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CD28 Signals in the Immature Immunological Synapse[1]

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T cell recognition of peptide-MHC complexes on APCs results in the aggregation of TCRs at a central supramolecular activation complex (c-SMAC) within a mature immunological synapse. T cells require a second "costimulatory" signal for activation, the most important of which, for naive T cells, is from CD28. However the time at which CD28-derived signals are induced relative to c-SMAC formation is not well understood. In this study, we have assessed the kinetics of CD28 localization and function relative to well-established aspects of c-SMAC formation. CD28 accumulates at the immature synapse alongside the TCR and is likewise enriched at the synapse at the onset of the calcium signal. In addition, using CD28 deficient or reconstituted murine cells in a single-cell recording approach shows that CD28 regulates this signal within seconds of a TCR-mediated rise in intracellular calcium levels. Finally, CD28 exerts effects on both the initiation and stabilization of the synapse in parallel with its effects on the downstream proliferation of T cells. Together, the data show that CD28 functions in the immunological synapse before the formation of the c-SMAC. ***The Journal of Immunology,*** **2004, 172: 5880–5886.**

Recent evidence has identified the formation of a novel signaling structure, termed the "immunological synapse," at the interface of the APC and T cell (1). Within the mature synapse, the TCR occupies a central zone, termed the central-supramolecular activating complex (c-SMAC),[4] with integrin and other molecules excluded to an outer peripheral zone (2). The function of this highly organized structure has remained controversial. Initially the c-SMAC was surmised to represent the structure necessary for initiating signals (2), but more recent data have demonstrated that both calcium signaling (3) and Lck phosphorylation (4) occur before the formation of the SMACs, when the immunological synapse is less mature. Therefore, it has been suggested stably formed signaling aggregates at the c-SMAC are necessary for long-lived signal generation (5, 6).

T cell-APC interactions follow a defined sequence of cellular and molecular interactions. These include an initial scanning of the APC by filopodial projections on the T cell, recognition by the T cell of cognate Ag presented in the context of the MHC, the generation of membrane-proximal TCR-derived signals, such as Lck phosphorylation and calcium influx, and the cessation of T cell crawling, which allows for the generation of a prolonged interaction between the T cell and the APC. Presumably, the prolonged interactions allow for the generation of the c-SMAC, for stable signals that mediate full T cell activation, and for efficient delivery of cytokines and other signals between APCs and T cells.

However, the generation of full T cell responses depends not only on signals that are mediated by the TCR, but also on second "costimulatory" signals, the most important of which for naive T cells are mediated by CD28. Indeed, T cells activated in the absence of CD28 display reduced proliferation, survival, and IL-2 production, as well as a defect in T_H2 differentiation (7–9). Data demonstrating recruitment of CD28 to the immunological synapse (10, 11) and a function for CD28 in the regulation of both the cortical cytoskeleton (12–14) and membrane microdomains (15), suggest that it regulates events that occur soon after T cell-APC contact. Nevertheless it has been proposed that CD28 accumulation at the synapse is also dependent on and occurs subsequent to initial TCR signaling (11). Thus the relationship between CD28 recruitment to the T cell-APC interface and its role in proximal signaling is poorly defined.

In this study we show that CD28 localization in the synapse drives signals that act in the first minute, before c-SMAC formation. We confirm that recruitment of CD28 to the T cell-APC interface is an early event in T cell activation, and demonstrate association with TCR clusters at the earliest time points at which these events are observed. Moreover, we demonstrate that this recruitment occurs before the calcium signal, and using CD28-deficient T cells, that engagement regulates both the initiation and maintenance of the calcium signal within the first 60 s of the onset of that signal. Moreover, CD28 signals contribute to the generation of stable T cell-APC couples. Our data support a model whereby CD28 recruitment to the synapse is critical in integrating early TCR signals, rather than being a phenomenon that is dependent on these signals.

Materials and Methods

Cell lines

The vector encoding the CD28 fusion molecule was generated by PCR using forward (CAGATCTCGAGATGACACTCAGGC) and reverse (GACCGGTGGATCCGGGCGGTACGC) primers to generate a CD28 molecule lacking a stop codon followed by subcloning of this in the *Bam*HI and *Age*I sites of either pE-green fluorescent protein (GFP)-N1 or pE-cyan fluorescent protein (CFP)-N1 (Clontech Laboratories, Palo Alto, CA). This

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[4] Abbreviations used in this paper: c-SMAC, central-supramolecular activation cluster; GFP, green fluorescent protein; CFP, cyan fluorescent protein; YFP, yellow fluorescent protein; DIC, differential interference contrast; MSCV, mouse stem cell virus.

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construct was then cloned into a mouse stem cell virus (MSCV)-based retroviral construct using the restriction enzymes *Bgl*II and *Not*I. For two-color experiments, these transfectants were superinfected with a retrovirus expressing yellow fluorescent protein (YFP) in a CD3ζ-YFP fusion molecule as previously described (16). A20 B cells (American Type Culture Collection, Manassas, VA) and transfected CHO cells (17) have been previously described.

Mice

BALB/c mice, 6–8 wk of age, were purchased from Charles River Breeding Laboratories (Wilmington, MA). Transgenic mice expressing the DO11.10 TCR (DO11), specific for the chicken $OVA_{323–339}$ peptide in the context of the MHC class II molecule I-A^d, were obtained from Dr. D. Loh (Hoffmann-LaRoche, Nutley, NJ). DO11 mice crossed with CD28-deficient mice were obtained from Dr. J. Bluestone (University of California, San Francisco, CA). All mice were bred and maintained in accordance with the guidelines of the Lab Animal Resource Center of the University of California, San Francisco. The mice were typed for the DO11 TCR by staining peripheral blood cells with Abs against CD4 and Vβ8 (BD PharMingen, San Diego, CA).

Generation of retrovirus

On the day before infection, 3×10^6 Phoenix E cells (from G. Nolan, Stanford University, Stanford, CA) were plated in 9 ml of DMEM (Life Technologies, Grand Island, NY), 10% FCS, 1 mM penicillin-streptomycin, supplemented with L-glutamine and HEPES, in 10-cm tissue culture dishes. The tailless (TL) construct was a gift from J. Imboden (University of California, San Francisco). Empty MSCV 2.2 vector (MSCV, 10 μg) or MSCV that contains the CD28 construct were transfected into the Phoenix E packaging cell line with 5 μg of pCL-Eco packaging vector (from L. van Parijs, MIT, Cambridge, MA). Medium was changed on the day following transfection, and supernatants were taken on days 2 and 3 after transfection and used directly for infection.

Retroviral infection of primary T cells

$CD4^+$ T cells were purified from the spleen and lymph nodes from either wild-type or CD28-deficient DO11.10 mice with $CD4^+$ Dynal beads (Dynal Biotech, Oslo, Norway) and Detachabead anti-CD4 (Dynal Biotech) as described (18). Before infection 2.5×10^5 $CD4^+$ T cells were cultured for 2 days with 2×10^6 mitomycin C-treated BALB/c splenocytes and 1 μg/ml OVA in 1 ml/well of 24-well plates. On days 3 and 4, 0.5 ml of medium was removed from each well and replaced with 1 ml of viral supernatant with 1 μg/ml polybrene (Sigma-Aldrich, St. Louis, MO). For double transfection experiments, supernatants from Phoenix cells transfected with different CD28 constructs were combined in a 1:1 ratio and were subsequently used for infection. Plates were centrifuged for 50 min at 2200 rpm at room temperature. Following centrifugation, 1 ml of the supernatant was removed and replaced with 1 μg/ml OVA peptide. On day 5 $CD4^+$ T cells were sorted on a MoFlo high-speed cell sorter (Cytomation, Fort Collins, CO) and both GFP^+ and their GFP^- control populations were collected for analysis.

Proliferation and cytokine assays of retrovirally infected cells

$CD4^+GFP^+$ cells were sorted and rested for 2 days in 50 μg/ml IL-2. Following rest, 2.5×10^4 $CD4^+$ T cells were cultured with 2.5×10^4 mitomycin C-treated CHO cells and 0.1 μg/ml OVA peptide in 96-well plates. After 24 h, 50 μl of supernatant was removed for IL-2 ELISA, cultures were pulsed for 8 h with 1 μCi of [^{3}H]thymidine (New England Nuclear, Boston, MA), and incorporated radioactivity was measured in a Betaplate scintillation counter (Wallac, San Francisco, CA). IL-2 production was measured by ELISA according to instructions provided by the manufacturer (BD PharMingen).

Imaging

For examining CD28 localization relative to calcium signaling CD28-GFP D10 cells were loaded with 1 μM fura 2 (Molecular Probes, Eugene, OR) for 20 min at 37°, washed, and placed on coverslips. After the T cells had settled, CH27 APCs pulsed with 1 μM Con A were added to the coverslips and cells were imaged every 30 s for 30 min. Differential interference contrast (DIC) images were used to follow the T cell-APC interactions over time and the ratio of fura 340:380 was used to determine the intracellular levels of free calcium. GFP images were taken at 1-micron intervals through the cell for 15 planes and these images were used to follow CD28 movement. For CD28-GFP/CD3ζ-CFP two-color imaging, fura loading was omitted as this interferes with CFP detection. Images in these experiments were typically collected every 30 s for 30 min.

For imaging calcium flux in retrovirally infected T cells on CHO APCs, 6×10^4 CHO I-A^d (with or without B7 as indicated) cells were placed in the four interior wells of eight-well-chambered coverslips on the day before imaging. As indicated CHO cells were pulsed with OVA peptide for 1 h and then medium replaced with imaging medium before imaging. Imaging medium is RPMI 1640 without phenol red (Life Technologies) supplemented as previously described and containing 10% FCS. T cells were labeled with fura 2, washed in room temperature imaging medium, and placed on the confluent CHO layer. Imaging started as soon as the first T cells came into the focus plane of the CHO cells.

Imaging was performed using a Zeiss Axiovert microscope and a ×40 objective. The microscope was fitted with a high-speed Piezzo electric z-motor (Physik Instruments, Karlsruhe, Germany), dual excitation and emission filter wheels (Sutter Instruments, Novato, CA), and a Princeton Instruments Intermline camera (Roper Scientific, Trenton, NJ). A high-intensity xenon light source (Sutter Instruments) was used, and data were streamed from the camera to the computer to eliminate excessive bleaching. Images were taken at intervals as described for figure. At each time point indicated, DIC and fura 340 and 380 images were collected as well as a 15 μm z-stack of GFP images separated by 1 μm where indicated. Microscope control, data acquisition, and image analysis were performed in Metamorph (Universal Imaging, Downingtown, PA).

Results

CD28 localization to the synapse is a proximal event in T cell activation

To study CD28 function, we first sought to establish the kinetics by which CD28 enters the synapse relative to the onset of signaling in the presence of a live APC. To this end, $CD4^+$ T cells from CD28-deficient DO11.10 TCR-transgenic mice were infected with retrovirus expressing a CD28-GFP fusion molecule and subsequently sorted for GFP^+ cells as previously described (19). Although preactivated cells are less dependent on CD28 than naive cells, throughout the course of the study, we used Ag concentrations that maintained the CD28-dependence of the cells.

The chimeric CD28-GFP molecule functioned similar to wild-type CD28 in its ability to induce T cell proliferation (Fig. 1*A*), and could be seen to cluster following streptavidin-cross-linking of biotin-labeled anti-CD28 Abs (Fig. 1*B*), supporting this validity of this chimera as a method to track endogenous molecules.

We imaged cells expressing this construct to record the movement of CD28 while simultaneously monitoring intracellular calcium levels in reactive T cells. As shown in Fig. 2*A*, before contact with an APC, CD28 was distributed evenly across the surface of the T cell. At the first visible moment of T cell-APC contact (there is a 30 s interval between frames), accumulation of CD28 to the interface was observed. This rapid accumulation intensified at the time of intracellular calcium rise (within 30 s of T cell contact with the APC in this example) and by 2 min had formed into a cohesive c-SMAC (Fig. 2*B*). This accumulation was typically maintained for at least an additional 15 min (data not shown). T cell contact with APC in the absence of antigenic peptide only occasionally (<10%, $n = 11$) gave accumulations lasting more than 1 min.

CD28 accumulates with identical spatial and temporal kinetics as CD3

The Ag-dependent accumulation and coalescence of CD28 at the synapse coincided with what is typically observed for MHC/TCR localization in APC-mediated activation (3, 12). This suggests that CD28 might track alongside the TCR and play a role during early signaling events. To define the temporal movement of CD28 to the synapse in relation to the TCR, we created CD28-CFP and CD3ζ-YFP retroviral constructs, with the goal of simultaneously expressing both constructs in CD28-deficient DO11 T cells. Fig. 1*C* demonstrates that this method allows molecules to be unambiguously identified with no detectable spectral overlap. Consistent with our findings in cells expressing CD28-GFP alone, there was a rapid accumulation

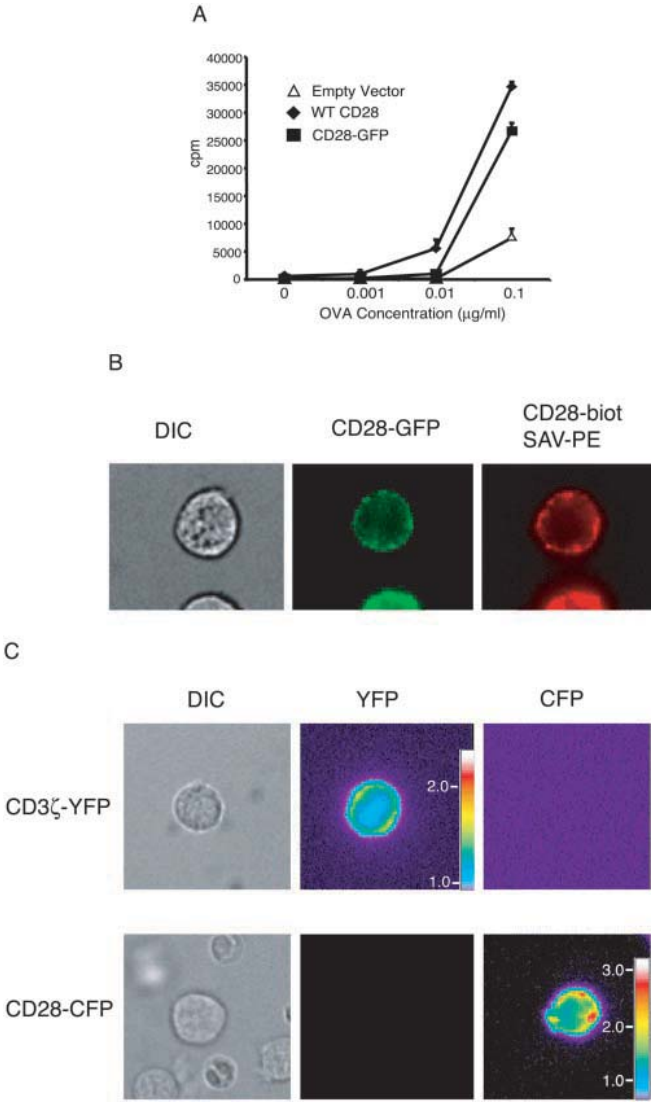

FIGURE 1. Functional properties of CD28 chimeric molecules. *A*, CD28-deficient DO11.10 T cells were induced to express empty vector, wild-type (WT) CD28, or the CD28-GFP construct by retroviral infection. The costimulatory activity of each construct was determined by its effect on regulating T cell proliferation in response to mitomycin C-treated splenocytes and OVA peptide. *B*, CD28-GFP was expressed in CD28-deficient DO11.10 T cells by retroviral transfection. To demonstrate that this construct can be used to document the movement of CD28 on the surface of the T cell, the cells were stained with biotin-labeled anti-CD28 Ab, which was subsequently cross-linked with PE-labeled streptavidin. The respective distribution of the molecules on the surface of the T cells is shown. *C*, T cells expressing either CD28-CFP or CD3ζ-YFP constructs used in Fig. 2 were imaged separately to verify that our data were not due to fluorescent cross-talk between the fluorophors.

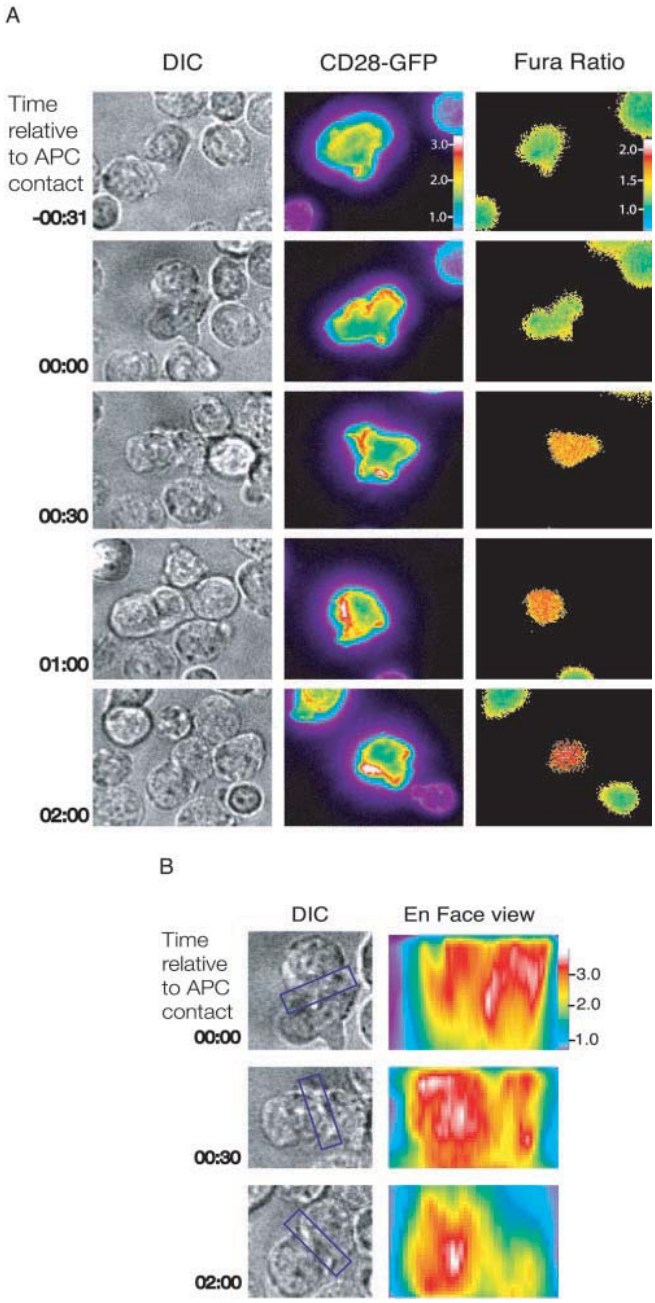

FIGURE 2. CD28 is rapidly recruited to the synapse. Peptide-pulsed A20 B cells were added to CD28-GFP-expressing CD28-deficient DO11.10 T cells loaded with the calcium dye fura 2. Cells were imaged every 30 s for 30 min. *A*, DIC images in the *left column* are used to follow the cells visually. The *middle column* shows the maximum intensity of GFP through all planes. The fura ratio of 340:380 displayed in the *right column* is used to determine the intracellular free calcium levels. Time from point of T cell-B cell contact is displayed to the *left* of the image. *B*, DIC images of the same cell shown in *A* are displayed with the area used for cross-sectional analysis of the T cell-APC interface highlighted by the box. The En Face view of the interface is shown in the *right columns*. The time from the moment of T cell-APC contact is shown on the *left* end of the image.

of CD28 to the area of T cell-APC contact (within 1 min, Fig. 3*A*). This accumulation consistently coincided both spatially and temporally with the accumulation of CD3ζ to the synapse. By 6 min after APC contact, both molecules formed cohesive clusters consistent with their incorporation in the c-SMAC (Fig. 3*B*).

CD28 increases the likelihood that a T cell will flux calcium after interaction with peptide-MHC complexes

The kinetics of CD28 recruitment suggest that this molecule may have a role in regulating early T cell signaling events initiated by the TCR. To test this hypothesis, we performed a series of experiments in a manner similar to that previously described. Preactivated $CD4^+$ T cells from wild-type or CD28-deficient DO11.10, TCR-transgenic mice were loaded with the calcium-sensitive dye fura 2. These cells were placed on coverslips containing OVA-pulsed CHO cells bearing both MHC and B7 molecules, and the percentage of cells fluxing calcium was determined. When scoring for the frequency at which any detectable calcium increase could

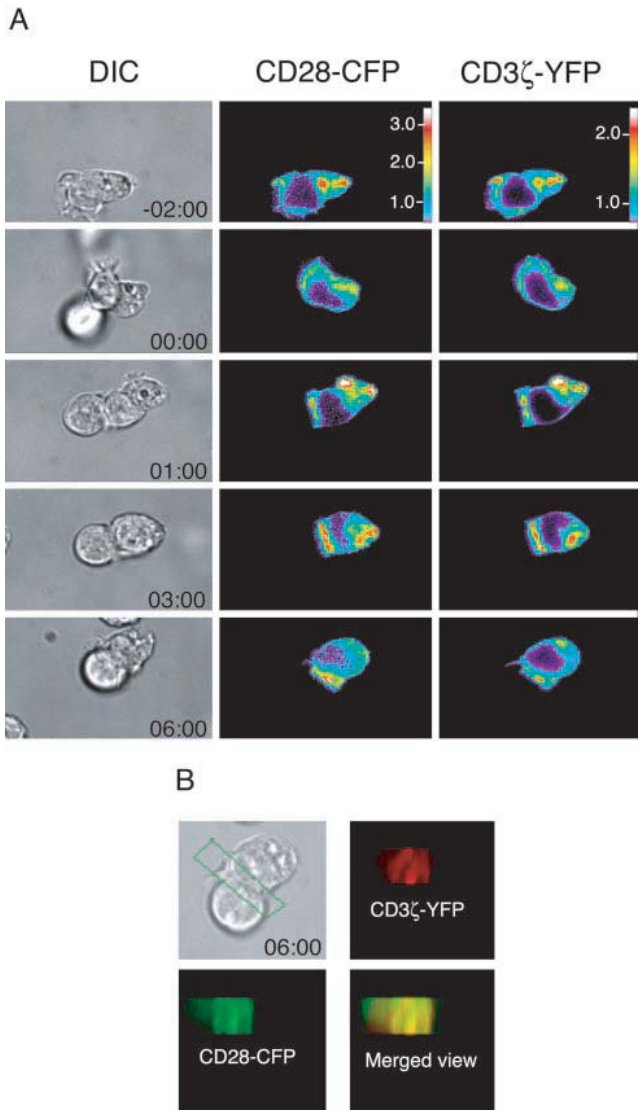

FIGURE 3. CD28 recruitment is coincident with the TCR. Peptide-pulsed A20 B cells were added to CD28-deficient DO11.10 T cells expressing both CD28-CFP and CD3ζ-YFP chimeric proteins. Cells were imaged every 60 s for 60 min. *A*, DIC images in the *left column* are used to follow the cells visually. The *middle column* shows a cross-sectional image of CD28-CFP through the midpoint of the cell. The same focal plane was used to acquire CD3ζ-YFP (*right column*). Time displayed in the *top middle* and *top right* is measured from point of T cell-B cell contact. *B*, At the 6-min time point, the cell shown in *A* was analyzed for the development of a c-SMAC. The En Face view of CD28-CFP and CD3ζ-YFP are shown at the *top right* and *bottom left*, respectively. An overlay of these two images is shown at the *bottom right*.

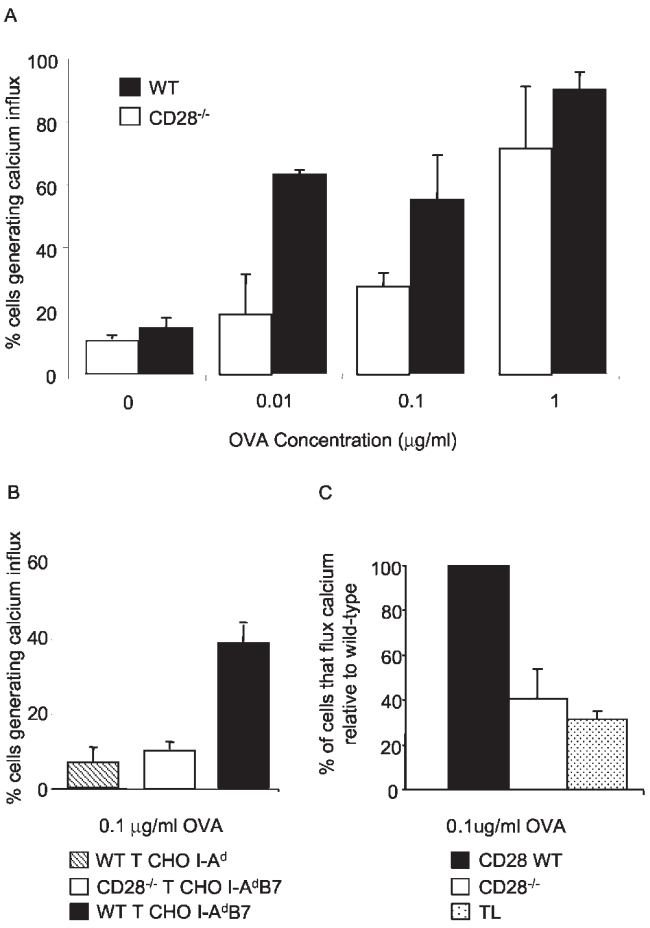

FIGURE 4. CD28 engagement increases the frequency of calcium influx in T lymphocytes. *A*, $CD4^+$ T cells from wild-type and CD28-deficient DO11 mice were infected with empty vector. Following sorting, T cells were placed on confluent layers of CHO $I\text{-}A^d$ B7 cells pulsed with varying concentrations of OVA peptide. Cells were imaged every 30 s for 30 min. Cells that remain in contact with a single CHO cell for at least 2 min are scored as to whether or not they flux calcium, indicated by an increase in intensity of the fura 340:380 ratio. *B*, The percentage of wild-type DO11 T cells fluxing calcium while interacting with CHO IA^d was compared with that of CD28-deficient and wild-type T cells interacting with 0.1 μg/ml OVA peptide-pulsed CHO IA^d B7. *C*, CD28-deficient DO11 T cells were reconstituted with empty vector ($CD28^{-/-}$), wild-type CD28 (CD28 WT), or a tailless construct (TL) as previously described. Cells were assayed for their ability to flux calcium. Results displayed are the average of the percentage of cells that flux calcium compared with the percentage of cells reconstituted with wild-type CD28 that fluxed calcium in a particular experiment. Results of 20–50 cells that settle on the CHO cell layer in each experiment are given for four independent experiments.

be observed, CD28-deficient cells were defective relative to wild-type cells (Fig. 4*A*). This difference was more pronounced at low Ag concentrations in which CD28 signaling has its most profound effects upon downstream proliferation and IL-2 production (8). The calcium influx observed was dependent upon the presence of Ag, as cells exposed to APCs in the absence of peptide showed greatly reduced ability to influx calcium (Fig. 4*A*). These differences did not result from inherent defects of the CD28-deficient cells because the calcium flux observed in wild-type T cells was also B7-dependent (Fig. 4*B*). In this series, wild-type cells placed on 0.1 μg/ml OVA-pulsed CHO cells expressing $I\text{-}A^d$ in the absence of B7 fluxed calcium at an equivalent level as CD28-deficient cells stimulated with CHO $I\text{-}A^d$ plus B7. This level was less than one-half the level seen by wild-type cells on CHO $I\text{-}A^d$ B7 cells.

To examine the role of signaling domains within the CD28 cytoplasmic tail in driving the calcium flux, we reconstituted CD28-deficient cells with the empty vector, wild-type CD28, or a cytoplasmic deletion mutant (CD28 TL), which contains the proximal four amino acids of the cytoplasmic domain but none of the putative signaling molecule binding motifs. Consistent with the data generated in preactivated cells from wild-type or CD28-deficient mice, cells infected with empty vector initiated a calcium influx at 40% the frequency of CD28-deficient cells expressing a wild-type CD28 construct when exposed to 0.1 mg/ml OVA-pulsed CHO cells bearing $I\text{-}A^d$ and B7 (Fig. 4*C*). Cells expressing the TL construct exhibited a similar frequency of calcium influx as those expressing empty vector (Fig. 4*C*) demonstrating that this phenomenon is mediated by signals that emanate from the CD28

cytoplasmic tail. Overall, these data suggest that CD28 signaling functions in generating initial calcium signals.

CD28 augments the duration and quality of the calcium signal

Although scoring for the frequency of calcium influxes demonstrates a clear signaling defect in CD28-deficient cells, we took advantage of the ability of this imaging approach to analyze individual cell couples to determine whether CD28 also regulates the nature or duration of the calcium influx upon encounter with Ag-pulsed APCs. CD28-deficient cells retrovirally infected with empty vector were not only less likely than cells bearing wild-type CD28 to initiate a calcium influx in response to Ag and APC stimulation, but those which did activate the calcium pathway also had an altered pattern of influx. Wild-type cells stimulated with 0.1 μg/ml OVA peptide generate a pronounced and sustained calcium influx (Fig. 5). In contrast, CD28-deficient cells typically displayed extremely transient calcium fluxes, lasting only two to three frames or <90 s (Fig. 5). Cells expressing the CD28 TL construct displayed calcium traces that were similar to CD28-deficient cells expressing empty vector (Fig. 5), suggesting that the cytoplasmic tail of CD28 is responsible for driving the sustained calcium response.

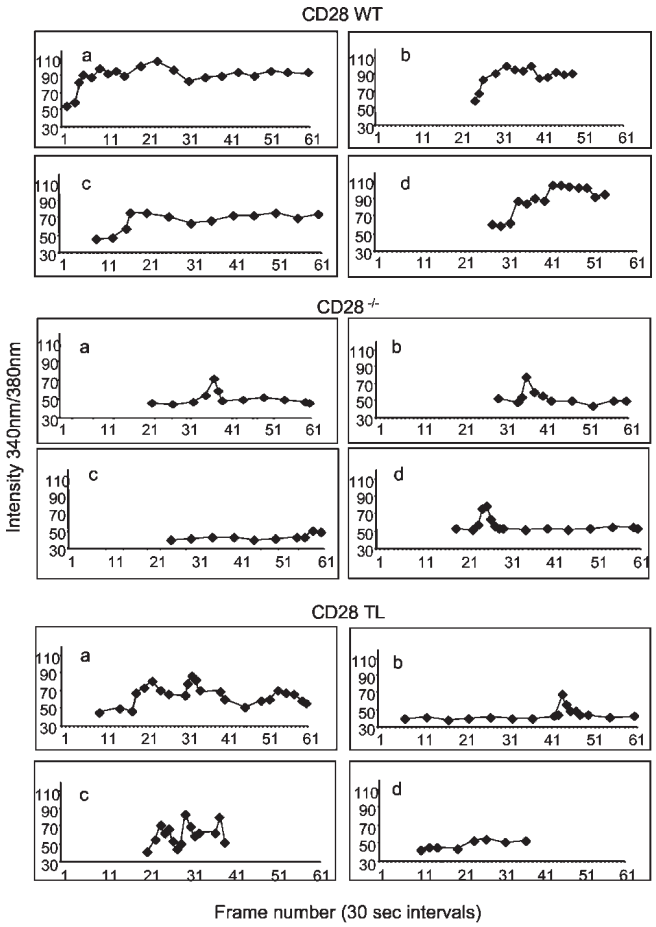

FIGURE 5. CD28 is required for sustained signaling in response to suboptimal TCR engagement. The ratio of emission intensities at 340 and 380 nm is plotted over time for CD28-deficient DO11 T cells expressing wild-type CD28, empty vector, or CD28 TL. Four representative cells (*a–d*) in which a calcium influx occurs are shown. Plots initiate when a T cell settles onto the CHO cell layer and end at the conclusion of the run or when a T cell moves out of the field of imaging.

CD28 signaling regulates the duration of T cell-APC interactions

Because intracellular calcium influx has been associated with the generation of the T cell stop signal (20), we measured the duration of T cell-APC interactions to determine whether the calcium defects we found in CD28-deficient cells correlated with an inability of these cells to generate prolonged couples with APCs. CD28-deficient DO11 T cells retrovirally induced to express either empty vector or wild-type CD28 were placed onto coverslips containing confluent CHO I-A^d B7 cells that had been pulsed with 0.1 μg/ml $OVA_{323–339}$ peptide. The cells were then imaged at 20-s intervals for 20 min, and the duration over which individual T cells associated with single APCs was determined. Over 300 cells were scored for each cell type. Compared with cells deficient in CD28, those bearing wild-type CD28 had increased ability to sustain prolonged interactions (Fig. 6). Expression of the CD28 TL construct did not rescue this defect (Fig. 6), consistent with the notion that signals mediated downstream of CD28, and not adhesive forces generated in our two ligand system, were responsible for prolonged stopping.

Abnormal calcium signals correlate with defective T cell functional responses

It is well recognized that T cell activation as measured by proliferation and cytokine production is dependent not only on the initiation of a calcium signal, but also on the kinetics and duration of that signal (21). To determine whether the partial or transient calcium fluxes seen in CD28-deficient cells correlated with defective T cell functional responses in our system, we restimulated cells expressing the CD28 mutant constructs with a 1:20 ratio of CHO I-A^d B7 to OVA peptide (Fig. 7). Consistent with their marked quantitative and qualitative calcium signal defects and the short interaction time with APCs, CD28-deficient cells expressing empty vector or the TL construct proliferated and produced IL-2 at levels significantly less than those expressing wild-type CD28.

Discussion

CD28 is a key regulator of CD4$^+$ T cell function. Nevertheless, the mechanism underlying its activity remains obscure. In this study we have used real-time molecular imaging to examine the timing of CD28 recruitment to the synapse, and its effect on the regulation of membrane-proximal T cell signaling and function. We show that CD28 localization to the immunological synapse between a

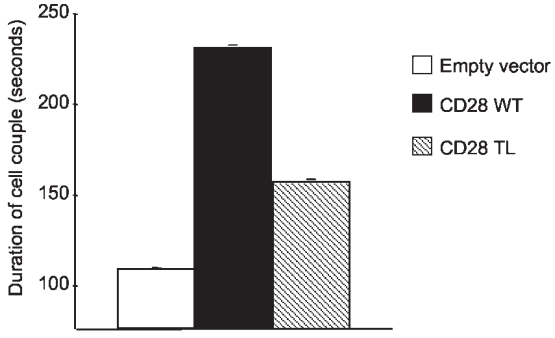

FIGURE 6. CD28 regulates T cell stopping. CD4$^+$ T cells from CD28-deficient DO11.10 TCR-transgenic mice were infected with empty vector, wild-type CD28, or CD28 TL constructs. Following sorting, cells were placed on confluent layers of CHO I-A^d B7 cells pulsed with 0.1 μg/ml OVA peptide as APCs. Cells were imaged every 20 s for 15 min. The average duration of the cell couple was determined. Over 300 T cell-APC interactions were scored for each cell type.

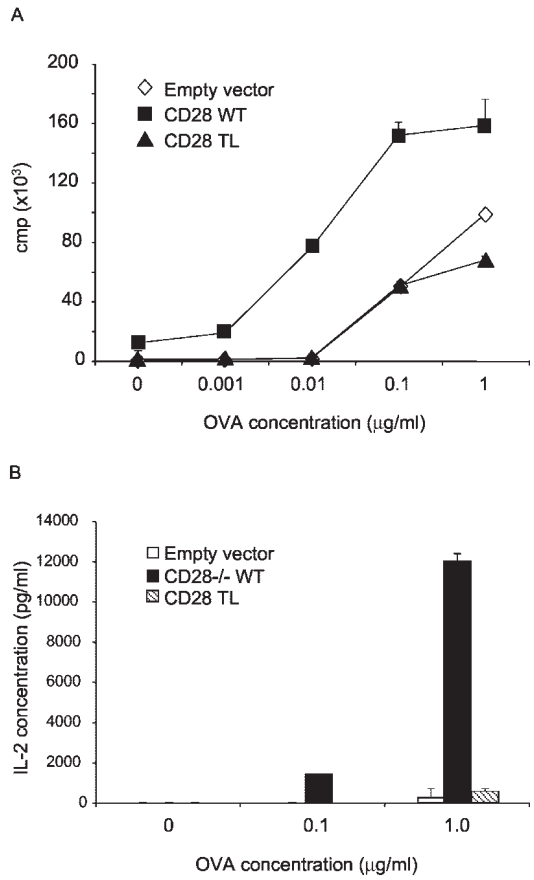

FIGURE 7. CD28 cytoplasmic domain mutants differentially regulate T cell proliferation and IL-2 production. CD28-deficient cells reconstituted with either empty vector, wild-type CD28, or CD28 TL were stimulated with a 1:20 ratio of CHO I-A^d B7 and OVA peptide. Proliferation (*A*) and IL-2 production (*B*) were measured as described in *Materials and Methods*.

live T cell and a live APC is an early event in T cell activation. It occurs coincidently with the recruitment of the TCR, and with the initiation of calcium signaling in both. Importantly, CD28 accumulation occurs before the development of a cohesive c-SMAC (Figs. 2 and 3). In addition, we demonstrate a proximal role for CD28 in mediating early biochemical and cellular functional consequences of T cell Ag recognition. It alters both the likelihood that cells will initiate a calcium signal (Fig. 4), regulates the nature of the signal at an individual cell basis (Fig. 5), and mediates T cell stopping (Fig. 6). This regulatory role on membrane-proximal signaling is critical in mediating downstream T cell functions (Fig. 7).

The finding of very rapid recruitment of CD28 to the T cell-APC interface is consistent with other studies in T cell blasts that demonstrate recruitment of CD28 to the immunological synapse within 30 s of encounter with an Ag-pulsed APC (10). In naive T cells, this recruitment, along with that of the TCR, may take a few minutes (11). Rapid recruitment may also explain how CD28 regulates the earliest stages of cytoskeletal rearrangement (13, 22) and membrane microdomains (15) in T cell-APC interactions. Overall, a comparison of CD3ζ distributions with those of CD28 suggests that at the time of encounter with Ag-bearing APCs, clustering of these molecules at the synapse occurs nearly simultaneously (Fig. 3).

We extend upon these results to demonstrate that this movement precedes or coincides with calcium influx (Fig. 2*A*), which in turn occurs before the development of a c-SMAC (Fig. 2*B*). This is an important result because it implies that CD28 can join a signalosome at a time when the earliest signals are being generated within a T cell as it responds to its cognate Ag presented by an APC. We therefore believe that the simultaneous recruitment of CD28 and the TCR to the same location within the synapse allows these molecules to cooperatively mediate membrane-proximal signals that occur before the development of the c-SMAC (3, 18, 23). Our data demonstrate that CD28 plays an important role in mediating one such signal: calcium influx into the T cell (Figs. 4 and 5). First, by increasing the frequency of calcium signaling, CD28 is likely to induce more cells within a population to respond and to increase the proliferation potential within the responding population (Fig. 4*A*). This effect predominates at physiological levels of Ag at which CD28 has its most prominent functional effects on T cells, and is consistent with a recent report demonstrating that CD28 plays an important role in inducing strong and durable calcium signals in Jurkat cells (24) and that blockade of CD28-B7 interactions diminishes MHC accumulation at the synapse (18). These data would seemingly conflict with data of Bromley et al. (11) that, using a lipid bilayer expressing ICAM-1, CD28, and I-E^k MHC molecules, showed no role for CD28 in augmenting the frequency of synapse formation in naive lymphocytes. Although it is possible the absence of ICAM expression in our APCs may explain the difference, we believe it is more likely that CD28 signaling is having a more profound effect on the signals that occur subsequent to the formation of the immature synapse, rather than on the formation of the signalosome itself.

Although our data show that CD28 signaling augments the proportion of responding cells, its role also extends to modulation of the magnitude of the calcium response of individual cells to Ag. In comparison to cells expressing wild-type CD28, the predominant pattern of calcium influx in cells incapable of generating CD28-mediated signals was both attenuated in amplitude and transient in duration (Fig. 5). Importantly, this qualitative regulatory effect of CD28 occurred only seconds after the initiation of the calcium signal, further supporting the notion that CD28 has a very early role in the regulation of T cell activation signals. The pattern of calcium flux that we observed in cells expressing the CD28 TL mutant is reminiscent of the transient responses seen in cells stimulated in the presence of antagonist peptide (25). In this latter study, these fluxes were not compatible with a productive activation signal as measured by proliferation. Similarly, expression of the CD28 TL construct was associated with profound defects in T cell functional responses (Fig. 7).

Because CD28 is a regulator of the earliest signals generated in a T cell, it should follow that it also modulates T cell functions soon after the T cell recognizes its cognate Ag on the surface of the APC. Negulescu et al. (20) have previously demonstrated that stopping/rounding is dependent on a strong calcium response. Using our ability to observe individual T cell-APC interactions by microscopy, we confirm that CD28-mediated signals play an important role in regulating the induction of prolonged T cell-APC contacts (Fig. 6). Other studies have demonstrated that in bilayers, CD80 cannot mediate adhesion of CD28-positive cells, suggesting that, in isolation, the affinity of this receptor-ligand pair does not mediate the generation of the T cell stop signal (11). Similarly, we find that for prolonged contacts, a TL construct that cannot mediate calcium signaling is mostly ineffective (Fig. 6). These data suggest that the intracellular signals mediated by CD28 are important for generating long-lived and productive cellular couples. This is consistent with data demonstrating that CD28 significantly augments conjugate formation between T cells and a B cell lymphoma cell line (24).

In summary, we have demonstrated that CD28 is rapidly recruited to the immunological synapse, and there regulates early T

cell signaling, such as the induction of a mature calcium influx, as well as proximal T cell functional responses such as stopping. These data strongly support a model whereby CD28 functions concomitantly with the TCR at the immunological synapse to generate the first signals that lead to T cell functional responses.

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