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CD5 Inhibits Signaling at the Immunological Synapse Without Impairing Its Formation

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Physiologically, Ag detection by T cells occurs at the immunological synapse (IS) formed at the interface with an APC. CD5 is considered as an inhibitory molecule for Ag receptor-mediated signals in T cells. However, the influence of CD5 at the IS on synapse formation and functioning has not yet been reported. We demonstrate here that CD5 is recruited and tightly colocalized with CD3 in different human and murine IS. Following transfection in a CD5-negative T cell line of CD5 fused to the green fluorescent protein, we show that CD5 recruitment includes a fast Ag-independent and a slower Ag-dependent component. In video-imaging recordings of doubly transfected cells, the movements of CD3 and CD5 show similar kinetics, and the amount of CD3 recruited to the synapse is unaffected by CD5 expression. Moreover, APC-T cell adhesion is unchanged in CD5-expressing cells. Despite this, the extent of tyrosine phosphorylation at the synapse and the amplitude of calcium responses induced by Ag recognition are both decreased by CD5. These inhibitions increase with CD5 membrane levels. They also require the pseudo-immunoreceptor tyrosine-based activation motif expressed in the cytoplasmic domain of the molecule. Thus, CD5 is rapidly recruited at the IS and lowers the T cell response elicited by Ag presentation by targeting downstream signaling events without affecting IS formation. The Journal of Immunology, 2003, 170: 4623–4629.
have analyzed in detail the kinetics of its recruitment and its Ag dependence. Simultaneous video recordings of CD5 recruitment together with either CD3 recruitment or Ca$^{2+}$ responses were performed. We conclude that a role of CD5 in IS formation or stability is unlikely, and that CD5 acts mostly or entirely by inhibiting signaling at the synapse. As in B cells (12), our results also suggest a role for the pseudo-immunoreceptor tyrosine-based activation motif (ITAM) of CD5 in this process.

### Materials and Methods

#### Antibodies

The following mouse mAbs were used: anti-human CD3 (UCHT1, ascite), anti-human CD5 (O490D, a gift from Dr. L. Boumsell, Institut National de la Sante et de la Recherche Médicale, Unité 448, Créteil, France), and anti-phosphotyrosine (4G10, Upstate Biotechnology, Lake Placid, NY), PE- or biotin-conjugated hamster anti-murine CD3e Abs (145-2C11) were purchased from BD PharMingen (San Diego, CA), Rhodamine Red-X-conjugated and PE-conjugated goat F(ab)2 anti-mouse IgG secondary Abs were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA) and Immunotech (Marseille, France), respectively. Streptavidin Alexa Fluor 488 conjugate was purchased from Molecular Probes (Eugene, OR).

#### Cells

The murine T cell hybridoma T8.1, provided by Dr. O. Acuto (Institut Pasteur, Paris, France), expresses a human CD4 and a chimeric human/Vα2 mouse (CuCDB) TCR specific for a tetanus toxin peptide (691-706-ascite; NeoSystem, Strasbourg, France; referred to as TTP) restricted to HLA-DRB1*1102 (21). The murine cell transfectants used as APC (L625.7) express HLA-DR*1102 and endogenous B7.1, ICAM-2, and, at a low level, ICAM-1 after, cells were left adhering for 8 min more, and then increasing shear flow was applied. Remaining cells were quantified for each condition, and results were expressed as the percentage of cells remaining adherent.

Plasmid constructs

CD5 was amplified by PCR using the primers 5′-TACCCGCGCCAGACTCTTCACCTG-3′ and 5′-GCGACCTCTTGACGCCCCATGC-3′ and the full-length cDNA of human CD5 into the pRC vector as a template. The PCR product was introduced in the TOPO TA cloning vector (Invitrogen, Leek, The Netherlands) according to the manufacturer’s instructions, then subcloned after EcoRI digestion into the pEFP-N1 or pEFP-N1 vector (Clontech Laboratories, Palo Alto, CA) in-frame with the N-terminal sequence of the green or cyan fluorescent protein (GFP or CFP). Constructs (CD5-GFP, CD5-CFP) were verified by sequencing. The CD3ζ-yellow fluorescent protein (YFP) retroviral construct was a gift from Dr. M. Malissen (Institut National de la Santé et de la Recherche Médicale-Centre National de la Recherche Scientifique, Marseilles-Luminy, France). The CD5 retroviral vector was constructed by inserting the full-length cDNA of human CD5 into a murine stem cell virus-retroviral vector (CD5-MSCV).

To make the desired deletion of the pseudo-ITAM motif (YSQPRPN SRLSAYPAL) in the CD5cytoplasmic domain, the following mutagenic oligonucleotides were used: 5′-GCCCTCACTGGAAGCCGAGGTTCTTC-3′ and 5′-GGACGGGATGGACAAACCTCTTTCCTTGAACGCCGAGG-3′, according to the manufacturer’s protocol of the Quick Change site-directed mutagenesis kit (Stratagene, La Jolla, CA), and using CD5-murine stem cell virus-retroviral vector as a template. The construct was verified by DNA sequencing.

Cell transfection

Stable T8.1 transfectants were obtained as follows: cells (10$^5$ in DMEM supplemented with 10% FCS) were electroporated in a Gene Pulse cuvette (Bio-Rad, Hercules, CA) with 5 μg of CD5-GFP or CD5-CFP plasmid at 960 μF at 250 V. Cells were cotransfected with 20 μg of Sro plasmid to confer puromycin resistance. Forty-eight hours after transfection, cells were cloned in 96-well tissue culture plates in DMEM containing 400 nM methotrexate, 1 mg/ml G418, and 1 μg/ml puromycin. Puromycin-resistant transfectants were then selected for CD5-GFP expression by flow cytometry (EPICSXL; Beckman Coulter, Miami, FL) and for equivalent expression of CD3, CD28, LFA-1, and CD43 markers with wild-type cells. T8.1 cells expressing both CD5-CFP and CD3ζ-YFP were obtained by infecting cells stably expressing CD5-CFP with a retrovirus (pMFG-B2) containing CD3ζ-YFP. Retroviral production was obtained by transfecting the Plat-E packaging cell line (22) using Eugene 6 (Roche, Meylan, France). After 24 h, supernatants were added, and cells were cultured for 24 h at 32°C. Supernatant was then added to T8.1 CD5-CFP cells for 24 h at 32°C, and YFP-positive cells were sorted with a fluorescence cell sorter (Elite; Beckman Coulter). Cells expressing different levels of CD5 were obtained by infecting T8.1 cells with the retrovirus construct containing CD5, followed by limited dilution cloning and analysis for CD5 expression by flow cytometry. 177 cells were transfected with 10 μg of CD5-GFP at 960 μF and 320 V and were used 24 h after transfection.

#### Cell preparation

CD5BL/6 splenic dendritic cells (DCs) were purified as previously described (23), and macrophages were purified by magnetic depletion of splenic T cells, B cells, and DCs with a mixture of appropriate rat anti-mouse receptor Abs, followed by incubation with Dynabeads (DynaBiotech, Lake Success, NY) coupled to anti-rat IgG. Splenic T cells from P14 TCR-transgenic mice were purified by negative depletion with a mixture of anti-CD45RB (Cedarlane, Hornby, Canada), anti-MAC1 (BD PharMingen), and anti-CD11c Abs (23).

Conjugate formation and fluorescence analysis

Twelve to 16 h before the experiment, L625.7 fibroblasts (3 × 10$^5$/ml) were plated in their culture medium with or without peptide Ag and incubated on glass coverslips at 37°C. Raji cells were incubated with 1 μM stock brefeldin A (tertiary butanol) and 1 μM anti-CD11c (clone UCHT1, as a control) in the wells of a 24-well plate with 0.5 × 10$^5$ T cells. Cells were then fixed with 3% paraformaldehyde in PBS for 10 min and, after several washes, incubated for 20 min with 0.1 M glycine in PBS. Cells were permeabilized, or not, in a PBS-0.1% Triton X-100 solution for 10 min and labeled in PBS-0.2% BSA with the different Abs. After washing, coverslips were mounted in Mowiol (Sigma-Aldrich). Immunofluorescence and transmission light images were acquired on an Eclipse TE300 inverted microscope (Nikon, Badhoevedorp, The Netherlands) equipped with a cooled CCD camera (CoolSNAPFx; Roper Scientific, Evely, France). Image capture and quantitative analysis were realized with Metafluor and Metamorph software (Universal Imaging, West Chester, PA).

#### Single-cell Ca$^{2+}$ and fluorescence video imaging

L625.7 or Raji cells were prepared and pulsed with Ag as described above and plated on glass coverslips mounted on 25-mm petri dishes. T8.1 or J77 cells (5 × 10$^5$) were incubated for 20 min at 37°C with 1 μM Fura-2/AM (Molecular Probes) and added to the APC in mammalian saline at 37°C. After macrophages and macrophages were incubated with 10-6 M LCMV gp33 peptide (Neo-system, Strasbourg, France) for 90 min at 37°C, then plated for 10 min on poly-L-lysine-coated glass coverslips. Splenic DCs and macrophages were incubated with 10-6 M LCMV gp33 peptide (Neo-system, Strasbourg, France), and then fixed with 3% paraformaldehyde in PBS for 10 min and, after several washes, incubated for 20 min with 0.1 M glycine in PBS. Cells were permeabilized, or not, in a PBS-0.1% Triton X-100 solution for 10 min and labeled in PBS-0.2% BSA with the different Abs. After washing, coverslips were mounted in Mowiol (Sigma-Aldrich). Immunofluorescence and transmission light images were acquired on an Eclipse TE300 inverted microscope (Nikon, Badhoevedorp, The Netherlands) equipped with a cooled CCD camera (CoolSNAPFx; Roper Scientific, Evely, France). Image capture and quantitative analysis were realized with Metafluor and Metamorph software (Universal Imaging, West Chester, PA).

#### Adhesion assay

Fibroblasts were laid on glass coverslips for 16–20 h in the presence of 1 μg/ml of soluble Ag. Cell density was adjusted so as to achieve a uniform monolayer. To quantify their adhesion, T8.1 cells labeled with 10 μM CFSE (Molecular Probes) and suspended in mammalian saline were introduced into a parallel plate flow chamber (Immunetics, Cambridge, MA) that was affixed to the coverslip bearing the fibroblast monolayer. In this system a flow rate of 2 ml/min resulted in a shear stress of 0.09 dynes/cm$^2$. Image acquisition was started as soon as the first CFSE-labeled T cells appeared in the chamber and was stopped after 2 min. The initial adhesion phase was then quantified by counting the number of cells remaining adherent and the total number of cells that had passed through the microscopic field. Thereafter, cells were left adhering for 8 min more, and then increasing shear stresses were applied, ranging from 0.09–4.5 dynes/cm$^2$. Remaining cells were quantified for each condition, and results were expressed as the percentage of cells remaining adherent.
Quantitative analysis of CD3 or CD5 recruitment at the synapse

Dynamic synaptic recruitment was assessed with the Metafluor software as the ratio between the fluorescent intensity in a region centered on the IS and the intensity over the whole cell. Results are expressed as the fold increase compared with ratio observed in the same regions before contact formation. For averaging purposes, time zero for each response was that of the initial detectable cell-cell contact. For synaptic phosphotyrosine signal and whole CD5 or CD5 ITAM synaptic recruitment, we measured the intensity ratio between a region in the synapse and a region of same area outside the synapse.

Statistics

Data are expressed as the mean ± SD, and the significance of differences between two series of results was assessed using Student’s t test.

Results

CD5 relocalization in IS formed with various T cell/APC systems

The distribution of CD5 molecule at the surface of unstimulated T cells is usually even, with no sign of spontaneous polarization or clustering. However, when examined after 15 min of APC-T cell interaction, CD5 was found clustered at the contact zone. Such a clustering was observed at the synapse formed between Jurkat T cells (J77 cells transiently transfected with a construction coding for a CD5-GFP fusion protein) and Raji B cells presenting the superantigen SEE (Fig. 1A). It was also observed in conjugates formed between P14 T cells, which express a transgenic TCR specific for lymphocytic choriomeningitis virus glycoprotein peptide gp33 and H-2D^d (24), and either dendritic cells (Fig. 1, A and D) or macrophages (Fig. 1C), both pulsed with gp33. A double labeling of CD5 and CD3 revealed a tight colocalization of the two molecules in the different cell systems. In most conjugates formed between Jurkat T cells and SEE-loaded Raji B cells, CD5 was clustered with CD3 in a small area in the center of the immune synapse (Fig. 1A). In P14-dendritic conjugate, either a densely packed localization (Fig. 1B) or a distribution over most of the contact zone (Fig. 1C) was observed. However, in both situations, CD3 and CD5 remained precisely colocalized.

Kinetics and level of CD5 recruitment at the IS and their Ag dependence

The recruitment dynamics of CD5 were further analyzed at the IS formed between T8.1 cells (a CD5^+ murine hybridoma T cells) stably transfected with the full-length CD5 molecule fused to GFP and MHC class II-expressing fibroblasts as APC (25, 26). Fig. 2A, left panel, shows the expression of CD5-GFP in T8.1-transfected cells compared
with T8.1 wild-type cells. Both GFP fluorescence and CD5 levels analyzed with a CD5-specific Ab were measured. By fluorescence microscopy, CD5-GFP appeared homogeneously distributed at the plasma membrane of single T8.1 cells (right panel).

In CD5-expressing T8.1 cells contacting Ag-pulsed L625.7 cells, a clear recruitment of CD5 coclustered with CD3 was observed at the contact zone (Fig. 2B), as in the other synapses described above. CD5 clustering was observed for at least 1 h of cell-cell contact. The kinetics of CD5 recruitment at the T cell-APC interface were next quantified by calculating for each time point on several cells the mean ratio of CD5-GFP fluorescence at the contact zone over that measured in the whole cell (Fig. 2C). CD5-GFP movements were observed in T cells interacting with APCs pulsed, or not, with 0.1 or 1 μg/ml antigenic peptide. With unpulsed APCs, a fast, but limited, recruitment of CD5 at the contact zone of T cells scanning the APC cell surface was observed (time constant (τ) = 1.8 ± 0.3 min). This recruitment was often unstable, a phenomenon that was manifest in single-cell videos (see movie 1), but not in the average shown in Fig. 2C. APCs pulsed with 0.1 μg/ml induced a recruitment that affected a larger amount of CD5 molecules and took more time to reach a plateau (τ = 5.3 ± 0.5 min). With APCs pulsed with a larger dose of Ag (1 μg/ml), an even larger proportion of CD5 accumulated at the synapse with similar kinetics (τ = 4.8 min ± 0.2). Under these conditions we calculated that 60 ± 10.6% of total CD5 (n = 40) accumulated in the IS. The Ag-dependent recruitment of CD5 was quite stable as shown in single-cell videos (see movie 2). Very similar kinetics was observed with Jurkat cells interacting with SEE-pulsed Raji cells (not shown).

**CD5 and CD3 recruitment at the IS**

As CD5 recruitment is influenced by Ag recognition, we next examined the relationship between CD3 and CD5 movements, monitored simultaneously. To this end we used T8.1 cells expressing

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**FIGURE 3.** CD3 and CD5 recruitment to the IS. **A,** Series of photographs showing the parallel recruitment of CD3-YFP and CD5-CFP in T8.1 cells (APC pulsed with 1 μg/ml TTP). **B,** Left, Quantification of CD3 and CD5 recruitment. The relative recruitment is defined as in Fig. 2. Time zero corresponds to contact formation. **Right,** Plot of the increase half-time for the synaptic recruitment of CD5 and CD3 in 26 individual cells. The dotted line corresponds to identical increase half-times. **C,** CD3 recruitment is not affected by CD5, as shown by the relative CD3 recruitment in T8.1 cells expressing, or not, CD5, after 5, 20, or 60 min of interaction with Ag-pulsed APCs. The data are representative of three experiments.

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*The on-line version of this article contains supplemental material.*
the IS, also demonstrated the parallel recruitment of CD5 and CD3 (Fig. 3B, left panel). The duration required for these increases to reach half-maximum values was then measured in 26 cells (Fig. 3B, right panel). In the majority of cells these durations were quite similar. In a few cases CD5 recruitment lasted slightly longer than CD3 recruitment. The average increase half-times were 3.7 ± 2.5 and 4.4 ± 3 min for CD3 and CD5, respectively; these values were not significantly different. In addition, the mean maximum fold increases at the IS were similar for CD5 and CD3 (2.4 ± 1.1 and 2.6 ± 0.9, respectively).

By comparing CD5− and CD5+ T8.1 cells, we also tested the possibility that CD5 recruitment could alter the level of CD3 at the synapse. Endogenous CD3 recruitment was assessed by immunofluorescence with a CD3ε-specific Ab. Conjugates formed between Ag-pulsed APCs and T8.1 cells were analyzed after 5, 20, or 60 min of interaction (30 conjugates for each condition). The fraction of recruited CD3 was not significantly different in T8.1 cells expressing, or not, CD5 (Fig. 3C). Moreover, at each time point, the number of conjugates showing CD3 clustering at the contact zone was unaffected by the presence of CD5 (data not shown). A similar conclusion was reached when following CD3ζ in cells expressing CD3ζ-YFP (data not shown).

**CD5 expression does not alter initial and late T cell adhesion to APCs**

Besides the CD3/TCR complex, various molecules, including adhesion molecules, are involved to initiate and stabilize the interaction of a T cell with an APC. We therefore analyzed the influence of CD5 expression on initial and late adhesion steps between T8.1 cells and L625.7 cells interacting in a parallel plate flow chamber. We first assessed the ability of CD5− and CD5+ T cells to stop rolling on a monolayer of Ag-pulsed fibroblasts under a low shear stress of 0.09 dynes/cm². The fraction of T cells arrested in a 2-min assay of rolling on APCs was not significantly different for

![Graph 1: Initial adhesion phase](image1)

**FIGURE 4.** CD5 expression does not alter cell adhesion to APCs. A, Initial adhesion measured the arrest of rolling of CD5− or CD5+ cells on a monolayer of Ag-pulsed APCs (1 μg/ml TTP). CFSE-labeled T cells were introduced into a parallel plate flow chamber with a flow rate of 2 ml/h (shear stress, 0.09 dynes/cm²). The total number of cells flowing through the chamber was counted on images taken every 1.2 s. The percentage of cells that remained bound was measured after 2 min of flow. B, Deadhesion step: after the first phase described above, cells were left still for 8 additional min. Then increasing flow rates were applied by 30-s steps (from 2 to 100 ml/h; i.e., 0.09–4.5 dynes/cm²). The number of cells remaining at the end of each step was measured and expressed as a percentage of adhering cells at the beginning of the deadhesion procedure. Data were averaged from three experiments.

Both CD5-CFP and CD3ζ-YFP, interacting with Ag-pulsed fibroblasts. Fig. 3A shows in a representative cell that the recruitment of CD3 and CD5 occurred with similar time courses. A quantitative analysis of the kinetics of CD3 and CD5 clustering, measured as the relative increase in CD5-CFP and CD3-YFP fluorescence at the synapse, also demonstrated the parallel recruitment of CD5 and CD3 (Fig. 3B, left panel). The duration required for these increases to reach half-maximum values was then measured in 26 cells (Fig. 3B, right panel). In the majority of cells these durations were quite similar. In a few cases CD5 recruitment lasted slightly longer than CD3 recruitment. The average increase half-times were 3.7 ± 2.5 and 4.4 ± 3 min for CD3 and CD5, respectively; these values were not significantly different. In addition, the mean maximum fold increases at the IS were similar for CD5 and CD3 (2.4 ± 1.1 and 2.6 ± 0.9, respectively).

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FIGURE 6. CD5 decreases the amplitude of the Ca\(^{2+}\) response induced by Ag presentation. A, Ca\(^{2+}\) response and CD5-GFP recruitment were followed in parallel during conjugate formation with an APC (L625.7 cells pulsed with 1 \(\mu\)g/ml TTP). The two panels show typical examples of the two types of kinetics observed for CD5 accumulation at the synapse occurring before (left) or together with (right) the onset of the Ca\(^{2+}\) response. B, APC-induced Ca\(^{2+}\) responses in CD5\(^{-}\) and CD5\(^{+}\) T8.1 cells. Top, Individual cell responses. Bottom, Averaged responses of 33 CD5\(^{-}\) T cells and 25 CD5\(^{+}\) T cells were calculated. For a better comparison of their time courses, the response in CD5\(^{-}\) cells scaled to the CD5\(^{+}\) response is also shown (dotted line). C, APC-induced Ca\(^{2+}\) responses in WT T8.1 cells (CD5\(^{-}\)) or in T8.1 cells expressing the wild-type CD5 molecule (CD5\(^{+}\)) or a CD5 molecule deleted of the pseudo-ITAM motif (CD5\(^{+}\) ΔITAM). For each cell type, the averaged response of 40 individual cells is plotted.

the two cell types, i.e., 13.2 ± 2.9% of CD5\(^{-}\) T cells and 17.4 ± 4% of CD5\(^{+}\) T cells (Fig. 4A). Next, cells that had been adhering for 10 min on Ag-pulsed APCs were submitted to an increasing shear stress (by steps, up to 4.5 dynes/cm\(^2\)) to assess the strength of their adherence. Again, the percentage of cells remaining on the monolayer under a fast flow was not significantly different regardless of whether T cells expressed CD5 (Fig. 4B).

**CD5 inhibition of T cell signaling in single-cell analysis**

Having established that CD5 is recruited at the IS, but does not alter its formation, we then analyzed CD5-mediated inhibition of T cell signaling induced by APC at the single-cell level. The level of tyrosine phosphorylation (\(\Phi\)-Tyr) and Ca\(^{2+}\) responses were monitored in wild-type CD5\(^{-}\) T8.1 cells and in T8.1 cells selected for intermediate or high levels of the molecule (Fig. 5A). \(\Phi\)-Tyr was assessed with the anti-phosphotyrosine mAb 4G10 in conjugates formed between the different T8.1 cells and Ag-loaded APCs. Fig. 5B shows typical conjugates obtained after 5 min of cell-cell contact. The amount of \(\Phi\)-Tyr was significantly lower in cells expressing CD5. To further analyze this inhibition, kinetic experiments were performed. They showed that both the percentage of cells with increased \(\Phi\)-Tyr (Fig. 5C) as well as the level of \(\Phi\)-Tyr at the IS (Fig. 5D) were reduced by CD5. This inhibition was of greater magnitude in cells with the highest expression of CD5. Thus, CD5 impairs Ag-induced tyrosine phosphorylation in T cells, correlating with the expression level of the molecule.

Next, we measured simultaneously, in Fura-2-loaded cells, the Ca\(^{2+}\) response and the synaptic recruitment of CD5 upon Ag presentation. In ~50% of the cases, CD5 was already concentrated at the synapse before the Ca\(^{2+}\) started to rise (Fig. 6A, left), whereas in the other half, high levels of CD5 recruitment coincided with the Ca\(^{2+}\) response (Fig. 6A, right). In addition, Ag-free L625.7 fibroblasts never triggered Ca\(^{2+}\) responses in T8.1 cells, whereas some extent of CD5 recruitment was observed under those conditions, as mentioned above. These findings demonstrate that the initial CD5 recruitment is not triggered by TCR-dependent signaling events (such as a Ca\(^{2+}\) rise). By comparing CD5\(^{-}\) and CD5\(^{+}\) T8.1 cells, we examined whether CD5 exerted an inhibitory effect on these APC-induced Ca\(^{2+}\) responses by affecting the probability of observing a response or by altering its amplitude or its time course. The percentage of responding T8.1 cells was 74 ± 7% for CD5\(^{-}\) and 70 ± 2% for CD5\(^{+}\) cells (n = 3 experiments), i.e., the probability of observing a Ca\(^{2+}\) response was unaffected by CD5 expression. However, as shown in Fig. 6B, CD5 expression resulted in a reduced amplitude of the Ca\(^{2+}\) responses in the numerous individual cells analyzed (upper panel) and in the mean responses without affecting their time course, as shown by the overlap of the two scaled responses (lower panel). Knowing that the pseudo-ITAM of CD5 is required to impair B cell receptor-induced Ca\(^{2+}\) in B cells (12), we finally tested its contribution to the inhibition of the Ca\(^{2+}\) response triggered by Ag presentation in T cells. The amplitude of the response was quite similar in CD5\(^{-}\) cells and in cells expressing the CD5 molecule lacking the pseudo-ITAM, well above the Ca\(^{2+}\) levels observed in cells expressing the wild-type receptor (Fig. 6C).

**Discussion**

We have analyzed here for the first time the involvement of CD5 at synapses formed between T cells and APCs. The colocalization of CD3 and CD5 was striking when CD3 was concentrated at the center of the synapse as well as when it spanned the whole contact area. This colocalization found at different human and murine IS fits well with two sets of results previously reported. First, a fraction of CD5 molecules was found associated with CD3 in coimmunoprecipitation experiments (16, 17). Second, based on a large set of experiments using CD3-CD5 co-cross-linking, it is considered that the inhibitory effect of CD5 can be exerted when CD3 and CD5 are brought into close proximity. Thus, the presence of CD5 in the close vicinity of CD3 in the IS fulfills a first condition allowing CD5 to exert a physiological function.

The kinetics of CD5 recruitment at the IS comprise a fast Ag-independent and a slower Ag-dependent component. Everything happens as if bringing the T cell and the APC in close contact, thanks to ill-defined adhesion molecules, was sufficient to rapidly induce (with a time constant of ~1.8 min) the recruitment of a limited amount of CD5 at the forming IS. It has been reported that T cell engagement and activation initiate before IS is fully formed (27). Therefore, CD5 probably controls TCR signaling from the
moment the T cell and the APC come into contact. However, some signals associated with Ag recognition are necessary to stabilize and further increase CD5 at the IS. This process requires a few minutes (time constant ~5 min for the IS under study). Interestingly, the amount of Ag on the APC seems to affect mainly the level of CD5 being recruited at the IS and to poorly affect its rapid initial increase after the contact. This is exactly what one would expect from a fine-tuner of TCR signaling.

By monitoring CD5 recruitment simultaneously with CD3 clustering, one can see that the two events occur in a very narrow window of time. The time constants of CD3 and CD5 recruitment are most often similar; in only one-quarter of the cases is CD3 recruitment slightly faster than that of CD5. Besides, CD3 clustering is clearly unaffected by the presence of CD5. Thus, CD5 does not act by slowing down CD3 recruitment to the synapse. These data are consistent with an independent recruitment of CD3 and CD5. This is not to contradict the above conclusion that efficient Ag presentation and TCR signaling contribute to the stabilization/enrichment of CD5 at the IS.

One could have expected that CD5 could act at the IS as one more adhesion molecule if its elusive ligand was present on L625.7 cells. This ligand is unlikely to be CD72, as initially proposed (11), but the presence or the absence of MHC-peptide complexes stimulatory TCR were able in vitro to alter their CD5 level depending on the presence of CD5 at the IS. This process requires a few minutes (time constant ~5 min for the IS under study). Interest-

References