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REVIEW

Concerning immune synapses: a spatiotemporal timeline [version 1; referees: 2 approved]

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Abstract

The term "immune synapse" was originally coined to highlight the similarities between the synaptic contacts between neurons in the central nervous system and the cognate, antigen-dependent interactions between T cells and antigen-presenting cells. Here, instead of offering a comprehensive molecular catalogue of molecules involved in the establishment, stabilization, function, and resolution of the immune synapse, we follow a spatiotemporal timeline that begins at the initiation of exploratory contacts between the T cell and the antigen-presenting cell and ends with the termination of the contact. We focus on specific aspects that distinguish synapses established by cytotoxic and T helper cells as well as unresolved issues and controversies regarding the formation of this intercellular structure.

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Introduction

The immune synapse (IS) is a central event in the development of the adaptive immune response that results in the activation of the T cell. The "synapse-like" nature of the intimate contact between the T cell and the antigen-presenting cell (APC) during T cell activation was initially proposed by Norcross in the early 1980s[1], although the term "immunological synapse" first appeared in a review by Paul and Seder in 1994[2]. The specifics of molecular segregation into activation clusters at the T cell:APC interface dates back to the seminal observations of Kupfer's group in 1998[3]. At the same time, Dustin and Shaw conjoined both concepts (the IS as the physical manifestation of T cell activation, and molecular segregation as the functional reflection of the T cell:APC interaction), adding crucial early data on the composition of the activation clusters[4]. The IS can be defined as a stimulus-driven, spatiotemporal segregation of molecules that participate in T cell activation. Segregation requires the establishment of an intimate contact between a T lymphocyte and an APC. The molecular redistribution is antigen dependent, requiring the interaction of an antigen-specific T cell receptor (TCR) with an antigen-loaded major histocompatibility complex (MHC) molecule. The features and outcome of the IS depend on the type of T cell and APC. The interaction of a CD4+ T helper (T_H) cell with an antigen-loaded MHC-II-bearing APC results in the specific recognition of the antigen and the activation of the T cell, i.e. proliferation, cytokine secretion, expression of effector molecules, etc. In the case of CD8+ T (CTL) cells interacting with cells displaying antigen-associated MHC-I, the outcome depends on the pre-exposure of the CTL to the antigen. Naïve CTL encountering specific antigens presented by APCs (e.g. dendritic cells [DCs] expressing antigen associated with class I via cross-presentation) are primed ("armed") to kill target cells and proliferate. Primed CTL also form transient IS with target cells (tumor cells or cells infected by a virus), resulting in specific killing.

The IS displays remarkable similarities with the neuronal synapse (NS), to which it owes its name. For spatial and functional reference, the APC is better compared to the pre-synaptic terminal, and the T cell to the post-synaptic terminal. The presynaptic portal provides the initiating signal, soluble in the NS (neurotransmitters), but membrane bound in the IS (antigen-bearing MHC). Upon ligation of the key receptor in the post-synaptic terminal (neurotransmitter receptors in the NS; TCR and its signaling co-receptor CD3 in the IS), downstream signaling ensues, including calcium mobilization, actin remodeling, and functional activation of the post-synaptic cell[1,5]. However, a unique feature of the IS consists of specific antigenic recognition, which is absent in the central nervous system (CNS). Another difference is the duration of the contact: whereas some NS can last for days, weeks, or even months, IS between CTL and target cells resolve in minutes, whereas between T_H cells and APCs they can last from several hours to two days[6,7]. This feature change implies a different meaning for the concept of plasticity. In the NS, it refers to the modifications to the post-synaptic terminal that involve the consolidation and adaptation of the post-synaptic terminal to the flux of signal stemming from the pre-synaptic portal. In the IS, plasticity follows contact resolution and could be used to describe the functional changes to the T cell caused by the establishment of a productive synapse. These include activation (T_H), activation (naïve CTL) or kill (primed CTL), and functional anergy

or apoptosis, e.g. during thymic selection of naïve T cells. A major manifestation of functional plasticity is the development of immunological memory, i.e. the generation of long-lived T cells primed to respond to a specific antigen that trigger a much faster and more efficient response to repeated exposure to the same antigen.

Overview of the spatiotemporal events of the IS

The study of the IS has focused on the establishment of hierarchical, spatiotemporally segregated events during the contact between the APC and the T cell. These events include the following:

1) Establishment of low-affinity, exploratory contacts between the T cell and the APC

2) Initial, scattered contact of the TCR with the antigen-loaded MHC on the APC, followed by initiation of TCR-dependent signaling pathways upon specific recognition of the MHC-peptide complex. Such activation is "umbrella shaped" (simultaneous activation and amplification of multiple pathways through different sets of effectors) and induces the activation of multiple effectors, including membrane-bound molecules, e.g. integrins, signaling adaptors, cytoskeletal elements, and transcription factors

3) Transactivation of adhesion molecules (integrins) that consolidate the interaction between the T cell and the APC. This step actually begins after initial TCR activation (step 2), but they evolve in parallel

4) Cytoskeleton- and signaling-dependent clustering of adhesion molecules and the TCR/CD3 complex at the contact interface between the T cell and the APC. In most cases, clustering is spatiotemporally segregated, i.e. the TCR/CD3 clusters and the integrin clusters, and their respective sets of adaptors, are separated

5) Signaling- and motor-dependent positioning of the secretory apparatus (including microtubules and microtubule-binding proteins) to the contact interface of the T cell

6) (Primed CTL only, also natural killer [NK] cells) Actin clearance at the center of the contact interface, enabling a tight association of the secretory apparatus with the plasma membrane

7-i) (T_H cells) Stabilization of the contact and transcriptional activation of the T cell, including cytokine production and the expression of activation markers

7-ii) (Naïve CTL) Stabilization of the contact, priming and activation

7-iii) (Primed CTL and NK cells) Degranulation and target cell killing

8) Termination of the contact

From this flowchart, it becomes obvious that a major difference between the IS established by CTL and that established by T_H cells is the overall duration of the process and its immediate repeatability. CTL contacts are quick (to eliminate target cells rapidly), and

CTLs can establish multiple IS with different target cells over short periods of time. Conversely, T_H cells establish prolonged IS and do not form consecutive IS once activated properly.

In the following sections, we will develop emerging concepts pertaining to each of these spatiotemporal events.

Exploratory contacts

Exploratory contacts are mediated by low-affinity interactions between specific ligands and receptors. A major factor is the glycocalyx, which establishes charge-dependent repulsive interactions between the APC and the T cell (reviewed in [8]). Additional contacts are mediated by glycosylation-dependent, low-affinity interactions, e.g. via galectins. For example, galectins bind TCR molecules with low affinity, thus the TCR does not activate[9]. Antigen-loaded MHC molecules successfully compete with galectin to trigger TCR/CD3 activation and subsequent cytoskeletal remodeling and transcriptional activation (see below). Chemokine receptors also participate in the formation and subsequent stabilization of the initial contacts and localize in the IS. Possible functions for chemokine receptors in this subcellular region are likely to involve co-stimulation, cell attraction, enhancement of actin polymerization, etc.[10]. Other exploratory contacts depend on specific protein-protein interactions, e.g. LFA-1 ($\alpha_L\beta_2$) (APC) with ICAM-3 (T cell)[11], and LFA-3 (APC) with CD2 (T cell). LFA-1 interacts with ICAM-3 while in a low-affinity conformation[12]. Likewise, LFA-3 interacts with CD2 with suitable low affinity[13], although the glycocalyces are likely to hinder their interaction sterically[14]. These contacts allow the transient interaction of the TCR with peptide-loaded MHC. If such interaction bears enough affinity, it overcomes the repulsive forces between the glycocalyces; if not, repulsion dominates and the unproductive contact between the mismatched T cell and APC is resolved.

TCR ligation and initial signaling

Successful interaction of the TCR/CD3 complex with peptide-loaded MHC initiates signaling. It is important to point out that very few TCR-MHC interactions are sufficient to trigger T cell activation[15]. Recent reviews have described the current viewpoints on TCR/CD3 signaling[16,17]. Here, we will focus on several aspects of TCR binding and initial signaling that are specific to IS formation and shape the rest of the process.

Productive TCR engagement promotes its immobilization and clustering in the contact area[18]. This is mediated in part by its interaction with the MHC on the APC, which restricts the possible lateral movement of the TCR to the interacting portion of the plasma membrane of the T cell with the APC. However, the TCR/CD3 complex appears more immobile and clustered than predicted by a model of free diffusion in a semi-planar layer[8], suggesting additional mechanisms of immobilization and aggregation. A crucial mechanism is the association of the TCR/CD3 complex with the actin cortex[19,20]. A recent study has shown that ligated TCR/CD3 molecules modify the flow of actin underneath them, indicating binding-dependent interactions between the TCR and cortical actin[21], which are essential for sustained TCR-dependent signaling[22]. Such interaction is not direct but relies on the recruitment of actin-binding adaptors, e.g. Nck[23].

Another important topic is cluster size. There is evidence of small (nanosized) TCR clusters even before their interaction with the MHC. These nanoclusters are continually generated throughout the plasma membrane of the T cell[24] and migrate and coalesce at the center of the contact to form micron-scale structures, termed central Supramolecular Activation Clusters (cSMACs) (Figure 1, top)[25], which concentrate signaling components (reviewed in [26]) as well as molecules involved in co-stimulation, e.g. CD28[27]. The mechanism of coalescence is also unclear, but it also depends on actin and TCR ligation[28]. Possible explanations involve increases in homotypic TCR lateral affinity, actin coalescence that would "drag" the TCR nanoclusters together, or changes to the size/position of the membrane nanoclusters based on alterations to the regional composition of the plasma membrane. The principles of spatiotemporal assembly of such structures remain unclear, mainly because of differences depending on the type of T cell and APC. In general, T cells that bear a higher basal activation state (e.g. leukemic T cells or memory T cells) form large clusters more readily than resting, naïve T cells. In the latter, TCR/CD3 clusters often remain small and sparse along the contact area between the T cell and the APC[29,30]. The difference could pertain to the expression of additional components in activated cells that promote, or facilitate, TCR/CD3 clustering in more pre-activated cells and/or that signals emanating from the TCR/CD3 are more intense in pre-activated cells owing to a higher activation baseline.

Adhesive interactions

TCR-dependent inside-out signals trigger the conformational extension of integrin LFA-1, enabling its interaction with APC-expressed ICAM-1 (reviewed in [31]). This process is similar to the inside-out signaling that activates integrins during extravasation[32], and it results in stable adhesion between the APC and the T cell.

TCR signals that mediate LFA-1 trans-activation go through several adaptor circuits, including Rap1-RapL-RIAM and SLP-76/ADAP/SKAP (Figure 2). Rap1 is a small Ras-like GTPase that is activated by RasGEFs triggered by the TCR, e.g. CalDAG-1. Active Rap1 forms a complex with RapL and RIAM that targets talin to the plasma membrane[33], where it promotes the conformational extension of LFA-1[34]. SLP-76/ADAP/SKAP-55 bind to the TCR effector LAT, triggering their association to RIAM, thereby participating in the delivery of talin to the integrin[35].

Another important molecule for the inside-out activation of LFA-1 via TCR is kindlin-3. Kindlin-3 mutations cause a severe form of immunodeficiency, named Leukocyte Adhesion Deficiency (LAD)-III[36,37]. LAD-III T cells do not migrate properly and activate poorly due to impaired adhesion mediated by LFA-1[38]. There are two possible mechanisms to explain the role of kindlin-3 in LFA-1 transactivation. One mechanism postulates that kindlin-3 triggers inside-out activation of LFA-1 by binding directly to the β chain cytoplasmic domain. The other mechanism suggests that kindlin-3 could facilitate the binding of talin, or its effect on the conformational extension of LFA-1 (reviewed in [39]). Recruitment of kindlin-3 to LFA-1 is likely mediated by its interaction with ADAP, as in platelet integrin $\alpha_{IIB}\beta_3$[40] (Figure 2).
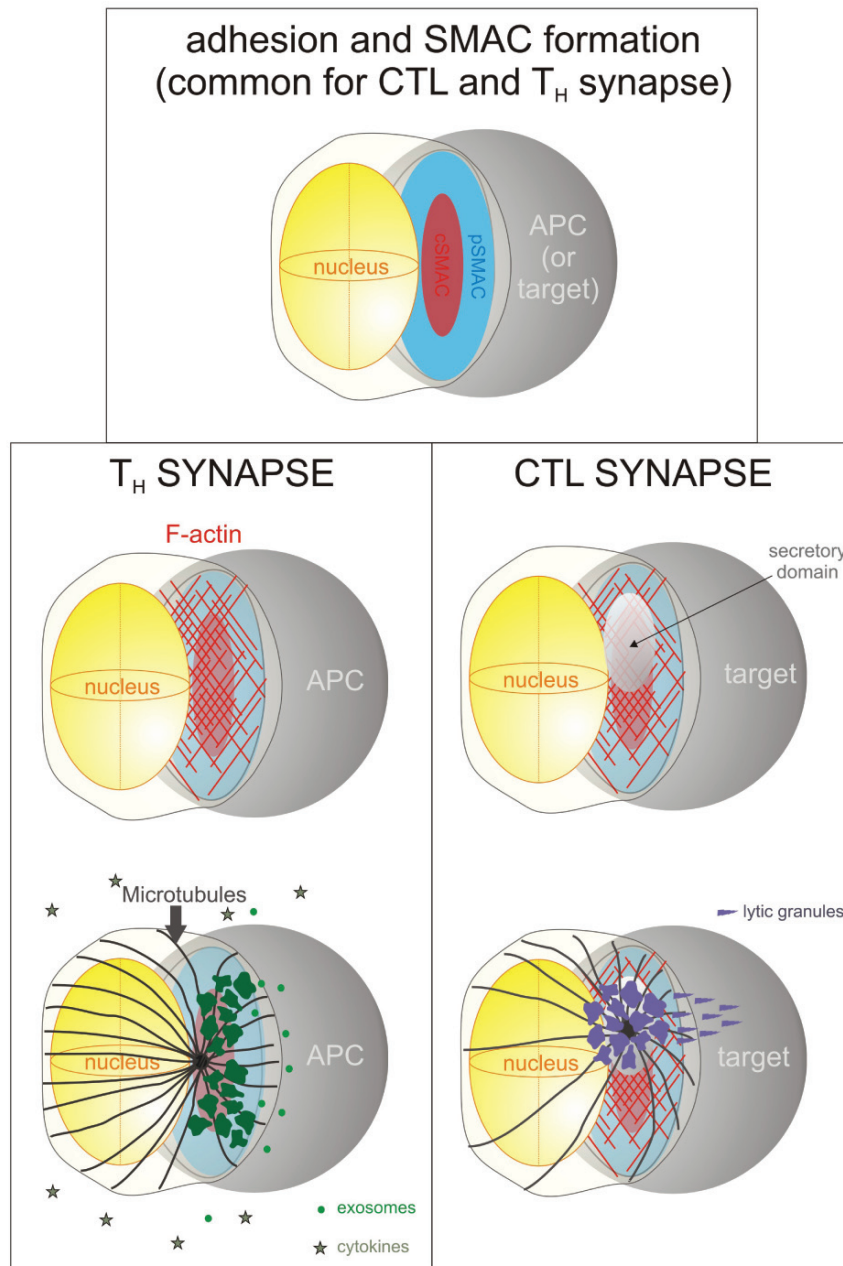
Figure 1. Key events during the formation of the immune synapse. *Top*, diagram represents the adhesion of the T cell (left) to the antigen-presenting cell (APC) (right), and the early formation of discrete domains, central supramolecular activation cluster (cSMAC) (red) containing the T cell receptor (TCR)/CD3 complex and signaling proteins, and the peripheral SMAC (pSMAC) (blue) displaying integrins and their adaptor proteins. *Bottom left column*, events in the T helper (TH) formation of a synapse with a professional APC, including F-actin accumulation (*top*, in red) and juxtaposition of the secretory apparatus (green) and the microtubule-directing centrosome (*bottom*, in black), resulting in the polarized secretion of exosomes (bright-green spheres) and the non-polarized secretion of cytokines (stars). *Bottom right column*, events in the CD8+ T (CTL) synapse, including F-actin accumulation and the formation of a secretory domain with weak actin presence (*top*) and the juxtaposition of the secretory apparatus (purple) and the microtubule-directing centrosome (*bottom*, in black), resulting in the highly polarized secretion of lytic particles that kill the target cell.
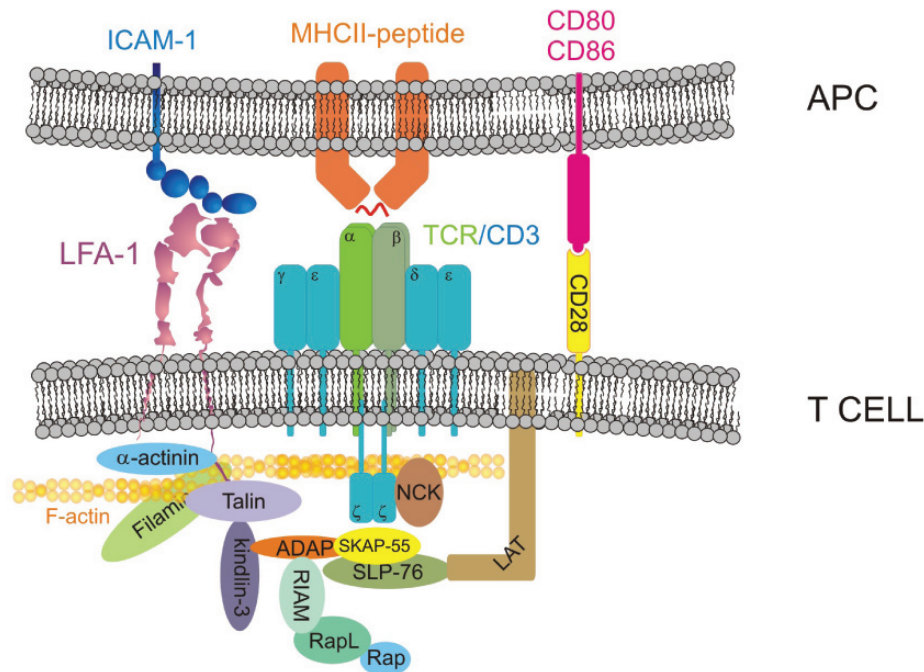
Figure 2. T cell receptor-dependent transactivation of LFA-1. Diagram depicts the major interactions involved in actin-dependent T cell receptor (TCR) and integrin immobilization at the immune synapse (IS), including the signaling modules involved in LFA-1 transactivation. The diagram focuses on the role of SLP-76/ADAP/SKAP-55 in recruiting kindlin-3 and RIAM in proximity to integrin, and the role of Rap/RapL/RIAM in promoting talin association with the β chain of the LFA-1 dimer.

LFA-1 is the predominant integrin that mediates the interaction of T_H cells with APC. It is also important for the formation of IS between CTL and target cells. However, it is unlikely that every target cell expresses ICAM-1, thus additional integrins may be implicated in the formation of IS. Prior studies have described possible roles for VLA-4 ($\alpha_4\beta_1$) and VLA-5 ($\alpha_5\beta_1$) in the IS (reviewed in 41), but their ligands as well as their redundant/unique functions with respect to LFA-1 remain unclear. Spatially, integrins localize throughout the contact area of the T cell and the APC. In activated cells (e.g. super-antigen-triggered clonal leukemic T cells), integrins localize in the outer edge of the contact zone, defining a peripheral SMAC (pSMAC) (Figure 1, top).

Actin reorganization at the IS

Outside-in signals stemming from the TCR and integrins promote actin polymerization and clustering at the T cell:APC interface (Figure 1). As discussed above, actin accumulation is fundamental for the clustering of the TCR and the integrins, forming a positive feedback loop. TCR/CD3 and integrins trigger actin polymerization through several pathways. A major pathway of TCR-mediated actin polymerization depends on the small GTPase Rac1. The TCR activates several Rac GEFs, including Vav1[42] and Tiam1[43]. Rac promotes branched actin accumulation by activating a multi-molecular complex that includes WAVE (Scar), HSP300, ABL2, SRA1, and NAP1. This complex associates with the Arp2/3 complex, triggering actin polymerization, as reviewed elsewhere[44]. Wiskott-Aldrich syndrome protein (WASP) is a protein related to

WAVE that also induces Arp2/3-dependent actin polymerization downstream of the TCR, but it is activated by the small GTPase Cdc42[45].

The contribution of other mechanisms of actin polymerization to the congregation of actin at the contact area with the APC is less clear. During the first steps of the formation of the IS, molecular regulators of actin assembly, e.g. ADF/cofilin, are involved in the dynamic reorganization and accumulation of actin at the contact region. For example, depletion of ADF/cofilin function in T cells enhances the accumulation of actin at the IS[46]. Formins, e.g. mDia, are barbed end nucleators that bind to the uncapped actin filament through one domain and to G-actin-loaded profilin through another, thereby catalyzing G-actin transfer from profilin to the barbed end. mDia-deficient T cells activate and migrate deficiently[47]. Finally, the Arp2/3 complex, which nucleates dendritic actin polymerization at the lamellipodium of migrating cells[48], also participates in the formation of actin lamellae at the IS, although differently shaped actin can accumulate at the IS in the absence of the Arp2/3 complex, in a formin-dependent manner[49].

Actin accumulation is also regulated by the function of actin-binding proteins involved in its cross-linking. For example, α-actinin and filamin accumulate at the IS and are required for proper T cell activation in response to antigen-loaded MHC[50,51]. It is important to note that these two actin cross-linkers also bind directly to the cytoplasmic tail of β integrins[52,53] (Figure 2), hence

they play a dual function facilitating actin and integrin accumulation at the synapse. Other cross-linkers, e.g. non-muscle myosin II (NMII), are also involved in the formation of efficient synapses. However, the role of NMII in IS formation is controversial. Some studies have shown that NMII affects TCR clustering into the cSMAC[54,55], likely due to impaired actin-dependent flux of the TCR towards the contact area[56], but other studies suggest a minimal involvement of this molecule in the formation of the IS[57,58]. The differences between these studies likely reside in the type of T cell and APC used. NMII may play an additional role by regulating the mechanics of the contact interface of the T cell and the APC. In this regard, changes to the rigidity of the APC surface (and NMII inhibition) affect T cell activation[59], indicating that the mechanics of the interfacing surfaces also play a role in the process.

Polarization of the secretory apparatus and the centrosome
TCR and integrin signaling promotes a dramatic redistribution of cellular components in the T cell, most notably the redistribution of the secretory apparatus (centrosome and Golgi, reviewed recently in [60]) and machinery involved in the generation of extracellular vesicles[61] towards the contact area with the APC (Figure 1, both columns). A major difference with the neuronal synapse is that the secretory apparatus of the APC does not polarize towards the postsynaptic cell (the T cell). This is a crucial event during this process that is often used as a marker of IS maturation. It depends on the activation of microtubule motors, e.g. dynein, which "reel in" the centrosome and the associated secretory elements towards the signaling area. This process has been reviewed in detail elsewhere[62–64]. In IS formed between CTL and target cells, this polarization ensures the rapid and specific lysis of the target cell (Figure 1, bottom right column, and next two sections). A major argument to explain the polarization of the secretory apparatus in T_H cells has emerged recently with the discovery of the unidirectional transmission of microRNA-containing exosomes from the T_H cell to the APC[65] (Figure 1, bottom left column), which could influence the activation state of the APC, inducing functional activation or anergy of the APC depending on the microRNAs contained in the exosomes.

Formation of a secretory domain in the CTL synapse
Actin accumulation at the IS facilitates the initial activation of the T cell by immobilizing receptors involved in the contact with the APC and sustaining localized signaling. However, it also constitutes a steric hindrance for polarized secretion. In the early 2000s, Griffiths' group described the clearance of a part of central actin in maturing cytotoxic IS (Figure 1, right column). Such a zone, containing less actin than its surroundings, coincided with the localization of intracellular granzyme[66], suggesting that the region of actin clearance acted as a gate that enabled efficient secretion towards the target cell. However, recent studies have indicated that very small openings in the cortical actin may be sufficient for efficient vesicle delivery[67,68]. The mechanism of actin clearance at the cytotoxic synapse remains unclear. A recent study indicates that coronin 1A is a key mediator of actin remodeling and clearance at the contact area to form the secretory domain[69]. The contribution of other actin mediators of depolymerization, e.g. cofilin, has been suggested but not directly demonstrated[70]. This scenario implicates

that the depolymerization signal stems from receptors localized at the CTL side of the IS. An intriguing possibility, untested yet, is that secretory granules directly depolymerize actin at the IS by carrying actin remodeling factors in their surface.

Target cell killing/T cell activation
In the case of pre-primed CTL-contacting target cells bearing antigen-loaded MHC-I, the subsequent steps of this process involve the secretion of granzyme- and perforin-loaded vesicles to kill the target cell (Figure 1, bottom right column). This has been reviewed in detail elsewhere[71–73]. Before that, naïve CTLs undergo priming (i.e. expression of lytic enzymes and their load into the secretory apparatus) at the secondary lymphoid organs (SLOs) when they enter into contact with mature DCs bearing suitable antigens associated with MHC-I. Direct priming occurs only when a) the pathogen infects and activates DCs directly and b) the pathogen-infected cell (or tumor cell) migrates directly into the SLO. Importantly, the establishment of IS between naïve CTLs and immature DCs leads to cross-tolerance, i.e. the inability of the CTL to activate properly[74]. This is likely an important mechanism of induction of tolerance involved in tumor evasion.

On the other hand, T_H–APC contacts trigger a transcriptional program that results in the activation of the T_H, including expression of activation markers, e.g. CD69 and CD25, and cytokine secretion, e.g. IFN-γ and IL-2 (Figure 1, bottom left column). The main function of these cytokines is to create an activating microenvironment for other immune cells in a paracrine manner. At the site of infection, these cytokines activate other effector cells, particularly macrophages involved in pathogen clearance, CTLs, and NK cells.

Additional molecules induced by the establishment of IS include mediators of cell proliferation downstream of NF-AT, AP1, and NF-kB (reviewed in [75]) as well as receptors implicated in the migration of the activated cell to the inflammatory site, e.g. CCR5[76].

IS termination
The specific signals that promote termination of the IS are unclear. In the case of IS of CTL with target cells, a clear candidate to promote termination of the contact is the flip-flop of the plasma membrane of the target cell due to the effect of the lytic enzymes secreted by the CTL. In such a mechanism, the CTL would recognize phosphatidylserine, annexin V, or other components of the inner leaflet of the plasma membrane of the target cell. In the case of naïve CTL or T_H cells, the mechanism is less clear but likely involves the exhaustion of the TCR recycling process over extended periods of stimulation[77]. Importantly, signaling molecules involved in the formation and function of the IS, e.g. PKCtheta, are also involved in synapse breakdown, constituting a possible mechanism of early remodeling of the IS[78].

Concluding remarks: towards the application of manipulating the IS in biomedicine
In recent years, the need for new therapies against multidrug-resistant tumors and the secondary effects of current therapies, e.g. chemotherapy, have led to the study and the development of

better "targeted" therapies with less deleterious side effects for patients. Therefore, enhancing the ability of the immune system to detect and remove pathological cells through recognition of tumor or different expression patterns of the target cells is a crucial step to develop better therapies. Another important issue is to counteract the evasive mechanisms developed by pathogens and tumor cells.

One approach aimed at improving the immune response against tumor cells consists of autologous or allogeneic tumor vaccination (Figure 3, top right). These approaches are aimed at generating strong CTL responses against tumor cells based on their specific molecular makeup. The underlying mechanism consists of vaccine-mediated CTL priming by vaccine-stimulated APC (mainly DCs), which would then home to the tumor and rapidly form an IS with the tumor cells, killing them. Several trials based on this approach are reviewed here[79]. Another possibility is the genetic immunization of patients (DNA vaccination) through DCs. The major limiting factor is the need for safe and specific carriers. An attractive

possibility is the use of *in vivo* DC-targeting liposomal DNA vaccine carriers[80].

Approaches aimed at suppressing the effects of the evasive maneuvers of tumor cells have also been tested in recent years (Figure 3, top left). For example, tumor cells are believed to promote the expression of CTLA-4, which is a molecule expressed by T cells that competes with CD28 for the co-stimulatory molecule CD80 (B7.1), thereby suppressing T cell activation. The US Food and Drug Administration (FDA) and the European Medicines Agency (EMA) have approved the use of a humanized monoclonal antibody against CTLA-4 for the treatment of late-stage melanoma[81]. Similar approaches have been developed for PD-1, which is another inhibitory receptor that suppresses T cell responses independent of CD28 but dependent of its ligands PD-L1 and PD-L2, which are abundantly expressed by several types of tumor cells[82]. A number of antibodies against PD-1 and PD-L1/2 are being developed by big pharmaceutical companies
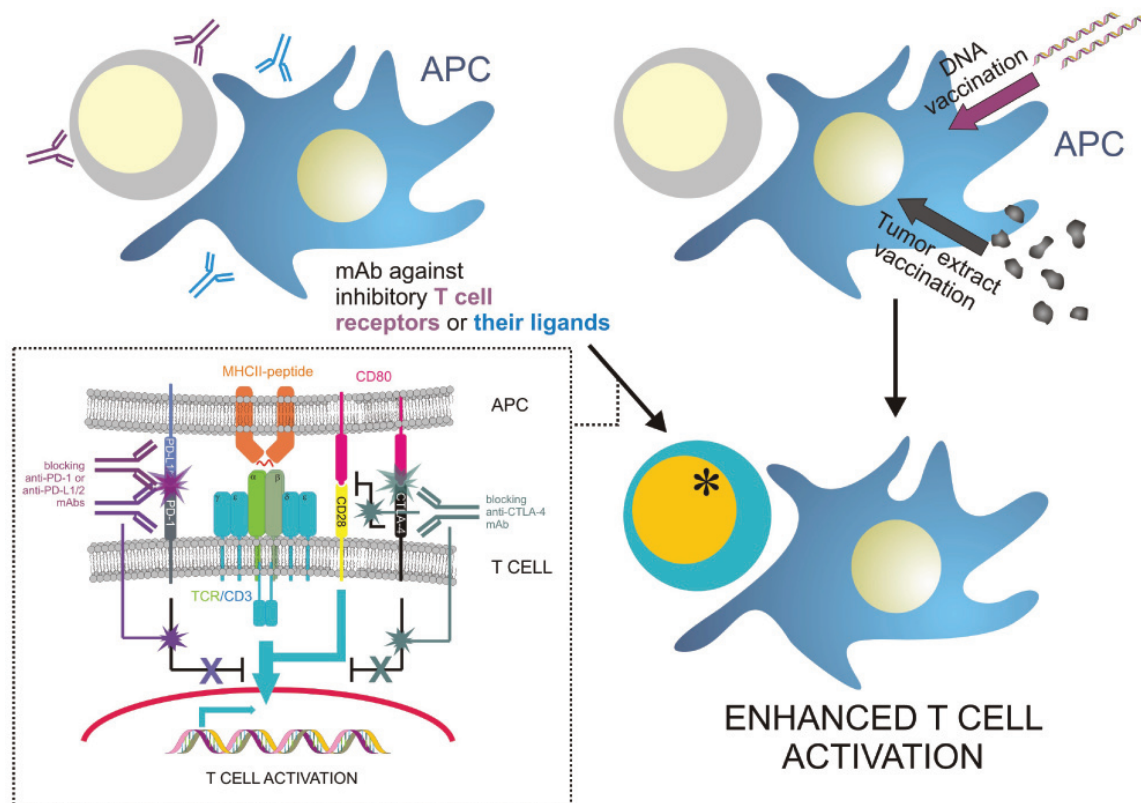

Figure 3. Therapy-based enhancement of immune synapse formation between T cells and tumor antigen-presenting dendritic cells. *Top left*, poorly responding T cells are treated with antibodies that block inhibitory molecules such as CTLA-4 and PD-1, or inhibitory ligands of the latter, e.g. PD-L1/2. *Bottom left inlay*, representation of the effect of anti-CTLA-4 blockade, which blocks inhibitory signals emanating from CTLA-4 that counteract TCR/CD3-dependent signals and also releases CD80 to co-stimulate via interaction with CD28; also depicted is the effect of anti-PD-1 or anti-PD-L1/2 monoclonal antibodies (mAbs), which prevent their interaction and the generation of inhibitory signals. *Top right*, direct vaccination of dendritic cells with tumor DNA or autologous or allogeneic tumor extracts. *Bottom right*, either treatment should enhance T cell response against tumor antigens.

aiming to find different anti-tumor therapies[83–85]. At a molecular level, CTLA-4 binding to CD28 disrupts TCR clustering, effectively destabilizing the IS[86]. Likewise, PD-1 accumulation at the IS recruits protein phosphatases, such as SHP-2, that quench the stimulating signals emanating from the synapse[87].

Clearly, these studies and novel forms of treatment are of outstanding importance in the development of new treatments for the more aggressive and less-tractable types of cancer and are likely the beginning of a new era of molecular treatment of cancer.

Competing interests
The authors declare that they have no competing interests.

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Version 1

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