Towards adoptive cellular therapy of chronic autoimmune arthritis

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TOWARDS ADOPTIVE CELLULAR THERAPY OF CHRONIC AUTOIMMUNE ARTHRITIS

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“De Heer is mijn herder,
mij ontbreekt niets”

(Psalm 23)

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CHAPTER I

General introduction

I. GENERAL INTRODUCTION

i. INTRODUCTION

Rheumatoid arthritis (RA) is the most common inflammatory disorder with joint involvement. The disease leads to severe disability as a result of inflammatory processes, and, subsequently, destruction of cartilage and bone in the joints. Although T cell-mediated autoimmune responses, cytokine networks and the destructive behaviour of rheumatoid synovium have been implicated in the pathogenesis of RA, recently, the role of pathogenic B cells producing autoantibodies is re-emerging as one of the most important factors in disease initiation and progression [5]. Many years of research and clinical practice have led to a better understanding of how the disease may develop, and how the disease can be treated. However, in a substantial number of patients, the disease cannot be controlled properly by conventional therapies, prompting clinicians and researchers to search for new therapeutic options.

This thesis will, therefore, study whether adoptive transfer of donor cells, either from the recipient itself or from a healthy donor, can be used to treat chronic autoimmune arthritis; the studies described in this thesis aim at treating disease through the eradication of self-reactive immune cells of the recipient and/or the induction of self-tolerance. In this chapter, an introduction will be given on RA and a mouse model for arthritis, collagen-induced arthritis (CIA). Furthermore, a rationale for adoptive cellular therapy to treat chronic autoimmune arthritis will be provided.

ii. RHEUMATOID ARTHRITIS

Rheumatoid arthritis (RA), being the most frequent amongst rheumatoid disorders, occurs in approximately one percent of the adult population worldwide, and is more common in females than in males [1, 39, 42, 135]. The main characteristic of the disease is arthritis in multiple joints, mainly hands and feet. Although a small percentage of the patients show (spontaneous or drug-induced) remission or stable disease, the majority of patients suffer from progressive disease throughout their lives. The disease is thought to be an autoimmune disease, meaning that the immune system aggressively reacts against self-antigens, instead of maintaining tolerance towards these antigens. The joints are the major sites of inflammation in RA, possibly because certain self-antigens are presented locally to the immune system within the synovial tissue.

However, extra-articular manifestations are quite common [150], and RA may, therefore, be considered a systemic autoimmune disease.

Treatment of RA comprises anti-inflammatory drugs, disease modifying anti-rheumatic drugs and cytokine-blocking agents like anti-tumour necrosis factor (TNF). These types of treatment do not always result in sufficient reduction of disease activity, while considerable side effects can be observed. Up till now, it is not known which series of events are responsible for pathology. Similarly, it is not known how clinical disease should be treated best.

iii. COLLAGEN-INDUCED ARTHRITIS

To answer questions about pathogenesis and effective treatment, it is necessary to use different animal models. Especially mouse models are commonly used, because mice have some relevant characteristics. The immune system of mice is very similar to humans. Besides that, the availability of knock-out (KO) and transgenic (Tg) mouse strains provides powerful tools to study the immune system in more detail. Concerning the study of autoimmune diseases, inbred mouse strains are available that are known for their susceptibility for (spontaneous) autoimmune disorders. These disorders resemble the disease in humans in several features such as T cell and/or B cell autoreactivity [24, 99, 103, 129, and reviewed in 95], and subsequent production of autoantibodies, e.g. anti-collagen type II (CII) antibodies in CIA [52, 59], anti-glucose-6-phosphate isomerase (GPI) antibodies in the K/BxN arthritis model [81, 103], anti-double stranded DNA and anti-nucleosomes antibodies in MRL/lpr and (NZB x NZW) F1 mice [24, 134]. Interestingly, anti-cyclic citrullinated peptide (CCP) antibodies appear to be highly specific for RA, and these antibodies have been suggested to contribute to disease in CIA as well [83]. Furthermore, histological abnormalities are very similar.

The mouse model used for the studies described in this thesis is CIA [reviewed in 95] which is considered to be a classical model for RA (Figure 1), although other mouse models [188], including spontaneous disease models [99, 103, 129], have been described. CIA is a severe, chronic autoimmune disease that progresses in time. In general, distal joints such as front and hind paws, and to a lesser extent knee joints, are clinically affected. Mice suffering from severe inflammation have stiff, painful and swollen joints due to infiltration by inflammatory cells including neutrophils, macrophages, and lymphocytes (i.e. T and B cells). These cells appear in the synovial

tissue as well as in the synovial fluid. Inflammation is followed by destruction of the normal joint architecture as a consequence of degradation of cartilage and bone. The disease is induced by immunization with (heterologous, e.g. bovine) CII that is mainly found in the (hyaline) cartilage of synovial joints. About 3-5 weeks after immunization, clinical arthritis develops while large amounts of anti-CII antibodies are readily detectable, even several weeks before clinical onset. It has been shown that anti-CII autoantibodies induce clinical disease as well as histological changes similar to those of immunized animals [59, 116]. The observation that the transfer of autoantibodies (i.e. serum from immunized animals or a cocktail of monoclonal antibodies) against anti-CII [52, 59] or other autoantigens like GPI [96, 102], can lead to full-blown synovial inflammation in naïve mice, suggest that these mouse models are useful to study B cell-dependent/antibody-mediated autoimmune diseases like RA.

iv. THE IMMUNE RESPONSE IN ARTHRITIS

Abundant data are now available from mouse models, including CIA. These animal studies shed light on the immunological events that take place during the development of clinical autoimmune disease, showing that B cells and their main products, i.e. autoantibodies, play an indispensable role, although CD4**+** T cells are also crucially involved in the initiation of the autoimmune response. The model described in Figure 2 can be used to explain how certain immunological events lead to clinical arthritis.

Under inflammatory conditions, professional antigen-presenting cells (APC), i.e. dendritic cells (DC), take up self-antigens in the periphery. Subsequently, DCs migrate to the draining lymph nodes where they present these antigens, after intracellular processing, to naïve CD4**+** T helper cells in the context of major histocompatibility complex (MHC) class II molecules (Figure 2A). MHC class II genes have been found to be strongly associated with disease. For the larger part, the susceptibility is dependent on MHC class II genes in CIA [reviewed in 95, 113], but also other mouse models of chronic arthritis, i.e. the K/BxN model as well as the proteoglycan-induced arthritis (PIA) [99, 103, 107, 108]. This association strongly suggests that CD4**+** T cells (i.e. T helper cells) are important in disease induction and/or progression as MHC class II molecules are involved in the presentation of antigen to CD4**+** T cells by mature professional APCs (i.e. DCs and macrophages).

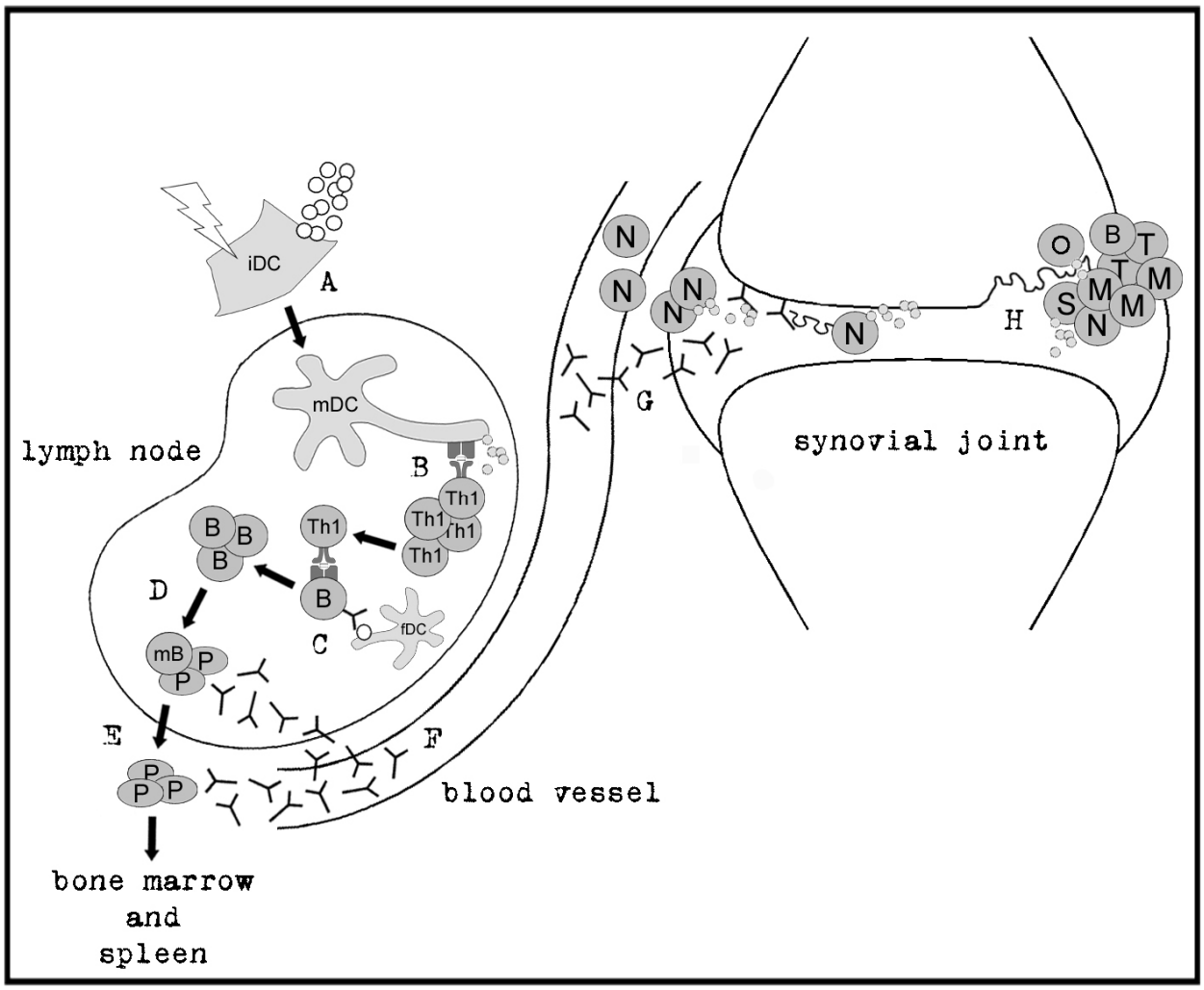
Figure 1: Similarities between collagen-induced arthritis and rheumatoid arthritis.

Characteristics [references]
Stiff, swollen and painful joints
Histopathology (including pannus formation, oedema, massive infiltration with leukocytes, i.e. neutrophils, T and B cells) [55, 187]
Destruction of cartilage and bone
Autoantibodies against different autoantigens (i.e. collagen type II, IgG and citrullinated proteins) [56, 58, 83, 111, 117, 121, 125, 130, 155, 158, 170]
Sex-linked susceptibility/influence by female sex hormones and pregnancy [51, 66, 171]
Association with MHC class II alleles [45, 53, 54, 187]
Pathogenic mechanisms underlying chronic joint inflammation (e.g. blockade of tumor necrosis factor-alpha or interleukin-1) [68, 69, 182]

Other immune-related genes like cytokines [87, 190], as well as ‘non-immune’ genes such as Ncf1 [43], also contribute to the susceptibility to autoimmune disease at the level of APCs and/or CD4**+** T cells as well. When naïve CD4**+** T helper cells specific for self-antigens encounter fully matured DCs that produce pro-inflammatory cytokines such as interleukin (IL)-23 and express co-stimulatory molecules like CD80 and CD86, they will be skewed towards a certain immunological phenotype that may result in autoimmunity. Indeed, CD4**+** T cells were shown to be crucial during initiation of the autoimmune response (Figure 2B), because depletion of CD4**+** cells could prevent onset of both clinical as well as immunological disease [82, 122]. In CIA, total spleen cells as well as CD4**+** T cells from diseased animals have been transferred into naïve recipients to induce disease, but this resulted in only, if any, mild arthritis [28, 57, 73, 114, 115, 146, 172, 181]. Some of these experiments showed that the addition of CII protein was needed to transfer disease after injection of total spleen cells or CD4**+** T cells from diseased animals immunized with CII, suggesting that antigen must be available to result in proper activation of autoreactive (memory) B cells [57, 114, 181]. In

fact, B cells need to take up, process and present the antigen via MHC class II molecules to CD4**+** T cells which in turn activate the B cells to produce pathogenic autoantibodies (Figure 2C). Depletion of CD4**+** T cells during the effector phase of arthritis (i.e. after onset of clinical disease) had no clear beneficial effects [82, 100, 183]. These data indicate that T cells are not likely to cause and maintain arthritis on their own. After activation-induced proliferation and the process termed ‘affinity maturation’ (due to somatic hypermutation on the DNA level), the autoreactive B cells differentiate into antibody-secreting cells, the so-called plasma cells (Figure 2D). A proportion of the B cells, however, will develop into memory B cells capable of proliferating rapidly upon secondary antigen encounter. Plasma cells reside mainly in bone marrow and spleen where they continuously produce antibodies, even independently from antigen availability (Figure 2E). Thus, CD4**+** T cells are indispensable for the induction of disease, but B cells by means of their autoantibody production may be the most important players in the effector phase of the immune response, i.e. necessary to develop clinical arthritis.

Figure 2: This figure summarizes which series of (immunological) events may account for the development clinical arthritis.



(A) Under pro-inflammatory conditions, immature (i)DC take up self-antigens (open circles) and migrate to the draining lymph node. (B) In the context of MHC class II molecules and pro-inflammatory cytokines (grey circles), fully matured (m)DC present self-antigens to CD4**+** T helper cells specific for self-antigens (Th1) resulting in autoimmunity. (C) Autoreactive B cells (B) take up, process and present the antigen (provided by follicular (f)DC via MHC class II molecules to CD4**+** T cells (Th1) which in turn activate the B cells to produce pathogenic autoantibodies. (D) After activation-induced proliferation and affinity maturation, autoreactive B cells differentiate into antibody-secreting cells, i.e. plasma cells (P), or develop into memory (m)B cells. (E) Plasma cells reside mainly in bone marrow and spleen where they continuously produce antibodies. (F) Autoantibodies can be readily detected in the circulation before onset of clinic disease as well as in the chronic phase of disease. (G) After binding of autoantibodies to their antigen such as cartilage within the joints, these newly formed immune complexes will then result in the activation of mast cells (M) and the complement system (grey circles). The subsequent release of chemotactic factors results in the infiltration of leukocytes such as neutrophils (N). These leukocytes are activated via crosslinking of FcRs on their cell surfaces, and produce pro-inflammatory cytokines and other soluble factors (grey circles). (H) The ongoing immune response which includes macrophages (M), neutrophils (N) as well as T and B cells, leads to damage of the normal joint architecture via the break-down of cartilage and bone by chrondrocytes (C) and osteoclasts (O) as these cells are activated by pro-

inflammatory cytokines (grey circles). Aberrant behaviour of (resident) synovial cells, i.e. fibroblast-like synoviocytes (S), and excessive bone remodeling by osteoblasts (O) result in additional damage of the joint.

Autoantibodies can be readily detected in the circulation before onset of clinic disease as well as in the chronic phase of disease (Figure 2F). The importance of B cells and their main products, i.e. autoantibodies, in the effector phase of disease has been shown in different mouse models. CIA does not develop in mice that lack B cells [141], which also holds true for the K/BxN arthritis model, a spontaneous model of chronic arthritis [82]. In these two models, it has been shown that the autoantibodies produced by B cells are crucial and sufficient for the induction of arthritis; the passive transfer of these autoantibodies resulted in clinical disease as well as pathological changes at the histological level [52, 59, 96, 102]. Furthermore, development of clinical arthritis is strongly hampered, if not completely abrogated, in mice lacking certain components of the complement system as well as in mice deficient for activating Fc-gamma receptors (FcR) [3, 27, 48, 49, 67, 72, 79, 173]. When present in the circulation, the autoantibodies will bind to their antigen, and, after that, the newly formed immune complexes will then result in the activation of, for example, mast cells as well as the complement system, i.e. the classical pathway via binding of C1q or the lectin pathway via mannose-binding lectin (Figure 2G). In brief, complement activation consists of a series of proteolytic cleavage of several components, leading to production of C5a which is a strong chemotactic factor for leukocytes such as neutrophils and macrophages. These infiltrating leukocytes will in turn be activated via crosslinking of FcRs on their cell surfaces, and produce pro-inflammatory cytokines (e.g. TNF-alpha, IL-1 beta) that will further orchestrate the ongoing inflammatory response in the joint. These data indicate that autoantibodies (of the IgG isotype) need to bind self-antigen in the joints (i.e. the local formation of immune complexes), and subsequently activate complement, thereby attracting effector cells such as neutrophils and macrophages. Finally, these effector cells will be activated via crosslinking of their FcRs by the immune complexes in the joint. Although activation of complement and FcR-mediated cellular activation seem to be two distinct immune processes, both mechanisms need to be fully intact to develop full-blown inflammation in the joints. As a result of the binding of these autoantibodies to their antigen, inflammatory processes dependent on activation of

complement and crosslinking of FcRs seem to be primarily responsible for the damage to joint tissue [44, 48, 67, 164, 165]. Finally, the autoimmune response may lead to damage of the normal joint architecture via the break-down of cartilage and bone by chrondrocytes and osteoclasts, respectively. These cells are activated by pro-inflammatory cytokines. Aberrant behaviour of (resident) synovial cells, i.e. fibroblast-like synoviocytes, has been implicated in the pathogenesis of 'end-stage' RA as well [149], while excessive bone remodeling by osteoblasts may result in additional damage of the joint (Figure 2H).

In summary, in different mouse models of arthritis, CD4**+** T helper cells are crucial in the induction phase, while B cells producing pathogenic autoantibodies are playing the most important role in the effector phase of (chronic) arthritis (Figure 3).

Figure 3: The importance of T cells, B cells and autoantibodies.

Characteristics/events [references]	Phenotype
B cell deficiency (i.e. no B cells and antibodies) [82, 141]	no autoantibodies no arthritis
Transfer of autoantibodies [52, 59, 96, 102]	arthritis
Deficiency for complement and Fc receptors [3, 27, 44, 48, 49, 67, 72, 79, 164, 165, 173]	no/less arthritis
Importance of MHC class II (i.e. antigen-presentation to CD4**+** T cells) [95, 99, 103, 107, 108, 113]	(dependent on MHC genotype)
Depletion of CD4**+** T cells during initiation phase (i.e. defective B cell help) [82, 122]	no autoantibodies no arthritis
Depletion of CD4**+** T cells during effector phase (i.e. effective B cell help) [82, 100, 183]	autoantibodies arthritis
Inability of CD4**+** T cells alone to transfer disease (i.e. no antigen > defective B cell help) [28, 57, 73, 114, 115, 146, 172, 181]	no arthritis
Ability of CD4**+** T cells to transfer disease in the presence of antigen (i.e. antigen > effective B cell help) [57, 114, 181]	arthritis

v. PATHOGENIC ROLE OF AUTOANTIBODIES

In humans, multiple autoantibodies against several autoantigens are often observed in patients with RA [reviewed in 154]. It is conceivable that the immune system is responding against other, i.e. currently unidentified, autoantigens as well. The underlying autoimmune response develops over time, responding to more and more self-antigens, eventually leading to clinical disease. For instance, accumulation of autoantibody responses before onset of clinical disease has recently been described in systemic lupus erythematosus (SLE) [2]. This knowledge may be crucial for our understanding of how clinical autoimmune disease develops; multiple antibody responses may be required for overt clinical disease. In agreement with this so-called 'multiple hit hypothesis', it is currently being hypothesized that the presence of multiple autoantibodies (e.g. anti-C1q and anti-DNA/histone antibodies) need to co-operate in the development of lupus nephritis (LN), a severe complication which is observed in patients with SLE [reviewed in 41].

In RA, anti-CCP antibodies and rheumatoid factors (RF) (i.e. immunoglobulins directed against the Fc portion of IgG molecules) are the most well-known autoantibodies, and are strongly associated with destruction of cartilage and bone [4, 7, 11, 156, 168]. RFs, one of the criteria for the classification of RA (i.e. revised American College of Rheumatology criteria, 1987), however, are not specific for the disease as patients with other immune-related diseases like SLE or infectious diseases have these antibodies as well [47, 88, 186]. Moreover, healthy individuals, particularly elderly, also have RFs [166], but no joint involvement. Similar to autoantibodies in SLE, it has been described that anti-CCP antibodies can be detected years before onset of clinical disease. It remains puzzling what is so specific about anti-CCP antibodies in the development of RA in humans as the process of citrullination occurs frequently during inflammation [10, 98], and also takes place under normal conditions in the skin [reviewed in 23]. Subclinical disease (i.e. inflammation at the histological level) cannot be excluded in these individuals, but the mere presence of anti-CCP antibodies cannot account for clinical arthritis; additional factors such as multiple autoantibodies and/or circulating immune complexes are likely to be involved as well. Indeed, a crucial role for circulating immune complexes in autoimmune arthritis has been suggested which was dependent on FcRs as well as neutrophils and mast cells [184, 185]. A significant proportion of patients may have other autoantibodies than those that are routinely tested for

diagnostic purposes, i.e. RFs, with unknown specificity. Thus, although detectable in the circulation, the precise role of anti-CCP antibodies, rheumatoid factors and/or anti-GPI autoantibodies (which are associated with extra-articular manifestations such as vasculitus [101, 157]) in the development of clinical arthritis remains unclear; their mere presence does not result in clinical disease. It has also been proposed that other pro-inflammatory signals are required for proper trafficking of arthritogenic autoantibodies as well, e.g. the transfer of anti-CII autoantibodies to wild-type mice only results in clinical disease when lipopolysaccharide (LPS) from E. coli is co-injected [147, 148]. Therefore, the co-existence of multiple autoantibodies, circulating immune complexes and/or additional pro-inflammatory stimuli may provide clues to explain how clinical disease is initiated. In conclusion, the pathogenic role of autoantibodies produced by autoreactive B cells has been well documented in a variety of mouse models of autoimmune disease; however, it remains unclear why the immune response against different (ubiquitously expressed) self-antigens leads to organ-specific autoimmune arthritis. Altogether, these data support the ‘multiple hit hypothesis’; clinical arthritis may develop as a consequence of a seropositive status, but the mere presence of arthritogenic autoantibodies does not explain the development of clinical disease.

vi. TREATMENT OF CHRONIC ARTHRITIS

Patients with chronic autoimmune disease, such as RA, are conventionally treated with non-specific immunosuppressive drugs. Almost all of these non-specific therapies for severe autoimmune disease are based on general suppression of the immune system, but are not curative (see Figure 4). Although beneficial to autoimmune disease, general immunosuppression also compromises immune responses against invading pathogens apart from other additional undesired side-effects. Although most patients with autoimmune disease respond well to conventional treatment, a significant proportion of patients suffer from severe, treatment-resistant progressive autoimmune disease. This emphasizes our lack of understanding of autoimmunity; we do not know how autoimmune diseases develop and progress [36]. Another example of this lack of understanding is the failure of many immunological approaches that are effective in animal models to modulate human autoimmune disease [36].

More specific immunological therapies have been developed recently, and this has led to a better understanding of the pathological immune responses in autoimmune

diseases. One of the best examples of these newly developed immunotherapies is the use of ‘biologicals’, i.e. therapeutic proteins such as antibodies or soluble receptors, in blocking pro-inflammatory cytokines like TNF. The blockade of TNF as well as IL-1 has resulted in marked beneficial effects in RA, inflammatory bowel disease, ankylosing spondylitis, and psoriasis [13, 34, 37, 77, 97, 110], indicating that cytokines are important mediators in chronic autoimmune responses. The major problem with these interventions, though, is that their benefit is achieved by interfering with processes that are also involved in host defence, and these types of extracellular molecules like cytokines and chemokines are often redundant in their biological properties. Most importantly, these molecules are ‘end products’ of cells that are somehow involved in the ongoing immune response, and intervention at this level will probably not result in long-standing remission of clinical disease. The underlying autoimmune response is, therefore, not dealt with as the cells that produce these mediators are left intact (see Figure 4). For the latter reason, the immune cells that are responsible for the induction and/or the maintenance of the chronic autoimmune response are, conceivably, the best therapeutic targets.

vii. B CELL DEPLETION

Recent (pre-)clinical studies have indicated that B cells may be the driving force behind autoimmunity instead of just being ‘workhorses’. In this respect, clinical trials using B cell depletion have provided important clues about RA as a B cell-mediated/antibody-dependent autoimmune disease. The biological used in these trials, named rituximab, is a chimeric monoclonal antibody against CD20, a B cell-specific marker expressed on the cell surface of B cells. Anti-CD20 treatment resulted in good clinical responses in a significant proportion of patients with RA, and in reductions of inflammatory biomarkers (e.g. C-reactive protein, CRP) [reviewed in 30]. These beneficial effects were often accompanied by a decrease in autoantibody levels, e.g. anti-CCP antibodies, in the circulation.

Several considerations and difficulties for targeting B cells in RA can be taken into account. First, depletion of B cells could result in the elimination of precursors of plasma cells that will produce pathogenic autoantibodies, and, as a result, ameliorate disease. The CD20 molecule is absent, however, on (long-lived) plasma cells which could be the most important targets in terms of autoantibody production. Still, studies in patients with

RA indicate that autoantibody levels often decrease significantly, suggesting that these autoantibody-producing cells are relatively short-lived as well as CD20**+**, and are continuously derived from an activated B cell precursor [30]. But, although this seems promising at first sight, when T cells are controlling the continuous activation of B cells and the subsequent production of autoantibodies, the autoantibodies are likely to be produced again as soon as B cells return. Second, if B cells are crucially involved in antigen-presentation to T cells [119, 191], and if removal of B cells will thus result in disease amelioration, then, again, disease would be expected to reappear. Third, when autoreactive B cells have entered a vicious cycle [31, 32, 89, 124], this clearly provides a rationale to deplete B cells. However, a major drawback of B cell depletion still remains that clinical responses are only temporarily achieved. Furthermore, repeated use of depleting doses of anti-CD20 may induce hypogammaglobulinaemia, thereby compromising humoral immunity against newly invading pathogens, while partial depletion does not seem to be a good therapeutic alternative.

In summary, B cells have been recognized as major players in the autoimmune response, probably being involved in the initiation and perpetuation of the ongoing pathogenic immune response, but the precise mechanisms of their continuous activation in human disease remain unclear. Therefore, B cell depletion, although clinically effective, might not be the ultimate therapy for severe and chronic autoimmune arthritis (see Figure 4).

viii. BONE MARROW TRANSPLANTATION

Hematopoietic stem cell transplantation (HSCT), a.k.a. bone marrow transplantation (BMT), has become an established treatment modality in the treatment of malignant disorders and inherited non-malignant immune diseases, and it has been successfully employed for some decades already. The treatment of human disease by allogeneic BMT was pioneered by Nobel Prize-laureate E.D. Thomas and colleagues in the 1950s. Later on, it has also been recognized as a potential treatment for severe and chronic autoimmune diseases which was based on anecdotal case reports of patients suffering from hematologic disorders, either malignant or drug-induced, and co-incidental autoimmune disease (including RA) who were effectively treated with allogeneic BMT with disease free remission even up to 20 years [65, 93, 94, 138]. Although not successful in all cases [105], its efficacy is probably best explained by high-dose pre-

transplant conditioning using chemo(radio)therapy: the removal of autoreactive lymphocytes, including B cells producing pathogenic autoantibodies, i.e. immunoablation. In the case of malignant diseases, pre-transplant conditioning using myeloablative therapy was used to strongly suppress the recipient's immune system which required rescue of hematopoiesis via infusion of donor bone marrow cells. Although myeloablation in the treatment of malignancies is most likely required for a good clinical response, this does not need to be true for severe autoimmune diseases [reviewed in 19, 20]. The rationale of BMT for the treatment of autoimmune diseases like RA is based on the concept of ablation of the recipient's immune system and the subsequent rescue or support with the infusion of bone marrow cells (i.e. 'resetting' the immune system, see Figure 4). Graft-derived immune cells may also contribute to the re-induction of tolerance to self-antigens, either directly via the so-called 'graft-versus-autoimmunity effect' (GVA) [50, 136] (analogous to 'graft-versus-leukemia effect' (GVL) first discovered in 1981) [60, 176] or indirectly via central and peripheral tolerance after donor-derived immune cells have newly developed. Case reports of autologous BMT (in which recipients receive their own bone marrow cells) have also been described for the treatment of co-incidental RA [22, 35], and other autoimmune disorders. Because of safety issues, as allogeneic BMT is associated with morbidity and mortality due to graft-versus-host disease (GVHD), most clinical studies have involved autologous BMT, leading to marked improvement of disease symptoms up to one to two years post-transplantation. Nevertheless, complete remissions are unusual, while relapses are common [reviewed in 19, 21, 25, 151, 163]. It has been suggested that escalating doses of high-dose immunosuppression result in improved clinical responses [121, 154]. These data indicate that effective eradication of immune cells of the recipient leads to good clinical responses. However, allogeneic BMT appears more effective than autologous BMT, which may be explained by the GVA effect [106, 137]. While the 'graft-versus-host' (GVH) alloresponses following allogeneic BMT may actively contribute to the eradication of recipient's immune cells, the co-infusion of autoreactive lymphocytes present within the graft and/or the 'intrinsic' (e.g. genetic) predisposition may explain why autoimmune disease re-develops after autologous BMT in most patients (see Figure 4).

Different obstacles preclude broad application of allogeneic BMT, despite its clinical efficacy. First, substantial pre-transplant conditioning is required to allow donor

engraftment and/or to prevent graft rejection by donor-reactive host cells, and this may result in significant tissue damage and even malignancies. Second, allogeneic bone marrow grafts are derived from a genetically (and thus immunologically) different person, and, as a consequence, GVHD is a major obstacle. GVHD can cause severe complications, and treatment-related mortality occurs in 10 to 35% of the cases. Intense immunosuppression using chemo(radio)therapy will, obviously, increase the risk of developing severe GVHD. Third, long-term post-transplant immunosuppressive drugs are often required, mainly due to GVHD. Again, this may result in substantial tissue damage, malignancies as well as compromised host defense against pathogens. Fourth, how allogeneic BMT would contribute to immunological tolerance towards self-antigens, and whether the concept of 'a non-susceptible donor' is just theoretical, remains elusive to date [19].

Allogeneic BMT as a treatment for autoimmune disease has been the subject of study in animals for a long time. Concerning animal models of arthritis, both spontaneous and induced models have been studied. A number of important observations have been made that may put into perspective at least some of the clinical data obtained in humans. In animals that spontaneously develop autoimmune disease, including lupus-prone MRL/lpr mice and insulitis-prone NOD mice, the disease cannot be cured with syngeneic BMT [75, 140], although it was prevented with congenic BMT from a nonautoimmune-prone donor strain [133]. In fact, the transplantation of bone marrow from autoimmune-prone mice to normal recipients leads to disease development [12, 86, 99, 127, 128, 131, 180]. Syngeneic BMT does result in some clinical responses [75, 140], but spontaneous autoimmune diseases are best treated, and even cured, with allogeneic BMT [62, 192]. Concerning animal models in which autoimmune disease is actively induced via immunization, including CIA: no evidence exist that 'induced' autoimmune disease can be transferred by syngeneic bone marrow alone [8]. The disease can be treated with syngeneic or 'pseudo-autologous' BMT (i.e. from syngeneic donors that were immunized), although with less efficacy than with allogeneic BMT [9, 17, 63, 74, 80, 153, 159, 160, 161, 162].

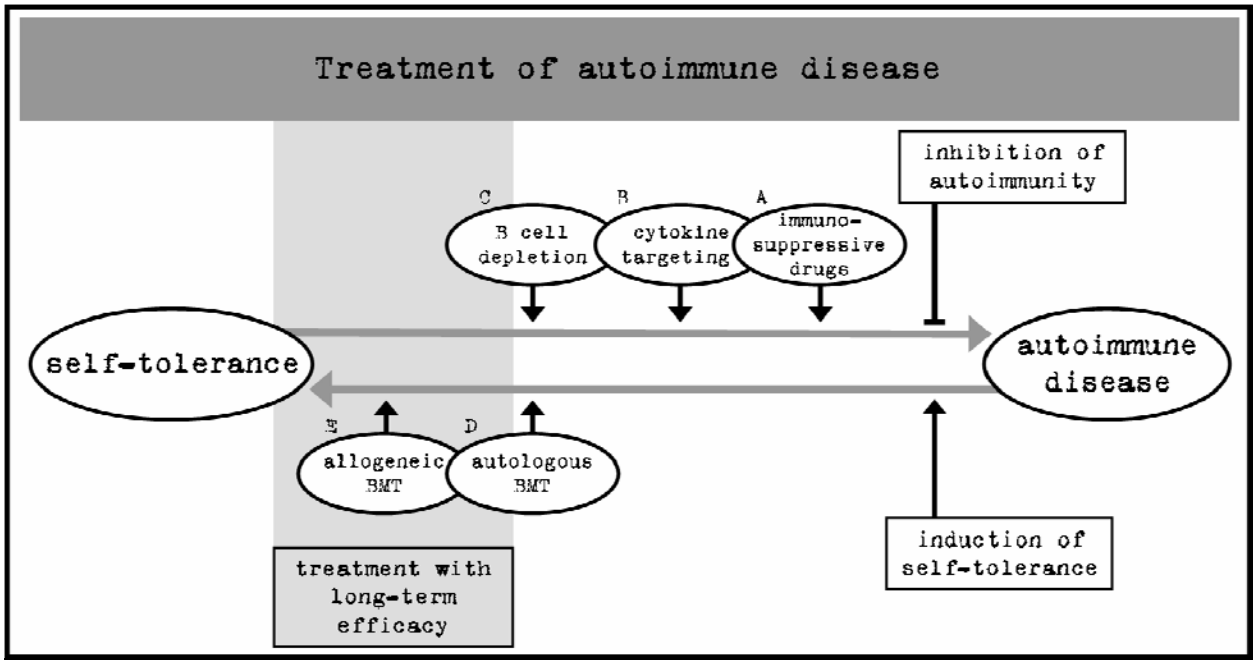
The aforementioned animal studies aimed at the removal of autoreactive lymphocytes via intense immunoablation, often using high-dose irradiation which is considered to be myeloablative as well. These types of conditioning regimens will, therefore, preclude the widespread application of allogeneic BMT to treat chronic autoimmune diseases.

Fortunately, however, a lot of progress has recently been made: the use of different so-called ‘nonmyeloablative’ conditioning regimens prior allogeneic BMT has shown to establish donor/host-specific tolerance without GVHD [64, 90, 84, 120, 142, 144, 177, 178]. These regimens, often using monoclonal antibodies to block co-stimulatory molecules or deplete host T cells, were originally developed to overcome graft rejection, either being solid organ grafts or bone marrow transplants [142]. Nonmyeloablative conditioning followed by allogeneic BMT, however, has also been shown to prevent as well as treat T cell-mediated autoimmune disease in mice [33, 91, 92, 118, 132, 189], but no data are currently available to show that B cell-mediated autoimmune disease, including chronic arthritis, can be treated with nonmyeloablative conditioning and allogeneic BMT. Nonetheless, some studies have focussed on mixed chimerism, i.e. the co-existence of both donor and host hematopoietic and immune cells instead of full donor chimerism [142], albeit after myeloablative conditioning and mixed bone marrow grafts. Again, these ‘allografts’ were able to prevent and/or arrest disease development [76, 174, 175], both T and B cell-mediated disorders such as insulitis and lupus-like autoimmune disease. However, despite the major advantages of nonmyeloablative conditioning regimens resulting in mixed chimerism, it is questionable whether mixed chimerism should be the ultimate goal in the treatment of chronic autoimmune diseases [162]. It seems obvious that mutual tolerance (i.e. donor-versus-host, and vice versa) is achieved; however, GVHD seems to be strongly associated with the GVA effect [50, 136], indicating that eradication of host immune cells is far more desirable. As mentioned above, after nonmyeloablative conditioning and allogeneic BMT, only T cell-mediated immunopathology could be effectively controlled, suggesting a role for T regulatory cells [46, 71, 85, 152]. On the other hand, B cell-mediated autoimmune disease involving plasma cells may not be effectively regulated in the present of T regulatory cells, although developing B cell responses in systemic autoimmune disease seem to be regulated to some extent by T regulatory cells [38]. Furthermore, these long-lived plasma cells are relatively resistant to chemo(radio)therapy, and are important contributors in different B cell-mediated autoimmune diseases [6, 61]. Recently, it has been suggested that, not only alloreactive T cells from donor-origin, but also donor NK cells can exert potent host-reactive alloresponses without causing GVHD, although maintaining the beneficial effects in terms of GVL responses [126]. Whether these types of alloresponses, i.e. donor T regulatory cells and donor NK cells, can be used to

control autoimmune disease in the setting of nonmyeloablation and allogeneic BMT is currently unknown. Still, it can be argued that complete eradication of autoreactive B cells as well as plasma cells will, most likely, result in the effective elimination of the effector phase of the autoimmune response.

In summary, syngeneic and autologous BMT seems less effective than allogeneic BMT in treating chronic autoimmune disease in humans as well as in animal models of autoimmune. Furthermore, the effective eradication of (autoreactive) host lymphocytes, including T and B cells, is associated with better clinical responses, and (beneficial) GVH alloresponses may contribute to this. However, GVHD remains a major obstacle, and, therefore, several studies have focussed on the development of less toxic conditioning regimens prior to transplantation aimed at donor/host-specific tolerance and mixed chimerism. A similar efficacy of allogeneic BMT to treat T cell-mediated autoimmune disease in the setting of mixed chimerism is observed, although it should be determined to what extent B cell-mediated autoimmune disease can be effectively treated via the induction of mixed chimerism. It can be questioned whether plasma cells producing autoantibodies are subject to regulation in the setting of mixed chimerism: it is to be expected that their elimination will lead to better clinical responses.

Figure 4: Treatment of autoimmune disease.



A series of (immunological) events take place, resulting in clinically overt autoimmune disease. To effectively treat, or even cure clinical disease, autoimmunity should be effectively inhibited and self-tolerance must be actively induced. (A) Immunosuppressive drugs often result in ineffective control of clinical disease as well as considerable side-effects on the long-term. (B) Biologicals that target pro-inflammatory cytokines, such as TNF, have shown to effectively suppress disease activity in a lot of patients; however, in chronic autoimmune disease, effector mechanisms are temporarily abrogated. (C) B cell-targeted therapy using depleting anti-CD20 antibodies induces disease remissions over prolonged periods of time. Despite effective removal of autoreactive B cells, the reoccurrence of B cell-dependent autoimmune responses after treatment appears inevitable. (D, E) Autologous and allogeneic bone marrow transplantation (BMT) following effective pre-transplant conditioning of the recipient does not only result in elimination of B cells, but also of other key players of the immune system, including T cells. When the recipient's immune system is replaced by that of donor's (in the case of allogeneic BMT), 'resetting' of the immune system may take place, resulting in the restoration/induction of self-tolerance.

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ix. OUTLINE OF THIS THESIS

The aim of the present studies is to investigate the therapeutic properties of adoptive cell therapy in chronic arthritis. The use of (autologous verus allogeneic) BMT, T regulatory and NK cells in the context of bone marrow transplantation are studied. Chapter II suggests that the outcome of high-dose chemotherapy and autologous BMT in patients with severe RA can be predicted by analyzing synovial biopsy specimens. More importantly, this study further indicates that effective ablation of the recipient's immune cells leads to better clinical responses. Chapter III provides a rationale for the future use of T regulatory cells in treating chronic autoimmune arthritis. Chapter IV shows that allogeneic BMT is highly effective in treating chronic autoimmune arthritis mediated by autoreactive B cells in the setting of nonmyeloablative conditioning, while Chapter V supports the notion that donor alloreactive NK cells play an important role in the beneficial effects after nonmyeloablation and allogeneic BMT, and supports the notion that sustained donor NK cell alloreactivity may be beneficial in the treatment of B cell-mediated autoimmune diseases such as RA.

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CHAPTER II

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TITLE

Outcome of intensive immunosuppression and autologous stem cell transplantation in patients with severe rheumatoid arthritis is associated with the composition of synovial T cell infiltration

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ABSTRACT

Objective: To determine clinical and immunological correlates of high dose chemotherapy (HDC) + autologous stem cell transplantation (ASCT) in patients with severe rheumatoid arthritis (RA), refractory to conventional treatment. Methods: Serial samples of peripheral blood and synovial tissue were obtained from seven patients with RA treated with HDC and autologous peripheral blood grafts enriched for CD34+ cells. Disease activity was assessed with the Disease Activity Score (DAS), serum concentrations of CRP, and human immunoglobulin (HIg) scans, and the extent of immunoablation was determined by immunophenotyping of peripheral blood mononuclear cells, and immunohistochemistry and double immunofluorescence of synovium. Results: Clinical responders (n=5) had a larger number of cells at baseline expressing CD3+, CD4+, CD27+, CD45RA+, CD45RB+, and CD45RO+ cells in synovium ($P<0.05$), higher activity on HIg scans ($P=0.08$), and a trend towards higher concentrations of CRP in serum than non-responders (n=2). Subsequent remissions and relapses in responders paralleled reduction and re-expression, respectively, of T cell markers. A relatively increased expression of CD45RB and CD45RO on synovial CD3+ T cells was seen after HDC+ASCT. No correlations were found between DAS and changes in B cells or macrophage infiltration or synoviocytes. Conclusion: HDC+ASCT results in profound but incomplete immunoablation of both the memory and naïve T cell compartment, which is associated with long-lasting clinical responses in most patients. The findings provide strong circumstantial evidence for a role of T cells in established RA, and demonstrate a role for the synovium in post-transplantation T cell reconstitution.

INTRODUCTION

High dose chemotherapy (HDC) followed by autologous stem cell transplantation (ASCT) is an experimental treatment for severe autoimmune diseases, including refractory rheumatoid arthritis (RA). A number of clinical studies have demonstrated long term responses in patients with RA previously refractory to disease modifying antirheumatic drugs (DMARDs) [1-9]. The rationale of this strategy is based on the concept of immunoablation by intense immunosuppression with subsequent regeneration of naive T lymphocytes derived from re-infused haematopoietic progenitor cells [10]. The mechanism by which HDC**+**ASCT exerts its antirheumatic effects has not yet been defined. It has been postulated that intensive immunosuppressive treatment followed by ASCT may be effective for the control of RA because the conditioning regimen deletes the relevant autoreactive lymphocyte population and the re-infused stem cells develop into a lymphocyte population that acquires self tolerance. Experimental studies of autoimmune disease in rodents have lent support to this concept, but no comprehensive studies have been done in humans.

We examined samples taken serially from synovial tissue and blood from seven patients with RA treated with HDC**+**ASCT in an attempt to unravel pathogenetic mechanisms in RA. We used lineage specific markers to analyse cellular infiltrates in the synovium, as well as activation and inflammatory markers, to assess disease activity at the tissue level.

PATIENTS AND METHODS

Patients: Seven patients with RA (mean age 49 years, range 35–55; disease duration 12 years, range 7-20) were treated at Leiden University Medical Centre (LUMC) with HDC**+**ASCT as part of a multicentre phase I/II trial [7]. The protocol was approved by the LUMC medical ethical committee and the patients gave written informed consent to undergo arthroscopic procedures as described below. All patients had an established diagnosis of RA according to American College of Rheumatology (ACR) criteria [10]. Eligibility criteria were destructive disease; failure to respond to more than 4 antirheumatic drugs, including maximal tolerable dose of methotrexate (MTX) and combination therapy; and a clinically inflamed knee. Tumour necrosis factor (TNF) blockade had also failed in four patients. Table 1 summarises measures of disease activity and concurrent DMARDs.

Detection of the monoclonal antibodies was performed using affinity purified and horseradish peroxidase (HRP) conjugated goat anti-mouse antibodies (Dako), rabbit anti-rat-HRP (Dako), and goat anti-rabbit-HRP (BD Pharmingen), the biotinyl tyramide/streptavidin-HRP amplification system (NEN Life Science Products Inc, Boston, MA, USA), and aminoethylcarbazole (AEC; Sigma, St Louis, MO, USA). The HRP conjugated antibodies were diluted in phosphate buffered saline (PBS)/bovine serum albumin (BSA) (1%) with 10% normal human serum (Bloedbank LUMC, Leiden, The Netherlands) as blocking serum, and incubated for 30 minutes. A biotinyl tyramide/streptavidin-HRP amplification system (NEN Life Science Products Inc, Boston, MA, USA), and AEC (Sigma, St. Louis, MO, USA) was used to enhance staining. A biotinyl tyramide solution was added and slides were incubated for 30 minutes, followed by subsequent incubation with streptavidin-HRP in PBS/BSA (1%) for 30 minutes. HRP activity was detected using hydrogen peroxide as substrate, and AEC as dye. After washing with distilled water, the sections were counterstained with Mayer's Hämalaunlösung (Merck), and mounted with Kaiser's glycerol gelatin (Merck).

All patients had a high Disease Activity Score (DAS) at baseline as defined by European League Against Rheumatism (EULAR) criteria [11]. Autologous haematopoietic stem cells were mobilised using a single infusion of cyclophosphamide 4 gr/m(**+**2) followed by filgrastim (granulocyte-colony stimulating factor; GM-CSF) 10 microgram/kg/day subcutaneously until leukapheresis. Immunomagnetic selection of CD34**+** cells from the leukapheresis product was performed using the Clinimacs Device

(Miltenyi Biotec, Munich, Germany). DMARDs were discontinued before mobilisation and not reinstituted until at least 3 months after transplantation if deemed necessary owing to recurrence or persistence of disease activity. Corticosteroids were tapered when possible. Non-steroidal anti-inflammatory drugs were continued in the lowest dosage needed to control pain and morning stiffness. The conditioning regimen consisted of intravenous cyclophosphamide 200 mg/kg followed by reinfusion of the CD34**+** enriched graft.

Clinical assessment: Clinical assessment was performed on the day synovial biopsies were obtained using the DAS [12]. DAS **=** (0.54 x Ritchie articular index (tender joint count)) **+** (0.065 x number of swollen joints) **+** (0.33 x ln(ESR)) **+** (0.0072 x patient disease activity visual analogue scale; VAS).

Human immunoglobulin (HIg) scan: HIg scans were performed at baseline and at 3 months after transplantation according to standard operating procedures [13]. Disease activity was assessed by scoring total uptake in joints by two independent observers (RV and MW) on a four point scale (0**=**no; 1**=**light; 2**=**moderate; 3**=**strong uptake).

Table 1: Drugs used at the time of arthroscopy.

	Before HDC+ASCT, at the time of 1st arthroscopy	3 months after HDC+ASCT at the time of 2nd arthroscopy	1 year after HDC+ASCT at the time of 3rd arthroscopy
Patient, response	DAS Drug, dose	DAS Drug, dose	DAS Drug, dose
1, good	5.88 Ciclosporin 300 mg/day Prednisone 10 mg/day Ketoprofen 400 mg/day	2.11 Prednisone 7.5 mg/day Ketoprofen 400 mg/day	2.78 Methotrexate 7.5 mg/week Ketoprofen 200 mg/day
2, good	4.31 Prednisone 10 mg/day Piroxicam 20 mg/day	0.89 None	2.62 None
3, good	4.99 Hydroxychloroquine 200 mg/day Ciclosporin 100 mg/day Ibuprofen 1200 mg/day	2.28 None	2.47 Ibuprofen 1200 mg/day
4, moderate	6.61 Methotrexate 17.5 mg/week Ciclosporin 150 mg/day Prednisone 10 mg/day Naproxen 1000 mg/day	3.85 Prednisone 7.5 mg/day Naproxen 1000 mg/day	4.60 Leflunomide 20 mg Prednisone 10 mg/day Naproxen 1000 mg/day
5, good	5.58 Methotrexate 15 mg/week Diclofenac 150 mg/day	2.05 Diclofenac 150 mg/day	2.78 Methotrexate 15 mg/week Ibuprofen 800 mg/day

6, no	4.71 Prednisone 10 mg/day Ibuprofen 1600 mg/day	5.16 Prednisone 5 mg/day Ibuprofen 1600 mg/day	4.67 Methotrexate 17.5 mg/week Prednisone 7.5 mg/week Ibuprofen 1600 mg/day Tramadol 150 mg/day
7, good	5.88 Methotrexate 20 mg/week Ciclosporin 100 mg/day Diclofenac 150 mg/day	2.11 Diclofenac 150 mg/day	3.26 Methotrexate 10 mg/week Diclofenac 150 mg/day

Four variable Disease Activity Score (DAS) = (0.54 x SQRT(Ritchie articular index)) + (0.065 x number of swollen joints) + (0.33 x ln(ESR)) + (0.0072 x patient disease activity VAS).

Synovial tissue: Synovial tissue specimens were obtained from patients by serial arthroscopy. The baseline arthroscopy was performed before mobilisation (n=7). The first arthroscopy after transplantation was performed at a mean interval of 86 days (range 60-127) after transplantation (n=7). The second arthroscopy was performed with a mean interval of 1.4 years (range 405-577 days) after transplantation (n=5). The arthroscopy procedure was performed in the inflamed knee. Five millimetre biopsy specimens of synovial tissue were taken. A minimum of 10 biopsy samples was obtained and analysed for each patient at each time point.

Immunohistochemistry: Sections of biopsy specimens taken at baseline and 3 months after transplantation were stained with monoclonal antibodies (Table 2). The following markers were investigated on the 1 year post-transplantation samples: CD3, CD4, CD8, CD27, CD45RA, CD45RB, CD45RO, CD55, and CD68. Immunohistochemical staining procedures were performed as follows. Slides were warmed up to room temperature, fixed in acetone (Merck) at room temperature for 10 minutes, and air dried. After each step, the sections were washed with PBS (Apotheek LUMC, Leiden, The Netherlands). All incubations were carried out at room temperature. Endogenous peroxidase activity was inhibited using 0.1% sodium azide (Merck) and 1% hydrogen peroxide (Merck) in PBS. The monoclonal antibodies were diluted in PBS with 1% BSA (ICN Biomedicals Inc, Aurora, OH), and incubated for 60 minutes. Interleukin (IL)-1-beta, IL-4, IL-10, IL-12, and interferon (IFN)-gamma were incubated for 18 hours. For control sections, the IgG1 isotype control (anti-KLH, Pharmingen) or PBS was applied.

Immunofluorescence double staining: To characterise subsets of CD3+ cells in the five responders, double staining procedures were performed with CD45RA, CD45RB, CD45RO, and CD27 (Table 3). The following combinations of markers were used in order to identify different cell types: naïve T cells (CD45RA+ and CD27+), memory T cells (CD45RO+), and early versus more mature T cells (CD45RB+) in combinations described in Table 3.

Table 2: Primary and secondary antibodies used for immunohistochemistry studies.

Clone	Specificity	Host	Source
Primary antibodies (unconjugated):			
UCHT-1	CD3	Mouse	Becton-Dickinson, San Jose, USA
MT-310	CD4	Mouse	Dako, Glostrup, Denmark
DK25	CD8	Mouse	Dako
CLB-CD27/1, 9F4	CD27	Mouse	CLB, Amsterdam, The Netherlands
4KB5	CD45RA	Mouse	Dako
PD7/26	CD45RB	Mouse	Dako
OPD4	CD45RO	Mouse	Dako
BRIC110	CD55	Mouse	CLB
M0718	CD68	Mouse	Dako
MACT-1	CD25	Mouse	Dako
ENA-1	CD62E	Mouse	Sanbio, Uden, The Netherlands
Greg 56	CD62L	Mouse	BD Pharmingen, Woerden, The Netherlands
FN50	CD69	Mouse	Dako
M704	HLA-DR	Mouse	Dako
HD37	CD19	Mouse	Dako
HB7	CD38	Mouse	Becton-Dickinson
AS10	IL-1-beta	Mouse	Becton- Dickinson
24910.1	IL-12	Mouse	R&D Systems, Abingdon, UK
MAB285	IFN-gamma	Mouse	Genzyme, Cambridge, USA
IP-300	TNF-alpha	Rabbit	Genzyme
JES 3-19F1	IL-10	Rat	Pharmingen
Secondary antibodies (conjugated):			
Goat anti-mouse HRP	Mouse Ig	Goat	Dako
Goat anti-rabbit HRP	Rabbit Ig	Goat	BD Pharmingen
Rabbit anti-rat HRP	Rat Ig	Rabbit	Dako

Table 3: Primary and secondary antibodies used for immunofluorescence double staining.

Clone	Specificity	Host	Source
Primary antibodies (unconjugated):			
4KB5	CD45RA	Mouse	Dako, Glostrup, Denmark
PD7/26	CD45RB	Mouse	Dako
OPD4	CD45RO	Mouse	Dako
UCHT-1	CD3	Mouse	Becton-Dickinson, San Jose, USA
Primary antibodies (conjugated):			
UCHT-1 FITC	CD3	Mouse	BD Pharmingen, San Diego, USA
M-T271 FITC	CD27	Mouse	Becton-Dickinson
Secondary antibodies (conjugated):			
R0270 TRITC	Mouse Ig	Rabbit	Dako

Microscopic analysis of immunohistochemically stained slides: Sections were coded and randomly analysed. All areas of each biopsy section were examined and histological features were scored semi-quantitatively by two observers (RV and RF, or RV and LvD), who were unaware of the clinical data. The expression of CD3, CD4, CD8, CD27, CD45RA, CD45RB, CD45RO, CD55, CD56, CD68, CD25, CD62E, CD62L, CD69, HLA-DR, CD19, CD38, IL-1-beta, IL-4, IL-12, IFN-gamma, TNF-alpha, and IL-10 was scored on a five point scale (0-4). A score of 0 was given to those sections with minimal infiltration and/or low expression, whereas a score of 4 represented large infiltration by numerous lymphocytes, macrophages, or a strong expression of a certain cell surface marker. For the evaluation of CD4**+** cells, only cells with lymphocyte morphology were included, because CD4 can be expressed on monocytes. The scoring is calibrated for each marker, and has been developed previously by examining more than 5 biopsy specimens of rheumatoid synovial tissues [14]. Inter-observer readings were identical or differed by only one point, and all differences that did occur were resolved by agreement.

Microscopic analysis of haematoxylin and eosin stained slides: Haematoxylin and eosin stained sections were scored for the degree of infiltration with lymphocytes, plasma cells, and polymorphonuclear cells on a five point scale (0-4) by two observers (RV and PMK), who were unaware of the clinical data. These quantitative scores corresponded with the numbers of cells per high power field (787.5x), as described earlier [15]. A score of 0 was given to those sections with minimal infiltration, whereas a score of 4 represented infiltration by numerous cells. A composite inflammation score was calculated by summing the scores for the four components: synovial lining hyperplasia, and infiltration with lymphocytes, plasma cells, and polymorphonuclear cells (range 0-14).

Microscopic analysis of immunofluorescence stained slides: Immunofluorescence double staining in the five responders was scored by counting at least 100 of single or double positive cells.

Peripheral blood T cell reconstitution analysis: Immunophenotyping studies were carried out on peripheral blood mononuclear cells obtained at baseline, and at 1, 3, 6, 9, and 12 months after transplantation. The following combinations of markers were used to identify different cell types: naïve CD4**+** and CD8**+** T cells, memory CD4**+** and CD8**+** T cells, and early versus more mature CD4**+** and CD8**+** T cells (see Table 4 for antibodies used).

Statistical analysis: Non-parametric techniques (Wilcoxon signed rank tests) were used to test whether measures of disease activity and immunological parameters after transplantation differed significantly from baseline. Differences between responders and non-responders were assessed using the Mann-Whitney U test. A Pearson correlation coefficient was calculated to assess the relationship between serum concentrations of C-reactive protein (CRP) and scores on HIg scans.

Table 4: Antibodies used for flow cytometric analysis of peripheral blood T lymphocytes.

Clone	Specificity	Host	Source
Primary antibodies (conjugated):			
RPA-T4 PE	CD4	Mouse	Becton-Dickinson, San Jose, USA
MT310 PE	CD4	Mouse	Dako, Glostrup, Denmark
RPA-T8 PE	CD8	Mouse	BD
3B5 PE	CD8	Mouse	Serotec, Düsseldorf, Germany
L48 FITC	CD45RA	Mouse	BD
PD7/26 FITC	CD45RB	Mouse	Dako
UCHL1 FITC	CD45RO	Mouse	Dako

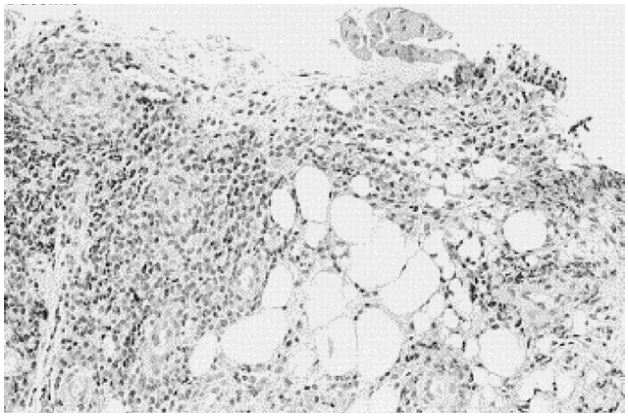
RESULTS

Clinical efficacy: The seven patients displayed a dichotomous clinical response to HDC+ASCT, with 5/7 patients attaining a good response based on the EULAR response criteria for disease activity (mean DAS from 5.33 to 1.89; P=0.04), at 3 months after transplantation. Of the two remaining patients (referred to as 'non-responders') one had an initially moderate response (DAS from 6.61 at baseline to 3.85 at 3 months), but then progressed, while the other failed to respond at all (DAS from 4.71 at baseline to 5.16 at 3 months). TNF blockade also failed in four patients. Two of these four patients responded favourably to HDC+ASCT. At the time of the second arthroscopy none of the seven patients were receiving DMARDs; however, these were re-instituted during the first year after transplantation in the two non-responders and in 3/5 responders because of flares. One year after transplantation the five responders underwent a third arthroscopy. The mean DAS in these patients at the time of the third biopsy was 2.78 (range 2.47-3.26, P=0.04 versus baseline) (Table 1).

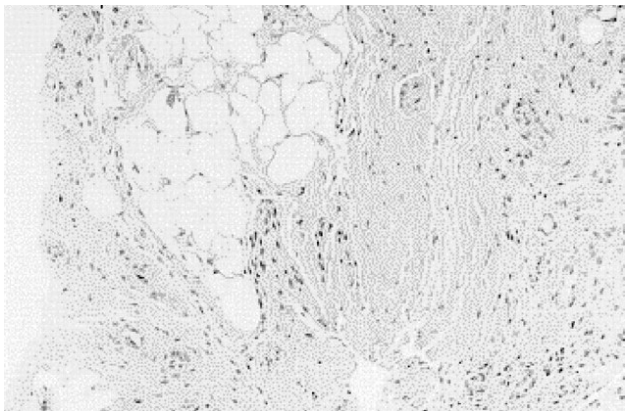
HIg scan: Baseline HIg scores and CRP concentrations were highly correlated (R=0.91, P<0.01), as were the changes after HDC+ASCT (R=0.91, P=0.01). The five clinical responders differed from the two non-responders in baseline HIg scan score (mean 2.4 vs 0.5, P=0.08) and serum concentrations of CRP (mean 54.2 g/l vs 34 g/l, P=0.44).

Haematoxylin and eosin stained slides: The mean inflammation score in the whole group decreased from 6.17 at baseline to 2.33 at 3 months after transplantation (P=0.06), and from 5.75 to 1.75 in the responders (P=0.14). A reduction of more than 50% in the inflammation score was seen in 4/7 patients after transplantation (Figure 1).

Figure 1: Synovial tissue taken from patient no. 2 (Table 1) before and 3 months after HDC+ASCT.



baseline


after transplantation

Infiltration with numerous lymphocytes and plasma cells before stem cell transplantation, which were almost absent after the transplantation.

Immunohistochemistry: Clinical responders had a high expression at baseline of CD3, CD4, CD27, CD45RA, CD45RB, CD45RO in synovium while the non-responders lacked a significant synovial T cell infiltrate (Table 5 and Figure 2). The expression of these markers decreased at 3 months after transplantation in the responders, and was significant for CD45RA and CD27 (P=0.04). When the changes between responders and non-responders were compared, significant differences were found for CD45RA and CD27 (P=0.05; Mann-Whitney U test) (Figures 2 and 3). Changes in other surface markers and cytokines were found, but these were not significant except for IL-10, which was significantly higher in the whole group at 3 months (P=0.04), and IL-1, which was significantly higher in non-responders at 3 months after transplantation (4.0 vs 0.60; P=0.02) (Table 5). Expression of IL-4 and TGF-beta was considered too low to allow meaningful analyses. At 1 year after transplantation, expression of CD3, CD4, CD27, CD45RA, CD45RO, but not CD45RB, had returned to baseline levels in the responders (Figure 3).

Table 5: Immunohistochemical scores at baseline and three months after HDC+ASCT for responders and non-responders.

	Responders (n=5)		Non-responders (n=2)	
	Baseline	At 3 months	Baseline	At 3 months
CD3	3.0	1.6	0.0	0.5
CD4	2.6	1.2	0.0	0.5
CD8	2.0	2.0	0.0	0.5
CD25	2.0	0.6	0.0	0.0
CD27	3.0	1.0	0.0	0.5
CD45RA	2.6	0.6	0.0	0.5
CD45RB	3.2	2.0	0.0	1.0
CD45RO	3.4	1.8	0.0	1.0
CD19	1.6	0.6	2.0	0.0
CD38	3.0	2.2	2.0	2.0
CD68 (lining)	1.8	2.4	0.5	1.5
CD55 (lining)	2.6	3.8	1.5	1.5
CD68 (sublining)	2.2	2.0	0.5	0.5
HLA-DR	2.6	2.6	0.0	1.5
CD62L	0.6	1.2	0.0	0.5
CD62E	0.4	0.2	1.0	0.0
CD56	2.2	1.8	1.5	3.0
IL-1-beta	1.0	0.6	0.0	4.0
TNF-alpha	2.1	1.8	1.0	3.3
IFN-gamma	2.0	2.6	1.0	2.0
IL-12	2.0	1.2	2.0	3.5
IL-10	1.2	2.3	1.5	3.5

Figure 2: Immunohistochemically stained synovial tissue in a clinical responder with CD3, CD27, CD45RA, and CD45RO, before and 3 months after HDC+ASCT.

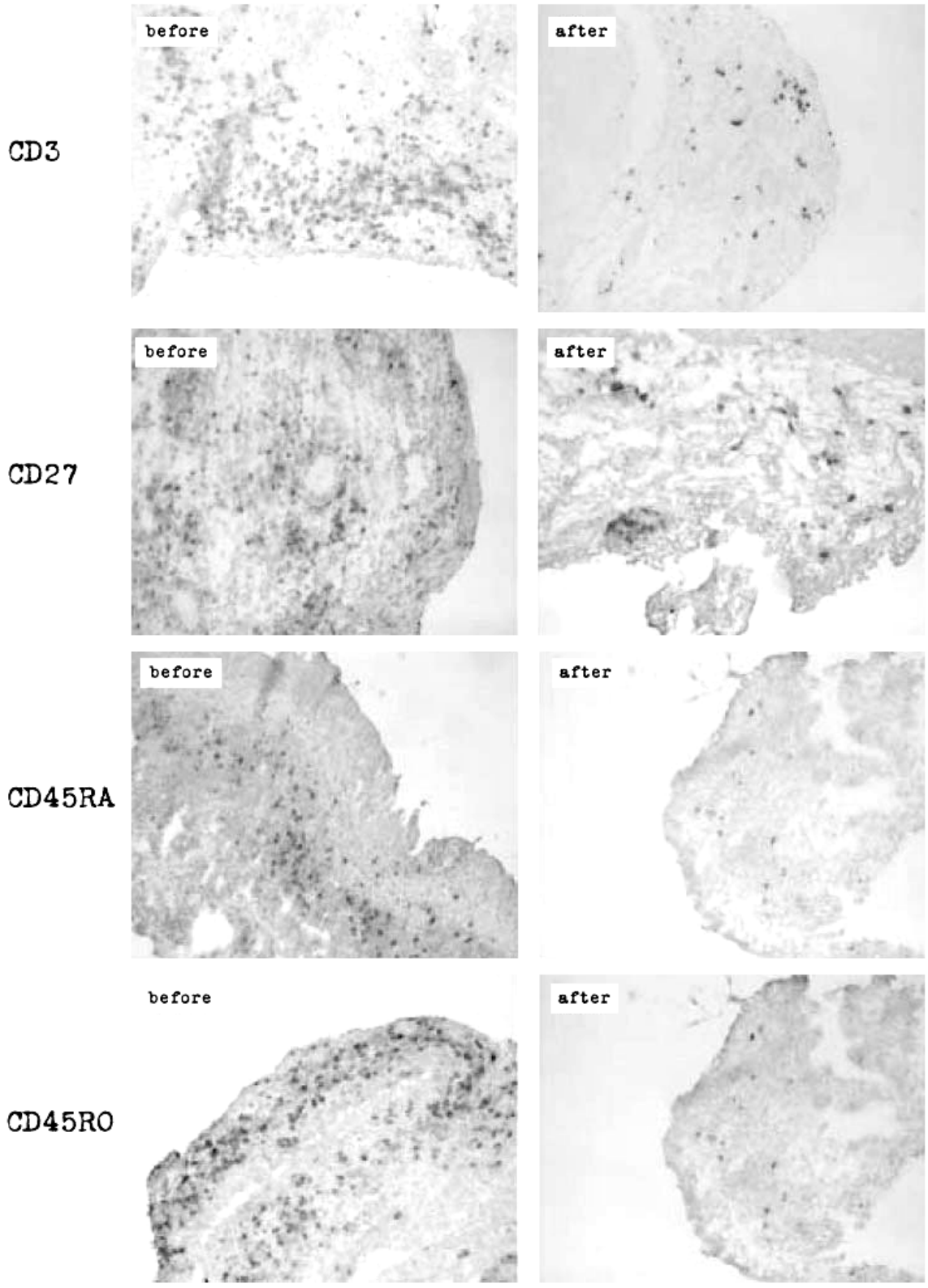
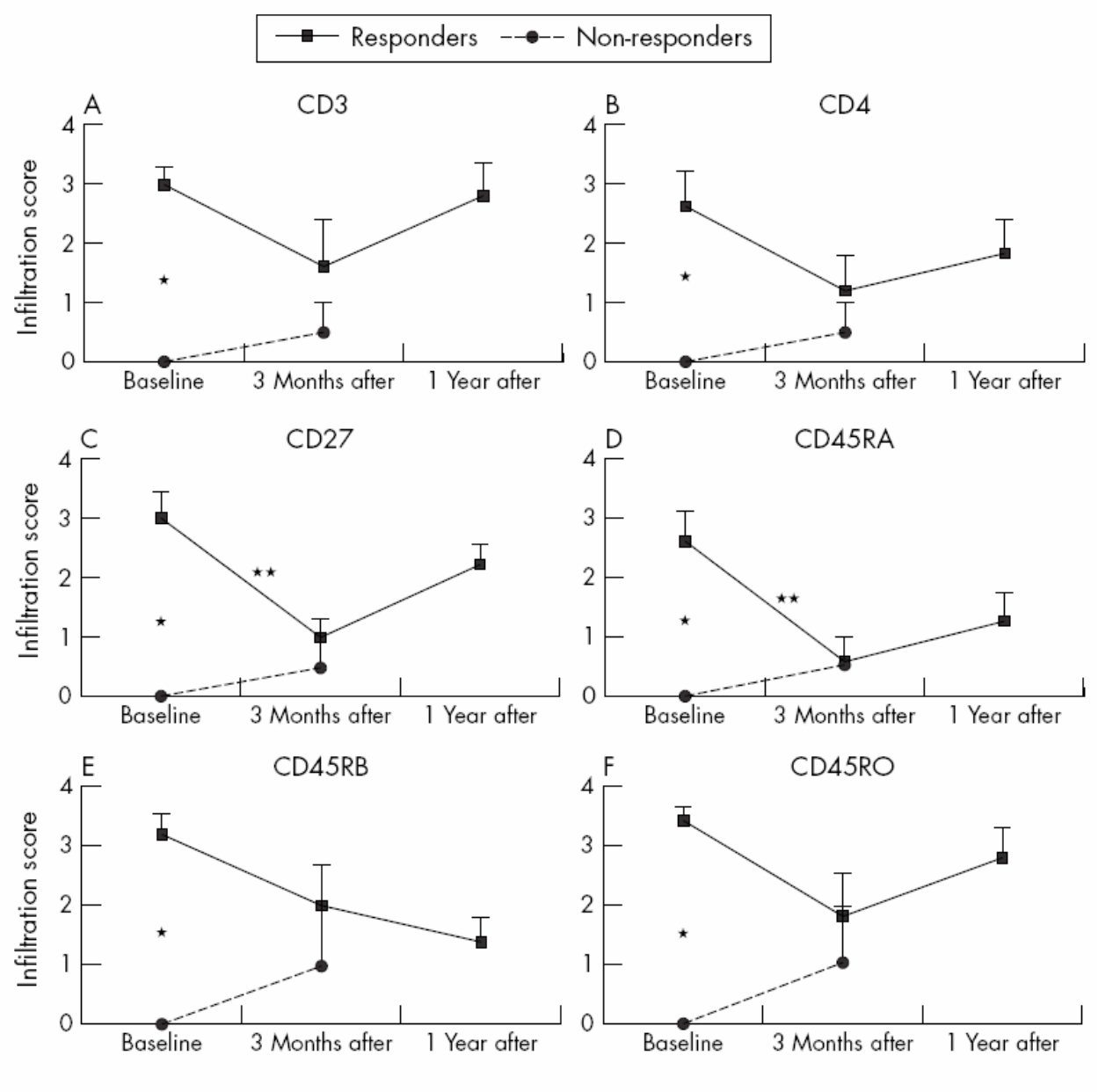
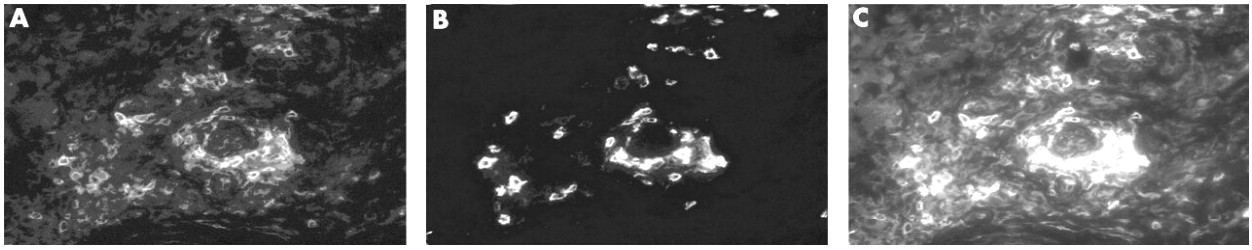


Figure 3: Semi-quantitative infiltration scores before, 3 months after, and 1 year after HDC+ASCT of responders and non-responders for CD3, CD4, CD27, CD45RA, CD45RB, and CD45RO. *P<0.05, significant baseline difference between responders and non-responders; **P<0.05, significant decrease at 3 months after transplantation in responders (i.e. expression of CD45RA and CD27).



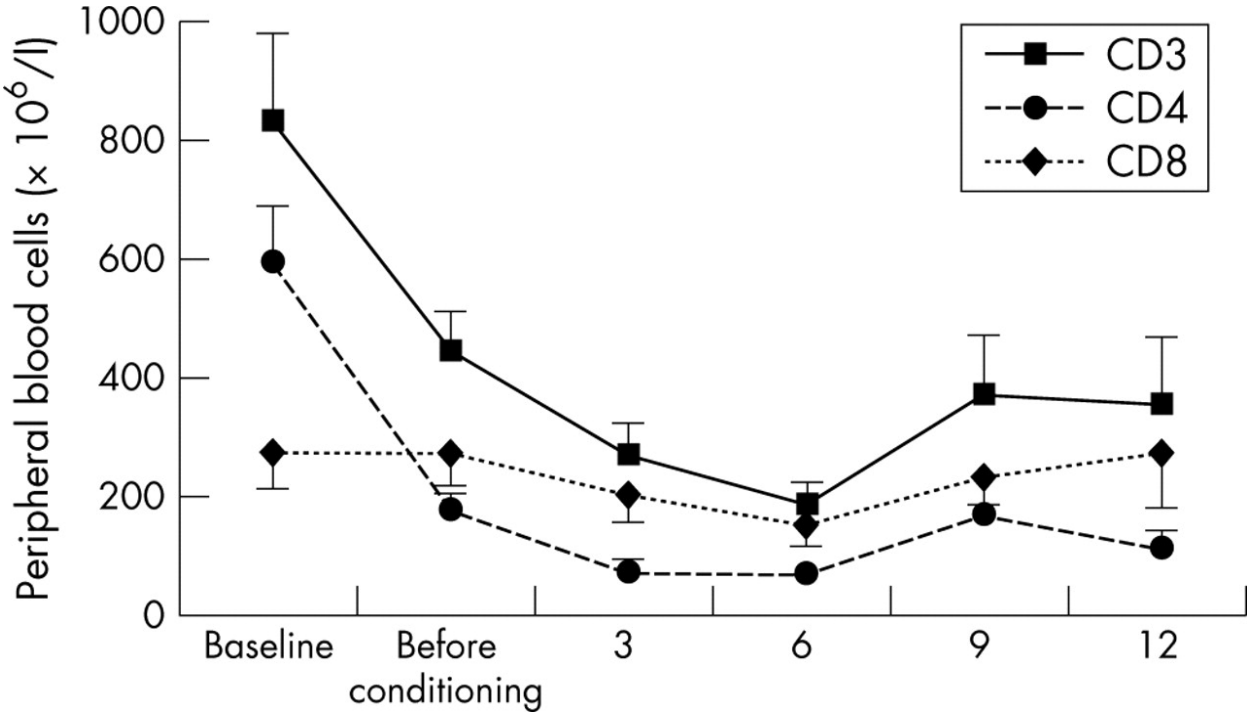
Immunofluorescence double staining: Immunofluorescence double staining was performed in the five responders to investigate co-expression of CD27 and CD45R isoforms on synovial CD3**+** T cells. At baseline 72% (range 56-86%) of CD27**+** cells were CD3**+** and 88% (range 57-99%) of CD45RO**+** cells were CD3**+** (Figure 4). Vice versa, of CD3**+** cells 68% (range 55-93%) were CD45RO**+**, 22% (range 5.5-32.7%) CD45RA**+**, and 34% (range 16.5-86%) CD45RB**+**. Three months after transplantation, CD45RA**+** was expressed on only 9% of CD3**+** cells, but CD45RO**+** on 90% of these cells (Figure 4). A high proportion of CD3**+** T cells co-expressed CD45RB (66%, range 48.9-72.4%, P**=**0.04) at 3 months after transplantation. One year after transplantation the distribution of CD45R isoforms on CD3**+** T cells had returned to baseline levels (Figure 6).

Figure 4: Co-expression of CD3 and CD45RO in RA synovial tissue before transplantation in a clinical responder.



CD3 (FITC**=**green) and CD45RO (TRITC**=**red) were detected using immunofluorescence techniques. (A) Rheumatoid synovial tissue showing CD3**+** cell infiltrate. (B) Rheumatoid synovial tissue showing CD45RO**+** cell infiltrate. (C) Double positive cells as depicted by yellow and orange, showing numerous double positive and only a few CD45RO single positive cells (red).

Figure 5: Absolute cell count in peripheral blood mononuclear cells in the five responders for CD3, CD4, and CD8.

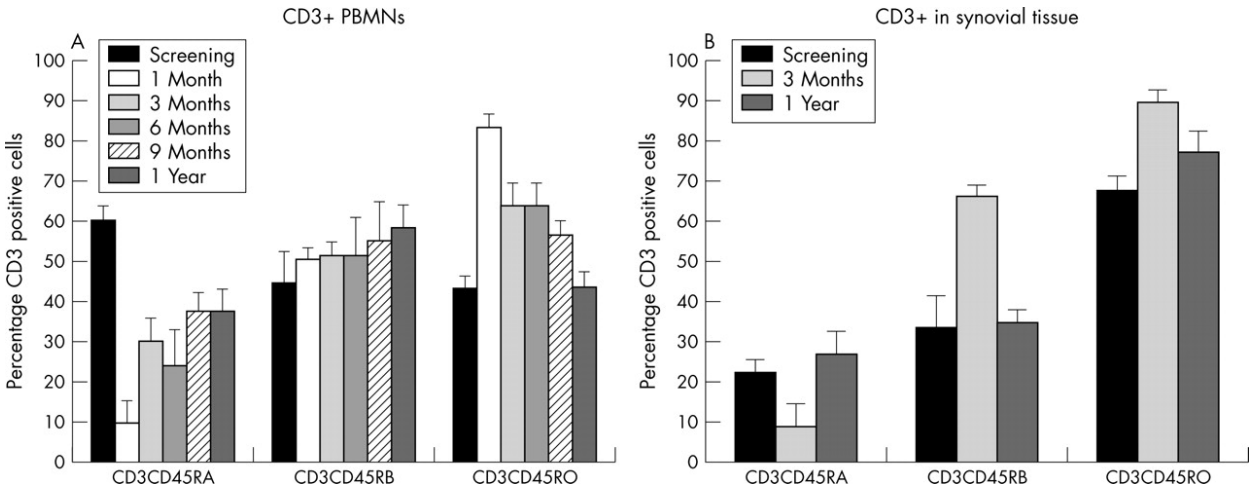


Absolute cell numbers were calculated by multiplying absolute lymphocyte count (10(+6)/l) by the percentage of each subset determined by flow cytometry after isolation of peripheral blood mononuclear cells by density gradient centrifugation.

Cell surface antigens in peripheral blood: To compare the reconstitution results of T cell subsets in the synovial tissue in the five responders, flow cytometric analyses of peripheral blood mononuclear cells in the five responders were performed focusing on CD45RA, CD45RB, and CD45RO expression on CD4+ and CD8+ cells. Absolute cell counts in peripheral blood after HDC+ASCT were characterised by prolonged lymphopenia of CD4+ T cells and transient expansion of CD8+ T cells (Figure 5). Numbers of B cells, monocytes, and natural killer (NK) cells decreased transiently, but had returned to baseline numbers by 3-6 months (data not shown). We then focused on the relative reconstitution of T cells in blood versus synovium. Figure 6 shows that a greater proportion of CD45RA+ T cells and a lower proportion of CD45RO+ T cells were present in peripheral blood than in synovial tissue at baseline. In the first months after transplantation the percentage of CD45RA in peripheral blood decreased and remained lower than baseline for the duration of the follow-up. A relative increase in CD45RO+ T cells was observed, which was significant up to 6 months after transplantation (P=0.03). This was mostly due to the transient peripheral expansion of CD8+ memory T cells

(data not shown). The percentage of CD45RB**+** cells remained relatively constant over time in peripheral blood, contrasting with the findings in synovial tissue described above.

Figure 6: The mean number of CD45RA**+**, CD45RB**+**, and CD45RO**+** cells expressed as a percentage of CD3**+** cells.



(A) Immunofluorescence double staining of peripheral blood mononuclear cells with CD3 plus CD45RA, CD45RB, or CD45RO in the five responders at screening and at 1, 3, 6, 9, and 12 months after HDC**+**ASCT. Results expressed as the percentage of CD3**+** cells expressing the CD45R isoform. (B) Immunofluorescence double staining of synovial cells with CD3 plus CD45RA, CD45RB, or CD45RO in the five responders at screening and 3 and 12 months after HDC**+**ASCT. Results expressed as the percentage of CD3**+** cells expressing the CD45R isoform.

DISCUSSION

This study was undertaken to advance our understanding of the immunological effects of HDC**+**ASCT in RA. This experimental treatment modality has been employed in recent years as a means to profoundly perturb the immune system of patients with severe autoimmune disease, including RA. Substantial improvements of disease activity have been reported in a number of studies, although treatment failures and relapses have also been seen (reviewed by Verburg et al) [9]. Data on immunological correlates of the clinical responses in RA are scarce, but the persistence of serum autoantibodies in many patients who have undergone transplantation suggested that eradication of autoreactive B cells was not achieved, possibly contributing to the failure to induce long term remission in these patients. An overall decrease in the titre of RF and anti-CCP IgM was seen at 3 and 6 months after transplantation. However, concentrations returned to pre-transplantation levels 1 year after transplantation.

Of all the measures investigated in this study those involving the T cell compartment were most pronounced, particularly in the patients with high inflammatory activity as measured by serum levels of CRP and uptake on HIg scans. Failure of TNF blocking treatment or any other clinical or laboratory variable was not predictive of clinical response. The induction of (partial) remission was, however, associated with strong baseline expression and subsequent reduction of CD3, CD4, and the differentiation markers CD27 and the CD45 isoforms (RA, RB, RO) in synovium, but expression of these markers had returned to baseline levels at the 1 year biopsy at a time when the disease had relapsed to varying extents in most patients. Although changes in other cell subsets occurred, no association was found between disease activity and the number of macrophages (CD68**+** cells), B cells (CD19**+** cells), plasma cells (CD38**+** cells), or fibroblast-like synoviocytes (CD55**+** cells). Marked expression of pro-inflammatory cytokines—notably of IL-1, remained detectable after transplant, in the two non-responders.

Of interest was the high proportion of CD45RB**+**CD3**+** T cells at 3 months after transplantation, and the gradual increase of this subset among peripheral blood T cells. This subset has recently been reported to be increased in the peripheral blood of patients with RA in comparison with healthy controls, reflecting accelerated differentiation of naive CD45RA T cells under the influence of inflammation [16]. Co-expression of high levels of CD45RB and CD45RO and loss of CD45RA on T cells has

been shown to reflect a phenotype typical of recently activated T cells [17]. The overrepresentation of T cells with a similar phenotype in the synovium at 3 months suggests either the selective migration of newly developed T cells expressing high levels of CD45RB from blood, and/or local differentiation and/or expansion in the synovium, probably under the influence of homeostatic pressures or a local inflammatory drive as suggested by the results on cytokine expression. Any of these possibilities might contribute to the prolonged depletion of T cells from peripheral blood, which is a feature of patients with RA after lymphocytotoxic treatment [18, 19, 20, 21, 22]. Why some patients have more abundant T cell infiltration at baseline and respond favourably is not known. It may be attributed either to differences in the pathogenesis or in fluctuations of disease state.

In non-responders an influx of inflammatory cells and cytokines after transplantation was observed possibly related to stopping DMARDs at the time of the first biopsy, whereas these DMARDs were not used at the second arthroscopy. The non-responders showed an increase in inflammatory cytokines; notably, IL-1 and TNF-alpha. Both cytokines are believed to have a central role in the pathogenesis of RA. The procedure in this subset of patients with HDC seems to worsen the disease rather than ameliorate it. In responders there was a general trend towards a decrease in all cytokine markers, except for IL-10 and IFN-gamma. The increase in IFN-gamma may be explained by a relative increase of CD45RB**+** cells, which were shown to produce IFN-gamma.

Our data, though for obvious reasons just a snapshot, provide strong circumstantial evidence for an active role of T cells in the perpetuation of disease activity [23]. Whether attracted to, or expanding in, the synovium specifically or non-specifically, interaction of T cells with residual lymphoid or myeloid cells or resident cells such as synoviocytes could turn a subclinical synovitis into a clinically manifest synovitis [24, 25]. The findings from our study on the central role of CD4**+** T cell infiltration add to the accumulating evidence from earlier case reports of patients with RA and juvenile idiopathic arthritis treated with HDC**+**ASCT, and patients with psoriatic arthritis treated with T cell depleting monoclonal antibodies [26, 27, 28].

Our study raises the important question as to whether more intense immunosuppression to the extent of myeloablation or post-transplantation immunosuppression is necessary to attain lasting remission. Preclinical studies and a recent registry analysis of transplants in human autoimmune disease have lent support

to this concept [29]. In none of our patients was complete ablation of the synovial T cell compartment achieved, at least not at the time points evaluated. Also, autoantibodies (rheumatoid factor and anti-CCP) remained detectable in serum taken at monthly intervals for 2 years (data not shown). Given the complex immune dysregulation in RA; notably, in the late stages, it is conceivable that a more comprehensive treatment may indeed be needed. The key goal from a T cell-centred perspective would be to induce sufficient immunoablation to allow re-generation of regulatory elements. HDC**+**ASCT may provide a brief opportunity to establish tolerance against selected autoantigens. Our data suggest such a strategy would not be effective in patients with a low synovial T cell load. Whether different pathogenetic mechanisms are involved in such patients remains to be determined, but a recent study does point in this direction [30]. A baseline synovial biopsy seems useful to discriminate between the two categories.

To our knowledge, this is the first comprehensive study to examine clinical and immunological correlates of HDC**+**ASCT in RA. Although the number of patients investigated was small, and technical issues precluded the use of in situ multiparameter staining of the synovial T cell population, the data disclose interesting aspects of pathogenetic mechanisms operative in RA. The association of clinical responses with T cell debulking in the joint, recurrence of disease activity with re-emergence of T cell infiltration of synovium, and the lack of a relation between disease activity and other cell types in blood or synovium lend support to the concept that T cells have a role in established disease. The changes in relative expression of the different CD45 isoforms in synovial tissue in comparison with blood suggest that T cell repopulation in the joint is dictated by local homoeostatic forces, selective homing, or antigenic stimulation. More complete eradication of the synovial T cell compartment or post-transplantation immunosuppression may be needed to induce more robust remissions, but whether the risks of these steps outweigh the risks of more intense immunosuppression remains to be determined.

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CHAPTER III

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TITLE

Effective treatment of collagen-induced arthritis by adoptive transfer of CD25**+** regulatory T cells

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ABSTRACT

Objective: Regulatory T cells play an important role in the prevention of autoimmunity and have been shown to be effective in the treatment of experimental colitis, a T cell-mediated and organ-specific disease. We previously demonstrated that intrinsic CD25**+** regulatory T cells modulate the severity of collagen-induced arthritis (CIA), which, in contrast to colitis, is a systemic antibody-mediated disease and an accepted model of rheumatoid arthritis. We undertook this study to determine whether regulatory T cells have the potential to be used therapeutically in arthritis. Methods: We transferred CD4**+**CD25**+** T cells into mice exhibiting arthritis symptoms, both immunocompetent mice and mice subjected to lethal irradiation and rescued with syngeneic bone marrow transplantation. Results: A single transfer of regulatory T cells markedly slowed disease progression, which could not be attributed to losses of systemic type II collagen-specific T and B cell responses, since these remained unchanged after adoptive transfer. However, regulatory T cells could be found in the inflamed synovium soon after transfer, indicating that regulation may occur locally in the joint. Conclusion: Our data indicate that CD25**+** regulatory T cells can be used for the treatment of systemic, antibody-mediated autoimmune diseases, such as CIA.

INTRODUCTION

Collagen-induced arthritis (CIA) in mice is a commonly used model for studying rheumatoid arthritis (RA) in humans. Although CIA is not identical to RA, the two diseases share many key features, such as synovitis, erosions of both bone and cartilage, and class II major histocompatibility complex-linked susceptibility [1]. CIA is induced through immunizations with bovine type II collagen (CII) emulsified in Freund's complete adjuvant (CFA), which leads to CII-specific antibody production. These antibodies are crucial and sufficient for disease induction. Therefore, CIA can be considered a systemic B cell-dependent and antibody-mediated autoimmune disease.

It is hypothesized that the bovine CII-specific antibodies recognize murine CII in articular cartilage, leading to the activation of the complement system and the consequent release of C5a (among other components), which acts as a chemoattractant for immune cells. In the joint, these cells are activated through Fc receptor (FcR) triggering, eventually resulting in chronic inflammation and destruction of the joints [reviewed in 2 and 3]. This concept is supported by the hampered ability of B cell-deficient, C3-deficient, C5a receptor-deficient, and FcR-deficient mice to develop disease [4, 5, 6, 7, 8, 9]. CD4**+** T cells are also important during the initiation of arthritis by virtue of their ability to help B cells, a finding supported by CD4**+** T cell depletion experiments using monoclonal antibodies (mAb) [10]. T cells are less crucial during the effector phase, since depletion of CD4**+** T cells in established arthritis does not influence disease severity [10]. Instead, the main effector cells appear to be innate immune cells, which are capable of maintaining joint inflammation and initiating erosions of cartilage and bone [3].

CD4**+**CD25**+** regulatory T cells are potent suppressors of T cell responses both in vitro and in vivo [11]. In vivo, they are important in the prevention of organ-specific autoimmune diseases, as demonstrated by experiments in which splenocytes that had been depleted of CD25**+** cells prior to adoptive transfer to immunocompromised mice caused the development of gastritis and thyroiditis as well as several other organ-specific autoimmune diseases [12]. Currently, research focuses on their therapeutic value, and they have been successfully used to treat established colitis, a T cell-mediated and organ-specific autoimmune disease caused by the transfer of CD4**+**CD45RB^{high} T cells to SCID mice [13]. Additionally, prophylactic treatment with CD4**+**CD25**+** regulatory T cells has been reported to diminish the severity of other T

cell-mediated autoimmune diseases, such as experimental autoimmune encephalomyelitis [14] and diabetes [15].

We have previously demonstrated that depletion of CD25**+** regulatory T cells before vaccinating mice with bovine CII results in an exacerbation of arthritis that is associated with an increase in CII-specific antibodies [16]. These data indicate that regulatory T cells are also involved in the control of systemic B cell-dependent and antibody-mediated autoimmune disease. However, it is not known whether regulatory T cells can also be used therapeutically in such diseases. To investigate the potential of treating CIA with CD25**+** regulatory T cells, we adoptively transferred CD4**+**CD25**+** regulatory T cells into mice during the early stage of arthritis. Our data indicate that CD4**+**CD25**+** regulatory T cells were able to diminish the clinical severity of arthritis despite a lack of reduction in systemic CII-specific T and B cell responses. Using immunohistochemistry, CD4**+**CD25**+** regulatory T cells were traced to the synovial tissue in affected joints, indicating that these cells may modulate inflammation locally.

MATERIALS AND METHODS

Mice: DBA/1 mice were bred at the animal facility of The Netherlands Organization for Applied Scientific Research-Prevention and Health (Leiden, The Netherlands) and then transferred to the animal facility at the Leiden University Medical Center (Leiden, The Netherlands). Mice that underwent bone marrow transplantation (BMT) were kept in filter-top cages under sterile conditions; otherwise, mice were kept under conventional conditions. Mice that underwent BMT were given an irradiated sterile diet (Hope Farms, Woerden, The Netherlands) and acidified water containing ciprofloxacin (85 mg/liter; Bayer, Leverkussen, Germany) and polymixin B (70 mg/liter; Bristol-Myers Squibb, Woerden, The Netherlands). Treatment and maintenance were in accordance with the national guidelines for animal care. The Experimental Animal Commission of the Leiden University Medical Center approved the experiments described in this article.

Induction and evaluation of CIA: Bovine CII (Chondrex, Redmond, WA) was dissolved in a 0.1 M acetic acid solution overnight at 4°C at a concentration of 2 mg/ml. The dissolved bovine CII (100 microgram/mouse) was emulsified with an equal volume of CFA (Difco, Detroit, MI), and a total of 100 microliter was injected subcutaneously into the base of the tail. Mice were examined 3 times each week beginning 2 weeks after immunization. The front and hind paws were evaluated for signs of arthritis. The severity of arthritis was graded for each paw using the following scoring system: 0**=**normal joint, 1**=**1 or 2 swollen joints, 2**=**more than 2 swollen joints, and 3**=**extreme swelling of the entire paw and/or ankylosis. An arthritis score (range 0-12) was assigned to each mouse by summing the scores of each paw. In all experiments except those involving BMT, mice were killed when they had 2 paws with maximal swelling, as dictated by the Experimental Animal Commission.

BMT: BMT was performed when more than 50% of the mice were clinically affected with arthritis. Bone marrow cells were collected by flushing femurs and tibiae of syngeneic donor DBA/1 mice with 1% bovine serum albumin (BSA)/phosphate buffered saline (PBS). For T cell depletion of the graft, cell suspensions were incubated first with fluorescein isothiocyanate (FITC)-conjugated anti-CD3 mAb and then with magnetic-activated cell sorting (MACS) anti-FITC microbeads (Miltenyi Biotec, Auburn, CA), and cells were removed by magnetic cell sorting. Prior to BMT, arthritic mice were subjected to lethal doses of total body irradiation (TBI; 9 Gy). On the day following TBI, purified CD4**+** T cell subsets (5 x 10(**+**5)) were injected intravenously (i.v.). The second day after TBI, an injection of T cell-depleted bone marrow cells (2.5 x 10(**+**6)) was administered.

Isolation, activation, and transfer of CD4**+** T cell subsets: Purified CD4**+** cells were isolated from spleens obtained from naïve DBA/1 mice, first by staining splenocytes with FTC-conjugated anti-mouse CD4 and then by using a MACS anti-FITC Multisort kit (Miltenyi Biotec), which allows for multiple positive selections of cells through the removal of microbeads from the cells. CD25**+** cells were isolated from the CD4**+** cells by first staining with biotin-conjugated anti-CD25 mAb (BD PharMingen, San Diego, CA) followed by incubation with MACS antibiotin microbeads (Miltenyi Biotec). CD4**+**CD25**+** T cells were then positively selected on a MACS LS-sized column, and the flow-through was collected as CD4**+**CD25- T cells. The purity of all cell subsets using this method was more than 95% as determined by fluorescence-activated cell sorting analysis (data not shown). Isolated cells were activated by overnight incubation on 24-well plates coated with 2 microgram/ml anti-CD3 (145-2C11) in the presence of 100 IU/ml IL-2 (Sanver Tech, Heerhugowaard, The Netherlands), in RPMI 1640 medium supplemented with 1 unit/ml penicillin, 1 microgram/ml streptomycin, 20 mM L-glutamine, 50 microM beta-mercaptoethanol, and 8% fetal calf serum (FCS). For i.v. injection, cells were suspended in 0.1% BSA/PBS, and a total volume of 200 microliter was injected per mouse. Transfer was performed when more than 50% of the mice had developed arthritis.

Isolation of messenger RNA (mRNA) and reverse transcription-polymerase chain reaction (RT-PCR) for Foxp3 expression in T cell subsets: Total RNA was isolated from T cell subsets (after overnight stimulation with anti-CD3 and IL-2) using RNA-Bee (Tel-Test, Friendswood, TX) according to the manufacturer's recommendations. RT-PCR was performed with 2 microgram of total RNA for complementary DNA (cDNA) synthesis. The following primers for Foxp3 expression were used: 5’-ACA-CCA-CCC-ACC-ACC-GCC-ACT-G-3’ (forward) and 5’-CAT-TTG-CCA-GCA-GTG-GGT-AG-3’ (reverse). For the generation of cDNA, a total of 40 cycles were run under the following conditions: 1 minute at 95°C, 1 minute at 57°C, and 1 minute at 72°C. The cDNA products were verified by sequencing.

Collagen-specific cell proliferation and suppression assay: Splenocytes were isolated from treated or naïve mice. Cells were re-stimulated with 20 microgram/ml bovine CII and cultured with Dulbecco's modified Eagle's medium (Life Technologies, Paisley, UK) supplemented with 1 unit/ml penicillin, 1 microgram/ml streptomycin, 20 mM L-glutamine, 50 microM beta-mercaptoethanol, and 8% FCS in 96-well round-bottomed plates at a concentration of either 2.5 x 10(**+**5) cells/well or 5 x 10(**+**5) cells/well.

Proliferation was measured 3-5 days later by addition of 0.5 microCi/well of tritiated thymidine (3H-TdR). Values are the mean (minus average medium values) and SEM of triplicate experiments. After isolating and activating CD4**+**CD25- and CD4**+**CD25**+** T cells, cells were cultured in 96-well round-bottomed plates in RPMI 1640 medium with equal numbers of freshly isolated splenocytes from bovine CII-immunized mice. Proliferation was stimulated by adding phytohemagglutinin (PHA) at a dilution of 1:400 and/or bovine CII at a concentration of 20 microgram/ml. Incorporation of 3H-TdR was measured after 4-6 days of culture. Values are the mean (minus average medium values) and SEM of triplicate experiments.

Measurement of serum collagen-specific antibodies: Antibodies were measured by enzyme-linked immunosorbent assay (ELISA). Immuno-Maxisorp Plates (96-well; Nunc, Roskilde, Denmark) were coated with ELISA-grade bovine or murine CII (Chondrex) overnight at 4°C. After washing with PBS-0.05% Tween 20, plates were blocked with PBS/10% milk for 2 hours at 4°C. Serially diluted mouse serum was then incubated on the washed plates overnight at 4°C. Plates were subsequently treated with horseradish peroxidase (HRP)-conjugated anti-mouse polyvalent immunoglobulins (Sigma, St. Louis, MO). Detection was performed using 3,3',5,5'-tetramethylbenzidine as a substrate (Sigma). The optical density was measured at 450 nm using a microplate reader (Wallac, Gaithersburg, MD) and the reader's software (MultiCalc; Wallac). Bovine or murine CII-specific antibody units were determined using a reference serum created from pooled sera of arthritic mice and assigned an arbitrary value of bovine or murine CII-specific antibody units.

Measurement of serum amyloid P (SAP): SAP was measured using ELISA. Sheep anti-mouse SAP (Calbiochem, San Diego, CA) was coated on 96-well Immuno-Maxisorp Plates overnight at 4°C. After washing with buffer (PBS-0.05% Tween 20), plates were blocked with PBS/10% milk for 2 hours at 4°C and washed again before the addition of diluted sera. Plates were incubated with sera overnight at 4°C and were washed again the following morning. Rabbit anti-mouse SAP (Calbiochem) was incubated for 2 hours at room temperature to detect the bound SAP. A secondary antibody was used (HRP-conjugated swine anti-rabbit; Dako, Heverlee, The Netherlands) to allow for detection. Substrate reactions were detected in the same manner as for the bovine or murine CII-specific antibody protocol.

Detection of cells in synovial tissues: T cell subsets were isolated and activated as described above. After activation, cells were stained with 5,6-carboxyfluorescein

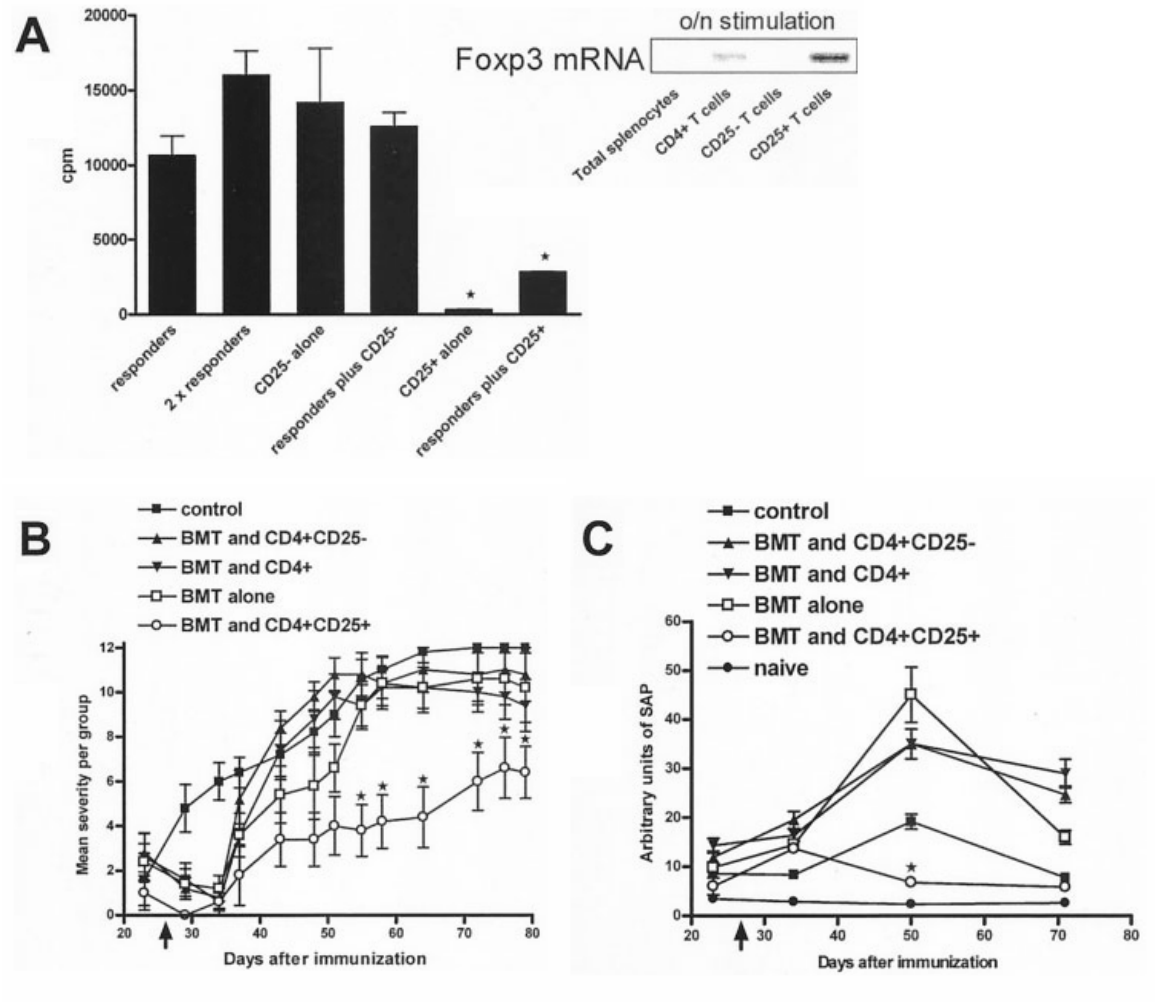
diacetate succinimidyl ester (CFSE; Molecular Probes Europe, Leiden, The Netherlands). Cells were suspended in 1% BSA/PBS containing 0.5 microM CFSE and then incubated for 10 minutes at 37°C in the dark. FCS was then added until a final concentration of 5% was reached. Cells were then washed twice with 1% BSA/PBS before i.v. transfer to arthritic mice. Between 1 and 2 days after transfer, mice were killed, and various tissues were sampled. Synovial tissue was harvested by first removing the surrounding muscle tissue and then removing the tissue lining the joint. Tissue samples were snap-frozen in Tissue-Tek (Sakura, Tokyo, Japan). In order to detect CFSE-labeled cells from the assorted tissues, we prepared acetone-fixed frozen sections. These sections were blocked with 10% mouse serum in PBS. Incubating the slides in a 1% H_2O_2/0.1% NaN3 solution for 20 minutes blocked endogenous peroxidase activity. CFSE**+** cells were detected using an HRP-conjugated anti-FITC antibody (Dako) as previously described [17]. A substrate reaction with 3,3'-diaminobenzidine (Dako) was then used to visualize the cells. Concomitant staining of CD4**+** T cells was performed by first staining with FITC-conjugated anti-mouse CD4 and then with the HRP-conjugated anti-FITC secondary antibody.

Statistical analysis: Significant differences in the severity of arthritis symptoms in CD25**+** T cell-treated and untreated mice on individual days and between levels of T cell proliferation were examined with Student's t-test. Significant differences between antibody titers and between SAP levels were determined by the Mann-Whitney U test. P values less than 0.05 were considered significant.

RESULTS

Reduction of arthritis severity in lymphopenic hosts by adoptive transfer of CD25**+** regulatory T cells: We have previously demonstrated that depletion of CD25**+** regulatory T cells 2 weeks before immunization with bovine CII leads to increased severity of arthritis symptoms in CIA [16]. Furthermore, we found that these magnified clinical manifestations were accompanied by augmented T and B cell responses against CII. These findings indicate that CD4**+**CD25**+** regulatory T cells are involved in the control of CII-directed responses in vivo.

Figure 1: Reduction in arthritis severity resulting from therapy combining the adoptive transfer of CD4**+**CD25**+** T cells and bone marrow transplantation (BMT).



(A) Pre-stimulated CD4**+**CD25**+** regulatory T cells (CD25**+**) were tested for their ability to suppress equal numbers of freshly isolated splenocytes stimulated with phytohemagglutinin (PHA) (responders; 10(**+**4) cells/well) in an in vitro assay. To eliminate the possibility that cells would be unable to proliferate due to

crowding, additional responder cells (2 x responders) were also tested in parallel with regulatory T cells. Pre-stimulated CD4+CD25- T cells (CD25-) were tested as a control. Values are the mean (minus average background values) and SEM, *P<0.005 versus responders. Also shown is Foxp3 mRNA expression in total splenocytes, CD4+ T cells, CD4+CD25- T cells, and CD4+CD25+ T cells after overnight (o/n) stimulation. In addition, beta-actin was used as a housekeeping protein (data not shown). (B) Mean disease severity in each group over time. Arthritis was induced through immunizations with bovine type II collagen (CII) emulsified in Freund's complete adjuvant. Upon reaching more than 50% disease incidence, groups of mice were lethally irradiated and underwent BMT, either alone or in combination with intravenous injection of subsets of CD4+ T cells (CD4+, CD4+CD25+ and CD4+CD25- cells; 5 x 10(+5) cells/mouse). The arrow indicates the time of treatment. Control mice were not irradiated and did not receive any treatments in addition to bovine CII immunizations and phosphate buffered saline. Mice that underwent BMT and were treated with CD4+CD25+ regulatory T cells had significantly lower disease severity (*P<0.05 versus all other groups). Values are the mean ± SEM (n=5 mice per group). The incidence and severity data are representative of 2 similar experiments. (C) Sera were collected in a similar experiment at a single time point before treatment and at 3 time points after treatment. The arrow indicates the time of treatment. Relative serum amyloid P (SAP) units were determined by enzyme-linked immunosorbent assay, both for naïve animals and for treated animals. *P<0.0001 versus controls. Values are the mean +/- SEM (n=8 mice per group). Results are representative of 2 experiments.

To investigate whether CD4+CD25+ regulatory T cells could be successfully used in a therapeutic setting, we studied the effects of infusions of CD4+CD25+ regulatory T cells in mice with early arthritis. In general, adoptive transfer of T cells for the treatment of disease appears most successful in immunocompromised hosts, presumably as a consequence of homeostatic proliferation and/or the absence of regulatory mechanisms [13, 18, 19]. For example, studies addressing the potency of adoptively transferred tumor-specific T cells are, in general, performed using tumor-bearing T cell-deficient mice. Indeed, studies in human cancer patients have shown that adoptive T cell therapy is most successful in lymphopenic patients [20]. Similarly, in a T cell-mediated colitis model, SCID mice have been used to study the potency of regulatory T cells [13]. Therefore, we chose to first examine the effects of regulatory T cells on chronic arthritis, as seen in CIA, in lymphopenic hosts.

Since CIA can only be induced in immunocompetent mice due to the necessity of T and B cells for disease initiation, we lymphodepleted bovine CII-immunized DBA/1 mice by lethal irradiation at the time the first clinical symptoms of arthritis appeared. In this way, a lymphopenic environment is created in mice that have already displayed high titers of CII-specific antibodies (data not shown) and that will have chronic arthritis as a result.

The next day, 5 x 10(+5) pre-stimulated CD4+CD25+ T cells were transferred, followed by an injection of syngeneic bone marrow cells to rescue the host. The pre-stimulated CD25+ regulatory T cells were capable of blocking PHA-induced T cell proliferation in vitro as well as bovine CII-specific proliferation (data not shown), and they expressed high levels of Foxp3 mRNA [21] (Figure 1A), demonstrating that the infused cells were effective suppressors. In contrast, similarly isolated and stimulated CD4+CD25- T cells used as a control did not display these suppressive capabilities (Figure 1A and reference 16). Although an initial decrease in clinical symptoms was noted soon after TBI in all irradiated groups (Figure 1B), most likely as a consequence of the irradiation, only mice treated with CD4+CD25+ regulatory T cells showed a long-lasting reduction in disease severity (Figure 1B).

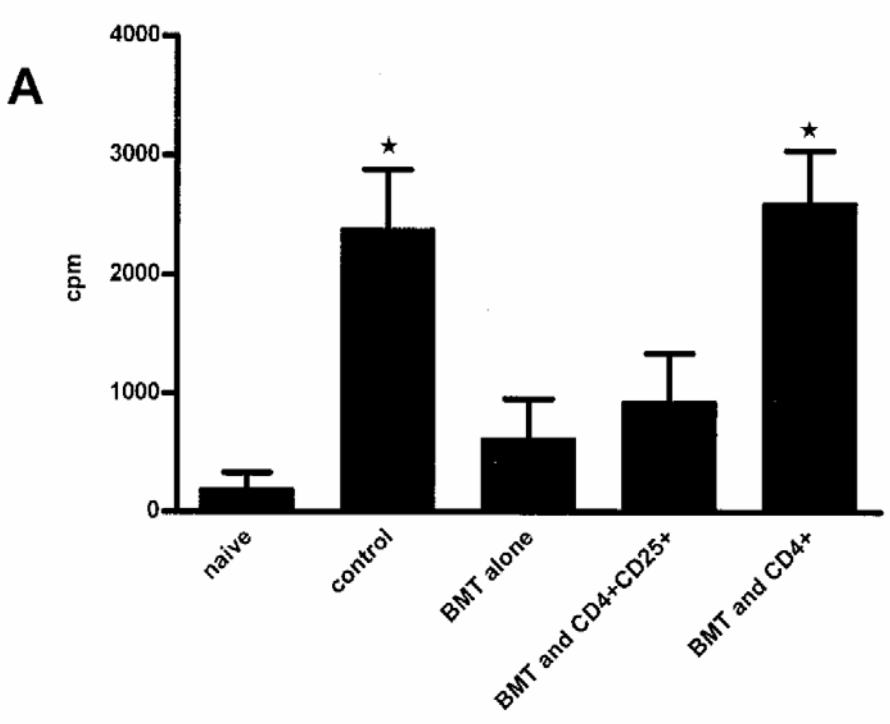
The finding that adoptively transferred CD4+CD25+ regulatory T cells are able to dampen ongoing inflammatory reactions was further strengthened by the results of analyses of the levels of SAP, an acute-phase protein detectable in sera during arthritis-associated inflammation as well as after lethal irradiation [22, 23]. All groups that underwent BMT without CD25+ regulatory T cells had increased levels of SAP, which occur as a result of TBI [24]. However, the SAP levels in mice that underwent BMT and were treated with CD4+CD25+ regulatory T cells were significantly lower than those detected in controls or mice receiving other treatments ($P<0.0001$, Figure 1C). Taken together, these observations demonstrate that CD25+ regulatory T cells can be used to decrease arthritis-associated inflammation as well as acute-phase responses.

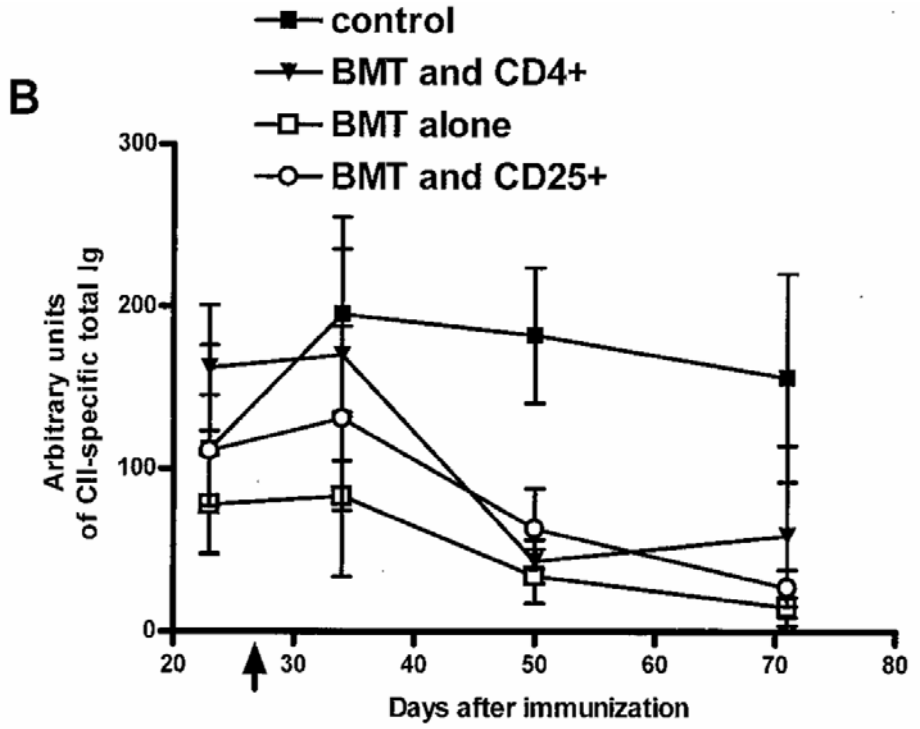
Lack of association between improved clinical outcome and CII-specific immunity: CII-specific T cells and antibodies play a crucial role in the development of CIA [reviewed in 3 and 25]. To clarify the mechanism responsible for our clinical observations, we examined both the CII-specific T cell responses and antibody titers in the treated mice. Because no differences were found with respect to disease progression and immunologic parameters between groups of mice treated with CD4+ or CD4+CD25- T cells, for these experiments we only used CD4+ T cells as a control for CD4+CD25+ T cells. In order to examine bovine CII-specific T cell responses, mice were killed at least 30 days after BMT and cell transfer. Spleens were harvested and analyzed in a proliferation assay against bovine CII.

As expected, bovine CII-specific proliferation was strongly diminished after lethal irradiation in mice that underwent BMT only, indicating that irradiation combined with BMT resulted in a strongly reduced T cell reaction. This overall immunocompromised

state was confirmed using a control antigen, keyhole limpet hemocyanin (KLH) (data not shown), as well as PHA (data not shown). In contrast, splenocytes from mice that underwent BMT and were treated with CD4**+** T cells still reacted significantly to bovine CII (Figure 2A). However, bovine CII-specific proliferation was strongly reduced in splenocytes from mice that underwent BMT and were treated with CD4**+**CD25**+** T cells. Together, these results imply that the infused CD4**+** T cells can be primed in vivo to respond against bovine CII, while the transferred CD4**+**CD25**+** T cells are hyporesponsive. More importantly, however, these findings indicate that the observed clinical effects do not correlate with proliferative responses against bovine CII.

Figure 2: Lack of correlation between clinical effects observed after regulatory T cell treatment and reduced CII-specific T cell and B cell responses.





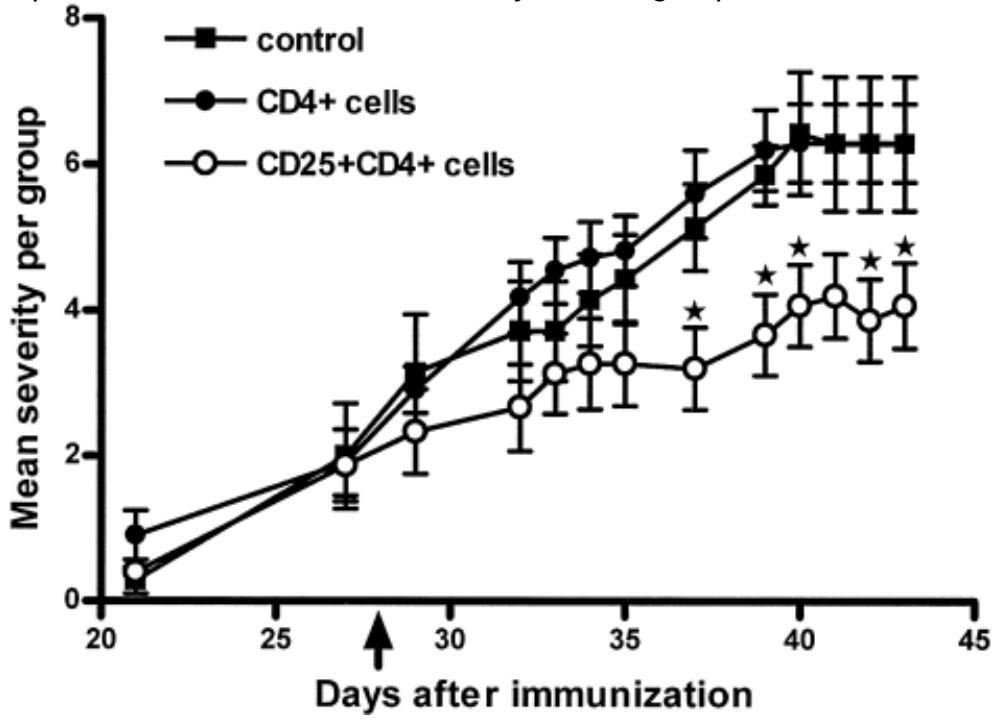
(A) Bovine CII-immunized mice were treated with combinations of BMT and CD4+ T cell subsets during disease onset. One month after treatment, splenocytes (5 x 10(+5)/well) were isolated from individual treated mice and untreated controls and tested for proliferative responses against bovine CII. Only splenocytes from control mice and from mice that underwent BMT and CD4+ T cell transfer responded to bovine CII significantly more than did splenocytes from naïve mice. Values are the mean (minus average medium values) and SEM of triplicate experiments. *P<0.05 versus naïve controls. Results are representative of 2 experiments. (B) At several time points, sera were obtained and examined for CII-specific antibodies (combined IgG, IgA, and IgM) by enzyme-linked immunosorbent assay. The arrow indicates the time of treatment. Values are the mean +/- SEM (n=8 mice per group). Results are representative of 3 independent experiments. See Figure 1 for definitions.

To determine whether the observed differences in severity between the groups were correlated with CII-specific antibody responses, we measured CII-specific antibody levels at several time points after immunization and/or treatment. All groups that underwent BMT displayed lower amounts of CII-specific antibodies irrespective of the additional therapy they received (Figure 2B). Taken together, these findings indicate that lethal irradiation followed by BMT results in a reduction of CII-specific T and B cell responses. However, as shown in Figure 1B, improvement in clinical symptoms was not associated with reduced CII-specific immune responses, indicating that the inhibition of

disease severity by the transfer of CD25**+** regulatory T cells is independent of CII-specific immunity.

Reduction of disease progression in nonlymphopenic hosts with arthritis by therapy with regulatory T cells: Although high-dose immunosuppression (i.e. lethal TBI) followed by syngeneic BMT and CD4**+**CD25**+** regulatory T cell transfer was highly effective in reducing arthritis severity, a less toxic treatment regimen is preferable. Therefore, we investigated the efficacy of CD4**+**CD25**+** regulatory T cell transfer without BMT in immunocompetent hosts. Initial experiments using 5 x 10(**+**5) cells/mouse showed little effect on disease progression (data not shown), but when 1 x 10(**+**5) CD4**+**CD25**+** regulatory T cells were adoptively transferred i.v. at the time arthritis appeared, the mice demonstrated significantly reduced arthritis symptoms (Figure 3). These results indicate that the treatment of arthritis by infusion of CD4**+**CD25**+** regulatory T cells positively affects clinical outcome when sufficient numbers of CD4**+**CD25**+** regulatory T cells are transferred.

Figure 3: Effectiveness of CD4**+**CD25**+** regulatory T cell transfer therapy in nonlymphopenic hosts. Mean disease severity in each group is shown over time.

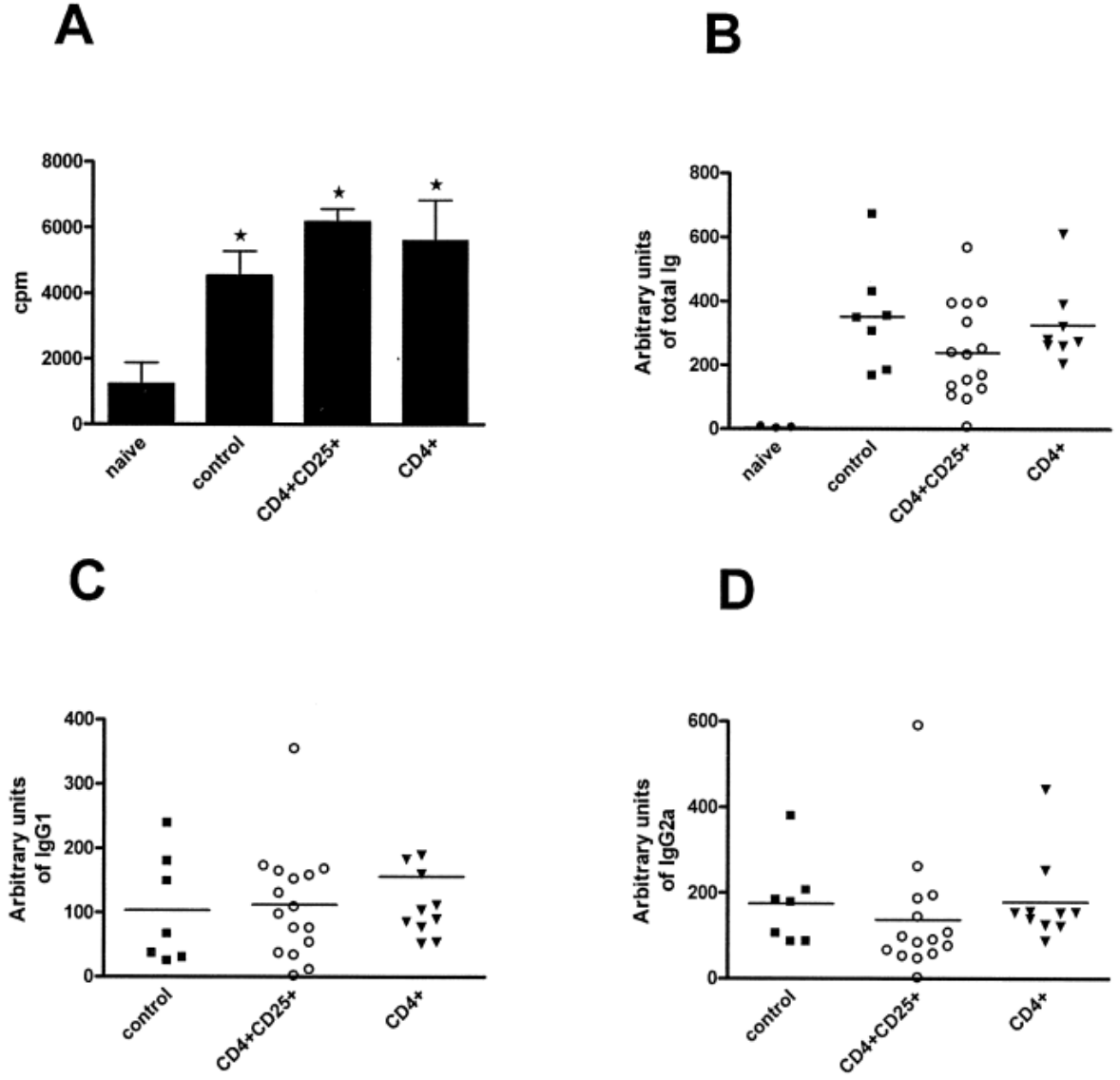


Bovine type II collagen-immunized mice were injected intravenously (i.v.) with either 10(+5) activated CD4+ T cells or 10(+6) CD4+CD25+ T cells (isolated from 20 mice) during disease onset. Control mice received only i.v. injections of phosphate buffered saline. The arrow indicates the time of treatment. Mice receiving CD4+CD25+ regulatory T cells had significantly reduced arthritis symptoms (*P<0.05 versus all other groups). Values are the mean +/- SEM (n=7-16 mice per group). Results are representative of 2 independent experiments.

Since CIA is mediated by bovine CII-specific immune responses and is crucially dependent on the presence of CII-specific autoantibodies, we wished to investigate whether adoptive transfer of CD4+CD25+ regulatory T cells leads to inhibition of CII-directed immunity in nonlymphopenic recipients. Therefore, we measured the bovine CII-specific T cell responses and determined CII-specific antibody titers in the different groups. In all groups, CII-specific proliferative responses were retained, and no apparent differences between groups were noted (Figure 4A). When we measured CII-specific antibody titers in sera obtained 43 days after immunization, a slight reduction of CII-specific antibodies was detected in mice given CD25+ regulatory T cells, but the difference did not reach statistical significance (Figure 4B). Since even a slight reduction in total CII-specific Ig titers may be indicative of significant changes in specific IgG isotypes, we examined CII-specific IgG1 and IgG2a titers. No significant differences between groups were detected for both isotypes (Figures 4C and D). Therefore, we consider it unlikely that a reduction in CII-specific T cell or antibody-mediated systemic immunity can account for the reductions in inflammation seen clinically.

Localization of adoptively transferred CD4+CD25+ regulatory T cells in inflamed joints: Because the improvements in disease outcome observed after transfer of CD25+ regulatory T cells did not correlate with differences in systemic CII-directed T and B cell responses, we wished to investigate whether the infused cells could exert their function locally by determining whether they had the capacity to travel to the inflamed joints as well as to the lymphoid organs. After labelling the cells with CFSE, we were able to show that pre-stimulated CD4+CD25+ regulatory T cells could easily expand both in naïve mice and in arthritic mice (data not shown). To investigate whether they localized in the inflamed knee joints, we collected a variety of organs as well as synovial tissue 1-2 days after adoptive transfer into arthritic mice. By using an HRP-labeled FITC-specific antibody that was cross-reactive with CFSE, we were able to visualize CFSE-labeled cells by immunohistochemistry [17].

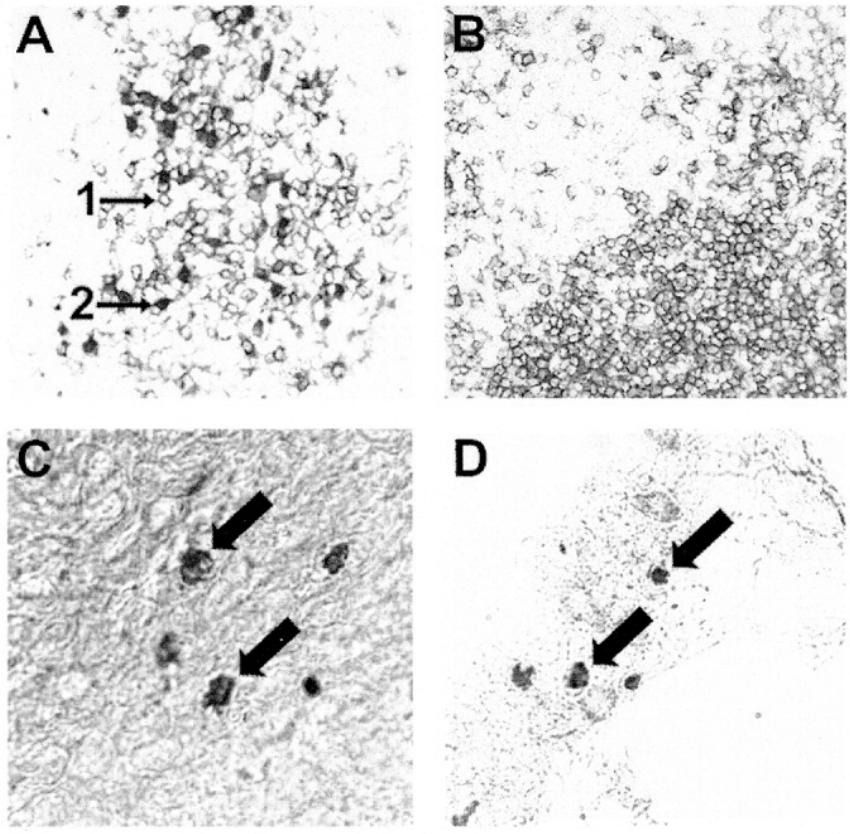
Figure 4: Lack of effect of adoptive transfer of CD4+CD25+ T cells on T and B cell responses to bovine type II collagen (CII).



(A) Splenocytes were isolated from individual naïve mice and mice that were immunized and treated with CD4+ T cell subsets (1 x 10(+6) cells/mouse). The splenocytes (5 x 10(+5)/well) were cultured for 5 days with bovine CII, and the proliferative response was measured. Splenocytes from all immunized mice recognized bovine CII significantly more than did splenocytes from naïve mice (*P<0.05). Values are the mean (minus average medium values) and SEM of triplicate experiments. Results are representative of 2 independent experiments. (B) Total IgG, IgA, and IgM specific for CII were measured both in naïve mice and in treated mice. No differences were found between the groups. (C) and (D) The same samples were also analyzed for CII-specific IgG1 and CII-specific IgG2a, respectively. Each symbol represents a measurement from an individual mouse; bars show the mean. Results are representative of 3 independent experiments.

As expected, CFSE-labeled CD25+ regulatory T cells localized to CD4+ cell-rich regions in the spleen (Figure 5A) and lymph nodes (results not shown), but were not found in the B cell areas of these organs. Transferred CD4+CD25+ T cells were also detected in joint-draining lymph nodes (popliteal), blood, liver, lungs, kidneys, and synovial fluid of arthritic mice (results not shown). More importantly, CD25+ regulatory T cells were also detected in inflamed synovial tissue (Figures 5C and D). Together, these results demonstrate that adoptively transferred regulatory T cells quickly appear in synovial tissue after injection (1-2 days), and they raise the possibility that the transferred CD25+ regulatory T cells control CIA locally in the inflamed tissue of the joint, rather than by inhibiting systemic CII-specific immunity.

Figure 5: Localization of adoptively transferred CD4+CD25+ regulatory T cells in inflamed synovial tissue.



Activated CD4+CD25+ regulatory T cells (5 x 10(+6)) were injected into arthritic mice. Spleens and synovial tissue were harvested and examined by immunohistochemistry. (A) Section of a spleen showing CD4+ T cells (indicated by cell surface staining; arrow 1) in the splenic white pulp, with darkly stained transferred CD4+CD25+ regulatory T cells (indicated by intracellular staining; arrow 2). (B) Darkly stained cells (indicated by intracellular staining) were not detected in T cell areas of the spleens of mice that

received no transferred cells. (C) and (D) Darkly stained, adoptively transferred CD4**+**CD25**+** T cells (arrows) in synovial tissue obtained from 2 arthritic mice. Results are representative of 3 independent experiments. (Original magnification: 250x in A and B; 400x in C and D.)

DISCUSSION

Successful treatment of autoimmune disease with CD25**+** regulatory T cells has been reported in an animal model of colitis in which the infusion of 1×10^6 regulatory T cells successfully treated early-stage disease induced through the transfer of CD4**+**CD45RB(high) cells to SCID mice [13]. Unlike colitis, which is a T cell-mediated, organ-specific autoimmune disease, CIA presents different challenges for CD25**+** regulatory T cells. CIA is a systemically induced autoimmune disease that is initiated mainly through CII-specific antibodies and is strongly influenced by innate immune cells [3]. In the colitis model, regulatory T cell therapy was successful in lymphopenic hosts, but the applicability of regulatory T cell therapy in nonlymphopenic hosts has yet to be analyzed. In general, adoptive cell therapies against, for example, tumors are most successful after immunodepletion, largely due to the elimination of regulatory mechanisms and/or the homeostatic proliferation of transferred cells [18]. These observations are consistent with our findings, since a transfer of 5 x 10(**+**5) regulatory T cells was sufficient to inhibit disease progression in hosts that were myeloablated following irradiation, but were insufficient in nonlymphopenic hosts. However, our data show that regulatory T cell therapy can be used successfully in nonimmunocompromised animals when more regulatory T cells (10(**+**6)) are transferred into mice with early arthritis.

The observation that regulatory T cell therapy can be an effective treatment in systemic autoimmune disease is important in the context of the observations that proinflammatory cytokines such as IL-6 can hinder the suppression mediated by regulatory T cells [26] and that regulatory T cells from RA patients display a compromised function that is reversed by anti-tumor necrosis factor (anti-TNF) treatment [27]. Mice with CIA, as well as RA patients, display highly elevated levels of proinflammatory cytokines, including IL-6, TNF-alpha, and IL-1 [3]. These cytokines lead to the production of acute-phase proteins such as CRP (CRP) in humans and SAP in mice [28]. TBI, which was used to generate lymphopenic mice, has also been reported to result in proinflammatory cytokine release and, as a consequence, in acute-phase protein production [29]. Despite high levels of proinflammatory cytokines such as IL-6, lymphopenic mice receiving CD25**+** regulatory T cells showed large reductions in serum levels of SAP compared with the levels in control groups, indicating that systemic autoimmune diseases associated with elevated levels of proinflammatory cytokines can be treated with CD4**+**CD25**+** regulatory T cells.

In vitro suppression assays with CD4+CD25+ regulatory T cells have indicated that these cells primarily require cell-cell contact in order to regulate [30, 31]. However, in vivo experiments have indicated that the secretion of antiinflammatory cytokines such as IL-10 may be necessary for sufficient regulation [11]. Therefore, CD25+ regulatory T cells may need to be in the direct vicinity of the cells responsible for the inflammatory responses. A direct cell-cell contact between transferred CD4+CD25+ T cells and CD11c+ cells (dendritic cells in the mouse) and pathogenic T cells has been observed during the treatment of colitis with CD4+CD25+ regulatory T cells [13]. Although the mode of action of regulatory T cell-mediated suppression of ongoing colitis is not known, these contacts are particularly noteworthy, since colitis is a T cell-mediated disease [32]. We also detected CD4+CD25+ regulatory T cells in the inflamed synovium after adoptive transfer (Figure 5). However, we consider it unlikely that regulatory T cells act in the CIA model via the suppression of T cell responses, since the effector phase of arthritis depends on immune responses that are independent of T cells [10, 33]. Likewise, we did not observe a decrease in systemic CII-specific antibody titers. Therefore, we believe that transferred CD4+CD25+ regulatory T cells are interacting locally with innate immune cells (e.g. mononuclear phagocytes and neutrophils), rather than with T cells or B cells.

Local control of innate immune cells by CD4+CD25+ regulatory T cells could be achieved in various ways. Local IL-10 delivery has been shown to decrease TNF-alpha and IL-1 production [34], and direct regulation through cell-cell contact between innate immune cells (which are abundantly present in inflamed joints) and CD4+CD25+ regulatory T cells is possible and has already been shown in a bacterially induced colitis model [35].

We have now demonstrated that the adoptive transfer of CD4+CD25+ regulatory T cells during the early phase of chronic arthritis can offer therapeutic benefits. Furthermore, we have found that these cells are capable of travelling to the joint, where they could conceivably have the potential to suppress inflammation locally. Although a number of practical difficulties still have to be overcome for clinical application, improvements in techniques for expansion of CD4+CD25+ regulatory T cells that would allow for multiple transfers of regulatory T cells [36], along with the detailed exploration of the interactions between CD4+CD25+ regulatory T cells and the immune cells of the joint, may allow for the development of optimized CD4+CD25+ regulatory T cell therapies for chronic arthritis and systemic autoimmune disease in general. However, such interventions will

represent a laborious treatment option, and it is therefore unlikely that adoptive cellular therapies will become part of the standard array of antirheumatic therapies. Nonetheless, our results do provide a rationale for targeting regulatory T cell activity in the treatment of patients with chronic systemic autoimmune diseases.

ACKNOWLEDGEMENTS

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CHAPTER IV

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TITLE

Control of systemic B cell-mediated autoimmune disease by nonmyeloablative conditioning and major histocompatibility complex-mismatched allogeneic bone marrow transplantation

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From animals to clinic: nonmyeloablative conditioning and allogeneic bone marrow transplantation in autoimmune disease / commentary by Richard K. Burt in Blood, 105:2623 (2005)

ABSTRACT

Systemic autoimmune disease can be controlled with conventional therapies in most patients. However, relapses are common, leading to progressive disability and premature death. Nonmyeloablative conditioning and allogeneic bone marrow transplantation (BMT) could be an effective treatment for severe autoimmune disease, because of mild toxicity of the conditioning and the potential benefits of donor chimerism. We examined the effects of this treatment in experimental autoimmune arthritis. Our results demonstrate the induction of complete donor chimerism and significant suppression of disease activity. No clinical graft-versus-host disease (GVHD) was observed. The beneficial effects were most likely caused by the elimination of plasma cells producing pathogenic autoantibodies, because these antibodies disappeared rapidly after BMT. Although this type of treatment was effective in organ-specific T cell-mediated autoimmune disease, the present study provides convincing evidence that nonmyeloablative conditioning and allogeneic BMT can effectively treat severe B-cell-mediated autoimmune disease with a systemic inflammatory component.

INTRODUCTION

Systemic autoimmune disease is characterized by immune dysregulation in which T or B cells play a pivotal role [1]. Preclinical studies and case reports indicate that myeloablative chemo(radio)therapy plus allogeneic bone marrow transplantation (BMT) may be an effective treatment for systemic autoimmune disease in humans [2-6]. The application of this treatment, however, is limited because of the risk for graft-versus-host disease (GVHD) and the toxicity of myeloablative therapy. Recently, preclinical protocols have been developed based on nonmyeloablative conditioning to achieve allogeneic donor chimerism without clinical GVHD [7-9]. Preliminary results indicate that this treatment modality may be effective in treating human autoimmune disease [1, 10]. The present study aimed to investigate the clinical and immunologic effects of allogeneic BMT after nonmyeloablation on experimental arthritis in mice, a systemic inflammatory B-cell-mediated autoimmune disease. More specifically, we wanted to study whether stable, long-term, and multilineage donor chimerism could be induced safely in mice with established polyarthritis and whether this treatment would result in the reduction of disease activity and serum levels of pathogenic autoantibodies produced by host plasma cells.

MATERIAL AND METHODS

Animals: Male DBA/1 and BALB/c mice (8-12 weeks of age) were obtained, housed, and fed as described earlier [11, 12].

Induction and clinical assessment of arthritis: Collagen-induced arthritis (CIA) was induced and evaluated as described by Morgan et al [12]. Mice with maximum scores of 12 were humanely killed.

Bone marrow transplantation: BMT was performed when more than 50% of the mice developed CIA. Mice were subjected to sublethal total body irradiation (TBI) of 6.0 Gy, and each received a single injection of anti-CD40 ligand (CD40L) monoclonal antibody (mAb) (MR1, 0.5 mg intraperitoneally) before BMT with 1.0 x 10(**+**7) total bone marrow (BM) cells intravenously collected from femurs and tibiae of donor mice.

Flow cytometric analysis for donor chimerism: The level of allogeneic donor chimerism was evaluated by flow cytometric analysis after staining of mononuclear cells with the following mAbs: biotinylated anti-H-2D(d) (donor), biotinylated anti-H-2D(q) (host), anti-CD3-fluorescein isothiocyanate (FITC), anti-CD4-allophycocyanin (APC), anti-CD8-alpha-APC, anti-Gr-1-FITC, anti-B220-FITC, and phycoerythrin (PE)-conjugated streptavidin (PharMingen, Erembodegem, Belgium).

Measurement of IgG2a and Igh-1a antibodies in serum: Anti-type II collagen (CII) antibodies were measured by enzyme-linked immunosorbent assay (ELISA) [12]. In the case of Igh-1a, the plates were incubated with biotinylated anti-Igh-1a (PharMingen) and subsequently with horseradish peroxidase (HRP)-conjugated streptavidin. Total IgG2a antibodies in serum were measured by sandwich ELISA using plates coated with polyclonal anti-mouse total immunoglobulin G (IgG) (DAKO, Heverlee, The Netherlands).

Measurement of serum amyloid P: Levels of serum amyloid P (SAP) were measured by sandwich ELISA using plates coated with polyclonal sheep anti-mouse SAP (Calbiochem, San Diego, CA). After incubation with serum, the plates were treated with polyclonal rabbit anti-mouse SAP (Calbiochem). Finally, the plates were incubated with HRP-conjugated polyclonal swine anti-rabbit immunoglobulin (DAKO).

Statistical analysis: Differences in disease severity, antibody levels, and SAP levels were analyzed using the nonparametric Mann-Whitney U test. P values less than 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

The aim of this study was to investigate the efficacy of allogeneic BMT in treating systemic autoimmune disease after nonmyeloablative conditioning. First, we studied whether allogeneic BMT after myeloablation using lethal TBI is effective in treating arthritis. These experiments indicated that allogeneic BMT was more effective than syngeneic BMT (data not shown). However, given that both GVHD and the toxicity associated with myeloablation preclude application in a clinical setting, these results prompted us to investigate the effects of allogeneic BMT after nonmyeloablation. To establish whether nonmyeloablative conditioning allows the development of complete donor chimerism without GVHD, we treated healthy DBA/1 mice with sublethal TBI (6.0 Gy) and a single injection of an anti-CD40L mAb (MR1), which is thought to result in tolerance or deletion of CD4**+** and CD8**+** host/donor-reactive T cells within a few days after BMT [9, 13, 14]. The mice subsequently received allogeneic bone marrow transplants from BALB/c donor mice. The induction of stable, long-term, and multilineage donor chimerism (greater than 95%) until day 300 after BMT was observed in mice treated with TBI and anti-MR1, but not in mice treated with TBI alone ($P<0.001$; Table 1), pointing to the importance of anti-MR1. No clinically overt GVHD (defined by skin abnormalities or weight loss) was observed, nor were significant histological abnormalities of liver, gut, and skin detected (data not shown). However, we could not exclude the presence of a subclinical host-versus-graft (HVG)/graft-versus-host (GVH) response. To address this point, we determined the levels of SAP, an acute-phase protein, after fully major histocompatibility complex (MHC)-mismatched allogeneic BMT. Mice treated with allogeneic BMT, but not those treated with syngeneic BMT, showed elevated SAP levels after treatment ($P=0.006$; Table 2), indicative of an alloresponse. These data show that complete donor chimerism can be induced using nonmyeloablation before allogeneic BMT, but they also point to the presence of an HVG/GVH response, though without clinical GVHD.

Table 1: Percentage of donor cells after nonmyeloablative conditioning using anti-CD40L mAb (MR1) before allogeneic BMT in healthy DBA/1 mice.

Time after allogeneic BMT (days)	% of donor cells, mean +/- SEM	
	TBI alone	TBI/anti-CD40L
42	27.8 +/- 10.6	83.0 +/- 1.3
50	29.2 +/- 13.4	84.8 +/- 6.6
57	29.2 +/- 8.0	90.0 +/- 3.9
64	20.1 +/- 19.8	83.7 +/- 4.9
77	3.4 +/- 1.5	78.3 +/- 11.0
106	0.4 +/- 0.1	93.9 +/- 1.7
169	N/A	97.0*
279	N/A	93.3*
295	N/A	97.0*

Percentages of total donor cells were analyzed by fluorescence-activated cell sorting (FACS) in peripheral blood (n=2 mice per group; *n=1). Level of allogeneic donor chimerism was significantly higher in mice treated with TBI plus anti-CD40L compared with mice treated with TBI alone ($P < 0.0001$). N/A indicates not available.

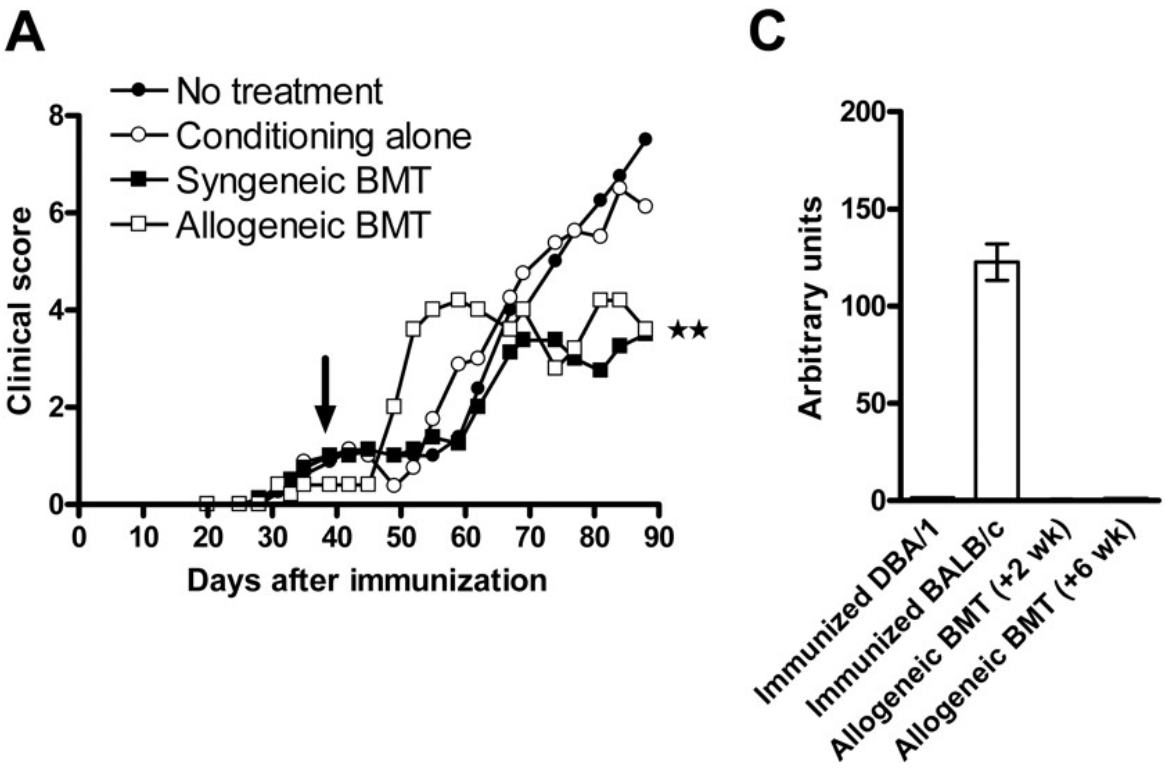
Table 2: Elevated SAP levels after allogeneic BMT, but not after syngeneic BMT, and conditioning alone at day +10 after treatment.

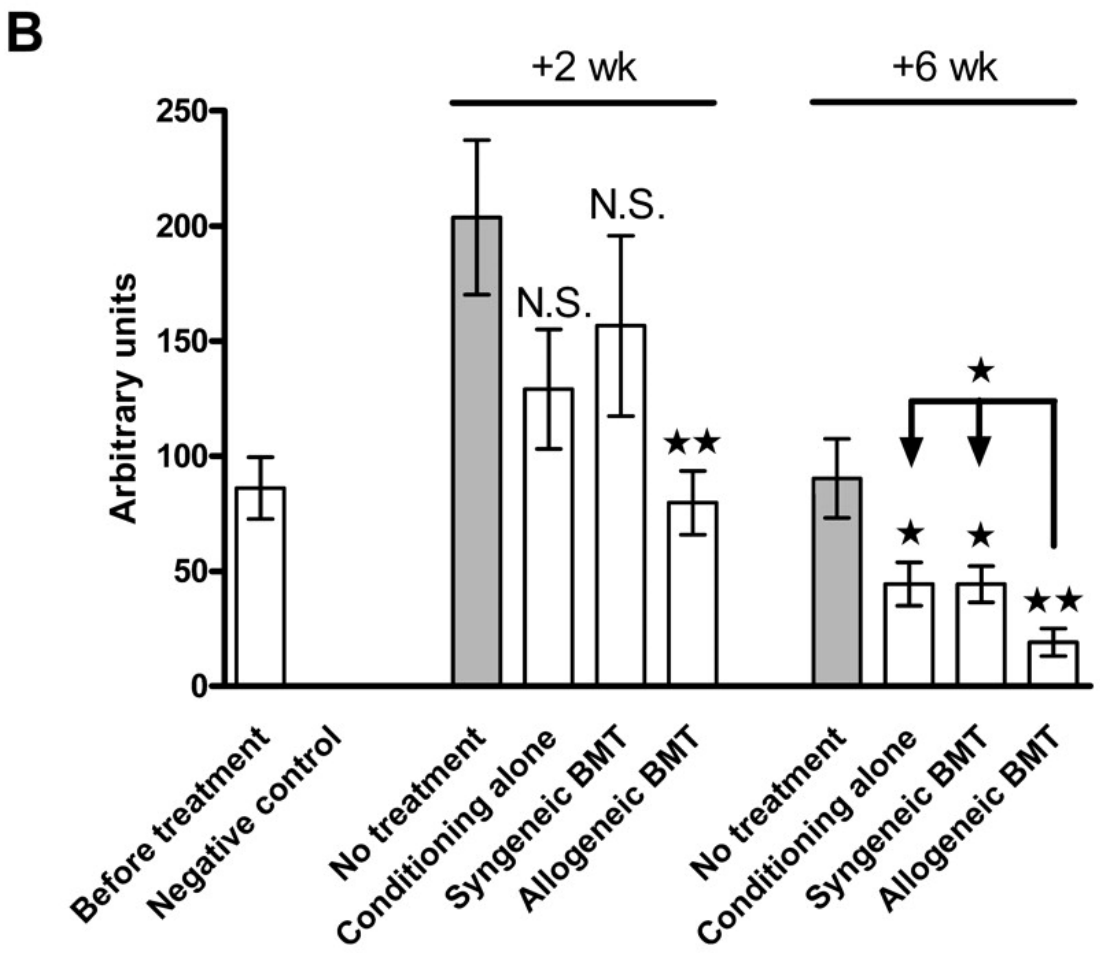
Groups of mice	Mean SAP levels +/- SEM	P-value
Positive control	88.9 +/- 2.5	N/D
Negative control	24.7 +/- 9.8	N/D
Conditioning alone	39.4 +/- 5.6	N/S
Syngeneic BMT	23.6 +/- 1.6	N/S
Allogeneic BMT	76.3 +/- 9.7	0.006

Arbitrary levels of SAP are expressed as mean +/- SEM (n=6 mice per group). N/S indicates not significant. SAP levels were determined using 2 reference sera created from naïve mice (negative control) or mice injected with CFA (positive control). P-values of treatment groups are calculated compared with negative control. N/D indicates not determined.

We then sought to assess the therapeutic effects of nonmyeloablative conditioning and allogeneic BMT in mice with established polyarthritis. Allogeneic and syngeneic BMT, but not conditioning alone, were able to arrest disease progression (P=0.007 and P=0.001, respectively, compared with untreated mice; Figure 1A). Of note, a temporary exacerbation of arthritis was consistently observed within 2 weeks after allogeneic BMT, followed by a steady and prolonged suppression of disease activity similar to that of syngeneic BMT (Figure 1A). In accordance with the results shown in Table 2, only allogeneic BMT recipients had elevated SAP levels during the exacerbation (data not shown), again pointing to the presence of a subclinical HVG/GVH response after allogeneic BMT.

Figure 1: Allogeneic BMT can effectively treat CIA, a severe B cell-mediated autoimmune disease.





(A) Allogeneic and syngeneic BMT have a suppressive effect on arthritis after nonmyeloablative conditioning (**P=0.007 and **P=0.001, respectively). Clinical data of arthritic DBA/1 mice (n=8 mice per group) treated with sublethal TBI of 6.0 Gy (day 37) plus a single injection of anti-CD40L mAb (0.5 mg intraperitoneally, day 38) and subsequently injected with 1.0 x 10(+7) total BM cells intravenously from syngeneic DBA/1 mice or fully major histocompatibility complex (MHC)-mismatched allogeneic BALB/c mice are shown (day 38; arrow indicates start of treatment). No statistical differences were observed at the time of treatment. Results from 1 of 2 experiments are shown. (B) Allogeneic BMT results in a marked decrease of pathogenic anti-type II collagen autoantibodies. Sera were taken at 2 and 6 weeks after BMT (days 52 and 81, respectively) and were tested by ELISA for the presence of anti-type II collagen antibodies (data are shown for the IgG2a isotype). Allogeneic BMT was more effective in suppressing the production of anti-type II collagen antibodies (**P<0.002) than syngeneic BMT or conditioning alone (*P<0.05) compared with untreated animals. Allogeneic BMT compared with syngeneic BMT and conditioning alone (*P=0.02) at 6 weeks after BMT. (C) Anti-type II collagen antibodies after allogeneic BMT are of recipient origin. None of the allogeneic BMT recipients developed anti-type II collagen antibodies of the Igh-1a allotype, demonstrating that the antibodies present after allogeneic BMT are of recipient origin. As positive and negative controls for the presence of Igh-1a, we immunized normal BALB/c and DBA/1 mice, respectively, with CII. Immunized BALB/c mice were able to produce CII-specific antibodies to the same extent as immunized DBA/1 mice without the clinical symptoms of arthritis (data not shown).

Complete and stable donor chimerism (greater than 95%) could be induced in healthy and arthritic mice in all cell lineages analyzed (Table 3), indicating that the presence of a systemic inflammatory autoimmune disease does not hamper the induction of allogeneic donor chimerism.

Table 3: Induction of complete donor chimerism is similar in arthritic and healthy mice.

	Mean % of donor cells +/- SEM			
	PB	SP	LN	BM
CD4+ T cells:				
Healthy	99.6 +/- 0.1	99.1 +/- 0.2	98.9 +/- 0.5	99.7 +/- 0.1
Arthritic	99.3 +/- 0.3	99.2 +/- 0.2	99.1 +/- 0.2	99.2 +/- 0.4
CD8+ T cells:				
Healthy	98.3 +/- 1.2	99.0 +/- 0.4	98.1 +/- 0.7	99.4 +/- 0.3
Arthritic	98.8 +/- 0.6	98.8 +/- 0.3	97.7 +/- 0.7	99.3 +/- 0.3
B cells:				
Healthy	99.6 +/- 0.1	99.7 +/- 0.1	99.2 +/- 0.2	99.4 +/- 0.2
Arthritic	99.7 +/- 0.1	99.7 +/- 0.1	99.3 +/- 0.2	99.8 +/- 0.1
Granulocytes:				
Healthy	97.4 +/- 1.3	98.2 +/- 0.4	N/A	92.8 +/- 1.4
Arthritic	98.4 +/- 0.3	98.4 +/- 0.4	N/A	93.7 +/- 1.2

Percentages of donor cells were analyzed using FACS in different cell lineages (i.e. CD3+ T cells, B220+ B cells, and Gr-1+ granulocytes) in the following lymphoid compartments: peripheral blood (PB), spleen (SP), lymph node (LN), and bone marrow (BM). Values are expressed as mean +/- SEM (n=8 mice per group). N/A indicates not available because of low numbers of Gr-1+ cells in lymph nodes.

Given that CII-specific serum antibodies which are produced by plasma cells are crucial and sufficient for the induction of CIA, we studied whether their presence or origin could explain the efficacy of allogeneic BMT. Therefore, sera were taken at 2 and 6 weeks after BMT and were tested for the presence of CII-specific IgG2a autoantibodies. Although differences between groups were already detectable 2 weeks after BMT, only allogeneic BMT was able to significantly suppress the production of CII-specific IgG2a antibodies, which was most evident at 6 weeks after BMT ($P<0002$; Figure 1B). This difference could not be explained by lower levels of total IgG2a in the sera of allogeneic chimeras, because no differences could be detected after syngeneic and allogeneic BMT (data not shown).

To exclude the possibility that the residual CII-specific antibodies resulted from a de novo autoimmune response by allogeneic donor cells against CII, we analyzed the presence of CII-specific Igh-1a, an IgG2a allotype found in BALB/c mice but not in DBA/1 mice. None of the allogeneic BMT recipients had developed CII-specific Igh-1a antibodies (Figure 1C), demonstrating that the antibodies present after allogeneic BMT are of recipient DBA/1 origin. Because serum antibodies in mice have a short half-life [15] and are produced predominantly by plasma cells, our data can be best explained by the disappearance of plasma cells producing CII-specific antibodies after allogeneic BMT. Allogeneic BMT is clearly correlated with a superior reduction of pathogenic antibody responses, so it is tempting to speculate that a graft-versus-plasma cell or a ‘cytokine storm’ related to allografting is responsible for the disappearance of anti-CII-producing plasma cells [16, 17]. Together, our data indicate that allogeneic BMT is highly effective in suppressing clinical disease and autoantibody production by host plasma cells without eliciting a de novo autoimmune response after BMT.

Although nonmyeloablative conditioning and allogeneic BMT has been successfully used in several animal models to prevent or treat (spontaneous) T cell-mediated autoimmune disease [1, 18, 19], our study provides convincing evidence that nonmyeloablative conditioning followed by allogeneic BMT can effectively treat severe B-cell-mediated autoimmune disease with a systemic inflammatory component.

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CHAPTER V

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TITLE

Targeting host B cell immune responses by persistent donor NK cell alloreactivity following nonmyeloablative stem cell transplantation

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ABSTRACT

The beneficial effects of allogeneic bone marrow transplantation (BMT) in the treatment of different hematological and immunological diseases are severely hampered by the risk of graft-versus-host disease (GVHD). Here, we show that haploidentical BMT after nonmyeloablative conditioning resulted in the early elimination of pre-existing antigen-specific host B cells and their main products, i.e. antibodies, in contrast to syngeneic BMT. Moreover, we show that sustained eradication of host immune cells by donor natural killer (NK) cells takes place after haploidentical BMT. Host spleen cells are still eliminated at 3 months after BMT, which could be attributed to donor NK cells. No GVHD was observed in any recipient of haploidentical BMT. Furtermore, our data show that clinical disease was effectively controlled by haploidentical BMT. Together, these data suggest that alloreactive NK cell responses are maintained without inducing GVHD. Moreover, these data indicate that NK cell-mediated alloreactivity towards host immune cells could be beneficial in the treatment of malignant and nonmalignant disorders in which B cells play a pathogenic role.

INTRODUCTION

Allogeneic bone marrow transplantation (BMT) is a potential treatment for different disorders, such as hematological malignancies and autoimmune diseases [1, 2]. The main limitations of this treatment are the conditioning-related toxicity, graft-versus-host disease (GVHD), and the toxicity related to post-transplant immunosuppressive medication [3]. Much progress has recently been made, especially in the development of so-called nonmyeloablative conditioning regimens that proved very promising in preventing T cell alloreactivity, and thus GVHD, as well as in the treatment of different chronic diseases [4, 5, 6]. These regimens do not seem to prevent activation of natural killer (NK) cells [5, 7], another cytotoxic cell lineage that can exert potent anti-host responses. Interestingly, donor NK cell alloreactivity after BMT has not been associated with GVHD. On the contrary, donor NK cells appear to be effective in preventing GVHD, probably because they are eliminating host professional antigen-presenting cells, presumably dendritic cells [7, 8].

Recently, we were able to show that systemic B cell-mediated autoimmune disease could be effectively treated with fully major histocompatibility complex (MHC)-mismatched allogeneic BMT [4]. The production of pathogenic autoantibodies was strongly inhibited compared to controls, suggesting that host B cells were eliminated as a result of the treatment. Therefore, we wished to investigate whether haploidentical (F1>P) BMT results in the effective eradication of host B cell responses as this treatment, in contrast to fully MHC-mismatched BMT, is not associated with GVHD. Finally, we also wished to investigate the efficacy of haploidentical BMT to control chronic autoimmune arthritis.

MATERIAL AND METHODS

Animals: Mice (8-12 weeks) were obtained, housed and fed as described [4, 5].

Immunization with ovalbumin (OVA): Mice were immunized with OVA emulsified in complete Freund's adjuvans (CFA), and boosted 3 weeks later. After seroconversion (i.e. the appearance of anti-OVA antibodies), the mice were subjected to BMT. Induction and clinical assessment of arthritis: Collagen-induced arthritis (CIA) was induced and evaluated as described by Morgan et al [9]. Mice with maximum scores of 12 were humanely killed. Paws of mice were scored for ankylosis by means of their ability to grasp a pencil.

Bone marrow transplantation: BMT was performed when more than 50% of the mice developed CIA. Mice were subjected to sublethal total body irradiation (TBI) of 6.0 Gy, and each received a single injection of anti-CD40 ligand (CD40L) monoclonal antibody (mAb) (MR1, 0.5 mg intraperitoneally) before BMT with 10 x 10(**+**6) total bone marrow (BM) cells intravenously collected from femurs and tibiae of donor mice. Following BMT, NK cells were depleted using anti-NK1.1 mAb (PK136, 0.25 mg intraperitoneally).

Flow cytometric analysis and ELISA: After BMT, peripheral blood and/or spleen cells were analyzed for host cells in different cell lineages as described [4, 5]. OVA-specific B cells were measured as follows. Spleen cells were harvested, and stained for B220. After fixation and permeabilisation with 4% paraformaldehyde and 0.02% saponine in PBS with 1% BSA and 0.02% sodium azide, respectively, cells were incubated with fluorescein isothiocyanate (FITC)-labeled OVA.

Measurement of serum antibodies: Anti-type II collagen (CII) antibodies were measured in enzyme-linked immunosorbent assay (ELISA) as described [4]. Anti-OVA antibodies (IgG2a) were measured in ELISA. Plates were coated with 2 microgram/ml of OVA. Anti-OVA antibodies were subsequently detected by HRP-conjugated goat anti-mouse IgG2a antiserum [4].

Measurement of serum amyloid P component: Levels of serum amyloid P component (SAP) were measured by sandwich ELISA as described [4].

Statistical analysis: Differences between groups of mice were analyzed using the nonparametric Mann-Whitney U test, and $P<0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Allogeneic bone marrow transplantation (BMT) is an established treatment for a variety of hematological and immunological disorders. It is postulated that the beneficial effects after myeloablation and haploidentical BMT are mediated by alloreactive donor NK cells, and host-reactive NK cell clones have been isolated from human transplant recipients up to three months post-transplantation [7, 10]. However, little is known about the ability of graft-derived NK cells to target host immune cells after nonmyeloablation and haploidentical BMT. Likewise, the persistence of donor NK cell alloreactivity in chimeric hosts is poorly defined in this setting [11].

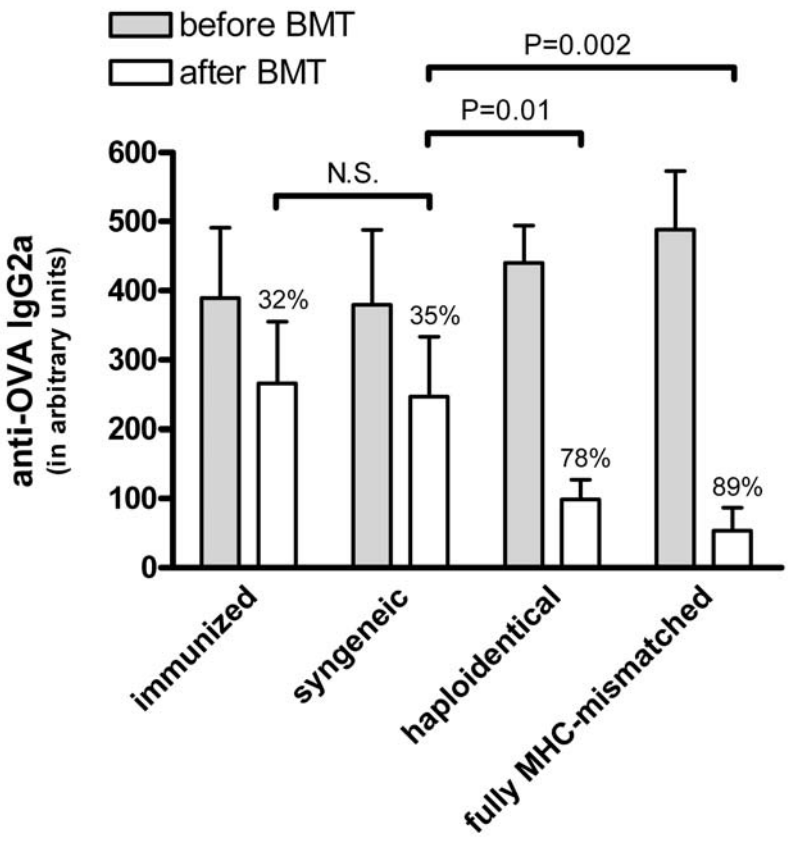
Recently, we hypothesized that NK cell-mediated anti-host responses, in the absence of graft-versus-host disease (GVHD), could be responsible for the beneficial effects observed in a mouse model for severe B cell-mediated autoimmune disease [4]. Therefore, we wished to analyze donor NK cell alloreactivity towards antibody-secreting B cells in more detail. To this end, we used a model of haploidentical (F1>P) BMT involving host B cells recognizing a model antigen, ovalbumin (OVA). In this model, donor T cells are tolerant for host cells [12, 13], but donor NK cells are activated as they lack inhibitory signals provided by self-MHC class I [14, 15]. We immunized DBA/1 mice with OVA, and after the appearance of high levels of anti-OVA antibodies (Figure 1A), the mice were subjected to nonmyeloablative conditioning and transplantion of 10 x 10(**+**6) BM cells from syngeneic, haploidentical or fully MHC-mismatched donors, i.e. DBA/1, (DBA/1 x BALB/c) F1 or BALB/c mice, respectively. As a control, one group of mice was left untreated. To confirm our earlier observations that allogeneic BMT suppresses the host antibody response [4], we measured the serum levels of OVA-specific antibodies before and after BMT. Our results show that antibody levels in syngeneic BMT recipients did not differ from controls, but were suppressed after allogeneic BMT (Figure 1A). In accordance with these data, OVA-specific B cells disappeared rapidly after allogeneic (i.e. both haploidentical and fully MHC-mismatched) BMT, but not after syngeneic BMT (Figure 1B). These findings indicate that pre-existing antigen-specific B cells of host origin are eliminated as a result of allogeneic BMT. Recipient mice had no signs of acute GVHD (i.e. no weight loss and/or detectable histologic abnormalities of liver, gut, and skin), supporting the notion that donor NK cells can mediate beneficial anti-host B cell alloresponses, without causing GVHD (data not shown). As donor NK cells are the only alloreactive cells in the setting of haploidentical BMT (i.e. F1>P), and as the eradication of host B cells in spleen is perforin-dependent

(data not shown), indicating an immune-mediated phenomenon, the observed elimination of pre-existing host B cells is most likely mediated by alloreactive donor NK cells.

Although the role of donor NK cells in anti-host responses after BMT has been evaluated shortly after transplantation, e.g. the conversion to full donor chimerism and graft-versus-leukemia effects [7], no data are available on the potency of donor NK cells at later time points after transplantation. Therefore, we wished to investigate whether anti-host responses by donor NK cells are still present at 3 months after haploidentical BMT. We subjected recipient C57BL/6 (Ly5.1**+**) mice to nonmyeloablative conditioning and transplantation of 10 x 10(**+**6) BM cells together with 25 x 10(**+**6) spleen cells, which were co-injected to facilitate the engraftment and outgrowth of donor cells by means of homeostatic proliferation [5]. C57BL/6 (Ly5.2**+**) and (C57BL/6 x BALB/c) F1 mice were used as congenic and haploidentical donors, respectively. As a control, congenic BMT was used to study physiological (i.e. non-immunological) mechanisms related to this transplantation protocol. Long-term and stable chimerism was achieved in different cell lineages, including granulocytes (Gr-1**+**), in all recipients (Figure 2A).

Figure 1: Haploidentical BMT results in a significant decrease in OVA-specific B cells in spleen as well as OVA-specific antibodies in serum.

A



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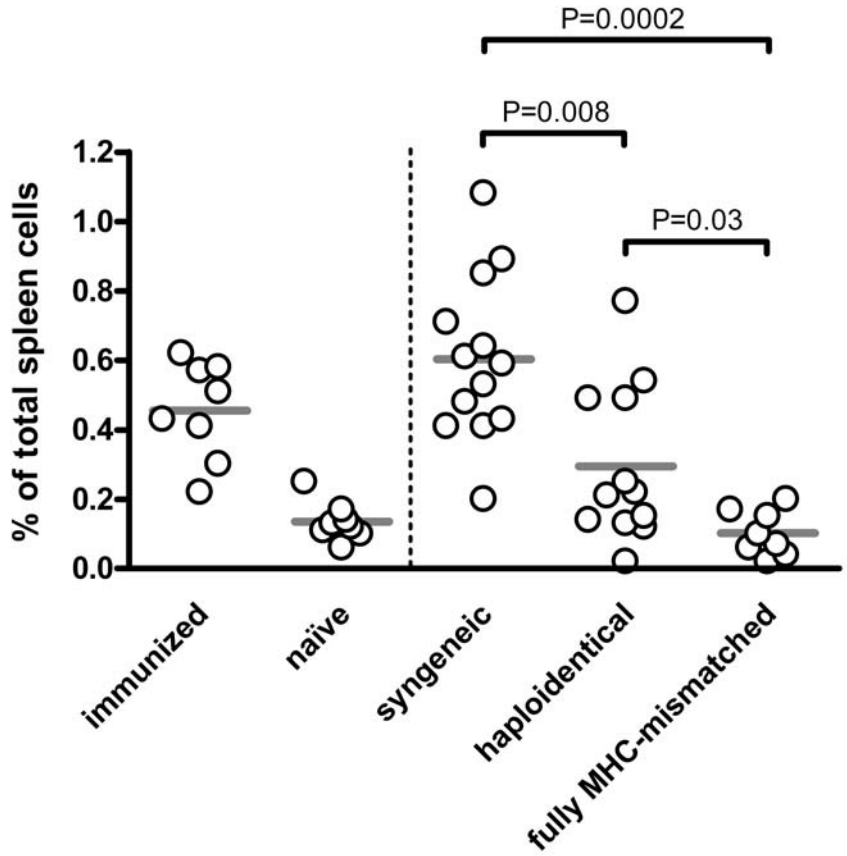
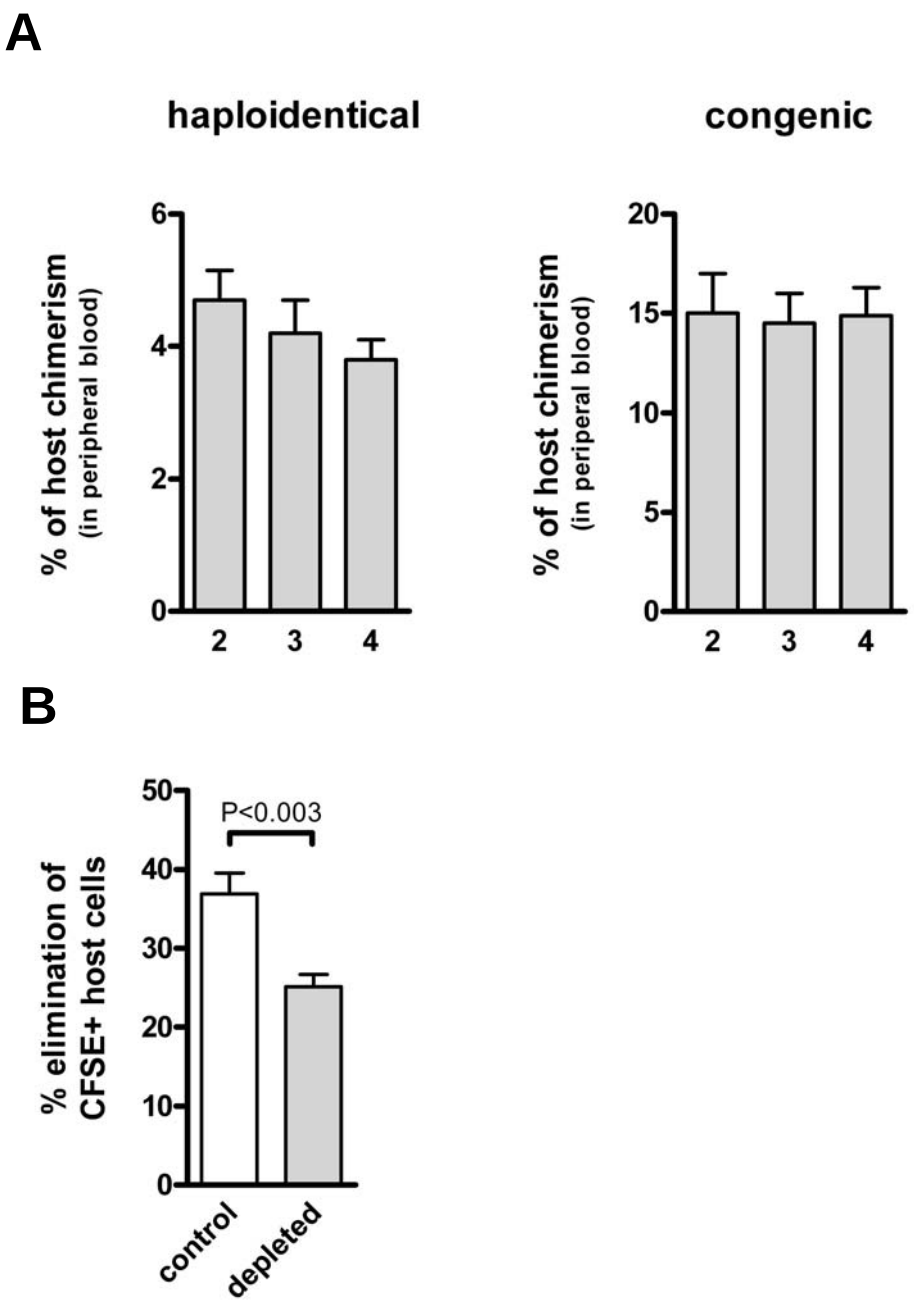


(A) DBA/1 mice (n>8 per group) were immunized with OVA, and were either left untreated or subjected to nonmyeloablative conditioning consisting of low-dose TBI of 6 Gy and a single injection of 0.5 mg of anti-CD40L antibodies (MR1) and transplantion of 10 x 10(**+**6) syngeneic, haploidentical or fully major histocompatibility complex (MHC)-mismatched BM cells from DBA/1, (DBA/1 x BALB/c) F1 or BALB/c donor mice, respectively. Sera were taken before and after BMT (**+**4 weeks), and tested by ELISA for the presence of anti-OVA IgG2a antibodies. At the start of the treatment, no differences in anti-OVA antibody titres were observed between the groups of mice. All groups displayed a decrease in anti-OVA antibodies after the time of treatment; syngeneic BMT showed a decrease in OVA-specific antibody titres of 35%, similar to that of controls (32%), while both haploidentical and fully MHC-mismatched BMT resulted in a strong decrease in antibodies against OVA (78% and 89%, respectively). However, only haploidentical and fully MHC-mismatched BMT resulted in a significant decrease in antibody levels compared to untreated controls, P**=**0.01 and P**=**0.002 respectively. (B) The percentage of OVA-specific B cells in spleen were analyzed after BMT (**+**2 weeks). Compared to syngeneic controls, both haploidentical and fully MHC-mismatched BMT recipients showed a significant decrease in the percentage of OVA-specific B cells, P**=**0.008 and P**=**0.0002 respectively.

However, the kinetics as well as the level of host chimerism differed between congenic and haploidentical BMT as: the level of host chimerism after haploidentical BMT was not only considerably lower, but was also further decreasing in time (Figure 2A). These findings indicate that mechanisms other than those related to the transplantation procedure as such, are still operative. Because donor NK cells are likely the predominant cell type that plays a role in haploidentical BMT, we wished to study the alloreactivity of donor NK cells directly. Therefore, 3 months after haploidentical BMT, differentially CFSE-labeled splenocytes from syngeneic (i.e. host) and haploidentical (i.e. control) mice were injected into chimeric hosts (i.e. 4-5% host Gr-1**+** cells). Prior to the challenge with CFSE-labeled splenocytes, NK cells in recipient mice were (or were not) depleted by anti-NK1.1 antibodies. At day **+**7, peripheral blood cells were analyzed for the presence of CFSE**+** cells, and the extent of host cell elimination was determined [5, 16]. Syngeneic (i.e. host) cells were eliminated in control mice, while this elimination was inhibited after depletion of NK1.1**+** cells (Figure 2B). Although the effect of depletion of NK1.1**+** cells is probably underestimated as not all F1-derived NK cells express NK1.1 (data not shown), these data indicate that host-reactive NK cells contribute to the eradication of host immune cells, and that this alloreactivity persists up to three months post-BMT.

Figure 2: Donor NK cells eradicate host spleen cells at 3 months after haploidentical BMT.



(A) C57BL/6 mice were treated with nonmyeloablative conditioning and congenic or haploidentical BMT. The presence of host cells was analyzed by FACS in different cell lineages. Data are shown for Gr-1**+** cells, i.e. granulocytes. (B) At 3 months after haploidentical BMT, chimeric recipient mice were injected with CFSE-labeled spleen cells from syngeneic (i.e. host) and haploidentical (i.e. control) mice. Before the adoptive transfer, donor NK cells witin the recipients were depleted with anti-NK1.1 (PK136) antibodies. PBS was used as a control. The presence of CFSE**+** cells in peripheral blood was analyzed by FACS **+**7 days after adoptive transfer.

Taken together, we provide evidence that sustained alloreactivity mediated by donor NK cells occurs at least up to 3 months after BMT. As the half-life of NK cells is estimated at

17 days [17], we postulate that donor NK cells that exert sustained anti-host responses were educated in the host. Furthermore, we show that NK cells are able to effectively eradicate host B cells with a subsequent decrease in the production of antigen-specific antibodies. Since the majority of spleen cells comprise of B cells (up to 60%), we hypothesize that sustained donor NK cell alloreactivity could be beneficial in the treatment of different malignant and nonmalignant disorders, including severe autoimmune diseases, in which B cells are known to be involved.

To investigate the latter aspect in more detail, we studied whether donor NK cell alloreactivity in the context of haploidentical BMT can effectively treat severe B cell-mediated autoimmune disease employing collagen-induced arthritis (CIA) in mice as a model. Susceptible male DBA/1 mice were immunized with bovine collagen type II (CII) as described earlier [4, 9]. After development of CIA, mice were subjected to sublethal total body irradiation (TBI) of 6.0 Gy and a single injection of anti-CD40 ligand (CD40L) monoclonal antibody (mAb) before BMT with 10 x 10(**+**6) total bone marrow (BM) cells from syngeneic, haploidentical or fully MHC-mismatched donors, i.e. DBA/1, (DBA/1 x BALB/c) F1 or BALB/c mice, respectively. One group of mice was left untreated as a control. In contrast to untreated mice, BMT was able to inhibit disease activity. In agreement with our earlier observations, fully MHC-mismatched BMT resulted in an initial exacerbation of clinical disease followed by prolonged suppression of disease activity (Figure 3A). This exacerbation correlated with elevated levels of serum amyloid P component (SAP), an acute-phase protein in mice (data not shown), resulting from systemic alloresponses within recipient mice after allogeneic BMT [4]. However, haploidentical BMT was associated with much less SAP levels in the circulation after transplantation (data not shown). Accordingly, this was associated with a less pronounced exacerbation of clinical disease (Figure 3A). Exacerbation of disease activity did not result in impairment of joint function (i.e. ankylosis) which was only observed in untreated control animals (Figure 3B). Interestingly, haploidentical BMT appeared as effective as in fully MHC-mismatched BMT in eliminating anti-CII antibody responses (Figure 3C). Thus, these data indicate that, in a BMT setting of F1>P, less adverse effects of a ‘cytokine storm’ (due to alloresponses) are observed, while NK cell-mediated alloreactivity is still in place resulting in the elimination of host B cells producing pathogenic antibodies.

Altogether, our data show that not only fully MHC-mismatched BMT is able to treat chronic autoimmune arthritis in mice [4], but that disease was also effectively inhibited

by haploidentical (F1>P) BMT. Although no increased beneficial clinical effects of haploidentical BMT over syngeneic BMT was observed within the time frame analyzed, we consider it likely that, in the long-term, haploidentical BMT will result in improved clinical outcome as haploidentical BMT was clearly associated with a more pronounced elimination of pathogenic antibodies as well as host B cells. In conclusion, our data indicate that donor NK cell alloreactivity towards host immune cells may be exploited in the setting of allogeneic BMT to treat autoimmune disease, such as chronic autoimmune arthritis in mice, in which B cells play a pathogenic role.

Figure 3: Haploidentical BMT results in the inhibition of disease activity and the elimination of autoantibodies in arthritic mice.

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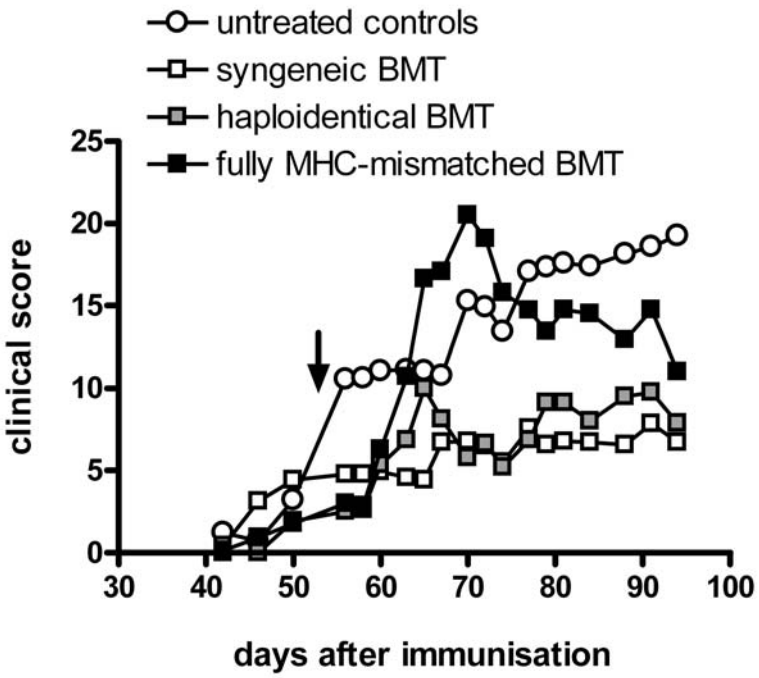

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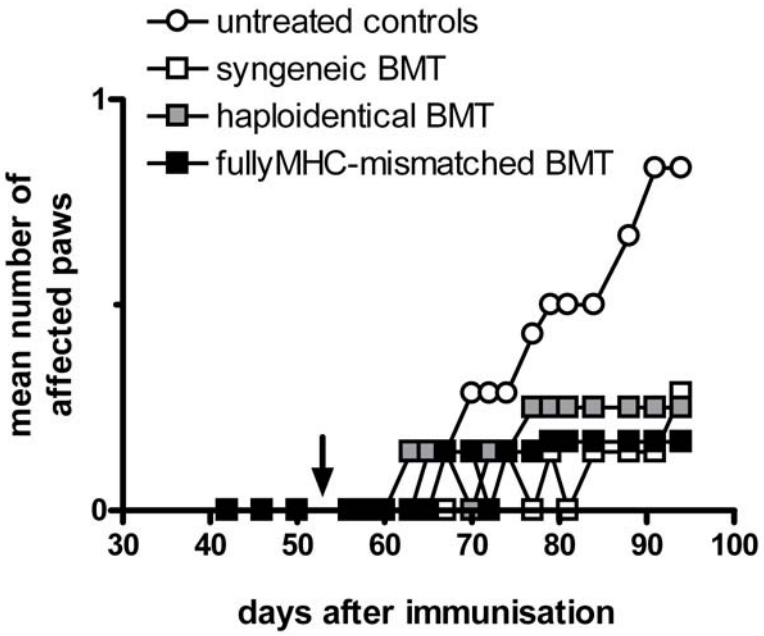

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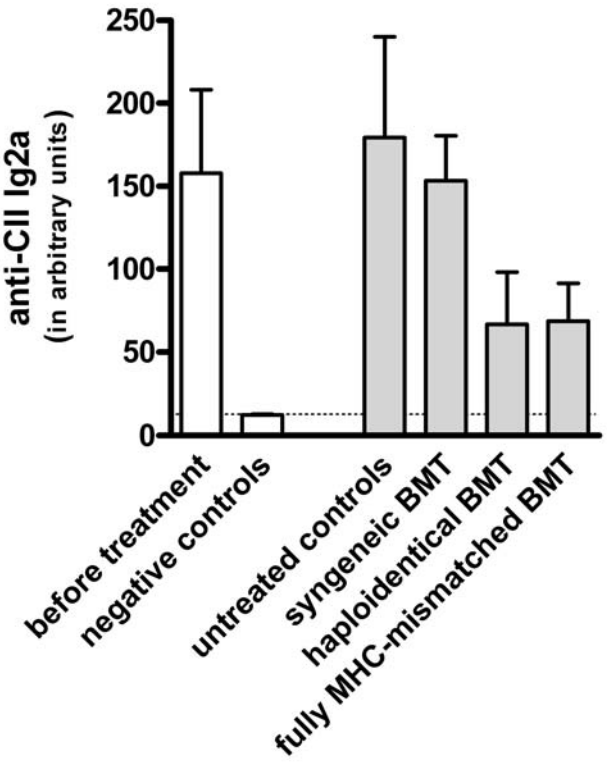

(A) Susceptible male DBA/1 mice (n>8 per group) were immunized with CII. After clinical disease developed, the mice were either left untreated or subjected to nonmyeloablative conditioning consisting of low-dose TBI of 6 Gy and a single injection of 0.5 mg of anti-CD40L antibodies (MR1) and transplantion of 10 x 10(**+**6) syngeneic, haploidentical or fully major histocompatibility complex (MHC)-mismatched BM cells from DBA/1, (DBA/1 x BALB/c) F1 or BALB/c donor mice, respectively. (B) Ankylosis of paws of arthritic mice was determined. (C) Sera were taken before and after BMT (**+**4 weeks), and tested by ELISA for the presence of anti-CII IgG2a antibodies. At the start of the treatment, no differences in anti-CII antibody titres were observed between the groups of mice.

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CHAPTER VI

General discussion

VI. GENERAL DISCUSSION

i. INTRODUCTION

There is a continuous debate about the balance between efficacy versus safety of adoptive cellular therapy to treat autoimmunity, including allogeneic BMT [16]. The treatment of severe autoimmune arthritis with conventional immunosuppression is not successful in controlling autoimmune disease in a substantial proportion of patients, and case reports have been described that strongly suggest that allogeneic BMT could be used to effectively treat RA (see Chapter I). Because of safety issues (i.e. allogeneic BMT is associated with (severe) GVHD), intense immunosuppression and autologous/syngeneic BMT in patients with refractory RA has been employed, although unsatisfactory. Nevertheless, long-term beneficial effects have been observed [78], and re-institution of conventional therapies showed increased benefit in these patients.

The data presented in this thesis, support the data from the literature that both allogeneic and autologous/syngeneic BMT suppress clinical disease (see Chapters II and IV). Allogeneic BMT, however, appears more effective as it results in the disappearance of pathogenic autoantibodies as well as B cells that produce these antibodies (see Chapters IV and V). Donor-derived NK cells contribute to these beneficial effects (see Chapter V), although their role needs further attention. Furthermore, adoptive transfer of (syngeneic) T regulatory cells proved beneficial (see Chapter III), thereby providing a rationale for their future use also in systemic autoimmune diseases that are mediated by B cells and autoantibodies. Altogether, our data suggest that (i) transplantation of BM cells, either from autologous, syngeneic or allogeneic donors, can be used to treat severe and chronic autoimmune arthritis, and that (ii) BMT can be used as a ‘platform’ to allow adoptive cellular immunotherapy employing T regulatory cells or alloreactive NK cells.

ii. BONE MARROW TRANSPLANTATION, DONOR NK CELL ALLOREACTIVITY AND T REGULATORY CELLS—TOWARDS ADOPTIVE CELLULAR THERAPY OF CHRONIC AUTOIMMUNE ARTHRITIS

The mechanisms that take place during initiation as well as progression of autoimmune disease in humans are difficult to elucidate. As a consequence, conventional immunosuppression as well as most experimental treatments mainly target pathogenic mechanisms operative in the effector phase (e.g. TNF-alpha [22]), while the other

disease-driving pathways are left alone. Although these types of therapies can be highly effective, one could argue that human autoimmune disease is best treated by 're-setting' the immune system. This may be effectively achieved via intense pre-transplant conditioning and subsequent donor BM cell infusion. Different animal data show that (spontaneous) autoimmune diseases are caused, prevented and/or cured by adoptive transfer of BM cells (see Chapter I). These data include mouse models of different systemic autoimmune diseases such as systemic lupus erythematosus, type I diabetes mellitus and rheumatoid arthritis. Concerning chronic autoimmune arthritis, the genetic contribution to disease susceptibility involves both immune and 'non-immune' molecules (see Chapter I), and may thus, genetically, reside within BM cells. In humans, however, the transfer of disease via allogeneic BMT has not yet been described for systemic autoimmune diseases like RA or SLE [23, 24]. Although an initial autoantibody response was observed after BM cells from a donor with autoimmune disease [24], the lack of disease transfer may be influenced by many (unknown) genetic and/or environmental parameters.

The use of BMT to treat autoimmune disease seems logical: (i) the pre-transplant conditioning leads to elimination of the recipient's immune system, including autoreactive lymphocytes; (ii) the recipient is infused with BM cells that will give rise to a new immune system; and (iii) the newly developed lymphocytes will be educated to be tolerant for self-antigens. However, the use of BMT still suffers from conceptual difficulties. First, the pathogenesis of chronic autoimmune diseases is largely unknown, and genetic and/or environmental factors that induce disease have remained elusive to date. Thus, whether BMT as such, i.e. the mere replacement of immune and BM cells, is the best way to treat or cure autoimmune disease is not yet known. Second, introducing BM cells from the recipient itself, i.e. autologous transplantation, is a relatively safe procedure in terms of GVHD. Animal models of (spontaneous) autoimmune diseases indicate, however, that the disease is transferred with autologous BM cells. Thus, the genetic susceptibility to develop disease is not dealt with. Furthermore, it is well-known that purging of the graft in humans (i.e. removal of contaminating lymphocytes such as T cells) is often incomplete, and autoreactive lymphocytes are subsequently re-introduced. Third, the patient's environment may be crucial in providing different stimuli that provoke autoimmunity. Infections seem to be important environmental factors, and have been associated with the onset of autoimmune disease, including type I diabetes mellitus (TIDM), multiple sclerosis (MS),

RA and SLE in humans [38, 39, 40, 41, 42, 43] as well as models of autoimmune disease in mice [44, 45, 46, 47]. In mice, it is even suggested that, despite the presence of genetically determined self-reactive T cells, stimulation of innate immunity by microbial products is required for overt autoimmune disease [44, 45, 46]. Thus, the patient's environment could, conceivably, provide triggers that re-induce disease in autologous BM recipients, because these triggers remain present, and are capable of doing so.

It is argued that allogeneic BMT provides a more powerful tool to conquer autoimmune disease, despite the risk of GVHD. First, concerning GVHD, it is believed that this type of alloreactivity can be held responsible for the beneficial consequences as well: the so-called 'graft-versus-autoimmunity' (GVA) effect. Although the severity of GVHD seems to be correlated with increased suppression of disease [48, 49, 50], other data indicate that the beneficial effects may be separated from those that have life-threatening consequences [51, 52, 70]. BM grafts containing alloreactive T and NK cells, as well as post-transplant donor lymphocyte infusions (DLI), mediate GVH responses against recipient's cells, including hematopoietic stem cells. It has been known for a long time that donor T cells strongly react to host cells. This alloreactivity is directed towards many vulnerable tissues of the recipient, including skin and gut, leading to pathogenic and even lethal consequences. However, it is currently thought that donor NK cells also react to hematopoietic and immune cells from the recipient, but not to tissue-resident cells in a pathogenic manner. They may even prevent GVHD, probably by depleting host APCs [51]. Second, when BM cells are harvested from a 'nonsusceptible' donor, the (re-)infusion of autoimmunity can be prevented. As mentioned earlier, evidence from animal models showed that the infusion of BM cells from autoimmune-prone donors led to the transfer of autoimmunity to the recipient, whereas the infusion of 'nonsusceptible' donors did not. Of course, the latter is no proof that this will never take place, but these data indicate that genetic predisposition is, at least partially, dependent on the source of the BM graft. However, these kinds of experiments in inbred mouse strains are difficult to translate to humans, and the definition of a 'nonsusceptible' donor, i.e. genotypically and/or phenotypically, is almost impossible. Humans donors should be genetically (and thus immunologically) related to some extent to perform the transplant procedure as safe as possible, i.e. without the risk of severe consequences of morbidity and mortality as a result of GVHD and/or long-term use of post-transplant immunosuppressive drugs.

Efforts to prevent GVHD, while leaving intact the proposed efficacy of allogeneic BMT, are currently being investigated.

In Chapter II, the effects of high-dose pre-transplant conditioning and autologous BMT to treat patients with severe RA are described. Our data suggest that (synovial) T cells are involved in perpetuation of disease, although it cannot be excluded that the infiltration by synovial T cells was just a consequence rather than a cause of synovial inflammation. As B cells producing autoantibodies are thought to play an important role as well, recent data are now available to put our data into perspective. It is known that the germinal center-like reactions that take place within the rheumatoid synovium depend on the presence of both B and T cells [53, 54]. In tonsils, data obtained using an ex vivo model suggest that the survival of plasma cells, either expressing CD20 or not, also depend on the presence of T cells [55]. Thus, as synovial B cells were still present after treatment, the local reappearance of T cells could, therefore, facilitate recurrent synovitis, e.g. by reactivating these B cells. With respect to autoantibodies in these patients, recent data from our group indicate that, after immunoablation and autologous BMT, reactivation of autoimmunity was accompanied by a newly generated IgG autoantibody response of low avidity. However, long-lasting humoral memory (against rubella and tetanus) was not eradicated by immunoablation, confirming that autoimmunity was independent of functional humoral memory responses [Y.K.O. Teng and co-workers, in press]. Together with data from another study showing that IgM autoantibodies are present in early as well as in longstanding RA [82], evidence is provided that autoreactive B cells are continuously activated in RA correlating with disease activity, in particular synovial inflammation. Although our study suggest a role for infiltrating T cells, the contribution of synovial B cells producing pathogenic autoantibodies and/or their interaction with newly arrived T cells cannot be excluded, and may even be equally important. It remains to be elucidated, when technically possible, what the relative contribution of these two lymphocyte subsets is in the re-occurrence of the autoimmune disease after high-dose immunosuppression and autologous BMT. Thus, our data indicate that the disappearance of synovial T cells after high-dose pre-transplant conditioning and autologous BMT is associated with the beneficial clinical effects observed.

In Chapter IV and V, our data from mouse experiments strongly suggest a strong potential for alloreactive donor NK cells in the treatment of chronic autoimmune arthritis. We observed the disappearance of (auto)antibodies as well as B cells producing these (pathogenic) antibodies, whereas our studies also indicated that donor-derived NK cells persistently mediate the eradication of host immune cells, including B cells. Of note, no GVHD was observed, probably as a result of effective inhibition of alloreactive T cells by anti-CD40L antibody treatment [59, 60, 61, 62], Alloreactive donor NK cells may provide a powerful tool to eliminate host immune cells as demonstrated earlier by Ruggeri and co-workers, both in human and mouse BMT recipients [51]. Transplantations were performed by using high numbers of CD34**+** cells to allow engraftment, combined with extensive T cell depletion to prevent GVHD, such that no post-transplant immunosuppression was needed [51]. Typically, such transplantations are associated with rapid recovery of NK cells and slow T cell recovery [85, 86, 87]. In the context of killer immunoglobulin-like receptor (KIR) ligand mismatches, no beneficial contribution of donor NK cells could be observed in all recipients of allogeneic BMT, indicating that graft composition and pre-transplant conditioning are important factors as the effects of donor NK cell alloreactivity in some recipients may have been obscured by donor T cells present in the graft [84]. Furthermore, other clinical data indicate that in vivo T cell depletion of the recipient can facilitate beneficial donor NK cell alloreactivity [88, 89]. Host-reactive NK cell clones have been identified in recipients up to 3 months post-transplantation [51, 90]. Likewise, our studies indicated that persistent donor NK cell alloreactivity towards host immune cells is taking place, but whether donor NK cells that were infused (i.e. 1-2% of donor cells) or those that newly developed from the graft (i.e. educated in the recipient) can be held responsible was not investigated. Nevertheless, recent data from mouse experiments have provided new insights in the turnover, survival and dynamics of NK cells in vivo. First, adoptively transferred NK cells undergo homeostatic proliferation in lymphopenic recipients [91, 92]. Next, in vivo experiments using 5-bromo-2-deoxyuridine (BrdU) showed that the half-life of NK cells is approximately 17 days, while the NK cell pool seems to be completely replaced within 40 days [92]. Finally, mouse experiments indicated that NK cells from MHC-deficient mice are defective in killing target cells [93], while becoming functionally competent through ‘licensing’ by self-MHC molecules [94]. These findings indicate that expression of MHC class I molecules within the host play an indispensable role in the functional development of NK cells. We, therefore, speculate that in our model of haploidentical

(F1>P) BMT infused as well as newly developed NK cells from donor-origin eliminate host immune cells. Infused donor NK cells may efficiently eliminate host B cells in the early time period following allogeneic BMT. Newly developing NK cells may be 'licensed' by self-MHC molecules expressed on (parental) BM stromal cells. After maturation, these donor-derived NK cells will be subsequently 'missing self' on host immune cells, including (long-lived) B cells such as plasma cells, leading to their persistent elimination in the periphery. Thus, donor NK cell alloreactivity after allogeneic BMT may be exploited to fully eliminate autoreactive host immune cells, i.e. the GVA effect, without developing, and possibly even preventing GVHD.

Interestingly, syngeneic BMT resulted in suppression of clinical disease, despite the fact that host B cells as well as their products, i.e. autoantibodies, were not eliminated as observed after allogeneic BMT. Conditioning alone did not result in suppression of the autoantibody response, but, in striking contrast to syngeneic BMT, inhibition of clinical disease was not observed. To put this into perspective, several data can be considered. First, normal levels of SAP, an acute-phase protein, were observed after syngeneic BMT, but not after conditioning alone, indicating that infusion of syngeneic BM cells can suppress systemic inflammatory responses after conditioning. Interestingly, it has recently been described that C3a, a chemotactic cleavage product of complement factor 3 (C3), potentiates engraftment of hematopoietic stem cells, whereas BM stromal cells that are 'coated' with iC3b, yet another cleavage product of C3, as a result of irradiation, may facilitate engraftment of stem cells as well. The homing potential of stem cells to BM may be enhanced via the priming of the infused BM by circulating C3a within the recipients suffering from a systemic inflammatory disease, and via tethering of stem cells unto (irradiated) BM stromal cells in recipients via complement receptor expression on hematopoietic stem cells such as C3 and C5L2 [79, 80, 81]. It is tempting to speculate that, on the one hand, syngeneic BMT, somehow, alleviates the effects of pro-inflammatory responses, including acute-phase responses after irradiation as well as autoimmune arthritis, while, on the other hand, inflammatory molecules (such as C3a and iC3b) present at the time of treatment contribute to its efficacy. Second, due to the limited follow-up of less than 2 months, it cannot be excluded that the positive clinical effects of syngeneic BMT are overestimated. The clinical data from patients with RA strongly suggest that autologous BMT is not curative; relapses after initial remission are common [76, 77]. Finally, the infused BM cells, including T and B cells, could compete with pathogenic T and/or B cells from the recipient by filling up 'space' and/or regulating

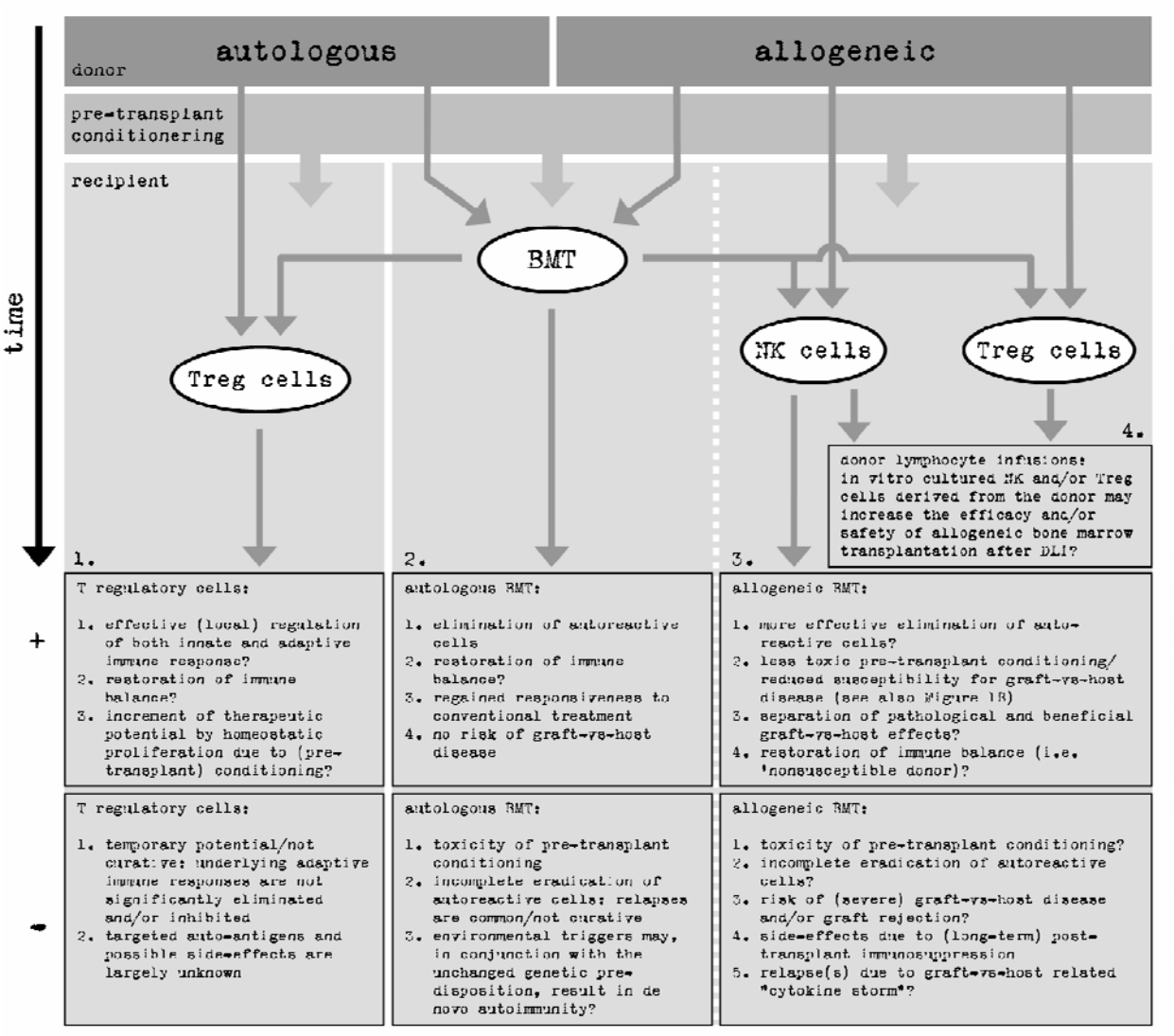
their homeostatic proliferation. Thus, our data suggest that syngeneic BMT positively affects ongoing (systemic) autoimmune responses.

In Chapter III, we investigated the therapeutic role of CD4**+**CD25**+** T regulatory cells, a recently described subset of T cells [83], in CIA, a mouse model of RA. T regulatory cells modulate (auto)immune responses [56, 57], and their potential to treat chronic autoimmune diseases (like RA) is now frequently studied. Our studies further substantiated the rationale of their potential use to treat autoimmunity. However, the future use of T regulatory cells in the treatment of autoimmunity also faces several difficulties. Most autoantigens recognized by the immune system are not known, while the precise mechanisms involved in their regulation have not yet clearly defined. Treatment with T regulatory cells may result in unwanted side-effects as well, e.g. suppression of innate and adaptive immune responses against pathogens. Other practical aspects such as costs and logistics may also hamper the widespread application of T regulatory cells in the near future. Still, the adoptive transfer of T regulatory cells recognizing disease-related autoantigens may be considered the most 'natural' way to treat autoimmune disease as well as to achieve the ultimate goal: the restoration of self-tolerance. Although antigen-specific interventions may be technically difficult to accomplish, the use of (polyclonal) T regulatory cells are promising. In mouse studies like ours, CD4**+**CD25**+** T regulatory cells are isolated from spleen and/or lymph nodes of donor mice, but T regulatory cells can also be generated, both in vivo and in vitro, using different approaches. For example, via tolerogenic DC vaccines [reviewed in 18], and the treatment with a certain 'tolerizing' monoclonal antibody against anti-CD3, a T cell-specific surface molecule that associates with the antigen-specific T cell receptor [reviewed in 19, 20]. So far, T regulatory cells have been studied in the treatment of a variety of T cell-mediated (autoimmune) diseases in mice, but whether T regulatory cells are also able to effectively treat chronic B cell-mediated autoimmune diseases was not yet been investigated. Although it has been shown that T regulatory cells can regulate autoantibody responses to some extent [5, 95, 96], to date, it is largely unknown what interactions between T regulatory cells and (autoreactive) B cells occur, and how these interactions would result in the restoration and maintenance of self-tolerance in the case of B cell-mediated autoimmune diseases such as chronic autoimmune arthritis. Interestingly, in concordance with our findings, more evidence is now available showing that local regulation of the immune response within the synovium can take place [31,

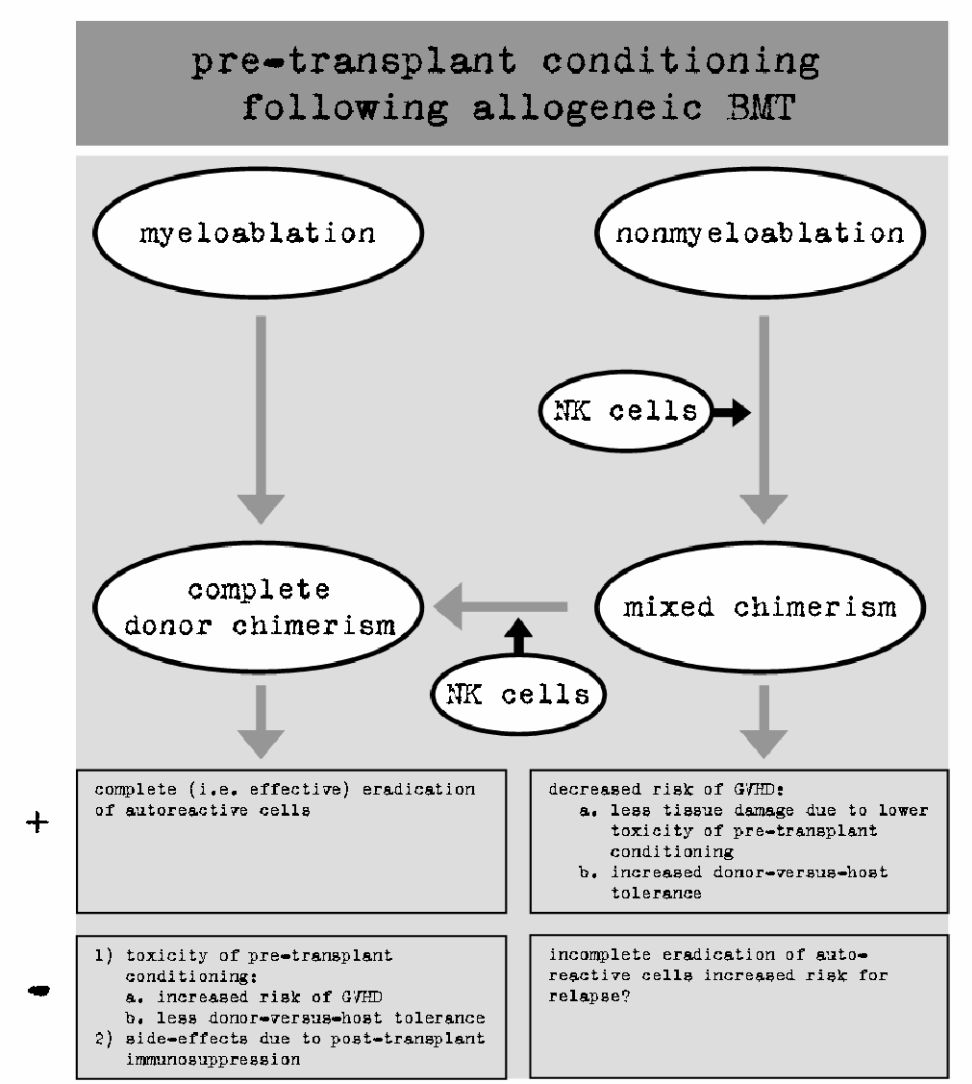
32]. Once autoantibodies are continuously produced by plasma cells, it will be interesting to determine whether, and if so, to what extent, effector mechanisms (i.e. activation of innate cells by immune complexes and/or complement, or even the molecular events of complement activation itself) are subject to regulation by T regulatory cells. With respect to the interference with innate processes during (synovial) inflammation, T regulatory cells have been shown to express CD46 [1, 2, 3, 4], a complement regulatory receptor for C3b and C4b, which is involved in the suppression ongoing inflammatory (autoimmune) responses [1, 4]. Furthermore, recent data from our group indicate a role for T regulatory cells secreting soluble TNF receptor II (sTNFRII) in the regulation of (systemic) inflammatory responses [G.J.D. van Mierlo and co-workers, submitted for publication]. Thus, T regulatory cells may ameliorate autoimmune arthritis via local interference of innate (e.g. complement-mediated events), but also via adaptive (e.g. regulating T-B cell interactions) immunity. Additionally, our studies indicate that lower numbers of T regulatory cells are needed to suppress clinical arthritis in lymphopenic recipients (because of pre-transplant conditioning prior to BMT) compared to nonlymphopenic animals. These data suggest that the efficacy of T regulatory cell-based immunotherapy could be enhanced when pre-transplant conditioning followed by BMT is used as a platform. In our studies, we made use of autologous T regulatory cells, but, with respect to our findings presented in Chapter IV and V, other pre-clinical studies support a future role of T regulatory cells in the context of allogeneic BMT [97, 98, 99, 100, 101, 102, 103, 104]. Of note, transferred T regulatory cells suppressed pre-existing autoimmune disease, while establishing transplantation tolerance [104]. This is of particular interest for the future use of allogeneic T regulatory cells (instead of those from autologous origin) as several studies are now available suggesting that T regulatory cells in human autoimmune diseases, including RA, are defective, i.e. functionally and/or numerically incapable of properly regulating local pathology [106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116].

Figure 1: Adoptive cellular immunotherapy in chronic autoimmune arthritis: a summary.

A



B



(A) In this figure, three types of adoptive cellular therapies which are discussed in this thesis: (1) T regulatory cells (Treg) from autologous origin, (2) bone marrow transplantation (BMT) from autologous origin, and (3) BMT from allogeneic origin (BMT). Furthermore, (4) some prospects of donor lymphocytes infusion (DLI) using allogeneic natural killer (NK) cells and/or Tregs are shortly referred to. Pre-transplant conditioning with BMT (either autologous or allogeneic) may serve as a 'platform' for additional adoptive cellular therapies (Treg, NK) to increase its efficacy. Pre-transplant conditioning facilitates the engraftment as well as the efficacy of BMT, Treg and/or NK cells after transfer (grey arrows). Pro's (**+**) and contra's (–) of each approach are summarized. (B) In the setting of allogeneic BMT, the toxicity of the pre-transplant conditioning (myeloablation versus nonmyeloablation) may, at least in part, determine the outcome after BMT in terms of chimerism. As lower toxicity of the regimen, i.e. nonmyeloablative conditioning, is associated with less GVHD and/or increased donor-versus-host tolerance, donor NK cells may be used to achieve complete donor chimerism. Thus, effective elimination of (autoreactive) host immune cells without the adverse side-effects of long-term immunosuppression, for example, which are frequently being observed after 'conventional' allogeneic BMT after myeloablation.

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iv. CONCLUSIONS

The different aspects of adoptive cellular immunotherapy in chronic autoimmune arthritis presented and discussed in this thesis are summarized in Figure 1. The data

presented in this thesis indicate that autologous as well as allogeneic BMT are effective in treating chronic autoimmune arthritis, and clinical efficacy is associated with elimination of (synovial) T cells and/or B cells producing pathogenic autoantibodies. Based on our studies, however, pre-transplant (nonmyeloablative) conditioning followed by allogeneic BMT resulting in total eradication of the host's hematopoiesis and immune system (i.e. complete donor chimerism) probably has superior curative potential due to the more effective elimination of host immune cells, including B cells producing pathogenic autoantibodies. Donor NK cell alloreactivity plays an important role in the beneficial effects after allogeneic BMT. Unlike donor T cells, alloreactive donor NK cells have not been associated with GVHD, and, indeed, no overt GVHD was observed. Nonetheless, donor NK cell alloreactivity, was persistently present up to 3 months after transplantation, and may be used for different types of clinical applications, e.g. hematologic malignancies, conditioning of the recipient, as well as in the treatment of systemic and chronic autoimmune disease like RA. Since clinical application of allogeneic BMT for chronic autoimmune disorders in humans still suffers major difficulties, e.g. GVHD and the availability of suitable (i.e. MHC-matched) allografts, our data also provide a rationale for the future use of T regulatory cells—alone or together with pre-transplant conditioning and/or autologous, syngeneic or allogeneic BMT. When efficacy, safety and feasibility have been properly evaluated in pre-clinical models, these approaches are potentially highly effective therapeutic tools for RA in the future.

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CHAPTER VII

Summary

VII. SUMMARY

In Chapter II, we demonstrated that the beneficial effects after autologous BMT in patients with RA, i.e. (partial) remission, were associated with changes in the expression of different immunological markers within the synovium, especially T cell markers. Before transplantation, clinical responders showed higher expression of T cell markers, including CD3 and CD4, and also displayed significant higher baseline expression of CD27, an activation/differentiation marker of both T and B cells. Furthermore, the expression of pro-inflammatory cytokines such as IL-1 also correlated with relapse. However, no differences were observed in the number of CD19**+** B cells and CD38**+** plasma cells, while the presence of resident synovial cells, i.e. CD68**+** macrophages and CD55**+** fibroblast-like synoviocytes, did not change either. These data indicate that the elimination (i.e. 'debulking') of T cells from the synovium by high-dose immunosuppression correlated best with a good clinical response. Likewise, the reappearance of T cells was associated with early relapse. These findings provide circumstantial evidence for a role of T cells in established RA.

In Chapter III, we were able to show that the adoptive transfer of (ex vivo activated) naturally occurring CD4**+**CD25**+** T regulatory cells resulted in significant and sustained suppression of clinical disease. Nevertheless, different parameters of systemic autoimmune response, i.e. antigen-specific T cell proliferation and serum levels of pathogenic antibodies, were not significantly influenced by this treatment. Of interest, our data show that a single infusion resulted in prolonged suppression of clinical inflammation. The mode of action was not thoroughly investigated, but the adoptively transferred T regulatory cells could be found in the inflamed synovium as well as the synovial fluid, suggesting that local immune regulation may take place. These results provide a rationale for the future use of T regulatory cells in the treatment of systemic autoimmune diseases.

In Chapter IV and V, we were able to demonstrate that allogeneic BMT following nonmyeloablative conditioning resulted in a significant suppression of disease activity. The beneficial effects were most likely caused by the elimination of B cells producing pathogenic autoantibodies because these antibodies disappeared rapidly after BMT. We postulated that a 'graft-versus-autoimmunity' (GVA) effect related to allografting is responsible for the disappearance of autoantibody-producing B cells (see Chapter IV). Indeed, antibody-producing B cells were eliminated within two weeks after treatment, and our data indicate a role for donor NK cells in this process. Furthermore, this host-

reactive NK alloreactivity was persistent in chimeric recipients, and host immune cells were eliminated up to three months post-transplantation (see Chapter V). These studies provide evidence that nonmyeloablative conditioning followed by allogeneic BMT can effectively treat severe B-cell mediated autoimmune disease characterized by systemic inflammation.

CHAPTER VIII

Samenvatting

VIII. SAMENVATTING

In Hoofdstuk I werd uiteengezet dat reumatoïde artritis (RA), de meest voorkomende ontstekingsziekte die de gewrichten aantast, kan leiden tot ernstige gebreken, bv. arbeidsongeschiktheid. De ontsteking aan de gewrichten resulteert niet zelden in de aantasting en afbraak van kraakbeen en/of bot. T-cellen en hun signaalstoffen zijn nauw betrokken bij het ontstekingsproces in RA, maar recent is de belangrijke, en misschien zelfs essentiële rol van B-cellen die autoantistoffen produceren aan het licht gekomen. Jaren van klinische praktijk en wetenschappelijk onderzoek hebben geleid tot een beter begrip van de ziekte, en hoe deze behandeld kan worden. Echter, de ziekte kan in een aanzienlijk deel van de patiënten niet of nauwelijks onder controle gehouden worden, zodat zowel artsen als onderzoekers op zoek zijn gegaan naar nieuwe mogelijkheden om ook deze groep patiënten te helpen.

Met het onderzoek beschreven in dit proefschrift is daarom getracht te onderzoeken of de adoptieve infusie van afweercellen, d.w.z. afkomstig van de ontvanger zelf of van een gezonde donor, gebruikt kan worden om langdurige autoïmmuunziekte zoals RA te behandelen. De studies die zijn beschreven in dit proefschrift hebben het voornemen om de ziekte te behandelen door de uitschakeling van zelf-reactieve afweercellen van de ontvanger, waaronder B-cellen, en de (her)introductie van 'tolerantie-voor-zelf'.

In Hoofdstuk II hebben we aannemelijk gemaakt dat de gunstige klinische effecten na autologe beenmergtransplantatie (BMT) in patiënten met RA zijn geassocieerd met sterke veranderingen in de expressie van verschillende, ontstekingsgerelateerde kernmerken. Deze verandering hebben we bestudeerd in het gewrichtskapsel, en het blijkt dat voornamelijk de kernmerken van T-cellen verschillen laten zien. Voor BMT hadden patiënten die goed reageerden op de behandeling een hogere expressie van T-cel kenmerken, inclusief CD3 en CD4, maar ook een hogere expressie van CD27, een activatie- en differentiatie-kenmerk van T-cellen, maar ook B-cellen. Daarnaast was de expressie van ontstekingsgerelateerde signaalstoffen waarvan het afweersysteem gebruik maakt, waaronder IL-1, gecorreleerd met klinische terugval. Echter, er werden géén verschillen geobserveerd in het aantal CD19**+** B-cellen of CD38**+** plasmacellen in het gewrichtskapsel. Ook de aanwezigheid van ontstekingscellen die daar reeds woonachtig zijn, d.w.z. CD68**+** macrofaag-achtige synoviocyten, liet geen verschil zien. Deze resultaten geven aan dat de uitschakeling van T-cellen in het gewrichtskapsel d.m.v. hoge-dosis immuunsuppressie sterk correleert met een goede klinische respons op de behandeling. Evenzo is de ‘terugkomst’ van T-cellen geassocieerd met een

snelle(re) klinische terugval. Deze bevindingen geven een indirect bewijs voor de belangrijke rol van T-cellen in patiënten met een langdurige ziektegeschiedenis.

In Hoofdstuk III toonden we aan dat de infusie van (buiten-het-lichaam geactiveerde) regulatoire T-cellen resulteerde in een aanzienlijke en langdurige onderdrukking van klinische ziekte. Niettemin bleken verschillende parameters van de onderliggende autoïmmuunziekte, d.w.z. specifieke T-cel reactiviteit en de hoeveelheid van ziekteveroorzakende autoantistoffen, onveranderd door de behandeling. Vooral interessant was de observatie dat slechts één infusie resulteerde in langdurige onderdrukking van klinische ontsteking. Ook al is de wijze waarop deze cellen de ziekte remmen niet in detail bekeken, de ingespoten regulatoire T-cellen konden teruggevonden worden op de plaats van ontsteking, nl. in het gewrichtskapsel. Samengevat suggereren deze bevindingen dat deze cellen lokaal hun functie uitoefenen en creëen ze een basis voor de toekomstige behandeling van autoïmmuunziekten, zoals RA, met regulatoire T-cellen.

In Hoofdstukken IV en V hebben we aangetoond dat allogene BMT na een zg. niet-myeloablatieve voorbehandeling resulteerde in de aanzienlijke onderdrukking van ziekteactiviteit. Dit gunstige effect werd hoogstwaarschijnlijk veroorzaakt door de uitschakeling van B-cellen die ziekteveroorzakende autoantistoffen maakten. Het bleek namelijk dat deze antistoffen snel verdwenen na de behandeling. Vervolgens postuleerden we dat een 'transplantaat-versus-autoïmmuunziekte' effect gerelateerd aan allogene BMT verantwoordelijk gehouden kon worden voor deze verdwijning van de autoantistof-producerende B-cellen (zie Hoofdstuk IV). Inderdaad werden deze B-cellen binnen twee weken na de behandeling uitgeschakeld, en onze resultaten geven reden om aan te nemen dat NK-cellen afkomstig van de donor hierin een belangrijke rol spelen. Deze NK-cel afhankelijke alloreactiviteit bleek gedurig, aangezien B-cellen van de ontvanger nog steeds uitgeschakeld werden op drie maanden na BMT (zie Hoofdstuk V). Deze studies leveren bewijs dat een niet-myeloablatieve voorbehandeling gevolgd door allogene BMT ernstige B-cel afhankelijke autoïmmuunziekte, zoals RA, kan behandelen.

Al met al kan geconcludeerd worden dat onze studies en die van anderen aanleiding geven om de adoptieve infusie van afweercellen (d.w.z. beenmergcellen, regulatoire T-cellen en donor NK-cellen) verder te onderzoeken en, waar mogelijk, reeds klinisch-experimenteel toe te passen in de behandeling van chronische autoïmmuunziekten zoals RA.

ABBREVIATIONS

APC	antigen-presenting cell
APC	allophycocyanin
BM	bone marrow
BMT	bone marrow transplantation
BrdU	5-bromo-2-deoxyuridine
CCP	cyclic citrullinated peptide
CFSE	5,6-carboxyfluorescein diacetate succinimidyl ester
CIA	collagen-induced arthritis
CII	collagen type II
CRP	C-reactive protein
DC	dendritic cell
DLI	donor lymphocyte infusion
DMARD	disease-modifying antirheumatic drug
FcR	Fc receptors
FITC	fluorescein isothiocyanate
GM-CSF	granulocyte-colony stimulating factor
GPI	glucose-6-phosphate isomerase
GVA	graft-versus-autoimmunity
GVH	graft-versus-host
GVHD	graft-versus-host disease
GVL	graft-versus-leukemia
HRP	horseradish peroxidase
HSCT	hematopoietic stem cell transplantation
IL	interleukin
KIR	killer immunoglobulin-like receptor
KO	knock-out
LN	lupus nephritis
LPS	lipopolysaccharide
MHC	major histocompatibility complex
MS	multiple sclerosis
MTX	methotrexate
PE	phycoerythrin
PIA	proteoglycan-induced arthritis
RA	rheumatoid arthritis
RF	rheumatoid factor
SLE	systemic lupus erythematosus
Tg	transgenic
TIDM	type I diabetes mellitus
TNF	tumour necrosis factor-alpha

CURRICULUM VITAE

De schrijver van dit proefschrift, Roelof Flierman, werd geboren in Sliedrecht (december 1976). Tijdens de afronding van zijn opleiding aan het Voorbereidend Wetenschappelijk Onderwijs (VWO) in Papendrecht (juni 1995), kwam zijn interesse voor de medische wetenschap aan het licht. Hij ging Biomedische Wetenschappen studeren aan de Universiteit Leiden (september 1995) en studeerde af met een onderzoeksstage en bijbehorende scriptie bij de afdeling Reumatologie onder leiding van Dr. Jaap M. van Laar in het Leids Universitair Medisch Centrum (LUMC, januari 2001). Andere onderzoeksstages bracht hij tot een goed einde onder de begeleiding van Dr. Peter J.K. Kuppen (afdeling Heelkunde, LUMC) en Dr. Maarten J.D. van Tol (afdeling Kindergeneeskunde, LUMC). Vanaf januari 2001 voerde hij het onderzoek, zoals beschreven in dit proefschrift, uit onder de begeleiding van Dr. Jaap M. van Laar en Dr. René E.M. Toes (afdeling Reumatologie, LUMC). De vraag of beenmergtransplantatie kan dienen als behandeling van chronische autoïmmuun-gemedieerde artritis stond hierbij centraal. Sinds september 2005 is hij werkzaam als post-doc onderzoeker onder de begeleiding van Prof. Dr. Mohamed R. Daha (afdeling Nierziekten, LUMC), en bestudeert hij welke factoren bijdragen aan het ontwikkelen van ernstige nierontstekingen in patiënten met systemische lupus erythematosus (SLE).

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