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Kevin M. Dennehy; ... et. al

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CUTTING EDGE

Cutting Edge: Monovalency of CD28 Maintains the Antigen Dependence of T Cell Costimulatory Responses[1]

Kevin M. Dennehy,[*†] *Fernando Elias,*[*] *Gabrielle Zeder-Lutz,*[‡] *Xin Ding,*[§] *Danièle Altschuh,*[‡] *Fred Lühder,*[*¶] *and Thomas Hünig*[2*]

CD28 and CTLA-4 are the major costimulatory receptors on naive T cells. But it is not clear why CD28 is monovalent whereas CTLA-4 is bivalent for their shared ligands CD80/86. We generated bivalent CD28 constructs by fusing the extracellular domains of CTLA-4 or CD80 with the intracellular domains of CD28. Bivalent or monovalent CD28 constructs were ligated with recombinant ligands with or without TCR coligation. Monovalent CD28 ligation did not induce responses unless the TCR was coligated. By contrast, bivalent CD28 ligation induced responses in the absence of TCR engagement. To extend these findings to primary cells, we used novel superagonistic and conventional CD28 Abs. Superagonistic Ab D665, but not conventional Ab E18, predominantly ligates CD28 bivalently at low CD28/Ab ratios and induces Ag-independent T cell proliferation. Monovalency of CD28 for its natural ligands is thus essential to provide costimulation without inducing responses in the absence of TCR engagement. *The Journal of Immunology,* **2006, 176: 5725–5729.**

The destructive potential of the immune system necessitates tight control of effector mechanisms by positive and negative regulatory receptors. CD28 and CTLA-4 comprise one such pair of costimulatory receptors controlling proliferation and differentiation of naive T cells (1, 2).

Almost all physiological responses of naive T cells require costimulation via engagement of the clonotypic TCR by the appropriate Ag/MHC and by CD28 and its ligands CD80 and CD86 (3). Under exceptional circumstances, triggering of the TCR/CD3 complex alone is sufficient to induce proliferation. Examples include stimulation with CD3-specific Abs at high surface density (4) or physiological responses to high avidity Ag from the noncytolytic lymphocytic choriomeningitis virus (3, 5). At the other extreme of costimulatory responses, CD28-specific Abs referred to as "superagonists" are able to induce proliferation without TCR engagement (6, 7). But the mechanism of superagonist Ab stimulation remains obscure, and there is no physiological equivalent of such T cell activation.

Although CD28 and CTLA-4 are both homodimers, they bind to their shared ligand, CD80, differently (Fig. 1*A*). The cocrystal structure of CTLA-4 and CD80 shows that the two Ig domains of CTLA-4 form a V structure in which each arm is accessible to bind separate CD80 molecules (8). Because CD80 is predominantly also a bivalent homodimer, CTLA-4 and CD80 form an unusually stable lattice structure. Biacore studies demonstrate that CD28, in contrast to CTLA-4, is functionally monovalent (9). To date there is no crystal structure of the CD28 homodimer. However, the structure of the human CD28 monomer in complex with a superagonist CD28 Ab Fab′ has recently been solved (10). Modeling based on this structure suggests that the CD28 Ig domains are arranged in a U structure. Although both arms are available for ligand binding, simultaneous binding of separate CD80 monomers would be prevented by a physical clash of the C-set domains. The CD28 homodimer is thus functionally monovalent, whereas CTLA-4 is bivalent.

In this study, we have addressed the influence of valency on CD28 function using CD28 chimeric molecules and superagonistic CD28-specific Abs. We show that monovalency of CD28 is essential for physiological costimulation. Monovalent ligation of CD28 induces efficient costimulation without inducing responses in the absence of TCR ligation, whereas bivalent ligation with natural ligands alone induces T cell activation. These results indicate that monovalency of CD28/ligand interactions is an important safeguard that keeps T cell activation under the control of the TCR under physiological conditions.

Materials and Methods

Abs, cell lines, and constructs

Abs to rat TCR$\alpha\beta$ (R73), and recombinant rat CTLA-4IgGFc have been described previously (11, 12). Recombinant mouse CD28IgGFc and human CD80IgGFc were from R&D Systems, sheep anti-mouse IgG was from Roche, and mouse anti-human IgG was from Dianova.

Chimeric CD28, comprising the extracellular region of rat CD28 (rCD28)[3] and the intracellular regions of mouse CD28 (mCD28), and chimeric mCD28,

*Institute for Virology and Immunobiology, University of Würzburg, Würzburg, Germany; †Institute of Infectious Disease and Molecular Medicine, University of Cape Town, Observatory, South Africa; ‡Unité Mixte de Recherche 7175, Centre National de la Recherche Scientifique, Ecole Supérieure de Biotechnologie de Strasbourg, Illkirch, France; §TeGenero Immunotherapeutics, Würzburg, Germany; and ¶Institut für Multiple Sklerose Forschung, Göttingen, Germany

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[2] Address correspondence and reprint requests to Prof. Thomas Hünig, Institute for Virology and Immunobiology, University of Würzburg, Versbacherstrasse 7, 97078 Würzburg, Germany. E-mail address: huenig@vim.uni-wuerzburg.de

[3] Abbreviations used in this paper: rCD28, rat CD28; mCD28, mouse CD28; RU, resonance unit(s).

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grafted with the human C′D loop (aa 60–65) and the rat ligand binding region (m/rCD28 1–66), have been described (7). To generate the CTLA-4mCD28 chimera, mCD28 intracellular domains amplified using 5′-TTTGGATCCTG GTCGTGGTTGCTGGAGT-3′ AND 5′-ATATAAGATCTTCAGGGGC GGTACGCTGCAAA-3′ were digested with *Bam*H1/*Bgl*11. The human CTLA-4 extracellular domain was amplified using 5′-CAGAATTCCACCAT GGCTTGCCTTGGATTTCAGCG-3′ and 5′-CAA**GGATCC**AGAGGAG GAAGTCAGAATCTGGGCACG-3′, digested with *Eco*R1/BamH1, and ligated with the mCD28 fragment into pczCFG5IEGN *Eco*R1/*Bam*H1. The chimera comprises CTLA-4 aa M1–I166 fused to CD28 aa L154–P218.

For generation of CD80mCD28 chimera, CTLA4mCD28 intracellular domains were amplified using 5′-CCAGAAGACCCTCCTGATTCAGACTTC CTCCTTTG-3′ and 5′-ATATAAGATCTTCAGGGGCGGTACGCTG CAAA-3′ and digested with Bbv11/Bgl11. The rCD80 extracellular domains were amplified using 5′-TTGAATTCCACCATGGCTTACAGTTGC CAGCTGA-3′ and 5′-ATAGGATCCAGGCAAACGGAATTGT-3′, digested with *Eco*R1/*Bbv*11, and ligated with the CTLA4mCD28 fragment into pczCFG5IZ *Eco*R1/*Bam*H1. The chimera comprises CD80 aa M1–P244 fused to CTLA-4 aa D159–I166 and CD28 aa L154–P218. Constructs in pczCFG5IZ/IEGN were transduced into 58 cells as described (7).

Stimulation, ELISA, and proliferation assays

For costimulation of cell lines (*lanes 2* and *3* in Fig. 1, *B* and *C*, and Fig. 2*B*), plates for nonadherent cells (Greiner Bioscience) precoated with sheep anti-mouse IgG were coated with 2 μg/ml TCR Ab, washed, and then coated with 10 μg/ml protein G′ (Sigma-Aldrich) to ensure immobilization of ligands containing human IgGFc. CD28 chimeras were ligated by the addition of 2 μg/ml CD80/CD28/CTLA4Ig fusion proteins to 4×10^5 cells/ml in solution immediately before plating. For stimulation of CD28 chimeras alone (*lane 4* in Fig. 1, *B* and *C*, and *lanes 4* and *5* in Fig. 2*B*), plates were coated with mouse anti-human IgG, washed, and then coated with 10 μg/ml protein G′ (Sigma-Aldrich) to ensure high surface density of ligands. Cells were mixed with 10 μg/ml soluble CD80/CD28/CTLA4Ig fusion proteins immediately before plating. IL-2 in the supernatant was assayed after 2–3 days using Opti-EIA kits (BD Biosciences).

For Fig. 4*C*, $CD4^+$ T cells purified by nylon wool passage (1×10^6/ml) were labeled with CFSE and stimulated with 10 μg/ml E18 conventional or D665 superagonistic CD28 Abs on plates precoated with sheep anti-mouse IgG. After 4 days, CFSE dilution was measured by flow cytometry. Stimulation of purified T cells with all superagonistic mAbs require the addition of 5–10 μg/ml mAb to cells in solution on plates, such as anti-mouse IgG-coated plates, that allow mAb immobilization at high density as described (6). For costimulation, CD28 mAbs can be added in solution at 10- to 100-fold lower concentrations to cells on TCR mAb-coated plates as described (13).

Generation of mouse anti-mCD28 Abs and surface plasmon resonance

$CD28^{-/-}$ mice (B6.129S2-Cd28^{tm1Mak}/J from The Jackson Laboratory) were immunized alternately with A20 cells expressing mCD28 i.p. and with recombinant mCD28Ig s.c. in TiterMax (Alexis). After boosting i.v. with CD28Ig, splenic cells were fused with X63Ag8.653 cells as described (7).

Abs were captured at 51–53 resonance units (RU) or 62–64 RU for D665 and E18, respectively, on an anti-mouse coated CM5 sensor surface (BR-1000-14; Biacore), and CD28Ig was repeatedly injected at concentrations of 1000 or 20 nM. Stoichiometry was calculated using the following equation: stoichiometry = $(RU_{Ag}/RU_{mAb}) \times (MW_{mAb}/MW_{Ag})$, where MW is molecular weight. Binding of CD28 at 1000 nM to D665 and E18 was comparable, with stoichiometries of >1.5 CD28 homodimers per Ab, indicative of monovalent CD28 binding. Based on the assumption that both Abs bind with a stoichiometry of 2 at 1000 nM, stoichiometries were corrected assuming that 80 and 65% of D665 and E18 paratopes, respectively, are active.

Results

Bivalent ligation of CD28 with natural ligands induces IL-2 production in 58 TCR$\alpha\beta$ cells

To study the effect of CD28 ligation valency on costimulation, we first generated an experimental system that mimics physiological costimulation. We used 58 cells transduced with rat TCR, which produce only low levels of IL-2 after TCR ligation with R73 Ab (Fig. 1*B*) (7). 58 cells do not express detectable endogenous CD28, making this cell line appropriate for investigating the signaling capacity of ectopically expressed CD28 constructs. Stimulation of 58 TCR$\alpha\beta$ cells ectopically expressing wild-type CD28 with recombinant CD80Ig did not induce

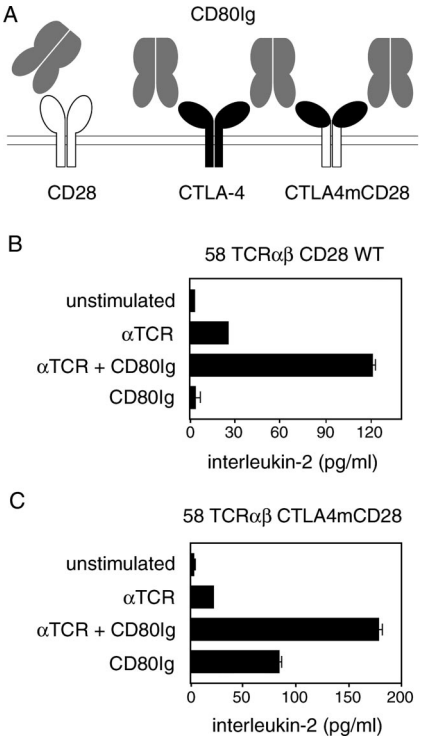

FIGURE 1. Bivalent ligation of CD28 with recombinant CD80Ig induces IL-2 production in 58 TCR$\alpha\beta$ cells. *A*, CD28 is monovalently ligated, whereas CTLA-4 is bivalently ligated by recombinant CD80Ig. The bivalent construct CTLA4mCD28 was generated by fusing the extracellular domains of CTLA-4 with the intracellular domains of CD28. *B* and *C*, 58 TCR$\alpha\beta$ cells expressing wild-type (WT) CD28 (*B*) or CTLA4mCD28 (*C*) were stimulated as indicated, and IL-2 production was measured by ELISA.

IL-2 production. However, costimulation via this receptor-ligand interaction induced high levels of IL-2 (Fig. 1*B*). Our system using 58 TCR$\alpha\beta$ cells expressing CD28 therefore corresponds to physiological costimulation with natural ligands in regard to the requirement for the ligation of both the TCR and CD28 in the induction of a functional response (6, 14).

Next, we generated a bivalent form of CD28. The extracellular domain of human CTLA-4 was fused with the transmembrane and intracellular domains of mCD28. The chimera, referred to as CTLA4mCD28, was expressed on 58 TCR$\alpha\beta$ cells at comparable levels to those of ectopically expressed wild-type CD28 on this cell line (data not shown). Stimulation of 58 TCR$\alpha\beta$ CTLA4mCD28 cells with TCR-specific Ab induced low levels of IL-2 production, which were markedly increased upon costimulation with CD80Ig (Fig. 1*C*). Importantly, stimulation of these cells with CD80Ig alone induced substantial IL-2 production. In this system, bivalent ligation alone is thus sufficient to induce cytokine production. Monovalent ligation of CD28 provides an efficient costimulatory stimulus but has no effect without TCR engagement.

A caveat of this interpretation is that we use an overexpression system to compare cell lines that may have differential sensitivity to costimulatory signals. To address these concerns, we designed an experimental system in which we directly compare differential ligation in the same cell line. By reversing the receptor-ligand pairs, we generated a CD28 chimera expressing the extracellular domains of CD80 fused to the intracellular domains of mCD28 (Fig. 2*A*). In contrast to Fig. 1, where we compare different CD28 constructs sharing the same ligand, we

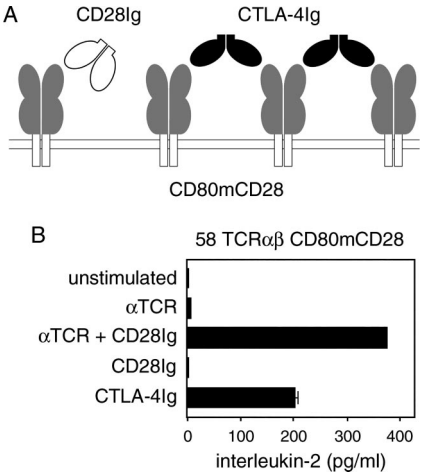

FIGURE 2. Ligation of CD28 with bivalent but not monovalent natural ligand pairs activates 58 TCR$\alpha\beta$ cells. *A*, A chimera comprising the extracellular domains of CD80 and intracellular domains of CD28 was stimulated with monovalent CD28Ig or bivalent CTLA-4Ig. IgGFc domains are not shown. *B*, 58 TCR$\alpha\beta$ cells expressing CD80mCD28 were stimulated as indicated, and IL-2 production was measured by ELISA.

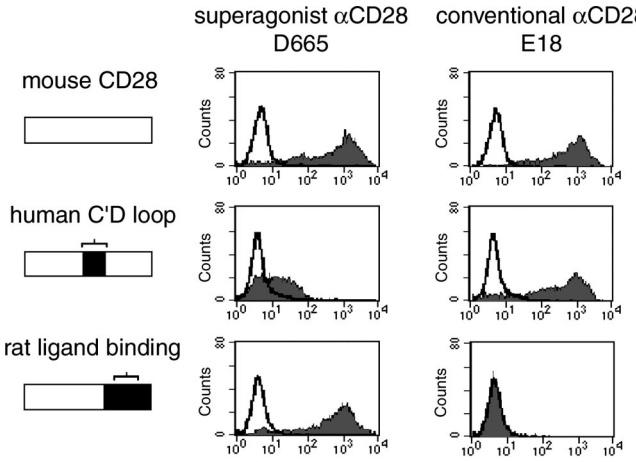

FIGURE 3. D665 Ab binds the C′D loop of mCD28, whereas E18 Ab binds to the ligand binding region. Binding of D665 (*middle panel*) and E18 (*right panel*) to L cells expressing CD28 constructs schematically depicted on the *left* is shown. Unshaded histograms show negative control staining.

now compare differential ligation of the CD80mCD28 construct by monovalent CD28Ig or bivalent CTLA4Ig ligands. Ligation with monovalent CD28Ig did not induce IL-2 production but provided an efficient costimulatory stimulus (Fig. 2*B*). By contrast, bivalent ligation with CTLA4Ig induced IL-2 production in the absence of TCR ligation.

Bivalent ligation of CD28 induces Ag-independent proliferative responses of primary peripheral T cells

To extend these findings to primary cells, we used novel Abs to mCD28 corresponding to previously described superagonistic and conventional Abs (6, 7, 13). Superagonistic Abs, such as 5.11A in human and JJ316 in rat, bind the laterally exposed C′D loop of CD28 and induce T cell proliferation without TCR ligation. Conventional Abs, such as JJ319 in rat and 37.51 in mouse, are strictly dependent on TCR ligation to induce T cell activation. For binding to CD28, both conventional Abs require integrity of residue 98 adjacent to the CD80 ligand binding loop and, thus, likely reflect natural ligand binding although with higher affinity. Two Abs (D665 and E18) that bound comparably well to mCD28 expressed on L cells (Fig. 3) were chosen. D665, hereafter referred to as a superagonistic Ab, induced proliferation of purified T cells, whereas E18, referred to as a conventional Ab, did not (data not shown, Fig. 4*C*). Binding of D665 to mCD28 grafted with the human C′D loop was severely diminished, whereas E18 binding was not affected. D665 therefore binds an epitope corresponding to that recognized by previously described CD28 superagonists (7). Binding of E18 to mCD28 grafted with the ligand binding region of rCD28 was abolished, but D665 binding was not affected. Given that the only difference between mouse and rat in the ligand binding region in the CD28 Ig domain is residue 98, E18 binds an epitope corresponding to that recognized by previously described conventional CD28 Abs.

We used surface plasmon resonance to assess the valency of CD28 for binding to D665 and E18 Abs. The two Abs were immobilized on a sensor surface at comparable levels and allowed to interact with recombinant CD28Ig homodimer injected on the surfaces at either high (Fig. 4*A*) or low (Fig. 4*B*) concentrations. At high concentrations, binding of CD28 to the two Abs was comparable with stoichiometries approaching 2, indicating that two CD28 homodimers are monovalently bound per Ab (Fig. 4*A*). Postinjection phases were similar, indicating similar dissociation rates and suggesting that the Abs have comparable affinity for CD28 (data not shown). At low concentrations, CD28 was bound by an E18 conventional Ab at a level approximately twice that of the D665 superagonist (Fig. 4*B*) with maximal stoichiometries of 1.6 and 0.7, respectively. These data indicate that under these conditions CD28 homodimers are predominantly ligated monovalently by the E18 conventional Ab but bivalently ligated by the D665 superagonist. The observation that the stoichiometry of the CD28/E18 Ab was <2 suggests that a proportion of CD28 may also be bivalently ligated by conventional Ab. The lower stoichiometry of 0.7 for CD28 complexed with the D665 superagonist, however, indicates that bivalent ligation of CD28 occurs more efficiently by the D665 superagonist than by the E18 conventional Ab.

To more rigorously test our earlier conclusion that superagonistic Abs induce T cell proliferation without the need for TCR ligation (6), we used C57BL/6 RAG$^{-/-}$ mice transgenically expressing OT-2 TCR in which the TCR repertoire is restricted to one specificity (IA^b/Ova). Stimulation with the D665 superagonist induced proliferation of transgenic T cells, and no proliferation was induced by stimulation with the conventional Ab E18. Because neither the Ag nor the presenting MHC class II molecule was present in these cultures, this experiment provides an example of an artificial ligand that bivalently ligates CD28 and induces Ag-independent proliferation of primary T cells.

Discussion

The surprising finding that CD28 is monovalent whereas CTLA-4 is bivalent raises a variety of questions on the mechanisms of T cell costimulation. We show here that CD28 must be monovalent to induce a costimulatory responses without inducing responses in the absence of TCR engagement.

We use superagonistic Abs to demonstrate that, if appropriately ligated, CD28 has the potential to induce Ag-independent

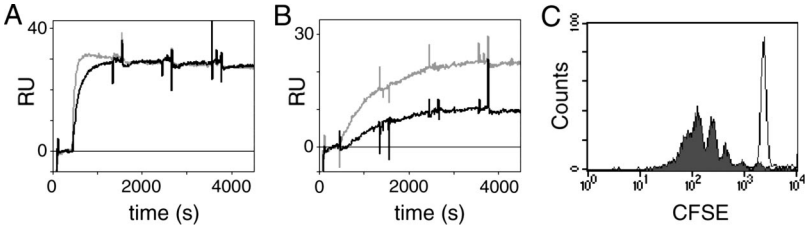

FIGURE 4. Superagonistic Ab D665, but not conventional Ab E18, bivalently ligates CD28 at low CD28/Ab ratios and induces Ag-independent proliferation of primary T cells. *A* and *B*, Recombinant CD28Ig at concentrations of 1000 nM (*A*) or 20 nM (*B*) was injected over a sensor surface with D665 (black line) or E18 (gray line) immobilized at comparable levels, and binding was measured over time. *C*, CFSE-labeled $CD4^+$ T cells from C57BL/6 $RAG^{-/-}$ OT-2 TCR transgenic mice were stimulated with D665 (shaded histogram) or E18 (open histogram), and proliferation was measured by CFSE dilution after 4 days.

T cell responses in primary peripheral T cells. We show in cell lines that such responses are also induced by bivalent ligation with natural ligands. By contrast, monovalent ligation with CD80Ig or the E18 conventional Ab induces efficient costimulatory responses without inducing responses in the absence of TCR ligation. We therefore conclude that monovalency of CD28 for its natural ligands is essential for its costimulatory function.

From the cocrystal structure of CD28 monomer and superagonistic 5.11A Fab′, it is evident that this Ab bivalently ligates CD28, likely forming a CTLA-4-CD80 lattice-like structure (10). Surface plasmon resonance data indicate that mCD28 is also bivalently ligated by the superagonistic Ab D665 and predominantly monovalently ligated by the E18 conventional Ab (Fig. 4). We note that some other conventional Abs bivalently ligate CD28Ig fusion proteins (9, 10). But it is not clear whether this observation also holds true for binding of these Abs to cell surface-bound CD28. For example, the conventional human CD28-specific Ab 7.3B6 was shown by cryoelectromicroscopy to ligate CD28Ig fusion proteins at an angle that may well be incompatible with the structural constraints imposed on neighboring CD28 homodimers by their insertion into the cell membrane. Differences in the efficiency with which D665 superagonistic and E18 conventional Abs bivalently ligate CD28 may provide an explanation for the mechanistic difference between stimulation with these Abs.

Because superagonists bind to a membrane proximal epitope and parallel to the cell surface, whereas conventional Abs bind a membrane distal epitope, Evans et al. (10) argue that the different topology of ligated Abs explain their different stimulatory capacities. Closer membrane approximation to the immobilizing surface allowed by superagonists bound to CD28 may prevent access of large phosphatases like CD45, allowing signaling to proceed. By contrast, the extended structure of conventional Abs bound to CD28 may prevent close membrane approximation and allow access of phosphatases that limit signaling. Our data do not support this interpretation, because our ligated monovalent and bivalent recombinant ligands share the same dimensions. We favor an interpretation in which the periodicity and increased stability of CD28 complexes in a lattice formed by bivalent ligation (but not necessarily by all Abs that bivalently ligate CD28) allow efficient assembly of signaling complexes. The mechanisms of superagonist Ab stimulation remain, however, a moot point.

Another argument supporting the importance of monovalent CD28 ligation comes from the comparison of the stability of CD28 and CTLA-4 bound to CD80. Differential engagement by CD80 results in an ~100-fold difference in stability (9). Given that CTLA-4 is expressed only at low levels on activated T cells or regulatory T cells, such differences in stability are likely to be essential for effective CTLA-4 function (15–17).

Lastly, early pharmacological studies suggested that the TCR/CD3 and CD28 signaling pathways are separate (18–20). However, a number of signaling molecules essential for TCR signaling, such as SLP-76 and Vav, are phosphorylated during superagonistic Ab stimulation (14). Moreover, although ligation of the TCR is not required for superagonistic signaling, expression of the TCR/CD3 complex is required (7). We therefore argue that CD28 is not an autonomous receptor. A likely explanation may be that CD28 signals amplify TCR signals downstream of TCRξ, ZAP70, and LAT (linker for activation of T cells) at the level of the SLP-76 signalosome (14). Monovalent ligation of CD28 with either natural ligands or conventional Ab may provide a physiologically relevant amplification system, allowing costimulatory signaling without inducing responses in the absence of TCR ligation. In contrast, bivalent ligation may induce an amplification loop that amplifies the tonic TCR signals, which are required by T cells (21, 22), to a level that suffices to trigger functional T cell responses. In this scenario, bivalent CD28 ligation would induce a physiological but dysregulated signaling pathway. In this respect, superagonistic Abs will be an invaluable tool to dissect TCR and CD28 signal integration.

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Disclosures

T.H. declares a commercial interest in TeGenero Immunotherapeutics AG, Würzburg, Germany.

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