CD28 between tolerance and autoimmunity: the side effects of animal models [version 1; peer review: 2 approved]

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Abstract
Regulation of immune responses is critical for ensuring pathogen clearance and for preventing reaction against self-antigens. Failure or breakdown of immunological tolerance results in autoimmunity. CD28 is an important co-stimulatory receptor expressed on T cells that, upon specific ligand binding, delivers signals essential for full T-cell activation and for the development and homeostasis of suppressive regulatory T cells. Many in vivo mouse models have been used for understanding the role of CD28 in the maintenance of immune homeostasis, thus leading to the development of CD28 signaling modulators that have been approved for the treatment of some autoimmune diseases. Despite all of this progress, a deeper understanding of the differences between the mouse and human receptor is required to allow a safe translation of pre-clinical studies in efficient therapies. In this review, we discuss the role of CD28 in tolerance and autoimmunity and the clinical efficacy of drugs that block or enhance CD28 signaling, by highlighting the success and failure of pre-clinical studies, when translated to humans.

Keywords
CD28; tolerance; autoimmunity; regulatory T cells; inflammation; mouse models
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Introduction

Shifting the balance toward restoration of immune tolerance could represent an important goal of the ongoing research in autoimmunity. Promising therapeutic strategies would be aimed to concomitantly dampen pathogenic inflammatory T-cell responses and induce/expand suppressive regulatory T (Treg) cells. Since its discovery in 1980–2 and based on the high homology between rodent (mouse and rat) and human CD2813, several in vivo animal models have been generated for understanding the role of CD28 in T-lymphocyte activation and differentiation. CD28 is constitutively expressed on both naïve and activated T cells. By binding its ligands B7.1/CD80 or B7.2/CD86 on the surface of professional antigen-presenting cells (APCs), through a MYPPPY motif within its extracellular immunoglobulin (Ig)-V-like domain14 and to B7-H2 through a region outside the MYPPPY motif15, CD28 delivers signals, which lower the T-cell receptor (TCR) activation threshold, thus leading to optimal cytokine production, cell cycle progression, and survival16. Furthermore, in the human system, CD28 is able to emanate TCR-independent autonomous signals, which account for its critical role in regulating pro-inflammatory cytokine/chemokine production and T-cell survival17. Finally, pre-clinical mouse models also showed a paradoxical function of CD28 in the development and homeostasis of CD4+CD25+ Treg cells18–20. Treg cells are negative regulators of T-cell signaling and contribute to T-cell anergy and to the maintenance of self-tolerance by suppressing autoreactive T cells20. Therefore, CD28 can either reduce or enhance the susceptibility to autoimmune diseases by altering T-cell effector and Treg cell compartments. However, the translation of knowledge from pre-clinical mouse models led to the development of CD28 signaling modulators that often failed when applied in clinical trials. Here, we discuss CD28 regulatory functions in mouse models of autoimmune diseases by showing the success and failure of pre-clinical studies when translated to humans.

CD28 role in autoimmune diseases: from animal models to human clinical trials

Owing to the high conservation between Mus musculus and Homo sapiens, mice represent the favorite experimental models used by immunologists. Most of the data on the pivotal role of CD28 in regulating tolerance and susceptibility to autoimmunity derive from mouse models, which have been extensively used for clarifying the pathogenic mechanisms of several autoimmune diseases as well as for identifying molecular targets to translate in clinical trials. These efforts led to the development of soluble CTLA-4-binding domain linked to the Fc region of Ig (CTLA-4Ig) able to efficiently bind B7 molecules (with a 20-fold higher affinity compared with the CD28Ig) and to block CD28/B7 interaction through the removal of its ligands from APC, a process known as trans-endocytosis, and by directly interfering with T/APC interaction18. The success obtained in animal models led to many pre-clinical and clinical trials in order to assess the potential use of CTLA-4Ig to ameliorate the onset, progression, and clinical course of human autoimmune diseases19. However, despite the positive results gained in non-human organisms, data from CTLA-4Ig clinical trials showed discrepant results that, for brevity, are discussed below for two major autoimmune diseases: rheumatoid arthritis (RA) and multiple sclerosis (MS).

Targeting CD28 in rheumatoid arthritis

RA is a chronic autoinflammatory disease affecting about 1% of the population and is characterized by the production of autoantibodies, inflammation in the joints with progressive articular destruction, and systemic cardiovascular and pulmonary disorders. Although the role for autoreactive T cells in the pathogenesis of RA has long been debated, these cells’ contribution to the disease has been established by the analysis of several murine models and pre-clinical and clinical interventions16. For instance, autoreactive T cells may help B cells to produce high-affinity autoantibodies as well as secrete inflammatory cytokines, thus contributing to synovial inflammation and osteoclast activation7. The pivotal role of CD28 in the pathogenesis of RA has been firstly shown in a collagen-induced arthritis (CIA) mouse model by the use of recombinant CTLA-4Ig. This molecule efficiently binds CD80 and CD86 and prevents access to these ligands, thus leading to the inhibition of lymphocyte expansion and pro-inflammatory cytokine production as well as to the induction of Treg cells by generating tolerogenic dendritic cells18. In 2005, a CTLA-4Ig compound, abatacept, was approved by the US Food and Drug Administration (FDA) for the treatment of RA, and its second-generation form, belatacept, which shows a higher-avidity binding for CD86, was approved by the FDA in 2011 for the prevention of acute rejection in adult patients who have had a kidney transplant. The results obtained from clinical trials showed that abatacept treatment of patients with established RA refractory to methotrexate or TNF therapy (or both) significantly reduced the progression of structural damage at 1 year. The evaluation of the safety and efficacy of treatment over 5 and 7 years demonstrated that abatacept was also well tolerated and provided several clinical benefits and sustained disease remission10,20. The efficacy of abatacept-mediated reduced inflammation and disease progression was also highlighted by the maintenance of clinical remission following the withdrawal of abatacept11,22 or by reducing abatacept dose23.

More recently, a novel CD28 antagonist Ab, FR104, which selectively and efficiently prevents CD28 interaction with B7 molecules without affecting the inhibitory signals transmitted through CTLA-4 and PD-L124, has proven to be as potent as abatacept in reducing clinical symptoms, inflammation, and Ab serum levels and more effective in suppressing the proliferation of autoreactive peripheral blood T cells in a rhesus monkey model of CIA25. Thus, blocking CD28 co-stimulatory signals has proven to be an effective therapeutic treatment for RA.

Targeting CD28 in multiple sclerosis

MS is an autoimmune chronic inflammatory disorder characterized by the infiltration of macrophages, autoreactive T cells, and B lymphocytes within the central nervous system (CNS), thus causing demyelination and remyelination events, which finally lead to the loss of sensory and motor functions. On the basis of the data obtained from MS patients and murine models of experimental autoimmune encephalomyelitis (EAE), two models for explaining the etiology of MS have been proposed26. In the CNS-extrinsic (peripheral) model, the priming and activation of autoreactive myelin-specific T cells likely occur in peripheral lymph nodes, where the dendritic cells may present myelin epitopes to naïve T cells. Differentiated autoreactive effector/memory T cells in turn cross the blood-brain barrier and migrate into
the CNS where they trigger an acute inflammatory response, thus mediating primary demyelination and axonal damage. For instance, in EAE, myelin-specific T-cell responses seem to initiate in the CNS-draining cervical lymph nodes, thus suggesting that myelin proteins are constitutively present in some lymph nodes. Several pieces of evidence support a function for myelin proteins—such as MBP (myelin basic protein), PLP (proteolipid protein), and MOG (myelin oligodendrocyte glycoprotein)—as relevant antigens in both EAE and MS. In contrast, the alternative intrinsic model predicts that events within the CNS trigger disease development, and the infiltration of autoreactive lymphocytes occurs as a secondary phenomenon. Independently of the mechanism, data demonstrated that CD4+ Th1 and Th17 subsets exert a central role in the pathogenesis of both EAE and MS.

The role of CD28 in MS pathogenesis has been extensively studied in animal models. Initial studies suggested that CD28/B7 interaction is essential for the development of EAE. However, data from Vogel et al. showed that the blockade of B7 by CTLA-4Ig or anti-B7 monoclonal antibodies (Abs) after T-cell priming led to severe CNS inflammation and demyelination and exacerbated EAE. These events correlated with the recruitment of interferon gamma (IFN-γ), interleukin-17 (IL-17), granulocyte-macrophage colony-stimulating factor (GM-CSF), and IL-10 producing CD4+ T cells in the popliteal lymph nodes and in the CNS. Furthermore, recent data from a randomized clinical trial of abatacept did not show any significant efficacy in reducing neuroinflammation in patients with relapsing-remitting (RR) MS. These discrepancies may be related to the ability of CTLA-4Ig to inhibit both co-stimulatory signaling through CD28 and co-suppressive signals mediated by CTLA-4. For instance, CTLA-4 knockout mice failed to develop EAE, an event associated with the expansion and activation of Treg cells. More recent data by Haastra et al. showing the reduction of both CNS inflammation and demyelination in human EAE in rhesus macaques following the administration of FR104 CD28 blocking Ab strongly support a crucial role for CD28 in regulating the expansion and inflammatory function of autoreactive T cells in MS. Finally, the identification of single-nucleotide polymorphisms within genes encoding molecules belonging to the CD28/CTLA-4/CD80/CD86 pathway associated with MS susceptibility and the age of onset highlights the relevance of co-stimulation in MS pathogenesis.

**CD28 in the regulation of tolerance: spotlight on regulatory T cell functions**

Despite the pivotal role of CD28 in favoring the proliferation, differentiation, and functions of conventional T cells, increasing evidence accumulated during the last two decades highlighted a critical function of CD28 in promoting the homeostasis and suppressor function of Treg cells. Depending on the context, CD28 can deliver either pro-inflammatory or anti-inflammatory signals. Indeed, CD28 is required both for efficient generation of Treg cells in the thymus and for Treg cell peripheral homeostasis, as shown by the initial demonstration that mice deficient in CD28 or CD80/CD86 exhibit a strong reduction of thymic Treg cells and develop diabetes in a non-obese diabetic (NOD) background. More recent data on conditional deletion of CD28 in FOXP3+ Treg cells showed a 25–30% decrease of thymic Treg cells, whereas the percentage of Treg cells in lymph nodes and spleens was unaffected, thus indicating that CD28 influences the cell number and turnover of thymic, but not peripheral, Treg cells. However, all mice developed signs of systemic autoimmunity, such as lymphadenopathy and splenomegaly, that could be prevented by supplementation with CD28-sufficient Treg cells. Moreover, they showed accumulation of activated T cells in the skin and liver and failed to suppress induced colitis and EAE. A more detailed characterization of the skin disease in these animals revealed that, in the absence of CD28, Treg cells failed to mature and differentiate from a quiescent/central to an effector phenotype that is characterized by the downregulation of the CCR7 chemokine receptor (lymphoid retention) and by the expression of the chemokine receptors required for skin homing, such as CCR6. More recently, the same group showed that CD28 was also essential for the numbers and function of follicular Treg cells, whose loss in a CD28-deficient mouse caused increased germinal center B cells and Ab production. Similarly, Franckaert et al. found that CD28 deficiency in Treg cells caused a severe autoimmune syndrome as a result of impaired Treg cell proliferation and functions. These data strongly suggest a role for CD28 in maintaining the homeostasis of both thymic and peripheral Treg cells and in sustaining their suppressive functions necessary to maintain immune tolerance in vivo. However, other studies displayed discordant results. Vahl et al. showed that the differentiation and maintenance of effector Treg cells as well as their suppressive functions were severely compromised by TCR ablation in mature Treg cells. Similar results were obtained by Levine et al., thus suggesting a critical role for continuous TCR signals in maintaining the suppressive function of Treg cells in vivo. Data from Dilek et al. showed that, in human Treg cells, blockade of CD28 interaction with CD80 or CD86 prolongs Treg cell/APC contacts and calcium mobilization without affecting cell motility. In contrast, in a mouse model, CD28 interaction with CD80 is critical for stopping motility and forming symmetrical immunological synapse in the presence of antigen. Finally, recent data from Kishore et al. showed a crucial role for CD28 signals in inducing the migration of Treg cells and for their redistribution from lymphoid tissues. This scenario was further complicated following the discovery, by the Hünig research group, of a class of CD28 superagonistic Abs (CD28SAs).

In rodent models, Hünig et al. found that CD28SAs, by binding the laterally exposed CD3 loop of the Ig-like domain of CD28 in a parallel manner, were able to expand Treg cells without any pro-inflammatory responses. The same group showed that in vivo treatment of EAE mice with CD28SAs protected them from the disease. This discovery led to a plethora of preclinical experiments in mouse models of RA, MS, Guillain–Barré syndrome, and type 1 diabetes (T1D) in order to evaluate the potential use of these CD28SAs to ameliorate the clinical course of human autoimmune diseases. The promising results obtained from these experimental models led to the generation of a fully humanized CD28SA, named TGN1412, which, in March 2006, was injected in six healthy young men. Surprisingly, the phase I clinical trial turned into a catastrophe because all volunteers experienced a rapid and massive cytokine release syndrome.
The ability of human CD28 stimulation to expand Treg cells has been supported by data showing that agonistic Abs\textsuperscript{27} and the natural ligands B7.1/CD80 and B7.2/CD86, in the presence of recombinant human IL-2, mediate \textit{ex vivo} expansion of human Treg cells\textsuperscript{35}. Other studies showed that human CD28 stimulation by either natural ligands or agonistic or superagonistic Abs induced a strong increase in pro-inflammatory cytokine production in CD4\textsuperscript{+} T lymphocytes from either healthy donors or patients with RR MS or T1D\textsuperscript{31,32}. Such a pro-inflammatory signature of human CD28 should be taken into account when stimulating T cells \textit{in vivo}, as re-emphasized by TGN1412 administration to a humanized mouse model in which it induced strong lymphopenia, pro-inflammatory cytokine production, and death within 2–6 hours\textsuperscript{33}. Thus, although more recent data from Tabares et al. showed that low doses of CD28SAbs increased the number of activated Treg cells without affecting pro-inflammatory cytokine production\textsuperscript{32} and no detectable inflammatory cytokines were found in the plasma of healthy volunteers in a new phase I trial\textsuperscript{33}, CD28SAbs must be used with great caution. For instance, two recent studies showed that effector T cells from patients with MS may also acquire resistance to Treg cell suppressive mechanisms in an IL-6 receptor-dependent manner\textsuperscript{44,45} and CD28 stimulation of peripheral CD4\textsuperscript{+} T cells from patients with RR MS strongly upregulates IL-6 production\textsuperscript{3}. Moreover, the non-physiologic activation by CD28SAbs fails to induce PD-1 on the cell surface, thus leading to the loss of a crucial negative feedback conferred by the PD-1/PD-L1 interaction\textsuperscript{9} that represents a key checkpoint of immune response by effecting its negative regulation mainly on CD28\textsuperscript{18}.

Conclusions

All these data suggest that, despite many similarities, a divergent evolution of about 65 million years may have generated significant differences between humans and mice that, if not taken into account, could determine new “errors in translation”. CD28 has a pivotal role in the orchestration of the immune response that makes it a precious target for the treatment of immune-based diseases, but caution is needed to translate experimental results from mice to humans because differences in CD28 functions and signaling capability might determine dramatic effects. For instance, our recent identification of a single amino acid variant within the cytoplasmic tail of human and rodent CD28 (P\textsuperscript{12} in human versus A\textsuperscript{218} in rodent) as a critical residue for human CD28 pro-inflammatory and signaling functions\textsuperscript{38} raises the question of whether or not rodents can be used as a model for the study of CD28-mediated functions and for the safety of new therapeutic approaches. Thus, new efforts to develop better \textit{in vivo} and \textit{in vitro} systems are required to take advantage of the great potential retained in the co-stimulatory pathway and to provide novel insights into CD28 biology and implications for therapies.

Author contributions

NP and MK contributed to reviewing and editing the manuscript. LT contributed to preparing the original draft and to reviewing and editing the manuscript.

Competing interests

The authors declare that they have no competing interests.

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