TREGS AND RHEUMATOID ARTHRITIS

Amy E. Anderson, John D. Isaacs*

Abstract

Regulatory T cells (Tregs) are a subset of T cells which are involved in peripheral immune tolerance. Their role in autoimmune disease, which occurs through a breakdown of tolerance, is of particular interest in trying to ascertain the mechanism(s) of disease progression. It is hoped that by understanding the role of Tregs in autoimmunity a reliable therapy may be developed to aid in both the treatment and, potentially, cure of disease. This review will focus on the naturally-occurring CD4+CD25+ regulatory T cell subset and their possible involvement in rheumatoid arthritis.

Keywords: Treg, CD4+CD25+, rheumatoid arthritis, autoimmunity, tolerance

Resumo

As células T reguladoras (Tregs) são um subtipo de linfócitos T envolvido na tolerância imunológica periférica. O seu papel nas doenças autoimunes, que surgem por um mecanismo de quebra de tolerância, reveste-se de particular interesse numa tentativa de esclarecer o(s) mecanismo(s) de progressão da doença. Espera-se que, compreendendo o papel das Tregs na autoimunidade, se possa desenvolver uma terapêutica fiável que ajude no tratamento e potencialmente na cura da doença.

Esta revisão foca o subtipo de células T reguladoras que ocorre naturalmente, as células T CD4+CD45+, e o seu possível envolvimento na artrite reumatóide.

Palavras-chave: Treg; CD4+CD25+; Artrite Reumatóide; Autoimunidade, Tolerância.

*Musculoskeletal Research Group, Institute of Cellular Medicine, Newcastle University, Newcastle upon Tyne, United Kingdom

Autoimmunity and tolerance

Autoimmune diseases, e.g. type 1 diabetes, and rheumatoid arthritis (RA), affect about 5% of the World's population. They arise due to a break down of immunological self-tolerance. Ordinarily there are a number of mechanisms in place which control self-reactive T cells. Clonal deletion (negative selection) is the primary mechanism and occurs in the thymus during the development of the immune system. This mechanism is referred to as central tolerance and involves the deletion of T cells expressing a TCR with a high avidity for autoantigen expressed on thymic APCs. However, this system is imperfect and some self-reactive T cells escape this elimination process, making their way into the periphery where they have the potential to cause devastating damage.

To regulate these potentially self-destructive T cells a number of mechanisms exist in the periphery. These mechanisms are broadly termed peripheral tolerance and include clonal anergy, peripheral deletion, immunological ignorance and a variety of regulatory T cells (Tregs). Clonal anergy occurs when T cells encounter processed antigen in the absence of co-stimulatory signals. Anergised T cells subsequently cannot become activated even in the presence of full activating signals and therefore cannot mount an immune response. Peripheral deletion contributes to the elimination of T cells with a high avidity for antigen and to the deletion of T cells when the immune response is no longer required. It occurs through activation-induced cell death, whereby T cells repeatedly stimulated by antigen are deleted by apoptosis. This mechanism limits hypersensitivity reactions to allergens and autoantigens. Immunological ignorance refers to the situation whereby self-reactive T cells, although capable of mounting an immune response to their autoantigen don't respond to, or "ignore", it. This can arise for two reasons. The first is that the autoantigen may be present in a too low concentration. All T cells have a threshold for receptor occupancy which is necessary to trigger a response. Very low

concentrations of antigen will simply not be sensed. The second reason involves the sequestering of antigens in locations which are not freely exposed to immunological surveillance, e.g. the eye or central nervous system. T cells will simply not have access to their autoantigen. The final and probably the major mechanism of peripheral tolerance is existence the regulatory T cell (Treg) subsets.

Naturally-occurring CD4+CD25+ Tregs

Development

The most studied subset of Tregs is the naturally--occurring CD4^{+}CD25^{+} population, which make up 5-10% of CD4^{+} T cells in peripheral blood. In humans, it is the 1-2% of cells which have high CD25 expression that have suppressive activity and as such some reports refer to CD4^{+}CD25^{+} naturally-occurring Tregs as CD4^{+}CD25^{bright} or CD4^{+}CD25^{high} Tregs. CD4^{+}CD25^{+} Tregs cells are thought to arise in the thymus during thymic development. In fact it has been shown that CD4^{+}CD25^{+} Tregs express self--reactive TCR.[1] High avidity interactions between the TCR and expressed autoantigen promote CD4^{+}CD25^{+} Treg selection.[2] Lower avidity interactions promote the development of CD4^{+}CD25^{-} effector T cells, whereas even higher avidity interactions lead to clonal deletion, as mentioned above. Recently, Vukmanovic-Stejic *et al.* have suggested that during adulthood a proportion of the Treg population is generated from highly differentiated memory CD4^{+} T cells.[3] This may explain why Tregs are maintained throughout life, despite a decrease in thymic output with age.

Properties and function

In vitro CD4^{+}CD25^{+} Tregs have properties of anergic cells upon stimulation characterised by low proliferation and low IL-2 production. However, it has been demonstrated that these cells have a high rate of proliferation *in vivo*.[3] Sakaguchi *et al.*, were the first to define CD4^{+}CD25^{+} Tregs.[4] They reported that the transfer of T cells depleted of the CD4^{+}CD25^{+} subset into athymic mice caused spontaneous development of various T cell-mediated autoimmune diseases, suggesting that CD4^{+}CD25^{+} Tregs are involved in the suppression of self-reactive effector T cells. *In vitro* assays have shown that CD4^{+}CD25^{+} Tregs are capable of suppressing polyclonal CD4^{+}CD25^{-} T cell proliferation and cytokine production, especially IL-2, in a dose-dependent man-

ner.[5-7] CD4^{+}CD25^{+} Tregs have also been shown to directly suppress monocytes and macrophages[8] and DCs[9], thereby affecting both innate and adaptive immune responses. Both CD4^{+}CD25^{+} Treg anergy and their suppressive function can be overcome by the addition of exogenous IL-2 or anti-CD28 antibody.[5-7]

Mechanisms of suppression and phenotypic identification

Suppression by CD4^{+}CD25^{+} Tregs is thought to be mediated by a cytokine-independent, cell contact–dependent mechanism that requires activation of the Treg via the TCR.[5,6] Although these cells require antigen-specific activation, they are generally able to suppress T cell responses through bystander suppression once activated. The exact factors which are involved in suppression have not been fully elucidated and the mechanism by which suppression is achieved is controversial. CD4^{+}CD25^{+} Tregs have been found to express IL-10, IL-4[5,10], TNF-α[5,10] and TGF-β[10] mRNA, but these cytokines were not detectable in anti-CD3-stimulated CD4^{+}CD25^{+} Treg culture supernatants.[5,6] Other studies have reported that CD4^{+}CD25^{+} Tregs do produce IL-10[11-13], IL-4[12] and TGF-β.[13,14] However, there is evidence which suggests that these cytokines do not play a role in CD4^{+}CD25^{+} Treg-mediated suppression as the addition of neutralising anti-IL-10, anti-IL-4 and anti-TGF- α antibodies to *in vitro* mixed cultures of CD4^{+}CD25^{-} and CD4^{+}CD25^{+} Tregs failed to reverse suppression.[5,6] It is widely agreed that CD4^{+}CD25^{+} Tregs do not produce IL-2.

Cytotoxic T lymphocyte-associated protein 4
As the mechanism by which CD4^{+}CD25^{+} Tregs exert their effect is thought to be cell-contact dependent the presence of certain surface markers may be important in both the function and phenotypic characterisation of these cells. CD4^{+}CD25^{+} Tregs express an array of surface molecules; however, the majority of these are not limited to CD4^{+}CD25^{+} Tregs. For example, CD25 and cytotoxic T lymphocyte-associated protein 4 (CTLA-4) are both constitutively expressed on CD4^{+}CD25^{+} Tregs but are also up-regulated on CD4^{+} T cells after activation. CTLA-4 binds to CD80/CD86 ligands on APC and transduces a negative signal that results in down-regulation of T cell activation. The role of CTLA-4 in CD4^{+}CD25^{+} Treg suppression is controversial. Some studies suggest that CTLA-4 is required for

suppressor function[15,16], whereas others have reported that it is not.[5] Interestingly, there are studies which report a genetic linkage between CTLA-4 and various autoimmune diseases, such as type 1 diabetes[17,18] systemic lupus erythematosus (SLE)[19], and Graves' Disease.[19,20] Genetic studies investigating the link between CTLA-4 polymorphisms and RA show conflicting results. However, CTLA-4 polymorphisms may play a role in RA susceptibility in the Asian population, but not the European population.[21]

Tumour necrosis factor receptor family – GITR and OX40
CD4+CD25+ Tregs also express high levels of the glucocorticoid-induced tumour necrosis factor receptor family-related protein (GITR). The ligand for GITR, GITR ligand (GITR-L), is expressed on APC and ligation of GITR to its ligand results in a co-stimulatory effect, enhancing CD4+ T cell proliferation. CD4+CD25+ Treg suppressor function has been shown to be abrogated by the addition of agonisitic anti-GITR antibodies.[22,23] However, it has been shown that GITR engagement on CD4+CD25- effector T cells, not CD4+CD25+ Tregs is responsible for the abrogation of suppression.[24] It should be noted that, like CD25 and CTLA-4, GITR is not CD4+CD25+ Treg-specific and is up-regulated on CD4+ T cells upon activation. Another member of the tumour necrosis factor receptor family with co-stimulatory properties, OX40 (CD134), has been implicated in murine Treg function.[25] However, like GITR this molecule is not CD4+CD25+ Treg-specific and is expressed on naïve T cells as well as being transiently expressed on activated T cells.

Lymphocyte activation gene-3
Lymphocyte activation gene-3 (LAG-3) is another surface molecule which has been proposed as a CD4+CD25+ Treg-specific marker and has been implicated in CD4+CD25+ Treg function.[26] LAG-3 is a CD4-related molecule that binds MHC class II. LAG-3 has been reported to be selectively up-regulated on CD4+CD25+ Tregs after activation and anti--LAG-3 antibodies inhibited suppression by CD4+CD25+ Tregs.[26] Interestingly, ectopic expression of LAG-3 on CD4+ T cells depleted of CD25+ T cells conferred regulatory activity to these cells.[26] However, it should be noted that the above study was carried out in a murine model and LAG-3 was detected not only in naturally-occurring CD4+CD25+ Tregs but also in an induced Treg sub-

set. Further work needs to be carried out to ascertain whether LAG-3 expression is relevant in human Treg biology and which Tregs subsets express this marker.

Neuropilin-1
Neuropilin-1 (Nrp1) is a receptor which is involved in axon guidance, angiogenesis, cell survival, migration and invasion. It has been reported that Nrp1 is constitutively expressed on the surface of CD4+CD25+ Tregs independently of their activation status, whereas Nrp1 expression is down-regulated in naïve CD4+CD25- T cells after activation.[27] Nrp1 is co-regulated with Foxp3 (see below) and CD4+ Nrp1^{high} T cells are able to suppress CD4+CD25- T cells. Therefore, Nrp1 may be considered a candidate surface marker of CD4+CD25+ Tregs.

Foxp3
Foxp3 is a member of the *forkhead box* family of transcription factors. Over the past few years work has demonstrated that Foxp3 is critical for the development and function of CD4+CD25+ Tregs and therefore this intracellular marker appears to be of great significance for both the identification and function of CD4+CD25+ Tregs. Brunkow *et al.* first identified Foxp3 as a gene which is mutated in the mouse strain, *scurfy*.[28] They reported that this mutation was responsible for the presence of the fatal autoimmune lymphoproliferative disease found in *scurfy* mice. A similar disease present in humans, called immune dysregulation, polyendocrinopathy, enteropathy X-linked syndrome (IPEX) has also been mapped to a number of different mutations in the human Foxp3 gene.[29,30] Further work revealed that Foxp3 was associated with CD4+CD25+ Tregs and was critical for both their development and function.[31-33]

In mice Foxp3 expression is limited to CD4+ CD25+ Tregs and unlike CD25 and other suggested Treg markers is not induced in CD4+CD25- effector cells upon activation.[31-33] However, Walker *et al.* demonstrated that in humans Foxp3 expression is induced in stimulated CD4+CD25- effector cells.[34] Expression of Foxp3 in these activated cells correlated with suppressive function. In agreement with this, a recent study has reported that activation-induced Foxp3 expression in human T cells leads to acquisition of a regulatory phenotype: these cells are able to suppress *in vitro* proliferation of autologous CD4+CD25- T cells.[35] In contrast to these observations, Wang *et al.* reported that Foxp3 is

transiently expressed in activated CD4+ T cells.[36] They demonstrated that induced Foxp3 expression leads to hyporesponsiveness in these cells but does not lead to acquisition of a regulatory phenotype. Interestingly, overexpression and ectopic expression of Foxp3 in non-regulatory T cells renders them suppressive.[31-33] The suppressive function of these cells is independent of CD25 expression, indicating that, at least in mice, CD25 is not required for regulatory activity.

Foxp3 has been implicated in the transcriptional regulation of cytokine genes and cell surface molecules. Two recent studies have identified Foxp3 binding regions in a number of genes.[37,38] Another recent paper has shown that Foxp3 expression in non-regulatory T cells leads to repression of genes, such as *IL-2* and *IFN-γ*, and induction of genes, such as *CD25*, *GITR*, and *CTLA-4*.[39] Interestingly, TGF-β has been shown to induce Foxp3 in CD4+CD25- T cells resulting in T cells with suppressive activity.[40-42] The latter study has shown that IL-2 is essential for this TGF-β-induced effect.

Therefore, Foxp3 is a master regulator in the development of Tregs and may programme the conversion of non-regulatory cells to Tregs possessing suppressive activity. However, Foxp3 may not be a specific marker for naturally-occurring CD4+CD25+ Tregs and caution should be used when interpreting human Foxp3 studies.

CD27 and CD127
Recently, two further surface molecules have been identified which could aid in the identification of CD4+CD25+ Tregs. The first was identified in T cells from the synovial fluid of juvenile idiopathic arthritis (JIA) patients.[43] It was found that CD27 could be used in conjunction with CD25 to identify Foxp3+ Treg cells: CD4+CD25+CD27+ expressing cells were found to express high amounts of Foxp3, did not produce IL-2, IFN-γ or TNF and suppressed T cell proliferation; whereas CD4+CD25+CD27- cells expressed low amounts of Foxp3, produced effector cytokines and did not suppress T cell proliferation. However, a more recent paper has found that CD27 may not be confined to Tregs and as such cannot be used to reliably identify naturally-occurring CD4+CD25+ Tregs.[44] The second recent marker to be identified is CD127 (IL-7 receptor).[45,46] It was found that CD127 expression is inversely correlated with Foxp3 and suppressive function in human CD4+ Treg cells. CD127 could prove useful in the search for a biomarker for the identification of human

Tregs if it is used in conjunction with other markers.

Type-1 T regulatory cells

Type-1 T regulatory (Tr1) cells are another distinct subset of regulatory T cells (reviewed by Roncarolo *et al.*, 2006)[47]. Tr1 cells are not produced in the thymus but are induced in the periphery by antigen stimulation via an IL-10-dependent process. It has been demonstrated that Tr1 cells can also be induced by immature and tolerogenic DCs.[48] Unlike naturally-occurring CD4+CD25+ Tregs, Tr1 cells do not express high levels of CD25 or Foxp3. They produce high levels of IL-10 and TGF-β, which are thought to play a role in their antigen-specific regulatory activity. Furthermore, it has been shown that Tr1 cells can modulate immune responses *in vivo* in autoimmunity, transplantation, and chronic inflammatory diseases.

T helper 3 cells

T helper 3 (Th3) cells are another subset of induced regulatory T cells which are important in mucosal immunity (reviewed by Faria and Weiner, 2006)[49]. Th3 cells are primarily induced after ingestion of foreign antigen via the oral route (oral tolerance). High levels of TGF-β in the gut help promote the differentiation of naïve T cells into Th3 cells. Like naturally-occurring CD4+CD25+ Tregs, they express CTLA-4 on their surface and CD25 and Foxp3 expression is up-regulated after restimulation. However, the main mechanism of suppression is through the production of TGF-β. Th3 cells are triggered in an antigen-specific manner, but they can suppress in an antigen-non-specific manner through bystander suppression. In addition, they have been shown to suppress systemic inflammatory autoimmune responses.

Other types of Tregs

Other cells which have regulatory functions include subpopulations of CD4+ and CD8+ T cells: γδ T cells have been implicated in tissue immunoregulation[50]; and CD8+CD28- Tregs play a role in suppression of autoimmunity.[51] Natural killer T (NKT) cells also have suppressive activity.[52] They produ-

ce regulatory cytokines, including IL-10, TGF-β, and can affect the Th1/Th2 balance. B cells subsets have also been implicated in immunoregulation.[53]

The remainder of this review will focus on naturally-occurring CD4+CD25+ Tregs and their role in autoimmune disease, in particular RA.

Evidence that CD4+CD25+ Tregs play a role in autoimmune disease

Animal models

Studies into the cause of autoimmune disease have demonstrated that it can be induced in normal animals by the elimination of particular subpopulations of CD4+ T cells and that the reconstitution of the eliminated population results in the prevention of that autoimmune disease. In an early study it was demonstrated that spleen cells, from which certain T cell subsets had been removed, could cause the development of organ-specific autoimmune diseases, such as oophoritis, gastritis, thyroiditis, and orchitis, when transferred to nude mice.[54] A T cell subpopulation, Thy-1+Lyt-1, 2, 3- cells, were shown to be responsible for the autoimmune disease induction. A different T cell subpopulation, Lyt-1+, 2, 3- cells, were shown to have suppressive activity and were able to inhibit disease induction when co-transferred with the Lyt-1, 2, 3- cells. Another early study using an autoimmune thyroiditis murine model reported that Lyt-1[dull] L3T4+ T cells were responsible for the induction of thyroiditis and Lyt-1^{bright} regulatory T cells were able to inhibit this induction.[55] In another murine study it was reported that CD4+ neonatal splenocytes and CD4+CD8- adult thymocytes were required for induction of autoimmune oophoritis and gastritis. Adult spleen cells did not elicit disease, but they prevented disease when co-transferred with neonatal spleen cells.[56]

Rat models have also been used to investigate the causes of autoimmune disease. Induction of autoimmune diabetes, insulitis, and thyroiditis in athymic rats by major histocompatibility complex compatible spleen cells was facilitated by prior *in vivo* depletion of RT6.1+ regulatory T cells.[57] In another rat model it was reported that CD45RB^{high}CD4+ T cells were responsible for the development of a severe wasting disease with inflammatory infiltrates in liver, lung, stomach, thyroid, and pancreas in athymic rats.[58] In contrast, CD45RB^{low}CD4+ T cells did not induce disease. Animals given unfractionated CD4+ T cells, containing approximately two-thirds CD45RB^{high} and one-third CD45RB^{low}, were protected from the wasting disease, and the incidence of organ-specific inflammation was reduced, suggesting that the CD45RB^{low}CD4+ T cells were able to inhibit the pathogenic CD45RB^{high}-CD4+ T cells.

Sakaguchi *et al.* were one of the first groups to demonstrate that CD4+CD25+ Tregs have a clear role in regulating autoimmune disease.[4] They reported that the elimination of CD4+CD25+ T cells by use of an anti-CD25 monoclonal antibody resulted in a wide range of organ-specific and systemic autoimmune diseases, such as thyroiditis and gastritis, as well as graft-versus-host disease-like wasting disease in normal mice. Reconstitution of mice with CD4+CD25+ T cells prevented the development of these autoimmune diseases. In a different study it was demonstrated that transfer of CD4+CD25+ Tregs from normal mice, which had had their anergic state and suppressive function abrogated by addition of high levels of IL-2 or anti--CD28 antibody, produced a variety of autoimmune diseases in syngeneic athymic nude mice.[6] Similar murine studies support the observation that CD4+CD25+ T cells can prevent the development of multiple-organ autoimmune disease.[10,59,60]

In terms of specific autoimmune disease CD4+CD25+ Tregs have been reported to play an important role in the regulation of diabetes. Both B7.1 (CD80)/B7.2 (CD86)-deficient and CD28-deficient NOD mice, which have decreased numbers of CD4+CD25+ T cells, develop spontaneous type 1 diabetes.[61] Transfer of the CD4+CD25+ Treg subset from control NOD mice into CD28-deficient mice can delay and even prevent diabetes from developing. Similar finding were made in a rat model of autoimmune diabetes.[62]

As already mentioned *scurfy* mice develop fatal autoimmune lymphoproliferative disease.[63] *Scurfy* mice lack CD4+CD25+ Tregs due to mutations in the Foxp3 gene.[28] Fontenot *et al.* reported that the adoptive transfer of CD4+CD25+ Tregs into neonatal *scurfy* mice prevented the development of the lymphoproliferative disease.[31]

In all of these animal models the development of disease involves manipulation of T cell homeostasis and is inhibited by a subpopulation of normal CD4+ T cells, mainly the CD4+CD25+ Treg population.

Human studies

Studies of CD4+CD25+ Tregs and their role in hu-

man autoimmune disease are less abundant than animal model studies. Human studies are limited to investigating frequencies and suppressive functions of CD4+CD25+ Tregs in patient samples. Unlike animal models, it is not currently possible to deplete or reconstitute Treg populations in human patients.

As already mentioned a disease, IPEX, similar to that found in *scurfy* mice, has been discovered in humans. IPEX is a rare, aggressive and fatal disease found in male children. It usually causes inflammatory bowel disease, neonatal diabetes, thyroiditis, and severe infection. A number of different mutations in the human Foxp3 gene are responsible for the development of this disease.[29,30] A recent study has shown that the number and phenotype of CD4+CD25+ T cells from IPEX patients are comparable to those of normal donors.[64] However, functional analysis revealed that CD4+CD25+ Tregs may either be normally suppressive or impaired to different degrees depending on: (a) the genotype of the target cells; (b) the type of *Foxp3* mutation; and (c) the strength of TCR activation. The authors concluded that *Foxp3* mutations in IPEX patients result in a range of biological abnormalities, leading to defective CD4+CD25+ Tregs and effector T cells, but not necessarily to a lack of differentiation of CD4+CD25+ Tregs.

Multiple sclerosis (MS) is an autoimmune disease thought to be mediated by T cells recognizing myelin protein peptides. A study by Viglietta *et al.* has shown that, although there is no difference in the frequency of CD4+CD25+ T cells in MS patients compared to healthy controls, there is a significant decrease in the suppressive activity of CD4+CD25+ Tregs from MS patients.[65] They demonstrated, through co-mixing experiments, that the decrease in regulatory activity was due to a defect in the CD4+CD25+ Treg population rather than the responder CD4+CD25- T cells being refractory to suppression.

Autoimmune polyglandular syndromes (APS) are a group of disorders in which multiple endocrine glands are damaged by an autoimmune mechanism. There are two types of APS: APS type I is caused by loss of central tolerance; whereas little was know of the etiology of APS type II (APS-II). It had previously been reported that in several murine models, depletion of CD4+CD25+ Tregs caused a syndrome resembling human APS-II with multiple endocrinopathies. Kriegel *et al.* therefore hypothesized that loss of active suppression in the periphery could be a major cause of APS-II.[66] They demonstrated that there was no difference in the frequency or surface phenotype or apoptosis rates of CD4+CD25+ Tregs in APS-II compared to controls. Like MS, it was shown that CD4+CD25+ Tregs from APS-II patients were defective in their suppressive activity and that this defect was persistent and not due to responder cell resistance.

Myasthenia gravis (MG) is an autoimmune disease which is characterised by fluctuating, sometimes fatal, muscle weakness. The thymus is believed to be the initiation site of pathogenesis. It has been reported that MG patients have normal numbers of CD4+CD25+ thymocytes but these Tregs have a severe functional defect in their regulatory activity together with a decreased expression of Foxp3.[67]

Type 1 diabetes is a T cell–mediated autoimmune disease. It has been reported that although levels of CD4+CD25+ T cells are normal in patients with recent-onset adult type 1 diabetes, the suppressive activity of the Tregs in this population is reduced compared with control subjects.[68] Interestingly, a higher proportion of the CD4+CD25+ T cells co-express the early activation marker CD69 and intracellular CTLA-4.

Together, the above studies have reported that in MS, APS-II, MG and type 1 diabetes there is no difference in the frequency of CD4+CD25+ Tregs in patient groups as compared to normal controls. The differences arise in the suppressive activity of CD4+CD25+ Tregs: the CD4+CD25+ Tregs from each patient group had reduced suppressive activity when compared to the normal control groups. Other studies have reported a decrease in CD4+CD25+ Treg frequency in human autoimmune disease as compared to normal controls: autoimmune lymphoproliferative syndrome[69]; SLE[70,71]; DiGeorge syndrome[72]; and Kawasaki disease.[73] The Kawasaki disease patients also had reduced mRNA expression levels of Foxp3, GITR and CTLA-4.[73] It has recently been reported that CD4+CD25+ Tregs from active SLE patients have decreased suppressive activity and reduced Foxp3 mRNA and protein levels as compared with normal controls and patients with inactive SLE.[74] There are also conflicting reports as to the frequencies of CD4+CD25+ Tregs in autoimmune disease. The above mentioned study on type 1 diabetes reported normal levels of CD4+CD25+ Tregs, whereas a different study reported that CD4+CD25+ Treg frequencies were lower in both newly diagnosed and

long-term type 1 diabetes patients as compared to normal controls.[75]

CD4+CD25+ Tregs and rheumatoid arthritis

RA is a chronic inflammatory autoimmune disease affecting about 0.8% of the UK adult population. Progression can be very rapid, and is characterised by swelling of the synovium and damage of the cartilage and bone around the joints, ultimately leading to joint destruction. Any joint may be affected but RA more commonly starts in the hands, feet and wrists. The exact etiology and development of RA is not fully elucidated, but the presence of inflammatory cytokines is thought to play a key role in the induction and maintenance of this disease. A study investigating the natural immune response against a candidate autoantigen in RA, human cartilage glycoprotein-39 (HC gp-39), has found that in normal controls the response is biased towards a regulatory (IL-10) phenotype, whereas in RA patients it is biased towards a pro-inflammatory Th1 (IFN-γ) phenotype.[76] This suggests that in a normal state the presence of HC gp-39-specific T cells may have an inhibitory effect on inflammatory responses in areas where HC gp-39 is present. In RA there appears to be a breakdown of tolerance, which causes the response to shift from a regulatory one to a pro-inflammatory one.

Animal models

There are two frequently studied murine models of RA: collagen-induced arthritis (CIA)[77,78] and antigen-induced arthritis (AIA)[79]. CIA is induced through immunisation of mice with bovine type II collagen (CII) emulsified in Freund's complete adjuvant. This leads to production of CII-specific antibodies, which are necessary for disease induction. Therefore, CIA is an antibody-mediated, B cell-dependent autoimmune disease. CIA is not identical to RA, but they do share many key features, such as synovitis, erosions of both bone and cartilage, and class II major histocompatibility complex–linked susceptibility. CIA is mediated by both cellular and humoral immune responses, whereas AIA is dependent on cell-mediated immunity, with a minor contribution of humoral immunity. AIA is induced by pre-immunisation of mice with methylated bovine serum albumin (mBSA) in complete Freund's adjuvant. The knee joint is then intra-articularly injected after 21 days with mBSA in saline. This results in a T cell-dependent disease in which an initial acute inflammatory reaction is followed by chronic disease, characterised by synovial hyperplasia, infiltration of mononuclear cells, and cartilage and bone destruction. The histopathological changes are similar to those that occur in RA. The AIA model results in 100% incidence of arthritis and has a major advantage over CIA in that the time point of induction of arthritis is known.

Morgan *et al.* used the CIA murine model to demonstrate that depletion of CD4+CD25+ Tregs prior to CII immunisation greatly increased both the severity and incidence of the disease and was associated with an increase in CII-specific antibodies.[80] Adoptively transferring CD4+CD25+ Tregs into CD25+-depleted mice reversed severity of the disease. In a later paper, the same group reported that CD4+CD25+ Tregs can be used therapeutically in CIA.[81] They adoptively transferred CD4+CD25+ Tregs into mice during the early stage of CIA and showed that disease progression was markedly slowed despite a lack of reduction in systemic CII--specific T and B cell responses. CD4+CD25+ Tregs were traced to the synovial tissue in affected joints, indicating that these cells may modulate inflammation locally in the joint. CD4+CD25+ Tregs and their role in AIA have also been investigated.[82] Depletion of CD25+ cells in immunised animals before arthritis induction led to an exacerbation of disease. Transfer of CD4+CD25+ Tregs into immunised mice at the time of induction of AIA decreased the severity of disease but was not able to cure established arthritis. Again, like the previous study, CD4+CD25+ Tregs were found to accumulate in the inflamed joint. It would appear that in murine models of RA CD4+CD25+ Tregs can migrate to sites of inflammation and play an important role in preventing the induction of disease, but are unable to cure established disease.

Human studies

Frequencies of CD4+CD25+ Tregs in the periphery and synovial fluid

A number of studies have examined CD4+CD25+ Tregs in human patients with RA and other types of inflammatory arthritis. As the two previously mentioned murine studies have shown that CD4+CD25+ Tregs can migrate to sites of inflammation it is important to investigate CD4+CD25+ Tregs from inflamed joints, as well as the periphery. Several studies have reported an enrichment of CD4+CD25+ Tregs in the synovial fluid (SF) of patients with RA and other types of inflammatory

arthritis.[83-89] This enrichment was demonstrated to be irrespective of disease duration, severity or drug treatment.[84,85] The SF CD4+CD25+ Tregs did display suppressive activity in both terms of proliferation and cytokine production.[83-89]

There is controversy surrounding the frequency of CD4+CD25+ Tregs in the peripheral circulation of inflammatory arthritis patients compared to normal controls: some studies report normal numbers;[83,86,87,90] some report an increase[88]; and others report a decrease.[84,89,91] Some of this variability may be explained by differences in disease stage and therapy. For example it has been shown that early active RA patients who had received no disease--modifying therapy had a smaller proportion of CD4+CD25+ Tregs in the peripheral blood than controls, whereas stable, well-controlled RA patients who were receiving therapy had similar numbers of CD4+CD25+ Tregs to controls.[89] There are also differences in the phenotypic definition of naturally-occurring Tregs in the above mentioned studies. Some studies used CD4+CD25+ Tregs, which included total CD25+ T cells, whereas some used only the CD4+CD25bright Tregs.

CD4+CD25+ Treg frequencies have also been found to be altered in JIA.[92] JIA and RA, although not the same disease, do have similar mechanisms of disease pathogenesis and clinical presentation. Persistent oligoarticular JIA (pers-OA JIA) is a subtype of JIA with a relatively benign, self-remitting course, while extended oligoarticular JIA (ext-OA JIA) is a subtype with a much less favorable prognosis. It has been shown that patients with pers-OA JIA had a higher frequency of CD4+CD25bright Tregs in the peripheral blood than ext-OA JIA patients. The numbers of SF CD4+CD25bright Tregs were equal in both groups and these cells displayed suppressive activity. Interestingly, the pers-OA JIA patients displayed a higher frequency of SF CD4+CD25int T cells, which were found to express Foxp3 mRNA and also displayed suppressive activity. It would therefore appear that there are more CD4+CD25+ Foxp3+ Tregs in the SF of pers-OA JIA patients. Therefore, CD4+CD25+ Tregs may play a role in determining disease outcome by directing the disease course to either a favorable or unfavorable one. The less favorable ext-OA JIA may be due to CD4+CD25+ Tregs being unable to migrate to or expand at sites of inflammation.

Characterisation of synovial fluid CD4+CD25+ Tregs
In RA and other inflammatory arthritis there ap-

pears to be two compartments of CD4+CD25+ Tregs: those in the peripheral blood and those at the sites of inflammation, usually in the SF. One important question is whether these two populations of CD4+CD25+ Tregs are phenotypically and functionally similar. It has been reported that the majority of CD4+CD25+ Tregs from inflamed joints are activated CD45RO+ memory cells expressing a variety of activation markers, such as HLA-DR and CD71[83] and are phenotypically dissimilar to peripheral blood CD4+CD25+ Tregs: SF CD4+CD25+ Tregs display a higher expression of CTLA-4 [83,87,88,92], GITR, CD69[87;88;92], OX40[87,88], HLA-DR[88,92], CD25[87], Foxp3 (mRNA)[92], and CD71 with a lower expression of CD62L.[83] These studies suggest that the CD4+CD25+ Tregs may undergo maturation in the joint. In correlation with this activated, mature phenotype, the SF CD4+CD25+ Tregs show increased regulatory activity compared with peripheral blood CD4+CD25+ Tregs.[43,88,92]

Are CD4+CD25+ Tregs defective in RA?
Another very important question is why, when CD4+CD25+ Tregs accumulate in joint and show enhanced suppressive activity, is RA able to progress? One study has reported that the CD4+CD25- responder T cells in the SF are activated and as such are less susceptible to suppression by CD4+CD25+ Tregs than their resting counterparts in the peripheral blood.[88] Importantly, this decreased susceptibility to suppression was observed when CD4+CD25+ Tregs from either peripheral blood or SF were used. Therefore, although CD4+CD25+ Tregs can migrate to inflamed areas and do possess suppressive activity, their ability to suppress arthritis may be limited by local responder T cells having a reduced susceptibility to regulation. It should be noted that a different study has also investigated the ability of CD4+CD25+ Tregs to suppress both peripheral blood and SF responder cells and has shown the degree of suppression was similar in one patient but actually lower for the peripheral blood responders in another patient.[83] These differences could be explained by differences in separation of the Treg populations: van Amelsfort *et al.* used MACS separation to obtain CD4+CD25+ Tregs, which included total CD25+ T cells, whereas Cao *et al.* used a FACS-sort to obtain only the CD4+CD25bright Tregs. The van Amelsfort study is in keeping with a previous study which reported that strongly activated CD4+ responder T cells are resistant to regulation by CD4+CD25+ Tregs, while weak-

ly stimulated CD4^{+} cells are sensitive to suppression.[93] Therefore, resistance to suppression is dependent upon the strength and duration of the stimulus: the stronger the TCR signal, the quicker and more fully the responder cells become refractory to suppression.

Ehrenstein *et al.* have reported that CD4^{+}CD25^{+} Tregs derived from peripheral blood of patients with active RA are defective in their ability to suppress pro-inflammatory cytokine production, but not proliferation.[90] As the presence of pro-inflammatory cytokines is very important in the development of RA this observation is of great significance. However, it should be noted that this finding differs from other studies using cells from SF.[84-86,88]

Another explanation as to why RA is able to progress is the cytokine profile in the joint and the effect this has on CD4^{+}CD25^{+} Tregs. The cytokine milieu of the RA joint includes many pro-inflammatory cytokines, such as TNF-α, IL-1, IL-6, GM-CSF, anti-inflammatory cytokines, such as IL-10 and TGF-β, and cytokine inhibitors such as IL-1RA and soluble TNF-R (reviewed in McInnes and Schett, 2007).[94] It would appear that there is imbalance between pro-inflammatory and anti-inflammatory cytokines in the joint. Therefore, although IL-10, for example, is expressed in the joint it is not present in a high enough concentration to mediate counter-regulatory activity against the dominant pro-inflammatory cytokine milieu.

It has been reported that the SF cytokine profile in early RA is transient and distinctly different from established RA.[95] The levels of a range of T cell, macrophage and stromal cell-related cytokines, including IL-2, IL-4, IL-13, IL-17, IL-15, bFGF and EGF, are significantly increased in early RA patients. Therefore, the disease stage may have an influence on the pro-inflammatory/anti-inflammatory cytokine balance and play an important role in disease development to persistent RA. For example, as has already been mentioned, both CD4^{+}CD25^{+} Treg anergy and suppressive function can be overcome by the addition of high levels of IL-2.[5-7] This may play a significant role in the inhibition of suppression by CD4^{+}CD25^{+} Tregs in early stages of the disease, which would allow responder cell activation and creation of a pro-inflammatory environment resulting in progression to established RA and joint destruction.

A recent study by van Amelsfort *et al.* has demonstrated that activated monocytes from the SF of RA patients produce both IL-7 and TNF-α and these two cytokines abrogate the suppressive activity of CD4^{+}CD25^{+} Tregs.[96] It was suggested that the effect of IL-7 may be to render the responder T cells resistant to suppression, since the CD4^{+}CD25^{+} Tregs have very low expression of the IL-7 receptor (CD127). IL-6 is also found in high levels in SF from the joints of patients with active RA.[97] Pasare and Medzhitov have demonstrated that IL-6, secreted by DCs upon TLR stimulation, has an effect on responder T cells and renders them resistant to CD4^{+}CD25^{+} Treg suppression.[98] In the above mentioned study by van Amelsfort *et al.* IL-6 was reported to have no effect on the suppressive activity of CD4^{+}CD25^{+} Tregs.[96] This may be explained by the fact that Pasare and Medzhitov have demonstrated that IL-6 alone is not sufficient for the inhibition of suppression and another unknown TLR-induced factor is also needed.[98] This unknown, TLR-induced factor may be missing from the SF in RA joints. Therefore, the interaction between CD4^{+} CD25^{+} Tregs and activated monocytes in the joint might lead to diminished suppressive activity of CD4^{+}CD25^{+} Treg cells, which contributes to the chronic inflammatory state that is RA.

The balance of evidence suggests that CD4^{+} CD25^{+} Tregs *per se* may not have defective suppressive activity in RA. The defect in peripheral tolerance may reflect the cytokine environment in the joint, which may render responder T cells resistant to suppression and/or have a direct effect on CD4^{+}CD25^{+} Tregs by inhibiting their suppressive activity.

Therapeutic potential of CD4+CD25+ Tregs in RA

Anti-TNF-α treatment and CD4+CD25+ Tregs

The animal models of autoimmune disease discussed above have demonstrated that adoptive transfer of CD4^{+}CD25^{+} Tregs can prevent or even cure certain autoimmune diseases. One very important question is: can CD4^{+}CD25^{+} Tregs be used or manipulated as a therapy for use in RA? Present RA treatments and their effect on CD4^{+}CD25^{+} Tregs have been studied. In the previously mentioned study by Ehrenstein *et al.* in which it was reported that CD4^{+}CD25^{+} Tregs from RA patients cannot suppress pro-inflammatory cytokine production, it was demonstrated that treatment with anti-TNF-α (infliximab) restored the capacity of CD4^{+}CD25^{+} Tregs to inhibit cytokine produc-

tion.[90] The clinical response to infliximab, but not conventional methotrexate treatment, correlated with an increased frequency of peripheral blood CD4+CD25+ Tregs, which in turn correlated with a reduction in C-reactive protein (a measure of disease activity). It was reported that TNF-α does not have a direct effect on CD4+CD25+ Tregs as exposure to a gradient of TNF-α concentration did not affect viability or function of these cells. However, in opposition to this finding it has been reported that TNF inhibits the suppressive activity and downregulates Foxp3 expression in both naturally-occurring CD4+CD25+ Tregs and TGF-β 1–induced CD4+CD25+ Tregs.[99] Treatment with infliximab was shown to increase Foxp3 expression by CD4+CD25+ Tregs and restore their suppressive activity. It has also been reported that CD4+CD25+ Tregs are apoptosis-prone[3,100] and that spontaneous apoptosis of CD4+CD25+ Tregs from active RA patients was increased, whereas the absolute number of peripheral blood CD4+CD25+ Tregs was reduced when compared to controls.[91] After infliximab treatment spontaneous apoptosis was reduced in the RA patients and CD4+CD25+ Treg numbers were equivalent to those of normal controls. The alteration and reversal in both spontaneous apoptosis and numbers of CD4+CD25+ Tregs was found to correlate with RA disease activity, as measured by C-reactive protein. This would seem to suggest that TNF-α can affect CD4+CD25+ Tregs and infliximab treatment reverses this. The Ehrenstein group has since gone on to demonstrate that infliximab therapy gives rise to a CD4+CD25hiFoxP3+ Treg population, which mediates suppression via TGF-β and IL-10.[101] *In vitro* studies have shown that this Treg population was induced from CD4+CD25- T cells and the process was dependent on TGF-β, suggesting that these Tregs may resemble Th3 cells. These induced Tregs can be distinguished from naturally-occurring CD4+CD25+ Tregs by their lack of CD62L expression. It was reported that the induced CD62L- Tregs have very potent suppressor activity, but the naturally-occurring CD62L+ T regs remain defective in infliximab-treated patients. Therefore, the induction of CD62L- Tregs could be used as a viable therapeutic tool for the restoration of tolerance in RA patients.

Adalimumab is another anti-TNF-α therapy used in the treatment of RA. Infliximab is a chimeric anti-TNF-α monoclonal antibody, whereas adalimumab is a fully human anti-TNF-α antibody. They are both able to block the interaction of TNF-α with its receptors; however their efficacy and side effects are not the same. Adalimumab has been found to increase numbers and improve the function of CD4+CD25+ Tregs in the peripheral blood of RA patients at day 15 of treatment.[102] However, after 6 months of treatment CD4+CD25+ Treg numbers had fallen but remained higher than pretreatment levels. In opposition to this another report studying the effect of adalimumab on CD4+CD25+ Tregs from RA patients demonstrated that the treatment had no effect on these cells.[103] Collectively, these studies suggest that TNF-α blockade, in addition to its anti-inflammatory effects, may play an important regulatory role in the amelioration of RA. This regulation may be through the induction of a CD62L- Treg population. It is of note that withdrawal of anti-TNF-α therapy from RA patients generally causes them to relapse[104], although a small study performed in patients with early RA suggested regulatory effects that lasted beyond the duration of treatment.[105] Therefore, patients who respond to therapy may require on-going treatment to maintain their response in the long term. It would appear that, at least in established disease, anti-TNF-α therapy provides immunosuppression rather than immunomodulation and, while this treatment helps control RA progression, it does not provide a cure for RA. Further investigation is required of its potential effects in patients with RA of recent onset.

Autologous stem cell transplantation and CD4+CD25+ Tregs

Autologous stem cell transplantation (ASCT) has been used in children to treat JIA with some promising results: 53% of the children achieved complete drug-free remission, some of whom had previously failed treatment with all licensed therapies, including anti-TNF-α; 18% showed a partial response; and 21% were resistant to the therapy.[106,107] In a recent study it was shown that the good outcome from ASCT in JIA may be linked to the restoration of immunologic self-tolerance.[108] There were reduced numbers of CD4+CD25+ Tregs before ASCT as compared to normal controls. After ASCT there was an increased frequency of Foxp3 expressing CD4+CD25+ Tregs with numbers returning to normal levels. It was shown that this recovery was due to a preferential homeostatic expansion of CD4+CD25+ Tregs during the lymphopenic phase of immune-reconstitution. It was also shown that ASCT induces reprogramming of autoimmune T

cells from a pro-inflammatory phenotype (IFN-γ and T-bet) to a tolerant phenotype (IL-10 and GATA-3 high). These data suggest that the restoration of the CD4+CD25+ immune regulatory network and reprogramming of autoreactive T cells is possible in some human autoimmune diseases.

Ex vivo generation of CD4+CD25+ Tregs

If CD4+CD25+ Tregs are to be used as a therapeutic tool it is important that these cells can be generated *ex vivo*. Several studies have demonstrated that human CD4+CD25+ Tregs can be expanded to large numbers when stimulated via CD3 and CD28 in the presence of high doses of IL-2.[109,110] The expanded CD4+CD25+ Tregs remained anergic and retained their suppressive activity. It has been reported that in the NOD murine model of diabetes the ability of expanded CD4+CD25+ Tregs to suppress diabetes in pre-diabetic and diabetic mice *in vivo* was greatly enhanced by using an autoantigen-specific stimulus.[111] Therefore, in contrast to the above mentioned study by Frey *et al.*, in which it was demonstrated that polyclonal CD4+CD25+ Tregs alone appear unable to counteract established and ongoing acute or chronic inflammation[82], expanded antigen-specific CD4+CD25+ Tregs cannot only suppress the development of disease but can also reverse it after disease onset. This provides hope that antigen-specific CD4+CD25+ Tregs can be used as a cellular therapy for autoimmune disease. These Tregs would only be activated in the target organ, thus focusing their effects. The auto-antigens involved in RA, once they are identified, could be used to increase the potency of CD4+CD25+ expanded Tregs. However, administration of large numbers of CD4+CD25+ Tregs should be approached with caution. The presence of such high numbers of regulatory cells may create an environment that favours development of more regulatory cells via infectious tolerance. High numbers of CD4+CD25+ Tregs could then cause non-antigen-specific bystander suppression, leaving the recipient vulnerable to infections or tumours.

In vivo expansion of CD4+CD25+ Tregs

There are practical and technical difficulties associated with the *ex-vivo* expansion of CD4+CD25+ Tregs. Any therapy destined for human use must be developed to a clinical-grade, compatible with good manufacturing practice requirements. Therapies which allow the *in vivo* expansion of CD4+CD25+ Tregs may be a better option. In two separate studies, anti-CD3 monoclonal antibodies were used to treat patients with recent-onset type 1 diabetes mellitus.[112,113] Treatment in both studies maintained or improved insulin production, which reduced the requirement for insulin doses for at least 12 months. No severe side effects occurred. In one of the studies clinical responses were associated with a change in the ratio of CD4+ T cells to CD8+ T cells.[112] This group went on to demonstrate that the increase in CD8+ T cells was due to the induction of a population of CD8+CD25+Foxp3+ Tregs, which were able to regulate autologous, antigen-specific CD4+ T cells in a cell contact–dependent manner.[114] They had previously shown that treatment with anti-CD3 monoclonal antibody also induces CD4+IL-10+ T cells, which may also play a role in tolerance.[115] A murine study has demonstrated that a combination of anti-CD3 monoclonal antibody with intranasal proinsulin can reverse recent-onset diabetes with higher efficacy than anti-CD3 monoclonal antibody alone and lead to the expansion of insulin-specific CD4+ Foxp3+ Tregs producing IL-10, TGF-β, and IL-4.[116] These cells could transfer tolerance to immunocompetent recent-onset diabetic mice and could also suppress autoaggressive CD8 responses. Therefore, anti-CD3 monoclonal antibody therapy could be a promising therapy for RA, especially if used in combination with an immunisation protocol, e.g. oral or intranasal immunisation with autoantigens.

CD28 superagonists are also capable of activating and preferentially expanding Tregs in various experimental animals and have been shown to have therapeutic effects in various models of autoimmunity (reviewed in Beyersdorf et al., 2005).[117] It should be noted, however, that in a recent phase I clinical trial the use of a CD28 superagonist caused a cytokine storm, characterised by a rapid induction of pro-inflammatory cytokines resulting in a systemic inflammatory response.[118] Therefore, the use of CD28 superagonists, while showing promising results in animal models, should be approached with great caution when considered for the treatment of human autoimmune diseases.

New therapies and CD4+CD25+ Tregs

There are several promising new approaches for tolerance induction, which have been studied in humans. The first used a mucosal peptide-specific immunotherapy to induce immunomodulation.[119] It was demonstrated that dnaJP1, a heat

shock protein-derived peptide, induces *in vitro* proliferative and pro-inflammatory T cell responses in a proportion of RA patients. Oral administration of this peptide over a six month treatment period, however, induced immune deviation from a pro-inflammatory (IFN-γ and TNF-α) to a regulatory (IL-4 and IL-10) response. As the peptide was orally administered there was the possibility that T cells may have been clonally deleted, however, this was ruled out. It was found that Foxp3 expression by CD4+CD25+ Tregs was increased suggesting that the treatment caused a shift from a pathogenic T cell phenotype to regulatory one although no clinical correlates were reported in this small study. In a different study, T cell vaccination was used to induce regulatory immune responses in RA patients.[120] Autologous pathological synovial T cells were rendered inactive by irradiation, and used for vaccination. This led to induction of IL-10-producing CD4+Foxp3+ Tregs and CD8+ cytotoxic T cells specific for the T cell vaccine. The observed regulatory immune responses collectively correlated with clinical improvement in treated patients.

Other potential therapies for the treatment of RA have been investigated in murine models. These include the use of immunomodulatory neuropeptides, vasoactive intestinal peptide (VIP)[121], andrenomedullin[122] and urocortin[123], to induce IL-10/ /TGF-β-producing CD4+CD25+Foxp3+ Tregs, which are able to suppress and ameliorate the progression of CIA; the use of the candidate autoantigen, BiP, to induce IL-4-producing Tregs, which are able to suppress established CIA[124]; and the use of immunoregulatory CD8+ T cells to suppress rheumatoid synovitis.[125]

Tolerogenic DCs may be another effective method of inducing Tregs, although not necessarily naturally-occurring Tregs. For example, it has been shown that VIP induces the generation of IL-10-producing tolerogenic DCs with the capacity to generate CD4+ and CD8+ Tregs.[126] The CD4+ Tregs resembled Tr1 cells, i.e. they were anergic and produced IL-10 and TGF-β, whereas the majority of CD8 Tregs were IL-10-producing CD8+CD28- T cells. Both CD4+ and CD8+ Tregs suppressed antigen-specific Th1-mediated responses. This therapy may be more successful in treating RA if the tolerogenic DCs are loaded with a target autoantigen, which when presented to naïve T cells could induce the development of antigen-specific Tregs.

Genetic manipulation of T cells to generate Tregs is also a promising therapeutic tool. It was re-ported to be successful in a murine model of type 1 diabetes.[127] Ectopic expression of Foxp3 by retroviral transduction was shown to confer a suppressor phenotype to naïve CD4+ T cells. Only antigen-specific Foxp3-transduced T cells and not polyclonal Foxp3-transduced T cells were effective in stabilising and reversing established disease.

In summary, there are many avenues for novel therapeutic treatments for RA and other autoimmune diseases involving CD4+CD25+ Tregs. However, more work is required to establish whether CD4+CD25+ Tregs can be used as a therapy for RA to not only decrease the severity and progression of disease but also to cure established disease. As it would appear that the cytokine environment in the joint plays a very important role in the inhibition of peripheral tolerance, any potential therapy for RA may need to take this into account. An appropriate regime may need not only to induce functioning Tregs but also to address the cytokine milieu of the joint.

Overall conclusions

CD4+CD25+ Tregs play a very important role in various autoimmune diseases. A number of animal studies have shown that elimination of CD4+CD25+ Tregs induces autoimmunity and that reconstitution of these cells inhibits it. Therefore, there is great potential to use these cells in a therapeutic regime for the treatment, and possible cure, of autoimmune disease, including RA. However, a number of issues must be addressed before a CD4+ CD25+ Treg therapy can be considered for use in humans: 1) a CD4+CD25+ Treg-specific marker, or a combination of markers, such as Foxp3 and CD127, must be agreed upon and used to ensure the purity and correct identification of CD4+CD25+ Tregs; 2) it must be established that administration or manipulation of CD4+CD25+ Tregs will only regulate specific, localised targets and not cause systemic immunosuppression. This may require the derivation of antigen-specific T-regs, only possible when an autoantigen is clearly defined; 3) when designing a therapeutic regime it may be prudent to use CD4+CD25+ Tregs in combination with another treatment as administration or manipulation of CD4+CD25+ Tregs alone may not be enough to cure established disease.

The *in vivo* induction of Tregs using, for example, anti-CD3 antibodies, or autoantigenic pepti-

des, provides an equally appealing approach which currently appears closer to clinical application.

Correspondence to:
John D. Isaacs
Musculoskeletal Research Group, Institute of Cellular
Medicine, Newcastle University, Newcastle upon Tyne,
United Kingdom
E-mail: j.d.isaac@newcastle.ac.uk

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