Summary & discussion

Chapter 8
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In this thesis, we have addressed aspects of two main arms of the adaptive immune system; the B cell and antibody arm and the T cell arm. This led to a division in the presentation of the results described in this thesis into two sections. In the first section, we present the results regarding the characterization of ACPA responses, B cells and ACPA secreting plasmablasts/-cells in RA as well as autoantibody responses and their regulation by an effective anti-rheumatic drug, abatacept, in the arthritis mouse model; Collagen Induced Arthritis (CIA). The second section is compiled of results obtained from studies examining the regulatory and other aspects of CD49b+CD4+ T cells on pro-inflammatory responses involved in the pathogenesis of arthritis.

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by inflammation of the synovial membrane of the joints, culminating in destruction of cartilage and deformity of the joints if remains untreated. Infiltration of inflammatory immune cells such as B cells and T cells into the inflamed joints is a characteristic feature of RA. These immune cells are in continuous interaction with each other and create a viscous circle that sustains persistent synovitis and damage to articular cartilage.

RA is regarded as an autoimmune disease that occurs when the immune system attacks body’s own components. In RA, the immune system specifically attacks the synovial membrane of the joints. The exact nature of the antigens triggering the autoimmune response in RA is not yet known. Nevertheless, the presence of auto-antibodies such as anti-citrullinated protein antibodies (ACPA) in most RA patients is an indisputable fact. ACPA have been revealed to have valuable diagnostic and predictive characteristics and display significant association with a severe disease course. This indicates that ACPA are involved in RA pathogenesis. Moreover, the strong association of shared epitopes-alleles with in particular ACPA positive RA favours the hypothesis that citrullinated antigens are targeted by the immune system leading to elicitation of an ACPA immune response associated with RA.

Insights into B cell responses is a way towards understanding RA

ACPA responses in RA

Several types of auto-antibodies have been revealed to be associated with RA including ACPA, Rheumatoid factor (RF) and anti-Carbamylated protein antibodies (CarP) [1-3]. With the discovery of ACPA, a considerable progress has been made in understanding the role of B cell responses in RA pathogenesis. ACPA can be detected in the circulation as well as the affected joints of patients with RA [4-6]. Commercial tests have been developed to detect antibodies targeting citrulline containing
antigens. One of these tests is the cyclic citrullinated peptide (CCP) 2 ELISA. This test aids in the diagnosis of RA and as CCP2 antibodies can be detected several years before the clinical onset of RA, it can also contribute to the prediction of RA leading to improved management of the disease. In parallel, various ELISA tests have been optimized to measure antibodies recognizing citrullinated proteins such as fibrinogen and vimentin in RA patients [7, 8]. The results obtained by these ELISA tests correlate very well with the results of CCP2 ELISA test. While the antigens used in ACPA ELISA tests are natural antigens that can be citrullinated in vitro using the PAD enzyme, the antigen used in CCP-2 assay is a synthetic peptide that is not naturally found in human protein.

This has led to debate about the biological relevance of the antibodies detected with CCP2 ELISA assay and also whether ACPA and CCP2 antibodies represent the same or two distinct antibody families. Therefore, in chapter 4, we wished to assess whether antibodies detected by CCP2 ELISA would recognize citrulline containing proteins previously detected in joints of patients with RA and to determine the extent of cross-reactivity of ACPA recognizing different citrullinated proteins. Furthermore, we estimated the amount of CCP2 antibodies present in the serum of RA patients.

To this end, we have first optimized a method that would allow for the isolation of sufficient amount of CCP2 antibodies in an antigen specific manner. CCP2 positive serum was incubated on CCP2 ELISA plate and affinity bound antibodies were eluted using an elution buffer. Testing for the functionality of the isolated CCP2 antibodies on CCP2 ELISA revealed that glycine is the best elution buffer to elute CCP2 antibodies. The amount of CCP2 antibodies was determined by incubating CCP2 positive serum on CCP2 plate, the unbound CCP2 antibodies in serum were subsequently transferred to next wells and this was repeated until no binding of CCP2 antibodies could be detected.

The level of CCP2 antibodies varied between patients and the amount of CCP2 antibodies present in the sera of patients with high titre CCP2 was estimated to reach up to 30 μg/ml. Considering the loss of antibodies during the purification procedure, this amount is probably an underestimation and the real amount might exceed 30 μg/ml. Furthermore, we have shown that CCP2 antibodies are able to recognize citrullinated proteins demonstrating that CCP2 antibodies are ACPA and that ACPA recognizing different citrullinated antigens display various degrees of cross-reactivity within individual patients.

This is in line with previous report showing that antibodies recognizing different citrulline containing antigens exhibit a certain level of cross-reactivity [9, 10]. Interestingly, distinct ACPA responses with no cross-reactivity were also found to be present in RA patients indicating that these antibody responses are likely derived from distinct B cells. Investigation into citrulline specific B-cell responses giving rise to the production of cross-reactive or distinct ACPA could provide major insight into the pathogenesis of RA.
The effect of Abatacept on antibody responses in the absence of CD4+ T cells

An important goal of conducting scientific research is to understand disease pathogenesis and to develop effective therapies to relieve disease symptoms or ideally to cure the disease. Although there is still a major need for drugs that can cure RA, a substantial success has been made in developing drugs to manage the disease. RA is a heterogeneous disease, which is reflected in the drug usage and response to therapies; as RA patients do not similarly respond to a specific therapy regimen. Abatacept is used in the clinic to treat RA patients failed to adequately respond to anti-tumor necrosis factor α (anti-TNFα) therapy [11, 12]. Abatacept is a recombinant CTLA-4-Ig fusion protein consisting of extracellular part of the human CTLA-4 and a fragment of the Fc part of the human IgG1 and is believed to exert its effect by interfering with the binding of B7 molecules CD80/CD86 to CD28 expressed by T-cells resulting in inhibition of T-cell activation [13].

The costimulatory signal delivered by CD28 is crucially important for the activation of naïve CD4 T cells during a primary response. However, it is still elusive whether CD28 is involved in the activation of already antigen experienced CD4 T cells, which are abundant in a fully established disease. Indeed, abatacept has been previously shown to prevent CIA in the early stages of arthritis development [14-16]. These notions suggest that abatacept might be beneficial as a CD4+ T cell inhibitor mainly during a primary response, when T cells are still naïve and need costimulation for their activation. Therefore we investigated in chapter 3 the effect of abatacept on disease activity and antibody responses in the absence of CD4+ T cells during the late stage of established CIA.

To this end, diseased mice were treated with Abatacept in the presence or absence of CD4+ T cells. Importantly, abatacept was effective in reducing disease activity when CD4+ T cells were depleted. This indicates that Abatacept, in addition to the inhibition of T cell costimulation, has additional mode of actions to decrease disease activity in CIA. A limitation of our study is, just like many other drugs used to in the treatment of RA, that the precise mode of action is not fully elucidated.

In our studies, abatacept treatment alone did not have an ameliorating effect on disease activity in established CIA. Although, most animal studies have investigated the effect of CTLA-4-Ig in a prophylactic setting, to our knowledge there is only one study that showed a beneficial effect of CTLA-4-Ig on established disease in CIA [14]. Dissimilarity in results between the two studies may be explained by differential timing of initiation of treatment and/or source of CTLA-4-Ig used. In the study of Webb et al, CTLA-4-Ig was given on the day that signs of clinical disease were observed and every other day until day 10 when the mice were sacrificed. In contrast, in our studies, CTLA-4-Ig treatment started after the initiation of clinical disease. The mice were monitored for a prolonged period of time after the treatment was ceased. In the study of Webb et al, a mouse CTLA-4-Ig was used, whereas in our studies, we have used a fully human CTLA-4-Ig (Abatacept), which may have incited an antibody response leading to neutralization of abatacept and loss of effectiveness. The absence of CD4+ T cells
may have prevented the initiation of antibody response against abatacept, resulting in clinical and immunological effects.

An important finding of our study is the observation that depletion of CD4+ T cells did not affect disease progression, suggesting that CD4+ T cells are dispensable for the progression of arthritis. This is in line with previous studies showing that anti-CD4 treatment is effective in preventing CIA if administered before the onset of clinical disease and has no significant effect once arthritis is already established [20, 21].

It has to be acknowledged that our results confirm the notion that abatacept acts as an inhibitor of T cell costimulation, which is manifested by complete depletion of CD4+ T cells upon administration of rat anti-CD4 antibody in combination with abatacept treatment that may have prevented the anti-Rat response by inhibiting costimulation of CD4+ T cells.

Analysis of the frequency of CD4+ T cells in the circulation and lymphoid tissue revealed that depletion of CD4+ T cells did not last for an extended period of time even when CD4 depleting antibody was weekly administered till the end of follow up. Therefore, we have used mice that were thymectomized at age of 6 weeks, so that CD4+ T cells are sufficiently present to induce arthritis and to be readily depleted with a depletion antibody once the disease is established to ascertain that no CD4+ T cells can return. Correspondingly, a combination of abatacept and CD4+ T cell depletion resulted in a significant reduction of disease activity. However, CD4 or abatacept treatment alone did not affect disease activity.

Antibodies are important for the development of arthritis. As such we also investigated whether abatacept would mediate antibody responses in the absence of CD4+ T cells. For this reason, total, collagen specific antibody levels were measured in sera obtained from mice that have received either CD4 depleting antibody alone, a combination of abatacept and CD4 depleting antibody or PBS. Abatacept treatment led to a decrease of total and antigen specific antibody production in the absence of CD4+ T cells. Likewise, we have shown that the levels of total and antigen specific IgG antibodies were decreased in the supernatant of ex-vivo cultured spleen and bone marrow cells obtained from mice treated with abatacept in combination with CD4 depleting antibody.

These results show that abatacept treatment is able to reduce total and antigen-specific antibody responses in the absence of CD4+ T cells, which allows to hypothesize that abatacept can have a direct effect on B cells. Several studies provide supportive evidence that abatacept modulates B cell responses. Scarsi, et al have found that abatacept can normalize the dysregulated B cell responses in RA. Treatment with abatacept for 6 months significantly reduced total serum levels of IgA, IgG and IgM and ACPA specific IgA and IgG levels. This was paralleled with a decrease in the level of switched
memory B cells. A significant reduction of B cells was also observed in synovial biopsies following 16 weeks of abatacept treatment [22].

However, in these studies, the beneficial effect of abatacept on auto-antibody production and B cell frequencies was achieved in the presence of CD4$^+$ T cells and is believed to be mediated via blockade of T cell costimulation. In contrast, our results reveal that abatacept can also affect auto-antibody responses in the absence of CD4$^+$ T cells. B cells have been shown to bind to CTLA-4-Ig via costimulatory molecules CD80 and CD86 expressed on their surface [23]. Furthermore, these costimulatory molecules can regulate IgG responses in activated B cells as stimulation of CD80/CD86 led to activation of several signalling pathways culminating in an augmented IgG production [24, 25].

**CD28 expressing B cells, plasmablasts/-cells and ACPA$^+$ plasmablasts/-cells in RA patients**

It is established that B cells are playing a prominent role in the development and progression of RA, primarily via production of auto-antibodies. In order to secrete antibodies, B cells undergo differentiation into antibody secreting cells, the so called plasma cells (PCs). Depending on their lifespan, they can be classified as short lived (SL) and long lived (LL).

It is believed that LLPCs are responsible for long term protection by continuously maintaining antibody levels in serum [26-28]. Unravelling molecular and/or cellular components that mediate the longevity of plasma cells would offer an opportunity for targeted intervention either by enhancing protection against pathogens or inhibiting auto-reactive B cell responses associated with RA. The costimulatory molecule CD28 has been shown to regulate the longevity of plasma cells in mice via interacting with members of B7 costimulatory family CD80 and CD86 expressed by DCs [29, 30].

As abatacept has proven great efficacy in patients with RA [11, 12] and is believed to bind B7 ligands to prevent interaction with CD28, we investigated in chapter 5 whether CD28 expressing B cells are present in patients with RA. Indeed, we have shown for the first time that CD28 expressing B cells and plasmablasts/-cells are present in peripheral blood and synovial fluid of RA. The frequency of CD28 expressing B cells and plasmablasts/-cells in peripheral blood was comparable between RA and SLE patients and healthy controls.

Interestingly, we have found a significantly higher proportion of CD20$^+$ B cells expressing CD28 in the synovial fluid of RA patients compared to peripheral blood. We have also demonstrated that ACPA$^+$ plasmablasts/-cells derived from the synovial fluid of RA patients express CD28 as well. However, CD28 expression was found not to be enriched on ACPA$^+$ plasmablasts/-cells compared to total CD28 expressing plasmablasts/-cells.

CD28 is known as a prototypic T cell costimulatory molecule and via binding to B7 family it enhances T cell activation and function. Our findings, on one hand provide supportive evidence that human
plasmablasts/-cells and B-cells can also express CD28 and on the other hand, show for the first time that human CD28 expressing B cells and ACPA\(^+\) plasmablasts/-cells are present in the circulation and at the site of inflammation of RA patients with a significant enrichment of CD28 expressing CD20\(^+\) B cells at the site of inflammation. The function of CD28 expressing plasmablasts/-cells and B cells in RA is not known.

Studies in mice and humans have provided evidence for a prominent role of bone marrow niches in supporting survival of plasma cells and long term antibody production. Results from our group and others [31-33] indicate that chronic inflammation can provide an environment for plasma cells to survive for an extended period of time. ACPA\(^+\) plasmablasts/-cells residing at the site of inflammation showed enhanced ability to produce pathogenic ACPA. These findings fuel the hypothesis that CD28 might also function as a survival factor for ACPA\(^+\) plasmablasts/-cells in the inflamed synovial tissue of RA patients, leading to enhanced ACPA production.

Since abatacept blocks the binding of CD28 to its ligands CD80 and CD86, it is tempting to speculate that CD28 expressing B cells are targeted by abatacept, which we have shown in this thesis to decrease auto-antibody production in the absence of CD4\(^+\) T cells in the mouse model of arthritis CIA. Importantly, it has been shown that abatacept treatment significantly reduced CD20\(^+\) B cells in the synovial tissue of RA patients.

Targeting auto-reactive ACPA\(^+\) plasmablasts/-cells expressing CD28 by abatacept might reduce their lifespan by disrupting CD28-dependent survival signal, leading to decreased production of auto-antibodies ACPA, which are associated with a worse disease and are believed to be involved in the pathogenesis of RA. Our findings contribute to the understanding of mode of action of abatacept by providing additional cellular component that can be targeted by abatacept.

**Modulation of Th-1 responses to restore immune tolerance**

Autoimmune diseases such as RA are characterised by loss of tolerance to self. This occurs when various mechanisms responsible for maintaining tolerance fail to operate optimally allowing for the emergence of an uncontrolled Th-1 response associated with the production of pro-inflammatory cytokines like IFN-\(\gamma\). T cells with regulatory or tolerance-inducing capacities are crucial for eliminating immune cells mediating damage to the body’s own components. Although the best known and well studied cells with regulatory potential are T reg cells [34-38], other T cell subtypes such as CD49b\(^+\)CD4\(^+\) T cells have also been revealed to be important in regulating Th-1 immune responses [39-43]. Their regulatory capacity is illustrated by studies with animal mouse models including CIA [40, 41], delayed hypersensitivity models [40] and diabetes [43].
These studies have demonstrated that CD49b<sup>+</sup>CD4<sup>+</sup> T cells can protect from disease development and also ameliorate an established disease. Importantly, the fact that a small number of adoptively transferred CD49b<sup>+</sup>CD4<sup>+</sup> T cells was sufficient to provide protection against arthritis further underscores the potent potential of these cells to regulate immune responses [41]. In CIA, adoptive transfer of CD49b<sup>+</sup>CD4<sup>+</sup> T cells resulted in a decrease in collagen type II specific antibodies of the IgG2a isotype [41], which is related to Th-1 immunity and an increase in the production of the anti-inflammatory cytokine IL-10.

This suggests that an IL-10 dependent mechanism is employed by CD49b<sup>+</sup>CD4<sup>+</sup> T cells to suppress pro-inflammatory immune responses in CIA. Therefore, we have performed in vitro studies to gain insights into the mechanisms potentially employed by CD49b<sup>+</sup>CD4<sup>+</sup> T cells to exert their regulatory effect in vivo (Figure 1). Interestingly, we have shown that CD49b<sup>+</sup>CD4<sup>+</sup> T cells alter the outcome of CD4<sup>+</sup> T cell response in an IL-4 dependent manner, by inducing an IL-10 response and inhibiting pro-inflammatory responses characterized by the secretion of IFN-γ [42]. This study has demonstrated that CD49b<sup>+</sup>CD4<sup>+</sup> T cells can directly modulate Th-1 immune responses (Figure 1C).

**Modulation of dendritic cell function and phenotype by CD49b<sup>+</sup>CD4<sup>+</sup> T cells**

LPS-matured DCs produce IL-12 that can induce an IFN-γ response. Accordingly, in chapter 6 we have investigated whether CD49b<sup>+</sup>CD4<sup>+</sup> T cells can reshape a Th-1 immune response in an indirect way via modulation of DC function (Figure 1B). Culturing CD49b<sup>+</sup>CD4<sup>+</sup> T cells with LPS-stimulated DCs led to a significant decrease in IL-12 production. Similarly, supernatant of CD49b<sup>+</sup>CD4<sup>+</sup> T cells culture was also able to decrease the production of IL-12 by DCs, suggesting that a soluble factor secreted by these cells was responsible for altering DC function. Neutralizing IL-10 but not IL-4 in the supernatant of CD49b<sup>+</sup>CD4<sup>+</sup> T cell culture prevented the inhibition of IL-12 production by DCs. DCs lacking the ability to produce IL-12 following co-culture with CD49b<sup>+</sup>CD4<sup>+</sup> T cell supernatant failed to induce IFN-γ production by CD4 T cells, which was subsequently restored upon addition of IL-12 or a blocking antibody for IL-10, unveiling another mechanism used by CD49b<sup>+</sup>CD4<sup>+</sup> T cells to modulate Th-1 immune responses.

Moreover, we investigated the effect of CD49b<sup>+</sup>CD4<sup>+</sup> T cells on DC-phenotype by measuring the expression level of costimulatory molecules including CD80, CD86 and CD40, MHC class II molecule and coinhibitory molecules PDL-1 and PDL-2. We observed increased expression levels of these molecules in particular the coinhibitory molecule PDL-2, which is crucially involved in negative regulation of T-cells and maintenance of immune tolerance [44, 45].
Summary & Discussion

Figure 1. Expansion and working mechanisms of CD49b⁺CD4⁺ T cells. (A) In vivo expansion of CD49b⁺CD4⁺ T cells upon vaccination of mice with immature dendritic cells. (B) CD49b⁺CD4⁺ T cells modulate CD4⁺ T cell response in an IL-10 dependent manner, by inhibiting DCs to produce IL-12 leading to the inhibition of IFN-γ production by CD4⁺ T cells. (C) CD49b⁺CD4⁺ T cells modulate CD4⁺ T cell response in a direct way via production of IL-4; they induce and inhibit IL-10 and IFN-γ production respectively. (D) CD49b⁺CD4⁺ T cells have also a modulation effect on CD8⁺ T cell responses by inducing production and expression of IL-10 and Foxp3 respectively and at the same time inhibiting proliferation and IFN-γ production. (E) Primarily results suggest an effect of CD49b⁺CD4⁺ T cells on B cells by increasing the expression of FcγRIIB.
Thus, CD49b+CD4+ T cells have potent capacities to modulate CD4+ T cell immune responses both directly and indirectly via modulation of DC function.

**Antigen specificity of CD49b+CD4+ T cells**

Repetitive injection of immature (im) DCs in mice induces CD49b+CD4+ T cells to vigorously expand and produce IL-10 (Figure 1A) [41]. The observations that imDCs derived from MHC class II knockout mice failed to induce the expansion of CD49b+CD4+ T cells [40] and that CD49b+CD4+ T cells display an antigen experienced phenotype [46] suggest that CD49b+CD4+ T cells recognize an antigen(s) in the context of MHC class II. Identifying the antigen(s) recognized by CD49b+CD4+ T cells would provide insights into their biology and facilitate enhancement of their regulatory function. In chapter 7, we wished to identify the antigen(s) recognized by CD49b+CD4+ T cells. Therefore, we studied the presence of CD49b+CD4+ T cells in young mice to gain knowledge of the nature of the antigen(s) they recognize. We have shown that CD49b+CD4+ T cells are present in two days old mice and have the tendency to reside in the liver. The observation that cultured imDCs, which were not loaded with a defined antigen, were also able to induce expansion of CD49b+CD4+ T cells, made us wonder about the source of the antigen(s) they present in vivo.

Considering our knowledge of imDC function in sampling and capturing antigens present in the surrounding environment, we hypothesized that cultured imDCs probably capture antigens from the culture environment and present them in vivo to CD49b+CD4+ T cells. ImDCs used to induce expansion of CD49b+CD4+ T cells in vivo were derived from bone marrow and were cultured for 7 days in medium supplemented with fetal calf serum (FCS). Therefore, we anticipated that FCS might contain the antigen(s) recognized by CD49b+CD4+ T cells. Indeed, FCS elicited a rigorous IL-10 response by CD49b+CD4+ T cells. Since FCS is composed of a large number of components, we separated FCS components by applying chromatography technology. Sequential separation starting with anion exchange chromatography and then followed by cation exchange chromatography resulted in the enrichment of antigens recognized by CD49b+CD4+ T cells.

The fraction enriched with CD49b+CD4+ T cell-inducing proteins was analyzed by mass spectrometry to delineate their molecular identity. As expected, this fraction contained various proteins, among them fibrinogen and fetuin, which we have chosen as candidate proteins owing to their availability and fetuin additionally to its anti-inflammatory property and its low molecular weight, which would be advantageous in the context of identifying the peptides recognized by CD49b+CD4+ T cells.

Both bovine fibrinogen and bovine fetuin were capable of promoting CD49b+CD4+ T cells to produce IL-10. However, the attempts to identify the peptides derived from fibrinogen and fetuin have failed. The ability of fibrinogen to elicit an IL-10 response by CD49b+CD4+ T cells was lost after digestion with various proteinases or after separation with C18 column, confirming the proteineous nature of the
antigens recognized by CD49b^+CD4^+ T cells. More research implementing new strategies and technologies is needed to identify the peptides recognized by CD49b^+CD4^+ T cells.

**Perspectives**

Currently RA is treated with various therapeutics that include disease modifying anti rheumatic disease (DMARD), non-steroidal anti-inflammatory drugs (NSAID), glucocorticoids (GCs), and biologic agents [47]. These therapeutics can arrest disease progression and relief some symptoms by reducing inflammation, without dealing with the cause of the disease. A considerable amount of patients with RA are refractory to a single or combined therapy with DMARDs and biologicals. Additionally, the use of these drugs has raised concerns of severe complications. All together, these observations underscore the need for therapies aiming at a prolonged or ideally a permanent restoration of self tolerance and at the same time maintenance of immune-competence.

DCs based immunotherapy fulfilling these criteria has emerged as a promising therapeutic approach to treat autoimmune diseases such diabetes [48]. DCs represent an attractive cell population for immunotherapy due to their capacity to present antigens to T cells, to regulate immune tolerance and induce an effective immune response [49]. Various protocols have been applied to generate DCs sharing similar tolerogenic properties such as reduced production of inflammatory cytokines, i.e IL-12, enhanced production of suppressive cytokines i.e IL-10 and induction of T cell hyporesponsiveness or IL-10 producing T cells.

Our studies focused on IL-10 producing CD49b^+CD4^+ T cells, which can be expanded by imDC vaccination. CD49b^+CD4^+ T cells are shown to have potent suppressive capability in CIA, which might have been achieved through modulation of Th-1 immune responses believed to play a role in arthritis, as we have shown in vitro that CD49b^+CD4^+ T cells modulate the outcome of CD4^+ T cell immune responses, converting IFN-γ producing cells to non-IFN-γ producing cells and/or IL-10 producing cells. It is believed that CD4^+ T cells are crucial for initiation of RA and B cells are key players in established disease.

However, CD4^+ T cells can still have a role in a progressed arthritis by sustaining the production of auto-antibodies by newly activated B cells. Indeed CD49b^+CD4^+ T cells have been shown to affect B cell responses by attenuating the levels of IgG2a, an isotype associated with the pathogenesis of arthritis, implying that CD49b^+CD4^+ T cells might either affect CD4^+ T-cells and/or have a direct effect on B cell responses [41]. Accordingly, preliminary data suggest that CD49b^+CD4^+ T cells can increase the expression of the inhibitory Fc gamma receptor IIB (FcγRIIB) in vitro (data not shown, Figure 1E), which upon triggering can lead to attenuation of antibody production.
We have learned that CD49b+CD4+ T cell responses are MHC class II dependent implying their recognition of an antigen(s) in the context of MHC class II. To further enhance the regulatory capacity of CD49b+CD4+ T cells, thorough understanding of their biology is required including identification of the nature of antigens they recognize. These antigens would be helpful in developing a therapy aiming at inhibiting a pro-inflammatory phenotype and enhancing a beneficial tolerogenic phenotype via targeting suppressive CD49b+CD4+ T cells. CD49b+CD4+ T cells are also present in humans. As mouse counterpart, they also possess suppressive functions and produce IL-10 [39, 50, 51]. Identification of the antigens recognized by these cells would facilitate the translation of murine results to human settings.

Immunotherapies employing T reg cells to restore immune tolerance have gained increasing attention and in vitro methods have been developed to obtain a sufficient number of antigen specific T-reg cells, which subsequently can be adoptively transferred into patients. These in vitro expansion methods raised concerns about the purity and the stability of T reg cells, which pose a major hurdle for the success of T-reg-based therapies. Using antigens able to induce in vivo expansion of cells possessing suppressive capacity like IL-10 producing T cells would provide an excellent alternative. Since the antigens recognized by CD49b+CD4+ T cells are not disease-specific, this treatment would be applicable in a wide range of autoimmune diseases without the need for identifying the triggering antigens.

We have shown that CD49b+CD4+ T cells modulate the outcome of CD4+ T cell immune response either directly or via modulation of DCs. The concept of antigen based immunotherapy started 100 years ago to treat allergies. The efficacy of antigen based immunotherapy can be enhanced by targeting CD49b+CD4+ T-cells-inducing-antigens to immature DCs by fusing them with antibodies against receptors expressed by DCs to enhance their uptake and processing. Such an approach using antibodies specific to DC-receptor; dendritic and epithelial cell receptor (DEC) 205 has been shown to be successful in suppressing Experimental Allergic Encephalomyelitis (EAE); a mouse model for multiple sclerosis [52]. In addition to the DEC205 receptor, targeting other receptors associated with promoting tolerance such as DC-asialoglycoprotein receptor (DC-ASGPR) can also be beneficial [53].

Auto-antibodies are a hallmark of RA and the list of auto-antibodies associated with RA is increasing [54]. ACPA in particular have attracted great attention due to their predictive, diagnostic and prognostic value. Increasing evidence suggests that ACPA are functionally involved in RA. Therefore, characterization of ACPA responses would help gain insight into the role of auto-antibodies in the pathogenesis of RA. In chapter 4, we provide evidence that anti-CCP2 are a collection of ACPA directed to different citrullinated proteins, with many antibodies exhibiting high level of cross-reactivity, whereas some are rather distinct and are specific for one antigen.
This finding obviates the confusion concerning the biological relevance of the antibodies detected with CCP2 ELISA based assay, in which a synthetic antigen with unknown sequence is used. Our results are in accordance with previous studies indicating that ACPA responses detected in patients with RA are cross-reactive. This was further confirmed by the study of Amara et al., in which they investigated citrulline specific antibodies generated from synovial fluid B-cells of RA patients [4].

Interestingly, the novelty of this study resides in the finding that some ACPA antibodies do not cross-react suggesting that they are most likely derived from distinct B cell responses. RA-associated-auto-antibody responses are consistently observed to emerge prior to disease onset and develop over time, starting with a response to a particular antigen and gradually expanding to reactivities to different antigens, a phenomenon known as epitope spreading. Although, it is thought that this phenomenon stabilizes at the onset of the disease, the question remains whether distinct ACPA reactivities we have detected in patients with an established RA would become cross-reactive at a certain time during progression.

Citrullination is a posttranslational modification that results in the generation of citrullinated antigens believed to represent an important group of auto-antigens in RA. In fact, significant progress has been made in identifying various citrullinated antigens in the joints of RA patients. Some citrullinated antigens exhibit high binding affinity to RA associated MHC class II molecule. Binding of citrullinated antigens to RA associated MHC class II molecules expressed by antigen presenting cells can lead to the induction of an immune response by CD4+ T cells, which subsequently can provide help for the activation of citrullinated antigen specific B cells and ACPA production. Indeed, citrullinated vimentin derived peptides have been shown to be recognized by T cells in HLA-DR4 positive patients implying the presence of auto-reactive T cells.

Citrullination can also occur under physiological conditions and inflammation and it remains a major challenge to determine whether citrullinated antigens are indeed responsible for triggering RA. The study of ACPA in individuals with arthralgia led to the observation that only a minority of arthralgia individuals having ACPA develop arthritis, while the majority of ACPA positive arthralgia individuals do not develop RA suggesting the requirement of other additional factors for the emergence of RA.

Abatacept is an effective therapy for the treatment of RA. The general belief holds that the mode of action of abatacept is based on the regulatory property of CTLA-4, which competes with the costimulatory molecule CD28 for binding to CD80/CD86 thereby preventing the activation of CD4+ T cells. The fact that abatacept is still able to reduce disease progression in the absence of CD4+ T cells in the CIA model strongly indicates that abatacept does not affect solely the costimulation of CD4+ T cells but might also exhibit additional mode of actions in established arthritis. This study calls for further research to unravel the additional mode of action of abatacept, which can lead to better understanding of disease etiology and development of tailored and effective therapies.
Our results are supported by increasing body of evidence showing that abatacept can exert its effect through different types of immune cells including monocytes, macrophages, osteoclasts [19] and B-cells [18]. Our studies show for the first time that abatacept treatment in the absence of CD4$^+$ T cells results in an attenuated B cell response, as manifested in a decrease in total and collagen specific antibody levels measured in serum as well as in supernatants of ex-vivo cultured spleen and bone marrow cells.

In principal, one could anticipate that abatacept would inhibit any interaction between CD28 and CD80/CD86 expressed by any type of cell. The finding that CD28 is also expressed by plasma cells and it functions as a survival molecule in mice prompted us to investigate whether CD28 expressing B cells are present in patient with RA. Indeed, in chapter 5, we show for the first time that a portion of synovial fluid B cells and ACPA$^+$ plasmablasts/-cells of RA patients express CD28, unraveling a novel cellular interaction that could be targeted by Abatacept.

CD28 has been shown to mediate longevity of bone marrow plasma cells in mice. Inflammation can also create an environment that supports the survival of plasma cells. This environment can be found in the inflamed synovial tissue of RA patients and indeed results from our group revealed that ACPA$^+$ plasmablasts/-cells from synovial fluid were able to survive for a prolonged period of time, which strongly suggests that this survival can also lead to sustained auto-antibody production and disease perpetuation.

In fact, ACPA$^+$ plasmablasts/-cells from the site of inflammation of RA patients produce increased levels of the ACPA auto-antibodies. The presence of CD28 expressing ACPA$^+$ plasmablasts/-cells in RA patients is an important finding that puts forward the need for investigations into the involvement of CD28 in conferring survival potential to auto-antibody producing B cells, which are playing an indisputable role in RA pathogenesis.

This finding is of a great relevance in auto-antibody mediated diseases like RA or vaccination. In this context, CD28 can be considered as a power switch that can be turned on or off depending on whether antibodies are desirable or not. As abatacept, which is a CTLA-4-Ig blocks the interaction between CD28 and the costimulatory molecules CD80 and CD86, by binding with high affinity to CD80 and CD86, we hypothesized that the reduced auto-antibody responses associated with clinical amelioration in RA patients after treatment with abatacept might be, in addition to inhibition of T-cell activation, a result of abatacept interfering with the survival of long lived plasma cells.

Identifying the role of CD28 expressed by auto-antibody producing B cells in RA would ultimately help to refine abatacept treatment and/or design a more efficient strategy that solely targets auto-antibody producing B cells responsible for the production of pathogenic auto-antibodies while maintaining B cells mounting protective immune responses against pathogens.
This strategy would be beneficial not only for patients with an established disease, but can also be used to prevent the development of RA in individuals with preclinical disease exhibiting high auto-antibodies titers and are at high risk for developing RA. This can be achieved by combining antagonist antibodies against CD28 and antigen specific auto-antibody producing B cells. Moreover, abatacept is also currently used in several clinical trials to test its efficacy in diseases other than RA such as type 1 diabetes and SLE. Therefore, understanding the working mechanisms of abatacept would not only benefit RA patients, but a wide range of patients suffering from diverse diseases.

In conclusion, we have shown that anti-CCP2 antibodies are a heterogeneous collection of antibodies directed to diverse citrulline-containing proteins (ACPAs), with some ACPA are crossreactive and some are distinct noncross-reactive. We have found that plasmablasts/-cells secreting ACPAs and B cells obtained from synovial fluid of RA patients express the prototypic T cell costimulatory molecule CD28.

We have also revealed that abatacept can modulate auto-antibody responses in the absence of CD4+ T cells in a mouse arthritis model CIA. Mice studies have shown that CD28 can be involved in the survival of long lived plasma cells which maintain longterm antibody production. Although, we have not shown that abatacept targets CD28 expressed by auto-antibody producing B cells, our findings indicate that humoral immunity in the form of auto-antibody production is affected by abatacept in a CD4+ T cell independent manner. Therefore, our results provide understanding of the mode of action of Abatacept and would open new avenues for research to improve the efficacy of Abatacept in a large spectrum of patients.

In addition, the results presented in this thesis also confirm the potent potential of CD49b+CD4+ T cells to modulate pro-inflammatory responses. We have shown that CD49b+CD4+ T cells inhibit the ability of LPS-stimulated dendritic cells to induce an IFN-γ response by CD4+ T cells via blocking IL-12 production in IL-10 dependent manner. These results have contributed to broaden our understanding of the mechanisms employed by CD49b+CD4+ T cells to suppress pro-inflammatory responses involved in arthritis pathogenesis. In the same context, we have presented data indicating that CD49b+CD4+ T cells can be found in young mice. This observation has prompted us to perform studies aiming at identifying the protein nature of the antigens responsible for the induction of IL-10 response by CD49b+CD4+ T cells.

We have demonstrated that bovine proteins like fibrinogen and fetuin, which are present in fecal calf serum can induce CD49b+CD4+ T cells to produce IL-10. However, the identity of the peptide(s) derived from these proteins is still unknown. Nevertheless, our results are paving the way towards unraveling the identity of these antigens that would facilitate the design of an antigen-based immunotherapy for RA.
Chapter 8

References


