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**T cell polarization and the formation of immunological synapses: from antigen recognition to virus spread**

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## **Abstract**

Soon after antigen recognition, T lymphocytes polarize towards antigen presenting cells (APC) and form immunological synapses. The formation of immunological synapses is a complex process that involves T cell signaling, as well as membrane, cytoskeleton and vesicular trafficking events. Immunological synapses are thought to be multitasking molecular arrangements that structure in time and space the complex communication between T lymphocytes and APCs. Lymphotropic viruses, such the human immunodeficiency virus (HIV), or herpes virus saimiri can impede the formation and function of immunological synapses hence downregulating the capacity of response of infected T lymphocytes. Moreover, retroviruses, like HIV-1, or the human T cell leukemia virus type 1 (HTLV-1), can subvert the mechanism of T cell polarization and immune synapse formation to form organized cell junctions through which these viruses can spread from cell to cell. By analogy to immunological synapses, these viral-induced cell-cell junctions have been called virological synapses. The understanding of the mechanism of generation of immunological synapses *versus* virological synapses is a fascinating field of study that we will discuss in this review.

## **Introduction**

Although resting circulating T lymphocytes may appear as rather spherical cells, they rapidly polarized in response to chemokines on the surface of endothelial cells and remain polarized during the process of transendothelium extravasation, further migration through lymphoid organs and antigen recognition on APCs. Cell polarization is characterized by an asymmetrical cell morphology and differential localization of cellular molecules and organelles between the two poles of the cell. Polarization of T lymphocytes during their different functional stages is a highly dynamic process that varies in nature and depends on distinct extracellular stimuli. Thus, T cells migrating in response to chemokines present a lamellipodium-like structure at the leading edge and a protrusion called uropod at the back. Close to the base of the uropod, it is located the microtubule organizing center (MTOC) and adjacent cellular organelles like the Golgi apparatus or the endosomal compartment. A variety of intracellular proteins are found asymmetrically distributed between the leading edge and the uropod. Upon antigen recognition, the T cell reorients the MTOC, the Golgi apparatus and the endosomal compartment towards the contact site, and a number of surface receptors, cytoskeletal and signaling molecules concentrate at the contact zone (reviewed in [1]).

As in other specialized cells, polarization of T lymphocytes is conditioned by distinct extracellular stimuli that are translated *via* regulatory molecules into cytoskeletal modifications that determine an asymmetrical intracellular organization. Polarization will in turn condition the functional status of a given cell. This review will focus on the mechanism of T lymphocyte polarization induced by antigenic stimuli, which is translated into a polarized molecular reorganization termed the immunological synapse. We will also discuss how viruses that infect T lymphocytes may interfere positively or negatively with this T cell polarization process in order to modulate the T cell response to antigen or facilitate virus transmission from cell to cell.

### **Antigen recognition induces T cell polarization towards the APC contact site.**

Seminal work from the eighties provided the first evidences of asymmetrical molecular distribution in T lymphocytes encountering antigen-bearing B lymphocytes or target cells. Thus, the microtubule organizing center (MTOC) was found oriented to the APC contact site and T cell antigen receptor (TCR), CD4 or CD8 co-receptors and adhesion molecules, like the integrin LFA-1, accumulated at the junction between T cells and antigen presenting cells. Filamentous actin and proteins interacting with the actin cytoskeleton, such as talin, were also

found concentrated at the cell-cell junction [2-7]. Together with the MTOC, the Golgi apparatus in the T lymphocyte was redirected to the APC. T cell helper cytokines and cytotoxic granules appeared to be secreted towards the APC and target cell respectively [8-10]. All this suggested that upon antigen recognition the site of contact between T lymphocytes and APCs becomes a specialized zone necessary for helper or cytotoxic T cell functions.

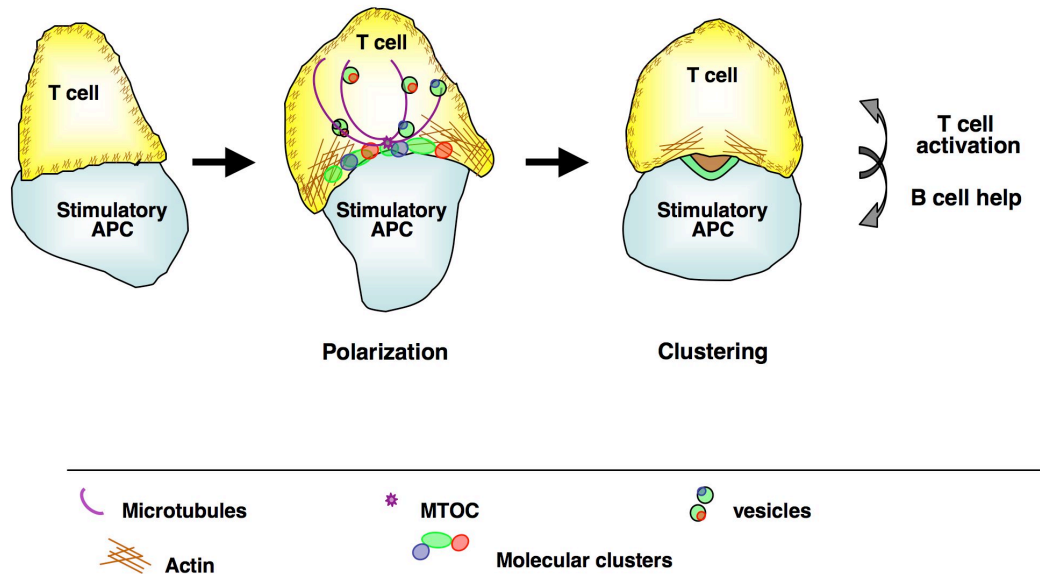
More recently, the development of microscopy imaging methods and the possibility to carry out three-dimensional representation of the T cell-APC junctions allowed a much refined spatial localization of a variety of molecules that were observed not only accumulated, but also segregated in central and peripheral supramolecular clusters that occupied distinct zones of the T cell-APC interface [11]. This gave rise to the new concept of immunological synapse as being a dynamic T cell-APC junction in which a variety of molecules from the T cell and the APC concentrate and reorganize themselves according to a certain spatial distribution [12]. The plethora of molecules that were observed to cluster in the immune synapse suggest that this supramolecular organization may help the T cell receptor to achieve its exquisite sensitivity to trigger and tune up T cell activation in response to a wide range of antigenic peptides. In addition, immune synapse organization may facilitate T cell effector functions by providing the context for polarized secretion of cytokines or cytotoxic granules (see reviews [13-18] (Fig 1).

However, the organization of the immunological synapse in central and peripheral supramolecular clusters [11,12] is not unique, and other types of spatial arrangements may take place. Depending on the type of cells involved and their state of activation (helper or cytotoxic T lymphocytes, thymocytes, NK cells and different types of APCs), the fine structure of immunological synapses may be different [19-22]. How the different types of molecules concentrate at the immune synapse, how they cluster and segregate, and what are the specific roles of molecular clusters in further T cell responses are today the subject of debate and active investigation.

### ***Polarization of the microtubule cytoskeleton***

The MTOC in T cells migrating in response to chemokines is located at the back of the cell, between the nucleus and the uropod [23]. Initial contact with APCs through the leading edge of T cells triggers early signaling (i. e.  $\text{Ca}^{2+}$  flux) [24,25] and the relocation of the MTOC towards the APC contact site in both helper and cytotoxic T lymphocytes [7]. In

cytotoxic T cells encountering specific target cells, the MTOC was seen to localize very close to the contact site and microtubules appeared to project and anchor on LFA-1-enriched areas at the periphery of the immune synapse [26-28].



**Figure 1**

**Figure 1. Antigen-induced T cell polarization and formation of the immunological synapse.** Upon antigen recognition on APCs, T cells polarize their microtubule and actin cytoskeleton, as well as vesicle traffic (Golgi, recycling endosomes and cytotoxic granules) towards the APC contact site. Clusters of receptors, adhesion molecules and signaling effectors generate in this dynamic cell junction that has been named the immunological synapse.

MTOC reorientation to the APC contact depends on TCR-mediated signaling. Functional ITAMs in the T cell receptor complex, Lck, Fyn and ZAP-70 protein tyrosine kinases activities, the LAT, SLP-76 and CD2AP adaptors, and the serine/threonine kinase ERK2 were shown to be necessary for MTOC reorientation [29-34]. Moreover, Rho and Rac GTPases also modulate microtubule dynamics [35,36], reviewed in [23,37]). The exact signaling pathway that links TCR signaling with MTOC reorientation is still unknown. Extrapolating from other cellular systems such as migrating astrocytes, it may be hypothesized that TCR would activate Cdc42 *via* Lck, ZAP-70, SLP-76 and the guanine nucleotide exchange factor Vav1. Activated Cdc42 could then recruit a signaling complex including Par6 and PKC $\zeta$ , which in turn would regulate the activity of the motor protein dynein. This could then modulate microtubule dynamics [38-40].

The regulation of microtubule dynamics triggered by T cell activation and leading to MTOC polarization is a complex process that implicates the shortening of microtubules facing the contact site and subsequent anchoring of the MTOC to the APC contact zone. Anchoring of microtubules to the immunological synapse seems to take place in the LFA-1-enriched peripheral area [27]. The signaling scaffold molecule ADAP, which connects TCR signaling with LFA-1 clustering, may play a role in MTOC polarization to the synapse. The recruitment of dynein to ADAP molecules previously clustered at the periphery of the synapse might provide the tension on microtubules, causing them to slide along the cortex approaching the MTOC to the synapse [41].

The way TCR signaling regulates microtubule dynamics during MTOC polarization is poorly defined. Microtubule dynamics may be modulated by post-translational modifications of tubulin and microtubule-associated proteins (MAPs). Increased tyrosine phosphorylation of  $\alpha$ -tubulin, a form of tubulin mainly found in the unpolymerized soluble fraction, was observed in response to TCR activation [42,43] and could modulate microtubule dynamics. Moreover, serine phosphorylation of some MAPs may modulate their affinity for microtubules and control microtubule stability. CD2 and CD28 stimulation phosphorylates Op18/stathmin, a cytosolic protein that interacts with tubulin dimers and controls microtubule depolymerization in a phosphorylation-dependent manner [44,45]. Microtubule motors may also modify microtubule reorientation [40], but how they may be modulated by T cell activation is at present unknown.

Moreover, acetylation of  $\alpha$ -tubulin plays a role in the stability of microtubules. Acetylated microtubules are enriched at the contact site between T cells and APCs. TCR engagement induces a transient deacetylation of microtubules at early times and enhanced acetylation at late times. Histone deacetylase 6 (HDAC6) is involved in tubulin acetylation changes during T cell polarization and immune synapse formation. HDAC6 translocates close to the T cell-APC contact area, and its over-expression impairs MTOC polarization and perturbs TCR and LFA-1 clustering at the synapse. This indicates that microtubule dynamics play a key role in MTOC polarization and immune synapse stabilization [46].

Finally, actin cytoskeleton dynamics may be involved in MTOC polarization. Thus, small GTPases, like Cdc42, or Rac1, known to modulate in various ways actin cytoskeleton dynamics (i. e. via WASP activation) were shown to control MTOC polarization in T lymphocytes, as well as in other cellular systems [35,36,47]. Moreover, actin nucleating

formins, such as diaphanous-1 (DIA1), or formin-like-1 (FMNL1), control antigen-mediated centrosome polarization [36].

Which might be the function of microtubule cytoskeleton polarization in antigen-induced T cell functions ? In contrast to the actin cytoskeleton, the integrity of microtubules is not essential for TCR-mediated signaling. However, microtubules may be necessary to optimize T cell activation, since a number of signaling molecules were found associated to the MTOC and relocate with it to the APC contact site. Moreover, the dynamics of microcluster formed by receptors and signaling molecules at the immunological synapse depend on microtubule integrity (see below). Finally, MTOC reorientation plays a critical role in polarized vesicular trafficking to the APC contact site. This concerns the Golgi apparatus, as well as recycling endosomes and secretory lysosomes, and is crucial for the delivery of cytokines to B lymphocytes by helper T lymphocytes, for the translocation of receptors and signaling molecules to the immune synapse, and for the targeting of cytotoxic granules to target cells (see below).

### ***Polarization of the actin cytoskeleton***

Antigen recognition and subsequent T cell activation involve a series of events that depend on membrane and actin cytoskeleton dynamics, namely cell motility, cell adhesion, polarization and receptor relocation. Initial cell contacts are very dynamic and involve transient relocation of adhesion molecules like ICAM-3 to the edges of small lamella that contact the APC [48]. If antigenic stimuli are present on the APC, T cells trigger intracellular signaling (i. e. intracellular calcium increase) and profoundly but transiently change their shape increasing the surface of contact with the APC. This leads to a partial engulfment of the APC [24,49,50], or to spreading on artificial activatory planar surfaces [12,51,52]. Ezrin and other actin-binding proteins, like coronin, concentrate at the T cell membrane protrusions that engulf the APC [49,50,53,54]. In addition, some molecules of the TCR signaling cascade, such as Vav and NEMO, also concentrate in those membrane protrusions [55,56]. A few minutes later, the lymphocyte rounds up and the surface of contact with the APC retracts. F-actin and the associated protein talin remain concentrated in the peripheral area of the T cell-APC contact [11,12]. Likewise, ezrin remains concentrated in the contact site, although not necessarily colocalizing with F-actin [50,57].

Interestingly, the increase and further retraction of the surface of contact between T lymphocytes and activatory APCs also takes place when planar lipid bilayers displaying



MHC/Ag and ICAM-1 adhesion molecules were used as surrogate APCs [12]. This indicates that the mechanisms that govern membrane extension and retraction at the site of contact with the APC are controlled within the T lymphocyte and may have a physiological meaning for the activated T cell. Worthnoting, T cell-APC contact retraction coincides with the coalescence and segregation of microclusters at the immune synapse [12,58], suggesting that these two processes may be related.

The dynamics of T cell-APC interactions depend on the nature of the APC (i. e. dendritic cell or B lymphocyte). These differences were observed in cells in culture as well as in lymphoid organs. Moreover, the end result in terms of membrane reorganization at the T cell-APC contact site, which is reflected by cluster segregation, is also distinct in T cell-dendritic cell than in T cell-B cell immune synapses [19,22,59,60]. It is likely that molecular interactions on the cell surface deliver distinct signals to the actin cytoskeleton on both the T cell and the APC, which then condition T cell motility, T cell-APC interactions and molecular clustering at the contact site.

The signaling pathways that connect T cell membrane receptors with the actin cytoskeleton have been extensively studied and overlap in part with, but also differ from, signaling pathways that control microtubule reorientation [37]. For instance, LFA-1 and CD8 engagement can induce actin cytoskeleton rearrangements without inducing MTOC reorientation, whereas TCR engagement leads to both actin cytoskeleton and MTOC reorientation involving protein tyrosine kinases like Lck, Itk and ZAP-70 and signaling adaptors, such as LAT, SLP-76 and Fyp/SLAP [30,32,49,51,61,62]. The tyrosine kinase Itk appears to play a key role in TCR-mediated actin polymerization at the immune synapse, by controlling the relocation of the guanine nucleotide exchange factor Vav-1 to the APC contact site [63-65]. Activation of Cdc42, likely by Vav-1, was observed at the immunological synapse and its spatial distribution in the cell could be associated with actin dynamics and TCR clustering [66].

Regulation of actin cytoskeleton dynamics by Cdc42 likely occurs through the binding and subsequent activation of the Wiskott-Aldrich-Syndrome protein (WASP) recruited at the synapse. WASP then regulates actin dynamics by interacting with the Arp2/3 complex, a set of proteins that control *de novo* nucleation and branching of actin filaments [67] and are involved in the formation of the F-actin rich lamellipodium that contact the APC [36]. Interestingly, recruitment of WASP to the immune synapse and WASP activation are two different events, the former requires the proline-rich region of WASP, whereas the latter

depends on the interaction with GTP-bound Cdc42, which in turn depends on Vav-1[68]. Recruitment of WASP to the synapse needs Nck and phosphorylated LAT and SLP-76 [69,70]. It has been proposed that the formation of a trimolecular complex between SLP-76, Nck and Vav-1 could bring together WASP with activated Cdc42 leading to WASP activation [69]. Interestingly, interaction of Nck with the TCR-CD3 complex [71] and SLP-76 recruitment to TCR signaling assemblies and microcluster structures at the immune synapse are among the first events observed after TCR engagement [58,72,73]. Therefore, Nck and SLP-76 may locally trigger WASP recruitment and activation, which in turn will locally increase actin dynamics *via* Arp2/3 necessary for the continuous formation of signaling clusters, membrane dynamics and cell spreading [12,36,51,74].

In addition, CD2 engagement may also recruit WASP to the immune synapse through the adaptors PST-PIP1 and CD2AP [33,75]. Therefore, WASP may act downstream of TCR and CD2 to promote actin polymerization at the T cell-APC contact site and immune synapse formation [63,69,75]. However, studies carried out on WASP-deficient cells from WAS patients, or from gene inactivated mice, did not always revealed a clear role of WASP on actin polarization and immune synapse formation [76,77], although defects on TCR or GM-1 clustering, cell shape and F-actin distribution in WASP-deficient cells were reported [78-80]. Moreover, the regulatory WASP-interacting protein (WIP), is also important for actin polymerization, cell spreading and transcription factor activation [81-83]. Therefore, WASP through its multiple molecular interactions appears to play key roles in T cell actin cytoskeleton dynamics, immune synapse formation and T cell activation.

Numerous other proteins interacting with the actin cytoskeleton were implicated in the regulation of actin dynamics downstream of the TCR or co-stimulatory receptors, and in the stabilization of the immune synapse. For instance, the membrane microfilament linkers ezrin and moesin undergo rapid and transient dephosphorylation on a threonine residue that modulates their conformation and ability to bind membrane and cytoskeletal partners [84,85]. This is thought to modulate cortical actin dynamics and molecular reorganization at the immune synapse [49,86-89]. Furthermore, the actin depolymerizing protein cofilin [90,91], the spectrin-ankyrin skeleton [92], the WAVE2 complex [93], filamin A [94,95], etc., also regulate cytoskeleton remodeling and immunological synapse formation downstream of TCR signaling. Some of these actin cytoskeleton-associated molecules may help the spatio-temporal organization of the different components of the T cell signaling machinery, thus integrating extracellular and intracellular signals in the immunological synapse [54,96].

Interestingly, different regulators of actin nucleation, namely the Arp2/3 complex and formins, differentially regulate cytoskeletal polarization in antigen-stimulated T cells. Whereas Arp2/3 appear fundamental for the formation of T cell lamellipodia at the APC contact site but does not influence MTOC polarization, formins are required for MTOC polarization, but dispensable for lamellipodia formation. Moreover, in cells in which Arp2 or Arp3 expression had been silenced, TCR stimulation leads to the formation of long F-actin-rich filopodia [36].

Dynamin-2, a member of the dynamin family of large GTPases, is ubiquitously expressed and regulates membrane vesiculation and endocytosis, as well as actin dynamics and cell motility, in various cellular systems. Dynamin-2 makes a functional link between the endocytic machinery and the actin cytoskeleton (reviewed in [97]). In T lymphocytes, dynamin-2 was shown to regulate actin polymerization at the immunological synapse and be necessary for T cell activation [98]. Dynamin-2 interacts with a variety of molecular partners including signaling molecules, such as Lck, Nck, GADS, CrkL, Vav-1, Grb2, as well as with regulators of actin dynamics, like cortactin or Abp1, or with endocytic adaptors like intersectin [97,98]. The cortactin homologue HCLS1 (or HS1), expressed in T cells, is phosphorylated in response to TCR stimulation, accumulates at the immunological synapse and is required for actin dynamics downstream of TCR signaling leading to IL2 gene activation. Interestingly, HS1 interacts with Vav-1 and is necessary for Vav-1 recruitment to the synapse [99]. In contrast to its role on the actin cytoskeleton, a role of dynamin-2 in endocytic events thought to occur at the immunological synapse has not been established yet. Yet, an inhibitory effect of a dominant negative mutant of dynamin-1 on anti-CD3-induced TCR down-regulation has been observed [100].

Some of these actin cytoskeleton-binding proteins are at the cross-roads between actin dynamics and membrane trafficking. For instance, dynamins interact with the endocytic adaptor intersectin [97]. Interestingly, intersectin-2 in T cells was shown to interact with WASP and to regulate TCR endocytosis [101]. Moreover, CD2AP, first described as linking the CD2 surface molecule to the actin cytoskeleton [33], plays a role in TCR down-modulation by regulating TCR degradation [102]. Finally, the F-actin binding protein mAbp1 (HIP-55), able to interact with dynamin 2, is also recruited to the immunological synapse and modulates TCR endocytic dynamics [103].

Therefore a complex network of signaling adaptors and regulators, connect TCR and co-receptor signaling with actin cytoskeleton dynamics. In turn, an increasing number of

actin-binding proteins appear necessary for the formation of immunological synapses and T cell activation. Interestingly, some key molecules, such as the GEF Vav-1 or the adaptor Nck, the actin regulator WASP, or the large GTPase dynamin 2 appear as knots in this network, being able to have multiple molecular interactions that regulate actin dynamics, immune synapse formation and TCR down-modulation.

### ***Polarization of intracellular vesicle trafficking***

Together with the microtubule cytoskeleton, intracellular vesicle trafficking polarizes towards the immunological synapse. Thus the Golgi apparatus of the T cell is reoriented towards the APC contact site upon antigen recognition [4]. This is thought to direct the secretion of cytokines to the APC. In this way, antigen-specific B lymphocytes presenting antigen to helper T cells, but not bystander B cells, will directly receive helper cytokines to proliferate and differentiate [8]. Worthnoting, T cells in contact with various APCs displaying distinct stimulatory capacities, make a choice and polarize their secretory machinery towards the APC presenting the stronger stimulus [104]. Moreover, the secretory apparatus in T cells appear to discriminate between helper and pro-inflammatory cytokines, polarizing the former, but not the later, towards the APC [105].

Cytotoxic T lymphocytes secrete cytolytic granules towards target cells in order to specifically kill virus-infected or transformed cells. Cytolytic granules are made of a particular type of lysosomal vesicles called secretory lysosomes [14]. The delivery of cytolytic granules towards the target cell contact is a tightly regulated vesicular traffic mechanism. It involves the microtubule-mediated transport of secretory lysosomes from peripheral areas of the T cell to the MTOC, which localizes very closed to the T cell-target cell contact zone, and the delivery of granules in a zone of the immune synapse situated between the central and the peripheral SMAC [26,28]. Several key proteins, Rab27a, adaptor protein-3 and hMunc13-4 ensure proper vesicular traffic of secretory lysosomes to the target contact site and docking and fusion at the immunological synapse. Mutation of some of these proteins lead to impaired cytotoxic function and are the cause of severe immunodeficiency syndromes [106-110]. Interestingly, granzyme-containing cytotoxic granules, Rab27a and hMunc13-4 are found initially in distinct vesicle populations. Munc13-4 is present in the Rab11 recycling endosomal compartment, its presence being necessary for the fusion of Rab11<sup>+</sup> and Rab27a<sup>+</sup>, and for the fusion of these endosomal vesicle with granzyme cytotoxic granules at the T cell-target cell contact site [111]. Cytotoxic T cells may simultaneously

encounter more than one target cell and, although they polarize the lytic granules towards the target displaying the stronger antigenic stimulus, they can also address some of the granules to other target cells and efficiently kill them [112].

Recycling endosomes of T cells are also rapidly polarized towards the APC contact site upon antigen recognition, and deliver surface receptors like the TCR [113], or CTLA-4 [114], and signaling molecules, such as the protein tyrosine kinase Lck [115], or the adapter molecule LAT [116] to the immunological synapse. Recycling endosomes that transport these various types of molecules may be compartmentalized in a way that the different receptors and signaling molecules do not mix in intracellular vesicular compartments.

Finally, the late endosomal compartment also appear to polarize and come close to the center of immunological synapse, leading to the idea that receptors clustered at the cSMAC may be sorted to the degradative pathway thus driving the exhaustion of early activation signaling of the engaged T lymphocyte [117].

Therefore, the immunological synapse appears to become an active zone for vesicle docking and fusion, where vesicle trafficking and fusion machineries meet in order to deliver effector proteins to the APC or target cells [26,28,105,108,111,113]. Distinct exocytic processes have to be combined with endocytosis of receptors and membrane-bound signaling molecules that, once activated, will be sorted to degradative compartments. It is at present unknown whether the delivery and fusion of vesicles from the various vesicular organelles is regulated by the same molecules and occurs in the same domains of the plasma membrane, or whether specialized sub-domains are generated to accomplish specific functions. Likewise, it is not determined whether exocytic and endocytic processes occur at distinct areas of the immune synapse.

### ***Polarization of signaling molecules***

The signaling machinery involved in antigen-mediated T cell activation also polarizes at the immunological synapse co-localizing in part with TCRs and the co-receptors that trigger this machinery. Dynamic arrays of molecular microclusters were shown to appear at the periphery of the synapse and move towards the center at the time that the T cell undergoes contraction [58,70,73,74,117,118]. Interestingly, the association of several signaling mediators to these microclusters, as well as their state of activation, evolve with time after initial TCR triggering, and correlate with their position in the contact zone. Thus, activated signaling molecules, often characterized by the phosphorylation of particular tyrosine

residues, were more often found within small microclusters at the periphery of the synapse, whereas inactive molecules were more often found in bigger clusters that coalesce close to the central areas of the synapse (cSMAC). Not all the molecules forming peripheral microclusters end up in the same areas. Thus, whereas the TCR $\zeta$  chain coalesces in the cSMAC, some signaling molecules like the tyrosine kinase ZAP-70, or the scaffold molecule SLP-76, disappear from the cell surface towards the cell interior before reaching the central cluster where TCR $\zeta$  accumulates [58,70,119]. It has been therefore proposed that early T cell signaling is generated at the periphery of the immunological synapse and exhausted in the center [117]. Worthnoting, the dynamic spatial pattern of receptors and signaling molecules within the immunological synapse appear to be important for the onset and exhaustion of signaling, since imposing geometric constraints on immunological synapse formation lead to altered TCR signaling [74].

Various modes of transport may bring the different signaling molecules to the immunological synapse. For instance, intracellular vesicles that partially overlap with recycling endosomes seem to transport membrane-associated signaling molecules, such as the tyrosine kinase Lck, or the adaptor LAT, to the synapse [115,116,120]. Other signaling molecules could approach the immunological synapse zone through their ability to bind to the microtubule cytoskeleton. For instance, various signaling molecules, like focal adhesion kinase (FAK), proline rich tyrosine kinase (PYK-2), Fyn, ZAP-70, Vav-1, and protein kinase C  $\beta$ (I) and  $\delta$ , were found associated with tubulin or the MTOC in T lymphocytes [121-125]. PYK-2 in T lymphocytes and NK cells translocates together with the MTOC to the T cell-APC contact site and becomes activated upon NK receptor engagement [122,126]. Paxillin may be the link between the microtubule cytoskeleton and some of the signaling molecules, since it can interact with  $\gamma$ -tubulin and with PYK-2, FAK and Lck [127,128]. The close proximity of the MTOC to the immunological synapse [28] could help the delivery of microtubule-associated signaling molecules to the immunological synapse where they could interact with TCR-initiated signaling complexes.

It is clear that protein and organelle polarization in the immunological synapse requires a coordinated ensemble of events that involves receptor and co-receptor signaling, microtubule and actin cytoskeleton remodeling and plasma membrane and intracellular vesicle dynamics. A challenge for the near future will be to understand how this coordination occurs at the molecular level. Interestingly, some lymphotropic viruses evolved to intersect some of these polarization mechanisms in order to modulate T cell responses and to spread

more efficiently from cell to cell. Understanding how viruses hijack T cell polarization mechanisms may help us, not only to understand the physiopathology of these infectious diseases, but also to gain insight in the complex process of T cell polarization.

### **Modulation of T lymphocyte polarization by lymphotropic viruses**

Viruses have to evade innate and adaptive immune responses to establish infection and disseminate *in vivo*. Concomitantly, viruses hijack the cellular machineries of target cells to replicate and/or persist. Some T lymphocyte lymphotropic viruses, such as retroviruses and some herpesviruses, can modulate intracellular signaling, interfere with intracellular trafficking and modify cytoskeleton organization. Therefore, they can impair the physiological immune synapse and usurp the mechanism of polarization to spread directly from cell to cell.

### ***Infection by lymphotropic viruses results in complex modulation of intracellular signaling, molecular trafficking and cytoskeleton organization***

Lymphotropic viruses are able to modulate intracellular signaling. Most of the times, this modulation results from the combination of several processes. Thus, specific interactions of viral products with different cellular molecules involved in the signaling pathway can lead to their inactivation, degradation and/or to their down-modulation in the case of surface receptors and/or to their retention in an intracellular compartment. Thus, modulation of signaling pathways by virus infection often results from both specific inactivation mechanisms and misrouting of targeted signaling molecules.

Because of its central regulatory role, T-cell receptor signal transduction is a common target of lymphotropic viruses. Herpesviruses and retroviruses have developed different mechanisms to modulate the TCR-induced pathways. For some of these viruses, it has been recently reported an effect of T lymphocyte infection on the formation of immunological synapses. We will mainly focus here on two documented examples, Human Immunodeficiency Virus type 1 (HIV-1) and Herpesvirus Saimiri (HVS), both infecting CD4<sup>+</sup> T lymphocytes.

## ***HIV-1***

As most viruses, HIV-1 reshapes the intracellular environment so as to optimize viral propagation and cell survival [129]. These changes lead to important alterations of T cell biology, and are accompanied by severe CD4+ T lymphocyte dysfunctions that take place during AIDS pathology [130]. HIV-1 infection of T lymphocytes is first characterized by a modulation of T cell activation capacities together with a virus-regulated apoptosis. Finally, a small proportion of T lymphocytes become latently infected quiescent cells through poorly defined mechanisms [131].

HIV-1 infected T cells improperly respond to TCR stimulation. Signaling pathways, transcriptional activation, as well as intracellular protein trafficking, are altered in HIV-1-infected T cells. Several HIV-1-encoded proteins are responsible for these alterations. Among them, Nef and Tat were reported to target T cell activation pathways at different levels [132,133], for a review see [134,135]. The accessory protein Nef, in particular, appears as a multifunctional factor implicated in virus pathogenicity *in vivo* [136], although not absolutely required for viral replication *in vitro* [137]. Nef is able to target both signaling and intracellular protein trafficking pathways through its interaction with intracellular trafficking adaptors and with various signaling effectors [135]. Thus, Nef interacts with various signaling molecules of the TCR signaling cascade, such as Vav1 [138], PKC $\theta$  [139], Erk [140], PAK-2 [141] and several src family kinases [142]. Nef interacts with an array of cellular partners *via* different domains [143]. However, the end result of these multiple Nef actions on T cell physiology may appear contradictory, since Nef inhibits some particular stages of T cell activation, while enhancing and/or potentiating others [144,145], for a review see [135].

Nef induces the down-regulation of various receptors from the cell surface and their concomitant accumulation in endosomal vesicles. For instance, CD4 [146], CD28 [147], MHC I and II [148,149], transferrin receptor (TfR) [150], CCR5 [151] are down-regulated, whereas DC-SIGN is up-regulated [152], and other surface molecules, like LFA-1, ICAM-1, ICAM-2 and the TCR remain unaffected or slightly upregulated [120]. Nef-induced down-regulation of all those surface receptors is unlikely the consequence of a non-specific blockade of T cell endosomal trafficking, since it is controlled by different Nef domains [146-148,150,153]. Moreover, the trafficking of molecules sharing the recycling endosomal pathway, like the TCR and the TfR [113], is differentially affected by Nef. Whereas TfR is down-regulated from the cell surface and strongly accumulated in endosomes [150], TCR are slowed down during their endocytic traffic, at both the internalization and the recycling steps

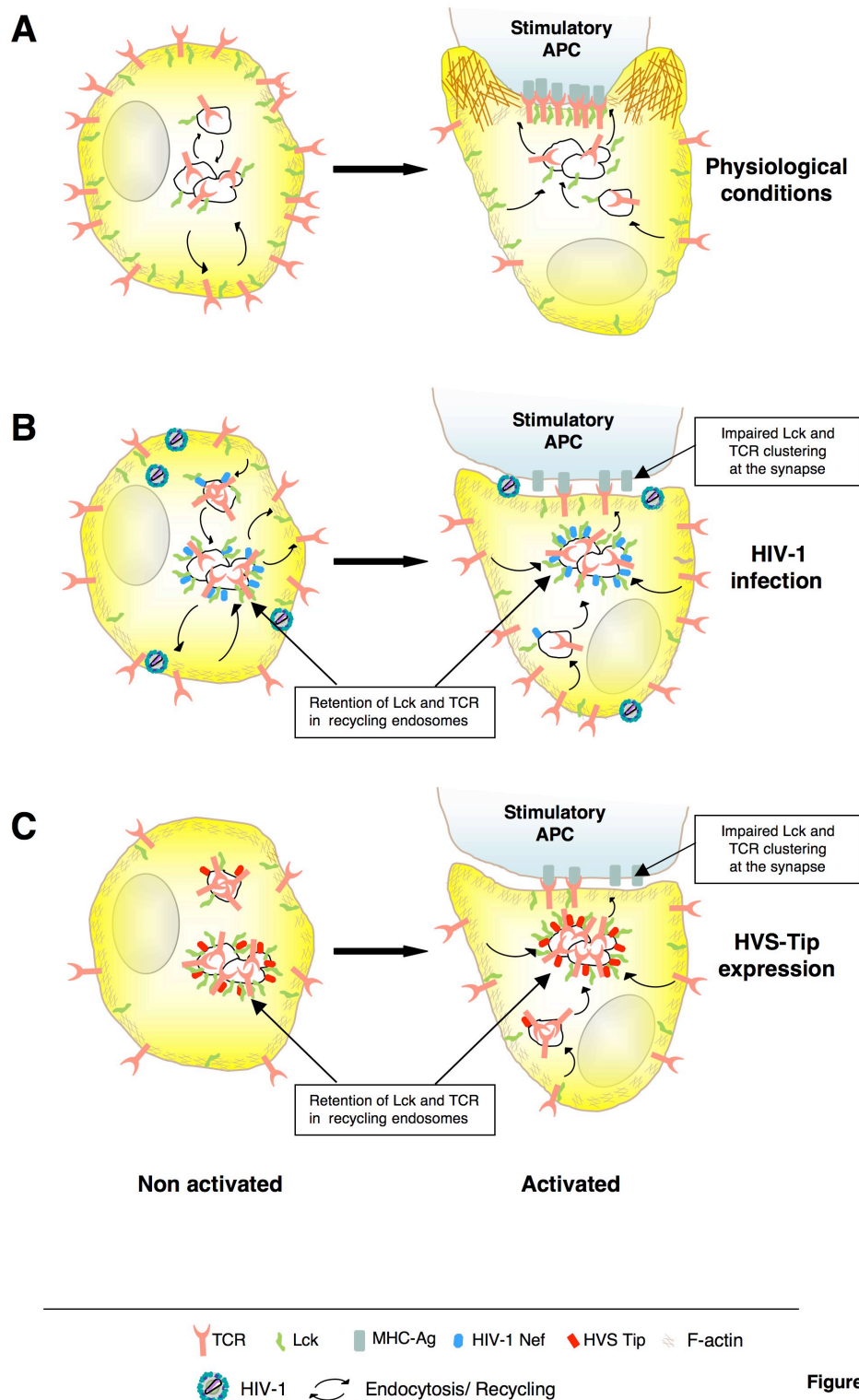


[120]. Therefore, HIV utilizes Nef to finely regulate the intracellular traffic of various key immune receptors, thus inhibiting T cell responses.

HIV-1 infection impairs the capacity of lymphocytes to form immunological synapses. This is due, at least in part, to the effect of HIV-1 infection on the intracellular trafficking of TCR and Lck. Thus, HIV-1-infected T cells, strongly accumulate Lck in the endosomal compartment. Moreover, TCR traffic is slowed down at both the endocytosis and recycling steps. As consequence, both TCR and Lck clustering at the synapse are strongly inhibited. Lck retention and immune synapse impairment are, to a large extent, caused by Nef, whose sole expression is sufficient to retain Lck in the recycling endosomal compartment and to inhibit the targeting of this kinase to the contact zone. Impaired immune synapse formation is concomitant with defective early TCR signaling [120]. Noteworthy, although CD4 can interact with Lck [154], HIV-induced accumulation of Lck in endosomes does not depend on CD4 down-regulation [120]. Interestingly, downstream signaling pathways leading to IL2 production are not inhibited, but rather up-regulated independently of synapse formation [120], suggesting dual effects of HIV infection on various stages of T cell signaling [134] (Fig 2).

In T cells expressing Nef and stimulated with anti-CD3 coated beads, a pool of Nef molecules appears to relocate, together with lipid rafts, to the contact zone with the stimulatory bead [155]. Concomitantly, an increase of late events in TCR signaling, namely activation of NFAT and NF $\kappa$ B transcription factors were observed. It has been hypothesized that a fraction of Nef located in rafts (around 10% of total Nef) could be involved in the subsequent activation of several molecules involved in TCR signaling and leading to IL2 transcription.

Interactions of HIV-1 with the actin cytoskeleton have been documented and Nef is emerging as an important modulator of cytoskeleton dynamics (reviewed in [156,157]. Through its direct interaction with Vav1, a Rac GTPase exchange factor, Nef might induce cytoskeletal rearrangements, morphological changes and further activation of the JNK/SAPK cascade [138]. Several mechanisms of Nef-induced actin cytoskeleton reorganization have been reported, but in cellular contexts unrelated to T cells. Depending on the cellular model, Nef induces F-actin polymerization or depolymerization. Furthermore, Nef, through its interaction with Dock-2-ELMO-1 complex activates Rac and inhibits chemotaxis [158], a process that also requires T cell polarization and actin remodelling. Finally, in T lymphocytes activated on anti-CD3 and anti-CD28-coated surfaces, expression of Nef alone, or viral



**Figure 2**

**Figure 2. Modulation of T lymphocyte polarization by lymphotropic viruses.** Some viruses that infect T lymphocytes, like HIV-1 and HVS, can subvert the mechanism of T cell polarization impeding the formation of the immunological synapse and down-regulating TCR signaling. (A): Under physiological condition T cells polarize their actin cytoskeleton and endosomal trafficking towards the APC contact site. This leads to the formation of clusters of receptors and signaling molecules. (B): In HIV-1 infected cells, the expression of the viral protein Nef, leads to retention of Lck and TCR in recycling endosomes and to impaired clustering and signaling at the synapse [120]. (C): In T lymphocytes infected with HVS, the expression of the viral protein Tip sequesters Lck and TCR in endosomes also preventing the formation of T cell-APC conjugates, clustering at the synapse and TCR signaling [165].

infection, inhibits actin cytoskeleton reorganization. Nef, through its interaction with PAK-2 interferes with actin cytoskeleton remodelling, inhibiting Rac-1 and N-WASP pathways [159]. Placed in a more physiological context, this inhibitory effect of Nef on actin remodelling could also have consequences on immune synapse formation [120,160].

HIV infection may also utilize and affect the microtubule cytoskeleton. Thus microtubules contribute to several steps of HIV-1 virus cycle. Many HIV-1 encoded proteins, including Vpr, Vpu, Tat, integrase and protease are reported to interact with some of microtubule components or to modulate them [161], for a review, see [157]. Because both MTOC and microtubules are strongly involved in immune synapse formation, HIV-1 induced modulation of these components may have important consequences on interactions between HIV-1-infected T lymphocyte and APCs. However, to date, this point has not been documented.

Therefore, HIV-1 has evolved to develop subtle mechanisms to alter the dynamics of various receptors, signaling and cytoskeletal components, impairing immune synapse formation and early T cell signaling, while enhancing late activation events, such as IL2 gene activation and cytokine production. The role of this dual regulatory action of HIV-1 is at present unknown, but it is tempting to speculate that it may be critical to keep a balance between T cell proliferation and apoptosis necessary for virus replication, transmission and persistence.

### ***Herpesvirus Saimiri***

Herpes viruses persists in its host through the ability to establish a latent infection and periodical reactivations that produce infectious virus. Herpesvirus Saimiri (HVS), an oncogenic simian gamma2-herpesvirus, persists in the T lymphocytes of its natural host (squirrel monkey) without any apparent disease. However, in several primate species other than their natural host, HVS may induce T cell lymphoma and leukemia (reviewed in [162,163]). HVS C strains immortalize human T lymphocytes and these transformed T cells retain many characteristics of intact T lymphocytes. Some functions in these immortalized cells are however perturbed, though less profoundly than those of HTLV-1 transformed cells. Thus, HVS infection and immortalization of target T lymphocytes induce an IL2 independent-growth of infected cells.

Some anchored proteins encoded by HVS, such as Stp and Tip, have been reported to play a key role in the ability of this virus to transform T cells *in vitro*. One of these proteins,

Tip (tyrosine kinase interacting protein), is responsible for the oncogenic potential of HVS strain C, it is expressed primarily during viral latency, but it is not required for viral replication [164]. Tip is constitutively present in lipid rafts, interacts with the protein tyrosine kinase Lck through its SH3 domain and sequesters it, together with TCR $\zeta$  and LFA-1 in an intracellular vesicular compartment [165-168]. In addition, Tip interacts with p80, a lysosomal protein that contains a WD repeat domain. P80 also interacts with Lck, being necessary for Lck intracellular sequestration, sorting to lysosomes and degradation [169]. In addition, Tip expression down-regulates TCR and CD4 surface expression. Interestingly, whereas Tip-mediated TCR down-regulation depends on Tip's interaction with p80, CD4 down-regulation depends on the interaction of Tip with Lck [166,168]. Likely as a consequence of these effects, Tip interferes with the TCR signaling cascade, and inhibits the formation of T cell-APC conjugates and immunological synapses [165], for a review see [163].

In apparent contradiction with the ability of Tip to inhibit TCR signaling, TCR signaling-deficient cells, lacking Zap70 kinase, recover CD2- and CD3-mediated activation when transformed by HVS [170]. It is tempting to speculate that HVS evolved in a way to inhibit early events of T cell signaling, at the latency state, while hijacking later TCR signaling events to induce tumoral growth of HVS-infected cells. This may be explained by the expression of other HVS-encoded products, such as ORF5 and ORF14 proteins, which contrary to Tip, are expressed during the viral replication cycle, but not during viral latency, and induce a strong increase in TCR signal transduction. Interestingly, ORF5 protein exhibits structural similarities with the signaling adaptor LAT, it is phosphorylated in response to TCR stimulation and interacts with the cellular SH2-containing signaling molecules Lck, Fyn, SLP-76 and p85. This interactions lead to T cell activation, as assessed by phosphorylation of signaling proteins (CD3 $\zeta$ , PLC $\gamma$ -1), mobilization of intracellular calcium, activation of transcription factors (AP-1, NFAT, and NF- $\kappa$ B), increase of CD69 surface expression and IL2 production [171]. Moreover, the ORF14 protein, a homolog of the mouse mammary tumor virus (MMTV) viral superantigen, is a secreted protein that induces polyclonal proliferation of CD4 T lymphocytes through its interaction with MHC-II molecules [172]. Therefore, ORF5 and ORF14 proteins, by interfering at different stages with TCR signaling, modulate T cell activation. However, their effect on synapse formation has not been documented.

Thus, a fine regulation of TCR signaling may occur at multiple points of HVS viral cycle. This may lead to a balance between increased TCR signaling during the replication

cycle, to facilitate viral production, and inhibition of TCR signaling during latency, to avoid host immune recognition and persistence of the viral reservoir.

Interestingly, two strains of Herpesvirus ateles (HVA), an HVS-related virus of spider monkeys that cause fulminant lymphoma in various new world primates, can transform T cells *in vitro* and *in vivo*. These strains of HVA encode Tio (“two-in-one”), a protein exhibiting similarities to the oncogenic proteins Tip and StpC from HVS strain C, in particular in its ability to interact with the src family kinases Lck, Src and Fyn [173,174]. Based on these properties, it is tempting to speculate that HVA infection and/or Tio expression by itself may impair the formation of immune synapses by a mechanism related to that mediated by HVS, although this is at present not known.

Finally, other lymphotropic herpesviruses, such as human herpes viruses 6 and 7 (HHV6, HHV7) interfere with TCR signaling and with the intracellular trafficking of receptors, such as TCR and CD4, without affecting Lck level [175,176], but the impact of these viruses on immunological synapse formation has not been reported.

In conclusion, impairment of the TCR transduction pathway, in particular Lck function is a common strategy employed by lymphotropic viruses to avoid host immune recognition and T cell activation during latency. By expressing Nef and Tip, respectively, HIV-1 and HVS down-regulate CD4 and Lck from the plasma membrane, accumulating them in endosomal compartments. In contrast, HIV-1 does not down-regulate TCR cell surface expression, whereas HVS does. In this respect, HVS resembles SIV and HIV-2 that down-regulate TCR by means of their respective Nef proteins [177], thereby exhibiting an alternative mechanism to avoid immunological synapse formation and modulate further T cell activation. Worth noting is that the capacity of SIV and HIV-2 Nef proteins to down-regulate TCR correlates with a lower pathogenicity of these viruses [177].

As viruses hijack cellular machineries of T cell activation and trafficking to replicate, they may also usurp some of the mechanisms of polarization that are involved in immune synapse formation. Thus, modulating T cell polarization may be an important strategy employed by viruses to escape host immune control and to transmit directly from cell to cell.

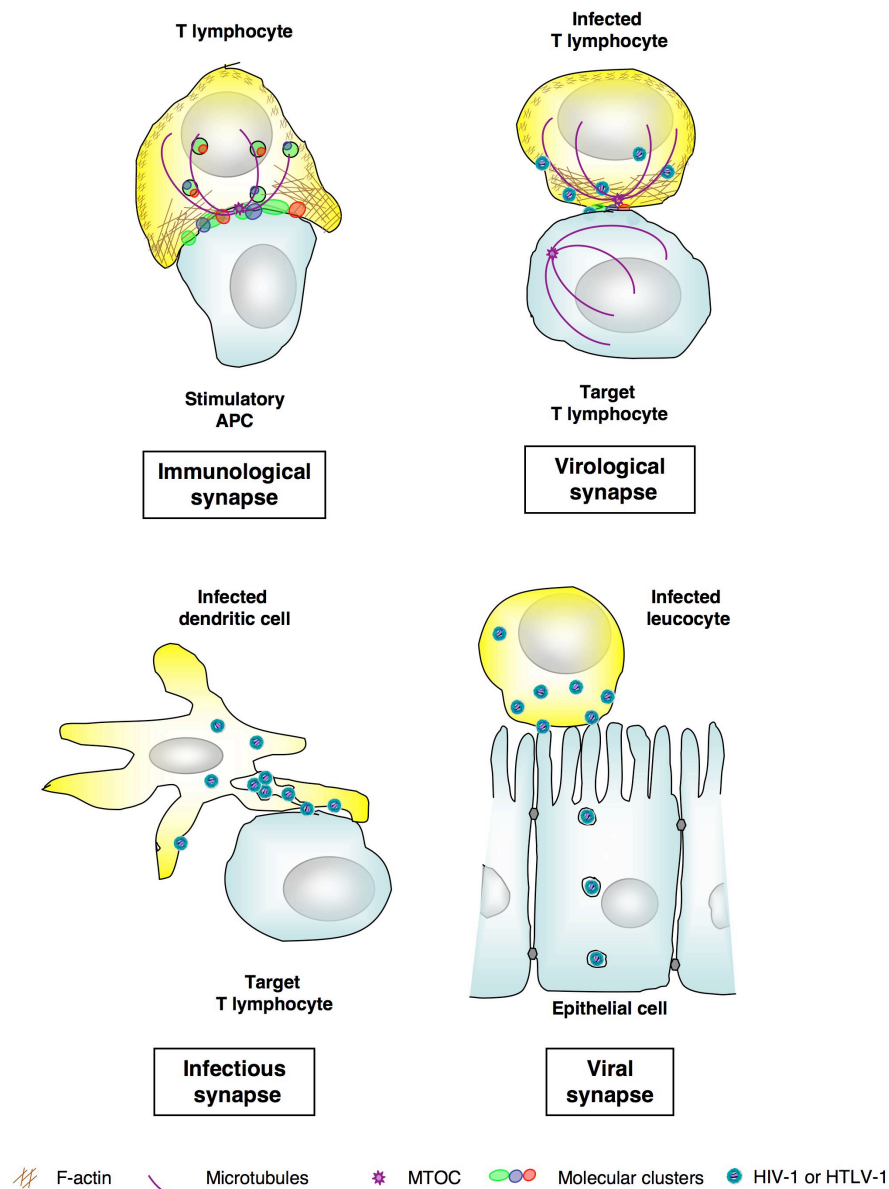
**Using the T cell polarization machinery to propagate from cell to cell. The generation of virological synapses by human lymphotropic retroviruses.**

It has long been established that viral infection begins with the entry of free virions into a target, non-infected, cell. This classical view has been changed by the observation that some viruses take advantage of the contact between cells to be transmitted without the need to release free virions into the extracellular milieu. In the latter case, virus evolved to condition the infected cell to direct virus egress towards cell-cell contacts [178]. Direct cell-to-cell transmission presents clear advantages since it obviates rate-limiting steps of the virus cycle by placing viruses in close proximity with their cell surface receptors, and by creating a confined space in which viruses can pass from cell to cell being protected from the immune system. In the case of lymphotropic retroviruses, such as the human immunodeficiency virus type 1 (HIV-1), or the human T cell leukemia virus type 1 (HTLV-1), the contacts generated during this cell-to-cell virus transmission share some molecular and organelle patterns with immunological synapses and thus, this contact has been termed as “virological synapse” [179-182]. It is important to note here that virological synapse is a general term that has been used by different authors to define organized cell contacts through which viruses can be transmitted from cell to cell. However, virological synapses can be formed between different cell types and can be induced by distinct viruses. Therefore, their molecular organization and mechanisms of generation are most likely distinct and are still poorly understood (Fig 3).

Transmission through cell-cell contacts has been described to take place during infection by HIV-1 [180,183-187] and HTLV-1 [179,188,189] reviewed in [181,182,190]. This mode of transmission is critical for HTLV-1 spread, since HTLV-1 infection by free virions is extremely inefficient [188,190,191]. Moreover, although HIV-1 can be propagated as free virions, cell-cell contacts strongly enhance virus replication [178,192-194]. In lymphoid organs, where immune cells concentrate and undergo multiple interactions to trigger and maintain immune responses, HTLV and HIV may easily spread from cell-to-cell. It is therefore not surprising that these retroviruses evolved to take advantage of physiological cell contacts to efficiently propagate among immune cells.

The generation of virological synapses is conditioned by the expression of various viral proteins within the infected cell. These proteins modulate the capacity of the infected cell to polarize in response to a contact with a non-infected cell, and facilitate on the target cell the clustering of viral receptors and adhesion molecules. Intracellular signaling is likely translated into the cytoskeletal rearrangements necessary for organelle polarization and molecular clustering in both the infected and the target cell. In the next paragraphs, we discuss the intracellular events that condition the formation of virological synapses between HTLV-1 and

HIV-1 infected cells and their targets, comparing them, when possible, with events involved in the formation of immunological synapses.



**Figure 3**

**Figure 3. Lymphotropic retroviruses usurp the mechanism of T cell polarization to propagate from cell-to-cell via virological synapses.** Infection of T lymphocytes by HIV-1 or HTLV-1 facilitate the polarization of the infected cell, the polarized egress of viruses and their transmission to a target cell. The contact sites between an HTLV-1 or HIV-1-infected T cell and a target T cell present similarities in molecular organization to immunological synapses and they were therefore called virological synapses [179,180]. HIV-1-infected dendritic cells accumulate HIV-1 in an intracellular vesicular compartment that is redirected to the contact site with a T lymphocyte forming an immune synapse with the dendritic cell. These were called infectious synapses [187,211]. Finally, HIV-1-infected mononuclear cells can form the so-called viral synapses with polarized epithelial cells, transmitting the virus to the epithelial cell and inducing virus transcytosis.

### ***Cell-cell contact, polarization and formation of virological synapses***

Similarly to immunological synapses, during HTLV-1 or HIV-1 spread *via* virological synapses the formation of stable cellular contacts were observed, where adhesion and cytoskeletal proteins clustered together with virus receptors. Both infected and target cells undergo reorganization of their contact sites in a way to stabilize cell-cell junctions and to facilitate the interaction between the virus particles and their receptors. Viral RNA and proteins accumulate in the contact zone and appeared to be transferred to the target cell through these organized junctions [179-182].

In HTLV-1-infected T cells, the MTOC, the core proteins Gag p19 and p15, the Env glycoprotein, the transactivator Tax and the integrin-microfilament linker protein talin polarize towards the cell-cell junction. Moreover, the HTLV-1 receptor GLUT-1 and Env-interacting protein neuropilin-1 concentrate at the target cell side of the contact [179,195,196]. The stability of cell-cell interaction in HTLV-1 virological synapses is likely the result of a process involving adhesive interactions up-regulated by intracellular signalling and the actin cytoskeleton, and facilitated by the expression of viral proteins, like Tax and p12/1 [179,197,198]. Moreover, Env glycoprotein expressed on the surface of HTLV-1-infected cells could interact with Glut-1 [199], neuropilin [196] and cell surface heparan sulphate proteoglycans [200-203] on target cells contributing to increase cell-cell adhesion and promoting virus cell-cell spread.

Polarization of infected cells during the formation of HTLV-1-induced virological synapses appears to be triggered by the conjunction of two signals, one induced by the interaction of the adhesion molecule ICAM-1 on the surface of the infected cell with its counter receptor the integrin LFA-1 on the surface of the target cell, and a second signal provided by the expression of the viral protein Tax [195,197]. In addition to its properties as transcriptional transactivator, the protein Tax seems to synergize with ICAM-1-mediated signalling in order to induce MTOC polarization. In addition to its main localization in the cell nucleus, Tax is also localized in the pericentriolar region and polarized together with the MTOC towards the stimulatory cell contact [195]. ICAM-1 and Tax mediated polarization signals depend on the integrity of microtubules and the activity of the GTPases Rac1 and Cdc42 [195]. Interestingly, Cdc42 and/or Rac1 are also involved in the control of MTOC polarization induced during the formation of immunological synapses [35,36], suggesting that both polarization pathways may converge on these regulatory molecules. However, it is at present unknown whether signals that connect ICAM-1 with these GTPases overlap with



those of the TCR. Interestingly, ICAM-1 ligation of HTLV-1-infected cells enhances the expression of viral genes, suggesting the existence of a positive feedback loop between cell-cell adhesion and HTLV-1 gene expression [195,204]. Therefore, a still not fully defined ensemble of cooperative interactions between viral proteins and cellular signaling pathways appear to regulate T cell polarization and adhesion events that generate HTLV-1 virological synapses.

Since HTLV-1 transmission is strictly cell contact-dependent [188-190], and HTLV-1 may infect other cell types, such as B cells, monocytes [205,206], dendritic cells [207], endothelial cells [208], epithelial cells [209,210], etc, it is possible that organized virological synapses also form between these other cells and chronically infected T lymphocytes. However, the characteristics of these other putative synapses are still poorly defined, but different cell types may utilize distinctly organized synapses. Moreover, the various surface molecules able to bind HTLV-1 Env glycoprotein, like Glut-1, heparan sulphate proteoglycans and neuropilin may be differently expressed on target cells and could differently promote HTLV-1 transmission between different cell types [196,199-203].

In the case of HIV-1, virus spread through virological synapses was shown to occur among T cells [180], between dendritic cells and T lymphocytes [187,211], between macrophages and T cells [184] and between macrophages and epithelial cells [185,212]. Formation of stable cellular contacts is likely to be different depending on the cell types involved, since the natural adhesive interactions between these cells are different. Moreover, the HIV receptors involved in the target cells may not be the same. Thus, CD4 and CCR5/CXCR4 are preferential receptors in T cells and macrophages, whereas epithelial cells appear to use Galactosyl ceramide and agrin as attachment receptors [185].

In virological synapses formed between HIV-1-infected and non-infected T cells, HIV receptors CD4 and CXCR4, talin, LFA-1 and F-actin concentrate at the target cell side, while related adhesion molecules and the viral proteins Env and Gag concentrate at the infected cell side. Molecular clustering at the virological synapse depends on actin and myosin dynamics. Env engagement to its receptors is necessary for this molecular reorganization, since blocking Env interaction to CD4 and CXCR4 reduces receptor polarization [180]. However, Env interaction with its counter-receptors may not be sufficient to drive virological synapse assembly, and it is likely that, similarly to immunological synapses, interactions between other surface molecules (i. e. LFA-1-ICAM-1) may provide the necessary adhesive forces and signalling to stabilize the cell junction [180]. One plausible hypothesis is that intracellular

signalling triggered by Env, together with ICAM-1, on the infected cell, and their counter receptors, on the target cell, induces polarized rearrangements of the actin cytoskeleton, which in turn promote molecular clustering and reinforced adhesion at the virological synapse. Indeed, electron microscopy analysis of virological synapses revealed areas of tight contact between both cell membranes, that resemble those observed in immunological synapses [22,26,180]. Some signalling and cytoskeletal events involved in HIV-1 virological synapses may be similar to those of immunological synapses. For instance, the protein tyrosine kinase ZAP-70, which controls MTOC polarization in immunological synapses [29], is necessary for HIV-1 Gag polarization to virological synapses and for HIV-1 transmission and efficient replication [194]. However, other key events observed in immunological synapses, such as TCR clustering, are absent in virological synapses, indicating that different signalling cascades may be involved.

In synapses formed between HIV-1-infected dendritic cells and noninfected T cells, a fast relocation of HIV virions to the cell contact was observed [187]. At the same time, the HIV receptors CD4 and coreceptors CCR5 and CXCR4, and the adhesion molecule LFA-1, cluster at the cell-cell contact site [187], reviewed in [181]. In infected dendritic cells, HIV-1 is stored in a tetraspanin-rich endosomal compartment, and targeted to the synapse *via* redirected trafficking of these endosomes [211]. This is reminiscent of the intracellular vesicular trafficking of MHC-II molecules, which occurs during the formation of the immunological synapse [213]. It is however a distinct vesicular pathway, since HIV-1-infected dendritic cells encountering T lymphocytes do not concentrate MHC-II molecules [211].

Finally, still a different type of virological synapse forms between HIV-1-infected peripheral blood mononuclear cells and epithelial cells, involving integrin interactions with the extracellular matrix and galactosyl ceramide and the heparan sulfate proteoglycan agrin as HIV-1 attachment receptors to the epithelial cells. This type of cell-cell contact induces HIV-1 transcytosis through the epithelial cells [185].

### ***Polarized budding and/or egress of lymphotropic retroviruses at virological synapses***

Virological synapses are cell contact sites where polarization and membrane trafficking events are orchestrated facilitating directed virus egress and transfer from cell to cell. Viral particles may bud directly at virological synapses, or they may bud at a different intracellular compartment and then exit the cell at the synapse. Importantly, the sites of budding may

depend on the retrovirus (HIV-1 or HTLV-1) and on the type of infected cell (i. e. T lymphocytes, macrophages, dendritic cells, etc).

The budding of HIV-1 and HTLV-1 is generally associated with consensus protein motifs, located at the virus late assembly domain (L domain) that interact with cellular proteins and lead to virus budding (reviewed in [214,215]). The L domain is encoded by the Gag polyprotein precursor and contains PPXY (X means any amino acid), PTAP, or LYPX(n)L motifs, that interact with cellular components of the endocytic-sorting machinery, like the ubiquitin ligase Nedd4, Tsg101, AP-2, or AIP1/Alix [216-224]. These cellular proteins are part, or interact with, the ESCRT complexes (endosomal sorting complex required for transport). ESCRT I, II and III complexes are involved in the sorting of ubiquitinated proteins into late endosomal compartments known as multivesicular bodies (MVBs) reviewed in [225]. Retrovirus Gag polyproteins can be ubiquitinated, which may promote their interaction with the ESCRT machinery [222,226]. Moreover, Gag can also interact, through the amino-terminal part of the matrix region, with the endocytic adaptor complex AP-3. This interaction is also necessary for targeting Gag to MVBs and for particle formation [227]. Therefore, retroviruses may follow this common cellular protein sorting pathway to be targeted to MVBs (reviewed in [214,215,228]. In addition of the interaction of Gag with intracellular sorting machinery, a tyrosine-dependent targeting signal located at the cytosolic domain of the HIV Env glycoprotein also appears to play a role in the control of polarized viral budding in polarized epithelial cells, as well as in lymphocytes [229-232]. Interestingly, this tyrosine polarization signal favours propagation of viral infection under conditions where virus transmission by cell-to-cell contact is thought to be predominant [232].

The MVB compartment is particularly developed in professional antigen presenting cells, such as dendritic cells or macrophages, where it plays an important role in the processing of extracellular protein antigens and in peptide antigen loading to MHC-II molecules. MVBs are enriched in several proteins of the tetraspanin family, like CD63, CD81, CD82, MHC-II molecules, some actin-binding proteins, some lipids like cholesterol and GM3, etc. [233,234] reviewed in [225]. Interestingly, MVBs can fuse with the plasma membrane releasing their vesicular content into the extracellular milieu. These vesicles have been called exosomes and may be involved in antigen presentation and stimulation to other immune cells [235,236].

HIV-1 was shown to bud into MVB structures in macrophages and to incorporate MVB proteins and lipids into virions [237,238]. This is likely due to the ability of Gag L domains to interact with components of ESCRT complexes. Virions then exit infected cells at a pole of the cell coinciding with sites of exosome exocytosis [237,239]. In T lymphocytes, the intracellular MVB compartment is not prominent and HIV-1 budding appears to take place at the plasma membrane. Thus, HIV-1 budding may take place at focal patches that display clusters of tetraspanins and late endosomal marker proteins. At these sites, HIV-1 Gag protein and exosome-like vesicles bud from the plasma membrane to the extracellular milieu [212,240-245]. Therefore, HIV budding, at the plasma membrane or into MVBs, could involve similar cellular mediators.

Contrary to HIV-1 whose budding can be readily observed by electron microscopy, the budding of HTLV-1 is an event much more difficult to observe. Therefore, the sites of HTLV-1 budding are less well defined. In chronically infected T lymphocytes, some viral particles budding from the plasma membrane were observed [246,247]. In fibroblast cells, HTLV-1 Gag protein was shown to interact with the ubiquitin ligase Nedd4.1, and with Tsg101, likely in late endosomes-MVBs [222]. Therefore, the molecular mechanism involved in HTLV-1 budding may be similar to that of HIV-1, involving ESCRT complexes either at the plasma membrane or in intracellular compartments.

Moreover, ERK-2-dependent phosphorylation of HIV-1 or HTLV-1 Gag protein was observed and shown to be necessary for efficient release of newly formed virus particles [248,249]. However, the exact mechanism by which phosphorylation modulates viral budding has not been completely elucidated.

Retroviruses may utilize proteins and lipids able to form particular membrane microdomains to bud and/or to be delivered to virological synapses. Thus, cholesterol and sphingolipid-enriched membrane microdomains, or membrane rafts, may be preferential sites for virus budding or release. HIV-1 virus particles contain GM1, a lipid enriched in membrane rafts. Moreover, in HIV-1 virological synapses, GM1, Env and Gag polarize together at the cell-cell interface. Depletion of cholesterol, which perturbs the integrity of membrane rafts, eliminates HIV-1 Gag clustering at cell contacts and the assembly of virological synapses [240,245].

Tetraspanin-enriched microdomains may also be preferential sites for HIV-1 budding, either at the plasma membrane of T cells, or in the MBV compartment of macrophages [237,241,244]. Moreover, HIV-1 particles are enriched in tetraspanins [237,238].

Interestingly, different tetraspanin-rich intracellular compartments are involved in the HIV-1 live cycle in different cell types. Thus, in dendritic cells, internalized HIV-1 accumulates in an endosomal compartment that is enriched in the tetraspanins CD9, CD81 and CD82, but contains little CD63 and the lysosomal protein LAMP-1 [211]. In contrast, in macrophages, the intracellular vesicular compartment where HIV-1 buds and concentrates is enriched in CD63, CD81, CD82 and LAMP-1 [237]. Worthnoting is that these two distinct tetraspanin compartments play different roles in the HIV-1 live cycle. While in macrophages the tetraspanin-rich compartment is a budding compartment for HIV-1, in dendritic cells, the tetraspanin rich compartment stores internalized virus and redirects them to virological synapses. Finally, tetraspanin seem also to play a role in HTLV-1 assembly. Thus, HTLV-1 Gag interacts with CD82 and therefore associates with tetraspanin microdomains [250,251].

In addition to viral particle budding, tetraspanins were also implicated in HIV-1 entry (reviewed in [252]. Silencing CD9 or CD81 with siRNA on the target cell enhances Env-mediated cell-to-cell membrane fusion. It is possible that decreasing either CD9 or CD81 expression could render CD4 molecules more available to interact with the HIV co-receptors, CXCR4 or CCR5, thus promoting the assembly of the complexes necessary for HIV virus entry [253]. In addition, tetraspanins may also modulate viral transcription as suggested by the observation that CD81 engagement increases HIV gene expression, possibly due to an increased translocation of transcription factors that positively regulate virus transcription, i.e. NF- $\kappa$ B, NFAT and AP-1 [254]. Moreover, CD82 interacts with HTLV-1 Env glycoprotein and influences its fusion capacity. It may be hypothesized that Env interaction with CD81/CD82 tetraspanins inside the infected cell may prevent Env to fuse with cellular membranes before budding and release of viral particles [255].

Virological synapses orchestrate cell polarization and virus egress, although the connexion between both processes is largely unknown. Microtubules and the actin-myosin cytoskeleton have been involved in the relocalization of viral proteins at the cell-cell interface and in the generation of virological synapses both during HTLV-1 and HIV-1 infection [179,195,197,212]. Furthermore, drugs or mutations that disrupt either actin or microtubules dynamics affect HIV intracellular transport [157,161,256]. Myosin has also revealed to be necessary for HIV budding [256]. The fact that isolated HIV particles contain proteins from the actin cytoskeleton and from microtubules further supports the role of the cytoskeleton in the polarization of viral proteins [238]. In addition, an actin ring is present at the virological synapse and actin disruption inhibits virus fusion with the membrane of the target cell

[253,257] reviewed in [258]. This suggests that the cytoskeleton besides participating on the polarization of viral proteins towards the synapse is also important for the virus entry in the target cell.

The connection between viral particles and the cytoskeleton is poorly known. Proteins interacting with viral proteins and the cytoskeleton may be good candidates to make this link. For instance, the targeting of HIV-1 virions to tetraspanin microdomains [211,237] may also promote its interaction with the cytoskeleton, since tetraspanins interact with the membrane and with microfilaments-associated proteins [259,260]. Moreover, HIV particles co-localize with microtubules [157] and HIV Gag has also been found to co-localize with actin in T cells and macrophages [157]. Furthermore, several proteins associated to microtubules, the actin cytoskeleton, or intermediate filaments co-purified with HIV-1 particles produced by infected macrophages [238], suggesting a tight relationship between HIV-1 particles and the cytoskeleton.

## **Conclusion**

A highly regulated process of T lymphocyte polarization towards antigen presenting cells takes place upon antigen recognition. T cell receptor, and a number of co-receptors, adhesion molecules and signaling and cytoskeletal components are engaged in this process ensuring the exquisite sensitivity of T lymphocytes to respond to foreign antigens. Lymphotropic viruses have evolved to subvert these mechanisms of polarization in order to down-regulate the responses of infected cells, and to propagate from cell to cell. In the case of HIV-1 an impairment of generation of immunological synapses by infected cells is concomitant with an enhanced ability of these cells to form virological synapses. One of the near future challenges will be to understand how these retroviruses balance these two polarization mechanisms to increase their chances to replicate and propagate in the infected organism. This will help us to understand the physiopathology of these viral infections and may provide insight into the mechanism of T cell polarization and immunological synapse formation.

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### **Figure legends:**

**Figure 1. Antigen-induced T cell polarization and formation of the immunological synapse.** Upon antigen recognition on APCs, T cells polarize their microtubule and actin cytoskeleton, as well as vesicle traffic (Golgi, recycling endosomes and cytotoxic granules) towards the APC contact site. Clusters of receptors, adhesion molecules and signaling effectors generate in this dynamic cell junction that has been named the immunological synapse.

**Figure 2. Modulation of T lymphocyte polarization by lymphotropic viruses.** Some viruses that infect T lymphocytes, like HIV-1 and HVS, can subvert the mechanism of T cell polarization impeding the formation of the immunological synapse and down-regulating TCR signaling. (A): Under physiological condition T cells polarize their actin cytoskeleton and endosomal trafficking towards the APC contact site. This leads to the formation of clusters of receptors and signaling molecules. (B): In HIV-1 infected cells, the expression of the viral protein Nef, leads to retention of Lck and TCR in recycling endosomes and to impaired clustering and signaling at the synapse [120]. (C): In T lymphocytes infected with HVS, the expression of the viral protein Tip sequesters Lck and TCR in endosomes also preventing the formation of T cell-APC conjugates, clustering at the synapse and TCR signaling [165].

**Figure 3. Lymphotropic retroviruses usurp the mechanism of T cell polarization to propagate from cell-to-cell via virological synapses.** Infection of T lymphocytes by HIV-1 or HTLV-1 facilitate the polarization of the infected cell, the polarized egress of viruses and their transmission to a target cell. The contact sites between an HTLV-1 or HIV-1-infected T cell and a target T cell present similarities in molecular organization to immunological synapses and they were therefore called virological synapses [179,180]. HIV-1-infected dendritic cells accumulate HIV-1 in an intracellular vesicular compartment that is redirected

to the contact site with a T lymphocyte forming an immune synapse with the dendritic cell. These were called infectious synapses [187,211]. Finally, HIV-1-infected mononuclear cells can form the so-called viral synapses with polarized epithelial cells, transmitting the virus to the epithelial cell and inducing virus transcytosis.

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