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Immunogenetic and immunological aspects of rheumatoid arthritis : DERAA and anti-citrulline reactivity can make the difference

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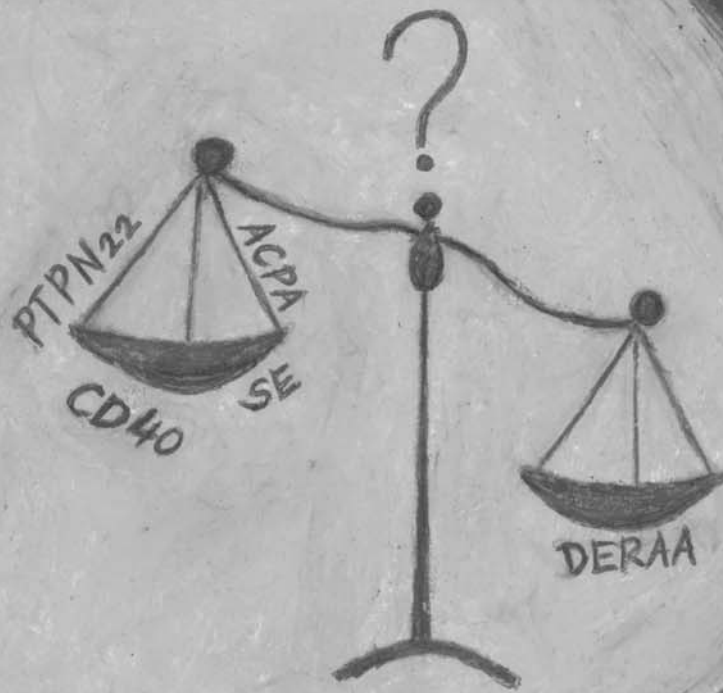
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Chapter 7

General Discussion



Many cell types take part in the immunological processes contributing to the synovial and systemic inflammation present in patients with rheumatoid arthritis (RA), including T cells and B cells. They exert their own functions and interact with each other, resulting in the chronic inflammation observed in RA. The research presented in this thesis focused on the identification of several risk factors and specific T cell responses that are thought to play a role in the pathogenesis of RA.

Four major topics were studied. Firstly, we showed that “DERAA”-containing HLA-DRB1 alleles are less frequently present in RA patients as compared to controls both when these alleles are inherited directly as well as when they are acquired as non-inherited maternal antigen (NIMA) (**Chapter 2 and 3**). Secondly, two naturally processed epitopes derived from the human citrullinated vimentin protein were identified in mice transgenic for the most frequent SE-containing HLA-DRB1 allele in Caucasians, HLA-DRB1*0401. IFN γ -production by CD4⁺ T cells against these peptides could be observed in RA patients (**Chapter 4**). These T cells could be involved in providing help to ACPA-producing B cells. Furthermore, we showed in **Chapter 5** that the C1858T polymorphism of the PTPN22 gene is not informative for the prediction of RA development in UA patients in addition to ACPA status, but does seem to affect ACPA-levels of RA patients. In **Chapter 6**, we observed that a newly identified risk factor for RA, CD40, also influences the severity of the disease as measured by radiological damage.

Regarding these findings, the following topics will be discussed in further detail in the different sections of this chapter:

1. “DERAA”-containing HLA-DRB1 alleles
 - a. The possible mechanism of the observed protection
 - b. Maternal microchimerism as mechanism of the observed NIMA effect
 - c. Associations of these HLA-DRB1 alleles with other diseases
2. PTPN22 and ACPA
3. The role of CD40 on ACPA production and RA development
4. Directions for further research

1a. Possible mechanism of DERAA protection

It has been shown by several groups that the frequency of “DERAA”-containing HLA-DRB1 alleles is reduced in RA patients as compared to healthy controls (1-4). We have described in **Chapter 2 and 3** of this thesis that this protective effect is present both when these HLA-DRB1 alleles are inherited, as well as when the gene products are

acquired as a non-inherited maternal antigen (NIMA). Although it is becoming increasingly clear that some HLA-DRB1 alleles confer protection to RA (5;6), it is unclear whether the entire “DERAA”-motif is essential for protection or whether only certain amino acids of this motif may confer the same effect. In contrast to several reports showing the protective effects by “DERAA”-containing HLA-DRB1 alleles on the development and severity of RA (1-4), other reports conclude that the amino acids “RAA” at position 72-74 in the third hypervariable region influence the susceptibility to RA development whereas the amino acids at position 70 and 71 modulate this effect (7;8). In these articles it is indicated that not only HLA-DRB1 alleles expressing the ⁷¹ERAA⁷⁴ sequence but also alleles that only contain the Aspartic acid (D) at position 70 have a lower frequency in RA patients as compared to healthy controls. The hypothesis that protection is mainly associated with the Aspartic acid (D) at position 70 is supported by Ruiz-Morales *et al.* (9) and Matthey *et al.* (10). A meta-analysis including large group sizes and different study populations has to be performed to elucidate which of the amino acids are essential for the observed protective effect. In the section below it is assumed that the “DERAA”-motif is responsible for the observed protection.

Table 1. Human proteins containing the amino acid sequence DERAA. The sequences are depicted as peptides with “DERAA” in the centre and 7 flanking residues on each side.

Protein name	Sequence
Vinculin	PNREEV DERAA NFENHSG
MHC	QKDI (L/F) LE DERAA VDTYCRH
Parkin	TTQAYRV DERAA EQARWEA
PER1 (period circadian protein)	SCLFQDV DERAA PLLGYLP

The mechanism by which the “DERAA”-containing HLA-DRB1 alleles influence the susceptibility to and the severity of RA is unknown, but it has been proposed that it is mediated by T cells recognizing peptides containing the “DERAA”-sequence presented by HLA-DQ molecules (11). As it is well-known that many peptides presented by HLA-class II molecules are derived from other HLA-molecules (12-18), it is hypothesized that the T cell repertoire of individuals carrying the “DERAA”-containing HLA-DRB1 alleles, in contrast to “DERAA”-negative individuals, is tolerized for “DERAA”-containing antigens. The “DERAA”-sequence has been

described to be only present in four human proteins (19): i.e. parkin, PER1, the “DERAA”-containing HLA-DRB1 molecules and vinculin (Table 1). Parkin and PER1 are both brain-specific proteins and are therefore probably not involved in the protection against RA. It has been shown that vinculin, a cytoskeletal protein, can be found under certain conditions (e.g. during apoptosis) on the cell surface, and that cross-priming to vinculin-specific cytotoxic T lymphocytes can occur (20;21). These findings indicate that vinculin-specific T cells can exist and thus are not tolerized in the thymus, despite the fact that vinculin is an abundantly expressed self-protein present in the cytoskeleton of every cell in the body. We have observed that PBMC from “DERAA”-negative individuals can respond with significantly more IFN γ to the “DERAA”-containing peptide derived from vinculin than those of “DERAA”-positive individuals (data not shown). It is hypothesized that the vinculin peptide is recognized as self in “DERAA”-positive individuals since the “DERAA”-containing HLA-DRB1 peptide is presented by another HLA molecule to the immune system, therefore resulting either in deletion of vinculin-reactive T cells or skewing of the cytokine profile to a suppressive profile without IFN γ .

Since it is not likely that IFN γ -producing T cells reactive against self-proteins are induced by such self-proteins, giving rise to autoimmunity, these vinculin-reactive T cells possibly result from e.g. an infection. Indeed, the phenomenon that molecular mimicry from a pathogen to a self-protein can lead to autoimmunity has been reviewed and observed several times (22-28). Many pathogens, such as Influenza, Measles, Bordetella Pertussis and Salmonella, can express proteins containing the “DERAA”-sequence. We therefore hypothesize that “DERAA”-directed T cells stimulated by a pathogen-derived “DERAA”-containing peptide accidentally can cross-react with human self-proteins, e.g. vinculin. Therefore, they aggravate an ongoing inflammation in the joints and play a role in the development of RA (Figure 1). These “DERAA”-(cross)reactive T cells only exist in “DERAA”-negative individuals (Figure 1A) in contrast to “DERAA”-positive individuals (Figure 1B) who are tolerant to the “DERAA”-sequence.

Since it was shown that the “DERAA”-containing peptide derived from the HLA-DRB1*0402 molecule can be presented by HLA-DQ8 (11), evidence supporting the hypothesis formulated above was obtained by analyzing T cell reactivity against the “DERAA”-containing peptide derived from Influenza A in DQ8-transgenic mice. Our preliminary data indicate that T cells specific for the “DERAA”-containing peptide derived from vinculin can be observed after immunization with this peptide, but not

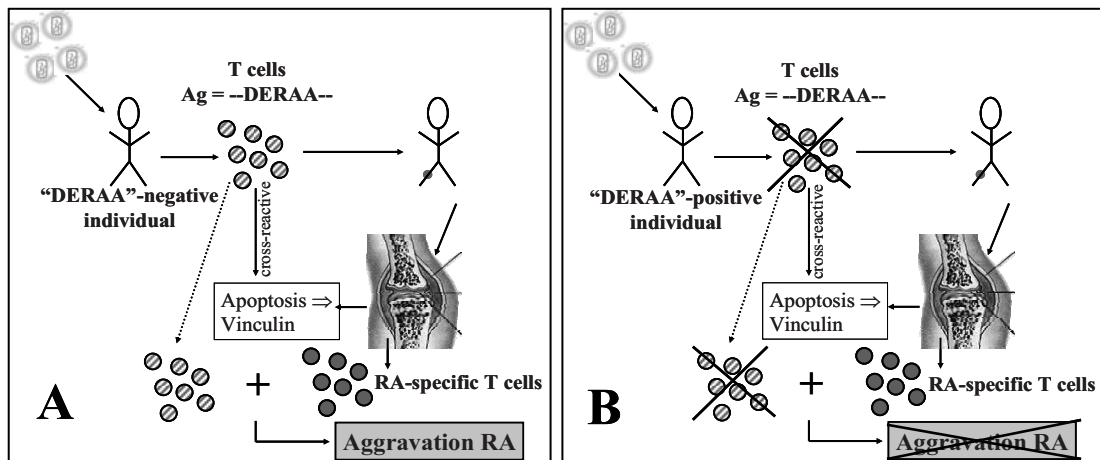


Figure 1. Proposed mechanism for the effect of "DERAA"-containing HLA-DRB1 molecules on RA. An individual is infected with a "DERAA"-containing pathogen. Depending on whether it is a "DERAA"-negative (A) or a "DERAA"-positive (B) individual T cells reactive with the "DERAA"-containing peptide processed from the pathogen are triggered or not. These T cells, which are only present in "DERAA"-negative individuals (A) can lead together with RA-specific T cells to the aggravation of the inflammation leading to the diagnosis of RA.

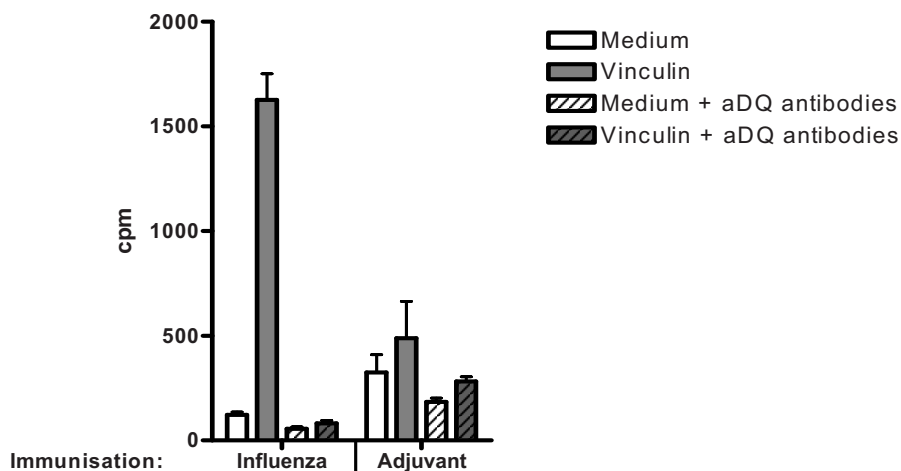


Figure 2. Crossreactivity to vinculin in DQ8-transgenic mice. Mice were immunized with the "DERAA"-containing peptide derived from Influenza (Influenza) or PBS in adjuvant (Adjuvant). Spleen cells were cultured for 4 weeks with the immunizing flu peptide and tested afterwards in a proliferation assay with 10,000 cells/well. No (open bars) or anti-HLA-DQ antibodies (hatched bars) were added to the wells without stimulation (white bars) or stimulated with the "DERAA"-containing peptide derived from vinculin (grey bars). Bars represent the mean amount of counts after addition of 3[H]-Thymidine overnight measured in triplicate with the SEM.

in mice immunized with a control peptide (data not shown). T cells triggered against the “DERAA”-peptide derived from Influenza A and cross-reactive with the vinculin-derived “DERAA”-peptide were HLA-DQ restricted (Figure 2, left part). These T cells are absent in mice immunized with adjuvant only (Figure 2, right). Together, these data suggest that T cells can show reactivity to the vinculin peptide after initial triggering against the “DERAA”-peptide derived from a pathogen, e.g. Influenza A.

When the decreased frequency of “DERAA”-containing HLA-DRB1 alleles in RA patients is studied in more detail, several articles showed that “DERAA”-containing HLA-DRB1 alleles protect against the development of ACPA⁺ RA (1;6). Since it has been shown that SE-containing HLA-DRB1 alleles associate with the production of ACPA and the so-called SE is located at the same position in the HLA-DRB1 molecule as the amino acids “DERAA”, this association has to be corrected for the presence of SE-containing HLA-DRB1 alleles. Lundstrom *et al.* showed that also after stratification for SE-containing HLA-DRB1 alleles, the frequency of HLA-DRB1*13 alleles (most frequent “DERAA”-containing HLA-DRB1 alleles) is significantly decreased in ACPA⁺ compared to ACPA⁻ RA patients (29), indicating that the “DERAA”-containing HLA-DRB1*13 alleles can protect against the development of ACPA. In a meta-analysis with RA patients and controls from four different countries (including our own EAC and BEST cohort) it was shown that HLA-DRB1*1301 alleles protect against the development of ACPA⁺ RA in contrast to ACPA⁻ RA after stratification for SE-containing HLA-DRB1 alleles. Since the HLA-DRB1*13 alleles account for 78-93% of the “DERAA”-containing HLA-DRB1 alleles present in the studied patients, the meta-analysis was still underpowered to prove or exclude HLA-DRB1*0103 and *0402 for the protective effect on ACPA⁺ RA (30). Therefore, future studies have to clarify which HLA-DRB1 alleles confer protection to ACPA.

1b. Maternal microchimerism as mechanism of the observed NIMA effect

When the protection induced by both inherited and non-inherited “DERAA”-containing HLA-DRB1 alleles on RA is working via molecular mimicry, it also could explain the mechanism of the NIMA effect.

In **Chapter 2 and 3** of this thesis we hypothesize that the observed NIMA effect of “DERAA”-containing HLA-DRB1 alleles is caused by maternal microchimerism. It has been shown in humans that there is long-term persistence of microchimerism (31) and that these microchimeric cells can differentiate into different tissue-specific cell types (32;33). It can be hypothesized that due to the presence of “DERAA”-containing

HLA-molecules on the surface of microchimeric maternal cells the child will recognize “DERAA”-containing antigens as self and therefore the observed T cell responses will resemble the reactivity of a “DERAA”-positive individual. This will result either in regulatory T cells or deletion of “DERAA”-reactive T cells, as occurs with the inherited effect of “DERAA”-containing HLA-DRB1 molecules.

It has been shown that *in utero* there is a much higher percentage of regulatory T cells in the fetus compared to after birth. T cell tolerance to alloantigens (e.g. NIMA) present *in utero* may, in some cases, be maintained after birth through the establishment of long-lived regulatory T cells (34). Following this scenario, the acquirement of “DERAA”-containing antigens *in utero* might result in the lifelong absence of pro-inflammatory “DERAA”-reactive T cells.

Another possibility is that the maternal microchimeric cells end up in the thymus serving as antigen presenting cell (APC), thereby inducing regulatory T cells or deletion of T cells, e.g. against “DERAA”-containing antigens. It is also hypothesized by Dutta and Burlingham that it is not the microchimeric APC themselves, but maternal antigen acquisition by host APC from these few microchimeric cells that drives the balance of T effector and regulatory T cells in favor of the latter (35).

1c. Effects of “DERAA”-containing HLA-DRB1 alleles in other diseases

Thus far we have focused on the association of “DERAA”-containing HLA-DRB1 alleles with RA. We wondered however whether the “DERAA”-containing HLA-DRB1 alleles might also be associated with other (immunological) disorders. Therefore a preliminary literature search was performed to analyze this. The most common “DERAA”-containing HLA-DRB1 alleles, HLA-DRB1*13 (from which approximately 97% will be “DERAA”-containing HLA-DRB1*13 alleles in the Caucasian population (36;37)), are associated with Hepatitis B (38-44) and –C infections (45-48), cervical cancer (49-54), HIV(55-57) and systemic lupus erythematosus (58-61). In all these different diseases, carriership of HLA-DRB1*13 protects either for the development of the disease or chronicity/ complications of the disease. Therefore, “DERAA”-associated protection does not seem to be specific for RA. It is unknown whether the “DERAA”-sequence of the HLA-DR13 molecules is directly involved in the observed protection. When future research indicates that these effects can be attributed to the presence of “DERAA” in the HLA-molecule, these observations can help to elucidate the mechanism by which these “DERAA”-containing HLA-DRB1 alleles can influence the immune response.

2. *PTPN22 and ACPA*

The most studied SNP of the *PTPN22* gene is the C1858T polymorphism. This single nucleotide change results in an amino acid substitution from Arginine to Tryptophan in the Lyp protein transcribed from the *PTPN22* gene. This substitution is located in a binding domain important for the function of Lyp, which acts as a Tyrosine phosphatase (62). The function of Lyp is the negative regulation of T cell receptor signaling, either direct or indirect, by influencing the phosphorylation status of different molecules involved in the signaling cascade that leads to T cell activation (63-69). The functional consequences of the amino acid change resulting from the C1858T polymorphism are not entirely clear as it has not only been shown that it leads to more T cell activation since there is less inhibition of the activation signal by Lyp (70), but also that cells from carriers of the T variant produce less cytokine (71). In mice it has been shown that a knock-out of *Pep*, the mouse ortholog of the Lyp protein, displays a hyperreactive T cell response (72). Lyp is expressed in different cell types and probably exerts different functions in e.g. T and B cells.

In **chapter 5** of this thesis, the predictive value of the C1858T polymorphism was studied next to ACPA status. Our study demonstrated an independent association of ACPA but not of the *PTPN22* C1858T polymorphism with progression to RA among patients presenting with UA, although the presence of this SNP is associated with an increased level of ACPA in ACPA⁺ patients. This finding was confirmed by another study (73). In many articles, it has been shown that the odds to be a carrier of the T variant of the C1858T polymorphism is about two times increased in ACPA⁺ compared to ACPA⁻ individuals, indicating that carriership of the T variant of the C1858T polymorphism is associated with ACPA production (74-77). The production of ACPA is also associated with the SE-containing HLA-DRB1 alleles. Intriguingly the association of *PTPN22* with RA is also present only in SE-positive individuals and not in SE-negative individuals (76), indicating that SE and the *PTPN22* allele are in the same biological pathway. As both genetic risk factors associate with ACPA⁺ disease, the contribution of *PTPN22* is probably found in setting the balance for ACPA production. It is therefore conceivable that *PTPN22* associates with ACPA production because it has a direct impact on the activity of the B cell receptor as it has been found that the T-variant of the C1858T polymorphism results in less B cell receptor signaling (78;79). More extensive studies have to show how these data relate to each other.

Thus, pathophysiologically both the SE-containing HLA-DRB1 alleles, ACPA and PTPN22 are players in the same pathway (see Figure 3), and by measuring ACPA the effect of the PTPN22 1858T-allele when present, is already included.

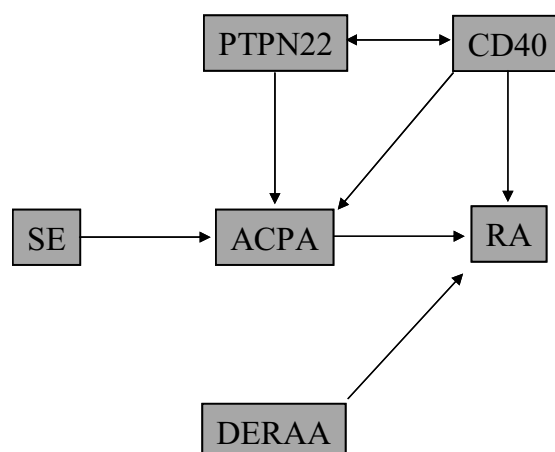


Figure 3. Schematic representation of the risk factors for RA that were studied and discussed in this thesis. The arrows indicate the direction of the putative interactions.

3. The role of CD40 on ACPA production and RA development

Recently, a SNP located in the intron of the gene encoding for CD40 was identified to associate with RA in a genome wide association study (GWAS) (80). The susceptibility allele of this SNP associates with less severity or progression of RA, as described in **chapter 6** of this thesis. CD40 is a well-known molecule expressed on B cells and other antigen presenting cells required for optimal cell activation by T cells. Its ligand, CD40L, is expressed on activated CD4⁺ T cells. Triggering of CD40 is involved in B cell proliferation, antibody production, class-switching and B cell memory formation. It is unknown what functional consequences the SNP has on the expression of CD40 protein, but the effect can be either on the B cell or other professional APC such as dendritic cells. Moreover, CD40 is also reported to be expressed on synovial fibroblasts.

Regarding the contribution of CD40 to the process of disease development of RA, it can either influence the production of ACPA and thereby the susceptibility and severity of RA or play an independent role on the pathogenesis of RA (Figure 3). There is only one article on the influence of CD40 on the production of ACPA. In this article it is

shown that B cells from the peripheral blood (both from RA patients and healthy controls), synovial fluid and bone marrow all start to produce ACPA in response to CD40 triggering. Although B cells from ACPA-positive individuals already produce ACPA without *in vitro* stimulation, the ACPA production is increased after CD40 stimulation (81). For rheumatoid factor (which are autoantibodies specific for the Fc portion of IgG) it has been shown that CD40 signaling plays a major role in the survival of rheumatoid factor producing B cells and therefore in the rheumatoid factor production (82).

CD40 signaling into the antigen presenting cell is mediated by the (de)phosphorylation of the Tyrosine kinases (83) on which Lyp also exerts its function. Therefore the CD40 signaling pathway and the effect of the PTPN22 SNP in B cells can probably influence each other, and it would be intriguing to know whether a similar relationship between CD40 and PTPN22 and/or SE-containing HLA-DRB1 alleles can be formed as described for the PTPN22-HLA-SE interaction.

On the T cell site CD40-CD40L interaction plays an important role in the amplification of the T cell response by a positive feedback loop for the production of co-stimulatory molecules on dendritic cells (84).

The role of CD40-CD40L interaction in arthritis has also been studied in different mouse models. It has been shown that blocking of the CD40L molecule results in prevention of collagen-induced arthritis (CIA), both measured in clinical scoring and in the absence of anti-collagen antibodies (85). The inhibition or prevention of arthritis in both the K/BxN model and DBA1 mice by prevention of the CD40-CD40L interaction has also been shown, but this does not affect established arthritis indicating that the CD40-CD40L interaction plays a role in the initiation rather than in the exacerbation phase of the arthritis (86-88).

CD40 is not only expressed on B cells but also on other antigen presenting cells. Therefore the influence of CD40 on RA may also be attributable to induction of fibroblast proliferation (89), the induction of TNF α production by synovial cells (90-92), chondrocytes (93) or osteoclasts (94).

A scheme showing the relationship of the risk factors studied in this thesis to RA is shown in figure 3.

4. Directions for further research

As already indicated in several parts of the discussion, future research has to be performed to elucidate the phenomena observed and studied in this thesis. Here I will indicate future directions that could be followed and that are not mentioned in the previous sections.

“DERAA”-containing HLA-DRB1 alleles protect against the development and severity of RA, both when they are inherited and when they are acquired as a NIMA. We observed that the effect of the “DERAA”-containing HLA-DRB1 alleles is of a similar strength when they are inherited compared to acquired as a NIMA. The underlying mechanism of the effect of “DERAA”-containing HLA-DRB1 alleles has been discussed in the previous sections but certainly is not proven up to now. The T cell reactivity observed against the peptide derived from the human cytoskeletal protein vinculin needs to be shown also for naturally processed peptides from the whole protein. The possibility of cross-reactivity of T cells triggered against a pathogen-derived peptide containing the “DERAA”-sequence, i.e. from Influenza A, with the “DERAA”-containing peptide derived from vinculin is demonstrated in DQ8-transgenic mice. It is important to know whether “DERAA”-specific T cells are triggered when a mouse or individual is infected with a “DERAA”-containing strain of the Influenza A virus. Furthermore, extensive studies for cross-reactivity of other pathogen-derived “DERAA”-containing peptides with the vinculin-peptide have to be performed both in mice and in humans. An extensive meta-analysis for the effect of “DERAA”-containing HLA-DRB1 alleles in other diseases and infections can probably help to elucidate the underlying mechanism.

An extensive family study in which individuals from three generations can be studied possibly will give more insight in the mechanism underlying the observed effect of “DERAA”-containing HLA-DRB1 alleles as a NIMA. It is important to know in which immunological status the acquirement of “DERAA”-containing antigens can still lead to a protective effect; is it necessary to acquire this NIMA in a fetal stage or is induction of the effect still possible when acquired in adulthood? The latter would have implications to treat “DERAA”-negative individuals with “DERAA”-containing molecules (e.g. by means of transfusing cells expressing the “DERAA”-containing HLA-DRB1 molecules) to induce protection against the development of RA in individuals who are at risk or ameliorate existing disease in “DERAA”-negative patients.

When more is known about the fine-specificity of ACPA, the interplay between T cells and their help to ACPA-producing B cells can be studied in more detail. This both accounts for “citrulline”-specific T cells and for T cells specific for a connected protein (e.g. vinculin) helping an ACPA-producing B cell. Since these studies rely on a delicate choice of at least HLA-type, ACPA-status and ACPA-specificity, large cohorts are necessary to perform these studies.

Both for the C1858T polymorphism of the PTPN22 gene and the CD40 SNP, functional studies are necessary to study what the precise effects are of the nucleotide change and whether the already studied SNPs are the most informative or whether they are in linkage disequilibrium with another SNP that is the causative SNP for the functional effect. After this, the cell type important for the observed effect has to be defined.

In conclusion, several different aspects playing a role in the pathogenesis of RA were studied in this thesis. Answers were found, opening new perspectives for further research, but also raising many new questions, waiting to be answered.

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