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A Monovalent Anti-Human CD28 Domain Antibody Antagonist: Preclinical Efficacy and Safety

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Targeting the CD28-CD80/86 pathway with an anti-CD28 antagonist is a promising alternative to current therapies for autoimmunity. However, attempts at generating conventional anti-CD28 mAbs lacking stimulatory activity has been challenging. In this study, we describe anti-human CD28 receptor antagonist domain Abs (dAbs) that are specific for human CD28. These dAbs are potent inhibitors of T cell activation, with an EC\textsubscript{50} of 35 ± 14 ng/ml for inhibition of proliferation. The EC\textsubscript{50} of 53 ± 11 ng/ml in an ex vivo CD28 receptor occupancy assay corresponds with in vitro functional activity, suggesting a direct correlation. The anti-CD28 dAb is equipotent in the inhibition of CD80- and CD86-mediated T cell proliferation and does not interfere with CTLA-4-mediated downmodulation of CD86 expression on APCs. The anti-CD28 dAbs are monomeric and do not demonstrate any evidence of agonism or costimulatory activity. In cynomolgus monkeys, the anti-CD28 dAb demonstrated pharmacodynamic activity, as measured by the inhibition of a T cell–dependent Ab response, without evidence of T cell depletion or cytokine release. Furthermore, there was a strong correlation between systemic exposure, duration, and extent of CD28 receptor occupancy, and pharmacodynamic activity. Taken together, these data support clinical evaluation of this novel anti-CD28 dAb for the treatment of autoimmune diseases. The Journal of Immunology, 2013, 191: 4599–4610.

Two signals that are delivered by APCs are required for T cell activation. The first signal is Ag specific and results from foreign peptides displayed on MHC molecules binding to the TCR. The second signal is a costimulatory signal that is Ag independent but works in concert with the Ag-specific signal. In the absence of this second costimulatory signal, Ag-specific T cells will either fail to respond and die or enter a state of unresponsiveness or anergy. Thus, under normal physiological conditions, costimulation is a key requirement for a T cell response to Ag. The first-identified and best-characterized costimulatory receptor on T cells is CD28, which binds to the B7 family members CD80 and CD86 on APCs. CD28 is expressed by most CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells and is required for both efficient cytokine production and proliferation in response to Ag (1, 2).

The role of CD28 signaling in autoimmunity and prevention of solid-organ transplantation has been informed by preclinical studies using CD28-deficient mice, anti-CD28 Fab fragments, or various CTLA-4Ig fusion proteins and clinical trials conducted with Orencia (abatacept; Bristol-Myers Squibb) and Nulojix (belatacept; Bristol-Myers Squibb) (3). Belatacept is a derivative of abatacept with a greater avidity for CD80 and CD86 (4). Abatacept has been approved for the treatment of rheumatoid arthritis (RA) and juvenile idiopathic arthritis, and belatacept for the prevention of renal transplant rejection.

CD28-deficient mice are healthy and fertile with normal T cell development (5) and show resistance to collagen-induced arthritis, chronic graft-versus-host reaction, experimental autoimmune encephalomyelitis (EAE), systemic lupus erythematosus, and experimental autoimmune neuritis (6–10). Because conventional anti-CD28 mAbs are agonistic in nature and therefore not well suited for evaluating the role of CD28 in preclinical models of autoimmunity, CTLA-4Ig has been commonly used to assess the CD28/B7 pathway in vivo. CTLA-4Ig binds to CD80 and CD86 and indirectly inhibits CD28 by blocking binding to its ligands. Altering the CD28/B7 pathway with CTLA-4Ig in the preventative mode in mice and rats completely inhibits the production of anticolonlagten IgG and disease onset in collagen-induced arthritis (11, 12). However, the impact on disease is less profound when CTLA-4Ig is administered to animals with established disease (therapeutic mode). This is likely due to the reduced importance of T cells in this phase of the animal model. In the NZB/NZW F\textsubscript{1} mouse model of systemic lupus erythematosus, murine CTLA-4Ig completely suppresses the development of anti-dsDNA Abs and reduces the rate of death, both in the preventative mode and in established disease (13). Murine CTLA-4Ig also suppresses antichromatin Ab production and glomerulonephritis in BXSX autoimmune disease–prone mice (14).

Although the examples above demonstrate that inhibition of the CD28/B7 pathway, either in the context of CD28-deficient (knockout) mice or through blockade of CD80/86 with CTLA-4Ig, work equally well in some preclinical disease models, it is...
also clear that differences in the interactions of CTLA-4ig versus CD28 with CD80 and CD86 might result in different outcomes of T cell activation. For example, in EAE, a single injection of CTLA-4ig in mice suppresses the first episode of disease but not the relapse phase (15). However, in this same study, multiple injections of CTLA-4ig prior to disease onset led to exacerbations of disease. In contrast, blockade of CD28 with a CD28 Fab fragment by: 1) pretreatment of myelin basic protein–primed T cell prior to transfer EAE; 2), treatment of animals prior to disease onset; or 3) treatment after onset of disease all led to an amelioration of clinical disease (16). There are at least three mechanisms by which CTLA-4ig could lead to exacerbation of disease: 1) CTLA-4ig binding to CD80 on APCs could prevent binding to programmed cell death ligand-1 (PD-L1) on T cells, thereby interfering with this coirhibitory pathway (17); 2) CTLA-4ig, by binding to CD80/CD86, could inhibit a CTLA-4–mediated negative signal into T cells (18); or 3) CTLA-4ig, by binding to CD80/CD86, could be interfering with the suppressive function of regulatory T cells (Tregs) (19). This suggests that an antagonist molecule that targets CD28 directly might be more efficacious compared with CTLA-4ig in certain disease contexts.

Consistent with this hypothesis is a recent report by Poirier et al. (20) demonstrating that a monovalent anti-human CD28 antagonist (CD28–specific single-chain Fv Ab fragment linked to α1-antitrypsin, sc28AT) synergizes with the in vitro suppressive activity of Tregs, whereas anti–CTLA-4 Ig Ab blocks the suppression. Furthermore, treatment of nonhuman primates with sc28AT plus tacrolimus, during and following kidney or heart allograft transplantation, results in the prevention of acute rejection, attenuation of chronic rejection, and the infiltration of functional Tregs into the grafts. Similarly, treatment of mice receiving cardiac allografts with a monovalent mouse anti-CD28 scFv (α28scFv) combined with either an anti-CD40L Ab or cyclosporine significantly increases the proportion of intragraft Tregs compared with recipients that received either treatment alone (21). As was seen in nonhuman primates, there is a good correlation between the number of intragraft Tregs and prolonged cardiac allograft survival in mice. In these studies, it was also demonstrated that the expression of intragraft programmed cell death-1 was increased, indicating that the programmed cell death-1/ PD-L1–negative signaling pathway remains intact and may even be enhanced in the presence of a CD28 antagonist.

In the current study, we describe the generation of monovalent anti-human CD28 domain Abs (dAbs) using phage display and affinity maturation through the diversification of a selected subset of amino acid residues. The anti-CD28 dAbs were formatted with polyethylene glycol (PEG) to increase their hydrodynamic size and extend their serum t1/2. These dAbs are potent inhibitors of T cell proliferation and cytokine production. Unlike abatacept, a CTLA-4 Ig fusion protein that binds with different affinities to CD80 and CD86 on APCs, the anti-CD28 dAb was equipotent in inhibiting both CD80- and CD86-driven T cell proliferation. In addition, the anti-CD28 dAbs were more potent and less variable across multiple T cell donors than CTLA-4ig in inhibiting a dendritic cell–driven MLR (DC-MLR). A comprehensive panel of in vitro assays was employed to ensure that the dAbs were completely devoid of agonist or coagonist activity. We also provide evidence that a monovalent anti-CD28 dAb does not interfere with Treg function. In addition, there was a good correlation among exposure, duration of CD28 RO, and suppression of a T cell–dependent Ab response in vivo studies, were based on dAb protein alone and did not include PEG. These dAbs have identical amino acid sequences in the Ag-binding domain but dAb-001 contains two additional amino acids (ST) at the N terminus remaining from the original expression vector. The two additional amino acids in dAb-001 did not significantly affect the behavior of dAb-001 versus dAb-002 when tested across various biochemical and in vitro assays. An irrelevant Vx dAb (Vx dummy dAb), formatted with 40-kDa branched PEG, was used as a negative control in some studies. Abatacept and belatacept were provided by Bristol-Myers Squibb. mAb 5.11A1 was used as a positive control in agonist assays. This Ab is a mouse anti-human CD28 superagonist Ab of the IgG1 subclass that was engineered to generate TGN1412, the humanized IgG4 version that was associated with cytokine storm in a phase I clinical trial. The immunogen keyhole limpet hemocyanin (KLH) was purchased from Pierce (Rockford, IL).

**Methods**

**Phage selection.** Phage particles from a large synthetic Vx dAb repertoire (24) based on a fully human scaffold encoded by germline κ and λ chain genes O12/02/DPK9 and Xε1 with the side chain diversity incorporated at positions in the Ag binding site were prepared and purified as described (25). This library was selected against recombinant biotinylated monomeric human CD28 fragment in solution, matured, and expressed as previously described (26).

**Surface plasmon resonance method for affinity measurements.** Kinetic analysis was performed on a BIACORE T1000 instrument (Biacore) at 25°C in 10 mM NaPO4, 130 mM NaCl (pH 7.4), and 0.05% Tween 20 buffer. After preconditioning the streptavidin chip (GE Healthcare) surface with 1 M NaCl and 50 mM NaOH, human CD28–biotinylated monomer was immobilized at a concentration of 2 μg/ml using a 9-s contact time at 20 μl/min to give 270 RU immobilized ligand. Samples of the PEGylated dAbs, in 20 mM NaPO4 and 150 mM NaCl (pH 7.2), were then injected for 3 min at 30 or 100 μl/min followed by 10 min of dissociation using the high-performance injection method. Analysis was performed using the BIACORE T100 evaluation software (Biacore) using a global analysis 1: Langmuir binding model. Calculation of the average kinetic parameters and SEs was determined using three titration series (30 μl/min, n = 2; 100 μl/min, n = 1).

**CD28-dependent cell-based luciferase reporter gene assay.** Jurkat-CA T cells (BMS, Hopewell, NJ) at 2 × 10^6 cells/ml in phenol-red-free RPMI 1640 containing 10% FCS, 1% l-glutamine, and 1% sodium pyruvate were combined with Raji B cells (ATCC CCL-86; American Type Culture Collection) at 2 × 10^5 cells/ml in a 96-well plate and 25 μl anti-CD3 Ab (clone UCHT1; final concentration of 0.1 μg/ml) followed by 25 μl serial dilutions of dAb. The plates were Incubated for 16–20 h at 37°C and allowed to cool to room temperature before the addition of 50 μl Steady-Glo (Promega, Madison, WI). Plate luminescence was measured in the TopCount (Packard Instruments, Meriden, CT) and data analyzed using XL Fit (Microsoft, Redmond, WA).

**Human DC-MLR.** Human T cells and monocytes were isolated by sheep RBC rosetting of PBMCs as previously described (27). DCs were generated from monocytes that were adhered to plastic and then cultured with GM-CSF and IL-4 for 6 or 7 d. Twenty-four hours prior to assay setup, LPS (1 μg/ml) was added to induce maturation. The CD28 dAbs, along with abatacept and belatacept controls, were titrated in half-log concentrations to evaluate their inhibition of a 1:10 ratio of DC to T cell interaction. Cytokine production was measured in supernatants by commercial ELISA. Proliferation was measured by [3H]thymidine incorporation, and EC50 values were generated from inhibition curves of each treatment using ExcelFit. Peripheral blood was obtained from healthy volunteers following written consent and approval by the Bristol-Myers Squibb institutional biosafety committee.

**Monkey MLR.** PBMCs were isolated from acid citrate dextrose-treated cynomolgus monkey blood using Ficoll diluted with 10% PBS. The
human cell line PM-LCL, an EBV-transformed human B cell line, was used as the APC. PM-LCLs, irradiated with 10,000 rad, were combined with cytonomologous PBMC, in the presence or absence of dAb, at a ratio of 1:25, in 96-well round-bottom plates and incubated at 37°C for 5 d. Pro- liferation was measured by \( ^{3}H \)thymidine incorporation and EC50 values for inhibition generated using XLI.Fit (Microsoft).

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dAb-001 and dAb-002. Because dAbs have terminal $t_{1/2}$ of $<45$ min in vivo (26), conjugation to PEG was used to increase the hydrodynamic size and in vivo serum $t_{1/2}$ (31).

In vitro characterization

The binding affinities of dAb-001 and dAb-002 for hCD28 were subnanomolar as determined by surface plasmon resonance (Table I). At a concentration 250-fold over the $K_{D}$ for hCD28, there was no detectable binding of these dAbs to another CD28 family member, human CTLA-4 (data not shown). In addition, evaluation of dAb binding by flow cytometry showed that binding to T cells is specific and that there was no apparent binding to monocytes, DCs, and neutrophils.

In primary functional assays, the dAb-001 and dAb-002 had $EC_{50}$s of 32–35 ng/ml (2.8–2.9 nM), respectively (Table II) for inhibiting human T cell proliferation in a DC-mediated MLR, with similar potency for inhibition of cytokine production. For comparison, the potency of abatacept, which blocks the CD28 pathway indirectly by binding to CD80 and CD86 molecules on the APC, is $\sim$2300 ng/ml (25 nM) in T cell proliferation (Table II). It has been reported that CD28 can also bind to ICOS ligand on APCs and act as a costimulus, with suboptimal concentrations of anti-CD3 mAb, to promote T cell proliferation (32). In assays in which suboptimal concentrations of immobilized anti-CD3 and ICOS ligand were coimmobilized at either 10 or 30 μg/ml, anti-CD28 dAb, at concentrations well above the $IC_{50}$ for inhibition of a DC-MLR, was unable to inhibit this response (data not shown).

Anti-CD28 dAb-001 and dAb-002 were also evaluated in the context of a cynomolgus monkey MLR. As the extracellular domain of cynomolgus monkey CD28 is 100% identical to human CD28, it was expected that both anti-CD28 dAbs would have $EC_{50}$ values similar to those seen in a human MLR. In the cynomolgus monkey MLR, an EBV-immortalized human B cell line was used as the APC to drive proliferation of monkey T cells. The $EC_{50}$s for dAb-001 and dAb-002 were $22 \pm 23$ and $23 \pm 29$ ng/ml, respectively, similar to the potency observed in the human MLR (Table II).

Differentiation of the impact of inhibiting CD28 directly (with an anti-CD28 dAb) versus indirectly by blocking CD80/CD86 on APCs (with CTLA-4Ig) was demonstrated by evaluating the CD80 versus CD86-mediated proliferation of T cells using CHO cells expressing either CD80 or CD86 on the cell surface. In this context, dAb-001 showed similar inhibition of CD80 versus CD86-driven T cell proliferation, with $EC_{50}$s of $0.4 \pm 0.05$ and $0.4 \pm 0.05$ nM, respectively (Fig. 1). Under these same assay conditions, abatacept has an $EC_{50}$ of 0.48 ± 0.14 nM for CD80 and 37.7 ± 18.6 nM for CD86-driven T cell proliferation, consistent with previous reports demonstrating that abatacept is $\sim$100-fold less potent against CD86-mediated responses (33). Even though belatacept is more potent than abatacept against CD86-driven T cell proliferation, it is still $\sim$5-fold less potent than dAb-001, with an $EC_{50}$ of 2.2 ± 1.5 nM, supporting the potential utility of an anti-CD28 dAb for targeting CD86-mediated responses.

### Table I. Kinetic parameters for the binding of anti-CD28 dAbs to biotinylated CD28 monomer as determined by surface plasmon resonance

<table>
<thead>
<tr>
<th>Biologic</th>
<th>$k_{a}$ (1/ms)</th>
<th>$k_{d}$ (1/s)</th>
<th>$K_{D}$ (nM)</th>
<th>$R_{max}$</th>
<th>$n$</th>
</tr>
</thead>
<tbody>
<tr>
<td>dAb-001</td>
<td>$7.9 \pm 2.6 \times 10^{4}$</td>
<td>$3.1 \pm 1.5 \times 10^{-5}$</td>
<td>$0.51 \pm 0.36$</td>
<td>$34 \pm 10$</td>
<td>2</td>
</tr>
<tr>
<td>dAb-002</td>
<td>$7.7 \pm 0.8 \times 10^{4}$</td>
<td>$2.7 \pm 0.5 \times 10^{-5}$</td>
<td>$0.36 \pm 0.08$</td>
<td>$76 \pm 10$</td>
<td>5</td>
</tr>
</tbody>
</table>

Data are means ± SEM unless otherwise indicated.

### Table II. Inhibition of T cell proliferation and cytokine release in a DC-MLR

<table>
<thead>
<tr>
<th>Biologic</th>
<th>Proliferation</th>
<th>IL-2</th>
<th>TNF-α</th>
<th>IFN-γ</th>
</tr>
</thead>
<tbody>
<tr>
<td>dAb-001</td>
<td>$32 \pm 14$</td>
<td>$24 \pm 3$</td>
<td>$30 \pm 7$</td>
<td>$54 \pm 23$</td>
</tr>
<tr>
<td>dAb-002</td>
<td>$35 \pm 14$</td>
<td>$26 \pm 7$</td>
<td>$24 \pm 6$</td>
<td>$52 \pm 18$</td>
</tr>
<tr>
<td>Abatacept</td>
<td>$2300 \pm 1300$</td>
<td>$460 \pm 150$</td>
<td>$860 \pm 740$</td>
<td>$1000 \pm 340$</td>
</tr>
</tbody>
</table>

$n = 10$ (MLR); $n = 6$ (cytokines).
In the more conventional costimulation assay, in which anti-CD3 was immobilized and anti-CD28 was added in solution to stimulate T cell activation, dAb-002 did not cause T cell activation as measured by proliferation or cytokine release. As expected, the anti-CD28 mAb 9.3 did enhance anti-CD3-mediated T cell proliferation and cytokine release (Fig. 4). In this same set of experiments, some activation was observed with the addition of the 9.3 Fab fragment, most likely by contamination with whole Ab that retained the ability to cross-link CD28 on the surface of the T cells.

Cross-linking assays

Finally, to evaluate the potential for anti-drug Abs to cross-link the dAb and subsequently lead to T cell activation, experiments were conducted using either an anti-V\(\kappa\) or anti-PEG Ab to capture dAb-002. Under these conditions, no significant proliferation (Fig. 5, top panel) or cytokine production (Fig. 5, middle and bottom panels) was observed in response to dAb-002. In contrast, T cell activation was observed when mAb 5.11A1 was captured with immobilized anti-mouse Fc (anti-\(\mu\)) Ab. These experiments were then expanded to determine if the V\(\kappa\) or anti-PEG–captured dAb-002 exhibited agonist activity in the presence of an anti-CD3 Ab. As was seen with the capture Abs alone, no significant proliferation (Fig. 6, top panel) or cytokine production (Fig. 6, middle and bottom panels) was observed in response to dAb-002. In contrast, T cell activation was seen when mAb 9.3 (anti-CD28) or mAb 5.11A1 were captured with immobilized anti-mouse Fc (anti-\(\mu\)) Ab or immobilized anti-V\(\kappa\). As others have observed (34), IL-2 appeared to be a less sensitive measure of T cell activation than either proliferation of IFN-\(\gamma\) production.

![Figure 1](http://www.jimmunol.org/) Anti-CD28 dAb-001 was equipotent at inhibiting either CD80- or CD86-driven T cell proliferation. T cell proliferation was initiated by the addition of anti-CD3 Ab plus CHO cells expressing either CD80 or CD86. In the presence of dAb-001, both CD80- and CD86-driven T cell inhibition were inhibited with identical EC\(_{50}\)s. In contrast, abatacept was very active against CD80-mediated T cell proliferation but not that mediated by CD86. Belatacept was more active than abatacept at inhibiting both CD80- and CD86-driven T cell proliferation, but remained less active against the CD86-mediated response. Data are mean ± SD of five experiments done with triplicate determinations.

![Figure 2](http://www.jimmunol.org/) Activation markers, proliferation and cytokine release from human T cells in response to dry-coated anti-CD28 dAb-002. Human T cells were added to wells coated with varying concentrations of V\(\kappa\) dummy dAb, dAb-002, or the superagonist Ab mAb 5.11A1 and cell activation determined by increased expression of the activation Ags CD69 (A) and CD25 (B); increased T cell proliferation (C); and increased IL-2 (D), IL-8 (E), IL-10 (F), TNF-\(\alpha\) (G), IFN-\(\gamma\) (H), and IL-6 (I) cytokine release. The concentration of dAb or mAb 5.11A1 added to each well for dry-coating is indicated in (A). Data are mean ± SD of 10 different blood donors.
CTLA-4–mediated downregulation of CD86

It is generally accepted that CTLA-4 expression on Tregs is involved both in vitro and in vivo suppression of autoreactive T cells (35). At least part of this effect is through the modulation of CD80/CD86 expression on DCs, which limits their ability to activate T cells via CD28. In contrast, blocking CTLA-4 directly with an Ab or indirectly through CTLA-4Ig binding to CD80/CD86 could potentially interfere with the function of Tregs. In the absence of any treatment (control), CTLA-4–expressing Jurkat cells block the upregulation of CD86 on DCs (Fig. 7). Similarly, addition of dAb-001 does not interfere with the ability of the Jurkat-CTLA-4 cells to cause the modulation of CD86 expression. In contrast, both belatacept and an anti–CTLA-4 Ab interfere with the ability of Jurkat-CTLA-4 cells to block CD86 upregulation. In these assays, belatacept treatment alone alters CD86 expression, likely through internalization of the receptor (P. Davis, unpublished observations).

Given that the extracellular sequence of the human CD28 receptor shares 100% homology with the cynomolgus receptor (36), the anti-CD28 dAbs were expected to be fully cross-reactive between human and cynomolgus monkey. The EC50 generated in whole blood for dAb-001 was 62 ± 28 ng/ml and for dAb-002 was 53 ± 11 ng/ml (Table III). These results agree with EC50 data generated from the in vitro DC-MLR assays (32 ± 14 and 35 ± 14 ng/ml, respectively; see Table II), suggesting that receptor occupancy (RO) of CD28 is strongly correlated with the inhibition of T cell proliferation/activation. The whole-blood RO values for cynomolgus monkey were 66 and 78 ng/ml for dAb-001 and dAb-002, respectively.

PK, CD28RO, and inhibition of KLH-induced TDAR in cynomolgus monkeys

Anti-CD28 dAb-001 was evaluated in monkeys to determine its PK properties, and to confirm intended pharmacologic effect and relationship to CD28 RO. In this study, selected endpoints (i.e., CD4+ T cell counts and cytokines) were also included to evaluate any potential for immunostimulation, given the experience with the anti-CD28 superagonist Abs. The plasma concentrations versus time profiles are shown in Fig. 8A. The t1/2 of anti-CD28 dAb-001, following single-dose s.c. administration of 0.05, 0.5, and 5 mg/kg, ranged from 46 to 63 h, with linear PK at the dose range studied.

The whole-blood CD4+ T cell CD28 RO versus time profiles for the anti-CD28 dAb-001 at three dose levels are shown in Fig. 9A. The extent and duration of the RO increased with dose, consistent with dose-dependent increases in drug exposure. The mean peak RO at 0.05, 0.5, and 5 mg/kg was achieved at 48 (89%), 4 (∼100%), and 2 h (∼100%), respectively, with the corresponding...
RO values decreased to <50% at 240, 504, and 672 h postdose. Furthermore, consistent with the PK and RO profiles, a dose-dependent suppression of the primary anti-KLH IgG response was observed with anti-CD28 dAb-001 (up to day 43) (Fig. 9B). As expected, the more prolonged duration of 100% RO observed with the increasing doses of anti-CD28 dAb-001 resulted in a greater and more sustained suppression of the KLH response.

Overall, these data demonstrate favorable PK properties of the anti-CD28 dAb-001 and confirm intended pharmacologic activity (immunosuppression) in monkeys that correlated closely with ex vivo CD28 RO. Additionally, there was no evidence of agonistic properties as identified by a lack of rapid and severe depletion of circulating peripheral blood CD4+ T cell levels (Fig. 8B), a feature of TGN1412 and other immunomodulatory CD3-specific Abs (37, 38), or stimulation of cytokine release in blood. Circulating IL-1β, IL-2, IL-5, and IFN-γ levels were at or below the level of detection for the ELISA assays, and IL-6 and TNF-α levels, if detectable, were transient and <100 pg/ml for IL-6 and <20 pg/ml for TNF-α, with no relationship to dose.

**Discussion**

It has been difficult to generate mAbs against CD28 that lack some level of agonist activity, whether it be by acting in concert with TCR activation or, in the most extreme case, an Ab that can stimulate T cells directly, as was the case with the CD28 super agonist TGN1412. As an alternative approach, a CTLA-4Ig fusion protein has provided the most effective blockade of the CD28 pathway, demonstrating efficacy in preclinical models of auto-

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**FIGURE 4.** Proliferation (A) and cytokine responses (B, C) of peripheral blood T cells to immobilized anti-CD3 in the presence of anti-CD28 dAb-002. Human T cells were added to wells coated with anti-CD3 and containing soluble CD28 dAb-002, anti-CD28 9.3 mAb or Fab fragment, or mAb 5.11A1. Supernatants were harvested at 24 h for cytokines and cells harvested on day 3 for proliferation. T cell activation in response to anti-CD3 alone is shown with the dashed line. The concentration of soluble dAb, 9.3 mAb, or Fab added to each well is indicated in (A).
immune disease as well as providing effective treatment in the clinic, where it is approved for the treatment of rheumatoid arthritis (Orencia; Bristol-Myers Squibb) and solid-organ transplantation (Nulojix; Bristol-Myers Squibb). There is, however, room for improvement by directly targeting the CD28 pathway in certain disease indications. For example, abatacept is \( \sim 100 \)-fold less potent at inhibiting CD86-dependent responses as compared with CD80-mediated responses. This may be acceptable for treating RA patients but is relatively ineffective in nonhuman primate models of transplantation (39), a condition that appears to be more dependent upon CD86-mediated costimulation than CD80-mediated responses. For this reason, we and others have continued to explore alternatives to conventional mAbs as antagonists of CD28.

Vanhohe et al. (40) generated a single-chain Fv Ab fragment from a high-affinity anti-human CD28 Ab (CD28.3) and fused it to human α1-antitrypsin (sc28AT) to increase the \( t_{1/2} \) in circulation. More recently, this same group described a humanized PEGylated anti-CD28 Fab Ab fragment (FR104) also derived from mAb 28.3 (22). Although these are relatively straightforward approaches for generating monovalent reagents from pre-existing Abs, they are limited by the affinity of the parental Ab and, in some cases, have even lower affinities than the parent (41). We chose an approach that would allow us to exploit phage libraries containing a diverse repertoire of human VH or Vk variable domains. These dAbs are naturally endowed with very useful characteristics such as good stability, the possibility of improved access/binding to epitopes not accessible to conventional Abs because of their small size (\( \sim 12 \) kDa), and can be easily engineered to achieve high target affinity. Several dAbs (nanobodies) are currently being evaluated in phase I and II clinical trials, including anti–TNF-α dAbs for RA/psoriasis and acute lung injury and anti–von Willebrand factor nanobodies.

FIGURE 5. Proliferation and cytokine responses of peripheral blood T cells to cross-linked anti-CD28 dAb-002 or mAb 5.11A1. Either no Ab or secondary Abs (anti-Vk and anti-PEG for dAb capture or anti-μ for TGN5.11A1 capture) were immobilized on 96-well plates and anti-CD28 dAb-002 or TGN5.11A1 added to these plates to allow Ab capture. T cells were then added, the supernatants harvested at 24 h for cytokines, and cells harvested on day 3 for proliferation. The "no x-link" wells show the response of T cells to soluble anti-CD28 dAb-002 or mAb 5.11A1 in the absence of cross-linking. The concentration of soluble anti-CD28 dAb-002 or mAb 5.11A1 added to each well is indicated in the top panel of the figure. Top panel, Proliferation; middle panel, IL-2; bottom panel, IFN-γ.
for thrombosis, acute coronary syndrome, and thrombotic thrombocytopenia purpura (42).

Like other Ab fragments, the anti-CD28 dAbs require formatting to ensure a prolonged $t_{1/2}$ in vivo. PEG linkage (PEGylation) is very efficient for increasing the $t_{1/2}$ and potentially reducing immunogenicity (42). However, PEGylation sometimes leads to a decreased affinity of the fragment for its target. The PEGylated anti-CD28 dAbs identified are the first examples of monovalent dAbs that exhibit subnanomolar binding affinity in the absence of dimerization. These dAbs are 10-fold more potent for binding to CD28 than the reported value for either sc28AT or FR104 (0.4–0.5 versus 8.5 and 4.6 nM, respectively) (22, 40). The dAbs were also more potent in functional assays such as binding to isolated human T cells (dAb EC$_{50}$ values of 62 and 53 versus 320 ng/ml for FR104) or in an MLR (dAb EC$_{50}$ values of 32 and 35 versus 160 ng/ml for FR104). These differences are even larger than they would appear when comparing these values relative to molecular mass (molecular masses of the protein portion of dAbs are $\sim$12 kDa versus that of the Fab, which is $\sim$50 kDa).

In vitro anti-CD28 dAbs inhibit T cell activation by preventing CD28 engagement with CD80 and CD86 while preserving the ability of CTLA-4 to bind to these same ligands. This is demonstrated by the ability of the dAbs to potently inhibit T cell proliferation and cytokine production in the context of a DC-MLR while not affecting the ability of CTLA-4–bearing cells to promote downregulation of CD86. CTLA-4 expression on conventional T cells downmodulates T cell activation by competing with CD28 for binding to CD80 and CD86 on APCs and is a suppressive mechanism in T cell homeostasis (33). In contrast, CTLA-4 expression on Tregs is important for the suppression of autoreactive T cells and the prevention of in vivo autoimmunity (19). Thus, our data suggest that an anti-CD28 Ab would prevent

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**FIGURE 6.** Proliferation and cytokine responses of peripheral blood T cells to cross-linked anti-CD28 dAb-002 or mAb 5.11A1 in combination with soluble anti-CD3. Secondary Abs were immobilized on 96-well plates and anti-CD28 dAb-002, 9.3 mAb, or TGN5.11A1 added to these plates to allow Ab capture. Anti-CD3 mAb (1 $\mu$g/ml) and T cells were then added, the supernatants harvested at 24 h for cytokines, and cells harvested on day 3 for proliferation. The 0 $\mu$g/ml bars show the response of T cells to soluble anti-CD3 mAb in the presence of secondary Ab alone. The concentration of either anti-PEG or anti-Vc captured anti-CD28 dAb–002, or anti-µ or anti-Vc captured mAb 9.3 or mAb 5.11A1 in each well is indicated in top panel of the figure. *Top panel*, Proliferation; *middle panel*, IL-2; *bottom panel*, IFN-γ.
conventional T cell activation without interfering with normal Treg function. This has been demonstrated in vitro by Poirier et al. (20), in which the addition of sC28AT did not interfere with the suppressive function of Tregs. Not surprisingly, the addition of an anti-CD28 dAb to the same type of in vitro Treg suppression assay yielded similar results (data not shown). The direct inhibition of CD28 should also maintain the interaction of PD-L1 with CD80, another negative regulatory pathway involved in modulating T cell activation (17). Sparing the suppressive activities imparted by both another negative regulatory pathway involved in modulating T cell CD28 should also maintain the interaction of PD-L1 with CD80, yielded similar results (data not shown). The direct inhibition of anti-CD28 dAb to the same type of in vitro Treg suppression assay suppressive function of Tregs. Not surprisingly, the addition of an anti–CTLA-4 Ab inter- actioned in vitro conditions. Since the TGN1412 clinical inci- dent was included in an attempt to recapitulate an inflammatory cytokines in humans (43, 44). Because of this concern, new in vitro cytokine release assays have been developed for hazard identification and to overcome potential limitations of standard preclinical safety tests (44). With this approach in mind, we carefully replicated the dry-coated condition that was de- scribed by Stebbings et al. (29) to demonstrate agonist activity of TGN1412 in vitro. This method has proven to be a more sensitive measure of agents on direct T cell agonism than the PBMC–endothelial cell coculture system introduced by Findlay et al. (34). Using this assay format with several enhanced readouts, we evaluated the potential agonist activity for the anti-CD28 dAbs using cells from 10 human donors by incorporating multiple cri- teria (cytokine release, cellular activation markers, and cellular proliferation). No stimulatory activity was observed with regard to human T cell proliferation, cytokine production, or cell-surface marker expression when evaluated at early (24 h) and later (72 h) time points.

Table III. dAb EC50 values in the whole-blood RO assay

<table>
<thead>
<tr>
<th>Biologic</th>
<th>Human EC50 (ng/ml) ± SD</th>
<th>Cynomolgus EC50 (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>dAb-001</td>
<td>62 ± 28</td>
<td>66</td>
</tr>
<tr>
<td>dAb-002</td>
<td>53 ± 11</td>
<td>78</td>
</tr>
</tbody>
</table>

n = 3 for dAb-001 and n = 10 for dAb-002 for human CD28 RO; n = 1 for cynomolgus monkey CD28 RO.

![FIGURE 7. The impact of anti-CD28 dAb-001 on CTLA-4–mediated downregulation of CD86 on DCs. Monocyte-derived immature DCs were matured in the presence or absence of anti–CTLA-4–expressing Jurkat cells, and the impact of the anti-CD28 dAb-001, belatacept, and anti– CTLA-4 on CD86 expression was determined by FACS. Error bars represent SD of triplicate wells. Values are the mean ± SD of triplicate determinations. Data shown are representative of at least two experiments.](image)

![FIGURE 8. PK and T cell counts following the s.c. administration of anti-CD28 dAb-001 in cynomolgus macaques. (A) Mean plasma levels of anti-CD28 dAb-001 following the administration of three different single s.c. doses of dAb-001 to groups of two male and two female cynomolgus monkeys. (B) Mean of CD4+ T cell counts for four monkeys following the s.c. administration of anti-CD28 dAb-001 or an irrelevant Vκ dummy dAb. Data are mean ± SEM.](image)
finding, Poirier et al. (22) reported no agonist activity with the monomeric Fab fragment, FR104. However, it was recently demonstrated that even a Fab fragment of TGN1412, dry-coated on plates could drive T cell proliferation (45). In this case the activity was attributed to the conformational impact of C-region on the binding epitope of the Fab, a region missing in the dAb but present in FR104, indicating that valency alone is not a guarantee that a molecule will lack agonist activity and suggested that cytokine release stimulated by TGN1412 is different from that of other molecules.

Consistent with a lack of agonist activity with either dAb-001 or dAb-002, there were no signs of T cell depletion, expansion, and/or cytokine release in cynomolgus monkeys dosed with these dAbs (data not shown). Even though it was reported that no signal was observed in cynomolgous monkeys following treatment with TGN1412, there were signs of CD4+CD28+ T cell and CD20+ B cell depletion observed by day 8 following the first i.v. injection (46). They also reported profound T cell expansion and parallel cell activation around day 15, consistent with the expected primary PD activity of TGN1412. Furthermore, studies in H2d Rag-/-γc-/- reconstituted with a human immune system and treated with the mouse precursor molecule of TGN1412, mAb 5.11A1 showed rapid and profound depletion of circulating T cells that persisted through 60 d (47). Thus, it is generally considered that the available preclinical evidence was suggestive of the potential for generalized T cell activation in humans with CD28 superagonism and that monitoring T cell counts in the clinic is paramount when targeting CD28.

Another point learned from the TGN1412 clinical trials is the importance of defining the relationship between the level of CD28 RO and corresponding level of T cell activation. In the case of an agonist, this would be translated into RO versus efficacy, but in the case of TGN1412, this could have led to a more fundamental understanding of how RO leads to T cell activation, thus informing clinical dose escalation. Unfortunately, the first-in-human dose of TGN1412 was at a level that was retrospectively calculated to result in at least 90% CD28 RO (38). For this reason, it was critical to understand the relationship between PK and RO measurements as well as the relationship between RO and in vivo PD effects of anti-CD28 antagonist dAbs.

In dAb-treated cynomolgus monkeys, there was a strong correlation between drug concentrations and CD28 RO. When dAb-001 plasma concentrations declined to <61 ng/ml (5 nM), the CD28 RO was <50%, suggesting a direct link between drug concentrations and RO. This result was consistent with the in vitro CD28 RO EC50 of 66 ng/ml (5.4 nM) observed in monkey whole blood. Furthermore, the extent of the RO correlated well with the degree of inhibition on the KLH-induced IgG response. When the CD28 RO at 0.5 and 5 mg/kg was maintained at >80% for 2 wk, the KLH-induced IgG response was suppressed by >80% on day 15. In contrast, when the CD28 RO at 0.05 mg/kg was well <30% on day 15, there was no significant inhibition on the KLH-induced IgG response. These results suggest that the CD28 RO is a suitable biomarker that links drug exposure and functional effects. Therefore, this measurement may have use in determining dose selection for the first-in-human study.

In summary, we describe the discovery and characterization of monovalent anti-CD28 dAbs formatted with PEG. These CD28 dAbs were shown to bind CD28 with high affinity and potently inhibit T cell proliferation and cytokine production in vitro as well as a TDAR to KLH in vivo in monkeys. Given the clinical incident with TGN1412 and heightened concerns of any potential for agonist activity, special attention was given to evaluating the CD28 antagonist dAbs, in combination with anti-CD3 Abs, or following cross linking, to confirm the lack of any potential for agonistic activity. Studies in monkeys demonstrated a strong relationship among CD28 dAb PK, CD28 RO, and PD effect, in the absence of any apparent agonist activity. Overall, these results present novel CD28-specific dAbs devoid of agonist activity that were designed to offer more selective costimulatory blockade and potentially enhanced clinical efficacy for treatment of autoimmune diseases.

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Disclosures
References


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