemic inflammatory disorder predominantly affecting synovial joints. In the past pivotal pathophysiological insight has been obtained by the identification of the anti-citrullinated protein antibodies (ACPA), a group of autoantibodies specifically directed to citrullinated proteins[1]. ACPA are highly specific for RA and directed against antigens that are also expressed in the inflamed joint[2].

It is now clear that RA represents two main syndromes, ACPA-positive- and ACPA-negative disease, that are most likely etiologically distinct as different genetic risk factors are underlying the two distinct disease phenotypes. This was first evidenced by the finding that different Human Leukocyte Antigen (HLA)-genes are predisposing to either ACPA-positive or ACPA-negative disease, and boosted by the results of Whole Genome Association (WGA)-studies[3-5]. In the recent past, the genetics of RA has undergone a revolution through the identification of several novel genetic risk factors. Although the identification of genetic regions triggering human disease represents a major step forward, one of the biggest challenges to date is to elucidate the biological pathways underlying these associations to obtain a thorough understanding of disease pathogenesis. At least 20% of all genetic variance contributing to RA is explained by the HLA class II region, which is considerably more than that of all known non-HLA common genetic variants together[6]. Thus, the HLA-region is by far the most important known genetic risk factor for RA. Nonetheless, the biological pathway(s) causing this association is/are still unknown. Therefore, one of the major questions in the field is how the HLA class II region is contributing to disease pathogenesis, how the immune response underlying the disease is generated and which role it is playing in disease induction and progression.

HLA-class II-proteins present antigenic peptides to T-cells, which will result in T-cell activation and induction of antigen-specific immune reactions. Activated T-cells can mediate a plethora of effects including provision of help to B-cells necessary for antibody isotype-switching, affinity-maturation and memory B-cell-formation. Although the association between HLA and RA is known for over 30 years, the biology explaining this association and the nature of the presented antigen is poorly understood. One of the prevailing ideas to date is the notion that the HLA-molecules that predispose to RA have an enhanced ability, in comparison to the HLA-alleles that do not predispose to ACPA-positive disease, to present citrullinated epitopes[7-8]. The HLA-molecules associating with RA are generally referred to as HLA-Shared-Epitopes (HLA-SE) as the HLA-DR-molecules encoded by the predisposing HLA-haplotypes share a common sequence in the peptide-binding groove of the molecule[9]. Through the ability of the HLA-SE-molecules to present citrullinated antigens, these molecules are best suited to activate T-cells reactive to citrullinated antigens, thereby facilitating the induction of T-cell responses that can provide help to B cells recognizing the same antigens. Whether this hypothesis

is correct is not known, but it has been shown that the pocket in the HLA-anti-gen-binding groove (the P4-pocket) that is, in part, shaped by the SE-sequence, is able to accommodate citrulline, but not arginine, the amino acid from which cit-rulline is formed. Nonetheless, it is not known whether these findings apply for all HLA-SE-molecules or for all citrullinated epitopes. To better understand the contri-bution of the HLA-SE-alleles in ACPA-positive RA, more refined insights the HLA-RA connection is important. Obviously, a similar notion applies for other rheumatic diseases where the contribution of the HLA-region to disease pathogenesis is also not well understood.

FINE MAPPING THE HLA LOCUS

The HLA locus is complex as it contains many genes that can be highly poly-morphic[10]. Also, this region is characterized by extensive linkage disequilibrium (LD) making it difficult to identify the responsible genes and/or causal variants. Recent studies have revealed relevant new insights into the association of the HLA-region with rheumatoid arthritis and several other rheumatic diseases[4, 8-12]. By fine-mapping of the HLA locus and imputation of amino acids (aa) sequences of HLA-molecules from SNP genotype data, HLA-genetics have been made feasible in large groups of patients and controls. This allowed new insights into the HLA-asso-ciation for many different diseases (detailed description in [4]).

Analysis of the association of individual imputed amino acids (aa) throughout the HLA-locus revealed specific positions in the HLA-DRB1-allele that associated best with disease. As the identified HLA-variants are part of peptide-binding grooves, these new findings were proposed to map causal variants in the disease-associating HLA-molecules. This approach has now been applied to both ACPA-negative[11, 12] and ACPA-positive RA [4, 11, 13], but also to other rheumatic diseases including Systemic Lupus Erythematosus (SLE) and Giant Cell Arteritis (GCA)[14-15]. Previ-ously, dominant risk genotypes of these diseases were documented on the basis of conventional HLA-genotyping. Susceptibility to ACPA-negative RA was shown to be associated with HLA-DRB1*03[3, 11], whereas ACPA-positive RA and GCA are most strongly associated with HLA-DRB1*04[16, 17]. SLE susceptibility in Koreans was reported to be linked to HLA-DRB1*15[18].

Intriguingly, the novel SNP fine-mapping data obtained for the HLA locus showed the strongest association with aa positions 11 and 13 of HLA-DRB1 for all these rheumatic diseases (summarized in Table 1). Importantly, positions 11 and 13, which are in tight LD, are critically relevant for shaping several peptide-binding pockets: Aa position 11 is involved in shaping peptide-binding pocket 6, whereas aa position 13 affects the shape of pocket 4[19]. Peptide-binding pockets are part of the peptide-binding groove of the HLA-molecule that are involved in epitope-pre-sentation. The interpretations of these combined findings often imply a dominant

	Pos1	Pos2	Pos3	Reference
ACPA- RA	11/13	-	-	[9, 10]
ACPA+ RA (Caucasians)	11/13	71	74	[6, 9, 11]
ACPA+ RA (Asians)	11/13	57	74	[11]
Giant cell arteritis	11/13	-	-	[13]
Systemic Lupus Erythematosus	11/13	26	-	[12]

Table 1: Summary of amino acid positions associated with risk to develop rheumatic diseases.

role for position 11/13 in driving the association with disease or implicate the iden-tification of causal variants in the HLA-region in relation to disease-susceptibility. For instance, when these aa positions where found in association with SLE, it was concluded that these aa positions at the epitope-binding groove of HLA-DRβ1 are responsible for most of the association between SLE and MHC. Likewise, when it was shown that the variants that associate with RA are the same variants that asso-ciated with risk for development of follicular lymphoma, the presence of a common HLA-DR antigen-driven mechanism for the pathogenesis of follicular lymphoma (FL) and RA was suggested[20].

PERSPECTIVE

If the interpretations, such as the one mentioned above, are correct, it would have an important impact on the design of experiments aiming to address the biological explanation for the HLA-disease association. For example, in the case of RA, less efforts should be devoted to the elucidation of the role of the shared epitope, but instead, the contribution of aa position 11/13 should be scrutinized. Aa positions 11 is not part of pocket 4 and does not interact with SE-residues. Therefore, these findings would imply that, at the molecular level, two different mechanisms might be operative that contribute to the development of ACPA-positive RA. Similar impli-cations would apply for other rheumatic diseases. To better understand the possible contributions of these aa associations and the findings generated by the described method, we reviewed variation between HLA-DRB1-alleles in closer detail.

An aa position can only be significantly associated with a disease if the particular position is polymorphic. HLA-DR molecules are heterodimers consisting of an alpha and a beta chain. For HLA-DR all the variation between donors is found in the beta chain. Figure 1A schematically depicts the variation in the HLA-DR b1 domain per amino acid of the top-15 most prevalent HLA-DRB1 chains present in the Cauca-sian population (together covering 91% of the variation in Caucasians[21]). Black dots indicate aa residues that are involved in shaping the peptide-binding pockets. As illustrated, most variation in HLA-DRB1 resides in residues critically engaged
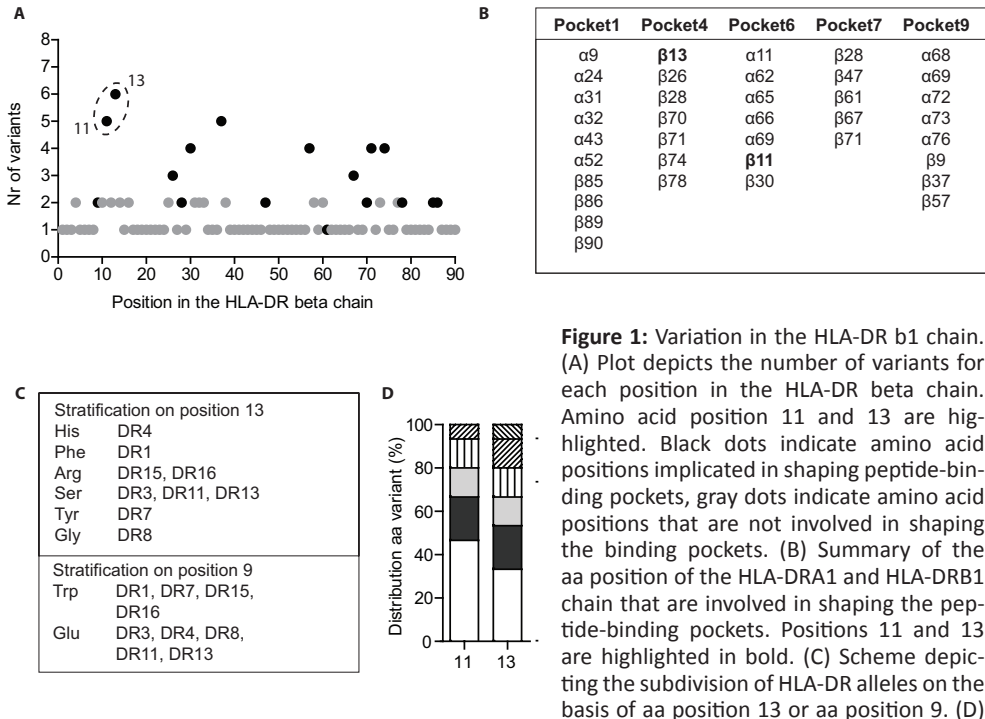
Figure 1: Variation in the HLA-DR b1 chain. (A) Plot depicts the number of variants for each position in the HLA-DR beta chain. Amino acid position 11 and 13 are highlighted. Black dots indicate amino acid positions implicated in shaping peptide-binding pockets, gray dots indicate amino acid positions that are not involved in shaping the binding pockets. (B) Summary of the aa position of the HLA-DRA1 and HLA-DRB1 chain that are involved in shaping the peptide-binding pockets. Positions 11 and 13 are highlighted in bold. (C) Scheme depicting the subdivision of HLA-DR alleles on the basis of aa position 13 or aa position 9. (D) Distribution of the aa variants at position 11 or position 13. All analyses were performed using the 15 most frequent HLA-DRB1 alleles in Caucasians (91% of all the variation).

in shaping peptide-binding pockets. The finding that the statistically significant amino-acid positions are those involved in peptide presentation is therefore not surprising and does not, per se, support a more important pathophysiological role of these particular amino acid positions compared to other non-associated positions.

HLA-crystallization and peptide-elution studies have shown that peptide-binding pockets are shaped by the combination of multiple different (polymorphic) amino acids (schematically depicted in Figure 1B) and that the combination of all these amino acids will eventually determine the size, charge and hydrophobicity of each peptide-binding pocket and thereby the ligands that can be presented by the HLA-molecule [22-27]. It is therefore surprising that aa positions 11 and 13 are so strongly associated with different rheumatic diseases compared to other aa positions that are also involved in shaping these pockets as one could assume that, in case a particular pocket is crucial to the presentation of a particular (set of) antigens, also the other aa involved in the formation of the pocket would influence the binding of such antigen(s). Recently, these studies were also conducted for non-rheumatic diseases: ulcerative colitis and FL [20, 28]. Again, aa positions 11 and 13 were most strongly associated with risk for these two diseases. Together,

these findings and considerations make it conceivable that the applied approach does not identify causal variants as implied, but may contain a bias towards these two aa residues.

Such bias could be introduced by the fact that some aa positions are more polymorphic than others. As depicted in Figure 1A, aa positions 11 and 13 are the most polymorphic amino acid positions in the HLA-DRB1-gene. It is conceivable that the applied approach biases towards amino acid positions that can subdivide the different HLA class II molecules into the largest number of groups, i.e. the most polymorphic amino acid positions. Thus, positions 11 and 13 are likely to be the best surrogate markers for defining HLA-genotype as they contain the most discriminative ability. This is illustrated in Figure 1C for ACPA-positive RA which is known to associate most strongly with HLA-DR4[29]. Stratification on position 13 generates 6 different groups on the basis of the 6 different aa that can be present in this position. HLA-DR4 will be in a group without other HLA-DR subtypes. Stratification on a less polymorphic position, e.g. position 9, subdivides the HLA class II molecules in only 2 groups. HLA-DR4 will now be in a subgroup with HLA-DR3, HLA-DR8, HLA-DR11 and HLA-DR13 that will all dilute the risk effect of HLA-DRB1*04. Therefore, a statistical approach based upon the presence of individual aa in certain positions will unlikely identify aa at position 9, as this position is relatively non-polymorphic. Nonetheless, in biological terms, this position shapes one of the pockets: pocket 9, and hence will impact HLA-ligand binding. Likewise, giant cell arteritis is also associated with HLA-DRB1*04 and therefore similar aa position will likely associate with this disease although the biological basis is likely different[16].

For ACPA-negative RA, HLA-DRB1*03 is the major risk determinant[3, 11, 30]. Stratification on the basis of aa position 13 will generate an HLA-DR3 subgroup with HLA-DRB1*11 and HLA-DR13, whereas stratification based on aa position 9 will generate a subgroup of HLA-DR3 with HLA-DR4, HLA-DR8, HLA-DR11 and HLA-DR13 (Figure 1C). Hence, stratification on aa position 13 will again be favored over less polymorphic positions, e.g. position 9. Finally, SLE is most strongly associated with HLA-DR15 in Asians and stratification on position 13 will generate an HLA-DR15 subgroup with DR16, whereas stratification on position 9 will generate a subgroup of HLA-DR15 with HLA-DR1, HLA-DR7 and HLA-DR16[18].

Stratification on aa positions 11 and 13 does not only generate the largest number of different groups, but also provides the best resolution due to the fact that the various aa variants predisposing to different rheumatic diseases are quite evenly distributed in size. This could further explain why these aa residues are so strongly associated with the different diseases using this statistical aa fine-mapping approach (Figure 1D).

Chapter 6

CONCLUSION

The development of new methods and tools for genetic data analyses in the HLA-region have been rewarding as they further refined the HLA-diseases associations by allowing inclusion of large datasets that lacked conventional HLA-genotyping data. Based on the analyses described above we feel, however, that conclusions on the biological role of amino acid positions obtained via this novel fine-mapping approach should be taken with caution as it appears that this approach may contain a bias towards particular aa positions that, at a single amino acid level, best mark the associated HLA-DRB1 genotype. These considerations are in agreement with MHC-ligand studies that clearly show that the combination of all residues shaping peptide-binding pockets will eventually determine the type of ligands that are presented by these molecules.

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Chapter 6

CHAPTER 7

The protective effect of HLA-DRB1*13 alleles during specific phases in the development of anti-citrullinated protein antibody positive rheumatoid arthritis.

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Submitted

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ABSTRACT

Objective HLA-DRB1*13 alleles are associated with protection from anti-citrullinated protein antibody (ACPA-)positive rheumatoid arthritis (RA). It is however unknown at which phase of disease development (seroconversion, ACPA maturation, disease onset or outcome) these alleles are most important. We therefore examined the effect of HLA-DRB1*13 on the presence of ACPA (:systemic autoimmunity associated with RA) in individuals with and without RA, on ACPA characteristics and clinical outcome measures.

Methods The effect of HLA-DRB1*13 on the presence of ACPA with of without RA was assessed in the Swedish twin registry (n=10748). ACPA characteristics were studied in ACPA-positive RA patients from the Swedish Epidemiological Investigation of Rheumatoid Arthritis (EIRA, n=1224) and the Dutch Leiden Early Arthritis Clinic (EAC, n=441). Disease activity at inclusion and disease outcome (DMARD-free sustained remission and radiographic progression) was assessed in more than 600 RA patients from the EAC.

Results HLA-DRB1*13 is associated with protection from ACPA-positive RA (prevalence 16% versus 28% in ACPA-negative RA), but not with significant protection (p-value 0.09) from ACPA (systemic autoimmunity associated with RA) (prevalence: 22%). HLA-DRB1*13 is associated with lower ACPA-levels (EIRA: 447 U/ml versus 691 U/ml, p-value: 0,0002) and decreased recognition of citrullinated vimentin and/or alpha-enolase (EIRA: p< 0.0001). HLA-DRB1*13 is not associated with disease activity or outcome.

Conclusion These data suggest that HLA-DRB1*13 mainly affects the onset of ACPA-positive RA in ACPA-positive non-RA individuals. HLA-DRB1*13 influences ACPA characteristics, but not disease course after seroconversion. This implies that therapeutic strategies aimed at emulating the HLA-DBR1*13 protective effect may be most effective in ACPA-positive healthy individuals at risk for RA.

INTRODUCTION

The risk of developing rheumatoid arthritis risk (RA) is influenced by both genetic and environmental factors, with the human leukocyte antigen (HLA) class II locus as the major genetic determinant [1, 2]. RA risk is mainly associated with the presence of HLA-DR molecules that carry the shared epitope (SE) motif, whereas HLA-DRB1*13 alleles (HLA-DRB1*13:01 and HLA-DRB1*13:02) are associated with protection against RA[3-5]. Interestingly, these alleles protect with an odds ratio of approximately 0,55 both in the absence and presence of SE-alleles, indicating that this protective effect is SE-independent [5].

Autoantibodies, in particular anti-citrullinated protein antibodies (ACPA), are an important hallmark of rheumatoid arthritis[6]. These autoantibodies recognize a broad variety of proteins that have undergone post-translational modifications converting arginine to citrulline residues[7, 8]. These autoantibodies can be found up to ten years before RA onset and are a strong predictive factor for RA development [9-11]. Prior to disease onset, the ACPA-response suddenly matures with a rise in ACPA levels, increased isotype usage and epitope-spreading to a broader range of citrullinated antigens (reviewed in [12]). Moreover, the presence of ACPA is associated with a more destructive disease phenotype[13].

HLA class II alleles, including the SE-alleles and HLA-DRB1*13 alleles, shape the risk to develop RA, and are predominantly associated with ACPA-positive RA [14, 15]. This suggests that ACPA-positive and ACPA-negative disease are two distinct diseases, and that these HLA-DRB1 alleles are specifically involved in the production of ACPA autoantibodies. Recently, several reports have investigated the contribution of SE-alleles during different phases of RA development. These studies suggest that SE plays a minor role in the development of ACPA in individuals without arthritis, but is more important for the transformation from ACPA-positive autoimmunity (in the absence of arthritis) to ACPA-positive disease[16-17]. Moreover, in ACPA-positive RA patients, SE-alleles associate with the recognition of a broader repertoire of fine-specificities and with specific ACPA fine-specificities, most notably epitopes on citrullinated α-enolase and vimentin [18-20]. However, SE-alleles do not associate with disease outcome[21-23]. Despite the strong protective association between HLA DRB1*13 alleles and ACPA-positive RA, it is unknown at which phases of disease development these alleles exert their effect. A better understanding of the protective effect of HLA DRB1*13 alleles might allow the development of therapeutic strategies aimed at imitating this effect. Ideally, the beneficial effect of these alleles could then be simulated in HLA DRB1*13-negative individuals by a therapeutic agent.

Therefore we now aimed to investigate the contribution of HLA-DRB1*13 during different phases of ACPA-positive disease. We therefore examined the effect of HLA-DRB1*13 on the presence of ACPA (:systemic autoimmunity associated with RA) in individuals with and without RA, the association between HLA DRB1*13 and

ACPA characteristics as well as clinical outcome measures (DMARD-free remission and radiographic progression) in established RA.

METHODS

Patients

This study made use of three independent cohorts: the Swedish TWINGENE cohort[24, 25], the Swedish Epidemiological Investigation of RA (EIRA[26]) and the Dutch Leiden Early Arthritis Clinic[27].

The TWINGENE cohort was used to study the influence of HLA-DRB1*13 on ACPA-positivity in subjects without RA (i.e. in non-RA individuals). This cohort consists of 12590 twins from the national Swedish twin registry. A twin was considered an RA patient if an RA diagnosis was listed in the patient's register at least once in 2009, or in prior years, as described previously [17]. The current analysis included 10748 individuals on whom HLA data was available: ACPA-negative non-RA twins (n=10454), ACPA-positive non-RA (called: healthy) twins (n=190) and ACPA-positive RA twins (n=104) of whom HLA-typing was available.

The EIRA was used to study the influence of HLA-DRB1*13 on characteristics of the ACPA-response. This Swedish cohort recruited incident RA cases and controls from 1996 to 2003 from the south and central regions of Sweden. All EIRA RA cases fulfil the 1987 ACR criteria for RA. Only HLA-genotyped, anti-CCP2 antibody positive EIRA RA patients were included in the current analysis (n=1224).

The Leiden EAC was used to study the influence of HLA-DRB1*13 on the ACPA-characteristics and on disease outcome measures. This cohort was initiated in 1993 and includes patients with a recent onset of arthritis (symptom duration < 2 years) from the Leiden area[27, 28]. Definitive diagnoses were determined after 1 year of follow-up by an experienced rheumatologist using existing classification criteria. Of the 1140 fulfilling the EULAR/ACR2010 criteria for RA, HLA-typing was available for 835 of which 441 patients were anti-CCP-2-positive. Disease characteristics such as the swollen joint count (SJC), score on health assessment questionnaire (HAQ), and joint damage as measured by Sharp-van der Heijde-score (SHS) were assessed at baseline and yearly thereafter[30].

Auto-antibodies

Anti-CCP-2 was measured by ELISA using the Immunoscan RA Mark 2-test; (Eurodiagnostica, Arnhem, The Netherlands; Eurodiagnostica AB, Malmö, Sweden) with a cut-off as specified by the manufacturer. In the EAC and the EIRA, antibodies against citrullinated and native vimentin (Vim59-74 in the EAC, Vim60-75 in the EIRA) or alpha-enolase (linear Eno5-20 in the EAC, cyclic Eno5-21 in the EIRA) peptides, as well as the corresponding arginine-containing control peptides, were determined by in-house ELISAs as previously described[12, 18, 19, 20, 22].

HLA genotyping

Genotyping was performed as previously described[17, 20, 30]. In the EAC a specific probe was used that can detect the presence of the SE sequence, classifying HLA-DRB1*01:01, *01:02, *04:01, *04:04, *04:05, *04:08 and *10:01 as SE-alleles. In the EIRA, HLA-DRB1*01:01, HLA-DRB1*01:02, HLA-DRB1*04 and HLA-DRB1*10 were classified as SE-alleles. In the TWINGENE cohort, HLA-DRB1 gene alleles were imputed from genome-wide datasets.

Remission

In the EAC, DMARD-free sustained remission was defined as previously described[31, 32. Briefly, three criteria needed to be fulfilled: no current use of DMARDs, no swollen joints, and classification as DMARD-free remission by the patient's rheumatologist. The absence of swollen joints had to be observed by a rheumatologist for at least one year after DMARD-therapy discontinuation.

Statistical analysis

To estimate the association of HLA-DRB1*13 with ACPA-positivity in twins with or without RA, a binominal logistic regression model was used to calculate odds ratios (ORs). Confidence intervals (CI) were adjusted due to clustering of twin data. P-values were estimated by Wald statistics and chi-square tests.

To determine the association of HLA-DRB1*13 with various ACPA-characteristics, ORs with 95% CI were calculated using logistic regression analyses with those negative for risk factors as reference. Stratification was performed as previously described [30] by analyzing the effect of HLA-DRB1*13 in both the SE-negative (x/x versus DRB1*13/x and DRB1*13/DRB1*13) and SE-positive stratum (SE/x versus SE/DRB1*13).

To analyze the rate of joint destruction, a multivariate normal regression model for longitudinal data with log-transformed SHS as response variable was used as previously described[33]. This method analyses al repeated measures at once and takes advantage of the correlation between these measurements. Analyses were adjusted for age, gender and different inclusion periods as proxy for treatment strategy [28].

Analysis of DMARD-free sustained remission was performed by Cox regression analysis, after verification that the proportional hazards assumption was satisfied. For patients who achieved remission the dependent variable is the "time-to-event", indicating the time until reaching remission. For non-remission patients the time to last follow-up was used, with a maximum of 15 years.

P-values <0.05 were considered significant. All reported p-values are two-sided. Analyses were performed using SPSS statistics, version 22.

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RESULTS

Influence of HLA-DRB1*13-status on the presence of ACPA in non-RA individuals and in RA patients

In order to investigate the effects of HLA-DRB1*13 alleles during different phases of RA development, we first examined whether HLA-DRB1*13 alleles prevent the first detectable stage of autoimmunity: the development of ACPA. To this end we used a cohort of 10.748 twins from the national Swedish twin registry[24, 25]. ACPA could be detected in 2.7% of these twins (n=294). Of those 294 ACPA-positive twins, 104 (35%) were diagnosed with RA[17]. The HLA-DRB1*13 frequency was compared in ACPA-negative non-RA individuals (27,6%), ACPA-positive non-RA individuals (22,1%) and ACPA-positive RA patients (16,3%). There was a significant protective effect of HLA-DRB1*13 in ACPA-positive RA patients in the TWINGENE cohort (OR=0.51, CI=0.30-0.87, p=0.01, Supplementary Table 1) as was previously demonstrated in other Caucasian cohorts[5]. However, there was no significant protective effect of HLA-DRB1*13 in ACPA-positive non-RA individuals (OR=0.74, CI=0.53-1.05 p=0.09,) (Table 1). However, there might be a trend towards protection, which could indicate that HLA-DRB1*13 might exert some protective effect also in the development of ACPA, but mainly protects from the development of ACPA-positive RA. Similar associations have previously been shown for the predisposing effect of the SE-alleles[17]. Stratifying the HLA-DRB1*13 analysis for SE status did not change the results (data not shown).

HLA-DRB1*13 also protects from ACPA-positive RA in the Japanese RA case-control study[4]. Recently, a report was published describing a large cohort (Nagahama-cohort) of Japanese volunteers without autoimmune diseases that were HLA-typed and serotyped for ACPA[34]. We have now extracted data from this report to make a similar comparison and to validate our findings (Table 1). In line with our data

TWINGENE		Non-RA			
		ACPA-	ACPA+	OR (95% CI)	P-value
DRB1*13	Neg	7566 (72,4%)	148 (77,9%)	REF	
	Pos	2888 (27,6%)	42 (22,1%)	0.74 (0.53-1.05)	0.09
NAGAHAMA		Non-RA			
		ACPA-	ACPA+	OR (95% CI)	P-value
DRB1*13	Neg	2495 (80,1%)	42 (75%)	REF	
	Pos	619 (19,9%)	14 (25%)	1.36 (0.74-2.51)	0.33

Table 1. HLA-DRB1*13 association with ACPA-positivity in non-RA patients.

EIRA	Anti-CCP-2 levels in U/ml			
Total	Median	IQR	Patient Nr.	P-value
DRB1*13 neg	690.64	224.94-1554.56	1100	REF
pos	446.50	105.94-907.78	126	0.0002
SE-negative stratum				
DRB1*13 neg	684.10	258.45-1514.04	143	REF
pos	387.00	102.00-656.09	46	0.0028
SE-positive stratum				
DRB1*13 neg	651.45	183.05-1593.90	568	REF
pos	454.49	109.88-1109.85	80	0.048
EAC	Anti-CCP-2 levels in U/ml			
Total	Median	IQR	Patient Nr.	P-value
DRB1*13 neg	609.93	193.30-1297.80	391	REF
pos	421.54	172.61-1060.61	50	0.30
SE-negative stratum				
DRB1*13 neg	545.87	191.94-1108.21	62	REF
pos	342.82	58.08-1012.72	26	0.48
SE-positive stratum				
DRB1*13 neg	626.53	180.81-1340.76	220	REF
pos	500.41	221.90-1749.70	23	0.68

Table 2. HLA-DRB1*13 association with anti-CCP-2 level in ACPA-positive RA patients.

from the TWINGENE cohort, HLA-DRB1*13 does not protect from the development of ACPA in Japanese individuals without autoimmune diseases (OR=1.36, CI=0.74-2.51, p=0.33). When compared to the TWINGENE cohort, the Nagahama cohort had a lower frequency of HLA-DRB1*13 alleles (20% versus 29%) and fewer ACPA-positive individuals (56 versus 190). Still, the data show that also in Japan, the presence of HLA-DRB1*13 alleles seem to protect from the development of ACPA-positive RA, but not from the development of ACPA. Hence data from two independent cohorts supports a protective effect of HLA-DRB1*13 on the transition from ACPA-positive autoimmunity to ACPA-positive RA.

Influence of HLA-DRB1*13 on ACPA levels

There are many data which have indicated that the transition from from ACPA-positive autoimmunity to ACPA-positive RA is marked by pronounced changes in the

EIRA		DRB1*13			
Total		**negative**	**positive**	**OR (95% CI)**	**P-value**
Vim and/or eno	Neg	272 (24.5)	54 (42.9)	REF	
	Pos	838 (75.7)	72 (57.1)	0.42 (0.29-0.62)	<0.0001
SE-negative stratum					
Vim and/or eno	Neg	64 (44.76)	25 (54.35)	REF	
	Pos	79 (55.24)	21 (45.65)	0.56 (0.26-1.18)	0.12
SE-positive stratum					
Vim and/or eno	Neg	147 (25.88	29 (36.25)	REF	
	Pos	421 (74.21)	51 (63.75)	0.60 (0.36-0.99)	0.047
EAC		DRB1*13			
Total		**negative**	**positive**	**OR (95% CI)**	**P-value**
Vim and/or eno	Neg	112 (40.1)	24 (60.0)	REF	
	Pos	167 (59.9)	16 (40.0)	0.45 (0.23-0.88)	0.02
SE-negative stratum					
Vim and/or eno	Neg	30 (68.2)	15 (78.9)	REF	
	Pos	14 (31.8)	4 (21.1)	0.57 (0.16-2.04)	0.39
SE-positive stratum					
Vim and/or eno	Neg	59 (37.1)	9 (45.0)	REF	
	Pos	100 (62.9)	11 (55.0)	0.72 (0.28-1.84)	0.36

Table 3. HLA-DRB1*13 association with ACPA fine-specificities towards vimentin and alpha-enolase.

nature of the ACPA-response. Around disease onset, the ACPA response changes: ACPA levels increase, the number of isotypes expand and the ACPA response "spreads" to other citrullinated epitopes[35]. Since HLA-DRB1*13 alleles appear to inhibit this transition from pre-disease to RA, we investigated whether HLA-DRB1*13 can also shape the ACPA-response by influencing ACPA characteristics. To avoid skewing of the results due to the inverse association between HLA DRB1*13 and ACPA-positive RA, we further focused our analyses on ACPA-positive RA patients in two independent cohorts, the Dutch Early Arthritis Clinic (EAC, n=441) and the Swedish Epidemiological Investigation of Rheumatoid Arthritis (EIRA, n=1224) cohort.

In the EIRA, HLA-DRB1*13 alleles are associated with lower ACPA-levels (p=0.0002) (Table 2). Since ACPA-levels have been described to be increased in patients positive for SE-alleles[14], we next investigated if the effect of HLA-DRB1*13 on ACPA levels

is SE-independent or explained by an underrepresentation of SE-alleles in HLA-DR-B1*13-positive individuals. To this end, the patients were stratified into an HLA-SE negative and -positive stratum. This revealed that ACPA-levels were decreased in HLA-DRB1*13-positive patients in both the SE-positive (p=0.048) and SE-negative (p=0.003) stratum. In the EAC, the same difference in ACPA-levels between HLA DRB1*13-positive and –negative patients was visible, with absolute ACPA-levels which were remarkably similar to the EIRA. However, the differences in EAC did not reach statistical significance, which may be explained by low statistical power (Table 2).

Together, these data indicate that HLA-DRB1*13 alleles associate with lower ACPA-levels independent of SE-status.

Influence of HLA-DRB1*13 on ACPA fine-specificities

A correlation between ACPA-levels and the number of recognized citrullinated epitopes has been reported. Based on the lower ACPA-levels in HLA-DRB1*13-positive ACPA-positive RA patients, we wondered if these patients would also have fewer ACPA-fine-specificities than HLA-DRB1*13-negative ACPA-positive RA patients. Since it has previously been shown that the presence of HLA SE-alleles in ACPA-positive RA patients is associated with increased presence of certain ACPA fine-specificities, in particular ACPAs targeting citrullinated vimentin and α-enolase, we focused on these two ACPA-reactivities [18-20, 21, 22]. Therefore, we subdivided ACPA-posi-

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Patient characteristic	DR13-negative n=391	DR13-positive n=50	P-value
Age in yrs, mean (SD)	54 (15)	55 (12)	0.54
Female gender n (%)	268 (69)	29 (58)	0.14
Symptom duration at baseline in months (SD)	7 (10)	7 (11)	0.77
ESR in mm/h, mean (SD)	40 (29)	36 (25)	0.29
CRP in mg/ml, mean (SD)	27 (33)	26 (29)	0.71
median (IQR)	16 (7-35)	11 (7-30)	0.43
IgM-RF positive, n (%)	339 (87)	40 (80)	0.20
SJC, mean (SD)	9 (8)	9 (7)	0.76
HAQ, mean (SD)	1 (0.7)	0.9 (0.7)	0.52
SHS, mean (SD)	9 (12)	7 (6)	0.26
median (IQR)	6 (2-11)	5 (3-11)	0.47

Table 4. HLA-DRB1*13 association with baseline characteristics of ACPA-positive RA patients.

tive RA patients in HLA-DRB1*13-negative and -positive subsets and determined the percentage of patients positive for one or both of these fine-specificities. In accordance with our hypothesis, the presence of HLA DRB1*13 is associated with a decreased recognition of these particular fine-specificities in both the EIRA cohort (OR=0.42, CI=0.29-0.62, p<0.0001) and the EAC (OR=0.45, CI=0.23-0.88, p=0.02) , Table 3). Next, we investigated whether the observed association is SE-independent by performing stratification. Again the recognition of these fine specificities was markedly lower in the DRB1*13-positive patients in all strata and in both cohorts, although this only reached (borderline) significance in the SE-positive stratum in the EIRA (Table 3), possibly due to lack of statistical power. Serum reactivity to citrullinated epitopes on collagen type II and fibrinogen was also analyzed in EIRA, but no associations with HLA DRB1*13 were found, similar to findings concerning SE-alleles.

Together these data show that the presence of HLA-DRB1*13 may be associated with differences in the ACPA-profile, based on the lower ACPA-levels and diminished presence of anti- vimentin and anti-alpha-enolase-reactivities observed in both the EIRA and the EAC cohort.
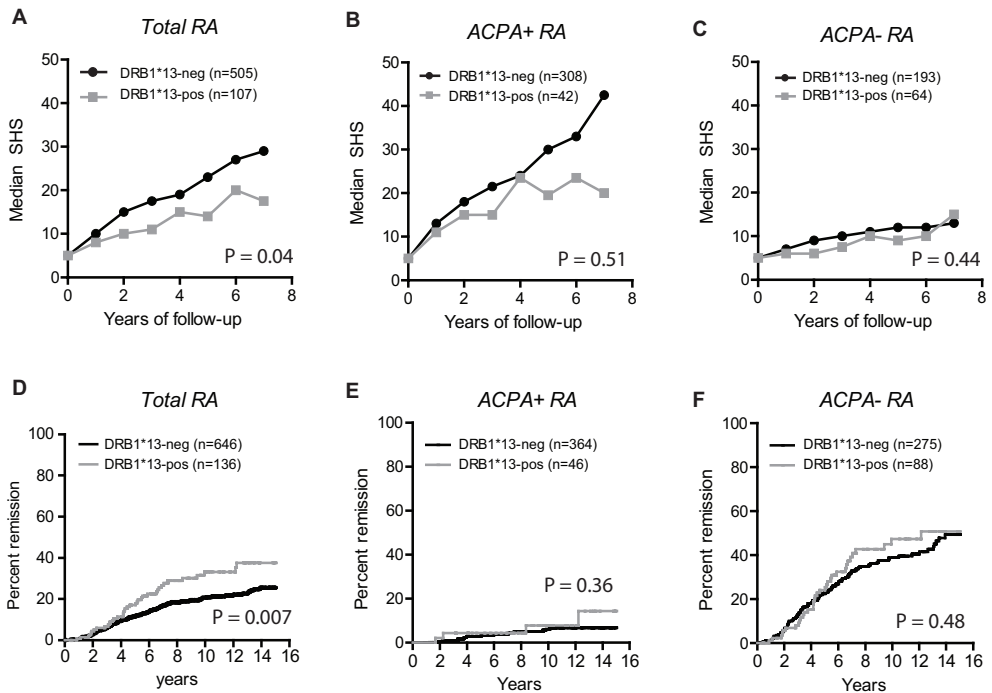
Figure 1. HLA-DRB1*13 association with radiographic progression (A-C) and clinical remission (D-F) in total RA patients (A,D), ACPA-positive (B,E) and ACPA-negative RA patients (C,D).

Influence of HLA-DRB1*13 on disease outcome

In order to complete our view of the role of the protective HLA DRB1*13 alleles in RA, we then analyzed the association of these alleles with clinical phenotype and disease outcome. To this end, we first compared the baseline characteristics in the EAC of HLA-DRB1*13-negative and HLA-DRB1*13-positive ACPA-positive RA patients, since the protective effect of the HLA DRB1*13 alleles is limited to ACPA-positive RA. This did not show any striking differences between the two subgroups (Table 4).

Concerning disease outcome we then assessed whether HLA-DRB1*13 is associated with radiographic progression or with DMARD-free sustained remission in the EAC. Previously, it was shown that ACPA-positive RA patients have a more rapid radiographic progression over time and a decreased chance of achieving sustained DMARD-free remission[13, 31]. In line with the decreased presence of ACPA in HLA DRB1*13-positive RA patients, we observed significantly less radiographic progression (p=0.04) and an increased risk for remission (p=0.007) in HLA-DRB1*13-positive RA patients when all RA patients were taken into account (Figure 1). To investigate whether this effect of HLA-DRB1*13 was independent of the presence of ACPA, we stratified for ACPA-status. Both in ACPA-positive and

Figure 2. HLA-DRB1*13 association with radiographic progression (A-D) and clinical remission (E-H) in ACPA-negative (C,D,G,H) and ACPA-positive RA patients. (A,B,E,F) in both SE-positive (B,D,F,H) and SE-negative (A,C,E,G) patients.

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ACPA-negative RA patients, the presence of HLA-DRB1*13 was then no longer asso-
ciated with radiographic progression of with remission (Figure 1).

To investigate whether the protective effect of HLA-DRB1*13 was dependent on
presence/absence of HLA-DRB1 SE, we stratified ACPA-negative and ACPA-positive
RA patients by SE and studied the effect of HLA-DRB1*13 on radiographic pro-
gression and remission (Figure 2). Again, the presence of HLA-DRB1*13 was not
associated with radiographic progression or with remission, both in the SE-negative
and the SE-positive stratum.

Together, these data suggest that neither risk (SE) nor protective (DRB1*13)
HLA-DRB1 alleles influence disease outcome measures independent of ACPA-status.

DISCUSSION

To enhance our understanding of HLA-mediated risk and protection in RA, it is
essential to grasp at what point during disease evolution these alleles exert their
effects. The contribution of the predisposing SE-alleles during different phases of
disease has been extensively studied [16-23]. However, this is the first report that
has studied the effect of protective HLA-DRB1*13 alleles during different phases of
disease development and in RA. Based on our data - generated from two large popu-
lation-based cohorts comprising ACPA-positive RA as well as ACPA-positive non-RA
individuals, and two large cohorts of ACPA-positive RA patients - we conclude that
HLA-DRB1*13 alleles mainly influence the risk of developing ACPA-positive RA,
rather than ACPA-positive autoimmunity (in the absence of clinical symptoms).
Further, that HLA-DRB1*13 alleles have some modifying effects on ACPA concen-
trations and ACPA fine-specificities, but not on bone destruction or chance of
DMARD-free sustained remission in response to treatment.

In the same twin cohort that was used in the present study, we recently des-
cribed the effect of SE-alleles on the development of ACPA-positive autoimmunity
in non-RA individuals versus the development of ACPA-positive RA[17], and we
could show that HLA-DRB1 SE-alleles were significantly associated with ACPA-po-
sitivity in non-RA individuals (OR=1.4), but that the HLA-DRB1 SE-association with
ACPA-positive RA was significantly stronger (OR=7.2). Interestingly, this observation
suggests that the most important effect of SE lies not in the evolution of anti-citrul-
line autoimmunity, but rather in determining the development of clinical symptoms
and disease. In contrast, smoking was associated with both ACPA-positivity and
ACPA-positive RA with equal strength (ORs approximately 1.3), indicating a more
important role for smoking and possibly also other environmental factors in the
initial development of ACPA.

Our data now indicate that similar to the SE-alleles, HLA-DRB1*13 alleles also mainly
influence the risk to develop arthritis rather than the risk of developing ACPA.

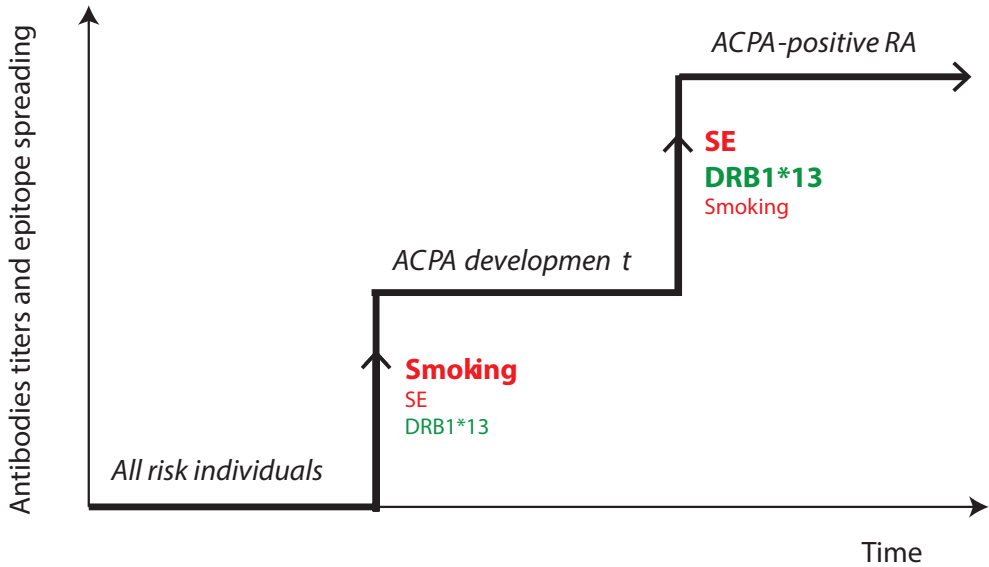These data support a previously postulated step-wise model for RA[17] (Figure 3)

Figure 3. Step-wise model of the development of RA. This figure is modified from reference 17.

that proposes that genotype is relatively more important for arthritis development. The fact that HLA-DRB1*13 appears to mirror the effect of the SE-alleles could indicate that these alleles act in the same pathophysiological pathway. Previous observations that SE-positive subjects have a reduced risk of developing ACPA-positive RA if they carry HLA-DRB1*13 alleles further supports this possibility[3-5]. Unfortunately, little is known about the molecular mechanisms underlying the association between HLA-DRB1*13 and ACPA-positive RA. Clues might come from previous work that indicate that a mother carrying protective HLA-DRB1 alleles can transfer RA protection to her child that does not carry such protective alleles[36]. This observation could support a role for microchimeric cells travelling from the mother to the child possibly influencing thymic selection of T-cells. Alternatively, specific antigen-presenting characteristics of the HLA DRB1*13-molecule might be involved.

In the present study, we specifically chose to examine the effects of the classical HLA DBR1*13 allele rather than look at specific amino acids within the HLA DR beta 1-chain which have been described to explain most of the association between MHC en seropositive RA[37]. The reasons for focusing on the classical HLA DRB1*13-allele were twofold. First of all, the protective effect of this allele for ACPA-positive RA has been well-described[4, 5], but it is unclear if this effect can be ascribed to certain amino acids, and if so, to which amino acids. Secondly, taking into account the biological and structural properties of HLA molecules, in which a large number of amino acids at various positions contribute to the peptide-binding groove, it seems wise to be cautious in inferring a causal role for a very limited number of amino

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acids. Our data support the idea that HLA-genotype plays that HLA-genotype plays only a modest role in established disease. Both the predisposing SE-alleles and the protective HLA-DRB1*13 alleles do not associate with disease outcome (e.g. clinical remission or radiographic progression) in ACPA-positive RA patients. These findings are in line with previous reports, which have also shown that the classical SE-alleles or distinctive predisposing amino acids (valine or leucine at HLA-DRB1 position 11) are not associated with disease outcome independently of ACPA [23, 31, 38]. HLA-DRB1*13 did associate with ACPA characteristics in the sense that HLA-DRB1*13-positive ACPA-positive RA patients have significantly lower ACPA levels and are less often positive for antibody responses towards citrullinated vimentin and alpha-enolase.

Our study has several limitations, the most important of which is the limited power of the studies concerning ACPA characteristics and outcome measures (radiographic damage and outcome measures) in HLA DRB1*13-positive RA patients. Due to the strong protective effect of HLA DRB1*13 on ACPA-positive RA, there is only a very small number of HLA DRB1*13-carrying individuals that develops ACPA-positive RA. This is also illustrated by the fact that homozygosity for HLA DRB1*13 hardly ever occurs among ACPA-positive RA patients (n=0 in the EAC, 4 in the EIRA). This makes it difficult to study ACPA characteristics and disease outcome in relevant subgroups of patients with sufficient power.

A further limitation is that only cross-sectional data from population-based cohorts (the TWINGENE sand the Nagahama study) are available to study risk factors for the presence of ACPA in non-RA individuals. Ideally, longitudinal data on the development and maturation of the autoantibody response in HLA DRB1*13-positive and –negative individuals would be used to reach more firm conclusions on the role of HLA DRB1*13 alleles during the different phases of disease development. The direction of the effect of HLA DRB1*13 alleles on the presence of ACPA in non-RA individuals differed in the TWINGENE (OR: 0.74) and Nagahama study (OR: 1.36), but was non-significant in both cohorts. This difference may indicate that the true effect might be associated with an odds ratio of approximately 1, which is in line with our conclusions that the HLA DRB1*13 alleles mainly affect the onset of RA in ACPA-positive individuals.

In conclusion, the protective HLA-DRB1*13 alleles can, like predisposing SE-alleles, influence ACPA characteristics, but (like SE) mainly have their effect in the timeframe between the development of ACPA and the onset of ACPA-positive disease. Previous studies have shown that ACPA can be detected more than 10 years before disease onset[10], thereby generating a window of opportunity, where at-risk individuals can be serologically identified based on the presence of ACPA and risk (HLA-)genotype. The protective effect of HLA-DRB1*13 in this timeframe suggests that pathways exist which might be targeted in order to prevent the onset of ACPA-positive disease. Understanding the molecular basis for HLA-DRB1*13-mediated protection from developing ACPA-positive disease could therefore potentially

offer new possibilities for autoantibody positive individuals at risk for RA.

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SUPPLEMENTARY INFORMATION

TWINGENE		ACPA- non-RA	ACPA+ RA	OR (CI)	P-value
DRB1*13	Neg	7566 (72,4%)	87 (83,7%)	REF	
	Pos	2888 (27,6%)	17 (16,3%)	0.51 (0.30-0.86)	0.01

Table S1. HLA-DRB1*13 association with ACPA-positive RA

CHAPTER 8

Crossreactivity to vinculin and microbes provides a molecular basis for HLA-based protection against rheumatoid arthritis.

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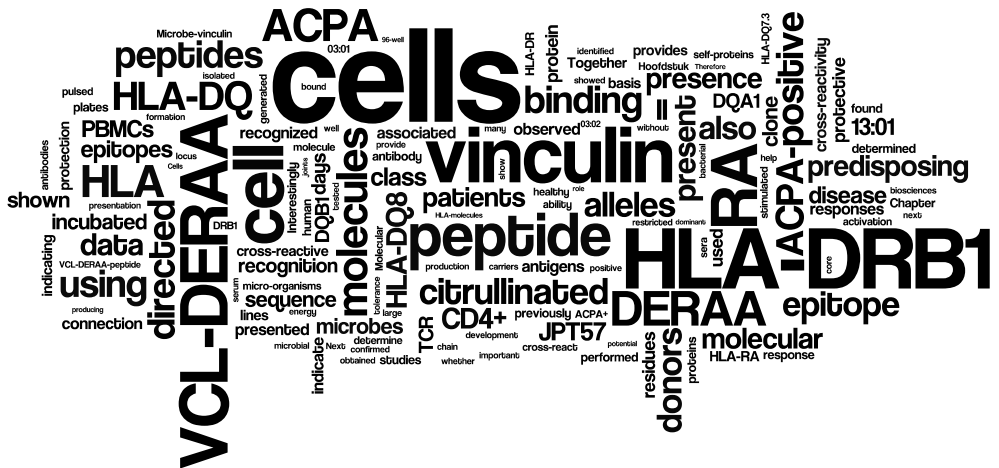
* Authors contributed equally

ABSTRACT

The HLA locus is the strongest risk factor for anti-citrullinated protein antibody (ACPA)+ rheumatoid arthritis (RA). Despite considerable efforts in the last 35 years, this association is poorly understood.

Here we identify (citrullinated) vinculin, present in the joints of ACPA+ RA patients, as an autoantigen targeted by ACPA and CD4+ T cells. These T cells recognize an epitope with the core sequence DERAA, that is also found in many microbes and in protective HLA-DRB1*13 molecules, presented by predisposing HLA-DQ molecules. Moreover, these T cells crossreact with vinculin- and microbial-derived DERAA epitopes. Intriguingly, DERAA-directed T cells are not detected in HLA-DRB1*13+ donors, indicating that the DERAA epitope from HLA-DRB1*13 mediates (thymic) tolerance in these donors and explaining the protective effects associated with HLA-DRB1*13.

Together, our data indicate the involvement of pathogen-induced DERAA-directed T cells in the HLA–RA association and provide a molecular basis for the contribution of protective/predisposing HLA-alleles.

INTRODUCTION

Rheumatoid Arthritis (RA) is a chronic autoimmune disease affecting synovial joints that can lead to severe disability. Pivotal pathophysiological insight has been obtained by the identification of Anti-Citrullinated Protein Antibodies (ACPA)[1,2]. These autoantibodies target proteins that have undergone a post-translational conversion of arginine to citrulline, catalyzed by peptidylarginine deiminases (PAD enzymes)[3]. ACPA are highly specific for RA, enriched in the joints of patients and can cross-react between citrullinated antigens that are expressed in the inflamed joints[4-8]. It is clear now that RA represents two main syndromes, ACPA-positive and ACPA-negative disease, each with distinct genetic and environmental risk factors and disease outcome[9-12]. Characteristics of ACPA (e.g. isotype usage and epitope spreading) indicate the involvement of CD4+ T cell help in shaping the ACPA response[13].

The most important genetic risk factor for ACPA+ RA is the HLA class II locus and risk is confined to a region with genes encoding for the beta chain of HLA-DR and the alpha and beta chain of HLA-DQ that are in tight linkage disequilibrium (LD) and inherit in haplotypes[14,15]. An understanding of the HLA class II association and the relative contribution of the HLA-DR and HLA-DQ locus has been lacking for the last 35 years.

Next to the association of the predisposing alleles to ACPA-positive disease, other HLA-molecules are associated with protection. These protective HLA alleles, mainly HLA-DRB1*13, carry the five amino acid sequence DERAA at positions 70-74 of the beta chain and protect also in the presence of predisposing alleles[16,17]. Intriguingly, protection by these alleles is transferred from mother to child, supporting an active protective role of these alleles, possibly via microchimeric cells influencing thymic selection of CD4+ T cells, and indicating a dominant role of HLA-DRB1*13 in disease protection[18].

HLA-derived peptides are a dominant peptide source presented by HLA class II molecules. We therefore proposed that the protective effect of HLA-DRB1*13 is explained by presentation of an HLA-DRB1*13 derived peptide in the context of other (predisposing) HLA class II molecules[19-21].

It was previously shown that degradation of HLA-DRB1*13 can result in the presentation of a peptide with the core sequence DERAA by other HLA class II molecules to CD4+ T cells[22]. This could allow for the negative selection of such "DERAA directed" CD4+ thymocytes. Interestingly, the DERAA sequence is also found in many microbes and in the self-protein vinculin. Vinculin is expressed in the synovium, and was recently shown to be citrullinated in the synovial fluid of an RA patient[4,23]. Likewise, T cells directed to vinculin are found under certain infectious conditions, indicating that T cell tolerance to vinculin is not absolute[24,25]. Molecular mimicry of self-proteins with pathogenic proteins was proposed as an important mechanism to break T cell tolerance[26,27]. Therefore, we postulate that upon priming

Chapter 8

of DERAA directed T cells by microbes expressing DERAA-containing proteins, T cells crossreactive to vinculin would be able to provide help to B cells reactive to citrullinated vinculin. This would ultimately result in the production of ACPA. In HLA-DRB1*13-positive donors these T cells are conceivably deleted, leading to protection against ACPA-positive disease.

METHODS

Cells and sera

HLA-typed buffy coats from healthy volunteers were obtained from the blood bank (Sanquin, The Netherlands). PBMCs and sera from RA patients were derived from patients participating in the Leiden Early Arthritis Clinic cohort [28]. All RA patients fulfilled the American College of Rheumatology (formerly the American Rheumatism Association) 1987 revised criteria for the classification of RA. A total of 178 RA patients were used in the current analyses. Patient samples were compared to 80 control samples from healthy individuals also living in the Leiden area. PBMCs were isolated using a standard Ficoll procedure. The protocols were approved by the Leiden University Medical Center ethics committee and informed consent was obtained.

Peptides

Peptides were synthesized according to standard Fmoc (N-(9-fluorenyl)methoxycarbonyl) chemistry using a SyroII peptide synthesizer (MultiSynTech, Witten, Germany). The integrity of the peptides was checked using reverse phase HPLC and MS. For vinculin ELISPOT experiments, HLA class II binding studies and T cell activation studies 15mer vinculin epitope $VCL_{622-636}$ (VCL-DERAA; REEVFDERAAN-FENH) was used. For ELISPOT assays against common pathogens, pathogenic peptides were used derived from measles virus (SSRASDERAAHLPTS), influenza A virus (VFEFSDERAANPIVP), human herpesvirus 7 (LAARADERAAFPDVG), B*ordetella pertussis* (SPNLTDERAAQAGVT), *Staphylococcus aureus* (QDMNDDERAALTMAM), *Haemophilus influenzae* (RFHGDDERAAKVYEN), *Salmonella enteritidis* (PLM-MDDERAAKVYEN) and *Propionibacterium acnes* (EEVFTDERAARLSHV). For the generation of T cell lines these 8 pathogenic peptides were pooled.

Cell lines

The following EBV-transformed lymphoblastoid B cell lines were used: BSM (DRB1*04:01;DQA1*03:01;DQB1*03:02), BOLETH (DRB1*04:01;DQA1*03:01; DQB1*03:02), BM21 (DQA1*05:01;DQB1*03:01), JSM (DQA1*03:02;DQB1*03:01), APD (DRB1*13:01;DQA1*01:03;DQB1*06:03), BC34 (DRB1*13:02;D-QA1*01:02;DQB1 *06:04), WT8 (DQA1*01:02;DQB1*06:02), KAS116

(DRB1*01:01;DQA1*01:02; DQB1*05:01) and 721.82 (DR/DQ-negative). Cells were maintained in IMDM (Lonza) supplemented with 10% heat-inactivated FCS.

HLA class II competitive peptide binding assay

Peptide-binding assays were performed, as described previously [29]. In short, cell lysates from HLA- class II homozygous B-lymphoblastoid cell lines were incubated on SPV-L3- (anti-HLA-DQ) or B8.11.2- (anti-HLA-DR) coated (10 µg/ml) FluoroNunc 96-well plates at 4ºC overnight. Titration ranges of the tested peptides (0 to 300 µM) were mixed with a fixed concentration (0.6 µM) of biotinylated indicator peptide and added to the wells. Bound indicator peptide was detected using europium-streptavidin (Perkin Elmer, Boston, MA) and measured in a time-resolved fluorometer (PerkinElmer, Wallac Victor2). IC50 values were calculated based upon the observed binding of the test peptide against the fixed concentration indicator peptide. The IC50 value depicts the concentration of test peptide required for a loss of 50% of the indicator peptide signal.

Enzyme-linked immunosorbent spot assay

PBMCs were incubated in 96-well Multiscreen HA plates (Millipore) precoated with 5 µg/ml anti-IFN-γ capture antibody (clone 1-D1K; Mabtech) at 5×10^5 cells/well and stimulated in X-VIVO medium (LONZA) with 5 µg/ml peptide or with a mix of recall antigens consisting of 0.75 Lf/ml tetanus toxoid (Netherlands Vaccine Institute), 0.5 µg/mL tuberculin purified protein derivative (Netherlands Vaccine Institute) and 0.005% Candida albicans (HAL allergy). After 24h incubation at 37ºC, plates were extensively washed and incubated with 0.3 µg/ml biotin labeled anti-IFN-γ detection antibody (clone 7-B6-1; Mabtech) for 2h at RT, with 1000x diluted ExtrAvidin-Alkaline phosphatase (Sigma-Aldrich) for 1h at RT and with 5-Bromo-4-chloro-3-indolyl phosphate/Nitro blue tetrazolium (Sigma-Aldrich). Spots were analyzed using the BioSys Bioreader 3000pro. Circular spots with a size of 80-450µm were included.

Flow cytometry

Polyclonal CD4+ T cell lines were generated by stimulating 3×10^6 PBMCs/well in a 24-well plate with 5 µg/ml peptide for 7 days in IMDM supplemented with 5% human serum (Sanguin). After 7 days 1.5×10^6 autologous PBMCs per well were plated in 24-well plates for 2h. After 2h non-adherent cells were removed and adherent cells were pulsed with 5 µg/ml peptide and used as feeders for 106 T cells. After 1h, 10 µg/ml brefeldin A was added. Cells were incubated overnight and used for intracellular cytokine staining. The cells were incubated with fluorochrome-conjugated antibodies recognizing CD4, (Clone RPA-T4; BD biosciences), CD14 (Clone 61D3; eBioscience) and CD25 (Clone M-A251; BD biosciences), after which they were permeabilized using CytoFix CytoPerm Kit (BD Biosciences). After washing, cells were incubated with PE-labeled anti-IFN-γ or matching isotype control. Cells

were taken up in 1% paraformaldehyde until flow cytometric acquisition. Flow cytometry was performed on FACS Calibur (BD biosciences) or LSR II (BD biosciences). Analysis was performed using FACS Diva (BD biosciences) and FlowJo software.

T cell cloning

JPT57 was generated from an HLA-DRB1*04:05/01:01;DQ8/DQ5 donor. PBMCs were cultured for 7 days in the presence of 10 μg/ml of VCL-DERAA peptide and restimulated with VCL-DERAA-pulsed antigen-presenting cells. After 1 week, cells were restimulated with 150 U/ml rIL-2. After two rounds of restimulation, T cell lines were tested for their specificity. The wells responding to VCL-DERAA peptide were cloned in a limiting dilution of 0.3 cells/well resulting in the isolation of clone JPT57.

D2C18 was generated from an HLA-DRB1*04:01;DQ2/DQ8 positive donor. CD4+ T cells and CD14+ monocytes were isolated from PBMCs using antibodies bound to magnetic beads from respectively Dynal and Miltenyi. CD4+ T cells were labeled with 1μM CFSE (Invitrogen) and incubated in a 2:1 ratio with CD14+ monocytes with 30 μg/ml PathMix. After 6 days, $CD3_{pos}CD4_{pos}CD25_{pos}CD14_{neg}DAPI_{neg}CFSE_{low}$ cells were sorted by FACS aria (BD). Isolated CD4+ T cells were rested in medium containing 20 IU/ml rIL-2 (Peprotech). After 3 days, cells were cloned in a limiting dilution of 0.3 cells/well resulting in the isolation of CD4+ T cell clone D2C18.

T cell activation

To determine IFN-γ production by T cell clones in response to peptide stimulation, 50.000 T cells were incubated in a 1:1 ratio with B-LCL lines pulsed with 10 μg/ml of peptide for 3-6h. After 3 days, supernatant was collected and an IFN-γ ELISA (eBioscience) was used to determine the concentration of IFN-γ. For measurement of T cell proliferation, 50.000 T cells were incubated in a 1:1 ratio with radiated (3000RAD) autologous PBMCs pulsed with 10 μg/ml of peptide for 3-6h in IMDM supplemented with 5% human serum. After 3 days, cells were cultured for 16–20h with [^{3}H]thymidine (0.5 μCi/well). ^{3}H incorporation was measured by liquid scintillation counting (1450 MicroBeta TriLux; PerkinElmer). Blocking experiments were performed by preincubating APCs for 1h with anti-DQ (SPVL3), anti-DR (B8.11.2) and anti-DP (B7.21) blocking antibodies.

Detection of anti-citrullinated vinculin antibodies

Citrullinated vinculin was generated by incubation of 50 μg vinculin protein (Sanbio) in a volume of 200 μl containing 0.1 M Tris-HCl pH 7.6, 0.15 M CaCl^2, and 10 U PAD4 (Sigma) for 4 h at 37 °C. Unmodified vinculin-protein was generated by incubation of vinculin with PAD4 without $CaCl_2$. Citrullinated and unmodified vinculin were loaded onto 10% SDS-polyacrylamide gels and transferred onto blotting membranes. Blots were blocked, washed and incubated in 1:500 diluted

serum overnight at 4°C. The sera were either ACPA-positive or ACPA-negative as determined by ELISA. Blots were incubated with HRP-labeled rabbit anti-human IgG (Dako) and visualized with chemiluminescence (ECL, Amersham). To analyze reactivity to vinculin by a monoclonal ACPA (anti-cFIB1.1, citrullinated fibrinogen), vinculin- or citrullinated vinculin-coated Nunc plates were incubated with 5 µg/ml anti-cFIB1.1 for 2h at room temperature [30]. Bound antibody was detected using HRP-labeled rabbit anti-human IgG (Dako) and visualized with ABTS. Peptide-ELISA was performed as described previously using biotinylated peptides coated on strep-tavidin precoated plates [31].

B cell activation by JPT57 cells

B cells were isolated from PBMCs of HLA-DQ8 positive healthy donors or ACPA positive RA patients by magnetic anti-CD19 beads (Invitrogen). B cells were cultured in IMDM supplemented with 10% FCS, penicillin, streptomycin and glutamax. From healthy subjects, 30,000 B cells were co-cultured with different number of JPT57 cells in the presence of 5µg/ml anti-IgM (JacksonImmunoresearch Laboratories) and with 10 µg/ml VCL-DERAA or 1 µg/ml PHA in round-bottom 96-well plates. After 7 days, IgG production by B cells was determined using a total IgG ELISA (Bethyl laboratories). From RA patients, 20,000 B cells were pulsed with 10 µg/ml VCL-DERAA peptide and co-cultured with 20,000 JPT57 cells in the presence of 5 µg/ml anti-IgM in round-bottom 96-well plates. After 7 days, ACPA production was determined by ELISA measuring reactivity against the CCP2-peptide in individual wells (EuroDiagnostica).

Energy minimization

Molecular simulations of HLA-DQ8(A1*0301/B1*302) and –DQ7.3 (A1*0302/B1*0301) complexed with various peptides, experimentally shown to bind to these molecules, were carried out as previously described using the Discover Suite (pro-grammes InsightII and Discover) of Accelrys (San Diego, CA, release of 2005) on a Silicon Graphics Fuel instrument, using a minimization approach previously des-cribed [32], i.e. one thousand steps of the steepest gradient method, followed by one thousand steps of the conjugate gradient method. Records of the energy of every step showed a continuous decrease in energy without any local minima and an energy asymptote for the last 300-400 steps of the conjugate gradient method. The base molecule was the crystal structure of HLA-DQ8 (A1*0301/B1*0302) with bound the insulin B11–23 peptide [33]. The region HLA-DQβ105-112 for which full coordinates were not available in the original data, was constructed by molecular replacement of the respective region from HLA-DR1 [34], after superposition of the β-plated sheet regions of the two molecules in the α1β1 domains. The binding registers of the vinculin and the bound microbial peptides were decided from the binding of truncated peptides as well as Arg-substituted peptides in presumed

anchor positions; energy minimization of successive registers confirmed the registers predicted from the binding data. The rotamers for the peptide residues were chosen from a library of rotamers provided by the software data base, in order to have no molecular clashes with the residues of HLA-DQ8. Minimizations were carried out either at pH 5.4 (endosomal pH) or 7.4 (extracellular). There are no similarly charged residues (e.g. Glu-Glu) with their charged groups so close to each other as to require that one of the residues be uncharged. Occasionally runs were performed on a Silicon Graphics Octane instrument with previous releases of the same software with very similar results. Figures are drawn using the WebLabViewer v.3.5 and DSViewerPro software of Accelrys, the latter currently freely available on the web. In the figures a side view of the bound peptide with the eye level placed at the
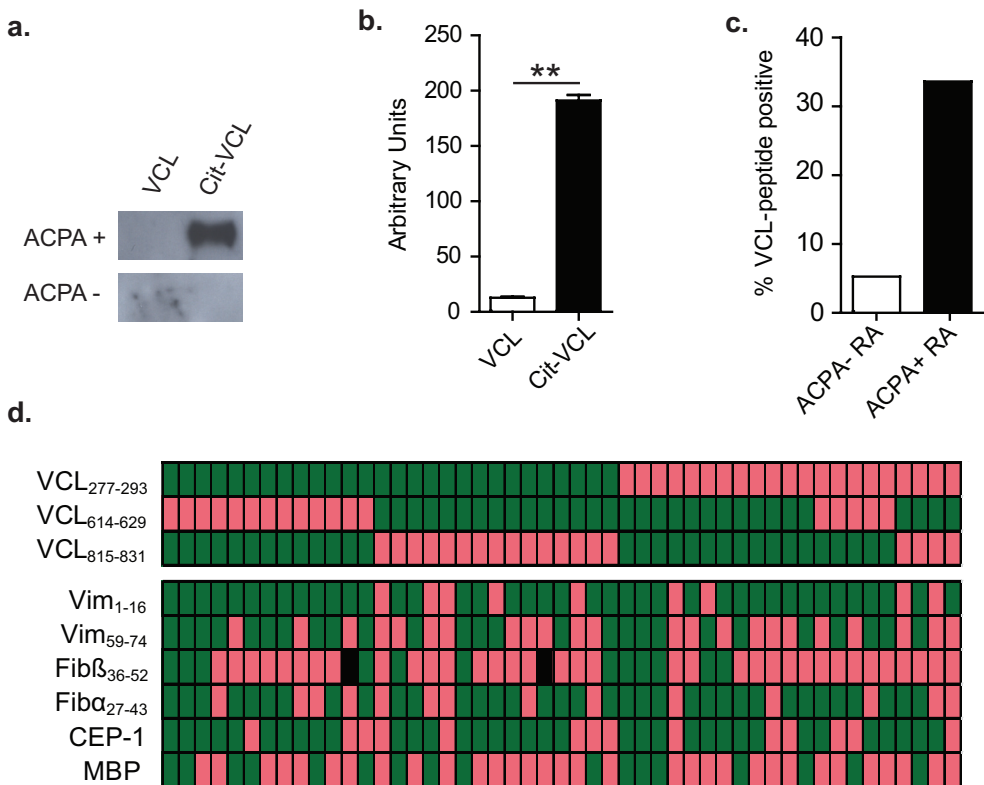
Figure 1. Citrullinated vinculin is a novel autoantigen targeted by ACPA. (a) Western blot of native and citrullinated vinculin protein, stained with serum of an ACPA-positive or an ACPA-negative RA patient. (b) ELISA of vinculin or citrullinated vinculin protein coated plates stained with ACPA monoclonal antibody anti-citFib1.1. Two-sided statistical analysis was performed using a student's t-test with ** indicating $P < 0.001$. (c) ELISA of citrullinated vinculin peptides VCL-CIT285$_{277-293}$, VCL-CIT622$_{614-651}$, VCL-CIT823$_{815-831}$ in serum of anti-CCP2 positive (n=140) versus anti-CCP2 negative (n=38) RA patients. Plot depicts the percentage of patient sera positive for one or more citrullinated vinculin peptides. (d) Heatmap depicting all patients positive for one or more citrullinated vinculin epitopes and their serum reactivity towards different citrullinated epitopes derived from vimentin, fibrinogen and alpha-enolase. Red = positive, green = negative, black is undetermined.

level of the peptide backbone and parallel to the HLA-DQ β-sheet floor is visualized with atomic charges coloured accordingly (red, acidic; blue basic, and with appropriate scales of grey for cases in between). In the supplementary figures, the α1β1 domains of the modelled HLA-DQ molecules in complex with given bound peptides are depicted in van der Waals surface representation, with color and depiction conventions identical to those for the other figures. Several visible residues from the HLA-DQ molecule in contact with the antigenic peptide and potential contact with a cognate TCR in canonical orientation are shown in stick form with a transparent surface (atomic color code: oxygen, red; nitrogen, blue; hydrogen, white; carbon, geen; sulfur, yellow). The antigenic peptide in the groove is shown in space-filling form.

Molecular Dynamics Simulations

The simulated system included the entire DQ8 molecule and the 13-residue peptide with the vinculin sequence Glu-Glu-Val-Phe-Asp-Glu-Arg-Ala-Ala-Asn-Phe-Glu-Asn, placed in a periodically replicated octahedral water box. The simulations were conducted with the molecular mechanics program CHARMM, version c37b2 [35]. The initial coordinates of all protein heavy atoms and the peptide mainchain heavy atoms were taken from the crystallographic structure of the DQ8:insulin complex (PDB entry 1JK8) [33]. The peptide side chain initial conformations were optimized with the program PROTEUS [36]. An additional control simulation studied the DQ8:insulin complex; the initial coordinates of the protein and peptide were taken from the corresponding crystal structure (PDB entry 1JK8) [33].

Protein atomic parameters were taken from the CHARMM36 all-atom force field [37, 38]. The interaction energies of selected peptide – protein residue pairs were computed by post-processing the MD trajectories in the GBSA approximation, as in [39] with the GBSW Generalized Born model [40, 41].

RESULTS

Citrullinated vinculin is a novel target for ACPA

We speculate that the protective effects of HLA-DRB1*13 on the development of ACPA-positive RA are related to a T cell response reactive to a sequence that is commonly present in the HLA-DRB1*13 molecule, micro-organisms and self-proteins that are targeted by ACPA. Indeed, a 5-amino-acid long HLA-DRB1*13-derived sequence (DERAA) is present in many micro-organisms and a few self-proteins. Of these self-proteins, the citrullinated protein vinculin attracted our attention. Therefore, we first analyzed whether citrullinated vinculin is recognized by ACPA. To this end, we citrullinated vinculin *in vitro* with PAD enzymes and tested both native and citrullinated vinculin for recognition with serum of an ACPA-positive RA-patient. We observed citrulline-specific recognition of vinculin by serum of an ACPA-positive RA
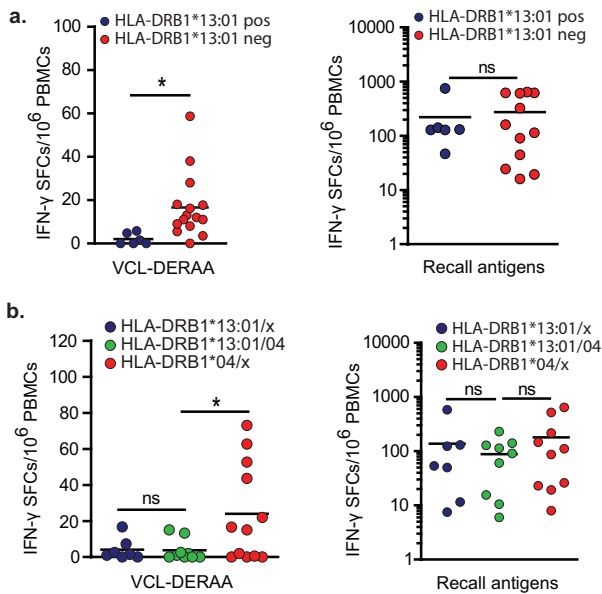
Figure 2. Citrullinated vinculin is a novel autoantigen targeted by CD4+ T cells in HLA-DRB1*13 negative donors. (a) IFN-γ ELISPOT of PBMCs from healthy individuals stimulated for 24h with VCL-DERAA peptide REEVFDERAANFENH or with a mix of recall antigens (tetanus, tuberculin, candida). Donors were stratified based on HLA-DRB1*13:01 status. (b) IFN-γ ELISPOT of PBMCs of HLA-DRB1*13:01 carriers, HLA-DRB1*04 carriers and HLA-DRB1*13:01/ DRB1*04 donors, stimulated for 24h with VCL-DERAA peptide or with a mix of recall antigens. Each dot represents a unique donor. Two-sided statistical analyses of ELISPOT data were performed using a Mann-Whitney U test with * indicating P < 0.05.

patient, but not with an ACPA-negative patient (Figure 1A). We further confirmed that citrullinated vinculin is a target of ACPA using an ACPA monoclonal antibody (Figure 1B) [30].

Recently, several reports showed the presence of citrullinated vinculin in the synovial fluid of ACPA+ RA patients. In addition, three sites of *in vivo* citrullination on this protein were identified: Arg285, Arg622 and Arg823 [4,23]. When we studied antibody responses to these citrullination sites, we could show recognition by 34% of tested sera from ACPA+ RA patients versus 5% of sera from ACPA- patients (Figure 1C). Sera of ACPA+ RA patients are highly (cross-)reactive towards multiple citrullinated antigens. Indeed, when we quantified responses to citrullinated alpha-enolase, fibrinogen, vimentin, and myelin basic protein in patient sera that react to citrullinated vinculin peptides, we could readily demonstrate additional reactivities indicating that ACPA are not exclusively directed against citrullinated vinculin as expected (Figure 1D). Together, these data show that citrullinated vinculin is a self-protein recognized by RA autoantibodies in a citrulline-dependent fashion.

Vinculin is an autoantigen recognized by T cells from HLA-DRB1*13-negative donors

We next wished to determine whether the DERAA-sequence from vinculin is recognized by human CD4+ T cells. To this end, PBMCs were stimulated with a 15-mer vinculin–peptide (VCL$_{622-636}$, REEVFDERAANFENH) (VCL-DERAA) for 24h. We observed a clear reactivity towards this epitope as determined in an IFN-γ-ELISPOT-assay (Figure S1). HLA-DRB1*13, especially HLA-DRB1*13:01, is strongly

associated with protection against ACPA-positive RA [17]. We hypothesize that HLA-DRB1*13 protects against ACPA-positive disease by affecting the generation of DERAA directed T cells. Therefore, we stratified donors for HLA-DRB1*13:01 status. We observed a striking difference in IFN-γ producing cells depending on HLA-DR-B1*13:01-status. The lack of reactivity in HLA-DRB1*13:01-positive donors was not due to a hampered ability of such donors to respond to T cell antigens as we observed strong response to microbial antigens in both HLA-DRB1*13:01 carriers and non-carriers when stimulated with recall antigens (Figure 2A). We further confirmed this finding in PBMCs stimulated for 4 days (Figure S2). The differential ability of HLA-DRB1*13:01-positive donors to respond to VCL-DERAA is most likely not due to a general deficiency to present the VCL-DERAA-epitope as these donors were heterozygous and thus expressed other HLA-molecules that could potentially present VCL-DERAA. Nonetheless, to further confirm that HLA-DRB1*13:01 affects the ability to generate VCL-DERAA directed T cell responses, we repeated the experiments in a set-up stratified for HLA using PBMCs from HLA-DRB1*04-, HLA-DRB1*13:01- and HLA-DRB1*04/*13:01 heterozygous donors. A significant reduction in IFN-γ producing cells was observed in both HLA-DRB1*13:01 carriers and DRB1*04/*13:01 heterozygous donors as compared to HLA-DRB1*04 carriers. Again, no difference was observed for recall antigens (Figure 2B). These data indicate that the lack of detecting VCL-DERAA directed T cells is not explained by the inability of HLA-DRB1*13 molecules to present VCL-DERAA, but rather the result of a dominant effect associated with the presence of HLA-DRB1*13.

Thus, vinculin is an autoantigen recognized by circulating VCL-DERAA directed CD4+ T cells. In HLA-DRB1*13:01 carriers these T cells are absent. This effect, like the protective effects of HLA-DRB1*13:01 on the development of ACPA-positive RA, was present in a dominant fashion, consistent with the notion that HLA-DRB1*13 affects the generation of DERAA directed T cells, possibly during thymic selection.
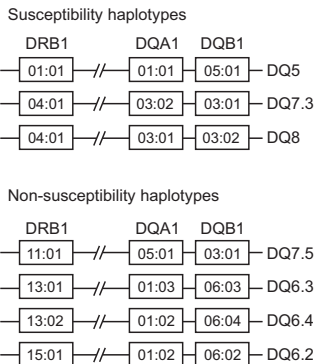
Presentation of the Vinculin-DERAA epitope is restricted to RA predisposing HLA-DQ molecules

HLA-DRB1 alleles predisposing to ACPA-positive RA in the Caucasian population are collectively called HLA shared epitope (SE) alleles [14,15,42]. The most common HLA-SE alleles are HLA-DRB1*04:01 and HLA-DRB1*01:01. Using HLA-DR binding assays, we examined the ability of VCL-DERAA to bind to these HLA-molecules, but we could not detect any binding of VCL-DERAA to these alleles (Figure 3A, Figure S 3A-B). The HLA class II region is known for its strong LD. Genes that encode for the HLA-DR-beta chains are inherited in haplotypes with genes that encode for the alpha and beta chain of HLA-DQ. The predisposing HLA-SE alleles are in tight LD with HLA-DQ5 (DQA1*01;DQB1*05:01), HLA-DQ7.3 (DQA1*03:02;DQB1*03:01) and HLA-DQ8 (DQA1*03:01;DQB1*03:02) (Figure 3B-C). In Figure 3C the distribution of HLA-DQ molecules in SE+ healthy donors is depicted, showing that these donors are HLA-DQ5, HLA-DQ7.3 or HLA-DQ8 positive. We therefore next studied

a.

Association	Subtype	HLA allele	IC50 (μM)
Susceptibility haplotypes	HLA-DR	DRB1*01:01	>300
		DRB1*04:01	>300
	HLA-DQ	DQ5	**39**
		DQ7.3	**11**
		DQ8	**14**
Non-susceptibility haplotypes	HLA-DR	DRB1*13:01	>300
		DRB1*13:02	>300
	HLA-DQ	DQ6.2	>300
		DQ6.3	>300
		DQ6.4	>300
		DQ7.5	>300

b.

Susceptibility haplotypes

DRB1	DQA1	DQB1	
01:01	01:01	05:01	DQ5
04:01	03:02	03:01	DQ7.3
04:01	03:01	03:02	DQ8

Non-susceptibility haplotypes

DRB1	DQA1	DQB1	
11:01	05:01	03:01	DQ7.5
13:01	01:03	06:03	DQ6.3
13:02	01:02	06:04	DQ6.4
15:01	01:02	06:02	DQ6.2

c.

HLA-SE+ACPA+ RA pts

HLA-DQ8 42%
HLA-DQ5 35%
HLA-DQ7.3 23%
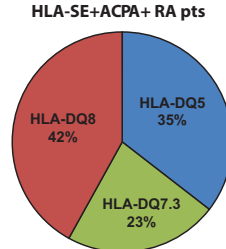
Figure 3. Presentation to vinculin-DERAA directed T cells is restricted to RA predisposing HLA-DQ molecules. (a) Affinity of VCL-DERAA for associated HLA class II molecules. Affinity was determined by a competitive binding assay using a high affinity biotinylated peptides. Results are depicted as IC50 value, the concentration of test-peptide (μM) where 50% of biotinylated peptide is bound. Experiments were performed at least three times and the average IC50 value between the different experiments is shown. (b) Schematic representation of the linkage disequilibrium between the HLA-DRB1, HLA-DQA1 and HLA-DQB1 gene in studied haplotypes associated or not associated with susceptibility to ACPA+ RA. (c) Distribution of HLA-DQ molecules in ACPA+ RA patients positive for HLA-DR shared epitope alleles (n=31).

the binding of VCL-DERAA to these HLA-DQ alleles and observed binding of the VCL-DERAA-peptide to HLA-DQ5, DQ7.3 and DQ8 (Figure 3A, Figure S3C-E). These data indicate that VCL-DERAA can be presented by HLA-DQ molecules in LD with predisposing HLA-SE alleles.

To obtain an indication whether the VCL-DERAA can be presented by more HLA molecules, we also studied the ability of additional HLA-DR and HLA-DQ molecules encoded by HLA-haplotypes that protect or have no influence on the development of ACPA-positive RA. We could not detect binding of VCL-DERAA to these HLA class II alleles (Figure 3A, Figure S3F-K). Likewise, using an IFN-gamma ELISPOT, VCL-DERAA directed T cells were absent in donors negative for predisposing HLA-DQ molecules (data not shown).

These data indicate that HLA-DQ-molecules that predispose to ACPA-positive disease present the VCL-DERAA-peptide, whereas the analyzed HLA-DQ and HLA-DR molecules not associated with disease do not present VCL-DERAA.

HLA-DQ restricted recognition of VCL-DERAA by T cells

To further confirm the presence of VCL-DERAA directed T cells and their HLA-restriction, we isolated CD4+ T cell clone JPT57, that was specific for VCL-DERAA. As shown in Figure S4, this clone proliferated readily and produced large amounts of

IFN-γ when stimulated with the VCL-DERAA-peptide.

When cultured with B cells from ACPA-positive RA patients pulsed with the VCL-DERAA epitope, the clone not only upregulated CD40L, but also enhanced the production of ACPA in culture, indicating that such T cells have a phenotype compatible with the ability to provide "help" to ACPA producing B cells (Figure S5).

To further confirm that HLA-DQ-molecules present the VCL-DERAA-peptide, we stimulated the clone with VCL-DERAA pulsed HLA-typed APCs pre-incubated with HLA class II blocking antibodies (Figure S6). In concordance with the binding studies, anti-HLA-DQ antibodies abrogated T cell recognition. Interestingly, we observed that JPT57 can recognize VCL-DERAA, presented by both HLA-DQ7.3 and HLA-DQ8 suggesting that this this epitope is presented in a similar binding register by these HLA-molecules (Figure S7).

Together, HLA-class II presentation and T cell recognition of VCL-DERAA was restricted to HLA-DQ molecules that are associated with RA susceptibility.

Identification of microbe-specific T cells targeting DERAA epitopes

We next investigated the presence of the DERAA sequence in micro-organisms. A Blast search showed that DERAA is found in 66% of bacteria and 12% of viruses (Figure 4A). This large number represents a major challenge to identify potential "cross-reactive micro-organisms". To select for relevant micro-organisms, we restricted the search to those micro-organisms that can cause disease or symptoms in humans and are present in the western world. This approach left us with 219 candidate sequences. We then synthesized 8 DERAA-containing peptides from common recall microbes including several bacteria (*P. acnes*, *S enteritidis*, *B. pertussis*, *S. aureus*) and viruses (measles virus, influenza A and human herpesvirus 7). Interestingly, all of these peptides were presented by HLA-DQ7.3 and DQ8 molecules showing that these HLA molecules can efficiently accommodate both the VCL-DERAA epitope as well as microbe-derived DERAA epitopes (Figure 4B).

To determine whether these microbial-derived epitopes are recognized by human T cells, we generated T cell lines by stimulating PBMCs from 3 healthy HLA-DQ8 positive donors for 7 days with a pool of the 8 pathogen-derived peptides (PathMix). Next, we determined the presence of PathMix specific CD4+ IFN-γ producing T cells. As shown in Figure 4C, such T cells were readily detectable. Using limiting dilutions, we isolated T cell clone D2C18, further confirming the presence of "microbial-DERAA-directed" T cells (Figure S8). Next, we also analyzed the presence of "microbial-DERAA directed" T cells by ELISPOT directly ex vivo, allowing the analyses of more donors at higher throughput. Interestingly, PBMCs from HLA-DRB1*13:01 carriers displayed a significantly reduced reaction against these peptides, indicating that the presence of HLA-DRB1*13 also affects the formation of T cell responses against DERAA-epitopes from microbes (Figure 4D).

Together, these data indicate that HLA-DQ alleles associated with ACPA-positive RA efficiently present both VCL-DERAA and microbe-derived DERAA epitopes and
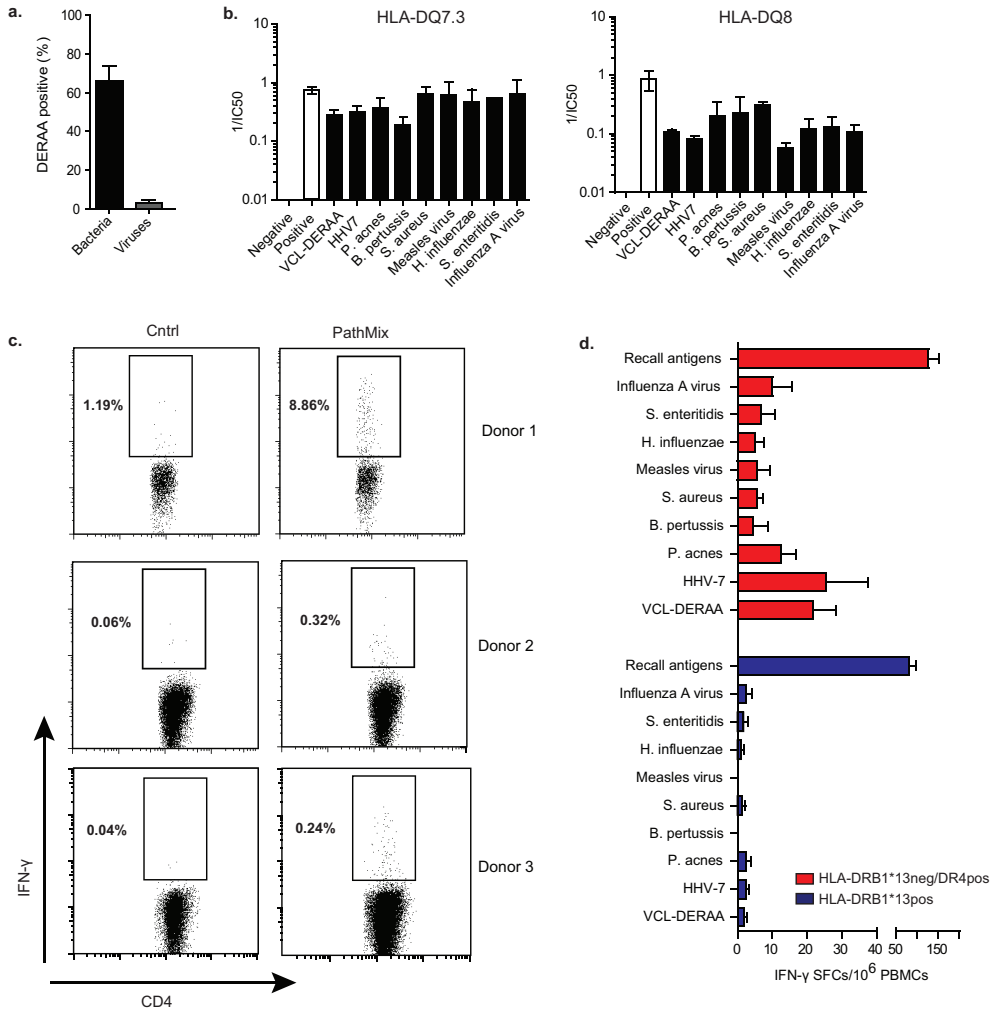
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Figure 4. Identification of pathogen-specific T cells targeting DERAA epitopes in HLA-DRB1*13 negative donors. (a) Frequency of bacteria or viruses positive for a DERAA containing protein as identified using the NCBI BLAST. List of all bacteria or viruses in blast database was and for three random sets of 50 bacteria/viruses a blast search for DERAA was performed. Error bars indicate the standard deviation between the three analyzed sets. (b) Competitive binding of pathogen-derived DERAA epitopes to HLA-DQ7.3 or HLA-DQ8. Binding experiments were performed at least three times and plots show pooled experiments, the error bars show the variation between the different experiments. (c) Flow cytometry staining of PathMix-directed T cell lines restimulated with PathMix pulsed or unpulsed APCs. Plots are gated on CD14neg/CD4pos/CD25pos T cells. (d) ELISPOT of PBMCs of healthy individuals of HLA-DRB1*13:01 donors (n=5) and HLA-DQ7.3/DQ8 donors (n=8) stimulated for 24 h with DERAA containing peptides and recall antigens. Error bars indicate the variation between different donors.

that microbe-specific T cells directed to DERAA epitopes are readily detected. The presence of HLA-DRB1*13:01 affects the formation of these T cell-responses.

Molecular modelling of HLA-DQ:VCL-DERAA complexes allows for predicting cross-eactive microbes

The data presented above demonstrate the presence of microbial- and VCL-DERAA directed T cells, but do not show if a single TCR can react with both epitopes. To facilitate the search for possible "crossreactive" DERAA-epitopes out of all microbe-derived DERAA-sequences, we first determined the binding register of VCL-DERAA using HLA class II binding assays with N- and C-terminally truncated and amino-acid substituted VCL-DERAA peptides as detailed in Figure S9-13. Together these experiments support VFDERAANF (anchors in bold) as the core binding register for both HLA-DQ7.3 and HLA-DQ8. All-atom molecular dynamics (MD) simulations showed that residues Val625 (P1), Glu628 (P4) and Phe633 (P9) make numberous intermolecular polar and non-polar interactions in the respective pockets. In Figure 5A-F we highlight key intermolecular interactions between the VCL-DERAA epitope and the HLA-DQ8 molecule. A detailed discussion of the MD results and a quantitative assessment of the intermolecular interactions can be found in the supplementary data (Figure S14-15 and Table S1). The MD simulations also indicated that the long protruding side-chains of Glu623 (P-2) Glu624 (P-1), Phe626 (P2), Asp627 (P3), Arg629 (P5), Asn632 (P8), Glu634 (P10) and Asn635 (P11) are exposed to solved and could potentially interact with crossreactive TCRs (Figure 5G). Subsequently we also further confirmed the obtained model using a second type of molecular modelling: energy minimization, that further confirmed possible TCR contact residues (Figure S16-17).

To functionally confirm whether a TCR would indeed interact with (some of the) potential TCR contact residues within the VCL-DERAA-peptide, we next determined how the epitope is recognized by the VCL-DERAA-directed T cell clone JPT57. Phe626 (P2) interacts with the JPT57 TCR as its removal results in a large decrease in recognition without affecting HLA-DQ8 binding. (Figure 5H, Figure S11). C-terminal truncations resulted in a large decrease in T cell recognition after removal of Glu634 (P10), showing that this residue is also important for JPT57 TCR interaction (Figure 5I). Thus, the data obtained using the VCL-DERAA directed T cell clone as a functional read-out, are in line with the HLA binding and molecular modeling studies indicating the sequence VFDERAANFE (anchors in bold) as the minimal epitope required for activation of JPT57. Next, we performed alanine substitutions within the minimal epitope to remove critical TCR interacting residues. Substituting Asn632 (P8) and Phe633 (P9) dramatically impacted T cell recognition, without affecting the binding affinity of VCL-DERAA (Figure 5J-K).

Together, these data indicate VFDERAANF (anchors in bold) as the most likely core binding register for HLA-DQ7.3 and HLA-DQ8 and Asn632 and Phe633 as important residues for JPT57-TCR recognition.
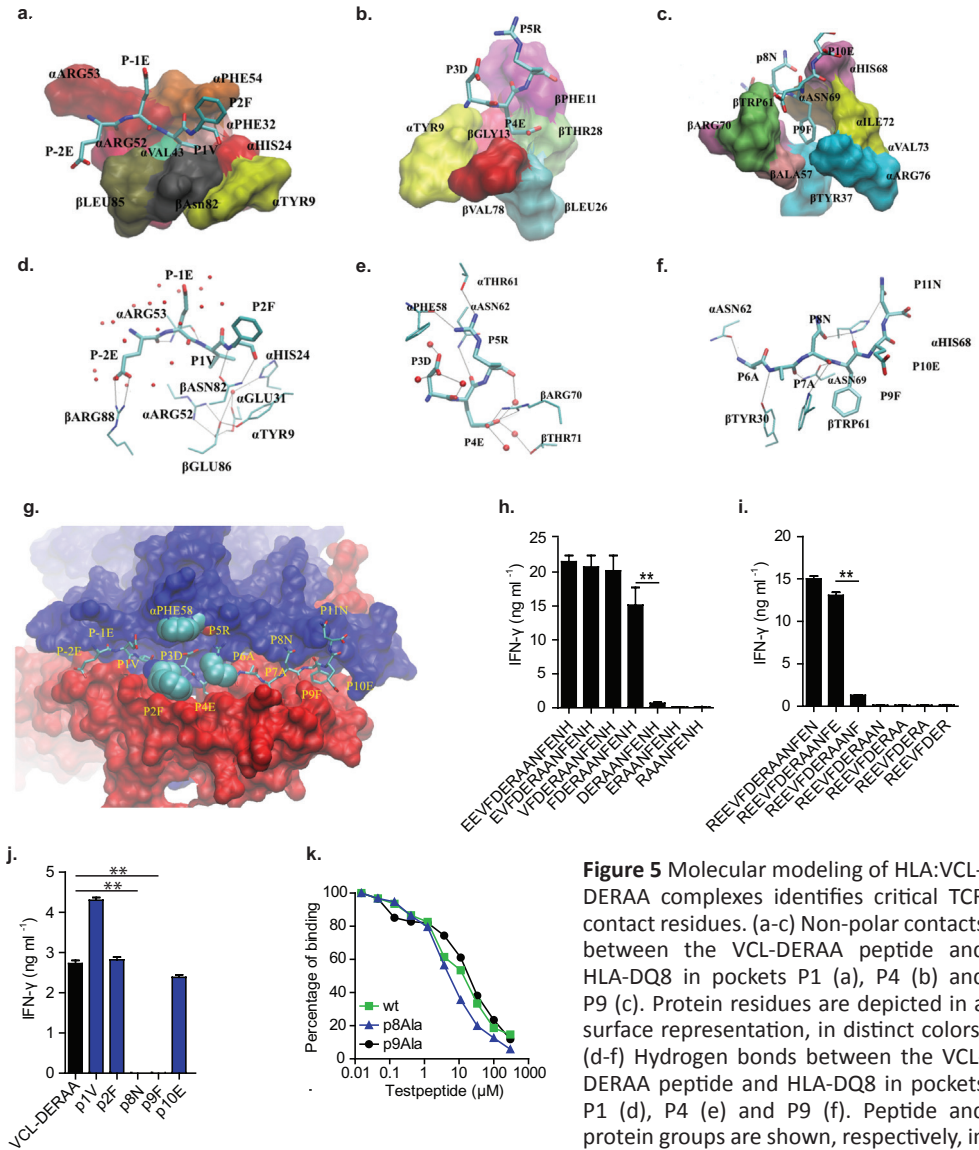
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Figure 5 Molecular modeling of HLA:VCL-DERAA complexes identifies critical TCR contact residues. (a-c) Non-polar contacts between the VCL-DERAA peptide and HLA-DQ8 in pockets P1 (a), P4 (b) and P9 (c). Protein residues are depicted in a surface representation, in distinct colors. (d-f) Hydrogen bonds between the VCL-DERAA peptide and HLA-DQ8 in pockets P1 (d), P4 (e) and P9 (f). Peptide and protein groups are shown, respectively, in thick and thin licorice representation, and water molecules as small spheres. Hydrogens are omitted, with the exception of the extra hydrogen in protonated αGlu31. (g) TCR view of the DQ8:VCL-DERAA complex.The peptide mainchain is shown in licorice; Domains α and β are shown in a surface representation, colored, respectively, in blue and red.Sidechains P-2E, P-1E, P2F, P3D, P5R, P8N, P10E and P11N are facing toward the TCR binding site. The P2F and αPhe58 sidechainsand the nonpolar moiety of P5Rform a nonpolar cluster, that faces toward the TCR binding site; the average P2F ring - αPhe58 ring distance is ~ 8 Å. (h-j) IFN-Ⅎ ELISA on supernatant of T cell clone JPT57 stimulated with unpulsed HLA-DQ8 positive APCs and N-truncated (h), C-truncated (i) or alanine-substituted (j) VCL-DERAA peptides. (k) Competitive binding of the VCL-DERAA peptide, and alanine substituted VCL-DERAA peptides to HLA-DQ8. Binding experiments were performed at least three times and plots show pooled experiments, the error bars show the varia-tion between the different experiments. Experiments involving JPT57 were performed three times and show a representative experiment. Two-sided statistical analysis was performed using a student's t-test with * indicating P < 0.05 and ** indicating P < 0.001.

TCR crossreactivity between vinculin and bacterial antigens

Identifying microbes that crossreact with vinculin was challenging due to the large number of potential candidates. Therefore, we used the data presented above to determine if a single TCR can crossreact both to DERAA-sequences from microbes and the self-protein vinculin. As we identified the Asn632 and Phe633 at P8 and P9 respectively, as important for JPT57-TCR interactions, we used the molecular modeling to predict which viral and bacterial epitopes are likely to bind to HLA-DQ8 and harbor P8Asn or P9Phe and are thus likely to crossreact with JPT57. From the 15 peptides identified (Table S2), three were able to activate JPT57 in a T cell stimulation assay at concentrations similar to those used for the vinculin-peptide. These epitopes were derived from *Campylobacter coli*, *Lactobacillus curvatus* and *Lactobacillus sakei* and cross-reacted with JPT57 in an HLA-DQ dependent manner
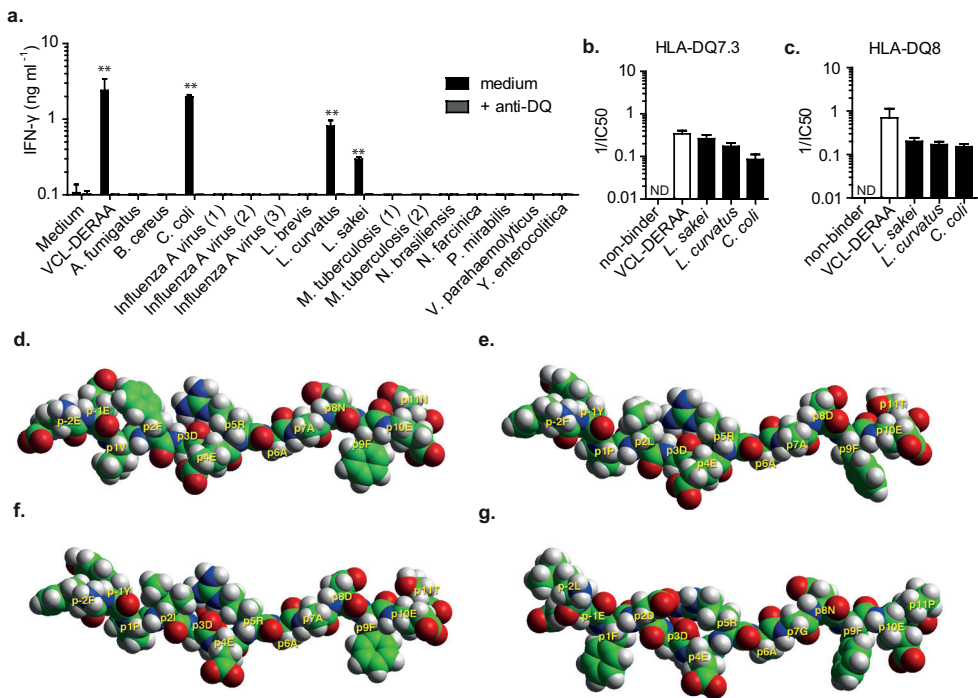
Figure 6 TCR crossreactivity of vinculin and bacterial DERAA epitopes. (a) IFN-γ ELISA on supernatant of T cell clone JPT57 stimulated with HLA-DQ8 positive B-LCLs and alanine-substituted VCL-DERAA peptides. This experiment was performed three times, the plot shows a representative experiment. Two-sided statistical analysis was performed using a student's t-test with * indicating P < 0.05 and ** indicating P < 0.001. (b-c) Competitive binding of HLA-DQ7.3 (b) and HLA-DQ8 (c) to a non-binding negative control peptide, an unbiotinylated positive control peptide and epitopes from *Lactobacillus curvatus*, *Lactobacillus sakei* and *Campylobacter coli*. Binding experiments were performed three times. The error bars show the variation between the different experiments. (d-g) Side views of HLA-DQ8 complexed with the vinculin-DERAA peptide (d) and peptides derived from *Lactobacillus curvatus* (e), *Lactobacillus sakei* (f) and *Campylobacter coli* (g) in space-filling mode at pH 7.4 (extracellular), obtained by energy minimization as described in the methods section. The peptide is shown in atomic color code: oxygen, red; nitrogen, blue; hydrogen, white; carbon, green; sulfur, yellow.

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(Figure 6A). Peptide-binding studies revealed that these three peptides bind with an intermediate binding affinity to HLA-DQ7 and HLA-DQ8 (Figure 6B-C).Molecular models of these bacterial peptides in HLA-DQ8 illustrate the similarities with VCL-DERAA (Figure 6D-G, Figure S18-20).

Thus, these data show that the RA susceptibility alleles HLA-DQ7 and HLA-DQ8 can present both VCL-DERAA and related microbe-derived epitopes to T cells and that such T cells can be cross-reactive to vinculin and bacterial epitopes, thereby providing an explanation for the presence of activated self-reactive CD4+ T cells directed to vinculin in peripheral blood.

DISCUSSION

The strong connection between the HLA locus and RA has been known for more than 35 years. The complex HLA class II associations and the diverse ACPA responses in RA patients suggest the presence of multiple etiological pathways. To unravel these pathways, identifying the relevant autoantigens is crucial. We here present evidence favouring the involvement of vinculin in the emergence of ACPA-positive disease. This cytoskeletal protein was recently found to be citrullinated *in vivo* in the synovial fluid. We now show that it is recognized by ACPA as well, thereby adding it to a still selective list of targets. Moreover, we identified an epitope from vinculin recognized by CD4+ T cells restricted to HLA-DQ molecules predisposing to ACPA-positive RA. The core amino acid sequence (DERAA) is also present in many pathogens and in HLA-DRB1*13, a molecule encoded by an HLA-locus associated with protection against ACPA-positive RA. We have also shown that a single TCR can recognize both a vinculin DERAA-epitope as well as DERAA-epitopes from microbes, indicating the crossreactive nature of "DERAA"-directed T cell responses. More importantly, such T cell responses appear absent from donors carrying HLA-DRB1*13 as DERAA directed T cell responses to either pathogen- or vinculin-derived DERAA epitopes were lacking in these subjects. Even donors that harbored HLA-DRB1*13 next to predisposing HLA-alleles were unable to respond to vinculin DERAA.

Together, these data indicate a novel pathway that explains several of the protective- and predisposing HLA-effects associated with ACPA-positive RA (Figure 7). In short, recognition of citrullinated vinculin by B cells will lead to the presentation of the "VCL-DERAA" epitope in the context of HLA class II molecules. The HLA-DQ-molecules genetically linked to the predisposing HLA-SE-molecules are particularly good in presenting DERAA-containing peptides. The DERAA directed T cells primed against various pathogens harboring DERAA-containing proteins cross-react with the VCL-DERAA peptide and provide help to the B cells, ultimately leading to a strong ACPA-response. Subjects born with HLA-DRB1*13, will present the HLA-DRB1*13-derived DERAA-peptide in the thymus, leading to tolerization
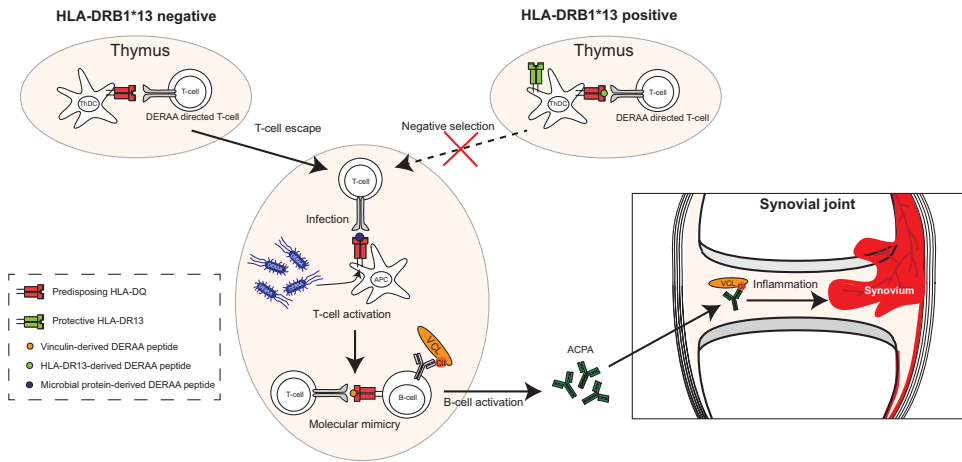
Figure 7 Schematic representation of the role of DERAA directed CD4+ T cells in ACPA-positive RA. DERAA directed T cells are restricted to RA predisposing HLA-DQ5, -DQ7.3 and −DQ8 molecules. In carriers of these HLA-DQ molecules, DERAA directed T cells can become activated upon contact with microbes. These activated T cells can subsequently cross-react with a DERAA epitope derived from vinculin resulting in the activation of citrulline-directed B cells and the production of ACPA directed to citrullinated vinculin or vinculin-linked proteins. Citrullinated vinculin is present in the synovial compartment and is a target of ACPA. Binding of ACPA to its target can induce antibody mediated effector mechanisms thereby contributing to synovial inflammation. In HLA-DRB1*13 positive individuals, a HLA-DRB1*13 derived DERAA epitope is presented by predisposing HLA-DQ molecules to CD4+ T cells resulting in their negative selection, thereby protecting against the development of ACPA-positive RA.

of the DERAA-reactive T cell response and hence the inability to provide help to ACPA-producing B cells via this pathway and thereby the emergence of ACPA-positive RA (Figure 7). The variation of HLA-DR and HLA-DQ molecules in the human population is enormous. We have shown that predisposing HLA-DQ molecules are particularly good at presenting the VCL-DERAA epitope. Interestingly, the absence of VCL-DERAA affinity for a wide variety of other tested HLA-DR or HLA-DQ molecules could indicate a selective presentation by these HLA risk-molecules. Next to role of predisposing haplotypes, we also focussed on the protective effect of HLA-DRB1*13 alleles. However, the DERAA sequence can also be found in other HLA-DRB1 alleles (*04:02, *11:02, *11:03), which are rare in Caucasian populations. These alleles have previously been implicated in protection from ACPA+ RA, but their allele frequency hampers functional studies [43-45]. Interestingly, it has been previously reported that the processing of these alleles results in the generation of a similar HLA-DERAA epitope suggesting that these alleles could all protect via the pathway that we have described [22].

The place in time at which HLA-DR13 mediates protection from the development of ACPA and/or ACPA-positive disease, or its relation to epitope spreading of the ACPA response is currently not known and would be relevant to determine in future studies. Recent evidence showed that the ACPA response matures prior to disease onset and that the HLA system could be involved in this [13, 46]. It is intriguing to

speculate that viral infections such as by DERAA containing microbes are involved in this expansion. Molecular mimicry of self-proteins with pathogenic proteins was proposed as a mechanism to break T cell tolerance, allowing the development of autoimmune disease [26,27]. Interestingly, the DERAA sequence is also present in proteins from many (common) microbes allowing priming of DERAA directed T cells. In mouse models, it was shown that low-avidity T cells to tissue-restricted antigens can persist without signs of anergy and unresponsiveness. Infection lowers the threshold for T cell activation resulting in the induction of autoimmunity and memory formation [47]. Infection could also induce autoimmunity via molecular mimicry of microbial proteins with self-proteins. We now identified crossreactive epitopes from the gut-residing bacteria L. sakei, L. curvatus and C. coli. It was shown that acute gastrointestinal infections can induce loss of T cell tolerance to (commensal) gut microbes, resulting in the activation of microbiota-specific T cells, their differentiation to inflammatory effector cells, and formation of memory T cells [48]. A recent study on the fecal microbiota of RA patients compared to controls demonstrated a significant increase in Lactobacillus species, together providing a rationale for a role of such bacterial species in the formation of DERAA-directed T cell responses [49].

Together, our study provides a mechanistic clue on the HLA-RA connection, including both predisposing and protective HLA-effect, and warrants further studies addressing the possibility to target DERAA-directed T cells in the prevention of ACPA-positive RA.

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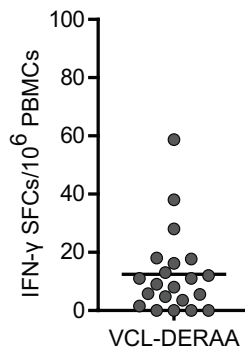
SUPPLEMENTARY INFORMATION

Figure S1. Vinculin is an autoantigen recognized by circulating CD4+ T cells. IFN-γ ELISPOT of PBMC from healthy individuals stimulated for 24h with VCL-DERAA peptide REEVFDERAANFENH. Each dot represents a unique donor.
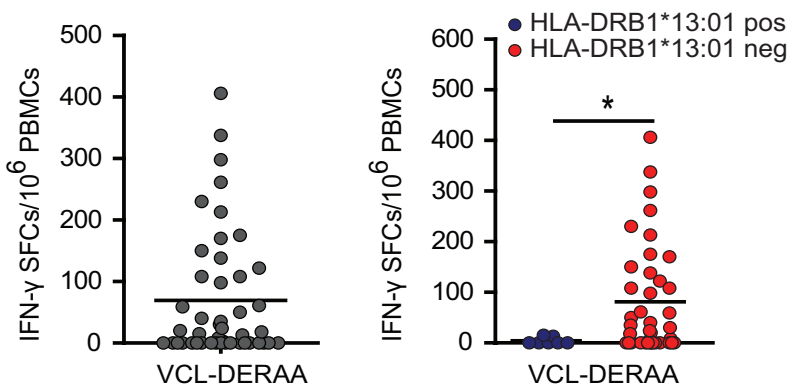
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Figure S2. Vinculin is an autoantigen recognized by circulating CD4+ T cells of HLA-DRB1*13 negative donors. IFN-γ ELISPOT of PBMC from healthy individuals stimulated for 4d with VCL-DERAA peptide REEVFDERAANFENH (Left panel). Stratification of donors in two distinct groups based on the presence or absence of HLA-DRB1*13:01 (Right panel). Each dot represents a unique donor. Two-sided statistical analyses of ELISPOT data were performed using a Mann-Whitney U test with * indicating P > 0.05.
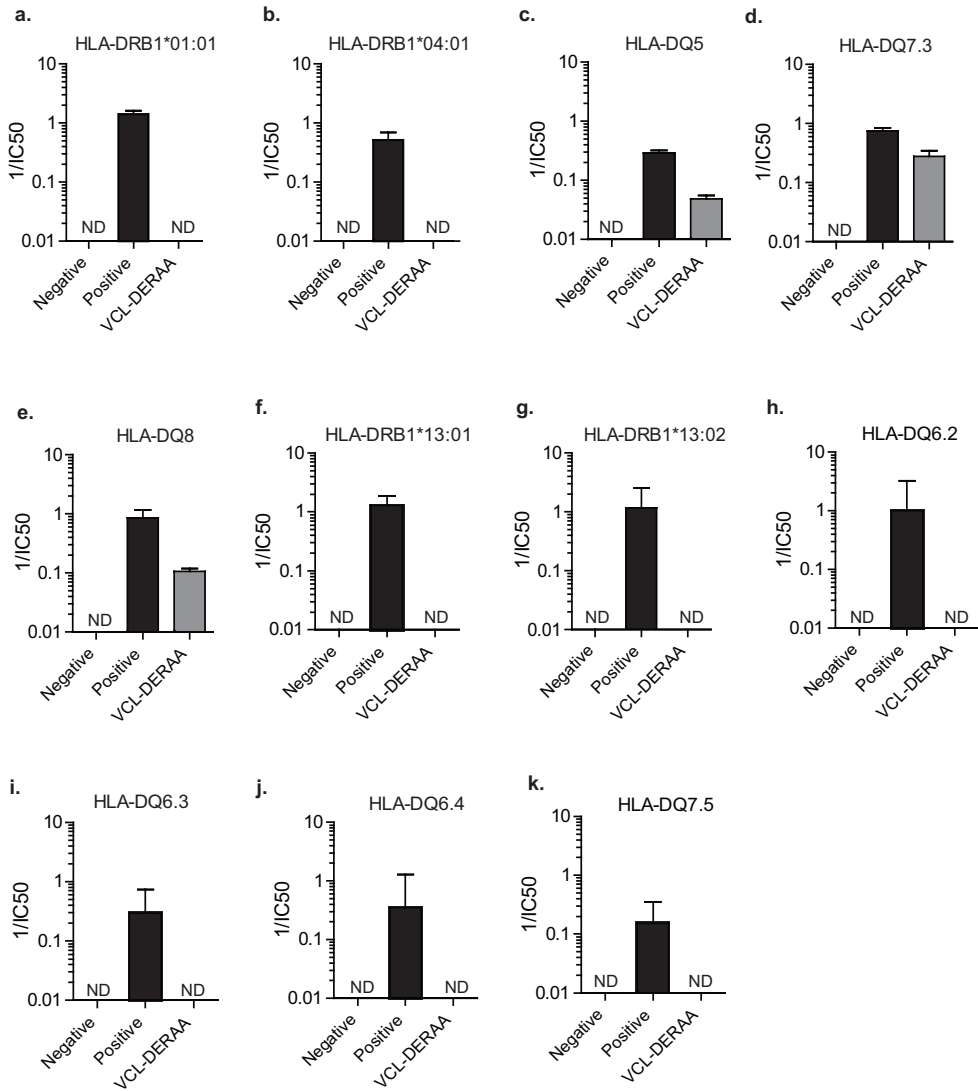
Figure S3. Presentation to vinculin-DERAA directed T cells is restricted to RA predisposing HLA-DQ molecules. Competitive binding of a non-binding negative control peptide, an unbiotinylated positive control peptide and the VCL-DERAA peptide to HLA-DRB1*01:01 (a), HLA-DRB1*04:01 (b), HLA-DQ5 (c), HLA-DQ7.3 (d), HLA-DQ8 (e), HLA-DRB1*13:01 (f), HLA-DRB1*13:02 (g), HLA-DQ6.2 (h), HLA-DQ6.3 (i), HLA-DQ6.4 (j) or HLA-DQ7.5 (k). IC50 is the concentration of test-peptide (µM) where 50% of bio-tinylated peptide is bound, ND = non-detectable (IC50 > 300 µM). All experiments were performed at least three times and bars show pooled experiments, the error bars show the variation between the different experiments.
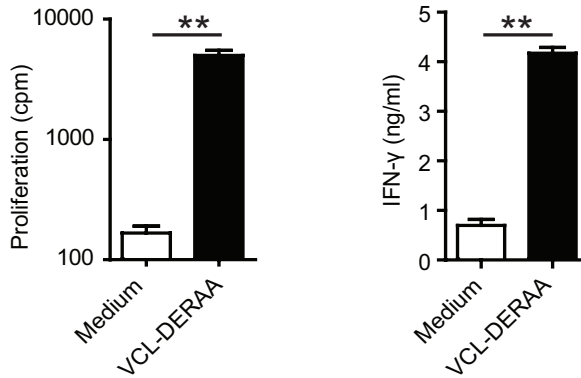
Figure S4. T cell clone JPT57 specifically recognized the VCL-DERAA epitope. 3H-thymidine incorporation and IFN-γ production upon stimulation of T cell clone JPT57 with unpulsed and VCL-DERAA pulsed autologous APCs. Data are representative of at least three independent experiments. Two-sided statistical analysis was performed using a student's t-test with ** indicating P < 0.001.
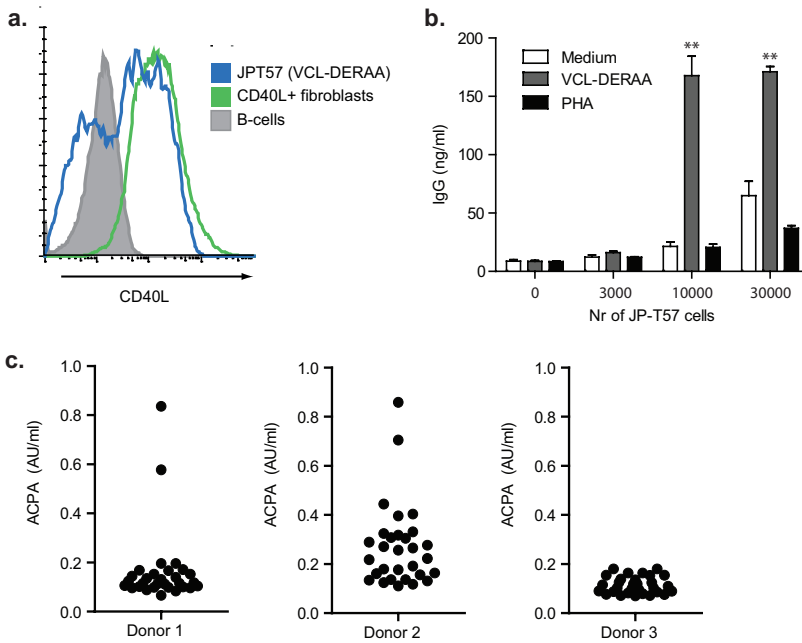

Figure S5. Activated JP-T57 cells can activate ACPA producing B cells (a) Flow cytometry analysis of CD40L expression on JPT57 cells stimulated with VCL-DERAA pulsed APCs, CD40L transfected fibroblasts and EBV transformed B cells. (b) Co-culture of VCL-DERAA pulsed HLA-DQ8 positive B cells from healthy subjects with increasing numbers of JPT57 cells in the presence of anti-IgM. The experiment was repeated three times and the plot shows a representative experiment. (c) Co-culture of VCL-DERAA pulsed HLA-DQ8 positive B cells from three ACPA positive RA patients with JPT57 (1:1) in the presence of anti-IgM. Activation of ACPA producing B cells was determined by CCP2 ELISA. Each dot represents a different well. Statistical analysis was performed using a student's t-test with ** indicating P > 0,001.
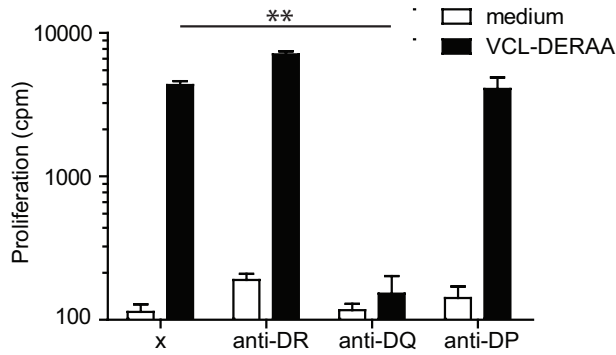
Figure S6. Vinculin-DERAA directed T cell clone JPT57 is HLA-DQ restricted. 3H-thymidine incorporation of JPT57 stimulated with feeders, pulsed or unpulsed with VCL-DERAA in the presence of HLA-II blocking antibodies. Data are representative of at least three independent experiments. Two-sided statistical analysis was performed using a student's t-test with ** indicating P < 0.001.

Figure S7. Vinculin-DERAA directed T cell clone JPT57 is restricted to HLA-DQ7.3 and HLA-DQ8. IFN-γ production by JPT57 cells stimulated with HLA-DQ7.3 or HLA-DQ8 homozygous EBV-transformed B cells. Data are representative of at least three independent experiments. Two-sided statistical analysis was performed using a student's t-test with ** indicating P < 0.001.
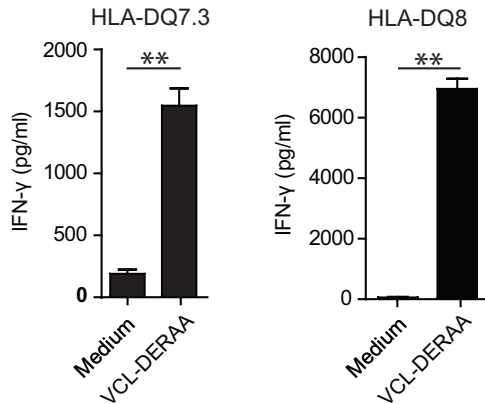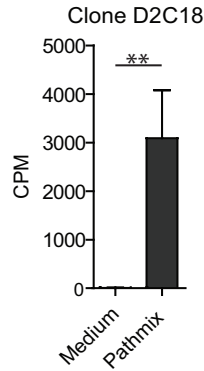
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Figure S8. Identification of a Pathmix directed T cell clone in an HLA-DRB1*13 negative donor. 3H-thymidine incorporation upon stimulation of T cell clone D2C18 with unpulsed and salmonella-DERAA pulsed autologous monocytes. The experiment was repeated two times and the plot shows a representative experiment. Two-sided statistical analysis was performed using a student's t-test with ** indicating P > 0.001.
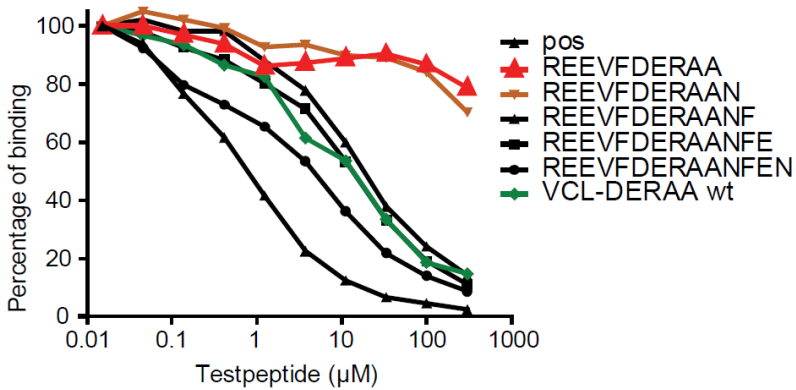

Figure S9. Truncation of Phe633 results in loss of binding to HLA-DQ8. Competitive binding of HLA-DQ8 to an unbiotinylated positive control peptide and C-truncated VCL-DERAA peptides. The experiment was repeated three times and plots show a representative experiment.

Figure S10. Arginine substuting Phe633 negatively impacts binding affinity to HLA-DQ8. Competitive binding of a negative control peptide, the native VCL-DERAA peptide and the VCL-DERAA peptide with Phe633 substituted to arginine. Experiments were performed three times and plots show a representative experiment.
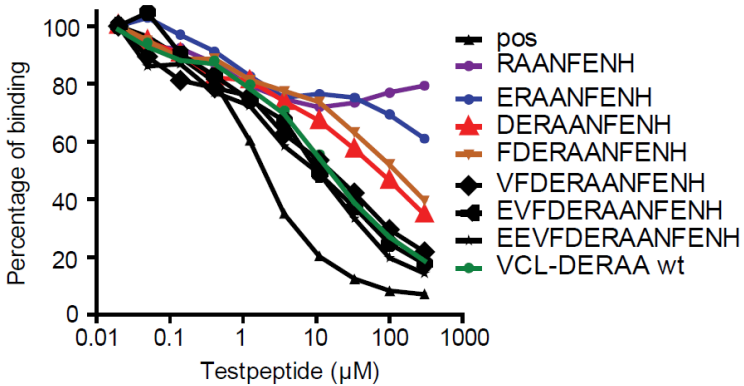
Figure S11. N-terminal truncation of Val625 lowers the binding affinity to HLA-DQ8. Competitive binding of HLA-DQ8 to an unbiotinylated positive control peptide and N-truncated VCL-DERAA peptides. The experiment was repeated three times and plots show a representative experiment.
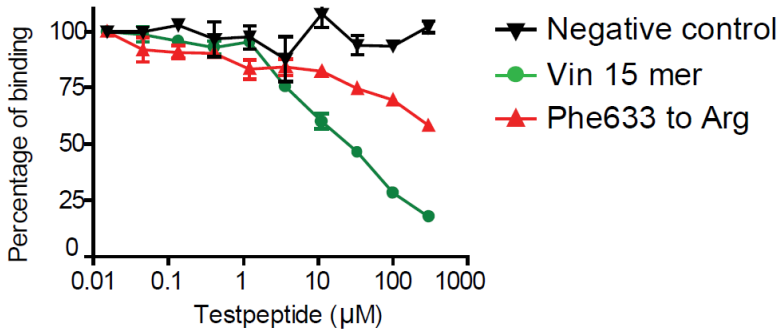
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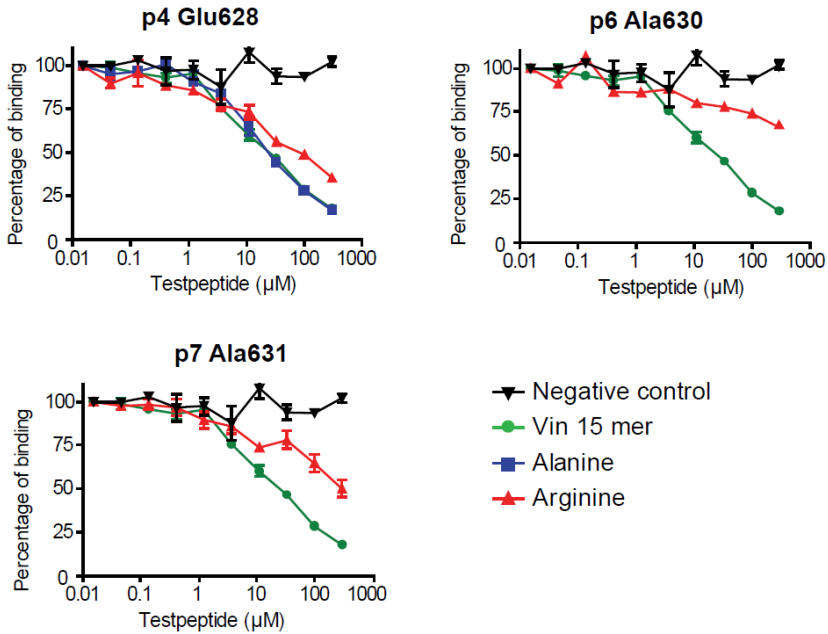
Figure S12. Arginine substitutions of residues implicated to interact with binding pockets negatively impacts binding to HLA-DQ8. Competitive binding of a negative control peptide, the native VCL-DERAA peptide and the VCL-DERAA peptide with Glu628, Ala630 and Ala631 substituted to alanine or arginine. Experiments were performed three times and plots show a representative experiment.
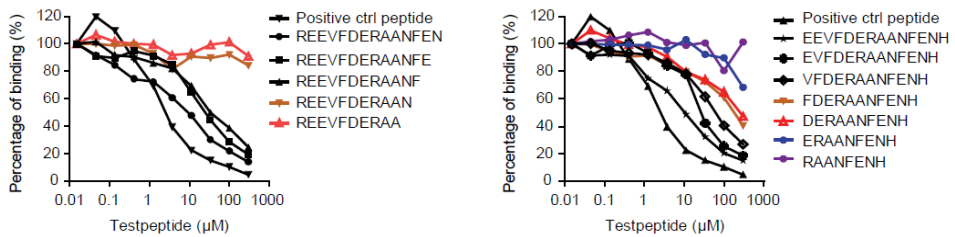
Figure S13. HLA-DQ7.3 presents VCL-DERAA in a similar binding register as HLA-DQ8. Competitive binding of HLA-DQ7.3 to an unbiotinylated positive control peptide and C-truncated and N-truncated VCL-DERAA peptides. The experiment was repeated three times and plots show a representative experiment.
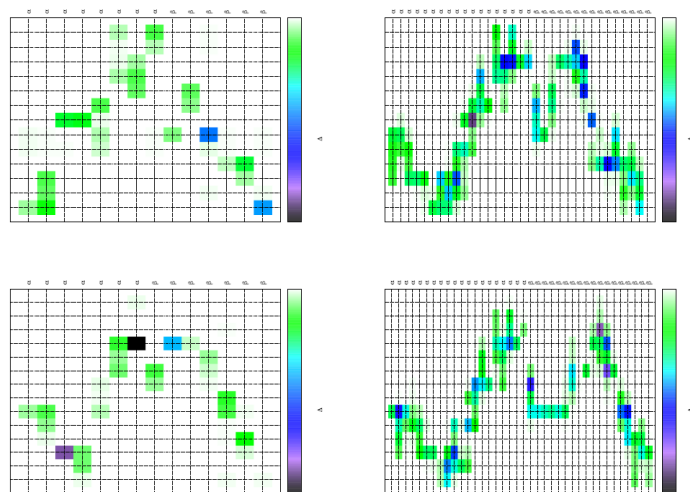
**Figure S14.** Interaction free-energies (in kcal/mol) for selected peptide – ligand residue pairs, averaged over the MD trajectories. The left and right panels correspond, respectively, to polar (GB+Coulomb) and non-polar (vW + SA) interactions, computed with Eq. (1) of the supplementary methods. Rows from top to bottom correspond, respectively, to the DQ8:VCL-DERAA and DQ8:insulin complexes.
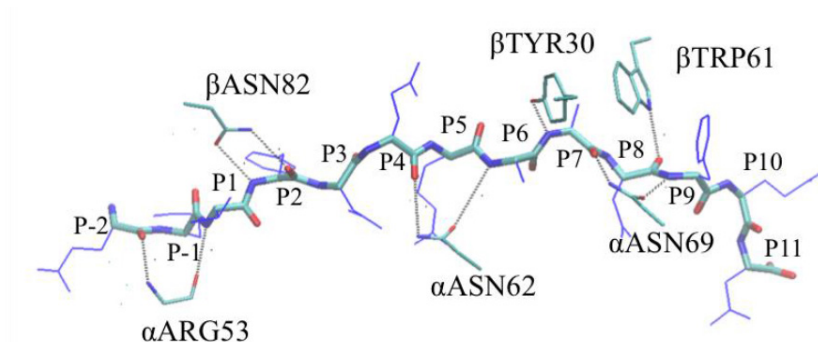
Figure S15. Hydrogen bonds between the VCL-DERAApeptide mainchain and DQ8.The peptide main-chain is shown in thick licorice and its sidechains are displayed in blue lines. DQ8 groups are shown in thin licorice. Hydrogen bonds with occupancy smaller than 20% are omitted. A comprehensive list of all hydrogen bonds is included in Table S1.
Figure

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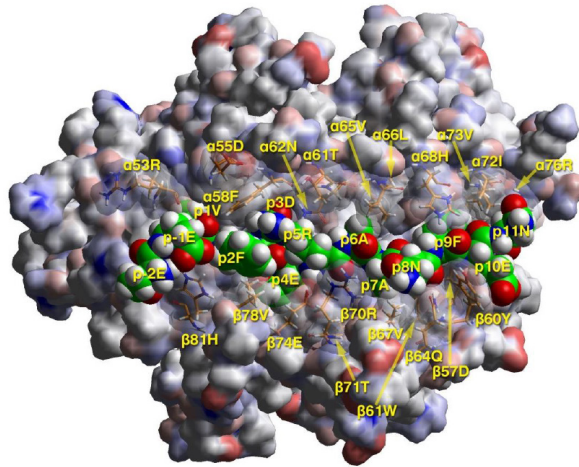
Figure S16. TCR view of the complex of HLA-DQ7.3 with the VCL-DERAA epitope in the groove at pH 7.4 (extracellular). The α1β1 domain of the modelled HLA-DQ molecule is in van der Waals surface representation, colored according to atomic charges (negative = red, positive = blue, neutral = gray, partial charges shades in-between). Several visible residues from the HLA-DQ molecule in contact with the antigenic peptide and potential contact with a cognate TCR in canonical orientation are shown in stick form with a transparent surface (atomic color code: oxygen, red; nitrogen, blue; hydrogen, white; carbon, green; sulfur, yellow). The antigenic peptide in the groove is shown in space-filling form, with identical colour conventions as in Fig. 6d-g. Anchors p1V and p9F point into the plane of the paper (screen) and are only partly seen. In vivo, p5R may not interact with p2F (cation-π interaction) because the former might more favorably interact with water molecules from the solvent. In this allele p4E makes an even weaker anchor than in DQ8, because of β13Gly→Ala and β26Leu→Tyr substitutions (DQ8→DQ7) that leaves less space available at the base of pocket 4.

Figure S17. TCR view of the complex of HLA–DQ8 with the VCL-DERAA epitope in the groove at pH 7.4 (extracellular). Depiction and colour conventions as in Figure S16. Anchors P1V and P9F point into the plane of the paper (screen) and are only partly seen. In vivo, P5R may not interact with P2F (cation-π interaction) because the former might more favorably interact with water molecules from the solvent.

Figure S18. TCR view of the complex of HLA-DQ8 with the *L. curvatus* epitope in the groove at pH 7.4 (extracellular). Depiction and colour conventions as in Figure S16. Anchors P1P and P9F point into the plane of the paper (screen) and are only partly seen.
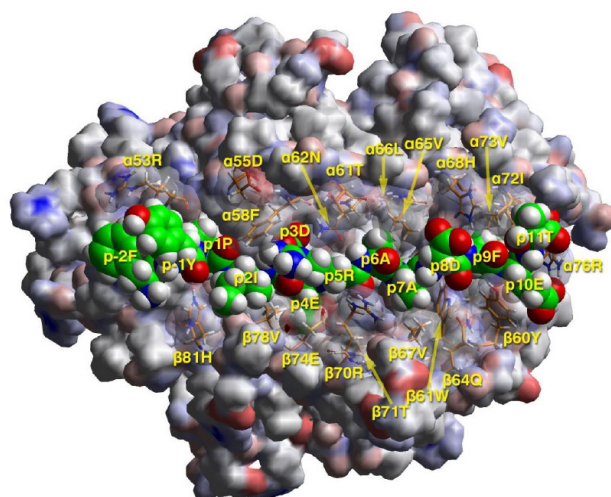
Figure S19. TCR view of the complex of HLA–DQ8 with the L. sakei epitope in the groove at pH 7.4 (extracellular). Depiction and colour conventions as in Figure S16. Anchors P1P and P9F point into the plane of the paper (screen) and are only partly seen.
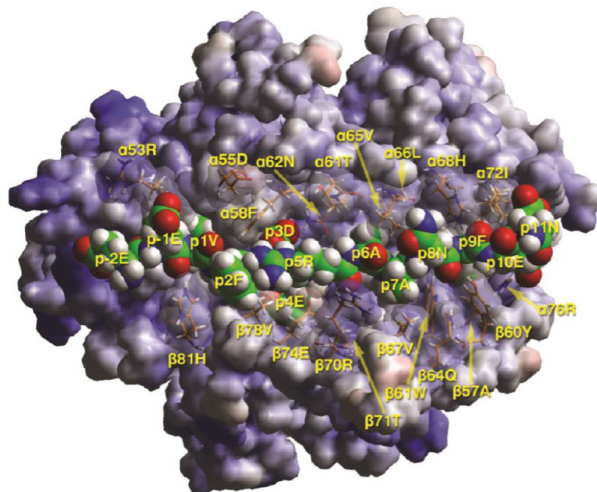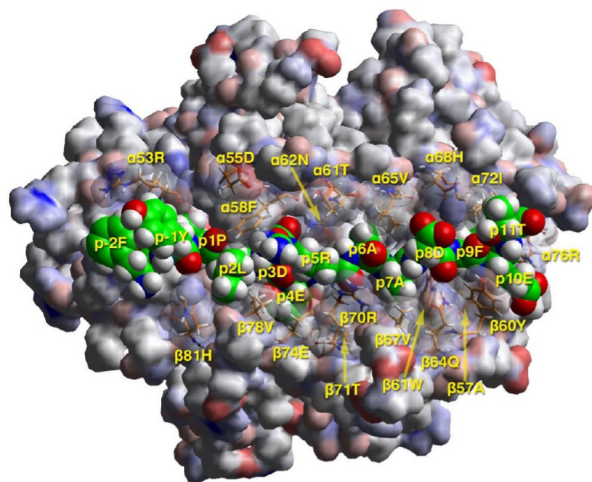
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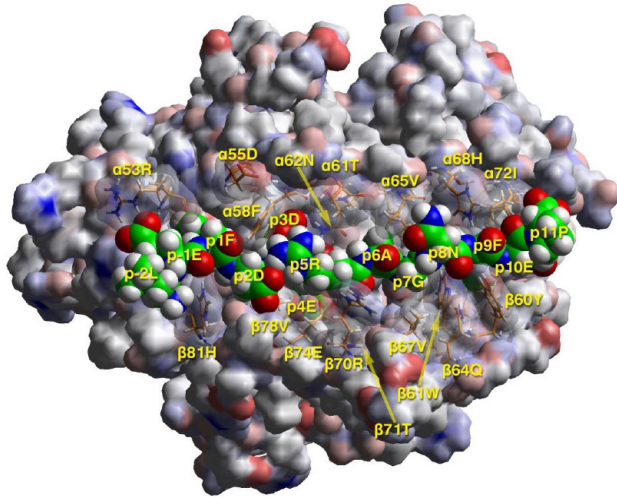
Figure S20. TCR view of the complex of HLA–DQ8 with the *C. Coli* epitope in the groove at pH 7.4 (extracellular). Depiction and colour conventions as in Figure S16. Anchors P1F and P9F point into the plane of the paper (screen) and are only partly seen.

Donor	Acceptor	Occupancy (%)
Water	**P-2E** (sc)	381
P-2E (Nter)	Water	188
βArg88 (sc)	P-2E(sc)	165
αArg53 (mc)	P-2E (mc)	42.7
αArg53 (sc)	P-2E (sc)	41.3
aPhe51 (mc)	P-2E (sc)	29.8*
αPhe51 (mc)	P-2E (Nter)	18.1*
Water	**P-1E**(sc)	535
Water	P-1E (mc)	62.9
P-1E (mc)	Water	56.7
βAsn82 (sc)	P-1E(mc)	20.5*
βHis81-Side	P-1E(mc)	16.6*
Water	**P1V** (mc)	79.3
P1V(mc)	αArg53 (mc)	76.9
P2F(mc)	βAsn82 (sc)	85.6
βAsn82 (sc)	P2F (mc)	68.5
Water	**P3D**(sc)	301
Water	P3D (mc)	64.2
αAsnN62 (sc)	P3D (sc)	46.9*
αTyr22 (sc)	P3D (sc)	36.6*
Water	**P4E**(sc)	269
βArg70 (sc)	P4E (sc)	160.4
βThr71 (sc)	E6 (sc)	64.7*
βThr28 (sc)	E6 (sc)	38.5*
αAsn62 (sc)	P4E(mc)	36

Donor	Acceptor	Occupancy (%)
P5R (sc)	Water	118
Water	P5R (mc)	98.3
P5R(sc)	αThr61 (sc)	76.9
P5R (sc)	αPhe58 (mc)	74.1
Water	**P6A**(mc)	118
P6A(mc)	αAsn62 (sc)	88.5
P7A(mc)	βTyr30 (sc)	65.4
P8N (mc)	Water	70.4
Water	P8N (sc)	70.4
Water	P8N (mc)	47.5
βTrp61 (sc)	P8N(mc)	41.9
P8N (sc)	Water	38.7
P8N (sc)	αAsn69 (sc)	35.0
αHis68 (sc)	P8N (sc)	19.2
Water	**P9F** (mc)	65.6
P9F(mc)	αAsn69 (sc)	38.8
Water	**P10E** (sc)	555
Water	P10E (mc)	62.8
P10E (mc)	Water	48.6
Water	**P11N** (Cter)	461
Water	P11N (sc)	117.5
P11N (mc)	Water	54.7
P11N (sc)	Water	44.0
P11N(sc)	αHis68 (sc)	21.3
αArg76 (sc)	P11N(sc)	33.2

Table S1. Statistics of intermolecular hydrogen bonds in the DQ8:VCL-DERAA complex. Hydrogen bonds were considered present if the D – A distance l_{DA} was smaller than 3.5 Å, and the angle $\theta_{D-H\cdots A}$ was larger than 150°. The (*) denotes water-mediated interactions; "sc" and "mc" denote side-chain and main-chain groups.

VCL-DERAA	p-2	p-1	p1	p2	p3	p4	p5	p6	p7	p8	p9	p10	p11
VCL-DERAA	**E**	**E**	**V**	**F**	**D**	**E**	**R**	**A**	**A**	**N**	**F**	**E**	**N**
Aspergillus fumigatus	V	T	A	E	D	E	R	A	A	M	F	F	R
Bacillus cereus	V	A	V	P	D	E	R	A	A	N	A	I	A
Campylobacter coli	L	E	F	D	D	E	R	A	G	N	F	E	P
Influenza A virus	F	E	F	S	D	E	R	A	A	N	P	I	V
Influenza A virus	F	E	L	S	D	E	R	A	A	N	P	I	V
Influenza A virus	F	E	L	S	D	E	R	A	A	N	P	V	V
Lactobacillus brevis	L	V	T	D	D	E	R	A	A	I	F	K	A
Lactobacillus curvatus	F	Y	P	L	D	E	R	A	A	D	F	E	T
Lactobacillus sakei	F	Y	P	I	D	E	R	A	A	D	F	E	T
Mycobacterium tuberculosis	A	L	P	F	D	E	R	A	A	V	F	L	R
Mycobacterium tuberculosis	A	M	P	F	D	E	R	A	A	V	F	L	R
Nocardia brasiliensis	A	L	P	F	D	E	R	A	A	I	F	L	R
Nocardia farcinica	A	L	L	A	D	E	R	A	A	L	F	A	R
Proteus mirabilis	V	I	T	D	D	E	R	A	A	V	F	Y	G
Proteus penneri													
Proteus stuartii													
Vibrio parahaemolyticus	Y	D	E	L	D	E	R	A	A	W	F	Y	E
Yersinia enterocolitica	L	E	D	Y	D	E	R	A	A	N	G	Y	D

Table S2. Microbe derived epitopes with molecular mimicry to vinculin-DERAA.

CHAPTER 9

The HLA-DRB1*14:02 allele predisposes indiginous North American populations to rheumatoid arthritis based on a unique molecular mechanism.

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Manuscript in preparation

* Authors contributed equally

ABSTRACT

HLA-DR*14:02 is an HLA molecule, prevalent in indigenous North Americans (INA), that contains the rheumatoid arthritis (RA) associated shared epitope (SE) sequence. This sequence forms an electropositive pocket that was shown to preferentially accommodate citrulline residues in contrast to RA-resistant HLA-molecules, providing an explanation for the link between the HLA-system, RA and citrullination. Recent studies suggested an additional role for specific HLA-DRB1-residues at position β11 and β13 that are not carried by HLA-DRB1*14:02. We show, using a large cohort of INA RA patients, a significant association between HLA-DRB1*14:02 and seropositive RA (OR=2.46). In contrast to other HLA-SE-alleles, HLA-DRB1*14:02 does not prefer citrulline in its electropositive pocket 4 or any of its other pockets as revealed by HLA-peptide-binding studies. Solved structures of HLA-DRB1*14:02 in complex with citrulline/arginine containing vimentin-epitopes revealed that citrulline and arginine residues are presented in a different orientation in pocket 4, allowing for the citrulline to interact with T cell receptors. The differences between the SE alleles are attributable to the β11/β13 polymorphisms influencing the packing of P4 residues

These findings deepen our understanding of the association between the HLA-system and RA by showing that different HLA-SE molecules present citrullinated ligands in structurally different ways, that nonetheless allow the unique presentation of citrullinated ligands to T cells.

INTRODUCTION

Rheumatoid arthritis is a chronic systemic inflammatory disease characterized by extensive inflammation of synovial joints[1]. The presence of Anti-citrullinated protein antibodies (ACPA) is an important hallmark of RA[2]. These autoantibodies specifically target proteins that have undergone a post-translational modification converting positively charged arginine residues in uncharged citrulline residues[3, 4].

The HLA class II locus is the most important risk factor for ACPA+ RA and contains genes encoding for the alpha and beta chains of HLA-DR and HLA-DQ molecules[5-7]. It was previously hypothesized that the contribution of the HLA class II region is explained by the HLA-DR locus when it was shown that RA risk associates with HLA-DRB1 alleles that encode the amino-acids (aa) sequences QKRAA, QRRAA and RRRAA in position 70-74. This sequence is known as the shared epitope (SE) motif [8, 9]. SE-residues β70, β71 and β74 shape peptide binding pocket 4 of HLA-DR molecules and it was proposed that the SE are directly involved in the presentation of a shared (type of) antigen to CD4+ T cells[8, 9]. To date, such an antigen has not been identified. However, accumulating data have shown that pocket 4 of different SE-alleles display an increased affinity for citrulline over arginine residues, thereby permitting enhanced presentation of citrullinated peptide-ligands[10-16]. Most of this work is centered around HLA-DRB1*04:01, the strongest risk allele in Caucasians.

Recently the genetic association of individual amino acids in the HLA-DRB1 locus with ACPA+ RA was studied in a large cohort of Caucasians. This study identified that next to SE-residues β70 and β71, also β11 and β13, that are in tight LD, are associated with RA risk[17]: β13His (e.g. present in SE-allele HLA-DRB1*04:01) and β13Phe (e.g. present in SE-allele HLA-DRB1*01:01) are most strongly associated with risk, while β13Ser is not associated with predisposition[17]. Interestingly, β13 is, like the SE-motif, involved in shaping the peptide binding properties of pocket 4. To understand and uncouple the relative contribution of the SE-motif and β13 to presentation of citrullinated peptides, we wished to study HLA-DRB1*14:02. HLA-DRB1*14:02 is an SE-allele that is rare in Caucasians, but prevalent in indigenous North Americans (INA). Interestingly, this SE-allele carries the non-predisposing β13Ser residue. We aimed to assess if HLA-DRB1*14:02 associates with seropositive RA as well as the relative capacity of the HLA-DRB1*14:02 to accommodate arginine and citrulline residues in the various peptide binding pockets, particularly in pocket 4.

METHODS

Patients

Study participants, both RA cases and controls, were recruited from INA popula-

tions in Central Canada (Cree, Ojiway, and Ojicree People) and Alaska Native people (from Southcentral and Southeast Alaska). The study and consent forms were approved by the Research Ethics Board of the University of Manitoba, and by the Alaska Area Institutional Review Board. In addition, written community agreements to undertake studies of RA risk were developed with communities in Manitoba (Norway House and St Theresa Point communities), and the study was approved by the tribal health organizations of the study communities in Alaska. The study participants signed consent documents after the study was explained to them in detail, with the help of an INA translator from their community, where necessary.

In total, 303 RA patients were recruited from the Cree/Ojibway/Ojicree population, along with 351 INA controls from the same geographic locations. In total, 69 RA patients were recruited from the Alaska Native population, along with 52 controls from the same geographic location. In all cases, RA patients met the 1987 ACR criteria for RA, and the controls had no personal or family history of RA or any related autoimmune diseases, including systemic lupus erythematosus, scleroderma, polymyositis, vasculitis, spondylarthritides, multiple sclerosis, inflammatory bowel disease, type 1 diabetes mellitus, or thyroid disease.

HLA-DRB1*1402 expression and purification

The extracellular domains of HLA-DRB1*1402 (HLA-DRA*0101 and HLA-DRB1*1402) were cloned into pHLSec for transient expression in HEK293S (GnTI-) cells[15, 18, 19]. HLA-DRB1*1402 was expressed with the class II associated invariant peptide (CLIP) covalently attached via a Factor Xa cleavable flexible linker to the N-terminus of the β-chain, which was preceded by an N-terminal strep II tag. In addition, the constructs contained enterokinase cleavable C-terminal fos/jun zippers that promoted dimerization, as well as a BirA biotinylation site and a His tag attached to the C-terminus of the β-chain.

HLA-DRB1*1402-CLIP was expressed and purified as previously described[15]. Briefly, HLA-DRB1*1402-CLIP was transiently expressed in HEK 293S (GnTI-) and purified via IMAC and size exclusion chromatography. HLA-DRB1*1402-CLIP was cleaved by Factor Xa for 6 h at 21 °C and then loaded with either Vimentin-64Arg$_{59-71}$ or Vimentin-64Cit$_{59-71}$ for 16 h at 37 °C with a 20-fold excess of the peptide in 100mM Sodium Citrate pH 5.4 in the presence of HLA-DM at a DR:DM ratio of 5:1. Peptide loaded HLA-DRB1*1402 was then purified via Strep-Tactin Sepharose (IBA; Göttingen), and the C-terminal fos/jun zippers removed via enterokinase (Genscript) digest. Cleaved and peptide loaded HLA-DRB1*1402 was then purified further anion exchange chromatography (HiTrap Q HP; GE Healthcare), buffer exchanged into 10 mM Tris pH 8, 150 mM NaCl, and concentrated to 6mg/mL for crystallization trials.

Crystallization and structure determination

Crystal trays were set up using the hanging-drop vapour diffusion method at 20°C. Protein and a mother liquor of 100 mM BTP pH 7.3, 22-28% (vol/vol) PEG3350, 0.2

M KNO_3 were mixed in a 1:1 ratio and plate like crystals typically grew within 5 days. Crystals were flash frozen in 16% ethylene glycol prior to data collection. Data were collected at the MX1 beamlines at the Australian Synchrotron and processed using the program Mosflm. The structures were determined by molecular replacement using the program Phaser and subsequently refined using Phenix and iterations of manual refinement using Coot. The structures were validated using MOLPROBITY.

Cell lines

The following EBV-transformed lymphoblastoid B cell lines were used: BC1402 (HLA-DRB1*14:02), BOLETH (HLA-DRB1*04:01), BSM (HLA-DRB1*04:01), and KAS116 (HLA-DRB1*01:01). Cells were maintained in IMDM (Lonza) supplemented with 10% heat-inactivated FCS.

Peptides

Peptides were synthesized according to standard Fmoc (N-(9-fluorenyl)methoxy-carbonyl) chemistry using a SyroII peptide synthesizer (MultiSynTech, Witten, Germany). The integrity of the peptides was checked using reverse phase HPLC and MS. For HLA-peptide affinity studies, we used Influenza-peptide $HA_{305-319}$ CPKYVK-QNTLKLATG. The anchors are depicted in bold. To assess citrulline or arginine accommodation, peptides with an arginine or a citrulline at the indicated anchor positions were generated. As negative control peptide, we have used a peptide that did not bind to the respective HLA molecules, the previously described A4KA5-peptide[20]. We also assessed the binding of vimentin peptides Vimentin-64Arg/Cit_{59-71} (GVYATRSSAVRLR) and Vimentin-71Arg/Cit_{66-78} (SAVRARSSVPGVR). The arginine's in bold were substituted for citrulline.

HLA class II competitive peptide binding assay

Peptide-binding assays were performed, as described previously[21]. In short, cell lysates from HLA-class II homozygous B-lymphoblastoid cell lines were incubated on B8.11.2- (anti-HLA-DR) coated (10 µg/ml) FluoroNunc 96-well plates at 4⬚C overnight. Titration ranges of the tested peptides (0 to 300 µM) were mixed with a fixed concentration (0.6 µM) of biotinylated indicator peptide and added to the wells. Bound indicator peptide was detected using europium-streptavidin (Perkin Elmer, Boston, MA) and measured in a time-resolved fluorometer (PerkinElmer, Wallac Victor2). IC50 values were calculated based upon the observed binding of the test peptide against the fixed concentration indicator peptide. The IC50 value depicts the concentration of test peptide required for a loss of 50% of the indicator peptide signal. IC50 values greater than 300 µM were classified as non-detectable binding affinity.

Statistical analysis

Wilcoxon's signed rank test was used to assess differences in IC50 values between

citrulline and arginine containing peptides. P-values below 0.05 were considered to be statistically significant. Logistic regression analysis was used to calculate odds ratios (OR) and 95% confidence intervals (CIs) for the development of RA. Healthy controls were used as comparator group.

RESULTS

SE-allele HLA-DRB1*14:02 is a risk factor for RA in INA.

HLA-DRB1*14:02 is rare in Caucasians, but prevalent in INA populations[22-24]. Interestingly, HLA-DRB1*14:02 carries both the predisposing SE-motif and the non-predisposing β13S-residue. Previously it was shown that the SE-alleles in INA, associate with RA risk. HLA-DRB1*04:04 is the most frequent SE-allele in these populations, followed by HLA-DRB1*14:02. Because HLA-DRB1*14:02 has the SE-sequence it was postulated that HLA-DRB1*14:02 is implicated in RA risk. However, this has not been shown as these studies lacked the proper size to provide a statistically significant answer to the association of HLA-DRB1*14:02 with RA risk[22, 24-26].

We have now analyzed a group of 344 INA RA patients and 352 controls, the largest cohort of HLA-typed INA RA patients and controls available. For 68% of the RA patients (241/352), RF and ACPA status was known. 90% (218/241) of RA patients were seropositive (positive for RF and/or ACPA). Both in total RA patients (OR = 2.48, 95% CI = 1.70-3.60, P< 0.0001) and in confirmed seropositive RA (OR = 2.46, 95% CI = 1.58-3.81, P=0.0001) patients, SE-positivity associated with RA risk (Table S1).

In total 293 of 344 RA patients carried a SE-allele, 176 carried a single SE-allele and 117 were SE homozygous. Thus, in total we identified 410 SE-alleles. 32% of these SE-alleles (132/410) were HLA-DRB1*14:02, and 61% HLA-DRB1*04, mostly HLA-DRB1*04:04 (185/410). The remaining SE-alleles were HLA-DRB1*01 and HLA-DRB1*10. To study the predisposing effect of HLA-DRB1*14:02 separately from the other SE-alleles, we stratified RA patients according to HLA-status (Table 1). Both in total and in seropositive RA patients, we demonstrated that HLA-DRB1*14:02 is a risk factor for seropositive RA independent of other SE-alleles (Table 1). In addition, the other SE-alleles (mainly HLA-DRB1*04) also associate with the risk to develop seropositive RA independent of HLA-DRB1*14:02 (Table 1).

Together these data show that HLA-DRB1*14:02 is a risk allele for the development of (seropositive) RA in INA.

HLA-DRB1*14:02 shapes the ACPA autoantibody response

Previously, we have shown that SE-alleles, mainly HLA-DRB1*04:01 and *01:01, influence both the level and epitope-recognition profile of the ACPA-response in Dutch RA-patients[27-29]. To investigate whether HLA-DRB1*14:02 has an impact

	HC, N (%)	RA, N (%)	OR (CI)	P-value	Seropositive RA, N (%)	OR (CI)	P-value
SE-	106 (30%)	51 (15%)	REF		32 (15%)	REF	
14:02+/ OtherSE-	64 (18%)	80 (23%)	**2.60 (1.63-4.15)**	**0.0001**	46 (21%)	**2.38 (1.38-4.12**	**0.0019**
14:02-/ OtherSE+	147 (42%)	178 (52%)	**2.52 (1.69-3.75)**	**<0.0001**	117 (54%)	**2.64 (1.66-4.19)**	**<0.0001**
14:02+/ OtherSE+	35 (10%)	35 (10%)	**2.08 (1.17-3.70)**	**0.0127**	23 (11%)	**2.18 (1.13-4.20)**	**0.02**

Table 1: Association of HLA-DRB1*14:02 with total and seropositive RA in INA RA patients and controls. Patients and controls were stratified according to the presence of SE. SE-positive individuals were further stratified in those that carry 14:02, those that carry other SE-alleles (DRB1*01:01, *01:02, *04:01, *04:04, *04:05, *04:08, *04:10, *04:13, and *10:01), or those that are positive for 14:02 and another SE-allele.

on the ACPA response, sera of 232 INA RA patients were tested in a multiplex ACPA antigen array[30]. We evaluated the depth of the ACPA response using an ACPA score (sum of all normalized ACPA titers divided by number of epitopes) as previously described [30]. These analyses revealed that HLA-DRB1*14:02-positive RA patients display a more diverse ACPA response, as compared to HLA-SE negative, but ACPA positive patients (data not shown).

Together, these data show that HLA-DRB1*14:02 is not only a risk factor for RA, but also that HLA-DRB1*14:02 impacts the constitution of the ACPA-response, mimicking the effects observed for other HLA-SE alleles.

HLA-DRB1*14:02 present arginine and citrulline residues with a similar peptide binding capacity.

SE-allele HLA-DRB1*14:02 associates with RA risk and a broadened ACPA-response despite the presence of the non-predisposing β13Ser-residue. The latter residue is known to influence the characteristics of binding pocket 4 and pocket 6. To better comprehend the ability of HLA-DRB1*14:02 to present citrullinated ligands, we next assessed the ability of HLA-DRB1*14:02 to accommodate arginine and citrulline in the various peptide binding pockets. For comparison, similar experiments were performed for HLA-DRB1*01:01 and HLA-DRB1*04:01, the two most common SE+ HLA-DR molecules in Caucasians[17, 31]. To systematically compare the capacity of the different binding pockets to accommodate citrulline and arginine residues, we made use of the influenza-derived $HA_{305-319}$ peptide. This peptide has previously been crystallized and studied in the context of different HLA-DR molecules and therefore its binding register is known[10, 20, 32, 33]. The accommodation of citrulline and arginine residues in peptide binding pockets was determined by substituting the pocket-interacting residues for arginine or citrulline. In doing so, the effect of an
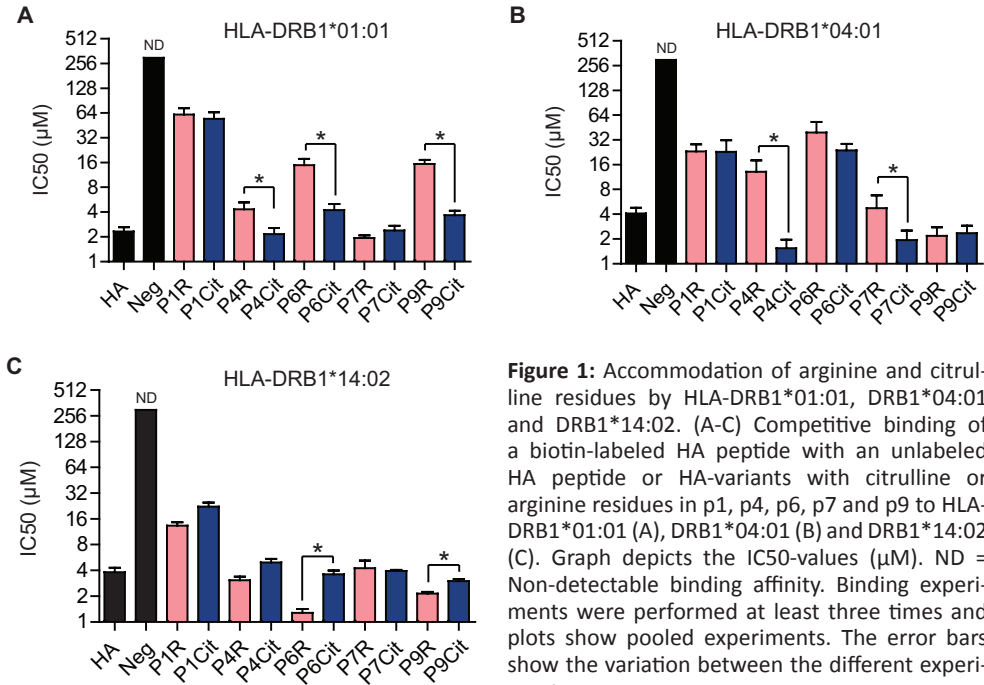
Figure 1: Accommodation of arginine and citrulline residues by HLA-DRB1*01:01, DRB1*04:01 and DRB1*14:02. (A-C) Competitive binding of a biotin-labeled HA peptide with an unlabeled HA peptide or HA-variants with citrulline or arginine residues in p1, p4, p6, p7 and p9 to HLA-DRB1*01:01 (A), DRB1*04:01 (B) and DRB1*14:02 (C). Graph depicts the IC50-values (μM). ND = Non-detectable binding affinity. Binding experiments were performed at least three times and plots show pooled experiments. The error bars show the variation between the different experiments.

arginine to citrulline conversion in the different position of the peptide (P1, 4, 6 and 9) interacting with the different binding pocket of the HLA-molecule is assessed, as previously described[10, 34-37]. Using this approach, we could demonstrate, as expected, that the conversion of arginine to citrulline at peptide-positions interacting with pocket 4 enhanced peptide binding affinity to HLA-DRB1*01:01 (Figure 1A) and *04:01 (Figure 1B). In addition, we also demonstrated a preference for citrulline in pocket 6 and 9 of HLA-DRB1*01:01 (Figure 1A) and in pocket 7 of *04:01 (Figure 1B). Intriguingly, our analyses of the HLA-DRB1*14:02 allele indicated that peptides containing an arginine or citrulline residue at a position interacting with peptide binding pocket 4 are presented with a similar binding affinity (Figure 1C). In addition, we could not demonstrate a preference for citrulline in any of the other peptide binding pockets. In contrast, for pocket 6 and pocket 9 we demonstrated an increased affinity for arginine residues (Figure 1C).

Thus using this approach, we confirmed previous findings describing a preference for citrulline in pocket 4 of HLA-DRB1*01:01 and HLA-DRB1*04:01. Moreover, our data indicate that HLA-DRB1*01:01 and *04:01 also prefer citrulline over arginine residues in other pockets albeit in a distinct pattern. In contrast, HLA-DRB1*14:02 displays no preference for citrulline residues in pocket 4 or any of its other peptide binding pockets.

To further support these data, we next analyzed the binding affinity of two previously studied vimentin peptides, Vimentin$_{59-71}$ and Vimentin$_{66-78}$, that carry a citrulline at a

position interacting with pocket 4[12, 15, 16, 38]. By using these vimentin-peptides, we demonstrated an increased affinity of the citrulline variant for SE-alleles HLA-DRB1*01:01 and *04:01, whereas HLA-DRB1*14:02 presented both the arginine and citrulline variant with a similar affinity (Table 2).

Thus, together these data indicate that different SE-alleles (HLA-DRB1*01:01, *04:01 and *14:02) have distinct capacities to present citrullinated ligands and, most importantly, that a preference for citrulline residues in pocket 4 is not a shared characteristic of all SE-alleles.

Structural determination of citrullinated ligand presentation by HLA-DRB1*14:02 shows a joint role for β11/13 and the SE-motif in the accommodation of arginine residues in pocket 4.

The HLA-DRB1 chain contains 12 different polymorphic residues that have been directly implicated in peptide accommodation[35]. HLA-DRB1*01:01 differs from *04:01 and *14:02 in 8 out of these 12 polymorphic residues (Figure 2A). These different residues are involved in shaping peptide binding pocket 4 (β13, β26, β28 and β71), pocket 6 (β11 and β30), pocket 7 (β28 and β71) and pocket 9 (β9 and β37, Figure 2B). Despite these differences, all these SE-alleles are strongly associated with susceptibility to seropositive RA[17, 27]. Minor differences in the polymorphic residues of MHC molecules have been described to greatly influence the nature of the ligand binding repertoire[36-40]. To determine how structural differences in pocket 4 of HLA-DRB1*14:02 explain our results, we aimed to solve the structure of HLA-DR*14:02 in complex with a citrullinated ad native epitope. We therefore cloned HLA-DR*14:02 and loaded the HLA-molecule with native Vimentin$_{59-71}$, Vimentin-64Cit$_{59-71}$, Vimentin-64,69,71Cit$_{59-71}$, Vimentin$_{66-78}$ and Vimentin71Cit$_{66-78}$. Interestingly, we could only make stable complexes of Vimentin$_{59-71}$ and Vimentin-64Cit$_{59-71}$ supporting our HLA-peptide binding studies (Figure S1, Table 2).

We next solved the structures of Vimentin-64Arg$_{59-71}$ (Figure 3A and 3C) and Vimentin-64Cit$_{59-71}$ (Figure 3B and 3D) in HLA-DRB1*14:02 (Table S2). Both these epitopes were bound in a linear extended manner with P1-Tyr, P6-Ser and P9-Arg occupying

Peptide	Variant	DRB1*01:01	DRB1*04:01	DRB1*14:02
Vim59-71	64Arg	43	>300	19
	64Cit	**34**	**197**	19
Vim66-78	71Arg	>300	187	>300
	71Cit	**51**	**21**	>300

Table 2: Presentation of (citrullinated) vimentin peptides by different SE-alleles. Competitive binding of a biotin-labeled HA peptide with unlabeled Vim$_{59-71}$-64Arg, Vim$_{59-71}$-64Cit, Vim$_{66-78}$-71Arg and Vim$_{66-78}$-71Cit to HLA-DRB1*01:01, *04:01 and *14:02. Table depicts average IC50-values (μM). ND = Non-detectable binding affinity. Binding experiments were performed at least three times and pooled experiments are shown.
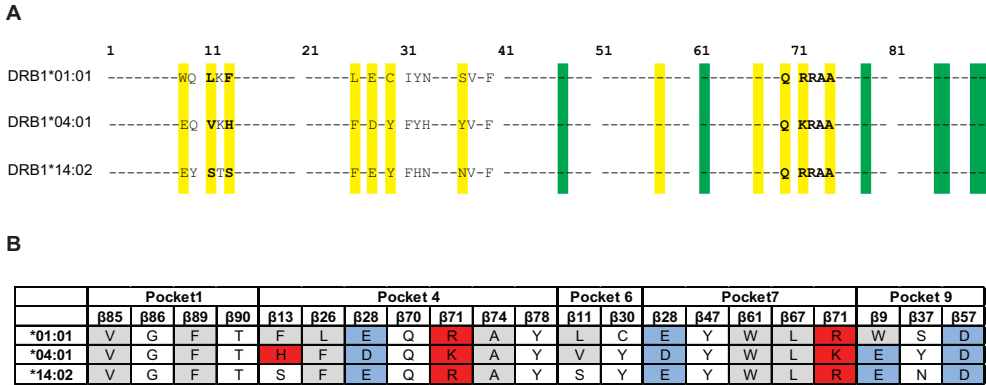
A

B

	Pocket1				Pocket 4							Pocket 6		Pocket7					Pocket 9		
	β85	β86	β89	β90	β13	β26	β28	β70	β71	β74	β78	β11	β30	β28	β47	β61	β67	β71	β9	β37	β57
*01:01	V	G	F	T	F	L	E	Q	R	A	Y	L	C	E	Y	W	L	R	W	S	D
*04:01	V	G	F	T	H	F	D	Q	K	A	Y	V	Y	D	Y	W	L	K	E	Y	D
*14:02	V	G	F	T	S	F	E	Q	R	A	Y	S	Y	E	Y	W	L	R	E	N	D

Figure 2: Characteristics of the tested SE-alleles. (A) Schematic representation of differences in amino acids between SE-alleles HLA-DRB1*01:01, *04:01 and *14:02. Positions important for interaction with the peptide side chains are highlighted in yellow and green. Polymorphic residues are highlighted in yellow. (B) Schematic representation of the differences in pockets between HLA-DRB1*01:01, *04:01 and *14:02. Aa residues are color coded according to their properties (white = hydrophilic, gray = hydrophobic, red = basic, blue = acidic).

respectively pocket 1, 6 and 9. P-1-Val, P2-Ala, P5-Ser, P7-Ala, P8-Val and P11-Arg represented potential TCR contact sites. These structures clearly show that for both ligands P4-Arg and P4-Cit are presented in an alternative orientation: The arginine is projecting inwards, whereas the citrulline projects outwards. In Figure 4A the interactions between the HLA-DR molecule and the P4-Arg residues are shown for Vimentin$_{59-71}$. The p4-Arg is buried in the pocket to avoid interactions with the positively charged β71Arg-residue. This orientation is promoted by β11Ser and β13Ser, two small hydrophobic residues that allow the accommodation of Arginine by providing necessary space. Larger residues in HLA-DRB1*04:01 (β11Val and β13His) and HLA-DRB1*01:01 (β11Leu and β13Phe) prevent the accommodation of arginine, thereby explaining observed differences between SE-alleles. β11Ser and β13Ser also contribute by providing hydrogen bonds with the P4-Arg residue. The arginine residue is further stabilized by an hydrogen bond with β30Tyr and a salt bridge with β28Glu.

In contrast to P4-Arg, the P4-Cit sits upright in the pockets, similar to what was shown for HLA-DRB1*04:01/04:04[15]. The citrulline forms hydrogen bonds with β71Arg and β70Gln. The upright position of P4-Cit could potentially be a target for citrulline-directed T cell receptors (Figure 4B).
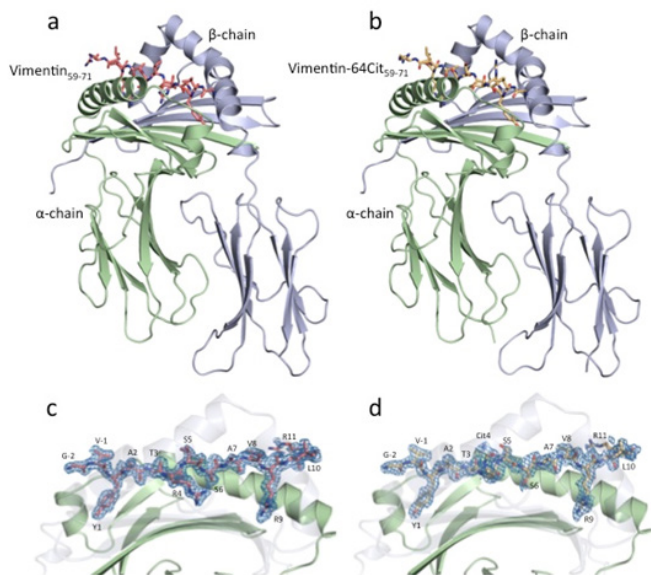
Together, these structures provide a detailed understanding of the accommodation of arginine and citrulline residues in pocket 4. Observed differences between SE alleles are explained by structural differences, in particular by residues β11 and β13. We believe that the upright position of citrulline in pocket 4 allows interactions with possible citrulline specific T cells, thereby providing a molecular basis for the association of HLA-DRB1*14:02 with seropositive RA.

DISCUSSION

The INA RA patient population is characterized by a high prevalence of ACPA. It was previously demonstrated that SE-alleles are a risk factor for RA in INA, but these studies were underpowered to draw conclusions on the contribution of individual SE-alleles to RA predisposition[22-24, 26]. We now demonstrated in a large cohort of INA, that SE-allele HLA-DRB1*14:02 is associated with predisposition to sero-positive RA. Interestingly, the presence of HLA-DRB1*14:02 is also reflected in a broader ACPA repertoire as was previously reported for other SE-alleles present in the Caucasian population.

More than 10 years ago, it was first proposed that the SE-alleles could predispose to RA through the increased ability to present citrullinated ligands via the exquisite ability of pocket 4 of HLA-DRB1*04:01 to interact with citrulline[16]. However, the amino acids encompassing the SE sequence are not only taking part in the formation of pocket 4, but also of pocket 7. Likewise, also other now performed a systematic analysis of the capacity of three different predisposing SE-alleles to present citrulline- and arginine residues in their different peptide binding pockets (HLA-DRB1*01:01, *04:01 and *14:02). Interestingly, all three SE-alleles show different patterns of citrulline "presentation". Our data document that HLA-DRB1*01:01 prefers citrulline residues in 4, 6 and 9, while HLA-DRB1*04:01 prefers citrulline over arginine in pocket 4 and 7. Interestingly, HLA-DRB1*14:02 has no preference for citrulline residues in any of its peptide binding pockets. When the sequences

Figure 3: Crystal structure of HLA-DRB1*1402 in complex with vimentin$_{59-71}$ and vimentin-64Cit$_{59-71}$ (A) HLA-DRB1*1402 in complex with vimentin59-71 and (B) vimentin-64Cit59-71. The α- and β-chains are shown in cartoon representation and coloured in light green and light blue respectively, while the vimentin peptides are displayed as sticks. The carbons are coloured deep salmon and light orange for vimentin$_{59-71}$ and vimentin-64Cit$_{59-71}$, respectively, nitrogens are coloured in blue and oxygens are coloured in red. Side view of (C) HLA-DRB1*1402-Vimentin$_{59-71}$ and (D) HLA-DRB1*1402-Vimentin-64Cit$_{59-71}$. The β-chain is transparent to help visualize the peptide. The unbiased 2Fo-Fc electron density map of the peptides is shown in blue and contoured to 1 σ.
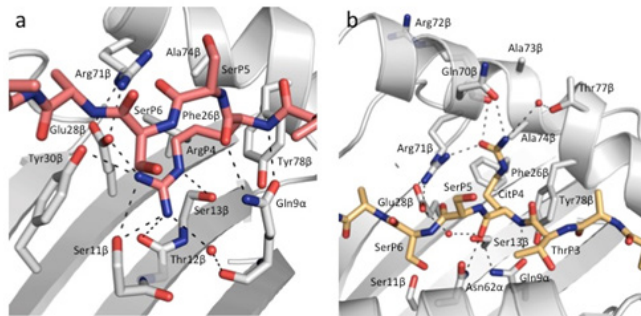
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Figure 4: Interactions in the P4 pocket of HLA-DRB1*1402 with vimentin59-71 and vimentin-64Cit$_{59-71}$ The P4 pocket of HLA-DRB1*1402 bound to (A) vimentin$_{59-71}$ and (B) vimentin-64Cit$_{59-71}$. The P4-Arg in the vimentin$_{59-71}$ peptide is buried in the P4 pocket, away from the electropositive Arg71β, and forms a salt bridge with Glu28β and a number of hydrogen bonds with Ser11β, Thr12β, Ser13β, Tyr30β, and a water-mediated hydrogen bond with the main chain carbonyl of Gln9α. The P4-Cit of the vimentin-64Cit$_{59-71}$ peptide adopts an upright conformation and forms hydrogen bonds with Arg71β and Gln70β and a water-mediated hydrogen bond with Thr77β. A water occupies the space below the P4-Cit.

of different SE-alleles are compared it becomes clear that HLA-DRB1*14:02 differs from other SE-molecules in residues important for shaping peptide binding pockets 4 and 6, most notably in β11 and β13[17, 35]. Indeed our structural analysis of the p4 pocket of HLA-DRB1*14:02 showed that differences in β11/β13 allowed for an enhanced affinity for arginine residues compared to the other tested SE-molecules. Together, these findings indicate that it is unlikely that a common mechanism related to binding of citrullinated residues in p4 or other pockets of SE-molecules explains the HLA-RA connection.

Intriguingly, however, our data also indicate that while the structural basis for citrullinated ligand presentation is different, the outcome for the presentation of citrullinated ligands is highly similar. We show that, although, HLA-DRB1*14:02 can accommodate both arginine and citrulline residues in pocket 4 with a similar affinity, the citrulline, in contrast to arginine, was accommodated in an upright position allowing interaction with possible citrulline-specific T cells. Thus, it is highly conceivable that the conversion of an arginine to a citrulline residue in pocket 4 generates a neo-epitope for HLA-DRB1*14:02-restricted CD4+ T cells, thereby mimicking the effects of citrullination for presentation of ligands in the context of HLA-DRB1*04:01 and 04:04[15]. We, therefore, believe our data provide a similar molecular basis for the HLA-DRB1*14:02-RA association as described for other SE-allomorphs and warrant future efforts to identify and characterize citrulline-directed T cells in HLA-DRB1*14:02-positive RA patients. Our data provide an important step forward for the structural requirements of an RA-inducing antigen that induces seropositive RA regardless of ethnic background.

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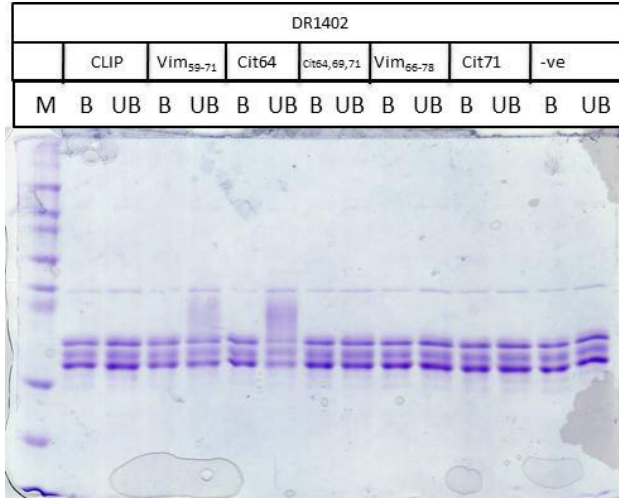
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SUPPLEMTENTARY INFORMATION

Figure S1: SDS resistance of HLA-DRB1*14:02 tetramers

	HC, N (%)	RA, N (%)	OR (CI)	P-value	Seropositive RA, N (%)	OR (CI)	P-value
SE-neg	106 (30%)	51 (15%)	REF		32 (15%)	REF	
SE-pos	246 (70%)	293 (85%)	**2.48 (1.70-3.60)**	**<0.0001**	186 (85%)	**2.46 (1.58-3.81)**	**0.0001**

Table S1: Association of the shared epitope with total and seropositive RA in INA RA patients and controls. Patients were stratified according to the presence of SE. SE-positive were classified as SE-positive when positive for DRB1*01:01, *01:02, *04:01, *04:04, *04:05, *04:08, *04:10, *04:13, and *10:01 and *14:02.

	HLA-DRB1*1402 Vimentin59-71	HLA-DRB1*14:02 Vimentin-64Cit59-71
Space group	P12(1)1	P12(1)1
Cell dimensions		
a,b,c (Å)	67.0, 77.7, 94.5	67.3, 76.9, 94.1
α,β,γ (°)	90, 109.5, 90	90, 109.5, 90
Resolution (Å)[a]	35.64-1.90 (2.0-1.90)	35.31-1.98 (2.09-1.98)
No. molecules in ASU	2	2
Total no. observations	262810 (35904)	229839 (31923)
No. unique observations	72027 (10379)	63444 (9163)
Multiplicity	3.6 (3.5)	3.6 (3.5)
R_{merge} (%)[b]	9.3 (37.9)	9.1 (30.8)
R_{pim} (%)[c]	5.6 (23.6)	5.6 (19.5)
<I/σI>	10.6 (3.3)	9.1 (4.3)
Completeness (%)	99.7 (98.9)	99.9 (99.3)
Refinement Statistics		
Non-hydrogen atoms	7465	7237
Macromolecule	6307	6258
Water	1026	901
Ligand	132	78
R_{factor}[d]/R_{free}[e]	17.0/20.2	19.0/23.3
Rms deviations from ideality		
Bond lengths (Å)	0.003	0.004
Bond angles (°)	0.86	0.89
Dihedrals (°)	14.0	13.2
Ramachandran plot		
Favoured regions (%)	98.3	98.7
Allowed regions (%)	1.7	1.3
B-factors (Å²)		
Average B-factors	20.4	22.0
Average macromolecule	18.1	20.6
Average ligand	40.9	33.4
Average water molecule	32.1	31.1

[a] Values in parentheses refer to the highest resolution bin. [b] $R_{merge} = \Sigma_{hkl} \Sigma_i \mid I_{hkl,\,i} - <I_{hkl}> \mid / \Sigma_{hkl} <I_{hkl}>$
[c] $R_{pim} = \Sigma_{hkl} [1/(N-1)]^{1/2} \Sigma_i \mid I_{hkl,\,i} - <I_{hkl}> \mid / \Sigma_{hkl} <I_{hkl}>$. [d] $R_{factor} = (\Sigma \mid \mid F_o \mid - \mid F_c \mid \mid) / (\Sigma \mid \mid F_o \mid)$ - for all data except as indicated in footnote e. [e] 5 % of data were used for the R_{free} calculation.

Table S2: Data collection and refinement statistics for HLA-DRB1*1402-Vimentin$_{59-71}$ and HLA-DR-B1*1402-Vimentin-64Cit$_{59-71}$

Chapter 9

CHAPTER 10

The increased ability to present citrulinated peptides is not unique to HLA-SE molecules: Arginine to citrulline conversion enhances peptide affinity for HLA-DQ molecules.

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Manuscript in preparation

ABSTRACT

Objective. Presentation of citrullinated neo-epitopes by HLA-DRB1 molecules that carry the shared epitope (SE) sequence was proposed to explain the association between HLA and seropositive RA. Although it is shown that several HLA-DRB1-SE molecules display enhanced binding affinities for citrullinated ligands, it is not known whether this feature is confined to these HLA-molecules. To better understand the HLA-RA-connection, we aimed to investigate if the enhanced capacity to present arginine to citrulline converted peptides is unique for HLA-SE alleles.

Methods. We selected four HLA-molecules (one HLA-DR and three HLA-DQ molecules) that could potentially prefer citrulline over arginine residues. The affinity of peptides containing arginine/citrulline residues at positions interacting with the various peptide-binding pockets was compared by HLA class II peptide affinity assays.

Results. HLA-DRB1*03:01 did not display an enhanced affinity for peptides containing a citrulline. In contrast, several peptide-binding pockets of the HLA-DQ molecules analyzed showed enhanced affinities for citrulline residues: i.e. Pocket 4, 7 and 9 for HLA-DQ2 and pocket 1, 6 and 9 for HLA-DQ7 and HLA-DQ8.

Conclusion. Arginine-to-citrulline conversion of peptides can also enhance the binding affinity for non-HLA-SE molecules and hence the capacity to present citrullinated neo-epitopes is not an unique feature of HLA-SE molecules.

INTRODUCTION

Rheumatoid arthritis (RA) patients can be divided in two distinct disease subsets based on the presence of anti-citrullinated protein antibodies (ACPA)[1, 2]. These autoantibodies target proteins that have undergone post-translational modifications converting positively charged arginine to uncharged citrulline residues[3].
The most important risk factor for ACPA+ RA is the HLA class II locus[2]. This locus contains genes encoding the beta chain of HLA-DR (HLA-DRB1) and the alpha and beta chain of HLA-DQ (HLA-DQA1 and HLA-DQB1) that inherit together in haplotypes. In 1987 it was proposed that risk is explained by the HLA-DRB1 locus, in particular by those alleles that carry the sequence QKRAA, QRRAA or RRRAA in position 70-74 of the DRB1 chain, also known as the shared epitope (SE) motif[4, 5]. About 80% of ACPA+ RA patients carry "HLA-SE" alleles, illustrating that these alleles are highly prevalent, but not required to develop seropositive disease[2].
HLA class II molecules are composed of an alpha and beta chain that create a ligand-binding groove that is involved in the presentation of peptides to CD4+ T cells. These ligands vary in length, but the part that interacts with this groove is 9 amino acids (aa) in length. The ligand-binding groove contains peptide-binding pockets allowing the interaction with amino acids from the peptide presented. These pockets accommodate the side chains of peptide residues 1, 4, 6 , 7 and 9 and are respectively named: pocket 1, 4, 6, 7 and 9[6].
The amino acid residues that shape the pockets are highly polymorphic, thereby dictating the preferences for particular amino acids that can be accommodated in the pockets of different HLA class II alleles. In this way, the compared characteristics of the peptide-binding pockets shape the repertoire of ligands presented by the HLA-molecule. The amino acids forming the SE motif are part of pocket 4 of the HLA-DRB1-molecule [4, 6]. It was proposed that the SE motif associates with risk because the positively-charged SE residues would prevent the accommodation of positively-charged arginine residues while favoring the accommodation of uncharged citrulline residues. The conversion of arginine to citrulline could thereby result in the presentation of a peptide that would otherwise not be presented [7]. Indeed, several studies have shown that pocket 4 of HLA-SE alleles HLA-DRB1*01:01, *04:01, *04:04 and *10:01 prefers citrulline over arginine residues[7-9]. It was postulated that this preference for citrulline over arginine residues would be unique to HLA-SE alleles, but this was never analyzed[7]. We aimed to study if also non-HLA-SE alleles can present citrullinated peptides with an enhanced affinity.

METHODS

Cell lines. Lysate of EBV-transformed lymphoblastoid B cell lines were used as a source for HLA-DQ/DR molecules: BOLETH (DQA1*03:01/DQB1*03:02), BSM (DQA1*03:01/DQB1*03:02), DUCAF (DRB1*03:01/DQA1*05:01/DQB1*02:01),

JSM (DQA1*03:02/DQB1*03:01) Cells were maintained in IMDM (Lonza) supplemented with 10% heat-inactivated FCS.

Peptides. Peptides were synthesized according to standard Fmoc (N-(9-fluorenyl) methoxycarbonyl) chemistry using a SyroII peptide synthesizer (MultiSynTech, Witten, Germany). The integrity of the peptides was verified using reverse phase HPLC and MS. For binding studies on HLA-DR3 molecules we used myoglobin-peptide LFRKDIAAKYK, for HLA-DQ2 deamidated alpha-gliadin-peptide LQPFPQPELPYPQ, and for HLA-DQ7 and HLA-DQ8 Herpes Simplex Virus 2 (HSV-2)-peptide $VP16_{432-444}$ VDMTPADALDDFD. The anchors are depicted in bold. These peptides were previously described in the context of the studies HLA-molecules[10-12]. To assess arginine or citrulline accommodation, peptides with an arginine or a citrulline at the indicated anchor positions were generated. As negative control peptides, we used peptides that do not bind to the respective HLA molecules. For HLA-DR3 we used AAAAKAAAAA, for HLA-DQ2 AKPFPQPEAPYKA, and for HLA-DQ7.3 and HLA-DQ8 AADTNRWSKMDAA.

HLA class II competitive peptide-binding assay. Peptide-binding assays were performed, as previously described[13]. In short, cell lysates from HLA- class II homozygous B-lymphoblastoid cell lines were incubated on SPV-L3- (anti-HLA-DQ) or B8.11.2- (anti-HLA-DR) coated (10 µg/ml) FluoroNunc 96-well plates at 4°C overnight. Titration ranges of the tested peptides (0 to 300 µM) were mixed with a fixed concentration (0.6 µM) of biotinylated indicator peptide and added to the wells. Bound indicator peptide was detected using europium-streptavidin (Perkin Elmer, Boston, MA) and measured in a time-resolved fluorometer (PerkinElmer, Wallac Victor2). IC50 values were calculated based upon the observed binding of the test peptide against the fixed concentration indicator peptide. The IC50 value depicts the concentration of test peptide required for a loss of 50% of the indicator peptide signal. IC50 values greater than 300 µM were classified as non-detectable binding affinity.

Statistical analysis. Wilcoxon's signed rank test was used to assess differences in IC50 values between citrulline and arginine containing peptides. P-values below 0.05 were considered to be statistically significant.

RESULTS

Presentation of arginine and citrulline residues by HLA-DR3
The genes encoding for HLA-DRB1, HLA-DQA1 and HLA-DQB1 are highly polymorphic and many different alleles have been identified in the human population. The residues within the HLA-molecules involved in shaping peptide-binding pockets

A

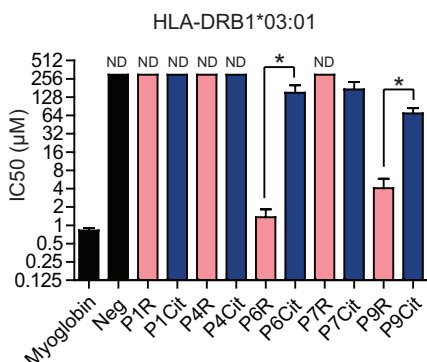|	Pocket1				Pocket 4							Pocket 6		Pocket7					Pocket 9		
	β85	β86	β89	β90	β13	β26	β28	β70	β71	β74	β78	β11	β30	β28	β47	β61	β67	β71	β9	β37	β57
*03:01	V	V	F	T	S	Y	D	Q	K	R	N	S	Y	D	F	W	L	K	E	N	D

B

Figure 1: Accommodation of citrulline and arginine residues by HLA-DR3 molecules. (A) Schematic representation of the peptide-binding pockets of HLA-DR3. Aa residues are color coded according to their properties (white = hydrophilic, gray = hydrophobic, red = basic, blue = acidic). (B) Competitive binding of a biotin-labeled myoglobin-peptide with an unlabeled myoglobin-peptide or myoglobin-variants with citrulline or arginine residues in p1, p4, p6, p7 and p9 to HLA-DR3. Graph depicts the IC50-values (µM). ND = Non-detectable binding affinity. Binding experiments were performed at least three times and plots show pooled experiments. The error bars show the variation between the different experiments.

are known[6]. To select for HLA-DRB1 alleles that could potentially prefer citrulline over arginine residues, we made use of MHC motif viewer[14], a web server that displays peptide-binding motifs for all HLA-DR alleles using a predictive algorithm. We searched for non-SE alleles that are common in Caucasians and that display a predicted preference for negatively-charged amino acids. In this way, we selected HLA-DR3 (HLA-DRB1*03:01), one of the most common HLA-DR-molecules in Caucasians, for further studies. HLA-DR3 was predicted to have a strong preference for negatively charged amino acids in pocket 4. Figure 1A depicts the amino acid residues of HLA-DRB1*03:01 involved in shaping the various binding pockets and indicates that pocket 4 has a net-positive charge, thereby explaining the predicted preference for negatively-charged aa residues.

To systematically analyze if arginine-to-citrulline conversions of peptides enhanced the affinity for HLA-DR3, we selected a myoglobin-peptide that was previously described to be accommodated by this allele and for which the peptide-binding register is known[11]. Next, we substituted each of the peptide positions interacting with peptide-binding pockets of the HLA-molecule for arginine or citrulline residues. The effect of these substitutions was subsequently studied in peptide-binding assays. In this way a systematic characterization of the ability of each of the peptide-binding pockets to accommodate arginine or citrulline residues was performed.

As depicted in Figure 1B, HLA-DR3 was unable to accommodate either arginine or citrulline in pocket 4. Also for the other peptide-binding pockets, arginine-to-citrulline conversion did not result in enhanced peptide-binding affinities. In contrast, in pocket 6 and 9 arginine- was preferred over citrulline-residues.

Together, these data indicate that HLA-DR3 does not prefer citrulline- over argini-

ne-residues in pocket 4 or any of its other peptide-binding pockets. It is therefore unlikely that HLA-DR3 would prefer peptides that contain a citrulline over their arginine counterparts.

Presentation of arginine and citrulline residues by HLA-DQ molecules.

Genes encoding for HLA-DR and HLA-DQ molecules are in tight linkage disequilibrium (LD) and inherit together in haplotypes. The ability of predisposing and non-predisposing HLA-DQ molecules to accommodate arginine and citrulline residues has not been studied, although it was described for different HLA-DQ molecules that they prefer negatively-charged amino acid residues, e.g. for HLA-DQ2 (DQB1*02:01/DQA1*05:01), HLA-DQ7 (DQB1*03:01/DQA1*03:02) and HLA-DQ8 (DQB1*03:02/DQA1*03:01). The latter two HLA-molecules are particularly interesting as these are encoded by genes that are in tight LD with SE alleles and hence also associate with RA-risk. Figure 2A shows the amino acids shaping the various peptide-binding pockets of these HLA-DQ molecules.

To systematically analyze if arginine to citrulline conversion of peptides enhanced their affinity for HLA-DQ molecules, we used a similar approach as applied for HLA-DR3. As the myoglobin-peptide cannot be presented by these HLA-DQ molecules, we arginine- or citrulline-substituted two previously described epitopes derived from alpha-gliadin (HLA-DQ2) and from herpes simplex virus VP16-protein (HLA-DQ7 and HLA-DQ8) for which the binding register has been established[10, 12], Our data show that all three HLA-DQ molecules preferred citrulline over arginine residues in different peptide-binding pockets of the HLA-molecules. HLA-DQ7 (Figure 2B) and HLA-DQ8 (Figure 2C) preferred citrulline over arginine residues in peptide-binding pocket 1, 6 and 9, whereas HLA-DQ2 (Figure 2D) molecules preferred citrulline residues in pocket 7 and 9.

Together these data demonstrate that an arginine to citrulline conversion can also enhance peptide-binding affinity for several HLA-DQ molecules thereby indicating that this property is not confined to HLA-SE alleles. These data are important as they indicate that also HLA-DQ molecules could contribute to the induction of citrullinated-peptide-directed T cell immunity thought to contribute to the auto-immune response underlying RA.

DISCUSSION

Over a decade ago, it was first demonstrated that arginine-to-citrulline conversion enhances the affinity of peptides for HLA-SE alleles[7]. It was suggested that this feature is unique to HLA-SE alleles and could thereby, explain the HLA-RA connection. We now also analyzed non-SE alleles and demonstrate that also several HLA-DQ molecules have the enhanced capacity to present citrullinated peptides

as we can clearly show that peptides that harbor a citrulline instead of arginine in positions interacting with peptide-binding pockets have an enhanced affinity.

Determining the capacity to present citrullinated neo-epitopes for all different HLA-molecules would require a large-scale approach given the fact that hundreds of different HLA-DR and HLA-DQ molecules have been described. However, the amino acid positions important for shaping peptide-binding pockets have been elucidated, thereby allowing us to predict HLA-DQ and HLA-DR molecules that are likely to display the capacity to present citrullinated neo-epitopes. In this study we
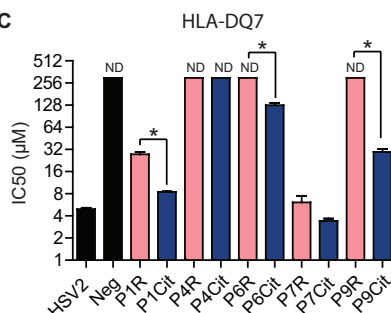
A

	Pocket1										Pocket 4						
	α9	α24	α31	α32	α43	α52	β85	β86	β89	β90	β13	β26	β28	β70	β71	β74	β78
DQ2	Y	H	Q	F	W	R	L	E	T	T	G	L	S	R	K	A	V
DQ7	Y	H	E	F	W	R	L	E	T	T	A	Y	T	R	T	E	V
DQ8	Y	H	E	F	W	R	L	E	T	T	G	L	T	R	T	E	V

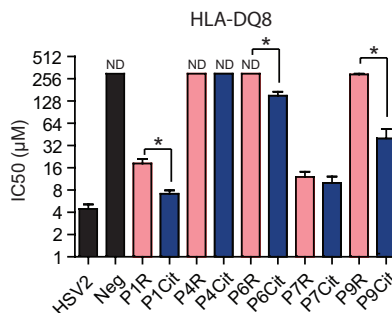|	Pocket 6							Pocket7					Pocket 9							
	α11	α62	α65	α66	α69	β11	β30	β28	β47	β61	β67	β71	α68	α69	α72	α73	α76	β9	β37	β57
DQ2	N	N	V	L	N	F	S	S	F	W	I	K	H	N	S	L	R	Y	I	A
DQ7	N	N	V	L	N	F	Y	T	Y	W	V	T	H	N	I	V	R	Y	Y	D
DQ8	N	N	V	L	N	F	Y	T	Y	W	V	T	H	N	I	V	R	Y	Y	A
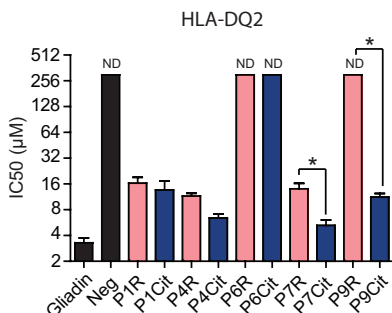
Figure 2: Accommodation of citrulline and arginine residues by HLA-DQ molecules (A) Schematic representation of the differences in peptide-binding pockets between HLA-DQ2, HLA-DQ7 and HLA-DQ8. Aa residues are color coded according to their properties (white = hydrophilic, gray = hydrophobic, red = basic, blue = acidic). (B-C) Competitive binding of a biotin-labeled VP16-peptide with an unlabeled VP16-peptide or VP16-variants with citrulline or arginine residues in p1, p4, p6, p7 and p9 to HLA-DQ7 (B) and HLA-DQ8 (C). (D) Competitive binding of a biotin-labeled alpha-gliadin-peptide with an unlabeled alpha-gliadin-peptide or alpha-gliadin-variants with citrulline or arginine residues in p1, p4, p6, p7 and p9 to HLA-DQ2. Graphs depict the IC50-values (µM). ND = Non-detectable binding affinity. Binding experiments were performed at least three times and plots show pooled experiments. The error bars show the variation between the different experiments.

focused on only a few SE-negative HLA class II molecules that are likely able to present citrullinated neo-epitopes. However, it is highly conceivable that also other unstudied HLA-DR or HLA-DQ molecules share similar capacities.

To the best of our knowledge the capacity of HLA-DQ molecules to present citrullinated ligands has not been studied. Interestingly, in a recent study, it was shown that T cells of collagen-type II (CII)-immunized HLA-DQ8 transgenic mice respond better to citrullinated CII than to native CII, which could be explained by an enhanced capacity of HLA-DQ8 to present citrullinated neo-epitopes[15]. Our data demonstrate that the presentation of citrullinated ligands by HLA-DQ molecules is not confined to those HLA-molecules associated with predisposition. The hypothesis that predisposing HLA-molecules associate with predisposition because of their capacity to present arginine-to-citrulline converted epitopes with an enhanced affinity is conceivably not explaining the complete molecular basis for the association between HLA-SE haplotypes and RA. It would, therefore, be interesting to better comprehend the additional contribution of the molecules encoded by the HLA-SE-haplotypes to seropositive RA. Together, this study provides a further refinement of the SE-hypothesis and the possible contribution of citrulline-specific T cells in the pathogenesis of ACPA-positive RA.

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CHAPTER 11

General discussion

ANTIGEN PRESENTATION

Expression of HLA class II molecules

HLA class II molecules are well-known for their role in the adaptive immune system. Particularly, these molecules are involved in presenting peptide-ligands that can be epitopes for CD4+ T cells. The expression of HLA class II molecules is restricted to professional antigen-presenting cells (e.g. macrophages, B cells and dendritic cells) and to specialized cells in the thymus involved in T cell education. In inflammation, these cells can increase their HLA-class II expression under the influence of IFN-gamma and IFN-gamma can also induce the expression of HLA-class II molecules by non-professional cells[1]. In **Chapter 2**, we now describe for the first time HLA class II expression by mast cells, which is sufficient to activate CD4+ memory T cells. The expression of HLA-class II molecules is largely influenced by the presence of IFN-gamma. Interestingly, we also show that mast cells can internalize antigen and process antigen to T cell epitopes. This is particular interesting because mast cells are located at strategic locations throughout the body (e.g. the skin and the gut) and can therefore be one of the first cells that present antigens to nearby memory CD4+ T cells.

Presentation of ligands by HLA class II molecules

Since the early 90s ligands have been eluted from different HLA class II molecules[2-7]. These studies have provided numerous information on the nature of HLA class II ligands and have clearly demonstrated that these ligands are very different from HLA class I ligands, both in their origin, the pathway of antigen-processing, as well as in length and in restriction. Also, these studies have provided insights in how structural difference between different HLA class II alleles are reflected in distinct peptide-binding repertoires. In **Chapter 3 and 4**, we describe two distinct HLA-class II molecules: HLA-DQB1*06:03 and HLA-DRB1*03:01 and correlate identified ligands to functional characteristics of the peptide-binding pockets.

In the last 25 years, there has been an enormous development in the instruments capable of measuring HLA-ligands. Whereas in the 90s, the number of ligands isolated from a single HLA class II molecules were generally below a hundred, in **Chapter 3**, we isolated over 13.000 ligands from a single HLA class II molecule, HLA-DRB1*03:01. This is the largest set of identified ligands that have ever been described and allowed us to perform analyses that were previously not possible. These analyses have provided novel insights into the nature of HLA class II ligands. For instance, we have shown that HLA class II ligands are not only conserved in amino acid positions involved in interacting with the HLA molecules peptide binding pockets, but also in N- and C-terminal residues projecting outside the HLA class II molecule.

TOWARDS A MODEL FOR RA RISK.

Rheumatoid arthritis patients are a highly heterogeneous population[8]. Interestingly, ACPA autoantibodies can subdivide RA patients in two distinct disease subsets, ACPA-negative and ACPA-positive RA patients. These patient subsets strongly differ with regard to important disease outcome measures (disease progression, chance for remission and radiographic damage), and risk factors (both genetic and environmental) (discussed in **Chapter 5**)[9-14].

The HLA class II locus is the most important risk factor for the development of RA, but the associated haplotypes differ between ACPA-positive and ACPA-negative RA (discussed in **Chapter 6**)[9, 11, 15, 16]. In Caucasians, HLA-DRB1*01/10:DQ5- and HLA-DRB1*04:DQ7/DQ8-encoding haplotypes are strongly overrepresented in ACPA-positive RA patients compared to healthy controls or ACPA-negative RA patients[17-19]. In addition to predisposing haplotypes, the HLA-DRB1*13:DQ6 haplotypes associates with protection from ACPA-positive RA[14, 20, 21].

Only a small percentage of donors carrying risk haplotypes will develop ACPA-positive RA. This shows that their presence is not sufficient for disease development and that other factors are required and these findings suggest that the development of ACPA+ RA involves multiple hits. This is further supported by reports demonstrating that ACPA-positivity can precede the onset of RA by up to ten years and that different environmental/genetic risk factors underlie the development of ACPA-positivity and the transition to ACPA-positive RA (**Chapter 7**)[22, 23].

Positivity for risk haplotypes is not required for the development of ACPA-positive RA as about 15% of patients are negative for these haplotypes. These patients are homozygous for a wide variety of other HLA-DR subtypes. The difference in the relative distribution of HLA-DR genotypes between ACPA+ RA patients and healthy controls (generally presented as odds ratios) could therefore best be explained by the sum of the risk to undergo the multiple hits required for disease development. This sum could be influenced by different factors (Figure 1):

(1)   Hit number. Etiological pathway in individuals positive for high-risk haplotypes possibly require less hits (or less rare hits) (Figure 1A).
(2)   Hit risk: Etiological pathways in those positive for high-risk haplotypes might require more prevalent hits (Figure 1B).
(3)   Multiple pathways: Individuals positive for high-risk haplotypes might develop ACPA-positive RA via more different routes (Figure 1C).

The distribution of HLA-DR haplotypes in ACPA-positive RA patients is most likely explained by a combination of the three factors (Figure 1D).

TOWARDS A MOLECULAR BASIS

For this thesis we aimed to further elucidate the molecular basis of RA with a specific focus on ACPA-positive RA.
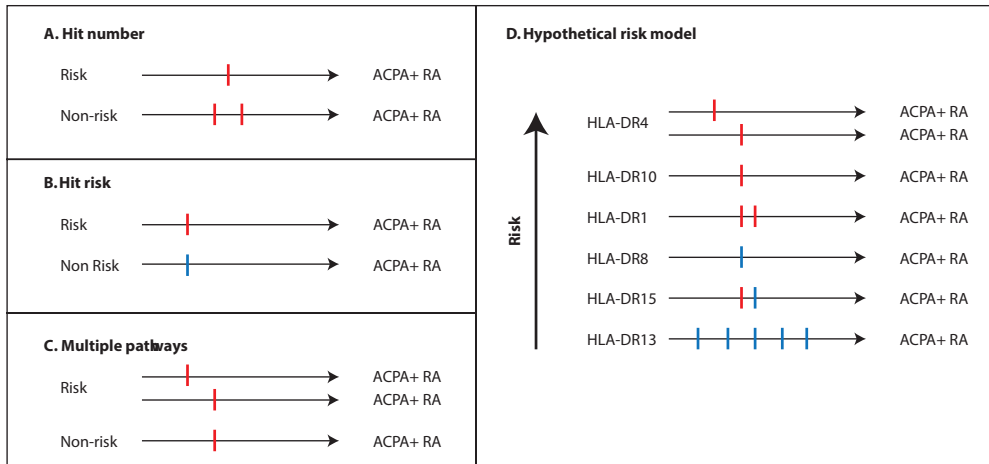
Figure 1: Schematic model explaining the HLA-RA connection. The risk of an HLA-genotype for RA development could be influenced by several factors including the number of required hits(A), the chance for a hit to occur (B) or the number of etiological pathways (C) Red lines indicate common hits, blue lines indicates rare hits.

Initial loss of B cell tolerance.

ACPA autoantibodies can be detected in the serum of healthy donors up to ten years before disease onset without any clinical signs of arthritis[23]. Interestingly, the ACPA-response of healthy donors is characterized by low levels, little cross-reactivity and low isotype usage[24]. Recently, it became increasingly clear that the HLA locus is playing only a minor role in the development of ACPA-autoantibodies as it was shown for both predisposing and protective HLA alleles that these do not associate with the presence of ACPA in healthy donors (**Chapter 7**)[22]. In contrast these HLA alleles associate more with the transition from ACPA-positive to ACPA-positive disease (**Chapter 7**). This indicates that the initial development of ACPA is triggered by HLA-independent risk factors. These independent risk factors could be genetic factors that shape the threshold for B cell activation (e.g. PTPN22) or perhaps factors that increase "citrulline load", e.g. polymorphisms in the PADI4 gene, anti-PAD antibodies that enhance PAD function, chronic infection with PAD-containing bacteria or environmental factors like smoking and silica exposure that promote citrullination in the lungs[13, 22, 25-28].

Loss of T cell tolerance

The transition from ACPA-positivity to ACPA-positive RA is characterized by strong changes in the ACPA-response, including a rise in ACPA-levels, isotype-expansion and a more diverse fine-specificity profile[24]. These changes in the ACPA-profile are likely the result of germinal center formation that are induced by T cell dependent B cell activation[29, 30]. A role for T cells during this phase is further

supported by the observation that the HLA class II molecules associated with RA are mainly involved during this particular phase. In this thesis, three different potential pathways were studied (**Chapter 8-10**) that are summarized in Figure 2. Interestingly, the molecular basis for all these pathways is different. The studied pathways will now be shortly summarized.

Molecular mimicry

In **Chapter 8**, we provide a first molecular basis for the role of both predisposing and protective HLA-alleles in ACPA-positive RA. As discussed, HLA-DRB1*13-positive donors are protected. HLA-DRB1*13 is particularly interesting as it was also shown to protect in the presence of shared epitope alleles indicating that HLA-DRB1*13 could act in the same biological pathway as risk alleles, possibly by preventing a particular hit to occur[14, 31]. In **Chapter 7**, we studied the protective role of HLA-DRB1*13 during different phases of disease development. We could show that HLA-DRB1*13 does not protect from ACPA-positivity, only from ACPA-positive disease. These data indicate that this allele exerts (part of) its effect in the time-frame between ACPA-positivity and the development of ACPA+ RA. In **Chapter 8**, we provide an explanation for the protective effect HLA-DRB1*13 alleles in the presence of SE alleles. We could show that predisposing HLA-DQ molecules (those in
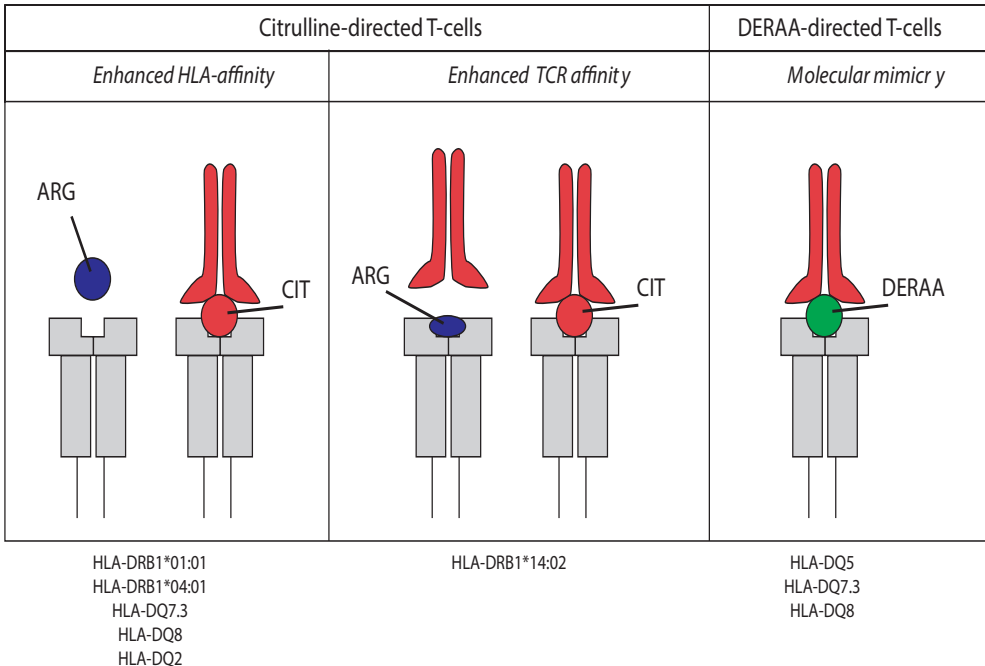

Figure 2: Summary of the studied molecular bases for the association between HLA, RA and citrullination

LD with SE-alleles) have an exceptional capacity to present a group of peptides with the core sequence DERAA that are derived from microbes, vinculin and from HLA-DRB1*13. Our data indicate a role for vinculin directed T cells, primed by pathogens in providing help to ACPA-producing B cells and a role for HLA-DRB1*13 in tolerizing this T cell population. Together this pathway explains part of the risk mediated by risk and protective haplotypes. Thus, this elegant pathway can explain a large part of the risk of SE-alleles and the protective effect of HLA-DRB1*13 alleles.

Enhanced HLA-affinity

It was previously shown for "Caucasian" SE alleles that these can present citrullinated peptides with an enhanced affinity over their native counterpart[32-38]. In **Chapter 10**, we show that this capacity is not specific for SE alleles as it can also be found for several predisposing and non-predisposing HLA-DQ molecules, thereby providing a further refinement of the association between HLA-SE alleles, RA and citrullination.

Enhanced TCR-avidity

In **Chapter 9**, we show that not all HLA-SE alleles can present citrullinated epitopes with an enhanced HLA-affinity. SE-allele HLA-DRB1*14:02, frequent in indigenous North Americans, has no enhanced affinity for citrullinated epitopes over their arginine counterpart. However, we could demonstrate that HLA-DRB1*14:02 presents citrulline residues in an orientation different from arginine residues, which could be a target of citrulline-directed T cells, thereby providing an alternative molecular basis for this HLA-SE allele.

CONCLUSION

In recent years, novel insights have been provided into the mechanisms underlying the association between the HLA system, RA and citrullination. Further elucidating the molecular basis for these associations will likely contribute to a better understanding of disease pathogenesis. The window between ACPA-positivity and disease development offers a unique opportunity for preventive treatment. As the HLA system plays a key role in disease development, elucidating the molecular basis for the association between HLA, ACPA and RA will be of pivotal importance. Excitingly, the data presented in this thesis together with other recent data, have provided novel insights into this long-known association.

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CHAPTER 12

Nederlandse samenvatting

PRESENTATIE VAN PEPTIDEN DOOR HLA KLASSE II MOLECULEN

HLA klasse II moleculen spelen een belangrijke rol in het adaptieve immuunsysteem. Deze moleculen zijn gespecialiseerd in het presenteren van eiwitfragmenten, ook wel peptiden of liganden genoemd, op het oppervlak van cellen. Deze HLA-peptide complexen kunnen vervolgens herkend worden door CD4+ T cellen. Indien de T-cel receptor het HLA-peptide complex met voldoende aviditeit herkent, kan dit resulteren in T-cel activatie en bijbehorende effectormechanismen, zoals het primen van cytotoxische T cellen of het geven van hulp aan B cellen. Er zijn 3 typen HLA-klasse II moleculen: HLA-DQ, HLA-DR en HLA-DP. De genen die coderen voor deze moleculen bevinden zich op chromosoom 6 en zijn zeer polymorf. Dit betekent dat er verschillen zijn in de HLA klasse II moleculen tussen verschillende individuen. Deze verschillen bevinden zich met name in het gebied van het HLA klasse II molecuul dat betrokken is bij het presenteren van peptiden. Verschillende HLA klasse II moleculen presenteren dan ook een verschillend peptide-repertoire.

HLA klasse II moleculen komen tot expressie op professionele antigeen-presenterende cellen als macrofagen, B cellen en dendritische cellen. Daarnaast kunnen HLA klasse II moleculen tot expressie komen in gespecialiseerde cellen in de thymus, waar ze een rol spelen bij de positieve selectie van HLA klasse II gerestricteerde T cellen en de negatieve selectie van auto-reactieve T cellen. Tot slot kunnen ook andere cellen tijdens inflammatie HLA klasse II tot expressie brengen, zoals geactiveerde T cellen en IFN-gamma gestimuleerde niet-hematopoietische cellen, zoals darmepitheelcellen, hepatocyten, pancreatische β-cellen en synoviocyten. In **Hoofdstuk 2** beschrijven we dat ook humane mestcellen in lage hoeveelheden HLA klasse II tot expressie kunnen brengen, waarmee ze CD4+ memory T cellen kunnen activeren. De mate van expressie wordt in grote mate beïnvloed door de aanwezigheid van IFN-gamma.

In **Hoofdstuk 3 en 4** hebben we HLA-peptide complexen geïsoleerd uit HLA klasse II homozygote cellen en de gepresenteerde peptiden geanalyseerd. In **Hoofdstuk 3** hebben we ons gefocust op HLA-DR0301 en in **Hoofdstuk 4** op HLA-DQ0603. Het motief waaraan peptiden moeten voldoen om gepresenteerd te kunnen worden verschilt erg tussen deze twee moleculen. Dit kan verklaard worden doordat deze twee HLA-moleculen erg verschillend zijn in diverse posities die een rol spelen bij HLA-peptide interacties. HLA-DQ0603 heeft bijvoorbeeld een elektropositieve p6 pocket, terwijl de p6 pocket van HLA-DR0301 elektronegatief is. De p6 pocket van DQ0603 heeft daarom een voorkeur voor zure aminozuren, terwijl de p6 pocket van DR0301 een voorkeur heeft voor basische aminozuren. Het gevolg is dat er weinig overlap is in de peptiden die gepresenteerd worden door deze twee moleculen.

Naast het bindingsmotief hebben we ook andere eigenschappen van de gepresenteerde peptides bestudeerd, zoals de lengte van de peptiden, fysisch-chemische eigenschappen en de eiwitten waarvan de peptiden afkomstig zijn. Deze studies hebben ons een goed beeld gegeven van de type peptiden die door HLA klasse II

moleculen gepresenteerd worden en hebben ons nieuwe inzicht verschaft in anti-geen-processing en antigeen-presentatie.

ACPA-POSITIEVE REUMATOIDE ARTHRITIS

Het HLA klasse II locus is sterk geassocieerd met het ontwikkelen van auto-immuun-ziekten. Dit suggereert een belangrijke rol voor antigeen-specifieke CD4+ T cellen in de ontwikkeling van deze aandoeningen. Tijdens ons onderzoek hebben wij ons met name gericht op reumatoïde artritis (RA). Dit is een systemische auto-immuun-ziekte waarbij met name de synoviale gewrichten sterk aangedaan zijn. De mate van ontsteking is sterk afhankelijk van de aanwezigheid van een groep antilichamen, ACPA genoemd, die specifiek gericht zijn tegen gecitrullineerde eiwitten. Citrulline-ring is een post-translationeel proces, waarbij arginine residuen worden omgezet in citrulline residuen. In de afgelopen 10 jaar is steeds meer duidelijk geworden dat er grote verschillen zijn tussen ACPA-negatieve en ACPA positieve RA patiënten. Zo verschillen deze patiënten in klinisch uitkomst, maar ook in genetische risicofac-toren. Zo is het HLA klasse II locus de belangrijkste risicofactor voor de ontwikkeling van RA, maar speelt het vooral een rol bij het ontstaan van ACPA-positieve RA. Het begrijpen van deze associatie kan waardevolle inzichten verschaffen in de pathoge-nese van ACPA-positieve RA.

Om ACPA-positieve RA goed te kunnen bestuderen is het belangrijk dat er goede tests zijn om ACPA te bepalen. In de kliniek wordt nu veel gebruik gemaakt van de anti-CCP-2 en anti-CCP-3 test. Deze tests kunnen met een hoge specificiteit ACPA+ RA patiënten identificeren. Echter, het is mogelijk dat toch een deel van de ACPA+ RA patiënten gemist wordt door de bestaande tests. Dit heeft geresulteerd in de ontwikkeling van multiplex-platforms, waarop serum van grote groepen patiënten getest kan worden op positiviteit voor een groot aantal gecitrullineerde antigenen. In **Hoofdstuk 5** analyseren we de waarde van een multiplex-assay in vergelijking met bestaande ACPA tests of als mogelijke toevoeging. Uit deze analyses is gebleken dat deze multiplex-assay weliswaar goed het onderscheid kan maken tussen gezonde controles en RA patiënten, maar niet in staat is om onderscheid te maken tussen RA patiënten en patiënten met een andere reumatische aandoening. Deze test bleek dan ook niet geschikt als vervanging van of als toevoeging op de bestaande tests.

EEN MOLECULAIRE BASIS VOOR DE ASSIOCIATIE TUSSEN RA, ACPA EN HLA.

De genen die coderen voor HLA klasse II moleculen bevinden zich in een regio op chromosoom 6 met een sterk linkage-disequilibrium. Dit betekent dat deze genen in haplotypen overerven. De genen die geassocieerd zijn met RA zijn met name de genen die coderen voor de alfa en de bèta ketens van HLA-DQ (HLA-DQA1 en HLA-DQB1) en voor de bèta keten van HLA-DR (HLA-DRB1). Tot op heden is het niet bekend of de genetische associatie verklaard wordt door HLA-DQ en/of HLA-DR.

Recentelijk zijn er diverse publicaties verschenen, waarbij middels een nieuw ont-wikkelde statistische methode, gekeken kan worden naar individuele aminozuren binnen het HLA-molecuul en de associatie van die aminozuren met ziekte, waar-onder met ACPA-positieve RA. De sterkst associërende aminozuren, geïdentificeerd met deze statistische methode, worden in causaal verband gebracht met de patho-genese van RA. In **Hoofdstuk 6** bediscussieerden we de uitkomsten en interpretatie van deze onderzoeken.

De analyses die we in **Hoofdstuk 6** uitgevoerd hebben tonen opnieuw aan hoe lastig het is om middels genetische studies causaliteit binnen de HLA locus te onderzoeken en ondersteunen het idee dat dit causale verband alleen middels func-tionele experimenten aan te tonen is. Het tweede deel van dit proefschrift is hier dan ook aan gewijd. De HLA klasse II locus speelt een dubbele rol in RA. Sommige haplotypen zijn sterk geassocieerd met predispositie (m.n. HLA-DRB1*04;DQ-B1*03;DQA1*03), terwijl anderen sterk geassocieerd zijn met bescherming (m.n. HLA-DRB1*13-DQB1*06-DQA1*01). In **Hoofdstuk 7 en 8** hebben we het bescher-mende effect bestudeerd. Dit alles met de gedachte dat als we bescherming begrijpen, we mogelijk ook tot belangrijke inzichten komen wat betreft predispo-sitie. In **Hoofdstuk 7** hebben we bestudeerd wanneer de HLA-DRB1*13 moleculen hun beschermend effect uitoefenen. Het is bekend dat ACPA antistoffen soms wel 10 jaar voor het ontstaan van symptomen in het bloed van donoren terugge-vonden kunnen worden. We hebben daarom bestudeerd of HLA-DRB1*13 vooral beschermt tegen het ontstaan van ACPA-positiviteit of tegen de transitie van ACPA-positiviteit naar ACPA+ RA. Uit deze studie blijkt dat HLA-DRB1*13 met name beschermt tegen de transitie naar ACPA-positieve RA. Dit is interessant omdat dit de fase is waar de ACPA respons een sterke maturatie laat zien, iets dat kan duiden op CD4-positieve T-cel hulp. Ook hebben de predisponerende haplotypen op dit moment hun effect, wat suggereert dat producten van de beschermende en pre-disponerende haplotypen deel uit kunnen maken van dezelfde route. Dit versterkte onze gedachte dat het begrijpen van het beschermende effect van HLA-DRB1*13 erg waardevol zou kunnen zijn. In **Hoofdstuk 8** proberen we het mechanisme hier-achter verder te begrijpen. In hoofdstuk 2 en 3, maar ook in eerdere studies, waar gekeken is naar peptiden gepresenteerd op HLA klasse II moleculen valt op dat veel gepresenteerde peptiden afkomstig zijn van andere HLA moleculen. Zo is er ook een peptide beschreven afkomstig van HLA-DRB1*13. Dit peptide heeft de ami-nozuursequentie DERAA, een sequentie die naast HLA-DRB1*13 ook voorkomt in veel ziekteverwekkers en in vinculine. Vinculine is een eiwit dat voorkomt in de gewrichten, gecitrullineerd kan worden en waarvan we hebben aangetoond dat het in gecitrullineerde vorm herkend kan worden door ACPA. In hoofdstuk 8 beschrijven we een populatie CD4+ T cellen die specifiek gericht zijn tegen het DERAA-fragment van vinculine. Deze populatie CD4+ T cellen is afwezig in beschermde HLA-DRB1*13 positieve donoren. Daarnaast laten we zien dat vinculine-DERAA specifieke T cellen kunnen kruisreageren met DERAA-bevattende peptiden van ziekteverwekkers.

Deze studie impliceert een rol voor vinculine-DERAA specifieke T cellen, geactiveerd tijdens infecties met DERAA bevattende ziekteverwekkers, in het ontstaan van antistoffen tegen gecitrullineerd vinculine. Wanneer we verschillende HLA-moleculen vergeleken op hun capaciteit om DERAA-peptiden te presenteren ontdekten we dat deze peptiden erg goed gepresenteerd kunnen worden op predisponerende HLA-DQ moleculen. We hebben daarmee een moleculaire basis ontdekt voor het ontstaan van ACPA-positieve RA, die zowel het beschermende als het predisponerende effect kan verklaren.

De groep ACPA+ RA patiënten is erg heterogeen, bijvoorbeeld in ziekteactiviteit en in ACPA karakteristieken (zie ook hoofdstuk 7), maar ook in genetische predispositie. Zo is bijvoorbeeld een deel van de ACPA+ RA patiënten niet positief voor predisponerende haplotypen. Dit suggereert dat de route, zoals beschreven in hoofdstuk 8 niet de enige route kan zijn. We zijn daarom ook naar andere mogelijkheden gaan kijken. Zo is er een theorie dat de genetische predispositie deels verklaard kan worden door de presentatie van gecitrullineerde peptiden. In **Hoofdstuk 9 en Hoofdstuk 10** hebben we hier verder onderzoek naar gedaan en op een systematische manier de capaciteit van verschillende HLA moleculen om gecitrullineerde peptiden te presenteren vergeleken. We vonden zowel HLA-DR als HLA-DQ moleculen in staat zijn om gecitrullineerde peptiden te presenteren. Voor HLA-DR vonden we dat gecitrullineerde peptiden goed gepresenteerd worden door predisponerende allelen, maar niet door alle predisponerende allelen. De capaciteit van predisponerende allelen om gecitrullineerde peptiden te presenteren wordt beïnvloed door bepaalde residuen in het HLA molecuul die betrokken zijn bij peptide-presentatie en waarvan eerder gesuggereerd is dat ze betrokken zijn in RA.

Samenvattend verschaffen we in dit proefschrift nieuwe inzichten in antigeen-presentatie en in de contributie van HLA moleculen in de pathogenese van ACPA-positieve RA. Het verder begrijpen van de HLA-associatie zal naar alle waarschijnlijkheid zeer belangrijke inzichten verschaffen in de pathogenese van RA en mogelijk in de toekomst kunnen leiden tot preventieve behandelingen voor risicogroepen.

Curriculum Vitae

Jurgen van Heemst werd op 20 juli 1986 geboren in Voorburg. In 2004 slaagde hij voor het gymnasium aan het Zandvlietcollege te Den Haag. Van 2004 tot 2010 studeerde hij Biomedische wetenschappen in het Leids Universitair Medisch centrum. Tijdens de bacheloropleiding heeft hij deelgenomen aan een uitwisselingsprogramma met het Karolinska Institutet in Stockholm waar hij een semester Biomedicine studeerde. Tijdens zijn masteropleiding heeft hij een stage gedaan in de groep van Thomas Rando aan de Stanford University School of Medicine in Palo Alto, Californië. Aansluitend is hij bij de afdeling Reumatologie van het LUMC begonnen aan zijn promotieonderzoek onder leiding van Prof.Dr. René Toes en Prof. Dr. T.W.J. Huizinga.

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