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**THE MHC AND THE RECOGNITION OF SELF AND ALTERED
SELF IN EXPERIMENTAL AND RHEUMATOID ARTHRITIS**

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“There can be no contentment but in proceeding”

Thomas Hobbes *Human Nature* VII.7

ABSTRACT

The Major histocompatibility complex (MHC) is a highly polymorphic and gene-dense region on chromosome 6 in humans and 20 in the rat. Genes in the MHC are the major risk factor for the development of autoimmunity. Rheumatoid arthritis (RA) is a common autoimmune disease with a strong association to a specific subset of *HLA-DRB1* alleles, which encode a shared amino acid motif in the HLA-DR β chain. This *shared epitope* (SE) contributes to the formation of the P4 pocket in the peptide-binding groove of the HLA-DR complex. Recently, two weaker associations to the MHC class I gene *HLA-B* and to a second MHC class II gene, *HLA-DPBI*, have been identified. Whereas the dissection of the genetic association between autoimmunity and the MHC has progressed rapidly in the last decade, the functional role of the MHC genes in the development of autoimmunity has not yet been resolved to the same extent. The work presented in this thesis represents some of our efforts to contribute to this understanding.

Paper I describes our attempts to induce arthritis with α -enolase and citrullinated α -enolase in HLA-DR4 transgenic mice. Based on the strong genetic association between antibodies to citrullinated α -enolase peptide 1 (CEP-1) and *HLA-DRB1* SE alleles, we aimed to identify if anti-CEP-1 antibodies can be induced in mice transgenic for a human *HLA-DRB1* SE allele, and if this would depend on a citrullinated *HLA-DRB1*0401* restricted T cell epitope. We could neither prove that native or citrullinated α -enolase induced arthritis nor did we observe a citrulline specific B or T cell response.

In *Paper II* we investigated the relevance and the extent of joint-directed anti-citrulline immunity in RA. We identified two citrullinated antibody epitopes on type II collagen (CII) present in 17% and 21% of RA patients. Anti-citrullinated CII reactivity partly overlapped with the reactivity towards CEP-1, however only antibodies directed to citrullinated CII bound to RA cartilage specimens. This suggests that antibodies directed to citrullinated CII may contribute to the inflammatory process in the joints.

Paper III-V summarizes our work concerning the influence of allelic variation in the rat MHC on T cell selection, MHC expression and susceptibility to autoimmune arthritis. Paper III introduces our panel of intra-MHC congenic strains and demonstrates that an interaction between the *RT1-A* genes in the MHC class I and *Tap2* in the MHC class II region regulates the negative selection of CD8 T cells. The *RT1-A* genes are part of a haplotype designated *T cell selection QTL-1* (*Tcs1*), whereas *Tap2* is in linkage disequilibrium with the MHC class II genes, in the locus designated *T cell selection QTL-2* (*Tcs2*).

In *Paper IV* we evaluated the impact of these two QTL on the regulation of Pristane-induced arthritis (PIA). Allelic variation in *Tcs1* did not influence PIA, whereas *Tcs2* regulated the onset and the severity of PIA. A comparison of the amino acid polymorphisms between the haplotypes as well as functional studies suggested a major contribution of the HLA-DQ homolog RT1-B to the development of PIA.

It has earlier been demonstrated that pristane-primed CD4 T cells transfer arthritis in naive recipients. To investigate how the MHC-II haplotype affects T cell priming and the subsequent PIA development, we characterized the T cell compartment after pristane administration. The frequency of Th1 cells correlated with an early onset of PIA and reduced arthritis severity in one haplotype was associated with a high ratio of T regulatory to T effector cells. These results are presented in *Paper V*.

LIST OF PUBLICATIONS

- I. *Human α -enolase is immunogenic, but not arthritogenic, in HLA-DR4-transgenic mice: Comment on the article by Kinloch et al.*
Sabrina Haag, Hüseyin Uysal, Johan Bäcklund, Jonatan Tuncel, & Rikard Holmdahl (2012). *Arthritis and Rheumatism*, 64(5), 1689–1691.
- II. *Mass spectrometric analysis of citrullinated type II collagen reveals new citrulline-specific autoantibodies, which bind to human arthritic cartilage*
Sabrina Haag, Nadine Schneider, Daniel E. Mason, Jonatan Tuncel, Ida E. Andersson, Eric C. Peters, Harald Burkhardt, & Rikard Holmdahl (2014). *Arthritis & Rheumatology (Hoboken, N.J.)*.
- III. *Natural Polymorphisms in Tap2 Influence Negative Selection and CD4 : CD8 Lineage Commitment in the Rat*
Jonatan Tuncel, **Sabrina Haag**, Anthony C. Y. Yau, Ulrika Norin, Amelie Baud, Erik Lönnblom, Klio Maratou, A Jimmy Ytterberg, Diana Ekman, Soley Thordardottir, Martina Johannesson, Alan Gillet, EURATRANS Consortium, Pernilla Stridh, Maja Jagodic, Tomas Olsson, Alberto Fernandez-Teruel, Roman A. Zubarev, Richard Mott, Timothy J Aitman, Jonathan Flint, & Rikard Holmdahl (2014). *PLoS Genetics*, 10(2), e1004151.
- IV. *A 0.2 Mb interval in within the MHC-II region controls onset and severity of pristane-induced arthritis in the rat*
Sabrina Haag^{*}, Jonatan Tuncel^{*}, Soley Thordardottir, Daniel E. Mason, Anthony C. Y. Yau, Doreen Dobritzsch, Eric C. Peters, & Rikard Holmdahl (2014).
Manuscript
- V. *T cell priming and Th lineage commitment in MHC class II congenic rats upon immunization with the hydrocarbon pristane*
Sabrina Haag^{*}, Jonatan Tuncel^{*}, & Rikard Holmdahl (2014).
Manuscript

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LIST OF ABBREVIATIONS

ACPA	Anti-citrullinated protein antibodies
AS	Ankylosing spondylitis
CII	Collagen type II
CCP	Cyclic citrullinated peptide
CD	Cluster of differentiation
CEP-1	Citrullinated α -enolase peptide 1
GWAS	Genome wide association study
HLA	Human leukocyte antigen
IFN- γ	Interferon- γ
IgG	Immunoglobulin G
IL	Interleukin
IHWS	International Histocompatibility Workshop
MHC	Major histocompatibility complex
MS	Multiple sclerosis
PAD	Peptidyl arginine deiminase
PIA	Pristane-induced arthritis
QTL	Quantitative trait locus
RA	Rheumatoid arthritis
RCS	Recombinant congenic strain
LD	Linkage disequilibrium
SE	Shared epitope
SNP	Single nucleotide polymorphism
Tcs	T cell selection QTL
Th	T helper (cell)
Treg	Regulatory T (cell)

1 A SHORT AND INCOMPLETE HISTORY OF SOME OF THE MOST FUNDAMENTAL DISCOVERIES IN IMMUNOLOGY

1.1 THE MAJOR HISTOCOMPATIBILITY COMPLEX

“It is clear that detailed knowledge of the system will accumulate slowly and may never reach completion” P. A. Gorer

1.1.1 The mouse MHC

The major histocompatibility complex (MHC) is a highly polymorphic and gene dense region on chromosome 6 in humans, on chromosome 17 in mice and on chromosome 20 in the rat. The identification of the MHC is based on early tumour transplantation studies in mice, which suggested that susceptibility or resistance to allogeneic tumours is genetically determined (1). The British physician and pathologist Peter A. Gorer was the first to describe, in 1936, inherited antigenic differences between three inbred mouse strains. He identified three antigens (*antigen I*, *antigen II*, and *antigen III*) with different expression levels between the three mouse strains; by grafting a tumour from the mouse strain carrying *antigen II* into mice lacking *antigen II* he could show that the tumour was rejected, while it grew well in the strain positive for *antigen II* (2, 3). Gorer later visited George D. Snell, a geneticist at the Jackson Laboratory, Bar Harbor, ME, who had shown that a tumour resistance gene, which he called *histocompatibility* or *H* gene was in strong linkage with a gene causing deformed tails in mice (*Fu*). With Gorer's antiserum against *antigen II* they could identify the locus encoding *antigen II* (4), which was consequently named *histocompatibility locus 2* or *H-2*. Based on the strength of its antigenicity, compared to other histocompatibility loci, the *H-2 locus* became the *major histocompatibility locus*. With the identification of the *D* and *K locus*, the two MHC class Ia loci, which are separated by the MHC class II and MHC class III loci in the mouse, within the *H2-locus*, the *H-2 locus* became the *H-2 complex* or the *major histocompatibility complex* (5).

1.1.2 The human MHC

The human MHC was discovered two decades after the H-2 complex, however the research developed in parallel and the discovery of the human MHC was not due to a direct search of the mouse equivalent. In both cases the identification of the first histocompatibility antigen in mouse and in human was based on serology. The first human histocompatibility antigen was described in 1958 by Jean Dausset and termed *MAC* according to the initials of the three key patients in his study. He showed that serum from patients that had received several blood transfusions, would react with other people's leucocytes, which causes them to clump (6). On the first International Histocompatibility Workshop (IHWS) (1964) researchers discussed the best techniques to identify HLA specificities. The results from 14 independent laboratories were reported on the second IHCW (1965), five groups came to the same classification of cells based on their leucocyte antigen specificity. The antigen, which was first described

as MAC by Dausset, was also identified by Payne as *LA2* (7) and van Rood as *8a* using leukoagglutination tests (8), by Dausset as *Da1* (9) and Terasaki as *Te2* (10) using lymphocyte cytotoxicity tests and by Shulman as *B1* (11) using complement fixation tests. On the first WHO nomenclature meeting 1968 the original identified MAC became HLA-A2, partly based on Payne and Bodmer's name *LA2* and Dausset's proposal "*Hu*". Payne and Bodmer had also identified the antigen *LA1* (7) – that is why the first identified antigen took the second place in the new nomenclature. The aim of the third IHCW (1967), organized by the Italian geneticist Ruggero Cepellini, was to study the genetics of the recently identified leucocyte antigens. The suggestion that a single chromosomal locus controls the identified antigens, which was based on data from Dausset et al. and van Rood et al. (8), was with the typing of blood from 11 families, including monozygotic twins, with antisera from the different laboratories, during the third IHCW fully established. The region, encoding these closely linked leucocyte antigens, was therefore designated *human leucocyte, locus A (HL-A)* and later changed to *human leucocyte antigen (HLA)* (5). The HLA antigens should later be divided in two classes, which originated from the observation that one class of antigens were expressed on virtually all nucleated cells, whereas the other class was present only on B cells, monocytes and dendritic cells. The designation of A, B, and C antigens as MHC class I and DR, DQ and DP as MHC class II was introduced by Jan Klein 1977 (12). Ceppellini had already 1967 introduced the term MHC haplotype to describe the genetic information of the two genetic loci.

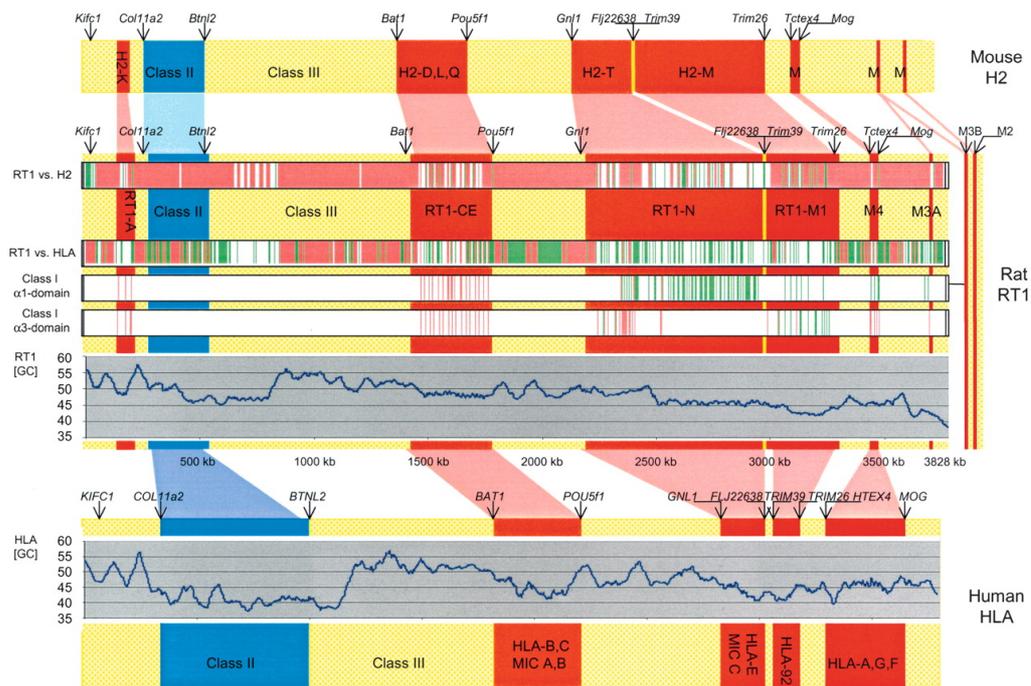


Figure 1. The mouse MHC in comparison to the rat and human MHC. Original illustration from Hurt, P. (2004). The Genomic Sequence and Comparative Analysis of the Rat Major Histocompatibility Complex. *Genome Research*, 14(4), 631–639. doi:10.1101/gr.1987704. The H-2-K and H-2-D are the two classical MHC class I loci in the mouse, the loci are separated by the MHC class II and MHC class III loci. In the human MHC the HLA-A and HLA-B loci are separated from the third MHC class I locus HLA-A. However in contrast to mouse and rat all MHC class I genes are located on one side of the MHC class III region. In the rat the classical MHC class I locus is located next to the MHC class II locus, the RT1-CE region only encodes non-classical MHC class I molecules.

1.1.3 The rat MHC

The work presented in this thesis is to a large extent based on the rat MHC. I will therefore revisit also the early work that led to the identification of the first rat histocompatibility antigens. The finding that the mouse H2-locus contained two independently segregating loci, *K* and *D*, had been first suggested in 1970 by Thorsby (13). For the HLA a two loci-model had already been suggested in 1966 (14) and had been proven shortly after (15). In the rat the first description of a histocompatibility locus already suggested the existence of two linked loci. By grafting skin from two different rat strains (Lewis and BN) on F2 hybrids R. E. Billingham et al. (1962) proposed a total of 14-16 independently segregating histocompatibility loci of which only 3-4 were classified as strong antigens (16). Joy Palm described in 1964 (17), utilizing serological typing of some of Billingham's F2 hybrids, that two earlier identified histocompatibility genes, *antigen 1* and *antigen 3*, were encoded by an "important histocompatibility locus" and that the two genes encoding *antigen 1* and *antigen 3* were in "the same chromosome linkage group" as another histocompatibility antigen *B* (18). Palm noted in the discussion of the aforementioned article that "the rat histocompatibility locus" identified in this study had striking similarity to the H-2 locus in the mouse. In a subsequent publication Elkins and Palm demonstrated that a single locus, the locus encoding the allelic variants *antigen 1* (Lewis), *antigen 3* (BN) and *antigen 5* (DA), elicits graft-versus-host reaction (GVHR), she termed this locus "Ag-B" (19).

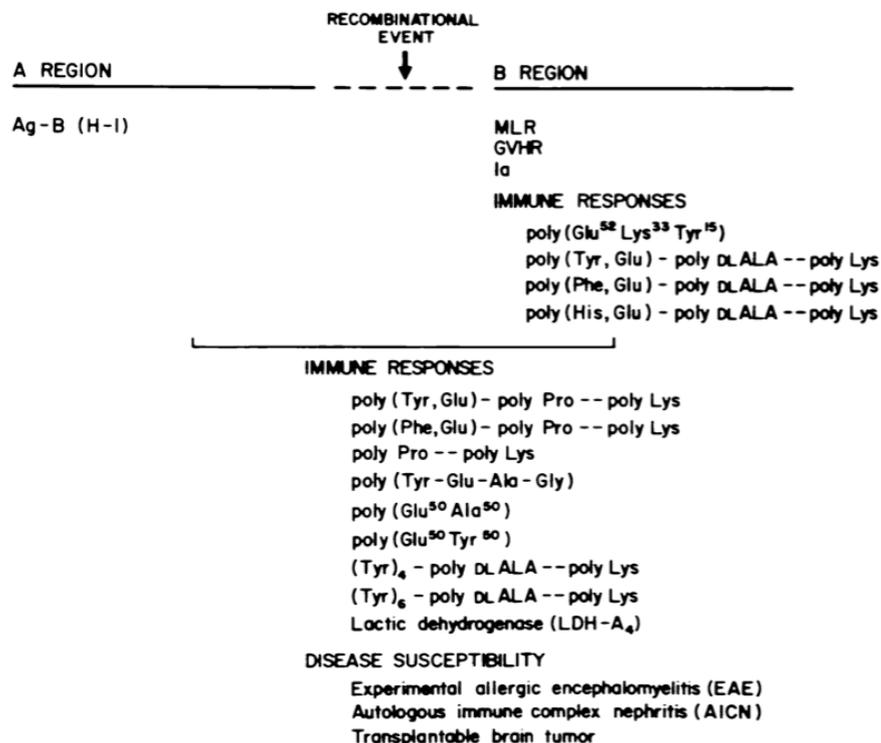


Figure 2. The rat MHC (*Ag-B*) as illustrated by Gill et al. 1978 in "The major histocompatibility complex--comparison in the mouse, man, and the rat. A review." *The American Journal of Pathology*, 90(3), 737. The *Ag-B* locus was based on recombinations observed in the MHC and the subsequent analysis of the recombinant strains divided in two sub-regions, denoted A and B. The A region was defined by the serologically defined histocompatibility antigens, whereas the B region controlled mixed-lymphocyte reactions and GVHR. The association between the B Region and the susceptibility to disease, such as experimental allergic encephalomyelitis, had not yet been established (20).

Bodgen and Aptekman had already earlier suggested the *R-1 hemagglutinin* (*B*) as a major histocompatibility antigen and designated the locus encoding this antigen as *R-1* (18). In addition to the antigens described by Bogden and Aptekman (*B-1, B-2, G*) and Palm (*antigen 1, 2, 3*) four hemagglutination antigens (*C, D, E, F*) had already been distinguished by Ray D. Owen 1948 (21). Furthermore, Stark et al. elaborated considerably on the serological and allelic complexity of histocompatibility antigens in the rat - and they also introduced their own *Rt H-1* terminology (22-24). The “*problem in nomenclature*” communicated by Joy Palm 1970 (24) could obviously not be resolved until 1978. Whereas Gill et al. (20) still referred to different nomenclatures in their review of the rat MHC in mouse, man and rat (Figure 2); a review by Dietrich Götze published in the same year was entitled “*The major histocompatibility complex of the rat, RT1*” (25) – this designation is valid until today (Figure 1).

1.2 SELF AND NON-SELF

“It remains to be seen whether this concept is of value” F. M. Burnet

The MHC was discovered based on the rejection of a tumour from one inbred mouse strain to another inbred mouse strain. Gorer had also already shown that the rejection of a tumour is accompanied by the development of antibodies against the not-shared antigen between tumour and host (26). The importance of his discoveries, however, are probably better illustrated by the difficulties of medical transplantation, which Peter B. Medawar devoted his research to after experiencing the horrific burn wounds of a pilot who crashed 1940 with his plane close to Medawar’s garden (27).

In the years after this plane crash Medawar worked together with the Scottish surgeon Thomas Gibson. They grafted skin transplants, on burn wounds of their first patient, either from the patient herself or from the patient’s brother. By following the wound healing with the two different transplants macro- and microscopically they identified that the skin from the brother, but not her own grafts were infiltrated by immune cells (28). To prove that the invading cells were actually the cause of the destruction (rejection) of a graft from a genetically different donor Medawar reproduced the observation from his first patient with a large number of transplantation experiments in rabbits (29, 30). Medawar received the Nobel Prize 1960 for his finding that immune cells cause the rejection of a graft and the discovery of a way to avoid incompatibility of grafts. Medawar’s discovery, how to circumvent rejection, published 1953 in *Nature*, was founded on earlier work by Ray D. Owen. Owen, as aforementioned, was one of the first to describe rat histocompatibility antigens, or blood groups in the rat. Owen stated in a review of his early work, that the findings in the rat were primarily driven to mimic natural parabiosis of fetal twin cattle by experimental parabiosis in the rat (31). The demonstration that even genetically not identical cattle twins share blood cells (32), was the work, which inspired Medawar, Rupert E. Billingham and Leslie B. Brent to experimentally prove that tolerance to a transplant can be acquired if tissue is shared as foetus (33). Noteworthy, Billingham should later after taking a position at the Wistar Institute in Philadelphia estimate the total number of histocompatibility loci in the rat.

At the time Medawar proofed acquired tolerance the rejection of grafts had not yet been linked to the major histocompatibility locus; the first histocompatibility antigen in man was described 1958 by Dausset (see above). It was the group of Paul L. Terasaki who showed first, 1969, that the transplantation of grafts between HLA matched sibling vs. non-matched was advantageous for the survival of the graft (34). Interestingly, Gorer, who died 1961, had already linked the rejection of tumours in incompatible mice to the H-2 locus, postulated that the same rules apply for the rejection of transplants (35). The idea to graft tissue from one human to the other dates back to a contemporary of Buddha (36), however, the need for transplants, in particular for skin transplants, in the 1940's was made acute by the war. Whereas, most doctors ascribed the rejection of grafts to surgical and not biological problems, the observation that own skin grafted better than skin from another person, raised the question why and how our body discriminates between what's self and what's not self.

Peter Medawar won the Nobel Prize for the discovery of "*acquired immunological tolerance*" together with the Australian Frank Macfarlane Burnet. Burnet together with Frank Fenner established 1949 the concept that our immune system discriminates self from non-self (37). Their hypothesis was built on the discovery from Owen, that there is an exchange of erythrocytes between non-monozygotic cattle twins due to shared vascularisation, presumably of the placenta (32). Burnet speculated that the twins in Owen's study acquired tolerance for each other since they were exposed to each other's cells as foetus and he expanded this hypothesis also to the human immune system (37). Burnet described his contribution to the discovery, which earned him the Nobel Prize "a very minor one – it was the formulation of an hypothesis that called for experiment" (38). And indeed, Burnet's and Fenner's publication from 1949 made Medawar aware of Owen's finding and spurred the experiments of Medawar and his colleagues, which proved the concept of "*immunological tolerance*" (39).

1.3 THE THYMUS

"*This would suggest that lymphocytes leaving the thymus are specially selected cells*" J. F. A. P. Miller

The concept of *acquired tolerance* implied that our immune system has to learn to not react against self. Today, we are associating this function instantly with the thymus. At the beginning of the 1960s, however, the thymus was the last organ of which the function was not yet known and Medawar considered "*the presence of lymphocytes in the thymus as an evolutionary accident of no very great significance*" (40). This judgement was based on observations, such as that the thymus contained large number of dead cells, that thymectomy did not impair immune responses, and that no germinal center and antibody formation had been observed after antigen exposure (40, 41). It was Jacques F. A. P. Miller, who revealed the immunological function of the thymus and proposed that "*the thymus at birth may be essential to life*" (40). He described that mice inoculated with a leukaemia-tissue filtrate would not develop lymphomas, when their thymus was removed after weaning (42). However, if a thymus would be transplanted to such mice, infected at birth with virus and thymectomised with four

weeks of age, these mice would subsequently develop lymphomas (43). Miller next performed skin transplantation experiments and demonstrated that mice thymectomized at birth did not reject skin grafts from another mouse strain (44). The thymectomized mice were further devoid of lymphocytes in the circulation (Figure 3), as well as in the lymphoid tissue (43). It was thereby established that the thymus is the origin of lymphocytes, and that these lymphocytes are involved in the rejection of non-self.

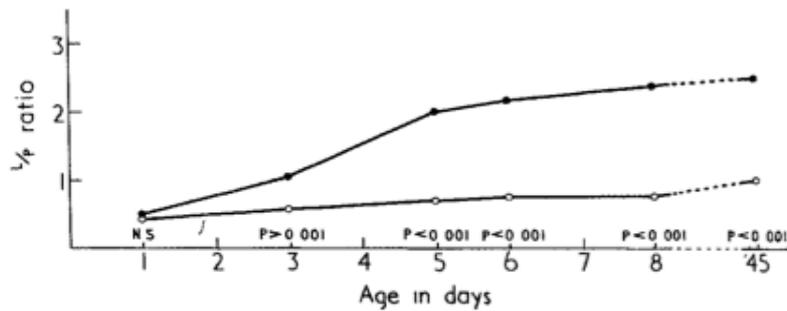


Fig. 1—Average lymphocyte:polymorph ratio of mice thymectomized in the neonatal period compared with sham-thymectomized controls. Statistical differences indicated.

○—○ thymectomized mice.
●—● sham-thymectomized mice.

Figure 3. The ratio of lymphocytes vs. polymorphic cells in the blood of sham-operated and thymectomized mice, which demonstrated the thymus as the origin of peripheral lymphocytes. Original illustration from Miller, J. F. A. P. (1961). Immunological function of the thymus. *The Lancet*, 748–749.

Miller's following experiment should be the crucial one, which linked the concept of *acquired tolerance* to the thymus. By transplanting thymic tissue from either syngenic or allogenic mice in thymectomized recipients, he could show that the recipients only accepted grafts if the skin and the thymus transplant were derived from the same mouse strain. He concluded from these experiments that the mice, which received a thymus transplant, were not deficient of lymphocytes, but tolerant to self (45). He already proposed in his publication what should later be referred to as *negative selection*; that tolerance must be related to a deletion or inactivation of self-reactive lymphocytes in the early stages of the thymus development.

In contrast to Medawar this concept found major support from Burnet, who stated 1962 in the *British Medical Journal* "It now appears likely that homeostatic control persist into independent life and everything points toward the vital concern of the thymus in the process." (41). He was referring back to Medawar's work, which ascribed the period when tolerance could be acquired to the foetal stage. He further supported Miller's definition of what he had termed "*selective immunological thymectomy*": the thymus is the origin of lymphocytes, from which they are released in the periphery after maturation and elimination of self-reactive lymphocytes (40, 41). Michael J. Bevan was the first to experimentally demonstrate, 1977, that there is a selection process of lymphocytes in the thymus. Experiments in bone marrow chimeras of two strains with different MHC haplotypes showed that MHC-restricted T cell responses are the consequence of *positive selection* of lymphocytes on radio-resistant cells in the thymus (46). That lymphocytes are in fact selectively eliminated before they are released to the

periphery, as suggested by Miller and Burnet at the beginning of the 1960s, was established as *negative selection* by John W. Kappler and Philippa Marrack 1987 (47).

1.4 THE ANTIBODY-PRODUCING CELL

“Functionally, we see this vertical division as immunological recognition and information on one hand, and specific antibody production on the other.” M. D. Cooper et al.

Shortly after Miller identified the thymus as the origin of small lymphocytes Max D. Cooper delineated, 1966, a *thymus-dependent tissue*, the white pulp, and a *bursa-dependent system* including lymphoid follicles and the plasma cells in the spleen of chickens (Figure 4). The *bursa-dependent lymphoid system* was functionally described as the immunoglobulin-producing system, whereas the *thymus-dependent lymphoid system* was linked to delayed hypersensitivity and graft-versus-host reactions and graft rejection (48).

1958, it had been established by Gustav V. J. Nossal and Joshua Lederberg that the magnitude of antibodies with specificity to self and non-self are produced upon encounter of a particular antigen by a single *antibody-producing cell* (49). This confirmed Burnet’s *clonal selection* theory and influenced immunologic research throughout the 1960s (50). With the discovery of the functional relevance of the thymus, it had also been described that the neonatal thymectomy of mice reduced antibody production towards certain antigens (45, 51). It was therefore not anticipated that the lymphocytes leaving the thymus are not the same as the cells producing antibodies. However, in the same year as Cooper published the thymus- and bursa-differentiation of the immune system in the chicken it was shown in the mouse, that thymocytes would not re-populate all areas in lymph nodes and the spleen of neonatally thymectomized mice (52). The areas restored by thymocyte transfer were therefore termed *thymus-dependent areas* (52).

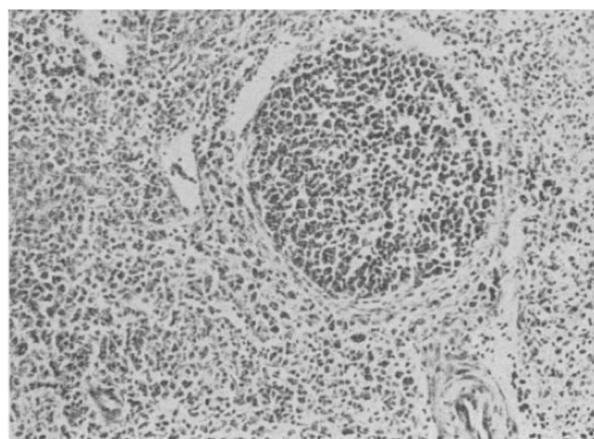


Fig. 3. Bursa-dependent follicle in the spleen showing the pyroninophilic cells (methyl green-pyronin; $\times c. 166$)

Figure 4. Lymphoid follicles dependent on the bursa of Fabricius in the chicken spleen. Original figure published by Cooper, M. D., Peterson, R., & Good, R. A. (1965). Delineation of the thymic and bursal lymphoid systems in the chicken. *Nature*.

Cooper concluded in his study revealing the function of the thymus- and bursa-dependent systems that the thymus has the same functional role in mammals and birds and thus suggested that a similar *morphologic and functional dissociation* must also exist in mammals (48). It was established 1968 by Miller and G. F. Mitchell, that thymus derived cells do not develop to antibody producing cells, but are important to initiate the differentiation of bone-marrow derived precursors to *antibody-producing cells* (53). These thymus- and bone marrow-derived cells, should later been termed T and B cells (54), respectively. The division of the adaptive immune system into two must have at least doubled the complexity of the still unresolved issue how our immune system distinguishes between self and non-self. I will focus in the following paragraphs only on the capacity of T cells to do so, and try to recapitulate the role of the MHC in this mechanism.

1.5 ALTERED SELF

“A central function of the major histocompatibility (H) antigens may be to signal changes in self to the immune system” P. C. Doherty & R. M. Zinkernagel

It was the work of Rolf M. Zinkernagel and Peter C. Doherty, which demonstrated that the histocompatibility genes control the immune response to pathogens (55, 56) – and have not just evolved to challenge transplant surgeons (57). Before Doherty and Zinkernagel’s discovery the MHC genes were recognized for their importance in transplantation. However, even though Miller had already described the thymus as the origin of the lymphocytes responsible for the rejection of transplants and David H. Katz and Baruj Benacerraf that the interaction between T and B cells is controlled by the H-2 complex (54) and more precisely by the *immune response (Ir)* genes (58), which should later be identified to be the MHC class II genes, the biological role of the MHC genes was not yet known (Figure 5).

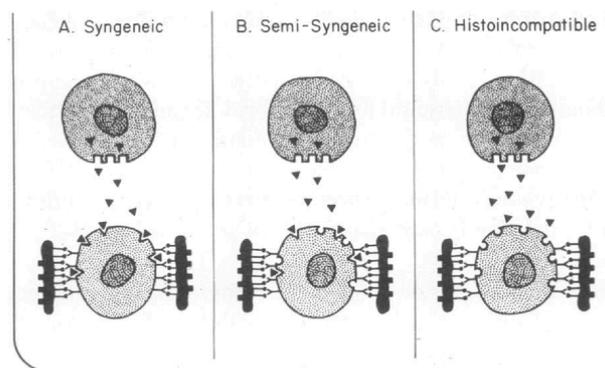


FIG. 5. Genetic requirements for physiologic cooperative interactions between T and B lymphocytes. Upper cell is the T lymphocyte, while lower cell is the B lymphocyte in all cases.

Figure 5. A schematic illustration of the interaction between T and B cells suggested 1973 by Katz, D. H., Hamaoka, T., Dorf, M. E., & Benacerraf, B. (1973). Cell interactions between histoincompatible T and B lymphocytes. The H-2 gene complex determines successful physiologic lymphocyte interactions. *Proceedings of the National Academy of Sciences of the United States of America*, 70(9), 2624–2628. In the case of syngenic or semi-syngenic B and T cells “the B cell “acceptor site” can recognize and bind the T cell product”. According to this model histoincompatible B cells lack the “acceptor site” and therefore do not respond to the T cell-derived antigen.

Zinkernagel and Doherty shortly after, 1974, clarify the role of the MHC in eliciting a T cell response. By co-culturing virus-infected macrophages with splenocytes from mice inoculated with the same virus they first identified that the anti-viral response is restricted to the MHC, and that lymphocyte and target tissue have to be close to each other to elicit an effector function (55). Based on these observations they built two hypotheses to explain the observed *MHC restriction* (59) (Figure 6).

The *intimacy model* suggested that the MHC genes are encoded on both T cell and target cell and that they have to interact with each other in addition to the interaction of a virus-specific receptor on the T cell and the viral antigen on the target cell. In contrast, the *altered-self model* proposed that the virus is not altering the target cell, but the histocompatibility antigen itself; since their experiment was done in heterozygous H-2^{k/b} mice this hypothesis also required that there are two distinct clones of T cells recognizing the viral-modified H-2^k and H-2^b molecule (60). That the recognition of *altered self* is mediated by a *single* receptor, and not two independent T cell receptors for antigen and MHC was suggested in 1984 by the discovery of the genes encoding the mouse (61) and the human T cell-receptor (62) by Mark M. Davis and Tak W. Mak, respectively. It was not before 1987 that the *altered self*-model found its major support in the electron density map of the HLA-A2 crystal (63).

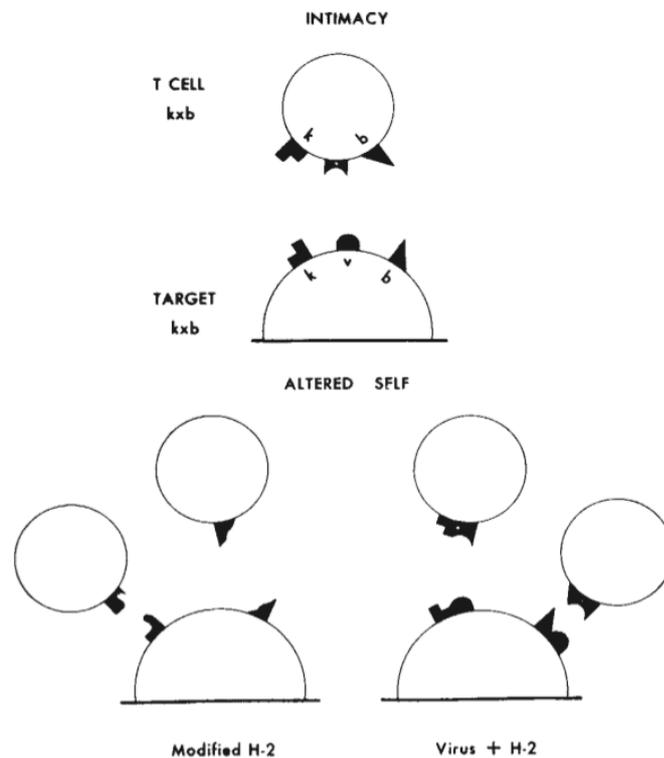


Figure 6. Doherty's and Zinkernagel's *intimacy* and *altered self* model as proposed in Zinkernagel, R. M., & Doherty, P. C. (1974). Immunological surveillance against altered self components by sensitised T lymphocytes in lymphocytic choriomeningitis. *Nature*, 251, 547–548. The intimacy model would depend on one T cell, specific for the viral antigen, whereas the altered self model would require at least two T cell clones, specific for the altered H-2 of the k and b haplotype in F1 mice.

The original definition of *altered self* by Zinkernagel and Doherty described that the MHC molecule is altered by an interaction of a viral antigen with the H-2 protein or by the structures coding for the MHC molecules. The first mechanism was later shown to be correct, however, the term has further evolved, and, as used in the title of this thesis, also refers to the modification of a self-peptide, which has been associated to allergy and autoimmunity (64).

1.6 THE POCKET

“The head of a moose (a large North American or Siberian elk)” P. Parham

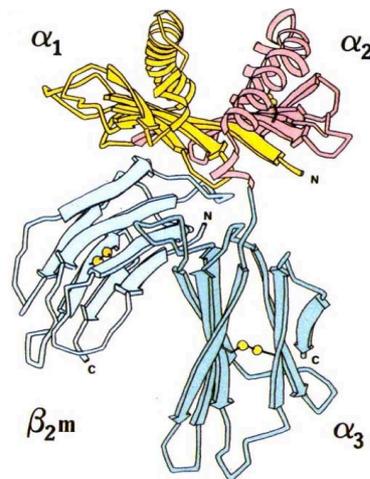


Figure 7. Original schematic representation of the crystal structure of HLA-A2 published by Bjorkman, P. J., Saper, M. A., Samraoui, B., Bennett, W. S., Strominger, J. L., & Wiley, D. C. (1987). Structure of the human class I histocompatibility antigen, HLA-A2. *Nature*, 329(6139), 506–512. The two α -helices of the α_1 and α_2 domains form the peptide-binding groove.

Supporting data for the concept that protein fragments (peptides) alter MHC molecules, which subsequently induce MHC-restricted T cell response had been obtained by many researchers in the beginning of the 1980s (65-67). Alain Townsend, for instance showed 1985, that fragments of the influenza nucleoprotein (68), and shortly after 1986, that indeed synthetic peptides are sufficient to trigger a cytotoxic T cell response (69).

However, as Pamela J. Björkman described in an essay looking back at her long endeavour crystallizing the first MHC molecule, for a structural biologist it was difficult to imagine that on protein should bind many different antigens. It was further not yet established that MHC molecules do present self and not only foreign antigens; since no foreign antigen was added to their purified HLA molecules they did not expect to identify a protein complex. Björkman even said that having been aware of such a heterogeneity 1979 could have been already the end of the attempts to obtain the structure (63). The structure of HLA-A2 with an “*unknown antigen*” in a groove between two α -helices was published 1987 in *Nature* (70). The first identified human MHC molecule, “MAC”, which became HLA-A2 after the reformation of the HLA nomenclature 1968, was with that the first crystallized MHC molecule. This might not

be pure coincidence, since HLA-A2 is the most common of the HLA-A antigens, carried by approximately 50% of Caucasian (71, 72).

Direct evidence that peptides occupy the groove of MHC class I and MHC class II molecules, which was first suggested by the HLA-A2 crystal structure, was in the following years found by many research laboratories and crystal structures of peptide/MHC complexes (see (63)). Grey et al. showed for instance, 1988, that purified MHC class II molecules are constitutively occupied with self-peptides (73). Two years later, Röttschke and Falk in Hans G. Rammensee's laboratory were the first to isolate and sequence naturally presented MHC-restricted peptides from the aforementioned influenza nucleoprotein (74). Interestingly, the identified peptides were even shorter in length than the synthetic peptides used by Townsend to elicit cytotoxic T cell responses (69, 74). The identification of the self and non-self peptide repertoire of potentially any MHC positive cell promised to be of great value, not only, for vaccine design, but also for the understanding and for the therapeutic intervention of autoimmunity.

1.7 AUTOIMMUNITY

"The finding of HL-A 27 in 96% of patients with classical ankylosing spondylitis and in 52% of their first-degree relatives is significant; but its clinical value and importance in aetiology remain to be assessed." D. A. Brewerton et al.

"Autoimmune diseases" was the title of the monograph of Ian R. Mackay and F. M. Burnet published 1962. The idea that the protection from non-self might be accompanied by failures, which can lead to autoimmunity was established with the demonstration that we reject non-self, but are tolerant to self (28). Based on the concept of *"acquired immunological tolerance"* Burnet suggested that there must be a homeostatic mechanism in healthy individuals, which deletes cells with *"self-reactive patterns"*. He further described a failure in homeostasis as a potential cause for autoimmunity, in his words: *"Failure of deletion of such cells, possibly associated with weakness of homeostasis, allows the development of a self-reactive or forbidden clone of cells, potentially capable of causing autoimmunity"* (75). This definition of autoimmunity is - with an additional level of complexity - still valid today, whereas Burnet's *"clonal selection theory"* was limited to *"the antibody-producing"* cell (76), the concept has been established also for T cells as outlined above. With the identification of *suppressor T cells* (77), the concept probably needed to be extended to that not only the elimination of *forbidden clones*, but also the failure in establishing a specific (suppressive) clone can cause autoimmunity.

Zinkernagel and Doherty established 1974 that the recognition of non-self is dependent on the MHC and Bevan demonstrated 1977 that also the positive selection of T cells - on self - is MHC restricted. That the failure in homeostasis distinguishing self from non-self, which can cause autoimmunity, is linked to the MHC was indisputably demonstrated already 1973. Lee Schlosstein, together with Paul I. Terasaki, Rodney Bluestone and Carl M. Pearson at UCLA as well as D. A. Brewerton et al. at Westminster Hospital in London had independently shown that a specific MHC serotype is overrepresented in patients with ankylosing spondylitis (AS) (72, 78). Both

groups determined the HL-A type of AS patients and controls. Schlosstein et al. had further included patients with rheumatoid arthritis (RA) and gout, whereas Brewerton et al. included first-degree relatives of the AS patients. 35 out of 40 AS patients in Schlosstein's, and 72 out of 75 in Brewerton's analysis were positive for HL-A W27. The frequency in controls, RA and gout patients was 8-9% in Schlosstein's study and only 4% among Brewerton's control samples. Although the association of AS to HL-A W27 was, 1973, not the first described association between HL-A antigens and disease, it was by far the strongest association described (Figure 8).

TABLE I—HL-A AND DISEASE ASSOCIATIONS

Disease	No. of studies	Antigen	Frequency in patients (%)	Frequency in controls (%)	Average relative risk	95% limits	Heterogeneous	χ^2	References
Ankylosing spondylitis	5	W27	90	7	141	80-249	No	290	20-22, 33
Reiter's disease	3	W27	76	6	46.6	23-94	No	116	23-24, 33
Acute anterior uveitis	2	W27	55	8	16.7	8-34	No	62	25-27
Psoriasis	6	HL-A13	18	4	5.0	4-7	Yes	120	28-33
		W17	29	8	5.0	4-6	No	143	
		W16	15	5	2.9	2-5	No	19	
		1*	HL-A8	47	21	3.3	2-6	—	
Graves' disease	6	HL-A8	78	24	10.4	8-14	No	224	34-35
Celiac disease	3	HL-A8	62	27	4.5	3-8	No	35	36-41
Dermatitis herpetiformis	5	HL-A8	52	24	4.6	3-6	No	103	42-44
Myasthenia gravis	2	W15	33	8	5.1	2-11	No	17	45-49
Multiple sclerosis	4	HL-A3	36	25	1.7	1-2	No	23	50-53, 85
		HL-A7	36	25	1.5	1-2	No	15	
Acute lymphatic leukaemia	7	HL-A2	63	37	1.7	1-2	Yes	15	17-18
		4c(W5)	25	16	1.6	1-2	Yes	14	
Hodgkin's disease	7	HL-A1	39	32	1.3	1-2	No	7	10, 55-62
		HL-A8	26	22	1.3	1-2	No	4	
Chronic hepatitis	1	HL-A8	68	18	9.5	5-20	..	35	63
Ragweed hayfever, Ra5 sensitivity† ..	1	HL-A7	50	19	4	1-12	..	7	64
Ragweed fever, allergen E‡	1	Multiple	65

* Data from ref. 34. † Patients = Ra5 sensitive, controls = Ra5 insensitive. ‡ Family study.
The relative risk is $\frac{pd(1-pc)}{pc(1-pd)}$ where pd = frequency in diseased and pc = frequency in controls.
The averages, 95% limits, heterogeneity, and χ^2 are calculated using standard weighting procedures.¹¹

Figure 8. Summary of HLA-associated diseases, including AS, in the year 1974. Original Table from McDevitt, H., & Bodmer, W. (1974). HL-A, IMMUNE-RESPONSE GENES, AND DISEASE. *The Lancet*, 303(7869), 1269-1275.

Schlosstein et al. as well as Brewerton et al. discussed the implications of their finding for the aetiology and the inheritance of AS. Schlosstein et al. ascribed the strong association identified to either an *immune-responsiveness gene* closely linked to HL-A or to cross-reactivity between the *etiologic agent* of AS with the W27 antigen. This findings were discussed before Doherty and Zinkernagel proposed their altered self model and before the association of a cytotoxic T cell response was linked to MHC class I and a T helper function linked to MHC class II, the *immune-responsiveness genes* (58). However, their argumentation, which was also supported by Hugh O. McDevitt and Walter F. Bodmer (79), suggested already what is today the major classification criterion for an autoimmune disease - the association to MHC class I and II genes. Schlosstein et al. concluded that this newly identified genetic marker would be useful develop new approaches to study AS. And indeed, the strong association of AS to HLA-B27, as it was termed after the 6th IHCW 1975 in Aarhus (80), stimulated the development of transgenic humanized animal models. After several attempts in mice, the introduction of HLA-B27 and human β_2 -microglobulin in rats lead to spontaneously developing disease, which resembled many features of the human disease (81).

Brewerton et al. discussed their finding more in relationship to its clinical usefulness. They acknowledged that the higher prevalence of HLA-B27 in first-degree relatives (52%) suggested that the antigen is inherited, however they clearly

differentiated the inheritance of HLA-B27 from the inheritance of the disease. They therefore excluded that the typing for HLA-B27 could be of use to predict AS, and they suggested instead that HLA-B27 could be used for the early diagnosis of AS. His rationale was based on the fact that the frequency of AS patients is much lower than the frequency of the HLA-B27 antigen in a Caucasian population. They calculated that only 8% of the man and 1% of the woman carrying HLA-B27 would develop AS. Brewerton et al. closed with the notion that there must be other factors – *genetic and environmental* – which contribute to the development of the disease.

Whereas to my knowledge no specific environmental factor has been associated with increased risk for AS (82), additional genetic risk loci have been demonstrated; e.g. two loci encoding the aminopeptidases (*ERAP1* and *ERAP2*) responsible for trimming peptides prior to loading and presentation by MHC class I molecules (83, 84). The association described between HLA-B27 positive AS and *ERAP1* is of interest in regards to Paper III presented in this thesis, in which we show that the selectivity of the peptide transporter in the endoplasmic reticulum regulates MHC expression and CD8 T cell selection (85). A contribution of an environmental factor to the susceptibility to autoimmunity has been identified for another rheumatic disease – Rheumatoid arthritis (RA). Interestingly, the increased risk to develop RA conferred by smoking is additive to the risk conferred by the HLA genotype (86, 87), and illustrates the strong relationship between genes and environment.

1.8 RHEUMATOID ARTHRITIS

“... , we must, for the moment, assume that an understanding of the function of genes in the HLA-D region will permit us to understand the mechanism of this type of association.” T. Sasazuki et al.

Rheumatoid arthritis was one of the diseases that Burnet and Mackay defined as an autoimmune disease in their aforementioned monograph *Autoimmune Diseases*. Burnet had defined an autoimmune disease “as a condition in which structural or functional damage is produced by the action of immunologically competent cells or antibodies against normal components of the body”. Mackay ascribed the clinical criteria, such as the presence of autoantibodies, the immune complex deposition, the infiltration of the target tissue with lymphocytes and plasma cells to be typical for an autoimmune disease (75).

The criteria described by Burnet and Mackay are today largely associated with a subclass of RA, namely the anti-citrullinated protein antibodies (ACPA)-positive disease (87). ACPA are not the only autoantibodies in RA, a class of antibodies, which recognize the Fc portion of IgG, has already been described at the end of the 1930s (88) and termed “until this factor is more completely characterized” as “Rheumatoid factor” (89). The presence of *Rheumatoid factor* was, before the inclusion of the more frequent and RA-specific ACPA (90), a classification criteria for RA (91) and associated with more severe progression of RA (92). Importantly, however the association between the HLA and disease, which is observed for most of the diseases recognized as autoimmune diseases, is predominantly observed for the ACPA-positive subset of RA (87, 93-95).

ACPA are observed in approximately 60% of RA patients (96), since ACPA were identified more than two decades (97) after the first MHC association was described (98), it underscores the strength of this association.

The association of AS to HLA-B27 was the strongest HLA/disease association observed at the beginning of the 1970s (Figure 8) and it was subsequently shown that also other rheumatic diseases have a weaker association to HLA-B27 (99). Interestingly, no significant association of HLA-A or HLA-B antigens was observed for RA (72, 100, 101). RA was in fact the first disease shown to be not associated to MHC class I, but to a MHC class II antigen (Figure 9) (98, 100-102). In contrast, the first association reported for Multiple sclerosis (MS) was to HLA-A3 and later to a HLA-B antigen (103-106). The first association of MS to a MHC class II antigen was identified by Jersild et al. and was confirmed by Terasaki et al. 1976 (107). Since the association to the MHC class II antigen was stronger than the association to HLA-A or HLA-B it had been concluded that the susceptibility gene for MS must be close to the HLA-D locus (Figure 9, today the MHC class II locus) and that HLA-A3, HLA-B7 and HLA-Dw2 are inherited as one haplotype (99, 106, 107).

The HLA-association pattern described for MS and RA are two good examples to introduce the phenomena referred to as *linkage disequilibrium* (LD); that is that a combination of alleles is inherited together more frequently than predicted by “*random matings*” (99). Based on similar findings for other autoimmune diseases, such as Diabetes mellitus and Myasthenia gravis Sasazuki et al. generalized that most of the diseases seem to be associated with the HLA-D region, and that initial association to HLA-A or HLA-B were the result of LD between the respective alleles. An exception of this generalization was the HLA-B27 association of AS and Reiter’s disease (99). The lack of association of MHC class I antigens to RA must mean that the HLA-A and HLA-B loci are not in LD with the disease associated HLA-Dw4 locus, in contrast to the MS-associated haplotype is in strong LD. It has recently been shown, that the MS-associated HLA-DR2 is indeed the haplotype with strongest LD among common Caucasian haplotypes (108). The strong LD in the MHC is and was a major complication and difficulty for the interpretation of MHC associated traits (95).

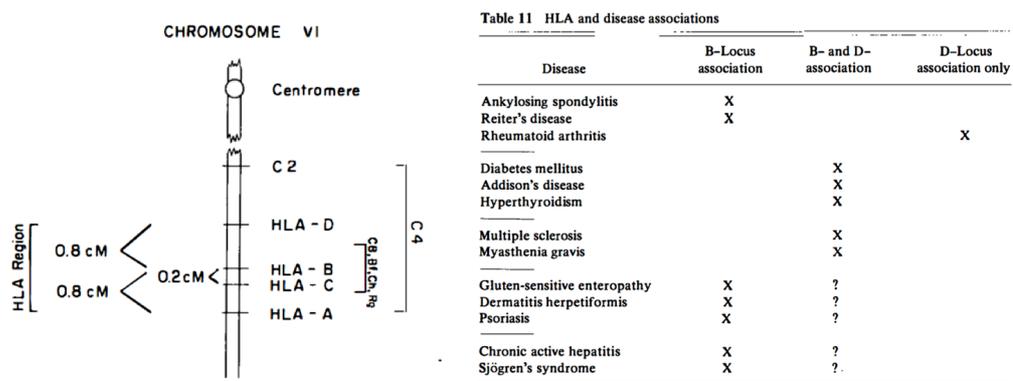


Figure 9. The human MHC on chromosome 6 and a Table summarizing the association of known autoimmune diseases to the HLA-B and HLA-D locus as illustrated by Sasazuki, T., & McDevitt, H. O. (1977). The association between genes in the major histocompatibility complex and disease susceptibility. *Annual Review of Medicine*.

More recently, 2012, has the imputation of the genotypes of the polymorphic alleles of the three MHC class I and the five MHC class II genes, as well as additional SNPs across the human HLA led to a major refinement of the association of RA to the MHC (95). Raychaudhuri et al. suggested that five amino acids in three proteins, encoded by *HLA-DRB1*, *HLA-B* and *HLA-DPB1* in the MHC largely explain the association between ACPA positive RA and the MHC (Figure 10). Two of the suggested amino acids were part of the “shared epitope” in the HLA-DR β chain defined by Peter K. Gregersen 1987 (109). Interestingly, in addition to the associated amino acids in the MHC class II molecules, a residue at the bottom of the HLA-B molecule was among the five identified amino acids (95).

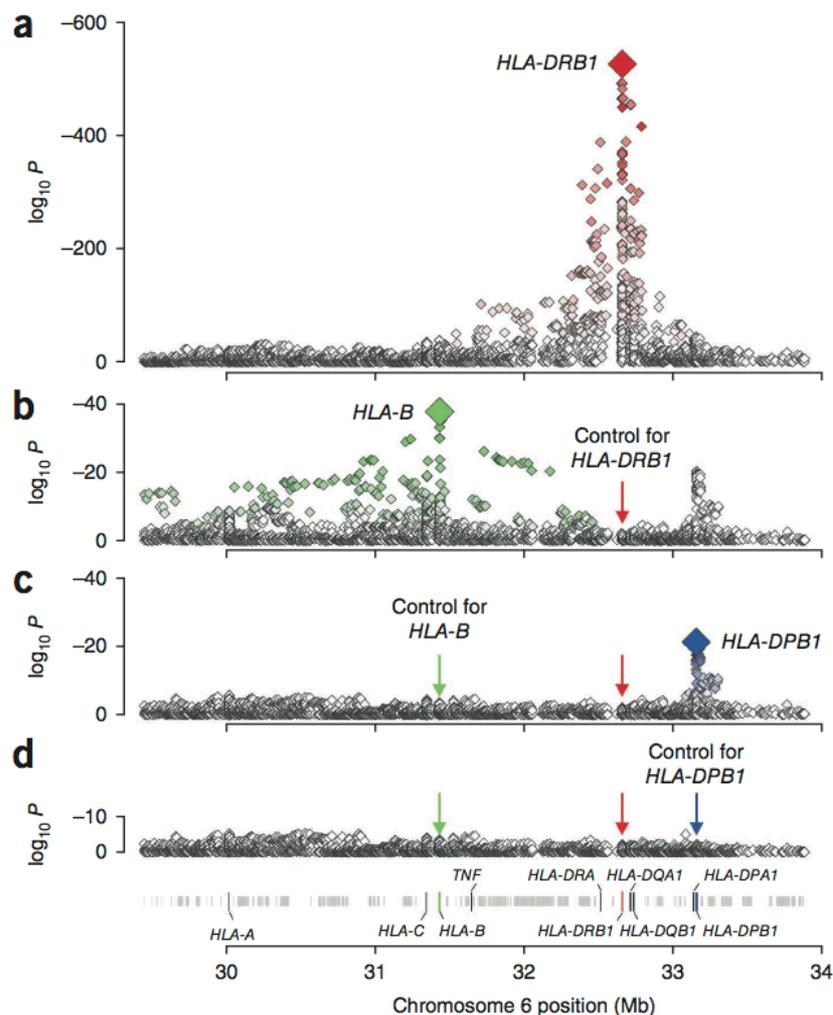


Figure 10. The RA-associated risk loci in the human MHC. After conditional analyses and controlling for the *HLA-DRB1* or *HLA-B* effect, an association to *HLA-B* and *HLA-DPB1* can be observed. Original figure of Raychaudhuri, S., Sandor, C., Stahl, E. A., Freudenberg, J., Lee, H.-S., Jia, X., et al. (2012). Five amino acids in three HLA proteins explain most of the association between MHC and seropositive rheumatoid arthritis. *Nature Genetics*, 44(3), 291–296. doi:10.1038/ng.1076

Even though “the function of genes in the HLA-D region” has been identified in the meanwhile, and enormous progress has been made in dissecting the contribution of the MHC to RA, the underlying mechanism of the association has not yet been elucidated. The identification of potentially causative risk variants in HLA-B and HLA-DP has –

even though being just three amino acids – increased the complexity. The strong association of ACPA-positive RA to SE alleles has directed research to identify citrullinated T cell epitopes, which could based on the neutral charge of citrulline be accommodated by the positively charged P4 pocket. Supporting evidence for the presentation of citrulline-containing peptides by SE molecules has been obtained (110, 111) and also the presence of cognate CD4 T cells has been reported (111-113).

The increasing understanding of the molecular basis behind the strong association of ACPA and HLA-SE alleles is promising, and will hopefully allow therapeutically intervention in the autoimmune response in RA. However, as illustrated by a recent GWAS in ACPA-negative RA, the mechanism of the HLA association and RA is probably more complex and not fully explained by the binding of citrullinated peptides to certain HLA-DR molecules. Bossini-Castillo et al. have identified an association of HLA-B and one of the shared epitope amino acids also to ACPA-negative RA (114). It will be interesting to see if this association is for example due to a subset of ACPA-negative RA patients, which are positive to citrullinated epitopes, which are not captured with the CCP peptide (115), or if the association is independent from the serological status.

There is no doubt that genetic variation in the MHC is the major risk factor for the development of autoimmunity; in RA the HLA explains 36% of the heritability of the disease (96), whereas the estimated heritability of the other 100 RA risk loci explain together 5% of heritability (116).

“It is clear that detailed knowledge of the system will accumulate slowly and may never reach completion” P.A. Gorer

2 PRESENT INVESTIGATIONS

2.1 PAPER I

Human α -enolase is immunogenic, but not arthritogenic, in HLA-DR4-transgenic mice: Comment on the article by Kinloch et al.

The aim of this study was to investigate the arthritogenicity of α -enolase, in particular, the citrullinated version of the protein. This is based on the fact that antibodies to citrullinated α -enolase peptide (CEP-1) are common in RA patients (117) and that the presence of anti-CEP-1 antibodies is strongly associated with HLA-DR shared epitope alleles (118). The Letter summarizes the different experimental set ups, in which we evaluated the arthritogenicity of α -enolase; e.g. we compared autologous vs. heterologous enolase, different adjuvants and two different DR4-transgenic mice. We further show the antibody response to enolase and CEP-1 from one of the performed experiments, as well as histology sections of joints to support our finding that immunization with α -enolase does not induce clinical signs of arthritis.

In addition to the data shown or described in the Letter we have identified the citrullination sites of *in vitro* citrullinated α -enolase, used for the immunization experiments, by high-resolution tandem mass spectrometry. We evaluated the T cell response to α -enolase and a selection of citrullinated and native α -enolase peptides and identified an immunodominant non-citrullinated T cell epitope. Interestingly, the identified T cell epitope, carrying another posttranslational modification, was also recognized by T cells in α -enolase immunized mice, and also induced a T cell response by itself. The nitrosylation of this T cell epitope further increased the binding affinity to HLA-DR4 molecules compared to the non-modified peptide. We further characterized the quality of the T cell response upon protein and peptide immunization and determined the IgG subclass profile of the anti-enolase antibodies.

2.2 PAPER II

Mass spectrometric analysis of citrullinated type II collagen reveals new citrulline-specific autoantibodies, which bind to human arthritic cartilage

Collagen type II (CII) is the major component of articular cartilage (119) and target of the autoimmune response in RA (120, 121). CII is arthritogenic in animal models (122), and anti-CII antibodies induce arthritis in mice (123). It has further been shown earlier that citrullination increases the recognition of an autoantibody epitope on CII in RA patients (124). The aim of this study was to investigate the broader relevance of CII as a joint-specific target of the anti-citrulline response in RA.

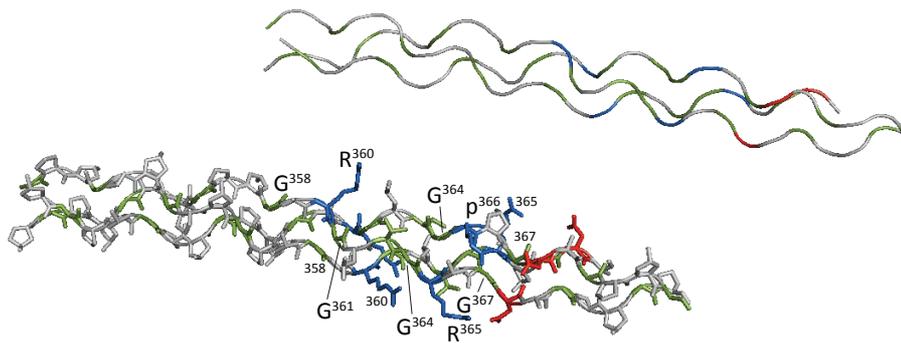


Figure 11. Representation of a CII-like triple helix based on a CII peptide (GARGLTGRpGDA), prolonged with Gly-Pro-HyP-repeats. Peptide extracted from the original crystal structure where it was in complex with an anti-CII antibody (PDB ID 2Y5T). CII is a homotrimer consisting of three α -chains, with repetitive Gly at every third position, such as G³⁵⁸, G³⁶¹, G³⁶⁴, G³⁶⁷. The side chains of R³⁶⁰ and R³⁶⁵ contribute to the hydrogen-bonding network, which stabilises the triple helix and R³⁶⁰ is further a major recognition site for the anti-CII antibody (125). In the study presented here R³⁶⁰ was identified as being citrullinated after PAD treatment, without a requirement for the denaturation of the triple helical conformation. R³⁶⁵ in contrast was not identified to be citrullinated in either of the conditions. All amino acid side chains were oriented outside of the triple helix (125), supporting our finding that most arginines could be citrullinated in the native conformation.

We identified potential citrullinated neoepitopes by high-resolution tandem mass spectrometry (MS) of *in vitro* peptidyl arginine deiminase (PAD) 2 treated CII, and showed that CII could be citrullinated in its native triple helical conformation. Based on the MS analyses, synthetic peptides were designed and analyzed for serum IgG reactivity in the Epidemiological Investigation of Rheumatoid Arthritis (EIRA) case-control cohort including 1949 RA patients and 278 healthy controls. We identified two new citrullinated B-cell epitopes close to the C-terminus of the CII triple helix that were recognized by autoantibodies in 21% and 17% of RA patients respectively. Affinity-purified antibodies from RA sera directed to these two epitopes, but not antibodies directed to CEP-1, bound to RA cartilage. We therefore propose that antibodies directed to citrullinated CII epitopes contribute to the induction and/or perturbation of joint inflammation in RA patients.

2.3 PAPER III

Natural Polymorphisms in Tap2 Influence Negative Selection and CD4:CD8 Lineage Commitment in the Rat

In paper III we assessed the impact of natural genetic variation in the MHC on MHC class I and class II expression and CD4:CD8 lineage commitment in two genetic models in the rat. We first mapped genome-wide Quantitative Trait Loci (QTLs) associated with variation in MHC class I and II protein expression and the ratio of CD4 and CD8 T cells in outbred Heterogeneous Stock rats. We identified 10 QTLs with genome-wide significance. QTLs for the individual traits co-localized within a region spanning the MHC on chromosome 20. To refine these overlapping QTLs for which the confidence intervals were determined to 4.1-9.7 Mb we generated a large panel of MHC-recombinant congenic strains (RCS). The panel derived from the MHC-recombinant strains DA.1I, DA.1F, DA.1H and DA.1U on a DA (RT1^a) background.

The respective haplotypes, indicated with capital letters in the recombinant strain designation, originate from the inbred rat strains BI (RT1ⁱ), AS2 (RT1ⁱ), KHW (RT1^h) and E3 (RT1^u). Applying a conventional intercross breeding we could refine the QTLs in the MHC to two adjacent intervals in the MHC-I and II regions, respectively. The size of the minimal QTL defined by congenic mapping was 0.282 Mb for QTL in the MHC-I region, termed *T cell selection QTL 1 (Tcs1)*, and 0.206 Mb for the *T cell selection QTL 2 (Tcs2)* in the MHC-II region.

Tcs1 regulated MHC class I expression and T cell numbers, and *Tcs2* controlled, the MHC class II expression and T cell numbers, but in addition also the expression of MHC class I molecules. The influence of both *Tcs1* and *Tcs2* on MHC class I expression and the frequency of T cells was due to an interaction between these two intervals. More precisely, this interaction affected the negative selection and lineage commitment of CD8 single-positive (SP) thymocytes. By comparing the sequence variation of the genes in *Tcs2* we could ascribe this phenotypic variations to the interaction between the transporter associated with antigen processing 2 (*Tap2*) in the MHC-II region and the classical MHC class I gene(s) (*RT1-A*) in the MHC-I region. The recombination between *RT1-A* and *Tap2*, which allowed the identification of this interaction, occurred in approximately 1 out of 500 rats. The MHC-Ia region in the rat is neighbouring the MHC-II region, whereas the MHC class Ia locus in humans is only neighbouring the MHC-III region (Figure 1/12). Allelic variants of *Tap2* have previously been shown to alter the peptide repertoire of MHC class I molecules and thereby to influence the antigenicity of MHC class I molecules (126, 127). This phenomena has been termed class-1 modification (cim) (Figure 12), we could in addition to the original cim-effect describe an inverse cim effect in the present study.

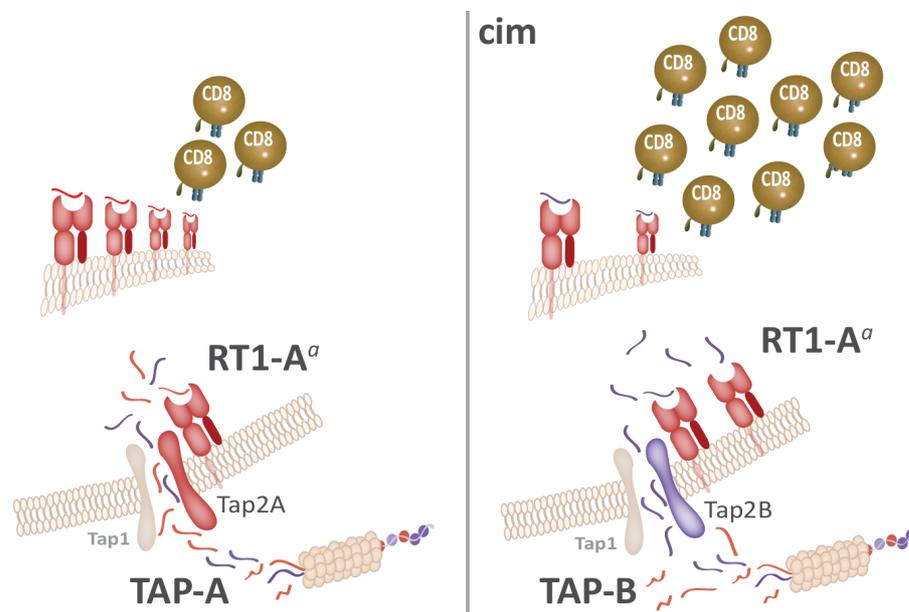


Figure 12. Illustration of the effect associated with the *Tap2* allele. Before the effect was ascribed to the *Tap2* gene, the locus causing a reduced expression of MHC class I (RT1-A) was termed cim (*class-1 modification*) and had been mapped to the MHC-II region (127, 128). The combination of a RT1-A molecule of the RT1^a haplotype with a *Tap2B* allele alters the CTL response as described by Livingstone et al., but also reduces the negative selection of CD8 T cells (85).

In summary, we have shown that a restricted peptide repertoire on MHC class I molecules, which is the consequence of the combination of a specific allelic variant of the *Tap2* gene and a specific *RT1-A locus*, reduces the negative selection of CD8SP cells. In more general terms, our study illustrates the significant impact of the LD or the loss of the LD in the MHC.

2.4 PAPER IV

A 0.2 Mb interval within the MHC-II region controls the onset and severity of pristane-induced arthritis in the rat

In paper IV we addressed the impact of the 0.2 Mb QTL in the rat MHC-II region, which influences MHC expression and T cell selection (85) on the development of pristane-induced arthritis (PIA). Pristane is a naturally occurring hydrocarbon (129-132), which induces in susceptible rats severe joint inflammation (133) and progresses into a chronic relapsing-remitting form of arthritis (134). Several quantitative trait loci (QTLs) have been mapped in PIA using arthritis susceptible DA and resistant E3 rats (135). One of the identified QTLs, which influenced onset and severity of PIA, mapped to chromosome 20 (*Pia1*) and spanned the MHC region (136). Using a panel of MHC RCS, we show here that genes responsible for *Pia1* are located within a 0.2 Mb interval in the MHC-II region. This interval contains 12 genes, which includes genes encoding the alpha and beta subunits of the two classical MHC class II molecules, RT1-B and RT1-D, as well as the non-classical RT1-DO (Figure 13).

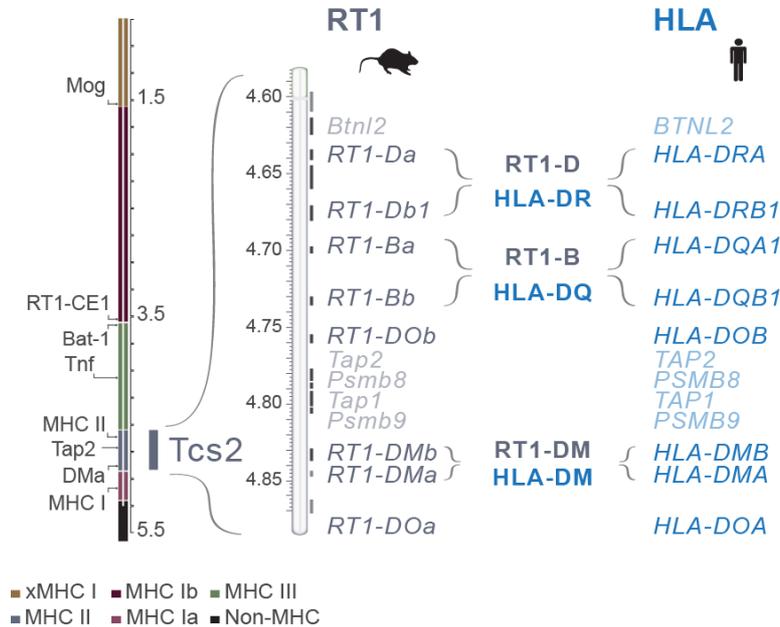


Figure 13. Comparison of the nomenclature of the genes in the rat (RT1) and human (HLA) MHC class II region. The human homologous genes to the rat genes are shown in blue, the corresponding proteins for the RT1-B, RT1-D and HLA-DM molecules are shown in between the gene designations. The organization of the genes in the HLA and RT1 in the MHC-II region is identical, however there are additional genes, such as *HLA-DRB5*, which encodes another beta chain, which forms a HLA-DR complex with the alpha chain encoded by *HLA-DRA*. The *RT1-DOb/RT1-DOa* and *HLA-DOB/HLA-DOA* genes encode the alpha and beta chain for the RT1-DO and HLA-DO molecule, respectively. The MHC class Ia locus is neighbouring the MHC class III locus in the HLA (Figure 1). For more detailed information see (137, 138), the *TAP2* gene is therefore not directly neighbouring the MHC class Ia genes.

As shown in Paper III, another gene in this interval, *Tap2*, influences T cell selection and the QTL was therefore denoted *T cell selection QTL 2 (Tcs2)* (Figure 13). We compared arthritis development in DA rats (RT1^a) with the *Tcs2* haplotypes RT1ⁱ, RT1^f, RT1^h and RT1^u. RT1^h fragments (DA.1HR10) and, to a lesser extent, RT1^u (DA.1UR10) suppressed the severity of the acute phase of arthritis, while fragments from the RT1^f haploypete (DA.1FR61) enhanced arthritis severity during the acute phase. The same association of haplotype and PIA severity was not seen in the chronic phase of PIA. The arthritis development of the RT1ⁱ haploypete (DA.1IR7) did not differ from the RT1^a haplotype (DA). The RT1-D locus is conserved between DA.1FR61 and DA, which suggest that RT1-B mediates the enhanced disease severity in DA.1FR61.

We therefore aimed to delineate the contribution of the two MHC II molecules to the development of PIA. Blocking of RT1-B but not RT1-D reduced acute and chronic PIA in all strains, suggesting that PIA is associated to RT1-B. While a low extracellular RT1-D expression supported the RT1-B association, expression differences between the strains of neither RT1-B nor RT1-D correlate with arthritis development. We further compared the MHC class II ligandome between the strains and identified multiple peptides from RA-related ubiquitous auto-antigens. While the quality of the source proteins of the eluted peptides did not differ between molecules or haplotypes, the peptide-binding motif of RT1-B showed marked differences to the RT1-D motif. Most noteworthy, the RT1-B P1 pocket was only in the RT1^f haplotype occupied by hydrophobic amino acids, while the pockets in RT1^{u,h} showed preferences for negative charged glutamic acid, and the RT1^a haplotype showed no binding preferences for P1 at all. Interestingly, we also identified haplotype-specific invariant chain-derived peptides on the disease-associated RT1-B molecule. Comparison of homology models of the MHC class II molecules showed that strains with less severe arthritis share amino acid variants in the RT1-B P1 pocket, which support the peptide binding data. In summary, we show that a 0.2 MB interval in the rat MHC II-region regulates the acute phase of PIA and our data strongly suggest that this phenotype is associated with amino acid polymorphism in RT1-B.

2.5 PAPER V

T cell priming and Th lineage commitment in MHC class II congenic rats upon immunization with the hydrocarbon pristane

In Paper V we characterized the T cell compartment in MHC class II congenic rats (introduced in Paper III) upon immunization with the hydrocarbon pristane. Pristane-primed CD4 and not CD8 T cells transfer arthritis to naïve recipients we therefore compared the priming of these autoreactive T cells between the RCS with different *Tcs2* haplotypes, and different severity of PIA (Paper IV). We analyzed the expansion of CD4 T cells in rats injected with pristane in the draining lymph nodes at different time points after immunization. We found no variation in the frequency or absolute number of activated/memory CD4 T cells between the strains. However, rats with a more severe disease phenotype had more proliferating CD4 T cells at day 5 and 8 after immunization. Hence, arthritis severity correlated with the expansion of

activated/memory CD4 T cells. We further addressed the proportion of Th1, Th17 and regulatory T cells, which revealed that reduced severity of arthritis is associated with an increase of Tregs and a Th17 bias. To support that a low Th1/Th17 ratio reduces the onset and severity of PIA we treated pristane-immunized rats with anti-IFN- γ and anti-IL-17 antibodies. Neutralization of IFN- γ , but not IL-17, during the priming phase ameliorated disease in all strains, while arthritis progression was largely dependent on IL-17. We therefore suggest that *Tcs2* regulates the early expansion of T cells and the balance of Th1 and Th17, which together determines the severity of acute arthritis.

3 DISCUSSION

The risk to develop CEP-1 and cyclic citrullinated peptide (CCP)-positive RA is significantly increased in smokers carrying one or two HLA-DRB1 SE alleles. Considering this strong interaction between genes and environment in RA, it might be plausible that the development of citrulline-specific anti α -enolase antibodies are also in an experimental system, such as in the mouse, dependent on one or many environmental factors. The contradictory findings concerning the arthritogenicity of citrullinated α -enolase observed by our group and Kinloch et al. might thus reflect such environmental differences between different animal facilities.

The strong association of anti-CEP-1 antibodies and *HLA-DRB1*04* alleles, which harbour a positively charged P4 pocket, implied the dependency of on a citrulline specific T helper response. In Paper II we describe the presence of two novel citrullinated autoantibody epitopes on CII. CII is an important autoantigen, since its tissue specific expression pattern, which is predominantly in articular cartilage, might explain the manifestation of RA in the joints. Our group has earlier identified an immunodominant T cell epitope on CII in *HLA-DRB1*04* positive RA patients; importantly, this epitope can be glycosylated at two positions, and T cell response can be detected to at least four different variants of this epitope. We therefore proposed that T cells specific to for example the glycosylated CII T cell epitope might give help to citrulline-specific B cells, which differentiate to plasma cells and produce antibodies, such as the citF4-directed antibodies described in Paper II (139). The deamidation of arginine to citrulline increased not only the recognition of the CII epitope described in Paper II, but also as earlier shown the recognition of the C1 epitope in RA patients (124). We have additional evidence that the increase in antigenicity by citrullination is not limited to these two epitopes, which together with the finding that at least one subset of these antibodies binds to RA cartilage strongly supports the earlier proposed self-perpetuating pathogenicity of the anti-CII response (139).

The role of T cells in the pathogenicity of RA is largely attributed to a T helper function, necessary to stimulate the production of autoantibodies, which is of course motivated by the strong genetic association between *HLA-DRB1* alleles and ACPA. However, T cells have been associated with many different, antibody independent, effector functions, which is also reflected by RA drugs, such as Abatacept (140). Paper IV and V describe the influence of the MHC class II haplotype on the disease development and T cell priming in an arthritis model, PIA, which can be transferred by CD4 T cells and is therefore not dependent on pathogenic autoantibodies. We could show that the activation and proliferation of T cells during the priming phase is dependent on the frequency of Th1 cells. Importantly, the variation in the frequency of Th1 cells was determined by genes in *Tcs2* (Figure 13). This suggests that variation in the self-repertoire of MHC class II molecules predisposes to a specific T helper polarization. Strains with a reduced frequency of Th1 cells after pristane administration developed less severe arthritis in the acute phase of the disease. Despite enormous efforts to minimize the disease associated *Tcs2* locus, the LD in the MHC class I region was too strong and we did not obtain a recombination between the *RT1-B* and *RT1-D*

genes, encoding the two principle MHC class II antigen-presenting molecules in the rat. Functional studies and comparison of sequence variation between the haplotypes suggests an important role of the RT1-B molecule.

In addition to the MHC class II genes *Tcs2* encodes four genes part of the MHC class I antigen presentation pathway, *Tap2*, *Psmb8*, *Tap1* and *Psmb9*. These genes are located in the same order in the human MHC class II locus (Figure 13). In Paper III we show that the *Tap2* allele regulates the thymic selection of CD8 T cells. This was revealed by a recombination between the neighbouring MHC class I and MHC class II loci. The human *TAP2* gene is not described to be as polymorphic as the rat homolog and is not in such strong LD as the rat *RT1-A* and *Tap2* genes.

4 CONCLUDING REMARK

The work included in this thesis touches on many different aspects in immunology and genetics with the MHC as the common denominator. I did not wanted to present an extended version of the individual introduction sections of the papers included in this thesis – I therefore revisited some of the discoveries, which coined our today's understanding of the MHC, T cell recognition and autoimmunity. Our findings did surely not reach the impact of the depicted discoveries; however, in particular, the discovery of the MHC illustrates that it is important to contribute with one's own findings to the common knowledge, since they might strengthen the findings of other researchers and thereby potentially contribute to a fundamental discovery. And whereas the identification of homology between systems supports a finding, the differences, which often remain, might be crucial to understand and dissect a functional mechanism.

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