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RECEPTOR LOCALIZATION AND DYNAMICS OF MURINE NATURAL KILLER CELLS AT SINGLE CELL LEVEL - USING ADVANCED FLUORESCENCE MICROSCOPY

Sunitha Bagawath Singh

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Cover page: STED image staining with NK1.1 on NK cells, presented deconvoluted and in 3D using Huygens image analysis software (front), and raw image (back)
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Receptor Localization and Dynamics of Murine Natural Killer cells at Single cell level - using Advanced Fluorescence Microscopy
THESIS FOR DOCTORAL DEGREE (Ph.D.)

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What is learnt is just a fistful,

what is not learnt, is the size of the world

Avvaiyar, 4th C Tamil poet

To my loving Grandma Rajammal, my life Dhriti & Drish

ABSTRACT

Natural Killer (NK) cells are immune cells and important for the defense against virally infected and malignant cells. NK cells are regulated by germline encoded activating and inhibitory receptors. Activating receptors specifically recognize ligands which are either encoded by infectious agents, or induced upon infection or cellular stress. Inhibitory receptors interact with self-ligands expressed on healthy cells, among them MHC class I. NK cells inspect the host cells by screening for alterations in activating and inhibitory ligand expression. The balance between input from activating and inhibitory receptors determines the NK cell response. NK cells undergo a process of functional maturation and acquisition of self-tolerance via sensing of the steady-state input through their receptors. This process is known as education. The cytotoxic activity of NK cells can be further increased by cytokines produced by other immune cells.

The aim of this thesis was to characterize the differences in receptor dynamics and localization between NK cells based on either cytokine activation or educational status. Fluorescence based advanced microscopy techniques were used to quantitate receptor dynamics and spatial organization.

In paper I, we investigated the influence of cytokine stimulation on the lateral diffusion of the inhibitory receptor Ly49A and its ligand MHC class I on NK cells within the cell membrane. The response to cytokine stimulation was heterogeneous among the NK cells. We characterized a subpopulation of NK cells with faster diffusion of both MHC class I and Ly49A. The receptor diffusion was established on primary NK cells using Fluorescence Correlation Spectroscopy. In paper II, a practical protocol for utilizing FCS on primary lymphocytes was presented. In Paper III, we showed that NKp46 and Ly49A were confined within microdomains on NK cells. The actin cytoskeleton and cholesterol composition of NK cells played important roles in initiating activating cell signaling. In Paper IV, we investigated the organization and clustering of activating and inhibitory receptors on educated and uneducated NK cells. We found that clusters of NKp46 and Ly49A were larger on uneducated NK cells. The nearest neighbour distances from activating to inhibitory receptors were not significantly different between educated and uneducated NK cells, thus the organization of inhibitory receptors in relation to the activating receptors do not seem to be of importance for the educational process. In summary, the findings in this thesis enlightens the importance of altered receptor dynamics and organization on NK cells depending on the state of activation and education. Furthermore, receptor dynamics could be an important aspect for understanding NK cell function.

LIST OF SCIENTIFIC PAPERS

I. Cytokines Induce Faster Membrane Diffusion of MHC Class I and the Ly49A Receptor in a Subpopulation of Natural Killer Cells
Sunitha Bagawath-Singh* Elina Staaf*, Arie Jan Stoppelenburg, Thiemo Spielmann, Taku Kambayashi, Jerker Widengren and Sofia Johansson
Front Immunol. Feb 4;7:16. 2016

II. Molecular Diffusion in Plasma Membranes of Primary Lymphocytes Measured by Fluorescence Correlation Spectroscopy
Elina Staaf, **Sunitha Bagawath-Singh**, Sofia Johansson
J Vis Exp. 1;(120) 2017.

III. Educated Natural Killer Cells Exhibit a More Dynamic Movement of NKp46 and a Higher Confinement of the Ly49A Receptor
E. Staaf*, P. N. Hedde*, **Sunitha-Bagawath Singh**, J. Piguet, E. Gratton, S. Johansson.
Submitted.

IV. Nano-structural Organization of Activating and Inhibitory Receptors in Murine Natural Killer cell Education
Sunitha Bagawath Singh, Jan Bergstrand, Daniel Rönnlund, Hans Blom, Jerker Widengren and Sofia Johansson.
Manuscript

* Authors contributed equally

CONTENTS

LIST OF ABBREVIATIONS

ADCC	Antibody-Dependent Cellular Cytotoxicity
CD	Cluster of Differentiation
CFSE	CarboxyFluorescein Succinimidyl Ester
Clr-b	C-type lectin related ligand b
DAP (-10/-12)	DNAX Activating Protein of (10kDa /12kDa)
DC	Dendritic cells
DNAM1 (CD226)	DNAX accessory molecule-1
F1 Hybrid	Filial 1 hybrid
FACS	Fluorescent Activated Cell Sorter
FCS	Fluorescence Correlation Spectroscopy
GFP	Green fluorescent Protein
H2	Mouse Histocompatibility Complex at gene locus 2
Ig	Immunoglobulin
IL	Interleukin
IFN	Interferon
ITIM	Immunoreceptor Tyrosine-based Inhibitory Motif
ITAM	Immunoreceptor Tyrosine –based Activation Motif
KIR	Killer Immunoglobulin-like receptors
KLRG1	Killer cell Lectin like Receptor G1
LFA-1	Lymphocyte function-associated antigen **1**
Ly49r	Ly49 family of activating or inhibitory Receptors
MHC	Major Histocompatibility Complex
mCMV	Murine Cytomegalovirus
mTOR	Mechanistic Target Of Rapamycin Kinase
ULBP1	UL16 binding protein 1
NCR	Natural cytotoxicity receptors

NKG2	Natural Killer Group 2
NKR-P1	Natural Killer Cell Receptor Protein-1
PMA	Phorbol 12-myristate 13-acetate
RAE1	Ribonucleic acid export 1
RPMI	Roswell Park Memorial Institute cell culture medium
SHP -1, -2	Src Homology region 2 domain-containing Phosphatase-1, -2
SLAM	Signaling Lymphocytic Activation Molecule
SPT	Single Particle Tracking
STED	STimulated Emission Depletion microscopy
STICS	Spatio Temporal Image Correlation Spectroscopy
TAP	Transporter associated with antigen processing
TIGIT	T cell immunoreceptor with Ig and ITIM domain
TRAST	Transient state microscopy
TIRF	Total internal reflection fluorescence
iMSD	image Mean Square Disaplacement

1 INTRODUCTION

1.1 NATURAL KILLER CELLS

In the 1970's, the standard assay for T cell cytotoxicity showed a persistently high background level in killing of tumor cells. This "background" killing was further characterized by Kiessling et al., who concluded that this killing was mediated by a lymphocytic cell other than T or B cells [1, 2]. At the same time, a similar study by Herberman *et al* showed that mouse non-T, non-B lymphocytes were reactive against syngeneic and allogenic tumors [3]. The above new subset of lymphoid cells with potent cytotoxic function was termed as "Natural killer" (NK) cells [1, 2]. Nowadays, NK cells are well defined by their ability to kill tumor cells and virally infected cells. NK cells are bone marrow derived lymphocytes possessing lytic granules and an array of receptors. They constitute around 3-5 % of murine splenocytes, and are also present in other lymphoid organs and tissues. NK cells have been characterized as being part of both the innate and adaptive immune system, mainly because: 1) NK cell receptors recognize healthy and unhealthy cells through their germline encoded receptors (discussed further below) which is a characteristic of the innate immune system, and 2) NK cells have been shown to mount better responses against recurrent infections by the same virus, reviewed in [4, 5], typically a feature of the adaptive immune system. NK cells respond to interferon-α and -β (IFN-α/β) and several other cytokines that are secreted e.g. during infections [6, 7]. Upon activation, NK cells secrete cytokines, chemokines and growth factors [5].

1.2 NK CELL MISSING-SELF RECOGNITION

George Snell in 1958 for the first time observed that tumor cells of parental origin were rejected to a higher extent than the native tumors, and showed that this was controlled by MHC class I linked genes [8]. This was puzzling since all the antigens of both the inbred parents should be present in the F1 hybrid off-spring, and there should thus not be anything "foreign" for T cells to react against. Since T and B cells were the only characterized lymphocytes at the time, it was unclear why an immune response was mounted against the parental cells. Later, subsequent studies from Cudkowicz and his associates demonstrated that transplants of paternal bone marrow into F1 hybrids were rejected [9]. The allograft reactivity was at the time hypothesized to be due to the presence of recessive tissue specific antigens. However, the rejection was independent of proliferation of host lymphoid cells and the thymus, which suggested it was independent of T cells [10]. The above two observations paved the way to a phenomenon termed Hybrid resistance. A few years later, after the

discovery of the NK cells, Kissling *et al* showed that NK cell activity and the rejection of tumors were associated with the H2 gene locus (which is the MHC in mice) correlating it to hybrid resistance [11]. However, the specificity of NK cell cytotoxicity was still undefined.

The puzzle of selective cytotoxicity was finally solved by the "missing-self" hypothesis that was proposed by Klas Kärre in 1981 [12, 13]. The hypothesis postulated that "absence or incomplete expression of host MHC class I molecules in a normal cell is sufficient to render it susceptible to NK cells" [14, 15].

Thus, NK cells recognize target cells by the lack or altered expression of MHC class I molecules. As was later delineated, this so-called missing-self rejection is based on a family of MHC class I specific inhibitory receptors, called Ly49 receptors in mice [16]. They mediate protection from NK cell killing to self MHC class I expressing cells, while cells without, or with a lower level of, MHC class I are killed [13, 15]. NK cells whose inhibitory receptors lack a specific ligand, either due to the allelic specificity of these receptors or that they develop in MHC deficient mice, are hyporesponsive [17-19]. This shows that other self-tolerance mechanisms, besides inhibitory receptors for MHC class I, exist as well.

1.3 NK CELL RECEPTORS

NK cell responses are mediated by cell surface receptors which can be broadly divided into activating and inhibitory receptors. Each category contains receptors with a large number of different specificities, to permit activating or inhibitory signals from a broad array of aberrant cells. The NK cell response is determined based on the balance between activating and inhibitory signals [20, 21].

1.3.1 MHC class I specific inhibitory receptors

There are three main families of inhibitory receptors which recognize MHC class I. They are the murine Ly49 receptors (Ly49r), the human KIR (killer immunoglobulin-like receptors), and the NKG2 inhibitory receptors which exist in both humans and mice. Inhibitory receptors signal through intracellular immunoreceptor tyrosine based inhibitory motifs (ITIM's), which are located at the cytoplasmic tail of the receptors. When tyrosine's in this motif are phosphorylated, SHP-1 or -2 is recruited and conveys the inhibitory signals further downstream [22].

Ly49r belong to C-type lectin family of receptors. They inhibit NK cells by binding to MHC class I molecules on target cells [16, 23]. Ly49r are expressed in a stochastic manner so that

individual NK cells may express none, one, or several receptors, in different combinations [24-26]. Individual Ly49r can also recognize more than one MHC class I allele [23, 27]. At least ten Ly49r have been found in the mouse population, although the number may vary in different individuals, typically between 5-8. The strength of these inhibitory receptor interactions with different MHC class I vary [23, 28]. Ly49A, which is the most well studied Ly49 receptor, interacts strongly with H2-D^d [23]. The majority of Ly49r are inhibitory, however, Ly49-D/-H/-P are activating receptors that signal through the adaptor molecule DAP12 [29, 30]. Recognition of MHC class I by Ly49r requires a peptide bound to the peptide-binding groove, but, with a few exceptions, the peptide specificity is not the main basis for specific interactions with Ly49r [31, 32]. Ly49A, and most likely other Ly49r as well, not only recognize MHC class I in *trans* but also binds in *cis* (interacting on the same cell) and that in turn reduces the activation threshold of the NK cell [33, 34].

CD94-NKG2/A/C/E receptors are heterodimers that are also C-type lectin receptors. They recognize non-classical MHC class I molecules. Their expression is variegated, as with the Ly49r [35]. NKG2A is the only inhibitory receptor in the NKG2 family. It binds to Qa-1b in the mouse, a non-classical MHC class I molecule [36]. NKG2A is the earliest inhibitory receptor expressed by NK cells during their development [37].

1.3.2 *Non-MHC class I specific inhibitory receptors*

Killer cell Lectin like Receptor G1(KLRG1) is an adhesion molecule, and an inhibitory receptor which binds to classical cadherins (E-, N- and R-) that are expressed on epithelial cells. Blocking of KLRG1 reinstates the killing of E-cadherin expressing target cells and crosslinking of KLRG1 inhibits IFN-γ production [38, 39].

NKRP1 is a C-type lectin receptor family. There are five receptors: NKRP1-A, -B/D, -C, -F and G (NKRP1-C discussed in activating receptors below). NKRP1-B/D is an inhibitory receptor that recognize a C-type lectin related ligand (clr-b) [40]. This was one of the first receptors with other specificity than MHC class I shown to initiate a missing-self response [5, 41, 42].

T cell immunoreceptor with Ig and ITIM domain (TIGIT), is an inhibitory receptor that recognize CD155 (Ig superfamily ligand) and inhibit NK cell responsiveness [43].

2B4 (CD244) belongs to the Signaling Lymphocytic Activation Molecule (SLAM) family of CD2 related receptors. They are composed of two extracellular Ig like domains and contain a cytoplasmic tail with multiple signaling motifs. 2B4 recognize the cognate SLAM molecule

CD48. Initially 2B4 was found to be an activating receptor on NK cells [44]. However, in the absence of the signaling adaptor SAP, 2B4 acts as an inhibitory receptor [45]. Thus, in resting NK cells, it is a non-MHC dependent inhibitory receptor [46], and associates with SHP-2 [47]. Tumors with high expression of CD48 are protected from NK cell killing [46]. SLAM6 is another family of the SLAM family, which also mediates both activating and inhibitory signals to NK cells. It mainly recognizes hematopoietic target cells [48].

1.3.3 Activating receptors

NK cells express different types of activating receptors. Their ligands are either induced or upregulated due to stress, e.g. during viral infections, or are virally encoded antigens. Most of these receptors signal through immunoreceptor tyrosine activating motifs (ITAM:s), either expressed in their own cytoplasmic tail, or by associating with adapter molecules. Natural cytotoxicity by resting NK cells require synergy of multiple receptors [49], however co-binding of adhesion molecules like LFA-1 may be sufficient for activation [50].

Natural cytotoxicity receptors (NCRs) is a family of receptors with a broad range of ligands. Apart from cellular ligands, which may be upregulated on tumor cells, they also interact with ligands from bacterial, viral, and parasite origin [51]. NKp46 is the only activating receptor in this family found in both humans and mice. In humans, along with NKp46, NKp44 and NKp30 are expressed on resting NK cells. Several virally induced ligands of NKp46 have been identified, for instance in refs [52-54].

NKRP1-A/-C/-F are the activating receptors in the NKRP1 family. NKRP1-C is a prototypical activating receptor in murine NK cells which is commonly cross-linked in *in vitro* functional assays (the most commonly used antibody against NKRP1-C is known as NK1.1). It associates with ITAM containing FcεRI-γ [55].

Like the Ly49r receptors, the activating receptors of the NKG2 family also signal through DAP-12 [56]. NKG2C, as NKG2A, binds to the non-classical MHC class I Qa-1 [57].

The NKG2D receptor mainly recognizes stress induced ligands and it is one of the most important receptors used by NK cells in their surveillance of tumorigenic cells [58]. NKG2D is known to stimulate macrophages, be co-stimulatory on CD8 T cells and have a significant role in NK cell killing [58, 59]. NKG2D signals via DAP-10 and DAP-12 in mice, but only via DAP-10 in humans [60-62]. There are two isoforms of NKG2D in the mouse, NKG2D-L with long N-terminal that can only bind to DAP-10 and NKG2D-S with short N terminal can bind to both DAP-10 and DAP-12 [63]. DAP-12 is capable of initiating both cytotoxicity and

cytokine release whereas DAP-10 can activate only cytotoxicity [64]. DAP-10 do not signal through ITAM, instead an SH2 domain in the cytoplasmic tail recruits the p85 subunit of PI(3) kinase for signaling [62]. The identified ligands of NKG2D include Mult-1, Rae-1 and H6 in the mouse, and MICA/B and ULBP1-6 in humans [61].

Ly49-D/-P/-H are the identified activating receptors of the Ly49 receptor family. Ly49H recognizes the mCMV-derived MHC class I like molecule m157. This interaction is sufficient to activate NK cells and eliminate mCMV [65-67]. Ly49P recognizes H2-D^k in combination with the mCMV-derived protein m04 [30]. Similarly, Ly49D binds to H2-D^d and induce rejection of allogenic (H2-D^d expressing) bone marrow transplants [68]. Interestingly, no autoimmune reaction mediated by Ly49D^+ was reported in H2-D^d mice [68, 69].

DNAM-1 (also known as CD226) is a co-stimulatory activating receptor and adhesion molecule that is expressed on murine NK cells [70, 71]. It interacts with the ligands CD155 and CD112 and activates NK cells via ITAM [72]. DNAM-1 is actively involved in synapse formation and co-localize with another adhesion molecule, LFA-1, to facilitate contacts with interacting cells [73]. Engagement of DNAM-1 to its ligands mediate cytotoxicity against tumors[74-76].

CD16, also known as the FcγRIIIa receptor, enables NK cells to eliminate antibody coated cells by ADCC (Antibody-dependent cellular cytotoxicity). CD16 may thus not be regarded as a "natural" cytotoxic receptor, since the specificity in its reactivity is in fact determined by B cells, which are part of the adaptive immune system [22]. CD16, in contrast to the natural cytotoxicity receptors, is sufficient to trigger NK cell degranulation on its own, and do not need additional co-stimulation [49].

1.4 NK CELL ACTIVATION

NK cell effector functions can be triggered without any prior sensitization, but the activation can be mediated in different ways depending on the maturation and activation status of NK cell [77]. Since some activating ligands are present on many normal cells, the lack or reduced expression of MHC class I on targets can be sufficient to trigger effector cell function [15, 78]. In other situations, effector function is triggered despite normal expression of MHC class I molecules, because target cells express abnormal levels of activating ligands, which can be pathogen encoded, stress induced, or tumor antigens expressed on unhealthy cells [79]. Most of the tumor induced ligands activate NK cells through the NKG2D receptor, while the NCRs

predominantly recognize pathogen associated ligands and cellular stress ligands [79]. Activation of NK cells by activating receptors require synergistic co-activation by more than one receptor [49].

NK cells can be pre-activated by cytokines which upregulates the cytotoxic potential [80, 81]. For instance, dendritic cells (DC) secrete cytokines which activate NK cells and stimulate IFN-γ secretion [82, 83]. NK cells can also be activated through direct interactions with antigen presenting cells [77, 80]. Priming by IL-15 that is *trans*-presented by DC is an important route of NK cell pre-activation [84]. *Trans* presentation of IL-15 is also important for NK responsiveness during inflammation [85].

A classical method for activation of NK cells *in vitro* is by culturing with IL-2. *In vivo* IL-2 is produced by activated T cells and boost NK cell responses, and regulatory T cells restrain NK cell cytotoxicity by limiting the IL-2 [86, 87]. In the mouse, bacterially activated DC's produce IL-2 and this increase NK cell activation [88, 89].

NK cells can also mediate antiviral defense in response to endogenous IFN-α/β that is secreted during viral infections and act either directly or indirectly on NK cells [90, 91]. During mCMV infection, $CD11b^{+}$ myeloid DCs mediate IFN-α/β dependent NK cell activation [92]. Chemokines are also known to activate NK cells, apart from inducing chemotaxis [93].

As described earlier in this thesis, NK cell activation can also occur via missing-self recognition. The final outcome in each target cell encounter is based on the combination of pre-activation stimuli, together with the sum of signals from activating and inhibitory ligands expressed by the specific target cell. All different ways of NK cell activation are schematically represented below in Figure 1.

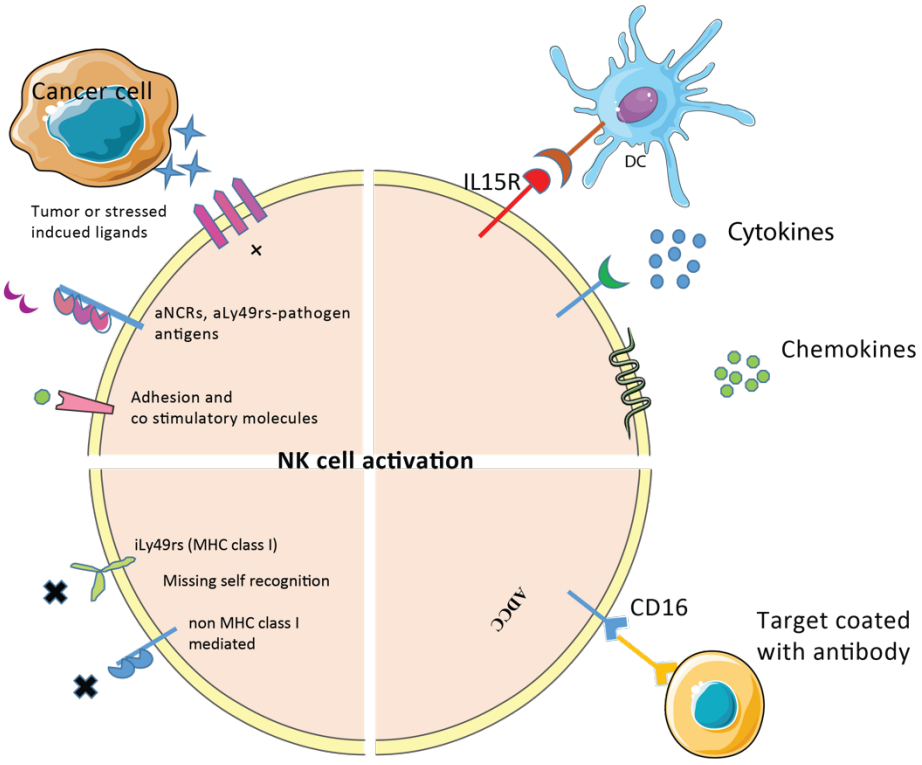

Figure 1: Schematic overview of NK cell activation. Described clock-wise from top right: activating ligand interactions with activating receptors, either pathogen-induced or on tumor target cells; soluble factors or direct interactions with antigen presenting or other bystander cells (pre-activation); activation triggered by ADCC; and missing self-recognition, both MHC class I and non-MHC class I mediated.

1.5 NK CELL EDUCATION

NK cells undergo a process of functional maturation which is known as education. The educational process also ensures that self-tolerance is maintained. According to the classical description of NK cell education, NK cells which express inhibitory receptors specific for self MHC class I after the educational process have full functional competence (they are responsive through missing-self recognition). However, in each individual, a subset of NK cells exists which either only express inhibitory receptors that lack MHC class I ligands in that host, or express no inhibitory receptors [18, 19]. Likewise, NK cells in mice lacking MHC class I altogether, and in $Tap^{-/-}$ humans exist in the host at a normal population size [17, 94, 95]. Thus, in these NK cells self-tolerance is obtained by other means [17, 19, 94, 95]. These NK cells are unable to kill cells via missing-self recognition of MHC class I, and are also generally more hyporesponsive, at least in the resting state (without pre-activation) [18, 19, 96].

The educational process, and its outcomes, has not yet been fully characterized at the molecular level. At least four different models have been put forward in order to explain the education phenomenon. The arming (licensing) model was proposed by Wayne Yokoyama. He proposed that NK cells by default are hyporesponsive and acquire responsiveness upon specific inhibitory receptor-MHC class I interactions [18]. The "disarming model", proposed by David Raulet, instead postulates that NK cells are by default functionally responsive and become hyporesponsive in the absence of inhibitory signals, in cases where overstimulation through activating receptors occurs [19]. According to the "*cis* model", proposed by Werner Held, NK cells acquire a fully functional phenotype by the interaction of Ly49r with MHC class I expressed on the same cell [97, 98]. Our group proposed the "rheostat model" that states that NK cells are not just in an 'on or off' state, but in a continuous state with different degree of responsiveness that depends on the qualitative and quantitative inhibitory input received via different NK cell receptors from the host environment [28, 99, 100]. This model was tested by experimental models allowing fine tuning of the responsiveness of mature NK cells by changing the inhibitory input in the environment [101]. It is important to note that the models are not necessarily mutually exclusive, the rheostat model is for instance compatible with both the licensing and the disarming model, since it does not address the specific mechanism, but rather address the temporal and quantitative aspect of education.

In the past few years, missing self-recognition based on non-classical MHC class I has also been shown to regulate NK cell education and function (reviewed in He., *et al,* [102]). NKG2A recognition of Qa-1 in the host enables the NKG2A positive cells to become efficient killers of target cells that lack this molecule [103]. Ly49A recognizes both the classical MHC class I molecule H2-D^d and the non-classical MHC class Ib molecule H2-M3. The latter interaction has been shown inhibit the effector function of $Ly49A^+$ NK cells. However, the strength of the H2-M3 education is low compared to classical education but still detectable [104].

MHC class I independent (non-MHC class I) mediated education has been demonstrated for several inhibitory receptor ligands. The first one was 2B4. Mice with either 2B4 or the ligand CD48 knocked out failed to reject CD48 deficient tumor cells [105, 106]. SLAM6, another receptor from the same family also confer enhanced responsiveness to non-hematopoietic targets by a process similar to classical NK cell education [48, 107]. Recently, TIGIT has been shown to play a key role in a classical education assay, $TIGIT^{-/-}$ NK cells had impaired recognition and killing of CD155 deficient targets [108]. Figure 2 summarizes the education

of NK cells mediated by MHC class I, non-classical MHC class I and non-MHC class I molecules.

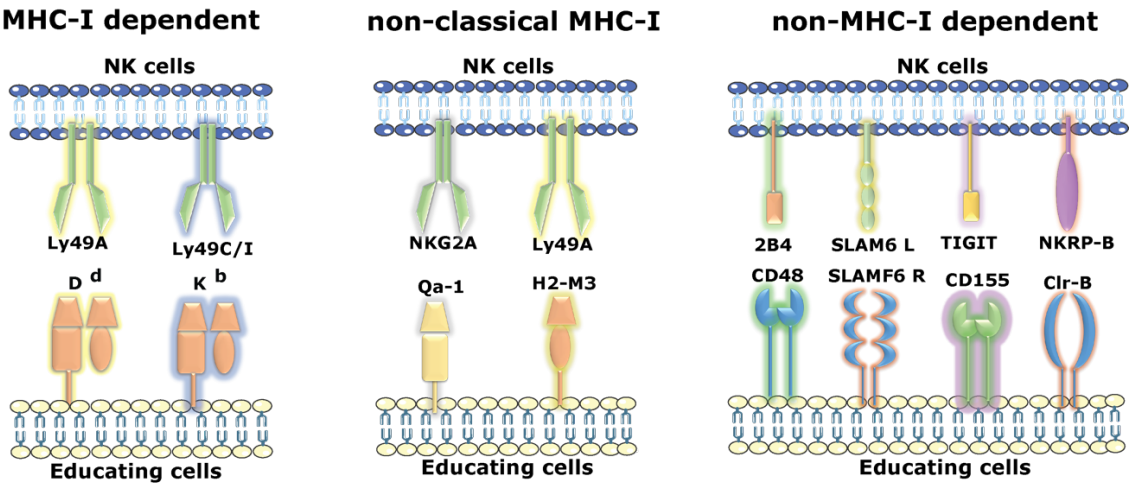

Figure 2 NK cell educating receptors with their cognate ligand for mice: MHC class I dependent, non-classical dependent and MHC class I independent interactions. Adapted from He. *et al.,*[102].

Furthermore, even though NK cell education has traditionally been regarded as mediated primarily through inhibitory receptors, more recent research has shown that NK cell education is regulated by a coordination of both activating and inhibitory signals. The total activation signal also sets the threshold for NK cell education, as continuous exposure to activating ligand leads to a down-modulation of response against this ligand [109-111].

It has also been shown that there is a difference in the threshold set for inhibition and education through inhibitory receptors, as the threshold for education is higher than the threshold for inhibition [112].

1.5.1 Signaling molecules in NK cell education

The arming model stated that education was mediated through inhibitory receptors. This hypothesis was further investigated by elucidating the importance of ITIM. ITIM deficient NK cells cannot be educated [18]. In addition, the lack of SHP-1 in NK cells alters the inhibitory receptor repertoire and impairs the NK cell function, which are both signs of an altered educational status of the NK cells [113, 114]. Thus, education seem to be dependent on the inhibitory signaling of Ly49r. Also, the absence of SHIP alters the NK receptor repertoire and cause hyporesponsiveness and failure to educate NK cells [115, 116]. However, this does not resolve the question of whether the licensing or the disarming model is the most physiologically relevant, since the absence of inhibitory signaling would lead to

hyporesponsiveness also according to the disarming model. It was also shown that educated NK cells are associated with a higher basal activity of the mTOR/Akt pathway [117].

1.5.2 Cellular and molecular correlates of NK cell education

Educated NK cells may be particularly important in certain clinical situations, e.g. in immunotherapy of cancer. It is therefore important to explore molecular markers or other cellular features that correlate with the educated state. A hallmark marker for NK cell education at the single cell level, other than the expression of a specific inhibitory receptor-MHC class I pair, is however yet to be identified. Currently NK cell education can be measured only by readouts from *in vivo* rejection, cytotoxicity, *in vitro* stimulation assays and the NK cell inhibitory repertoire. The cytotoxic assays are cumbersome to perform. In addition, the NK cells are typically lost in the process, which means they cannot be used for subsequent clinical applications, or even further characterization [28]. Two adhesion molecules, KLRG1 and DNAM-1, are preferentially expressed on MHC class I educated NK cells, indicating their association with education [118, 119]. However, it is only the frequency of positive cells that increase, so they are not markers of education at the single cell level. One study elucidated the difference between educated and uneducated NK cells in terms of receptor organization. Activating receptors were confined in membrane nanodomains on educated NK cells, while in uneducated NK cells they were dispersed and only hindered by the actin meshwork [120]. In a microchip based single cell cytotoxic assay, it was shown that educated NK cells displayed higher dynamic migration and made more contacts with target cells [121].

1.6 RECEPTOR CLUSTERING AND DYNAMICS IN IMMUNE CELLS

Receptors have a natural tendency to form clusters on the cell membrane. Recent advancements in technology have increased our understanding of the mechanisms involved in immune cell functions, both at a large scale and the microscale level. In T cells, the TCR complex form microclusters both before and upon engagement with its ligand. This T cell receptor clustering is important for initiation of and sustained cell signaling, by recruiting ZAP-70 [122, 123]. Similarly, in B cells, the immunoglobulin IgG1-B cell receptor form microclusters which initiate cell signaling [124]. The NK cell receptors are organized in nanoscale clusters and the phosphorylation of the activating signaling molecule ZAP70 and the inhibitory SHP-1 is favored in larger sized clusters [125].

Cytoskeleton networks and lipid rafts have been shown to play an important role during the immune synapse formation and modulate the spatiotemporal characteristics of receptors. The

role of the cytoskeleton in regulating receptor compartmentalization, dynamics and clustering have been discussed extensively [126]. It was shown in mature B cells that the diffusion of the BCR is highly restricted by the cytoskeleton. Alteration of the cytoskeleton in this case induces BCR mobility, which allowed formation of larger BCR clusters and hence induced activating signaling [127]. In another study, LFA-1 molecules on resting B cells were restricted, but after PMA (Phorbol 12-myristate 13-acetate) activation, the diffusion of LFA-1 was increased 10-fold, which increased LFA-1 signaling [128]. But there are also actin independent cell surface receptors, for example the CD19 coreceptor of BCR was less affected by actin alterations [129]. Role of intact actin cytoskeleton was shown to be important for nanoscale clustering of inhibitory receptors on human NK cell line[130]. A small adaptor protein Crk associated with cytoskeleton scaffold complexes in NK cell signaling. Crk was also shown to be important for movement of activating microclusters[131]. Moreover, Actin has important roles in integration of inhibitory and activating signals of NK cells and also in micro to nano meter scale organization of receptors[132]. Dynamics and clustering which are predominantly modulated by the cytoskeleton and exploring the receptor dynamics could be beneficial in understanding NK cell education and stimulation.

1.7 PRINCIPLES OF FLUORESCENCE MICROSCOPY TECHNIQUES USED IN THE THESIS

1.7.1 Fluorescence microscopy

Fluorescence microscopy is a sensitive and specific imaging technique, capable to resolve fine structures in biological specimens. Over the past years, several advanced fluorescence microscopy techniques have evolved, which have further extended the possibilities to characterize cellular features and mechanisms. To acquire data, the molecules of interest must typically be fluorescently labeled, although sometimes auto-fluorescent compounds can be used. Fluorescence is the emission of light from a fluorescent compound, which occurs within nanoseconds after light-induced electronic excitation of the compound. For excitation to occur, the photon energy of the excitation light should match the energy difference between the ground electronic state (S_0) and the excited electronic state (S_1). The return of the electron to the ground state can occur via different pathways. The most direct pathways give rise to emission of a fluorescence photon. Because of losses of vibrational energy upon excitation and de-excitation, the fluorescence photons have less energy than the excitation

light photons, and the wavelength of the emitted fluorescence light is therefore red-shifted, compared to the excitation light. The relaxation from S_1 to S_0 can also take place without emission of fluorescence. The excitation energy is then lost into heat. Another possible pathway is so-called intersystem crossing into a triplet state (T_1), whereby one of the outer electrons in the fluorophore changes its spin direction. The T_1 state has a lifetime of microseconds to milliseconds and has a very weak emission (phosphorescence). It can thus be considered as a non-fluorescent, dark state. The electronic state transitions within a fluorophore following excitation can be illustrated by a Jablonski energy diagram (Figure 3A).

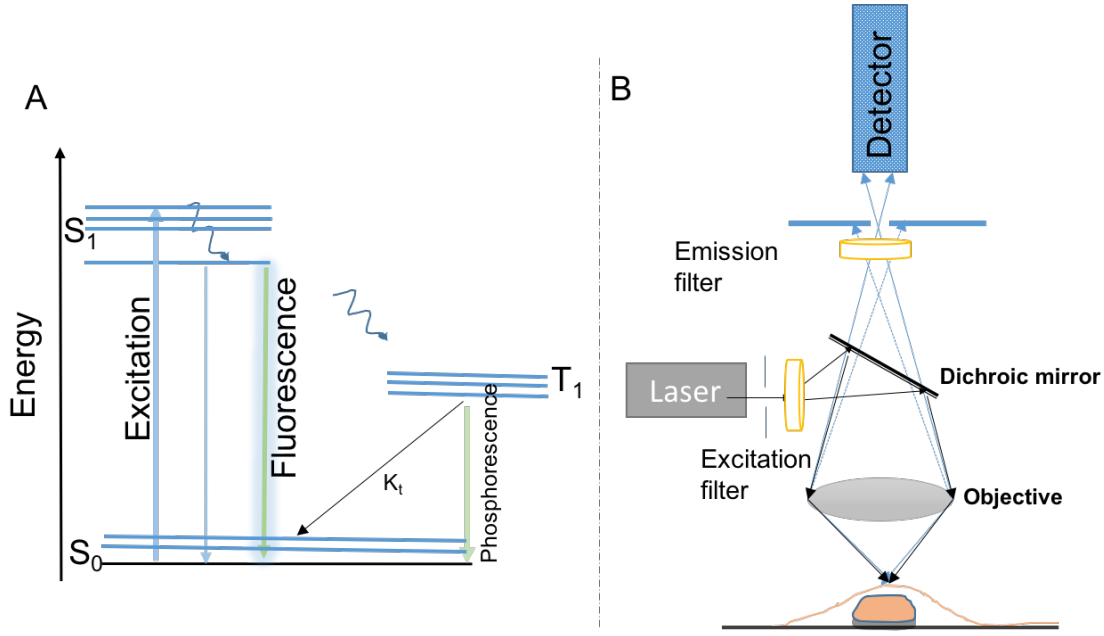

Figure 3: Simple illustration of (A) Jablonski energy diagram (B) working principle of confocal fluorescence microscopy: black lines for excitation laser, blue lines represent emitted fluorescence. Dotted lines indicate out-of-focus/scattered/fluorescent light.

In Figure 3B, a simple depiction of the principle of fluorescence microscopy is illustrated. The beam of an excitation laser is reflected by a dichroic mirror (which reflects the shorter wavelength of the laser light) and is then focused by the objective so that the focal plane is within the sample. Electrons excited by the laser emit light (fluorescence) which is collected by the same objective, transmitted through the dichroic mirrors (which transmits the longer wavelength light of fluorescence), passes through a pinhole, an emission filter, and is captured by a detector.

1.7.2 Fluorescence Correlation Spectroscopy (FCS)

FCS is based on correlation analysis of temporal fluctuations of the fluorescence intensity. It is a quantitative technique with single molecule sensitivity. It can measure the diffusion and concentration of molecules and protein complexes in absolute numbers. It is based on detecting the minute fluctuations in fluorescence intensity that occur when individual labelled molecules diffuse in and out of a small detection volume. By monitoring the amplitude of the fluctuations over time, the average number of fluorescent molecules residing within the focus can be determined. Also, the diffusion properties of these molecules can be determined from the duration of the fluorescence intensity fluctuations [133]. FCS can in principle be combined with any type of fluorescent microscope, one such recent combination was with STED microscopy, known as STED-FCS, where the molecule or lipid membrane dynamics can be observed in nanodomains [134].

1.7.3 STimulated Emission Depletion microscopy (STED)

The resolution of regular confocal microscopy is restricted by the diffraction of light, to around 250-300 nm. In recent years this resolution limitation has been overcome by the development of several super-resolution techniques. One of them is STED microscopy, invented by professor S. Hell [135]. With STED microscopy, it is possible to resolve fluorescent structures in a sample which are less than 40 nm apart. This is achieved by using a pair of synchronized laser pulses. The first laser beam (the excitation pulse) excites the fluorescent probes in the sample (from S_0 to S_1), within a region that cannot be made smaller than 250-300 nm, given by the diffraction limit of light. After a few picoseconds, the excitation laser is followed by a second laser (depletion pulse) with a red-shifted wavelength. The depletion pulse is designed in a doughnut shape, where at the center, the depletion intensity is zero. The depletion pulse induces stimulated emission (from S_1 to S_0), so that fluorescence emission is out-competed in the peripheral parts of the excitation beam. Thus, the fluorescence emission is confined to a smaller spot, thereby increasing the resolution.

1.7.4 Transient state microscopy (TRAST)

Transient state microscopy (TRAST) was developed in professor Jerker Widengren's lab, KTH, Stockholm [136]. This method is based on recording the transition of fluorophores into transient dark states, upon excitation by a time-modulated laser beam. The intersystem crossing rate, or transit probability, of S_1 into the triplet state is much lower than the de-excitation from S_1 to S_0. However, the triplet state is relatively long-lived, and under continuous excitation the T_1 population increases with time and finally reaches a level where

a steady-state population is established. The triplet relaxation rate (k_t) from T_1 to S_0 is strongly dependent on the oxygen concentration. In most biological systems k_t increases linearly with the oxygen concentration in the micro-environment:

$$k_t = k_0 + k_Q * [O2]$$

where k_t is the triplet relaxation rate, k_0 is a small intrinsic rate (not oxygen dependent), k_Q is a quenching constant and [O2] is the oxygen concentration. The higher the oxygen concentration, the larger the quenching of the triplet state (i.e. triplet relaxation rate is higher) [137].

2 AIM

The overall aim of this thesis was to study the spatial organization and temporal dynamics of NK cell receptors, with focus on characteristics that can be studied and quantified using different fluorescence based techniques. The studies address comparisons of NK cell populations in steady-state, but with different intrinsic activity levels, based either on previous cytokine activation or education.

Paper 1. To characterize the expression and membrane dynamics of an MHC class I molecule and a inhibitory receptor (Ly49A) after cytokine stimulation of NK cells.

MHC class I molecules are known to be upregulated on the cell surface with cytokine stimulation. The impact of this on the dynamics of the inhibitory receptor (Ly49A) and MHC class I was assessed on activated NK cells.

Paper 2. To develop a fluorescence correlation spectroscopy technique on primary NK cells for quantifying the molecular diffusion and concentration on the cell membrane. This technique could be easily adapted for other immune cells.

Paper 3. To investigate if there is any difference in the molecular diffusion pattern of activating and inhibitory receptors on the cell membrane between educated and uneducated NK cells.

A novel technique, TIRF-iMSD, was established to study the diffusion patterns of receptors.

Paper 4. To investigate the correlation of activating and inhibitory receptor organization and clustering with NK cell education.

3. MATERIALS AND METHODS

3.1 MICE

Mice were bred and maintained at the MTC animal facility and the Comparative Medicine Wallenberg laboratory animal facility according to governmental guidelines and regulations. Experimental procedures were approved by the local ethical committee, ethical permit number N70/15, N418/12 and N419/12 for all the studies. All the mice used in this thesis were from C57BL/6 background and usually from 8 to 12 weeks old. Two mouse strains were used as a model to study education, here called H2-D^d and $MHC^{-/-}$mice. H2-D^d mice express only one of the MHC class I allelic products, the D^d allele molecule. This is an educating ligand for the inhibitory receptor Ly49A. The strength of Ly49A–H2-D^d binding is high and this interaction may thus have a high impact on education [28]. $MHC^{-/-}$ mice do not express any MHC class I molecules and thus none of the MHC class I specific inhibitory receptors expressed in these mice mediates education [17]. Study III and IV are focused on comparing the Ly49A positive subpopulation of NK cells from H2-D^d and $MHC^{-/-}$ mice, as a model to compare educated versus uneducated NK cells.

3.2 IMMUNO-FLUORESCENT LABELING

Most of the techniques used in the studies were based on advanced fluorescent microscopy and carried out on freshly isolated primary live or fixed NK cells. Due to the long exposure times involved in both live cell and super resolution imaging, it was a requirement for the probes to have high fluorescence quantum yield and photostability. In study I, II and III, due to the unavailability of antibodies with such suitable fluorescent tags, purified primary antibodies were conjugated to dyes by ourselves in the lab. The conjugation was based on the amino-reactive reagent N-hydroxysuccinimidyl (NHS)-esters. This reactive group forms chemically stable bonds between the probe and the antibody. For study IV, we employed secondary antibodies that were conjugated to recommended fluorescent probes for STED microscopy against primary antibody for activating and inhibitory receptors of NK cells.

3.3 MICROSCOPY TECHNIQUES

3.3.1 Fluorescence correlation spectroscopy (FCS)

FCS facilitates quantification of concentrations and diffusion coefficients of proteins on live cell membranes. The technique was adapted to study freshly isolated primary cells. In study I, FCS was used to characterize MHC class I molecules and inhibitory Ly49A

receptors on the membrane of resting and cytokine stimulated NK cells. NK cells were enriched from freshly isolated splenocytes and cultured with IL-2 and IFN-α/β for 4 hours at 37 °C. A short time of stimulation was chosen to see whether cytokines already induced changes to the membrane protein dynamics at this time point, before extensive proliferation. MHC class I was present in abundance compared to Ly49A, so, MHC class I was chosen for the green channel (excitation at 488 nm, argon laser) and Ly49A was placed in the red channel (excitation at 633 nm, helium–neon laser). Cells were measured in suspension in a 1:1 mix of transparent RPMI (to avoid background from phenyl red) and PBS with 1 % fetal bovine serum. This mixture was found to minimize cell movement during the measurements.

3.3.2 STED microscopy

To quantitate the cluster density, size, and distance between activating and inhibitory receptor clusters, we opted for dual color super resolution STED microscopy, with a resolution as high as 35-40 nm. STED does not require any photoswitchable or photoactivated dyes, it simply works with high quantum yield dyes with good spectral separation. The organization of receptors were compared on educated and uneducated NK cells, hence the Ly49A positive subpopulation was sorted by flow cytometry from the two chosen mouse strains prior to imaging. Images were acquired on fixed cells that were mounted to the microscope slide. The tedious part of the STED experiments was optimization of sample preparation, to establish a working protocol with no unspecific binding of antibodies, low background and high signal for dual color STED images of activating and inhibitory receptors co-stained on NK cells. The two color STED images were established using two pulsed diode lasers for excitation at 532/590 nm and 640 nm for Alexa flour 594 and Abberior star 635p dyes, respectively.

3.3.3 TIRF – iMSD

Total internal reflection fluorescence (TIRF) microscopy is utilized to study proteins close to or at the cell membrane. This technique combined with Spatio-Temporal Image Correlation Spectroscopy (STICS)-image Mean Square Displacement (iMSD) analysis enables to identify the type of movement that is the most prominent among the labeled proteins on the cell membrane. The advantage of this method is that slow movements are also accounted for, unlike the FCS method. The fraction of receptors that are completely immobile during the whole measurement period is however removed also in TIRF-iMSD.

3.3.4 TRAST microscopy

Transient state imaging was used to test whether educated and uneducated NK cells could be differentiated based on their metabolic states. Ly49A sorted NK cells from both mouse strains were labeled with CFSE at a very low concentration of 0.1 nM. Healthy and live cells were chosen for measurement using bright field view of the cells. The electrons were driven into a dark triplet state by using a 488nm excitation laser (210 mW). The duration of the excitation pulses was systematically changed via an acousto-optical modulator (AOM), which lead to different fractions of the fluorophores populating the triplet state, and in turn lead to systematic changes in the average fluorescence intensity recorded from the fluorophores. The changes in the average fluorescence intensity versus the duration of the excitation pulses were plotted, and fitted to electronic state models. Thereby, the transition rates into the dark state(s) can be calculated, including k_t. Any photo-bleaching during measurement was taken into account by additional control measurements. The strong dependence of k_t on the ambient oxygen concentration provide an indirect measure of the local oxygen concentration (and thus consumption) in the cell [138].

3.4 IMAGE ANALYSIS

All microscopy data was analyzed by customized MATLAB scripts for efficient and standardized analysis. The scripts were written by software programmers. For study I and II, the script was based on fitting the experimental data to chosen mathematical models. For each fit, diffusion coefficients and counts per molecule are extracted for the moving entities. In study III, the images of 6000 frames were loaded into MATLAB and a square Region of Interest of 3.2 x 3.2 μm^2 was selected and analyzed. The experimental data was fitted to all proposed models of movement and output parameters for each model was generated. In study IV, clusters were identified by setting a threshold. Above this threshold, the intensities were counted as a cluster. The size of a cluster intensity profile was set by the full width half maximum. That is, the border of the cluster is defined as being where the intensity has reached half of the maximum intensity within that cluster, moving outwards from the highest recorded intensity for each cluster. Since the activating and inhibitory receptors do not have direct contact but may communicate through signaling molecules, the nearest neighbor distance between receptors is calculated, rather than direct overlap. Due to steric hindrance of the secondary antibody labeling, it is also very difficult to detect direct fluorescent overlap using super resolution microscopy [139].

4. RESULTS AND DISCUSSION

The main aims of this thesis can be categorized into two parts. First, it was to elucidate the influence of cytokine stimulation on dynamics of NK cells receptors. Second, to investigate the temporal dynamics and spatial organization of receptors on educated and uneducated NK cells. We also investigated the metabolic state of NK cells and if it was altered depending on the educational status.

Paper I

This paper demonstrated that the molecular dynamics of the studied MHC class I molecule and the Ly49A inhibitory receptor were altered within a few hours of cytokine stimulation. Ly49A interacts with the MHC class I molecule H2-D^d in *trans,* as well as in *cis,* on the same NK cell [34]. It was shown in a transfected cell line system that the level of MHC class I expression influences the amount of *cis* interaction between Ly49A and H-$2D^d$ [140]. During viral infections, type I interferons are produced, which mediates various immunostimulatory functions [141]. These interferons upregulate MHC class I expression on lymphoid cells [142]. On the other hand, for *in vitro* assays and clinical applications IL-2 is widely used for activating NK cells. Less was known about how IL-2 affects MHC class I expression levels. We thus started by investigating if these cytokines influence MHC class I expression levels on NK cells at different time points, and if this would lead to an alteration in the fraction of Ly49A receptors bound in *cis*. This was first addressed by stimulating splenocytes and by gating on NK cells they were analyzed for MHC class I and Ly49A expression levels at the cell surface, using flow cytometry. We observed upregulation of MHC class I with IL-2 and IFN-α/β stimulation already at the earliest time point of 4 hours (Figure 4). However, this stimulation effect could be either direct or indirect, since T cells and other cells in the culture could have mediated the cytokine effect on NK cells. Later, enriched NK cells were stimulated with the same concentrations of cytokines and we could still observe a similar effect (data not shown).

We used two different antibodies to study how cytokine stimulation affected the total cellular expression level of Ly49A, as well as the amount of Ly49A which was “free” (not bound in *cis*) (Figure 4). Surprisingly, despite the dramatic upregulation of MHC class I in response to cytokines, the fraction of free Ly49A receptors compared to total Ly49A did not seem to change dramatically over the time period we studied.

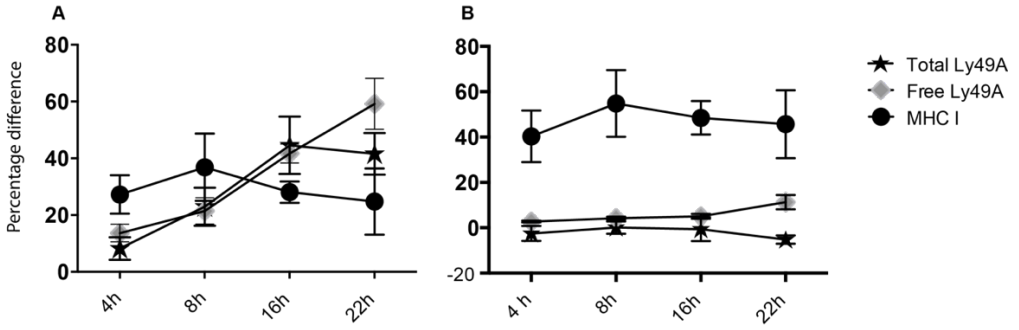

Figure 4. Upregulation of MHC class I and Ly49A with cytokine stimulation. Splenocytes activated with IL-2 and IFN-α/β cytokines at different time points. NK cells are gated on the $NK1.1^{+}CD3^{-}$ lymphocyte population. Percentage difference was calculated with respect to control that was incubated at 37C without any cytokines **(A)** IL-2 activated NK cells **(B)** IFN-α/β activated NK cells.

Since MHC class I was upregulated already at the earliest time point, we investigated if this short period of stimulation was enough to alter the molecular dynamics of $H\text{-}2D^{d}$ and Ly49A using the FCS method. Data revealed that MHC class I molecules diffused significantly faster on stimulated NK cells with both IL-2 and IFN-α/β, while Ly49A diffused significantly faster only with IFN-α/β stimulated NK cells (Paper I). Ly49A diffusion was in general twice as fast as that of MHC class I. The IL-2 stimulation did not alter the diffusion rate of Ly49A in total Ly49A positive NK cells, but interestingly when performing a multivariate analysis, it was shown that a subpopulation of NK cells that displayed faster diffusion of MHC class I also exhibited faster diffusion also of Ly49A. The FCS data distribution indicates clearly, that upon cytokine stimulation the NK cells respond heterogeneously. That was evident from the spread of the stimulated cells in the diffusion coefficient and also in the brightness of the analyzed MHC class I and Ly49A. Thus, it was shown with multivariate analysis that there were around 29 % of IL-2 and 31 % of IFN-α/β stimulated cells which contributed to a distinct subpopulation among stimulated NK cells. Previously, in line with this it was shown that human NK cells react heterogeneously in their cytotoxic response and a subpopulation of IL-2 activated cells have increased their cell size and was able to kill several targets after a few days of activation [143]. This subpopulation of NK cells could be efficient in killing target cells. Thus, it would be interesting to further characterize the subpopulation of NK cells from our study, by combining FCS with microchip based method that is well established in professor Björn Önfelt's group which could facilitate isolation of these distinct individual NK cells. Another possible speculation could be that this percentage of 29-31 % may indicate the percentage of Ly49A single positive subset from total Ly49A population in homozygous $H2\text{-}D^{d}$ mouse that would enable faster diffusion on the cell membrane due to less crowding. For a future direction, it would be interesting to characterize the molecular dynamics of adhesion

molecules on NK cells after cytokine stimulation which could give an insight on the role of faster molecular dynamics for better conjugation with target cells. In summary, a better understanding of how cytokines influence molecular expression and dynamics at the NK cell surface could be of potential importance in order to better understand target cell interaction for effective cytotoxic activity and cell trafficking.

Paper II

Fluorescence correlation spectroscopy (FCS) has significantly contributed to our understanding of cell biology. It can provide information about concentration and diffusion of molecules, and even biomolecular interactions (if extended to Fluorescence Cross-Correlation Spectroscopy). Two important advantages of this technique compared to other methods for studying protein concentrations and diffusion rates are that it provides single molecule sensitivity and it works well for low concentrations. So far, the utmost majority of the applications of FCS were carried out on cell line systems [144]. Guia *et al*. however in a study on educated and uneducated NK cells using FCS revealed that the activating receptors are confined in microdomains on educated NK cells [120]. In paper II, we adapted the method and provided a protocol for measuring FCS on primary NK cells. There are earlier studies on MHC class I topographical distribution and mobility [145, 146]. In a previous study on cell lines, the diffusion rate of MHC class I molecules on the cell membrane was 0.9 $\mu m^2/s$ [140], and in line with that, we also observed a similar diffusion coefficient of MHC class I (0.95 $\mu m^2/s$) on primary NK cells (Paper I). By first verifying the sensitivity and specificity against fluorescently tagged versions of the protein of interest, as was done in ref [140], the use of antibodies for detection in primary cells can be validated. In paper I, we also utilized this method to find the binding affinity of YE1/48 clone antibody to Ly49A receptors on a Ly49A-GFP transfected cell line. By adapting our protocol of immunostaining and sample preparation, FCS can be implemented for any immune cells to study diffusion rates and concentration of the molecules.

In extension to the published data, we applied this method to differentiate molecular dynamics of educated and uneducated NK cells by immunostaining an activating receptor, NKp46, on H2-D^d and $MHC^{-/-}$ NK cells. Autocorrelation curves were acquired on Ly49A positive NK cells (Ly49A was stained with another fluorophore, $Ly49A^+$ cells were identified visually in the microscope). It was found that NKp46 diffused faster on educated NK cells (Figure 5). Interestingly, along with this difference in the diffusion rate of NKp46, there was also some speculation from the preliminary NKp46 STED images (paper IV) which gave rise

to the idea that NKp46 may follow some differential pattern in their organization on the cell membrane, depending on education (discussed further below). The faster diffusion of NKp46 on educated NK cells may help the NK cell to screen more efficiently for activating ligands on target cells.

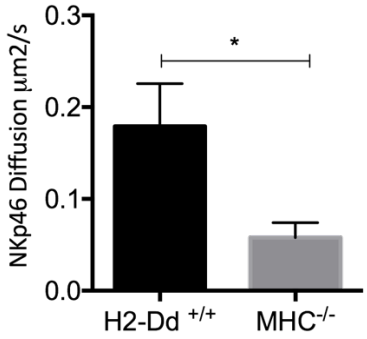

Figure 5. Increased diffusion rate of NKp46 on educated NK cells. NK cells were enriched from H2-D^d and $MHC^{-/-}$ mice and immunostained for NKp46 and Ly49A receptors. Comparison between two unpaired groups by Mann-Whitney test, * $p < 0.05$.

Paper III

Having a basic understanding of NKp46 diffusion dynamics and its organization on NK cells from our FCS studies, we established a novel technique, TIRF-iMSD, which combined TIRF microscopy and spatio-temporal image correlation spectroscopy (STICS) with image mean square displacement (iMSD) analysis. The TIRF-iMSD analysis yields a quantitative description of the dynamics at each pixel and distinguishes different diffusion patterns exhibited by the molecule. To validate this new analysis approach, the images were also analyzed by another method, single particle tracking (SPT), which enables tracking of the diffusion pattern of individual molecules. With the SPT technique, it was observed that NKp46 and Ly49A were transiently confined in microdomains on the NK cell membrane, and that NKp46 diffused faster on educated NK cells. With TIRF-iMSD it was found that NKp46 resided in larger microdomains compared to uneducated NK cells (confirmed by SPT), and the transit time through domains were shorter. Ly49A on the other hand, were observed in smaller domains and diffused slower on educated NK cells, but still dwelled in domains for a shorter time. NKp46 residing in larger microdomains as well as exhibiting faster diffusion on educated NK cells could facilitate the formation of larger and thus more stable conjugates with target cells, since receptors during synapse formation are recruited to the synapse from other parts of the cell. These observations could be related to a study that

reported that NKG2A$^+$ educated NK cells form more long-lasting target cell conjugates [121]. The observation that Ly49A diffuse faster on uneducated NK cells, may be interpreted as a way to effectively interrupt the conjugate formation with target cells. ITIM bearing receptors inhibit the adhesion of NK cells to target cells [147], and it was also shown that uneducated NK cells form fewer stable conjugates [121, 148].

The actin meshwork restricts the diffusion of activating receptors on uneducated NK cells [120], it however remained unclear if the restriction of activating receptors results in hypo-responsivity. The role of the actin meshwork in educated NK cells also remained unclear. We disrupted the actin cytoskeleton and measured the diffusion of the activating receptor NKp46 using TIRF-iMSD. It was observed that the actin cytoskeleton disruption abrogated the cell signaling in response to activating receptor crosslinking, which showed that the actin meshwork plays an important role in activating signal initiation. This is in line with what has been previously shown in T cells [149, 150] and it has also been discussed that actin cytoskeleton is the point for modulation of protein dynamics and clustering in B cells [126, 129]. Next, we examined if the receptor diffusion could be altered by addition of cholesterol and thereby influence the NK cell response. The cholesterol addition decreased the dwell time of NKp46 in microdomains and also led to a decrease in the cell response to NKp46 crosslinking. The cholesterol addition might have restricted the transition of activating receptors from one domain to another domain, which disturbed the cell signaling. Alteration of cholesterol and the actin composition on the NK cell membrane, leading to a perturbed cell signaling via the NKp46 receptor, indicates that NK cell receptors might be regulated by several topological factors of cell membrane.

Paper IV

For the past few years, more interest has been directed towards receptor organization in resolution down to the nanometer scale. We were interested in finding out if education of NK cells has altered the organization of activating and inhibitory receptors on their cell membrane. This was addressed with STED microscopy. We imaged inhibitory and activating receptors on murine educated and uneducated NK cells. Firstly, we measured the cluster density and size of the receptor clusters. Interestingly the cluster size of inhibitory receptor Ly49A and NKp46 was larger on uneducated NK cells (Paper IV). The cluster sizes of NKRP1C and NKG2D did on the other hand not change with educational status). It is shown that the size of activating and inhibitory clusters controls the recruitment of signaling molecules and thereby affect cell signaling [125]. The larger clusters could thus be an indication for different threshold levels for signaling between educated and uneducated NK

cells. The larger size of Ly49A clusters in uneducated MHC$^{-/-}$ NK cells could simply be due to the higher expression level. This does nevertheless not exclude that an educational effect is mediated through larger receptor clusters, since the two phenomena, receptor expression level and educational status, has not been studied in isolation. This is due to that the same factor, namely the presence of a specific MHC class I ligand, mediates both effects. It would have been interesting to measure the cluster size of two inhibitory receptors within the same mice, where one has an educating MHC class I ligand and the other does not. This comparison would however be connected with the same issue, that the expression level and education cannot be differentiated.

Regarding the larger clusters of the activating NKp46 receptor, it has been shown that the clustering of TCR is important for cell signaling and activation leading to recruitment of ZAP-70 and further with multi-molecular complexes [122]. It is interesting to note that the clustering effect of activating receptors varied depending on their function. NKp46 is from the NCR family of activating receptors which recognize pathogen associated antigens and it is important for NK cells to respond to infection, which occurs irrespective of their educational status. It has even been shown in some circumstances of infection that uneducated NK cells respond better than educated, since the educated NK cells have some extent of inhibition via MHC class I expressed on target cells (reviewed in [151]).

Next, we measured the nearest neighbor distance between activating and inhibitory receptors to investigate if this distance is an important factor in education. The “licensing” model of NK cell education implies that a licensing signal is transmitted from the inhibitory receptors, allowing activation to proceed upon encounters with target cells expressing activating ligands. It was thus interesting to investigate whether inhibitory receptors need to be in close contact with activating receptors for licensing to occur. Another hypothesis could be that inhibitory receptors need to be close to activating receptors in resting cells for an immediate inhibition of activation upon target cell encounter. In this case the inhibitory receptors would be closer to the activating receptors than expected from a random distribution in both educated and uneducated NK cells. To test these hypotheses, we wanted to measure the distance from each activating receptor cluster to its closest inhibitory receptor cluster (A to B), also known as the nearest neighbor (NN) distance. We also measured the NN distance from each inhibitory to its closest activating receptor (B to A). The distance from activating to inhibitory receptors was however not significantly different between educated and uneducated cells on average. The only difference observed was in the distance from Ly49A to NKp46 receptors, which was most likely due to the difference in cluster density. This

indicated that the distance from activating to inhibitory receptors is not important for the educational effect, nor is influenced by education. In the future, it would be important to study the localization of signaling molecules like SHP-1, 2 and SHIP and other activation associated molecules which are shown to be vital for NK cell activation. These signaling molecules might need be in closer proximity to induce an impact on activation or inhibition, rather the than distance between receptors.

Distance between (µm)	**NKp46 (A) and Ly49A (B)**			**NKG2D (A) and Ly49A (B)**		
	H2-D^{d}	**MHC$^{-/-}$**	**p value***	**H2-D^{d}**	**MHC$^{-/-}$**	**p value***
	Median	**Median**		**Median**	**Median**	
A-B	94,09	82,82	0,062	241,1	217,9	0,958
B-A	96,94	122,22	**0,004**	98,75	97,12	0,496
B-B	151,2	123,6	**0,002**	153,6	148,7	0,638
A-A	155,1	163,2	0,062			
*Mann Whitney test						

Table 1: Summary results of the nearest neighbor distance measured on dual color STED images. Table includes the median value and P value from Mann Whitney statistical test.

Interestingly, the NKp46 receptor had shown a difference in the organization and diffusion on NK cells correlated with education (from study II and III). We thus wanted to investigate if the distribution of this receptor is random or organized differently. To address this, we generated simulated cells with the same cluster density and cell size, and then measured the Nearest Neighbor distance and compared with experimental (NK) cells (shown in Figure 6).

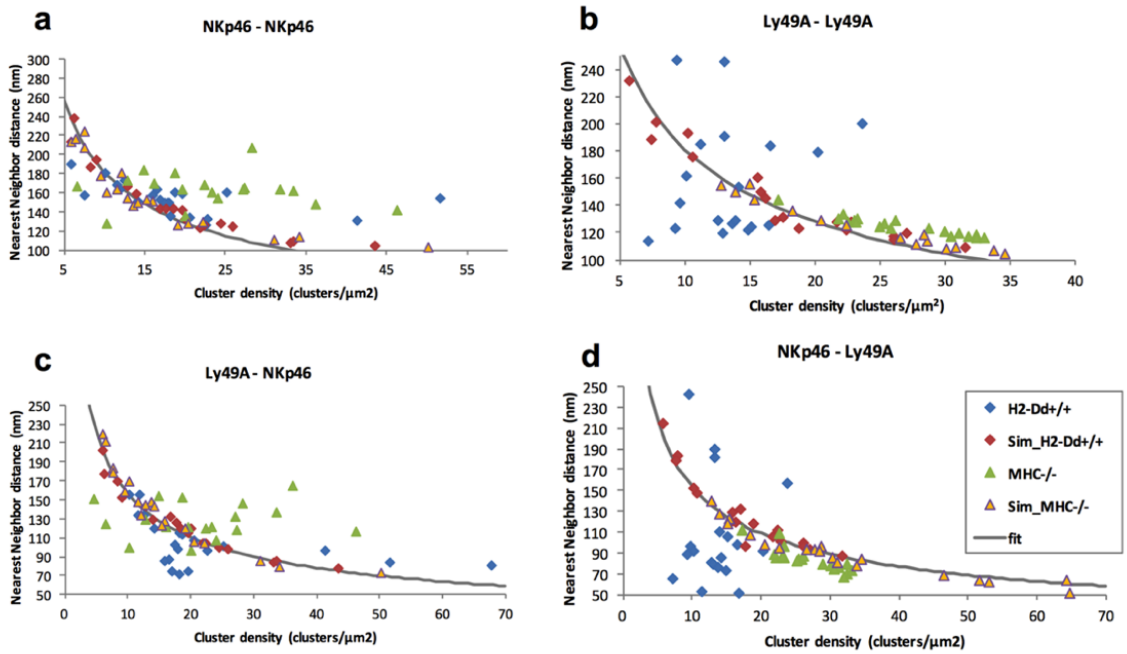

Figure 6: **Nearest neighbor distance from the 1st receptor to the 2nd (mentioned from left to right in the table headings), plotted against the density of the 2nd receptor.** Distance between receptors on simulated and experimental cells (a) NKp46 to NKp46 (b) Ly49A to Ly49A (c) Ly49A to NKp46 (d) NKp46 to Ly49A. The x axis show the density of NKp46 in (a) and (c), and of Ly49A in (b) and (d). The line indicates the curve fitting for the simulated cells (random distribution).

The NKG2D and the NKRP1-C receptors followed a random distribution pattern in both mice (data not shown). But preliminary results indicated that the organization of NKp46 and Ly49A receptors did not simply follow a random distribution when taking the receptor density of each cell into account (Figure 6). The nearest neighbor distance between each NKp46 receptor cluster was similar in all cells, regardless of their density (Fig. 6a). The nearest neighbor distance between each Ly49A receptor have on the other hand a scattered pattern of distribution in H-2D^d single mice (Fig. 6b). Since all these NK cells were educated, this indicates that another factor than education affects the distribution of Ly49A in a non-random fashion in H-2D^d mice, whereas it follows a more random pattern in MHC$^{-/-}$ mice. The distance between activating NKp46 receptors might on the other hand be kept at a relatively fixed distance, regardless of receptor density, although this deviation from random was weaker that the scattered pattern of Ly49A.

Overall, our data indicated that the distance between activating and inhibitory receptors is not a major governing factor for education. To conclude, the organization of the different receptors were not completely random. The organization of Ly49A may be influenced by other molecules, for example it could be an adhesion molecules like LFA-1, or the expression of other inhibitory receptors, since our measurements were done on the total Ly49A$^+$

population. Rather than the distance between inhibitory and activating receptors the cluster size, especially of the inhibitory receptors, may be an important factor for NK cell education. Lastly, it is also important to elucidate whether the clustering of receptors lead to better signaling for NK cells. Results from our study have indicated the importance of the cytoskeleton for cell signaling. Based on these findings, it would be an interesting idea to differentiate the actin structure on educated and uneducated NK cells using STED.

Metabolic state of NK cells

Recent years has become an era of research on cellular metabolism and its association with diseases (e.g. cancer) and specialized (e.g. immune) cell functions. Upon activation, NK cells increase glucose metabolism through glycolysis (reviewed in [152]). We wanted to investigate if NK cells in the resting state can be in different metabolic states, depending on their educational status? This was addressed by measuring the oxygen consumption by cells using the TRAST microscopy method. As a control, IL-2 stimulated NK cells from H-2D^{d} and MHC$^{-/-}$ mice were also measured in the experimental setup. Interestingly, the metabolic rate of resting educated NK cells was higher compared to uneducated NK cells, whereas this effect was not seen in the cytokine stimulated NK cells.

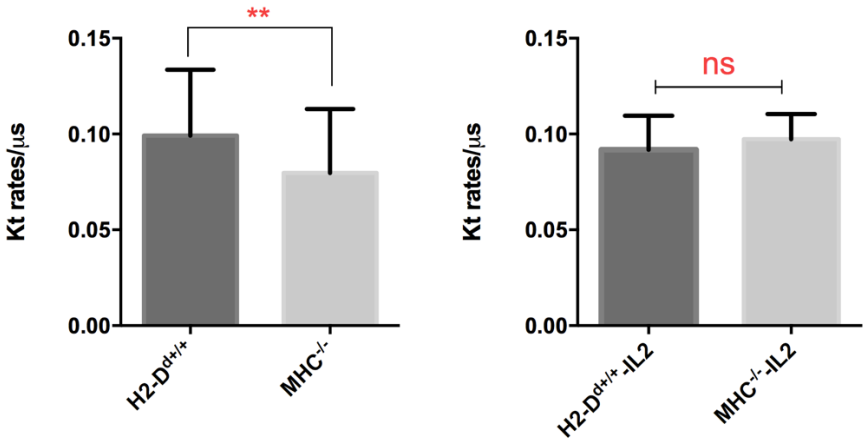

Figure 7. Kt rates measured per microseconds on educated (H2-D$^{d+/+}$) and uneducated MHC$^{-/-}$ NK cells. Data presented is a compilation from two individual experiments, n= 64 cells. Unpaired t test was performed, **p<0.01

This difference in NK cell metabolism associated to education are well in line with recent findings that educated NK cells have higher basal activity of the mTOR pathway, a central coordinator of the metabolism. NK cells undergo metabolic reprogramming during activation [153]. CD56(bright) NK cells were shown to be more metabolically active than CD56(dim) NK cells and metabolic reprogramming supported the IFN-γ production [154]. Using

transient state microscopy, we could easily distinguish the metabolic state of the NK cells at the single cell level. This is an advantage compared to alternative methods to measure oxygen consumption, which often require large numbers of cells. Furthermore, this technique can be used in combination with specific staining of certain cell organelles, to better distinguish the metabolic activity differences within different subcellular compartments.

5. CONCLUSION AND FUTURE DIRECTIONS

In summary, this thesis aimed to explore how the receptor dynamics and clustering are influenced upon education and cytokine stimulation of NK cells. The results from the studies have indicated that the receptor spatiotemporal organization could be an influential factor for determining the effective response of NK cells.

Conclusions from the studies are stated below:

Cytokine stimulated NK cells were shown to have a heterogeneous membrane dynamics and exhibit a subpopulation with higher membrane dynamics. In addition, we also showed that a few hours of cytokine stimulation is sufficient to influence the receptor levels and dynamics of NK cells (Paper I).

The activating and inhibitory receptors of NK cells were confined in microdomains. Activating and inhibitory receptors exhibit differential confinement and diffusion on educated NK cells. The actin and cholesterol composition of NK cells can influence the receptor dynamics and cell signaling (Paper III).

Receptor clustering was different on educated and uneducated NK cells, NKp46 and Ly49A clusters were larger on uneducated NK cells. The inhibitory and activating receptors were not in closer proximity in educated NK cells compared to uneducated NK cells. The distribution of NKp46 and Ly49A clusters was nevertheless modulated on the NK cell membrane in a non-random fashion (Paper IV).

Educated resting NK cells had a higher metabolic rate compared to uneducated NK cells, and this difference was not observed with cytokine stimulation of NK cells 'activation overriding education' effect (unpublished study).

Based on the results, a few interesting thoughts that could be tested: 1) To differentiate actin and lipid structures on educated and uneducated NK cells, which could be a potential regulator of receptor clustering and dynamics. 2) Cytokine stimulated and educated NK cells may share similar receptor dynamic properties, which could be characterized using our experimental approaches. 3) Difference in metabolic state of NK cells and receptor expression on educated NK cells, there could be difference in protein turnover via autophagy. This could be tested using mouse models for education.

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7. References

1. Kiessling, R., et al., *"Natural" killer cells in the mouse. II. Cytotoxic cells with specificity for mouse Moloney leukemia cells. Characteristics of the killer cell.* Eur J Immunol, 1975. **5**(2): p. 117-21.
2. Kiessling, R., E. Klein, and H. Wigzell, *"Natural" killer cells in the mouse. I. Cytotoxic cells with specificity for mouse Moloney leukemia cells. Specificity and distribution according to genotype.* Eur J Immunol, 1975. **5**(2): p. 112-7.
3. Herberman, R.B., et al., *Natural cytotoxic reactivity of mouse lymphoid cells against syngeneic and allogeneic tumors. II. Characterization of effector cells.* Int J Cancer, 1975. **16**(2): p. 230-9.
4. Paust, S., B. Senman, and U.H. von Andrian, *Adaptive immune responses mediated by natural killer cells.* Immunol Rev, 2010. **235**(1): p. 286-96.
5. Gabrielli, S., et al., *The Memories of NK Cells: Innate-Adaptive Immune Intrinsic Crosstalk.* J Immunol Res, 2016. **2016**: p. 1376595.
6. Orange, J.S. and C.A. Biron, *Characterization of early IL-12, IFN-alphabeta, and TNF effects on antiviral state and NK cell responses during murine cytomegalovirus infection.* J Immunol, 1996. **156**(12): p. 4746-56.
7. Freeman, B.E., et al., *Cytokine-Mediated Activation of NK Cells during Viral Infection.* J Virol, 2015. **89**(15): p. 7922-31.
8. Snell, G.D. and R.B. Jackson, *Histocompatibility genes of the mouse. II. Production and analysis of isogenic resistant lines.* J Natl Cancer Inst, 1958. **21**(5): p. 843-77.
9. Cudkowicz, G. and M. Bennett, *Peculiar immunobiology of bone marrow allografts. I. Graft rejection by irradiated responder mice.* J Exp Med, 1971. **134**(1): p. 83-102.
10. Cudkowicz, G. and M. Bennett, *Peculiar immunobiology of bone marrow allografts. II. Rejection of parental grafts by resistant F 1 hybrid mice.* J Exp Med, 1971. **134**(6): p. 1513-28.
11. Kiessling, R., et al., *Evidence for a similar or common mechanism for natural killer cell activity and resistance to hemopoietic grafts.* Eur J Immunol, 1977. **7**(9): p. 655-63.
12. Kärre, K., *On the immunobiology of Natural Killer Cells.*, in *Tumor Biology*. 1981, Karolinska Institute.
13. Kärre, K., et al., *Selective rejection of H-2-deficient lymphoma variants suggests alternative immune defence strategy.* Nature, 1986. **319**(6055): p. 675-8.
14. Kärre, K., *NK cells, MHC class I molecules and the missing self.* Scand J Immunol, 2002. **55**(3): p. 221-8.
15. Kärre, K., *Natural killer cell recognition of missing self.* Nat Immunol, 2008. **9**(5): p. 477-80.
16. Karlhofer, F.M., R.K. Ribaudo, and W.M. Yokoyama, *MHC class I alloantigen specificity of Ly-49+ IL-2-activated natural killer cells.* Nature, 1992. **358**(6381): p. 66-70.

17. Liao, N.S., et al., *MHC class I deficiency: susceptibility to natural killer (NK) cells and impaired NK activity.* Science, 1991. **253**(5016): p. 199-202.

18. Kim, S., et al., *Licensing of natural killer cells by host major histocompatibility complex class I molecules.* Nature, 2005. **436**(7051): p. 709-13.

19. Fernandez, N.C., et al., *A subset of natural killer cells achieves self-tolerance without expressing inhibitory receptors specific for self-MHC molecules.* Blood, 2005. **105**(11): p. 4416-23.

20. Malarkannan, S., *The balancing act: inhibitory Ly49 regulate NKG2D-mediated NK cell functions.* Semin Immunol, 2006. **18**(3): p. 186-92.

21. Regunathan, J., et al., *NKG2D receptor-mediated NK cell function is regulated by inhibitory Ly49 receptors.* Blood, 2005. **105**(1): p. 233-40.

22. Long, E.O., et al., *Controlling natural killer cell responses: integration of signals for activation and inhibition.* Annu Rev Immunol, 2013. **31**: p. 227-58.

23. Hanke, T., et al., *Direct assessment of MHC class I binding by seven Ly49 inhibitory NK cell receptors.* Immunity, 1999. **11**(1): p. 67-77.

24. Raulet, D.H., et al., *Specificity, tolerance and developmental regulation of natural killer cells defined by expression of class I-specific Ly49 receptors.* Immunol Rev, 1997. **155**: p. 41-52.

25. Kubota, A., et al., *Diversity of NK cell receptor repertoire in adult and neonatal mice.* J Immunol, 1999. **163**(1): p. 212-6.

26. Held, W. and B. Kunz, *An allele-specific, stochastic gene expression process controls the expression of multiple Ly49 family genes and generates a diverse, MHC-specific NK cell receptor repertoire.* Eur J Immunol, 1998. **28**(8): p. 2407-16.

27. Michaelsson, J., et al., *Visualization of inhibitory Ly49 receptor specificity with soluble major histocompatibility complex class I tetramers.* Eur J Immunol, 2000. **30**(1): p. 300-7.

28. Johansson, S., et al., *Natural killer cell education in mice with single or multiple major histocompatibility complex class I molecules.* J Exp Med, 2005. **201**(7): p. 1145-55.

29. Smith, K.M., et al., *Ly-49D and Ly-49H associate with mouse DAP12 and form activating receptors.* J Immunol, 1998. **161**(1): p. 7-10.

30. Kielczewska, A., et al., *Ly49P recognition of cytomegalovirus-infected cells expressing H2-Dk and CMV-encoded m04 correlates with the NK cell antiviral response.* J Exp Med, 2009. **206**(3): p. 515-23.

31. Orihuela, M., D.H. Margulies, and W.M. Yokoyama, *The natural killer cell receptor Ly-49A recognizes a peptide-induced conformational determinant on its major histocompatibility complex class I ligand.* Proc Natl Acad Sci U S A, 1996. **93**(21): p. 11792-7.

32. Correa, I. and D.H. Raulet, *Binding of diverse peptides to MHC class I molecules inhibits target cell lysis by activated natural killer cells.* Immunity, 1995. **2**(1): p. 61-71.

33. Doucey, M.A., et al., *Cis association of Ly49A with MHC class I restricts natural killer cell inhibition.* Nat Immunol, 2004. **5**(3): p. 328-36.

34. Scarpellino, L., et al., *Interactions of Ly49 family receptors with MHC class I ligands in trans and cis.* J Immunol, 2007. **178**(3): p. 1277-84.

35. Sternberg-Simon, M., et al., *Natural killer cell inhibitory receptor expression in humans and mice: a closer look.* Front Immunol, 2013. **4**: p. 65.

36. Vance, R.E., et al., *Mouse CD94/NKG2A is a natural killer cell receptor for the nonclassical major histocompatibility complex (MHC) class I molecule Qa-1(b).* J Exp Med, 1998. **188**(10): p. 1841-8.

37. Sivakumar, P.V., et al., *Cutting edge: expression of functional CD94/NKG2A inhibitory receptors on fetal NK1.1+Ly-49- cells: a possible mechanism of tolerance during NK cell development.* J Immunol, 1999. **162**(12): p. 6976-80.

38. Ito, M., et al., *Killer cell lectin-like receptor G1 binds three members of the classical cadherin family to inhibit NK cell cytotoxicity.* J Exp Med, 2006. **203**(2): p. 289-95.

39. Robbins, S.H., et al., *Cutting edge: inhibitory functions of the killer cell lectin-like receptor G1 molecule during the activation of mouse NK cells.* J Immunol, 2002. **168**(6): p. 2585-9.

40. Iizuka, K., et al., *Genetically linked C-type lectin-related ligands for the NKRP1 family of natural killer cell receptors.* Nat Immunol, 2003. **4**(8): p. 801-7.

41. Rahim, M.M., et al., *The mouse NKR-P1B:Clr-b recognition system is a negative regulator of innate immune responses.* Blood, 2015. **125**(14): p. 2217-27.

42. Carlyle, J.R., et al., *Missing self-recognition of Ocil/Clr-b by inhibitory NKR-P1 natural killer cell receptors.* Proc Natl Acad Sci U S A, 2004. **101**(10): p. 3527-32.

43. Stanietsky, N., et al., *The interaction of TIGIT with PVR and PVRL2 inhibits human NK cell cytotoxicity.* Proc Natl Acad Sci U S A, 2009. **106**(42): p. 17858-63.

44. Garni-Wagner, B.A., et al., *A novel function-associated molecule related to non-MHC-restricted cytotoxicity mediated by activated natural killer cells and T cells.* J Immunol, 1993. **151**(1): p. 60-70.

45. Tangye, S.G., et al., *Cutting edge: human 2B4, an activating NK cell receptor, recruits the protein tyrosine phosphatase SHP-2 and the adaptor signaling protein SAP.* J Immunol, 1999. **162**(12): p. 6981-5.

46. Lee, K.M., et al., *2B4 acts as a non-major histocompatibility complex binding inhibitory receptor on mouse natural killer cells.* J Exp Med, 2004. **199**(9): p. 1245-54.

47. Schatzle, J.D., et al., *Characterization of inhibitory and stimulatory forms of the murine natural killer cell receptor 2B4.* Proc Natl Acad Sci U S A, 1999. **96**(7): p. 3870-5.

48. Wu, N., et al., *A hematopoietic cell-driven mechanism involving SLAMF6 receptor, SAP adaptors and SHP-1 phosphatase regulates NK cell education.* Nat Immunol, 2016. **17**(4): p. 387-96.

49. Bryceson, Y.T., et al., *Synergy among receptors on resting NK cells for the activation of natural cytotoxicity and cytokine secretion.* Blood, 2006. **107**(1): p. 159-66.

50. Bryceson, Y.T., H.G. Ljunggren, and E.O. Long, *Minimal requirement for induction of natural cytotoxicity and intersection of activation signals by inhibitory receptors.* Blood, 2009. **114**(13): p. 2657-66.

51. Kruse, P.H., et al., *Natural cytotoxicity receptors and their ligands.* Immunol Cell Biol, 2014. **92**(3): p. 221-9.

52. Mandelboim, O., et al., *Recognition of haemagglutinins on virus-infected cells by NKp46 activates lysis by human NK cells.* Nature, 2001. **409**(6823): p. 1055-60.

53. Chisholm, S.E. and H.T. Reyburn, *Recognition of vaccinia virus-infected cells by human natural killer cells depends on natural cytotoxicity receptors.* J Virol, 2006. **80**(5): p. 2225-33.

54. Jarahian, M., et al., *Modulation of NKp30- and NKp46-mediated natural killer cell responses by poxviral hemagglutinin.* PLoS Pathog, 2011. **7**(8): p. e1002195.

55. Arase, N., et al., *Association with FcRgamma is essential for activation signal through NKR-P1 (CD161) in natural killer (NK) cells and NK1.1+ T cells.* J Exp Med, 1997. **186**(12): p. 1957-63.

56. Lanier, L.L., et al., *Association of DAP12 with activating CD94/NKG2C NK cell receptors.* Immunity, 1998. **8**(6): p. 693-701.

57. Vance, R.E., A.M. Jamieson, and D.H. Raulet, *Recognition of the class Ib molecule Qa-1(b) by putative activating receptors CD94/NKG2C and CD94/NKG2E on mouse natural killer cells.* J Exp Med, 1999. **190**(12): p. 1801-12.

58. Guerra, N., et al., *NKG2D-deficient mice are defective in tumor surveillance in models of spontaneous malignancy.* Immunity, 2008. **28**(4): p. 571-80.

59. Jamieson, A.M., et al., *The role of the NKG2D immunoreceptor in immune cell activation and natural killing.* Immunity, 2002. **17**(1): p. 19-29.

60. Gilfillan, S., et al., *NKG2D recruits two distinct adapters to trigger NK cell activation and costimulation.* Nat Immunol, 2002. **3**(12): p. 1150-5.

61. Lanier, L.L., *NKG2D Receptor and Its Ligands in Host Defense.* Cancer Immunol Res, 2015. **3**(6): p. 575-82.

62. Wu, J., et al., *An activating immunoreceptor complex formed by NKG2D and DAP10.* Science, 1999. **285**(5428): p. 730-2.

63. Diefenbach, A., et al., *Selective associations with signaling proteins determine stimulatory versus costimulatory activity of NKG2D.* Nat Immunol, 2002. **3**(12): p. 1142-9.

64. Zompi, S., et al., *NKG2D triggers cytotoxicity in mouse NK cells lacking DAP12 or Syk family kinases.* Nat Immunol, 2003. **4**(6): p. 565-72.

65. Daniels, K.A., et al., *Murine cytomegalovirus is regulated by a discrete subset of natural killer cells reactive with monoclonal antibody to Ly49H.* J Exp Med, 2001. **194**(1): p. 29-44.

66. Arase, H., et al., *Direct recognition of cytomegalovirus by activating and inhibitory NK cell receptors.* Science, 2002. **296**(5571): p. 1323-6.

67. Smith, H.R., et al., *Recognition of a virus-encoded ligand by a natural killer cell activation receptor.* Proc Natl Acad Sci U S A, 2002. **99**(13): p. 8826-31.

68. George, T.C., et al., *Positive recognition of MHC class I molecules by the Ly49D receptor of murine NK cells.* J Immunol, 1999. **162**(4): p. 2035-43.

69. George, T.C., et al., *Tolerance and alloreactivity of the Ly49D subset of murine NK cells.* J Immunol, 1999. **163**(4): p. 1859-67.

70. Tahara-Hanaoka, S., et al., *Identification and characterization of murine DNAM-1 (CD226) and its poliovirus receptor family ligands.* Biochem Biophys Res Commun, 2005. **329**(3): p. 996-1000.

71. Gilfillan, S., et al., *DNAM-1 promotes activation of cytotoxic lymphocytes by nonprofessional antigen-presenting cells and tumors.* J Exp Med, 2008. **205**(13): p. 2965-73.

72. Bottino, C., et al., *Identification of PVR (CD155) and Nectin-2 (CD112) as cell surface ligands for the human DNAM-1 (CD226) activating molecule.* J Exp Med, 2003. **198**(4): p. 557-67.

73. Shibuya, K., et al., *Physical and functional association of LFA-1 with DNAM-1 adhesion molecule.* Immunity, 1999. **11**(5): p. 615-23.

74. Pende, D., et al., *PVR (CD155) and Nectin-2 (CD112) as ligands of the human DNAM-1 (CD226) activating receptor: involvement in tumor cell lysis.* Mol Immunol, 2005. **42**(4): p. 463-9.

75. Carlsten, M., et al., *DNAX accessory molecule-1 mediated recognition of freshly isolated ovarian carcinoma by resting natural killer cells.* Cancer Res, 2007. **67**(3): p. 1317-25.

76. El-Sherbiny, Y.M., et al., *The requirement for DNAM-1, NKG2D, and NKp46 in the natural killer cell-mediated killing of myeloma cells.* Cancer Res, 2007. **67**(18): p. 8444-9.

77. Horowitz, A., K.A. Stegmann, and E.M. Riley, *Activation of natural killer cells during microbial infections.* Front Immunol, 2011. **2**: p. 88.

78. Oberg, L., et al., *Loss or mismatch of MHC class I is sufficient to trigger NK cell-mediated rejection of resting lymphocytes in vivo - role of KARAP/DAP12-dependent and -independent pathways.* Eur J Immunol, 2004. **34**(6): p. 1646-53.

79. Chan, C.J., M.J. Smyth, and L. Martinet, *Molecular mechanisms of natural killer cell activation in response to cellular stress.* Cell Death Differ, 2014. **21**(1): p. 5-14.

80. Newman, K.C. and E.M. Riley, *Whatever turns you on: accessory-cell-dependent activation of NK cells by pathogens.* Nat Rev Immunol, 2007. **7**(4): p. 279-91.

81. Malhotra, A. and A. Shanker, *NK cells: immune cross-talk and therapeutic implications.* Immunotherapy, 2011. **3**(10): p. 1143-66.

82. Jinushi, M., et al., *Critical role of MHC class I-related chain A and B expression on IFN-alpha-stimulated dendritic cells in NK cell activation: impairment in chronic hepatitis C virus infection.* J Immunol, 2003. **170**(3): p. 1249-56.

83. Ferlazzo, G., et al., *Distinct roles of IL-12 and IL-15 in human natural killer cell activation by dendritic cells from secondary lymphoid organs.* Proc Natl Acad Sci U S A, 2004. **101**(47): p. 16606-11.

84. Lucas, M., et al., *Dendritic cells prime natural killer cells by trans-presenting interleukin 15.* Immunity, 2007. **26**(4): p. 503-17.

85. Luu, T.T., et al., *Independent control of natural killer cell responsiveness and homeostasis at steady-state by CD11c+ dendritic cells.* Sci Rep, 2016. **6**: p. 37996.

86. Bihl, F., et al., *Primed antigen-specific CD4+ T cells are required for NK cell activation in vivo upon Leishmania major infection.* J Immunol, 2010. **185**(4): p. 2174-81.

87. Gasteiger, G., et al., *IL-2-dependent tuning of NK cell sensitivity for target cells is controlled by regulatory T cells.* J Exp Med, 2013. **210**(6): p. 1167-78.

88. Granucci, F., et al., *Early IL-2 production by mouse dendritic cells is the result of microbial-induced priming.* J Immunol, 2003. **170**(10): p. 5075-81.

89. Granucci, F., et al., *A contribution of mouse dendritic cell-derived IL-2 for NK cell activation.* J Exp Med, 2004. **200**(3): p. 287-95.

90. Biron, C.A., et al., *Natural killer cells in antiviral defense: function and regulation by innate cytokines.* Annu Rev Immunol, 1999. **17**: p. 189-220.

91. Martinez, J., X. Huang, and Y. Yang, *Direct action of type I IFN on NK cells is required for their activation in response to vaccinia viral infection in vivo.* J Immunol, 2008. **180**(3): p. 1592-7.

92. Andoniou, C.E., et al., *Interaction between conventional dendritic cells and natural killer cells is integral to the activation of effective antiviral immunity.* Nat Immunol, 2005. **6**(10): p. 1011-9.

93. Maghazachi, A.A., A. Al-Aoukaty, and T.J. Schall, *CC chemokines induce the generation of killer cells from CD56+ cells.* Eur J Immunol, 1996. **26**(2): p. 315-9.

94. Zimmer, J., et al., *Activity and phenotype of natural killer cells in peptide transporter (TAP)-deficient patients (type I bare lymphocyte syndrome).* J Exp Med, 1998. **187**(1): p. 117-22.

95. Ohlen, C., et al., *Prevention of allogeneic bone marrow graft rejection by H-2 transgene in donor mice.* Science, 1989. **246**(4930): p. 666-8.

96. Anfossi, N., et al., *Human NK cell education by inhibitory receptors for MHC class I.* Immunity, 2006. **25**(2): p. 331-42.

97. Chalifour, A., et al., *A Role for cis Interaction between the Inhibitory Ly49A receptor and MHC class I for natural killer cell education.* Immunity, 2009. **30**(3): p. 337-47.

98. Bessoles, S., et al., *Education of murine NK cells requires both cis and trans recognition of MHC class I molecules.* J Immunol, 2013. **191**(10): p. 5044-51.

99. Brodin, P., K. Karre, and P. Hoglund, *NK cell education: not an on-off switch but a tunable rheostat.* Trends Immunol, 2009. **30**(4): p. 143-9.

100. Brodin, P., et al., *The strength of inhibitory input during education quantitatively tunes the functional responsiveness of individual natural killer cells.* Blood, 2009. **113**(11): p. 2434-41.

101. Wagner, A.K., et al., *Retuning of Mouse NK Cells after Interference with MHC Class I Sensing Adjusts Self-Tolerance but Preserves Anticancer Response.* Cancer Immunol Res, 2016. **4**(2): p. 113-23.

102. He, Y. and Z. Tian, *NK cell education via nonclassical MHC and non-MHC ligands.* Cell Mol Immunol, 2017. **14**(4): p. 321-330.

103. Yawata, M., et al., *MHC class I-specific inhibitory receptors and their ligands structure diverse human NK-cell repertoires toward a balance of missing self-response.* Blood, 2008. **112**(6): p. 2369-80.

104. Andrews, D.M., et al., *Recognition of the nonclassical MHC class I molecule H2-M3 by the receptor Ly49A regulates the licensing and activation of NK cells.* Nat Immunol, 2012. **13**(12): p. 1171-7.

105. Lee, K.M., et al., *Requirement of homotypic NK-cell interactions through 2B4(CD244)/CD48 in the generation of NK effector functions.* Blood, 2006. **107**(8): p. 3181-8.

106. Vaidya, S.V., et al., *Targeted disruption of the 2B4 gene in mice reveals an in vivo role of 2B4 (CD244) in the rejection of B16 melanoma cells.* J Immunol, 2005. **174**(2): p. 800-7.

107. Chen, S., et al., *The Self-Specific Activation Receptor SLAM Family Is Critical for NK Cell Education.* Immunity, 2016. **45**(2): p. 292-304.

108. He, Y., et al., *Contribution of inhibitory receptor TIGIT to NK cell education.* J Autoimmun, 2017. **81**: p. 1-12.

109. Fauriat, C., et al., *Education of human natural killer cells by activating killer cell immunoglobulin-like receptors.* Blood, 2010. **115**(6): p. 1166-74.

110. Joncker, N.T. and D.H. Raulet, *Regulation of NK cell responsiveness to achieve self-tolerance and maximal responses to diseased target cells.* Immunol Rev, 2008. **224**: p. 85-97.

111. Tripathy, S.K., et al., *Continuous engagement of a self-specific activation receptor induces NK cell tolerance.* J Exp Med, 2008. **205**(8): p. 1829-41.

112. Jonsson, A.H., et al., *Effects of MHC class I alleles on licensing of Ly49A+ NK cells.* J Immunol, 2010. **184**(7): p. 3424-32.

113. Viant, C., et al., *SHP-1-mediated inhibitory signals promote responsiveness and anti-tumour functions of natural killer cells.* Nat Commun, 2014. **5**: p. 5108.

114. Lowin-Kropf, B., et al., *Impaired natural killing of MHC class I-deficient targets by NK cells expressing a catalytically inactive form of SHP-1.* J Immunol, 2000. **165**(3): p. 1314-21.

115. Wang, J.W., et al., *Influence of SHIP on the NK repertoire and allogeneic bone marrow transplantation.* Science, 2002. **295**(5562): p. 2094-7.

116. Gumbleton, M., E. Vivier, and W.G. Kerr, *SHIP1 intrinsically regulates NK cell signaling and education, resulting in tolerance of an MHC class I-mismatched bone marrow graft in mice.* J Immunol, 2015. **194**(6): p. 2847-54.

117. Marcais, A., et al., *High mTOR activity is a hallmark of reactive natural killer cells and amplifies early signaling through activating receptors.* Elife, 2017. **6**.

118. Corral, L., et al., *NK cell expression of the killer cell lectin-like receptor G1 (KLRG1), the mouse homolog of MAFA, is modulated by MHC class I molecules.* Eur J Immunol, 2000. **30**(3): p. 920-30.

119. Wagner, A.K., et al., *Expression of CD226 is associated to but not required for NK cell education.* Nat Commun, 2017. **8**: p. 15627.

120. Guia, S., et al., *Confinement of activating receptors at the plasma membrane controls natural killer cell tolerance.* Sci Signal, 2011. **4**(167): p. ra21.

121. Forslund, E., et al., *Microchip-Based Single-Cell Imaging Reveals That CD56dimCD57-KIR-NKG2A+ NK Cells Have More Dynamic Migration Associated with Increased Target Cell Conjugation and Probability of Killing Compared to CD56dimCD57-KIR-NKG2A- NK Cells.* J Immunol, 2015. **195**(7): p. 3374-81.

122. Neve-Oz, Y., et al., *Mechanisms of localized activation of the T cell antigen receptor inside clusters.* Biochim Biophys Acta, 2015. **1853**(4): p. 810-21.

123. Yokosuka, T., et al., *Newly generated T cell receptor microclusters initiate and sustain T cell activation by recruitment of Zap70 and SLP-76.* Nat Immunol, 2005. **6**(12): p. 1253-62.

124. Liu, W., et al., *Intrinsic properties of immunoglobulin IgG1 isotype-switched B cell receptors promote microclustering and the initiation of signaling.* Immunity, 2010. **32**(6): p. 778-89.

125. Oszmiana, A., et al., *The Size of Activating and Inhibitory Killer Ig-like Receptor Nanoclusters Is Controlled by the Transmembrane Sequence and Affects Signaling.* Cell Rep, 2016. **15**(9): p. 1957-72.

126. Mattila, P.K., F.D. Batista, and B. Treanor, *Dynamics of the actin cytoskeleton mediates receptor cross talk: An emerging concept in tuning receptor signaling.* J Cell Biol, 2016. **212**(3): p. 267-80.

127. Treanor, B., et al., *The membrane skeleton controls diffusion dynamics and signaling through the B cell receptor.* Immunity, 2010. **32**(2): p. 187-99.

128. Kucik, D.F., et al., *Adhesion-activating phorbol ester increases the mobility of leukocyte integrin LFA-1 in cultured lymphocytes.* J Clin Invest, 1996. **97**(9): p. 2139-44.

129. Mattila, P.K., et al., *The actin and tetraspanin networks organize receptor nanoclusters to regulate B cell receptor-mediated signaling.* Immunity, 2013. **38**(3): p. 461-74.

130. Pageon, S.V., et al., *Superresolution microscopy reveals nanometer-scale reorganization of inhibitory natural killer cell receptors upon activation of NKG2D.* Sci Signal, 2013. **6**(285): p. ra62.

131. Liu, D., M.E. Peterson, and E.O. Long, *The adaptor protein Crk controls activation and inhibition of natural killer cells.* Immunity, 2012. **36**(4): p. 600-11.

132. Lagrue, K., et al., *The central role of the cytoskeleton in mechanisms and functions of the NK cell immune synapse.* Immunol Rev, 2013. **256**(1): p. 203-21.

133. Haustein, E. and P. Schwille, *Fluorescence correlation spectroscopy: novel variations of an established technique.* Annu Rev Biophys Biomol Struct, 2007. **36**: p. 151-69.

134. Mueller, V., et al., *FCS in STED microscopy: studying the nanoscale of lipid membrane dynamics.* Methods Enzymol, 2013. **519**: p. 1-38.

135. Hell, S.W. and J. Wichmann, *Breaking the diffraction resolution limit by stimulated emission: stimulated-emission-depletion fluorescence microscopy.* Opt Lett, 1994. **19**(11): p. 780-2.

136. Sanden, T., G. Persson, and J. Widengren, *Transient state imaging for microenvironmental monitoring by laser scanning microscopy.* Anal Chem, 2008. **80**(24): p. 9589-96.

137. Spielmann, T., et al., *Transient state monitoring by total internal reflection fluorescence microscopy.* J Phys Chem B, 2010. **114**(11): p. 4035-46.

138. Spielmann, T., et al., *Transient state microscopy probes patterns of altered oxygen consumption in cancer cells.* FEBS J, 2014. **281**(5): p. 1317-32.

139. Xu, L., et al., *Resolution, target density and labeling effects in colocalization studies - suppression of false positives by nanoscopy and modified algorithms.* FEBS J, 2016. **283**(5): p. 882-98.

140. Stromqvist, J., et al., *A modified FCCS procedure applied to Ly49A-MHC class I cis-interaction studies in cell membranes.* Biophys J, 2011. **101**(5): p. 1257-69.

141. Hervas-Stubbs, S., et al., *Direct effects of type I interferons on cells of the immune system.* Clin Cancer Res, 2011. **17**(9): p. 2619-27.

142. Lindahl, P., et al., *Interferon treatment of mice: enhanced expression of histocompatibility antigens on lymphoid cells.* Proc Natl Acad Sci U S A, 1976. **73**(4): p. 1284-7.

143. Vanherberghen, B., et al., *Classification of human natural killer cells based on migration behavior and cytotoxic response.* Blood, 2013. **121**(8): p. 1326-34.

144. Machan, R. and T. Wohland, *Recent applications of fluorescence correlation spectroscopy in live systems.* FEBS Lett, 2014. **588**(19): p. 3571-84.

145. Damjanovich, S., et al., *Distribution and mobility of murine histocompatibility H-2Kk antigen in the cytoplasmic membrane.* Proc Natl Acad Sci U S A, 1983. **80**(19): p. 5985-9.

146. Capps, G.G., et al., *Short class I major histocompatibility complex cytoplasmic tails differing in charge detect arbiters of lateral diffusion in the plasma membrane.* Biophys J, 2004. **86**(5): p. 2896-909.

147. Burshtyn, D.N., et al., *Adhesion to target cells is disrupted by the killer cell inhibitory receptor.* Curr Biol, 2000. **10**(13): p. 777-80.

148. Thomas, L.M., M.E. Peterson, and E.O. Long, *Cutting edge: NK cell licensing modulates adhesion to target cells.* J Immunol, 2013. **191**(8): p. 3981-5.

149. Beemiller, P. and M.F. Krummel, *Regulation of T-cell receptor signaling by the actin cytoskeleton and poroelastic cytoplasm.* Immunol Rev, 2013. **256**(1): p. 148-59.

150. Kumari, S., et al., *T cell antigen receptor activation and actin cytoskeleton remodeling.* Biochim Biophys Acta, 2014. **1838**(2): p. 546-56.

151. Tu, M.M., A.B. Mahmoud, and A.P. Makrigiannis, *Licensed and Unlicensed NK Cells: Differential Roles in Cancer and Viral Control.* Front Immunol, 2016. **7**: p. 166.

152. Gardiner, C.M. and D.K. Finlay, *What Fuels Natural Killers? Metabolism and NK Cell Responses.* Front Immunol, 2017. **8**: p. 367.

153. Donnelly, R.P., et al., *mTORC1-dependent metabolic reprogramming is a prerequisite for NK cell effector function.* J Immunol, 2014. **193**(9): p. 4477-84.

154. Keating, S.E., et al., *Metabolic Reprogramming Supports IFN-gamma Production by CD56bright NK Cells.* J Immunol, 2016. **196**(6): p. 2552-60.