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

N Beyersdorf, T Hanke, T Kerkau, T Hüning

This paper reviews the existing evidence regarding the use of superagonistic anti-CD28 antibodies (CD28 superagonists) for therapeutic manipulation of regulatory T cells (T<sub>reg</sub> cells). The molecular properties of superagonistic anti-CD28 antibodies allow the generation of a strong activating signal in mature T cells, including T<sub>reg</sub> cells, without additional stimulation of the T cell receptor complex. CD28 superagonist administration in vivo leads to the preferential expansion and strong activation of naturally occurring CD4<sup>+</sup>CD25<sup>+</sup>-FoxP3<sup>+</sup> T<sub>reg</sub> cells over conventional T cells. In animal models, both prophylactic and therapeutic administration of a CD28 superagonist prevented or at least greatly mitigated clinical symptoms and induced remission. Adoptive transfer experiments have further shown that CD28 superagonists mediate protection by expansion and activation of CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> cells. Therefore, superagonistic anti-CD28 antibodies offer a promising novel treatment option for human autoimmune diseases and the first clinical trials are eagerly awaited.

According to the paradigm of costimulation, which was initially adopted from existing concepts of B cell activation, a T cell needs two signals to become fully activated. Physiologically the “first signal” arises from the interaction of T cell receptor (TCR) molecules with peptide/major histocompatibility complex (MHC) complexes on antigen presenting cells (APCs). The “second signal” is provided by the engagement of a so called costimulatory receptor. The first to be discovered and still the most prominent of these costimulatory receptors is CD28. The physiological ligands for CD28, B7.1 and B7.2, are only expressed at high levels by the APC upon prior activation, thereby controlling the initiation of the T cell response.

To mimic physiological T cell activation in vitro, monoclonal antibodies (mAbs) with specificity for the TCR complex and CD28 have proved extremely useful. Neither anti-TCR mAbs alone nor “conventional” anti-CD28 mAbs by themselves suffice to fully stimulate T cells, whereas a combination of both efficiently induces T cell proliferation and cytokine secretion (fig 1). Interestingly, however, there is a subclass of CD28 specific antibodies, the CD28 superagonists, which are capable of fully activating T cells without additional stimulation of the TCR (fig 1). Moreover, superagonistic anti-CD28 stimulation works not only in vitro but also in vivo as administration of a CD28 superagonist to either rats or mice (unpublished data) induces a transient but significant increase in overall T cell numbers. In contrast with—for example, anti-CD3 stimulation—in vivo superagonistic anti-CD28 antibodies do not unleash a toxic “cytokine storm”. Therefore, the lymphocytosis induced by CD28 superagonists appears to be benign and well tolerated.

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 (MYPPP Y 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<sup>99</sup>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 homeostasis 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<sup>+</sup>CD25<sup>+</sup> regulatory T cells (T<sub>reg</sub> cells). The primary task of T<sub>reg</sub> 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 T<sub>reg</sub> cells versus pathogenic autoreactive T cells leads to the initiation of autoimmunity (fig 3). Accordingly, B7 deficient mice, which are devoid of T<sub>reg</sub> 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; T<sub>conv</sub>, conventional T cell; T<sub>reg</sub>, regulatory T cell
Apart from CD28, the cytokine IL-2 has been identified as being crucially involved in the generation and homeostasis of Treg cells.\(^9\) IL-2 and CD28 were both shown to be essential for thymic development of T reg cells and their peripheral maintenance.\(^9\) Further analysis of the role of CD28–B7 interactions in Treg cell survival revealed that, on the one hand, Treg cells themselves need to receive a signal through CD28, and,\(^11\) on the other hand, CD28 signalling induces IL-2 production by CD25\(^\text{low}\) conventional T cells (Tconv) which in turn stimulates Treg cells.\(^10\) In contrast to their "anergic" phenotype upon in vitro stimulation, Treg cells, in fact, have a high, CD28 dependent, turnover under steady-state conditions in vivo.\(^7\) These findings nourished the idea of employing CD28 superagonists in protocols aiming at the therapeutic expansion and activation of Treg cells both in vitro and in vivo.

**EX VIVO EXPANSION OF T reg CELLS BY CD28 SUPERAGONISTS AND IL-2**

As T 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 Treg cells. One way of making use of T reg cells therapeutically is to expand them ex vivo and to reinfect them into animals or patients with autoimmune diseases. As re-transferred T reg cells probably survive in the host for long periods of time, one advantage of adoptive cellular immunotherapy with T reg cells may be the induction of long term peripheral tolerance. Another advantage is that one can generate T reg cells with specificity for the relevant autoantigen allowing antigen specific therapy.\(^12\)\(^13\)

We used CD28 superagonists in conjunction with IL-2 to expand highly purified rat and human CD4\(^+\)CD25\(^+\) T cells in vitro. Expansion of rat T reg cells was up to 5\(^*\)10\(^6\)-fold and the cells could be kept in culture for more than 100 days (unpublished data). The expanded T 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+CD25+ cells, as cultured T conv cells did not acquire a suppressive phenotype. Importantly, the expansion of rat T reg cells was superior to that of T conv cells (unpublished data). This is a big advantage over other, less robust, protocols for in vitro T 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 conv cells.\(^15\)
IN VIVO EXPANSION AND ACTIVATION OF CD4+CD25+ TREG CELLS

Adoptive cellular immunotherapy with ex vivo expanded Treg cells recognising 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+CD25brightCTLA-4hiFoxP3hi cells (unpublished data)—a phenotype defining “natural” Treg cells.10,11 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).

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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 mark 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.

**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 Treg 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 Treg cells with CD28 superagonists. Therefore, superagonist-anti-CD25 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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