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POSTTRANSLATIONAL MODIFICATION OF COLLAGEN TYPE II

EFFECTS ON ANTIGEN SPECIFIC T-CELL TOLERANCE AND AUTOREACTIVITY IN COLLAGEN-INDUCED ARTHRITIS

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"Life is like riding a bicycle. To keep your balance you must keep moving."

Albert Einstein

To my family.

ABSTRACT

Rheumatoid arthritis (RA) is a common chronic inflammatory disease affecting peripheral joints in approximately 1% of the world population. Immunization of susceptible strains with CII, leads to development of collagen-induced arthritis (CIA), an animal model for RA. The aim of this thesis was to investigate mechanisms involved in regulation of immunological T-cell tolerance in CIA by studying availability of joint-specific CII for presentation to autoreactive T cells in healthy as well as pathological settings.

This work shows that transgenic expression of heterologous CII can inhibit expansion and Th1/Th17-skewing of antigen-specific T cells upon immunization with heterologous CII. The strength of tolerance induction was found to be dependent on the abundance of the self-antigen, the genetic background of the mice, as well as the presence or absence of posttranslational modifications on CII. Data indicate that joint-specific antigens are readily available for presentation in draining lymph nodes to induce immunological tolerance. Furthermore, a defect in thymic tolerance induction suggests that certain CII modifications are presented differentially depending on the location in the organism (Paper IV).

To obtain these results, established mouse systems were refined by generating a T-cell receptor specific antibody (Paper I) or by breeding diverse mouse and human transgenes on genetic backgrounds with different susceptibilities (Paper II & III).

Even though it is accepted that T cells play an important role in arthritis development, it remains controversial where and how they contribute to pathogenic mechanisms after loss of tolerance. In summary, this thesis describes a series of new mouse models that will aid to further elucidate the arthritogenic action of T cells in disease relevant sites. This will hopefully enlarge the mechanistic framework for further investigation of human disease pathogenesis, which might lead to new therapeutic strategies to promote self-tolerance in diseased individuals.

LIST OF PUBLICATIONS

I. Merky, P., Batsalova, T., Bockermann, R., Dzhambazov, B., Sehnert, B., Burkhardt, H., and Bäcklund, J.
Visualization and phenotyping of pro-inflammatory antigen-specific T cells during collagen-induced arthritis in a mouse with a fixed collagen type II specific transgenic TCR beta chain.
Arthritis research & therapy 2010, **12**(4):R155.

II. Batsalova, T., Dzhambazov, B., Merky, P., Bäcklund, A., and Bäcklund, J.
Breaking T cell tolerance against self type II collagen in HLA-DR4-transgenic mice and development of autoimmune arthritis.
Arthritis Rheum 2010, **62**(7):1911-1920.

III. Merky, P., Batsalova, T., Dzhambazov, B., Förster, M., Yamada, H., Holmdahl, R., and Bäcklund, J.
Tolerance to glycosylated self-collagen type II is regulated in the periphery and leads to protection from collagen-induced arthritis
In manuscript

IV. Merky, P., Holmdahl, R., and Bäcklund, J.
AIRE-expression is specifically associated with controlling tolerance to non-glycosylated collagen type II in collagen-induced arthritis
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LIST OF ABBREVIATIONS

ACPA	anti-citrullinated protein antibody
AICD	antigen-induced cell death
Aire	autoimmune regulator
APC	antigen presenting cell
APECED	autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy
CFA	complete Freund's adjuvant
CIA	collagen-induced arthritis
CII	collagen type II
COMP	cartilage oligomeric matrix protein
CTLA-4	cytotoxic T lymphocyte antigen-4
DC	dendritic cell
EAE	experimental autoimmune encephalomyelitis
Foxp3	forkhead box P3
HEL	henegg lysozyme
HLA	human leukocyte antigen
IDO	indoleamine 2,3-dioxygenase
IFA	incomplete Freund's adjuvant
IFN	interferon
IL	interleukin
LCMV	lymphocytic choriomeningitis virus
LPS	lipopolysaccharide
MHC	major histocompatibility complex
MMC	mutated mouse collagen
MMP	matrix metalloproteinase
MS	multiple sclerosis
mTEC	medullary thymic epithelial cell
OVA	ovalbumin
PAMP	pathogen-associated molecular pattern
PD-1	programmed death-1
PRR	pattern-recognition receptor
RA	rheumatoid arthritis
RAG	recombination activating gene
RANKL	receptor activator for nuclear factor κ B ligand
RF	rheumatoid factor
ROS	reactive oxygen species
TCR	T-cell receptor
TGFbeta	transforming growth factor
TLR	Toll-like receptor
TRA	tissue restricted antigen
Treg	regulatory T cell
TSC	T-cell epitope in systemic collagen
VCAM-1	vascular cell adhesion molecule-1

INTRODUCTION

Organisms are constantly challenged by pathogens encountered in the air, food and water or by malignancies of cells within the individual itself, i.e in cancer. To fight these exogenous and endogenous dangers, a complex defense system has developed which can be divided into two lines, the more primitive innate immune system and the highly evolved adaptive immune system. These two lines of defense work in concert to provide a high degree of protection for vertebrate species.

Innate immunity confers immediate non-specific protection against a majority of the pathogens that we are confronted with, and consists of several protective features. First, the skin and the mucosal surfaces are the primary line of defense against intruders. Behind this strong barrier enforced with anti-microbial enzymes, a number of phagocytic cells, such as blood monocytes, neutrophils and tissue macrophages engulf cellular debris and microbes from infected tissues. Pattern-recognition receptors (PRRs) on the surface of these cells recognize a variety of evolutionary conserved microbial products, such as LPS, double-stranded RNA or flagellin, which are also called pathogen-associated molecular patterns (PAMPs). Engagement of these receptors activates antigen-presenting cells and initiate phagocytosis of the invading pathogens. The complex cell machinery breaks down and processes the pathogenic structures to finally present them to the second line of defense, the adaptive immunity. A key feature of the adaptive immune system is its memory, exerted through highly antigen specific T cells and antibody producing B cells allowing a rapid elimination of pathogens upon re-infection.

A major challenge for the immune system is to distinguish between foreign and self, which can have very similar structures. During cell development, T-cell and B-cell receptors undergo random rearrangements of receptor genes generating a gigantic panel of specificities. These include both receptors binding to pathogens that might not have been encountered yet as well as receptors with potential self-specificity. These self-reactive cells can induce an attack against the host if a favorable inflammatory environment is provided and cause what is termed autoimmunity. However, thanks to diverse regulatory checkpoints, several factors have to coincide for autoimmune disease to be induced. Nonetheless, around 5-10% of the world population is suffering from autoimmune disorders [1, 2] causing chronic morbidity and disability, which becomes a burden to healthcare systems around the world.

Rheumatoid arthritis (RA) is one of the most common autoimmune diseases affecting approximately 1% of the population worldwide [3]. There are multiple mechanisms leading to RA, some of which have been found and further investigated in animal studies. My thesis is aiming at shedding light on factors and mechanisms involved in regulation of immunological T-cell tolerance in CIA, a common animal model for RA. More specifically, in the presented work the interaction of the immune system with CII, the major protein in joint cartilage, has been studied to better understand the availability of joint-specific antigens for presentation to autoreactive T cells in healthy as well as pathological settings. To prepare the reader for the discussion of the findings made within this thesis, this introduction will first shortly describe general aspects of T cell tolerance and then give an overview on RA and its animal model CIA, the main model used in my thesis.

IMMUNE TOLERANCE

To prevent autoimmunity, lymphocytes undergo tolerization processes ensuring that functional receptors are being expressed on the cell surface without being responsive for self-antigens. Regarding T cells, tolerance induction takes place at two different maturation states. First, the T-cell precursors undergo a selection process in the thymus, referred to as central tolerance, where the majority of the self-reactive T cells is deleted. However, this process is not absolute because in some cases potentially pathogenic self-reactive T cells were found to escape central tolerance. Therefore a series of different peripheral tolerance mechanisms are coping with these mature T cells to avoid activation and immunopathology.

CENTRAL TOLERANCE

The thymus is the site of T-cell development and maturation. A three-dimensional sponge-like network of epithelial cells, dendritic cells (DC) and macrophages build the educational matrix for T cells. At first, a small number of T-cell progenitors being negative for the co-receptors CD4 and CD8 enters the thymus. Upon expression of the recombination activating gene (RAG), T cells begin to rearrange their T-cell receptor (TCR) β loci. In the case of conventional αβ-T cells, a rearranged TCR β-chain is eventually jointly expressed with a surrogate pre-TCR α-chain. A functional pre-TCR provides the double negative (DN) T cells with a survival signal, inducing massive proliferation together with upregulation of CD4 and CD8 and rearrangement of the TCR α loci [4]. After this "β-selection", the motile $CD4^+CD8^+$ double positive cells migrate to the cortical region where the positive selection takes place. This process, is responsible for creating a self-MHC-restricted T-cell repertoire by ensuring that only those T cells survive, which recognize self-MHC molecules [5]. Low affinity TCR engagement induces survival and further maturation whereas TCRs with no or too low affinity for self-peptide-MHC complexes die by neglect [6]. Positively selected T cells ultimately develop into either CD4 or CD8 single positive cells, depending on their specificity for MHC class II or I, respectively. In addition, RAG transcription is suppressed to prevent further rearrangement of TCR genes [7].

In a next step, potentially autoreactive single positive (SP) T cells are deleted by negative selection in the thymic medulla. T cells that bind with high affinity to self-peptide-MHC complexes are deleted by apoptosis [8]. Presentation of self-antigens by

thymic APCs occurs if the particular antigens are either expressed in the thymus [8] or transported from the tissue of origin into the thymus [9, 10]. Interestingly, it was shown that the thymic medulla, and more specifically the medullary thymic epithelial cells (mTECs) are capable of expressing a large spectrum of tissue-restricted antigens (TRA). Thus the mTECs can drive negative selection of antigens that would otherwise be secluded in specific organs or only secreted in specific situations [11-14]. Thymic expression of a wide array of TRAs is dependent on the transcription factor autoimmune regulator (Aire) and Aire-deficiency leads to organ-specific autoimmune disease [15, 16]. However, mTECs by themselves are poor mediators of negative selection [17] and partly have to rely on cross-presentation by bone-marrow derived DCs for which they act as a TRA pool [18]. In addition, SP $CD4^+$ and $CD8^+$ thymocytes were shown to reside in the medulla for up to 2-3 weeks and so enable the scanning of a multitude of self-antigens on thymic APCs [19].

The process of positive and negative selection is controlled by a delicate balance of affinity of the TCR and the avidity of interactions, meaning the number of TCRs in contact with self-peptide-MHC complexes [20, 21]. Differential activation and localization of signaling molecules in the cell defines a very narrow affinity threshold, which determines the selection outcome for a given TCR specificity [22]. This means that self-reactive T cells have only a very small window to potentially escape negative selection. On the other hand, it has also been shown that strong selection of a TCR can drive thymocytes towards differentiation into the regulatory T cell lineage (Treg). The exact molecular mechanisms are not understood yet. It seems that not only the affinity threshold of the TCR/self-peptide-MHC interaction may induce either Treg differentiation or clonal deletion, but also the actual expression pattern of co-stimulatory molecules on APCs, and the cytokine environment [23].

Nonetheless, negative selection is incomplete because circulating T cells that are reactive with self-antigens in peripheral blood are detect in organ-specific autoimmune diseases, such as type I diabetes and multiple sclerosis (MS) [24, 25]. Moreover, autoreactive T cells, such as CII-specific T cells, can be found in healthy individuals, indicating that additional mechanisms in the periphery suppress the onset of autoimmune disease.

PERIPHERAL TOLERANCE

Thymic selection effectively deletes premature T cells that express TCRs with high affinity for self-peptide-MHC complexes. Therefore, peripheral tolerance mechanisms are critical to keep mature T cells bearing TCRs with relatively low affinity for self-peptide-MHC complexes under control. This was recently shown in an elegant study using double-transgenic mouse model expressing a TCR β-chain originating from an ovalbumin (OVA)-specific $CD8^+$ T cell in concert with a rat insulin promoter-dependent and membrane-bound OVA transgene exclusively expressed in the pancreas, the kidney and in mTECs [26]. The advantage of using a transgenic TCR β-chain is to allow rearrangement with endogenous TCR α-chains resulting in a detectable polyclonal $CD8^+$ T cell population with heterogeneous affinity for OVA-MHC (A similar strategy was used in paper I [27] included in this thesis, although in a different context). Thus, although low-affinity OVA-MHC-specific $CD8^+$ T cells could be detected in the periphery in relatively high numbers in these mice, no signs of diabetes appeared in naïve as well as virus or *Listeria*-infected mice. Infecting these mice with *Listeria monocytogenes* genetically modified to express the OVA sequence resulted in rapid development of diabetes driven by low-affinity $CD8^+$ effector T cells. This demonstrates that the interplay between central and peripheral tolerance is eliminating or keeping T cells in check that could potentially be primed with endogenous levels of TRAs. If, however, higher levels and/or mimic of self-antigens activate these T cells the system fails and autoimmunity can develop. Some examples of overlapping mechanisms of peripheral tolerance will be described below, including ignorance, clonal anergy and clonal suppression.

Clonal ignorance is achieved when the expression site of the autoantigen is anatomically separated from potentially autoreactive cells or when the antigen is inappropriately presented for immune activation. Naïve T cells are circulating from blood to secondary lymphoid organs, and back to the blood through the efferent lymph. It is basically only in the secondary lymphoid organs where the naïve T cells scan interdigitating DCs for the presence of pathogen-derived peptide-MHC complexes. Hence, they are secluded from non-lymphoid peripheral tissues, where the chance of encountering a tissue-resident cell that expresses sufficient levels of TRA is higher. To illustrate this, naïve TCR-transgenic lymphocytic choriomeningitis virus (LCMV) glycopeptide specific $CD8^+$ T cells remain unresponsive to pancreatic islet cells engineered to express the LCMV glycoprotein [28]. Despite the presence of

autoreactive T cells, these mice do not develop diabetes. However, infection of these mice with LCMV results in priming and infiltration of the autoreactive $CD8^+$ T cells into the pancreas, that target the β cells.

The situation changes once the naïve T cell has encountered its antigen. The engagement of the TCR and the co-stimulatory molecules with peptide-MHC complexes on APCs mediate activation and maturation of the naïve T cells. Upregulation and downregulation of defined receptors and molecules follows, which change the circulation pattern of the antigen-primed T cells. They now migrate through most tissues of the body, preferentially to local sites of inflammation, thereby increasing the risk of encountering sites with high TRA expression. However, this does not necessarily lead to immune activation because T cell unresponsiveness (anergy) occurs when T cells are activated by APCs through TCR engagement in absence of co-stimulatory signals, including for example B7/CD28 pathway [29]. An APC type of major importance in regulation of immune responses is the dendritic cell (DC). Different types of DCs are found in different tissues and they act as sentinels detecting "danger signals" by constantly processing available antigens through MHC class II complexes or MHC class I complexes as a result of cross-presentation [30]. This ensures that debris from apoptotic and necrotic cells but also pathogen-derived proteins will be presented to T cells upon DC maturation. Activation of Toll-like receptors (TLRs) by microbes or necrosis, and proinflammatory cytokines are ways of triggering DC maturation [31]. Subsequently, DCs up- and downregulate a battery of molecules that influences their migration pattern, the co-stimulatory capacity, and secretion of proinflammatory cytokines. However, if this maturation is incomplete DCs adopt a tolerogenic phenotype instead. An illustration of this comes from a study, where DCs were given the experimental antigen hen egg lysozyme (HEL) without inducing maturation, i.e. there was no detectable upregulation in MHCII and CD80 expression [32]. When these semi-mature DCs stimulated naïve HEL-specific TCR-transgenic $CD4^+$ T cells, initially there was a proliferative expansion. However, a coupe of days later most of these HEL-specific $CD4^+$ T cells had disappeared and the remaining cells were refractive to additional HEL stimulation. In summary, these data suggest that in the absence of "danger", lymph node and spleen resident DCs can induce tolerance by functionally inactivating T cells that ultimately can lead to peripheral deletion from secondary lymphoid organs where their cognate antigen is presented.

Co-stimulatory molecules, including CTLA-4 and PD-1 on T cells have also been shown to be crucial in controlling immune responses in the periphery. CTLA-4 deficient mice were found to develop spontaneous lymphoproliferation and autoimmunity [33]. CTLA-4 downregulates immune responses after the acute phase by outcompeting CD28 for binding to the costimulatory molecules CD80/86 on APCs [34, 35], thus slowing down proliferation and expansion of effector T cells. CTLA-4 also prevents activation induced cell death (AICD) by inhibiting upregulation of FasL [36], which allows memory T cells to remain in the system. An alternative function for CTLA-4 in T cell downregulation may be through the interaction with CD80 on DCs, which can induce production of indoleamine 2,3-dioxygenase (IDO). The catabolizing enzyme IDO depletes the essential amino acid tryptophan from the surrounding tissue and strongly inhibits naïve T cell activation [37]. IDO-expressing DCs can promote Treg differentiation and induce PD-1 expression on Tregs to allow bystander suppression [38].

PD-1 appears to be expressed in different developmental stages of T cells as well as on a variety of other immune cells. PD-1 promotes both Treg development and function and maintains T cells in an anergic state [39]. For example, PD-1 seems to block inhibitors of cell migration that are required to allow T cells to engage activating TCR contacts with APCs [40]. Blocking of PD-1 and its ligand PD-L1 repressed T cell migration and prolonged T cell-DC contact, which triggered TCR signaling and enhanced T cell cytokine production. The result of this treatment was reactivation of anergic T cells leading to impaired peripheral tolerance with development of type I diabetes. In summary, data suggest that CTLA-4 signaling may terminate proliferation and promote anergy induction, whereas PD-1 ligation controls previously tolerized autoreactive T cells in the periphery by keeping them in an anergic state.

Clonal suppression is mediated by different subsets of Tregs, such as natural killer (NK) T cells, $CD8^+$ and $CD4^+$ T cells. Most attention has been given to the $CD4^+Foxp3^+$ Tregs that are divided into natural Tregs, mostly generated in the thymus, and induced Tregs, whose differentiation from naïve T cells is driven by TGF-β in the periphery [41]. The importance of Foxp3 in Treg development and function has been illustrated with the scurfy mouse, which harbors a loss of function mutation in the FOXP3 gene [42]. Similarly as in humans with IPEX (Immune dysfunction/Polyendocrinopathy/Entheropathy/X-linked; also mutated in FOXP3 gene), these mice suffer from fatal autoimmune lymphoproliferative disease [43]. The

mode of action of Tregs is to suppress the activation of T cells by direct cell-to-cell contact and by secreting anti-inflammatory cytokines, including IL-10 and TGF-β. A major molecule involved in cell-cell-contact is CTLA-4, which is constitutively expressed on murine Tregs [44, 45]. The direct interaction of CTLA-4 on Tregs with CD80/86 on DCs may block DC accessibility to effector T cells [46], modulate DC phenotypes by inhibition of CD80/86 upregulation on immature DCs that experience antigenic stimulation, or downregulate the expression of CD80/86 on mature DCs [47]. Furthermore, as mentioned previously, interaction of the Treg CTLA-4 with CD80/86 on DCs may also induce IDO production in the DCs, thereby depriving the surrounding with the essential amino acid tryptophan [37, 47]. Foxp3$^+$ Tregs were also shown to deplete the surrounding of IL-2, upon which they are highly dependent on for their survival [48]. Taken together, the data on regulatory T cells that has accumulated over the last decade suggests a key role of Tregs in peripheral self-tolerance and immune homeostasis.

Most of our knowledge about tolerance has been established in TCR transgenic systems. However, it is unclear to which extent these findings can be applied to physiological polyclonal conditions, where clonal populations of naïve T cells have been estimated to be much smaller [49]. It is easy to imagine that a massive increase of precursor frequencies of a given antigen specific transgenic T cell and the level of expression of the cognate antigen in a host can distort the picture. Therefore, it may be that some observations on incomplete tolerance or even autoimmunity originating from double transgenic animal systems are artifacts from an overburden of the natural tolerance mechanisms.

AIRE IN TOLERANCE INDUCTION

Discovery of AIRE in humans

The autoimmune regulator gene (AIRE) was first positionally cloned in autoimmune polyendocrine syndrome type I (APS I) and autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) patients [50, 51]. The disease APECED is rare and characterized by a set of three syndromes including chronic mucocutaneous candidiasis, hypoparathyroidism, and Addisons's disease. Often APECED patients develop additional clinical manifestations, such as thyroiditis, type 1 diabetes, ovarian failure, alopecia or hepatitis [52]. APECED is characterized by circulating autoantibodies against self-antigens expressed in affected tissues. Most

autoantibodies have been found to be specific for enzymes in neurotransmitter and hormone synthesis [53]. Recently, antibodies against IFN-α subtypes and IFN-ω have also been discovered, which are thought to be valuable diagnostic markers for APECED [54]. Despite being a monogenic disease, there is a striking variation in clinical symptoms in patients suffering from APECED, even in twin-siblings with identical mutations, suggesting that additional genes and/or environmental factors might be involved. However, since the available studies on APECED are based on small patient numbers from genetically isolated groups it is difficult to pinpoint these factors.

After the discovery of the Aire gene in humans, the mouse models for APECED were created [15, 16, 55-59]. All these Aire-deficient mice have greatly contributed to the understanding of AIRE function.

Role of Aire in central tolerance

Aire has a major role in the negative selection of T cells by inducing TRA expression in mTECs [15]. This was confirmed in studies using transgenic mice harboring transgenic T cells specific for a defined antigen, which was expressed under the Aire-regulated insulin promoter [60, 61]. In Aire-deficient mice, there was an increase of antigen specific T cells in the periphery due to impaired deletion in the thymus [62, 63]. In addition, Aire was shown to exert its effect in a dose dependent manner, where AIRE homozygous mice displayed a less efficient thymic deletion than AIRE heterozygous mice [64]. Surprisingly, it also appeared that next to regulating the expression of TRAs in mTECs, AIRE had an additional function in thymocyte deletion. This became clear when negative selection of antigen specific T cells was impaired in Aire-deficient mice even though the antigen was normally expressed in the thymus [61]. Correspondingly, other Aire-deficient mice displayed circulating antibodies against the self-antigens α-fodrin [55] and pancreas-specific protein disulfide isomerase [58] that are expressed in the thymus in an Aire-independent manner. In both of these studies the autoantibodies did not seem to be pathogenic but reflected B cell activation through primed and expanded T cells.

Data supporting alternative roles of Aire in tolerance induction revealed that a whole battery of additional genes is expressed under the control of Aire that is not linked to expression of peripheral-tissue antigens. Many of these loci encoded proteins involved in antigen processing/presentation and thymocyte trafficking (chemokines and

cytokines) [61]. These changes might very well influence thymocyte access and attachment to mTECs, as illustrated by the reduced expression of the chemokine CCL22 in Aire-deficient mTECs [61]. CCL22 is required to attract thymocytes to the medulla to finalize their maturation process [65]. In contrast, CCL19 and CXCL10 are upregulated in the Aire-deficient cortico-medullary junction and aid SP T cells to leave the thymus through the blood vessels [66]. Taken together, these reports indicate that Aire might play multiple roles in central tolerance.

Role of Aire in peripheral tolerance

Although Aire is principally expressed in the thymus, it has also been detected in peripheral tissues. The function of this expression in peripheral lymphoid organs has however been controversial [15, 67-69]. Two recent reports offered arguments in favor of a contribution of Aire in establishing peripheral tolerance. The first study identified a fraction of nonhematopoietic cells in the mesenteric lymph nodes that express Aire and a repertoire of TRAs, which were mostly overlapping with those of mTECs. Presentation of a transgenically targeted antigen by these stromal cells led to activation and subsequent deletion of T cells [69]. However, it was not investigated if the array of TRAs is Aire-dependent. The second study found stromal cells in peripheral lymph nodes, spleen, and Peyer's patches, that expressed Aire and mediated deletion of autoreactive T cells [70]. The TRA repertoire in these cells appeared to be more restricted and overlapped only minimally with the TRA repertoire of mTECs. As in the first study, T cells encountering the transgenically targeted antigen on these stromal cells underwent activation followed by death. Whereas the first study proposed the peripheral tolerance to be a backup for central tolerance because of the overlapping TRA repertoires, the second study rather suggests a complementary role due to the mainly distinct TRA repertoires in the thymus and the periphery.

Several reports have suggested a contribution of Aire to hematopoietic cells function in the periphery. Strikingly, Aire-deficient mice were found to have an increased proliferative response upon immunization with the foreign HEL antigen [16]. This might be caused by the increased number of peripheral APCs, which also display an altered phenotype in both Aire-deficient mice and APECED patients. These APCs expressed higher levels of vascular cell adhesion molecule-1 (VCAM-1), which in turn partly provided them with an increased ability to activate naïve T cells [71]. Also, aging Aire-deficient mice were found to develop increased levels of various autoantibodies,

marginal zone B-cell lymphoma, and liver infiltrates of B cells [72], indicating an overstimulation of B cells. This overt activation of B cells could be explained by recent findings showing that Aire is involved in regulation of the IFNγ signaling pathway in peripheral DCs. The absence of Aire leads to augmented signaling downstream of the IFNγ receptor and increased production of the cytokine B-cell-activating factor of the TNF family (BAFF) by the DCs. BAFF specifically binds to B cells and is required for maturation and plasma cell survival. Hence, the higher BAFF levels can explain the enhanced activation of B cells [73]. Taken together, the deficiency of Aire may lead to breakdown of several central and peripheral tolerance mechanisms. Therefore, Aire-deficient mice provide a unique tool for investigating mechanisms behind autoimmune diseases.

RHEUMATOID ARTHRITIS

Rheumatoid arthritis (RA) is a common chronic inflammatory disease affecting peripheral joints in approximately 1% of the world population. RA is considered an autoimmune disease because of the presence of autoantibodies such as rheumatoid factors (RF) and anti-citrullinated protein antibodies (ACPAs), which can be detected years before clinical manifestation of RA [74, 75]. As for many autoimmune diseases women are more affected than men. Furthermore, the incidence of RA generally increases with age. Compared to a healthy joint, a typical rheumatic joint features immune complexes in the articular cartilage layers and variable numbers of macrophages, T cells and plasma cells in the synovium, often accompanied by fibrosis and synovial hyperplasia. This condition leads to gradual destruction and deformation of the joint cartilage and the underlying bone in mostly hands, feet and spine. However, larger joints like knee and shoulder can also be affected, and in some cases extra-articular manifestations in vasculature and organs such as the lung can be observed.

The etiology of RA is largely unknown, but genetic factors as well as environmental factors are believed to increase the risk. First historical evidence for RA dates back several thousands of years and can be located to North America. Interestingly, the incidence of RA to date is still very high in this region and reaches up to 5% in certain groups [76]. The first signs of RA in Europe can be found in paintings of the 17th century created by Dutch artists, such as Van Gogh. However, about 300 years had to pass from the first case report in the 17th century until RA was exactly defined and distinguished from diseases with similar symptoms, such as osteoarthritis, systemic lupus erythematosus, and others.

RISK FACTORS OF RHEUMATOID ARTHRITIS

Estimations from twin-studies revealed a 60% chance of inheritability of RA [77], showing that genetic factors have a high influence on the development of the disease. With a 30% contribution to the total genetic risk, the HLA (human leukocyte antigen) locus was found to be the most important. Within the HLA gene cluster, the HLA-DRB1 alleles encoding the β-chain of the class II molecule HLA-DR are associated with higher risk. A number of RA associated alleles share a conserved amino acid motif that constitutes an α-helical domain shaping one side of the antigen-presenting peptide groove of the DRβ-chain. This motif, called the shared epitope, is

thought to be involved in presentation of arthritogenic peptides to T cells in different stages [78].

PTPN22, a gene encoding the intracellular tyrosine phosphatase Lyp in lymphocytes, was also found to be associated with RA, however to a lesser extent than the HLA locus. A mutation in PTPN22 leads to a stronger negative regulation of T cell activation [79, 80]. This might result in a failure of deleting autoreactive T cells during thymic selection due to increased threshold for negative selection. Alternatively, it might influence the activation of Tregs and hamper suppressor functions [80]. Interestingly, the PTPN22 polymorphism was also shown to have effects on B cell numbers and the stimulation level through the B cell receptor [79].

Recently, a T cell-related RA associated SNP on STAT4 has been indentified in a North American population [81]. STAT4 is expressed in lymphoid and myeloid tissues and is a transcription factor involved in development of Th1 and Th17 responses [82].

Apart from genetic factors, a number of environmental factors have been indentified as risk factors for RA. One of the most prominent environmental risks is smoking, which has been shown to correlate in a dose dependent manner with development of RF [83, 84]. An insteresting study also suggested that smoking might be inducing citrullination of proteins and by that formation of ACPAs in RA patients carrying the shared epitope [85]. Citrullination and other posttranslational modificiations will be discussed in more detail below.

Infectious agents were also suspected to favor RA development. Up to 20% of early RA patients have serological indication for recent infection of for example Epstein-Barr virus (EBV) and Parvovirus or bacteria, such as *Streptococcus, E. coli* or *Mycoplasma*. However, none of these infectious organisms could be conclusively pinpointed as being the cause, indicating that the total infection status might be more important than a single agent in the early phase of disease [86].

Advancing age is a strong and inevitable risk factor for developing RA [87] because the mean onset of disease is around the age of 50 and the incidence is increaseing with progessing age. The process of aging affects all aspects of immunity but especially the adaptive immune system. An aged immune system is less efficient in mouting adaptive immune responses through rapid clonal expansion of antigen specific T cells. Due to the reduced thymic activity in adults, the constantly diminishing lymphocyte pool is replenished by homeostatic proliferation of mature cells instead of

novel cell generation as in childhood. Firstly, this leads to faster senescence of the cells which is reflected in the shortening of the telomeric ends of chromosomes. Interestingly, RA patients have been shown to have a significantly older immune system than comparable healthy individuals and importantly, the age phenotype could be observed prior to arthritis onset [88]. Secondly, homeostatic proliferation occurs under selective pressure with potential loss of TCR diversity. Thus it can be hypothesized that lymphopenia, peripheral repertoire selection and reduced diversity provide prerequisites for autoimmune deviations [89]. Remarkably, senescent T cells have been shown to change their phenotype by downregulating co-stimulatory molecules, such as CD28 and CD40L and upregulating other stimulatory molecules including killer cell immunoglobulin-like receptors and others. Thus, senescent T cells inappropriately express a set of molecules that allows them to receive various unconventional costimulatory signals in the synovial membrane, which may be enough to maintain a chronic autoreactive T-cell response.

PLAYERS IN ARTHRITIS PATHOGENESIS

In RA, most of the aberrations of the immune system are systemic, but the main target organs in established disease are the joints. It is still unclear how this joint-specificity is achieved. As mentioned earlier, an arthritic joint is characterized by synovial inflammation and destruction of joint cartilage and bone mediated by local production of proinflammatory cytokines and matrix metalloproteinases (MMP). The healthy synovium consists of a thin layer of macrophage-like and fibroblast-like synoviocytes. These cells ensure production of extracellular matrix, provide a smooth and low-resistance surface at the joint interface and allow diffusion of nutrients to the cartilage. The synovium in RA on the other hand, forms an inflamed invasive tissue packed with immunocompetent cells. T cells are the most abundant cells making up 30-50% of the arthritic synovium with a majority being $CD4^+CD45RO^+$ memory cells and a small number being $CD8^+$ cells [90]. Approximately 15-20% of RA patients have lymphoid follicle-like structures with germinal centres in the synovium that provide a potent milieu for antigen recognition by T and B cells presented by follicular and myeloid DCs [91]. The antigen presented in these structures does not need to be locally expressed, but can be caught by follicular DC from the bloodstream and transported into the synovial tissue by migrating DCs. Thus, the availability of such ectopic lymphoid structures may be an important factor in sustaining a self-directed immune

response in the tissue [92]. An additional layer covering the described cell groups is made up of infiltrating and activated macrophage-like and fibroblast-like cells. This intimal lining of the synovium produces a whole range of proinflammatory cytokines, chemokines and growth factors, which in turn activates the local fibroblast-like synoviocytes to produce cytokines such as IL-6 and MMPs. This inflammatory network recruits more cells to the joint including macrophages, osteoclasts and invasive fibroblast like synoviocytes, which shapes an invasive tumor like structure called the pannus with highly erosive effect on cartilage and bone structure. Interestingly, the pannus contains relatively few T and B cells (reviewed in [90]).

DIAGNOSIS AND TREATMENT

RA can be considered a collection of symptoms, differing in severity and progression. In a joint effort the American College of Rheumatology (ACR) and the European League Against Rheumatism (EULAR) have recently introduced a new set of classification criteria for RA [93] to replace the traditional classification criteria, which have been defined over 20 years ago [94] and are widely used in the clinics. Modern and early detectable serological (ACPAs and RFs) and acute phase parameters (ESR and CRP), which were shown to have high specificity for RA have been exchanged for older long term parameters such as radiographic detection of erosive joints. This should facilitate the identification of patients in early stage of disease whereby increasing the benefit from early therapeutic intervention. Over the past decade a set of disease-modifying anti-rheumatic drugs (DMARDs) has greatly ameliorated disease symptoms in RA patients. In particular metothrexate in combination with TNF-α blockers has brought benefit to a subgroup of patients [95]. Other cytokine blockers are reaching the market, such as anti-interleukin (IL)-6 [96] and anti-IL-15 therapy [97] which have promising anti-inflammatory capabilities. Other successful therapy strategies are aimed at blocking T-cell co-stimulation with a recombinant protein comprising CTLA-4 fused to immunoglobulin (Ig), [98] or by B cell depletion with an anti-CD20 antibody [99]. Although these therapies are greatly alleviating symptoms and dampening disease progression, one has to keep in mind that they are not curing the disease.

AUTOANTIGENS AND POSTTRANSLATIONAL MODIFICATION IN RA

The first autoantigen that was suggested for RA has been immunoglobulins (IgG) because of the occurrence of rheumatoid factor (RF), an antibody reactive with

the Fc portion of IgG, in the serum and synovial fluid of RA patients [100]. However, RF is not specific for RA but can also be detected in other autoimmune diseases, infections and even healthy individuals. Therefore, it is unlikely that IgG is the pathogenic antigen driving the destructive autoimmune inflammation in RA. Other antigens of clinical relevance have been shown to be either ubiquitously expressed molecules, including glucose-6-phosphoisomerase [101], heterogeneous nuclear ribonucleoprotein-A2 [102], the stress protein BiP [103] or joint-specific proteins, such as aggrecan [104], human cartilage gp39 [105], and collagen type II [106]. Given the heterogeneity of clinical and pathological aspects of RA, it seems likely that different antigens are dominating in different subgroups of RA patients.

Recently, it was discovered that a whole range of autoantibodies in RA patients was citrullinated protein-specific, including citrullinated fibrinogen, vimentin, fibronectin, α-enolase and again CII. The process of citrullination (deamination of arginine into citrulline) is a posttranslational modification occurring naturally on many different proteins and is necessary for physiological processes, such as gene regulation and brain development to name a few. However, apoptosis and inflammatory conditions, such as during RA, are thought to activate further pathologic citrullination, which allows for accumulation of citrullinated proteins. The exact implication of these proteins in the autoimmune pathology is not known. Nonetheless, ACPAs are of great value for diagnosis of disease because of their high specificity for RA (96%) [107, 108].

There is a plethora of posttranslational modifications and they are of major importance for the well functioning of an organism. They involve modification of amino acids (arginine/citrulline), addition of chemical groups (acetylation, phosphorylation) and sugar moieties (glycosylation), cleavage, and other changes on proteins. Stress conditions as inflammation can trigger production of radicals and reactive oxygen species that can induce various uncontrolled modifications. This has very important implications in autoimmune diseases, since these modifications may create neo-self-antigens against which the immune system is not tolerized [108]. Glycosylation of CII, an additional important posttranslational modification in RA and its animal model CIA, will be discussed in more detail below.

THE NEED FOR ANIMAL MODELS

It is difficult to understand mechanisms of RA and to develop optimal treatments by studying the disease in humans although this would obviously be the most direct approach. Investigations on initial immune responses setting off the disease are basically impossible because at the time of diagnosis the subclinical disease course might have been going on without obvious symptoms for some time. Individual medication, enormous genetic and environmental variation between persons, and ethical issues on human experimentation are other drawbacks for this type of research. Therefore, animal models are helpful tools to circumvent some of these problems by controlling environmental conditions in animal houses and reducing genetic complexity by the use of inbred strains. Another advantage of using mouse models in particular is the possibility of generating knock-out/in and transgenic mice to test the molecule of interest in a given disease. However, one should not forget that none of the animal models for RA truly reflects the human disease, but the models imitate diverse features and can be used as tools to understand particular pathways.

Many mouse models have been used to study the central role of $CD4^+$ T cells in promoting and controlling crucial steps in autoimmune responses due to the strong linkage between MHC class II, CTLA-4, PTPN22, and RA. Among those, CIA is the most widely used animal model for RA.

COLLAGEN-INDUCED ARTHRITIS

CIA was first described over 30 years ago in rats, mice, and primates [109-111]. CIA is induced by intradermal injection of heterologous CII in adjuvant in susceptible animals. The effect of the immunization can be detected in the secondary lymphoid tissues some days later and three to six weeks after immunization mice develop arthritis with swelling and redness in the peripheral joints. In contrast to RA, murine CIA is mostly an acute inflammation, which resolves 2-4 weeks later whereas chronic relapsing disease is only observed in certain mouse strains after immunization with homologous CII [112-114], or rat CII with a subsequent booster injection [115], or in IL-4 deficient mice when immunized with heterologous CII in Incomplete Freund´s Adjuvant (IFA) [116]. As in RA, the inflammation starts with infiltration of macrophage-like ($CD11b^+$) cells expressing high levels of MHC class II and $CD4^+$ T cells into the marginal zone of the joint [117]. By the time when clinical arthritis

becomes apparent, edema formation is accompanied by massive infiltration of granulocytes. Finally, pannus tissue forms along the marginal zone by abnormal proliferation of activated macrophages, fibroblasts, T cells and DCs [118]. At this stage bone and cartilage destruction becomes apparent, as detected through the release of cartilage oligomeric matrix protein (COMP) in serum [119] and leads to deformed and stiff joints because of uncontrolled neo-formation of bone tissue.

As for RA, susceptibility to CIA is genetically associated with the MHC class II region. Susceptible mouse strains express the MHC class II haplotypes H-2^q or H-2^r [120]. Unlike the shared epitope in humans, these two murine MHC molecules however do not share the same sequence specificity. Mice with the H-2^q haplotype develop arthritis upon immunization with rat, bovine, human or chick CII. In contrast, H-2^r expressing mice are more restricted and develop disease only after bovine and porcine CII immunization. Humanized transgenic mice expressing HLA-DR4 and DR1 were shown to develop arthritis with a very similar CII binding pattern as the mouse H-2^q counterpart [121-123]. These data not only show that the mouse model shares many similarities with the human disease but also point to a direct link between the HLA-DR alleles associated with susceptibility to RA and the development of immunity to CII in RA patients.

Other non-MHC genes have also been identified as regulating arthritis in murine CIA, including Ncf1. The Ncf1 gene encodes the p47phox protein of the phagocytic nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex and has first been positionally cloned in rats [124]. Rats, carrying a certain Ncf1 mutation that causes low production of reactive oxygen species (ROS), were shown to develop more severe arthritis caused by a changed oxidation status of arthritogenic T cells [125]. Similar results were obtained in mice with a comparable Ncf1 mutation [126, 127]. Thus, the reduced ROS production lowers the threshold for T cell activation and promotes CIA susceptibility.

COLLAGEN TYPE II

CII is the major component of hyaline cartilage and is exclusively expressed in the cartilage and the vitreous body of the eye. Chondrocytes are responsible for the production of CII. Between the actual synthesis of the three α1(II)-chains and the formation of the triple helical structure, CII is undergoing posttranslational modifications on defined amino acids [128, 129]. These include proline and lysine,

which can be hydroxylated when positioned N-terminally to glycine. In a next step, hydroxylysine can be further glycosylated in maximally two steps resulting in four different posttranslational variants: lysine (K), hydroxylysine (HyK), β-D-galactopyranosyl-hyrdoxylysine (GalHyK) and α-D-glucopyranosyl-(1>2)-β-D-galactopyranosyl-hyrdoxylysine (GlcGalHyK). However, the degree of hydroxylation and glycosylation of CII is varied and dependent on the functional state of the chondrocyte.

The immunodominant T cell epitope in T cell tolerance

In H-2^q expressing mice, the immunodominant T cell epitope of CII has been identified and is located within the region of the residues 260-270 of CII (CII260-270) [130, 131]. This sequence is identical between rat, human, bovine and chick CII but differs in one amino acid at position 266 in mouse CII, where a glutamic acid on heterologous CII is exchanged for an aspartic acid in mouse [130]. This difference was found to critically influence the binding affinity to the MHC class II molecule and may offer an explanation as for why the heterologous CII is a stronger inducer of disease than the corresponding mouse CII peptide with the lower affinity [132]. In addition, this low affinity-binding between the self-CII peptide and the MHC molecule may represent a typical self-antigen that is recognized by autoreactive T cells escaping tolerance, as discussed above.

Two different transgenic mice have been established expressing the heterologous CII260-270 epitope in a mutated mouse CII protein restricted in cartilage (MMC mouse) or systemically in type I collagen (TSC mouse). These constructs allow T cells in these mice to interact with self-CII and to become tolerized to the immunodominant T cell epitope present on heterologous CII. Consequently, only autoreactive T cells became activated in CII-primed MMC and TSC mice and differences between transgene-positive and transgene–negative mice were either directly or indirectly related to T cell tolerance. The data demonstrated that the immune system indeed interacts with self-CII under physiological conditions. However, this interaction did not necessarily lead to complete T cell tolerance and protection from CIA [133]. Instead, the level of T cell tolerance and CIA-susceptibility seemed to be influenced by the availability and the location of the antigen. TSC mice, which express the heterologous CII260-270 epitope more ubiquitously, were completely tolerized, whereas the MMC mice with the joint-restricted self-CII epitope expression exhibited

incomplete tolerance. CII-specific MMC T cells displayed a reduced proliferative capacity while still producing detectable IFN-γ levels and aiding B cells to produce class-switched anti-CII antibodies. Although MMC mice were less susceptible to CIA, some still developed arthritis with similar severity as non-transgenic littermates. Comparable results were also observed in transgenic mice expressing human CII in a cartilage-restricted fashion. Although reduced susceptibility to CIA after immunization with human CII was observed, some mice still developed arthritis [134].

Importantly, the MMC mouse model also revealed that posttranslational modifications of the CII260-270 epitope strongly influence the level of T cell tolerance to self-CII. More specifically, tolerance to self-CII in H-2^q mice was found to primarily affect T cells specific for the non-glycosylated version of the CII260-270 epitope [135]. The remaining autoimmune response in MMC mice was strongly biased towards the galactosylated CII260-270 peptide, which was also correlated with the development of CIA in MMC mice [136, 137]. In line with the mouse data, RA patients expressing the shared epitope were also found to predominantly respond to the galactosylated CII260-270 peptide [138].

Initially, this suggested that the non-modified CII peptide would be more accessible *in vivo* in the MMC mouse for induction of T cell tolerance, compared to the galactosylated CII-peptide. However, CII prepared from both healthy rats and humans was found to be uniformly galactosylated [139, 140]. Furthermore, by crossing MMC mice with a TCR transgenic mouse specific for the hydroxylated CII-peptide it was shown that T cells remained unaffected, suggesting that the hydroxylated CII-peptide is not available for immune recognition and tolerance induction in the naïve MMC mouse [139]. Hence, it is still uncertain how or where partial tolerance to self-CII is induced and which posttranslational modifications of CII tolerize or trigger arthritogenic T cells.

T CELLS AND B CELLS IN ARTHRITIS

The role of T cells in both RA and CIA has regained interested with the discovery of IL-17, a T-cell derived proinflammatory cytokine involved in joint inflammation and destruction. Previously, RA and CIA were regarded as Th1-driven diseases supported by the predominance of IFNγ and a lack of Th2 cytokines, such as IL-4. Although not conclusively demonstrated, an active role for CII-specific T cells during clinical arthritis in CIA was indirectly supported by their presence in the arthritic joints [141, 142], and also a number of studies have shown amelioration of disease by

reducing Th1 cytokines [143-145] or increasing Th2 cytokines [145], which were found to antagonize Th1 cytokine production. However, determining the role of Th1 and Th17 cells in mediating effector functions and regulating the initiation or progression of CIA and RA is challenging as the lymphoid response varies over time and in between lymphoid organs and the joints [146, 147]. The role of IFNγ and IFNγ-signalling in CIA and other organ-specific autoimmune diseases is complex and has now been associated with both proinflammatory and anti-inflammatory functions [148-153]. Furthermore, the prerequisite of an IL-12-mediated Th1 establishment in autoimmune inflammation was questioned in several studies [154-156]. It soon became clear that the heterodimer IL-12 (IL-12p40/IL12-p35) is sharing a subunit with IL-23 (IL-12p40/IL-23p19) [157], which explained the discrepancy seen in the different knockout mice affecting the IL-12/IFNγ pathways, such as IFNγ$^{-/-}$, IL-12p40$^{-/-}$, and IL-12p35$^{-/-}$ mice. Therefore, it appeared that many autoimmune mechanisms that previously had been attributed to IL-12 (CIA, EAE) were actually caused by effects of IL-23.

Today, we know that IL-23 is required for expansion of the newly established Th17 cell subset and that a combination of the cytokines TGF-β, IL-6 and IL-1 is required for differentiation of these cells (reviewed in [158, 159]). IL-17 was shown to be important in development of CIA, since IL-17$^{-/-}$ mice were protected from disease [160]. The precise molecular effects of IL-17 in arthritogenesis are not well understood but it was found to be associated with the process of bone destruction. Th17 cells are thought to promote joint degradation by induction of MMPs and RANKL expression on T cells and synovial fibroblasts. RANKL and proinflammatory cytokines including IL-17, TNF-α, and IL-1 were found to drive osteoclast differentiation and bone erosion [161]. In addition, IL-17 was revealed to recruit neutrophils and monocytes by inducing various chemokines, which in turn mediate inflammation in RA [162]. However, other reports suggested that Th1 cells are more important than Th17 cells in inflamed joints of RA patients [163]. Also, the conclusion was drawn that Th1 and Th17 cells are relatively plastic, as shown in a mouse study where differentiated Th17 cells rapidly responded to IL-12 *in vitro*, by upregulating IFNγ production and downregulating IL-17 expression [164]. Moreover, Th17 cells seem to be associated with Tregs, as suggested by the common use of TGF-β for induction and the close relation ship of the Th17-associated transcription factor RORγt and the Treg factor Foxp3 [159].

Besides producing proinflammatory and regulatory cytokines, T cells are also believed to provide help to B cells to produce autoantibodies in arthritis [165]. The RA synovium contains approximately 5% of B cells, which are thought to undergo clonal expansion triggered by antigen-driven maturation. This may lead to local production of RF, ACPAs, and anti-CII autoantibodies in many patients [90]. Interestingly, B cells also have a reciprocal role on T cells by regulating T-cell infiltration into the synovial tissue. This was concluded from experiments using severe combined immune deficiency (SCID) mice that were transplanted with RA-synovium and adoptively transferred with human RA T cell clones. Depletion of B cell originating from the transplant prior to transfer resulted in a diminished infiltration and activation of T cells [166]. In line with this, depletion therapy of B cells in RA patients using anti-CD20 antibodies has a strong ameliorating effect on the arthritis symptoms [99]. Of note, RA patients frequently display antibody titers against CII, reminiscent of the observation made in mice during CIA. The transfer of human sera containing high levels of anti-CII antibodies can induce arthritis in mice [167]. Even though the role of these antibodies in human arthritis is unknown, these experiments suggest that the anti-CII antibodies have the potential to initiate an articular inflammatory response. In accordance with this potential function is the observation that anti-CII antibodies can be detected in RA cartilage, but not in osteoarthritic cartilage [168].

In CIA, several studies have revealed the importance of B cells in arthritis pathogenesis. Adoptively transferred CII-specific T cells alone were not potent enough to induce clinically apparent arthritis although microscopic changes in the joints were detected [169], and B-cell deficient mice were protected from CIA [170]. Moreover, arthritis could be induced in normal mice by injecting serum from arthritic mice or a cocktail of monoclonal anti-CII antibodies [171, 172]. Along with the fact that development of arthritis was only reported in mice immunized with native CII and not with the single immunodominant T cell peptide, this indicates that the availability of B-cell epitopes on the administered CII is crucial for the production of arthritogenic anti-CII antibodies that bind to the triple helical structure.

Despite an obvious role of anti-CII antibodies in arthritis it is not clear which factors are important for antibody pathogenicity. Both genetically susceptible as well as non-susceptible mouse strains were reported to produce anti-CII antibodies, however the latter did not develop arthritis [152, 173, 174]. Data on this matter suggest that a combination of antibody specificity for various CII-epitopes, the antibody isotype and

the quantity of antibody strongly modulates disease induction [172]. These factors were shown to influence the interaction of the CII-antibodies with complement cascade components as well as with Fc receptors on phagocytes [175-177].

PRESENT STUDY

Paper I. Visualization and phenotyping of proinflammatory antigen-specific T cells during collagen-induced arthritis in a mouse with a fixed collagen type II-specific transgenic T-cell receptor β-chain

The use of TCR transgenic mice has proven a powerful tool for investigating the nature of self-reactive T cells in tolerance and autoimmunity. To investigate the role of antigen-specific T cells in CIA the Vβ12-transgenic mouse was previously generated [178]. This mouse expresses a transgenic TCR β-chain with specificity for CII, which may combine with any endogenous TCR α-chain, leading to increased immunity to CII and increased susceptibility to CIA [136]. However, the frequency and distribution of CII-specific T cells in the Vβ12-transgenic mouse has not been determined.

The aim of paper I was to establish a system enabling identification of CII-specific T cells in the Vβ12-transgenic mouse in order to determine to what extent the transgenic expression of CII-specific β-chain would skew the response towards the immunodominant galactosylated T-cell epitope and to use this system to monitor these cells throughout development of CIA. To this end we have generated and thoroughly characterized a clonotypic antibody, which recognizes a TCR specific for the galactosylated CII(260-270) peptide in the Vβ12-transgenic mouse. We found that the Vβ12-transgenic mouse expresses several related but distinct T-cell clones specific for the same galactosylated peptide. The clonotypic antibody could specifically recognize the majority of these. Clonotypic T cells occurred at low levels in the naïve mouse, but rapidly expanded to around 4% of the $CD4^+$ T cells, whereupon the frequency declined with developing disease. Combinatorial analysis with the clonotypic antibody, the early activation marker CD154 (CD40L), and cytokine production revealed an early Th1-biased response in the draining lymph nodes that would shift to also include Th17 around the onset of arthritis. Data showed that Th1 and Th17 constitute a minority among the CII-specific population, however, indicating that additional subpopulations of antigen-specific T cells regulate the development of CIA. Thus, this study presents a new tool that will greatly facilitate further investigation of the different subsets of CII-specific T cells during development and regulation of CIA at different time points and in different tissues, including joints.

Paper II. Breaking T-cell tolerance against self type II collagen in HLA-DR4-transgenic mice and development of autoimmune arthritis

RA is associated with DRB1-genes encoding HLA-DR1 and HLA-DR4 molecules. Because of the presence of anti-CII specific autoantibodies and CII-specific T cells in many RA patients, CII has been proposed as a possible autoantigen in RA. Furthermore, the DR4 molecule in both humans and humanized DR4-transgenic mice presents almost the same immunodominant peptide to CII-specific T cells as the murine MHC class II A^q-molecule. Importantly, posttranslational modifications of the immunodominant T-cell epitope have a great impact on CII-specific T cell reactivity and may be a possible cause for the tolerance breakdown to self-antigens. To investigate T cell tolerance to self-CII, A^q- and DR4-expressing mice harbouring a transgene for heterologous CII (of rat or human origin) have been established earlier [133, 136, 138]. Upon immunization with heterologous rat CII, the A^q-expressing mice display incomplete tolerance to self-CII, characterized by reduced proliferative T cell response to CII while retaining their ability to produce proinflammatory cytokines and giving B-cell help. However, DR4-transgenic mice expressing human CII in a cartilage-specific manner would exhibit total tolerance to self-CII without any signs of arthritis. However, to be able to study the interaction of the immune system with joint-derived self-antigens and the impact of posttranslational modifications in establishing immunologic tolerance, it is favourable to have a weaker tolerance effect in the humanized system.

Therefore, efforts have been made in paper II to establish a new animal model in DR4-transgenic mice in which T-cell tolerance to self-CII could be broken and allow for development of autoimmune arthritis. To achieve this goal DR4-transgenic mice expressing either the entire human CII protein (HuCII) or the immunodominant T-cell epitope of heterologous CII (MMC) in joint cartilage were established on different genetic backgrounds, and susceptibility to CIA was tested. We found that HuCII mice displayed stronger T-cell tolerance to heterologous CII than did MMC mice. On the B10-background, arthritis developed only in MMC mice with a defective oxidative burst. However, MMC mice on the C3H background were susceptible to arthritis also with a functional oxidative burst. With regards to posttranslational modifications, significant recall responses in tolerized mice were detected only against the non-glycosylated CII250-270 epitope. Although the recognition of the CII260-270 epitope

was heterogeneous, the majority of T cells in DR4 mice specifically recognized the non-glycosylated side chain of the critical lysine at position 264. These data showed that arthritis susceptibility is tightly controlled by the genetic background and by the source of the transgenic element for expressing the heterologous CII peptide as a self-CII protein in the joint. In contrast to CIA in A^q-expressing mice, the non-glycosylated CII260-270 epitope was clearly immunodominant in both tolerized and non-tolerized DR4 mice.

Paper III. Tolerance to glycosylated self-CII is regulated in the periphery and leads to protection from collagen-induced arthritis

Immunization of susceptible strains with CII leads to development of CIA. To further define the interaction between the immune system and self-antigens in cartilage, we generated a novel T cell receptor (TCR) transgenic mouse, denoted HCQ.3, and crossed it to the earlier described MMC and TSC mice [133]. The transgenic TCR is highly specific for the galactosylated immunodominant T cell epitope of CII (CII260-270). The MMC- and TSC mouse express the heterologous CII260-270 in cartilage-restricted and systemic fashion, respectively. The amino acid exchange from aspartic acid (mouse CII) to glutamic acid (rat CII) at position 266 increases binding to the MHC class II, which results in more effective presentation of the peptide in vivo. As a result, the MMC mouse and TSC mouse are protected from CIA on the B10.Q background and show strong T cell tolerance to CII.

In paper III, we have thoroughly characterized the immune response of the HCQ.3 mouse to CII and investigated the tolerance induction mechanisms in the context of MMC and TSC. Transfer experiments showed that CII-specific T cells interact rapidly with CII in the peripheral joint draining lymph nodes. This interaction did not result in complete deletion of autoreactive T cells as CII-specific T cells were maintained in MMC mice. HCQ.3 mice were more susceptible to CIA than non-transgenic littermates. Still, the CII-skewed TCR repertoire was not sufficient to break tolerance in MMC and TSC double transgenic mice, even though significant pro-inflammatory Th1 and Th17-responses against self-CII could be documented. Instead arthritis protection was associated with a significantly decreased anti-CII antibody response in MMC and TSC expressing mice. Due to the increased frequency of CII-specific T cells in naïve HCQ.3 mice, this model is very suitable for investigating

induction and maintenance of tolerance towards self-CII in a normal, non-inflamed environment, in contrast to the Vβ12-transgenic mouse in paper I. By further increasing arthritis susceptibility in these mice, it will also become possible to investigate how breaking T cell tolerance to self-CII may cause development of autoimmune arthritis in HCQ.3-MMC double transgenic mice.

Paper IV. AIRE-expression is specifically associated with controlling tolerance to non-glycosylated collagen type II in collagen-induced arthritis

Development of CIA is dependent on T-cell recognition of the CII260-270 peptide. Transgenic expression of the heterologous CII epitope in cartilage in the MMC mouse induces tolerance to self-CII after immunization with rat CII as shown in paper II and III. Although the role of CII as a relevant autoantigen in RA is unclear, CII may still serve as an excellent model autoantigen for understanding how the immune system interacts with joint-derived self-antigens in order to establish immunologic tolerance and for understanding how posttranslational modifications may influence these processes. An intriguing problem that remains to be solved is how T cell tolerance to the autoantigen CII is achieved. It was suggested that CII may be expressed in human and mouse mTECs and that Aire drives expression of a wide array of TRAs in these cells. Deficiency of Aire in humans leads to a multi-organ autoimmune disease, APECED. Mice lacking Aire develop similar symptoms and exhibit a decreased expression of TRAs in mTECs. Taken together, these facts lead to an interesting possibility that Aire might be controlling central tolerance induction to CII.

The aim of paper IV was to investigate how tolerance to self-CII is achieved. Introduction of the Aire-deficiency in MMC-mice was found to overcome arthritis resistance in B10.Q.MMC mice. Development of arthritis in Aire-deficient B10.Q.MMC mice was associated with a specific loss of tolerance towards the non-glycosylated version of the CII260-270 epitope, whereas T cell tolerance towards the glycosylated version remained intact. Although we failed to identify Aire-dependent expression of CII within the thymus, these findings clearly show that tolerance to non-glycosylated and glycosylated self-CII is regulated by distinct mechanisms. Furthermore, this finding helps to explain the earlier enigma as to why T cell tolerance primarily affects T cells specific for non-glycosylated self-CII, despite the fact that self-CII derived from healthy cartilage is only available in its glycosylated form.

CONCLUDING REMARKS

RA, as far as it concerns our current understanding, is an autoimmune disease with involvement of many different cell types, which are in continuous communication with each other. It is however not clear how the generally systemic aberrations of the immune system translate into joint-specific pathology. The inflamed joint synovium is a dynamic tissue with high density of MHC class II molecules expressed on DCs, macrophages, fibroblasts, B cells, and mast cells, which all can communicate with T cells. The role of T cells in RA patients is not fully understood, and it is likely that several autoantigens are involved in arthritis development. Although the role of CII as a relevant autoantigen in RA is unclear, CII may still serve as an excellent model antigen for understanding how the immune system interacts with joint-derived self-antigens. Even though it is accepted that T cells play an important role in arthritis development, it remains controversial where and how they contribute to pathogenic mechanisms after loss of tolerance.

In this thesis we were able to contribute to the understanding of tolerance mechanisms in CIA. We show that the strength of tolerance induction is dependent on the abundance of the self-antigen, the genetic background of the mice, as well as the presence or absence of posttranslational modifications on CII. Moreover, data indicate that joint-specific antigens are readily available for presentation in draining lymph nodes to induce immunological tolerance in the periphery in a non-inflammatory environment. Furthermore, a defect in thymic tolerance induction suggests that certain CII modifications are presented differentially depending on the location in the organism. Thus, the variation of the posttranslational modification levels of antigens over time and location may have important implications in tolerance to self-antigens. This holds true for a healthy milieu as well as in a stressed environment, such as during inflammation, infection, trauma or aging, where inappropriate posttranslational modifications can lead to accumulation of neo-epitopes. These could induce priming of naïve T cells and promote induction of autoimmune responses.

Still, important questions in tolerance induction remain unanswered. What are the regulatory processes utilized by the joint-resident cells to inhibit activation of CII-specific T cells and subsequent inflammation? Are these processes imposed by APCs and/or Tregs and which molecules and signals could be involved? Are the T cells directly involved in arthritogenic mechanisms in the joints or do they trigger other cells

systemically and their own infiltration into the joints is only a secondary effect?

I am confident that the new mouse models developed in the presented thesis will help to answer some aspects of these questions and to further elucidate the arthritogenic action of T cells in disease relevant sites. This will hopefully enlarge the mechanistic framework for further investigation of human disease pathogenesis, which might lead to new therapeutic strategies to promote self-tolerance in diseased individuals.

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