T Lymphocyte Costimulation Mediated by Reorganization of Membrane Microdomains

Antonella Viola,* Susanne Schroeder, Yoichi Sakakibara, Antonio Lanzavecchia

Although dispensable, costimulation through CD28 facilitates activation of naïve T lymphocytes. CD28 engagement led to the redistribution and clustering of membrane and intracellular kinase-rich raft microdomains at the site of T cell receptor (TCR) engagements. Although not affecting TCR down-regulation, this process led to higher and more stable tyrosine phosphorylation of several substrates and higher consumption of Lck. These results may provide a general mechanism for amplifying receptor signaling by reorganization of membrane microdomains.

The outcome of TCR stimulation is regulated by the simultaneous engagement of the costimulatory molecule CD28. Mice lacking CD28 can still mount T cell responses but require repeated stimulations with high doses of antigen (*1*). In vitro studies demonstrated that in the absence of CD28 engagement, T cells require a very high TCR occupancy and a prolonged stimulation, whereas when costimulated through CD28 they respond more rapidly to lower levels of TCR occupancy (*2*, *3*). Although CD28 can specifically enhance distinct signaling pathways leading to gene transcription (*4*–*6*), the mechanism by which CD28 facilitates T cell activation remains elusive. One possibility is that CD28 might exert its costimulatory action by acting as a general amplifier of early TCR signaling.

To study the mechanism of CD28 costimulation, we stimulated human resting T cells with surrogate antigen-presenting cells (APCs), consisting of beads coated with various concentrations of antibody to CD3 (anti-CD3) in the presence or absence of anti-CD28 (*7*). Resting T cells proliferated in response to beads coated with anti-CD3 plus anti-CD28, but not to beads coated with anti-CD3 alone (Fig. 1A). An enhancing effect of CD28 engagement was also evident when early tyrosine phosphorylation events were measured. Stimulation of resting T cells with anti-CD3– plus anti-CD28–coated beads induced more tyrosine phosphorylation on multiple proteins and, as compared with beads coated with anti-CD3 alone, shifted the dose-response curve to at least 10-fold lower levels of anti-CD3 (Fig. 1B). The different responses did not reflect different numbers of TCR triggering events because the same number of TCR-CD3 complexes was down-regulated in the absence or presence of costimulation (Fig. 1C). These results confirm previous experiments showing that engagement of CD28 increases the T cell response (*2*) as well as early tyrosine phosphorylation (*8*) and show that this effect is not due to higher numbers of TCRs triggered. To explain this paradox we considered the possibility that the increased tyrosine phosphorylation observed when CD28 was engaged might be due to the presence of phosphorylated molecules for a longer time.

To fully activate T cells, antigen-stimulated TCR signaling needs to be sustained for up to several hours (*9*), which requires ongoing TCR triggering. As soon as the serial triggering process is blocked, signaling, as measured by Ca^{2+} increases or acid production, is terminated within minutes (*10*). To measure the lifespan of phosphorylation induced by TCR triggering, we stimulated T cells with beads and analyzed tyrosine phosphorylation before and at various times after the addition of the powerful tyrosine kinase inhibitor {4-amino-5-(4-methyphenyl)-7-(t-butyl)pyrazolo [3,4-d]pyrimidine} (PP1) (*11*). In the absence of costimulation through CD28, all the tyrosine-phosphorylated substrates disappeared rapidly after the addition of PP1 and were undetectable within less than 30 s. In contrast, when costimulation was provided, tyrosine phosphorylation was more persistent because it was still detectable after 2 min (Fig. 2A). This increased stability was observed for all of the most prominent tyrosine-phosphorylated substrates including LAT, an adapter molecule that links TCR to downstream effectors (*12*, *13*). In addition, CD28 engagement resulted in increased degradation of Lck (Fig. 2B), indicating that more kinase is recruited and consumed by the same number of triggered TCRs.

The above results show that tyrosine phosphorylation, which is rapidly induced after TCR engagement (*14*), is very transient because it is rapidly lost as soon as kinases are inhibited. CD28, rather than acting on a distinct pathway, acts early on by enhancing global aspects of TCR signaling. Costimulation results in higher steady-state levels of phosphorylation because of increased recruitment of Lck and increased stability of phosphorylation, possibly as a result of lower activity or accessibility of phosphatases.

To explain how CD28 might globally increase TCR-induced Lck consumption and substrate phosphorylation, we considered the possibility that CD28 induces a reorganization of the signaling machinery. The plasma membrane is composed of discrete lipid microdomains in which membrane molecules are differentially partitioned (*15*). These sphingolipid-cholesterol–rich rafts concentrate glycophosphatidylinositol-linked proteins, glycosphingolipids, as well as several molecules involved in signal transduction such as Lck, LAT, Ras, and guanosine triphosphate–binding proteins (G proteins) (*16*). In polarized cells rafts are concentrated at the apical surface, whereas in nonpolarized cells they are dispersed over the cell surface as small domains of <70 nm (*17*). We tested whether CD28 engagement affects the distribution of

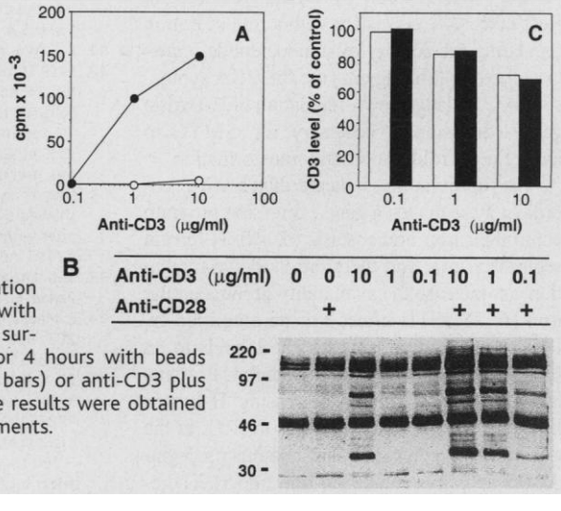

Fig. 1. Costimulation by CD28 promotes T cell activation and enhances TCR-induced phosphorylation, but does not increase the number of TCRs triggered. (**A**) Proliferative response of resting $CD4^+$ T cells cultured with polystyrene beads coated with various concentrations of anti-CD3 with (●) or without (○) anti-CD28. (**B**) Patterns of tyrosine phosphorylation induced in resting T cells by stimulation with beads coated with anti-CD3 with or without anti-CD28. (**C**) Levels of surface TCRs in T cells stimulated for 4 hours with beads coated with anti-CD3 alone (open bars) or anti-CD3 plus anti-CD28 (filled bars). Comparable results were obtained in at least five independent experiments.

Basel Institute for Immunology, Grenzacherstrasse 487, CH 4005 Basel, Switzerland.

*To whom correspondence should be addressed. E-mail: viola@bii.ch

rafts using as a raft marker fluorescein isothiocyanate (FITC)–labeled cholera toxin (CTx) B subunit, which binds the GM1 glycosphingolipid (*18*) (Fig. 3, A to C). In unstimulated resting T lymphocytes the surface distribution of GM1 appeared homogeneous, and this pattern did not change when the cells were stimulated with anti-CD3–coated beads. By contrast, when CD3 and CD28 were simultaneously engaged, within 15 min GM1 had redistributed to form a dense cap that contacted the stimulating bead, indicating that rafts had aggregated in the zone of contact. A time-dependent increase in CTx binding was measured by flow cytometric analysis in T cells conjugated with anti-CD3 plus anti-CD28 beads (Fig. 3D). Whereas in effector T cells GM1 was present at high levels only on the cell surface, in resting T cells it was mainly intracellular and was present only at low levels on the cell surface (Fig. 3E).

The above results indicate that CD28 engagement promotes redistribution of rafts at the TCR contact site, a process that may amplify and sustain TCR-induced signaling by segregating strategically important molecules. We therefore asked whether passive clustering of rafts and triggered TCRs would provide costimulation to resting T cells (*19*). Both CTx or a monoclonal antibody to the GPI-anchored protein CD59 provided efficient costimulation when immobilized on culture wells together with anti-CD3 (Fig. 4A). However, when presented on anti-CD3–coated beads, no costimulatory effects were observed (Fig. 4B). These results suggest that passive aggregation of rafts by cross-linking of rafts-associated molecules can provide effective costimulation under the favorable conditions afforded by a large surface area of interaction. However, under less favorable conditions provided by the beads, GM1 and CD59, in contrast to CD28, failed to reorganize rafts into an active configuration. Indeed, GM1 was not redistributed in T cells stimulated by anti-CD3– plus anti-CD59–coated beads (*13*).

Kupfer *et al.* have shown that T cells polarize toward the APCs, thereby forming a highly structured synapse. This process involves rearrangements of the T cell's cytoskeleton as well as redistribution of surface molecules. Some molecules, such as TCR, CD4, CD2, and CD28, are enriched in a central zone, whereas others, such as LFA-1 and CD45, remain outside (*20*). TCR triggering leads to T cell polarization (*21*) and to a redistribution of membrane molecules that is mediated by CD2 (*22*). Our

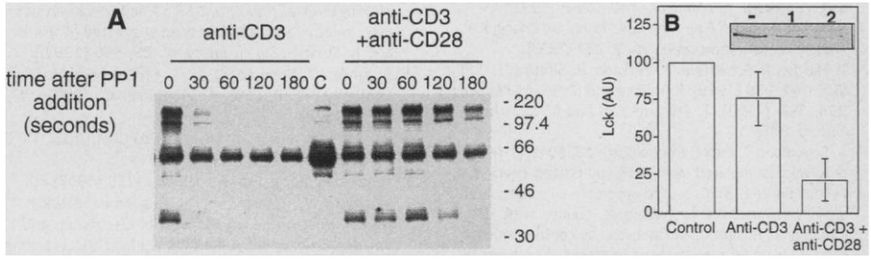

Fig. 2. Increased stability of TCR-induced phosphorylation and increased activation and consumption of Lck induced by costimulation through CD28. (**A**) Resting T cells were stimulated with beads coated with anti-CD3 (10 μg/ml) with or without anti-CD28. C, unstimulated control cells. The pattern of tyrosine phosphorylation was determined at various times after the addition of 10 μM PP1. Comparable results were obtained in six independent experiments. (**B**) Lck consumption after T cell activation. (**Inset**) Lck was measured by protein immunoblotting in T cells stimulated for 1 hour with anti-CD3 (lane 1) or anti-CD3 plus anti-CD28 (lane 2). Histograms represent the mean values of four experiments. Error bars are standard deviations. AU, arbitrary units.

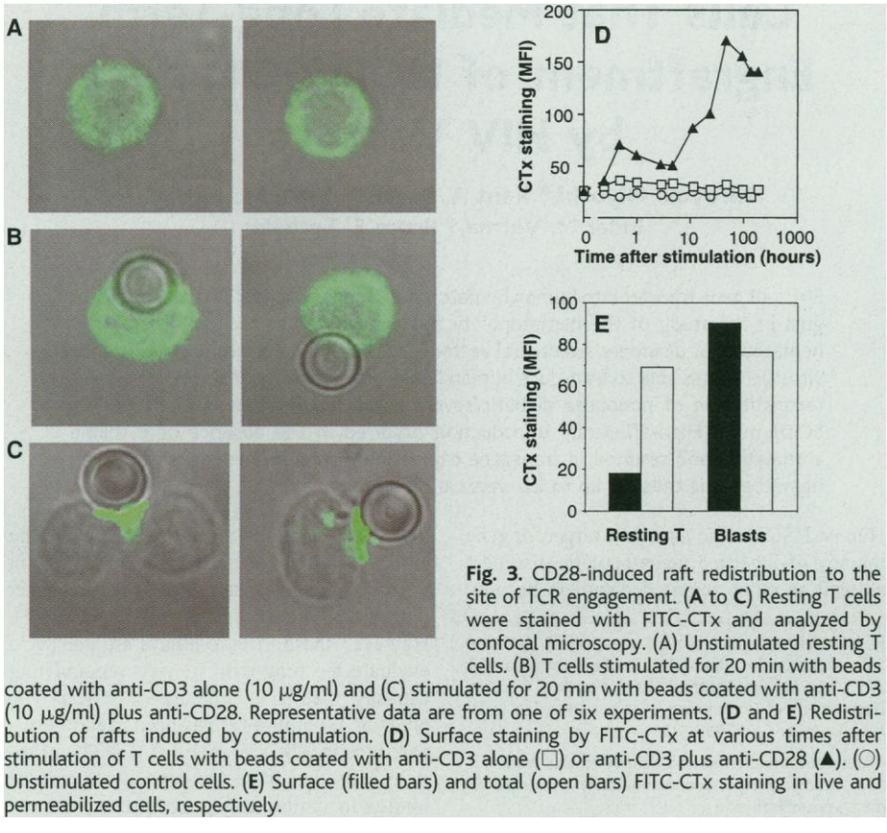

Fig. 3. CD28-induced raft redistribution to the site of TCR engagement. (**A** to **C**) Resting T cells were stained with FITC-CTx and analyzed by confocal microscopy. (A) Unstimulated resting T cells. (B) T cells stimulated for 20 min with beads coated with anti-CD3 alone (10 μg/ml) and (C) stimulated for 20 min with beads coated with anti-CD3 (10 μg/ml) plus anti-CD28. Representative data are from one of six experiments. (**D** and **E**) Redistribution of rafts induced by costimulation. (**D**) Surface staining by FITC-CTx at various times after stimulation of T cells with beads coated with anti-CD3 alone (□) or anti-CD3 plus anti-CD28 (▲). (○) Unstimulated control cells. (**E**) Surface (filled bars) and total (open bars) FITC-CTx staining in live and permeabilized cells, respectively.

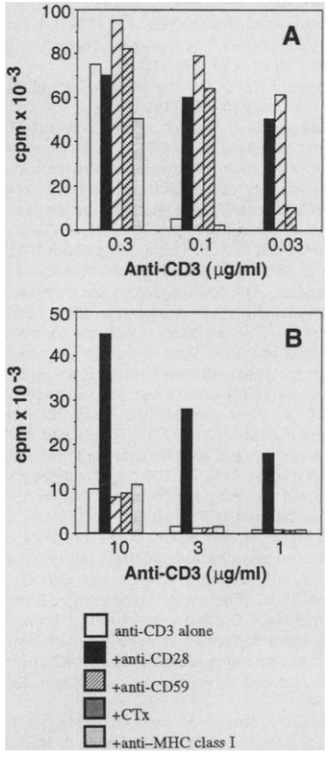

Fig. 4. Costimulation provided by passive clustering of rafts. (**A**) Proliferative response of T cells stimulated with various concentrations of plastic-bound anti-CD3 alone or with anti-CD3 plus anti-CD28, anti-CD59, CTx, or anti–MHC class I. (**B**) Proliferative response of T cells stimulated by beads coated with various doses of anti-CD3 alone or with anti-CD3 plus anti-CD28, anti-CD59, CTx, or anti–MHC class I. Comparable results were obtained in three experiments.

results are consistent with this model, but in addition demonstrate that ongoing TCR triggering is required to sustain the signaling process. We have shown here that CD28 participates in the organization of the immune synapse by recruiting rafts into it (*23*). The fact that cross-linking of the rafts produced levels of costimulation comparable to that of CD28 engagement suggests that the costimulatory effect of CD28 in resting T cells is mediated to a large extent by its effect on raft redistribution.

Recent evidence suggests that membrane compartmentalization between rafts and nonrafts is required for efficient T cell activation (*24*). The recruitment of rafts to the site of TCR engagement may represent a general mechanism by which costimulation can increase the signaling process. This may result from increased recruitment of kinases and segregation of phosphorylated substrates from phosphatases (*25*).

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7. Human peripheral blood $CD4^+$ T cells were sorted by negative selection. Polystyrene Latex microsheres (Polysciences) were coated with various concentrations of anti-CD3 (clone TR66) in phosphate-buffered saline, followed by coating with anti-CD28 (clone CD28.1) at 10 μg/ml. T cells (5×10^4) were cultured with 10^5 beads in 200 μl of RPMI 10% fetal calf serum in round-bottom microplates. TCR down-regulation was measured after 4 hours and thymidine incorporation after 72 hours as described (*2*). Costimulation was not observed when anti-CD3 and anti-CD28 were presented on separate beads nor when antibodies to major histocompatibility complex (MHC) class I was used instead of anti-CD28 (A. Viola, unpublished data). To detect tyrosine phosphorylation, 10^6 T cells and 10^6 beads were centrifuged and the sediment was incubated for 5 min at 37°C in 100 μl of culture medium, followed by lysing for 30 min at 4°C in 1% NP40 buffer [20 mM tris-HCl (pH 7.5), 150 mM NaCl, 1 mM $MgCl_2$, 1 mM EGTA] in the presence of protease and phosphatase inhibitors [aprotinin (Sigma, 10 μg/ml), leupeptin (Sigma, 10 μg/ml), 1 mM Pefabloc-SC (Boehringer Mannheim), 50 mM NaF, 10 mM $Na_4P_2O_7$, and 1 mM $NaVO_4$]. Immunoblottings were performed as described with anti-phosphotyrosine mono-clonal antibody 4G10 (*26*).
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19. T cells were stimulated in tissue culture wells that had been coated with anti-CD3 at various concentrations followed by addition of antibodies to CD28 (CD28.1), CD59 (p282, Pharmingen), MHC class I (W6/32) (all at 10 μg/ml), or CTx (Sigma, 20 μg/ml).
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Transduction of Human $CD34^+$ Cells That Mediate Long-Term Engraftment of NOD/SCID Mice by HIV Vectors

Hiroyuki Miyoshi,* Kent A. Smith,* Donald E. Mosier, Inder M. Verma,† Bruce E. Torbett

Efficient gene transfer into human hematopoietic stem cells (HSCs) is an important goal in the study of the hematopoietic system as well as for gene therapy of hematopoietic disorders. A lentiviral vector based on the human immunodeficiency virus (HIV) was able to transduce human $CD34^+$ cells capable of stable, long-term reconstitution of nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice. High-efficiency transduction occurred in the absence of cytokine stimulation and resulted in transgene expression in multiple lineages of human hematopoietic cells for up to 22 weeks after transplantation.

Human HSCs are an attractive target for gene therapy of inherited hematopoietic disorders as well as other acquired disorders because these cells have the ability to regenerate the entire hematopoietic system. A number of in vitro assays have been established to detect pluripotent human hematopoietic cells (*1*). However, these in vitro assays are unable to evaluate the long-term in vivo repopulating capacity that is a hallmark of HSCs. The NOD/SCID mouse (*2*) has been used to evaluate human HSCs in vivo (*3*). $CD34^+$ primitive cells that have the capacity to initiate long-term multilineage engraftment in these

H. Miyoshi and I. M. Verma, Laboratory of Genetics, Salk Institute for Biological Studies, La Jolla, CA 92037, USA. K. A. Smith, D. E. Mosier, B. E. Torbett, Department of Immunology, Scripps Research Institute, La Jolla, CA 92037, USA.

*These authors contributed equally to this work.
†To whom correspondence should be addressed. E-mail: verma@salk.edu