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Title: Immune regulation by CD49b+ CD4+ T cells & modulation of B cell responses
Issue Date: 2016-11-29
Chapter 1

Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune disease characterized by inflammation in the joint, leading to the damage of cartilage and bone. It affects approximately 1% of the general population [1].

The pathogenesis of RA is not fully understood. Infiltration of immune cells such as B cells, T cells, dendritic cells (DCs) and neutrophils, into the synovial tissue, together with a systemic presence of auto-antibodies constitute the hallmark of RA. The infiltrating immune cells are considered to be an important source of cytokines, such as interleukin-6 (IL-6), tumor necrosis factor α (TNF-α) and other pro-inflammatory cytokines. The produced cytokines will provide an inflammatory loop, by activating immune cells, stromal cells or osteoclasts and/or by attracting additional immune cells, which will ultimately result in severe inflammation in the joints.

In addition, the presence of auto-antibodies in most RA patients and the efficacy of B cell-depleting therapies points to a prominent role of B cells in the pathogenesis of RA. Several antibodies have been associated with RA, however the antibodies with the highest specificity for RA are those targeting antigens containing the amino acid citrulline, which is generated during posttranslational modification of arginine mediated by peptidylarginine deiminases (PADs) enzymes (Figure 1).

Studies to unravel the etiology of RA have led to the identification of genetic as well as environmental factors that might have a crucial role in predisposition to RA.

Figure 1. Citrullination of arginine is mediated by peptidylarginine deiminase (PAD) enzymes. Citrullination is a post-translational modification that converts arginine into citrulline and is catalyzed by PAD enzymes in the presence of calcium. Adapted from Bax M, Huizinga TW, Toes RE. The pathogenic potential of auto-reactive arthritis. Semin Immunopathol. 2014 May; 36 (3): 313-25.
**Genetic risk factors for RA**

Studies in twins have provided evidence for the involvement of genetic factors in the development of RA [2]. Genetic factors have been estimated to contribute with 50 to 60% to RA [2, 3]. The human leukocyte antigen (HLA) region, which is encoded by major histocompatibility complex (MHC) is the most important genetic risk factor for RA and contributes with 30 to 50% to the overall genetic RA susceptibility. A conserved amino acid sequence QKRAA, QRRAA, or RRRAA situated in the third hypervariable region of DRB1 chain is shared between HLA alleles of the DRB1 gene. This sequence is called the shared epitope (SE). SE-alleles were found to be associated in particular with ACPA positive RA [4].

Genome wide association studies have considerably contributed to the expansion of the list of genes related to RA [5]. In addition to HLA-DRB1, over 100 genetic risk factors conferring susceptibility to RA have been identified, including tumor necrosis factor receptor associated factor 1/complement 5 (TRAF1/C5) [6, 7], protein tyrosine phosphatase non-receptor type 22 (PTPN22) [8, 9], PAD type 4 (PADI4) [10], Cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) [11] and CD28 [5]. TRAF1/C5 encompasses two genes that lie adjacent to each other on chromosome 9: TRAF1 and C5. TRAF1 gene encodes for TRAF1 that by forming a complex with other members of TRAF proteins associate with TNFR superfamily and facilitates the signaling via TNFR superfamily. C5 encodes for the complement component 5. A polymorphism situated in the PTPN22 gene has been revealed to confer high risk to especially antibody positive RA.

PTPN22 encodes a cytoplasmic tyrosine phosphatase involved in various signalling pathways and has an important role in downregulating T cell activation by binding to Src homology (SH)3 domain of the tyrosine kinase Csk [8, 12]. It is thought that PTPN22 polymorphism associated with RA alters the binding site of the PTPN22 molecule leading to reduced binding to Csk and ultimately to reduced activation threshold. These genes are mainly associated with antibody positive disease and confer modest level of risk. Despite increasing evidence for the importance of genetic factors in the risk for developing RA, the mechanisms by which these genes contribute to the disease are still elusive.

The association of HLA-alleles with RA points to a contribution of T cells to disease susceptibility [13].

**Environmental risk factors for RA**

Smoking is the main environmental risk factor for RA [14-18]. Like most genetic risk factors, smoking is strongly associated with seropositive RA. The contribution of smoking to the risk of developing RA has been estimated to account for up to 25% in general population, which can further increase to up 35% in ACPA positive RA. Smoking has a dose dependent effect on the development of RA as the intensity and the duration of smoking were found to be related to increased risk for RA [16]. After 20 years of...
smoking cessation, the risk conferred by smoking in past smokers has been reported to be decreased to the level estimated in the general population. Exposure to cigarette smoke has been revealed to increase the induction and development of disease in a mouse model of arthritis. This does not only confirm the association studies, but also provides biological evidence for the involvement of smoking in arthritis development [19].

One mechanism by which smoking might contribute to RA is by inducing the expression of PADI, the enzyme responsible for the generation of citrullinated antigens targeted by ACPA. This hypothesis is based on studies suggesting that the prevalence of citrullination is augmented in the lungs of smokers [20]. The strong association between smoking and RA has led to the hypothesis that RA might initiate in the lungs during the preclinical phase that precede the onset of the disease [21]. Cigarette smoking in association with HLA-DRB1 has been shown to increase the risk for developing RA especially in seropositive RA [22-26].

**Humoral immunity**

**B cells**

B cells are key players of humoral immunity. The most important function of B cells is the production of antibodies. Antibodies have a key role in protecting the host from various infectious diseases. In addition to protection, antibodies can also cause damage to the host by targeting normal constituents of the body as it is believed to be the case for RA.

B cells are derived from multipotent stem cells in the bone marrow. Before leaving the bone marrow and entering the circulation, B cells undergo a series of maturation stages culminating in the generation of functional B cell receptors (BCRs) that confer B cells the ability to recognize a huge diversity of antigens. B cells with BCRs reacting with high affinity to self-antigens are cleared by clonal deletion. However, some B cells bearing self-reactive BCRs find their way to the periphery and remain silent. They can become active if peripheral tolerance is breached and contribute to autoimmune responses associated with autoimmune diseases like RA.

Encounter with an appropriate antigen in the T cell region of the secondary lymphoid organs, instigates naïve B cells to proliferate and differentiate into memory B cells and antibody secreting plasma cells. Some plasma cells have the ability to survive for a very long time or even a life time and they are therefore named long lived plasma cells (LLPC).

**Memory B cell**

A memory system is characterized by the ability to conserve the information of the antigen that induced the primary immune response and to provide long lasting protection after antigenic
resolution. After the resolution of the immune response, memory B cells are maintained in an inert state and need an antigenic stimulation for their activation. Upon activation, memory B cells rapidly differentiate into plasma cells that produce and secrete antibodies. Memory B cells are, amongst others, identified based on previous antigenic experience that is manifested by isotype switching and/or somatic hypermutation of immunoglobulins.

The expression of TNF-receptor family member CD27 is predominantly used to identify human memory B cells [27]. CD27 expression in B cells is induced following activation and correlates with the presence of mutations in the variable region of the immunoglobulin genes [27, 28]. Based on the expression of CD27, peripheral blood B cell populations are typically composed of 40-50% CD27 expressing memory cells and 50-60% CD27 naïve B cells [29]. Memory B cells can be divided into four subpopulation according to the expression of the surface immunoglobulin IgM and IgD: CD27+ IgM+ IgD- (40%) class switched cells, CD27+ IgM+ IgD+ (40%), CD27+ IgM+ IgD- (20%) and very rare CD27+ IgM- IgD+ (<1%).

**Plasma cells and Long lived plasma cells**

Plasma cells are the final product of B cell differentiation and their main function is antibody production. B cell differentiation gives rise to plasmablasts; the precursors of plasma cells. Although these two B cell populations are similar in the context of antibody production, they differ in their proliferation and migratory abilities and the expression of certain molecules. Downregulation of B cell markers including CD19, CD20, CD22, MHC class II, upregulation of CD138 and B lymphocyte-induced maturation protein (Blimp)-1 and loss of proliferation and migratory capacities constitute the characteristics of plasma cells [30].

LLPCs are plasma cells that have the potential to survive for a very long time and provide persistent antibody titre in the blood without the need for a permanent presence of an antigen [31-33]. Based on studies in mice, most LLPCs reside within the bone marrow niches; however a small proportion can also be found in the spleen. The longevity of LLPCs is determined by both the support provided by the bone marrow niches and molecular competence to receive this support [34]. Bone marrow niches can support LLPCs via cell-cell contact and/or production of survival factors.

Several factors have been described to contribute to the persistence of plasma cells in the bone marrow in mice as well as humans including TNF-α, IL-6, IL-5, signalling via CD44, CXCL12 [35, 36] and a proliferation-inducing ligand (APRIL). CXCL12 is expressed by bone marrow stromal cells and is also considered to be an important factor involved in mediating the migration of plasma cells to the bone marrow [37]. The observation that plasma cells can be found in close proximity of the bone marrow stromal cells expressing CXCL12, underscores the importance of the CXCL12-CXCR4 axis in the determination of plasma cell migration fate [38].
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The survival niche is not homogenously composed of only non-hematopoietic cells, but several types of hematopoietic cells like DCs, monocytes/macrophages and basophils have been reported to be key components of plasma cell survival niches [39-41]. Under chronic inflammatory conditions, plasma cells can also be found in the inflamed tissues like the synovial tissue of RA patients [42, 43].

CD28, which was known as an essential costimulatory molecule for the activation of T cells, is also expressed by plasma cells in humans as well as mice. Recent studies suggest that CD28 has an intrinsic role in the survival of LLPCs and maintenance of antibody production [44, 45]. Triggering of CD28 via interaction with the ligands CD80 and/or CD86 provides a survival signal to LLPCs and maintains antibody production. In an indirect way, CD28 can also mediate the survival of LLPCs by targeting DCs to produce the survival factor IL-6 [44].

Auto-antibodies in Rheumatoid arthritis

The presence of auto-antibodies in RA patients has been known for a very long time, with reumatoid factor (RF) antibodies targeting the Fc part of the immunoglobulin IgG as the first antibody type identified and used for diagnostic purposes. This was followed by the discovery of another family of auto-antibodies Anti-Citrullinated Protein Antibodies (ACPA), which together with RF are part of the American College of Rheumatology/ the European League Against Rheumatism (ACR/EULAR) 2010 classification criteria [46].

Recently several new categories of antibodies in RA patients have been reported including Anti-Carbamylated Proteins (CarP) [47] and anti-PAD antibodies [48]. The former recognizes carbamylated proteins containing a post-translationally acquired homocitrulline-residue that is generated from a lysine residue upon exposition to cyanate and the latter targets PAD4, which is the key enzyme for the generation of citrullinated proteins. The relevance of anti-PAD to the pathogenesis of RA is supported by studies demonstrating their ability to activate PAD4 by increasing calcium sensitivity [48].

ACPA

ACPA are antibodies directed against proteins containing the post-translationally acquired amino acid citrulline. Citrullination is a posttranslational modification whereby the amino acid arginine is modified into the amino acid citrulline. This process is mediated by PAD, enzymes that are encoded by genes harbouring genetic variations that have been identified as risk factor for RA-development [10]. Both the RA-specificity of the ACPA-response and the genetic associations in the genes encoding the enzymes creating the antigens recognized by ACPA, implicate a contribution of the “anti-citrulline” response to RA pathogenesis. ACPA can be present years before the manifestation of the clinical symptoms associated with RA [49] and can be detected systemically, as well as locally in synovial fluid [50, 51]. However, their presence is relatively more abundant in synovial fluid compared to serum.
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Despite several studies extensively characterizing the ACPA response and the citrullinated antigens present in the joints of RA patients (e.g. vimentin and fibrinogen) [52, 53], the antigen(s) responsible for the induction of the citrullinated protein-specific B cell response are yet unknown.

Interestingly, the presence of ACPA is predictive for a more aggressive disease course as reflected by severe joint damage [54, 55]. ACPA also have an important diagnostic value for RA. They can be detected by commercial assays such as the Cyclic Citrullinated Peptide (CCP)-2 based enzyme-linked immunosorbent assay (ELISA). By using the CCP-2 test, ACPA appear to be present in a high percentage of RA patients and to display a high disease specificity which is significantly higher than RF IgM [56]. Cross reactivity of ACPA has been reported by different groups [57-59] highlighting the importance of citrulline in the recognition of ACPA. This leads to suggest that citrulline might function as a hapten capable of inducing immune responses to multiple proteins.

Epitope spreading is the expansion of specificities of an immune response over time towards different epitopes. Analyses of sera from individuals at different stages of the disease suggested that the fine-specificity of ACPA changes and broadens before the onset of the disease, leading to an ACPA response against a large number of antigens in the established disease. Thus ACPA epitope spreading (Figure 2a) appears to take place many years before the clinical manifestation of RA and comes to a halt when the disease is established. At the time of disease onset, an increase in the recognition of citrullinated antigens in patients with undifferentiated Arthritis (UA) is associated with a rapid progression to RA within one year of follow up [60, 61].

Like epitope spreading, ACPA levels display a similar pattern of development with an increase before disease onset and stabilization in established disease. Epitope spreading before the clinical manifestation of the disease is not unique for RA, it has also been described in Systemic Lupus Erythematosus (SLE). This has led to the hypothesis that autoimmune diseases might be triggered by a limited number of auto-antigens and that the number auto-antigens/epitopes recognized broadens during the progression of the disease.

The ACPA repertoire uses antibodies displaying different forms of the Fc part including IgM, IgA and IgG, all of which have been shown to be specific for RA [62, 63] (Figure 2b). The number of isotypes is associated with radiographic progression in ACPA positive RA patients [64]. Of interest, analysis of the presence of IgA-, IgM- and IgG-ACPA in serum samples collected prior to the onset of symptoms demonstrated that full isotype usage of ACPA, like fine specificity and ACPA levels, also occurs many years before the onset of RA [65]. ACPA are specific for RA patients, however a relatively high percentage (19 %) of healthy North American first nation individuals is positive for ACPA. Interestingly, the ACPA isotype usage of healthy individuals is limited compared to patients with RA [66].
Figure 2. The ACPA response broadens as evidenced by an increase in fine specificity and isotype usage during the development of RA. (a) ACPA target various citrulline-containing antigens such as citrullinated fibrinogen, citrullinated vimentin and citrullinated α-enolase. (b) ACPA repertoire consists of different isotypes including IgA, IgG and IgM, that can activate the immune system by inducing distinct effector functions. Adapted from Willemze A, Trouw LA, Toes RE, Huizinga TW. The influence of ACPA status and characteristics on the course of RA. *Nat Rev Rheumatol*. 2012 Jan 31; 8 (3): 144-52
These observations, together with the beneficial effects observed with B cell depleting (anti-CD20) therapy [67] fuel the hypothesis that ACPA and/or ACPA-producing B cells may have a pathogenic role in RA. In addition to the production of auto-antibodies targeting citrullinated antigens, B cells can contribute to RA pathogenesis via other mechanisms such as the presentation of citrullinated antigens. Indeed, B cells derived from immunized mice have been described to possess the capacity to present citrullinated peptides to CD4+ T cells [68, 69].

The presence of citrulline specific T cells has also been reported in patients with RA [70, 71]. This, in addition to the observations that different ACPA isotypes can be detected and that ACPA generated from synovial fluid B cells of ACPA+ RA patients are highly mutated, indicate that ACPA producing B cells receive T cell help to undergo isotype switching and somatic hypermutation [59] (Figure 3).

Figure 3. ACPA producing B cells receive help from T cells to produce ACPA. Although it is not yet fully understood how the ACPA response is initiated, the following scenario is proposed; ACPA production by B cells depends on the help of T cells, which are activated by dendritic cells through presentation of peptides in the context of HLA class II molecules. B cells that have internalized citrullinated antigens (e.g. in the form of citrullinated peptide complexes), present a peptide in the context of HLA molecules encoded by predisposing HLA-alleles to T cells leading to the production of ACPA. Adapted from Willemze A, Trouw LA, Toes RE, Huizinga TW. The influence of ACPA status and characteristics on the course of RA. Nat Rev Rheumatol. 2012 Jan 31; 8 (3): 144-52.
Figure 4. Potential pathogenic roles of ACPA. The joints of RA patients are infiltrated by activated immune cells such as B cells, macrophages, T cells, and neutrophils. The presence of highly mutated ACPA and the enrichment of ACPA-producing B cells in the joints of RA patients point towards ongoing antigen-driven immune responses. ACPA have been proposed to contribute to RA pathogenesis via different mechanisms: activation of macrophages via FcγRs to produce TNF-α, activation of osteoclasts, activation of complement, and activation of neutrophils to release neutrophil extracellular traps (NETs) that has been reported to enhance inflammation. Release of NETs leads to the expulsion of inflammatory factors and citrullinated proteins, which by becoming accessible for ACPA, enhance the formation of ACPA-citrullinated proteins complexes leading to further exacerbation of inflammation. Adapted from Catrina AI, Ytterberg AJ, Reynisdottir G, Malmström V, Klareskog L. Lungs, joints and immunity against citrullinated proteins in rheumatoid arthritis. Nat Rev Rheumatol. 2014 Nov; 10 (11): 645-53

The high prevalence of auto-antibodies can give rise to the formation of antibody-antigen complexes. Indeed, immune complexes (ICs) can be found in the circulation as well as in the affected joints of RA patients [50, 51, 72]. Their presence is associated with a more severe disease suggesting a possible contribution of ICs in the pathogenesis of RA [54, 55]. ICs can elicit effector functions through interaction with Fc gamma receptors (FcγRs). In vitro formed ICs containing ACPA directed against citrullinated fibrinogen as well as ICs obtained from synovial fluid of RA patients have an inflammatory potential via their ability to activate macrophages to produce the pro-inflammatory cytokine TNF-α in an FcγR-dependent manner. The IgG content of the ICs was found to correlate with TNF-α levels,
pointing to a contribution of FcγRs [73-75]. Production of pro-inflammatory cytokines by triggering FcγRs on immune cells represents a mechanism through which ACPA could perpetuate chronic inflammation of the joints. Additional mechanisms by which ACPA may contribute to RA pathogenesis have also been proposed (Figure 4).

Cell mediated immune responses in RA

Dendritic cells

DCs are highly specialized antigen presenting cells that have attracted considerable attention over the past years due to their key role in orchestrating immune responses. DCs are widely distributed through lymphoid and non-lymphoid tissues and they are strategically located at sites potential pathogens use to enter the body. The development of DCs initiates in the bone marrow. DC progenitors give rise to DC precursors that leave the BM and circulate in the blood to settle in different tissues as immature DC (imDC) [76]. DCs exist in two functional states: steady state immature DCs and mature DCs. This distinction is based on phenotypic and functional changes DCs undergo during their conversion from immature to mature DCs. imDCs are equipped with strong endocytic and phagocytic abilities and function therefore as sentinels of the immune system that patrol and capture antigens. Captured protein antigens are processed to peptide antigens prior to loading onto MHC class II molecule and presented to T cells [77, 78]. Following encounter with an antigen, DCs enter the process of maturation, which is reflected by powerful ability to prime a T cell response. DCs maturation can be accompanied by upregulation of the expression of surface molecules; CD80, CD86, CD83, MHC class II molecule and a poor phagocytic activity [79]. Upon maturation DCs migrate to the lymph nodes to present the captured antigen to T cells (Figure 5).

DCs are suggested to have an important role in the activation of self-reactive T cells allowing the emergence of autoimmune diseases. On the other hand, DCs in the steady state protect mice from autoimmunity. Selective depletion of DCs leads to high prevalence of auto-reactive CD4+ T cells resulting in devastating autoimmunity [80]. The fate of the antigen-specific T cells is directed by DCs depending on the type of the antigen presented. Foreign antigens presented in the context of danger signals such as signals mediating inflammation and tissue destruction, elicit T cell activation. While self-antigens presented under physiological conditions are thought to induce tolerance by either eliminating or suppressing T cell responses [81, 82]. DCs responsible for the induction of tolerance are called tolerogenic DCs (TolDCs).
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Figure 5. Maturation of dendritic cells. Dendritic cells (DCs) arise from haematopoietic stem cells (HSC) that reside mainly in the bone marrow. DCs are recruited to peripheral tissues and are the ‘sentinal guard’ of the immune system; they continuously sample antigens from the surrounding environment and process them in endosomes. Upon antigen-uptake, DCs migrate to the draining lymph nodes and mature phenotypically by upregulating the expression of CD80, CD86, CD40, MHC class II molecules. Functionally matured DCs produce cytokines such as interleukin-12 (IL-12), IL-10 and tumour-necrosis factor (TNF) and activate immune cells. Adapted from Hackstein H, Thomson AW. Dendritic cells: emerging pharmacological targets of immunosuppressive drugs. Nat Rev Immunol. 2004 Jan; 4 (1):24-34.

Tolerogenic DCs

TolDCs are present in the blood as well as lymphoid and non-lymphoid tissues. Due to their scarcity, methods have been developed to readily generate them in vitro by using blood or bone marrow (BM) derived precursors in combination with cytokine growth factors. Targeting DCs in the steady state or injection of imDC has been proven to be a powerful mean to induce a suppressive environment [83-86]. Injection of imDCs leads to the induction of specific IL-10 producing T cells with suppressive activity [85, 86]. In addition, DCs are able to promote tolerance by deletion of self-reactive T cells in processes involving negative co-stimulatory molecules such as program death (PD)-1 and CTLA-4 or by producing soluble molecules such as IL-10 [87], TGF-β [88] and IDO [89]. DCs have the potential to determine the differentiation fate of T cells depending on the local cytokine environment. In the
presence of IL-12 naïve CD4+ T cells differentiate toward the pro-inflammatory Th-1 response, into Th-17 in the presence of IL-6 and into T reg cells in the presence of TGF-β.

The importance of DCs in RA is illustrated by the association of RA with certain HLA alleles. It is believed that a self-antigen is presented by antigen presenting cells leading to the activation of self-reactive T cells and therefore induction of RA development. Different subsets of DCs, especially the ones with potent capability to initiate an inflammatory response are found to be present among the immune cells infiltrating the synovial tissue, fuelling the hypothesis that DCs represent key mediators of initiation and perpetuation of RA. To restore immune tolerance in autoimmune diseases like RA, TolDC-based immunotherapies that would target the pathogenic autoimmune response and leave the protective immunity intact have been developed.

Therefore different strategies have been described to generate TolDCs in vitro including short stimulation of DCs with LPS [90] and treatment of DCs with cytokines like IL-10, TGF-β and TNF-α, with inhibitors of NF-κB signalling pathway such as BAY 11-7085 compound [91], microRNA 23b [92] and Dexamethasone and/or vitaminD3 [93]. Another attractive way to achieve tolerogenicity is genetic modification of DCs, which either allows constitutive expression of immunoregulatory cytokines and molecules such as IL-10 [94], IL-4 [95, 96], Indoleamine 2,3-dioxygenase (IDO) [97], apoptosis inducing ligands FasL [98] and TNF-related apoptosis-inducing ligand (TRAIL) [99] or blockade of immunostimulatory molecules such as CD80, CD86 [100] and IL-12 [101].

Tolerogenic DCs have been shown to be effective in controlling pathogenic T cell responses in animal models of autoimmune diseases like arthritis models [91, 95, 98, 102, 103]. TolDCs have proven to be effective in preventing and controlling arthritis [91, 95-98, 102-104]. This has encouraged the implementation of these findings into the human setting and the development of TolDC protocols to treat RA patients. Encouraging results have been obtained from treating HLA-DRB1 SE+ early RA patients using autologous monocyte derived DCs treated with BAY 11–7082 and loaded with citrullinated peptide antigens [105] (Figure 6). Another group has also developed a protocol for generation of TolDC by treating DCs with Dexamethasone and vitaminD3 together with a Toll like receptor (TLR)-4 agonist. These TolDCs were found to exhibit phenotypic characteristics of tolerogenic DCs with a reduced expression of costimulatory molecules and production of pro-inflammatory cytokines. While they were revealed to have a poor ability to activate autologous antigen specific T cells, they were effective in suppressing proliferation and production of pro-inflammatory cytokines of activated T cells. These observations combined with results obtained from a clinical trial, in which TolDC treated with BAY 11-7082 and loaded with citrullinated peptide antigen were used to treat early RA patients, are potentially paving the way to a wide-scale usage of TolDC in the clinic.
The procedure of the first-in-human trial of antigen specific DC (Rheumavax) immunotherapy in RA patients. Early RA patients positive for the shared epitope containing HLA-DRB1 were injected a single dose of citrillinated peptides exposed DCs, which were generated from monocytes in the presence of IL-4, granulocyte-macrophage colony stimulating factor (GM-CSF) and Bay11-7082. The biological efficacy of DC immunotherapy have been assessed a month after the injection. Rheumavax did not induce severe side-effects and some trends were observed indicating a reduction in the numbers of effector T cells and increase in the numbers of T reg cells in peripheral blood. The level of pro-inflammatory cytokines such as IL-15 reduced following Rheumavax injection. Rheumavax potentially impacted on clinical disease activity, as the DAS28 scores were attenuated in patients with active disease at the baseline. Adapted from Buckland J. Rheumatoid Arthritis: First-in-human phase I trial of DC immunotherapy of early RA. Nat Rev Rheumatol. 2015 Aug; 11 (8): 443

Following recognition of the antigen presented by DCs, T cells enter the state of activation and start to proliferate and differentiate into distinct effector T cells. T cells are equipped with a receptor; T cell receptor (TCR), that enables them to recognize antigens. TCRs consist of heterodimeric glycoproteins and depending on the type of these glycoproteins, T cells can be divided into two types: αβ T cells express TCRs composed of α and β chains and γδ T cells express TCRs consisting of γ and δ chains. Most T cells are αβ T cells and are studied at a large scale. In contrast γδ T cells are not prevalent and their function is still elusive. αβ T cell repertoire consists of two compartments; CD4⁺ T cells and CD8⁺ T cells. A main difference between these two T cells populations is the class of MHC they interact with in order to recognize antigens. CD4⁺ T cells recognize antigens presented by DCs on MHC class II, whereas CD8⁺ T cells recognize antigens in the context of MHC class I.
Genetic studies point to the involvement of $\text{CD4}^+$ T cells in the development of RA [13]. In addition, studies in animal models of arthritis and the infiltration of T cells into the synovial tissue of patients with RA underscore the important role of $\text{CD4}^+$ T cells in the pathogenesis of arthritis. Adoptive transfer of $\text{CD4}^+$ T cells derived from arthritic animals into healthy animals resulted in the induction of arthritis with severe tissue damage. It is believed that auto-reactive $\text{CD4}^+$ T cells are crucial, especially in the initiation of RA [106].

**CD8$^+$ T cells**

CD8$^+$ T cells are best known for their crucial role in eradicating viruses and eliminating tumor cells. Recognition of antigens presented on MHC class I induces naive CD8$^+$ T cells to expand and differentiate into either cytokine producing effector cells or cytotoxic T cell (CTLs) and subsequently to migrate to the site of target cells. Upon binding to the target cell bearing MHC class I/peptide, CTLs exert their killing activity through secretion of cytotoxic granules containing perforin and granzyme B and through induction of Fas signalling leading to the initiation of apoptosis. Perforin is believed to facilitate the entrance of granzyme B into the cytosol of the cell by forming pores in the target cell plasma membrane.

Differentiation, activation status and cytokine profile determine the classification of CD8$^+$ T into naive or antigen experienced populations including effector, central memory and effector memory. Effector CD8$^+$ T cells have potent cytotoxic activity, express high levels of perforin and produce low levels of cytokines. Memory CD8$^+$ T cells lack the ability to kill target cells, proliferate and produce cytokines following antigen stimulation. Effector memory CD8$^+$ T cells have strong ability to produce high levels of cytokines, have medium levels of perforin and exert less potent cytotoxic activity.

The expression of certain surface molecules allows for the discrimination between different subtypes. Naive CD8$^+$ T cells express CD27, CD28, CD45RA, CD62L and CCR7. Effector CD8$^+$ T cells express low levels of CD28 and are negative for all other cell surface markers. Central memory CD8$^+$ T cells express all these molecules except CD45RA and CCR7. Effector memory CD8$^+$ T cells lack the expression of CD62L and CCR7 and express low levels of CD28 and can express variable levels of CD45RA [107, 108].

**CD4$^+$ T cells**

CD4$^+$ T cells have the ability to differentiate into distinct effector cells depending on the cytokine present during an encounter with an antigen. The type of pathogens to which they are targeted, cytokine production and transcription factors expression determine the characteristics of these effector cells. T helper (Th)-1 cells protect the host from intracellular infection, express T-box transcription factor (T-bet) and produce IFN-γ, TNF-α, IL-1 and IL-12. Th-2 cells express Trans-acting T cell-specific transcription factor (GATA)-3, secrete IL-4, IL-5 and IL-13 and are involved in the
protection against helminthes. Regulatory T cells (T reg cells) produce TGF-β and IL-10 and Forkhead box P3 (Foxp3) is their lineage specific transcription factor. Th-17 cells target mainly extracellular pathogens and fungal infection, express transcription factors retinoic acid receptor-related orphan receptor-γt (RORγt) and signal transducer and activator of transcription 3 (STAT3) and produce IL-17 (Figure 7).

It is believed that RA is associated with a Th-1 response characterised by the production of IFN-γ. This notion is not consistent with the finding that blocking IFN-γ receptor causes worsening of arthritis. Analysis of cytokine profile of synovial T cells during early and late stage of RA showed that during an established disease mainly IFN-γ, TNF-α and IL-10 were expressed, while the expression of IL-2, IL-4, IL-13, IL-17, and IL-15 was dominated during early disease indicating that distinct Th cell responses might act during different stages of the disease. Since the discovery that Th-17 cells are key inflammatory players in multiple inflammatory diseases like RA [109], the focus has now shifted from Th-1 to Th-17 cells.

Increasing evidence indicates that Th-17 cells have a pathogenic role in arthritis. Both the expression of IL-17 and IL-17 producing CD4⁺ T cells are observed at the site of inflammation of RA patients [110] and the levels of IL-17 in the supernatants of the synovial tissue culture are found to be highly correlated with disease activity. Activation of anti-apoptotic pathways in inflammatory cells [111] and causing bone damage by induction of osteoclasts formations [112] represent additional Th-17 mediated-mechanisms that might play a role in the pathogenesis of RA. Animal models have also provided evidence for the pathogenic effect of Th-17 cells in RA. Blocking IL-17 protected mice against arthritis by preventing joint inflammation and bone erosion in antigen induced arthritis model [112].

**CD4⁺ regulatory T cells**

CD4⁺ regulatory T cells constitutively express CD25, the high affinity receptor for IL-2 and Foxp3, which is the key transcription factor for the development, maintenance and function of T reg cells. There are two types of T reg cells; natural T reg cells originate in the thymus and adaptive or induced T reg cells are generated in the periphery from naive CD4⁺ T cells. Loss or inhibition of T reg cell activity by deficiency in IL-2, CD25, and Foxp3 or by neonatal thymectomy leads to the emergence of autoimmunity with fatal consequences, illustrating the pivotal role of T reg cells in the regulation of tolerance and immune homeostasis.

Likewise, in humans the dysfunction of Foxp3 as a result of mutations in the Foxp3-encoding gene leads to the development of the severe autoimmune disease immunodysregulation, polyendocrinopathy and enteropathy, x-linked syndrome (IPEX).
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Figure 7. Differentiation of naïve T cells into different T helper cells. Dendritic cells (DCs) activate naïve CD4⁺ T cells to differentiate into different T helper cell subsets depending on local cytokine environment. This polarization is regulated by different transcription factors. Each T-helper subset has a characteristic cytokine signature; while in the presence of TGF-β, CD4⁺ T cells differentiate into T reg cells expressing the anti-inflammatory TGF-β and transcription factor Foxp3, in the presence of both TGF-β and IL-6, CD4⁺ T cells can be polarized into Th-17 cells, which produce IL-17 and express transcription factors RORγt and STAT3. DCs can also promote the differentiation towards Th-1 and Th-2, which are characterized by the production of IFN-γ and IL-4 respectively. Adapted from Weiping Zou and Nicholas P. Restifo. TH17 cells in tumor immunity and immunotherapy. Nat Rev Immunol. 2010 Apr; 10 (4): 248-256.

Moreover, induction of Foxp3 expression converts naïve CD4⁺ T cells into T reg-like cells with in vivo and in vitro suppressive potential, underlining its crucial role in promoting the development of T regs [113]. Foxp3 is an invaluable marker in mice T reg cells, nonetheless in humans Foxp3 is also expressed by CD4⁺ T cells without regulatory function and therefore is not a specific marker for human T regs. Over the past few years, major progress has been made in understanding how T regs suppress immune responses. Some of the mechanisms underlying the suppressive function of T regs are suppression of T cells, suppression by modulation of DC maturation and function and suppression by inhibitory molecules and cytokines.
CD4+ regulatory T cells modulate T cells

It has been shown that T reg cells have the ability to inhibit proliferation and cytokine production of effector CD4+ T cells [114], to prevent the differentiation of CD8+ T cells into cytolytic effector cells [115] and to kill effector T cells in a granzyme B [116], perforin [116-118] or cyclic adenosine monophosphate (cAMP) dependent and independent manner [119]. Furthermore, T reg cells have the potential to confer suppressive properties to CD4+CD25- T cells, which in turn suppress syngeneic CD4+ T cells by producing immunosuppressive cytokines like IL-10 [120, 121]. The observation that T reg cells can suppress proliferation of effector T cells in the absence of APC, raised the suggestion that T reg cells suppress effector T cells in a cell-cell dependent manner [122]. By expressing high levels of CD25, T reg cells induce cytokine deprivation by consuming IL-2 produced by effector T cells thereby preventing their proliferation and differentiation [123].

CD4+ regulatory T cells modulate DCs

To gain more insights into the function of T reg cells, their effect on DCs have been evaluated. T reg cells and DCs have been observed to form close contact in vivo indicating that these cell populations communicate with each other to suppress immune responses. Indeed T reg cells have been shown to abrogate antigen presenting capability of DCs, thereby reducing or constraining their potent ability to stimulate T cells. This occurs through downregulation of costimulatory molecules CD80 and CD86 expression or inhibition of pro-inflammatory cytokine secretion [124-127]. T reg cells can downregulate the expression of costimulatory molecules expressed by DCs in a CTLA-4 dependent manner. Blocking CTLA-4 prevented T reg cells from downregulating CD80 and CD86 and rendering DCs poor inducers of T cell proliferation [125]. T reg cells express Lymphocyte antigen activation gene 3 (Lag-3); a CD4 homologue that binds with high affinity to MHC class II molecule. Interaction of Lag-3 with MHC class II expressed on the surface of DCs is another way to suppress maturation and immunostimulatory capacity of DCs [128].

Furthermore, T reg cells alter DC function in a way that favours tolerogenic properties by inducing secretion of the suppressive cytokine IL-10 [129] and expression of IDO, which is an enzyme that converts the essential amino acid tryptophan into kynurenine allowing creation of a toxic microenvironment for likely proliferating immune cells. The induction of IDO has been found to be dependent on CTLA-4 expressed on T regs [130]. Killing of DCs by secretion of perforin [118] and maintaining normal numbers of DCs constitute additional mechanisms employed by T reg cells to control immune responses.
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Molecules and cytokines mediating the suppression capacity of regulatory CD4+ T cells

Searching for a specific molecule that can simplify the identification of T reg cells have lead to the characterization of various T reg cells associated molecules and cytokines including CTLA-4, TGF-β, IL-10 and IL-35. T reg cells treated with neutralizing antibody against CTLA-4 or T reg cells derived from CTLA-4 deficient mice maintain their suppressive ability in \textit{in vitro} suppression assay. However some \textit{in vivo} studies suggest that CTLA-4 is important for the suppressive function of T reg cells [131]. Moreover, T reg cells are prominent in producing cytokines with suppressive properties such as IL-10 and TGF-β. Although these cytokines are essential for the regulation of immune responses, their requirement for the suppressive function of T reg cells remains a subject of debate. IL-35 is preferentially produced by T reg cells and its expression is under the control of Foxp-3. IL-35 is composed of two subunits; Epstein-Barr virus induced gene 3 (EBI3) and p35. Genetic abrogation of these subunits causes a significant reduction of T reg cell suppressive activity \textit{in vivo} as well as \textit{in vitro}. Ectopic expression of IL-35 on the other hand promotes the conversion of naïve T cells into T cells with suppressive activity.

It is believed that T reg cells in RA display an aberrant suppression function and become unable to control the inflammatory immune responses associated with the disease. T reg cells from patients with RA failed to suppress the production of pro-inflammatory cytokines such as IFN-γ and TNF-α and to convey suppressive activity to T cells [132]. TNF-α was held responsible for the defects in T reg cell function, as neutralization of TNF-α restored the suppressive activity of T reg cells [132]. Accordingly, TNF-α has been shown to mediate impaired function of T reg cells in RA patients by inhibiting Foxp3 phosphorylation [133, 134].

Furthermore, defects in CTLA-4 such as reduced expression and inability to regulate TCR signalling are suggested to contribute to the aberrant function of T reg cells in patients with RA [135]. In addition, depletion of T reg cells before the induction of CIA resulted in a severe disease [136] suggesting an essential role of T reg cells in the protection from arthritis. However these studies have been contradicted by other studies suggesting that the function as well as the number of T reg cells is not affected in patients with RA when compared to T reg cells of healthy donors. It is also noteworthy to mention that T reg cells are detected in synovial fluid of RA patients and that their frequency is found to be higher compared to peripheral blood [137-139]. It is plausible to think that they could behave differently at the site of inflammation. However it appears that the suppressive function of T reg cells at the site of inflammation is normal or even enhanced. T reg cells derived from synovial fluid of patients with RA are suggested to exhibit a highly activated phenotype and display increased suppressive capacity.
Outline of the thesis

An important goal in the study of a disease is to increase the understanding how the disease emerges as well as to develop therapeutic strategies in order to improve the life of diseased individuals. Therefore the overall aim of this thesis is to get insight into B cell and auto-antibody responses as well as immune regulation in arthritis.

Chapter 2 is a review summarizing the finding on auto-antibodies in Arthritis and their relation to Fcγ receptors.

In Chapter 3 we focused on the immune-regulatory potential of a accepted medication effective in RA Abatacept, which is a CTLA-4-Ig based. It is widely believed that Abatacept is effective due to its ability to inhibit the co-stimulation and subsequently the activation of T cells, through binding with high affinity to the ligands of the co-stimulatory molecule CD28. This is based on studies testing Abatacept in preventive settings in mice and rats. However in humans Abatacept is used to treat an established disease, when the activation of memory T cells is less dependent on the CD28 co-stimulation, suggesting that the mode of action of Abatacept may not solely depend on the inhibition of T cell activation. For this purpose we studied the effect of Abatacept in the absence of CD4+ T cells in an established CIA model in mice.

The studies presented in Chapter 4 describe the presence and reactivity of anti-CCP antibodies in order to obtain more insight into regulation and occurrence of this prominent auto-antibody system in RA. RA is characterized by the presence of antibodies CCP2 or ACPA, which are detected in ELISA based assays using citrullinated peptides or proteins as antigens respectively. Although ACPA are detected in the serum of CCP2 positive patients, it was still not known whether CCP2 are ACPA (i.e. anti-Citrullinated Protein Antibodies). Therefore we investigated whether CCP2 are ACPA and evaluated the extent of cross-reactivity of ACPA antibodies. In addition we estimated the concentration of CCP2 antibodies in the serum of CCP2 positive RA patients.

As extension of the studies presented in chapter 3 and 4, the studies presented in Chapter 5 describe the expression of CD28 on human B cells, including ACPA-producing human B cells: CD28, which is a key co-stimulatory molecule, is also expressed by plasma cells and has been shown to be involved in the regulation of longevity of long-lived plasma cells in mice. Since plasma cells and auto-antibodies are crucial for the pathogenesis of RA, together with the fact that Abatacept is an effective drug to treat RA, we studied the expression of CD28 on plasmablasts/cells in peripheral blood of patients with RA and evaluated whether its expression is altered compared to SLE patients and healthy donors. We also investigated this expression by plasmablasts/cells and ACPA-expressing plasmablasts/cells at the site of inflammation.
The studies presented in Chapter 6, focused on a particular set of CD4+ T cells with immune-regulatory properties: CD49b+CD4+ T cells (which are also named DX5+CD4+ T cells) are potent regulatory T cells, which have been shown to reduce collagen induced arthritis, diabetes and delayed type hypersensitivity reactions in mice. CD49b+CD4+ T cells also modulate Th-1 outcome in vitro by inhibiting the production of IFN-γ and inducing IL-10 production in IL-4 dependent manner. In this study we further characterized these cells by studying their effect on DCs phenotype and function.

As follow-up of the studies presented in chapter 6, in Chapter 7 we investigated the antigenic factor inducing the expansion of CD49b+CD4+ T cells that occurs following repetitive injections of immature DCs in mice. DCs lacking the expression of MHC class II molecule failed to induce CD49b+CD4+ T cell expansion. Considering the fact that the activation of CD4+ T cells occurs through the simultaneous engagement of T cell receptor, that interacts with the antigen-MHC class II complex and a co-stimulatory molecule, together with the expression of activation markers, indicate that these cells recognize an antigen (s) in the context of MHC class II. In this study we assessed the presence of CD49b+CD4+ T cells in young mice and we wished to determine the nature of the antigen (s) recognized by CD49b+CD4+ T cells.

In Chapter 8 the results of this thesis are summarized and discussed and perspectives are provided.

In Chapter 9 the results of this thesis are summarized in Dutch.
Chapter 1

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Introduction


Chapter 1


Introduction


