Superagonistic anti-CD28 antibodies: potent activators of regulatory T cells for the therapy of autoimmune diseases

N Beyersdorf, T Hanke, T Kerkau, T Hüning

MOLECULAR PROPERTIES CHARACTERISING SUPERAGONISTIC ANTI-CD28 ANTIBODIES

As both conventional and superagonistic CD28 specific antibodies, in principle, induce an agonistic signal on interaction with the CD28 molecule, it was important to compare the molecular properties of these two classes of antibody.

Mapping of the binding motifs of anti-CD28 mAbs to the CD28 molecule has revealed that all the conventional antibodies studied bound to a membrane-distal part of CD28, which is close to the B7 binding site (MYPPPY motif) (schematically depicted in fig 2A). In contrast, superagonistic anti-CD28 antibodies that recognise rat, human, or mouse CD28 (unpublished data) bind to a lateral, membrane-proximal loop of the molecule (C”D loop). Crystallographic analyses of the interaction of a superagonistic anti-CD28 antibody with the extracellular domain of human CD28 further showed that this interaction leads to linear complex formation (schematically presented in fig 2B). Although conventional anti-CD28 antibodies were also able to bind CD28 bivalently, the resulting complexes were not linear in structure but “tangled”. This suggests that in the physiological setting of membrane bound CD28 molecules only CD28 superagonists are capable of mediating formation of complexes by linearly arraying CD28 molecules (fig 2B, C).

Complex formation induced by CD28 superagonists, presumably, leads to the aggregation of stimulatory signalling components such as phosphatidylinositol 3-kinase (PI3K) and growth factor receptor-bound protein 2 (Grb2) and thus to the generation of a strong activating signal.

CD28 SIGNALLING AND IL-2 ARE OF PARTICULAR IMPORTANCE FOR THE GENERATION AND MAINTENANCE OF REGULATORY T CELLS

Not only the activation of T cells but also their differentiation in the thymus, and the homoeostasis of the peripheral T cell pool, are subject to tight control mechanisms. To study the impact of CD28 on T cell homeostasis, mutant mice, which were deficient for CD28 and/or ligand B7 molecules, were generated. Interestingly, the absence of CD28 signalling in vivo did not affect overall T cell differentiation and numbers but led to a specific loss of CD4+CD25+ regulatory T cells (Treg cells). The primary task of Treg cells is to keep autoreactive T cells that have escaped negative selection in the thymus at bay in the periphery (fig 3). Conversely, a functional and/or numerical deficit in Treg cells versus pathogenic autoreactive T cells leads to the initiation of autoimmunity (fig 3). Accordingly, B7 deficient mice, which are devoid of Treg cells, showed a higher incidence and an earlier onset of symptoms in a model of human type 1 diabetes.

Abbreviations: AA, adjuvant arthritis; APC, antigen presenting cell; EAE, experimental autoimmune encephalomyelitis; TCR, T cell receptor; Tconv, conventional T cell; Treg, regulatory T cell
Apart from CD28, the cytokine IL-2 has been identified as being crucially involved in the generation and homeostasis of T\textsubscript{reg} cells.\textsuperscript{9,10} IL-2 and CD28 were both shown to be essential for thymic development of T\textsubscript{reg} cells and their peripheral maintenance.\textsuperscript{9–11} Further analysis of the role of CD28–B7 interactions in T\textsubscript{reg} cell survival revealed that, on the one hand, T\textsubscript{reg} cells themselves need to receive a signal through CD28, and,\textsuperscript{11} on the other hand, CD28 signalling induces IL-2 production by CD25\textsuperscript{low} conventional T cells (T\textsubscript{conv}) which in turn stimulates T\textsubscript{reg} cells.\textsuperscript{10,11} In contrast to their ''anergic'' phenotype upon in vitro stimulation, T\textsubscript{reg} cells, in fact, have a high, CD28 dependent, turnover under steady-state conditions in vivo.\textsuperscript{7} These findings nourished the idea of employing CD28 superagonists in protocols aiming at the therapeutic expansion and activation of T\textsubscript{reg} cells both in vitro and in vivo.

**EX VIVO EXPANSION OF T\textsubscript{reg} CELLS BY CD28 SUPERAGONISTS AND IL-2**

As T\textsubscript{reg} cells seem to be one of the cornerstones of peripheral self-tolerance, novel therapies should aim at re-establishing the balance of autoreactive effector T cells and T\textsubscript{reg} cells. One way of making use of T\textsubscript{reg} cells therapeutically is to expand them ex vivo and to reinfuse them into animals or patients with autoimmune diseases. As re-transferred T\textsubscript{reg} cells probably survive in the host for long periods of time, one advantage of adoptive cellular immunotherapy with T\textsubscript{reg} cells may be the induction of long term peripheral tolerance. Another advantage is that one can generate T\textsubscript{reg} cells with specificity for the relevant autoantigen allowing antigen specific therapy.\textsuperscript{12,13}

We used CD28 superagonists in conjunction with IL-2 to expand highly purified rat and human CD4\textsuperscript{+}CD25\textsuperscript{+} T cells in vitro. Expansion of rat T\textsubscript{reg} cells was up to $5 \times 10^6$-fold and the cells could be kept in culture for more than 100 days (unpublished data). The expanded T\textsubscript{reg} cells displayed enhanced suppressive activity on a per cell basis compared with freshly isolated regulatory T cells. Regulatory T cell activity, however, remained restricted to the progeny of purified CD4\textsuperscript{+}CD25\textsuperscript{+} cells, as cultured T\textsubscript{conv} cells did not acquire a suppressive phenotype. Importantly, the expansion of rat T\textsubscript{reg} cells was superior to that of T\textsubscript{conv} cells (unpublished data). This is a big advantage over other, less robust, protocols for in vitro T\textsubscript{reg} cell expansion where the starting population of regulatory T cells needs to be extremely pure so as not to be overgrown by contaminating T\textsubscript{conv} cells.\textsuperscript{13}

![Figure 1](https://www.annrheumdis.com)

**Figure 1**  Two classes of CD28 specific monoclonal antibodies (mAbs): “conventional” and “superagonistic”. Conventional anti-CD28 mAbs are, only in the context of costimulation, capable of driving interleukin (IL)-2 production and T cell proliferation. In contrast, superagonistic anti-CD28 antibodies do not depend on exogenous T cell receptor (TCR) triggering for full T cell activation.

![Figure 2](https://www.annrheumdis.com)

**Figure 2**  Bivalent linear complex formation could be the molecular clue to superagonistic CD28 stimulation. Only CD28 superagonists (red), but not conventional anti-CD28 antibodies (yellow) or B7 molecules (green), bind to a lateral motif of the CD28 molecule (\textsuperscript{A}).\textsuperscript{4} Therefore, only CD28 superagonists are capable of forming linear complexes with CD28 molecules (\textsuperscript{B, C}). In these complexes activating signalling components, presumably, get aggregated, which is sufficient to surpass the threshold for T cell activation.
IN VIVO EXPANSION AND ACTIVATION OF CD4+CD25+ Treg CELLS
Adoptive cellular immunotherapy with ex vivo expanded Treg cells recognizing the dominant autoantigen is, in principle, an elegant form of therapy in animal models. But, of date, it has not been feasible in most human autoimmune diseases where the relevant autoantigens have not been identified, despite intensive research. Moreover, as the ex vivo expansion of T cells under good manufacturing practice conditions is expensive and consumes a lot of resources it is unlikely to be broadly applicable in large cohorts of patients.

We thus monitored CD4+CD25+ T cells after direct application of superagonistic anti-CD28 mAbs to either rats or mice (unpublished data) in vivo. Application of CD28 superagonists in a dose range from 0.5 mg/kg to 5 mg/kg body weight transiently increased the proportion of CD25+ cells among rat CD4+ cells from about 5% to 20% (unpublished data). Absolute cell numbers were also significantly increased over the whole dose range—that is, up to 20-fold. But here a clear dose–response effect was observed with higher dosages of CD28 superagonist leading to a stronger increase in Treg cell numbers (unpublished data). Importantly, we could segregate expansion of Treg cells from induction of gross lymphocytosis by applying low doses of CD28 superagonists (e.g. 0.5 mg/kg body weight per rat).

Phenotypically, CD28 superagonist administration in vivo leads to the preferential expansion of CD4+CD25bright CTLA-4high FoxP3+ cells (unpublished data)—a phenotype defining “natural” Treg cells.15–16 CD28 superagonists, in fact, induce a true expansion of pre-existing CD4+CD25+ cells among rat CD4+ cells from about 5% to 20% (unpublished data). Absolute cell numbers were also significantly increased over the whole dose range—that is, up to 20-fold. But here a clear dose–response effect was observed with higher dosages of CD28 superagonist leading to a stronger increase in Treg cell numbers (unpublished data). Importantly, we could segregate expansion of Treg cells from induction of gross lymphocytosis by applying low doses of CD28 superagonists (e.g. 0.5 mg/kg body weight per rat).

Functional analyses of in vivo CD28 superagonist-expanded CD4+CD25+ T cells revealed a dose dependent increase in suppressive activity per cell compared with CD4+CD25+ Treg cells from untreated animals (unpublished data). However, CD4+CD25− cells, even after CD28 superagonist stimulation in vivo, were not able to suppress the proliferation of other T cells in vitro. In summary, CD28 superagonist administration in vivo leads to the preferential expansion and strong activation of naturally occurring CD4+CD25+ Treg cells with a Treg phenotype, not recruited into the pool of CD4+CD25+ T cells.11

Moreover, Treg cells are particularly dependent on signals leading to the preferential expansion of Treg cells over Tconv cells upon superagonistic anti-CD28 stimulation observed in healthy animals gave rise to the concept that CD28 superagonists might provide a novel form of therapy for autoimmune diseases. Efficacy of CD28 superagonist therapy has so far been evaluated in animal models of both peripheral and central nervous system inflammation as well as in a model of human rheumatoid arthritis.

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The neuroinflammatory models employed were experimental autoimmune encephalomyelitis (EAE) of the Lewis rat (monophasic), relapsing EAE of the Dark Agouti (DA) rat and experimental autoimmune neuritis (EAN) of the Lewis rat. EAE is used as an animal model for human multiple sclerosis and EAN resembles many of the clinical and pathological features of Guillain–Barré syndrome, an acute and mostly self-limiting form of peripheral nervous system inflammation in humans. Administration of a CD28...
superagonist in either of these animal models prevented or at least greatly mitigated clinical symptoms when given prophylactically—that is, before the animals showed signs of clinical disease (unpublished data). Importantly, protection from disease was not restricted to prophylactic administration of the CD28 superagonist. Even after the onset of clinical symptoms therapeutic CD28 superagonist administration rapidly stopped disease progression and induced remission. For successful therapy, as for Treg cell expansion, low doses of CD28 superagonist (0.5 mg/kg body weight) were sufficient (unpublished data).

To assess the therapeutic efficacy of CD28 superagonists in an animal model of human rheumatoid arthritis, adjuvant arthritis (AA) was induced in Lewis rats by immunisation with heat-killed mycobacteria. Signs of systemic inflammation, such as weight loss, and local arthritic alterations to the joints marked clinical disease. Therapeutic administration of a CD28 superagonist to animals already displaying clinical symptoms halted arthritis progression (unpublished data). Furthermore, rats treated with CD28 superagonists stopped losing and actually resumed gaining weight. Clinical efficacy was paralleled by histological preservation of joint architecture, whereas histological analysis of the ankle joints of untreated animals showed all the stigmata of arthritis (unpublished data). Therefore, CD28 superagonist therapy in this arthritis model was capable of halting clinical disease symptoms and histological manifestations of joint destruction.

PROTECTION FROM AUTOIMMUNITY IS, INDEED, MEDIATED BY CD28 SUPERAGONIST ACTIVATED T_{reg} CELLS

The therapeutic efficacy of CD28 superagonists in these models of human autoimmune diseases could, in principle, be independent of the observed expansion and activation of T_{reg} cells by CD28 superagonists. For example, activation induced cell death at pathogenic effector T cells could be the primary mode of action of superagonistic anti-CD28 therapy. However, we observed that neither was there increased apoptosis of central nervous system infiltrating T cells after CD28 superagonist therapy of animals with EAE nor did CD28 superagonist stimulation in vitro or in vivo induce apoptosis of effector T cells in the same animal model (unpublished data). Therefore, activation induced cell death of effector T cells does not seem to substantially contribute to the efficacy of superagonistic anti-CD28 therapy.

To obtain proof that protection from autoimmunity was mediated by CD28 superagonist-activated CD4+CD25+ T_{reg} cells we performed adoptive transfer experiments with purified in vivo CD28 superagonist activated T_{reg} cells. Donor animals were primed with a high dose of CD28 superagonist three days prior to isolation of CD4+CD25+ and CD4+CD25− T cells from the peripheral lymph nodes. On the same day of adoptive transfer of either pure T_{reg} cells or T_{conv} cells the recipient animals were also immunised with myelin basic protein in adjuvant for EAE induction. Transfer of CD28 superagonist-activated T_{reg} cells clearly protected recipient animals from clinical signs of EAE (unpublished data). Importantly, CD4+CD25− T_{conv} cells purified from the same animals were not protective, even when administered at a 10-fold excess compared to the regulatory T cells.

In summary, CD28 superagonists were proved to be highly efficacious in treating autoimmunity in a number of different animal models, including EAE and adjuvant arthritis of the Lewis rat. Adoptive transfer experiments further showed that CD28 superagonists mediated protection by expansion and activation of CD4+CD25+ T_{reg} cells.

APPLICATION OF CD28 SUPERAGONISTS IN HUMAN AUTOIMMUNE DISEASES

Our preclinical data obtained in animal models of human autoimmune diseases indicated that CD28 superagonists effectively target CD4+CD25+ regulatory T cells leading to the amelioration of autoimmunity in vivo. Significantly, therapeutic regimens over a broad dose range were never accompanied by detectable side effects. Thus, both the pharmacodynamics and the toxicological profile of CD28 superagonists support broad applicability of this class of antibody in humans.

Since correlative deficits in T_{reg} cell numbers and/or function have also been found in patients with multiple sclerosis or rheumatoid arthritis, such patients should clearly benefit from a boost in T_{reg} cells with CD28 superagonists. Therefore, superagonist-anti-CD28 antibodies constitute a promising novel treatment option for human autoimmune diseases and the first clinical trials are eagerly awaited.

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Competing interests: T Kerkau and T Hüning declare a commercial interest in TeGenero ImmunoTherapeutics AG.

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