



Review

Calcium, cancer and killing: The role of calcium in killing cancer cells by cytotoxic T lymphocytes and natural killer cells[☆]



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ABSTRACT

Killing cancer cells by cytotoxic T lymphocytes (CTL) and by natural killer (NK) cells is of vital importance. Cancer cell proliferation and apoptosis depend on the intracellular Ca^{2+} concentration, and the expression of numerous ion channels with the ability to control intracellular Ca^{2+} concentrations has been correlated with cancer. A rise of intracellular Ca^{2+} concentrations is also required for efficient CTL and NK cell function and thus for killing their targets, in this case cancer cells. Here, we review the data on Ca^{2+} -dependent killing of cancer cells by CTL and NK cells. In addition, we discuss emerging ideas and present a model how Ca^{2+} may be used by CTL and NK cells to optimize their cancer cell killing efficiency. This article is part of a Special Issue entitled: 12th European Symposium on Calcium.

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1. Introduction

To eliminate cancer cells from the human body is one of the most important challenges for the immune system. Ca^{2+} plays a dual role in this process considering its involvement in proliferation/activation and apoptosis of both cancer and immune cells. Cytosolic Ca^{2+} signals are controlled by several mechanisms that can be grouped in three main general categories: 1. Ca^{2+} import into the cytosol, 2. Ca^{2+} export out of the cytosol, and 3. Ca^{2+} buffering in the cytosol. Together, these mechanisms control cytosolic Ca^{2+} signals and thereby regulate proliferation, activation and apoptosis. In general, transient small elevations (low to medium nM) of cytosolic Ca^{2+} will increase cell proliferation whereas sustained substantial elevations (high nM to μM) may induce apoptosis [1]. Thus, Ca^{2+} has the potential to modulate proliferation and apoptosis of cancer cells, and at the same time, Ca^{2+} modulates proliferation, apoptosis and the effector efficacy of immune cells.

One of the emerging hallmarks of cancer is how cancer evades immune surveillance. The different aspects of immune evasion, for instance immune-editing, are discussed in detail in many reviews including the “Hallmarks of Cancer: The next generation” review by Hanahan and Weinberg [2]; we will not discuss these important issues in this review because no well-defined role for Ca^{2+} during immune evasion has been reported yet. We will, however, discuss the kinetic aspect of killing

cancer cells by the immune system, because it is most likely Ca^{2+} dependent. Whereas the recognition of cancer cells by immune cells is probably not Ca^{2+} dependent, the effector functions of the immune cells, characterized by the efficacy and the speed of cancer cell killing through immune cells, greatly depend on Ca^{2+} [3]. Thus, immune evasion of cancer cells may also occur in a “kinetic” manner, meaning that the speed and efficacy of cancer cell killing through immune cells are modulated in ways that will slow the overall killing speed. These aspects and their Ca^{2+} dependence will be discussed in this review.

2. Calcium channels

Ca^{2+} signaling in tumor cells and in T cells is strongly dependent on the activity of Ca^{2+} channels. Highly selective Ca^{2+} channels can be grouped into three main channel types: voltage-gated Ca^{2+} channels [4], TRPV5 and TRPV6 channels [5] and ORAI channels [6]. Voltage-gated Ca^{2+} channels have a major role in neuronal excitation and muscle contraction, however some reports have been published pointing towards potential involvement in cancer [7,8]. In the immune system, a potential role of voltage gated Ca^{2+} channels is controversially discussed [9,10]. TRPV5 and TRPV6 are the only highly Ca^{2+} selective ion channels of the TRP channel family, they are considered to be important for Ca^{2+} uptake in epithelial kidney and intestinal tissues [11]. There are several reports indicating a role of TRPV6 in cancer, in particular in prostate cancer [12,13]. TRPV5 and TRPV6 have probably no significant role for immune cell function, at least not in T cells because they are not consistently expressed [14]. ORAI channels are store-operated Ca^{2+} channels which form CRAC channels (see details below). They have also been implicated in

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several types of cancer [15,16], but most importantly, CRAC/ORAI channels form the major Ca^{2+} entry pathways in immune cells [1,6]. Since the major focus of this review on how Ca^{2+} influences the potential and efficacy of CTL and NK cells to kill cancer cells, these channels need to be introduced in some detail.

The concept of store-operated Ca^{2+} entry was originally introduced by Casteels, Droogmans and Putney [17,18]. CRAC channels were discovered already in 1992 in mast cells and T cells [19–21] but it took a long time to finally unmask their molecular basis. In 2005, STIM was discovered as the activator molecule of CRAC [22–24], and in 2006, ORAI was found to form the main subunit of CRAC channels [25–27] and was also established as the pore-forming unit [28,29]. In humans and other vertebrates, two STIM homologues (STIM1, STIM2) and three ORAI homologues (ORAI1, ORAI2, ORAI3) exist [6]. STIM1-activated ORAI1 channels are considered to form the major part of the channels responsible for CRAC currents. CRAC channels and also their molecular counterpart ORAI1 are store-dependent, highly Ca^{2+} selective Ca^{2+} inwardly rectifying with a very low single channel conductance.

For the development of T, B, and NK cells, STIM1/ORAI1 activity is not necessary, since their numbers appear almost normal in KO/transgenic mice and deficient patients [25,30–35]. For regulatory T cells (Tregs), developmental defects have been reported in mice [32]. Whereas development of most immune cells types appears normal, lack of STIM1/ORAI1 activity results in loss of T cell function and a severe combined immunodeficiency in patients [25,33]. In mouse T cells, similar phenotypes have been reported [31,32]. In addition, it has been shown that STIM1/ORAI1 activity-deficient NK cells have a drastically reduced store-operated Ca^{2+} entry and cannot kill target cells [36]. In conclusion, the STIM1/ORAI1 combination is very important for T and NK effector cell function, but not required for their development.

3. Cancer and calcium

According to the first “hallmarks” review by Hanahan and Weinberg [37], cancer is promoted through six biological capabilities which are “sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis”. These hallmarks are linked to genomic instability and tissue inflammation. In the second “hallmarks” review, the reprogramming of energy metabolism and the evasion from the immune system were added to this list as emerging hallmarks [2].

How does calcium influence cancer cells? Cytosolic Ca^{2+} concentrations can be modulated directly by channels and transporters that either import Ca^{2+} into the cytosol or export it from the cytosol. Import is mainly mediated by ion channels and many Ca^{2+} /cation channels have been postulated to modulate cancer cell function. Of the three types of highly selective Ca^{2+} channels, voltage-gated Ca^{2+} channels, TRPV5/6 and ORAI channels, all of them have been implicated in cancer as already state above [7,8,12,13,15,16,38]. The studies range from correlations of Ca^{2+} channel expression in cancerous tissue compared to normal tissue to more functional studies which show correlations between ion channel expression/function and cellular functions such as cell proliferation. Activity of Ca^{2+} channels will mainly influence cytosolic Ca^{2+} , which modulates many cellular functions including proliferation and apoptosis [1]. Non-selective cation channels like many of the other TRP channels besides TRPV5 and TRPV6 have also been correlated with cancer growth [12]. K^+ and Cl^- channels control the membrane potential and thereby the open probability of the voltage-gated Ca^{2+} channels and the driving force for all Ca^{2+} /cation channels. It is thus not surprising that there is a vast amount of literature pointing towards a role of mostly K^+ but also some Cl^- channels for cancer growth [8,39]. Regarding K^+ and Cl^- channels, the main hypothesis is that they regulate cytosolic Ca^{2+} through membrane potential changes. In addition, the activity of

Ca^{2+} , K^+ , Cl^- and cation channels may also control the concentration and ion strength in the cytosol. For K^+ , Na^+ or Cl^- , concentration changes are less prominent compared to for Ca^{2+} whose concentration may change by orders of magnitude inside cells. Osmotic changes through ion concentration changes may also control cell volume regulation. In addition, channels and transporters control cellular pH. All these factors might affect cancer cell growth and function [8,39], however, much work remains to be done to prove causality of ion channel and Ca^{2+} signal modulation for cancer cell growth and function. Nevertheless, Ca^{2+} channels are already major prospective pharmacological targets to manipulate cancer cell proliferation and apoptosis.

4. The immune response and calcium

When normal cells are transformed into cancerous cells, they are usually attacked by the immune system. In the main part of this review we will focus on the involvement of Ca^{2+} for a productive and efficient immune response against cancer [1], which is dependent on proliferation, maturation/activation and effector functions of immune cells.

4.1. Proliferation and maturation of CTL and NK cells

Dendritic cells constantly scan the human body and phagocytose foreign antigens. They process antigens and present them or parts of them on their surface. After entering secondary lymph nodes, dendritic cells present foreign antigens to T cells and matching naïve T cells including cytotoxic T lymphocytes (CTL) are recognized; they proliferate, mature and gain effector status. They will then leave the lymph nodes and circulate with the aim to find, in our case, cancer cells. The concept of DC-T cell interaction in secondary lymph nodes is quite well understood, however, in vivo 2-photon imaging during the last decade has added interesting new insights [40,41]. In one of the first papers, Mempel et al. could for instance identify three different phases of CD8^+ -DC interaction in lymph nodes, one stable phase flanked by two transient phases [42].

Natural killer (NK) cells are enriched in sinusoidal regions of the liver and in the red pulp of the spleen under resting conditions [43]. During infection they proliferate and mature from a resting to an effector state, which increases their responsiveness and killer effectiveness. They also infiltrate lymphoid and non-lymphoid tissues to kill targets. Whereas naïve CTL cannot kill because they do not express perforin, resting NK cells express low levels of perforin compared to more matured effector NK cells, but they can also kill [43]. The local environment is probably quite important for priming NK cells and recent data have shown that they continuously mature further and are probably even capable of generating memory [44], with the memory cells being less killing competent as effector NK cells, similarly to the quite well established memory concept in the T cell compartment [43].

Together, CTL and NK are well suited to attack cancer because they can complement each other. For instance, if cancer cells down regulate MHC I to escape the attack by CTL, this would favor recognition by NK cells. Vice-versa, antigen-loaded MHC I expression inhibits NK cell killing but is a prerequisite for CTL-dependent killing.

4.1.1. The calcium dependence of proliferation and maturation of CTL and NK cells

Up to this point very little work has been attributed to the Ca^{2+} dependence and maturation of CTL and NK cells. Whereas the development of T cells, including CD8^+ and NK cells, does not seem to depend much on STIM1/ORAI1-mediated Ca^{2+} entry [25,30–35], proliferation, maturation and activation of T cells strongly depend on CRAC channel activity, formed by STIM1-activated ORAI1 [45–49]. In T helper cells, the Ca^{2+} dependence of proliferation is firmly established, and in addition the maturation of naïve to effector cells is quite Ca^{2+} dependent,

considering for instance that Feske et al. have shown that about 2/3 of activation or repression of gene expression in T cells is Ca^{2+} dependent [46]. Thus the number of armed effector T cells critically depends on Ca^{2+} influx through CRAC/ORAI channels, highlighting the great importance of Ca^{2+} channels for an effective immune response against cancer. Considering the presence of store-operated Ca^{2+} entry in CTL and NK cells and the expression of STIM-activated CRAC/ORAI channels, it is very likely that proliferation and maturation of CTL and NK is similarly Ca^{2+} dependent as has been shown for T helper cells. However, this hypothesis needs still testing.

4.2. Finding cancer cells

Once armed effector T cells leave the lymph node, they should find or, in the ideal case, even be enriched in cancer tissue. Convective transport in the blood stream will bring effector cells to all parts of the human body, however, they have to infiltrate the endothelial tissue of the blood vessels to reach cancer tissue. Not an easy task as concluded by Constantin and Laudanna [50] “for a cell to stop its motion under ‘frantic’ flow conditions, such as those encountered in blood vessels”.

In case of inflammation, which is a condition that also requires immune cell action, the enrichment of CTL and other immune cells in tissues is quite well understood [51]. Immune cells interact with endothelial cells, they are captured, they roll, are activated and arrested, which is followed by strengthening adhesion between immune cells and endothelial cells, and the immune cells finally migrate through the endothelial layer in a paracellular or transcellular manner and can thus be enriched in inflamed tissues. Capture and rolling of immune cells are facilitated by slower blood flow in inflamed tissue due to blood vessel relaxation and an increased expression of selectins on endothelial cells in inflamed tissue which can interact with several surface receptors on immune cells including P-selectin glycoprotein ligand, E-selectin ligand 1 and CD44 (see [51] for details). Endothelial integrins also influence immune cell rolling but in addition they are the key molecules to establish firm adhesion between endothelial cells and immune cells. Arrest and activation of immune cells, which are required for their subsequent transmigration through the endothelial cell layer into the tissue, is initiated by chemokines and chemoattractants [51,52]. The importance of extracellular chemokines on the endothelial surface for lymphocyte adhesion has been recently extended by Shulman et al. [53] who found that T cells express integrins on their surface that can bypass chemokine signals but are still arrested firmly on the endothelial surface. Transendothelial migration of T cells did depend, however, on intracellular chemokines stored in endothelial vesicles highlighting once more their role for adhesion and transmigration.

Chemokines and chemoattractants activate G-protein-coupled receptors in T cells, which modulate integrin affinity for its ligands through signal cascades which are not completely understood but do involve changes in cytosolic Ca^{2+} concentrations. The best studied lymphocyte integrin is the $\alpha_2\beta_2$ integrin LFA-1 (lymphocyte function-associated antigen 1). LFA-1 is kept in an inactive state, the bent state [54] on circulating lymphocytes. Its activation into the extended state is achieved following receptor activation for instance through chemokines and chemoattractants, so called “inside-out” signaling because inside signals change the extracellular (“out”) LFA-1 conformation from the bent to extended state. Active integrins vice versa can also signal to the inside, a mechanism called “outside-in” signaling. In contrast to the bent state, the extended state has an intermediate to high affinity for ICAM-1 on endothelial cells and it will also facilitate the lateral membrane motility of LFA-1 into clusters [55]. LFA-1 is also important for (the initiation of) integrin-dependent cell transmigration through the endothelial cells layer and integrin-dependent migration in tissues. In three dimensional tissue like most inflammatory or cancer environment, integrin mediated migration is probably less important and immune cells use integrin-independent flowing and squeezing

amoeboid-like migration driven solely by actin-network expansion [56] through actin-treadmilling controlled by a MEK-cofilin signaling module, which does not operate in two-dimensional but only in three-dimensional conditions [57].

In inflamed tissue, chemokines and chemoattractants probably help to enrich immune cells including CTL and NK cells, but in case of cancer, it appears more likely that immune cells may reach cancerous tissue just through random migration. Enrichment of immune cells in cancerous tissue could be achieved by a mechanism where cancer works as an anchor, meaning that immune cells do not easily leave cancer tissue once they invaded it because of their many interactions with it.

4.2.1. The calcium dependence of finding cancer cells

There are several ways that Ca^{2+} may contribute to the efficiency of finding cancer cells, for example during immune cell migration/chemotaxis and adherence to the target cells as well as during tumor cell recognition. First of all, the integrin LFA-1 and Ca^{2+} can interact in different ways:

1. Ley et al. [51] have combined the current knowledge into a putative model of LFA-1 activation: Inside-out signaling through G-protein coupled receptors induces the activation of the PLC signaling cascade with its two arms, generation of DAG and an increase of the cytosolic Ca^{2+} concentration through Ca^{2+} release from stores and subsequent Ca^{2+} influx through STIM-gated CRAC/ORAI Ca^{2+} channels. DAG, Ca^{2+} and Ca^{2+} -calmodulin together activate GTPases which directly or through intermediates act on LFA-1. By modulation of Ca^{2+} signals, immune cells could thus modify LFA-1 activation, which in turn would influence immune cell arrest and migration and thereby the number of immune cells in cancer tissue.
2. In addition, LFA-1 contains a Ca^{2+} coordination site in its short disulfide-bonded genu loop which is preserved during activation from the bent to the extended state [54]. Whether this coordination site is modulated in any way is presently unknown. Since extracellular Ca^{2+} under most conditions should be quite constant, one would not predict that this coordination site plays a modulatory role in LFA-1 activation. However, in case external Ca^{2+} concentrations change in cancer tissue (see below), LFA-1 function could be changed through this mechanism.
3. Outside-in signaling through LFA-1 contributes to Ca^{2+} signaling. It activates Ca^{2+} entry in T cells [58]. Interestingly, chemokine activation of LFA-1 recruits the MTOC and mitochondria to the immune synapse [59], where mitochondria control the activity of CRAC/ORAI Ca^{2+} channels in T cells [60,61]. This mechanism could also be relevant at the contact point between endothelial cells and T cells and would in this case influence cytosolic Ca^{2+} signals in T cells. This may be highly relevant for T cell arrest and transmigration through the endothelial layer. Sustained Ca^{2+} signals are correlated with T cell arrest during positive selection [62] and it is reasonable to assume that high Ca^{2+} signals are also relevant for T cell arrest at the endothelial cell layer.

Cytosolic Ca^{2+} signals are also well known to influence cell migration in many cell types including immune cells. There is a large body of evidence that many Ca^{2+} permeable ion channels are relevant for cell migration. Small, partly local cytosolic Ca^{2+} signals are usually needed for optimal cell migration [63]. Thus, by influencing ion channel expression or localization, cells could modulate their migration behavior and speed, and this may in turn influence the efficiency of the immune attack against cancer.

4.3. Immune synapse formation and killing

After successful migration to the cancer tissue and infiltration of the tissue, which depends largely on tumor antigens, CTL and NK

cells interact with cancer cells. A major problem is of course to recognize cancer cells as targets that need to be eliminated, because cancer cells try to escape detection by several mechanisms including surface receptor regulation including adhesion receptors, MHC or Fas. We do not want to discuss these very important issues here because nothing is to our knowledge known about a potential Ca^{2+} dependence of these mechanisms. Thus, we assume that either CTL or NK cell can recognize particular cancer cells. That means, they will form an immune synapse with the cancer cells with the goal to kill them. The two most important killing mechanisms are the release of lytic granules filled with perforin, which can perforate membranes, and granzymes, which have proteolytic activity, at the IS and the activation of Fas–FasLigand receptors, also called death receptors [64]. These two mechanisms can probably work in parallel, however, little quantitative data is available.

In a first approximation, release of lytic granules in CTL and NK cells appears not to be that different from vesicle release in other eukaryotic cell types [65–67]. While certain molecular details are probably different between CTL/NK and neurons/chromaffin cells (the latter are often used as a model of an electrically excitable cell to study secretion), the molecular toolbox appears to be quite similar, a clear indication for similarity of the actual release event between immune cells and neurons. Considering the very different functions of the cells, there are phenotypic differences:

1. The kinetics are different. Neurons respond on a millisecond time scale [68], chromaffin cells usually at on a second time scale [69,70], whereas CTL and NK cells work most usually on a minute time scale [71–73].
2. A neuronal synapse is mostly stationary, which means that vesicles can be accumulated and even be pre-docked at the same subcellular localization within the synapse to facilitate a fast and reliable synaptic transmission. In chromaffin cells, it may not always be that important where exactly vesicles are released. In CTL/NK cells, it is very important that lytic granules are release at the immune synapse, but the location of the immune synapse is not predefined and depends on the cell-cell contact area. Thus, lytic granules have to be transported to the immune synapse on demand.
3. How foolproof should vesicle release or lytic granule release from CTL or NK cells be compared to other eukaryotic cells to secure proper killing of the respective targets?

Next to the perforin/granzyme dependent target killing through the release of lytic granules, CTL can also kill in parallel by activating Fas receptors on target cells through Fas ligand (FasL) binding. Fas is a member of the tumor necrosis factor (TNF) receptor family, which all contain death domains and trigger apoptosis through the activation of caspases including caspase 3, either in a mitochondrial-dependent or – independent way. Fas-dependent apoptosis of target cells is somewhat slower than lytic granule-dependent killing and believed not to be Ca^{2+} dependent [64,74] which is why it will not be considered in more detail in this review.

4.3.1. The calcium dependence of immune synapse formation and killing

Contact between killer cells and cancer cells is achieved 1) through surface receptors like T cell receptors of CTL, which specifically interact with antigen-presenting MHC on cancer cells and 2) through adhesion molecules like the integrin LFA-1 on T cells with its respective adhesion receptor counterparts on cancer cells (Fig. 1A). An IS is usually formed if TCR binds to the MHC-antigen receptor of the respective cancer cell with high enough affinity. In contrast, NK cells will bind with high enough affinity to the targets and are able to kill them if more activating rather than inactivating receptors are present on their surface and if they are more efficiently activated by target surface receptors than the inhibitory ones.

IS formation in T cells is characterized by TCR and LFA-1 accumulation at the contact point between killer cell and target cell as well as by actin

cytoskeleton rearrangement towards the IS [64], the latter of which is not shown in Fig. 1A for simplicity. The microtubule organization center (MTOC), Golgi apparatus, mitochondria and lytic granules also polarize to the IS. Ca^{2+} influx is not necessary for IS formation as indicated by actin accumulation in the complete absence of extracellular Ca^{2+} , however, T cell polarization is not complete under these conditions because mitochondria are not relocalized to the IS if Ca^{2+} entry through ORAI channels is completely blocked [75]. This is not surprising because motor-based transport of mitochondria requires Ca^{2+} elevations which cannot be maintained by the transient Ca^{2+} release from internal stores but requires Ca^{2+} influx across the membrane. Thus, certain functional implications of IS formation require Ca^{2+} entry, however a (less functional) IS may be formed without the need of any Ca^{2+} influx.

A potential role for Ca^{2+} in target cell contact has been described for thymocytes [62]. The rise of intracellular Ca^{2+} through Ca^{2+} oscillations was necessary and sufficient to immobilize thymocytes as if Ca^{2+} provided a STOP signal during positive selection. Thus Ca^{2+} could have a function to determine the length of IS formation; whereas high Ca^{2+} immobilizes CTL and NK cells, lowering Ca^{2+} again may provide a signal to break the symmetry of the IS, which is considered to be the signal to restrict IS duration. Thus, Ca^{2+} may play a role to control IS duration and enforce kinetic synapses (also called kinapses), meaning that cells are mobile during IS formation [64]. Kinapses would of course be the most efficient way for CTL to kill target cells if contact time is sufficient to secure lytic granule exocytosis. This is an interesting, but as of yet not experimentally tested, role for Ca^{2+} influx in CTL function.

By analyzing ORAI1-deficient NK cells from a patient, Maul-Pavicic et al. [36] observed that, while store-operated Ca^{2+} entry was deficient (see below), NK-activating and inhibiting receptor expression was mostly normal, perforin expression was normal, cytotoxic granule polarization was not impaired, and inside-out signaling for LFA-1 activation was normal. This indicates quite normal NK–target cell “contact signaling” in the absence of large Ca^{2+} rises, similarly to CTL. Therefore, we conclude that Ca^{2+} is not required to make the initial contact between CTL and NK cells, it is however required for downstream signaling following contact formation.

In case of CTL, TCR stimulation induces Ca^{2+} store depletion and Ca^{2+} influx (Fig. 1A). Zweifach clearly demonstrated that this influx is dependent on store depletion and that it resembles the hallmarks of CRAC channels found in T helper cells and mast cells [76]. This Ca^{2+} entry is required for target cell lysis by CTL [3,77]. Following the discovery of STIM and ORAI in 2005 and 2006, it was shown that CTL and NK cells express STIM1 and ORAI1 and that STIM1/ORAI1 dependent Ca^{2+} entry is present in CTL and NK cells [31,35,36,78]. Very importantly, Maul-Pavicic et al. [36] showed that the cytotoxicity of NK cells and their target killing potential greatly depend on STIM1/ORAI1 dependent Ca^{2+} entry.

To discuss the Ca^{2+} dependence of target cell killing in detail it is necessary to review the actual killing process. We focus on the lytic granule-based killing because it is certainly Ca^{2+} dependent, whereas Fas–FasL dependent killing is probably not at all Ca^{2+} dependent [64,74]. Following IS formation, lytic granules have to be transported to the IS to release perforin and granzymes at the IS to kill target cells. Transport of lytic granules to the IS has received great attention during the last couple of years. Griffiths and colleagues have put forward a model, in which lytic granule transport to the IS is mediated by MTOC docking at the IS [73,79,80]. While MTOC docking at the IS is not necessary for lytic granule exocytosis [81], the MTOC–IS interaction model is attractive because it could secure directed and efficient lytic granule release at, and only at, the IS. However, two recent papers have challenged the MTOC–IS docking model. Our lab has shown that Vti1b-dependent vesicle tethering between lytic granules and TCR-containing vesicles is required for docking of lytic granules at the IS but that the MTOC is still 0.5 μM away from the IS [72]. Furthermore, Kurwska et al. have presented compelling evidence that kinesin-1 (in a complex with Slp3 and Rab27a) is required for the

terminal transport of lytic granules to the IS, a process that appears to exclude MTOC-IS docking [82,83]. We illustrate both models and a hybrid of both models in Fig. 1B. In the hypothetical hybrid model, tethered LG-TCR vesicles are transported in a dynein-dependent way towards the MTOC and in a kinesin-1-dependent way from the MTOC to the IS. In this model, the MTOC is polarized towards the IS as has been reported by many labs including ours but not as close as reported by Griffiths and colleagues.

Once transported and accumulated at the IS, lytic granules have to fuse with the plasma membrane to release perforin and granzymes at the IS to kill the target cell but not innocent bystander cells. The molecular mechanism of exocytosis in CTL is only partially understood. Most of the molecular players have been identified from patients with a dysfunction in CTL and/or NK-mediated killing which can result in familial or acquired hemophagocytic lymphohistiocytosis (FHL or HL) [43,65,66,84–86]. Next to perforin, which is mutated in FHL2 [86],

Munc13-4 (FHL3) [87], syntaxin11 (FHL4) [88] and Munc18-2 (FHL5) [89] are involved in lytic granule docking, priming or fusion. In addition, Vti1b and Vamp8 are important for the killing capacity of CTL [72,90].

Obviously, CTL and NK cells are not the best-studied membrane and vesicle fusion systems. In many other eukaryotic cells, fusion has been studied in much more detail [67] and many of the molecular players have been identified and detailed mechanisms have been proposed. Südhof and Rothman [67] have recently summarized membrane fusion as follows: SNARE proteins act as force generators, with t-SNAREs on the target membrane and v-SNAREs on the vesicles, SM (Sec1/Munc18-like) proteins are shaped like clasps and control the fusiogenic action while regulators like complexin and synaptotagmin, the latter of which is regarded as one of the Ca^{2+} sensors, control the timing of exocytosis. Before vesicles can fuse with the plasma membrane they are docked and primed by docking and priming factors like Munc18 and Munc13 [91,92].

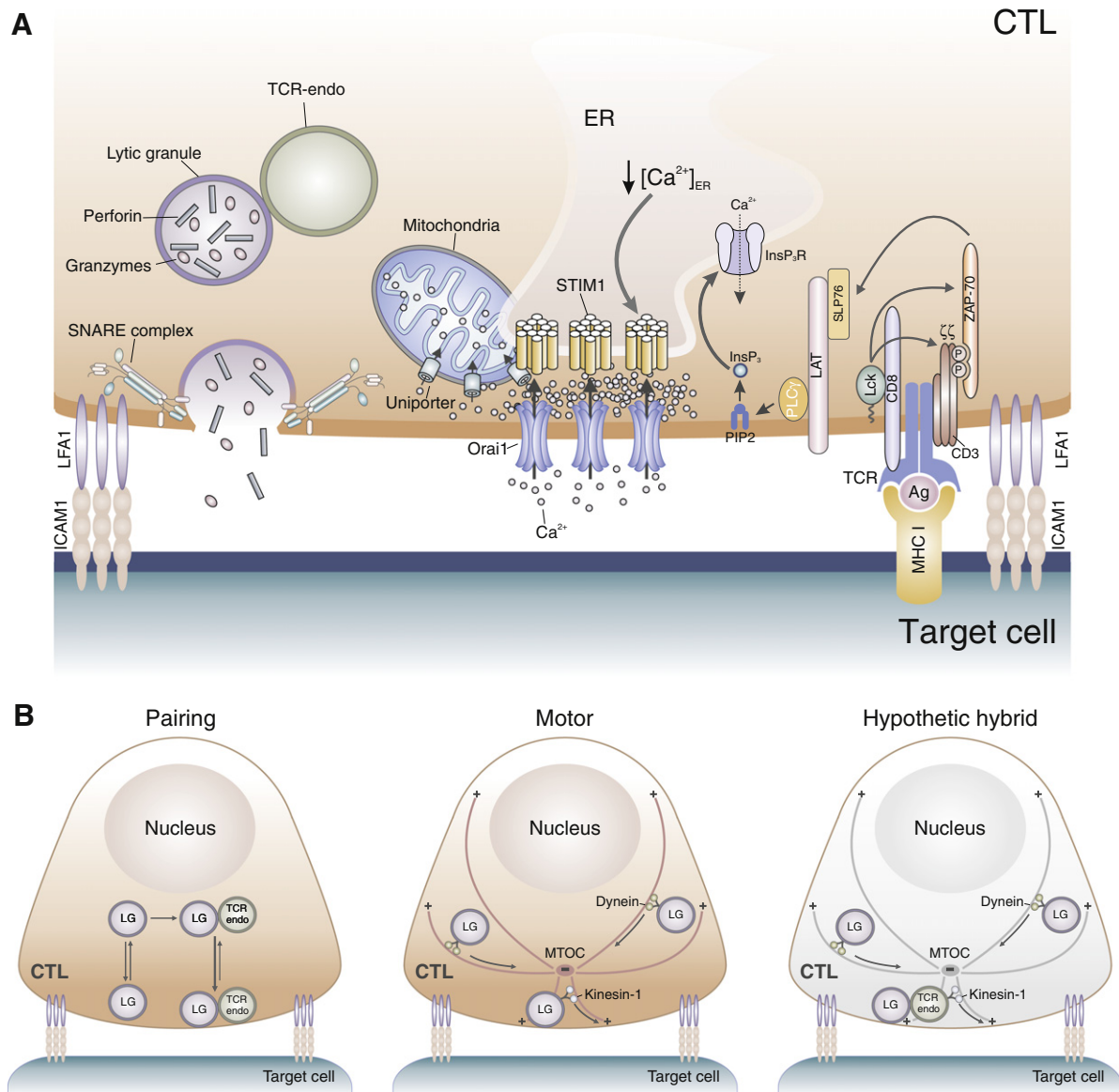


Fig. 1. The immune synapse between CTL and target (cancer) cells. (A) TCR activated Ca^{2+} entry and lytic granule release. TCR stimulation through a well-known signaling cascade involving ZAP-70, Lck, LAT, PLC γ and IP $_3$ induces Ca^{2+} depletion from the ER store. Ca^{2+} de-binding induces STIM1 multimerization and in the ideal case twelve STIM1 molecules activate one hexameric ORAI1 channel [100]. Ca^{2+} entry through ORAI1 channels at the IS is heavily controlled by closely mitochondria, which control Ca^{2+} dependent ORAI1 inactivation. Lytic granule fusion is controlled by SNARE complex, triggered upon Ca^{2+} entry. (B) Models to enrich lytic granules at the IS. Our lab has shown that Vti1b-dependent vesicle tethering between lytic granules and TCR-containing vesicles is required for docking of lytic granules at the IS (Pairing) [72]. Kinesin-1 (in a complex with Slp3 and Rab27a) is required for the terminal transport of lytic granules to the IS, a process that appears to exclude MTOC-IS docking (Motor). We illustrate both models and a hybrid of both models in Hypothetic hybrid. In this model, tethered LG-TCR vesicles are transported in a dynein-dependent way towards the MTOC and in a kinesin-1-dependent way from the MTOC to the IS.

Comparing the SNARE and Munc proteins involved in lytic granule release from CTL or NK cells to the proteins described in the well-studied eukaryotes [65], it appears reasonable to assume that molecular mechanisms are similar in CTL/NK cells and other eukaryotic cell types. Considering that CTL have on average only about 18 perforin-containing lytic granules in the resting state [72], that CTL may only release one or a few lytic granules per target cell killing, and that timing with a precision of seconds or even milli-seconds may not be required at the IS, some differences between the molecular mechanisms in CTL and NK cells compared to other eukaryotic cells are however expected.

Which of the above-mentioned processes in CTL or NK are Ca^{2+} dependent? At present nothing is known about a potential Ca^{2+} dependence of vesicle transport to the IS. The transport of other organelles to the IS is however Ca^{2+} dependent. Mitochondrial polarization to the IS is Ca^{2+} dependent [75]. The molecular mechanism of Ca^{2+} -dependent motor-assisted transport of mitochondria along microtubules has been unmasked by Wang and Schwarz [93]. They showed that the EF hands of the mitochondrial Ca^{2+} -binding Rho-GTPase Miro mediates the Ca^{2+} dependent arrest of mitochondrial motility. Ca^{2+} binding permits Miro to directly interact with the motor domain of kinesin-1 which prevents the motor-microtubule interaction and subsequently arrests mitochondria. Considering the dynein and kinesin-1 dependent transport of lytic granules along microtubules [82] (compare also Fig. 1B), the Rho GTPase Miro may modify lytic granule transport the same way as mitochondrial transport to the IS. If true, lytic granule accumulation at the IS could be modulated by the internal Ca^{2+} concentration.

Lytic granule fusion is the other target for Ca^{2+} control. While the Ca^{2+} dependence of lytic granule fusion is undisputed in CTL and NK cells [3,36,77], the exact mechanisms and their Ca^{2+} dependence have to be elucidated (compare [65] for a detailed and critical discussion). Here, we discuss a model that illustrates the importance of Ca^{2+} influx through ORAI channels for CTL or NK cell killing. Fig. 2A illustrates the normal ORAI function in killing. TCR stimulation, through a well-known signaling cascade involving ZAP-70, Lck, LAT, PLC γ and IP $_3$, induces Ca^{2+} store depletion (compare Fig. 1A). Ca^{2+} de-binding induces STIM1 multimerization and in the ideal case twelve STIM1 molecules activate six ORAI1 subunits, which are assumed to form one functional channel [100]. Ca^{2+} entry through ORAI1 channels at the IS is heavily controlled by closely mitochondria [94,95], which modulate Ca^{2+} dependent feedback inhibition of ORAI1. If ORAI2 and ORAI3 also accumulate at the IS and are controlled by mitochondria has not been investigated yet. Normal (modest) entry of Ca^{2+} through ORAI channels induces the fusion of a few (in this case two) lytic granules per target cell in a SNARE-dependent manner similar to other eukaryotic cells [65–67]. Considering the average number of 18 lytic granules per CTL [72] (only 6 are shown for simplicity in Fig. 2A), one CTL could serially kill up to 9 target cells (3 are shown in Fig. 2A) without refueling their lytic granule pool (Fig. 2A). If ORAI-dependent Ca^{2+} influx is now dramatically decreased, Ca^{2+} dependent lytic granule exocytosis would be impaired. It has been recently shown by Maul-Pavicic et al. [36] that the absence of ORAI in primary human NK cells from a patient leads to reduced Ca^{2+} signals and decreased lytic granule exocytosis. This is in a way a “trivial” case: No functional ORAI1 – no Ca^{2+} entry (Fig. 2B). Interestingly, CTL could also use the STIM1-ORAI1 protein ratio to modulate exocytosis. It has been shown that one ORAI1 channel (with its six subunits [100]) needs twelve STIM1 molecules to carry the maximum CRAC current

[96,97]. By reducing STIM1 expression, Ca^{2+} entry decreases, but interestingly also by increasing ORAI1 expression, Ca^{2+} entry decreases almost to not detectable levels because too many ORAI1 channels compete for the insufficient number of STIM1 and no ORAI1 channel obtains the optimum of twelve STIM1 molecules. Thus, by shifting the STIM1 to ORAI1 expression ratio, Ca^{2+} influx can be greatly decreased resulting in decreased lytic granule exocytosis. CTL could also delocalize their mitochondria away from the IS, thereby reducing Ca^{2+} entry [61,94]. All these maneuvers, by decreasing local Ca^{2+} at the IS, would decrease lytic granule release and inhibit cancer cell killing (Fig. 2B). Using the reverse maneuvers, Ca^{2+} influx could be maximized. For instance, by optimizing the STIM1-ORAI1 ratio, CTL or NK cells may significantly increase their Ca^{2+} entry, and this could result in an increased lytic granule release. This may result in lytic granule depletion with the result that only the first or first few target cells could be killed (Fig. 2C). Thus Ca^{2+} influx in CTL or NK cells should be adjusted to the need of the immune response against cancer to secure efficient serial target cell killing.

5. Conclusions and perspective

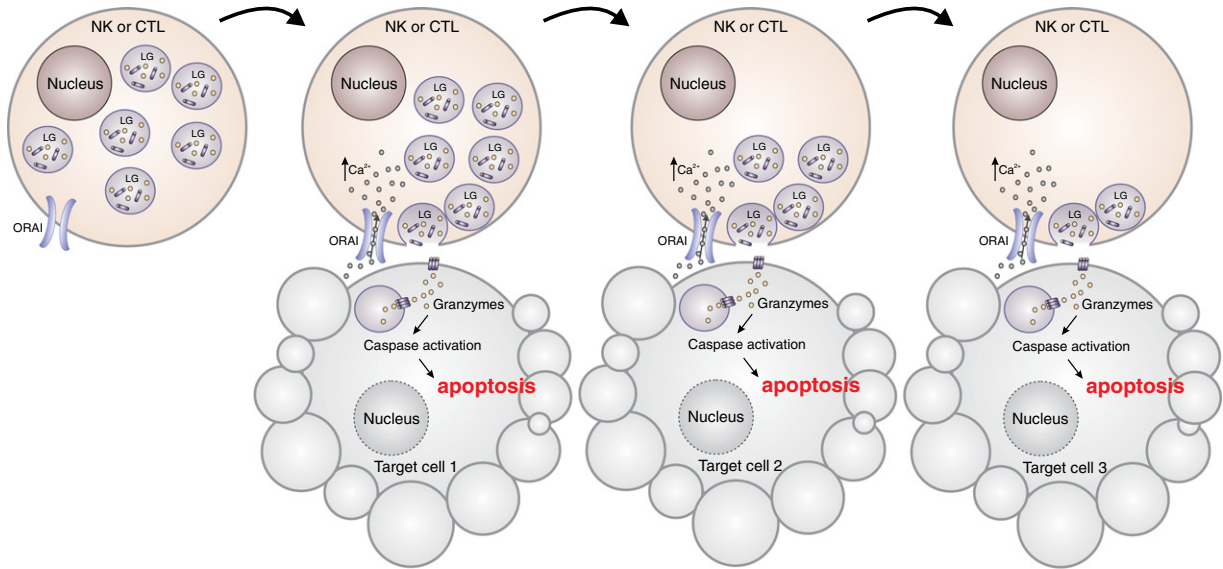
Several steps during the killing of cancer cells by CTL and NK cells are Ca^{2+} dependent. While it is reasonable to assume the Ca^{2+} dependence of finding cancer cells, lytic granule transport, and IS duration considering the combination of several published reports and the approximation from other T cell subtypes or other organelles, these concepts need to be rigorously tested in the future. The Ca^{2+} dependence of lytic granule exocytosis has been clearly demonstrated but needs to be analyzed in more quantitative detail. In this regard it is of particular interest to define the Ca^{2+} channels responsible for Ca^{2+} influx at the IS in CTL (in NK, ORAI1 is clearly very important for lytic granule exocytosis), but also to define the different STIM-ORAI ratios not only for STIM1 and ORAI1. This could be an interesting mechanism to modulate the killing efficiency of CTL and NK cells. The immune system would hopefully try to optimize this ratio, while cancer may try to de-optimize it. Another very important aspect of understanding the role of Ca^{2+} to efficiently kill cancer cells is the quantification of external Ca^{2+} in cancerogenous tissue. At the moment it is assumed that the free external Ca^{2+} concentration is close to the free serum Ca^{2+} , which is around 1.2 mM, but nobody appears to know if this is really true. Fluctuations from this value may greatly influence cancer cell killing by CTL and NK and CRAC channels are well-suited to modulate killing because the KD for Ca^{2+} permeation is in this range [98,99]. This means that small variations in external Ca^{2+} could significantly alter Ca^{2+} signals and Ca^{2+} dependent target cell killing. Controlling external Ca^{2+} could thus greatly influence tumor growth but also CTL and NK cell killing efficiency and this should be tested in the future.

Abbreviations

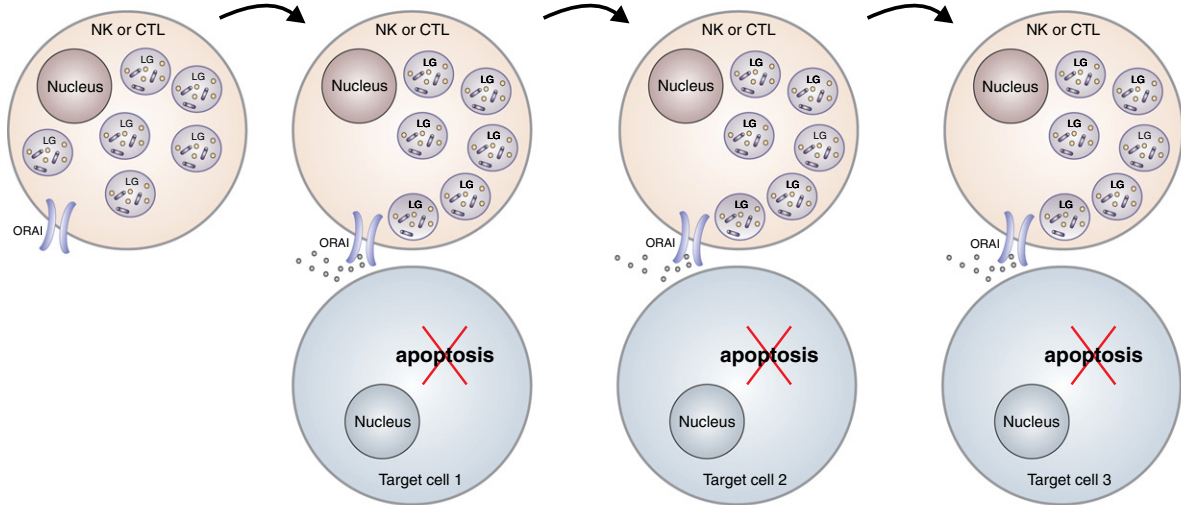
APC	antigen-presenting cell
[Ca^{2+}] _i	intracellular Ca^{2+} concentration
CRAC channel	Ca^{2+} release-activated Ca^{2+} channel
ER	endoplasmic reticulum
CTL	cytotoxic T cells
IS	immune synapse
LFA-1	lymphocyte function-associated antigen 1
NFAT	nuclear factor of activated T-cells
TCR	T cell receptor

Fig. 2. The role of the Ca^{2+} influx magnitude through ORAI channels for efficient target cell killing. (A) Balanced vesicle release. When the Ca^{2+} entry is optimized, the appropriate number of lytic granules is released upon target cell engagement, assuring proper serial killing of several target cells. (B) No vesicle release. When Ca^{2+} influx is impaired, for example by shifting the STIM1 to ORAI1 expression ratio or by dysfunctional ORAI1 channels, no lytic granules are released, resulting in dysfunctional target cell cytotoxicity. (C) Unbalanced vesicle release. If Ca^{2+} influx is too large, lytic granule release could be increased with the enhanced Ca^{2+} entry. This may result in lytic granule depletion with the result that only the first or first few target cells could be killed until new lytic granules are being produced.

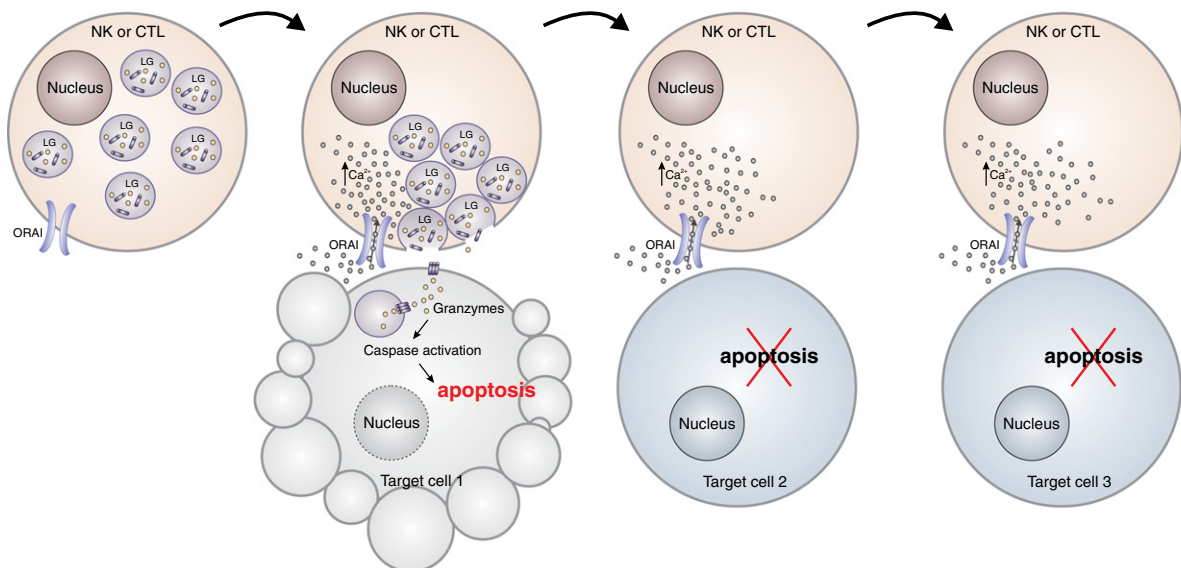
A balanced vesicle release



B no vesicle release



C unbalanced vesicle release



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