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Visualizing Intermolecular Interactions in T Cells

Nicholas R.J. Gascoigne, **Jeanette Ampudia**, **Jean-Pierre Clamme**, and **Guo Fu**
Department of Immunology and Microbial Science, The Scripps Research Institute, 10550 North Torrey Pines Rd, La Jolla, CA, 92037, USA

Carina Lotz
Department of Hematology, University Medical Center Utrecht, Lundlaan 6, 3584EA, Utrecht, The Netherlands

Michel Mallaun
Department of Biomedicine, University Hospital Basel, Switzerland

Nathalie Niederberger
Serono Pharmaceutical Research Institute, Geneva, Switzerland

Ed Palmer
Department of Biomedicine, University Hospital Basel, Switzerland

Vasily Rybakin
Department of Immunology and Microbial Science, The Scripps Research Institute, 10550 North Torrey Pines Rd, La Jolla, CA, 92037, USA

Pia P. Yachi
Applied Molecular Evolution, 3520, Dunhill Street, San Diego, CA 92121, USA

Tomasz Zal
Department of Immunology, University of Texas MD Anderson Cancer Center, 7455 Fannin, Houston, TX 77030, USA

Abstract

The use of appropriate fluorescent proteins has allowed the use of FRET microscopy for investigation of intermolecular interactions in living cells. This method has the advantage of both being dynamic and of working at the subcellular level, so that the time and place where proteins interact can be visualized. We have used FRET microscopy to analyze the interactions between the T cell antigen receptor and the coreceptors CD4 and CD8. This chapter reviews data on how these coreceptors are recruited to the immunological synapse, and how they interact when the T cell is stimulated by different ligands.

1 Introduction

Fluorescent proteins based on the *Aequoria victoria* green fluorescent protein (GFP) and improvements in microscopy have made it possible both to locate cells in living tissues and to locate proteins within living cells. Within immunology, this has made great contributions to studies of subcellular localization and translocation during, for example, T cell activation. Use of different fluorescent proteins or complementary fragments of fluorescent proteins has allowed analysis of intermolecular interactions using Förster resonance energy transfer

(FRET) or bimolecular fluorescence complementation (BiFC). Our work has used GFP variants primarily to investigate the movements and interactions of T cell receptor (TCR) components and the coreceptors CD4 and CD8.

Early immunofluorescence studies of T cells interacting with antigen-presenting cells (APC) showed that the CD4 or CD8 coreceptors became concentrated at the interface between the cells (Kupfer and Singer 1989). Later improvements in imaging technology made it possible to identify features of this interface in some detail. These revealed that the TCR and signaling components such as protein kinase Cθ became concentrated in a central region of the interface – the central supramolecular activation complex (cSMAC) surrounded by a region where intracellular adhesion molecules were concentrated, the peripheral (p)-SMAC, with other proteins such as the phosphatase CD45 pushed out to a distal (d)-SMAC (Grakoui et al. 1999; Johnson et al. 2000; Monks et al. 1997, 1998). The whole structure has become known as the immunological synapse (IS). However, time-lapse studies using cell–cell interactions or experimental systems where T cells interact with purified proteins associated with a supported lipid bilayer have demonstrated that the immunological synapse as originally defined, described above, is a mature version of a structure that in fact is extremely dynamic (Grakoui et al. 1999; Hailman et al. 2002; Revy et al. 2001; Ritchie et al. 2002; Trautmann and Valitutti 2003; Zal et al. 2002). Indeed, recent data cast doubt on the idea that the immunological synapse is itself a signaling structure. These experiments indicate that signaling occurs in small accumulations of TCRs (microclusters) at the distal area of the synapse, and that these are brought to the cSMAC to be internalized and degraded (Campi et al. 2005; Varma et al. 2006). Other experiments showed that initial phosphorylation of TCR by the kinase Lck occurs at the distal region of the immunological synapse (Lee et al. 2002), although signaling is within the immunological synapse at later time points (Cemerski et al. 2008).

Even relatively early experiments showed that the immunological synapse structure of cSMAC and pSMAC forms dynamically over time, with cell adhesion molecules starting centrally and then switching places with the TCR to end in the mature structure (Grakoui et al. 1999). Experiments with dendritic cells rather than B cells or lipid bilayers as the antigen-presenting surface, revealed that coreceptors are recruited to the interface between T cell and dendritic cell even when antigen is not present (Zal et al. 2002), and that the interaction between T cell and nonstimulatory dendritic cell is sufficient to cause a small Ca^{2+} response in the T cell (Revy et al. 2001). It has become clear that, even in the absence of overt activation of the T cell, some information is transferred, such that interactions with endogenous peptides allow T cells to get survival signals, or thymocytes to get signals for positive selection (Goldrath and Bevan 1999; Stefanova et al. 2002). The original definition of the immunological synapse is rather too restrictive to cover all of the different interfaces between T cells and APCs, or their form at different stages. To quote US President Bill Clinton, *it depends on what IS is*. We prefer a broad definition, that the immunological synapse is the interface between a T cell and APC, whether or not antigen is involved, and whether or not the T cell becomes activated.

2 Molecular Recruitment to the Immunological Synapse

2.1 T Cell Receptors and Coreceptors

Mutagenenesis of the *A. victoria* green fluorescent protein and discovery of fluorescent proteins in other taxa resulted in different colored versions which could report localization of two or more proteins inside cells. A very useful resource identifying different FPs and their uses is available (Shaner et al. 2005). Our experiments have predominantly used the enhanced cyan and yellow derivatives of GFP: CFP and YFP. These were chosen because, having distinguishable but overlapping spectra, they were well suited as a FRET donor and

acceptor pair to investigate intermolecular interactions as well as for conventional tracking of receptor movement to the immunological synapse. We were particularly interested in the dynamics and localization of interactions between the TCR and CD4 duo of MHC Class II-binding receptor and coreceptor. We prepared gene constructs encoding CFP fused to the CD3ζ subunit of the TCR–CD3 complex and YFP fused to CD4, which were expressed in a T cell hybridoma with defined TCR that was deficient for CD4 expression (Zal et al. 2002). Thus, the transfected cells expressed only the fluorescent CD4. Although the cells expressed endogenous, nonfluorescent CD3ζ, this was at a relatively low level, such that expression of TCR on the cell surface was weak, as CD3ζ is limiting for TCR expression (Klausner et al. 1990). CD3ζ–CFP expression therefore raised cell surface TCR expression to a level comparable to normal T cells (Zal et al. 2002). We demonstrated that the chimeric proteins were functional and that they allowed the transfected cells to respond to antigen similarly to cells expressing nonfluorescent versions of CD4 and CD3ζ.

Time-lapse movies of fluorescent T cells interacting with APCs showed that recruitment of CD4 to the immunological synapse is almost instantaneous, with concentrations of CD4 discernible within 10 s of contact between the T cell and the APC. This concentration of CD4 built up over the next minute and, over a period of several minutes, the contact between T cell and APC was seen to be very dynamic. Although TCR–CD3 also became concentrated at the synapse, this was to a much smaller degree than for CD4, 1.3- to 3-fold higher than on the rest of the cell surface compared to 4- to 8-fold for CD4. Recruitment of CD4, but not CD3ζ, was found when the T cell interacted with APCs that were not loaded with antigen. We found that this did not occur with all the different APCs, but that it occurred with mature dendritic cells and with a subset of B cell tumors. It did not occur with immature dendritic cells nor with macrophages, certain B cell tumors, or MHC class II-transfected fibroblasts. This nonantigen-specific recruitment of coreceptor was inhibited with antibodies against various cell surface molecules, including anti-MHC class II and CD4, as well as the B7.1, B7.2-blocker CTLA4-Ig, and antibodies against the cell adhesion molecules LFA1 and ICAM1. In contrast, anti-CD45 enhanced the CD4 recruitment. We found that CD4 recruitment to the immunological synapse in the absence of antigenic stimulation was slower than that seen when antigen was present, showing that recognition of antigen increased the rate of CD4 translocation to the immunological synapse (Zal et al. 2002).

Similar experiments were performed with MHC class I-restricted cells expressing CD3ζ–CFP and CD8α–YFP, or normal CD8α plus CD8β–YFP (Yachi et al. 2005, and unpublished). As with CD4^{+} cells, the coreceptor was recruited rapidly to the immunological synapse, although here we did not notice a significant difference between the presence or absence of antigen, except that the conjugates formed more slowly and were less frequent in the absence of antigen (Fig. 1). Again, coreceptor concentration was much more evident than TCR concentration.

Experiments with MHC class I-restricted T cells have a technical advantage over those using class II-restricted T cells in that we can use the cell line RMA-S as the APC, which can be loaded with defined peptides in a well-controlled manner. RMA-S is a thymoma cell line that is deficient in transporter of antigen processing (Tap)-2 expression. Because of this, it is unable to load most peptides onto class I molecules, and its cell surface MHC class I expression is thus much lower than that of its parental cell line, RMA. Early flow cytometry data showed class I-expression on RMA-S to be 5–10% of the amount of class I on RMA (Ljunggren and Karre 1985; Townsend et al. 1989), and we estimate that there are ~550 molecules of H2-K[b] on the cell surface in the absence of added peptides (Yachi et al. 2007).

In fact, the class I molecules associated with β2-microglobulin are transported to the cell surface, but the vast majority are unstable because they lack bound peptide. At below-physiological temperatures, the molecules remain stable, so it is possible to culture the cells at 32°C in the presence of suitable peptides, which then become integrated into the MHC molecule. When the temperature is raised to 37°C, class I molecules into which peptide has been folded remain stable and can therefore be recognized by T cells, while the other, "empty," class I molecules are degraded. Thus, it is possible to control both the specific peptides that are presented by class I molecules on the cell surface, and the total amount of MHC class I, which we used to dissect the forces that drive TCR and CD8 recruitment to the immunological synapse. We found that either antigenic peptide or a peptide that is not recognized by the T cell's TCR allowed CD8 to be recruited to the immunological synapse and concentrated there (Fig. 2). The amount of CD8 recruited to the immunological synapse correlated with the quantity of these peptides loaded into the MHC class I molecules, indicating that the CD8 recruitment is caused by the noncognate interaction between CD8 and class I (Yachi et al. 2005). Recent experiments performed with a T hybridoma that does not express TCRα or β-chains, but which we transfected with CD8α and CD8β–YFP, demonstrated that the CD8 recruitment to the synapse was independent of TCR signaling (Yachi, unpublished).

2.2 Signaling Proteins

We are currently extending our studies to analyze various aspects of signal transduction in the immunological synapse. Analysis of protein kinases C originally indicated that only PKCθ was associated with the immunological synapse (Monks et al. 1997, 1998). The recruitment of PKCθ required signaling through the kinase Lck and costimulation (Huang et al. 2002). We recently identified PKCη as another PKC that is strongly and specifically recruited to the synapse (G. Fu and N. Niederberger, in preparation). Interestingly, PKCη is upregulated during thymocyte positive selection (Mick et al. 2004; Niederberger et al. 2005), and is seen in synapses formed by mature thymocytes, whereas PKCθ is expressed from earlier stages of development and is found in the IS of both immature $CD4^+$ $CD8^+$ and mature thymocytes (Fu and Niederberger, in preparation). We are currently testing the hypothesis that PKCθ and PKCη have some redundant functions in thymocyte development.

3 Molecular Interactions in the Immunological Synapse

3.1 FRET Analysis

By choosing enhanced CFP and YFP as our fluorescent reporters, we could employ FRET to detect molecular range proximity between these fluorophores. FRET is the nonradiative transfer of energy between an excited molecule and a nonexcited molecule. For this to happen, the donor must have an emission spectrum that overlaps with the acceptor's excitation spectrum, and the fluorescent cores of the molecules must be within about 10 nm of each other. Thus, this is a useful technique to analyze whether molecules are in close proximity with each other. However, there are certain difficulties in using the method in live cells. When the two fluorescent moieties are on separate molecules, it is important that sufficient acceptors are available (typically 1:1 to 3:1 acceptor to donor ratio) and that the dependence of FRET on acceptor concentration is determined to differentiate between FRET due to specific interactions and crowding effects.

The simplest method for analysis is the "donor recovery" method, where an image of the donor, for example, CFP, is taken before and after photobleaching of the acceptor (YFP). If FRET occurs, then the donor image taken after the photobleaching will be brighter by the amount of energy that was transferred to the acceptor before photobleaching. This method is unsuitable for living cells where the relevant molecules are likely to move between taking

the two images. For live imaging, we therefore used an extended ratiometric method where images for emission at both CFP and YFP wavelengths were taken (Zal and Gascoigne 2004; Zal et al. 2002). This is best performed with simultaneous imaging of CFP and YFP emissions to minimize movement artifacts (Zal and Gascoigne 2004). If FRET occurs, then the YFP emission increases while the CFP decreases during CFP excitation. In addition, the YFP-only image and correction factors for bleed-through between the channels are necessary to deduce FRET between independently expressed donors and acceptors. This method is suitable for FRET analysis of live cells, but corrections must also be made for the photobleaching that occurs when taking z-stacks and during time-lapse imaging (Zal and Gascoigne 2004).

3.2 TCR–Coreceptor Interactions

We used FRET imaging to investigate the interaction between the TCR–CD3 complex and CD4 or CD8 during antigen recognition. Many studies have addressed whether TCR and coreceptors are associated, but the data are highly equivocal, with some showing a constitutive interaction (Anderson et al. 1988; Arcaro et al. 2001; Beyers et al. 1992; Doucey et al. 2003; Gallagher et al. 1989; Kwan Lim et al. 1998; Rojo et al. 1989; Suzuki et al. 1992; Takada and Engleman 1987), and others showing an antigen-induced interaction (Anel et al. 1997; Block et al. 2001; Mittler et al. 1989; Osono et al. 1997). Some of these studies used cocapping as a measure of interaction (Anderson et al. 1988; Kwan Lim et al. 1998; Rojo et al. 1989; Takada and Engleman 1987), which may be flawed by the molecules residing on the same membrane micro-domains, and almost all used activation by antibodies rather than by MHC–peptide complexes on an APC. Thus, our studies allowed us to investigate the induction of the interaction during recognition of antigens presented on APC. We found that, for both CD4 and CD8, the FRET signal between TCR and CD4 or CD8β increased significantly during antigen recognition, indicating that the TCR–coreceptor interaction is induced during TCR recognition of antigen (Yachi et al. 2005; Zal et al. 2002). Figure 1 shows the FRET response between TCR and CD8 by antigen (OVA), but not by a nonstimulatory peptide (VSV). A certain low level constitutive association between the molecules is indicated by a low background FRET signal (~2%), but the interaction is strongly enhanced by antigen recognition.

Interestingly, the time course of the FRET signal was different with different systems. In the case of the CD4–TCR interaction, we found that FRET was induced clearly within 3 min, and that it remained strong for up to 30 min (Zal et al. 2002). In the CD8–TCR experiments, we found that FRET was clearly above background levels (averaged over the whole of the IS) within 5 min, and peaked at around 10 min, then declined (Yachi et al. 2005, 2006). These experiments were performed using RMA-S cells as APCs, but in other experiments using APC such as EL4, where processing of peptides and MHC class I expression is normal, we did not see such a steep decline in the FRET signal (unpublished).

3.3 TCR:CD8 Interactions Induced by Positive and Negative Selecting Ligands

We used FRET microscopy to analyze the recruitment of TCR and CD8, and their interaction in the immunological synapse, during recognition of ligands of different types by the OT-I TCR for which the affinities for a variety of K^b–peptide complexes have been determined (Alam et al. 1996, 1999; Gascoigne et al. 2001; Rosette et al. 2001). These different ligands have defined biological activities, as agonists, weak agonists, and antagonists of mature cells, and as negative and positive selectors of thymocytes in fetal thymus organ culture (FTOC) (Hogquist et al. 1994; Jameson et al. 1994). We found that, as expected, CD8 recruitment was not affected by the different ligands, although TCR recruitment was affected – weaker ligands caused less CD3ζ to be concentrated at the immunological synapse (Yachi et al. 2006). The induction of CD3ζ–CD8β FRET followed

different time courses for negative selectors (strong agonists) than for positive selectors (weak agonists and antagonists). Negative selecting ligands showed peak FRET at 10 min, whereas the positive selectors peaked at 20 min. The negative selectors showed a faster induction of FRET that then faded out, whereas the positive selectors gave a slower induction of FRET. The magnitude of the FRET response was not as clearly correlated with the bioactivity, as a weak agonist that is a positive selecting ligand gave a strong FRET signal but with the slower time course found with other positive selectors (Yachi et al. 2006).

More recently, we have expanded the analysis of ligands for the OT-I TCR to include those that straddle the rather sharp boundary between positive and negative selection. The weakest ligand that induces only negative selection, and the strongest that causes only positive selection (Daniels et al. 2006), induced similar levels of CD3–CD8 FRET, yet were separated in their time courses (Mallaun et al. 2008): the positive selector showed a slower induction of FRET than the negative selector. Despite considerable variability in the strength of FRET induced, the differences in the timing of peak FRET were maintained between the positive and negative selecting ligands.

Most interestingly, there was one ligand that could induce either positive or negative selection in FTOC – at higher concentration it induced negative selection, but at lower concentration it induced positive selection. This switch occurred abruptly within a 10-fold concentration range (Daniels et al. 2006). In this case, the induction of FRET between TCR and CD8 was induced with kinetics similar to the negative selecting ligands, but FRET remained at a high level for longer, into the time-range when the positive selectors induce FRET (Yachi, unpublished).

From these results, we infer that the time course of the induction of the intimate CD8–TCR interaction is related to the signal eventually transduced by the TCR, or vice versa. When we analyzed the induction of phosphorylation of Erk in the cells responding to the positive and negative selecting ligands, we found that phospho-Erk was induced more rapidly and strongly at the immunological synapse by the negative selectors than by the positive selectors (Yachi et al. 2006). In another series of experiments, phospho-Erk and other members of the Ras/MAP kinase signaling pathway were found predominantly at the cell surface after stimulation with negative selectors, but were found in an intracellular compartment (apparently the Golgi apparatus) with the positive selectors (Daniels et al. 2006). Phosphorylated JNK was only found intracellularly. This separation of ERK and JNK signals in negative but not positive selection signaling is believed to underlie the induction of differentiation or apoptosis by the two types of signal (Daniels et al. 2006).

3.4 Antagonism of T Cell Activation

T cell activation by antigen can be antagonized by other MHC–peptide complexes, usually, but not necessarily, closely related to the antigen's sequence. The mechanisms by which this works have been a subject of much debate. The original definition of a TCR antagonist came from studies on altered peptide ligands for an MHC class II-restricted T cell (Evavold and Allen 1991), and many examples are now known. For example, in the MHC class I-restricted T cell OT-I, changing one of the potential TCR-contacting residues (residues 1, 4, 6, or 7 of the 8-amino acid peptide antigen) was often sufficient to change the peptide from antigen (agonist) to antagonist (Hogquist et al. 1994; Jameson et al. 1993, 1994; Rosette et al. 2001). In other cases, the change was to a weak agonist or to a nonstimulatory peptide. As mentioned earlier, the antagonists were also found to induce positive selection (Hogquist et al. 1994; Jameson et al. 1994; Rosette et al. 2001). They showed weaker affinity and usually faster dissociation kinetics than did the strong agonists (Alam et al. 1996, 1999; Gascoigne et al. 2001; Rosette et al. 2001).

We made use of FRET microscopy to investigate how antagonism of T cell activation affects the interaction of coreceptor with TCR. We stimulated an MHC class II-restricted T cell hybridoma that responds to a self-antigen derived from a complement protein (Zal et al. 1994) with the antigenic C5 peptide. Two antagonist peptides have been identified for this TCR, each differing from the antigen by a single amino acid (Volkmann et al. 1998; Zal et al. 2002). Addition of either antagonist peptide, or a peptide that does not stimulate this TCR (but which binds to the same MHC molecule), did not affect the recruitment of the TCR (measured by CD3ζ–CFP) to the synapse (Fig. 3). The CD4–YFP recruitment to the synapse was also similar to when the cells were stimulated with antigen alone. We had earlier found that stimulation with the antagonist peptide alone led to a very weak FRET signal, much weaker than that obtained with agonist (Zal et al. 2002), and that this is also the case for antagonists in the OT-I system (Yachi et al. 2006). When both agonist and antagonist ligands were present, the FRET signal induced by agonist was abrogated (Fig. 3), but this did not occur when the nonstimulatory peptide was used in place of the antagonists. This demonstrated that the antagonists somehow inhibited the close range interaction between the TCR and the coreceptor, despite not affecting the ability of either of these molecules to be recruited to the immunological synapse (Zal et al. 2002). Since the efficiency of FRET is linearly dependent on the proportion of donor (TCR) that is in complex with the coreceptor (CD4 or CD8), we have interpreted this finding as supporting a model where the stability of the coreceptor–TCR interaction is dependent on the quality of TCR binding to the MHC–peptide. Moreover, our data indicate that MHC molecules are bound first by the coreceptor, irrespective of the peptide, and then by TCR. The half-life of TCR binding to agonists is usually longer than that of antagonists (Alam et al. 1996; Gascoigne et al. 2001; Kersh et al. 1998; Lyons et al. 1996). We envisage that the coreceptor–MHC complexes in the immunological synapse have even more dramatic differences in the kinetics of their interaction with TCR depending on the peptide than it is expected based on the measurements of MHC–TCR interaction without coreceptor in solution. In other words, we suggest a threshold-setting function for the coreceptor that helps discriminate between MHC-peptides that otherwise interact with TCR with only slightly different kinetics. Since the antagonists were present in the experiment at a higher concentration than the agonist, they would bind TCR more frequently than the agonists (due to higher frequency of the ligands) but TCR would fall off more rapidly. The overall signal for T cell activation would depend on the accumulation of sufficient TCR–coreceptor complexes in a short time.

4 Variability in the Form of the T Cell:APC Interface

4.1 Nanotubes

The ability to image cell surface molecules during interactions between cells led to many surprises, for example, the structure of the immunological synapse itself, and the choreography of different proteins entering and exiting the synapse at different times. The form of the interaction site between T cell and APC alters over time, with the earliest interaction showing very active membrane spreading, then a period of contraction when the mature immunological synapse is formed. At later time points, we noticed very fine processes joining T cells to APCs, often as the T cells detached and started to move away from the APC (Zal et al., unpublished) (Fig. 4). However, they could also form early in T:APC interactions or could be intermediates between periods of full immunological synapse-like contact. The point of contact of these membrane tethers to the APC showed concentrations of TCR and coreceptor. Similar structures were identified in *Drosophila* wing buds, providing cell–cell interactions between cells in different regions of the imaginal disk, and in mouse limb buds. These were termed cytonemes (Ramirez-Weber and Kornberg 1999). Similar "nanotubes" formed between mammalian cells in culture were shown to allow transport of organelles and membrane vesicles from cell to cell (Rustom et al. 2004). In immune system cells, nanotubes have been shown to allow intercellular transfer of cell

membrane proteins (Onfelt et al. 2004). More recently, nanotube connections between T cells have been found to be routes for HIV transfer as well as information transfer (Sowinski et al. 2008). The emerging data on nanotubes have recently been reviewed (Davis and Sowinski 2008;Gerdes and Carvalho 2008). The resolution and sensitivity of two-photon microscopy is not yet sufficient to be able to identify these kind of structures in the immune system *in vivo*. However, it is very tempting to speculate that they could be involved in the scanning activity of T cells migrating through the lymph node, for instance, in the early phase of T cell stimulation by antigen where T cells do not make long-term contacts with APC (Mempel et al. 2004). They may conceivably retain these fine contacts with numerous APCs that they have previously contacted, perhaps adding signals until they are sufficiently stimulated to make a full stop response and make a stable synapse.

Nanotubes are still very poorly understood; much remains to be learned about their functional importance, particularly *in vivo*. This section serves to illustrate the idea that imaging of the immune response gives an opportunity to discover surprising and perhaps delightful facts about the cells that could not have been predicted by other methods.

5 Concluding Remarks

Imaging of molecules in living cells has led to many exciting discoveries about how cells interact. These could not have been predicted by any knowledge that was available before the advent of high resolution microscopic techniques. We are confident that improvements in resolution, especially to the single molecule level, and newer techniques, such as total internal reflection microscopy, will lead to more surprises about cellular interactions and signaling. Use of multiphoton methods for *in vivo* studies will also be more useful when we can image cell surface molecules rather than simply labeled cells. FRET as a method for investigating intermolecular interactions is a useful technique but currently suffers from relatively low dynamic range. This is expected to improve as newer fluorescent protein FRET partners are developed. More importantly, because of the extensive "image math" that must be performed for each subcellular region, such as the IS, FRET is extremely low throughput. Significant improvements in image analysis software are needed to overcome this problem.

Acknowledgments

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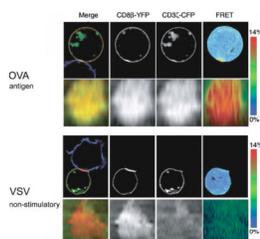
Fig. 1.
OT-I T cell recognizing antigen (OVA, *top panels*) or a nonstimulatory peptide (VSV, *lower panels*). The *top set of images of each panel* shows a single z-slice through a T cell:APC couple, showing, from left to right, a merged color image (CD8β–YFP in *red*, CD3ζ–CFP in *green*, the APC labeled with Cy5, shown in *blue*), CD8β–YFP in monochrome, CD3ζ–CFP in monochrome, and the FRET image, heat-colored to represent the FRET efficiency (using the *scale* shown on the *far right*). Reproduced from (Yachi et al. 2005)
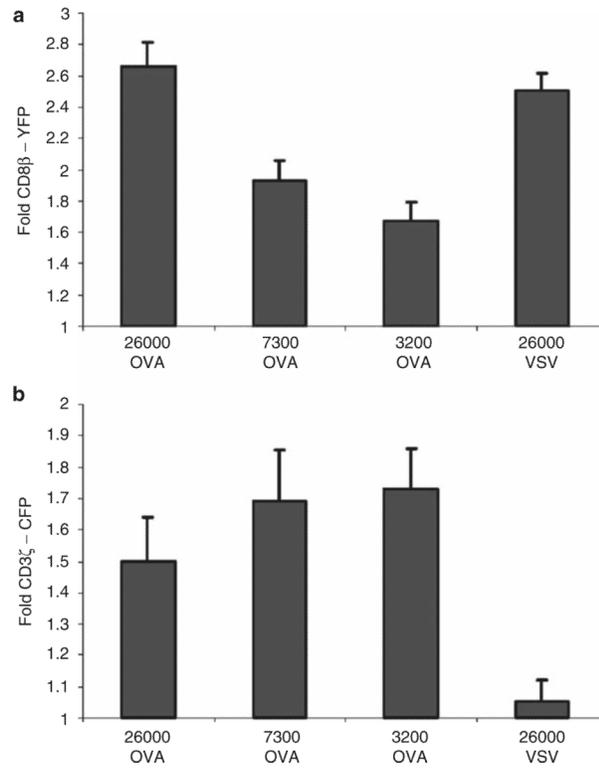
Fig. 2.
Recruitment of CD8β–YFP and CD3ζ–CFP in response to different quantities of peptide (OVA or VSV) presented on RMA-S cells. The *numbers* refer to the numbers of H2K^b molecules on the cell surface or the RMA-S cells. Reproduced from (Yachi et al. 2005)
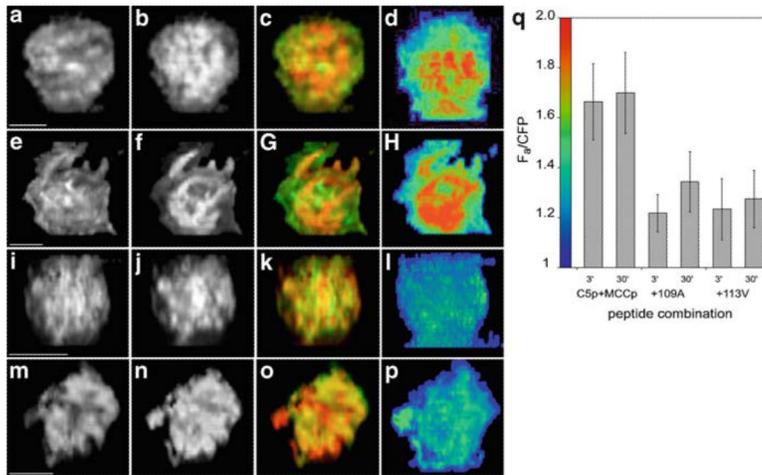
Fig. 3.
Antagonists of the TCR inhibit the close interaction between TCR and CD4. These images all show en face projections of the immunological synapse. (**a–d**) 3-min time point, (**e–h**) 30-min time point for antigen plus nonstimulatory peptide. (**i–j**) 3-min time point for antigen plus antagonist 109A, (**m–p**), the same at 30 min. *Left panel* (**a**) and below show CD3ζ–CFP images. (**b**) and images below show CD4–YFP. (**c**) and pictures below show merged false color images showing CD3ζ–CFP in *green* and CD4–YFP in *red. Right panel* (**d**) and below are FRET images, heat-colored as in Fig. 1.(**q**) shows FRET signals obtained from multiple measurements in the IS with antigen plus the nonstimulatory peptide MCC, or the C5 antagonists 109A and 113V. Reproduced from(Zal et al. 2002)
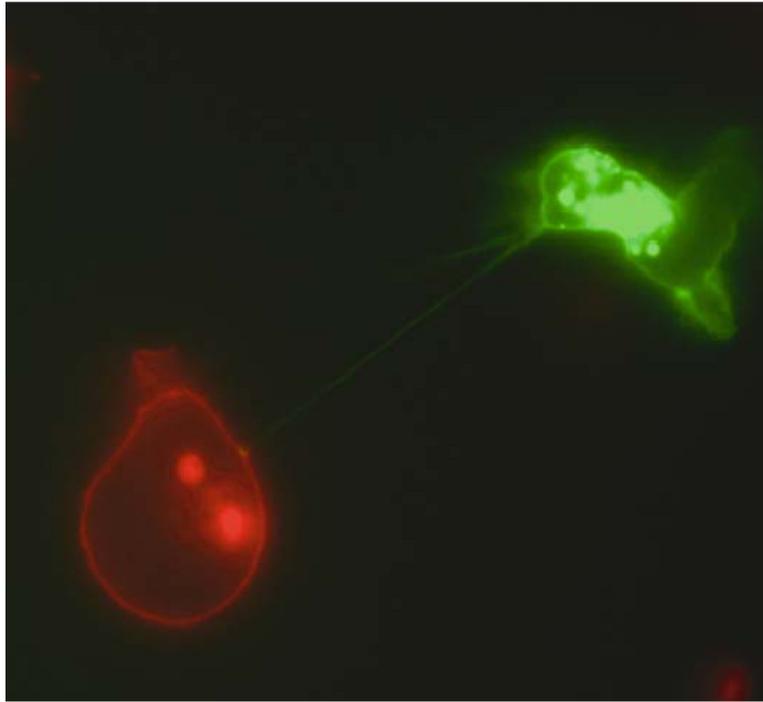
Fig. 4.
A nanotube extending from a CD4$^+$ T cell expressing CD3ζ–CFP(*green*) interacting with an antigen-expressing APC (*red*). This is from a movie lasting about 30 min. The T cell started in synapse with the APC, then moved away. It remained tethered for the duration of the movie