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Kupfer-type Immunological Synapses *in vivo*: Raison D'être of SMAC

Izaskun Mitxitorena, Elena Saavedra, Carlos Barcia, Ph.D.

Department of Biochemistry and Molecular Biology, Institute of Neuroscience & School of Medicine, Universitat Autonoma de Barcelona, Lab M2-107, Bellaterra, Cerdanyola del Valles, Barcelona, Spain.

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Corresponding Author: Carlos Barcia
Institute of Neuroscience
Department of Biochemistry and Molecular Biology
School of Medicine, Lab M2-107
Autonomous University of Barcelona
Cerdanyola del Vallès,
08193, Barcelona, Spain
carlos.barcia@uab.es

Abstract

T cells engage with antigen-presenting cells to form immunological synapses. These intimate contacts are characterized by the complex arrangement of molecules at the intercellular interface, which has been described as the supramolecular activation cluster (SMAC). However, due to T cells functioning without SMAC formation and the difficulties of studying these complex arrangements *in vivo,* its biological importance has been questioned. In light of recent data, we focus this review on the putative functionality of SMACs in T-cell synaptic contacts *in vivo* and emphasize the therapeutic potential of SMAC manipulation in immune-driven diseases.

Immunological Synapse Formation and SMAC arrangement

Immunological synapses (IS) are critical intercellular communications between specific immune cells and antigen-presenting cells (APC)[1]. This particular engagement between both counterparts requires intimate contact between the aforementioned cells and includes multiple factors and complex signaling cascades of activation [1,2]. T-cell ISs have been largely studied and represent the best-known IS type [3], although ISs may also be established by different types of effector cells, such as NK or B cells [4-6]. The formation of an IS involves the T-cell recognition of specific antigens that are presented by APCs. Major Histocompatibility Complexes (MHC) display antigens at the APC cell surface, which are detected by T-cell receptor (TCR) molecules that are displayed on the T-cell membrane [7]. The interaction between the antigen-MHC and the TCR induces the TCR signaling cascade [8], thus initiating the activation of the T cell, which is characterized by the phosphorylation and polarization of tyrosine kinases such as lymphocyte-specific protein tyrosine kinase (Lck) and zeta-chain-associated protein kinase 70 (ZAP-70) at the interface [9,10] (**Figure 1**). In mature IS formation, the process of activation involves severe changes to the micro-anatomical configuration of the T cell that are characterized by rearrangement of the actin cytoskeleton and are driven by the microtubules organizer center (MTOC), which becomes polarized toward the APC and participates in the organization of secretory domains [11-14]. The polarization of the T cell is also accompanied by the rearrangement of lymphocyte function-associated antigen 1 (LFA-1) molecules that segregate three-dimensionally at the IS interface and specifically bind to the APC's intercellular adhesion molecule 1 (ICAM-1) [15,16]. This binding of LFA-1/ICAM-1 takes place at the interface, and LFA-1/ICAM-1 complexes rearrange micro-anatomically,

forming a ring-shaped area named the peripheral supramolecular activation cluster (pSMAC), which surrounds a characteristic central accumulation of TCRs, known as the central supramolecular activation cluster (cSMAC) [15] (**Figure 1 and Box**). This way, a "bull's eye" characteristic structure is formed, where an outer ring contains the adhesion molecules, and an inner area contains the signaling molecules. In cytolytic T cells, the cSMAC may also contain secretory domains that usually encompass an area of smaller size and is located near the TCR signaling central cluster, where lytic granules of effector molecules are concentrated and released [6,17,18]. Importantly, LFA-1 molecules are linked to talin proteins, which are key integrins involved in cell migration and cellular junction because they are linked to the actin-myosin cytoskeleton through vinculin [15,19,20].

Visualization of SMACs *in vivo*

The initial description and most of the studies on the microanatomy and function of ISs have been performed *in vitro* [1,15,21]. Although the knowledge on ISs has substantially grown and successfully improved based on *in vitro* experiments, the functionality of ISs in living organisms has barely been explored. A criticism often rises considering that *in vitro* environments are different from those in tissue. Cultures and planar bilayers are isolated, two-dimensional milieus, whereas tissues are three-dimensional environments in which cells receive information and signals from different planes and directions involving diverse biological systems. Thus, research of ISs *in vivo* is an important matter for a complete understanding of T-cell biology.

Formation of SMAC *in vivo* has been demonstrated using high-resolution confocal microscopy of labeled, fixed tissue with multiple fluorescence-specific

antibodies. The formation of the CD3/TCR central cluster (cSMAC) and/or the peripheral segregation of LFA-1 (pSMAC) are observed in different tissues, such as the brain and secondary lymphoid tissues [22,23]. ISs are stable and preserved structures in mammals. As described *in vitro*, ISs show a flat interface *in vivo*; and cSMAC and pSMAC are formed in all species studied so far. From rodents [22] to primates [24], including humans [25], the formation of SMAC seems to be consistently involved in mammalian immune responses.

However, despite the good level of resolution, this *in vivo* technique has the limitation of picturing static events. High-resolution confocal images in fixed tissue represent a scenery taken at a certain and specific moment and do not resolve the dynamics of the IS. Two-photon microscopy in living animals will be the ideal technical approach to show the dynamics of IS formation *in vivo,* but some issues must still be solved. Currently, multi-photon microscopes are able to image several hundreds of microns deep into tissue; however, the resolution of the anatomical details is still not sufficient to distinguish the micro-anatomy of the IS at the SMAC level. In addition, observations are hampered by the parenchyma's high auto-fluorescence and by the reduced number of fluorophores that are available to detect molecule arrangements *in vivo* in time-lapse, live imaging. Two-photon microscopy studies in tissue, especially in lymph nodes, have shown the dynamics by which T cells engage APCs (i.e., dendritic cells), but no micro-anatomical details of the SMAC were given [26,27]. Currently, time-lapse studies of the microanatomy of complete SMAC formation, containing the central and peripheral clusters, have not been yet performed in living tissue. Notably, however, a successful attempt was performed regarding visualization of the dynamics of the formation of the TCR central cluster using a two-photon microscope in lymph nodes in

live mice. In a study by Friedman et al., some features of the TCR dynamics *in vivo*, as well as the behavior of TCR accumulation, were revealed [28]. In addition Azar et al., using linker for activation of T cells (LAT)-EGFP labeled T cells, were able to detect the *in vivo* formation of central and peripheral clusters of LAT at the IS interface in lymph nodes, which may underlie some insights into the molecular distribution of SMACs [29]. The next scientific challenge is the combination of different fluorophores to observe the dynamics of the peripheral SMAC in relation to the central TCR cluster and how the formation of these structures affects immune responses in healthy subjects and experimental models of diseases.

Function of SMAC *in vivo*

Previous observations have shown that SMAC formation is not required for TCR signaling or for the effectiveness of cytotoxic T cells [6,30]. These results question the biological importance of SMAC formation. Why is such an enormous and complex arrangement in the cell needed? Why invest such a large amount of energy and effort? pSMAC and cSMAC formations were first observed in brain tissue, in the context of the clearance of virus-infected cells [22]. In this case, the formation of SMACs preceded the elimination of viral-infected cells in immune-competent animals that were primed with an adaptive immune response [22]. In this context, the percentage of ISs forming SMACs and engaged with virus-infected cells was approximately 60% in a specific time window, before complete viral clearance [31,32]. These results indicate that a large percentage of SMAC formation may be essential for viral clearance in tissue, suggesting its biological significance [31]. In the same scenario of viral clearance, the secretory domain that was

observed at the immunological synaptic interface was characterized by the formation of interferon-gamma (IFN-γ) and perforin clusters, which conveys that both effector molecules and their polarization at the synaptic interface may be necessary phenomena for the elimination of virus-infected cells [31]. In fact, IFN-γ- or perforin-deficient mice are unable to eliminate virus-infected cells from the brain [33]. However, whether completely mature SMAC rearrangements will take place at the interface seems to depend upon multiple factors. For example, IFN-γ appears polarized in Kupfer type (with SMAC) and non-Kupfer type (without SMAC) synapses [31], which indicates that the formation of mature synapses with SMAC does not precede the formation of the secretory domain; therefore, SMAC formation may not be strictly necessary for the release of effector molecules and elimination of target cells. In fact, although cytotoxic ISs restrict killing to antigenic target cells, IFN-γ signaling is also detected in non-antigenic bystander cells [34], suggesting a certain leakage or multidirectional diffusion of the cytokine, which implies defective SMAC formation.

On the other hand, secretory effector molecules have a different pattern of segregation that is independent of c- and pSMAC formation. Therefore, different cytokines show different patterns of secretion in T cells. For example, IFN-γ and interleukin 2 (IL-2) are polarized and secreted to the synaptic interface, while TNF-α and chemokine (C-C motif) ligand 3 (CCL3) are secreted multi-directionally [35]. These established patterns of secretion indicate a different behavior of T-cells that depends on the context of the immune response. Thus, the need for complex SMAC rearrangement may not always be required.

These results indicate that SMAC arrangement could be necessary to directionally secrete specific molecules towards the APC without altering adjacent cells, thus safely channeling intercellular communication [13,36] (**Figure 2**). Outer ring LFA-1/ICAM-1 adhesion allows for the formation of a shielded micro-chamber, which is an intercellular space that is kept isolated from the surrounding environment. This flat interface feature is possible due to rearrangement of the actin cytoskeleton, which forms a consistent and renewable scaffold that is oriented to the interface [37,38]. Most likely, the reason for these interface arrangements may be for maximal reduction of the surface at the intercellular contact, which could result in more effective communication and less chance of membrane and receptor miss-folding. In that intercellular space, cytotoxic compounds, such as effector molecules, can be safely delivered, and signaling only occurs with the contacting cell, without damaging the surrounding healthy cells that are not involved in the immunological response. Therefore, the formation of SMACs may represent a highly evolved and specific immune response that only has an effect on target cells and does not affect bystander cells. Thus, it can be hypothesized that the SMAC is a necessary structure to channel cytokines and other effector molecules in an extremely selective manner (**Figure 2**).

Overall, T-cell synaptic contacts may be necessary for an effective immune response, but, the formation of SMACs may depend on the immunological context and the effector molecules that are delivered. It is, therefore, tempting to speculate that the ideal situation may be SMAC formation because it would preserve the surrounding tissue and result in a more specific and safe response. As a drawback, an immune response with SMAC formation is most likely slower and requires high-energy waste. Thus, if the

immune response needs to be faster and inexpensive, it should be carried out without SMAC.

In summary, *in vivo* studies of Kupfer-type ISs exhibit a complex scenario for further research. Multiple types of intercellular combinations, involving diverse cytokine release and adaptable immune responses within different tissues, are important variables that should be considered for future research, although the visualization and unraveling of the IS function will only be fully achieved *in vivo* if new, specific approaches are designed that selectively inhibit IS formation in the tissue of a living organism.

A therapeutic view of the immunological synapse

Because the formation of the SMAC may be an important part of the specificity and effectiveness of the T-cell response, manipulation of ISs represents a promising tool from a therapeutic point of view. It presents an advantage whereby we could specifically inhibit or activate the different immune responses according to therapeutic needs, as multiple targets could potentially be aimed to hinder or empower IS formation. In fact, immunotherapy is a therapeutic field that has lately been developed and is becoming promising, particularly for cancer. Specific drugs, usually artificially made antibodies, have been designed to empower anti-tumor immunity, and most of them intervene at the synaptic level (**Figure 3**).

One of the most hopeful approaches to directly stimulate the formation of specific ISs between T and tumor cells is the development of bi-specific T-cell engager (BiTE) antibodies. These monoclonal antibodies target the TCR/CD3 complex and tumor antigens, such as CD19, epithelial cell adhesion molecule (EpCAM) or epidermal growth

factor receptor (EGFR). This way, the antibodies promote the synaptic interaction between tumor cells and T cells and induce the activation of cytolytic T cells. This engagement-induced tumor-cell death leads to T-cell accumulation in the tumor microenvironment and reduces tumor cell proliferation *in vivo* [39].

Another successful approach to modify synaptic contacts is based on the development of antibodies that are able to antagonize receptors that inhibit the immune response. A successful case is that of ipilimumab, an antibody that binds an inhibitory T-cell protein called cytotoxic T lymphocyte antigen 4 (CTLA-4). CTLA-4 is expressed in activated T cells and is recruited to the cSMAC in competition with the T-cell activation molecule, CD28 [40,41]. The binding of ipilimumab interferes with CTLA-4-mediated T-cell suppression at the cSMAC, therefore, facilitating active synaptic interactions between T cells and target cells, which results in a more aggressive immune response against the tumor. Ipilimumab has been tested in patients with melanoma (Yervoy®), and it has been proven to be effective in specific cases because it removes melanoma without tumor recurrence [42-44]. Analogously, therapeutic blockade of programmed cell death 1 (PD-1), which is also localized at the cSMAC, increases T-cell motility and cytotoxic effectiveness, thus improving viral clearance [45]. Indeed, the combination of both, CTLA-4 and PD-1 blockade, has been proven to be effective toward tumors by increasing the cytolytic T-cell population and reducing regulatory T cells [46].

In this context, optimization of the cytolytic arm seems to be the primary therapeutic strategy to eliminate tumors because the tumorigenic microenvironment facilitates a pro-inflammatory response that promotes tumor growth. In the case of CNS tumors, particularly in human glioma, the formation of SMAC has been studied in depth. In

glioma tissue, mature ISs are established between T-cells and tumorigenic cells, although at a low rate [25]. However, SMAC analyses performed in murine experimental models of glioma have shown that the formation of Kupfer-type synapses does not predict the elimination of the tumor [47], which is different from the process of viral clearance [31]. This feature may be characteristic of tumors because the multidirectional delivery of cytotoxic compounds could theoretically be the fastest and most effective way to destroy tumors in an environment where the majority of bystander cells should be rapidly eliminated. However, because T cells form SMACs, they may still be needed in a sufficient quantity for the recognition of specific antigens to take place. This fact supports the idea that SMACs would only be formed when the tissue in the vicinity must be preserved. These concepts may open new avenues of research regarding the formation of SMAC or bonafide ISs.

On the other hand, tumor development and other immune-mediated degenerative diseases might be a consequence of defective SMAC formation. This alteration may be reflected in altered immune responses due to deficient recognition of the antigen, anergy or exhaustion of the T-cell response, either of the regulatory or cytolytic response. In line with this, a recent study showed for the first time that alterations in SMAC formation in T cells can be a crucial element in immune disorders. In this report, CD4 T cells obtained from patients with multiple sclerosis and type-1 diabetes were exposed to antigens from influenza virus. Both CD4-T-cell groups showed divergent formation of SMAC when compared with normal T cells obtained from healthy patients [48]. These differences included deficient SMAC-structure formation regarding the proper CD3/TCR or MHC accumulation and ICAM-1/LFA-1 segregation, a distinct motility of T cells, and altered

timing and velocity of SMAC formation. Importantly, a deficiency in SMAC formation sets the possibility for alteration in cellular communication and could explain how T cells might escape the negative selection that takes place in autoimmune diseases.

Another example regarding the X-linked lymphoproliferative syndrome, which is characterized by fatal responses to Epstein-Barr virus infection, has recently been reported. This syndrome is caused by mutations affecting the adaptor SAP (signaling lymphocytic activation molecule (SLAM)-associated membrane protein), which is a molecule involved in correct arrangement of the synaptic contact. In fact, SAP-deficient cytotoxic T lymphocytes exhibit abnormal actin organization and reduced centrosome docking at T-cell–B-cell ISs [49]. These results demonstrate that correct assembling of T cells with their target cells and the micro-anatomical arrangement of SMACs and their associated organelles is a fundamental process in the immune response.

We are beginning to understand how malfunction of SMAC formation may induce different immune-mediated diseases. The understanding of this process *in vivo* as well as the specific mechanisms occurring during SMAC formation in tissues within different immune scenarios will be crucial to propose molecular targets that restore the correct arrangement of Kupfer-type ISs.

Box

The term immunological synapse has been used to generally define communications between immune cells, although it is also specifically and more accurately referred to as the formation of the characteristic interface with complex rearrangements of molecules and compounds called SMAC (Supra-Molecular Activation Cluster). Synapses that form

SMACs are considered mature immunological synapses and, in some publications, to honor its discoverer, immunological synapses are classified as Kupfer-type or non-Kupfer-type immunological synapses according to the presence or absence of the “bull’s eye” formation at the interface, respectively.

Figure Legends

Figure 1. T-cell immunological synapse forming a SMAC (Kupfer-type). T cells recognize antigens that are presented by the MHC of an APC through the TCR/CD3 complex. Then, T cells are activated through phosphorylation of tyrosine kinases such as Lck and ZAP-70, which are polarized to the T-cell/APC interface. This activation leads to dramatic changes in the cell, including the rearrangement of adhesion molecules, such as LFA-1, which are segregated towards the interface to bind ICAM-1 of the APC and form the peripheral activation cluster (pSMAC). On the other hand, TCR/CD3 molecules are aggregated at the center of the interface and form the central SMAC (cSMAC). In addition, cytotoxic granules are delivered to the center of the interface and form the secretory domain.

Figure 2. Hypothetical strategies for cytolytic T-cell responses in tissue. A. Unidirectional secretion of effector molecules after immunological synapse formation. T cells (red) form mature immunological synapses after antigen recognition and subsequent apposition to an APC (blue). LFA-1 adhesion molecules are segregated at the external border of the interface (red), forming the pSMAC, whereas TCR (green) is concentrated at the center of the interface, forming the cSMAC, where the cytolytic granules (yellow arrow) may be delivered in one specific direction. With this strategy, the APC (blue) can be specifically eliminated without damaging bystander cells (light brown cells). B. Multidirectional secretion of effector molecules without bona fide synapse formation. T cells (red) may not form mature immunological synapses after antigen recognition; thus,

the strict apposition to antigen-presenting cells (blue) may not be necessary. LFA-1 molecules (red) do not arrange as pSMAC, and TCR does not concentrate at the center of the interface, forming the cSMAC. Cytolytic granules (yellow arrows) may be delivered multi-directionally. With this strategy, bystander APCs (blue) can be eliminated discretionally.

Figure 3. Therapeutic targets at the immunological synapse. CTLA-4 competes with CD28 for CD80/CD86. Bound CTLA4-CD80/CD86 complexes are recruited to the cSMAC, whereas unbound CD28 is segregated to the pSMAC. PD1 molecules bind to PDL1 and are recruited to the cSMAC. The binding of CTLA4-CD80/CD86 inhibits T-cell activation. Thus, CTLA-4 blocking antibodies hamper binding to CD80/CD86, which facilitates the binding of CD80/CD86 with CD28 and impedes CTL inhibition. Similarly, PD1-blocking antibodies obstruct the inhibition of T cells at the synaptic interface.

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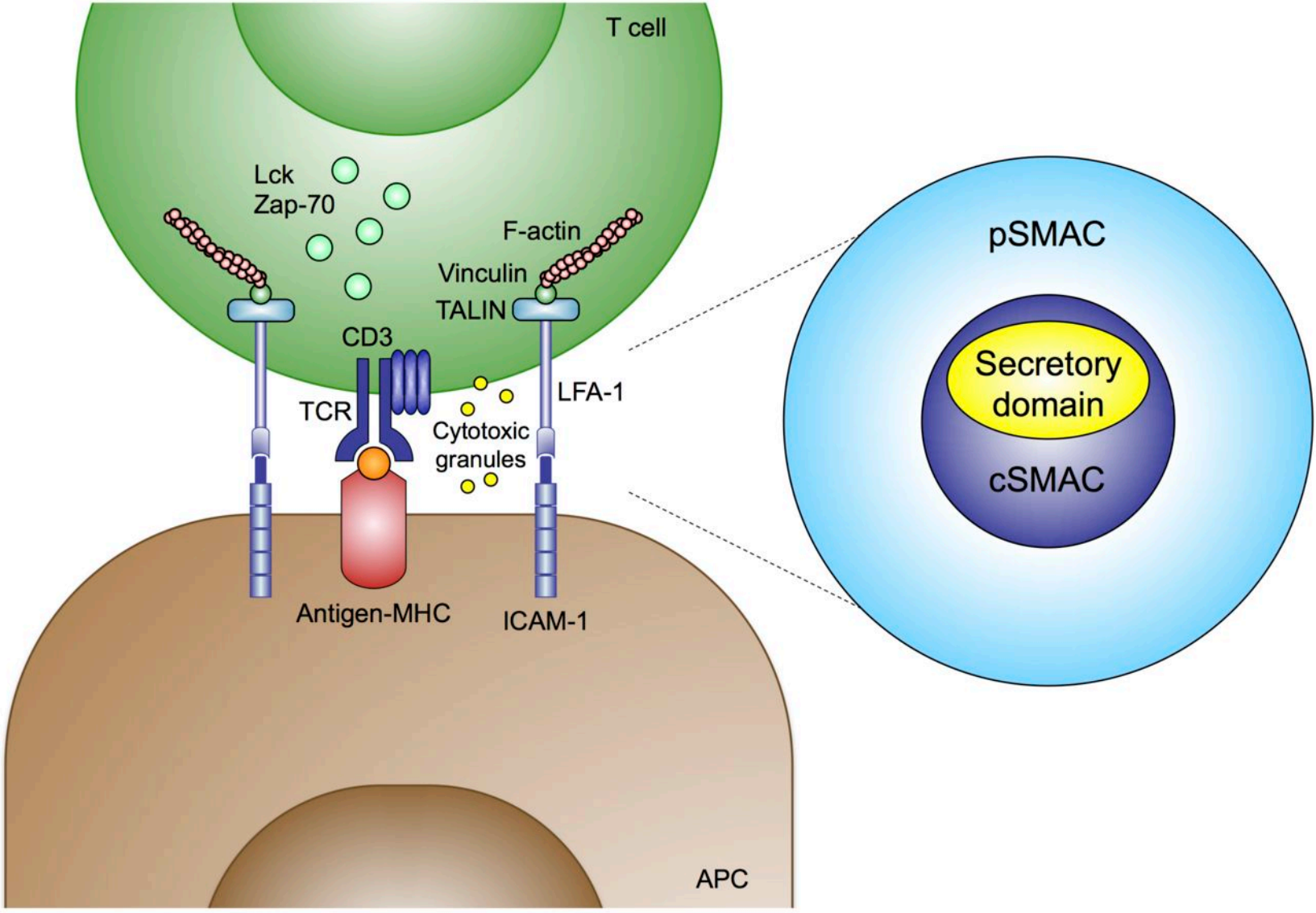
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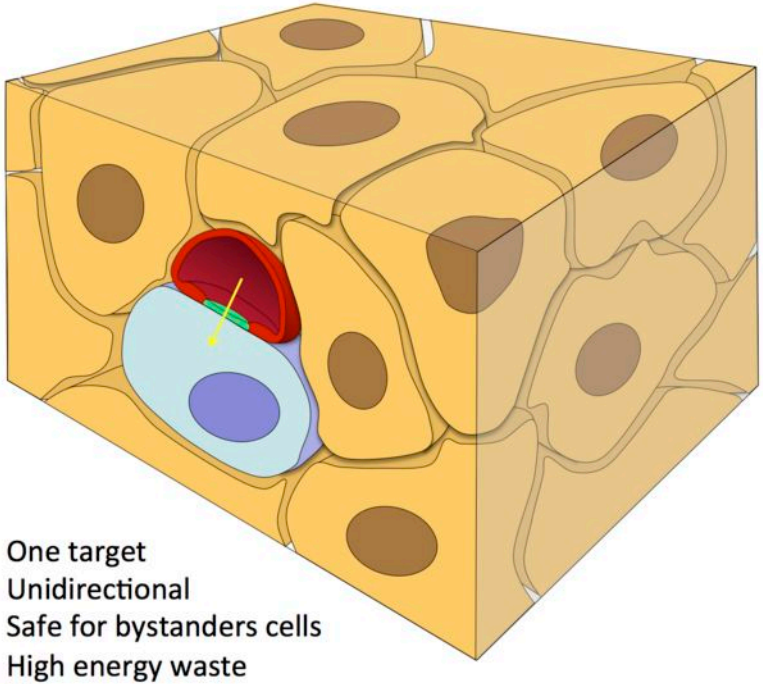
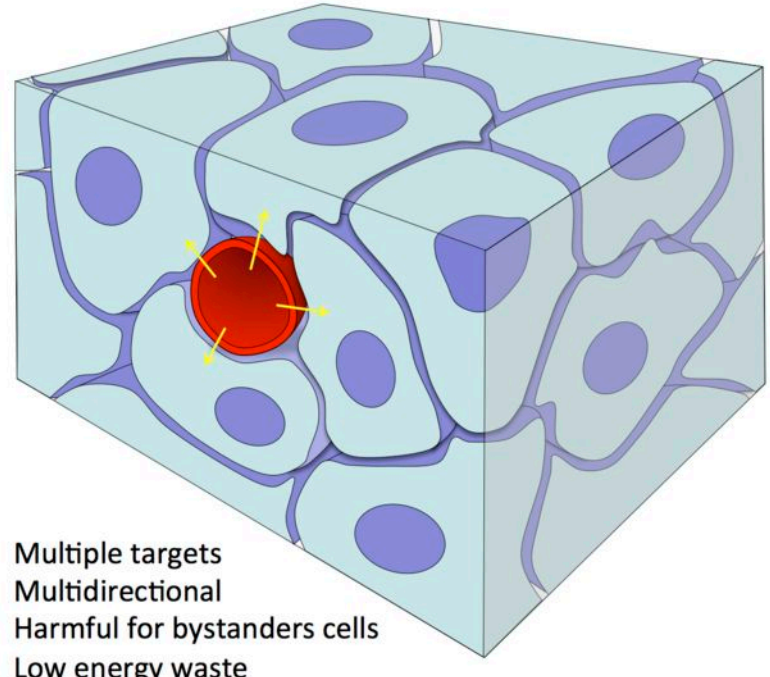
Secretory
domain
cSMAC

A

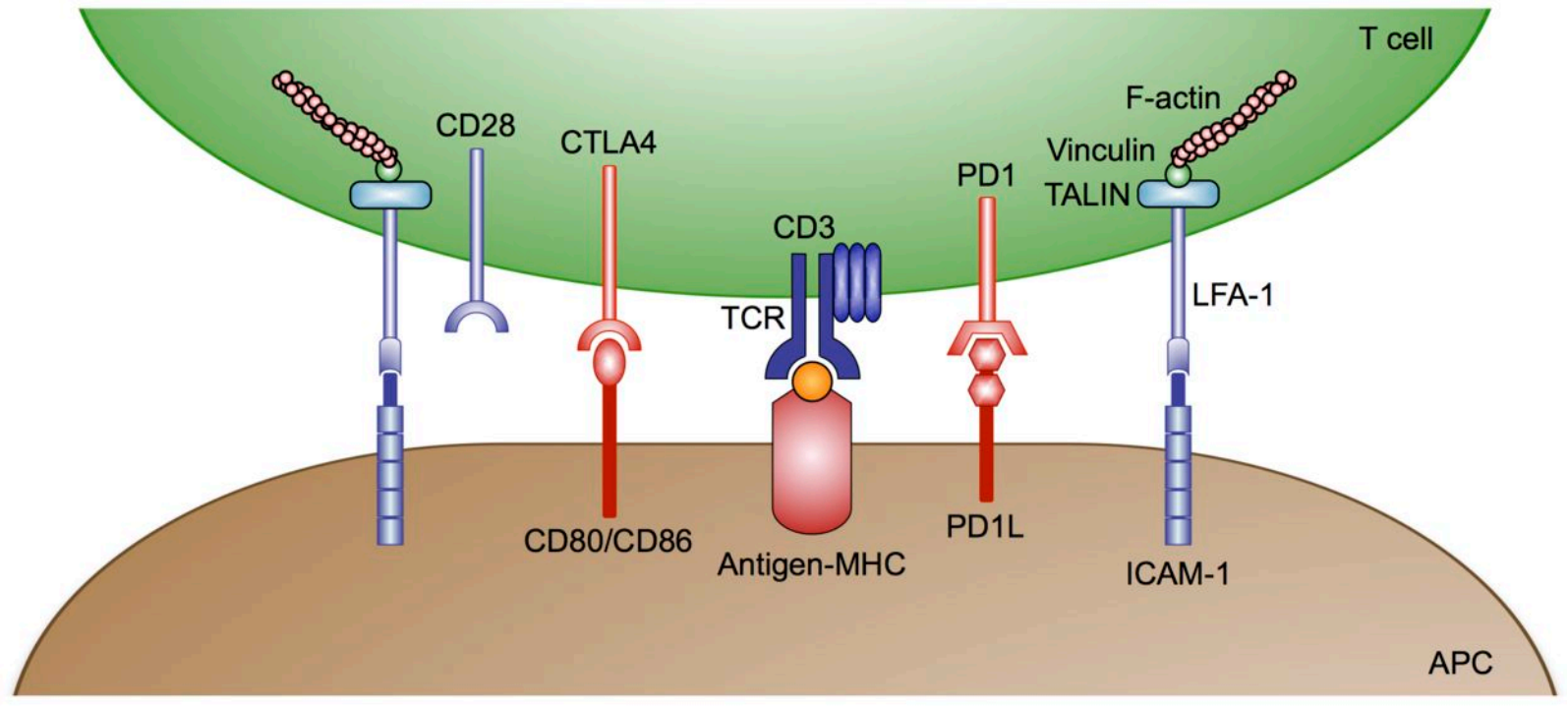


One target
Unidirectional
Safe for bystanders cells
High energy waste
Slow process
(i.e. Punctual infection)

B



Multiple targets
Multidirectional
Harmful for bystanders cells
Low energy waste
Fast process
(i.e. Tumors, massive infection)


T cell
CD28
CTLA4
CD3
TCR
PD1
F-actin
Vinculin
TALIN
LFA-1
CD80/CD86
Antigen-MHC
PD1L
ICAM-1
APC