The EMBO Journal (2010) 29, 4035–4047 | © 2010 European Molecular Biology Organization | All Rights Reserved 0261-4189/10
www.embojournal.org

THE EMBO JOURNAL

Adhesion shapes T cells for prompt and sustained T-cell receptor signalling

Rita Lucia Contento[1,2,*], Silvia Campello[1,3], Anna Elisa Trovato[1], Elena Magrini[1], Fabio Anselmi[1] and Antonella Viola[1,2,*]

[1]Istituto Clinico Humanitas IRCCS, Rozzano, Milan, Italy and [2]Department of Translational Medicine, University of Milan, Rozzano, Milan, Italy

During T-cell migration, cell polarity is orchestrated by chemokine receptors and adhesion molecules and involves the functional redistribution of molecules and organelles towards specific cell compartments. In contrast, it is generally believed that the cell polarity established when T cells meet antigen-presenting cells (APCs) is controlled by the triggered T-cell receptor (TCR). Here, we show that, during activation of human T lymphocytes by APCs, chemokines and LFA-1 establish cell polarity independently of TCR triggering. Chemokine-induced LFA-1 activation results in fast recruitment of MTOC and mitochondria towards the potential APC, a process required to amplify TCR Ca^{2+} signalling at the upcoming immunological synapse, to promote nuclear translocation of transcriptional factor NFATc2 and boost CD25 expression. Our data show that the initial adhesive signals delivered by chemokines and LFA-1 shape and prepare T cells for antigen recognition.

The EMBO Journal (2010) **29,** 4035–4047. doi:10.1038/emboj.2010.258; Published online 15 October 2010
Subject Categories: cell & tissue architecture; immunology
Keywords: immunological synapse; LFA-1; mitochondria; T-cell polarity

Introduction

Cell polarity defines a structural and functional asymmetric compartmentalization of proteins and/or organelles. It is essential for the proper functions of many cell types, and failure in establishing or maintaining polarity has severe consequences, such as defective cell development and uncontrolled cell proliferation (Bilder, 2004). Both lymphocyte migration and activation require the compartmentalization of membrane receptors and signalling molecules in specific cell locations.

During migration, polarity refers to the ability of cells to change their morphology in response to chemoattractants, and to maintain a stable asymmetric shape with two poles: the leading edge, which protrudes at the cell front, and the rear edge (termed uropod in leukocytes), which retracts (Sanchez-Madrid and del Pozo, 1999). This process is initiated by chemokine receptor signalling and adhesive interactions with the extracellular matrix.

T-cell stimulation by antigen-presenting cells (APCs) also results in a general and specific relocation of receptors, signalling molecules and organelles at the immunological synapse (IS). However, the induction of this cell polarity in T lymphocytes is thought to be dependent on stimulation of the T-cell receptor (TCR) by antigenic complexes. Although long-lasting interactions between T cells and APCs are dependent on antigens (Skokos *et al*, 2007; Henrickson *et al*, 2008), antigen-specific interactions are preceded by antigen-independent, chemokine-promoted adhesive contacts between T cells and APCs, which are important for T cells to scan the surface of their cellular partners (Revy *et al*, 2001; Montoya *et al*, 2002; Friedman *et al*, 2006). APC scanning is thought to be an important step in T-cell activation, allowing TCRs to interact with the few specific peptide–MHC complexes. In addition, in T cells, LFA-1 cross-linking is sufficient to induce locomotion, which is accompanied by polarization of the MTOC and signalling molecules to the cell uropod (Volkov *et al*, 2001). Thus, we decided to clarify the role of adhesive versus antigenic interactions in the establishment and meaning of T-cell polarity towards APCs.

We focused our attention on mitochondria, highly mobile and dynamic organelles that, in migrating T cells, are recruited to and accumulate at the uropod (Campello *et al*, 2006). It has been recently shown that stimulation of T cells by beads coated with anti-CD3 antibody induces mitochondria redistribution to the bead-cell contact region (Quintana *et al*, 2007). This process seems to be required to allow proper TCR-induced Ca^{2+} influx in T cells, because mitochondria can sequester Ca^{2+} and thus keep Ca^{2+} release-activated Ca^{2+} (CRAC) channels open (Hoth *et al*, 1997; Quintana *et al*, 2007; Schwindling *et al*, 2010). As anti-CD3 stimulation induces accumulation of mitochondria towards the coated beads, it has been suggested that signals delivered by the triggered TCR are responsible for mitochondria accumulation under the IS. However, in physiological conditions of T-cell activation, several receptors, including chemokine receptors and adhesion molecules, are actively involved in the process of IS formation.

Here, we show that LFA-1 triggers localization of mitochondria at the T-cell IS independently of TCR stimulation and through a mechanism requiring chemokine receptor signalling. Importantly, we found that, by recruiting mitochondria to the IS, LFA-1 sustains and amplifies the upcoming TCR-induced Ca^{2+} signalling, indicating that establishment of T-cell polarity is pivotal to a prompt and sustained TCR signalling and induces nuclear translocation of the transcriptional factor NFATc2 and CD25 expression.

*Corresponding authors. RL Contento or A Viola, Department of Translational Medicine, Istituto Clinico Humanitas IRCCS, via Manzoni 113, Rozzano, Milan 20089, Italy.
Tel.: +39 028 224 5118; Fax: +39 028 224 5101;
E-mail: Rita_Lucia.Contento@humanitasresearch.it or Antonella.Viola@humanitasresearch.it
[3]Present address: Department of Cell Physiology and Metabolism, University of Geneva, Geneva 1211, Switzerland

Received: 6 August 2010; accepted: 24 September 2010; published online: 15 October 2010

Results

Mitochondria translocation to the IS does not require TCR triggering

To study mitochondria translocation towards the IS, we initially used Jurkat T cells stably expressing a mitochondrially targeted red fluorescent protein (mtRFP) and B cells pulsed or not with the bacterial superantigen staphylococcus enterotoxin E (SEE). We found that, independently of TCR triggering, mitochondria accumulated under the IS (Figure 1A; Supplementary Video 1; Supplementary Figure S1). Real time, time-lapse microscopy of T cells interacting with pulsed or unpulsed B cells confirmed that TCR stimulation was not required for mitochondria recruitment to the IS (Figure 1B and C; Supplementary Videos 2 and 3). Moreover, these experiments showed that mitochondria recruitment to the contact region between T cells and APCs is a very dynamic process, and that, when the T cell moves onto the APC, organelles are promptly re-oriented towards the adhesive interaction (Supplementary Video 4).

Mitochondria translocation to the IS depends on LFA-1 binding to ICAM

In order to identify which molecule is responsible for the observed mitochondria recruitment to the IS, we focused our attention on LFA-1, a main player during the initial contacts between T cells and APCs (Sims and Dustin, 2002).

We blocked LFA-1 functions by pre-incubation of T cells with neutralizing antibodies against the two subunits of LFA-1 (CD11a and CD18) and found that, in these conditions, polarization of T-cell mitochondria towards the B cell was reduced, regardless of TCR stimulation (Figure 2A).

To exclude the possibility that the observed antigen-independent T-cell polarization was a specific feature of Jurkat T cells, we validated our findings using human primary resting T cells (Figure 2B).

To further confirm the role of LFA-1 in controlling mitochondria compartmentalization, and to estimate the percentage of cells with polarized mitochondria in the complete absence of LFA-1 binding—a condition that is unlikely achieved with blocking antibody—we incubated human T cells with a murine fibroblast cell line stably expressing the human molecules ICAM-1, ICAM-2, ICAM-3, MHC class II (DR α, β 1*0101) or MHC class II plus ICAM-1. In both Jurkat (Figure 3A) and primary (Figure 3B) T cells, polarity was established towards fibroblasts expressing ICAM-1 or ICAM-2, independently of MHC expression. In agreement with the previous experiments, the presence of TCR ligands did not affect the recruitment of T-cell mitochondria to the IS. The different effect of ICAM-1/ICAM-2 or ICAM-3 on LFA-1-induced mitochondria polarization is consistent with previous reports showing that the LFA-1-binding sites for the three ICAM molecules are different (Binnerts *et al*, 1994) and that these three ligands induce different LFA-1 functions (Bleijs *et al*, 1999).

To formally prove that LFA-1 signalling alone can induce mitochondria polarization in T cells, we analysed the localization of mitochondria along *z* axis in T cells seeded onto microscope slides coated with poly-L-lysine, which does not trigger LFA-1 binding, or ICAM-2. To overcome the absence of chemokines, which are required to activate LFA-1 (Constantin

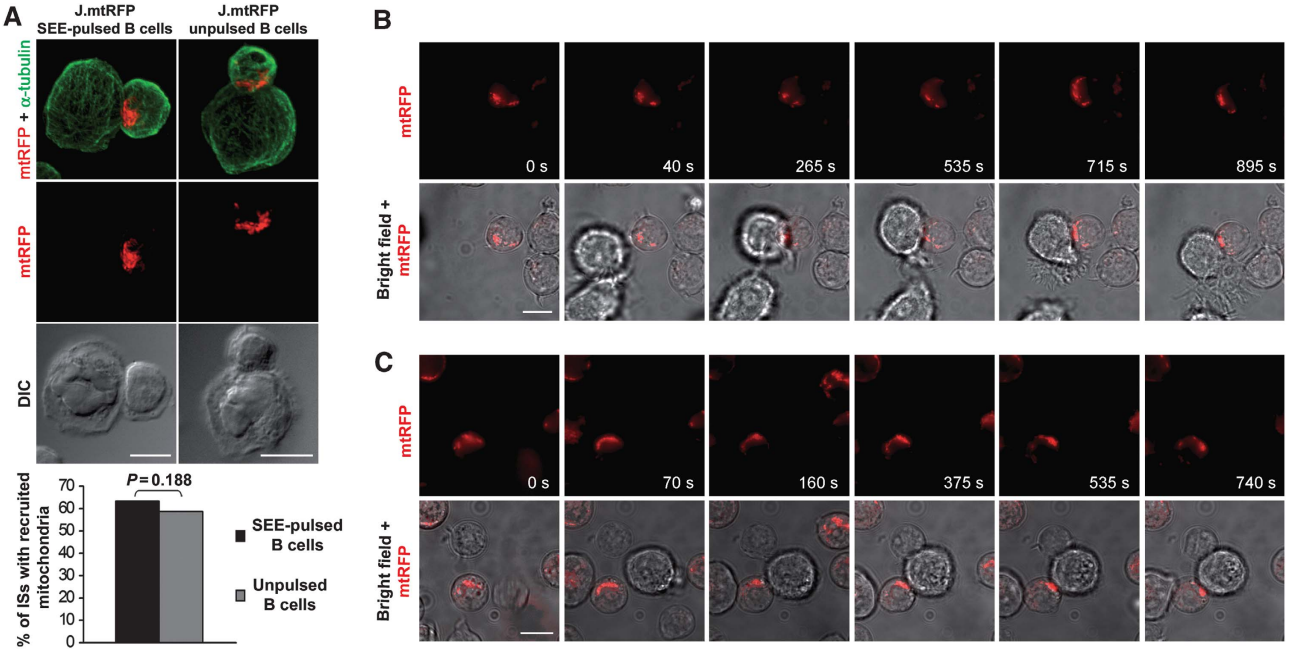

Figure 1 Mitochondria accumulate under the IS independently of TCR triggering. (**A**) Jurkat T cells stably expressing the mitochondrial marker mtRFP were incubated with SEE-loaded or unloaded B cells for 15 min. Images of mtRFP fluorescence from randomly selected conjugates (at least 180 for each condition, out of five independent experiments) were acquired, classified and analysed statistically as described in Materials and methods. Representative images of T cells with mitochondria recruited or not to the IS are shown in Supplementary Figure S1. Graph shows the quantitative analysis of mitochondria accumulation at the IS. Fluorescence (z projection of the stacks) and DIC representative images are shown. mtRFP was colour-coded red; α-tubulin, green. Bars, 10 μm. (**B**, **C**) Jurkat T cells expressing the mitochondrial marker mtRFP were plated with SEE-pulsed (**B**) or unpulsed (**C**) B cells and monitored by time-lapse microscopy for mitochondria relocation. Representative images taken from the digital movies (Supplementary Videos 2 and 3) at the indicated times are shown. Bars, 10 μm.

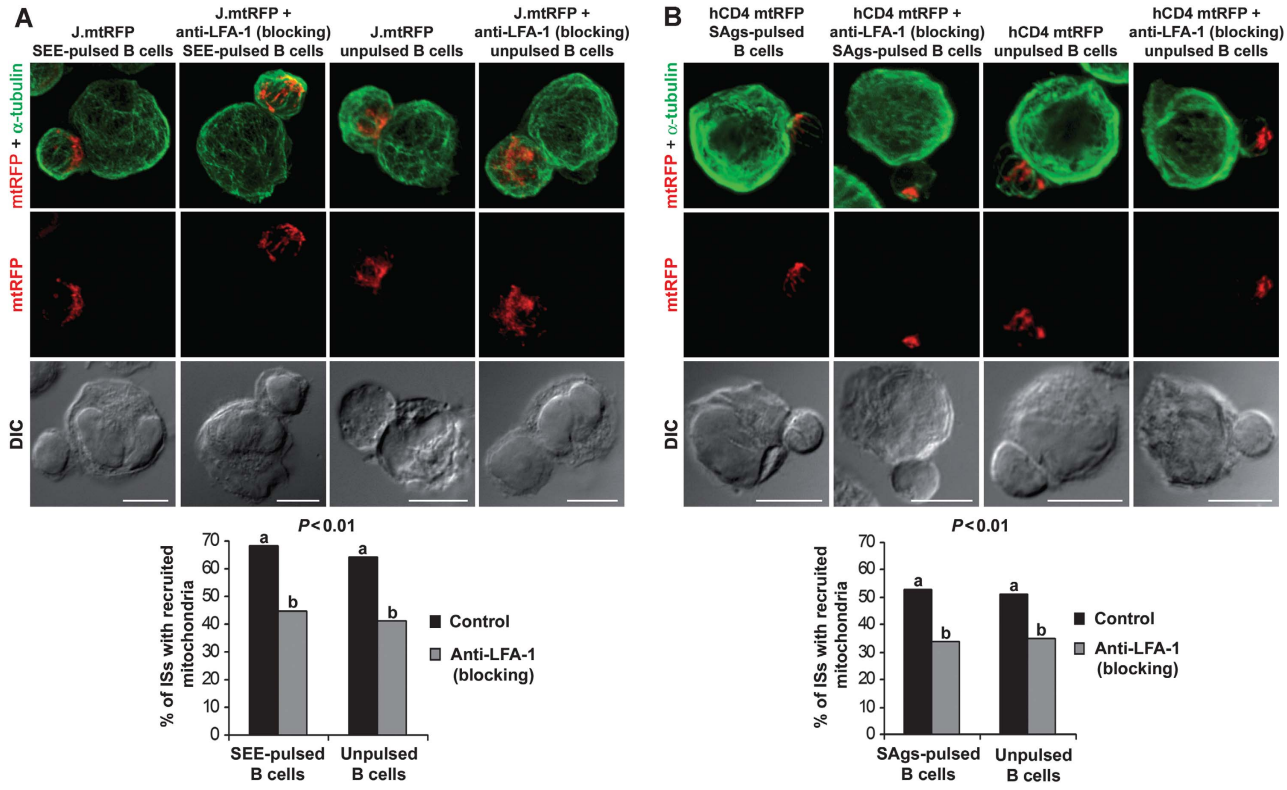

Figure 2 Mitochondria recruitment to the IS depends on LFA-1. Jurkat T cells stably expressing the mitochondrial marker mtRFP (**A**) or primary T cells transfected with mtRFP (**B**) were incubated with B cells, pre-pulsed or not with SEE (**A**) or superantigens (SAgs) (**B**), for 15 min. Where indicated, T cells were pre-treated with anti-LFA-1 neutralizing antibodies. Images from randomly selected conjugates (at least 149 for each condition, out of four (**A**) or two (**B**) independent experiments) were acquired, analysed and classified as described in Materials and methods. Graphs show the quantitative analysis of mitochondria accumulation at the IS. Data were statistically analysed as described in Materials and methods and columns with different letters are significantly different from each other. Fluorescence (z projection of the stacks) and DIC representative images are shown. mtRFP was colour-coded red; α-tubulin, green. Bars, 10 μm.

et al, 2000) and were provided by B cells or fibroblasts in the previous experimental settings (Molon *et al*, 2005), we stimulated T cells with 100 nM CXCL12 before seeding them onto slides. We observed clear recruitment of mitochondria towards the site of LFA-1 engagement in the presence of CXCL12 only (Figure 3C; Supplementary Video 5), suggesting that the presence of chemokines is required for LFA-1-induced T-cell polarity towards the APCs.

Finally, we analysed the localization of mitochondria along *z* axis in T cells seeded onto borosilicate slides (that are different from the previous slides and allow optimal antibody binding) coated with anti-CD3, anti-LFA-1-blocking or anti-LFA-1-activating mAbs. T cells seeded onto anti-LFA-1-activating mAb showed a statistically significant higher recruitment of mitochondria towards the slides than T cells seeded onto anti-CD3 or anti-LFA-1-blocking mAbs (Figure 3D).

Altogether, these data indicate that T cells polarize their mitochondria towards cells producing chemokines and expressing ICAM-1 or ICAM-2, independently of MHC expression.

LFA-1-driven mitochondria translocation to the IS requires microtubule integrity and depends on chemokine receptor signalling

Interactions of mitochondria with the cytoskeleton are crucial for normal mitochondrial functions and for localization of the organelles at the proper sites of action within cells (Boldogh and Pon, 2007). Several studies have revealed a role for microtubule motors in mitochondrial motility in different cell types, such as neurons and epithelial cells (Boldogh and Pon, 2007). In agreement, mitochondria are transported along microtubules to the cell uropod of migrating lymphocytes (Campello *et al*, 2006). To examine the role of the tubulin cytoskeleton in the observed LFA-1-induced mitochondria translocation, we allowed conjugates to form between B cells and T cells that had been pre-treated with the microtubule-depolymerizing drug colcemid. Unsurprisingly, we found that microtubule integrity was required for mitochondria recruitment to the IS (Figure 4; Supplementary Video 6) or towards slides coated with anti-LFA-1-activating mAb (Supplementary Figure S2).

As control, we analysed MTOC localization under the IS and we found that, as expected, it was inhibited by colcemid treatment. Interestingly, and in agreement with all experiments reported above, MTOC polarization did not require TCR stimulation in our experimental setting (Figure 4; Supplementary Video 6). This result seems to be in contrast with previous studies showing a role of TCR signalling in inducing MTOC translocation towards the IS (Kupfer *et al*, 1987; Blanchard *et al*, 2002; Martin-Cofreces *et al*, 2008). We speculated that these conflicting results on the role of LFA-1 or TCR in the establishment of T-cell polarity may be due to the presence or absence of chemokines in the experimental settings. Indeed, in our experiments, we have used APCs that had been previously screened for their chemokine production (Molon *et al*, 2005), and chemokines released by APCs are

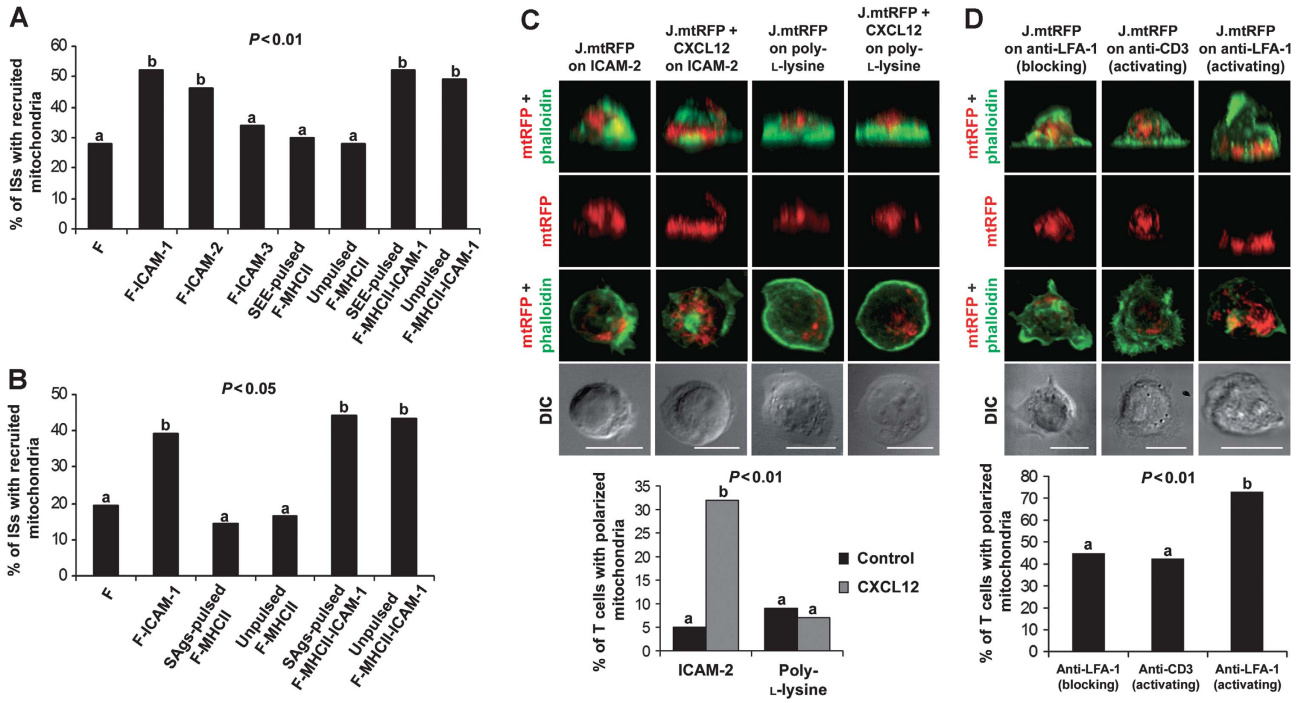

Figure 3 Mitochondria translocation requires LFA-1 triggering. (**A**, **B**) Jurkat T cells stably expressing mtRFP (**A**) or primary T cells transfected with mtRFP (**B**) were incubated for 15 min with murine L cells expressing, or not (F), human ICAM-1 (F-ICAM-1), ICAM-2 (F-ICAM-2), ICAM-3 (F-ICAM-3), MHC class II (F-MHCII) or MHC class II plus ICAM-1 (F-MHCII-ICAM-1). F-MHCII and F-MHCII-ICAM-1 were pre-pulsed or not with SEE (**A**) or superantigens (SAgs) (**B**). Images from randomly selected conjugates (at least 480 (**A**) or 69 (**B**) conjugates for each condition, out of three (**A**) or two (**B**) independent experiments) were acquired, analysed and classified as described in Materials and methods. Graphs show the quantitative analysis of mitochondria accumulation at the IS. (**C**, **D**) Jurkat T cells stably expressing mtRFP, pre-treated or not with 100 nM CXCL12 for 5 min, were plated for 15 min at 37°C on (**C**) microscope slides coated with 0.05 mg/ml poly-L-lysine or 20 μg/ml human ICAM-2/Fc or (**D**) borosilicate slides coated with 20 μg/ml blocking anti-LFA-1, activating anti-CD3 or activating anti-LFA-1 antibodies. (**C**, **D**) Randomly selected cells (at least 48 for each condition) were analysed by confocal microscopy. Graphs show the quantitative analysis of mitochondria translocation towards the slide. Fluorescence and DIC representative images are shown: lateral view (first and second row) and top view (third row) of three-dimensional reconstruction of the fluorescence stacks. mtRFP was colour-coded red; actin, green. Bars, 10 μm. (**A**–**D**) All data were statistically analysed as described in Materials and methods and, in all graphs, columns with different letters are significantly different from each other.

known to regulate local T-cell adhesiveness through LFA-1 activation (Constantin *et al*, 2000; Shamri *et al*, 2005). In support of this hypothesis, we found that in T cells in which the chemokine receptor signalling was inhibited by incubation with pertussis toxin (Goldman *et al*, 1985), TCR triggering supported MTOC and mitochondria recruitment to the IS (Supplementary Figure S3).

Phosphatidylinositol 3-kinase (PI3K) has a central function in integrin-mediated cellular responses in a variety of cells (Cantley, 2002), including T lymphocytes (Sanchez-Martin *et al*, 2004). Moreover, PI3K is required for integrin-induced, microtubule-mediated cell polarity of several cell types, including neurons (Shi *et al*, 2003) and epithelial cells (Liu *et al*, 2004). Thus, we asked whether PI3K was also implicated in integrin-driven polarization of T cells towards the cell partner. PI3K inhibition by wortmannin impaired mitochondria recruitment to the IS, both in Jurkat T cells (Figure 5A) and in human primary resting T cells (Figure 5B). PI3K signalling is induced by several types of receptors and thus, to verify that the effect of wortmannin on mitochondria translocation was dependent on LFA-1, the same experiment was repeated using primary human T cells conjugated with murine fibroblasts expressing human ICAM-1 alone or in combination with human MHCII, as already described for Figure 3B. This experiment confirmed that LFA-1-driven mitochondria recruitment to the IS requires PI3K signalling (Figure 5C). To further dissect the role of PI3K in the process and to discriminate between its requirement for chemokine-induced LFA-1 activation or LFA-1-driven mitochondria translocation, we induced T-cell polarization towards borosilicate slides using either ICAM-2 plus CXCL12 or anti-LFA-1-activating mAb. We found that wortmannin reduced mitochondria relocation only in T cells stimulated by ICAM-2 plus CXCL12, indicating that PI3K is required for LFA-1 activation—as already reported (Constantin *et al*, 2000)—but not for LFA-1-induced mitochondrial polarity (Figure 5D).

Time-dependent contribution of TCR and chemokine receptors to mitochondria recruitment to the IS

Prompted by the observation that TCR triggering may induce LFA-1 activation in the absence of chemokine signalling, we decided to further investigate the effects of chemokine receptors and TCR in modulating LFA-1 activity (Kinashi, 2005), and thus mitochondria recruitment to the IS. Therefore, we pre-treated T cells with pertussis toxin and/or with PP2 that specifically inhibits src kinases downstream of TCR signalling (Hanke *et al*, 1996). According to previous experiments (Supplementary Figure S3), we found that either chemokine receptor signalling or TCR signalling alone were both able to induce mitochondria recruitment through LFA-1 activation (Figure 6A). These data show that during the initial T-APC interactions, both chemokine receptors and TCR may

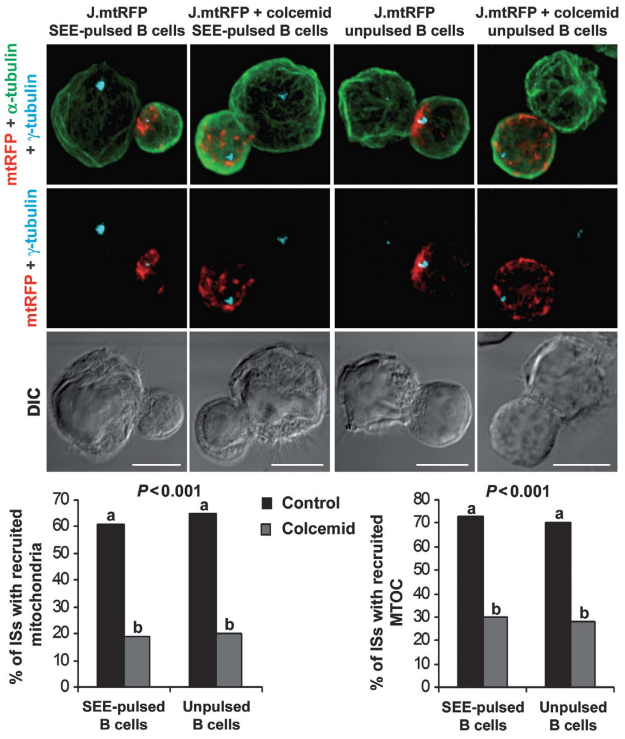

Figure 4 Mitochondria recruitment to the IS depends on microtubule integrity. Jurkat T cells stably expressing the mitochondrial marker mtRFP were incubated with B cells, pre-pulsed or not with SEE, for 15 min. Where indicated, T cells were pre-treated with 10 μM colcemid. Images from randomly selected conjugates (at least 274 for each condition, out of two independent experiments) were acquired, analysed and classified as described in Materials and methods. Graphs show the quantitative analysis of mitochondria and MTOC recruitment to the IS. Data were statistically analysed as described in Materials and methods and columns with different letters are significantly different from each other. Fluorescence (z projection of the stacks) and DIC representative images are shown. mtRFP was colour-coded red; α-tubulin, green; γ-tubulin, cyan. Bars, 10 μm.

activate LFA-1 and thus similarly trigger mitochondria recruitment, without additive effect when both the receptors are engaged.

Although adhesive interactions are responsible for the initial T-cell-APC contacts, formation of stable IS and T-cell activation require TCR triggering by peptide–MHC complexes. Signals delivered by the triggered TCR—including Ca^{2+} entry—have to be sustained over time to promote transcription and cell cycle progression (Gallo *et al*, 2006). Thus, we hypothesized that mitochondria accumulation under the IS was long lasting and that TCR signalling might have a function in controlling the organelle positioning over time. To verify this hypothesis, we analysed the recruitment of mitochondria to the IS of T cells incubated with pulsed or unpulsed APCs for 5 min or 3 h (Figure 6B). While TCR triggering did not impact mitochondria localization after 5 min of conjugate formation, on the long term (3 h) it increased the percentage of conjugates with polarized mitochondria in T cells. This result is even more convincing when considering the analysis of mitochondria translocation over time (Figure 6C). Although single cell analysis showed that mitochondria recruitment to the IS occurs in <2 min after establishment of conjugates (Supplementary Videos 2 and 3), in cell population experiments, translocation of the organelles was evident (46% of conjugates) 5 min after T-APC co-incubation, but it peaked (61% of conjugates) at 15 min, likely because of the fact that cells are not synchronous and that, in contrast to the single cell experiments, time for formation of conjugates is included in the analysis. The recruitment of mitochondria at 5 and 15 min was likely ascribable to LFA-1, because TCR stimulation did not increase it. However, 3 h after T-APC co-incubation, the percentage of cells still displaying mitochondria under the IS was significantly higher in T cells stimulated through both LFA-1 and TCR (58% of conjugates) than through LFA-1 alone (46% of conjugates) (Figure 6C). These results indicate that TCR signalling has a function in stabilizing mitochondria localization under the IS.

The role of TCR in activating LFA-1 is well established (Kinashi, 2005). We wondered if the TCR-induced stabilization of mitochondria at the IS involves the TCR inside-out signalling to LFA-1. Therefore, we blocked (or not) LFA-1 only after the initial 15 min of T-APC interaction and we allowed these conjugates to interact for more 3 h, after which we analysed the mitochondria recruitment to the IS. During the initial phase of this experimental setting (first 15 min), LFA-1 could, therefore, induce mitochondria relocation to the IS, as shown in Figures 2 and 3. During the rest of the incubation period, TCR should be able to stabilize and maintain mitochondria positioning at the IS. We found that TCR signalling did not stabilize the organelles at the IS in the presence of anti-LFA-1-blocking antibodies (Figure 6D), showing that the effects of TCR signalling on mitochondria localization during long-lasting cellular interactions still depend on LFA-1. Interestingly, the fact that TCR signalling is required to stabilize mitochondria at the IS indicates that during long-lasting cellular interactions, the chemokine receptor signalling is not sufficient to control LFA-1-induced mitochondria stabilization at the IS (Figure 6B–D).

TCR-induced CRAC triggering retains mitochondria at the IS

Although intracellular Ca^{2+} is required for mitochondria relocation inside the cells, using anti-CD3-coated beads, it has been shown that mitochondria translocate towards the bead and not to the calcium influx source (Schwindling *et al*, 2010). We therefore speculated that sustained calcium influx through CRAC channels would be required for mitochondria stabilization at the IS. We analysed mitochondria translocation towards the IS in conjugates formed between pulsed or unpulsed B cells and T cells expressing the wild type or the dominant negative form of the CRAC-channel subunit ORAI1. The ORAI1-E106A mutant is a dominant negative, non-conducting CRAC channel that inhibits native CRAC currents (Lioudyno *et al*, 2008) (Supplementary Figure S4). We found that LFA-1-induced mitochondria translocation was not affected by ORAI1-E106A expression, whereas TCR-mediated, long-term retention of the organelles at the IS required sustained CRAC currents (Figure 6E). To further confirm that the ORAI1-dependent calcium influx was not involved in LFA-1 triggered translocation of mitochondria, we completely blocked the ORAI1 channels by treating T cells with 2-APB (Prakriya and Lewis, 2001). We found that 2-APB did not inhibit the recruitment of mitochondria at the IS induced by LFA-1 (Supplementary Figure S5).

Altogether, these data indicate that LFA-1 triggers translocation of mitochondria towards the IS independently of TCR

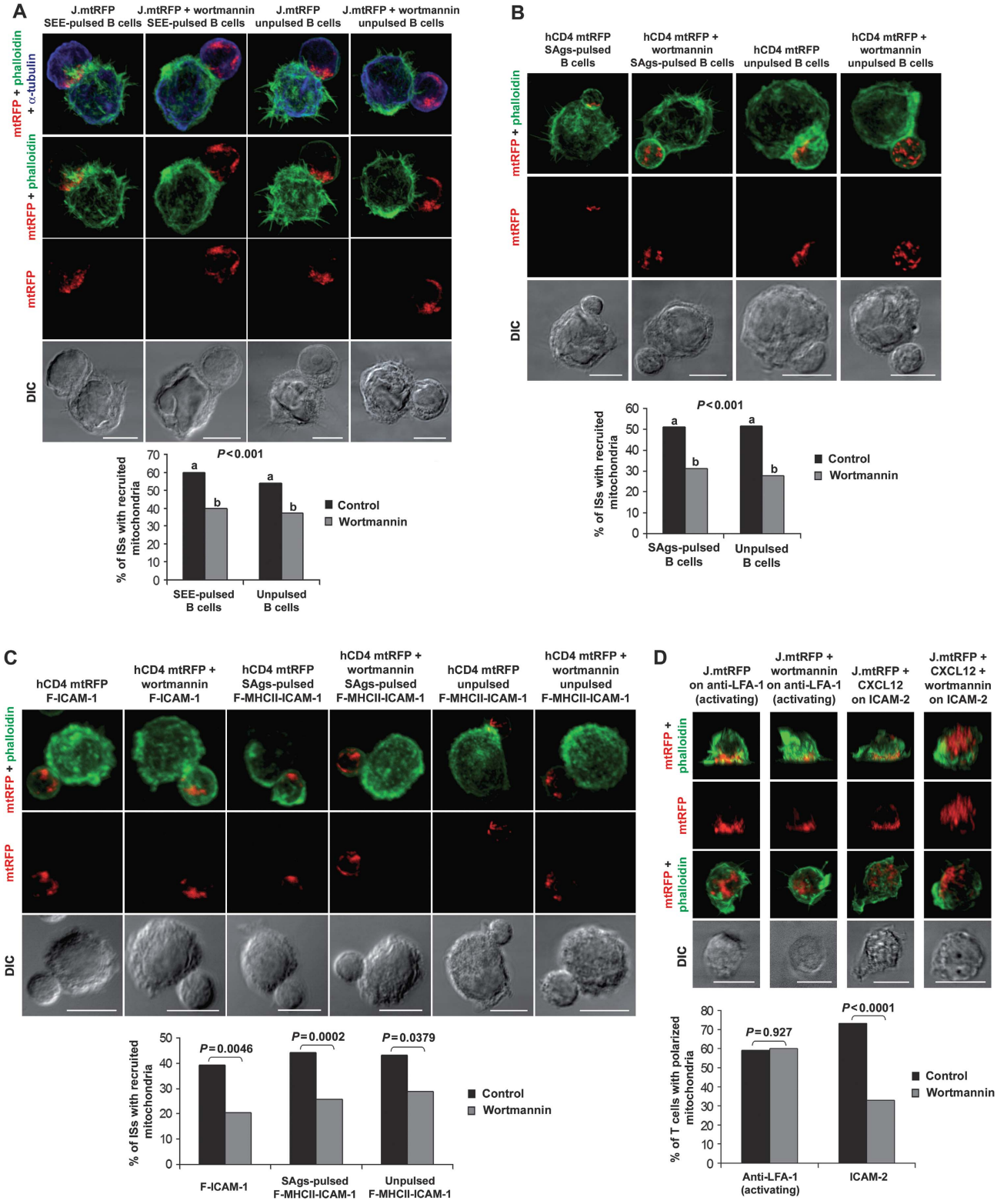

Figure 5 Mitochondria recruitment to the IS requires PI3K signalling. (**A**) Jurkat T cells stably expressing mtRFP were incubated with B cells, pre-pulsed or not with SEE, for 15 min. (**B**, **C**) Primary T cells transfected with mtRFP were incubated for 15 min with B cells (**B**) or murine L cells expressing human ICAM-1 (F-ICAM-1) or MHC class II plus ICAM-1 (F-MHCII-ICAM-1) (**C**), pre-pulsed or not with superantigens (SAgs). (**D**) Jurkat T cells stably expressing mtRFP were plated for 15 min at 37°C on borosilicate slides coated with 20 μg/ml activating anti-LFA-1 mAb or human ICAM-2/Fc (in this latter case, T cells were pre-treated for 5 min with 100 nM CXCL12). Where indicated, T cells were pre-treated with 1 μM wortmannin. Images from randomly selected conjugates or cells (at least 269 (**A**), 183 (**B**), 69 (**C**) conjugates or 44 (**D**) cells for each condition) were acquired, analysed and classified as described in Materials and methods. Graphs show the quantitative analysis of mitochondria accumulation at the IS. (**A**–**D**) Fluorescence (z projection of the stacks) and DIC representative images are shown. mtRFP was colour-coded red; actin, green; α-tubulin, blue. Bars, 10 μm. (**D**) First and second rows show lateral view of three-dimensional reconstruction of the fluorescence stacks. (**A**, **B**) Data were statistically analysed as described in Materials and methods and columns with different letters are significantly different from each other. (**C**, **D**) Owing to the different types of stimuli (different APCs in (**C**), ICAM and mAb in (**D**)), wortmannin-treated cells were statistically compared (χ^2 test) to their specific controls.

stimulation. However, TCR-induced CRAC triggering is important to sustain their recruitment over time.

LFA-1 amplifies TCR-dependent Ca^{2+} signalling and boosts T-cell activation by recruiting mitochondria to the IS

Several studies have suggested that the sub-cellular localization of mitochondria is critical for Ca^{2+} signalling (Hoth *et al*, 1997; Rizzuto *et al*, 1998; Quintana *et al*, 2007). These organelles buffer intracellular Ca^{2+} and sustain Ca^{2+} influx through membrane channels that would otherwise be inhibited by high intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) (Lawrie *et al*, 1996; Hoth *et al*, 1997). On the basis of the experiments reported above, we speculated that the adhesion-induced mitochondria translocation towards the potential APC would be pivotal to prepare the T cell for the upcoming TCR Ca^{2+} signalling.

To test this hypothesis, Ca^{2+} fluxes were analysed in conjugates between T cells, incubated or not with anti-LFA-1-blocking antibodies, and unpulsed B cells. In these conditions, the TCR is not triggered (no TCR ligand is present), as indicated by the lack of Ca^{2+} oscillations in T–B cell conjugates (Figure 7A). Moreover, by performing our analysis on conjugates only, we excluded any alteration of the results imputable to the adhesive properties of LFA-1. After 5 min—time required to obtain recruitment of mitochondria to the IS in about 50% of conjugates that had not been incubated with anti-LFA-1-blocking antibody—addition of SEE induced a rapid increase of $[Ca^{2+}]_i$, indicating TCR triggering (Figure 7A). However, Ca^{2+} influx was reduced in conjugates formed with T cells having LFA-1 blocked, suggesting, but not proving, that our hypothesis was correct.

To formally prove that the LFA-1-induced enhancement of TCR Ca^{2+} influx was operated by mitochondria, we applied the same experimental protocol described above to T cells pre-treated with ruthenium 360 that selectively blocks the mitochondrial Ca^{2+} uptake (Kirichok *et al*, 2004), but does not interfere with mitochondria recruitment to the IS (Supplementary Figure S6). We found that the two treatments—blockade of LFA-1 or of mitochondrial Ca^{2+} uptake—reduced TCR-induced Ca^{2+} influx to a similar degree (Figure 7B). Moreover, the ability of LFA-1 to potentiate TCR signalling was reduced in cells having mitochondria unable to buffer Ca^{2+} (Figure 7B). Considering the essential role of chemokine in activating LFA-1 (Constantin *et al*, 2000; Shamri *et al*, 2005) and recruiting mitochondria to the IS

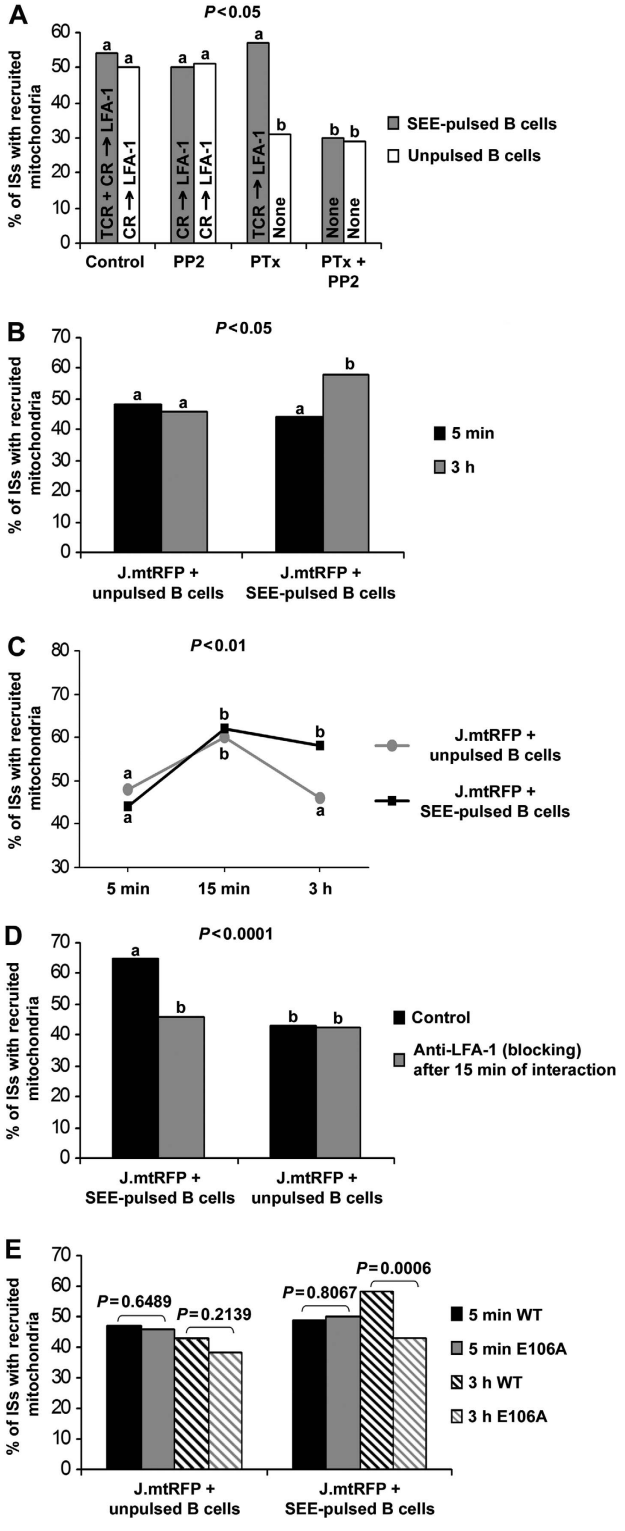

Figure 6 Different contribution of chemokine receptors and TCR to mitochondria recruitment to the IS. (**A**) Jurkat T cells stably expressing mtRFP, pre-treated or not with 1 μg/ml pertussis toxin and/or 50 μM PP2, were incubated with B cells pre-pulsed or not with SEE for 15 min. Text inside the graph bars indicates for each condition which signalling pathway (from TCR and/or chemokine receptors (CR)) is inducing LFA-1 activation. (**B**) Jurkat T cells stably expressing the mitochondrial marker mtRFP were incubated with SEE-loaded or unloaded B cells for 5 min or 3 h. (**C**) The graph condenses the kinetics of mitochondria translocation towards the IS in the presence or absence of TCR triggering. The statistical differences indicated by letters refer to each condition over time. (**D**) Jurkat T cells stably expressing mtRFP were incubated with B cells pre-pulsed or without SEE for 3 h. After 15 min of interaction, where indicated, anti-LFA-1-blocking antibodies were added. (**E**) Jurkat T cells stably expressing the mitochondrial marker mtRFP were transfected with CFP-ORAI1 or CFP-ORAI1-E106A and then incubated with SEE-loaded or unloaded B cells for 5 min or 3 h. Statistical differences between ORAI1 and ORAI1-E106A are indicated in the figure. (**A**–**E**) Images from randomly selected conjugates (at least 120 (**A**), 359 (**B**), 140 (**D**) or 171 (**E**) for each condition) were acquired, analysed and classified as described in Materials and methods. Graph shows the quantitative analysis of mitochondria accumulation to the IS. Data were statistically analysed as described in Materials and methods and columns with different letters are significantly different from each other.

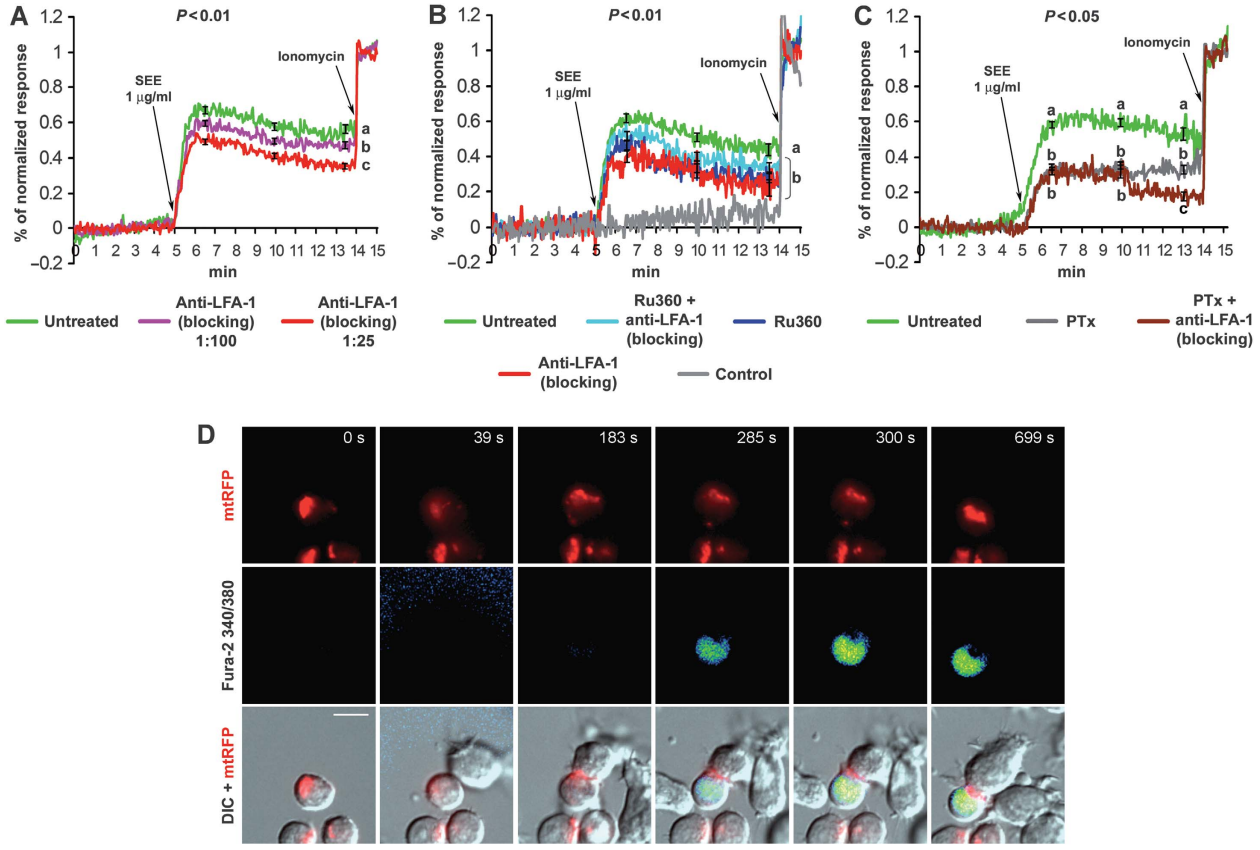

Figure 7 LFA-1-induced translocation of mitochondria to the IS amplifies TCR Ca^{2+} signalling. (**A**–**C**) Free $[Ca^{2+}]_i$ was measured by FACS in Fluo-4/Fura-Red-loaded Jurkat T cells conjugated with unpulsed B cells. SEE was added 5 min after conjugate formation. Ionomycin was used as positive control for the maximal Ca^{2+} influx. Where indicated, T cells were treated with anti-LFA-1 neutralizing antibodies and/or 50 μM ruthenium 360 or pertussis toxin (PTx). Data are representative of one out of three experiments. 'Control' indicates Jurkat T cells analysed in the absence of B cells. The results are expressed as percentage of normalized response, calculated as indicated: [(mean of Fluo-4/Fura-Red ratio)−(mean of Fluo-4/Fura-Red ratio during T-cell stimulation with unpulsed B cells before SEE addition)]/[(mean of Fluo-4/Fura-Red ratio during the response to 1 μg/ml ionomycin)−(mean of Fluo-4/Fura-Red ratio during T-cell stimulation with unpulsed B cells before SEE addition)]. For each graph, the curves were statistically compared using two-way ANOVA, at three representative time points (indicated by standard error (s.e.) bars in the graphs). Curves with different letters are significantly different from each other at each time point. (**D**) Jurkat T cells expressing the mitochondrial marker mtRFP were plated with SEE-pulsed B cells and monitored by time-lapse microscopy for mitochondria relocation and calcium signalling. Representative images taken from the digital movies (Supplementary Video 7) at the indicated times are shown. Bars, 10 μm.

(Supplementary Figure S3; Figure 6A), we analysed the role of chemokine-mediated LFA-1 activation in enhancing TCR calcium signalling. We found that TCR-induced calcium influx is significantly reduced in T cells lacking chemokine receptor signalling and stimulated by APCs, and that—in contrast to what was observed in control cells—anti-LFA-1-blocking antibodies did not further reduce it, suggesting that the inhibition of chemokine receptor signalling has already blocked the ability of LFA-1 to enhance TCR-induced calcium influx (Figure 7C). Interestingly, at later time points, pertussis toxin-treated cells showed a significantly higher calcium influx than cells treated with pertussis toxin plus anti-LFA-1, suggesting that TCR signalling may replace the chemokine signalling and partially restore LFA-1 functions, even if not immediately (Figure 7C). This experiment shows that the amplification of calcium signalling by LFA-1 requires chemokine-mediated LFA-1 activation.

Our data indicate that LFA-1, activated by chemokines, enhances TCR signalling by recruiting mitochondria to the upcoming IS and suggest that induction of T-cell polarity towards the IS precedes TCR-induced Ca^{2+} influx. To verify this hypothesis, we used real time, time-lapse microscopy to simultaneously analyse mitochondrial movements and Ca^{2+} influx in T cells interacting with APCs. Indeed, we found that massive Ca^{2+} influx in T cells follows mitochondria recruitment to the IS (Figure 7D; Supplementary Video 7).

In T cells, transcription and cellular activation require a sustained Ca^{2+} influx across the plasma membrane through CRAC channels (Gallo *et al*, 2006). Thus, we hypothesized that the calcium buffering operated by mitochondria at the IS should have an important function in sustaining T-cell activation.

We analysed the localization of the HA-tagged form of NFATc2, the main transcription factor controlled by intracellular calcium in T cells (Jain *et al*, 1993). We measured the nuclear translocation of NFATc2 in Jurkat T cells treated or not with ruthenium 360 and/or anti-LFA-1-blocking antibodies and conjugated with SEE-pulsed B cells. We found a strong correlation between the nuclear localization of NFATc2 and the recruitment of mitochondria to the IS. In fact, the NFAT nuclear translocation index was significantly higher in T cells with mitochondria recruited to the IS than in cells with

mitochondria not recruited (Figure 8A). Importantly, in the presence of anti-LFA-1-blocking antibodies, this correlation was maintained (because mitochondria were functional), but the overall percentage of ISs with recruited mitochondria was reduced (as well as the number of cells with nuclear NFATc2). Most interestingly, NFAT nuclear translocation was reduced—and equal to that of cells with evenly distributed mitochondria—in T cells with organelles polarized, but dysfunctional because of treatment with ruthenium 360 (Figure 8A). This experiment clearly indicates that NFAT translocation depends on mitochondria recruitment to the IS and on their ability to buffer calcium at that specific location.

Finally, we analysed the ability of LFA-1 to enhance CD25 expression—a key marker of T lymphocyte activation—in human primary resting T cells. As APCs, we used murine fibroblasts that selectively express human MHCII and/or ICAM-1 in order to activate TCR and/or LFA-1 (as described in Figures 3B and 5C). We found that co-engagement of TCR and LFA-1 was able to increase the expression of CD25 (Figure 8B). Most importantly, this costimulatory function of LFA-1 depended on the ability of mitochondria to buffer calcium because it was significantly reduced by ruthenium 360 (Figure 8B).

Altogether, these data show that the recruitment of mitochondria to the IS and their role in sustaining TCR-induced calcium influx are pivotal to support the nuclear translocation of NFAT and thus facilitate T-cell activation.

Discussion

Lymphocyte polarization is accompanied by rapid cytoskeletal rearrangements, assembly of specialized lipids and receptors at the plasma membrane (Viola and Gupta, 2007), and non-random redistribution of organelles, such as centrosome, the Golgi apparatus and mitochondria (Barreiro *et al*, 2007).

Mitochondria, highly mobile and dynamic organelles, can accumulate in sub-cellular regions requiring high metabolic activity, where they provide ATP (Li *et al*, 2004; Campello *et al*, 2006). In addition to their role in ATP delivery, mitochondria are critical regulators of $[Ca^{2+}]_i$. These organelles serve as a powerful buffer of $[Ca^{2+}]_i$ and sustain Ca^{2+} influx through membrane channels that would otherwise be inhibited by high $[Ca^{2+}]_i$ (Lawrie *et al*, 1996; Hoth *et al*, 1997). Interestingly, the ability of mitochondria to regulate Ca^{2+} signalling depends on their sub-cellular localization (Hoth *et al*, 1997; Rizzuto *et al*, 1998; Quintana *et al*, 2007). Moreover, it has been proposed that actin morphological changes, following the IS formation with anti-CD3-coated beads, bring mitochondria close to the plasma membrane and modulate Ca^{2+} influx through CRAC channels (Quintana *et al*, 2009).

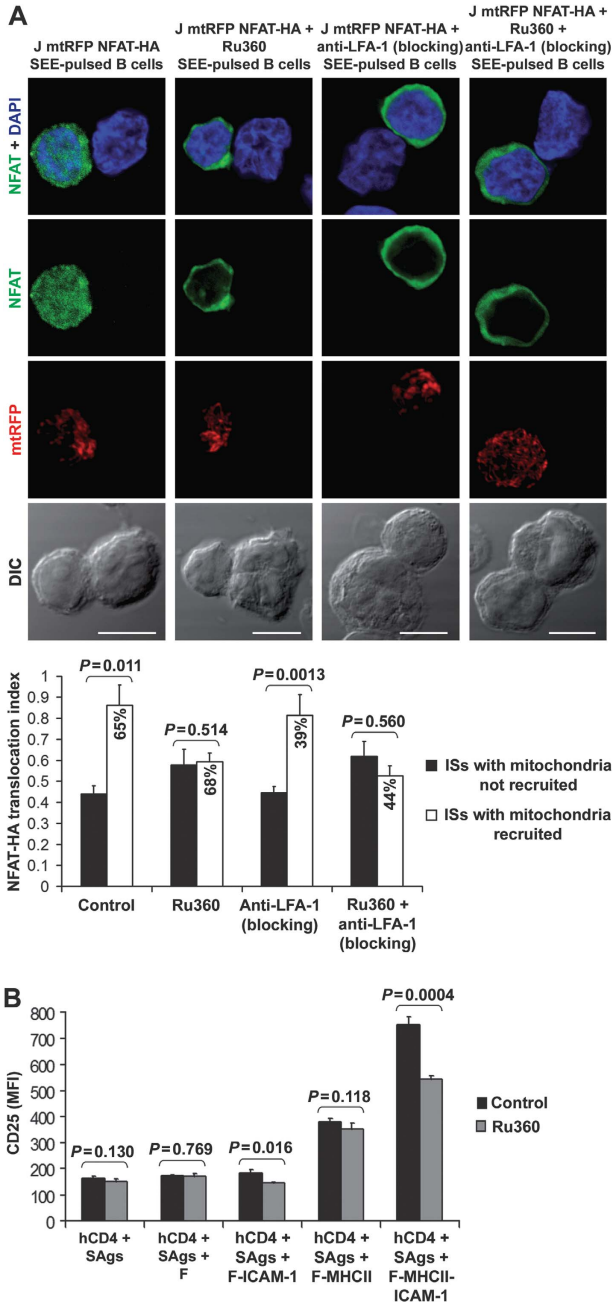

Figure 8 LFA-1, by recruiting mitochondria to the IS, sustains nuclear NFAT translocation and CD25 up-regulation. (**A**) Jurkat T cells were transfected with HA-NFATc2 and the mitochondrial marker mtRFP and then incubated with SEE-pulsed B cells for 15 min. Where indicated, T cells were pre-treated with anti-LFA-1-blocking antibodies and/or ruthenium 360. Images from randomly selected conjugates (at least 40 for each condition) were acquired, classified and analysed as described in Materials and methods. Numbers in the bars indicate the percentages of ISs with recruited mitochondria for each condition. Graph shows the quantitative analysis of NFAT-HA translocation to the nucleus (mean ± s.e.). The NFAT-HA translocation index of unstimulated cells was 0.33 ± 0.02 (mean ± s.e.). Data were statistically analysed with Mann–Whitney test. The percentage of T cells with NFAT translocation index >0.66, considering all ISs independently of mitochondria position, was 43% (control), 26% (Ru360), 28% (anti-LFA-1) and 29% (Ru360 + anti-LFA-1)). Fluorescence (z projection of the stacks for mitochondria and single slice for NFAT and DAPI) and DIC representative images are shown. mtRFP was colour-coded red; NFAT-HA, green; DAPI, blue. Bars, 10 μm. (**B**) Primary T cells were incubated for 18 h in the presence of superantigens (SAgs) with murine L cells expressing, or not (F), human ICAM-1 (F-ICAM-1), MHC class II (F-MHCII) or MHC class II plus ICAM-1 (F-MHCII-ICAM-1). Where indicated, T cells were pre-treated with ruthenium 360 and also incubated with APCs in the presence of ruthenium 360. Cells were then harvested, stained for CD4 and CD25 and analysed by FACS. Graph shows the mean fluorescence intensity (MFI) of CD25 (± s.d.). Statistical analysis was performed using Student's *t*-test.

During migration and activation of T cells, mitochondria accumulate in specific active regions to exert distinct functions. In migrating T cells, mitochondria concentrate at the cell uropod to provide ATP and fuel the cell motor of migrating leucocytes (Campello *et al*, 2006). Here, we show that during stimulation of T cells by APCs, mitochondria accumulate at the IS, where they buffer Ca^{2+}, prevent CRAC-channel inhibition and sustain TCR signalling, NFAT nuclear translocation and CD25 expression. These results confirm and extend previous observations obtained in Jurkat T cells stimulated by anti-CD3-coated beads (Hoth *et al*, 1997; Quintana *et al*, 2007; Schwindling *et al*, 2010). Most importantly, we have shown that this mobilization of mitochondria towards the APC is orchestrated by chemokine receptors and LFA-1. Our data indicate that, as soon as the T cell contacts the APC membrane, chemokine receptors trigger LFA-1 activation that, in turn, delivers signals that change T-cell polarity and recruit mitochondria to the potential, upcoming IS. However, our data do not exclude a role of the TCR in this process. We found that the TCR signalling has a function in stabilizing mitochondria under the IS, but its contribution to the establishment of T-cell polarity becomes evident only over time. Thus, chemokines and TCR seem to have distinct roles in establishing and maintaining cell polarity, respectively. Nevertheless, the effect of both receptors on mitochondria localization is strictly dependent on integrin functions and, on the basis of our data, we suggest that, at the IS, the requirements for LFA-1 activation are finely tuned over time.

This primary role of LFA-1 in the orchestration of T-cell polarity is in accordance with the general role of integrins in controlling polarity in various types of cells. The orientation of the mitotic spindle and of the MTOC are two key integrin-dependent events in epithelial and neuronal cells (Arimura and Kaibuchi, 2005; Streuli, 2009). Here, we have shown that adhesion induces polarization of the T-cell MTOC towards the APC, and thus allows recruitment of mitochondria under the upcoming IS. Although previous studies have proposed a role of TCR signalling in inducing MTOC translocation towards the IS (Kupfer *et al*, 1987; Blanchard *et al*, 2002; Martin-Cofreces *et al*, 2008), several other data indicate that this process depends on adhesion (Barreiro *et al*, 2007). First, as mentioned above, integrins control MTOC polarization in most—if not all—cell types. Second, LFA-1 interacts directly with tubulin-binding proteins, such as Mac-MARCKS (Zhou and Li, 2000) and AKAP450 (El Din El Homasany *et al*, 2005), and, at the IS, microtubules are anchored at LFA-1 clusters (Kuhn and Poenie, 2002). In natural killer cells, LFA-1 controls MTOC and cytotoxic granules polarization towards the IS (Barber *et al*, 2004; Liu *et al*, 2009). Finally, LFA-1 interaction with its ligand ICAM-1 is sufficient to trigger reorientation of the MTOC towards the virological synapse formed between human T-lymphotropic virus type-1-infected and uninfected T cells (Barnard *et al*, 2005).

Chemokines released by APCs deliver costimulatory signals to T cells (Molon *et al*, 2005), and regulate local T-cell adhesiveness through LFA-1 activation (Constantin *et al*, 2000; Shamri *et al*, 2005). We propose that the conflicting results reported on the role of LFA-1 or TCR in the establishment of T-cell polarity may be due to the lack of chemokines in the specific experimental system used to activate T cells. Indeed, we have shown that LFA-1 activation by chemokines is required for mitochondria attraction towards the adhesive contacts and that, when chemokine receptor signalling is inhibited, MTOC and mitochondria recruitment to the IS are supported by the triggered TCR. However, chemokines are absent in most of the systems used to study the IS, such as the lipid bilayer or plate-coated antibodies. Moreover, not all the APCs secrete chemokines efficiently and, in our experiments, we have used APCs that had been previously screened for their chemokine production (Molon *et al*, 2005). Thus, in the absence of chemokines, it is not surprising that the activation of LFA-1—and its polarizing properties—depends on TCR triggering (Katagiri *et al*, 2000).

Our data unveil, to our knowledge, a novel feature of LFA-1 during T-cell activation. By attracting mitochondria towards the upcoming IS, LFA-1 prearranges T cells for prompt responses. In T cells, a transient increase in $[Ca^{2+}]_i$ is not sufficient to activate transcription, which requires a more sustained Ca^{2+} influx through CRAC channels (Gallo *et al*, 2006). However, CRAC channels are inactivated by intracellular Ca^{2+}, and Ca^{2+} buffering by mitochondria is required for a sustained CRAC activity (Demaurex *et al*, 2009). Thus, integrin-mediated adhesive contacts that, in a physiological context—few antigenic complexes—precede TCR triggering and clustering (Montoya *et al*, 2002), prepare T cells for the upcoming TCR signalling, sustain Ca^{2+} influx through CRAC channels, promote NFAT nuclear translocation and boost CD25 expression. Our data also propose a possible mechanism for the reported ability of chemokines to prime T cells for synapse formation and costimulate *in trans* T-cell activation (Friedman *et al*, 2006).

The role of LFA-1 as enhancer of T-cell activation proposed in this study is in agreement with previous reports (Van Seventer *et al*, 1992; Doucey *et al*, 2003; Graf *et al*, 2007; Suzuki *et al*, 2007). As far as Ca^{2+} is concerned, adhesion can amplify TCR signalling through different mechanisms including prolonged inositol phospholipid hydrolysis (Van Seventer *et al*, 1992; Randriamampita *et al*, 2003), filling of intracellular Ca^{2+} stores (Randriamampita *et al*, 2003) or cyclic AMP increase (Conche *et al*, 2009). Here, we provide an additional mechanism for LFA-1-induced TCR signalling amplification, and we propose that the first adhesive contacts between T cells and APCs prepare and arrange the TCR signalling microenvironment by controlling the spatial organization of mitochondrial functions.

In metazoans, all cellular processes are finely tuned by integrin-mediated adhesive interactions. This is particularly true in the case of the immune cells, because the immune response organization requires exchanging information through direct communication between cells. Our study has shown that integrins establish T-cell polarity when adhesive contacts with potential APCs are initiated and that, by doing this, they shape T cells for prompt and sustained TCR signalling.

Materials and methods

See also Supplementary data.

Cell cultures, constructs and transfections

The Jurkat T cell line J.E6-1 (Jurkat cell clone purchased by the American Type Culture Collection), the EBV-B 221 cell line and primary resting $hCD4^+$ T cells were cultured in RPMI 1640 medium (Lonza) supplemented with 10% FCS, 2 mM L-glutamine, sodium pyruvate and non-essential amino acids. Jurkat E6-1 cells stably

expressing mtRFP (J.mtRFP) (Campello *et al*, 2006) were maintained as above with the addition of 2 mg/ml G418 (Gibco). L cells murine fibroblast were cultured as described in Supplementary data.

Primary resting hCD4$^+$ T cells were sorted by negative selection using RosetteSep kit (StemCell Technologies). Healthy donors' blood was provided by the Desio Hospital (Milan, Italy) that approved the use and obtained informed consent from all subjects.

CFP-ORAI1 and CFP-ORAI1-E106A in pIRESneo were a kind gift of DL Gill (Philadelphia, PA). pTO-HA-STREPIII-GW-FRT-EF1a-NFATc2 was a kind gift of G Baier (Innsbruck, Austria). The mtRFP construct has been already described (Campello *et al*, 2006).

Primary resting hCD4$^+$ T cells were transiently transfected using an electroporation system (Amaxa Biosystems) according to the manufacturer's guidelines. Jurkat cells were transiently transfected using a Bio-rad electroporation system.

Immunofluorescence confocal microscopy

For experiments with primary resting hCD4$^+$ T cells, B cells or L cells were suspended at 10^7 cells/ml and incubated (or not) with 1 μg/ml of bacterial superantigens (SAgs) SEA, SEB and SEE at 37°C for 2 h, mixing every 30 min. For experiments with Jurkat cells, B cells were loaded with 1 μg/ml SEE. Pulsed cells were washed and incubated at 37°C for 5, 15 min or 3 h with equal amount of Jurkat cells or with double amount of primary resting hCD4$^+$ T cells.

In some experiments, before conjugate formation, T cells were incubated with 10 μg/10^6 cells anti-CD11a plus 10 μg/10^6 cells anti-CD18 neutralizing mAbs at 4°C for 30 min, or with 10 μM colcemid or 1 μM wortmannin at 37°C for 30 min, or with 1 μg/ml pertussis toxin at 37°C for 4 h and/or with 50 μM PP2 at 37°C for 60 min, or with 50 μM 2-APB at 37°C for 5 min, or with 50 μM ruthenium 360 at room temperature for 45 min. Control cells were mock-treated accordingly. All of these substances, except colcemid, were also present during the conjugate formation. The percentage of T cells conjugated with B cells in the presence of each substance was analysed by FACS (Supplementary Table I).

Cells were adhered to microscope slides coated with 0.05 mg/ml poly-L-lysine, fixed with 4% paraformaldehyde, washed and either permeabilized with 0.1% Triton/PBS and stained with primary and secondary reagents, or directly mounted with vectashield mounting medium, with or without DAPI (Vector Laboratories). For the experiments of mitochondria polarization towards coated slides, J.mtRFP cells were treated or not with 100 nM CXCL12 for 5 min at 37°C, and then overlaid onto microscope slides coated with 0.05 mg/ml poly-L-lysine or with 20 μg/ml ICAM-2/Fc (100 μl/well 6 mm diameter). For experiments of mitochondria polarization towards antibody-coated slides, chambered eight-well Lab-Tek borosilicate coverglasses (Nalge Nunc Inc.) were treated with 20 μg/ml anti-CD3 or anti-CD11a (clone 38 or MEM-83) or ICAM-2/Fc in PBS overnight at 4°C and blocked with PBS containing 1% BSA for 3 h. Where indicated, cells were pre-treated with colcemid, wortmannin or CXCL12 as described above. For γ-tubulin staining, cells were post-fixed in methanol at −20°C and then rehydrated in PBS before permeabilization.

Specimens were acquired with fine focusing oil immersion lens ($\times$ 60, NA 1.35) in optical sections of 0.3 μm using an FV1000 laser scanning confocal microscope (Olympus), and operating in channel mode with 405, 488, 543 and 633 nm excitations. The resulting fluorescence emissions were collected using 425-to-475 nm (for DAPI or CFP), 500-to-550 nm (for AlexaFluor488), 565-to-615 nm (for mtRFP) and 655-to-750 nm (for AlexaFluor647) band-pass filters. Differential interference contrast (DIC, Nomarski technique) was also used. The z stacks were acquired with resolution of 1 Airy unit to allow three-dimensional reconstructions.

Analysis of fluorescence images and fluorescence quantitation

Digital images were processed using the NIH-ImageJ 1.37c and Adobe Photoshop 9.0.2 programs. Three-dimensional reconstructions were performed with the Imaris software (version X64 6.2 1; Bitplane AG).

The results are expressed as the percentage of conjugates with mitochondria or MTOC redistributed to the T-cell-APC contact area or towards the slide. Image analysis was performed blind to the treatment conditions. For each experimental condition, images were randomly taken from different wells of the microscope slide, observed and classified by three different operators. Cells were scored polarized when (1) at least 66% of the mitochondria fluorescence was localized in the half-part of the cell close to the area in contact with the APC or the slide and (2) mitochondria were localized close to the plasma membrane (representative images of T cells with mitochondria recruited or not to the IS are shown in Supplementary Figure S1). MTOC was considered recruited when the γ-tubulin spot was localized in the third part of the cell proximal to the APC. The results were confirmed by performing a mathematical analysis on a random sample of images, using Image J software (as described in Supplementary data).

To quantify the NFAT-HA translocation to the nucleus, boxes were drawn around the nucleus and the cytoplasm of the T cell, and a background area was drawn in the nucleus of the B cell. The translocation index was calculated as indicated: [mean fluorescence intensity (MFI) in the nucleus–background]/[MFI in the cytoplasm–background]. Quantitative analysis of MFI was performed with the Image J program.

FACS Ca^{2+} measurements

A total of 10^7 J.mtRFP cells were suspended in 2 ml RPMI containing 1% FCS, 0.125% pluronic acid, 4 μg/ml Fluo-4 and 10 μg/ml Fura-Red (Molecular Probes). After 30 min at 37°C, cells were washed and resuspended in HBSS containing 1% FCS, and kept at room temperature protected from light. In some experiments, after loading with Fluo-4 and Fura-Red, J.mtRFP were incubated with anti-CD11a plus anti-CD18 neutralizing mAbs and/or 50 μM ruthenium 360, at room temperature for 45 min. In some experiments, J.mtRFP were first incubated with 1 μg/ml pertussis toxin at 37°C for 4 h, then loaded with Fluo-4 and Fura-Red and treated or not with anti-CD11a plus anti-CD18 neutralizing mAbs, at room temperature for 30 min.

For each experiment, 2×10^6 labelled T cells were spun down with unpulsed B cells to produce conjugates. Cells were resuspended by vortexing, warmed up to 37°C and immediately analysed. Fluo-4 positive cells exhibiting a forward scatter corresponding to T cell–APC duplexes were gated and Ca^{2+} ratio (Fluo-4/Fura-Red) was measured over time using an FACSCanto (BD Biosciences) and analysed with Flow-Jo Software.

FACS measurements of CD25 expression

Primary resting hCD4$^+$ T cells, treated or not with 50 μM ruthenium 360 at room temperature for 45 min, were plated at a 4:1 ratio with L cells stably expressing (or not) the human molecules ICAM-1, MHC class II or MHC class II plus ICAM-1, in the presence or not of SAgs. After 18 h, cells were collected and stained with commercial anti-hCD4 and anti-hCD25 mAbs, and CD25 expression was measured using an FACSCanto (BD Biosciences) and analysed with Diva Software.

Statistical analysis

All our experiments on mitochondria localization were statistically analysed using the program XLSTAT 2009. As our variables are not continuous, but categorical (each cell can be either polarized or not), the results are expressed as a proportion and thus the appropriate statistical analysis was performed through the statistical test for proportions comparison (Bewick *et al*, 2004). Proportions were calculated on cumulative frequencies for each condition. Statistical analysis was performed using χ^2 test. For multiple comparisons (more than two conditions), we applied the Marascuilo Procedure (NIST/SEMATECH e-Handbook of Statistical Methods, http://www.itl.nist.gov/div898/handbook/prc/section4/prc474.htm, accessed 24 August 2009) when the χ^2 test indicated that at least two proportions were significantly different. *P*-values are indicated in each figure.

All other experiments were statistically analysed using the program GraphPad Prism 4, applying, where required, two-way ANOVA, Mann–Whitney test or Student's *t*-test.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

Acknowledgements

We thank Dr Gottfried Baier, Dr Yvette van Kooyk, Dr Loretta Tuosto and Dr Donald L Gill for providing reagents and Dr Achille

Anselmo, Dr Chiara Buracchi and Mr Andrea Doni for assistance. We are grateful to Dr Francisco Sanchez-Madrid, Dr Giovanna Capizzi, Dr Emanuela Morenghi and Dr Thomas Hoefer for helpful discussions and Dr Marinos Kallikourdis, Dr Adelaida Sarukhan, Dr Santos Manes and Dr Tullio Pozzan for critical reading of the paper. The research leading to these results has received funding from EMBO Young Investigator Programme, the European Community's Seventh Framework Programme (FP7/2007-2013) under Grant agreement no. 201106 and E-Rare 2007. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the paper.

Author's contributions: RLC, SC and AV designed the study. RLC, SC and AET performed and analysed immunofluorescence experiments. EM and FA performed time-lapse experiments. RLC performed calcium experiments and the statistical analysis of all data, and wrote the paper. AV wrote the manuscript and provided funds. All authors contributed to critical revision of the paper.

Conflict of interest

The authors declare that they have no conflict of interest.

References

Arimura N, Kaibuchi K (2005) Key regulators in neuronal polarity. *Neuron* **48:** 881–884

Barber DF, Faure M, Long EO (2004) LFA-1 contributes an early signal for NK cell cytotoxicity. *J Immunol* **173:** 3653–3659

Barnard AL, Igakura T, Tanaka Y, Taylor GP, Bangham CR (2005) Engagement of specific T-cell surface molecules regulates cytoskeletal polarization in HTLV-1-infected lymphocytes. *Blood* **106:** 988–995

Barreiro O, de la Fuente H, Mittelbrunn M, Sanchez-Madrid F (2007) Functional insights on the polarized redistribution of leukocyte integrins and their ligands during leukocyte migration and immune interactions. *Immunolog Rev* **218:** 147–164

Bewick V, Cheek L, Ball J (2004) Statistics review 8: qualitative data—tests of association. *Crit Care (London, England)* **8:** 46–53

Bilder D (2004) Epithelial polarity and proliferation control: links from the Drosophila neoplastic tumor suppressors. *Genes Dev* **18:** 1909–1925

Binnerts ME, van Kooyk Y, Simmons DL, Figdor CG (1994) Distinct binding of T lymphocytes to ICAM-1, -2 or -3 upon activation of LFA-1. *Eur J Immunol* **24:** 2155–2160

Blanchard N, Di Bartolo V, Hivroz C (2002) In the immune synapse, ZAP-70 controls T cell polarization and recruitment of signaling proteins but not formation of the synaptic pattern. *Immunity* **17:** 389–399

Bleijs DA, de Waal-Malefyt R, Figdor CG, van Kooyk Y (1999) Co-stimulation of T cells results in distinct IL-10 and TNF-alpha cytokine profiles dependent on binding to ICAM-1, ICAM-2 or ICAM-3. *Eur J Immunol* **29:** 2248–2258

Boldogh IR, Pon LA (2007) Mitochondria on the move. *Trends Cell Biol* **17:** 502–510

Campello S, Lacalle RA, Bettella M, Manes S, Scorrano L, Viola A (2006) Orchestration of lymphocyte chemotaxis by mitochondrial dynamics. *J Exp Med* **203:** 2879–2886

Cantley LC (2002) The phosphoinositide 3-kinase pathway. *Science (New York, NY)* **296:** 1655–1657

Conche C, Boulla G, Trautmann A, Randriamampita C (2009) T cell adhesion primes antigen receptor-induced calcium responses through a transient rise in adenosine 3′,5′-cyclic monophosphate. *Immunity* **30:** 33–43

Constantin G, Majeed M, Giagulli C, Piccio L, Kim JY, Butcher EC, Laudanna C (2000) Chemokines trigger immediate beta2 integrin affinity and mobility changes: differential regulation and roles in lymphocyte arrest under flow. *Immunity* **13:** 759–769

Demaurex N, Poburko D, Frieden M (2009) Regulation of plasma membrane calcium fluxes by mitochondria. *Biochim Biophys Acta* **1787:** 1383–1394

Doucey MA, Legler DF, Faroudi M, Boucheron N, Baumgaertner P, Naeher D, Cebecauer M, Hudrisier D, Ruegg C, Palmer E, Valitutti S, Bron C, Luescher IF (2003) The beta1 and beta3 integrins promote T cell receptor-mediated cytotoxic T lymphocyte activation. *J Biol Chem* **278:** 26983–26991

El Din El Homasany BS, Volkov Y, Takahashi M, Ono Y, Keryer G, Delouvee A, Looby E, Long A, Kelleher D (2005) The scaffolding protein CG-NAP/AKAP450 is a critical integrating component of the LFA-1-induced signaling complex in migratory T cells. *J Immunol* **175:** 7811–7818

Friedman RS, Jacobelli J, Krummel MF (2006) Surface-bound chemokines capture and prime T cells for synapse formation. *Nat Immunol* **7:** 1101–1108

Gallo EM, Cante-Barrett K, Crabtree GR (2006) Lymphocyte calcium signaling from membrane to nucleus. *Nat Immunol* **7:** 25–32

Goldman DW, Chang FH, Gifford LA, Goetzl EJ, Bourne HR (1985) Pertussis toxin inhibition of chemotactic factor-induced calcium mobilization and function in human polymorphonuclear leukocytes. *J Exp Med* **162:** 145–156

Graf B, Bushnell T, Miller J (2007) LFA-1-mediated T cell costimulation through increased localization of TCR/class II complexes to the central supramolecular activation cluster and exclusion of CD45 from the immunological synapse. *J Immunol* **179:** 1616–1624

Hanke JH, Gardner JP, Dow RL, Changelian PS, Brissette WH, Weringer EJ, Pollok BA, Connelly PA (1996) Discovery of a novel, potent, and Src family-selective tyrosine kinase inhibitor. Study of Lck- and FynT-dependent T cell activation. *J Biol Chem* **271:** 695–701

Henrickson SE, Mempel TR, Mazo IB, Liu B, Artyomov MN, Zheng H, Peixoto A, Flynn MP, Senman B, Junt T, Wong HC, Chakraborty AK, von Andrian UH (2008) T cell sensing of antigen dose governs interactive behavior with dendritic cells and sets a threshold for T cell activation. *Nat Immunol* **9:** 282–291

Hoth M, Fanger CM, Lewis RS (1997) Mitochondrial regulation of store-operated calcium signaling in T lymphocytes. *J Cell Biol* **137:** 633–648

Jain J, McCaffrey PG, Miner Z, Kerppola TK, Lambert JN, Verdine GL, Curran T, Rao A (1993) The T-cell transcription factor NFATp is a substrate for calcineurin and interacts with Fos and Jun. *Nature* **365:** 352–355

Katagiri K, Hattori M, Minato N, Irie S, Takatsu K, Kinashi T (2000) Rap1 is a potent activation signal for leukocyte function-associated antigen 1 distinct from protein kinase C and phosphatidylinositol-3-OH kinase. *Mol Cell Biol* **20:** 1956–1969

Kinashi T (2005) Intracellular signalling controlling integrin activation in lymphocytes. *Nat Rev Immunol* **5:** 546–559

Kirichok Y, Krapivinsky G, Clapham DE (2004) The mitochondrial calcium uniporter is a highly selective ion channel. *Nature* **427:** 360–364

Kuhn JR, Poenie M (2002) Dynamic polarization of the microtubule cytoskeleton during CTL-mediated killing. *Immunity* **16:** 111–121

Kupfer A, Swain SL, Singer SJ (1987) The specific direct interaction of helper T cells and antigen-presenting B cells. II. Reorientation of the microtubule organizing center and reorganization of the membrane-associated cytoskeleton inside the bound helper T cells. *J Exp Med* **165:** 1565–1580

Lawrie AM, Rizzuto R, Pozzan T, Simpson AW (1996) A role for calcium influx in the regulation of mitochondrial calcium in endothelial cells. *J Biol Chem* **271:** 10753–10759

Li Z, Okamoto K, Hayashi Y, Sheng M (2004) The importance of dendritic mitochondria in the morphogenesis and plasticity of spines and synapses. *Cell* **119:** 873–887

Lioudyno MI, Kozak JA, Penna A, Safrina O, Zhang SL, Sen D, Roos J, Stauderman KA, Cahalan MD (2008) Orai1 and STIM1 move to the immunological synapse and are up-regulated during T cell activation. *Proc Natl Acad Sci USA* **105:** 2011–2016

Liu D, Bryceson YT, Meckel T, Vasiliver-Shamis G, Dustin ML, Long EO (2009) Integrin-dependent organization and bidirectional vesicular traffic at cytotoxic immune synapses. *Immunity* **31:** 99–109

Liu H, Radisky DC, Wang F, Bissell MJ (2004) Polarity and proliferation are controlled by distinct signaling pathways downstream of PI3-kinase in breast epithelial tumor cells. *J Cell Biol* **164:** 603–612

Martin-Cofreces NB, Robles-Valero J, Cabrero JR, Mittelbrunn M, Gordon-Alonso M, Sung CH, Alarcon B, Vazquez J, Sanchez-Madrid F (2008) MTOC translocation modulates IS formation and controls sustained T cell signaling. *J Cell Biol* **182:** 951–962

Molon B, Gri G, Bettella M, Gomez-Mouton C, Lanzavecchia A, Martinez AC, Manes S, Viola A (2005) T cell costimulation by chemokine receptors. *Nat Immunol* **6:** 465–471

Montoya MC, Sancho D, Bonello G, Collette Y, Langlet C, He HT, Aparicio P, Alcover A, Olive D, Sanchez-Madrid F (2002) Role of ICAM-3 in the initial interaction of T lymphocytes and APCs. *Nat Immunol* **3:** 159–168

Prakriya M, Lewis RS (2001) Potentiation and inhibition of Ca(2+) release-activated Ca(2+) channels by 2-aminoethyldiphenyl borate (2-APB) occurs independently of IP(3) receptors. *J Physiol* **536:** 3–19

Quintana A, Kummerow C, Junker C, Becherer U, Hoth M (2009) Morphological changes of T cells following formation of the immunological synapse modulate intracellular calcium signals. *Cell Calcium* **45:** 109–122

Quintana A, Schwindling C, Wenning AS, Becherer U, Rettig J, Schwarz EC, Hoth M (2007) T cell activation requires mitochondrial translocation to the immunological synapse. *Proc Natl Acad Sci USA* **104:** 14418–14423

Randriamampita C, Boulla G, Revy P, Lemaitre F, Trautmann A (2003) T cell adhesion lowers the threshold for antigen detection. *Eur J Immunol* **33:** 1215–1223

Revy P, Sospedra M, Barbour B, Trautmann A (2001) Functional antigen-independent synapses formed between T cells and dendritic cells. *Nat Immunol* **2:** 925–931

Rizzuto R, Pinton P, Carrington W, Fay FS, Fogarty KE, Lifshitz LM, Tuft RA, Pozzan T (1998) Close contacts with the endoplasmic reticulum as determinants of mitochondrial Ca2+ responses. *Science (New York, NY)* **280:** 1763–1766

Sanchez-Madrid F, del Pozo M (1999) Leukocyte polarization in cell migration and immune interactions. *EMBO J* **18:** 501–511

Sanchez-Martin L, Sanchez-Sanchez N, Gutierrez-Lopez MD, Rojo AI, Vicente-Manzanares M, Perez-Alvarez MJ, Sanchez-Mateos P, Bustelo XR, Cuadrado A, Sanchez-Madrid F, Rodriguez-Fernandez JL, Cabanas C (2004) Signaling through the leukocyte integrin LFA-1 in T cells induces a transient activation of Rac-1 that is regulated by Vav and PI3K/Akt-1. *J Biol Chem* **279:** 16194–16205

Schwindling C, Quintana A, Krause E, Hoth M (2010) Mitochondria positioning controls local calcium influx in T cells. *J Immunol* **184:** 184–190

Shamri R, Grabovsky V, Gauguet JM, Feigelson S, Manevich E, Kolanus W, Robinson MK, Staunton DE, von Andrian UH, Alon R (2005) Lymphocyte arrest requires instantaneous induction of an extended LFA-1 conformation mediated by endothelium-bound chemokines. *Nat Immunol* **6:** 497–506

Shi SH, Jan LY, Jan YN (2003) Hippocampal neuronal polarity specified by spatially localized mPar3/mPar6 and PI 3-kinase activity. *Cell* **112:** 63–75

Sims TN, Dustin ML (2002) The immunological synapse: integrins take the stage. *Immunol Rev* **186:** 100–117

Skokos D, Shakhar G, Varma R, Waite JC, Cameron TO, Lindquist RL, Schwickert T, Nussenzweig MC, Dustin ML (2007) Peptide-MHC potency governs dynamic interactions between T cells and dendritic cells in lymph nodes. *Nat Immunol* **8:** 835–844

Streuli CH (2009) Integrins and cell-fate determination. *J Cell Sci* **122:** 171–177

Suzuki J, Yamasaki S, Wu J, Koretzky GA, Saito T (2007) The actin cloud induced by LFA-1-mediated outside-in signals lowers the threshold for T-cell activation. *Blood* **109:** 168–175

Van Seventer GA, Bonvini E, Yamada H, Conti A, Stringfellow S, June CH, Shaw S (1992) Costimulation of T cell receptor/CD3-mediated activation of resting human CD4+ T cells by leukocyte function-associated antigen-1 ligand intercellular cell adhesion molecule-1 involves prolonged inositol phospholipid hydrolysis and sustained increase of intracellular Ca2+ levels. *J Immunol* **149:** 3872–3880

Viola A, Gupta N (2007) Tether and trap: regulation of membrane-raft dynamics by actin-binding proteins. *Nat Rev Immunol* **7:** 889–896

Volkov Y, Long A, McGrath S, Ni Eidhin D, Kelleher D (2001) Crucial importance of PKC-beta(I) in LFA-1-mediated locomotion of activated T cells. *Nat Immunol* **2:** 508–514

Zhou X, Li J (2000) Macrophage-enriched myristoylated alanine-rich C kinase substrate and its phosphorylation is required for the phorbol ester-stimulated diffusion of beta 2 integrin molecules. *J Biol Chem* **275:** 20217–20222