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Assembly and function of the natural killer cell immune synapse

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1. ABSTRACT

During the natural killer (NK) cell immune response, cytoskeletal components, adhesion and signaling molecules and ligand-specific receptors are involved in the formation of the natural killer cell immune synapse (NK-IS), a highly organized supramolecular structure assembled at the interface of the interacting cells that regulates the NK cell activation and decides the target cell fate. In this review the current state on knowledge about the organization and physio-pathological function of the inhibitory, cytotoxic and activating NK-IS is presented. Moreover, it briefly summarizes microspectroscopy techniques suitable for live cell imaging of the dynamic biochemical processes, which achieve the coordinated NK cell immune responses.

2. INTRODUCTION

Natural killer (NK) cells are essential cytotoxic lymphocytes of the innate immunity able to directly lyse tumour cells, virus infected cells and other pathogens. Furthermore, by secreting cytokines and chemokines, as well as interacting with other immune cells, such as T lymphocytes or dendritic cells (DC), they also regulate both the innate and the adaptive immune responses (1, 2). NK cell function is regulated by cytokines, chemokines, the contact with the extracellular matrix (ECM), and a balance of intracellular signals triggered from the cell surface by a diverse group of activating and inhibitory receptors upon the specific binding of ligands expressed on surrounding cells (3). As predicted by the *missing self* hypothesis, inhibition is mainly mediated by the specific binding of the

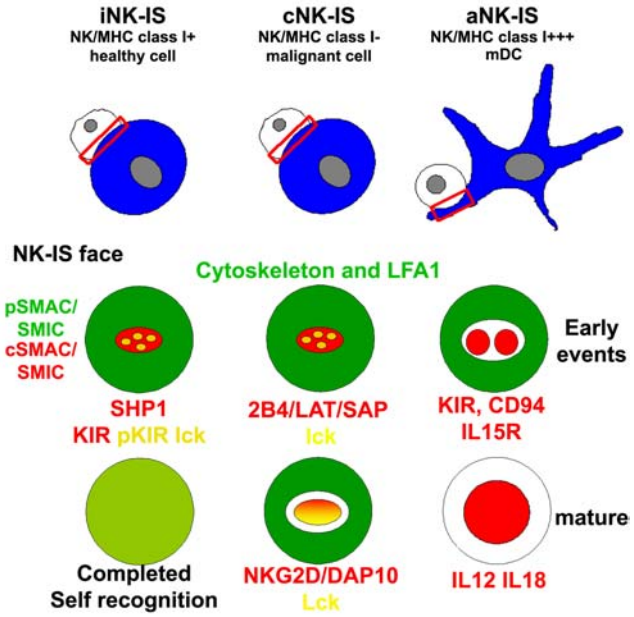

Figure 1. Organization of the cNK-IS, iNK-IS and aNK-IS. Schematic representation of the synapse face arranged inside the red boxes. Main components present at the central (c)SMAC/SMIC and peripheral (p)SMAC/SMIC are detailed at the early events and mature state of the different synapses described in the text.

MHC class I molecules by the killer cell immunoglobulin-like (Ig-L) receptors (KIR) and the NKG2A/CD94 lectin-like (L-L) receptor heterodimer (4). These receptors switch activating signals off by recruiting the SH2-containing protein tyrosine phosphatase SHP-1 to an immunoreceptor tyrosine based inhibitory motif (ITIM) placed in their cytoplasmic tails (5, 6). A wide repertoire of Ig-L and L-L activating receptors, which bind different adaptor molecules in order to form activating complexes, has been described. The activating receptor complexes in the Ig-L group include the natural cytotoxicity receptors (NCR), NKp46/CD3ζ, NKp30/CD3ζ and NKp44/DAP12, the Fc receptor CD16/CD3ζ/FcγR, the signaling lymphocyte activating molecules (SLAM), 2B4/LAT (Linker for activation of T cells), NTB-A and CRACC, and the activating KIR. The L-L group is mainly represented by NKG2D/DAP10, CD94/NKG2C/DAP12 and NKp80 (7-9). Upon the binding of the specific ligands, activating signals are triggered by immunoreceptor tyrosine based activation motifs (ITAMs) present at the cytoplasmic tail of the adaptor molecules DAP12, CD3ζ and FcγR, by the PI3K and Grb2 binding motif present in DAP10, by the tyrosine based motifs present at the cytoplasmic tail of LAT and by the immunoreceptor tyrosine-based switch motifs (ITSM) present at the cytoplasmic tail of 2B4, NTB-A and CRACC. The cellular ligands described for some of these receptors and the signaling molecules recruited upon ligand binding are summarized in the table 1.

In 1999 two important contributions were made by Sentman and co-workers (10): First, inhibitory receptors altered the interaction with target cells, a fact that showed the significance of receptor-ligand binding for the fate of cells after the contact. Secondly, cytotoxic and non-cytotoxic interactions were simultaneously performed by single NK cells, which demonstrated the existence of a spatio-temporal regulation of activating and inhibitory signals during the NK cell response. These results posed a new question: How do surface receptors and signaling molecules achieve this spatio-temporal regulation that eventually decides the fate of the target cells? In answering this question, the immune synapse (IS) (11) has emerged as a highly organized supramolecular cluster at the interface of contacting immune cells that allow the intercellular communication needed for a coordinated immune response to be obtained (12). This review will focus on the organization and physiological significance of the NK cell immune synapse (NK-IS) established at the cytotoxic and non-cytotoxic interactions formed during the NK cell "social" life.

3. ASSEMBLY OF THE NATURAL KILLER CELL IMMUNE SYNAPSE (NK-IS)

Receptor tyrosine phosphorylation of ITIMs and ITAMs by Lck is an early common phenomenon necessary for both the inhibition and activation of NK cell effector functions respectively (13). However, the different spatio-temporal organization of supramolecular activating or inhibitory clusters (called SMAC or SMIC respectively) will regulate the NK cell cytotoxic activity and therefore the eventual fate of the target cell (14, 15). Recently, the activating, non-cytotoxic, NK-IS formed between resting NK cells and mature (m) DC has been described (16). The organization of inhibitory (i), cytotoxic (c) and activating (a) NK-IS (Figure 1) is described below.

3.1. The inhibitory NK-IS

The NK-IS was first described in 1999 during the *missing-self* recognition (17). Exogenously expressed KIR

and their specific MHC class I ligands clustered and co-localized at the contact area formed during NK cell-Target cell interactions. Importantly, later studies done by Dupont and co-workers have shown that very early after the interaction of primary activated NK cells with MHC class I positive cells, the constituted iNK-IS is characterized by a central single cluster of the phosphatase SHP-1 and KIR (cSMIC) surrounded by a peripheral ring of talin and the αLβ2 integrin leukocyte function associated antigen 1 (LFA-1) (pSMIC) (14, 15, 18). The activating signaling molecule Lck is seen dispersed in two or three clusters at the cSMIC by this time, consistent with the requirement of early tyrosine phosphorylation for KIR inhibition. However, this accumulation is rapidly dissolved to multiple small clusters. Other signaling molecules such as ZAP-70, SLP-76, PLC-γ, Itk or PKC-θ are not recruited to the iNK-IS. The microtubule organizing centre (MTOC) and secretory lysosomes (SL) are not polarized to the iNK-IS while talin accumulation is dissolved within 5 min of interaction. Then, although inhibition mediated by KIR involves the cortical cytoskeleton remodelling for the active formation of SMIC of signaling molecules, non-cytotoxic conjugates are short lived probably due to an absence of further downstream signaling. In fact, talin, Lck and SHP-1 do not show significant accumulation after 10 min of non-cytotoxic interactions, which suggests completion of the self recognition (Figure 1).

Recently Davis and co-workers have shown that although inhibitory KIR are clustered across the iNK-IS, phosphorylation of these receptors are restricted to discrete microclusters along with Lck clusters (figure 1) (19). Thus, one hypothesis for NK cell inhibition could be that Lck recruited to the membrane by activating receptors would be able to trans-phosphorylate KIR molecules engaged with the specific MHC class I molecule at the iNK-IS. Thus, the inhibitory action of KIR would be locally focused at the early interface of the NK cell-target cell interaction where activating signals are triggered (Figure 2A). Only the absence of MHC class I on target cells would then allow the eventual formation of the cytotoxic NK-IS (Figure 2B-3).

3.2. The cytotoxic NK-IS

The cNK-IS organization resembles the one previously described for cytotoxic T lymphocytes (CTL), which is characterized by a cSMAC that contains a secretory domain, along with a signaling region where the T cell receptor (TCR) accumulates, and a pSMAC enriched in f-actin. CTL acts to clear virally infected and tumour cells via this so called secretory synapse (20). The supramolecular organization at the early and mature cNK-IS is described below.

3.2.1. Early events during the cNK-IS assembly

The early events during the NK cell immunosurveillance are summarized in figure 2B. The first step during the formation of the cNK-IS is the adhesion process mainly mediated by LFA-1. Signaling mediated by LFA-1 induces the tyrosine phosphorylation and activation of Vav-1, which lead to the polymerization of actin and clustering of lipid rafts (21, 22). These events are essential for the final assembly of the mature cNK-IS. In fact, activation of Vav-1/Rac pathway, which precedes actin polymerization, also regulates the generation of cell-mediated killing by CTL (23). Early recruitment of the SLP-76 has also been observed preceding the polarization of the MTOC and SL that occurs within the first 5 min (18). The recruitment of the SLP-76 could be involved in the "inside-out" signaling for integrin activation, consequently sustaining the adhesion during cytotoxic interactions (24). Remarkably, early talin and LFA-1 accumulation is a common phenomenon initiated during conjugate formation, independently of the nature of the target (Figure 2A-B), however, as pointed out in section 3.1, KIR inhibitory action block further downstream signals leading to unstable, short lived conjugates (see also section 4).

Another early event is the formation of Lck small clusters at the synapse (presumably recruited to activating receptors), that will eventually culminate after 5 min of interaction with the formation of one unique cluster of this signaling molecule at the cSMAC surrounded by a ring of talin and f-acting at the pSMAC of the mature cNK-IS (Figure 1). Lck phosphorylation of ITAMs at the cytoplasmic tail of activating receptors lead to the recruitment and activation of Syk kinases, which subsequently induces the activation of PI3K and the eventual polarization of the MTOC and SL, the activation of SLP-76, WASP and Vav-1 complexes, and the phosphorylation of LAT and other adaptor molecules (25-27).

Another important molecule during the early events is the receptor 2B4 (28). Accumulation of 2B4 at the synapse occurs just 30 sec after the cell-cell contact preceding the MTOC polarization and remarkably remains at the synapse over 20 min of interaction. This early accumulation of 2B4 underscores the role of this molecule in cell-cell adhesion and NK cell activation. In addition, the adaptor molecule SAP (SLAM-associated protein) also accumulates at the cNK-IS and co-localizes with 2B4 during this prolonged time, which demonstrates the relevance of this adaptor molecule for the cytotoxic process in NK cells (29). In this regard, SAP is encoded by the *SH2D1A* gene, which is mutated in patients with the inherited immunodeficiency X-linked lymphoproliferative disease (XLP), a human disorder characterized by a dysfunction of the T/B-cell interactions and NK cell function, leading to a reduced ability to control the Epstein-Barr virus (EBV) infection (30, 31). NK cells deficient in SAP are not able to properly trigger activating signals upon 2B4 engagement with CD48. It has been postulated that SAP competes for the binding of SHP-1 to the cytoplasmic tail of 2B4, allowing the activating signals to be properly triggered (Figure 2B) (32, 33). Moreover, SAP is also necessary for the recruitment of Fyn to the cytoplasmic tail of 2B4 (34). The essential role of SAP on the cytotoxic synapse formation has been demonstrated in SAP-deficient CTL. These cells present a defective polarization of perforin, GM1 and 2B4 to the synapse, which leads to a defective lytic activity against EBV infected cells (35). Thus, SAP may also control the assembly of the cNK-IS

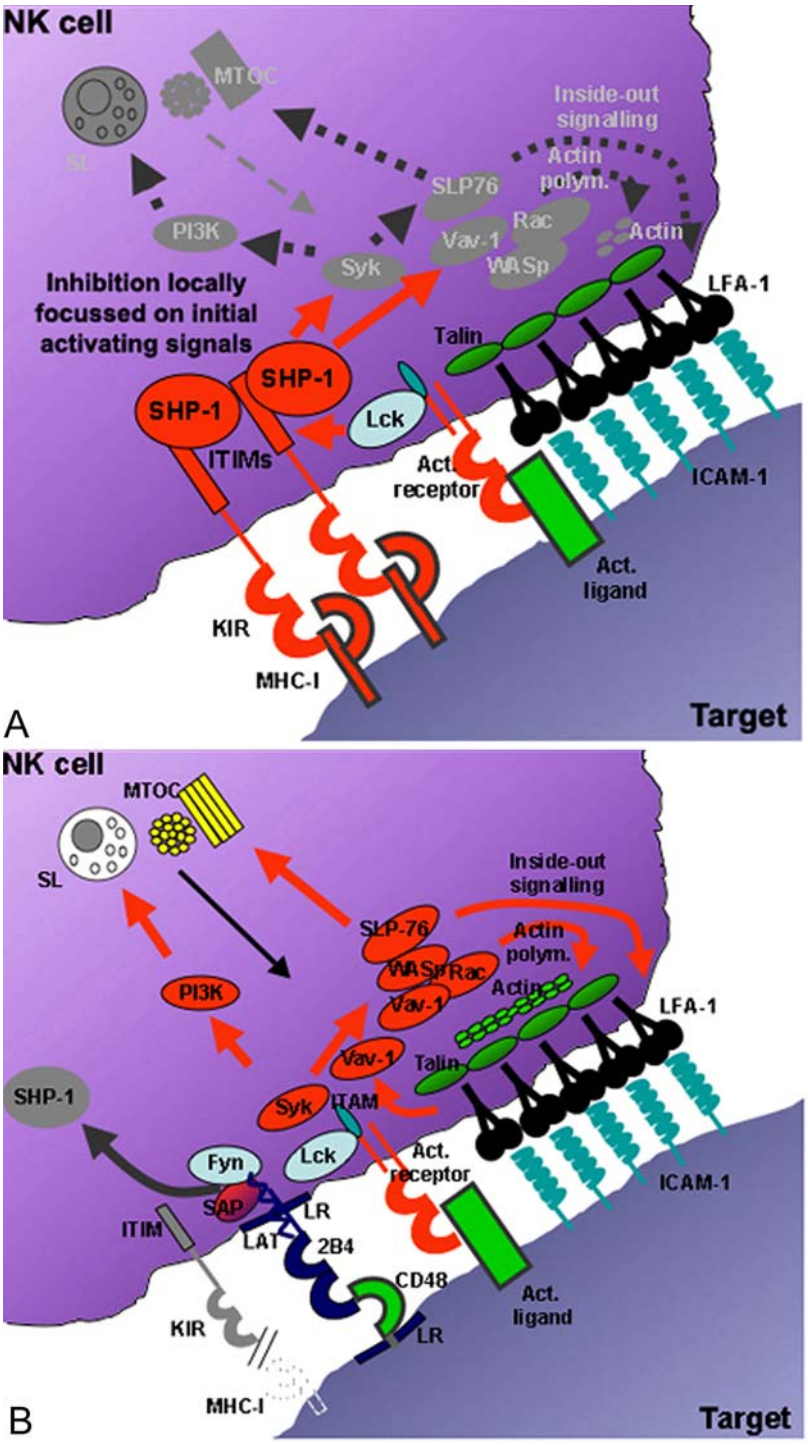

Figure 2. Early events during the NK cell *missing-self* recognition (A) or activation (B). **(**A) Early Adhesion events and activating (act.) ligand recognition induce the activation of Lck, which trans-phosphorylates in turn the ITIM motifs present at the KIR upon MHC class I engagement. As a consequence a docking site for SHP-1 is created. SHP-1 desphosphorylates and inactivates Vav-1, which is essential for actin polymerization, promoting then the early detachment from the target cell. Grey objects show the blocked pathways upon Vav-1 desphosphorylation. (B) The absence of physiological levels of MHC class I on target cells avoids the recruitment of KIR and SHP-1 and then signals for cytoskeleton rearrangement and inside-out integrin signaling freely occurs. The MTOC and SL can then polarize to the contact site for the eventual establishment of the mature cNK-IS. LR: lipid rafts. Red arrows: On going pathways. The black arrow indicates the directional movement of the MTOC and SL to the cSMAC. SAP competes the SHP-1 binding to 2B4 (grey arrow).

Table 1. Activating receptor complexes

Estructure	Receptor	Adaptor molecule	Signaling molecules	Cellular Ligand
L-L	NKG2D	DAP10	PI3K, Grb2	MICA-B, ULBPs
	CD94/NKG2C	DAP12	Lck, Syk	HLA-E
	NKp80	-	-	AICL
Ig-L	NKp30	CD3ζ	Lck, Syk	Viral hemaglutinins
	NKp44	DAP12	Lck, Syk	Viral hemaglutinins
	NKp46	FcεRIγ, CD3ζ	Lck, Syk	-
	KIR2DS	DAP12	Lck, Syk	HLA-I
	2B4	LAT	EAT-2, SAP, Fyn, 3BP2 Grb2, Plcγ	CD48
	NTB-A	-	EAT-2, SAP	NTB-A
	CRACC	-	EAT-2, SAP?	CRACC

The two families of receptors are colour-coded (L-L: lectin-like receptors; Ig-L: Immunoglobulin-like receptors). Signaling molecules recruited to the cytoplasmic tails and the specific cellular ligands involved in the triggering of the NK cell cytotoxic activity are enumerated in the fourth and fifth columns. The adaptor molecules DAP12, FcεRIγ and CD3ζ contain ITAM motifs, which are phosphorylated by Lck upon ligand binding. This process generates a docking site for the recruitment of Syk kinases. DAP10 contains the YxNM motif for the binding of PI3K and Grb2. Currently there is controversy about the recruitment of SAP by the ITSM motifs present in CRACC. The adaptor molecule LAT recruits PLCγ and Grb2 upon the engagement of CD48 by 2B4.

since it is able to bind 2B4 and block the binding of SHP-1. Another human disease, the Wiskott Aldrich syndrome (WAS), is characterized by an altered formation of the cNK-IS (36). As in the case of SAP, the WAS protein (WASp) also accumulates at the cNK-IS and is, along with SLP-76 and Vav-1, an important signaling molecule, promoting the polymerization of actin at the early cNK-IS (figure 2B).

3.2.2. Supramolecular organization of the mature cNK-IS

The NK cell cytotoxic process is mediated by the polarized exocytosis to the synaptic cleft of SL containing perforin, granzyme B (GB) and Fas-L, which trigger the apoptotic pathways in target cells by the binding of GB receptors and Fas (37-40). Kinesin driven SL moves along the microtubules and reach the MTOC at the contact site. MTOC polarization to the synapse assures the secretion of SL to a specific target cell by the restriction of the delivery of lytic substances into the cSMAC (38). This space is confined by the pSMAC, the area enriched in talin, actin and LFA-1 that is also responsible for the adhesion platform required for stable conjugates to be obtained (figure 3). The recruitment of the MTOC starts within the first 5 minutes of the interaction, before the redistribution of talin to the pSMAC and the mature synapse is constituted. Interestingly, a fraction of signaling molecules clustered at the cSMAC in the mature cNK-IS, including Lck, Fyn, ZAP-70, Pyk2, paxilin and Grb2, is localized around the MTOC (14, 41-43). Others such as Jnk, Rac, Cdc42 and Vav-1 are localized on the microtubules (44-46). It is then plausible to speculate that the reorientation of the MTOC is also able to transport signaling molecules to the synapse and therefore helps to keep high concentrations of these molecules at the contact site, which is essential for sustaining activating signals and the eventual killing of the target. An important role of some of these signaling molecules in MTOC polarization to the synapse has also been demonstrated (47).

By contrast to CTL, that trigger the lytic process upon engagement of only one receptor, NK cells express many different activating receptors (Table 1). The behavior of only a few of them during cNK-IS organization has been studied. That is the case of NKG2D in activated human NK cells (48). Experiments done in the NKL cell line and primary activated NK cells have shown that the expression of the NKG2D ligand MICB on susceptible MHC class I negative target cells induce the clustering of the NKG2D/DAP10 receptor complex at the cSMAC surrounded by a ring of f-acting at the pSMAC (Figure 1), which suggests that the clustering at the cSMAC is mainly mediated by the signaling triggered by this receptor. Since lipid rafts accumulate at the NK-IS independently of the NKG2D ligand expression on target cells, it is plausible to speculate the existence of different mechanisms for the recruitment of lipid rafts and NKG2D to the NK-IS. Recently, the PI3K binding site present in DAP10 (YxxM) has been found to be required for the recruitment of this molecule to the cNK-IS (49). Interestingly, this motif is necessary for the NKG2D endocytosis in B cells lines and activated NK cells as well as for the traffic of DAP10 to SL polarized to the cNK-IS (50) (Roda-Navarro and Reyburn, in preparation). Upon target cell recognition, endosomes containing NKG2D/DAP10 are polarized toward the intercellular contact and interestingly only the engagement of NKG2D by MICB expressed on target cells trigger the polarized exocytosis of this intracellular NKG2D/DAP10 at the cNK-IS (Roda-Navarro and Reyburn, in preparation). We then propose this polarized exocytosis as a new mechanism for receptor recruitment to the synapse, that may constitutes a checkpoint for NK cell activation, in a similar way that CTLA-4 is secreted in SL to the T cell synapse in a signal strength-dependent manner (51). The polarized exocytosis of SL containing NKG2D/DAP10 may also help the unidirectional delivery of lytic substances to MICB expressing target cells, as was previously postulated for the TCR at the cytotoxic synapse constituted by CTL and APC (52, 53).

cNK-IS formed between resting ex-vivo isolated NK cells and MHC class I negative target cells have also been found however the reduced lytic activity shown by these cells. Initially, perforin was found at the cSMAC surrounded by integrins, f-acting and CD2 at the pSMAC of this mature synapse observed after 30 minutes of interaction. Moreover, pSMAC organization and perforin polarization to the cSMAC were found to be sequential

processes, which require actin and tubulin, respectively (54). In other experiments, cell conjugates formed between NK cells and target cells expressing the 2B4 ligand CD48 showed the accumulation of the activating receptor complex 2B4/LAT at the cSMAC, surrounded by a pSMAC characterized by the accumulation of talin in the NK cell and ICAM-1 on the target cell. Moreover, CD48 also accumulated at this synapse (29). The topography of this synapse observed at 5 minutes of the conjugate formation is consistent with the recruitment of 2B4 and LAT to lipid rafts observed in different systems, and with the organization of signaling molecules (cSMAC) and LFA-1 (pSMAC) observed at the cNK-IS in activated NK cells (14, 55).

3.3. The activating NK-IS

The IS has been described during the interaction between CD4+ helper, CD8+ CTL and B cells with antigen presenting cells (APC) (11, 56, 57) as a platform for the extended interactions required to trigger the differentiation of naïve T and B cells to their effector state. Recently it has become clear that resting NK cells require activation by DC to differentiate to their full function effector state (58, 59). This process is dependent on the contact between NK and DC cells, which importantly can be observed *in vivo* in sites of inflammation and lymph nodes (60, 61). Upon activation by DC, NK cells can efficiently produce cytokines, proliferate and reach the highest cytotoxic state. This process is especially important in the $CD56^{bright}$ $CD16^{-}$ subset that only acquires cytotoxicity upon prolonged activation (61). Among the factors required for NK cell activation by DC are the IFN-α, IL12, IL18 and IL15 and, interestingly, the polarization to the interface of NK-DC contacts of IL12, IL18 and adhesion molecules has been observed after long interaction time (Figure 1) (59, 62).

Notwithstanding prior data, the early events occurring at the NK-IS formed between resting NK cells and MHC class I^{bright} mDC have only been investigated in a recent study (16). This study shows the formation of a regulatory NK-IS, which does not trigger cytolysis. Resting NK cell activation is mediated by the rapid organization of this NK-IS containing central but separated clusters of inhibitory KIR, CD94 and IL15-R surrounded by a ring of talin and LFA-1 at the periphery (Figure 1). The inhibitory receptor/MHC class I interaction assures the protection of the mDC from the NK cell lytic activity while the IL15-R/IL15 binding induce NK cell survival. Although the resultant NK cell activation, as demonstrated by CD69 induction and triggering of calcium fluxes, this synapse presents features of an iNK-IS such as un-polarized Perforin and clustering of KIR and MHC class I that do not allow the cytolitic process to be triggered. Therefore, this activating interaction should be defined as a new NK-IS, namely here the activating NK-IS.

A reciprocal regulatory function in NK cell homotipic contacts, and in NK-macrophages interactions have also been described (63, 64). Overall, the aNK-IS constituted with different immune cells contributes to the NK cell differentiation from the naïve to the effector state able to exert an efficient Natural Killing. Thus, the aNK-IS is a nascent research field that deserves future work.

4. PHYSIOLOGICAL SIGNIFICANCE OF THE NK-IS

The IS seems to be a platform where surface receptors and intracellular signaling molecules arrive at so as to exert their coordinated functions in order to achieve the cellular response: differentiation from naïve to effector states, *missing self* recognition, or the spatially restricted cytotoxicity observed in CTL and NK cells. Therefore, the functional impact of the IS vary depending on the nature of contacting cells and the physiological circumstances where it takes place.

As previously discussed, the NK cell tolerance toward healthy tissues is achieved by the *missing self* recognition. Ligation of either inhibitory KIR or the CD94/NKG2A heterodimer abolishes the redistribution of the multimolecular signaling complex associated with lipid rafts to the cNK-IS (65, 66). Vav-1 dephosphorylation has been proposed as the mechanism for inhibition of the cellular cytotoxicity (67). In this regard, the NK-IS organizes in close proximity activating receptors, necessary for Lck activation, and inhibitory receptors, which recruit SHP-1 upon being trans-phosphorylated by Lck. The close proximity of SHP-1 and Vav-1, which becomes phosphorylated upon LFA-1/ICAM-1 engagement, allows its desphosphorylation (figure 2A). Conversely, the absence of SHP-1 will allow the acting polimeryzation and the inside-out integrin signaling needed for the recruitment of lipid rafts and activating receptors, and for the formation of the tight adhesion, respectively.

The aberrant response of NK cells lacking components of the organized cNK-IS in a healthy situation, such as SAP or WASp (35, 36), clearly demonstrate the significance of the synapse for a normal NK cell immune response to be obtained. The most recent contribution to the field showing that the synapse organization is directly responsible for the cellular response has been the description of the aNK-IS (section 3.3), where clusters of the KIR/MHC-I engagement promotes the target cell protection simultaneously with the NK cell activation by the recruitment of IL15-R (16).

It is also proposed that the IS offers checkpoints for lymphocyte inhibition and activation. The intensity and quality of the signal expressed on the target cell will regulate the recruitment of inhibitory and activating signaling molecules to the synapse. Regulated, polarized exocytosis to the synapse of molecules such as CTLA-4 in T cells and NKG2D/DAP10 in NK cells (51) (Roda-Navarro and Reyburn, in preparation) suggests one cellular mechanism for the achievement of these checkpoints. This is possible because of the MTOC and SL polarization to the synapse. Interestingly, when one NK cell simultaneously interacts with a susceptible and a resistant target cell the MTOC only polarizes to the former target, which enables its selective cytolysis by the regulated delivery of SL (38, 68). This constitutes the cellular mechanism explaining how activation and inhibition are spatially and temporally restricted (10). Therefore, the NK-IS is responsible for this spatial and temporally confined NK cell function.

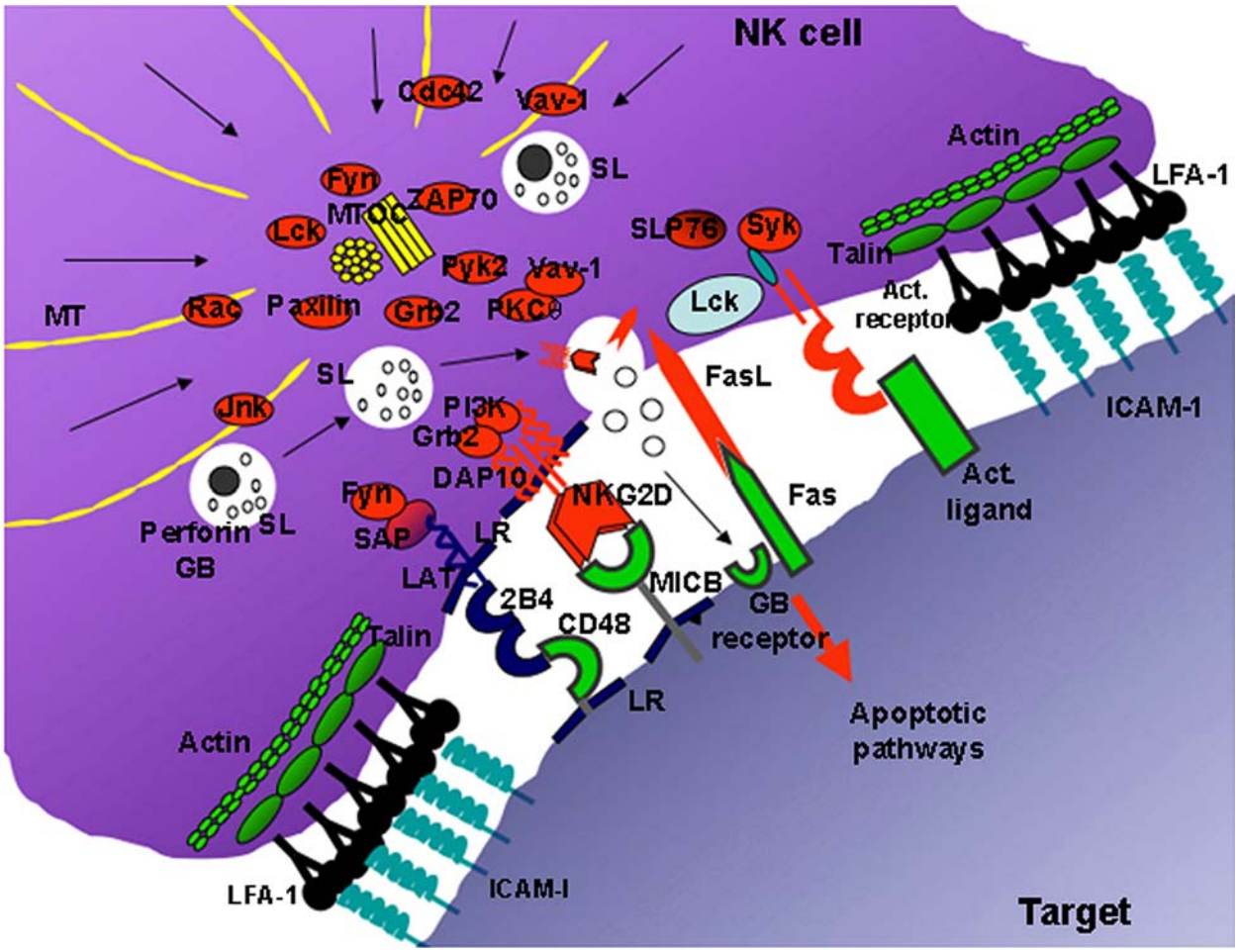

Figure 3. Organization of the mature cNK-IS. The MTOC has already polarized to the contact site along with the associated signaling molecules, and the ring of integrins and cytosqueletal components surrounding activating receptors is already organized. Polarized secretion of SL containing perforin, granzyme B (GB), Fas-L and NKG2D/DAP10 to the cSMAC triggers the apoptotic pathways in the target cell upon the engagement of GB receptors and Fas. Black arrows indicate the directional movement of signaling molecules on the microtubules (MT) and SL to the cSMAC. LR: lipid rafts.

As a phenomenon associated to the synapse assembly, the intercellular transfer of proteins is a new and potentially fruitful research field (extensively reviewed in (69-71). It has been proposed to be involved, for example, in the initiation and termination of immune responses or in the regulation of the extent of the lymphocyte response (48, 72, 73). Acquisition of external proteins can also alter functional capabilities or add to the cell new features (74). For example, NK cells are infected by the EBV only upon synaptic acquisition of the receptor for the virus entry CD21 (75). Then, pathogens can also take advantage of the synapse assembly. This is also shown by the viral synapse organized in lymphocytes infected with the HTLV-1 virus, which favors the spreading of the infection (76).

Immune cells can be connected through long distances by the formation of membrane connective structures (MCS)/nanotubes (10, 29, 48, 77-79). In NK cells it has been demonstrated that these structures are formed in cytotoxic interactions when a NK cell detaches from the target cell (29). The strong adhesion between the NK and the target cell may be the force originating these membrane tethers. Interestingly, MCS/nanotubes are not observed in non-cytotoxic interactions. Although it is not clear what is the function of these structures, surface receptors, intracellular organelles and calcium fluxes are transported inside them (48, 78-81). Importantly, activating receptors transfer from NK cells to target cells at the distal end of MCS/nanotubes, which indicates that they can offer a mechanism for the intercellular protein transfer (29, 69). Finally, viral proteins are also transported through nanotubes formed during the detachment of T cell conjugates (77).

5. MICROSPECTROSCOPY TECHNIQUES FOR THE STUDY OF THE NK-IS

3D fluorescence imaging microscopy has been mainly used to get descriptive information about the organization of the IS, including the NK-IS. This technology is limited by a spatial resolution of a few hundred nm, due to the diffractive nature of the light, and

an acquisition time that depends on the illumination system used. Since the activity of proteins in living cells is confined by spatial and temporal restrictions, microscopy approaches improving the spatial resolution beyond the diffraction limit and adjusting the acquisition times to the biological process are necessary. Microspectroscopy, which combines different microscope set-ups with fluorescence biosensors, is already giving us information about the spatio-temporal regulation of protein activities, protein-protein interactions, and the environment surrounding proteins in living cells (82).

Fluorescence Resonance Energy Transfer (FRET) measurements provides a useful tool for detection and quantification of protein interactions in living cells. Interacting proteins should be located in close proximity with a maximun separation of around 6 nm (82). This resolution may help not only to precisely know where signaling pathways are switched on and off, but also the activation state of enzymes regulated by, for example, postranslational modifications (83, 84). Although there are different methods to measure FRET, quantifying the lifetime of the donor chromophore by Fluorescence Lifetime Imaging Microscopy (FLIM) is the most robust method described (85). Importantly, FLIM is also suitable to get information about the environment where proteins reside. Advantages of FRET are illustrated in different works: The well-established clustering of lipid rafts at IS has been recently challenged by Nichols and co-workers in a study where the clustering of GPI-anchored proteins at T cell synapse was not detected by this approach (86). The spatially restricted phosphorylation of KIR in microclusers at the iNK-IS (section 3.1) has also been observed using this approach (19).

Other techniques such as Total Internal Reflection Microscopy (TIRFM) (87) and Stimulated Emission Detection (STED) (88) are able to obtain a resolution of about 100 nm, and therefore are useful for studying processes like the organization of membranes and endo- or exocytosis during endosome trafficking.

The diffusion properties, interaction and aggregation of molecules can be studied by Fluorescence Correlation and Cross-Correlation Spectroscopy (FCS and FCCS) (89, 90). These techniques along with Fluorescence Recovery after Photobleaching (FRAP) (91) are suitable to study the dynamics of molecules in living cells. Although only average values can be obtained by these methods, Single Particle Tracking (SPT) (92) allows the trajectory of a particle, such a quantum dot bound to a small number of molecules (1-10), to be studied.

New Electron Microscopy (EM) methodologies such as GRAB (GFP Recognition After Photobleaching) (93) and Wet Scanning EM (94) have been described. The former detects electrodense precipitates formed by GFP upon radiation exposure and produces high quality EM to reveal detailed spatial information, whilst the latter allows the examination of fully hydrated samples with a resolution up to 20 nm.

Improvements to the imaging techniques, combined with powerful computer methods for data analysis will be required to understand the dynamic functional connectivity among the components of signaling networks, which regulates the IS organization allowing then the coordinated immune response.

6. PERSPECTIVE

Almost 10 years after the discovery of the NK-IS, many of the contributions published are fair descriptions of its supramolecular organization. Today there are several major challenges to be addressed in this research field:

- *To understand the molecular mechanisms for the IS assembly*. Endosome polarized exocytosis described for the TCR, CTLA-4 and Fas-L (37, 51, 52), and association with lipid rafts or tetraspanins (95-97), which help the lateral organization of signaling molecules, have been proposed as mechanisms for the IS assembly. Another hypothesis proposed is that the distribution of surface molecules into distinct domains is determined by the length of the extracellular domain (98, 99). The recruitment of signaling molecules by these and other mechanisms, such as cytosolic diffusion and motor proteins, require further study.

- *To understand the significance of the IS and the intercellular protein transfer*. At this point, protein transfer, that increases the cellular proteome, is especially intriguing. As previously discussed, proteins acquired promote changes in cellular behaviour, can be re-presented to other cells to terminate or sustain immune responses, or can help to promote viral infection propagation. Moreover, synaptic intercellular transfer of molecules could assists cell-cell dissociation in order to get an efficient immune response (29, 100). All these hypotheses should be further tested.

- *To verify, in vivo, the observations performed by in vitro studies*. The T cell synapse has been observed to form *in vivo* during the killing of virus infected astrocytes by CTL (101, 102). In addition, MHC class I acquisition by NK cells has also been observed to occur in vivo (103). However, it has not been studied so far the NK cell immune response in the 3D environment found in tissues and whether synaptic protein transfer and MCS/nanotubes formation play any essential role in vivo. How NK cells are activated/primed by contact with other cells, ECM components, or gradients of soluble factors during the recruitment to inflamatory sites also deserve further investigation.

- *To study the spatio-temporal regulation of the activitiy of signaling components (kinases, phosphatases, GTPases, etc…), and describe and quantify the functional connections among them*. This will facilitate our understanding of how the NK cell immune response is orchestrated and how this precise regulation is altered during pathological processes.

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Abbreviations: EBV: Epstein-Barr virus; Ig: Immunoglobulin; ITSM: immunoreceptor tyrosine-based switch motif; KIR: killer cell Ig-like receptor; MHC: major histocompatibility complex; SH2: Src-homology 2 domain; XLP: X-linked lymphoproliferative disease

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