THE USE OF REGULATORY T CELLS FOR THE TREATMENT OF ATHEROSCLEROSIS

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ABSTRACT: Natural and adaptive regulatory T cells play an important role in modulating the immune responses which contribute to the development of atherosclerosis in animal models. Naturally occurring Treg, constitutively expressing high levels of CD25 and the transcription factor forkhead box P3 (Foxp3), are generated in the thymus and account for 5-10% of peripheral CD4+ T cells in mice and humans. The CD4+CD25+FoxP3+ Treg have a crucial role in controlling immune homeostasis, maintaining self tolerance and preventing autoimmunity. FoxP3 is critical for the function of the naturally occurring Treg, as spontaneous or experimental Foxp3 gene mutations abolish Treg function, leading to subsequent severe lymphoproliferative disorders. The level of Foxp3 expression and the extent of post-translational Foxp3 acetylation seem to be important factors governing the suppressive activity of the naturally occurring Treg. The natural and the adaptive Tregs might play distinct immunosuppressive roles in vivo. Natural Tregs are constitutively present at all times in healthy individuals and maintain a steady state of immunological tolerance to self antigens. On the other hand, adaptive Tregs seem to be induced as a consequence of antigen exposure during ongoing immune and inflammatory responses, as a negative feed-back for controlling the amplitude and the duration of these immune responses. Whereas natural Tregs are responsible for background systemic immune suppression, adaptive Tregs are induced when and where additional immunosuppression is needed for controlling an ongoing immune response. Another important issue to be clarified is the influence of regulatory T cells on the composition of the atherosclerotic plaques, as the results published so far have been fairly inconsistent and even contradictory. Whereas the decrease in plaque area was paralleled in most of the studies by significant reductions in the numbers of infiltrating T cells and macrophages, others failed to detect any differences. The regulatory T cells appeared to promote collagen accumulation in the lesions in some of the studies, whereas other investigators reported an increase in SMC proliferation and matrix synthesis in mice with defective Treg function. Although these results are difficult to compare, as the experimental settings differ markedly, plaque composition directly correlates with the incidence of acute cardiovascular events and will need to be carefully monitored in future studies. The encouraging results obtained so far have opened the way for designing new therapeutic protocols able to restore the balance between effector and regulatory T cells in atherosclerosis.

Keywords: atherosclerosis, regulatory T cells, FoxP3, CD4+, CD25+

CELULELE T REGULATOARE ÎN TRATAMENTUL ATEROSCLEROZEI

Rezumat: Celulele T naturale și adaptative joacă un rol important în modularea răspunsului imun care contribuie la dezvoltarea aterosclerozei pe modelele experimentale animale. În mod natural celulele T regulatoare exprimă nivelele ridicate ale CD25 și ale factorilor de transcripție FOXP3 sunt generate în timus și reprezintă 5-10% din celulel T CD4+ la șoareci și oameni. Celulele T regulatoare CD4+CD25+FOX3P au un rol crucial în controlul homeostazei imune, menținând toleranța self-ului și prevenind autoimunitatea. FoxP3 este foarte importantă pentru funcționarea celulelor T regulatoare deoarece mutațiile spontane sau provocate ale genei FoxP3 abolesc funcția celulelor T regulatoare ducând...
INTRODUCTION

Regulatory T cells (Treg) are T lymphocytes that have a crucial role in keeping the immune system in check by controlling immune responses against self and non-self antigens. The existence of “suppressor” T cells was first documented in the 1970s, but advancement of research in the field was hampered by the lack of specific molecular markers identifying this particular lymphocyte population. In 1995, a paper by Sakaguchi and colleagues demonstrated the crucial importance of a distinct CD4+ T cell subset expressing high levels of CD25, the alpha subunit of the IL-2 receptor, for prevention of autoimmunity. CD25 subsequently emerged as a reliable marker for identification and isolation of CD4 T cells with regulatory properties. During the past decade, our understanding of regulatory T cells has increased tremendously and new subsets of CD4 and CD8 T cells with regulatory ability have been described. The prospect of harnessing the potent immunosuppressive properties of the regulatory T cells for treatment of diseases with an immune component is extremely attractive and the first clinical trials are currently underway. However, there are still a number of unanswered questions regarding the mechanisms involved in the generation, activation, maintenance, migration and function of these cells that would need to be addressed before Treg can be confidently introduced into the clinical practice. Two main regulatory T cell subsets have been described: the naturally occurring and the adaptive or induced regulatory T cells. These cells differ markedly in their origin, phenotype, antigen specificity and regulatory mechanisms, but both subsets have been shown to efficiently control the development of immune-mediated diseases in experimental models.

NATURALLY OCCURRING REGULATORY T CELLS

Naturally occurring Treg, constitutively expressing high levels of CD25 and the transcription factor forkhead box P3 (Foxp3), are generated in the thymus and account for 5-10% of peripheral CD4+ T cells in mice and humans. The CD4+CD25+Foxp3+ Treg have a crucial role in controlling immune homeostasis, maintaining self-tolerance and preventing autoimmunity. Foxp3 is critical for the function of the naturally occurring Treg, as spontaneous or experimental Foxp3 gene mutations abolish Treg function, leading to subsequent severe lymphoproliferative disorders. The level of Foxp3 expression and the extent of post-translational Foxp3 acetylation seem to be important factors governing the suppressive activity of the naturally occurring Treg.

Similar to conventional T cells, regulatory T cells require activation by TCR engagement in the secondary lymphoid organs. Once activated, Treg can inhibit activation and proliferation of effector T cells in the lymphoid tissue or they can migrate to the inflammation site and suppress their effector functions. Treg can interact directly with the target lymphocytes or they can modulate the activity of APCs (antigen-presenting cells).

As Treg form only transient interactions with the effector T cells in vivo, their influence on the APCs seems to mediate most of their suppressive functions. This can
explain why regulatory T cells specific for a certain antigen are able to suppress effector T cells with multiple antigen specificities, a phenomenon called bystander regulation. Treg employ multiple cell contact-dependent and paracrine mechanisms for their suppressive function in vivo. The soluble mediators, that have been shown to be involved in Treg function are TGFβ, IL-10, IFNγ, and IL-35. In its membrane bound form, TGFβ also plays an important role in Treg mediated cell contact-dependent suppression.

**ADAPTIVE REGULATORY T CELLS**

Adaptive (or induced) regulatory T cells can be generated in vivo and in vitro under certain conditions of antigen exposure and cytokine milieu. CD4+CD25+Foxp3− T cells can upregulate Foxp3 expression and become CD4+CD25+Foxp3+ regulatory T cells by antigenic stimulation in the periphery in the absence of an adequate co-stimulatory signal or in the presence of TGFβ. Tr1 cells are antigen-specific T cells with regulatory properties which secrete high amounts of IL-10 upon activation. These cells are present in humans and unmanipulated animals and can be generated under experimental conditions by antigen exposure in the presence of IL-10. Th3 cells are TGFβ-secreting regulatory T cells identified in Peyer's patches and in the mesenteric lymph nodes following induction of oral tolerance to various antigens. Other minor populations of T cells with regulatory activity include NK T cells, CD8+CD28− T cells and CD4−CD25− double negative (DN) T cells. The Tr1 and Th3 regulatory T cells lack specific surface markers and can only be identified by the pattern of their cytokine secretion. They need to encounter their cognate antigen for activation, but suppress pathogenic immune responses through a paracrine bystander regulation mechanism, mediated by the inhibitory cytokines IL-10 (Tr1) and TGFβ (Tr1 and Th3).

The natural and the adaptive Tregs might play distinct immunosuppressive roles in vivo. Natural Tregs are constitutively present at all times in healthy individuals and maintain a steady state of immunological tolerance to self antigens. On the other hand, adaptive Tregs seem to be induced as a consequence of antigen exposure during ongoing immune and inflammatory responses, as a negative feed-back for controlling the amplitude and the duration of these immune responses. Whereas natural Tregs are responsible for background systemic immune suppression, adaptive Tregs are induced when and where additional immunosuppression is needed for controlling an ongoing immune response.

**REGULATORY T CELLS IN ATHEROSCLEROSIS**

Atherosclerosis is currently defined as a chronic inflammatory disease of the artery wall with important innate and adaptive immune components. Similar to autoimmune diseases, the adaptive immune response in atherosclerosis is mainly triggered by autoantigens. Oxidised LDL (oxLDL) is the principal candidate antigen in atherosclerosis, as 10% of plaque T cells were shown to be specific for oxLDL epitopes and the hyperlipidemic atherosclerosis prone mice have important circulating levels of anti-oxLDL antibodies. Other antigens involved in pro-atherogenic immune responses are heat shock protein 60 (HSP60) and â2-glycoprotein 1 (â2GPI). It is well documented that IFNγ-producing Th1 cells are the most prevalent adaptive immune component present in the atherosclerotic lesions and they play a very important role in plaque initiation and progression. IL-5 and IL-4 produced by Th2 cells were shown to downregulate the activation of atherogenic Th1 lymphocytes and induce production of atheroprotective IgM antibodies. Nevertheless, IL-4 deficiency in LDLR−/− mice leads to decreased lesion formation, indicating that Th2 cells might also be pro-atherogenic. As regulatory T cells control the adaptive immune system and they have been shown to be able to suppress the development of various autoimmune diseases such as type 1 diabetes, rheumatoid arthritis or multiple sclerosis, it has been hypothesized that they also play an important role in atherosclerosis.

Using a series of adoptive transfer experiments, Ait-Oufella and colleagues have demonstrated for the first time the involvement of naturally occurring CD4+CD25+Foxp3+ regulatory T cells in atherosclerosis. Immunodeficient ApoE−/−Rag2−/− mice reconstituted with Treg deficient splenocytes have accelerated disease progression in comparison with mice reconstituted with wild-type cells. Additional transfer of CD4+CD25+ Treg restored the regulatory T cell compartment in the spleen and inhibited disease progression. Similar results were obtained by adoptive transfer of CD4+CD25+ T cells into immunocompetent ApoE−/− mice. Conversely, treatment with a depleting anti-CD25 antibody reduced Treg numbers and accelerated atherogenesis in ApoE−/− mice. Depleting the CD25+ cells did not have any effect on atherogenesis in mice lacking a functional TGFβ receptor, suggesting an
important role for this cytokine in Treg mediated control of atherosclerosis.

Subsequent studies have shown that unspecific interventions leading to increased regulatory T cell numbers and suppressive activity inhibit atherosclerotic disease progression in ApoE-/- and LDLR-/- mice. Treatment with antibodies against the TCR receptor-associated surface molecule CD3 was shown to restore tolerance and induce disease remission in animal models of diabetes through a mechanism involving generation of TGF-β-secreting CD4+ regulatory T cells. Similarly, anti-CD3 treatment increased Treg numbers and reduced plaque development in LDLR-/- mice on a high fat diet. The transient T cell depletion recorded in these mice might favour preferential expansion of regulatory T cells by a mechanism similar to tolerance induction in mouse models of organ transplantation following treatment with non-depleting anti-CD4 antibodies and donor-specific antigens.

Two recently published studies have demonstrated that treatment with the immunomodulatory compounds CD31 receptor globulin and measles virus nucleoprotein reduce the development of atherosclerotic plaques in ApoE-/- mice. Although the parallel contribution of other mechanisms could not be excluded, the anti-atherosclerotic effect of these interventions was attributed to increased numbers of circulatory CD4+CD25+FoxP3+ Treg and the induction of a Tr1 type-like immune response, respectively. Tr1 cells secrete large amounts of their signature cytokine, IL-10, which was previously shown to have potent anti-inflammatory and anti-atherosclerotic properties. Adoptive transfer of OVA-specific Tr1 cells repeatedly stimulated in vivo with their cognate antigen leads to an increase in IL-10 production in the spleen and inhibits atherosclerosis in ApoE-/- mice.

Not only the absolute numbers but also the suppressive function and the migratory capacity of regulatory T cells are important for an efficient control of immune responses. It has been indicated that oxLDL is able to downregulate FoxP3 expression and has an inhibitory effect on regulatory T cell function, which may lead to a disturbed local balance between effector and regulatory immune responses in atherosclerotic plaques. The cytokine-like hormone leptin, secreted mostly by adipocytes, also seems to interfere with the immunomodulatory properties of Treg. Leptin deficient LDLR-/- mice have increased numbers and enhanced function of naturally occurring Treg, associated with marked inhibition of atherogenesis. These effects of leptin on Treg might represent one possible explanation for the high prevalence of atherosclerosis-related cardiovascular disease in individuals suffering from the metabolic syndrome, characterised by obesity and dyslipidemia. Another important component in the homeostasis and function of regulatory T cells is the inducible costimulatory molecule (ICOS), a member of the CD28 family. Interference with the co-stimulatory signals delivered by the ICOS-ICOS ligand pathway led to impaired Treg responses and accelerated lesion development in irradiated LDLR-/- mice reconstituted with ICOS deficient bone marrow.

Continuous clearance of proinflammatory apoptotic debris by macrophages and dendritic cells maintains the natural homeostasis of the vascular wall. Lactadherin (milk fat globule-EGF factor B) is a mediator required for efficient phagocytosis of apoptotic cells. Impaired scavenger function in LDLR-/- mice reconstituted with lactadherin deficient bone marrow leads to accelerated atherogenesis and enhanced accumulation of apoptotic debris in the plaques. This effect is paralleled by reduced IL-10 production in the spleen and the inability of dendritic cells isolated from these mice to provide adequate support for Treg in suppression assays in vitro. The interplay between effector and regulatory T cell-associated chemokines is another important factor that controls the immune balance within the vascular wall. Deficiency in the effector T cell-specific chemokine IP-10 (CXCL10) in CXCL10-/-/ApoE-/- mice favours overexpression of the Treg-associated chemokines CCL17 and CCL22. Compared to their ApoE-/- controls, the CXCL10-/-/ApoE-/- mice have smaller atherosclerotic lesions with reduced inflammatory infiltrate and increased expression of Treg-specific markers. Blocking IP-10 in vivo might represent a viable approach for tipping the balance towards preferential recruitment of regulatory T cells to the atherosclerotic plaques.

Taken together, these results demonstrate that regulatory T cells control the adaptive immune responses that promote atherosclerotic plaque progression in vivo. Atherogenesis was shown to be inhibited by transfer of polyspecific Treg populations or by stimulating the regulatory arm of the immune system in vivo. These proof-of-principle studies open new perspectives towards using these mechanisms for the treatment of atherosclerosis-related cardiovascular disease. However, in view of a potential Treg based therapy applicable in humans, these results have to be interpreted with due caution. Unspecific generalised suppression of the immune system is known to favour the development of infections and malignancies, as seen in transplant patients undergoing immunoregulatory...
therapeutic regimens. Therefore, cellular therapy using Treg or general stimulators of Treg activity is unlikely to become a clinical reality, unless it can be specifically targeted to the atherosclerosis-prone regions of the vascular wall. Ideally, the therapeutic regulatory T cells should only be activated by specific components confined to the atherosclerotic plaque, which would limit their activity to the site of interest and reduce their systemic side effects. There are encouraging results from several studies (detailed below) which indicate that such an effect is possible, paving the way towards a potential regulatory T cell therapy against atherosclerosis.

Oral tolerance to antigens has been shown to have beneficial therapeutic effects in various animal models of autoimmune disease and clinical trials using oral tolerance protocols for treatment of arthritis, multiple sclerosis and diabetes are currently ongoing. Mucosal administration of high antigen doses leads to deletion of antigen specific T cells, whereas low doses seem to promote a suppressive effect mediated by regulatory T cells. Two consecutive studies by van Puijvelde et al. have described Treg-mediated anti-atherogenic effects triggered by the induction of oral tolerance to oxLDL and HSP60 respectively. Oral administration of oxLDL or HSP60 leads to expansion of an antigen-specific population of TGFâ-secreting cells, presumably Th3, and increased numbers of CD4+CD25+FoxP3+ natural Treg in the mesenteric lymph nodes and spleen. The regulatory T cells infiltrated the plaques, as assessed by increased mRNA expression of the Treg markers CD25, Foxp3 and CTLA-4, and presumably played an instrumental role in lowering lesion area by 30% in the antigen-treated animals compared to the PBS controls. Similar results were obtained by adoptive transfer of an in vitro expanded HSP60 specific CD4+CD25+ Treg population in ApoE/- mice. The effects observed in the latter study were shown to be dependent on antigen specific activation, as adoptive transfer of equal numbers of OVA-specific Treg did not influence disease progression. Although a detailed analysis of Treg dynamics in vivo was not performed in this study, one can speculate that the presence of HSP60 in the atherosclerotic plaques and draining lymph nodes has lead to specific activation and local proliferation of the injected Treg. More detailed studies will have to be performed in order to determine if the immunosuppressive effects were confined to the atherosclerotic compartment and whether the adoptively transferred or in vivo generated regulatory T cells infiltrate the plaques themselves or induce the expansion of other Treg populations, leading to systemic immune suppression.

Another important issue to be clarified is the influence of regulatory T cells on the composition of the atherosclerotic plaques, as the results published so far have been fairly inconsistent and even contradictory. Whereas the decrease in plaque area was paralleled in most of the studies by significant reductions in the numbers of infiltrating T cells and macrophages, others failed to detect any differences. The regulatory T cells appeared to promote collagen accumulation in the lesions in some of the studies, whereas other investigators reported an increase in SMC proliferation and matrix synthesis in mice with defective Treg function. Although these results are difficult to compare, as the experimental settings differ markedly, plaque composition directly correlates with the incidence of acute cardiovascular events and will need to be carefully monitored in future studies.

**CONCLUSION**

In conclusion, natural and adaptive regulatory T cells play an important role in modulating the immune responses which contribute to the development of atherosclerosis in animal models. Additionally, it has been shown that the number and suppressive properties of regulatory T cells inversely correlate with the incidence of acute coronary syndrome in humans. Once activated, regulatory T cells specific for a defined plaque antigen might be able to suppress effector T cells with various specificities in the atherosclerotic lesions, through bystander regulation. The ability of Treg to induce bystander regulation might prove to be extremely important for their therapeutic function, as multiple antigens and immune pathways are involved in the development of atherosclerosis. The encouraging results obtained so far have opened the way for designing new therapeutic protocols able to restore the balance between effector and regulatory T cells in atherosclerosis. However, as it is unlikely that a generalised immune suppression approach would be suitable for treatment of this slowly progressing disease, there is an acute need for efficient approaches designed to specifically and exclusively target the function of the regulatory T cells to the atherosclerotic compartment.
REFERENCES


REFERENCES (CONTINUED)


