

# The tyrosine phosphatase CD148 is excluded from the immunologic synapse and down-regulates prolonged T cell signaling

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CD148 is a receptor-like protein tyrosine phosphatase up-regulated on T cells after T cell receptor (TCR) stimulation. To examine the physiologic role of CD148 in TCR signaling, we used an inducible CD148-expressing Jurkat T cell clone. Expression of CD148 inhibits NFAT (nuclear factor of activated T cells) activation induced by soluble anti-TCR antibody, but not by antigen-presenting cells (APCs) loaded with staphylococcal enterotoxin superantigen (SAg) or immobilized anti-TCR antibody. Immunofluorescence microscopy revealed that the extracellular

domain of CD148 mediates its exclusion from the immunologic synapse, sequestering it from potential substrates. Targeting of the CD148 phosphatase domain to the immunologic synapse potently inhibited NFAT activation by all means of triggering through the TCR. These data lead us to propose a model where CD148 function is regulated in part by exclusion from substrates in the immunologic synapse. Upon T cell–APC disengagement, CD148 can then access and dephosphorylate substrates to down-regulate prolongation of signaling.

## Introduction

The activation of a T cell requires the complex interactions of a vast number of signaling molecules and eventually results in proliferation, differentiation, and secretion of cytokines such as interleukin 2 (IL-2). The initial triggering events are regulated by the Src and Syk families of protein tyrosine kinases (Kane et al., 2000; Lin and Weiss, 2001b). These kinases phosphorylate a large number of substrates leading to the further activation of other enzymes and allow for molecules with no intrinsic enzymatic activity to function as adaptors for the assembly of signaling complexes (Tomlinson et al., 2000). One such adaptor is the linker for the activation of T cells (LAT) (Zhang et al., 1998). LAT is a transmembrane adaptor protein containing multiple sites of tyrosine phosphorylation, which allow for the recruitment of many essential signaling molecules, including phospholipase C $\gamma$ -1 (PLC $\gamma$ -1), Grb2, and Gads (Finco et al., 1998; Zhang et al., 1999, 2000; Tomlinson et al., 2000; Lin and Weiss, 2001a). Multiple groups have demonstrated that signals emanating from the T

cell receptor (TCR) must be sustained for prolonged periods of time, on the order of hours, for T cells to proliferate (Iezzi et al., 1998; Lee et al., 2002). IL-2 production also requires a similar length of time; for instance, 6 h of stimulation with Con A and PMA were required for commitment to maximal IL-2 production in Jurkat T cells (Weiss et al., 1987).

In order for a productive T cell–antigen-presenting cell (APC) conjugate to form, a number of cell surface molecules must bind their respective ligands. During this cell–cell interaction, many proteins localize to the area of cell–cell contact, whereas others are excluded. Studies originally performed in mouse T cell clones demonstrated that the contact point between the T cell and the APC is a highly ordered structure (Monks et al., 1998). This structure has become known as the immunologic synapse (Bromley et al., 2001). To mimic what was seen in mouse T cell clones, we used the Jurkat–superantigen (SAg) system, where Jurkat T cells are stimulated with staphylococcal enterotoxin SAg presented on B lympho-

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Abbreviations used in this paper: APC, antigen-presenting cell; c-SMAC, central supramolecular activation cluster; ERM, ezrin/radixin/moesin; IL-2, interleukin 2; LAT, linker for the activation of T cells; MHC, major histocompatibility complex; NFAT, nuclear factor of activated T cells; PBMC, peripheral blood mononuclear cell; p-SMAC, peripheral SMAC; SAg, superantigen; SE, staphylococcal enterotoxin; TCR, T cell receptor.

blastoid cells. SAg function by simultaneously binding to the  $\beta$  chain of the TCR and major histocompatibility complex (MHC) class II molecules on the APC. This interaction has a similar activation time course as that seen with peptide loaded onto MHC molecules (Lavoie et al., 1999). Molecules known to be excluded from the synapse in mouse clones, such as CD43 and CD45, are also excluded in the Jurkat–SAg system (Johnson et al., 2000; van der Merwe et al., 2000; Delon et al., 2001; Bunnell et al., 2002).

Many groups have studied the signals involved in activating T cells, but considerably less is known about how a T cell terminates its response to stimulation. A class of molecules thought to play a role in the down-regulation process is tyrosine phosphatases. CD148, also known as Dep-1 and Byp, is a transmembrane tyrosine phosphatase that is up-regulated on T cells after activation (Tangye et al., 1998a). The extracellular domain consists of eight fibronectin type III domains, with multiple glycosylation sites that add to the size of the molecule (Gaya et al., 1999). Because of its bulky extracellular domain, it is likely to be excluded from the immunologic synapse, similar to other proteins with bulky extracellular domains, such as CD43 and CD45 (Johnson et al., 2000; van der Merwe et al., 2000; Delon et al., 2001). Previous work has shown that CD148 is a negative regulator of TCR signaling, as the activity of the TCR-induced transcription factor NFAT (nuclear factor of activated T cells) was reduced in CD148-expressing cells (Tangye et al., 1998b; Baker et al., 2001). CD148 can act as a negative regulator by causing the specific dephosphorylation of LAT and PLC- $\gamma$ 1 (Baker et al., 2001). However, in these studies, cells were only stimulated with soluble anti-TCR antibodies that do not mimic the physiologic interaction that leads to the formation of an immunologic synapse.

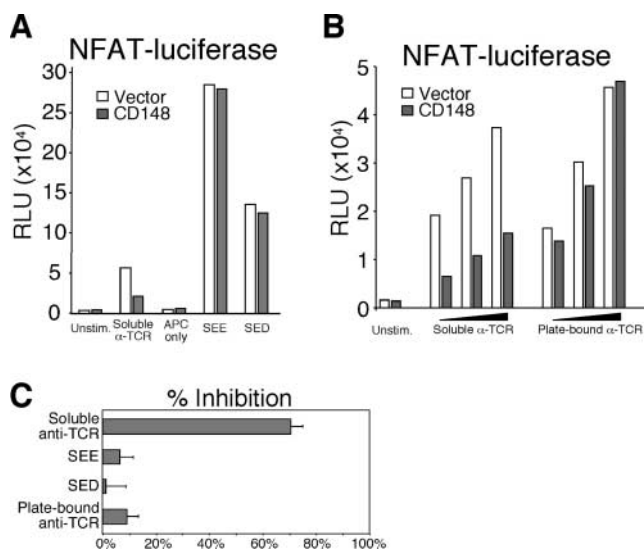
To further study the role of CD148 in T cell signaling, we investigated whether expression of CD148 inhibits T cell activation induced not only by soluble anti-TCR antibody (Ab) stimulation, but also with either SAg and APCs or immobilized anti-TCR Ab. We found that at 8 h after stimulation, CD148 only inhibits T cell activation stimulated with soluble anti-TCR. Immunofluorescence revealed that CD148 is excluded from the immunologic synapse and would therefore be unable to interact with substrates localized to the synapse, such as LAT (Tanimura et al., 2003). This exclusion is mediated, at least in part, by the large extracellular domain of CD148. Forced targeting of the CD148 phosphatase domain to the synapse results in the potent inhibition of NFAT activation independent of the technique used to trigger the TCR. These data lead us to propose a model whereby CD148 activity during early T cell–APC interactions is regulated by exclusion from the immunologic synapse. After T cell–APC disengagement, CD148 is no longer excluded by the synapse and can then access and dephosphorylate substrates to down-regulate prolongation of the response. Exclusion from the synapse could provide an important level of regulation, to prevent the premature termination of signaling pathways.

## Results

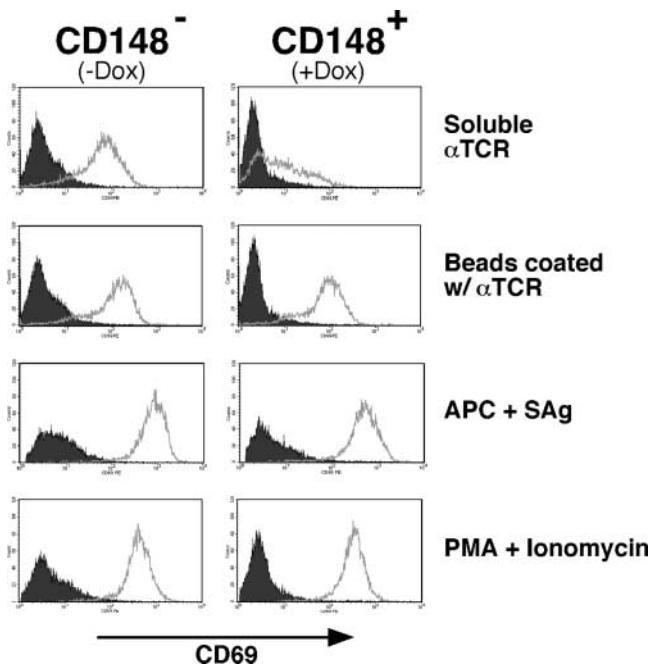
### Differential effects of CD148 depending on stimuli

To examine the negative regulatory role of CD148 on TCR signaling using more physiologic stimuli, we used APCs

loaded with staphylococcal enterotoxin (SE) SAg. The SAg-coated APCs provide a localized stimulus to which a T cell will polarize to after appropriate TCR stimulation. These changes can be visualized as directed actin polymerization and microtubule organizing center reorientation (Lowin-Kropf et al., 1998). Jurkat T cells were transfected with wild-type murine CD148 together with an NFAT–luciferase reporter construct. Cells were stimulated the next day with either soluble anti-TCR (C305 mAb) or two types of SAg (SED or SEE) with Daudi B lymphoblastoid cells serving as APCs. As previously reported, expression of CD148 in Jurkat T cells inhibited soluble anti-TCR–induced NFAT activation by  $\sim$ 60–70% (Baker et al., 2001). Interestingly, stimulation with either SAg was not associated with substantial decreases in the NFAT response when CD148 was expressed (Fig. 1 A). A potential concern when comparing soluble anti-TCR with SAg and APCs is that APCs present a number of additional ligands to the T cell besides the SAg bound to MHC class II molecule during the interaction. To rule out the possibility that these other interactions accounted for the differences seen, we coated plates with the same anti-TCR used for the soluble mAb stimulation. Stimulation with immobilized anti-TCR mimics the polarized stimulus provided by the SAg and APC. As seen with the SAg and APC stimulation, NFAT activation in response to various concentrations of plate-bound anti-TCR stimulation was not substantially inhibited by the expression of CD148 (Fig. 1 B). The average percent inhibition induced by the various TCR stimuli is shown in Fig. 1 C. Thus, it seems



**Figure 1. Expression of CD148 only inhibits NFAT activation induced by soluble anti-TCR.** Jurkat T cells were transfected with 2  $\mu$ g of pcdef3-mCD148 or vector control, along with 15  $\mu$ g of a 3xNFAT–luciferase reporter and rested overnight. (A) Cells were then stimulated with soluble anti-TCR (1:2,000 dilution of ascites; C305), or with the indicated SAg (250 ng/ml) and APCs. (B) Cells transfected as before were stimulated with increasing amounts of soluble anti-TCR or plate-bound anti-TCR. For A and B, the mean of triplicate samples in one representative experiment is shown. (C) The mean percent inhibition for each stimulus is shown from multiple experiments. Error bars represent standard errors of the mean for at least four separate experiments, each done in triplicate. Relative light units were normalized to PMA/ionomycin.

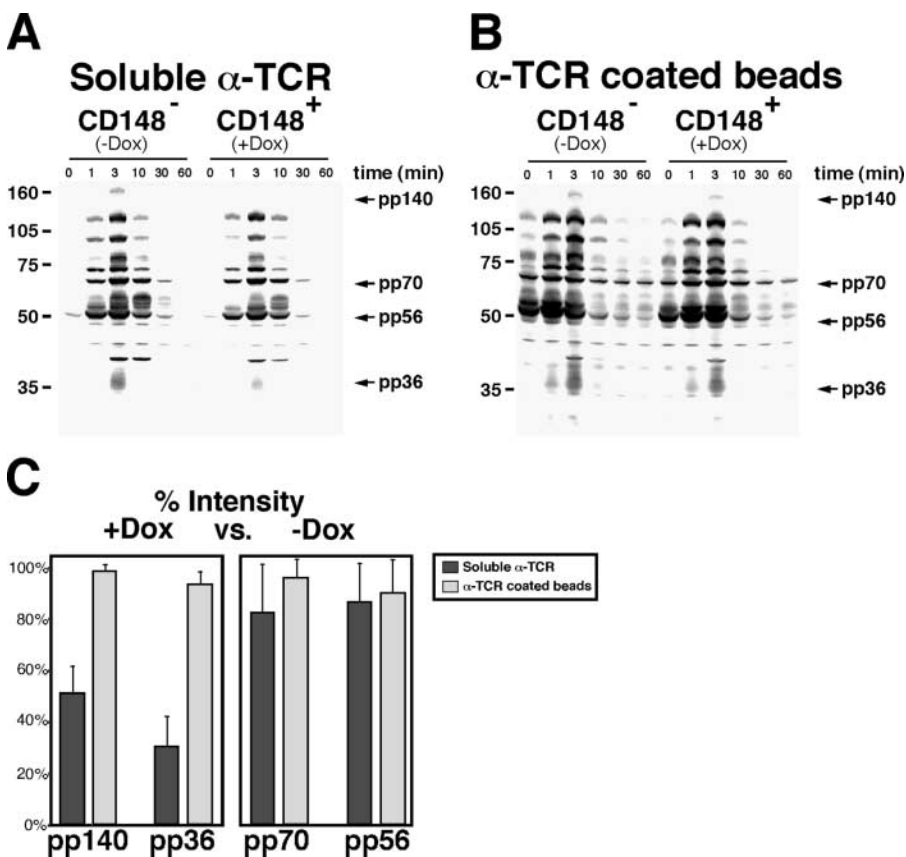


**Figure 2. Only soluble anti-TCR-induced CD69 up-regulation is inhibited by CD148 expression.** Tet-inducible CD148-expressing Jurkats (clone L12) were induced with 1  $\mu$ g/ml doxycycline for 48 h before stimulation. Cells were then stimulated with soluble anti-TCR, bead-coated anti-TCR, or SAg (1  $\mu$ g/ml SEE) with Daudi B cells serving as APCs, overnight. Cells stimulated with 20 ng/ml PMA + 1  $\mu$ M ionomycin served as positive controls. Cells were then stained with anti-CD69-PE and analyzed by flow cytometry. For SAg and APC stimulation, T cells were gated with anti-CD5-FITC. The shaded histogram represents unstimulated samples or those stimulated with APCs only.

from these data that CD148 preferentially inhibits a nonpolarizing, soluble, anti-TCR stimulation. In contrast, CD148 expression has little effect on polarizing stimuli such as SAg with APCs or plate-bound anti-TCR.

To further examine the differences in T cell activation seen with the various methods of triggering through the TCR, we used a previously characterized CD148-inducible system in Jurkat T cells (Baker et al., 2001). The early activation marker CD69 is known to be up-regulated in T cells stimulated through the TCR. As shown in Fig. 2, expression of CD148 substantially inhibited the subsequent up-regulation of CD69 induced by an overnight stimulation with soluble anti-TCR. However, in cells stimulated with anti-TCR-coated beads or SAg and APCs, expression of CD148 did not inhibit the up-regulation of CD69 (Baker et al., 2001). These data demonstrate that endogenous responses reflecting T cell activation are also unaffected by CD148 expression if cells are stimulated by immobilized anti-TCR or SAg and APCs.

Previous studies have demonstrated that inducible expression of CD148 leads to a specific loss of LAT and PLC $\gamma$ -1 phosphorylation induced by soluble anti-TCR stimulation (Baker et al., 2001). To investigate whether immobilized anti-TCR stimulation results in the same phosphorylation pattern, we stimulated Jurkat T cells that express CD148 with either soluble or bead-coated anti-TCR Ab for various periods of time. Stimulation with soluble anti-TCR in the presence of CD148 resulted in decreased tyrosine phosphorylation levels of pp140 (PLC $\gamma$ -1) and pp36 (LAT), compared with cells not expressing CD148, as previously reported (Fig. 3 A) (Baker et al., 2001). Quantitation of



**Figure 3. CD148 does not affect tyrosine phosphorylation induced with immobilized anti-TCR.** Tet-inducible CD148-expressing Jurkats were induced as before. Cells were washed once and rested in PBS for 30 min before stimulation with either (A) soluble anti-TCR or (B) bead-coated anti-TCR for the indicated time points. Cells were then lysed in buffer containing 1% NP-40, and proteins were separated by SDS-PAGE. Tyrosine-phosphorylated proteins were then visualized by Western blotting with an antiphosphotyrosine mAb followed by a goat anti-mouse-HRP secondary. (C) Average percent intensity of the indicated phosphotyrosine bands from three separate experiments is shown comparing soluble to bead-coated anti-TCR stimulation. Blots were developed on a Kodak Imaging Station, and bands were quantitated using Kodak 1D imaging software.

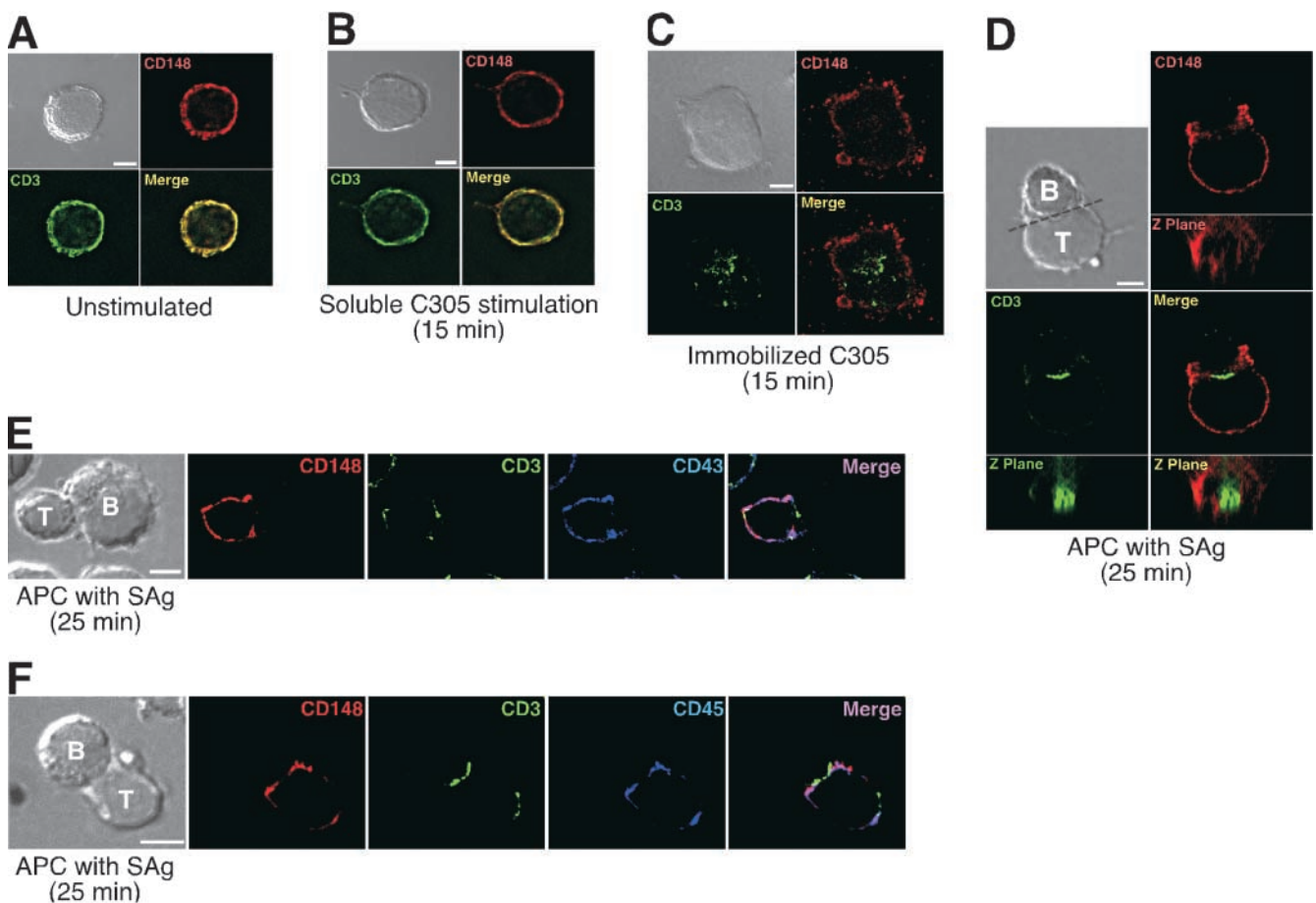


phosphotyrosine bands from three separate experiments showed that phosphorylation of pp140 and pp36 was decreased by  $\sim 50$  and 70%, respectively (Fig. 3 C). Interestingly, cells expressing CD148 did not show decreased phosphorylation of pp140 and pp36 when stimulated with anti-TCR-coated beads (Fig. 3, B and C). The tyrosine phosphorylation states of other bands, such as pp70 and pp56, were similar for either stimulus independent of CD148. These data show that the initial events in proximal TCR signaling, as assessed by tyrosine phosphorylation, are affected differently by CD148, depending upon whether soluble or immobilized anti-TCR is used.

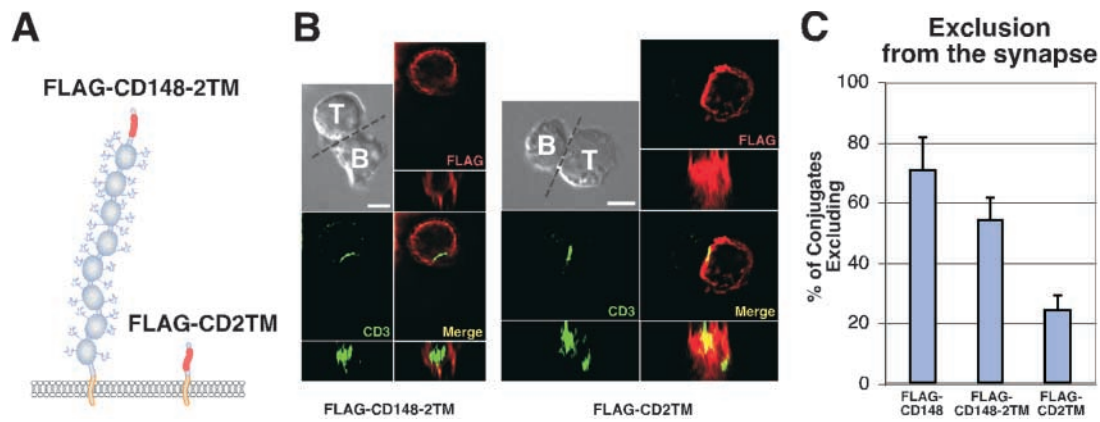
### CD148 is excluded from the immunologic synapse

To study the cellular localization of CD148 during the response to the various TCR stimuli, immunofluorescence microscopy was performed with antibodies specific to CD148 and the CD3 $\epsilon$  chain of the TCR. In resting Jurkat T cells inducibly expressing CD148, both CD148 and CD3 colocalize at the cell surface (Fig. 4 A). Similarly, after soluble anti-TCR mAb stimulation, CD148 and CD3 still show almost com-

plete colocalization with very little compartmentalization of green and red staining (Fig. 4 B). In cells dropped onto coverslips coated with anti-TCR Ab, the cells initially make contact and then begin to spread, as previously reported by others (Bunnell et al., 2002). After 15 min, the cells were visualized at the cell-coverslip interface with the same antibodies as before. In striking contrast to a soluble anti-TCR stimulation, cells stimulated by immobilized anti-TCR showed small clusters of CD3 in the center of the cell-coverslip interface, whereas CD148 accumulated at the edges of the spread cell (Fig. 4 C). This pattern of CD148 staining is similar to those previously described for CD45 and CD43 using Ab-coated slides (Bunnell et al., 2002). When T cells stimulated with SAg and APCs were visualized, CD3 clustered tightly at the interface of the T cell and APC. Staining of CD148 showed that it was largely excluded from this region of CD3 staining (Fig. 4 D). To further confirm the exclusion of CD148 from the immunologic synapse, localization of CD148 was compared with that of CD43 and CD45, two molecules previously demonstrated to be excluded from the synapse (Johnson et al., 2000; van der Merwe et al., 2000; Delon et al., 2001).



**Figure 4. CD148 does not colocalize with CD3 and is excluded from the immunologic synapse upon triggering with a polarizing stimulus.** Cells were induced to express CD148 and stimulated with (A) PBS, (B) soluble anti-TCR (C305), (C) anti-TCR (C305)-coated coverslips, or (D) APCs with SAg (1  $\mu\text{g}/\text{ml}$  SEE). Immunofluorescence was performed using anti-hCD148 and anti-CD3 $\epsilon$  and the appropriate secondary antibodies. CD148 is shown in red and CD3 is shown in green. For D, the lower panel for each stain represents the reconstructed Z stack contact view at the dotted line from the DIC image. (E) Jurkat cells were transfected with mCD148 and truncated hCD25, or (F) mCD148, mCD45RO, and truncated hCD25. Transfections were enriched for CD25 $^+$  before conjugate formation. Cells were stained for mCD148 (red), CD3 (green), and either (E) hCD43 (blue) or (F) mCD45 (blue). Bars, 5  $\mu\text{m}$ .



**Figure 5. The extracellular domain of CD148 plays a role in exclusion from the immunologic synapse.** (A) A representation of the chimeric molecule containing a FLAG epitope and the full-length mCD148 extracellular domain fused to a truncated hCD2 transmembrane domain (FLAG-CD148-2TM) was compared with an extracellular domain deletion chimera (FLAG-CD2TM). (B) Cells were transfected with one of the constructs along with truncated hCD25 and enriched for CD25<sup>+</sup> cells before conjugate formation. Conjugates were fixed and stained with anti-FLAG and anti-CD3 $\epsilon$ , shown in red and green, respectively. The lower panel for each of stain represents the reconstructed Z stack contact view at the dotted line from the DIC image. Bars, 5  $\mu$ m. (C) Quantitation of the exclusion of each chimeric molecule out of the immunologic synapse was performed. The graph depicts the mean of two or three experiments, where between 30 and 60 conjugates for each construct were analyzed for each experiment. Error bars represent the standard error of the mean.

As shown in Fig. 4 (E and F), both CD43 and CD45 colocalized with CD148, demonstrating that CD148 is indeed excluded from the immunologic synapse.

### The extracellular domain mediates exclusion from the synapse

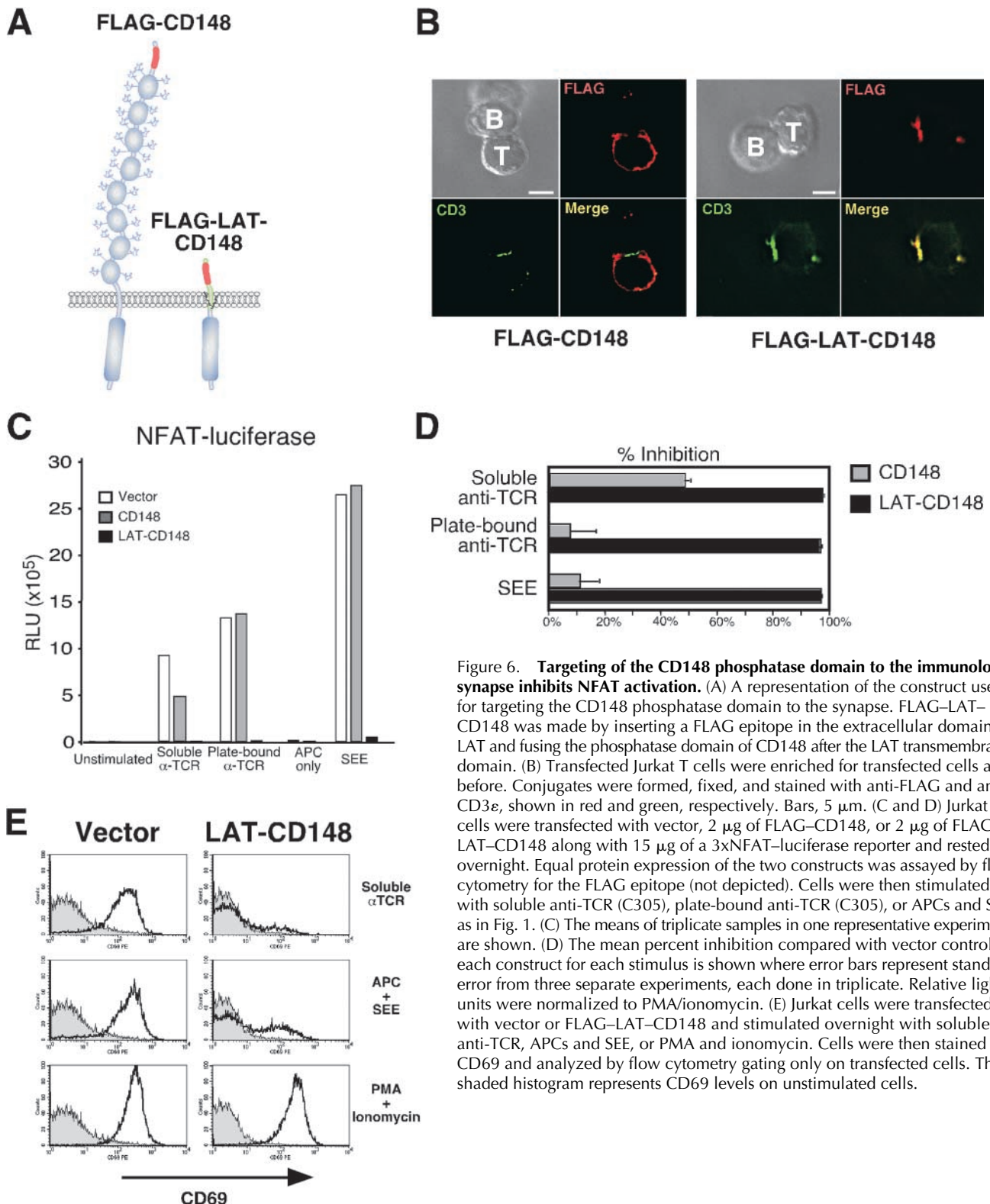
To examine the mechanism that mediates the exclusion of CD148 from the synapse, the entire extracellular domain of CD148, with a FLAG epitope inserted just COOH terminal to the signal peptide, was fused to the transmembrane domain of CD2 (FLAG-CD148-2TM). This chimeric molecule was truncated after the sixth cytoplasmic amino acid to prevent its association with CD2AP, as previous reports had shown that CD2AP plays a role in receptor patterning and cell polarization (Dustin et al., 1998). The localization of FLAG-CD148-2TM protein was compared with a protein that only has the FLAG epitope on the extracellular portion of the molecule fused to the same CD2 transmembrane domain (FLAG-CD2TM) (Fig. 5 A). The endogenous CD148 signal sequence was used in both constructs for consistency. Transfected Jurkat T cells were mixed with Daudi B cells preincubated with SAg as before. Conjugates were visualized by immunofluorescence of the FLAG epitope (in red) and CD3 (in green) (Fig. 5 B). The chimera containing the full-length extracellular domain of CD148 was generally excluded from the site of CD3 staining, whereas the molecule containing only the FLAG epitope in the extracellular domain showed considerable colocalization with CD3. Quantitation of multiple conjugates fixed at a 25-min time point determined that the FLAG-CD148-2TM chimera was excluded a bit less frequently than wild-type CD148, however much more frequently than just the FLAG-CD2TM (Fig. 5 C). Conjugates were scored as “excluded” if CD3 staining was tightly localized at the site of T cell-APC contact and if the majority of FLAG staining did not colocalize with CD3. These data demonstrate that the extracellular domain does indeed play a major role in the exclusion of

CD148, but suggest that other factors may also be involved in exclusion of CD148 from the immunologic synapse.

### Targeting of the CD148 phosphatase domain to the synapse inhibits signaling

To determine if exclusion from the immunologic synapse could limit the ability of CD148 to down-regulate NFAT, we constructed a chimeric molecule that would target the CD148 phosphatase domain into the synapse (Fig. 6 A). A FLAG-tagged LAT extracellular and transmembrane region was used because previous studies had demonstrated that LAT colocalizes with CD3 (Harder and Kuhn, 2000; Bunnell et al., 2002; Tanimura et al., 2003). Immunofluorescence with antibodies to the FLAG epitope and the CD3 $\epsilon$  chain of TCR was performed to demonstrate that the chimera was indeed targeted to the immunologic synapse (Fig. 6 B). It is interesting to note that the efficiency of mature synapse formation was decreased in cells expressing FLAG-LAT-CD148 compared with wild-type FLAG-CD148 (unpublished data).

To determine if targeting of the CD148 phosphatase domain to the synapse had a functional effect on T cell signaling, an NFAT-luciferase reporter was cotransfected. Similar to Fig. 1, the wild-type FLAG-CD148 construct inhibited NFAT activation induced by soluble anti-TCR stimulation, whereas plate-bound anti-TCR or SAg and APC stimulation was only slightly affected. When the FLAG-LAT-CD148 chimera was expressed at the same level as the full-length FLAG-CD148 molecule, as measured by FACS<sup>®</sup> staining for the FLAG epitope (unpublished data), it completely inhibited NFAT activation for all of the stimuli used. The mean percent inhibition of NFAT activation is depicted in Fig. 6 D. When transfected cells were also assayed for CD69 up-regulation in response to stimuli, the expression of FLAG-LAT-CD148 dramatically inhibited CD69 up-regulation in response to soluble anti-TCR and APCs with SEE (Fig. 6 E). These experiments demonstrate that when the phosphatase domain of CD148 is not excluded from the site



**Figure 6. Targeting of the CD148 phosphatase domain to the immunologic synapse inhibits NFAT activation.** (A) A representation of the construct used for targeting the CD148 phosphatase domain to the synapse. FLAG-LAT-CD148 was made by inserting a FLAG epitope in the extracellular domain of LAT and fusing the phosphatase domain of CD148 after the LAT transmembrane domain. (B) Transfected Jurkat T cells were enriched for transfected cells as before. Conjugates were formed, fixed, and stained with anti-FLAG and anti-CD3 $\epsilon$ , shown in red and green, respectively. Bars, 5  $\mu$ m. (C and D) Jurkat T cells were transfected with vector, 2  $\mu$ g of FLAG-CD148, or 2  $\mu$ g of FLAG-LAT-CD148 along with 15  $\mu$ g of a 3xNFAT-luciferase reporter and rested overnight. Equal protein expression of the two constructs was assayed by flow cytometry for the FLAG epitope (not depicted). Cells were then stimulated with soluble anti-TCR (C305), plate-bound anti-TCR (C305), or APCs and SAg as in Fig. 1. (C) The means of triplicate samples in one representative experiment are shown. (D) The mean percent inhibition compared with vector control of each construct for each stimulus is shown where error bars represent standard error from three separate experiments, each done in triplicate. Relative light units were normalized to PMA/ionomycin. (E) Jurkat cells were transfected with vector or FLAG-LAT-CD148 and stimulated overnight with soluble anti-TCR, APCs and SEE, or PMA and ionomycin. Cells were then stained for CD69 and analyzed by flow cytometry gating only on transfected cells. The shaded histogram represents CD69 levels on unstimulated cells.

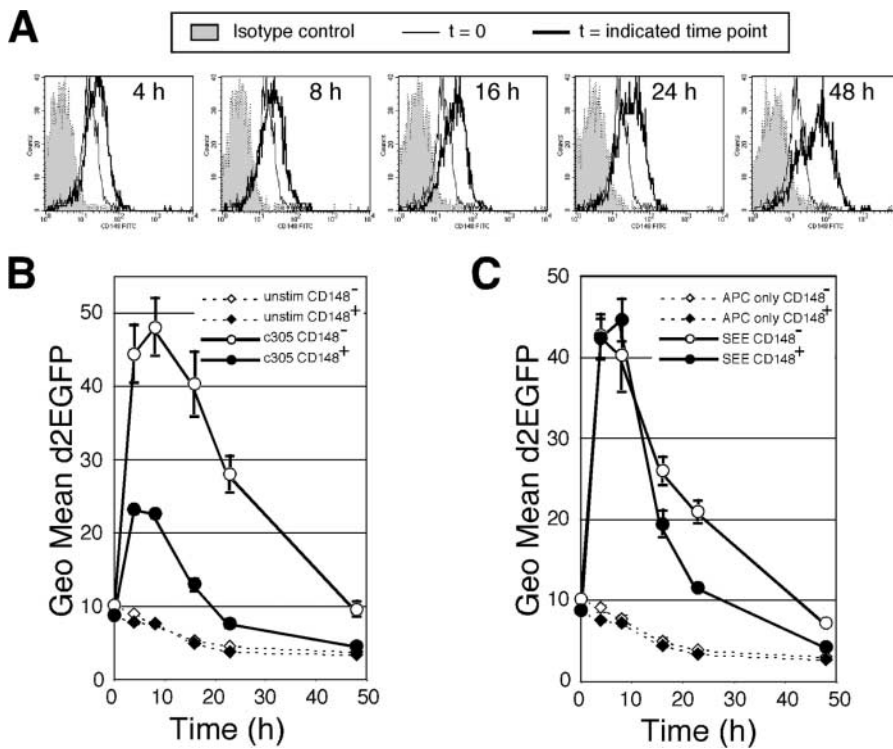
of the immunologic synapse, it can potently inhibit TCR-induced activation.

#### CD148 down-regulates prolonged T cell signaling

As expression of CD148 did not have an effect on SAg- and APC-induced NFAT activation, presumably due to exclu-

sion from potential substrates associated with the TCR complex, we wanted to test whether CD148 plays a role in down-regulating prolonged T cell signaling events. Previous studies had demonstrated that stimulation of primary human T cells with either phytohemagglutinin or anti-CD3 antibodies induces the up-regulation of CD148 after 48 h





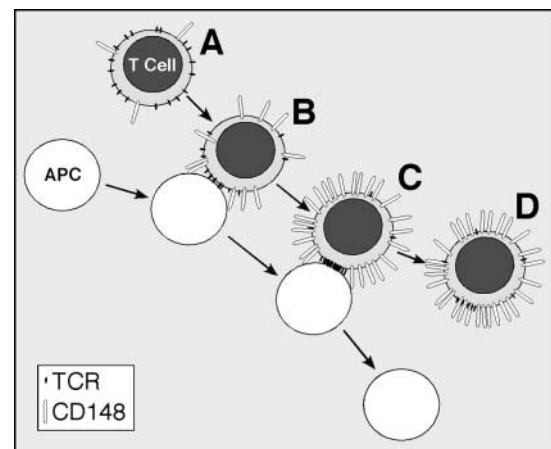
**Figure 7. Expression of CD148 inhibits prolonged NFAT signaling in T cell-APC conjugates.**

(A) Human PBMCs were isolated and stimulated with a cocktail of SEA, SED, and SEE (300 ng/ml each). Cells were stained at the indicated time points with anti-CD148 and anti-CD4. Histograms represent CD148 expression gated on CD4<sup>+</sup> cells. Shaded gray is the isotype control at each time point, whereas the  $t = 0$  (thin line) and the indicated time point (thick line) are overlaid. (B and C) Tet-inducible CD148 Jurkats were induced for 48 h and then transfected with the 4xNFAT-d2EGFP reporter construct and truncated CD25 as a transfection marker. Cells were rested for 6 h and then stimulated with the indicated stimulus. Cells were analyzed by FACS<sup>®</sup> at various time points by gating on CD25<sup>+</sup>, 7-AAD<sup>-</sup> cells (live cell marker) and reading out the geo mean fluorescence of the d2EGFP. (B) Cells with or without Dox were either mock stimulated or stimulated with soluble anti-TCR. (C) Cells with or without Dox were stimulated with either Raji B cells alone or Raji B cells with 250 ng/ml SEE. Shown is the mean from three separate experiments where error bars indicate the standard error.

(Tangye et al., 1998a). To more carefully study the time course of CD148 up-regulation using a more physiologic stimulus, we stimulated primary human CD4 T cells with a mixture of SAGs (SEA, SED, and SEE) and assayed for CD148 expression by FACS<sup>®</sup> at 0, 4, 8, 16, 24, and 48 h after stimulation. As shown in Fig. 7 A, freshly isolated CD4 cells express low levels of CD148. However, after stimulation, CD148 levels are increased, even at 4 h, and continue to rise. CD148 expression was highest at 48 h.

As expression of CD148 continued to increase past 8 h, we hypothesized that CD148 may play a role in down-regulating the prolonged signaling events after the T cell has become activated. To test this hypothesis, an NFAT reporter construct was generated that expresses a destabilized form of EGFP with a half-life of 2 h (4xNFAT-d2EGFP). This destabilized form of GFP contains a PEST domain that has been shown to target the protein for more rapid degradation (Li et al., 1998). Response of the 4xNFAT-d2EGFP reporter could then be monitored at later time points by the loss of GFP fluorescence. As earlier data showed that expression of CD148 in SAG- and APC-induced T cell activation had no effect on activation up to 8 h and due to the nonsynchronous nature of the inducible system, the reporter was transfected into either CD148-expressing or nonexpressing cells. When reporter-transfected Jurkat T cells inducibly expressing CD148 were stimulated with soluble anti-TCR, NFAT activation was suppressed at all time points, similar to that seen with the NFAT-luciferase construct at 8 h (Fig. 7 B). When these same cells were stimulated with SAG and APCs, early NFAT responses were similar regardless of CD148 expression, as seen before. However, at time points after 8 h, cells expressing CD148 down-regulated NFAT ac-

tivation more rapidly than those not expressing CD148 (Fig. 7 C). These data indicate that CD148 does indeed play a role in down-regulating the prolonged signaling events after SAG and APC stimulation.



**Figure 8. Model of CD148 function in T cells.** (A) A mature resting T cell in the periphery expresses low levels of CD148 at the cell surface. (B) When the T cell encounters an APC presenting the proper peptide or SAG, the T cell engages the APC and the TCR clusters at the site of APC contact. During this engagement, the T cell receives the proper signals for the up-regulation of CD148. (C) A fully mature synapse stabilizes the T cell-APC contact and prevents the up-regulated CD148 molecules from dephosphorylating its substrates within the immunologic synapse. (D) Once the T cell has finished receiving all of the signals required for proliferation and IL-2 secretion, it releases the APC, allowing CD148 to redistribute, access its substrates, and down-regulate the sustained signaling events that are taking place at the synapse.

## Discussion

Based on this study, we propose the following model for the role of CD148 in TCR signaling (Fig. 8). Resting T cells express low levels of CD148 at the cell surface. Upon engagement of the TCR with the appropriate SAg or peptide-loaded MHC molecule, signals are generated to up-regulate the expression of CD148. The low amounts of CD148 that are present at the cell surface are excluded as the immunologic synapse forms. More importantly, the newly synthesized CD148 that is made in response to the initial T cell activation is excluded as well. This is critical, as we know that a T cell must receive a continuous signal, on the order of hours, to proliferate and generate IL-2. If CD148 were not segregated away from potential substrates in the synapse, premature termination of T cell response would occur. Once the T cell has disengaged from the APC, CD148 can access its substrates, leading to the dephosphorylation of LAT, PLC- $\gamma$ 1, and other substrates, thereby down-regulating prolonged T cell activation.

To date, the studies investigating the role of CD148 in TCR signaling have relied solely upon soluble stimuli such as phytohemagglutinin, PMA and ionomycin, or soluble anti-TCR Ab (Tangye et al., 1998a,b; Baker et al., 2001). These techniques, though effective in activating T cells, do not take into consideration T cell polarization after receptor engagement. To further investigate the function of CD148 in TCR signaling, we used more physiologic, polarizing stimuli. In experiments involving plate-bound anti-TCR mAb, bead-coated anti-TCR mAb, and APCs presenting SAg, expression of CD148 had only a minimal effect on TCR signaling events such as CD69 up-regulation and NFAT activation at early time points. Previous studies have demonstrated that these polarizing stimuli induce the formation of an immunologic synapse-like structure enriched in the TCR and downstream signaling molecules (Lowin-Kropf et al., 1998; Roumier et al., 2001; Bunnell et al., 2002). During T cell engagement by an APC with SAg, CD148 was excluded from the immunologic synapse, leading to the notion that this serves to sequester CD148 from substrates that are localized within the synapse, such as LAT. In contrast, when the phosphatase domain of CD148 was targeted to the synapse, NFAT activation was completely inhibited.

The data presented here support a model proposed by van der Merwe et al. (2000), termed the kinetic-segregation model of TCR triggering. This model suggests that the role of the immunologic synapse is to create a "prosignaling" environment by the exclusion of active tyrosine phosphatases and the enrichment of tyrosine kinases. In this model, the initial interaction between the APC and TCR is regulated by multiple small close-contact zones within the contact area that result in the "small-scale" segregation of molecules (van der Merwe et al., 2000). Areas such as these could explain why the tyrosine phosphorylation induced with anti-TCR-coated beads is not affected by CD148 even at very early time points (1–3 min). These time points are well under the amount of time it takes to form a mature immunologic synapse (Bromley et al., 2001).

For the purposes of this study, the term immunologic synapse is used to describe the area of TCR enrichment. The

immunologic synapse was originally described as being composed of two parts: the central supramolecular activation cluster (c-SMAC) and the peripheral SMAC (p-SMAC) (Monks et al., 1998). The c-SMAC contains proteins such as the TCR complex, CD28, PKC- $\theta$ , and src family kinases. The p-SMAC, consisting of proteins such as CD2, LFA-1, and talin, appears as a ring that surrounds the c-SMAC. Most studies of the immunologic synapse have been done using mouse T cell clones that respond to APC loaded with a specific peptide (Bromley et al., 2001; van der Merwe, 2002). As a reactive peptide for Jurkat T cells is not known, the SAg system was used to mimic peptide-dependent interactions between the TCR and MHC. In the case of Jurkat cells stimulated with SAg, we were never able to clearly discriminate between c-SMAC and p-SMAC structures as described in mouse clones and preactivated T cells. In general, the site of APC contact in this system showed colocalization of molecules from both the c-SMAC and p-SMAC (unpublished data). This may be a characteristic of Jurkat cells interacting with SAg and APCs, or it may be a feature of T cells stimulated with SAg. It is important to note that proteins such as CD43 and CD45, excluded from the synapse in mouse clones, were also excluded in the Jurkat–SAg system.

With the elucidation of the ezrin/radixin/moesin (ERM)-mediated mechanism for CD43 exclusion from the immunologic synapse, the model of purely size-based exclusion has become less favored. However, many groups have demonstrated that altering the size of extracellular domains can alter the function of proteins such as CD45 and CD48 (Wild et al., 1999; Irls et al., 2003). The attachment of the CD148 extracellular domain onto the TM of CD2 alone was enough to cause the exclusion of the chimera from the immunologic synapse, albeit not to the same extent as native CD148. When cells expressing the FLAG–CD148–2TM construct were dropped onto anti-TCR-coated coverslips, the chimera was not excluded (unpublished data). One possible interpretation is that dropping cells onto a coverslip does not confer the same spatial constraints that are involved in T cell–APC conjugates. Another is that a possible ligand on the APC may be involved in the exclusion from the synapse. These data suggest that the extracellular domain plays an important role, but other factors requiring the cytoplasmic domain also contribute to the exclusion of CD148 from the synapse. As CD148 has a very similar distribution on the cell surface to that of CD43, an obvious candidate for one of these other factors involved in the exclusion of CD148 was that of the ERM family. Previous studies have mapped the ERM protein's association with CD43, CD44, and ICAM-2 to a series of positively charged amino acids in the juxtamembrane cytoplasmic domain (Yonemura et al., 1998). CD148 does have a similar stretch of residues, but almost all transmembrane proteins do, presumably as a stop-transfer sequence for the transmembrane domain. Experiments attempting to coimmunoprecipitate ezrin with CD148 did not reveal any association of the two proteins (unpublished data). Moreover, the FLAG–LAT–CD148 chimera still contains this string of charged residues on the cytoplasmic face of the TM domain. This construct localized to the synapse, implying that the charged residues do not play a critical role



in the exclusion of CD148. Another possible mechanism mediating the exclusion of CD148 from the synapse is the PDZ binding motif present at the very COOH terminus of CD148, which has been demonstrated to bind syntenin (Iuliano et al., 2001). When the GYIA motif was deleted, this truncated CD148 functioned similarly to wild-type CD148 in its ability to down-regulate NFAT in response to stimulation with soluble anti-TCR mAb. The PDZ mutant also did not inhibit the immobilized anti-TCR or SAg and APC stimulation, nor was it localized differentially by immunofluorescence microscopy compared with wild-type CD148 (unpublished data).

The role of the immunologic synapse in T cell–APC interactions has remained a controversial topic. From studies examining tyrosine phosphorylation by biochemistry and synapse formation by microscopy, it is clear that signaling precedes the formation of the synapse (Lee et al., 2002). Observations such as these have led us to propose that the synapse is a mechanism whereby the cell can prevent the premature termination of a productive signaling event, as inhibitory proteins such as CD148 become up-regulated. Once the T cell has obtained the signal it requires for proliferation and other effector functions, it disengages from the APC, which then allows CD148 to redistribute and gain access to and dephosphorylate substrates. This segregation of phosphatases from their substrates is potentially yet another layer of regulation, beyond protein expression, enzymatic activation, or protein–protein interactions, for the control of T cell activation.

## Materials and methods

### Antibodies and cells

TCR stimulation was performed with the anti-Jurkat TCR V $\beta$ 8 monoclonal antibody (mAb) C305 (Weiss and Stobo, 1984). The antiphosphotyrosine (4G10) and anti-FLAG (M2) mAbs are from Upstate Biotechnology and Sigma-Aldrich, respectively. 7-AAD, anti-CD5 (L17F12), anti-CD25 (2A3), anti-CD69 (L78), anti-CD3 $\epsilon$  (SK7), and anti-mCD45 (30F-11) are from BD Biosciences. Anti-human CD148 (A3) has been previously described (Tangye et al., 1998a). Anti-mouse CD148 (8A-1) was generated by immunizing a hamster with cells expressing murine CD148 and screening hybridomas for antibodies specific to the extracellular domain of murine CD148. Anti-human CD43 was a gift from L. Lanier (University of California, San Francisco). Streptavidin conjugated to Alexa 488, 555, and 647 were from Molecular Probes, and goat anti-mouse IgG-Cy5 and donkey anti-rat IgG-Cy3 were from Jackson ImmunoResearch Laboratories. Jurkat T cells, Daudi B cells, and Raji B cells were maintained in RPMI 1640 supplemented with 10% FBS, 2 mM glutamine, penicillin, and streptomycin. Tetracycline-inducible CD148 cells (clone L12) were previously described (Baker et al., 2001). For PBMC (peripheral blood mononuclear cell) separation, buffy coats from whole blood were layered onto Ficoll-Hypaque (Sigma-Aldrich) and centrifuged at 350 g for 30 min at RT. Cells were washed and resuspended in RPMI 1640 with 10% FBS, 2 mM glutamine, penicillin, and streptomycin.

### Plasmids

pcdef3-mCD148 was constructed by removing the mCD148 cDNA from pME18S-Byp (a gift from H. Umemori (University of Tokyo, Tokyo, Japan) and inserting it into pcdef3 vector cut with EcoRV. This version of the mCD148 cDNA has a T175S, compared with the published sequence. FLAG-mCD148 was generated by inserting a FLAG epitope between Ala38 and Ala39 (after the signal sequence) in pcdef3-mCD148. Residue numbers for FLAG-mCD148 do not include the amino acids from the FLAG epitope. FLAG-CD148-2TM was created by fusing the FLAG-mCD148 extracellular domain at Asp870 to Ile210 of hCD2. The FLAG-CD2TM construct was made by fusing the signal sequence and FLAG epitope of FLAG-mCD148 at Ala48 to Gly207 of hCD2. Both CD2-containing constructs were truncated at Arg241. The FLAG-LAT-CD148 con-

struct was made by first inserting a FLAG epitope after the start methionine of human LAT. The fusion joined LAT (Pro33) to mCD148 (Phe896) with a Ser linking the two fragments. Fusion constructs were made by multistep PCR, and all final products were sequenced. The 3xNFAT-luciferase plasmid was previously described (Shapiro et al., 1996). Murine CD45RO was expressed in a pcdef3 expression construct. The 4xNFAT-d2EGFP was made by replacing the EGFP from a 4xNFAT-EGFP with the d2EGFP (CLONTECH Laboratories, Inc.). The original 4xNFAT-EGFP was made by placing four copies of the NFAT/AP-1 binding site from IL-2 promoter in pEGFP-1 (CLONTECH Laboratories, Inc.).

### APC and SAg stimulation

Equal numbers of Daudi or Raji B cells were used as APCs. Cells were loaded with the indicated staphylococcal enterotoxin (SE) SAg for 30 min before stimulation. SEE, SEA, and SED were purchased from Toxin Technology, Inc. For PBMC stimulation, a mixture of SEA, SED, and SEE were used at 300 ng/ml of each type. Cells were centrifuged to the bottom of a round bottom tube to initiate stimulation and left undisturbed until time of harvest.

### Transfection and enrichment

Jurkat cells were transfected as previously described (Baker et al., 2001). For enrichment of transfected cells, cells were cotransfected with a truncated CD25 construct. 18 h later, cells were labeled with anti-human CD25 MACS beads (Miltenyi Biotec) for 20 min on ice and then washed. Cells were positively selected with an LS column and MACS magnet (Miltenyi Biotec). Enriched cells were resuspended in media and allowed to rest for 2 h.

### Lysates and Western blots

Cells were lysed in 1% NP-40 and visualized by Western blotting as previously described (Lin and Weiss, 2001a). For quantitation of Western blots, bands were quantitated on a Kodak Imaging Station using Kodak 1D image analysis software version 3.5 (Eastman Kodak Co.).

### Immunofluorescence

Cells were placed onto poly-L-lysine-coated slides and allowed to settle for 5 min. Conjugates were made by preloading Daudi B cells with 1  $\mu$ g/ml of SEE for 30 min before mixing 1:1 with T cells in complete media. Cells were then gently centrifuged for 30 s and placed at 37°C for 25 min. Conjugates were gently resuspended and allowed to settle on slides as before. Paraformaldehyde was added to 3% final concentration for 30 min. Cells were then blocked in 1% BSA and 10% rabbit serum in PBS. Cells were stained with the indicated antibodies, followed by the appropriate secondary antibodies when necessary. Slides were visualized on a Marianas Turn-Key system from Intelligent Imaging, and images were analyzed using SlideBook software. Images were deconvolved by nearest neighbor and exported as TIFF files.

### Reporter assays

Jurkat cells were transfected as before with 20  $\mu$ g of a 3xNFAT-luciferase reporter construct and the indicated CD148 construct. Expression of the CD148 constructs was examined by FACS<sup>®</sup>. Stimulation for luciferase assays was performed for 8 h. Cells were harvested, lysed, and assayed for luciferase activity as previously described (Shapiro et al., 1996). For NFAT activation time course experiments, CD148 was induced on clone L12 for 48 h before transfection. 15  $\mu$ g of a truncated CD25 construct was cotransfected with 20  $\mu$ g of the 4xNFAT-d2EGFP reporter. The cells were allowed to recover for 6 h before stimulation. NFAT activation was assessed as the geometric mean fluorescence intensity of CD25<sup>+</sup>, 7-AAD<sup>-</sup> cells.

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