

Imaging Molecular Recognition by the Inhibitory Human Natural Killer Cell Receptor KIR2DL1

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Submitted for the Degree of Doctor of Philosophy

2012

Abstract

Natural Killer (NK) cells are lymphocytes that identify and kill virus-infected and tumour cells dependent on recognition via numerous activating and inhibitory surface receptors. The mechanisms by which NK cells can integrate activating and inhibitory signals at the immune synapse remain unclear, although a correlation has been established between a cell's response and the organisation of receptors into specific molecular patterns at the synapse. Here, I used three novel imaging techniques to investigate the spatial and temporal dynamics of molecular recognition by human NK cells. The inhibitory NK cell receptor KIR2DL1 was tagged with the photoswitchable protein tdEosFP, which can be irreversibly switched from a green to red fluorescent state using ultraviolet light. First, tdEosFP was used to visualise the localisation and organisation of KIR2DL1 at the synapse at unprecedented resolution using photoactivated localisation microscopy. KIR2DL1 was organised in pre-existing protein clusters with a diameter of 84.2 ± 5.4 nm. Upon receptor ligation, clusters became smaller by 15% and denser by 27%, in an actin-independent manner. Surprisingly, the ligation of other receptors such as NKG2D and CD94/NKG2A affected the nanoscale organisation of KIR2DL1 by increasing the cluster density by 18-19% and decreasing the average cluster diameter by 20%. Thus, the distribution of KIR2DL1 is directly influenced by other receptors. Second, the movement of photoswitched molecules was temporally and spatially resolved and the diffusion coefficient of KIR2DL1 in the membrane of NK cells determined to be $0.167 \pm 0.077 \mu\text{m}^2 \cdot \text{s}^{-1}$. KIR2DL1 was continuously recruited to an immune synapse, where it remained stable, unless a second synapse was formed, in which case KIR2DL1 was able to traffic between synapses. Finally, a system using a photocleavable peptide to temporally control NK cell inhibition was established. This enables the timing of inhibitory signalling mediated by KIR2DL1 to be controlled. Together, these complementary approaches provide important new insights on how the nanoscale organisation of KIR2DL1 at the synapse can reflect different stimulatory conditions and novel spatio-temporal information on KIR2DL1 recruitment to the synapse.

Author declaration

The data presented in this thesis are my own work, with the following exceptions described below.

- The TIRF/PALM microscope used in Chapter 3 was built by Stephen Rothery (FILM Facility, Imperial College London) and Mirella Koleva (Tony Magee lab, Imperial College London). Stephen assisted with the optimisation of PALM imaging.
- In Chapter 4, MRes student Kathryn Lagrue contributed to the imaging of KIR2DL1 in the photoswitching experiments, providing additional n numbers for Figures 4.6C, 4.8C and 4.10C. Kathryn also performed the measurements for the validation of the analysis method, presented in Figure 2.8B.
- In Chapter 4, Gerardo Aquino (Systems Biology, Division of Molecular Biosciences, Imperial College) performed the mathematical modelling, generating the graphs used in Figures 4.7, 4.9 and 4.11.
- In Chapter 5, the synthesis of the CNB-caged photocleavable peptide was performed in collaboration with David Trentham and John Offer (National Institute for Medical Research, London). Figure 5.5 was obtained as a result of this collaboration.

Sophie Paeon

Publications

Paeon, S. V., et al. (in preparation) The nanoscale organisation of KIR2DL1 is directly influenced by the ligation of other NK cell receptors (*working title*)

Fadda, L., Borhis, G., Ahmed, P., Cheent, K., **Paeon, S. V.**, Cazaly, A., Stathopoulos, S., Middleton, D., Mulder, A., Claas, F. H., Elliott, T., Davis, D. M., Purbhoo, M. A. & Khakoo, S. I. (2010) Peptide antagonism as a mechanism for NK cell activation. *Proceedings of the National Academy of Sciences of the United States of America* 107 (2), 10160-10165.

Purbhoo, M. A., Liu, H., Oddos, S., Owen, D. M., Neil, M. A., **Paeon, S. V.**, French, P. M., Rudd, C. E. & Davis, D. M. (2010) Dynamics of subsynaptic vesicles and surface microclusters at the immunological synapse. *Science Signaling* 3 (121), ra36.

Acknowledgements

Thank you to my supervisor, Professor Dan Davis, for giving me the opportunity to study in your lab. Over the years, your guidance, your enthusiasm, and the freedom you have given me in my research have all contributed to making me the scientist I am today. Thank you also for your incredibly quick response to emails! I would also like to thank you for reading my thesis so quickly.

I am deeply grateful to all the members of the Davis lab, past and present, who have supported me and provided an engaging and enjoyable research environment. I am indebted to Philipp Eissmann, who mentored me during the first year of my PhD, and to Marco Purbhoo and Alice Brown for interesting discussions. I would also like to acknowledge the contribution of Kathryn Lagrue, an MRes student that I supervised during my third year who has now joined the lab as a PhD student. Special thanks to Anne Aucher and Dominika Rudnicka. As well as sharing long and frequent discussions about our work, we also became close friends outside the lab. Your friendship and support, especially in the last year of my PhD, were invaluable.

Thank you also to my advisors, Professors Salim Khakoo and Murray Selkirk, for your guidance and support. Also a big thank you to Professor Tony Magee for our informal discussions on my research and your advice. Thanks also to all my collaborators for your various important contributions to my work.

I would like to thank Martin Spitaler and Steve Rothery from the FILM imaging facility for their involvement in the PALM project. Our long discussions at our Wednesday meetings (usually with chocolates!) were extremely useful for the progress of the project. I would like to particularly thank Steve for his work on building the microscope set-up with Mirella Koleva and helping me to achieve beautiful PALM images. Without Steve, there would be no PALM!

Thanks to all my friends outside the lab for your support and for providing a change of scenery every now and then! In particular, I would like to thank Naïma for our daily email exchanges, which have helped to keep me motivated when things were not going according to plan, and for our long and fun evenings at Wagamama... I would also like to acknowledge the Body Combat classes that I regularly attended at the Imperial College sports centre, which helped to keep me fit and on the right side of sane during my PhD.

I could never have reached this goal without the fantastic love and emotional support of my wonderful family; my brother Alexandre and my sister Katherine, who have always greatly encouraged their baby sister; my entertaining little nieces and nephews (in particular Giselle, who was born in the first week of my PhD); and especially my parents Sylvia and Daniel. There is too much to be grateful for, so I just want to say, thank you for everything – it has meant so much to me!

And finally, to my amazing boyfriend Bojan. Thank you for your support and understanding. We have shared this journey all the way, and will hopefully become Doctors together. Thank you for pulling me back up when I was feeling down, for making me laugh when I really needed it, for critically reading my reports and manuscripts, for always being there for me, and above all, for making me feel so special. I would not have managed without you.

Funding was provided by the Biotechnology and Biological Sciences Research Council (BBSRC).

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Abbreviations

221	721.221 EBV-transformed B cell line
ADCC	Antibody-dependent cellular cytotoxicity
ANKD	Absolute NK cell deficiency
AOTF	Acousto-optical tunable filter
APC	Antigen-presenting cell
β_2m	β_2 -microglobulin
BNM	<i>t</i> -butyl 2-nitromandelate
BSA	Bovine serum albumin
CmnCbz	5-(carboxy-methoxy)-2-nitrobenzyl-oxycarbonyl
CNB	α -carboxy-2-nitrobenzyl
CNKD	Classical NK cell deficiency
CTLA-4	Cytotoxic T lymphocyte antigen 4
DAP10	DNAX activating protein of 10 kDa
DC	Dendritic cell
DCM	Dichloromethane
DMAP	4-(dimethyl)amino-pyridine
DMF	Dimethylformamide
EM-CCD	Electron-multiplying charge-coupled device
EosFP	Eos fluorescent protein
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
FACS	Fluorescence-activated cell sorting
FasL	Fas ligand
FCS	Foetal calf serum
FLAP	Fluorescence localisation after photobleaching
FLIM	Fluorescence lifetime imaging microscopy
FLIP	Fluorescence loss in photobleaching
FNKD	Functional NK cell deficiency
FRAP	Fluorescence recovery after photobleaching
FRET	Förster resonance energy transfer
FWHM	Full-width half maximum

GFP	Green fluorescent protein
GM-CSF	Granulocyte macrophage-colony stimulating factor
GSD	Ground state depletion
HLA	Human leukocyte antigen
HPLC	High-performance liquid chromatography
IFN-γ	Interferon- γ
ICAM-1	Intercellular cell adhesion molecule 1
IL	Interleukin
ILT2	Immunoglobulin-like transcript 2
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibition motif
KIR	Killer Immunoglobulin-like receptor
Lat A	Latrunculin A
LC-MS	Liquid chromatography-mass spectrometry
LFA-1	Leukocyte function-associated molecule 1
LIR	Leukocyte Immunoglobulin-like receptor
MAPK	p38 mitogen-activated protein kinase
MCC	Moth cytochrome c peptide
mEos	Monomeric Eos fluorescent protein
MHC	Major histocompatibility complex
MIC	MHC class I chain-related
MIP	Macrophage inflammatory protein
MTOC	Microtubule organising centre
NCR	Natural cytotoxicity receptor
NK	Natural killer
NKG2D	NK group 2 member D
NPE	Nitrophenylethylurethane
n.s.	Non-significant difference
NVOC	6-nitroveratryloxycarbonyl
PA-GFP	Photoactivatable green fluorescent protein
PALM	Photoactivated localisation microscopy
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PFA	Paraformaldehyde

PI	Propidium iodide
PI3K	Phosphoinositide 3-kinase
PKC-θ	Protein kinase C-θ
PLC-γ	Phospholipase C-γ
PLL	Poly-L-lysine
PSF	Point spread function
PVR	Poliovirus receptor
PVRL2	Poliovirus receptor ligand 2
RANTES	Regulated upon Activation, Normal T cell Expressed, and Secreted
SEM	Standard error of the mean
SH2	Src homology 2
SHIP	SH2 domain-containing inositol phosphatase
SHP-1/SHP-2	SH2 domain-containing tyrosine phosphatases 1/2
SIM	Structured illumination microscopy
SLP-76	SH2 domain-containing leukocyte protein of 76 kDa
SMAC	Supramolecular activation cluster (cSMAC – central SMAC; pSMAC – peripheral SMAC; dSMAC – distal SMAC)
SNR	Signal-to-noise ratio
SPEM	Saturated patterned excitation microscopy
STED	Stimulated emission depletion
STORM	Stochastic optical reconstruction microscopy
TAP	Transporter associated with antigen processing
TCR	T cell receptor
tdEosFP	Tandem dimer Eos fluorescent protein
TFA	Trifluoroacetic acid
TIR	Total internal reflection
TIRF	Total internal reflection fluorescence
TLC	Thin layer chromatography
TNF	Tumour necrosis factor
TRAIL	TNF-related apoptosis-inducing ligand
ULBP	UL16-binding protein
UV	Ultra violet
ZAP-70	Zeta-chain-associated protein kinase of 70 kDa

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Chapter 1 - Introduction

The human immune system responds to challenges in a highly complex manner. All cells and processes involved in immunity are tightly regulated by activating and inhibitory signalling events. Although many key molecules involved in immune cell surveillance have been identified and characterised, the advent of new imaging techniques has started to reveal in more detail how immune cells communicate with each other and how they recognise signs of disease through specific receptors expressed at their surface. This investigation focuses on Natural Killer (NK) cells, and explores the dynamics and nanoscale organisation of the inhibitory receptor KIR2DL1 by exploiting novel techniques and super-resolution imaging.

1.1 Natural Killer cells and the immune synapse

1.1.1 Natural Killer cells and their role in immunity

Natural Killer (NK) cells are large granular lymphocytes (white blood cells) that play an important role in the immune system and account for 5-15% of peripheral blood lymphocytes. They participate in early defence against viral infections, intracellular pathogens and tumours by recognising 'abnormal' and diseased cells. The term 'natural killer' cells was coined when lymphocytes with a 'natural' ability to kill were isolated from mice (Kiessling et al., 1975b; Herberman et al., 1975b, 1975a) and then from humans (Pross and Jondal, 1975). NK cells were first characterised by Kiessling and Herberman, who described them as a sub-population of bone marrow-derived lymphocytes, which are distinct from T and B cells in that they can exhibit cytotoxic behaviour against tumour cells in the absence of prior immunisation or priming by specific antigen (Kiessling et al., 1975a; Herberman et al., 1975a). Human NK cells can be distinguished from other immune cells by being CD3⁻CD56⁺ cells (Cooper et al., 2001), i.e. they do not express CD3 like T cells, but stain positive for the adhesion molecule CD56; although more recently, the activating receptor NKp46 was identified as a common NK cell-specific marker conserved across species (Walzer et al., 2007).

NK cell effector functions

Thirty years of work on NK cells have shown that they play an essential role in the early stages of an immune response. Indeed, NK cells have the ability to detect and kill sensitive target cells such as pathogen-infected cells and tumour cells. They distinguish healthy from abnormal somatic cells using a large array of germline-encoded activating and inhibitory receptors, which can detect cellular changes induced by infection, transformation or stress (Lanier, 2005).

The main mechanism for NK cell cytotoxicity is the directed release of lytic granules containing perforin and granzymes at the site of contact with the target cell (Kägi et al., 1994). The exact mechanism of granule delivery to the target cell remains controversial, but it is thought that perforin inserts itself into the target cell membrane, forming pores of up to 20 nm through which granzymes can penetrate the cell and initiate apoptosis (programmed cell death) (Liu et al., 1995). NK cells are also able to induce target cell death through perforin-independent pathways via other factors, such as members of the Tumour Necrosis Factor (TNF) family, including TNF- α , TNF-related apoptosis-inducing ligand (TRAIL) and Fas ligand (FasL) (Kashii et al., 1999; Screpanti et al., 2001). These proteins initiate target cell death by apoptosis or necrosis.

As well as mediating natural cytotoxicity, the role for which they were named, NK cells can secrete soluble pro-inflammatory cytokines and chemokines, such as the anti-viral interferon- γ (IFN- γ). In addition to its pro-inflammatory and anti-viral properties, IFN- γ is capable of shaping the adaptive immune response by promoting helper T cell differentiation (Martín-Fontecha et al., 2004; Morandi et al., 2006) and can directly activate neutrophils and macrophages. NK cells are also a major source of the inflammatory cytokine TNF- α , which can initiate a cytokine cascade during an inflammatory response. NK cells also secrete granulocyte macrophage-colony stimulating factor (GM-CSF), interleukin (IL)-5, IL-13, macrophage inflammatory proteins (MIP)-1 α and -1 β , and the chemokine RANTES (Regulated upon Activation, Normal T cell Expressed, and Secreted) (Fauriat et al., 2010).

NK cell functions can be regulated by direct interactions with other cells of the immune system, such as T cells, dendritic cells (DCs) and macrophages (Lucas et al., 2007) or by the cytokine microenvironment. Several cytokines are known to promote NK cell effector functions, including IFN- α and - β , IL-12, IL-18 and IL-15 (Walzer et al., 2005), although the most important is IL-2, which enhances NK cell proliferation, cytotoxicity and cytokine secretion (Trinchieri, 1989).

Bridging innate and adaptive immune responses

NK cells have long been considered as components of the innate immune system. Some studies have provided evidence that NK cells can also regulate the adaptive immune system by directly interacting with and activating macrophages (Nedvetzki et al., 2007), DCs (Gerosa et al., 2002) and T cells (Zingoni et al., 2004). NK cells also secrete cytokines and chemokines that recruit and activate other hematopoietic cells (Trinchieri, 1989; Biron et al., 1999).

More recent studies have revealed features of NK cells that bear similarities to adaptive immunity, by showing that they exhibit signs of 'immunological memory'. Using mice, Sun and co-workers showed that NK cells can become more protective against viral infections after an initial exposure (Sun et al., 2009). In another investigation, NK cells in mice devoid of T and B cells were capable of mounting vigorous recall responses to hapten-based contact sensitizers (O'Leary et al., 2006). 'Memory' NK cells have been detected in mice for at least a month after initial infection in several studies (O'Leary et al., 2006; Sun et al., 2009; Cooper et al., 2009; Paust et al., 2010), and thus, NK cells appear to remember their past. However, the recent evidence for immunological memory in NK cells raises many new questions and much work remains to be done in this field. NK cells are emerging as a cell type that bridges the innate and adaptive arms of the immune system (Vivier et al., 2011).

Natural Killer-like T cells

It is important not to confuse NK cells and Natural Killer-like T (NKT) cells. NKT cells share properties of both T cells and NK cells, but originate from a separate lineage (Makino et al., 1995). NKT cells are a rare subpopulation, representing approximately 0.2% of peripheral T cells, and their classification remains complicated. NKT cells were first isolated in 1987 by three different groups, and were characterised by low levels of T cell receptor (TCR), lack of CD4 or CD8 proteins and the presence of NK cell-specific markers (Budd et al., 1987; Fowlkes et al., 1987; Ceredig et al., 1987). NKT cells specifically recognise glycolipid antigens presented by CD1d and are thereby involved in the recognition of bacterial glycolipids (Borg et al., 2007; Pellicci et al., 2009). They have also been shown to produce immunoregulatory cytokines, such as IL-4, IL-21, TNF and IFN- γ (Hammond et al., 1998; Wilson et al., 1998; Terabe et al., 2000; Harada et al., 2006; Coquet et al., 2007). NKT cells

constitute a clearly distinct subpopulation from T and NK cells, performing different immune functions.

NK cells and disease

The importance of NK cells is illustrated by human diseases in which NK cells are absent or defective. These diseases are grouped under the term NK cell deficiencies, including classical NK cell deficiency (CNKD), which is defined by the lack of NK cells, functional NK cell deficiency (FNKD), where NK cells are present but exhibit defects in cytotoxicity or other functions, and absolute NK cell deficiency (ANKD), which is characterised by a complete absence of NK and NKT cells (Orange, 2006). Patients diagnosed with NK cell deficiencies appear more vulnerable to chronic infections, especially with Herpes viruses, and show an increased risk of developing cancer. Although isolated NK cell deficiencies are extremely rare and remain poorly understood, these diseases highlight the important contributions that NK cells make in the defence against disease and in the maintenance of good health (Orange, 2006).

The identification of mechanisms underlying NK cell functions has led to new clinically relevant approaches using NK cells in anti-tumour therapy. The most successful example to date involves two monoclonal antibodies against CD20 on B cells, Rituximab and Trastuzumab, which have been used to treat B cell malignancies and early stage breast cancer. These antibodies bind to CD20 on tumour cells and their Fc portion is recognised by the activating receptor CD16 on NK cells, triggering the elimination of the tumour cells via antibody-dependent cellular cytotoxicity (ADCC). Indeed, treatment with these antibodies has been shown to correlate with increased NK cell activity and increased NK cell numbers (Beano et al., 2008).

Two main approaches are used to exploit NK cells in anti-tumour therapy, although many more approaches have been explored and recently reviewed (Chan et al., 2008). The first approach is based on the activation of endogenous NK cells against tumours, which can be achieved by blocking inhibitory NK cell receptors to effectively enhance NK cell cytotoxicity, by administering cytokines that enhance NK cell functions (e.g. IL-2, IL-15 and/or IL-21), or by using drugs that induce the expression of stress ligands on tumour cells (Chan et al., 2008; Colonna, 2008). The second approach is based on the adoptive transfer of NK cells, either by transferring donor NK cells to HLA-haploidentical hosts to promote NK cell reactivity against host tumour cells, or by using NK cells transduced to express chimeric receptors that can activate NK cells (Chan et al., 2008; Colonna,

2008). The possibility of combining multiple approaches make NK cells an exciting field of research for the discovery of new effective therapies.

1.1.2 NK cell recognition of target cells

The activation of NK cell effector functions is tightly regulated by a dynamic equilibrium between signals from activating and inhibitory cell surface receptors that can be engaged simultaneously, depending on the presence and quantity of specific ligands in their environment (Vivier et al., 2004; Lanier, 2005). During their development, T and B lymphocytes undergo a process of somatic recombination and hypermutation in order to generate a wide array of receptors specific to a range of antigens. In contrast, NK cells express a large repertoire of invariant, germline-encoded receptors. Subsets of NK cells can express just one or a random assembly of the different activating and inhibitory receptors. Target cells generally express both types of ligands recognised by activating and inhibitory receptors, and signals are integrated within the NK cell to result in the appropriate cellular response. Using this large repertoire of receptors, NK cells can discriminate between healthy and aberrant cells by using different strategies, such as ‘induced-self’ and ‘missing-self’ modes of recognition (Figure 1.1).

Activating receptors and ‘induced-self’ recognition

NK cells express a variety of activating receptors and, unlike inhibitory receptors, most NK cells express all of the activating receptors, allowing them to recognise a variety of targets, such as non-self (viral) ligands or stress-induced self-ligands. NK cell activation generally arises from the concerted action of multiple surface proteins, not just activating receptors but also cytokine receptors and adhesion molecules (Colucci et al., 2002). Using a minimalist cell system to dissect the requirements for NK cell activation, Eric Long and colleagues transfected insect cells to express various combinations of activating ligands and adhesion molecules, and showed that some activating receptors on freshly isolated human NK cells require simultaneous stimulation through other receptors, and are not sufficient to trigger efficient activation on their own (Bryceson et al., 2005).

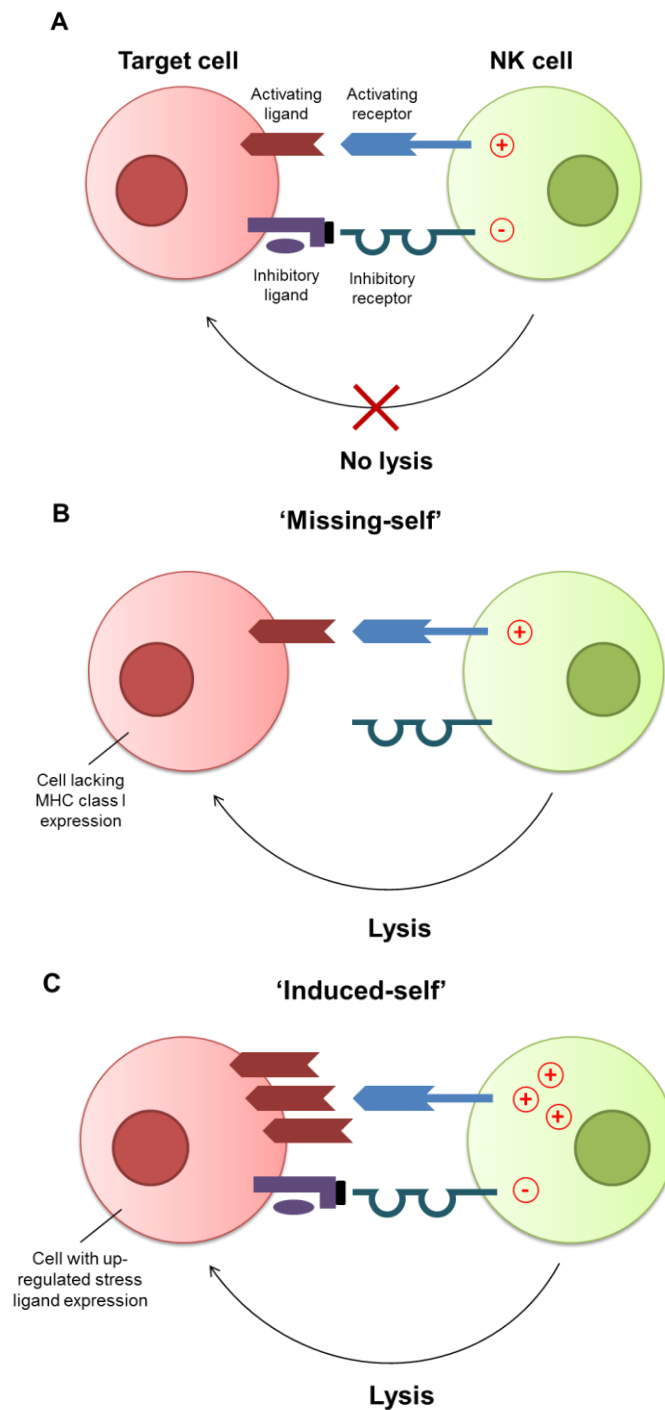


Figure 1.1: Recognition of target cells by NK cells. (A) Upon interaction with a normal, healthy, autologous target cell, an NK cell might receive activating signals; however, due to the expression of appropriate self-MHC class I alleles on the target cell, the NK cell receives inhibitory signals through its inhibitory receptors and does not lyse the target cell. (B) **'Missing-self'**: If the target cell loses its expression of MHC class I molecules, as a result of transformation or viral infection, the MHC-binding inhibitory receptor on the NK cell is not engaged. In this case, the NK cell does not receive inhibitory signals. Activating signals dominate and the NK cell lyses the target cell. (C) **'Induced-self'**: If the target cell upregulates stress ligands on its surface, owing to viral infection or transformation, then activating signals predominate and the target cell is lysed. Based on (Raulet and Vance, 2006).

One well characterised NK cell activating receptor is the NK group 2 member D (NKG2D), a dominant activating receptor critical for immunosurveillance. NKG2D is a C-type lectin-like type II transmembrane immunoreceptor (Houchins et al., 1991) that recognises a diversity of ligands associated with tumour transformations, viral infections and cellular stress. In humans, NKG2D ligands include two proteins encoded by major histocompatibility complex (MHC) class I chain-related (MIC) genes, MICA and MICB (Bahram et al., 1996; Bahram and Spies, 1996), and six UL-16 binding proteins, ULBP1-6 (Cosman et al., 2001; Carayannopoulos et al., 2002; Eagle et al., 2009). These 'self' ligands are generally absent from cells or expressed at very low levels, but are up-regulated when cells are transformed, virally-infected, or stressed as in the case of DNA damage or heat shock. As such, NKG2D is considered to mediate killing via recognition of 'induced-self'. In this mode of recognition, host proteins that are specific ligands of activating NK cell receptors are up-regulated at the surface of cells that are stressed and need to be eliminated. Importantly, NKG2D-mediated activation is able to override inhibitory signals induced by the presence of MHC class I molecules on a target cell (Bauer et al., 1999; Cerwenka et al., 2001; Diefenbach et al., 2001).

Another important, but more recently discovered type of activating NK cell receptor is the family of natural cytotoxicity receptors (NCRs), such as NKp30 (Pende et al., 1999), NKp44 (Cantoni et al., 1999) and NKp46 (Pessino et al., 1998). No host ligands have yet been identified for NKp44 and NKp46, but B7-H6 expressed on human tumour cells was identified as a ligand for NKp30 (Brandt et al., 2009), emerging as another example of 'induced-self' recognition. Interestingly, *in vitro* studies have shown that NKp44 and NKp46 can recognise haemagglutinins on influenza virus particles, resulting in NK cell activation, whereas NKp30 cannot (Arnon et al., 2001; Mandelboim et al., 2001). NKp46 has also been implicated in the recognition and enhanced killing of cells undergoing mitosis (Nolte-'t Hoen et al., 2007). Cells in mitosis undergo heightened surveillance by NK cells, which might represent a new way in which NK cells can control the expansion of rapidly dividing cells (Nolte-'t Hoen et al., 2007), although the mechanism remains unclear. The search for NCR ligands is ongoing.

NK cells express other important activating receptors that recognise non-self proteins or stress-induced self-ligands, as summarised in Table 1.1. An interesting activating receptor is CD16 (or FcγRIIIA), which binds the Fc portion of antibodies and activates NK cells to lyse antibody-coated opsonised target cells in a process called antibody-dependent cellular cytotoxicity (ADCC) (Lanier et al., 1985; Perussia, 1998).

Receptor	Ligand(s)	References
NKG2D	MICA, MICB ULBP1-6	(Houchins et al., 1991)
NCRs: - NKp30 - NKp44 - NKp46	B7-H6 Viral haemagglutinins Viral haemagglutinins	(Pende et al., 1999) (Cantoni et al., 1999) (Pessino et al., 1998)
2B4	CD48	(Eissmann et al., 2005)
DNAM-1	PVR nectin-2	(Bottino et al., 2003)
CD28	B7 ligands (CD80 and CD86)	(Galea-Lauri et al., 1999) (Wilson et al., 1999)
CD44	hyaluronate	(Galandrini et al., 1994)
BY55	HLA-C	(Le Bouteiller et al., 2002)
CD16 (FcγRIIIA)	Fc portion of antibodies	(Lanier et al., 1985)
CD2	CD58	(Siliciano et al., 1985) (Dustin et al., 1987)

Table 1.1: Activating receptors expressed by NK cells. DNAM-1, DNAX accessory molecule 1; PVR, poliovirus receptor.

Complex signalling events occur downstream of activating receptors, leading to NK cell activation. Most NK cell activating receptors, such as NCRs and CD16, mediate signalling through a common immunoreceptor tyrosine-based activation motif (ITAM), which consists of the consensus sequence YxxL(x)₆₋₈YxxL (in amino acid one-letter code, where x denotes any amino acid) (Reth, 1989). These receptors lack their own cytoplasmic signalling domains and instead couple with adaptor molecules containing an ITAM, such as DAP12, CD3ζ and FcεRly (Lanier, 2003). Ligation of one of these ITAM-bearing receptor complexes leads to the recruitment and activation of the tyrosine kinases Syk and Zeta-chain-associated protein kinase of 70 kDa (ZAP-70). Events in the downstream signalling cascade include the phosphorylation of SH2 domain-containing leukocyte protein of 76 kDa (SLP-76), phosphoinositide 3-kinase (PI3K), phospholipase C-γ (PLC-γ), the recruitment of the adaptor protein growth factor receptor-bound protein 2 (Grb2), linker for activation of T cells (LAT), Vav-1 and Vav-2 proteins, the elevation of intracellular calcium levels, and the activation of Rho, Ras, p38 mitogen-activated protein kinase (MAPK) and extracellular signal-regulated kinase (ERK).

NKG2D however mediates activation through an ITAM-independent pathway. Human NKG2D associates with DNAX activating protein of 10 kDa (DAP10), an adaptor signalling molecule, via a

charged residue in its transmembrane domain (Wu et al., 1999). The short cytoplasmic tail of DAP10 contains a YxxM motif, where x denotes any amino acid. Upon engagement of NKG2D by its ligands, phosphorylation of DAP10 leads to the recruitment of PI3K and Grb2 (Chang et al., 1999; Wu et al., 1999). These events result in downstream signalling via the guanine nucleotide exchange factor Vav-1 (Graham et al., 2006).

Both ITAM-dependent and DAP10-mediated signalling converge on a common NK cell activation pathway, centred on the activation of Rac GTPase, MAPK and ERK (Vivier et al., 2004) (Figure 1.2). These activating pathways result in the rearrangement of the actin cytoskeleton, polarisation and secretion of lytic granules, and/or secretion of cytokines.

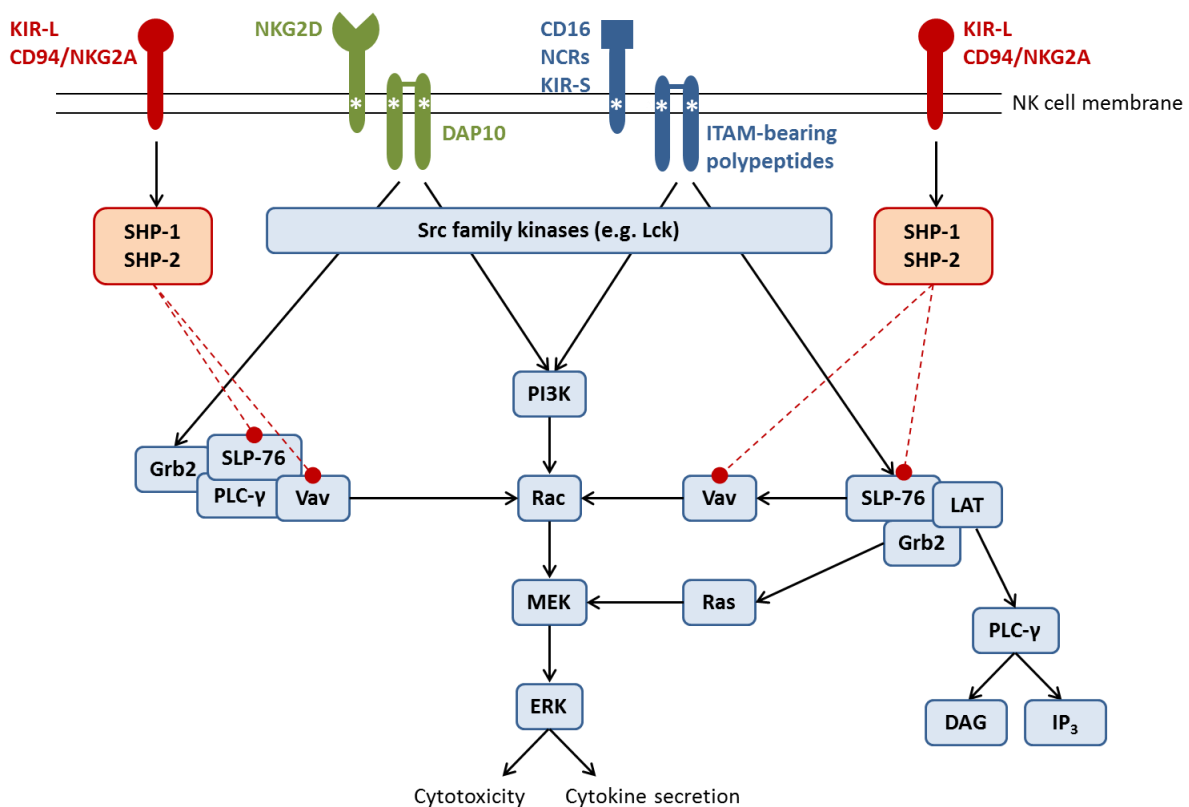


Figure 1.2: NK cell effector signalling pathways. NK cells express activating receptors, which associate with ITAM-bearing molecules (blue) or DAP10 (green), as well as inhibitory MHC class I-specific receptors (red). Asterisks indicate the presence of a transmembrane charged amino acid. Inhibitory KIR (KIR-L) receptors contain an intracellular ITIM domain, whereas activating KIR-S receptors have short cytoplasmic domains and no intrinsic signalling properties. ITAM-dependent and DAP10-dependent activating signalling pathways are initiated as described in the main text (blue), leading to actin rearrangement and NK cell activation. Inhibitory signals are propagated downstream of ITIM-bearing molecules by SHP-1 and SHP-2 tyrosine phosphatases (red), whose substrates include Vav and SLP-76. Simplified diagram based on (Vivier et al., 2004).

Inhibitory receptors and 'missing-self' recognition

Although NK cell cytotoxicity can be induced through activating receptors upon engagement of their ligands on target cells, recognition of target cells by NK cells was initially described by the 'missing-self' hypothesis and the identification of inhibitory receptors expressed on NK cells. NK cells can discriminate healthy cells from abnormal cells according to the 'missing-self' hypothesis. 'Missing-self' recognition refers to the ability of NK cells to recognise and eliminate target cells that have down-regulated surface expression of MHC class I molecules, which would otherwise mark them as healthy 'self' cells (Kärre et al., 1986; Ljunggren and Kärre, 1990). MHC class I molecules are expressed by the majority of human cells and they present cell-derived antigens to T cells, thus facilitating immunosurveillance by T cells for signs of intracellular infections or transformation. As a consequence, many viruses and tumour transformations have evolved immunoevasion strategies in which MHC class I proteins are down-regulated from the cell surface, thus avoiding antigen presentation and rendering them invisible to T cell surveillance. These evasion strategies have been extensively reviewed (Früh et al., 1999; Jonjić et al., 2008; Garrido et al., 2010).

In the 'missing-self' model, NK cells can also eliminate cells bearing a foreign MHC class I molecule (Höglund et al., 1988; Ohlén et al., 1989). In this scenario, cells that express non-self MHC are killed. This model includes MHC molecules that are from the same host species but not from the same individual and has been used to explain graft rejection (Ohlén et al., 1989).

The 'missing-self' hypothesis can be explained by the fact that NK cells express inhibitory receptors that recognise self MHC class I molecules as markers of healthy cells (Karlhofer et al., 1992; Moretta et al., 1993). As such, the absence or down-regulation of MHC class I on target cells renders them susceptible to lysis by NK cells, whereas the presence of self MHC class I inhibits NK cell-mediated killing.

Inhibitory receptors expressed by human NK cells are summarised in Table 1.2. They include the human killer immunoglobulin-like receptors (KIR), which recognise classical MHC class I proteins (human leukocyte antigen (HLA)-A, -B, -C) (Colonna and Samaridis, 1995; Wagtmann et al., 1995a, 1995b), the CD94/NKG2 receptors, which recognise the non-classical MHC class I protein HLA-E (Braud et al., 1998; Vance et al., 1998), and the leukocyte immunoglobulin-like receptors (LIR, also known as ILT and LILR), which interact with a broad range of classical and non-classical MHC class I molecules (Samaridis and Colonna, 1997; Cosman et al., 1997; Vitale et al., 1999). Structurally, KIRs and LIRs are quite similar as both families are immunoglobulin-like type-I transmembrane receptors, whereas CD94/NKG2 receptors are C-type lectin-like type-II transmembrane heterodimers. NK cells

can also express other inhibitory receptors, which recognise non-MHC class I ligands. NK cells express an array of inhibitory receptors, which enables them to recognise many types of MHC class I molecules and to efficiently survey the body's cells for 'missing-self'.

Receptor	Ligand(s)	References
KIRs	HLA-A, -B, -C	(Colonna and Samaridis, 1995) (Wagtmann et al., 1995a, 1995b)
CD94/NKG2A	HLA-E	(Braud et al., 1998) (Vance et al., 1998)
LIRs	broad range of MHC class I	(Samaridis and Colonna, 1997) (Cosman et al., 1997)
CD66 (CEACAM-1)	Non-MHC ligands	(Markel et al., 2002)
TIGIT	PVR PVR ligand 2 (PVRL2)	(Stanietsky et al., 2009)
KLRG1	cadherins	(Ito et al., 2006)
LAIR-1	collagen	(Meyaard et al., 1997)
NKR-P1A	LLT-1	(Lanier et al., 1994)

Table 1.2: Inhibitory receptors expressed by NK cells. CEACAM-1, carcinoembryonic antigen-related cell adhesion molecule; TIGIT, T-cell immunoreceptor with immunoglobulin and ITIM domains; KLRG1, killer cell lectin-like receptor G1; LAIR-1, leukocyte-associated immunoglobulin-like receptor-1; NKR-P1A, NK cell receptor protein 1A; LLT-1, lectin-like transcript 1.

Most inhibitory NK cell receptors share a common immunoreceptor tyrosine-based inhibition motif (ITIM) in their cytoplasmic domains, which delivers the inhibitory signal. An ITIM motif contains the consensus sequence I/V/LxYxxL/V, where x denotes any amino acid (Wagtmann et al., 1995b; Burshtyn et al., 1996).

After receptor engagement of their ligands, inhibition is initiated by ITIM tyrosine phosphorylation by Src kinases such as Lck, followed by recruitment of Src homology 2 (SH2) domain-containing tyrosine phosphatases SHP-1 and SHP-2 or the SH2 domain-containing inositol phosphatase (SHIP) (Binstadt et al., 1996). SHP phosphatases initiate a signalling cascade that results in the dephosphorylation and hence inactivation of key components in the signalling pathway for activation, such as ZAP-70 (Binstadt et al., 1996), PLC- γ (Valiante et al., 1996), SLP-76 (Binstadt et al., 1998) and Vav-1 (Stebbins et al., 2003) (Figure 1.2).

Inhibitory NK cell receptors are very heterogeneous and a single individual can possess NK cells carrying various combinations of inhibitory receptors (Parham, 2005). In addition, the KIR receptor family displays large haplotypic and allelic variability, leading different individuals to have very distinct NK cell receptor repertoires (Parham, 2005).

NK cell 'education'

The activation of NK cells must be tightly regulated to avoid autoimmune attack of normal tissues and at the same time to react appropriately in response to diseased host cells. NK cell-mediated killing of healthy cells is prevented in part through the action of inhibitory receptors. However, certain NK cells do not express MHC class I-specific receptors or express receptors for the wrong MHC class I ligand. Surprisingly, these cells were found to respond poorly to stimulation, despite expressing a full range of functional activating receptors (Fernandez et al., 2005; Kim et al., 2005). This hyporesponsiveness can explain the absence of auto-aggression by NK cells that are not inhibited by MHC class I-mediated recognition. NK cell self-tolerance has been shown to be caused by changes in the responsiveness of activating receptors to stimulation and is determined by recognition of MHC class I molecules (Fernandez et al., 2005; Kim et al., 2005; Anfossi et al., 2006). The functional adaptation of NK cells to recognition of MHC class I has been termed NK cell 'education' (Ohlén et al., 1989). The mechanisms, timing and location of events underlying NK cell education remain controversial and constitute an area of very active research (Held et al., 2011).

1.1.3 The immune synapse

NK cell responses are dependent on the integration of signals from multiple simultaneously engaged activating and inhibitory receptors. Now that many of the key molecules involved in NK cell recognition have been identified, mainly by biochemical and genetic approaches, a major new challenge is to understand their spatial localisation and dynamics within the cell membrane when encountering ligands on target cells. The advent of fluorescence microscopy and the recent progress in fluorescence labelling have enabled the visualisation of protein localisation within NK cells. Importantly, fluorescence microscopy led to the discovery of the formation of a polarised contact site between immune cells and other cells (Kupfer et al., 1983), termed an immunological, or immune, synapse. This name was adopted because the immune synapse, like a neural synapse,

consists of a junction formed between two cells that allows polarised communication and signalling between the cells (Norcross, 1984).

The immune synapse is currently defined as the interface between two contacting cells, including at least one immune cell, where signalling events resulting from this contact are dependent on the spatial organisation and temporal dynamics of proteins involved in the interaction. The formation and spatial organisation of immune synapses were first observed by confocal microscopy in the late 1990s (Monks et al., 1998; Grakoui et al., 1999; Davis et al., 1999; Delon and Germain, 2001). Since then, it has become apparent that they are critical for cell-cell communication and appropriate immune effector responses (Stinchcombe et al., 2001; McCann et al., 2003; Lee et al., 2003; Varma et al., 2006). If there is an error in communication, an immune cell might mistakenly launch an attack on healthy cells, as occurs in autoimmune diseases, or allow diseased cells such as cancer cells to escape detection. Immune synapses are therefore very complex and highly regulated structures that control immune responses and it is therefore not surprising that the mechanisms of immune synapse formation have received great attention in the fields of immunology and medicine.

The T cell immune synapse

T cells play a crucial role in the body's adaptive immune response – they amplify or suppress immune responses by secreting cytokines or promoting co-stimulation (in the case of CD4⁺ helper T cells) or by destroying cognate-antigen-bearing cells (CD8⁺ cytotoxic T cells). T cell activation is initiated by engagement of the T cell receptor (TCR) with an antigenic peptide fragment (derived from a pathogen or an unhealthy cell) displayed by an antigen-presenting cell (APC) in the context of MHC class I or class II proteins. The formation of an immunological synapse was first described between T cells and B cells *in vitro* (Monks et al., 1998) and has since been proven to play a central role in T cell effector functions. Importantly, T cell immune synapses have also been observed *in vivo* by two-photon microscopy (Bousso et al., 2002; Miller et al., 2002; Stoll et al., 2002).

Two landmark studies pioneered research into the spatio-temporal organisation of proteins at the immune synapse. In the first study, Monks, Kupfer and colleagues imaged the organisation of proteins at the interface between a CD4⁺ helper T cell and an APC (B cell) in fixed immunolabelled conjugates (Monks et al., 1998). They found that key molecules involved in the T cell immune synapse are organised in distinct micrometer-scale domains within the interface, known as supramolecular activation clusters (SMACs) (Figure 1.3). Signalling molecules such as the TCR and

protein kinase C- θ (PKC- θ) were accumulated in a central cluster (central SMAC; cSMAC), surrounded by a ring of the adhesion molecules leukocyte function-associated molecule 1 (LFA-1) and talin (peripheral SMAC; pSMAC). Large molecules like CD43 and CD45 were found to be excluded from the immune synapse and located in a region distal to the synapse (distal SMAC; dSMAC) (Monks et al., 1998; Freiberg et al., 2002).

In the second study, the authors used supported lipid bilayers as artificial APCs, in which they had inserted fluorescently-labelled peptide-MHC and the intercellular cell adhesion molecule 1 (ICAM-1). They then imaged live T cells interacting with these bilayers in order to monitor the dynamic organisation of the synapse over time (Grakoui et al., 1999). They found that, directly following cell-cell contact, the TCR-peptide-MHC complex initially accumulated in a ring surrounding a central cluster of adhesion molecules, such as LFA-1 and ICAM-1 interactions, to form an immature T cell synapse (Grakoui et al., 1999). Within minutes, this situation was reversed to form a mature immune synapse, consisting of the organisation of proteins into SMACs as described by Kupfer's group (Monks et al., 1998) and confirmed by other studies (Freiberg et al., 2002).

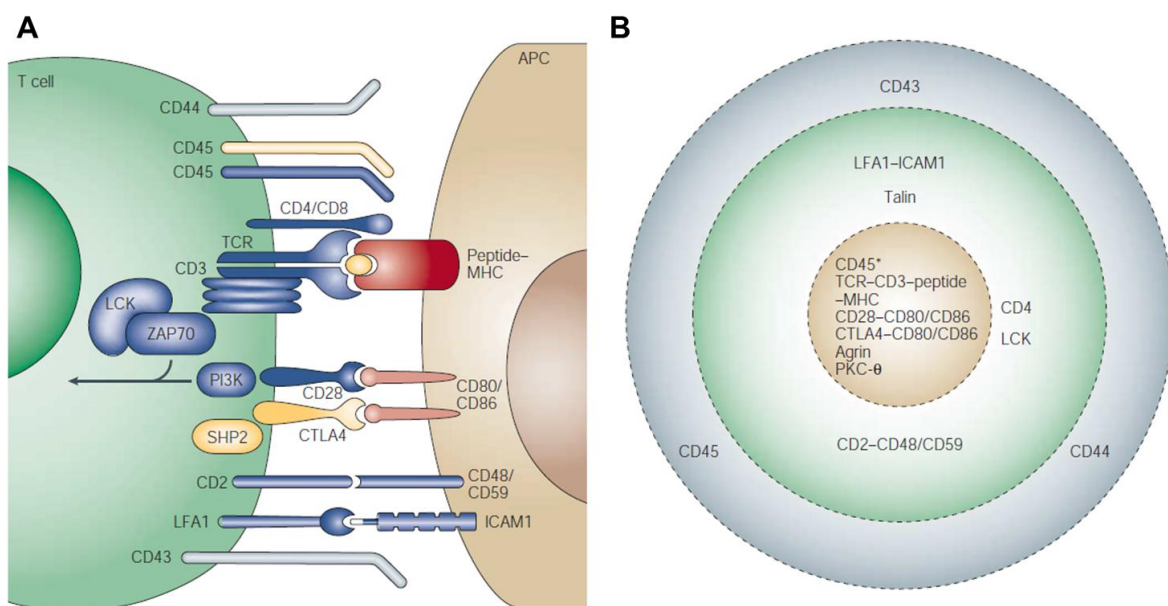


Figure 1.3: Mature T cell immune synapse. (A) Side view showing a selection of key ligand pairs and signalling molecules involved in T cell recognition. The stimulatory peptide-MHC complex is shown in red, activating and co-stimulatory molecules are blue, inhibitory molecules are shown in yellow and molecules that do not contribute to signalling are grey. The arrow indicates downstream signalling leading to T cell activation. **(B)** *En face* view of a mature T cell synapse showing the characteristic bull's eye patterning, including the cSMAC (yellow), the pSMAC (green) and the dSMAC (blue), as well as the key molecules and ligand pairs enriched within each zone. From (Huppa and Davis, 2003).

The organisation of the immune synapse has been shown to be strongly dependent on actin cytoskeletal rearrangements, since intact actin dynamics are necessary for central accumulation of TCR at the synapse and for T cell proliferation (Tskvitaria-Fuller et al., 2003). In addition, the microtubule organising centre (MTOC) rapidly reorients itself towards the T cell/APC interface upon synapse formation (Tskvitaria-Fuller et al., 2003).

The first typical immune synapses were described as stable structures exhibiting the prototypical “bull’s eye” pattern, with distinct cSMAC and pSMAC regions (Monks et al., 1998; Grakoui et al., 1999) and are now referred to as mature synapses, occurring predominantly with B cells as APCs. More recently, other types of molecular arrangements at the contact zone have been characterised, such as multifocal synapses, with several partially organised clusters without clearly distinguishable SMACs, or unstructured synapses (Cemerski and Shaw, 2006; Friedl et al., 2005; Brossard et al., 2005). Nowadays the term immune synapse encompasses all types of protein organisation and is used to describe structures where there is an enrichment of receptors at the contact site. The morphology of the synapse strongly depends on the cell types involved, the duration of the interaction, and the intensity of the stimulation (Blanchard and HIVROZ, 2002; Brossard et al., 2005; Friedl et al., 2005).

Various functions have been proposed for the three-dimensional organisation of the T cell immune synapse, including targeted cytokine secretion, and a role in enhancing, balancing and terminating signalling (Lee et al., 2003). The immune synapse machinery has also been exploited by viruses for the formation of virological synapses, which promote polarised viral propagation (Igakura et al., 2003; McDonald et al., 2003).

More recently, high resolution imaging studies have shown that T cell activation is accompanied by the formation of small aggregates of protein within the immune synapse termed microclusters (Bunnell et al., 2002; Varma et al., 2006; Bunnell et al., 2006). TCR ligation results in cluster formation and rapid co-accumulation of tyrosine kinases and critical adaptor proteins, such as SLP-76 (Bunnell et al., 2002). TCR signalling is thus initiated in peripheral TCR microclusters (Bunnell et al., 2002; Campi et al., 2005; Yokosuka et al., 2005) and is terminated when the microclusters move towards the centre of the synapse (Yokosuka et al., 2005; Varma et al., 2006; Bunnell et al., 2006). It has been proposed that the cSMAC is a site for TCR degradation (Lee et al., 2002, 2003). Continuous formation of TCR microclusters and movement from the dSMAC to the cSMAC are essential for sustained TCR signalling (Varma et al., 2006). In studies using supported planar bilayers to mimic target cells, it was shown that the initial size of TCR microclusters was proportional to the density of

agonist peptide-MHC in the lipid bilayer (Yokosuka et al., 2005). Each TCR microcluster was shown to contain approximately 100 TCRs (Yokosuka et al., 2005).

Signalling molecules involved in T cell activation also form microclusters, including SLP-76, LAT and ZAP-70 (Bunnell et al., 2002; Yokosuka et al., 2005; Varma et al., 2006; Campi et al., 2005; Purbhoo et al., 2010). The localisation, dynamics and interactions of these microclusters are important for regulating T cell signalling by sequestering signalling proteins into distinct compartments and promoting functional interactions (Purbhoo et al., 2010). Importantly, ligation of the inhibitory receptor Immunoglobulin-like transcript 2 (ILT2) was shown to inhibit formation of SLP-76 microclusters (Purbhoo et al., 2010). Similarly, signalling through the inhibitory receptor cytotoxic T lymphocyte antigen 4 (CTLA-4) inhibited clustering of ZAP-70 (Schneider et al., 2008), suggesting that the action of inhibitory receptors expressed on T cells may be upstream of the formation of protein microclusters.

These studies demonstrate the importance of protein organisation and segregation within the immune synapse for efficient T cell signalling. Overall, it seems that interactions between receptors, kinases and adaptor proteins implicated in immune cell signalling are controlled by the dynamics of molecular microclusters rather than by protein-protein interactions alone.

The NK cell immune synapse

Although immunological synapses were first seen in T cells, a variety of relevant synapse structures have since then been observed between other types of immune cells. Indeed, immune synapses have also been described between NK cells and their targets (Davis et al., 1999).

Complex protein interactions take place at the immune synapse between NK cells and target cells that are essential for determining the outcome of the contact. When a target cell is healthy, inhibitory signals will dominate the interaction and a so-called inhibitory synapse is formed (reviewed in (Eissmann and Davis, 2010)). In the opposite scenario, where the target cell is diseased, the balance of signals is tipped towards activation and the formation of an activating or lytic synapse (reviewed in (Orange, 2008)), leading to lysis of the target cell. The two different types of immune synapses will form depending on the repertoire of receptors expressed on the NK cell and the quality and quantity of ligands expressed on the target cell. Interestingly, a third type of immune synapse has emerged with the discovery of 'regulatory' synapses formed between NK cells and DCs (Borg et al., 2004) or macrophages (Nedvetzki et al., 2007).

The majority of NK cell effector functions require direct cell-cell contact, such as an immune synapse. Another recently characterised mode of communication between NK cells and target cells is via long membranous tethers that connect two cells, called membrane nanotubes (Onfelt et al., 2004; Chauveau et al., 2010). Interactions mediated by nanotubes are functional, since NK cells were able to lyse target cells at a distance through these nanotubes (Chauveau et al., 2010). Importantly, NK cell nanotubes formed small sub-micron scale junctions where activating receptors and signalling proteins accumulated; these were named nanotube synapses (Chauveau et al., 2010). Within the scope of this thesis, the focus will remain on direct close-range intercellular NK cell synapses.

The activating NK cell immune synapse

An activating NK cell immune synapse can be defined as the cytolytic synapse that forms between an NK cell and a susceptible target cell where positive signals dominate the interaction and lead to target cell death. Activation of NK cell cytotoxicity involves a tightly coordinated series of events, from initial contact and adhesion of NK cells with target cells, immune synapse formation and cytoskeletal rearrangements, to lytic granule polarisation and exocytosis, ending with immune synapse disassembly (Orange et al., 2003; Davis, 2002; Davis and Dustin, 2004; Orange, 2008; Krzewski and Strominger, 2008). Activation of NK cells provides a 'stop signal', which induces symmetrical spreading of NK cells and halted NK cell migration, resulting in the formation of a large stable NK synapse (Culley et al., 2009) (Figure 1.4).

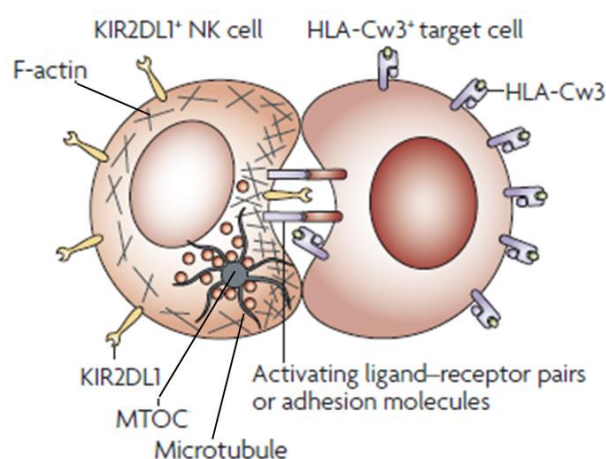


Figure 1.4: Activating NK cell immune synapse. In this example, the target cell expresses HLA-Cw3, which is not recognised by KIR2DL1. Activating receptors and ligands accumulate at the SMAC, whereas KIR2DL1 and HLA-Cw3 do not. F-actin accumulates at the NK cell synapse and the MTOC polarises towards the SMAC. Adapted from (Orange, 2008).

Formation of a structured and dynamic immune synapse ensures that an individual NK cell can precisely and accurately target a diseased cell it is interacting with without affecting neighbouring healthy cells that it might be contacting simultaneously. Signalling is spatially restricted towards a target cell, whereby an effector response can be initiated locally at the site of a specific immune synapse and inhibitory signalling can occur locally at another immune synapse at the same time (Eriksson et al., 1999).

Numerous molecules participate in the formation of the immune synapse, including receptors, signalling molecules, cytoskeletal components and cellular organelles. The first step in the formation of a lytic immune synapse consists of the initial engagement of receptors and adhesion molecules (such as LFA-1), and the lateral translocation of these proteins towards the immune synapse where they accumulate, followed by the rapid segregation of adhesion molecules to the pSMAC and receptors to the cSMAC (Vyas et al., 2001, 2002). Downstream signalling molecules are also recruited to the synapse, such as Src kinase, SLP-76, LAT, ZAP-70, PLC- γ and Vav-1, triggering the polymerisation and reorganisation of actin. This rearrangement of the actin cytoskeleton is vital for the supramolecular organisation of activating receptors at the synapse and for directed secretion of lytic granules towards the target. In the presence of actin inhibitors, actin accumulation at the immune synapse and NK cell cytotoxicity are decreased (Orange et al., 2002, 2003). Rearrangement of the actin cytoskeleton is also involved in the recruitment and accumulation of activating receptors at the synapse (Orange et al., 2003) and this receptor clustering is vital for robust signalling within the NK cell (Giurisato et al., 2007). Sustained receptor signalling initiates downstream signalling cascades, leading to the polarisation of the MTOC and subsequent recruitment of lytic granules towards the synapse, a pre-requisite for NK cell cytotoxicity (Jiang et al., 2000; Chen et al., 2006, 2007; Li et al., 2008). Lytic granules are thought to move along microtubules to the MTOC as it polarises towards the synapse and MTOC polarisation itself requires the reorganisation of the actin network (Wülfing et al., 2003; Orange et al., 2003; Graham et al., 2006). In addition, super-resolution images published in a recent study revealed that the cortical actin mesh present within the synapse is remodelled, whereby the periodicity of the actin mesh is increased, creating 'granule-penetrable' holes that are permeated by lytic granules, which are then released into the synaptic cleft (Brown et al., 2011). These findings were in contrast to the previously broadly accepted hypothesis that actin was totally cleared from the sub-synaptic area in order to allow release of lytic granules.

Involvement of lipid rafts in immune synapse formation

A potential mechanism that has been proposed for the rapid recruitment of proteins to the immune synapse is inclusion of these proteins in 'lipid rafts', both in T and NK cells. Lipid rafts are membrane microdomains containing specific lipids (namely sphingolipids, cholesterol and glycolipids) that are packed into more highly ordered regions than the rest of the membrane (Simons and Ikonen, 1997; Rietveld and Simons, 1998; Brown and London, 2000). The existence of these membrane domains remains highly controversial, mainly due to limitations in the methods available to isolate and study rafts. Most techniques rely on indirect isolation of rafts, such as detergent extraction, but direct visualisation of rafts in living cells has proved difficult (Munro, 2003). However, there is some evidence that lipid rafts do exist and that they are dynamic assemblies where signalling proteins are concentrated, and negative regulators such as tyrosine phosphatase CD45 are excluded (Leupin et al., 2000). Many proteins involved in signalling at the synapse have been shown to associate with lipid rafts isolated by density gradient centrifugation, including Lck (Kabouridis et al., 1997) and LAT (Zhang et al., 1998). A common way of visualising rafts by fluorescence microscopy has been to stain cells with a fluorophore conjugated to cholera toxin B subunit, which binds GM1 ganglioside, a marker of glycolipid-enriched rafts (Schön and Freire, 1989). Using this technique, the polarisation of lipid rafts to the immune synapse has been implicated in NK cell activation and has been shown to be blocked by the engagement of inhibitory receptors (Lou et al., 2000; Watzl and Long, 2003). These studies and others support a functional involvement of lipid rafts in signalling at the immune synapse.

Size-dependent kinetic segregation model

Another mechanism that might be important for the supramolecular organisation of proteins at the synapse is the spontaneous segregation of receptors and ligands spanning similarly sized intercellular distances. Based on the crystal structures, the intermembrane distance for receptor-ligand pairs would be approximately 15 nm, whereas integrin-ligand pairs would span approximately 40 nm. These two types of interactions are clearly segregated in the immune synapse according to their size, and a combination of experimental data and mathematical modelling has revealed that membrane-bending elasticity can drive size-dependent exclusion of specific proteins within immune synapses (Brzostek et al., 2010; Köhler et al., 2010; Alakoskela et al., 2011; Burroughs et al., 2011),

without the need for any additional mechanisms to explain the protein patterning found at synapses. This kinetic segregation based on size was also described in T cells (Choudhuri et al., 2005).

Overall, the formation and the organisation of lytic NK cell synapses share similarities with T cell synapses, including in the downstream signalling events that lead to activation. NK cells have also been shown to form inhibitory immune synapses where activation is blocked.

The inhibitory NK cell immune synapse

Upon encounter with a resistant target cell bearing cognate MHC class I (or other inhibitory ligands), NK cells engage their inhibitory receptors, which accumulate at the interface. The clustering of inhibitory receptors on the NK cell, which is mirrored by inhibitory ligands on the target cell, leads to the formation of an inhibitory immune synapse that prevents the lysis of the target cell (Davis et al., 1999). Resting NK cells contain lytic granules and pre-formed stores of perforin, and they express germline-encoded activating receptors – they are thus armed to kill target cells at short notice. The role of inhibitory immune synapses is therefore to prevent inadvertent killing of healthy cells.

The molecular composition and spatio-temporal events associated with the formation of non-cytolytic, inhibitory immune synapses are very different compared to activating NK cell synapses (Davis et al., 1999; Carlin et al., 2001). The first inhibitory immune synapses to be characterised were formed between NK cells and EBV-transformed B cells expressing MHC class I proteins recognised by the inhibitory receptors expressed on the NK cells (Davis et al., 1999) (Figure 1.5).

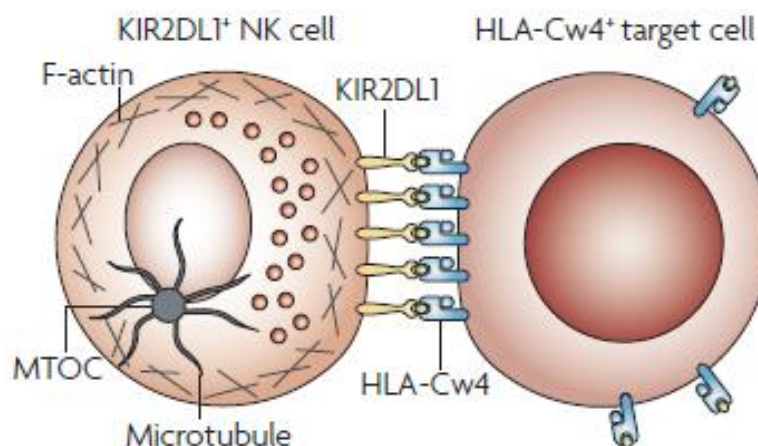


Figure 1.5: Inhibitory NK cell immune synapse. In this example, the target cell expresses HLA-Cw4, which is a cognate ligand for KIR2DL1. KIR2DL1 and HLA-Cw4 accumulate at the NK cell synapse. F-actin does not accumulate at the synapse and the MTOC does not polarise. From (Orange, 2008).

The formation of an inhibitory synapse begins with the engagement of adhesion molecules and inhibitory receptors with their ligands. Inhibitory receptors and their ligands rapidly and spontaneously cluster at the inhibitory immune synapse in a process triggered by receptor-ligand binding alone. Unlike receptor clustering in activating synapses, the accumulation of inhibitory proteins does not require downstream signalling or the engagement of adhesion molecules (Fassett et al., 2001; Faure et al., 2003). In addition, this accumulation is not dependent on the remodelling of the actin cytoskeleton or on any other, ATP-driven processes (Davis et al., 1999; Carlin et al., 2001; Standeven et al., 2004; Almeida and Davis, 2006), but appears to be driven by thermodynamic processes instead (Davis et al., 1999; Vyas et al., 2002; Fassett et al., 2001; Faure et al., 2003; Almeida and Davis, 2006). This is in contrast to the recruitment of the inhibitory T cell receptor CTLA-4, which is proportional to the intensity of the TCR signal (Egen and Allison, 2002). This difference might reflect an important distinction in the mechanisms by which these different receptors exert their inhibitory action.

Activating receptors have also been seen to initially accumulate at an inhibitory synapse (Schleinitz et al., 2008), but directed inhibitory signalling terminates or prevents NK cell activation by specifically excluding membrane-proximal intracellular proteins required for activation. NK cell inhibition seems to be mediated by a block at an early stage of the activation signalling pathway, since the engagement of inhibitory receptors prevents actin cytoskeletal rearrangements (Krzewski et al., 2006; Masilamani et al., 2006; Stebbins et al., 2003) and induces exclusion of lipid rafts from the synapse (Masilamani et al., 2006). Inhibition has been shown to be mediated by SHP dephosphorylation of key components in the signalling pathway for activation, such as Vav-1 (Stebbins et al., 2003). The spatial location of SHP-1 in the synapse is critical for distinguishing non-cytolytic from cytolytic interactions since, within the first minute of conjugation, SHP-1 will cluster in the periphery of lytic synapses, whereas it clusters in the centre of inhibitory synapses (Vyas et al., 2002). Further signalling through clustered activating receptors is therefore abrogated, resulting in the target cell being spared. The NK cell immune synapse is therefore the key site where inhibitory and activating signals are integrated to result in the appropriate NK cell response.

1.1.4 The inhibitory receptor KIR2DL1

The human killer cell immunoglobulin-like receptors (KIR; also known as CD158 and p58) are a family of transmembrane proteins expressed on NK cells and a subset of T cells that recognise MHC class I molecules (Wagtmann et al., 1995a; Colonna and Samaridis, 1995). The majority of normal

nucleated cells in the body express MHC class I molecules, with the exception of cells found in immunoprivileged sites such as the central nervous system and the placenta.

The KIR family is encoded by 14 highly polymorphic genes (KIR2DL1-5, KIR3DL1-3, KIR2DS1-5 and KIR3DS1). Distinct members of the family can transduce either activating or inhibitory signals depending on their structure. The naming of the KIR family members is based upon the number of extracellular immunoglobulin-like domains (2D for two domains, 3D for three domains) and on the length of the cytoplasmic tail (L for long and S for short) (Marsh et al., 2003). Inhibitory KIRs such as KIR2DL1, KIR2DL2/3, KIR3DL1, and KIR3DL2, contain either two or three immunoglobulin-like extracellular domains (KIR2D or KIR3D respectively) and long cytoplasmic domains. Interestingly, KIR receptors that induce activating signals generally have short cytoplasmic domains (e.g. KIR2DS1) (Moretta et al., 1995). Instead, they possess a positively charged residue in their transmembrane region, enabling them to associate with adaptor molecules such as DAP12 homodimers to trigger downstream signalling and activate NK cells (Lanier et al., 1998).

Allele-specific recognition of MHC class I molecules by KIR receptors

Individual KIR receptors recognise different subsets of MHC class I alleles and inhibitory KIR always have higher affinity for MHC class I molecules than activating KIR (Stewart et al., 2005). Members of the KIR family discriminate between two groups of HLA-C alleles: group 1 (including alleles HLA-Cw1, Cw3, Cw7 and Cw8) versus group 2 (which includes alleles Cw2, Cw4, Cw5 and Cw6) (Moretta et al., 1993; Wagtmann et al., 1995b). MHC class I molecules are a complex of two non-covalently associated subunits: the polymorphic heavy chain α (containing three α domains) and the invariant light chain β_2 -microglobulin (β_2m) (Barnstable et al., 1978b). MHC molecules associate with a short peptide, which is presented in their peptide-binding groove.

Ligand recognition is mediated by the KIR extracellular domains, which comprise either two or three immunoglobulin-like domain structural units (designated D1 and D2 in most KIR2D receptors). The D1 and D2 domains interact with the exposed surface of the HLA molecule at a site overlapping the C-terminal end of the bound antigenic peptide. Certain bound peptides can therefore influence the affinity of inhibitory KIR receptors for their MHC ligands. More specifically, residues at positions 7 and 8 of the peptides can dramatically influence KIR-HLA binding (Rajagopalan and Long, 1997).

Crystal structures have identified 16 amino acids in the D1 and D2 domains of KIR2DL receptors that mediate direct contact with residues in the $\alpha 1$ and $\alpha 2$ domains of HLA-C molecules (Boyington et al.,

2000). Differences in these 16 residues form the basis for the distinction among HLA allotypes by KIR receptors. For example, residues at positions 77 and 80 in HLA-C are critical for defining the two groups of allotypes, which are either serine and asparagine (respectively) in the group 1 HLA-C allotypes, or asparagine and lysine in the group 2 allotypes (Biassoni et al., 1995). In addition, a single residue in the D1 domain of KIR (at position 44) has been found to be sufficient to determine the ability of the KIR receptors to discriminate between HLA-C group 1 (with a methionine residue at position 44) and group 2 (with lysine at position 44) (Winter and Long, 1997).

KIR2DL1 is a member of the KIR family that recognises group 2 HLA-C allotypes (Wagtmann et al., 1995b). Studies on KIR2DL1 have mainly focussed on its interactions with the group 2 HLA-C molecule HLA-Cw4 as a prototype. KIR2DL1 consists of two extracellular immunoglobulin-like domains, a transmembrane domain, and a cytoplasmic tail containing two ITIM motifs that initiate inhibitory signalling (Figure 1.6). The crystal structure of the KIR2DL1-HLA-Cw4 complex has been solved and reveals that the lysine in position 80 of HLA-Cw4 mediates specificity (Fan et al., 2001).

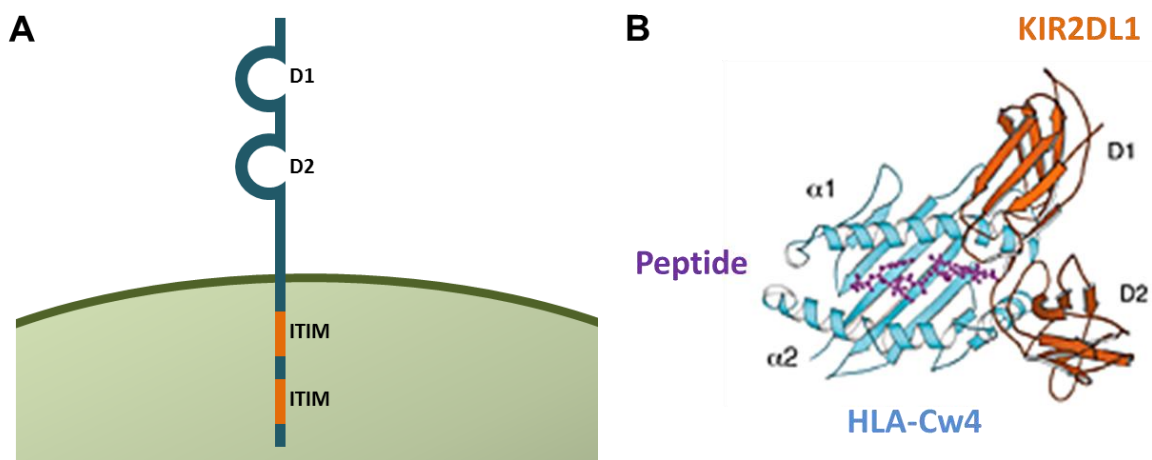


Figure 1.6: Structure of the inhibitory NK cell receptor KIR2DL1. (A) The inhibitory receptor KIR2DL1 contains two immunoglobulin-like extracellular domains (D1 and D2), a transmembrane region, and two ITIM motifs which transduce inhibitory signalling. (B) Ribbon diagram of the crystal structure of KIR2DL1 bound to HLA-Cw4. KIR2DL1 is shown in orange. The heavy chain and β_2m of HLA-Cw4 are in blue and its bound peptide is shown in purple. Adapted from (Fan et al., 2001).

Supramolecular organisation of KIR2DL1 at the immune synapse

The spatial organisation of KIR2DL1 and its ligands at the inhibitory immune synapse has been studied in some detail using confocal microscopy. According to gel-shift assays, surface plasmon resonance experiments and their crystal structures, KIR2DL1 and HLA-C interact at a molar ratio of 1:1 (Fan et al., 1996; Valés-Gómez et al., 1998; Fan et al., 2001). When imaged by confocal microscopy, their localisation at the immune synapse overlaps (Davis et al., 1999; Carlin et al., 2001). In initial experiments, it was discovered that KIR2DL1 on the NK cell and HLA-C on the target cell accumulated in a ring at the synapse surrounding a central accumulation of LFA-1 and its ligand ICAM-1 (Davis et al., 1999). Subsequent studies revealed that the inhibitory synapse is a dynamic structure and that KIR2DL1-HLA-C patterns change over time (Carlin et al., 2001; Almeida and Davis, 2006; Oddos et al., 2008). High-resolution imaging of the interface between NK cells expressing KIR2DL1 and target cells expressing HLA-Cw6 demonstrated that over time small clusters of KIR2DL1 moved from the synapse periphery to the centre (Oddos et al., 2008).

HLA-C and KIR2DL1 can adopt different patterns at the synapse, such as a homogeneous distribution without segregation, a ring-shaped distribution, a multifocal distribution, or a distribution containing multiple exclusions (Carlin et al., 2001; Almeida and Davis, 2006). The synaptic organisation of HLA-C correlates with its level of expression on the target cell and directly influences NK cell inhibition, with a tendency towards ring formation with higher levels of surface HLA-C on the target cell, although overall, the multifocal distribution seems to predominate (Almeida and Davis, 2006). These different patterns of proteins at the immune synapse can therefore transmit information about the density of ligand on the target cells, possibly by regulating the proximity of activating and inhibitory receptors, and reflect a functional role for receptor clustering, since a certain threshold level of MHC class I on target cells is required for NK cell inhibition (Almeida and Davis, 2006; Almeida et al., 2011).

As in T cells, research has shown that the formation of protein microclusters within the NK cell synapse is important for efficient signalling, although functional microclusters have mainly been investigated in inhibitory rather than activating NK cell synapses. For example, KIR2DL1 has been shown to form microclusters within the inhibitory synapse (Oddos et al., 2008). Only a small fraction of KIR2DL1 receptors gets phosphorylated, and intriguingly, rather than being homogeneous across the synapse, KIR2DL1 phosphorylation is spatially restricted to discrete microdomains within the immune synapse (Treanor et al., 2006). In addition, signalling molecules such as Lck have also been shown to form microclusters within the inhibitory immune synapse (Treanor et al., 2006). These microclusters could be formed through protein-protein interactions or interactions with the

cytoskeleton. A hypothesis that has emerged for efficient KIR-mediated inhibition is that activating receptors recruit Lck, which may facilitate the transphosphorylation of KIR2DL1 receptors if they are engaged by HLA-C on the opposing cell (Treanor et al., 2006). The spatial confinement of inhibitory receptor phosphorylation would thereby promote focussed inhibitory action to the specific sites where activating signals have been initiated.

In summary, the supramolecular organisation of the immune synapse has emerged as an important factor for the outcome of an NK cell-target cell interaction and has been described in some detail at the micrometer scale. A major challenge that remains is to understand how the organisation of the immune synapse relates to the integration of activating and inhibitory signals that govern the NK cell's responses. Specifically, it is important to determine the spatio-temporal dimensions of events and interactions occurring at the synapse at the single-molecule level, especially since protein microclusters are emerging as important aspects of NK cell signalling. To better understand how signals are integrated, it has become important to observe the organisation and proximity of inhibitory and activating receptors, as well as downstream signalling molecules, and to investigate the mechanisms that can influence this organisation. Importantly, an emerging paradigm in immune cell signalling is that interactions occurring between receptors and signalling proteins are controlled in part by the dynamics of molecular clusters rather than by isolated protein-protein interactions as previously imagined. A better understanding of these dynamic protein microclusters requires an investigation at the single molecule level. A lot can and has been learnt from simply imaging what happens at the immune synapse using conventional microscopy techniques. The view of a concentric organisation of proteins at the immune synapse has begun to change drastically with the use of high-resolution microscopy. However, new imaging methods are necessary to advance our understanding of the immune synapse and probe deeper into the organisation and dynamics of proteins within this structure.

1.2 Super-resolution microscopy

Fluorescence microscopy has become an essential tool for biologists to study cellular events in real time and in living cells with high molecular specificity, low background noise and minimal invasiveness. Many new fluorescence microscopy techniques are emerging, driven by the need to visualise events at the molecular level to improve our knowledge and understanding of cellular mechanisms such as the integration of signals at the immune synapse.

1.2.1 Fluorescent probes

A fluorophore is a molecule that emits fluorescence upon excitation by light of an appropriate wavelength. A large range of fluorescent probes is now available to researchers for labelling given molecules with high specificity, allowing them to be imaged with high contrast in living cells.

Fluorescent dyes

Fluorescent dyes are chemical compounds designed to specifically stain given molecular species in order to localise their presence inside a cell or inside specific cellular compartments. Such dyes include DAPI, DRAQ5 and Hoechst, which stain DNA and can be used to localise the nucleus, and DiD, DiO and DiI, which stain lipids and can be used to visualise the cellular plasma membrane.

Some fluorescent dyes are designed as sensors. For example, the membrane-staining dye di-4-ANEPPDHQ can distinguish between ordered and disordered lipid phases; it has different emission spectra in these two types of lipid domains, and is thus able to specifically localise cholesterol-enriched lipid rafts (Jin et al., 2005). Another commonly used sensor dye is Fluo-4, whose brightness increases with Ca^{2+} concentration.

However, these dyes generally do not stain one specific molecule; rather they locate general cellular compartments or events. They are therefore inappropriate for tracking specific proteins of interest.

Immunofluorescence labelling

Some small, bright and stable fluorophores that do not exhibit specific binding properties are used for immunofluorescence labelling. One of these fluorophores, for example Alexa Fluor, cyanine (Cy) or Atto dyes, can be conjugated to an antibody that binds to a given molecule with high specificity. The dye-conjugated antibodies are then used to label the sample. Generally, staining with a primary antibody specific for the molecule of interest precedes staining with a conjugated secondary antibody, which binds to the primary antibody and amplifies the signal. Although this binding is highly specific, the preparation of samples generally requires permeabilisation and fixation, which are incompatible with live cell imaging and only produce snapshots of protein localisations.

Fluorescent proteins

The discovery and isolation of green fluorescent protein (GFP) from the jellyfish *Aequoria victoria* (Shimomura et al., 1962; Chalfie et al., 1994) have revolutionised biology, permitting the visualisation of protein localisation, dynamics and interactions in living cells in real-time. Most proteins can be tagged with GFP, resulting in the expression of a fusion protein that can be used as a fluorescent reporter to study the dynamics of the protein of interest (Chalfie et al., 1994). The development of GFP variants by genetic engineering and the discovery of orange to near infra-red fluorescent proteins (Baird et al., 2000) has provided researchers with a range of robust fluorescent proteins covering the entire visible spectrum (Giepmans et al., 2006; Shaner et al., 2007). In addition, the discovery of photoactivatable and photoswitchable fluorescent proteins has extended the fluorescent protein toolbox available to researchers and has opened up possibilities for new exciting imaging techniques, as will be described later.

Quantum dots

Quantum dots are nanometer-sized semiconductor crystal structures with narrow emission spectra, making spectral separation of multiple dots relatively easy. They are very bright and stable, being almost insensitive to photobleaching. They can be used to bind specific proteins by coating them

with antibodies. However, they are quite large and can disrupt protein functions (Bruchez et al., 1998; Alivisatos, 2004).

In parallel to the development of new fluorophores, major advances have been made in fluorescence imaging techniques and microscopy systems.

1.2.2 Confocal fluorescence microscopy and the diffraction barrier

In fluorescence microscopy, a beam of light of a specific wavelength is directed onto a biological sample containing molecules of interest that have been appropriately and individually labelled with fluorophores. When fluorescent molecules absorb a photon of light, they emit a photon of longer-wavelength light about one nanosecond later (Figure 1.7). The light emitted by excited fluorophores is collected by the microscope and directed towards a detector for measurement after using a filter to eliminate light of unwanted wavelengths. These photons are collected by the detector, which then converts these detection events into electrical signals that can be quantified to generate an image of the protein distribution in the sample.

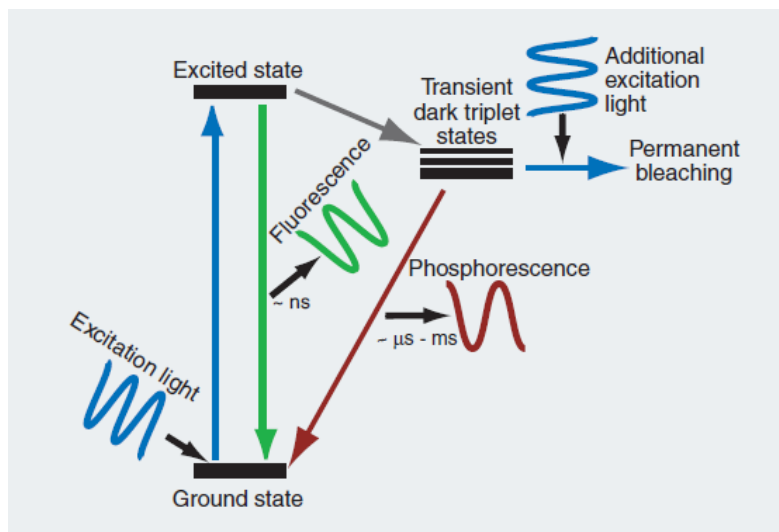


Figure 1.7: Jablonski energy diagram representing the electronic states and transitions of a fluorophore. In a resting fluorophore, electrons populate the ground state. When illuminated with light of appropriate wavelength, the fluorophore will absorb a photon and be promoted to the excited state. Fluorescence occurs through the radiative relaxation of electrons from the excited state to the ground state. Alternative routes such as phosphorescence are also shown in this diagram. From (Evanko, 2009).

Confocal microscopy

In wide-field fluorescence (or epifluorescence) microscopy, the whole field of view is uniformly illuminated and photons from the entire image are collected at once to record the localisation of the fluorophores. Illumination of the sample and collection of the fluorescence signal are usually performed using the same lens. The fluorescence signal from the sample is projected onto a wide-field detector to generate a fluorescence image (Figure 1.8). However, the fluorescence signal is emanating from the whole sample, creating high levels of background noise from the out-of-focus planes of the sample.

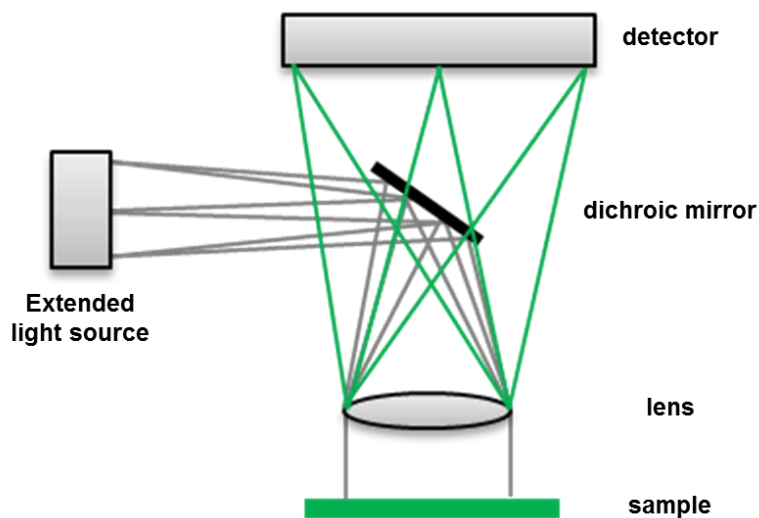


Figure 1.8: Schematic representation of a fluorescence wide-field microscope. The sample is uniformly illuminated by a light source at the excitation wavelength (grey). The light emitted from the sample (green) is collected on a wide-field detector, producing a fluorescence image of the sample.

Sectioned microscopy techniques overcome this problem by building up an image of a thinner z-plane pixel by pixel, using a principle proposed as early as 1943 (Koana, 1943). The most commonly used type of sectioned microscope is the laser scanning confocal microscope (Cremer and Cremer, 1978). In a typical laser scanning confocal fluorescence microscope (Figure 1.9), fluorophores are excited by a point illumination (generally a laser source) that is scanned across the sample, usually line by line, and the fluorescence signal is detected by a point detector (in general this is achieved by using a pinhole in front of the detector). A computer reconstructs the planar image pixel by pixel in real time. The combination of selective illumination and point detection leads to the rejection of out-of-focus light emitted by the sample and results in a clearer image. If images are collected at

multiple confocal planes, a three-dimensional image of the fluorescent sample can be reconstructed. The development of laser scanning confocal microscopy has therefore provided a technique for studying biological structures in greater detail (Brakenhoff et al., 1985).

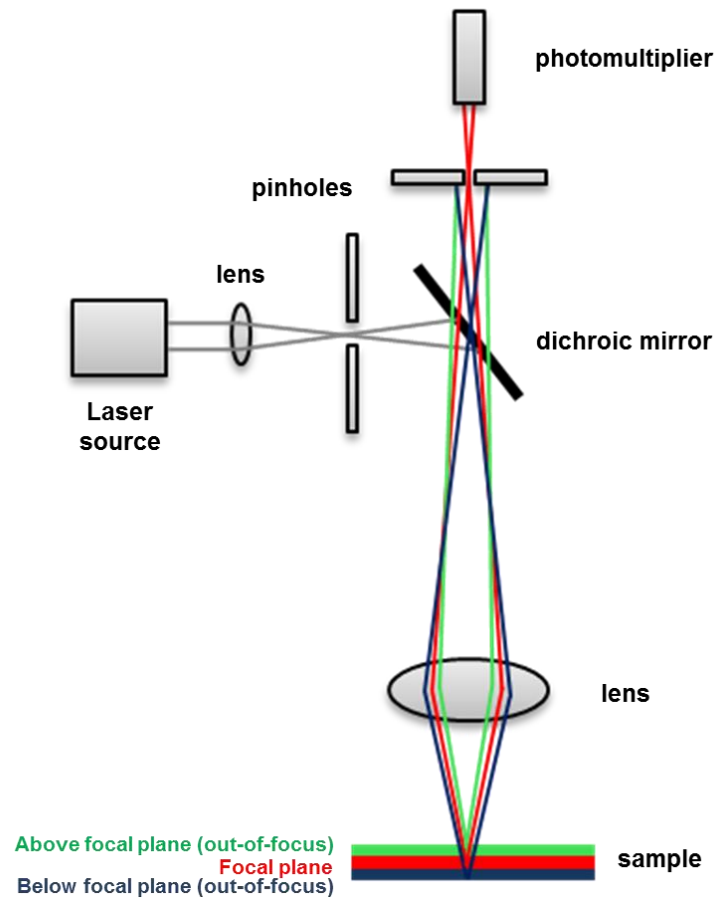


Figure 1.9: Schematic representation of a fluorescence confocal microscope. The excitation beam (grey) originates from a point light source and is focussed onto the sample to create an excitation spot. The fluorescence emitted by this spot (red) is collected on a point detector through a confocal pinhole. The spot is scanned across the sample to generate a fluorescence image of the sample. The combination of point excitation and point detection (produced by the presence of pinholes) rejects signal coming from out-of-focus planes (green and blue).

Resolution and the diffraction barrier

Optical resolution is the minimal distance separating two distinct fluorophores that allows them to be discriminated as individual objects. The aim of fluorescence microscopy is to generate magnified images of the sample at the highest resolution possible, providing as much detail as possible about the sample. In fluorescence microscopy, the resolution of an image depends on the signal-to-noise

ratio (SNR) in the image and the point-spread function (PSF) of the microscope. The SNR of an image is dictated by sample properties, such as brightness of the fluorophore and staining specificity, and varies between samples and acquisitions. The PSF is the response of a microscope to a point object, i.e. it is the smallest representation of a single object that the imaging system can form (Figure 1.10). The degree of spreading of a PSF reflects the quality of the microscope. If the PSFs of two neighbouring fluorophores are overlapping, the microscope is unable to resolve them as two distinct objects.

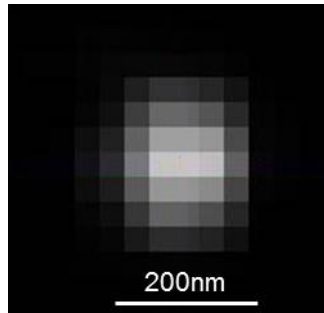


Figure 1.10: Diffraction-limited image of a point source. The point spread function (PSF) is the smallest representation of a point object that a microscope can form. In most confocal microscopes, the size of the PSF is limited to ≈ 200 nm in the lateral plane.

The resolution of fluorescence microscopes is limited by diffraction. In 1873 the German physicist Ernst Abbe observed that light focussed through a lens always forms a blurred PSF, which limits the resolution to the diameter of the PSF. If light travelled in a straight line and detectors were ultra-sensitive, it would be possible to discriminate all the individual fluorophores in an image. However, due to diffraction of light resulting from interactions with the surrounding physical environment it travels through, light from a single fluorophore is never projected onto an image as a discrete point.

The mathematical expression of what is now known as Abbe's diffraction barrier states that the resolution of a fluorescence microscope (r) is limited to:

$$r = \frac{\lambda}{(2n \sin \alpha)}$$

in the lateral plane; where λ is the wavelength of the emitted light, n is the refractive index of the medium in which the sample is placed, and α is the angular aperture of the microscope.

This diffraction barrier has limited the detail that could be obtained about cellular structures, signalling pathways and protein interactions and dynamics. A standard confocal microscope,

equipped with a lens with high numerical aperture, can generate images with lateral and axial resolutions of approximately 250 nm and 1 μm respectively. Since elements making up most biological structures exist at scales smaller than this limit, they cannot easily be distinguished as individual molecules. It is therefore desirable to achieve higher resolution when studying these structures. The aim of high- and super-resolution microscopy techniques is to improve the resolution of images using various approaches.

1.2.3 High-resolution imaging techniques

Many important biological processes occur at or near the cellular plasma membrane or at the interface between two cells. It is difficult to image these events in detail, since axial resolution remains poor in confocal fluorescence microscopy and details near the cell surface are hidden by fluorescence emanating from the rest of the cell. Several methods based on traditional wide-field and confocal microscopy have been developed to allow high-resolution visualisation of these events.

Total Internal Reflection Fluorescence Microscopy

Total internal reflection fluorescence (TIRF) microscopy (also known as evanescent wave or evanescent field microscopy) is based on classical wide-field microscopy where fluorophores near the cell surface are selectively excited whilst the fluorescence from intracellular regions is minimised (Axelrod, 1981, 1989, 2001). This is achieved by irradiating the sample at a critical angle so that the light is totally reflected at the glass coverslip-aqueous sample interface. In this way, no direct illumination reaches the sample, but instead an evanescent wave is created on the opposite side of the interface, within the sample (Figure 1.11). This evanescent wave usually penetrates approximately 100 nm into the sample, thus providing better sectioning strength than a confocal microscope. Only fluorophores that are present within this evanescent field are excited, thereby minimising background fluorescence and allowing imaging of processes occurring at or near the cell membrane.

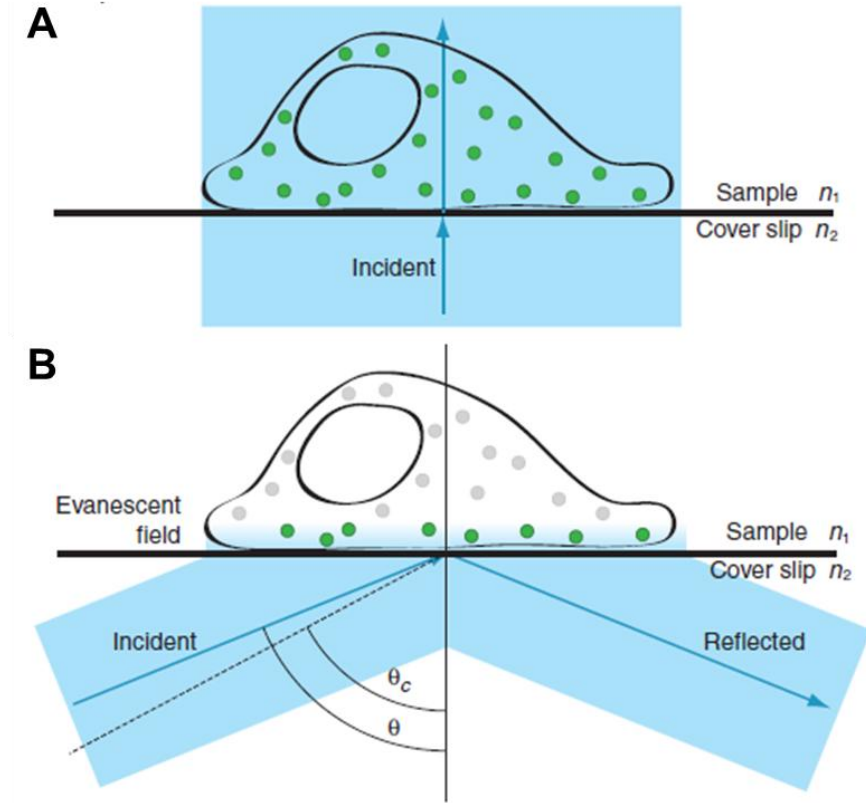


Figure 1.11: The physical basis underlying epifluorescence and TIRF illumination. (A) Epifluorescence. The incident excitation beam travels through the coverslip-sample interface in a coherent beam, exciting all the fluorophores in the sample. **(B) TIRF.** The excitation beam travels from the left at an incident angle θ , which is greater than the critical angle θ_c (dashed line). The excitation beam is reflected off the coverslip-sample interface, generating an evanescent wave on the opposite side of the interface, within the sample. Only fluorophores that are within the evanescent field are excited, as indicated in green. From (Mattheyses et al., 2010).

The light beam only undergoes total internal reflection (TIR) if the angle of incidence of the excitation light (θ) is greater than the critical angle (θ_c), which is given by:

$$\theta_c = \sin^{-1} \left(\frac{n_1}{n_2} \right)$$

where n_1 and n_2 are the refractive indices of the sample and the coverslip respectively. Therefore, the refractive index of the sample must be less than that of the coverslip in order to achieve TIR.

To achieve optimal TIRF, all of the light incident on the interface should be at the same angle. A uniform angle of illumination can be obtained by focussing the laser beam on the back focal plane of the objective, where the light is collimated and emerges onto the interface in a coherent beam, i.e. with all its rays parallel, at the same angle and in phase.

TIRF microscopy has been used in many different types of biological studies, shedding light for instance on cytoskeletal dynamics (Schmoranzer and Simon, 2003; Krylyshkina et al., 2003), endocytosis (Rappoport, 2008), exocytosis (Schmoranzer et al., 2000; Allersma et al., 2006), cell-substrate contacts (Axelrod, 1981; Choi et al., 2008) and membrane-proximal signalling (Haugh et al., 2000; Dyachok et al., 2006; Purbhoo et al., 2010). The applications of TIRF microscopy in cell biology, the physical basis of TIR and the experimental set-up required for TIRF microscopy were recently reviewed (Mattheyses et al., 2010).

TIRF microscopy is therefore a very powerful tool for imaging processes occurring at or near the cell membrane at an interface with a coverslip. Importantly, many membrane-proximal events occurring at the immune synapse were revealed by imaging the interface between an immune cell and an artificial substrate mimicking a target cell (such as antibody-coated glass slides or artificial glass-supported lipid bilayers) using TIRF microscopy. TIRF microscopy has also been exploited for super-resolution single molecule imaging, as will be described below.

Optical tweezers

Optical trapping using laser tweezers is a technique that allows manipulation of live cells and small objects in all dimensions (Ashkin et al., 1986; Ashkin, 1997). The principle of optical trapping was first introduced by Ashkin, when he performed an experiment where small latex beads were unexpectedly pulled into a tightly focussed laser beam (Ashkin, 1970). This phenomenon was then exploited to create a method by which small objects can be manipulated. A laser light beam is focussed by sending it through a microscope objective and small (nanometer- or micrometer-sized) dielectric particles are attracted to the narrowest point of the focussed beam, the beam waist, which contains a very strong electric field gradient. The force exerted on the particles is sufficient to manipulate and stably trap them as desired.

Optical tweezers have become a commonly used tool in cell biology. The biological applications are vast. For example, optical trapping has been used to study macromolecular motors such as ribosomes in RNA translation or myosin and kinesin in cell motility (Svoboda et al., 1994), to measure the viscoelastic properties of DNA (Bustamante et al., 2000) and to investigate optically guided neuronal growth (Carnegie et al., 2008). Other useful applications in immune cell biology include the use of optical tweezers to artificially promote the formation of multiple synapses between one effector cell and several target cells. For instance, NK cells were made to contact a

sensitive target cell whilst already forming an inhibitory synapse with a resistant cell – these experiments provided important new information on the integration of signals at the synapse and how signalling is spatially restricted to an immune synapse (Eriksson et al., 1999).

Another important application of optical tweezers is to visualise the immune synapse between two living cells *en face*. Indeed, imaging intercellular interactions is generally achieved by acquiring image stacks by confocal microscopy and subsequently reconstructing the desired optical slice through the sample, an approach that is both time-consuming and only provides poor resolution. Therefore, using an optical tweezers system to manipulate cell conjugates to reposition them so that the immune synapse lies on a horizontal plane (as depicted in Figure 1.12) provides a fast method of visualising the organisation and dynamics of proteins at the immune synapse between two live cells at higher resolution (Oddos et al., 2008; Brown et al., 2011).

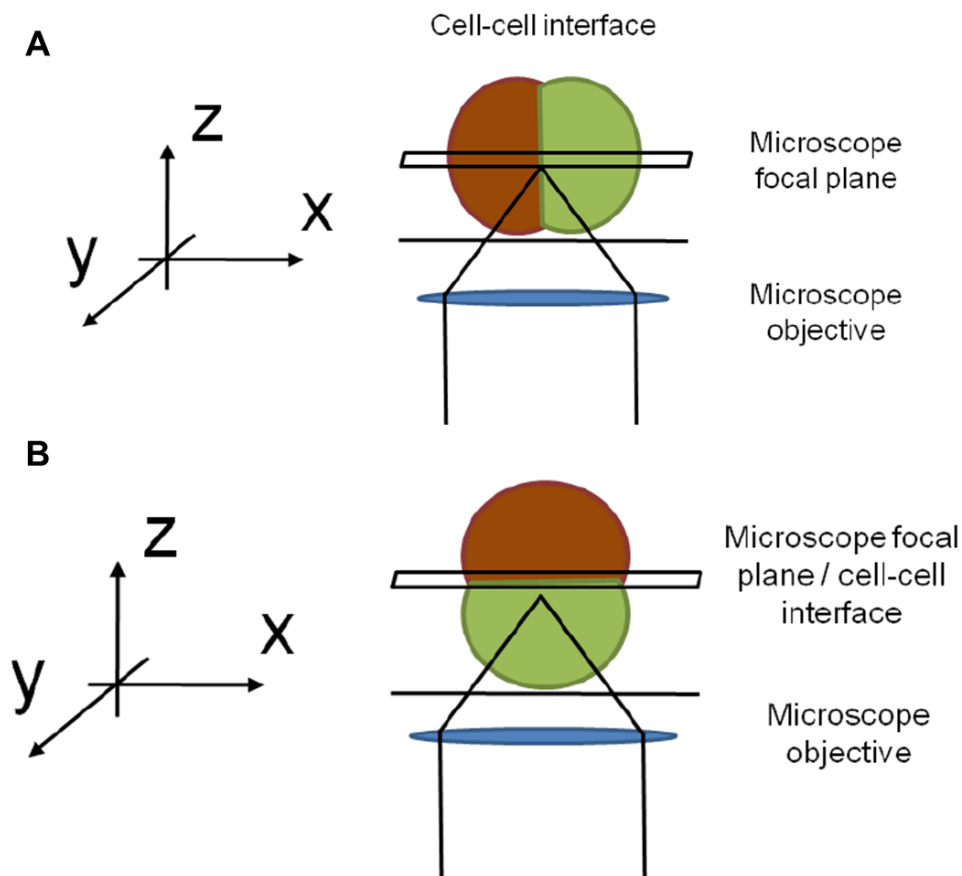


Figure 1.12: Using optical tweezers to image the immune synapse. (A) Geometric arrangement of the cell-cell interface with regards to the confocal plane in a confocal microscope. In this configuration, the immune synapse is perpendicular to the focal plane of the microscope objective. **(B)** Geometric arrangement of the cell-cell interface with regards to the confocal plane in a confocal microscope after manipulation of the conjugate with optical tweezers. In this configuration, the immune synapse is parallel to the focal plane of the microscope objective. From (Oddos, 2010).

TIRF microscopy and optical tweezers combined with confocal microscopy provide the tools to study the interface between a cell and a surface or between two cells at higher resolution. However, the diffraction limit still applies and it is necessary to utilise novel, more advanced, super-resolution techniques to investigate the organisation of proteins at these interfaces at the single molecule level.

1.2.4 Super-resolution imaging techniques

Recently, powerful new fluorescence imaging techniques have been emerging; they break the diffraction barrier using different approaches. These super-resolution imaging techniques generally exploit the presence of dark and bright states in fluorophores and can be grouped into two main classes: ensemble techniques, where resolution is improved by minimising the size of the PSF, and single-particle localisation techniques, where resolution is improved by localising single molecules. Using these techniques, a fluorescence microscope is in theory capable of unlimited “resolution”, in this context meaning that fluorophores can be localised with very high accuracy (as opposed to true imaging resolution).

Ensemble techniques

Ensemble super-resolution techniques aim to improve the resolution of images by minimising the size of the PSF during acquisition, thereby generating super-resolution images at the population level. This is achieved by manipulating the emission pattern of fluorophores using different approaches. Two of the main ensemble super-resolution techniques are described here.

Structured illumination microscopy (SIM) uses full-field illumination of the sample with spatially structured light, which can provide additional information about the distribution of fluorophores in the sample. SIM uses patterned lines of light that are translated and rotated, generating a series of images with high spatial frequency information. Such a pattern is usually obtained by passing the laser beam through a mobile optical grating before projecting it onto the sample through the objective (Heintzmann and Cremer, 1999; Gustafsson, 2000, 2005). If a sample contains fine structures (such as cytoskeletal filaments) and is illuminated with the patterned lines of light, interference patterns (called moiré fringes) arise, which have coarser features than the original

structure and are recorded onto an image. By imaging the sample with the structured light pattern in different orientations, the acquired images can be reconstructed into a high-resolution image using computer algorithms (Figure 1.13). A theoretical two-fold improvement in lateral resolution can be obtained using this approach.

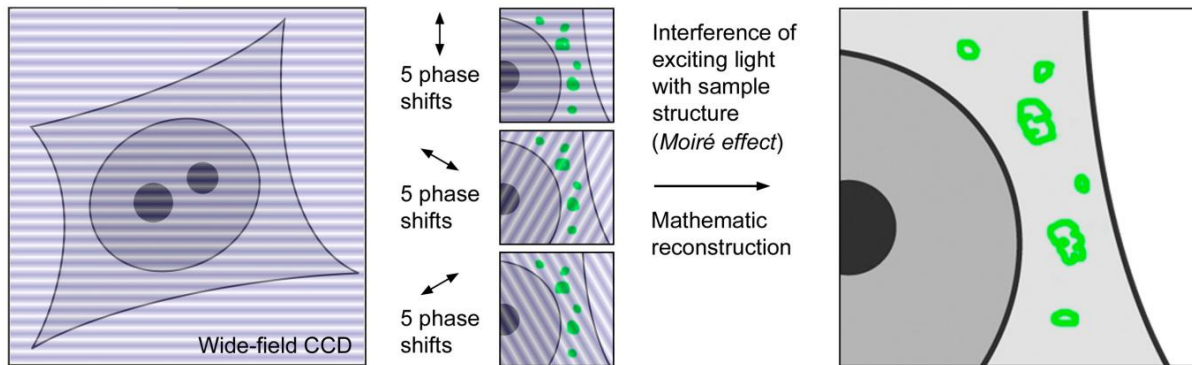


Figure 1.13: Principle of Structured Illumination Microscopy (SIM). The sample is excited by patterned illumination, obtained by passing the light through an optical grating to generate stripes of sinusoidal interference. In combination with interference from sample structures that are below the diffraction limits, this generates Moiré fringes. The image detected by the CCD camera contains high spatial frequency information about the sample. A mathematical reconstruction from a series of 15 raw images per slice generates a high-resolution image. From (Schermelleh et al., 2010).

SIM is an attractive candidate for cell biological applications as standard dyes and staining protocols can be used, as long as the fluorophores are sufficiently stable to avoid photobleaching during the additional exposure cycles. SIM has successfully been used to visualise mitochondria, tubulin filaments and kinesin dynamics in living cells (Hirvonen et al., 2009; Kner et al., 2009). Importantly, SIM was the main technique used to show how the actin cytoskeletal mesh underlying the immune synapse in NK cells is remodelled to allow the passage of lytic granules to the synaptic cleft (Brown et al., 2011). Variants of SIM that are currently being developed include three-dimensional SIM (3D-SIM) (Gustafsson et al., 2008; Schermelleh et al., 2008) and saturated SIM (SSIM) (Heintzmann et al., 2002) amongst others.

In stimulated emission depletion (STED) microscopy, the PSF is shaped by controlling the de-excitation of previously excited fluorophores, a phenomenon termed stimulated emission depletion. This technique was first developed and successfully implemented by Hell and colleagues (Hell and Wichmann, 1994; Klar et al., 2000; Dyba and Hell, 2002; Dyba et al., 2003). In this technique, the sample is illuminated and excited with a laser at a focal spot, as in confocal microscopy, but this focal spot is surrounded by a doughnut of longer wavelength laser light (called the STED beam), which

depletes or de-excites the fluorophores surrounding the focal spot back to the ground state. Fluorescence from the central region is detected. The spot is scanned across the sample to generate a super-resolution sub-diffraction image (Figure 1.14). A resolution of 30-80 nm can be achieved with this approach (Hell and Wichmann, 1994; Dyba and Hell, 2002). The choice of an appropriate dye is crucial for STED microscopy, as the wavelength of the depletion laser should not overlap with the excitation range of the dye, which would result in unwanted excitation in the doughnut region of depletion.

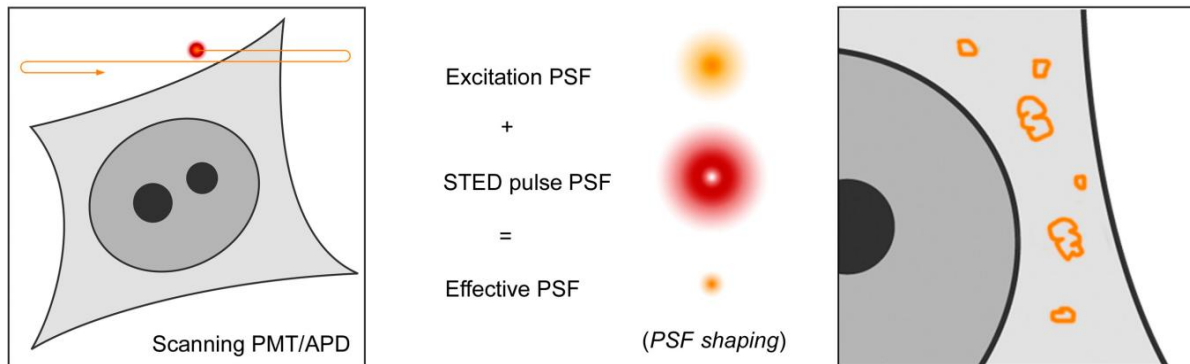


Figure 1.14: Principle of Stimulated Emission Depletion Microscopy (STED). The sample plane is scanned by two overlapping laser beams. The first laser excites the fluorophores; the second laser of longer wavelength drives the fluorophores back to the ground state by stimulated emission depletion. The depletion laser is given a doughnut shape, leaving only a small volume from which light can be emitted. This method basically remodels the PSF to a volume that is below the diffraction limit. From (Schermelleh et al., 2010).

STED microscopy has successfully been applied to solving key problems in biology. For instance, it has been used to study the distribution of proteins at neuronal synapses (Willig et al., 2006) and at insect neuromuscular junctions (Kittel et al., 2006) as well as the organisation of SNARE proteins in the plasma membrane (Sieber et al., 2006). STED microscopy has been improved with the development of continuous wave STED (CW-STED), which implements continuous wave lasers for both excitation and depletion beams (Willig et al., 2007), two-colour STED (Donnert et al., 2007), *in vivo* STED (Schröder et al., 2009) and live-cell STED imaging (Hein et al., 2008, 2010). Other techniques using similar approaches include ground state depletion (GSD) (Hell and Kroug, 1995) and saturated patterned excitation microscopy (SPEM) (Heintzmann et al., 2002).

Single-particle localisation techniques

Another approach that has been used to improve resolution is to first localise single molecules and then reconstruct a super-resolution image from multiple frames, as achieved by single-particle localisation super-resolution techniques. The basic principle underlying these techniques is to switch photoactivatable or photoswitchable fluorophores from a non-fluorescent to a fluorescent state (or from a fluorescent state to another fluorescent state of a different wavelength) by exposure to a specific wavelength.

Photoactivated localisation microscopy (PALM) and stochastic optical reconstruction microscopy (STORM) are the two main techniques relying on this principle and were invented at about the same time. PALM and STORM were both developed using the concept that an isolated fluorophore, even though subject to the diffraction barrier and therefore producing a diffraction limited spot on a detector, could be precisely localised by calculating the centroid of the PSF in the image (Thompson et al., 2002) (Figure 1.15).

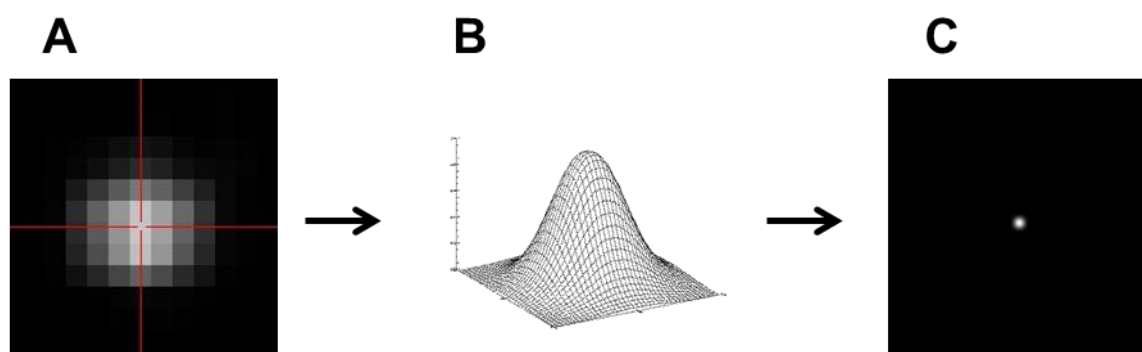


Figure 1.15: Particle localisation by Gaussian fitting. (A) An isolated fluorophore, producing a diffraction-limited PSF, can be subjected to (B) mathematical fitting such as fitting to a Gaussian distribution, to find the centroid of the PSF, resulting (C) in the accurate localisation of the fluorophore.

By exploiting the properties of photoactivatable and photoswitchable fluorophores, individual molecules are switched on stochastically in space. To ensure that overlapping fluorophores are not activated at the same time, the activating beam is kept at low power. These fluorophores are imaged until they are bleached, and the cycle is repeated until the positions of sufficient molecules have been determined. By acquiring a large number of frames and localising the centre of the PSF of each particle detected in every frame, a full super-resolution image can be reconstructed (Figure 1.16).

Rather than re-engineering the PSF as in STED microscopy, PALM and STORM achieve image resolution beyond the diffraction limit by extensive post-acquisition image processing.

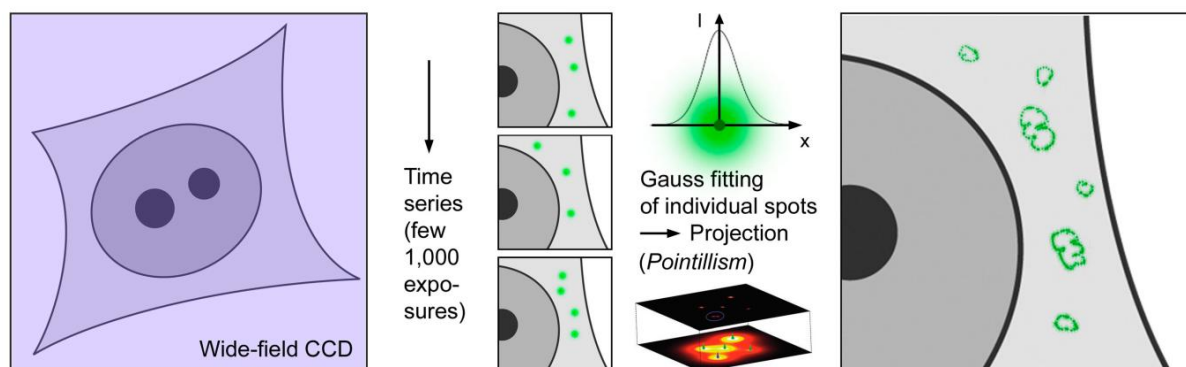


Figure 1.16: Principle of single-molecule localisation techniques (PALM and STORM). Only a subset of fluorophores are emitting (active) in every frame. This is generally achieved by using photoactivatable or photoswitchable fluorophores. These molecules are detected by the camera as diffraction-limited spots, from which the lateral position of the fluorophore is determined with high accuracy by mathematical fitting. A final super-resolution image is reconstructed from thousands of frames, each with a different subset of emitters. From (Schermerle et al., 2010).

PALM and STORM differ in the mechanism of fluorophore photoswitching. Whilst PALM exploits photoactivatable and photoswitchable fluorescent proteins, STORM was first demonstrated using pairs of photoswitchable synthetic fluorescent dyes. STORM was first developed by conjugating photoswitchable dye pairs (Cy3-Cy5) to DNA molecules to obtain nanometer-scale localisation accuracy (Rust et al., 2006). Since then, STORM has been primarily implemented using photoswitchable fluorescent dyes for antibody labelling and is therefore limited to imaging of fixed cells. However, other improvements have been made to this technology with the possibility of multi-colour STORM (Bates et al., 2007) as well as three-dimensional STORM (Huang et al., 2008). A variant of STORM is direct STORM (dSTORM), based on the reversible photoswitching of synthetic organic fluorophores into a long-lived dark state (Heilemann et al., 2008). dSTORM extends this technique to conventional dyes, such as Cy5 and Alexa Fluor 647. The reversible photoswitching of standard dyes is achieved by specific buffer conditions, for instance the presence of glutathione (Heilemann et al., 2008; van de Linde et al., 2011).

PALM was first demonstrated using the photoactivatable fluorescent proteins Kaede and dEosFP to image specific target proteins in various cellular structures such as mitochondria, lysosomes, lamellipodia and the plasma membrane (Betzig et al., 2006). Almost at the same time, a similar (almost identical) technique was developed by another group and called fluorescence

photoactivation localisation microscopy (fPALM) (Hess et al., 2006). Since PALM can be performed using fluorescent proteins, one of the main advantages of this method is that it is compatible with genetically-encoded labelling and live cell imaging. However, the majority of studies still resort to fixing cells, due to the large number of frames required in order to obtain one super-resolution image. In addition, the image resolution achievable with PALM is comparable to that of electron microscopy (approximately 30 nm), whilst being specific for only the desired target protein. The applications of PALM microscopy have broadened with the development of two-colour PALM (Shroff et al., 2007) and efficient live cell PALM imaging (Shroff et al., 2008).

The main advantages and disadvantages of the four super-resolution imaging techniques described here are summarised in Table 1.3.

Technique	Advantages	Limitations
SIM	- Three-dimensional resolution improvement - Possible to image deep within a cell - Conventional dyes can be used (if they are sufficiently photostable) - Straightforward sample preparation	- At best two-fold improvement in resolution - Complex post-acquisition processing of data (introduction of artefacts can occur and needs to be controlled) - Long acquisition and processing times
STED	- Fast acquisition and potential for live cell imaging - Potential for extremely high resolution (5 nm) - Great depth penetration and potential for three-dimensional imaging - No need for post-acquisition data processing (minimal artefacts)	- Problems with photobleaching in biological samples (due to the high power of the depletion beam) - Only some conventional dyes and fluorescent proteins can be used - Acquisition times are generally slowed down due to the need to collect sufficient photons when much of the PSF is being depleted
STORM	- Potential for very high resolution (10-20 nm) - Three-dimensional resolution improvement is possible when combined with TIRF or a cylindrical lens - Conventional dyes can be used	- Long acquisition times - Complex and time-consuming post-acquisition image analysis - Possibility of artefacts due to free dye or free labelled antibody - Limited to imaging fixed cells
PALM	- Potential for very high resolution (10-20 nm) - Three-dimensional resolution improvement is possible when combined with TIRF or a cylindrical lens - Fluorescent proteins are used, which is compatible with live cell imaging	- Long acquisition times - Complex and time-consuming post-acquisition image analysis - Lower photon counts from fluorescent proteins might lead to poorer resolution improvement

Table 1.3: Advantages and limitations of super-resolution techniques.

High-resolution high-speed imaging of events occurring at the immune synapse has mainly been achieved using TIRF microscopy. With this technique, researchers are able to produce informative images of the interactions occurring between an immune cell and an underlying substrate mimicking a target cell, such as an antibody-coated slide or a lipid bilayer. In addition, the recent advent of novel super-resolution imaging techniques has revolutionised fluorescence microscopy, allowing events to be observed in more detail than ever before. In particular, super-resolution imaging has started to reveal important information about the organisation of signalling proteins into clusters and the role of cluster dynamics in cell signalling. It therefore becomes apparent that the tools are now at our disposal to image the organisation of proteins at the immune synapse at the single molecule level.

1.3 Aims

NK cells are important players in the body's defence against tumours and pathogens. Activation of NK cells is tightly controlled by the balance of activating and inhibitory signals at the interface with a target cell: the immune synapse. The recruitment of receptors and signalling proteins to the immune synapse and the supramolecular distribution of proteins within the synapse have emerged as crucial factors for efficient signalling and the initiation of appropriate effector responses. A major gap in the knowledge is how activating and inhibitory signals are integrated at the molecular level both in time and space. Recent advances in imaging techniques have revealed that the formation and dynamics of protein microclusters play a major role in orchestrating signalling within the immune synapse, but research in this field has been limited by the resolution of conventional microscopes.

The main aim of this thesis was to investigate the involvement and distribution of the inhibitory NK cell receptor KIR2DL1 at the immune synapse using novel microscopy techniques. More specifically, the aim was to better characterise the molecular organisation of KIR2DL1 at the immune synapse, the dynamics of KIR2DL1 recruitment to the synapse, and the temporal aspects of signal integration.

The specific objectives of this thesis were:

- To image the nanometer scale organisation of the inhibitory receptor KIR2DL1 in the membrane of NK cells using a super-resolution microscopy technique called PALM and to probe some of the mechanisms that can influence this organisation (Chapter 3)
- To determine the diffusion coefficient of KIR2DL1 and to investigate the dynamics of KIR2DL1 recruitment to the immune synapse at the micrometer scale (Chapter 4)
- To develop a cellular system in which the inhibition of NK cells can be temporally controlled using a photocleavable peptide (Chapter 5).

A panel of complementary techniques was used to address these aims, including super-resolution microscopy, confocal microscopy, flow cytometry, molecular biology techniques and other assays.

Chapter 2 - Materials and Methods

2.1 Cells and cell culture

2.1.1 Immortalised cell lines

Various immortalised cell lines were used in this study. Most cell lines were cultured in R10 medium, which consists of RPMI-1640 (GIBCO-BRL, Gaithersburg, MD) supplemented with 10% heat-inactivated foetal calf serum (FCS), 2 mM L-glutamine, and 1 mM penicillin/streptomycin (all Invitrogen, Paisley, UK) at 37°C in a 5% CO₂ atmosphere, unless stated otherwise. A summary of all effector and target cell lines used in this work is presented in Tables 2.1 and 2.2, detailing their culture medium and source.

Effector cell lines

Various immortalised NK cell lines were used as effector cells (Table 2.1). The EBV-immortalised human NK cell line YTS (Yoneda et al., 1992) has been transfected to express an ecotropic receptor to facilitate retroviral transduction (Cohen et al., 1999). YTS cells expressing KIR2DL1 (YTS-KIR2DL1) and YTS cells expressing a GFP-tagged KIR2DL1 (YTS-KIR2DL1-GFP) were available in the lab, as well as the human NK cell leukaemia line NKL (Robertson et al., 1996). NK-92C1 cells are stably transfected to express IL-2 so no additional IL-2 was required in the medium. YTS and NKL cells were transfected to express KIR2DL1-tdEosFP (called YTS-KIR2DL1-tdEosFP and NKL-KIR2DL1-tdEosFP respectively), as described below.

Cell Line	Culture medium	Supplements	Source
YTS	R10	-	(Yoneda et al., 1992)
YTS-KIR2DL1	R10	-	(Cohen et al., 1999)
YTS-KIR2DL1-GFP	R10	-	(Borszcz et al., 2003)
YTS-KIR2DL1-tdEosFP	R10	-	Generated for this study
NKL	R10	100 U.ml ⁻¹ IL-2 (Roche, Basel, Switzerland)	(Robertson et al., 1996)
NKL-KIR2DL1-tdEosFP	R10	100 U.ml ⁻¹ IL-2	Generated for this study
NK92-C1	Advanced MEM (GIBCO)	10% FCS 10% horse serum Glutamine Penicillin/streptomycin	ATCC, Rockville, MD

Table 2.1: Effector cell lines. The cell lines listed above are NK cell lines used as effector cells in this thesis.

Target and control cell lines

Target and control cell lines that were used in this study are summarised in Table 2.2. The HLA-A/B/C-negative human B lymphoblastoid cell line 721.221 (hereafter referred to as 221) (Shimizu et al., 1988) and 221 cells stably transfected to express HLA-Cw4 or HLA-Cw6 (221-Cw4 and 221-Cw6 respectively) were available in the lab, as were 221 cells expressing MICA (221-MICA) and 221 cells expressing MICA and HLA-Cw6 (221-MICA-Cw6). The murine RMA-S cell line was a kind gift from Keith Gould, Imperial College London. RMA-S cells were transfected to express Cw3-GFP or Cw4-GFP, as described below. RMA-S cells transfected to express β_2 -microglobulin (RMA-S- β_2 m) were a kind gift from Eric Long (National Institutes of Health, Maryland, USA), along with RMA-S- β_2 m-Cw4 cells, which additionally express HLA-Cw4. T2 cells were a kind gift from Denis Hudrisier (IBPS and Paul Sabatier University, Toulouse, France). T2 cells were transfected to express Cw4-GFP (T2-Cw4-GFP), as described below. The murine mastocytoma cell line P815 was available in the lab.

Cell Line	Culture medium	Supplements	Source
221	R10	-	(Shimizu et al., 1988)
221-Cw4	R10	-	(Biassoni et al., 1995)
221-Cw6	R10	-	(Davis et al., 1999)
221-MICA	R10	-	Davis Lab (unpublished)
221-MICA-Cw6	R10	-	Davis Lab (unpublished)
RMA-S	R10	-	(Ljunggren and Kärre, 1985)
RMA-S-Cw3-GFP	R10	1.5 mg.ml ⁻¹ geneticin (Invitrogen)	Generated for this study
RMA-S-Cw4-GFP	R10	1.5 mg.ml ⁻¹ geneticin	Generated for this study
RMA-S-β2m	R10	0.5 mg.ml ⁻¹ geneticin	(Anderson et al., 1993)
RMA-S-β2m-Cw4	R10	0.5 mg.ml ⁻¹ geneticin 10 µg.ml ⁻¹ mycophenolic acid (GIBCO) 10 µg.ml xanthine sodium salt (Sigma-Aldrich)	(Rajagopalan and Long, 1997)
T2	R10	-	(Alexander et al., 1989)
T2-Cw4-GFP	R10	1.2 mg.ml ⁻¹ geneticin	Generated for this study
P815	R10	-	ATCC, Rockville, MD

Table 2.2: Target cell lines. The cell lines listed above are cell lines used as target cells in this thesis. All are human cell lines, except for the RMA-S cell line and its derivatives and the P815 cell line, which are derived from the mouse.

Retrovirus producer cell line

Phoenix human embryonic kidney HEK 293T (hereafter Phoenix), an adherent cell line used for helper-free production of retroviruses (Kinsella and Nolan, 1996), was grown in R10 medium.

2.1.2 Primary NK cells and NK clones

Peripheral blood mononuclear cells (PBMC) from normal healthy volunteers were obtained by separation on a Ficoll-paque gradient and primary NK cells were isolated from PBMC by negative

selection with an NK isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). Primary NK cells were maintained in DMEM (GIBCO), supplemented with 10% human serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 1 mM penicillin/streptomycin and 1 mM non-essential amino acids. NK clones were established by limiting dilution cloning in U-bottomed 96-well plates of freshly isolated primary NK cells in the presence of irradiated RPMI8866 feeder cells and irradiated PBMC. Cells were seeded in the same medium with F-12, mercaptoethanol, phytohaemagglutinin (PHA) and IL-2. Clones were expanded and used in subsequent assays.

2.1.3 Transfections and cell sorting

Retroviral transductions

YTS and NKL cells were transfected to express KIR2DL1-tdEosFP by retroviral transduction using the retroviral vector PINCO (Grignani et al., 1998). Amphotropic Phoenix 293 packaging cells were transfected with 20 µg DNA using 40 µl Lipofectamine LTX (Invitrogen) in 2 ml Opti-MEM medium (incubated at room temperature for 20 minutes to allow liposome formation before adding to the cells). Fresh R10 medium was given to the cells after incubating them at 37°C overnight, and the cells were further incubated at 37°C for production of virus particles. The viral supernatant was removed from the Phoenix cells, sterile filtered and used to infect YTS and NKL cells by three consecutive spinfections in 6-well plates. Briefly, 10^6 YTS or NKL cells were placed in a well in 1 ml of R10 medium (in the presence of IL-2 for NKL cells) and 5 ml of viral supernatant were added along with polybrene ($5 \mu\text{g} \cdot \text{ml}^{-1}$) and Hepes (10 mM final concentration; GIBCO). NK cells were subjected to three sequential centrifugations (1600 rpm, 45 minutes, 30°C) followed by an incubation at 37°C of 2, 4 and 6 hours respectively after each centrifugation. At the end of this process, the transfected cells were cultured in R10 medium in 6-well plates until they had recovered, and Phoenix cells were discarded.

Electroporations

RMA-S cells were transfected to express Cw3-GFP and Cw4-GFP and T2 cells were transfected to express Cw4-GFP. pcDNA3.1 plasmids encoding GFP-tagged Cw3 and Cw4 were available in the lab

(kind gift from Philipp Eissmann). The cells were transfected with 100 µg of linearised plasmids by electroporation at 250 V, 975 µF and grown in R10 medium without antibiotics. One day later, cells were selected with 1.5 mg.ml⁻¹ geneticin. Cells were screened by flow cytometry for GFP expression and subcloned in U-bottomed 96-well plates.

Cell sorting

Clones with the highest protein expression were expanded and enriched by fluorescence-activated cell sorting (FACS). Propidium iodide (PI) was added to the samples prior to cell sorting. PI is a marker of dead and apoptotic cells; during cell sorting cells that were PI-positive were excluded from collection by gating.

2.2 Molecular biology

2.2.1 Cloning tdEosFP

The pcDNA3-Flag1-tdEosFP vector was purchased from MoBiTec (Göttingen, Germany). The tdEosFP protein was inserted into the PINCO-KIR2DL1 retroviral vector in two steps (Figure 2.1). First, tdEosFP was inserted into the pEGFP-N1 vector in order to exploit some useful restriction sites. To summarise, pcDNA3-Flag1-tdEosFP and pEGFP-N1 were digested with *XhoI* and *ApaI* at 37°C for two hours and pEGFP-N1 was dephosphorylated. The digestion products were run on a 1% agarose gel and extracted using QIAquick gel extraction kit (Qiagen). tdEosFP was ligated into pEGFP-N1 using Rapid DNA ligation kit (Roche). The ligation product was transformed into XL2 blue competent cells, which were plated on agar plates made with kanamycin. The following day, colonies were picked and grown in LB medium containing kanamycin, whilst shaking at 37°C. The amplified DNA was purified using QIAprep Spin Miniprep kit (Qiagen). In the second step, tdEosFP was inserted into PINCO-KIR2DL1-GFP by replacing GFP. The pEGFP-N1-tdEosFP construct was digested with *BglII* and *PspOMI* and PINCO-KIR2DL1-GFP was digested with *BamHI* and *NotI*. These restriction enzymes have compatible cohesive ends, respectively. The digestion products were re-ligated to obtain the desired construct: PINCO-KIR2DL1-tdEosFP. DNA sequences were analysed by MWG Eurofins.

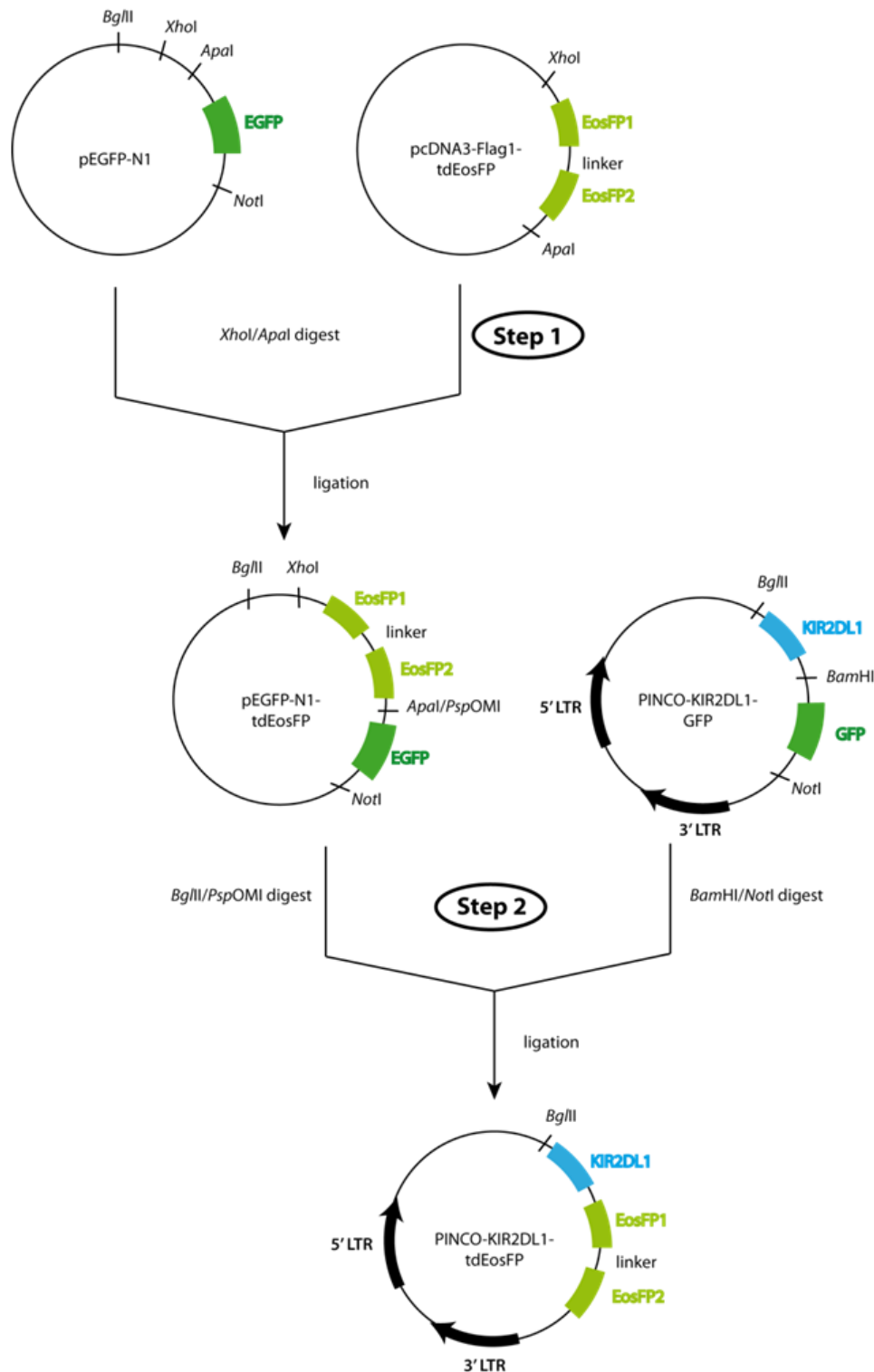


Figure 2.1: Cloning strategy for KIR2DL1-tdEosFP. **Step 1:** Cloning tdEosFP into pEGFP-N1 vector. pEGFP-N1 and pcDNA3-Flag1-tdEosFP were digested with *ApaI* and *XhoI* restriction enzymes and tdEosFP was ligated into pEGFP-N1 vector. **Step 2:** Cloning tdEosFP into PINCO-KIR2DL1 vector. pEGFP-N1 was digested with *BglII* and *PspOMI*. PINCO-KIR2DL1-GFP was digested with *BamHI* and *NotI* to remove GFP and tdEosFP was ligated into PINCO-KIR2DL1. *BglII* and *PspOMI* have compatible ends with *BamHI* and *NotI*, respectively.

2.2.2 Linearisation of DNA

Electroporation efficiency can be improved by linearising the plasmid DNA used for transfecting cells. This was done with pcDNA3.1 plasmids encoding Cw3-GFP and Cw4-GFP. Briefly, 100 µl of plasmid DNA were mixed with 12 µl digestion buffer, 5 µl restriction enzyme (in this case *Bgl*II) and 3 µl dH₂O, and then incubated overnight at 37°C. The next day, the digested DNA was dephosphorylated by mixing it with 12 µl dephosphorylation buffer, 2.5 µl alkaline phosphatase and 15.5 µl dH₂O and incubating it at 37°C for 10 minutes. Finally, the linearised DNA was purified using the QIAquick gel extraction kit and a small sample was analysed by gel electrophoresis on a 1% agarose gel to check that the linearisation was successful.

2.3 Antibodies

Various antibodies were used for flow cytometry and imaging experiments. Flow cytometry and immunofluorescence imaging were performed using a combination of primary antibodies followed by fluorophore-conjugated secondary detection antibodies (Table 2.3).

Antigen	Clone	Isotype	Conjugate	Concentration	Source
Isotype control	MOPC-21	IgG1	Purified	10 µg.ml ⁻¹	BD Biosciences
Isotype control	20102	IgG2a	Purified	10 µg.ml ⁻¹	R&D Systems
KIR2DL1	EB6	IgG1	Purified	10 µg.ml ⁻¹	Beckman Coulter
NKG2D	149810	IgG1	Purified	10 µg.ml ⁻¹	R&D Systems
MHC class I	W6/32	IgG2a	Purified	10 µg.ml ⁻¹	ATCC, Rockville, MD
CD80	L307.4	IgG1	Purified	10 µg.ml ⁻¹	BD Biosciences
Mouse IgG (H+L)	ND	Goat	Cy5	10 µg.ml ⁻¹	Jackson ImmunoResearch
Mouse IgG (H+L)	ND	Goat	Alexa633	10 µg.ml ⁻¹	Invitrogen

Table 2.3: Antibodies for the study of protein surface expression on cell lines. The antibodies listed above were used to determine the surface expression of specific proteins on various cell lines by flow cytometry. The anti-KIR2DL1 antibody was also used to detect KIR2DL1 in imaging of fixed cells. Unless otherwise specified, all antibodies are mouse monoclonal. The two last antibodies were used as secondary antibodies in flow cytometry and imaging experiments respectively. All the antibodies were diluted in FACS buffer or PBS at the specified concentrations and used to analyse the cells.

For imaging cells by PALM, antibodies were used to coat glass slides to ligate specific proteins (Table 2.4). Anti-KIR2DL1 antibody supernatants were a kind gift from Stipan Jonjić (University of Rijeka, Croatia).

Antigen	Clone	Isotype	Concentration	Source
Isotype control	MOPC-21	IgG1	3 or 10 $\mu\text{g}.\text{ml}^{-1}$	BD Biosciences
Isotype control	20102	IgG2a	3 $\mu\text{g}.\text{ml}^{-1}$	R&D Systems
KIR2DL1	EB6	IgG1	3 $\mu\text{g}.\text{ml}^{-1}$	Beckman Coulter
CD28	CD28.2	IgG1	1.5 or 3 $\mu\text{g}.\text{ml}^{-1}$	BD Biosciences
CD11a (LFA-1)	G43-25B	IgG2a	1.5 or 3 $\mu\text{g}.\text{ml}^{-1}$	BD Biosciences
NKG2D	149810	IgG1	3 $\mu\text{g}.\text{ml}^{-1}$	R&D Systems
CD94	HP-3D9	IgG1	3 or 10 $\mu\text{g}.\text{ml}^{-1}$	BD Biosciences

Table 2.4: Antibodies used to coat slides for PALM microscopy. The antibodies listed above were used to coat the surface of Labtek slides, as described in Section 2.6. All antibodies are mouse monoclonal. All the antibodies were diluted in PBS at the specified concentrations and used to coat the slides on a background of poly-L-lysine (PLL).

Although it is not an antibody, phalloidin (conjugated to the fluorescent dye Atto-647N) was used to stain cells to detect filamentous actin.

2.4 Functional assays

2.4.1 Flow cytometry

NK cell lines were analysed for surface expression of the inhibitory receptor KIR2DL1, the activating receptor NKG2D and other receptors by flow cytometry. Target cells were analysed by flow cytometry for surface expression of MHC class I molecules and, in certain experiments, the activating ligand CD80, as specified in the Results sections.

Briefly, 2×10^5 cells were loaded into V-bottom 96-well plates and washed once. The cells were incubated with primary antibodies at specified concentrations in 50 μl of FACS buffer (PBS containing 2% BSA and 0.01% NaN_3) for 45 minutes on ice. Samples were washed three times with 200 μl FACS buffer, then resuspended in 50 μl of FACS buffer with secondary antibody at the specified dilution

and incubated on ice for 45 minutes. After staining, samples were washed a final three times in 200 μ l of FACS buffer and transferred to FACS microtubes for data acquisition on a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA).

Cells transfected with GFP- or tdEosFP-tagged constructs were analysed for fluorescence in the green channel (acquisition channel FL-1). Cells stained with primary and secondary antibodies were analysed in the red channel (acquisition channel FL-4). In general, 7500 live cells, as determined in the forward scatter/side scatter plots, were collected for each condition for each experiment. Data analysis was performed using FlowJo software version 7.2.4 (TreeStar).

2.4.2 Cytotoxicity assay

The susceptibility of target cells to NK cell cytotoxicity was assessed in 5-hour [^{35} S]-methionine release assays performed at several effector to target ratios in triplicate. NK cells were washed and resuspended at the desired concentration and plated in V-bottomed 96-well plates in a volume of 50 μ l. Target cells were incubated overnight with [^{35} S]-labelled methionine. Target cells were washed three times and resuspended in R10 medium at a final concentration of 10^5 cells per ml and 50 μ l of target cells were added to the NK cells. The assay was incubated at 37°C for 5 hours and [^{35} S] release was measured in a scintillation counter (TopCount NXT, Perkin Elmer). Maximal [^{35} S] release was determined by lysing target cells in 0.1 M sodium hydroxide (NaOH) and spontaneous release was measured by incubating target cells in medium. Spontaneous release was <20% of the maximal release. The percentage of specific lysis was calculated as defined in equation 2.1.

Equation 2.1:

$$\text{Specific lysis (\%)} = \frac{(\text{experimental release} - \text{spontaneous release})}{(\text{maximum release} - \text{spontaneous release})} \times 100$$

2.5 Work with the photocleavable peptide

2.5.1 Peptide synthesis

Solid-phase peptide synthesis

Peptides were synthesised by Fmoc solid-phase peptide synthesis (Chan and White, 2000) at the NIMR (National Institute for Medical Research, London, UK) in collaboration with David Trentham and John Offer (Figure 2.2). Briefly, amino acid residues were weighed out and sequentially added to an insoluble Wang resin (Novabiochem) preloaded with leucine. After each coupling reaction, the resin and the growing peptide inside the filter vessel were washed with dichloromethane (DCM) and dimethylformamide (DMF) solvents and deprotected with piperidine. Trifluoroacetic acid (TFA) was used for final cleavage of the resin and side-chain deprotection. Reagents were added and removed under computer control using a peptide synthesiser (CSBio Co.).

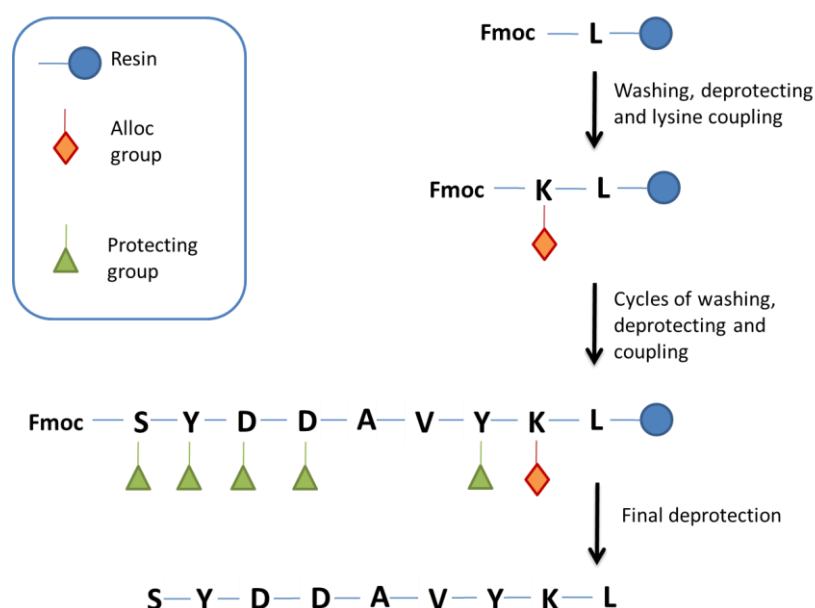


Figure 2.2: Solid-phase peptide synthesis. Amino acids were sequentially added to an insoluble polymeric resin support from the C-terminus to the N-terminus of the peptide. For synthesising the control peptide SYDDAVYKL, the process initiated with a leucine bound to a resin support. The Fmoc (fluoren-9-ylmethoxycarbonyl) group is for temporary N- α -protection. This complex was washed and deprotected to remove the Fmoc group, then coupled to lysine. Further cycles of washing, deprotecting and coupling followed until the complete sequence was obtained. Side-chain protecting groups are used to mask the highly reactive functional groups present in certain side-chains. Final deprotection removed the side-chain protecting groups and the resin, yielding the desired peptide.

The purity of the peptides was assessed by reverse phase high-performance liquid chromatography (HPLC) and mass spectrometry.

Synthesis of photocleavable peptide

For synthesising the photocleavable peptide, an existing protocol describing the synthesis of an α -carboxy-2-nitrobenzyl (CNB)-caged glycine (Corrie et al., 2008) was adapted and optimised (Figure 2.3).

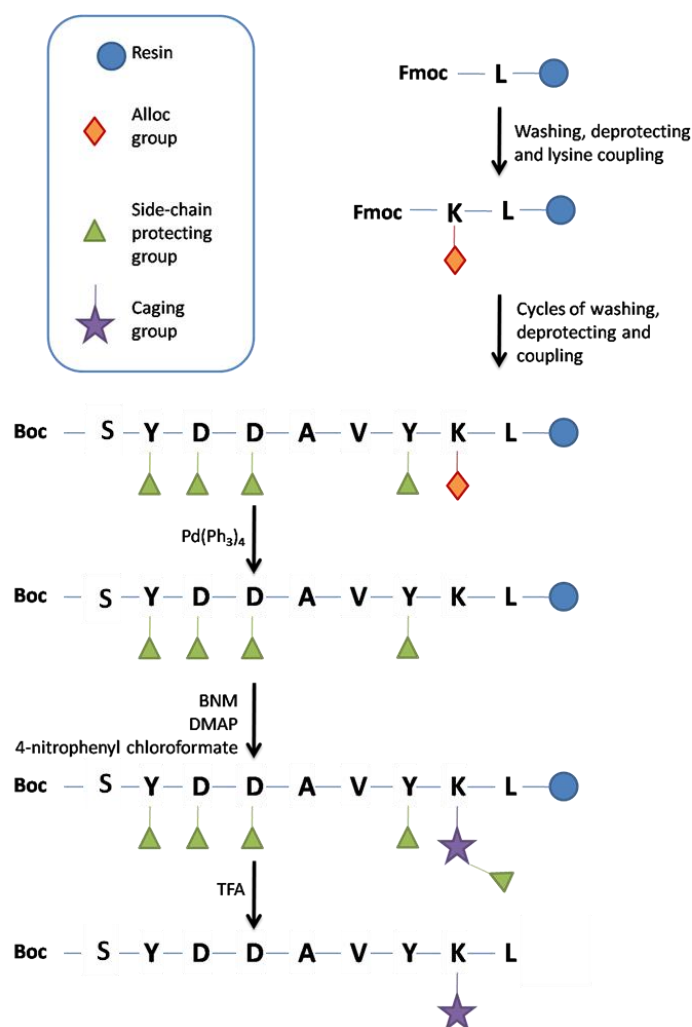


Figure 2.3: Synthesis of the CNB-caged photocleavable peptide. This synthesis protocol was based on solid-phase peptide synthesis. The Fmoc and Boc (tert-butoxycarbonyl) groups are for temporary N- α -protection. After coupling all the desired residues onto the peptide, only the Alloc (allyloxycarbonyl) protecting group on the lysine side-chain was removed by treatment with Pd(Ph₃)₄ (palladium tetrakisphenylphosphine). This side-chain was then free to react and the CNB group was added. TFA was used for final deprotection to remove all side-chain protecting groups and the resin support.

To summarise, a solution of *t*-butyl 2-nitromandelate (BNM) and 4-(dimethyl)amino-pyridine (DMAP) in dry tetrahydrofuran was treated with 4-nitrophenyl chloroformate and stirred for 3 hours in the dark at room temperature, then rotary evaporated and resuspended in 1 ml of dry DMF. This mixture was added to the resin solution of the QYDDAVYKL peptide with a deprotected lysine residue and stirred overnight in the dark at room temperature. At each step of the reaction, the starting compounds and reaction products were analysed by thin layer chromatography (TLC) and the final product was analysed with the ninhydrin assay. To dissociate the peptide from the resin and deprotect the side-chains, the resin was filtered, washed with DMF and DCM, then dried in a vacuum desiccator. The solution was then treated with TFA and triethylsilane in the presence of water for 45 minutes before the resin was filtered off and the TFA was removed from the solution by volatilisation with a nitrogen stream. The peptide was precipitated with ether, centrifuged, re-dissolved in acetonitrile and water and filtered prior to analysis by liquid chromatography-mass spectrometry (LC-MS).

Thin layer chromatography

Thin layer chromatography (TLC) was used to analyse the compounds present in the reaction mixture at each step of the synthesis reaction. Solvents used were ethyl acetate and petroleum ether. Samples were loaded onto TLC silica slides, which were dipped in solvent. The solvent is drawn up the plate by capillary action. Different compounds travel at different rates and spots were visualised under a blacklight (UV 254 nm).

Flash chromatography

The photocleavable peptide on the resin was purified by flash chromatography. The sample was dried by rotary evaporation and run on a flash chromatography column. The column was prepared as described (Still et al., 1978) with cotton wool, small silica gel particles and a binary solvent system consisting of ethyl acetate and petroleum ether. Pressurised nitrogen was used to drive the solvent through the column. 10 ml fractions were collected and analysed by UV spectrophotometry. The fractions containing the desired product were pooled for further purification and final deprotection.

Ninhydrin assay

The ninhydrin assay is a spectrophotometric method based on the formation of a blue colour by the reaction between ninhydrin (2,2-dihydroxyindane-1,3-dione) and compounds with free amino groups (Figure 2.4). The assay was performed as previously described (Spies, 1957) to determine whether the free amine group on the peptide had reacted. 20 µl of sample (or water for negative control or 4 mM L-glutamate for standard) were placed in a photometer tube and 200 µl of ninhydrin solution were added. The tubes were covered with aluminium caps and the contents mixed. The tubes were then heated for 20 minutes in a boiling water bath and 0.5 ml of diluent (water/ethanol at a 50/50 ratio) were added to each tube. The samples were analysed by UV spectrophotometry starting 15 minutes after the tubes were removed from the water bath, and the colour (detected at 570 nm) is known to remain stable for one hour (Spies, 1957).

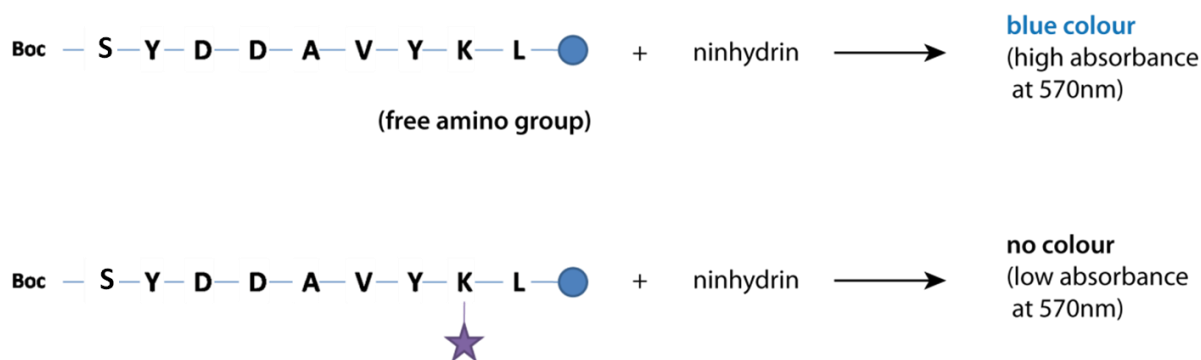


Figure 2.4: Ninhydrin assay. Ninhydrin is a compound that reacts with free amino groups, resulting in the formation of a blue colour detectable at 570 nm by UV spectrometry. Here, the ninhydrin assay was used to detect whether the amino group of the lysine residue on the peptide had reacted with the caging group. If the caging group were present on the peptide (bottom), the solution would have a much lower absorbance at 570 nm than the non-caged peptide, which has a free amino group on the lysine (top).

Purchase of photocleavable peptide

A peptide with the sequence SYDDAVYKL and a 5-(carboxy-methoxy)-2-nitrobenzyl-oxycarbonyl (CmnCbz) caging group on the lysine was purchased from Cambridge Research Biochemicals. This was mainly due to time restrictions and insufficient yield obtained from the peptide synthesis.

2.5.2 Liquid chromatography-mass spectrometry

Liquid chromatography-mass spectrometry (LC-MS) is a powerful tool for analysing chemical compounds as it combines physical separation (by liquid chromatography) with mass analysis (by mass spectrometry). Analysis of the peptides by LC-MS was performed in the Department of Chemistry (Imperial College), in the laboratory of Edward Tate and Robin Leatherbarrow, with the help of William Heal. LC-MS analysis and purification were performed on a Waters HPLC system (Waters 2767 autosampler for sample injection and collection; Waters 515 HPLC pump to deliver the mobile phase to the source; XBridge C18 column (Waters, 4.6 mm D × 100 mm for analytical and 19 mm D × 100 mm for preparative); Waters 3100 mass spectrometer with ESI and Waters 2998 Photodiode Array (detection over 200–600 nm)).

Peptide analysis

Samples were pipetted into a 96-well plate, which was loaded into the LC-MS spectrometer. Compounds were eluted over a gradient of 5%-98% methanol in water over 15 minutes. All solvents used were supplemented with 0.1% formic acid and degassed with helium.

Peptide purification

The control peptide was purified by LC-MS. A small sample was analysed by LC-MS to identify the peak corresponding to the peptide. Appropriate fractions containing the identified peak were collected, then pooled, reduced and dried by rotary evaporation. The dried peptide powder was resuspended in DMSO and re-analysed by LC-MS to check purity.

Data processing

All LC-MS spectra were analysed using the MassLynx software (Waters). For each sample, the diode array, positive ion and negative ion spectra can be visualised. In addition, specific peaks can be selected in these spectra to visualise the UV and mass spectra specific to the compounds present in these peaks. Quantification of the peaks was obtained by integrating the area under the peak. A calibration curve was established from three independent experiments with six different

concentrations of the CmnCbz-caged photocleavable peptide. This curve was then used to calculate the concentration of photocleavable peptide in a given solution from the integrated peak area measured on the chromatogram.

2.5.3 Methods used for photolysis

Several light sources were tested for cleaving the photocleavable peptide to yield the native peptide. The peptide samples were diluted in DMSO. After each test, the sample was analysed by LC-MS and the resulting spectra were compared to the LC-MS data from the control and photocleavable peptides. The calibration curve described above was used to quantify the cleaving efficiency. The percentage of cleaving was calculated using Equation 2.2.

$$\text{Equation 2.2: \% cleaving} = \frac{(\text{starting concentration} - \text{final concentration})}{\text{starting concentration}} \times 100$$

The first two tests were carried out on inverted microscopes (Zeiss) with a x63 objective, using two different light sources (X-Cite Light Source and HBO lamp).

X-Cite light source

The first photolysis test was performed on an inverted microscope, using the illumination from a high pressure metal halide lamp and a specific filter to get a peak at 350 nm. The samples of photocleavable peptide were pipetted into a glass-bottomed flat-bottomed 96-well plate and illuminated for different exposure durations (10s, 1 minute, 5 minutes, 10 minutes, or not at all).

HBO Lamp light source

Photolysis was also attempted on a different inverted microscope using the illumination from a high pressure mercury vapour lamp and a DAPI filter (excitation peak $\approx$ 360 nm). One sample of photocleavable peptide was loaded into a glass-bottomed flat-bottomed 96-well plate and illuminated for 5 minutes.

Nd YAG pulsed laser

Photolysis was also tested using a Nd YAG pulsed laser in Marina Kuimova's laboratory in Chemistry (Imperial College). Nd YAG (neodymium-doped yttrium aluminium garnet, Nd: $\text{Y}_3\text{Al}_5\text{O}_{12}$) is a crystal commonly used in solid-state lasers (Geusic et al., 1964). This light source has a wavelength of 1064 nm, but this can be frequency-tripled to 355 nm due to the third harmonics properties of this laser. The sample was placed in a cuvette and irradiated over increasing time scales. The sample was analysed by UV spectrophotometry following each irradiation, using DMSO as a blank control. The final photolysis product was also analysed by LC-MS.

Mercury discharge UV lamp

The final light source used to cleave the peptide was a HPW125 mercury discharge blacklight source (Philips) with an almost monochromatic UV output centred around 365.5 nm. The sample was placed in a 96-well plate under the lamp and illuminated for 30 minutes. The distance between the sample and the lamp was approximately 5 cm. In this case, the light source was diffuse whereas using the other three light sources gave a focussed light beam.

2.5.4 Acid stripping and peptide loading

Target cells were incubated with exogenous peptide to stabilise MHC class I molecules containing this peptide at the cell surface. RMA-S and T2 cells are transporter associated with antigen processing (TAP)-deficient cells and these cells therefore cannot transport endogenous peptides to the cell surface and do not express stable MHC class I molecules at their surface. 221-Cw4 cells are able to process endogenous peptides to present at their surface. These cells were therefore stripped of surface MHC class I molecules by acid treatment prior to peptide loading. This was done to ensure that the only peptide presented by these cells would be the exogenous peptide added after acid treatment.

Peptide stabilisation of MHC class I on acid-stripped cells

Acid treatment was performed as described previously (Betser-Cohen et al., 2006). To summarise, 5×10^6 221-Cw4 cells were resuspended in 0.25 ml of 1% BSA and 300 mM glycine-HCl buffer (pH 2.4)

for two minutes at room temperature. The suspension was neutralised by adding RPMI 1640 medium. The cells were pelleted and pooled in 5 ml of RPMI containing $10 \mu\text{g} \cdot \text{ml}^{-1}$ brefeldin A (Sigma-Aldrich). 2×10^6 acid-stripped cells were aliquoted, centrifuged for 5 minutes at 3000 rpm and resuspended in R10 medium with $5 \mu\text{g}$ of $\beta_2\text{m}$ (Sigma-Aldrich) and $1 \mu\text{g}$ of brefeldin A, with or without peptide ($100 \mu\text{M}$ final concentration). The cells were incubated at 26°C for four hours, washed once in RPMI 1640 medium and resuspended in R10 medium for use in subsequent experiments.

Peptide stabilisation of MHC class I on TAP-deficient cells

Peptide stabilisation of MHC class I on RMA-S-Cw4-GFP, RMA-S- $\beta_2\text{m}$ -Cw4 cells and T2-Cw4-GFP cells was performed following two different protocols. Peptide loading experiments with RMA-S-Cw4-GFP and RMA-S- $\beta_2\text{m}$ -Cw4 cells were carried out as described previously (Rajagopalan and Long, 1997). Briefly, cells were plated at 2.5×10^5 cells per well in a 48-well plate in a final volume of 0.5 ml. Cells were incubated at 26°C for 24 hours with peptide ($100 \mu\text{M}$ final concentration) added at the onset of culture and again 8 hours later. Cells were harvested and washed in RPMI for use in subsequent experiments.

For T2-Cw4-GFP cells, 3×10^6 cells were centrifuged and resuspended in $500 \mu\text{l}$ of R10 medium with peptide ($100 \mu\text{M}$ final concentration) in a 48-well plate. The cells were incubated at 26°C for four hours, washed once and resuspended in R10 for use in subsequent experiments.

2.5.5 Cytotoxicity assays with peptide-loaded cells

When peptide-loaded cells were used for cytotoxicity assays, they had to be labelled with radioactive ^{35}S -methionine at an appropriate moment in the protocol. 221-Cw4 cells were labelled with ^{35}S -methionine overnight and washed three times in RPMI 1640 medium before the acid treatment and the four-hour incubation with peptide at 26°C . In the case of RMA-S-Cw4-GFP and RMA-S- $\beta_2\text{m}$ -Cw4 cells, the cells were labelled after the first 8 hours of incubation with peptide, at the same time as new peptide was added before the overnight incubation. T2-Cw4-GFP cells were labelled with ^{35}S -methionine overnight then washed three times before the four-hour incubation with peptide. After the incubation, the cells were added to effector cells in 96-well plates for the cytotoxicity assay, as described above.

2.6 Imaging

2.6.1 Confocal microscopy

Live cell confocal fluorescence microscopy

For live cell microscopy, cells were imaged in eight-well chambered borosilicate coverglasses (LabTek, Nunc) pre-coated with $10\ \mu\text{g}\cdot\text{ml}^{-1}$ fibronectin (Sigma). Cells were imaged by confocal microscopy using an SP5 inverted confocal microscope (TCS SP5 RS, Leica Microsystems) with a 63x water immersion objective with a numerical aperture of 1.2. Simultaneous imaging of different fluorophores was performed by sequential line scanning. Live cell images were acquired at 37°C and 5% CO₂.

Immunostaining for fixed cell imaging

For immunostaining, 2×10^5 cells were gently resuspended in PBS and transferred to poly-L-lysine (PLL)-coated 8-well LabTek chamber slides. After an incubation of 15 minutes that allowed the cells to settle, they were fixed in 4% paraformaldehyde (PFA) in PBS at room temperature for 20 minutes, then gently washed with PBS and permeabilised with 0.1% Triton X (Sigma) in PBS for 4 minutes. Cells were washed again once in PBS then blocked with blocking buffer (5% BSA in PBS) for 15 minutes at room temperature. Samples were labelled with primary antibody at a specified concentration in blocking buffer for one hour at room temperature. After washing three times in PBS, samples were stained with secondary detection antibody diluted in blocking buffer for one hour at room temperature. Samples were washed a final three times, covered in 200 μl cold PBS and stored in the dark at 4°C until imaged. During all washing and labelling steps, a minimal amount of fluid was always maintained in the chambers to ensure that the surface or the cells were never exposed. Samples were imaged by confocal microscopy. For experiments with Latrunculin A (Lat A, Tocris Bioscience), 2.5×10^5 cells were incubated with Lat A for one hour at 37°C, before fixing and staining them with Phalloidin-Atto647N on PLL-coated slides.

Photoswitching experiments

For the photoswitching experiments, YTS-KIR2DL1-tdEosFP and 221-Cw4 cells were gently added to fibronectin-coated chamber slides at a 1:1 ratio just before imaging. During live cell acquisitions, a small region of the YTS-KIR2DL1-tdEosFP membrane was selected and the tdEosFP molecules in this region were photoconverted from green to red fluorescence (FRAP Wizard, Leica). This photoswitching involved firing the 405 nm UV laser (at 80% laser power) at a specific region of the cell membrane for 20 frames. The cells were then imaged for a further 20 minutes at one frame every ten seconds to follow the movement of the photoswitched molecules. The green emission from tdEosFP was visualised using the 496 nm laser line (fluorescence was collected between 500 and 550 nm) and the red emission wavelengths were visualised with the 543 nm laser line (fluorescence was collected between 575 and 650 nm).

2.6.2 Photoactivated localisation microscopy (PALM)

Sample preparation

LabTek slides were washed in 1 M HCl for 15 minutes then washed in 70% ethanol and dried. The slides were coated with 0.01% filter sterilised PLL for 20 minutes and dried. For samples with antibody-coated surfaces, the slides were coated with antibody at specified concentrations by overnight incubation at 4°C with 150 µl of antibody solution (antibody diluted in PBS). The slides were then washed three times and blocked with Hepes medium (consisting of 500 µl of 1 M Hepes in 25 ml of R10 medium, pre-warmed to 37°C) for 30 minutes at 37°C. Cells were allowed to settle on the surface for 15 minutes at 37°C. Cells were fixed with 6% PFA in PBS for 20 minutes at room temperature then washed three times with PBS and covered in 200 µl cold PBS and stored in the dark at 4°C until imaged. For experiments with Lat A, 2.5×10^5 cells were incubated with Lat A for one hour at 37°C, before fixing them. During all washing and labelling steps, a minimal amount of fluid was always maintained in the chambers to ensure that the surface or the cells were never exposed. Samples were imaged by PALM microscopy.

PALM/TIRF imaging

PALM images were acquired on an inverted widefield fluorescence microscope (Zeiss, Jena, Germany) with TIRF illumination, using a 100x oil-immersion objective with a numerical aperture of 1.45. Photoconversion of tdEosFP was achieved using a Polychrome Light Source (Polychrome IV; TILL Photonics, Eugene, OR) set to 390 nm, and the red-converted form of tdEosFP was imaged with the 561 nm laser line. For each PALM acquisition, 2×10^4 raw images were acquired with an electron-multiplying charge-coupled device camera (EM-CCD camera C9100-13; Hamamatsu Photonics, Hamamatsu, Japan) with an exposure time of 30 ms, gain of 1 and sensitivity gain set to maximum (1200). Brightfield images were acquired with an exposure time of 108.5 ms, gain of 1 and sensitivity gain set to minimum (4). The final image pixel diameter was 156 nm. The TIRF beam was focussed at low laser power at the beginning of each session of PALM imaging. Cells were imaged in TIRF mode to visualise the distribution of proteins solely at or near the plasma membrane.

2.7 Data analysis

2.7.1 Confocal imaging data analysis

Confocal microscopy images were analysed using specific software (LAS AF, Leica Application Suite, Advanced Fluorescence, Leica, and ImageJ, US National Institutes of Health, Bethesda, MA).

Pearson's correlation coefficients (R_r) were calculated (ImageJ plug-in, "Intensity Correlation Analysis"). Values for R_r range from -1 (anticorrelation) to 1 (total correlation), where a value of 0 means absence of correlation between the two channels examined. Mander's overlap coefficients (R) were obtained (ImageJ plug-in) and provide another quantification of colocalisation between two channels. The values for R range between 1 and 0, where 1 indicates high colocalisation and 0 indicates low colocalisation.

Brightness, contrast and levels of images were adjusted in Photoshop (Adobe) to improve depiction of images for printing and ease of visualisation, although raw data was used for analysis.

2.7.2 PALM data analysis

PALM image reconstructions

Super-resolution PALM images were reconstructed from 2×10^4 raw images (QuickPALM software, available as a plug-in for ImageJ (Henriques et al., 2010)) (Figure 2.5).

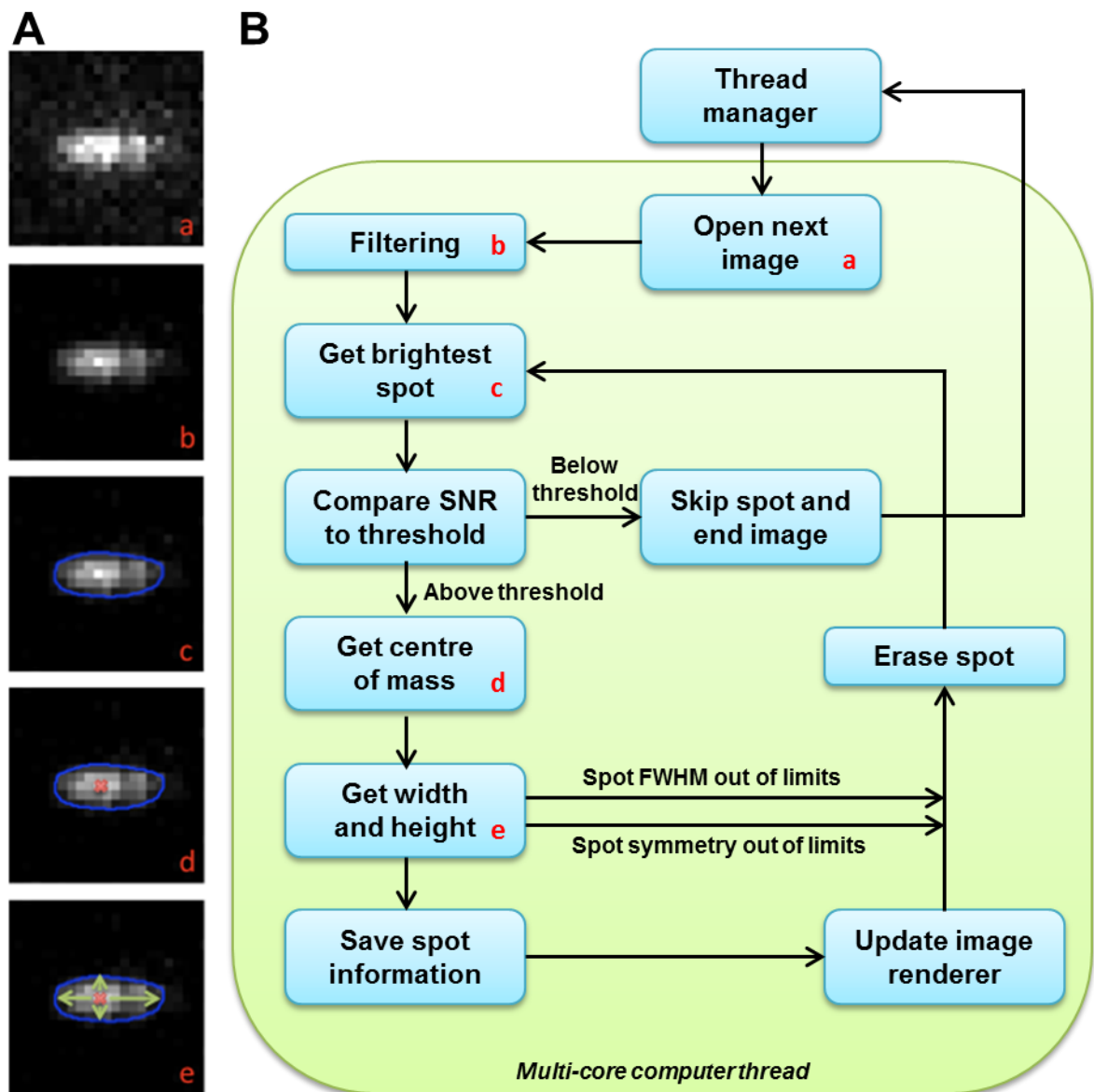


Figure 2.5: Flow-chart of particle detection, localisation and visualisation within QuickPALM. Images in **(A)** represent the corresponding steps in the flow-chart **(B)**, labelled with the same inset letter (red). Adapted from (Henriques et al., 2010).

For each reconstruction, specific parameters were measured in the dataset to optimise the detection of ‘real’ events. The signal-to-noise ratio (SNR) is defined as in Equation 2.3.

Equation 2.3:
$$\text{SNR} = \frac{\text{mean pixel value of signal}}{\text{standard deviation of noise}}$$

The mean pixel value of the signal and the standard deviation of the noise were measured for several frames in each dataset, generally a frame from the beginning, a frame from the middle, and a frame from the end of the dataset, and averaged to choose the appropriate SNR for the reconstruction of this dataset. The SNR values ranged from 5 to 12, with the majority of reconstructions performed with a SNR of 8 or 9. The full-width half maximum (FWHM) of an event was measured (plug-in for ImageJ, “plot FWHM”) and was another parameter specified specifically for the reconstruction of each dataset. Again, several events were measured for each dataset and the average was used for the reconstruction. The FWHM value was constant across datasets at about 3 pixels.

The PALM image is built up molecule by molecule and hence contains detailed information about the molecular distribution of proteins at the plasma membrane. The x and y coordinates of all the detected events are recorded in a Particles Table (in Excel format, QuickPALM).

Cluster analysis

The table containing the x and y coordinates of events can be used to construct two-dimensional spatial point patterns of the molecular distribution of the protein of interest. Cluster analysis, based on Ripley’s K function, can be applied to these patterns to quantify the distribution and heterogeneity of molecules (Owen et al., 2010). Ripley’s K function evaluates a complete map of all interparticle distances over the studied region, and compares the observed distribution with that expected from complete spatial randomness (Ripley, 1977, 1979).

Regions of 3 µm x 3 µm were selected for analysis. The regions were selected if they were wholly contained within the cell boundaries and did not exhibit any major structural artefacts that would interfere with the analysis, such as cell edges, membrane ruffles or lamellipodia, or other evident inhomogeneities. Several non-overlapping regions (between 1 and 6 regions) were selected from each cell. Cluster analysis was performed for each region (“SpPack”, an add-in for Excel (Microsoft) that can calculate the Ripley’s K function for spatial point data (Perry, 2004; Perry et al., 2006)). This

function calculates the level of molecular clustering in a region – for each point in the region it counts how many other points are contained within concentric circles centred on that point. Ripley's K function is calculated using Equation 2.4.

$$\text{Equation 2.4: } K(r) = A \times \Sigma \left(\frac{\delta_{ij}}{n^2} \right) \text{ where } \delta_{ij} = 1 \text{ if } \delta_{ij} < r, \text{ otherwise } 0.$$

Where A is the area, n is the number of points, r is the spatial scale (i.e. radius) for the K function calculation, and δ_{ij} is the distance between points i and j . This function counts the number of other points encircled by concentric circles centred on each point in the region. This function is then linearised to generate the $L(r)$ function as described in Equation 2.5.

$$\text{Equation 2.5: } L(r) = \sqrt{\frac{K(r)}{\pi}} = \sqrt{\frac{A \Sigma \left(\frac{\delta_{ij}}{n^2} \right)}{\pi}}$$

For a random distribution of points, $L(r) = r$ and it is common to plot $L(r) - r$ against r , where a random distribution would have $L(r) - r = 0$ for all r . This means that for spatial scales at which the points are more clustered than would be expected in a random distribution, $L(r) - r$ is positive, whereas $L(r) - r$ is negative if the distribution is less clustered than would be expected for randomly distributed events.

In order to visualise the molecular distribution and extract further information from the data, the regions were also analysed using a more elaborate cluster analysis programme (Figure 2.6 and Appendix 1; a kind gift from Dylan Owen, University of New South Wales, Australia) that runs in MATLAB (MathWorks, Natick, MA). The programme calculates the Ripley's K function at a specific spatial scale, as defined below. To negate edge effects, the programme uses slightly larger regions than selected for the analysis, then weights the edge points and crops the image edges after the calculation.

The main outputs of the cluster analysis programme are a cluster map, a binary map and a table. In the cluster map, the $L(r)$ values generated for each point are interpolated onto a surface plot as the z-axis (defined by a colour scale). This cluster map is assigned a binary threshold value to create a binary map, from which information about the clusters can be extracted (see below). The table contains the x and y coordinates of all the points in the selected region as well as a few interesting numbers, such as the total number of molecules in that region, the number of molecules in this

region that are “in” clusters (i.e. above the cluster threshold set to produce the binary map) and the percentage of molecules that are in clusters.

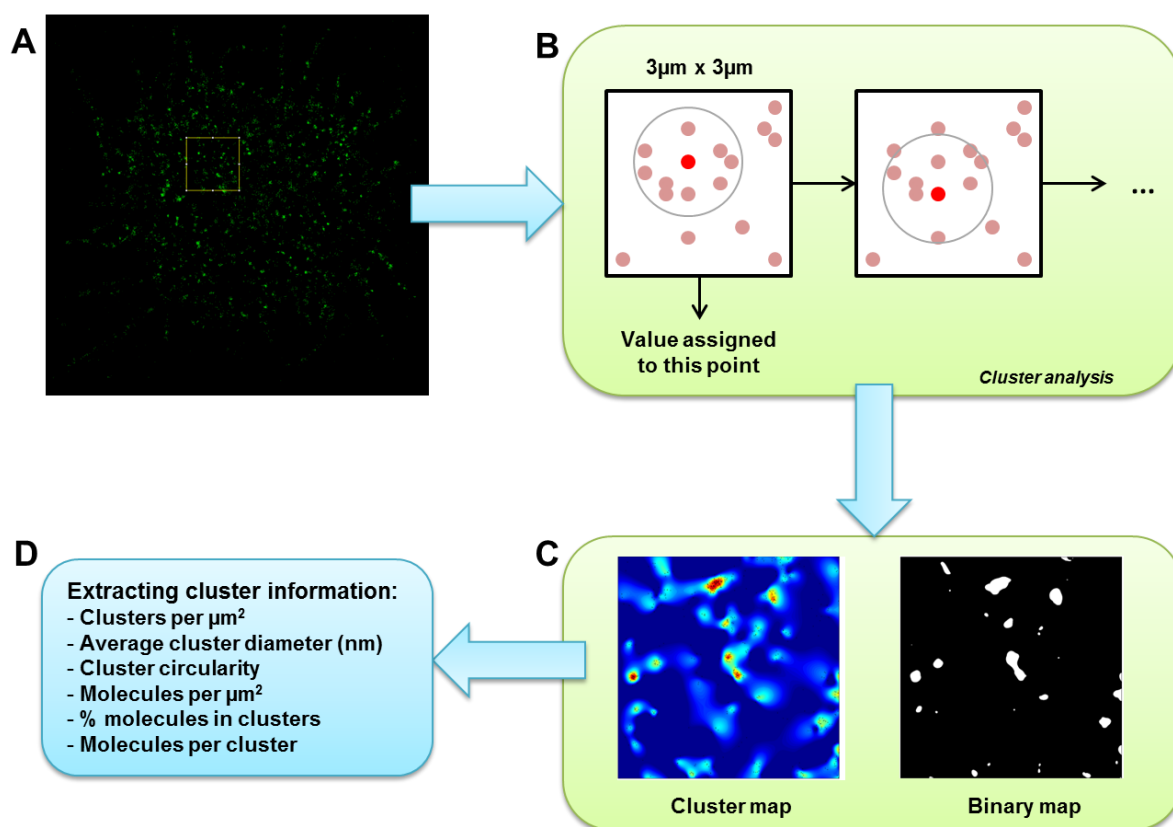


Figure 2.6: Flow-chart of PALM data analysis using quantitative cluster mapping. (A) From the reconstructed PALM image, regions are selected for quantification by cluster analysis. (B) The software quantifies the level of clustering by counting the number of molecules detected within a certain radius of a specific point. This counting is repeated for every point in the region. (C) The cluster analysis generates a cluster map showing levels of clustering and a binary map with clusters that have been subjected to a threshold. (D) The results obtained from the cluster analysis can be used to calculate important information about the molecular distribution.

To run the programme, certain parameters need to be set by the user. These include the x and y lengths of the region that will be analysed (or more specifically the length of the side from the centre coordinate, i.e. half the length, 1500 nm in this case). The programme was set to examine clustering at a spatial scale of 50 nm, i.e. it was calculating $L(50)$ for each point. In addition, the maximum value of the cluster-map colour scale was set to 300, which was determined from the maximum $L(50)$ values observed in typical samples. A binary threshold was applied to the $L(r)$ component of the cluster map to produce a binary map. The threshold was set at $L(50) = 120$, which corresponded to approximately 30% of the maximum $L(50)$ value in a typical sample. Areas of the

region where $L(50) > 120$ were considered to be clustered and were assigned the value of one on the binary map. Areas of the map where $L(50) < 120$ were not considered as clusters and were assigned the value of zero. These parameters were maintained throughout the analysis of all subsequent samples and regions.

Extracting cluster information

Binary cluster maps were analysed to obtain further information on the clusters (ImageJ). A watershed function was applied to the binary threshold maps to separate closely spaced clusters from each other and the “Analyse Particles” function was then applied to extract quantitative information about each cluster in each region. The information extracted from the images included the total number of clusters within the region, and the area, perimeter and circularity (which is a function of area and perimeter) of each cluster. Along with the other information provided directly by the cluster analysis, this analysis allows the user to determine several key values about the clustering in each region: the number of clusters per μm^2 , the average diameter and the circularity of clusters, the total number of molecules per μm^2 and the percentage of molecules in clusters, and the number of molecules per cluster (which can be calculated from the other values using Equation 2.6).

Equation 2.6:

$$\text{Number of molecules per cluster} = \frac{\text{Percentage of molecules in clusters} \times \text{Total number of molecules}}{\text{Number of clusters}}$$

2.7.3 Photoswitching data analysis

Image processing

Imaging data obtained from the photoswitching experiments were processed and analysed (ImageJ). Since images were acquired for 20 minutes at a frame rate of one frame every ten seconds, there were approximately 120 frames per acquisition. In this time, the cells often underwent some movement across the field of view. To correct for this movement, the frames within each image sequence were aligned (plug-in for ImageJ, “StackReg”, Philippe Thévenaz, Swiss Federal Institute of Technology Lausanne, Switzerland).

The images were then assigned a threshold to minimise background fluorescence before further analysis. Measurements in the red channel were taken at the region where the tdEosFP molecules were photoconverted to red during the acquisition as well as the region of the membrane directly opposite (Figure 2.7). The regions in which fluorescence was monitored were chosen to be tall, thin rectangles to take into account any remaining cell movement and changes in cell shape that might have occurred during the acquisition and that would skew the analysis. Within these regions, the measurement that was taken was “area”, which measures the number of fluorescent pixels in a region.

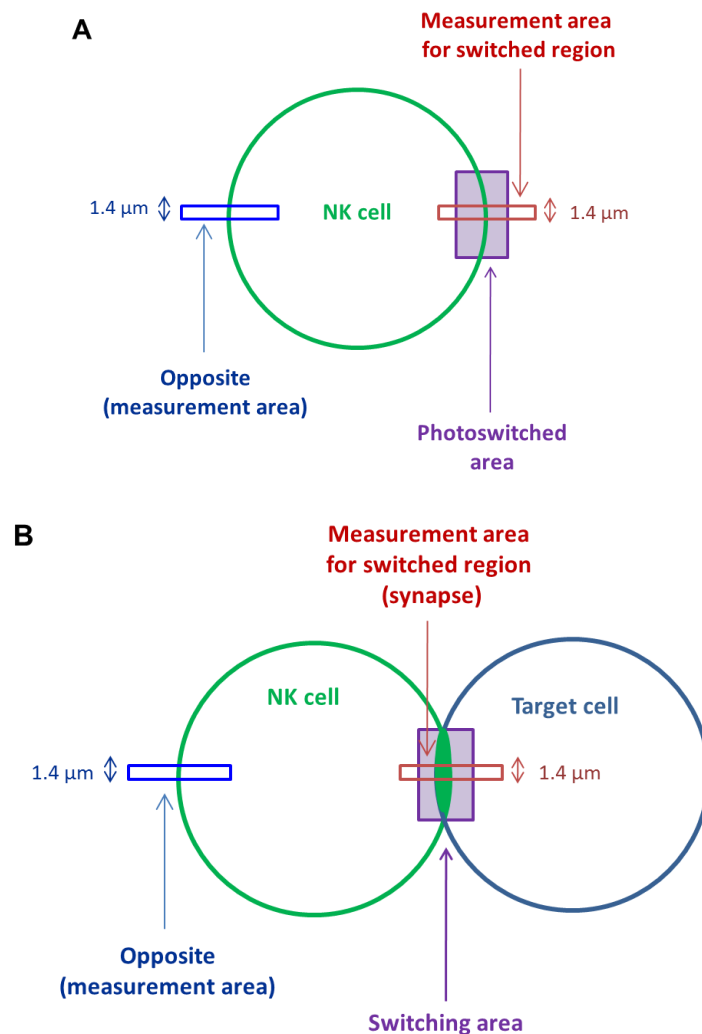


Figure 2.7: Photoswitching experiments. (A) tdEosFP molecules were photoswitched from green to red fluorescence in the region indicated by the purple box. Measurements were carried out in this region as well as the region directly opposite. (B) When a synapse was formed with a target cell, tdEosFP molecules were photoswitched either within the synapse region (purple box) or in the region opposite the synapse (not shown here). Measurements were carried out in the synapse region and the region opposite.

This method of measurement was validated by measuring the length of membrane detected within the two monitoring regions in a typical image sequence containing 122 frames (Figure 2.8A). The standard deviation and standard error of the mean were very small (<0.6% of the mean value), indicating little variance (Figure 2.8B). This indicates that for each region that is selected, the length of membrane that is being analysed for the whole image sequence remains constant. After validation, this method of analysis was applied to all cells in further experiments.

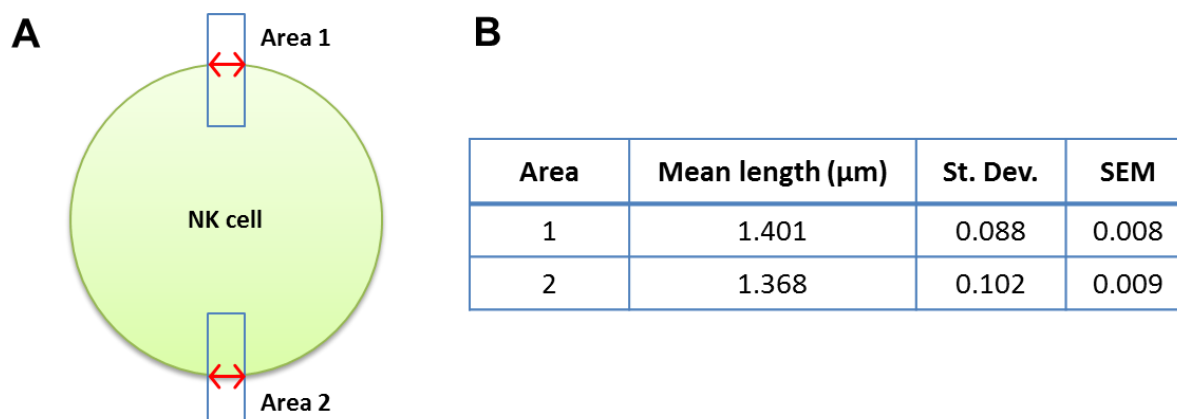


Figure 2.8: Measurement regions in photoswitching experiments. **(A)** Measurements were carried out in long thin rectangles directly opposite each other on the cell membrane. **(B)** Validation of the measurement method. The length of membrane (indicated by red arrows in the diagram) within the rectangles was measured over a sequence of 122 frames and the results are presented here. St. Dev. is the standard deviation; SEM is the standard error of the mean.

The data were normalised to facilitate comparison between cells; the measurement at the first time point after photoswitching was set as 100% and all other measurements in the same cell were compared against this value. This normalisation allowed the comparison of different cells that might have had different initial levels of red fluorescence after photoswitching.

Mathematical modelling

All mathematical modelling was kindly carried out by Robert Endres and Gerardo Aquino (Systems Biology, Division of Molecular Biosciences, Imperial College). The mathematical simulations were performed using the algorithm platform Mathematica 8.0 (Wolfram Research). All modelling was based on the basic diffusion equation (equation 2.7) in Cartesian coordinates.

Equation 2.7:
$$\frac{dp(x,y,t)}{dt} = D \Delta p(x,y,t) = D \left(\frac{\partial^2 p(x,y,t)}{\partial x^2} + \frac{\partial^2 p(x,y,t)}{\partial y^2} \right)$$

Where $p(x,y,t)$ is the density of receptors at the point with coordinates x,y at time t , D is the diffusion coefficient, and Δ the Laplacian operator, which in Cartesian coordinates is just the sum of the second order partial derivatives, as seen in the term on the right.

Since we are considering the diffusion of molecules on a cell surface (which can be approximated to a spherical surface) rather than on a planar surface, the diffusion equation can be conveniently expressed in spherical polar coordinates (equation 2.8) to describe movement on a spherical surface.

Equation 2.8:

$$\frac{dp(\varphi,\theta,t)}{dt} = D \Delta p(\varphi,\theta,t) = D \left[\frac{1}{r^2 \sin \theta} \frac{\partial \left(\sin \theta \frac{\partial p(\varphi,\theta,t)}{\partial \theta} \right)}{\partial \theta} + \frac{1}{r^2 \sin^2 \theta} \frac{\partial^2 p(\varphi,\theta,t)}{\partial \varphi^2} \right]$$

Where $p(\varphi,\theta,t)$ is the density of receptors at the point on the sphere of azimuth angle φ and inclination θ , r is the radius of the sphere, i.e. in this case the radius of the cell, and Δ is the spherical Laplace operator.

The assumptions in this model are that (i) the radius r of the cell is constant; and (ii) the diffusion rate will be the same all around the cell. A schematic representation of the model (Figure 2.9) shows an example of the time it would take for the photoswitched molecules to move around the cell if the diffusion coefficient of the receptor was $0.25 \mu\text{m}^2 \cdot \text{s}^{-1}$. This model was fit to the experimental data to extract the diffusion coefficient.

To define the spherical diffusion model, complex mathematical algorithms were designed to extract the diffusion coefficient. To summarise briefly, the Gaussian profile of the initial conditions at time 0 was expressed as a superposition of the spherical harmonics function Ψ , as previously described (Bülow, 2004). This expansion allows one to describe the evolution of the initial conditions induced by free diffusion over the whole area of the sphere at a generic time as a function of only two parameters: the diffusion coefficient D and a normalisation factor N . If this function is integrated over the area of the measurement region, it is then possible to fit this equation to the experimental data using the method of minimum least squares fit. An algorithm was used that changes D and N until the difference (squared) between the numerical simulations and the experimental data is

minimised. If the radius r of the cell is known, the final value of the diffusion coefficient D can be extracted, in the appropriate unit ($\mu\text{m}^2.\text{s}^{-1}$). The radii of all cells analysed were measured for this purpose; they ranged from 15-20 μm , averaging at 17 μm .

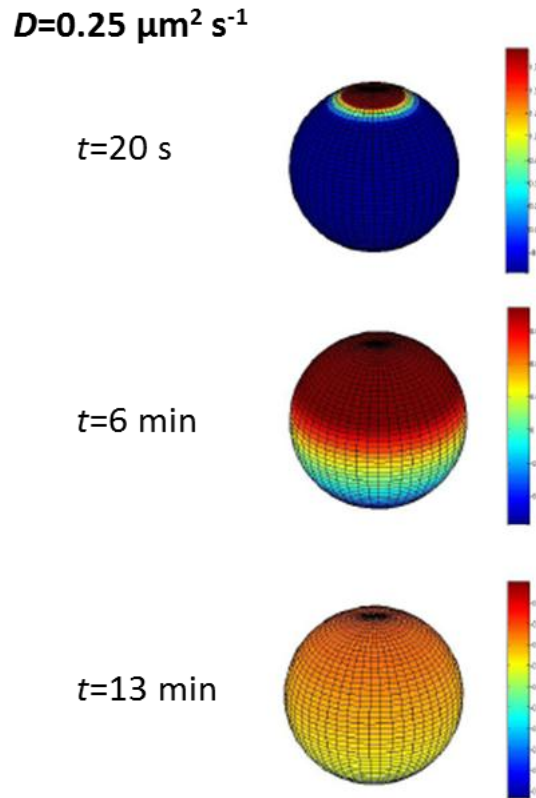


Figure 2.9: Schematic representation of spherical diffusion. In this model, fluorescent molecules are highly concentrated at the top pole of the sphere (representing a cell). Over time, the molecules diffuse around the sphere, resulting in a homogeneous distribution of molecules all around the sphere. The example given here is with a theoretical diffusion coefficient of $0.25 \mu\text{m}^2.\text{s}^{-1}$ and the molecular distribution reaches equilibrium after 13 minutes. Diagram from Gerardo Aquino.

In the situation where a target cell is present and an immune synapse has formed, free diffusion can no longer be assumed and further modelling was applied. Two hypothetical models were studied. In the first instance, both models were described on a squared lattice with periodic conditions (Figure 2.10) for a qualitative study before attempting to fit the data to a spherical model for a quantitative study. In the first, more general model, the hypothesis was that the diffusion coefficient within the synapse (D_s) was inferior to the diffusion coefficient in the rest of the cell (D), implying that the receptors are moving more slowly within the synapse (Figure 2.10A). In the second, more specific model, a force is present that physically traps the receptors within the synapse (Fig 2.10B). The

magnitude of this force is hypothesised to be of the same order of magnitude as the receptors' momentum due to thermal fluctuations since some receptors are thought able to escape the synapse.

2.7.4 Statistical analysis

Statistical analysis performed on the cluster information generated from the PALM imaging was a two-tailed unpaired student's t-test to test for significance. Mean values are shown and error bars represent the standard error of the mean (SEM). In the statistical analysis, differences were defined as non-significant when $p > 0.05$ (n.s.), statistically significant when $p < 0.05$ (indicated by a single asterisk *), $p < 0.01$ (**) and $p \leq 0.0001$ (***). All graphs were prepared and statistical analyses were performed in Prism (Graph Pad).

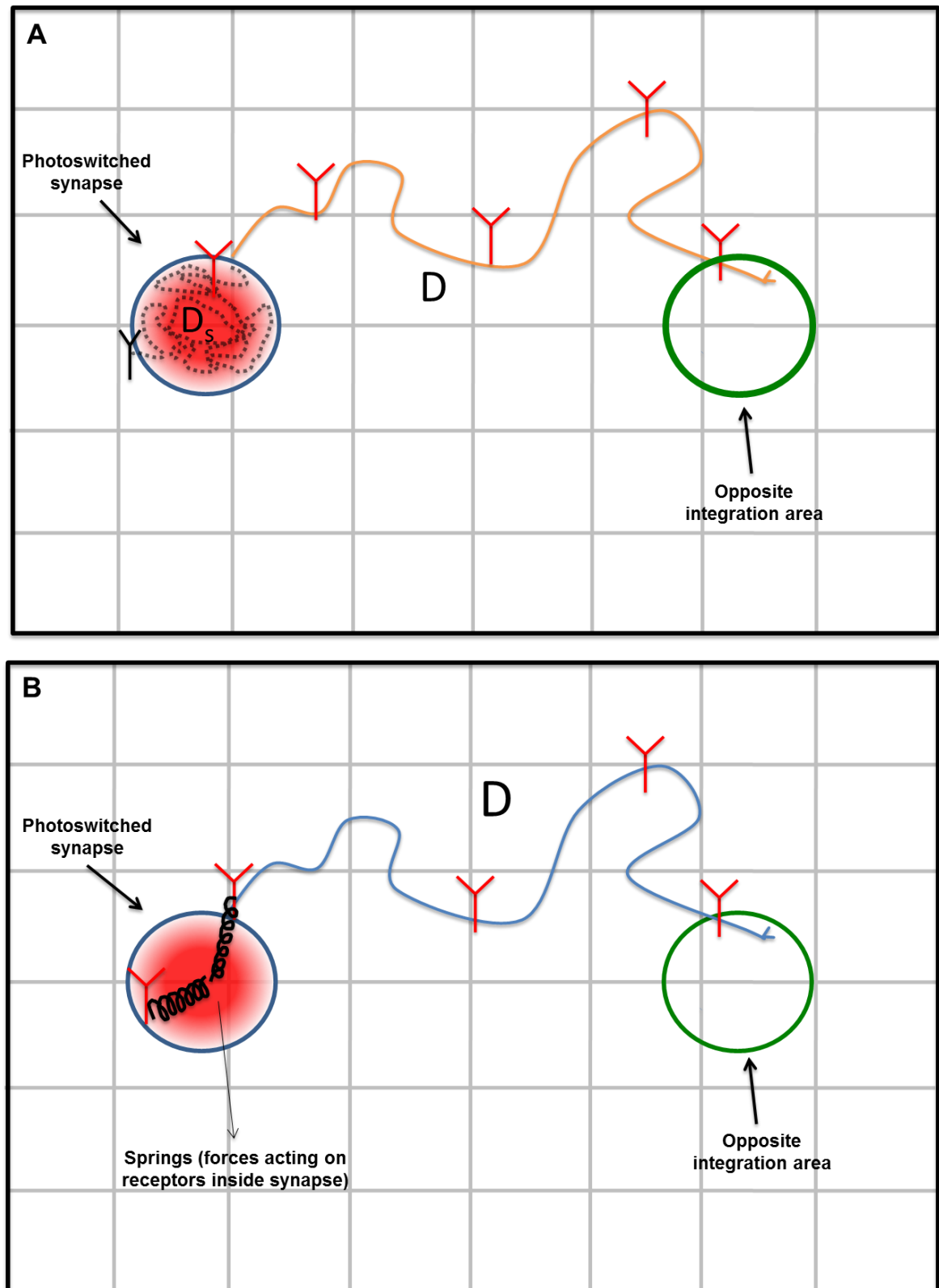


Figure 2.10: Two models of receptor mobility in the presence of a target cell. Both models are described on a square lattice. **(A)** Model where the receptors have reduced mobility within the synapse ($D_s < D$). **(B)** Model where attractive forces are entrapping the receptors within the synapse. Diagrams from Gerardo Aquino.

Chapter 3 - Imaging the nanoscale organisation of KIR2DL1 using Photoactivated Localisation Microscopy (PALM)

3.1 Introduction

3.1.1 Photoactivated Localisation Microscopy (PALM)

PALM is a super-resolution microscopy technique that relies on single-particle localisation (Betzig et al., 2006). To achieve nanometer scale resolution, PALM exploits the properties of photoactivatable or photoswitchable fluorescent proteins. The basic principle behind PALM is the stochastic photoactivation of subsets of fluorescent proteins by UV light, followed by the precise localisation of single activated molecules by finding the centre of their diffractive PSF (Figure 3.1). These molecules are then photobleached using high laser power. The cycle of photoactivation-localisation-photobleaching can be repeated with 10^4 - 10^5 molecules to obtain a reconstructed PALM image (Betzig et al., 2006). A typical acquisition contains several thousand frames, with sparse subsets of molecules being turned on and off – this gives the impression of ‘blinking’ molecules in the image sequence.

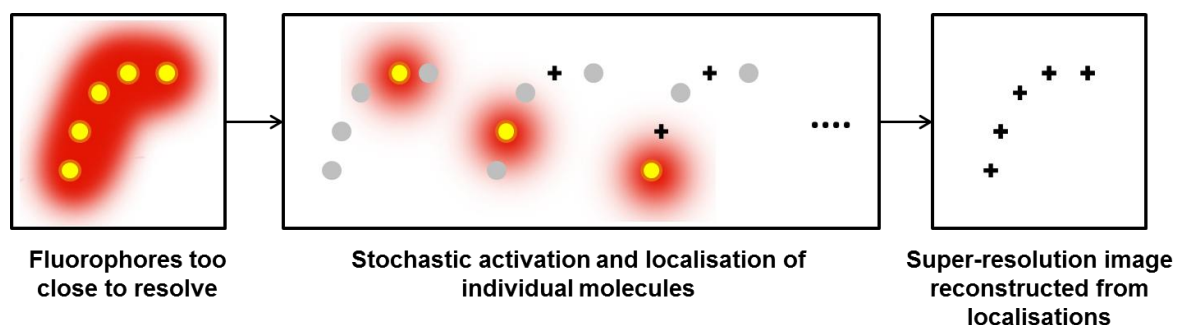


Figure 3.1: Principle of photoactivated localisation microscopy (PALM). PALM relies on the use of photoactivatable or photoswitchable fluorescent proteins. Only a subset of fluorophores are activated in every frame. These molecules are detected as diffraction-limited spots, which can be localised with high accuracy by mathematical fitting. A final super-resolution image is reconstructed from thousands of frames, each with a different subset of emitters.

The localisation of molecules and image reconstruction can be performed in real time during acquisition on certain set-ups, although these steps are generally carried out post-acquisition using specialised software such as QuickPALM (Henriques et al., 2010), or rapidSTORM (Wolter et al., 2010).

The centre of each PSF in each frame is determined by statistical curve fitting of the PSF intensity distribution to a Gaussian function or a centre-of-mass distribution. The centre of the fitted curve localises the lateral (x,y)-position of the original molecule to within tens of nanometres. By processing the entire stack of images from a dataset, a coordinate map containing the location of every sampled molecule can be compiled and used to derive a super-resolution image of the specimen.

The localisation precision, or how accurately the position of a molecule can be determined from its recorded diffraction-limited PSF, is dependent on several factors, mainly the number of photons collected from each molecule. The localisation precision is increased when more photons are emitted by a molecule, as the fitting of the PSF can be calculated more accurately from a higher photon flux density. It is therefore important to select a fluorescent probe with a high quantum yield and high photon emission rates.

3.1.2 The photoswitchable protein tdEosFP

EosFP, named after Eos the goddess of dawn in Greek mythology, is a fluorescent protein isolated from the stony coral *Lobophyllia hemprichii* that is capable of an irreversible photoconversion from green fluorescence (516 nm) to red fluorescence (581 nm) upon near-UV irradiation at ≈ 390 nm (Wiedenmann et al., 2004; Nienhaus et al., 2005). Wild-type EosFP is tetrameric, but single point mutations yielded functional monomers with identical photoswitching properties (Wiedenmann et al., 2004). However, monomeric EosFP (mEos) does not mature at 37°C and variants that can be used in mammalian cells in physiological conditions have been developed (Nienhaus et al., 2006; McKinney et al., 2009; Hess, 2009). The tdEosFP construct is a tandem dimer in which two EosFP subunits are connected by a 12-amino acid linker (Nienhaus et al., 2006). tdEosFP has the same photoswitching properties as wild-type EosFP (Figure 3.2) and is well expressed at 37°C following fusion with other proteins (Nienhaus et al., 2006).

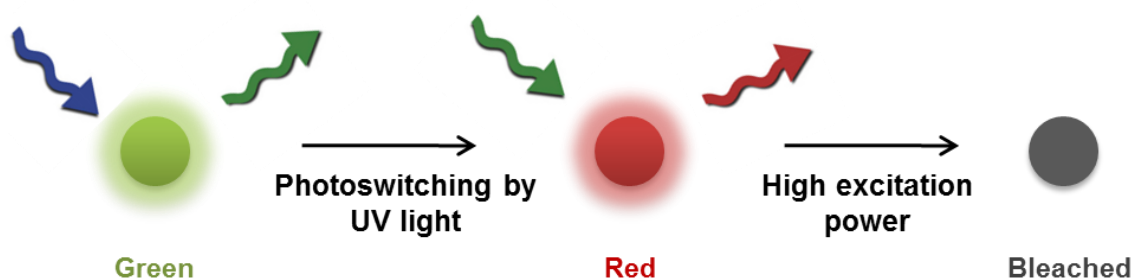


Figure 3.2: The photoswitchable fluorescent protein tdEosFP. The photoswitchable protein tdEosFP emits green fluorescence (516 nm). Upon illumination with UV light, tdEosFP undergoes a structural change defined by a cleavage of the peptide backbone. This break modifies the chromophore contained within the structure, resulting in tdEosFP emitting red fluorescence (581 nm). With high levels of excitation laser, tdEosFP is permanently photobleached.

The structural basis for the photoconversion of EosFP has been described by comparing the x-ray crystal structures of the green and red forms of EosFP (Nienhaus et al., 2005). In the green form, fluorescence is mediated by the typical green chromophore 4-(*p*-hydroxybenzylidene)-5-imidazolinone. This green chromophore forms autocatalytically from the tripeptide consisting of amino acids histidine (His), tyrosine (Tyr) and glycine (Gly) at positions 62, 63 and 64 respectively, and is tightly encased within the structure. After photochemical modification the overall three-dimensional structures of the green and red forms remain conserved (Nienhaus et al., 2005). It has been suggested that the mechanism of photoconversion involves His-62, since the mutation of this residue to other amino acids resulted in a loss of photoconversion ability (Wiedenmann et al., 2004). Indeed, absorption of a photon of UV light resulted in a rearrangement of the structure, mainly defined by a cleavage of the peptide backbone between the alpha carbon and the alpha nitrogen of His-62, as previously inferred (Mizuno et al., 2003). This cleavage generates the formation of the red chromophore 2-[(1*E*)-2-(5-imidazolyl)ethenyl]-4-(*p*-hydroxybenzylidene)-5-imidazolinone (Nienhaus et al., 2005). Superposition of the structures of the two species highlights their similarities and demonstrates that a small structural change can result in drastically altered photophysical properties.

The tdEosFP fluorescent protein is a good candidate for PALM microscopy as it has a high quantum yield in the red form. In the context of fluorescence, the quantum yield of a given fluorophore essentially represents the emission efficiency of the fluorophore, which can be calculated by dividing the number of photons that are emitted by the number of photons that are absorbed.

The green form of tdEosFP has a quantum yield of ~ 0.7 (Wiedenmann et al., 2004), which is comparable to that of wild-type GFP (0.77). More importantly, the red form of tdEosFP has a quantum yield of ~ 0.6 (Wiedenmann et al., 2004), which is higher than the quantum yield of most red fluorescent proteins. In addition, tdEosFP has a higher contrast ratio than many other fluorescent proteins typically used in PALM imaging (Shroff et al., 2007). The contrast ratio is defined as the ratio in fluorescence intensity between the activated and inactive states of the probe at the excitation wavelength of the activated form – in this case the ratio in emission of red fluorescence between the red and green forms of the protein. tdEosFP has an extremely high photon count compared to other known photoconvertible fluorescent proteins (Betzig et al., 2006; Shroff et al., 2007), which increases the localisation precision achievable by PALM. Together, these factors make tdEosFP a good choice for achieving maximum localisation precisions by PALM imaging.

3.1.3 TIRF/PALM microscopy

PALM was implemented and is frequently used in combination with TIRF microscopy (Betzig et al., 2006; Rust et al., 2006). Background fluorescence can be significantly minimised by using PALM in a TIRF configuration as fluorophore excitation is restricted to the evanescent wave at or near the interface between the cell and the coverslip. This is crucial for PALM, since background fluorescence could interfere with or mask the PSFs from individual fluorophores. In addition, TIRF microscopy is a wide-field technique; therefore many molecules can be imaged simultaneously, leading to faster acquisition rates. TIRF microscopy is ideal for studying structures that lie close to the membrane, such as focal adhesions and cytoskeletal components, as well as the organisation of membrane proteins themselves. However, TIRF is not an absolute requirement for PALM imaging if using specimens such as bacteria that are sufficiently thin (~ 200 nm) so that the localisation of single molecules is not affected by background fluorescence. For example, chemotaxis clusters were imaged by PALM in *Escherichia coli* using a conventional wide-field microscope (Greenfield et al., 2009).

PALM is a powerful single-molecule localisation technique that can be used to investigate the distribution of proteins within cell membranes. Combining PALM and TIRF allows the visualisation of membrane proteins at the single-molecule level with reduced background fluorescence interference. In this thesis, PALM imaging was carried out in a TIRF configuration. From here onwards, reference

to PALM imaging will imply that the imaging was done on a TIRF set-up (TIRF/PALM microscopy) as opposed to simple TIRF imaging or PALM imaging on a wide-field set-up.

3.1.4 Applications of PALM microscopy

PALM imaging has many applications in all fields of biology and has been successfully used in cell biology, neurobiology, microbiology and immunology.

In cell biology, for example, imaging by PALM provided important new insights into the dynamics of focal adhesion complexes during their initiation, maturation and disassembly (Shroff et al., 2008) and into the nanoscale molecular architecture of integrin-based cell adhesions (Kanchanawong et al., 2010). In neuroscience, PALM was used to investigate the organisation of pre- and post-synaptic proteins at neuronal synapses (Dani et al., 2010). In microbiology, PALM has mainly been used to shed light on the distribution of specific proteins within bacterial cells (Biteen et al., 2008; Greenfield et al., 2009).

Importantly, immunologists have started to realise the potential of PALM microscopy for imaging the organisation of proteins at the immune synapse. More specifically, PALM has been applied to the study of protein microclusters at the T cell synapse (Lillemeier et al., 2010; Owen et al., 2010; Williamson et al., 2011; Sherman et al., 2011). These studies mainly focussed on the distribution and interaction of TCR and LAT microclusters during T cell activation. Previous studies based on conventional imaging techniques such as confocal fluorescence microscopy assumed that many signalling molecules, including TCR and LAT, colocalised in protein microclusters during early stages of T cell activation (Bunnell et al., 2002; Campi et al., 2005; Yokosuka et al., 2005; Varma et al., 2006). In addition, based on electron microscopy studies, TCR and LAT, amongst other plasma membrane proteins, have been proposed to pre-cluster in resting cells (Schamel et al., 2005).

When observed at higher resolution using PALM, TCR and LAT were indeed found to be pre-clustered in quiescent T cells (Lillemeier et al., 2010). Upon activation of T cells, these small aggregates concatenated to form microclusters, but instead of being overlapping, TCR and LAT were found in distinct separate microclusters (Lillemeier et al., 2010). Another study based on PALM imaging confirmed that LAT was found in pre-existing nanoclusters in resting T cells (Sherman et al., 2011). Two-colour PALM extended this investigation to demonstrate that although TCR and ZAP-70 clusters were colocalised, both were mainly distinct from LAT clusters, with very small regions of overlap that could potentially represent “hot spots” for LAT phosphorylation by ZAP-70 (Sherman et

al., 2011). In addition, upon T cell activation the enzyme PLC- γ colocalised with LAT, whereas SLP-76 molecules localised to the periphery of LAT clusters (Sherman et al., 2011).

However, another recent study combining PALM, live PALM and dSTORM experiments suggests that TCR signalling may depend on LAT molecules associated with subsynaptic intracellular vesicles rather than LAT clusters already present in the plasma membrane (Williamson et al., 2011). Indeed, Williamson, Owen and colleagues demonstrated that signal-relevant LAT clusters appeared at the synapse after recruitment from membrane-proximal subsynaptic vesicles and that pre-existing LAT clusters in the membrane did not participate in T cell signalling (Williamson et al., 2011). These results are in accordance with a previous investigation where TIRF microscopy and experiments with optical tweezers revealed that motile intracellular vesicles containing LAT were repeatedly moving to the membrane, where they showed decreased motility and interacted with SLP-76 clusters (Purbhoo et al., 2010). In this study however, the authors show that both vesicular LAT and surface clusters of LAT are important for signalling (Purbhoo et al., 2010). Nonetheless, taken together, these studies have led to the proposal of a new model to explain the assembly of TCR-proximal signalling components, in which vesicle traffic to and from the membrane is important for the recruitment and disassembly of signalling complexes. This model extends previous understanding and goes beyond the linear and probably oversimplified signalling cascade generally believed to lie downstream of TCR ligation.

This type of study stresses the importance of using super-resolution microscopy techniques when trying to understand cluster formation and signalling interactions. However, super-resolution imaging has not been applied to NK cell signalling and much about signalling protein organisation, cluster interactions and mechanisms for signal integration in NK cells remains generally unexplained. It remains to be seen how receptors and signalling molecules distribute themselves upon activation or inhibition of NK cells and how they interact in space at the single molecule level to promote downstream signalling. The efficient integration of activating and inhibitory signals within an NK cell may require specific localisation and proximity of proteins, which might be reflected by the presence of dynamic protein clusters that orchestrate signalling.

3.1.5 Aims

NK cell signalling pathways have been studied extensively using biochemical and genetic approaches to identify the key molecules involved in signalling. However, the localisation of the signalling

components and their interactions remain to be deciphered, for example with super-resolution imaging techniques.

Therefore, the main goal of this investigation was to determine the nanoscale organisation of KIR2DL1 at the NK cell synapse. In addition, the organisation of KIR2DL1 was analysed under different conditions. The specific aims of this study were as follows:

- (i) To set up an appropriate cell system for PALM imaging to visualise the organisation of KIR2DL1 in the membrane of NK cells; and to optimise sample preparation and image acquisition
- (ii) To determine the nanoscale organisation of KIR2DL1 in the membrane of resting NK cells
- (iii) To determine how the organisation of KIR2DL1 is changed upon ligation of KIR2DL1
- (iv) To study how the organisation of KIR2DL1 is affected by the disruption of active cellular processes such as actin cytoskeleton polymerisation
- (v) To investigate how the organisation of KIR2DL1 is affected by the ligation of other receptors including activating receptors and other inhibitory receptors.

3.2 Setting up the cell system

3.2.1 Cell system

To visualise the organisation of KIR2DL1 in the membrane of NK cells, the NK cell lines YTS and NKL were transfected to express KIR2DL1 fused to the photoswitchable protein tdEosFP. These cells, termed YTS-KIR2DL1-tdEosFP and NKL-KIR2DL1-tdEosFP respectively, were then used for PALM imaging on glass slides, as depicted in Figure 3.3. The slides were coated with different antibodies to mimic target cells expressing different ligands on their surface. The cells were allowed to settle on the slides before being fixed with PFA.

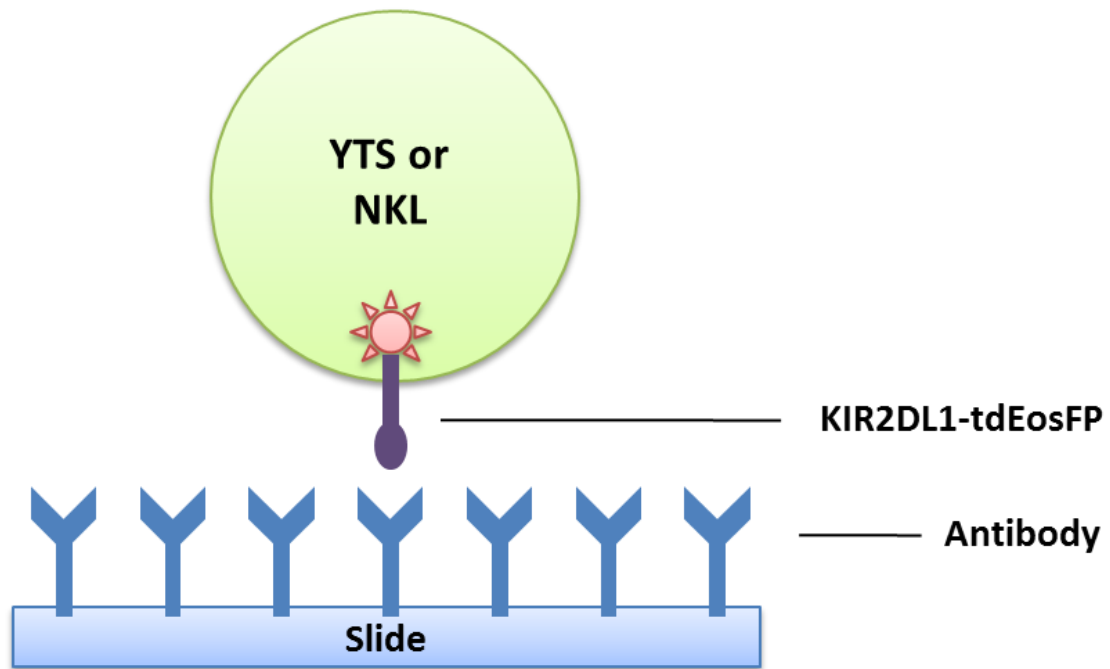


Figure 3.3: Schematic diagram depicting a sample for PALM imaging. NK cells (YTS or NKL) transfected to express KIR2DL1 fused to tdEosFP were allowed to settle on slides coated with PLL and antibody, before being fixed with PFA.

3.2.2 Cloning KIR2DL1-tdEosFP

As described in the Materials and Methods chapter, tdEosFP was cloned into the PINCO-KIR2DL1 retroviral vector in two steps (Figure 2.1). Briefly, in the first step the tdEosFP sequence was introduced into an intermediate cloning vector pEGFP-N1. pEGFP-N1 and pcDNA3-Flag1-tdEosFP were digested with the restriction enzymes *Apal* and *XhoI* and the samples were run on an agarose gel (Figure 3.4A). The appropriate bands were extracted from the gel and purified, and then tdEosFP was ligated into the pEGFP-N1 vector. A test digest was performed on the resulting construct to verify that tdEosFP was indeed present (Figure 3.4B).

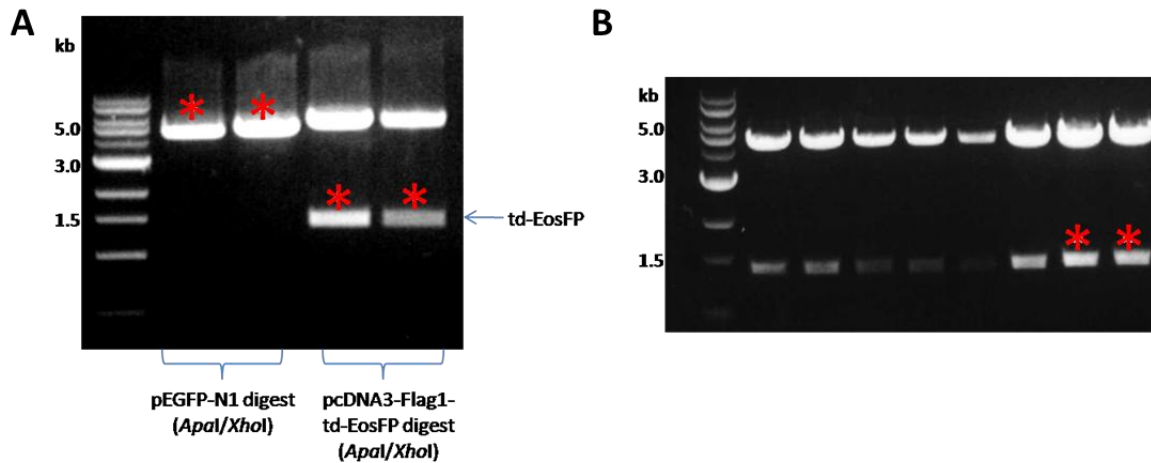


Figure 3.4: Cloning tdEosFP into pEGFP-N1. (A) pEGFP-N1 and pcDNA3-Flag1-tdEosFP vectors were digested with the restriction enzymes *ApaI* and *XhoI*. The bands marked by a red asterisk were extracted from the gel and purified. (B) The tdEosFP digest was ligated into the pEGFP-N1 vector. A test digest with *ApaI* and *XhoI* on the resulting MiniPreps confirmed the presence of tdEosFP in the construct. Samples marked by a red asterisk were used for subsequent cloning steps.

In the second cloning step, tdEosFP was inserted into the retroviral vector PINCO-KIR2DL1. pEGFP-N1-tdEosFP was digested with *BglII* and *PspOMI* and tdEosFP was extracted from the gel (Figure 3.5A). PINCO-KIR2DL1-GFP was digested with *BamHI* and *NotI* to remove GFP (Figure 3.5B). Finally, tdEosFP was ligated into PINCO-KIR2DL1 to obtain the final vector containing KIR2DL1-tdEosFP.

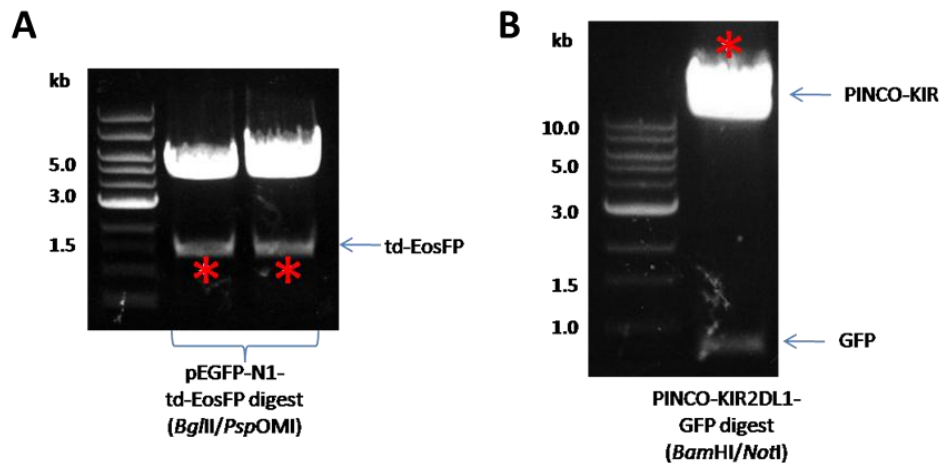


Figure 3.5: Cloning tdEosFP into PINCO-KIR2DL1. (A) The pEGFP-N1 construct containing tdEosFP was digested with the restriction enzymes *BglII* and *PspOMI*. The bands marked by a red asterisk, corresponding to tdEosFP, were extracted from the gel and purified. (B) PINCO-KIR2DL1-GFP was digested with *BamHI* and *NotI* to remove GFP. The band marked by a red asterisk corresponds to the resulting PINCO-KIR2DL1 vector. tdEosFP was ligated into PINCO-KIR2DL1 and the constructs were checked by sequencing.

3.2.3 Obtaining the YTS-KIR2DL1-tdEosFP and NKL-KIR2DL1-tdEosFP transfectants

The retroviral vector containing KIR2DL1-tdEosFP was transfected into the NK cell lines YTS and NKL using the retroviral transduction method. The cells were analysed by flow cytometry to determine whether the transfection was successful (Figures 3.6A & 3.7A). The presence of the construct was detected by the green fluorescence of tdEosFP. Since the percentage of positive cells was very low (less than 3%), the cells were enriched by FACS and then re-analysed by flow cytometry. The resulting cultures contained a higher fraction (more than 80%) of positive cells (Figures 3.6B & 3.7B) and the expression of KIR2DL1-tdEosFP remained stable in these cells over time, which was ideal for performing repeated imaging experiments with the same cells.

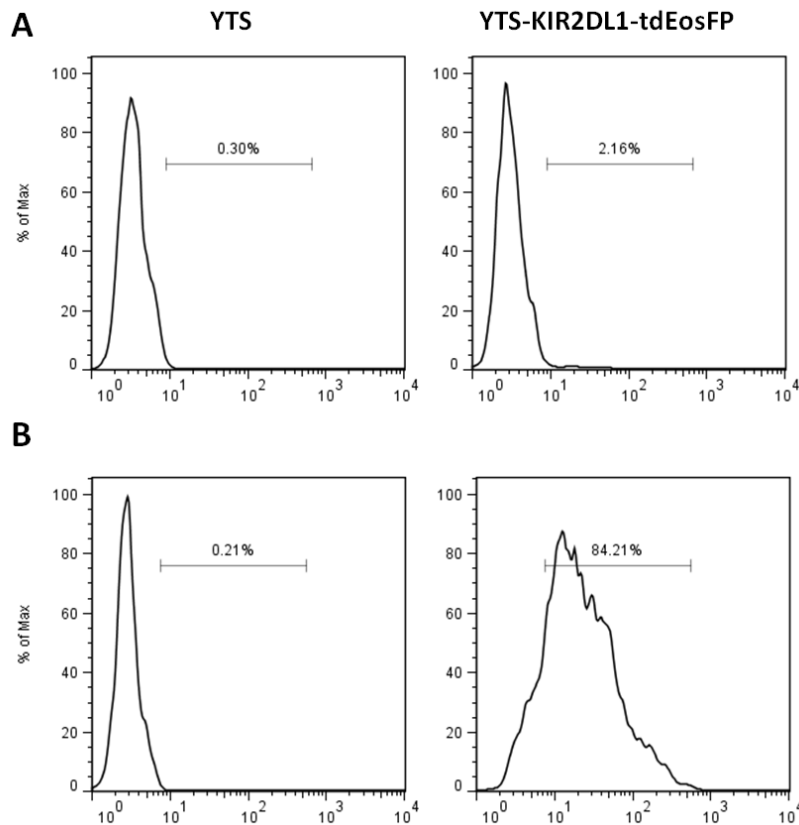


Figure 3.6: Transfecting YTS cells to express KIR2DL1-tdEosFP. (A) YTS cells transfected to express KIR2DL1-tdEosFP were analysed by flow cytometry for tdEosFP fluorescence and compared to untransfected YTS cells. **(B)** YTS cells expressing KIR2DL1-tdEosFP were enriched by FACS and re-analysed by flow cytometry after cell sorting. Based on the distribution of untransfected YTS cells, a gate was drawn to quantify GFP-positive cells. The percent values represent the percentage of cells that were found within the gate.

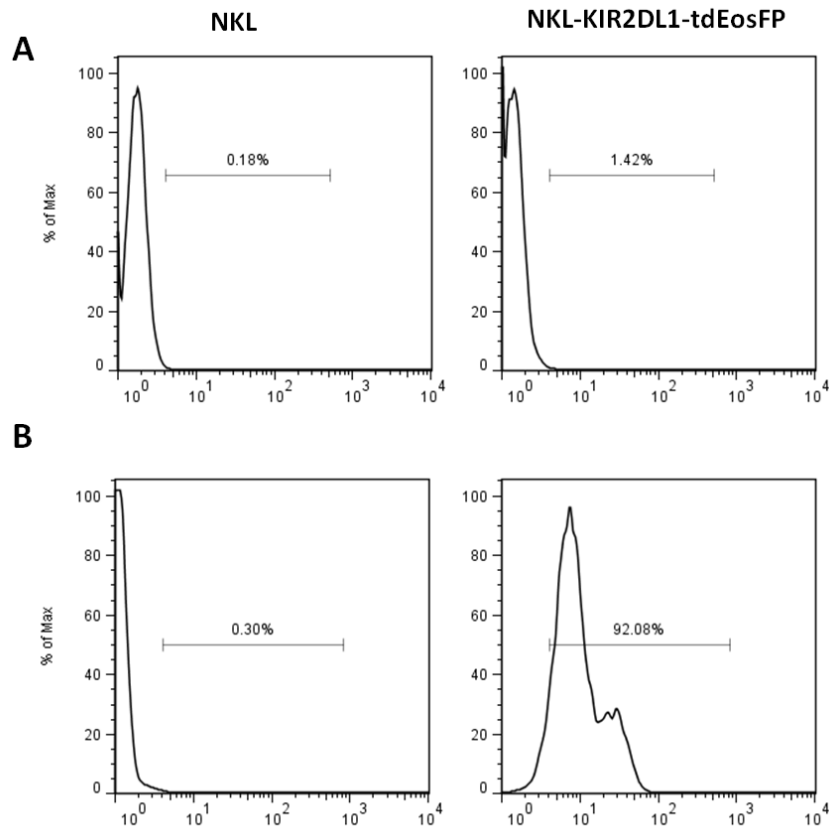


Figure 3.7: Transfecting NKL cells to express KIR2DL1-tdEosFP. (A) NKL cells transfected to express KIR2DL1-tdEosFP were analysed by flow cytometry for tdEosFP fluorescence and compared to untransfected NKL cells. **(B)** NKL cells expressing KIR2DL1-tdEosFP were enriched by FACS and re-analysed by flow cytometry after cell sorting. Based on the distribution of untransfected NKL cells, a gate was drawn to quantify GFP-positive cells. The percent values represent the percentage of cells that were found within the gate.

To detect the presence of KIR2DL1 protein in these cells, YTS-KIR2DL1-tdEosFP and NKL-KIR2DL1-tdEosFP cells were further analysed by flow cytometry and immunostaining. The cells were stained with an anti-KIR2DL1 antibody (EB6) and a secondary detection antibody and analysed by flow cytometry (Figures 3.8 & 3.9) to check whether KIR2DL1 could be detected in the cells. Indeed, YTS-KIR2DL1-tdEosFP and NKL-KIR2DL1-tdEosFP cells were shown to express KIR2DL1 at their surface. KIR2DL1 levels detected in YTS-KIR2DL1-tdEosFP cells were comparable to those found in the cell line used as a positive control, YTS-KIR2DL1.

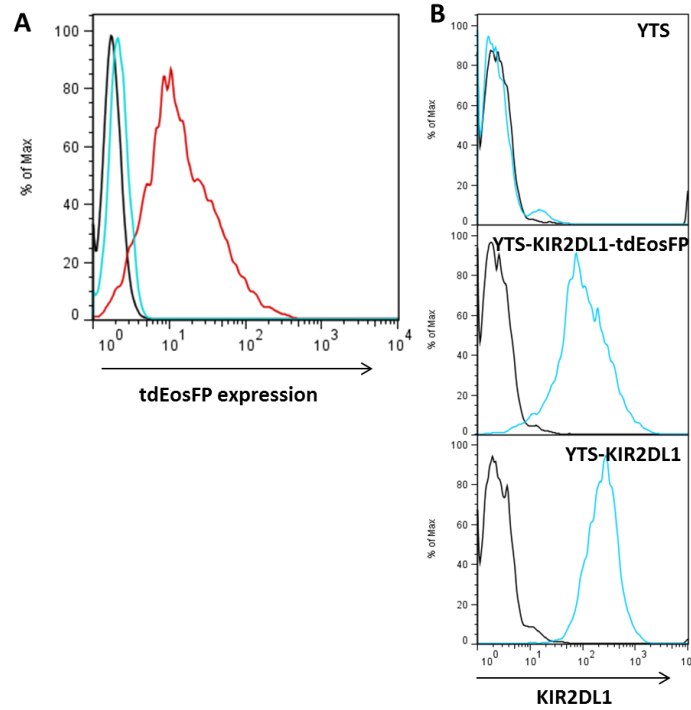


Figure 3.8: tdEosFP fluorescence and KIR2DL1 surface expression on YTS-KIR2DL1-tdEosFP cells. (A) YTS-KIR2DL1-tdEosFP cells (red) were analysed by flow cytometry for tdEosFP fluorescence and compared to YTS (black) and YTS-KIR2DL1 cells (blue) as negative controls. (B) YTS-KIR2DL1-tdEosFP cells were analysed by flow cytometry for surface expression of KIR2DL1. Specific staining (blue) is compared to isotype control (black). YTS and YTS-KIR2DL1 cells were used as negative and positive controls, respectively.

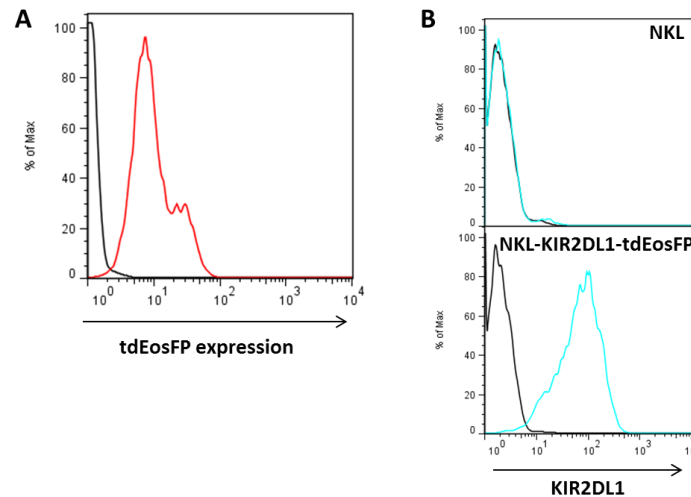


Figure 3.9: tdEosFP fluorescence and KIR2DL1 surface expression on NKL-KIR2DL1-tdEosFP cells. (A) NKL-KIR2DL1-tdEosFP cells (red) were analysed by flow cytometry for tdEosFP fluorescence and compared to NKL cells (black) as a negative control. (B) NKL-KIR2DL1-tdEosFP cells were analysed by flow cytometry for surface expression of KIR2DL1. Specific staining (blue) is compared to isotype control (black). NKL cells were used as a negative control.

The YTS-KIR2DL1-tdEosFP cells were also fixed and stained with EB6 and then imaged by confocal microscopy to determine whether KIR2DL1 colocalised with tdEosFP (Figure 3.10). The secondary antibody used to visualise KIR2DL1 (Alexa 633) was detected in the red channel and the fluorescence emanating from tdEosFP was visualised in the green channel. The values for the Pearson's correlation coefficient (Rr) and Mander's overlap coefficient (R) were close to 1, indicating reliable colocalisation between the two channels. Untransfected YTS cells were used as a negative control and no fluorescence was detected in these samples (Figure 3.10A).

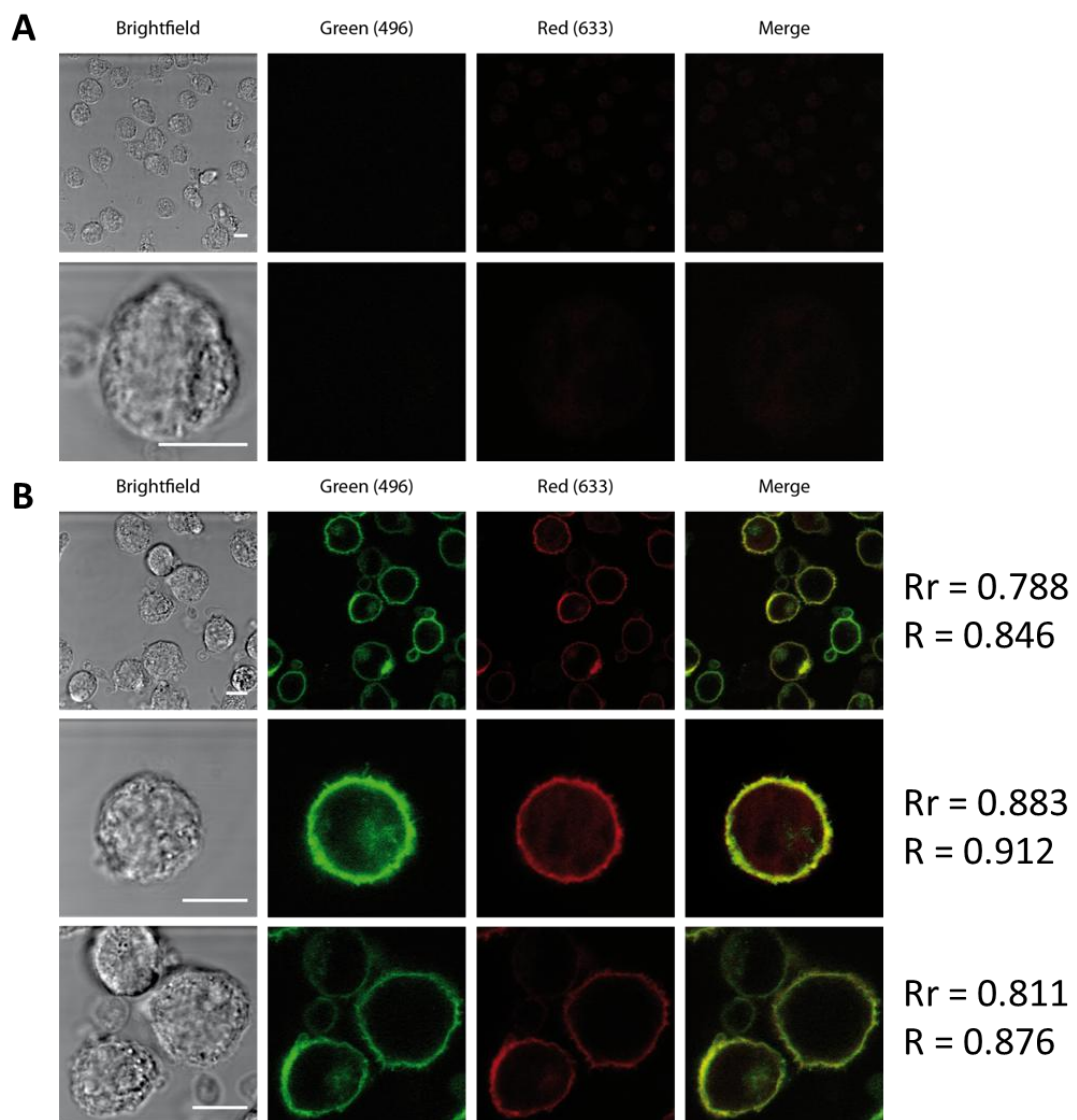


Figure 3.10: tdEosFP and KIR2DL1 colocalise in the membrane of YTS-KIR2DL1-tdEosFP cells. (A) YTS and **(B)** YTS-KIR2DL1-tdEosFP cells were fixed and stained with anti-KIR2DL1 EB6 antibody and Alexa 633 secondary antibody. Pearson's correlation coefficient (Rr) and Mander's overlap coefficient (R) are shown to the right of the corresponding images. Scale bars represent 10 μ m.

These experiments were also carried out using an anti-KIR2DL1 antibody supernatant obtained from Stipan Jonjić (Figure 3.11; anti-KIR2DL1 supernatants were screened for optimal detection of KIR2DL1 and compared to commercial EB6 antibody, see Appendix 2). These results show that a membrane protein could be detected in these cells using anti-KIR2DL1 antibodies, suggesting that KIR2DL1 was present in these cells, and that tdEosFP fluorescence was detected at the cell membrane, where KIR2DL1 is expressed.

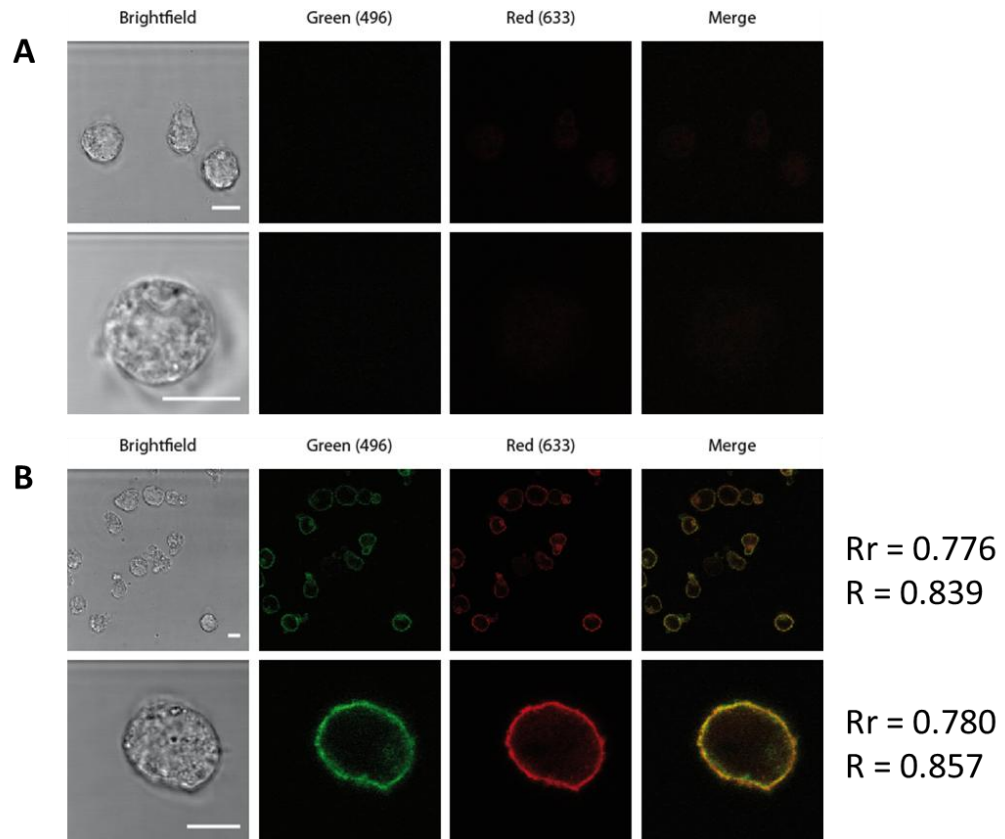


Figure 3.11: tdEosFP and KIR2DL1 colocalise in the membrane of YTS-KIR2DL1-tdEosFP cells, as confirmed with a different anti-KIR2DL1 antibody. YTS (A) and YTS-KIR2DL1-tdEosFP cells (B) were fixed and stained with anti-KIR2DL1 antibody supernatant (from Stipan Jonjić) and Alexa 633 secondary antibody, then imaged by confocal microscopy. Pearson's correlation coefficient (Rr) and Mander's overlap coefficient (R) are shown to the right of the corresponding images. Scale bars represent 10 μ m.

3.2.4 Functionality of the KIR2DL1-tdEosFP construct

Functionality of KIR2DL1

Since the KIR2DL1-tdEosFP construct was specifically generated for this study, it was necessary to show that the fusion protein resulting from the expression of the DNA construct was able to signal normally. For this purpose, YTS-KIR2DL1-tdEosFP and NKL-KIR2DL1-tdEosFP cells were tested in cytotoxicity assays with target cells expressing MHC class I proteins compared to target cells that do not express MHC class I molecules (Figure 3.12).

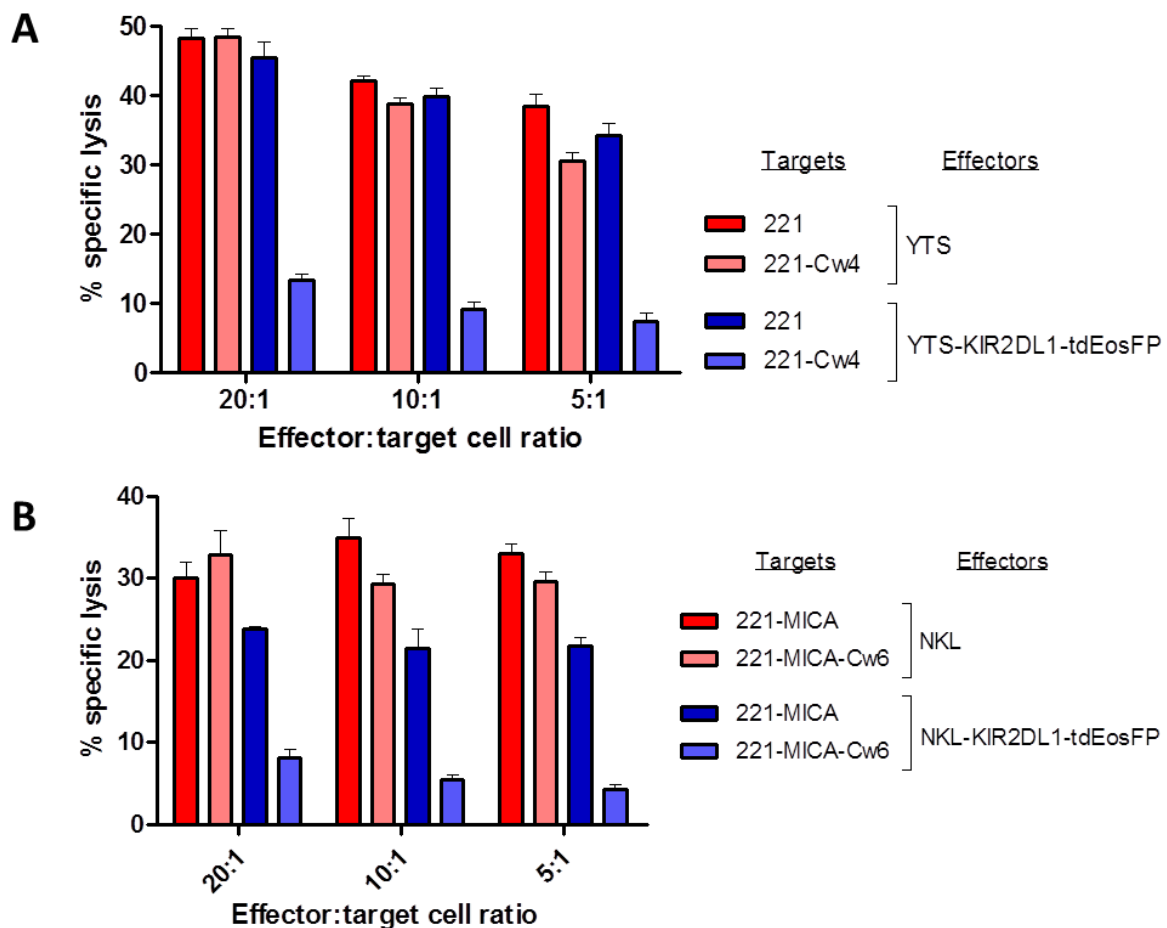


Figure 3.12: KIR2DL1 is functional in YTS-KIR2DL1-tdEosFP and NKL-KIR2DL1-tdEosFP cells. (A) YTS and YTS-KIR2DL1-tdEosFP cells were tested in cytotoxicity assays with 221 and 221-Cw4 target cells. **(B)** NKL and NKL-KIR2DL1-tdEosFP cells were tested in cytotoxicity assays with 221-MICA and 221-MICA-Cw6 target cells. The interaction between KIR2DL1 and Cw4 or Cw6 resulted in inhibition of NK cell cytotoxicity, showing that KIR2DL1 is functional in the transfected cells.

As described in the introduction, KIR2DL1 recognises specific MHC class I molecules, which include HLA-Cw4 and HLA-Cw6. In this assay, the target cells expressed one of these proteins, which should cause a decrease in susceptibility to lysis by the NK cells. Indeed, 221-Cw4 cells were significantly less susceptible to lysis by YTS-KIR2DL1-tdEosFP cells compared to 221 cells, which were efficiently killed by YTS-KIR2DL1-tdEosFP cells (Figure 3.12A). YTS cells were used as a positive control and efficiently killed 221 and 221-Cw4 cells at comparable levels. These results were observed at all three effector to target ratios used in the assay. In a similar fashion, 221-MICA-Cw6 cells were significantly less killed by NKL-KIR2DL1-tdEosFP than 221-MICA cells, with NKL cells used as a positive control (Figure 3.12B). NKL cells mainly kill via recognition through the activating receptor NKG2D; therefore the target cells used in this assay expressed MICA, one of the main ligands for NKG2D. These results demonstrate that the cells expressing KIR2DL1-tdEosFP were inhibited in the presence of MHC class I and thus the KIR2DL1-tdEosFP construct is functional.

Functionality of tdEosFP

In addition, it was essential to confirm the photoswitchable properties of the tdEosFP protein in the transfected cells. YTS-KIR2DL1-tdEosFP and NKL-KIR2DL1-tdEosFP cells were imaged by confocal microscopy. The cells were seen to express green fluorescence, confirming the flow cytometry results. Cells were then illuminated with the 405 nm UV laser for one minute and imaged again. The images clearly show that after exposure to UV light, the amount of green fluorescence was reduced in the cells, whereas the cells were now strongly visible in the red channel (Figure 3.13).

These preliminary photoswitching images and the results described above demonstrate that the YTS-KIR2DL1-tdEosFP and NKL-KIR2DL1-tdEosFP cells express a functional KIR2DL1 protein fused to tdEosFP that can be successfully photoconverted from green to red fluorescence upon illumination with UV light. These cells were therefore well suited for performing PALM microscopy under the appropriate imaging conditions.

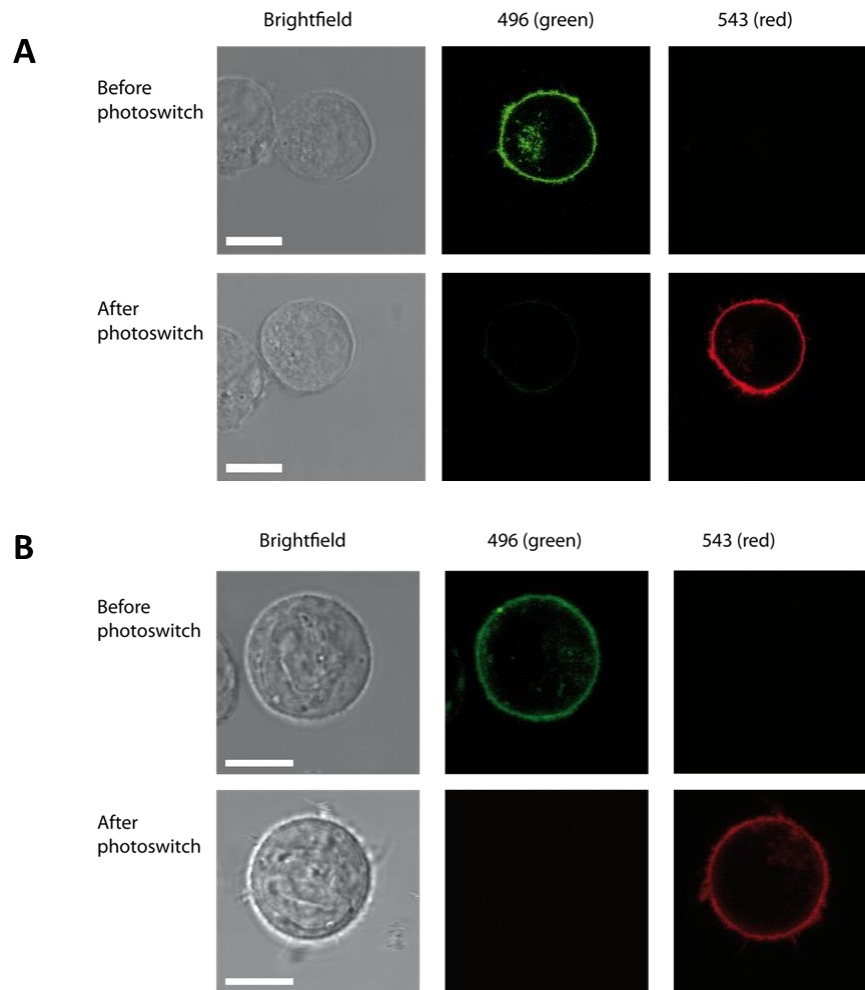


Figure 3.13: tdEosFP can be photoswitched from green to red fluorescence in YTS-KIR2DL1-tdEosFP and NKL-KIR2DL1-tdEosFP cells. (A) YTS-KIR2DL1-tdEosFP cells and **(B)** NKL-KIR2DL1-tdEosFP cells were imaged by confocal microscopy before (top panel) and after (lower panel) illumination with UV light.

3.3 Optimising PALM imaging

3.3.1 The set-up of the microscope

Members of the Imperial College Facility for Imaging by Light Microscopy (FILM facility), Martin Spitaler and Stephen Rothery, in collaboration with PhD student Mirella Koleva (co-supervised by Tony Magee and Mark Neil, Imperial College), were leading the work on setting up a microscope on which PALM (and STORM) microscopy can be performed. The final physical set-up of the microscope is depicted in Figure 3.14.

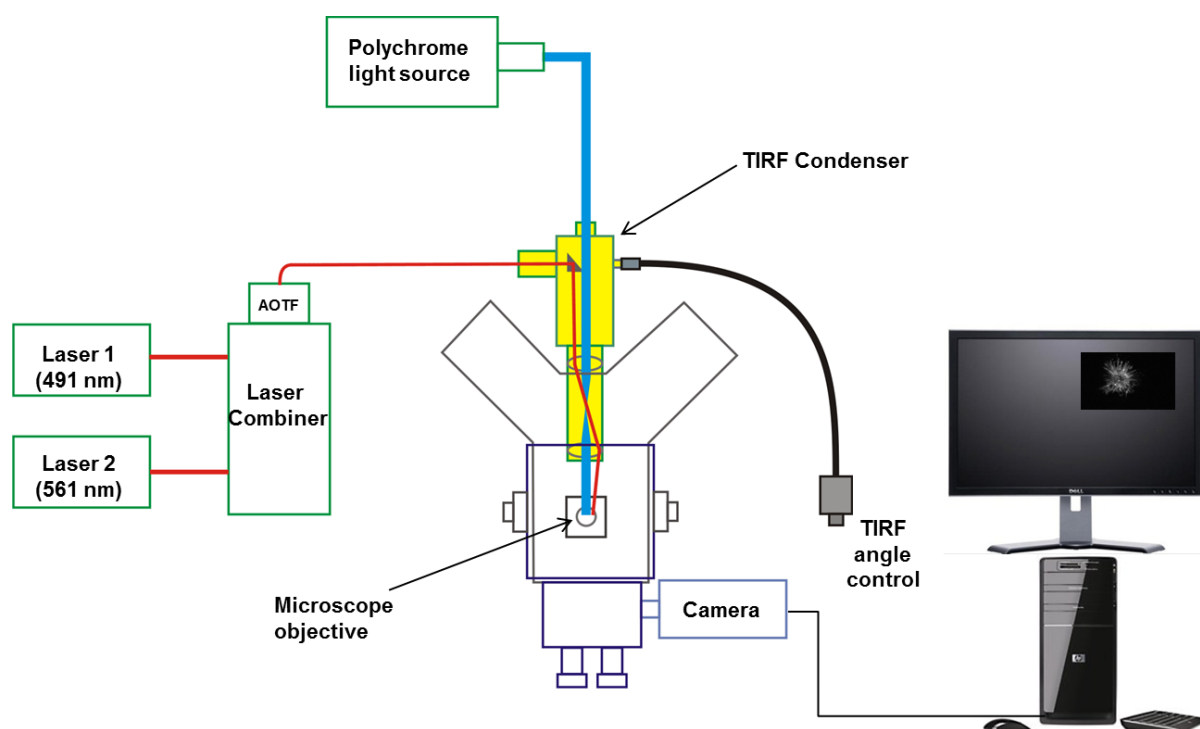


Figure 3.14: Schematic representation of the microscope set-up used for PALM imaging. Light emitted from the laser light sources is controlled using an acousto-optical tunable filter (AOTF). Within the TIRF condenser, the light beam is angled as controlled with the manual TIRF angle control, before reaching the sample. Illumination from the polychrome light source is used to photoconvert tdEosFP molecules within the sample. Fluorescence emission is recorded by a very sensitive camera and images are directly transmitted to the computer during acquisition. Based on a diagram from Stephen Rothery.

A widefield microscope was adapted by installing the necessary components for acquiring PALM datasets. The appropriate lasers were installed: laser 1 with a wavelength of 491 nm (for detecting the green form of tdEosFP) and laser 2 with a wavelength of 561 nm (for detecting the red form of tdEosFP). The laser beams are combined in a laser combiner and controlled using an acousto-optical tunable filter (or AOTF), which can be used to open or close the appropriate laser shutters. The TIRF condenser allows the easy adjustment of the laser beam to the correct illumination angle in the TIRF objective – this is achieved using a manual TIRF angle control, which adjusts the TIR angle as it is turned. The Polychrome Light Source is a monochromator, i.e. an optical device which can emit light of a specific wavelength (as selected on the Polychrome Manual Controller) chosen from a wide range of wavelengths, and was used for generating UV illumination to photoswitch tdEosFP molecules. The intensity of the beam is mechanically selectable. Images are recorded using a very fast and very sensitive camera and are then transmitted directly to the computer. With this microscope set-up it was possible to obtain high-resolution TIRF images (Figure 3.15). The next step

was to determine the settings and imaging protocol to achieve super-resolution PALM imaging on the same set-up.

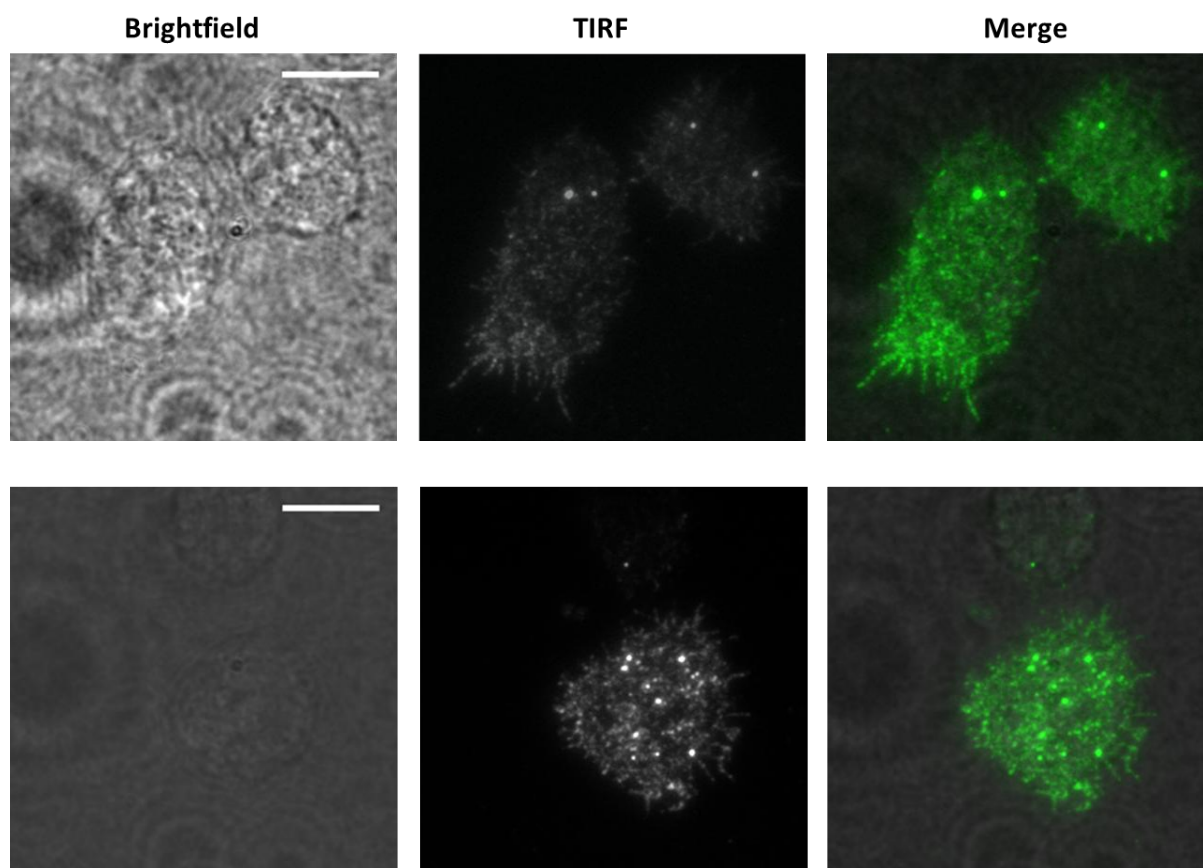


Figure 3.15: Preliminary TIRF images. NKL-KIR2DL1-tdEosFP cells were imaged by TIRF microscopy using the microscope set-up described above. In TIRF microscopy, only fluorophores close to the slide (within approximately 100 nm) are selectively illuminated. Hence, the fluorescence in the images above represents tdEosFP molecules that are at the interface of the cell with the slide. Scale bars represent 10 μm .

3.3.2 Optimisation of PALM imaging

Optimisation of PALM acquisitions

Initial PALM imaging experiments were carried out in order to optimise the acquisition settings on the microscope. YTS-KIR2DL1-tdEosFP cells were used in these preliminary experiments. The aim here was to get the right balance between photoswitching and photobleaching to achieve the best single-molecule localisation images using PALM. By carefully controlling the photoswitching laser

intensity as well as the excitation laser intensity, the rate at which molecules are activated and photobleached can be controlled. In an ideal PALM dataset, the number of active emitters in each frame is low enough to ensure that the distance between each active molecule and its nearest neighbour exceeds the lateral diffraction limit (i.e. 200-250 nm). High levels of excitation power are used to photobleach the molecules quickly, whereas the photoswitching beam is kept at low power to obtain non-overlapping subsets of activated molecules. Precisely controlling the density of fluorescent probes detected in each frame allows single-molecule imaging and localisation. In the set-up described above, this was achieved by using a laser power of 3 mW for the excitation laser (561 nm) and a power of 400 μ W for the photoswitching laser (UV). These measurements were taken at the back focal plane of the microscope and a non-negligible fraction of this light is reflected off the interface when imaging in TIRF mode.

The images were captured using a very sensitive camera and it was vital to optimise the camera acquisition settings to achieve the maximum signal-to-noise ratio (SNR) in each frame. The gain, sensitivity gain and exposure time were optimised for samples prepared with YTS-KIR2DL1-tdEosFP and the same settings were used for all subsequent experiments. Briefly, the gain was tested for values 1 and 2; the sensitivity gain was tested for values ranging from 175 to 1200; the exposure time was tested for durations of 0.01 s to 1 s. The final camera settings used were a gain of 1; a sensitivity gain of 1200 and an exposure time of 0.03 s. These settings generated the best SNR. In addition, this exposure time enabled the camera to detect the majority of the events occurring when using the aforementioned laser powers.

Optimisation of sample preparation

The methodology for sample preparation was also optimised. Different types of dishes and coating protocols were tested. For PALM, it is very important that the cells attach tightly to the slide and do not move, as cell drift would have a large impact on the localisation precision of the PALM acquisitions. The dishes used were MatTek and LabTek dishes. Different coatings were tested, including fibronectin (10 μ g.ml⁻¹), non-filtered and sterile filtered PLL (0.01% and 0.1%) and concentrated PLL (1%). The cells attached best on LabTek slides coated with filter-sterilised PLL (0.01%). All samples were then prepared on LabTek slides pre-coated with 0.01% sterile filtered PLL. For samples on antibody-coated slides, the antibodies were added on a background of PLL.

3.3.3 Controlling for drift

Due to the high localisation precision achievable by PALM, it is very important to minimise drift during PALM acquisitions. There are two types of drift that can affect the localisation of detected molecules: stage drift and cell drift.

Stage drift

Stage drift occurs when the stage starts to move in one direction due to fluctuations in temperature or physical vibrations. To minimise these short-term fluctuations, several important measures were included in the microscope set-up design, such as a draught-proof enclosure, covering the stage and objectives, and placing the microscope on a vibration isolation table. However, to ensure that the stage drift was indeed minimised by these precautions, several image sets were acquired with samples containing beads.

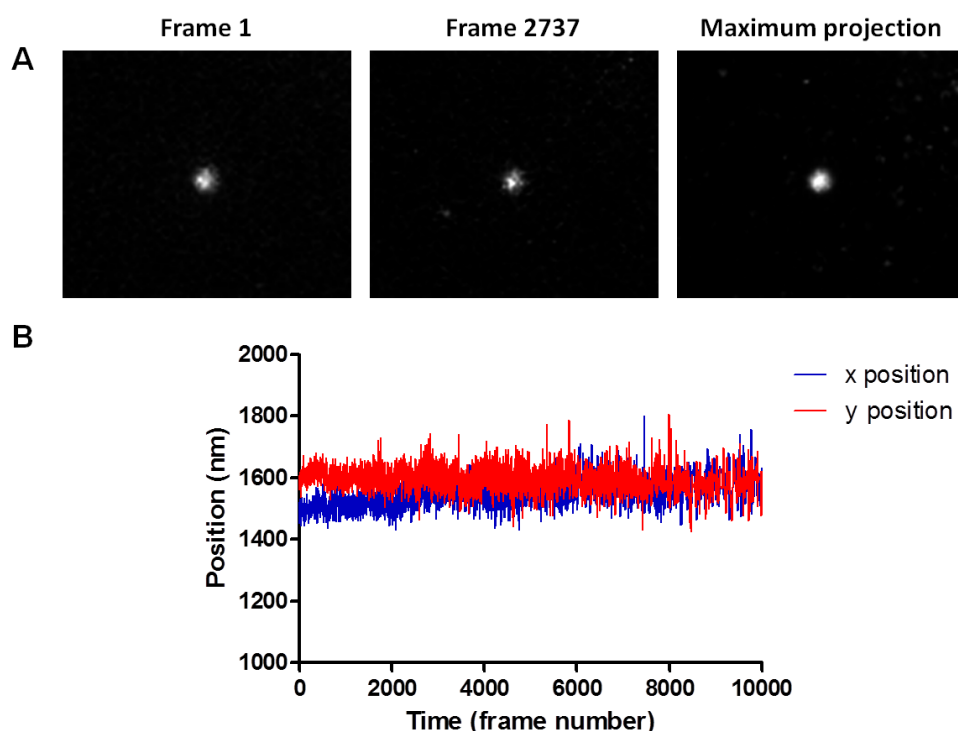


Figure 3.16: Testing stage drift by imaging beads. Beads (1 μm diameter) were imaged by TIRF microscopy over long periods of time equivalent to a PALM acquisition. **(A)** In this example, two frames from the acquisition are shown, as well as the maximum projection obtained from all the frames in the acquisition. **(B)** The centroid position of the bead (both in x and y coordinates) was plotted against time (measured in frame numbers).

Each dataset contained 2×10^4 frames, like a typical PALM dataset, and the movement of the beads was studied. In all cases, there was minimal movement of the beads observed, as visualised in a maximum projection of all frames from the dataset. For example, the maximum projection of the bead in Figure 3.16A does not show any evident drift – the bead's image in the maximum projection is not elongated in any direction, as would be the case if it had moved during the acquisition. In addition, the centroid position of the bead was plotted against time in Figure 3.16B and remained constant. Due to time restrictions, there was insufficient time to put in place a protocol using beads as fiduciary markers (to correct for drift during image reconstructions) and all experiments assumed that stage drift was minimal, as shown in the preliminary experiments described here.

Cell drift

Cell drift occurs when a cell itself is moving within the sample. This can happen if the cells have not properly attached to the slide. To minimise problems due to cell drift, a brightfield image was taken before and after each PALM acquisition. These two images were then superimposed to detect whether the cell had moved during the acquisition. In this indirect way, it could be determined whether the cell had moved or not during the acquisition for each PALM dataset. If the cell had not moved, the dataset was used in further analysis. If the cell had moved, the dataset was discarded. For example, in Figure 3.17 the cell did not move during the acquisition, as can be seen by the superposition of the two images – the whole cell becomes yellow, indicating a merge of the signals from the two brightfield images (pseudo-coloured as red and green respectively). The pre- and post-acquisition brightfield images were analysed in this way for each dataset before it was used for PALM reconstructions. Cells where green and red signal became apparent in the merged image, indicating cell drift, were discarded from further analysis.

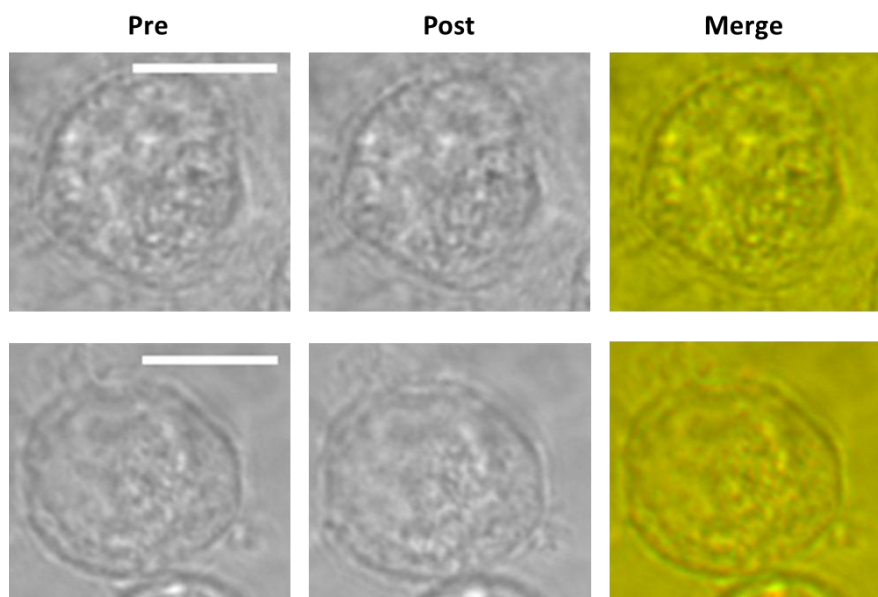


Figure 3.17: Testing cell drift by imaging cells in brightfield mode before and after PALM acquisitions. Cells were imaged in brightfield mode before and after each PALM acquisition to check for cell drift. Two examples are shown here, where the merged green-red image shows that the cells perfectly overlap in the pre- and post-acquisition images (as shown in yellow). Scale bars represent 10 μm .

3.3.4 PALM reconstructions and cluster analysis

PALM image reconstructions

Using the determined conditions for PALM imaging, PALM images were acquired as described in the Materials and Methods chapter. For each PALM reconstruction, a dataset containing 2×10^4 frames was processed using a reconstruction software (QuickPALM (Henriques et al., 2010)). Most PALM reconstruction algorithms rely on fitting Gaussian profiles to the detected diffraction-limited spots, allowing high-accuracy localisations but requiring several hours of processing time for a single dataset. The novel high-speed reconstruction algorithm used in this study uses a different approach. The QuickPALM algorithm is based on the classical Högbom 'CLEAN' method (Högbom, 1974) for detecting particles. This method was originally used in astronomy to detect individual stars in the night sky. In this reconstruction software, the spot-finding step is followed by an adapted centre-of-mass algorithm that computes the spot position and defines the spot shape. During image processing, a recorded PSF was determined to be a

valid single molecule event if the SNR was higher than the user-specified input value for SNR. Spots that were too close together in the same frame and appeared as one distended PSF were excluded from the reconstruction. In a first optimisation approach, different values were entered for the SNR and the full-width half maximum (FWHM). Finally, the best method for getting accurate reconstructions was to measure and calculate the SNR and FWHM for each dataset.

The reconstruction software (QuickPALM) provides a reconstructed PALM image as well as a table containing localisation information for each spot. Figure 3.18 shows a TIRF image and the corresponding PALM image for the same field, as obtained with the reconstruction software, with enlarged regions highlighting the resolution improvement seen with PALM. The particles table is essentially a collection of discrete molecular coordinates and contains all the important information describing a PALM dataset. Structural information of the labelled sample is therefore described as a discontinuous pattern of single molecular spots. This representation, in the style of pointillism, can compromise the representation of continuous biological structures such as actin filaments or microtubules. This pointillism is a direct consequence of the Nyquist-Shannon sampling theorem, which states that the labelling density should be double the spatial resolution in order to accurately detect underlying structural features, i.e. the sample should be labelled so that the distance separating two events corresponds to half the resolution (Shannon, 1949). For fine, continuous biological structures, it is difficult to achieve this level of labelling density, so the reconstruction of these structures contains gaps. However, PALM is an ideal method for the study of protein microclusters, as the particle coordinates generated by PALM can be quantitatively assessed by spatial point pattern analyses.

Resolution improvement by PALM

When comparing images obtained by TIRF and PALM microscopy, it became apparent that PALM could generate images of higher resolution, especially when zooming into a specific region of the cell membrane (Figure 3.18A). Indeed, in PALM images, every single dot corresponds to a single molecular event detected during acquisition and hence represents a single molecule. In contrast, the TIRF images appear blurred and less detailed. To directly compare the resolution obtainable from TIRF and PALM, profile plots are shown in Figure 3.18B. Critically, the use of PALM microscopy

revealed finer details of the distribution of KIR2DL1. PALM microscopy therefore provides the ability to detect KIR2DL1 molecules at higher resolution than previously obtainable with widefield or TIRF microscopy.

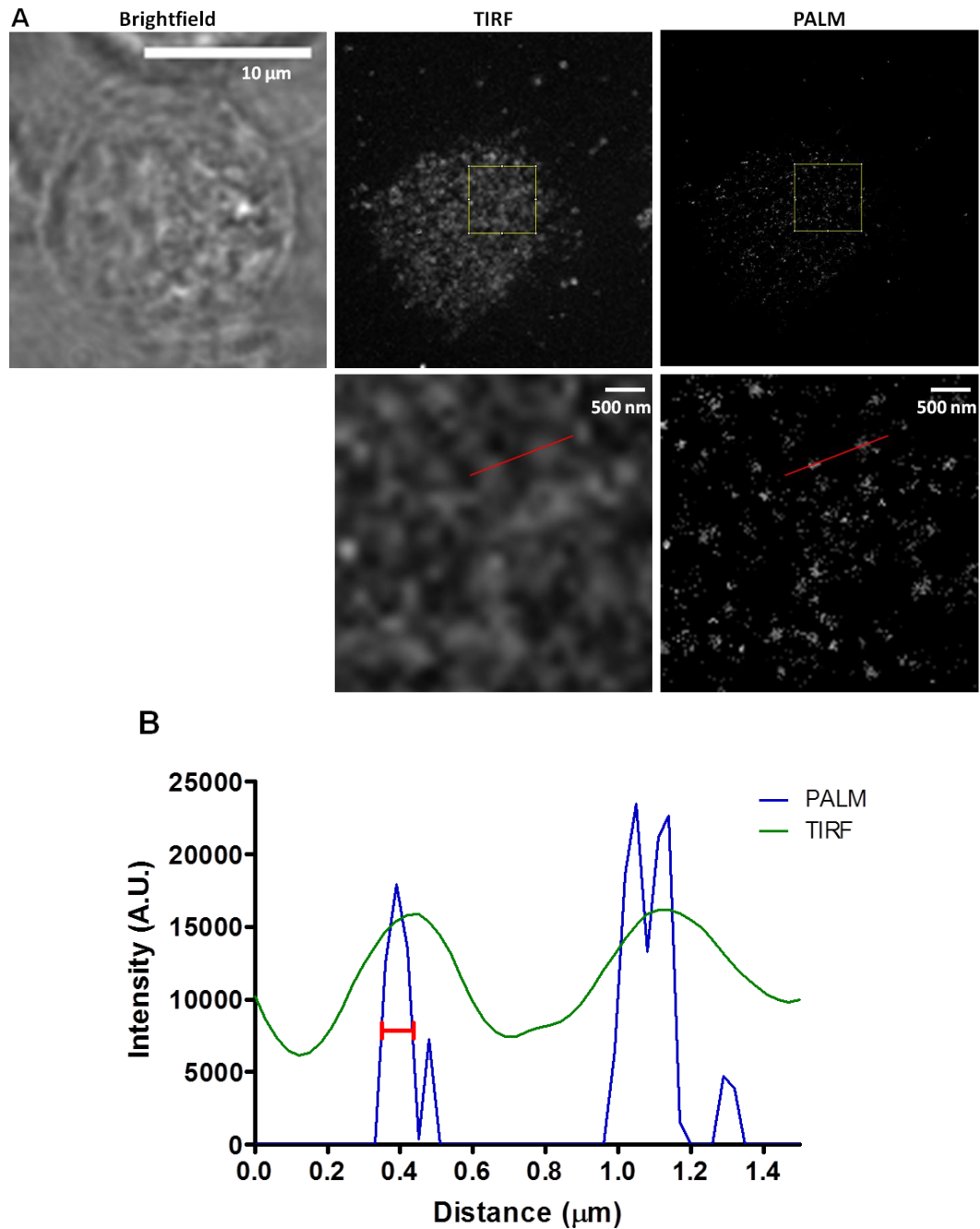


Figure 3.18: Resolution improvement with PALM. (A) Top row: Full-frame images of the same cell imaged in brightfield, by TIRF microscopy and by PALM. Scale bars represent 10 μm . **Bottom row:** The same region was selected in the TIRF and PALM images (yellow box) and the enlarged region is shown in the bottom row. Scale bars represent 500 nm. **(B)** Plot profiles to directly compare the TIRF and PALM regions marked by a red bar in (A). Estimation of the FWHM (red bar on the graph) from the Gaussian fit of the PALM data gives a resolution of approximately 80 nm.

Cluster analysis

The cluster analysis method used in this study is based on Ripley's K function. Ripley's K function is a second-order point pattern analysis that centres circles of sequentially increasing radius on each point and essentially counts the number of other points in this circle, which is then normalised to the average molecular density of the entire region (see Materials and Methods for more details). This method essentially quantifies the level of clustering in a region. This can be plotted as in Figure 3.19B. When $L(r) - r$ is positive, the points are more clustered than expected from a random distribution; when $L(r) - r$ is negative, the distribution is less clustered than would be expected from randomly distributed events.

Clustering was sampled on a scale of 50 nm because this represents approximately half the size of the peak clustering spatial scale observed in the Ripley's K function plot for KIR2DL1 in resting NK cells (Figure 3.19B). In these cluster colour maps, high levels of clustering are represented in red, whereas low levels of clustering are in blue. A threshold was applied to cluster maps to create binary maps, from which information about the clusters can be extracted. Figure 3.19C shows an example of a cluster map with its corresponding binary map. The main parameters that can be extracted from these analyses about the distribution of KIR2DL1 are as follows: the number of clusters per μm^2 , the average diameter and the circularity of clusters, the total number of molecules per μm^2 and the percentage of molecules in clusters, and finally the number of molecules per cluster. Comparing these parameters under different conditions could potentially reveal how the organisation of protein microclusters is altered at the nanoscale level in different situations.

3.4 Results

3.4.1 Basal level of clustering in non-stimulated YTS and NKL cells

The organisation of KIR2DL1 was investigated in resting YTS-KIR2DL1-tdEosFP and NKL-KIR2DL1-tdEosFP cells. Cells were incubated for 15 minutes on slides pre-coated either with PLL or with an isotype control antibody such as IgG1 on a background of PLL, then fixed with paraformaldehyde. Resting cells were imaged and analysed by TIRF microscopy and PALM (Figure 3.19A). The images, both by TIRF and by PALM, show that KIR2DL1 was clustered even in

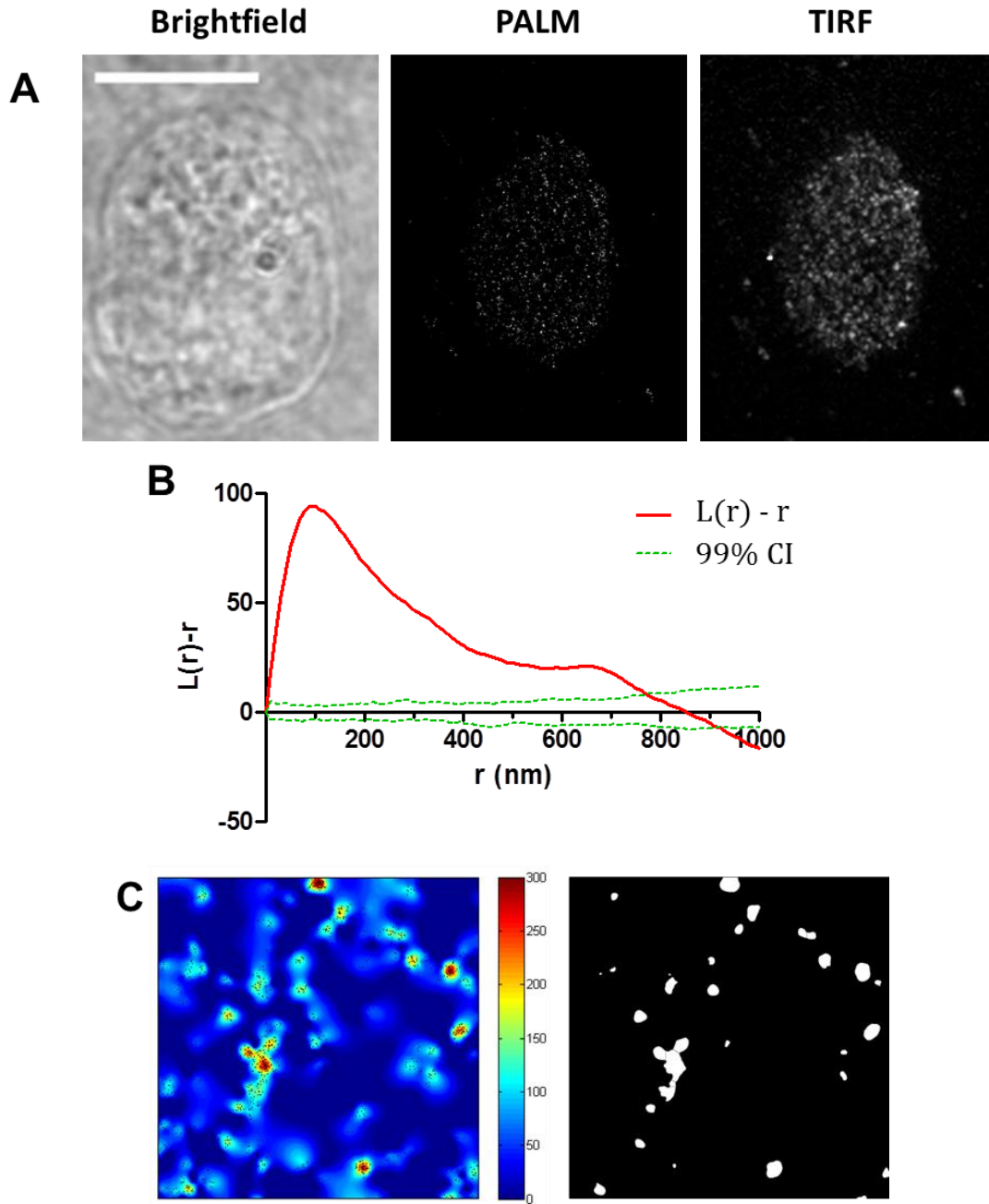


Figure 3.19: KIR2DL1 is pre-clustered in non-stimulated NK cells. (A) YTS-KIR2DL1-tdEosFP cells were imaged by PALM on PLL-coated slides. Scale bar represents 10 μm . (B) Ripley's K function plot (red) was generated for KIR2DL1-tdEosFP in YTS cells imaged on PLL-coated slides. This plot was generated from all points in a region, describing spatial clustering across the entire region. The peak of this curve indicates the spatial scale at which molecules are considered to be clustered. The point at which the curve crosses the x-axis indicates the maximum spatial scale at which molecules are clustered. Confidence interval curves (green) were obtained as described in the main text. (C) Cluster map of the same membrane region (left) with colour indicating the degree of local clustering, from low (blue) to high (red), and corresponding binary map (right). These maps correspond to a $3 \mu\text{m} \times 3 \mu\text{m}$ region selected from the full-frame image.

non-stimulated, resting NK cells. The distribution of KIR2DL1 was analysed by Ripley's K function. These plots confirmed that KIR2DL1 was highly pre-clustered in the plasma membrane of resting cells and that the majority of the clusters were below the diffraction-limited resolution of conventional fluorescence microscopy (Figure 3.19B). KIR2DL1 clustering was compared against the clustering of randomly distributed molecules, indicated by the dashed lines representing the 99% confidence intervals (99% CI). These confidence intervals were obtained by running 100 rounds of stochastic simulations of random distributions with the same number of events as the dataset studied. The value for $L(r) - r$ is much higher for the data than for the random simulations. $L(r) - r$ rises sharply as the distance from a specific point (r) increases, indicating that molecules are surrounded by many close neighbours. This clustering effect peaks at a spatial scale of about 100 nm and beyond approximately 100 nm, KIR2DL1 molecules become less clustered. Negative values for $L(r) - r$ are observed beyond approximately 700-800 nm and indicate dispersal of KIR2DL1 clusters. In addition, the cluster and binary maps of regions selected within the cell also show a basal level of KIR2DL1 clustering in these cells (Figure 3.19C). These results support the hypothesis that the protein KIR2DL1 is pre-clustered in the membrane of resting NK cells on control surfaces.

Ripley's K function is a good method for obtaining a global view of molecular clustering in a certain region, but as described above, further key information about the clusters can be extracted from an extended cluster analysis using data from a large number of regions rather than just one region. Figure 3.20 displays the various measurements of KIR2DL1 clustering obtained from cluster analysis of the PALM data tables. These results show that KIR2DL1 molecules were present at the membrane surface of resting YTS-KIR2DL1-tdEosFP cells (on PLL-coated slides) at a density of 97.7 ± 15.2 (mean $\pm$ SEM) molecules per μm^2 ($n=25$). From this total population, $39.2 \pm 2.7\%$ of all molecules were contained within clusters. KIR2DL1 cluster density was measured as 3.29 ± 0.22 clusters per μm^2 .

Cluster circularity was 0.75 ± 0.01 , where a perfect circle has a circularity value of 1. These values remained reasonably constant in all experimental conditions and this meant that an approximation of cluster diameter could be determined by assuming that all the clusters were circular. Hence, cluster diameter was calculated according to Equation 3.1.

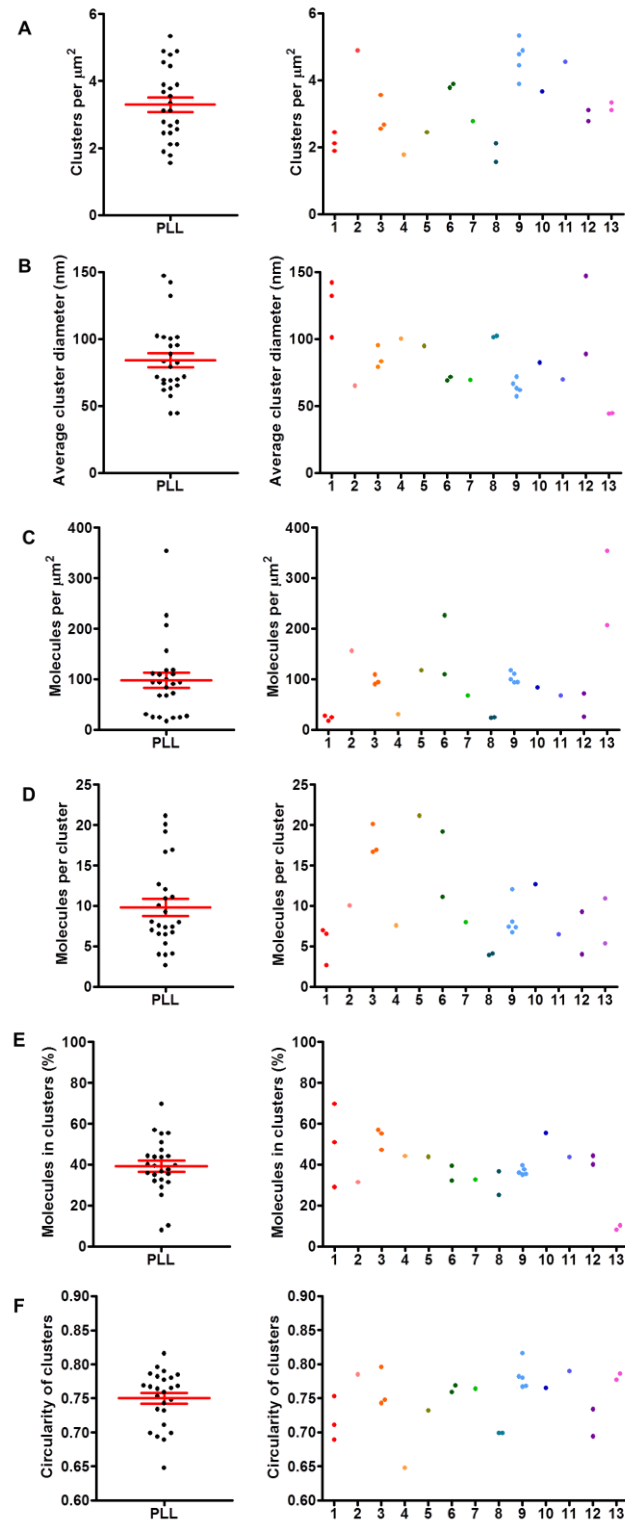


Figure 3.20: Heterogeneity within and between cells. YTS-KIR2DL1-tdEosFP cells were imaged by PALM on PLL-coated slides. Cluster analysis of regions from the PALM images generates information about **(A)** the cluster density, **(B)** the average cluster diameter, **(C)** the molecular density, **(D)** the number of molecules per cluster, **(E)** the percentage of molecules contained in clusters and **(F)** the circularity of clusters. For each panel, the graph on the left represents the summarised data, whereas in the graph on the right, data from each different cell are grouped together in the same colour.

Equation 3.1: $d = 2 \sqrt{\frac{A}{\pi}}$

Where d is the cluster diameter and A is the cluster area. The average cluster diameter was 84.2 ± 5.4 nm. Similar numbers were obtained for all these parameters in experiments with YTS-KIR2DL1-tdEosFP cells on IgG1-coated slides and with NKL-KIR2DL1-tdEosFP cells on PLL-coated and IgG1-coated slides.

In Figure 3.20, the data for YTS-KIR2DL1-tdEosFP cells imaged on PLL were studied in further detail to gather information on data heterogeneity between cells and between regions of the same cell. One to six regions were selected from each cell for the cluster analysis. In Figure 3.20, the regions from the same cell are shown in the same colour, with 13 cells studied in total. There was some variability for regions analysed from the same cell, whereas the data were more heterogeneous over several cells, as quantified in Table 3.1. In order to compensate for this variability, high n numbers were used in all subsequent experiments.

Parameter	Average	Standard deviation within cells	Standard deviation between cells
Clusters/ μm^2	3.29 ± 0.22	0.36	1.09
Average cluster diameter	84.2 ± 5.4	17.9	27.0
Molecules/ μm^2	97.7 ± 15.2	50.4	79.2
Molecules/cluster	10.6 ± 0.52	3.3	5.3
% molecules in clusters	39.2 ± 2.7	8.9	13.5
Circularity of clusters	0.75 ± 0.01	0.02	0.04

Table 3.1: Variability between cells and between regions of the same cell. The average values of different parameters describing the nanoscale organisation of KIR2DL1 are given, along with the standard deviation calculated for regions of the same cell (and averaged across several cells) and the overall standard deviation of the data from all cells.

To conclude, in resting, unstimulated NK cells, KIR2DL1 molecules were found to be present in pre-existing nanoclusters. The clusters of KIR2DL1 were in the order of 85 nm in size and contained approximately 40% of the total KIR2DL1 protein.

3.4.2 KIR2DL1 clusters get smaller and denser upon receptor ligation

Organisation of KIR2DL1 on anti-KIR2DL1-coated slides

The next aim was to determine whether changes in the organisation of KIR2DL1 clusters could be detected using PALM. YTS-KIR2DL1-tdEosFP and NKL-KIR2DL1-tdEosFP cells were imaged by PALM on slides coated with a monoclonal anti-KIR2DL1 antibody (EB6) in order to directly ligate KIR2DL1. Brightfield, TIRF and PALM images are shown in Figure 3.21A. Visual inspection of the cluster maps and binary maps suggests that the size of clusters was decreased in samples on EB6-coated slides compared to the isotype control (Figure 3.21C). To test whether or not the organisation and clustering of KIR2DL1 were affected by receptor ligation, the PALM data were further analysed.

The total concentration of KIR2DL1 molecules at the interface with the slide was higher on EB6-coated slides than on slides coated with isotype control (Figure 3.22B). In cells imaged on EB6-coated slides, a density of 164.0 ± 13.8 molecules per μm^2 was detected ($n=54$), compared to 81.2 ± 6.9 molecules per μm^2 on IgG1-coated slides ($n=33$). This difference was statistically significant ($p<0.0001$), suggesting that significantly more KIR2DL1 molecules are recruited to the immune synapse when KIR2DL1 is engaged.

The density of clusters was 3.24 ± 0.10 clusters per μm^2 on IgG1-coated slides, compared to 3.27 ± 0.09 clusters per μm^2 on EB6-coated slides ($p = 0.8347$; Figure 3.22A). Thus, the cluster density was not affected by receptor ligation. However, other changes in cluster properties were affected. Notably, the number of molecules per cluster increased from 9.45 ± 0.67 in resting cells to 12.00 ± 0.59 in cells on EB6-coated slides ($p = 0.0064$; Figure 3.22D), indicating that the clusters are more dense when KIR2DL1 is ligated. In addition, the average cluster diameter decreased by 15% in cells on EB6-coated slides (71.7 ± 2.0 nm) compared to cells on IgG1-coated slides (83.9 ± 3.4 nm; $p = 0.0011$; Figure 3.22C).

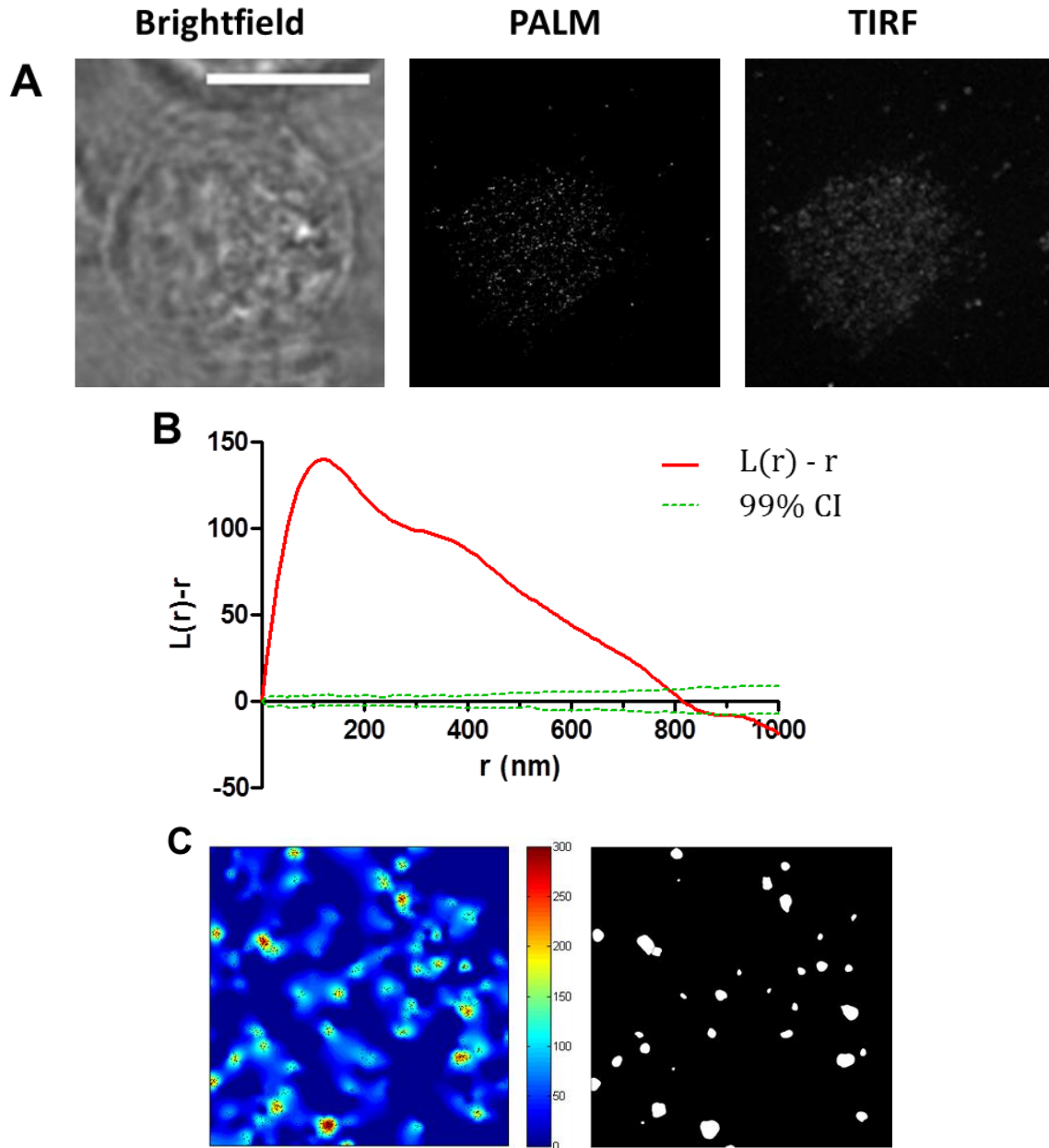


Figure 3.21: Clustering of KIR2DL1 in YTS cells on EB6-coated slides. (A) YTS-KIR2DL1-tdEosFP cells were imaged by PALM on slides coated with anti-KIR2DL1 antibody (EB6). Scale bar represents 10 μm . (B) Ripley's K function plot (red) was generated for KIR2DL1-tdEosFP in YTS cells imaged on EB6-coated slides. This plot was generated as described in the main text. (C) Cluster map of the same membrane region (left) with colour indicating the degree of local clustering, from low (blue) to high (red), and corresponding binary map (right).

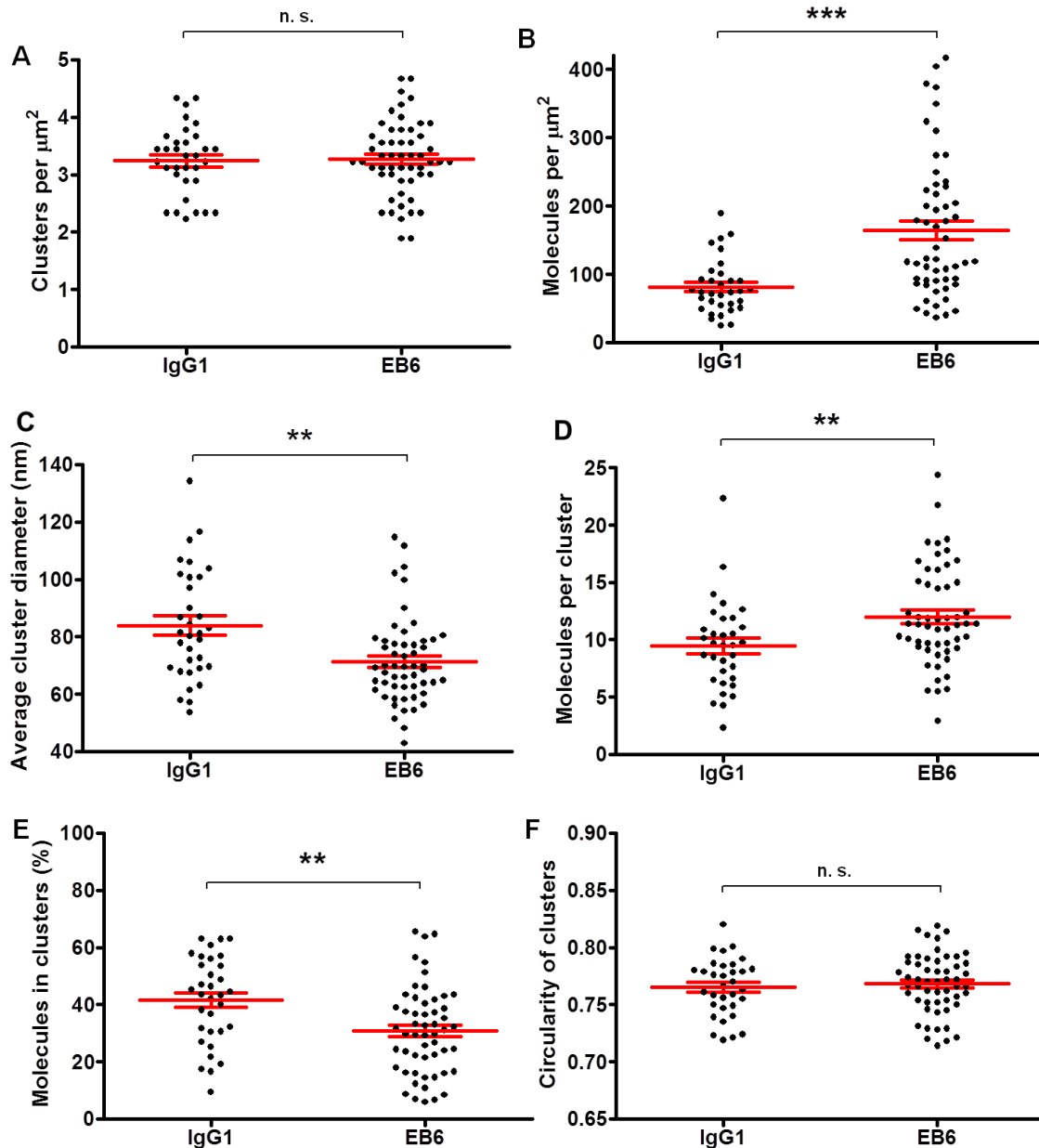


Figure 3.22: KIR2DL1 clusters get smaller and denser upon ligation by monoclonal antibody in YTS cells. YTS-KIR2DL1-tdEosFP cells were imaged by PALM on EB6-coated slides. Cluster analysis of regions from the PALM images generates information about **(A)** the cluster density, **(B)** the molecular density, **(C)** the average cluster diameter, **(D)** the number of molecules per cluster, **(E)** the percentage of molecules contained in clusters and **(F)** the circularity of the clusters. Significance was determined using the student's t-test.

The percentage of KIR2DL1 molecules that contribute to clusters was decreased, from $41.6 \pm 2.6\%$ in resting cells to $30.8 \pm 2.1\%$ in cells on EB6-coated slides ($p = 0.0018$; Figure 3.22E). Clusters remained round in shape after receptor ligation (circularity: 0.77 ± 0.004 in resting cells

versus 0.77 ± 0.003 in cells on EB6-coated slides; $p = 0.5758$; Figure 3.22F). Cluster circularity remained constant in all subsequent imaging experiments.

Similar changes in cluster properties were found when NKL-KIR2DL1-tdEosFP cells were imaged by PALM on IgG1- and EB6-coated slides (Figures 3.23 & 3.24). The only different result was that no significant change was detected in the number of molecules per cluster after KIR2DL1 ligation in NKL-KIR2DL1-tdEosFP cells.

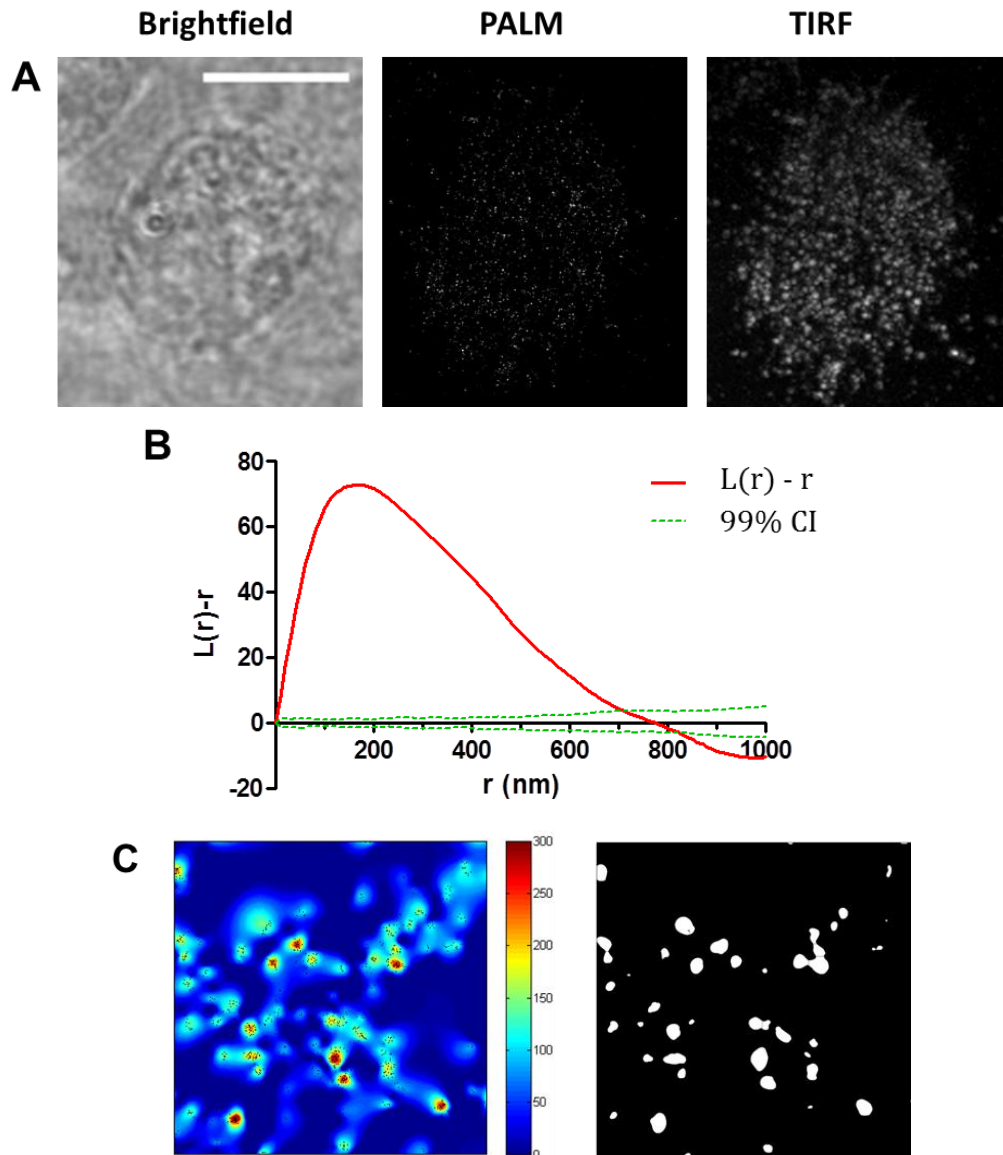


Figure 3.23: Clustering of KIR2DL1 in NKL cells on EB6-coated slides. (A) NKL-KIR2DL1-tdEosFP cells were imaged by PALM on slides coated with anti-KIR2DL1 antibody (EB6). Scale bar represents 10 μm . **(B)** Ripley's K function plot (red) was generated for KIR2DL1-tdEosFP in NKL cells imaged on EB6-coated slides. This plot was generated as described in the main text. **(C)** Cluster map of the same membrane region (left) with colour indicating the degree of local clustering, from low (blue) to high (red), and corresponding binary map (right).

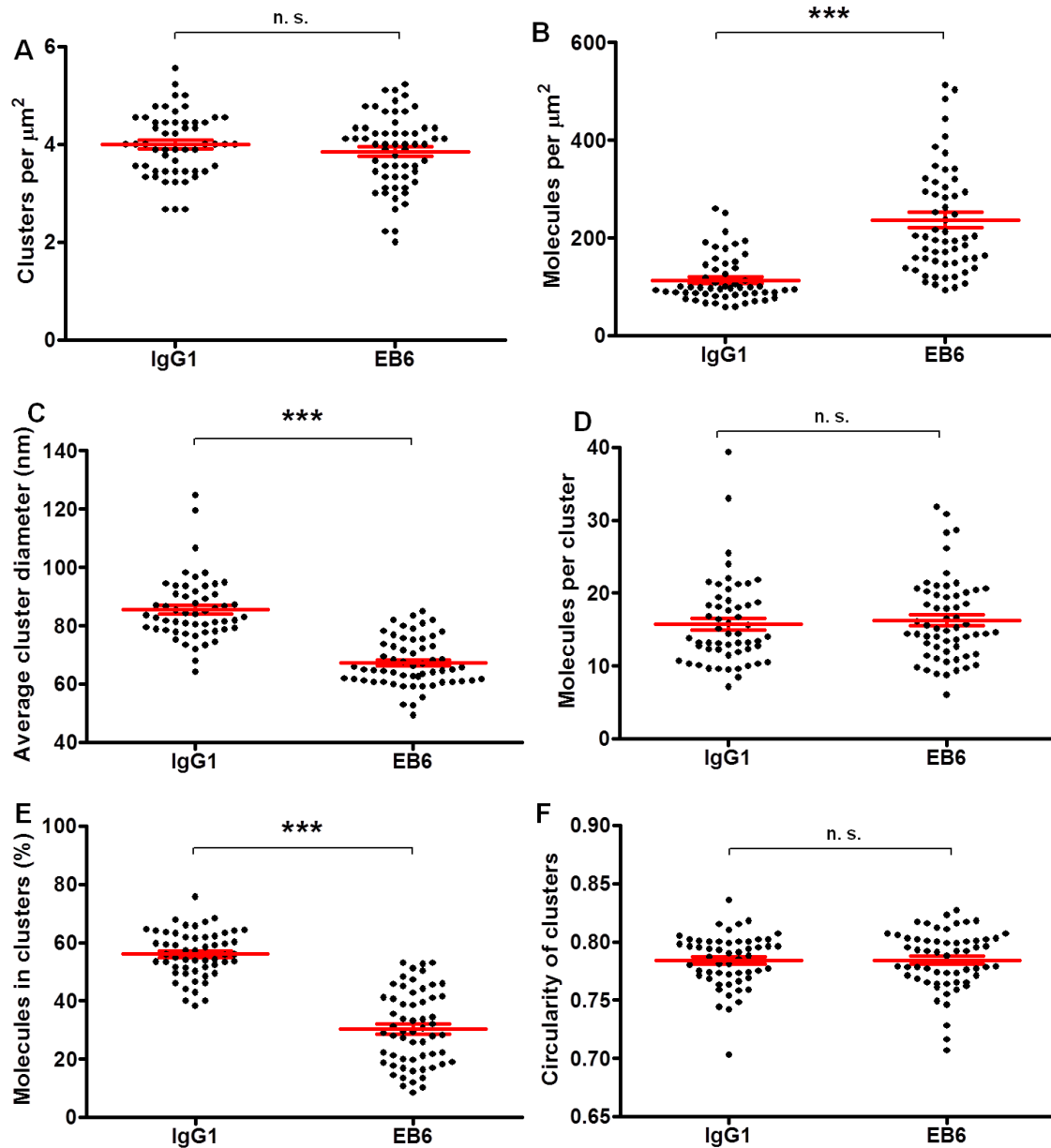


Figure 3.24: KIR2DL1 clusters get smaller and denser upon ligation by monoclonal antibody in KNL cells. NKL-KIR2DL1-tdEosFP cells were imaged by PALM on EB6-coated slides. Cluster analysis of regions from the PALM images generates information about **(A)** the cluster density, **(B)** the molecular density, **(C)** the average cluster diameter, **(D)** the number of molecules per cluster, **(E)** the percentage of molecules contained in clusters and **(F)** the circularity of the clusters. Significance was determined using the student's t-test.

Overall, these data show that KIR2DL1 clusters get smaller and denser upon receptor ligation. These results are important because they show that (i) nanometer-scale changes in KIR2DL1 organisation can be detected using this methodology and (ii) direct ligation of KIR2DL1 by monoclonal antibody causes a re-organisation of its distribution at the single molecule level.

Organisation of KIR2DL1 on anti-CD28-coated slides

As a control, YTS-KIR2DL1-tdEosFP cells were imaged by PALM on slides coated with an antibody against a different receptor present on YTS cells - CD28. YTS and YTS-KIR2DL1-tdEosFP cells were analysed by flow cytometry for the surface expression of CD28 (Figure 3.25). CD28 was indeed expressed on these cells, although it was absent from NKL cells, as expected. CD28 is an activating NK cell receptor, but ligation of CD28 alone is not sufficient for activating NK cell cytotoxicity. Activation requires co-ligation of the adhesion molecule LFA-1 (Azuma et al., 1992; Burshtyn et al., 2000). Ligation of CD28 should not activate the cells and was therefore used as a control to see the effect of ligating a different receptor.

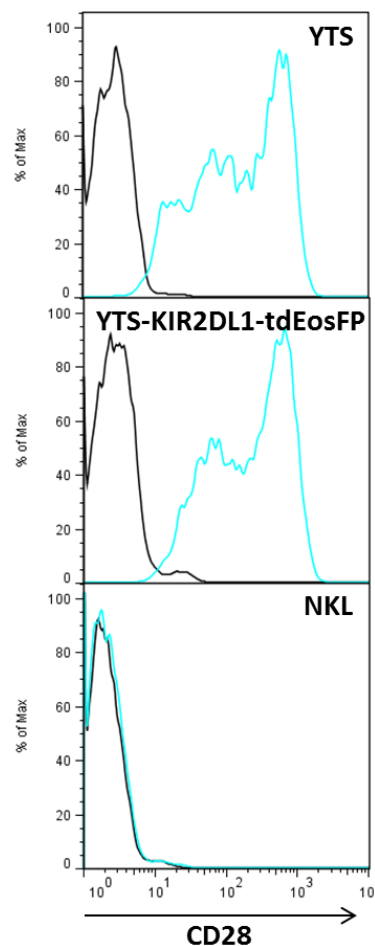


Figure 3.25: Surface expression of CD28 on NK cells. YTS, YTS-KIR2DL1-tdEosFP and NKL cells were analysed by flow cytometry for surface expression of CD28. Specific staining (blue) is compared to isotype control (black).

The ligation of CD28 in YTS-KIR2DL1-tdEosFP cells did not alter the organisation or the cluster properties of KIR2DL1 (Figure 3.26), although there was a slight increase in the molecular density of KIR2DL1 (from 81.2 ± 6.9 molecules per μm^2 in resting cells to 106.2 ± 8.4 molecules per μm^2 in cells on anti-CD28-coated slides; $p = 0.0379$; $n = 50$). Overall there was very little effect on the organisation of KIR2DL1 when the CD28 receptor was ligated.

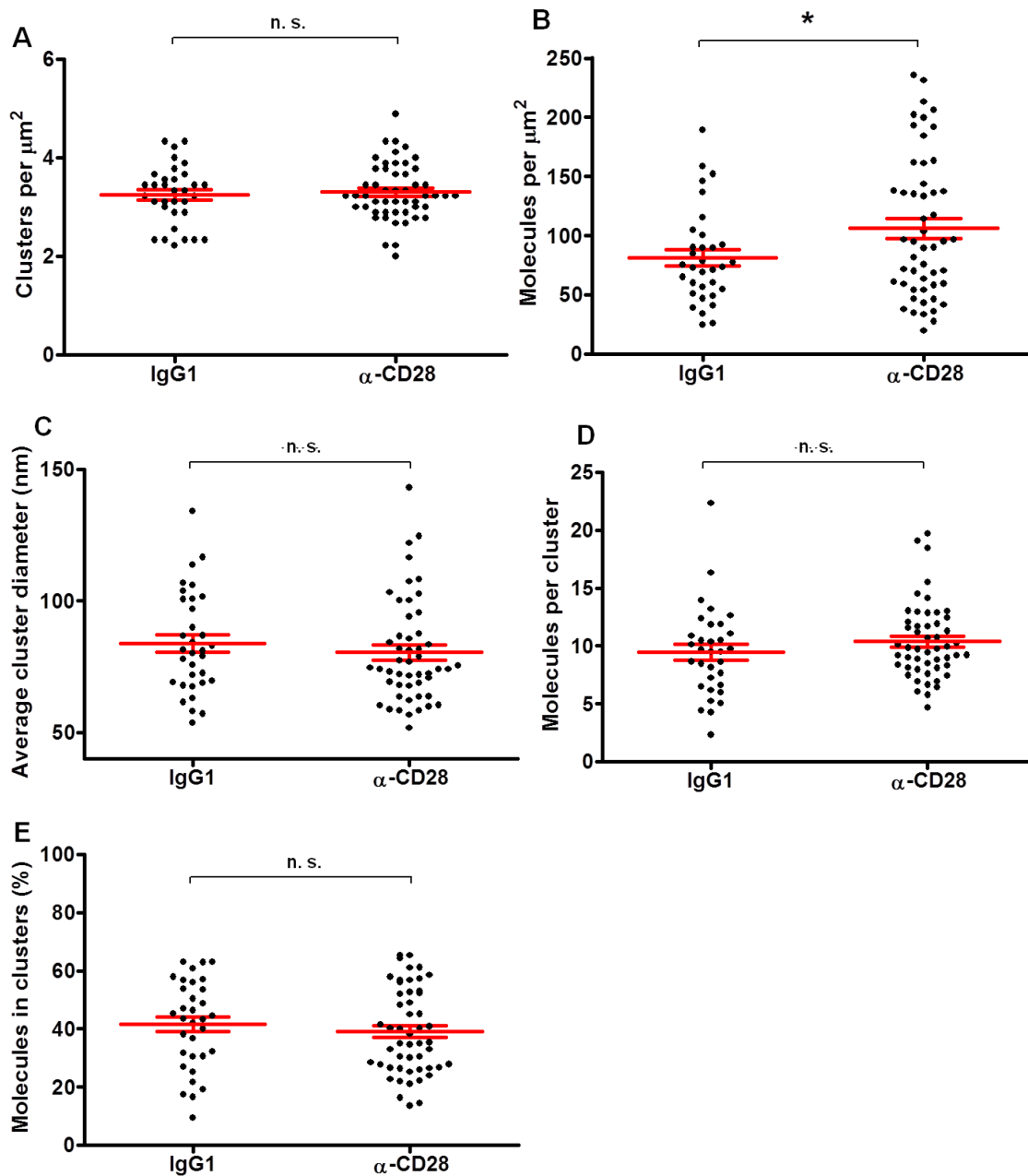


Figure 3.26: No changes in KIR2DL1 clustering occur upon CD28 ligation. YTS-KIR2DL1-tdEosFP cells were imaged by PALM on slides coated with anti-CD28 antibody. Cluster analysis of regions from the PALM images generates information about **(A)** the cluster density, **(B)** the molecular density, **(C)** the average cluster diameter, **(D)** the number of molecules per cluster, and **(E)** the percentage of molecules contained in clusters. Significance was determined using the student's t-test.

3.4.3 Actin polymerisation is not required for the nanoscale organisation of KIR2DL1

To test whether actin polymerisation was required for the change in the organisation of KIR2DL1 induced by the anti-KIR2DL1 antibody EB6, YTS-KIR2DL1-tdEosFP cells were treated with Latrunculin A (Lat A), a drug which inhibits actin polymerisation.

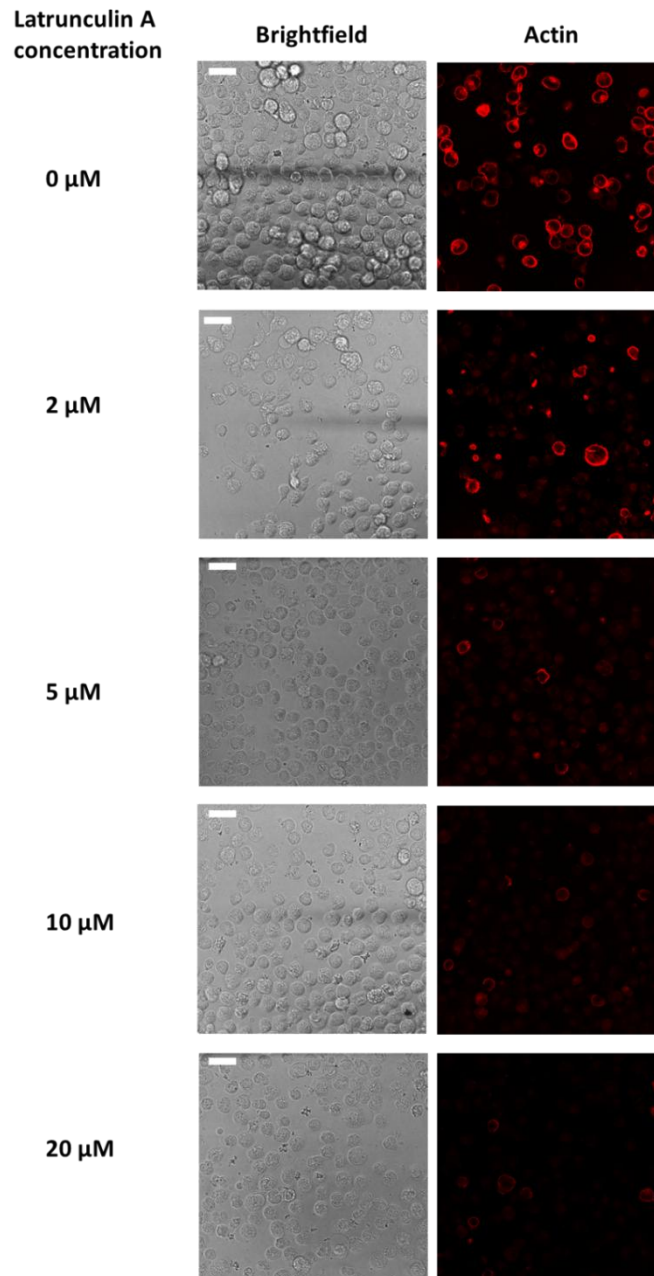


Figure 3.27: Latrunculin A destroys the integrity of the actin cytoskeleton. YTS-KIR2DL1-tdEosFP cells were treated with different concentrations of Latrunculin A and were then fixed and stained with Phalloidin-Atto647N. The cells were then imaged by confocal microscopy. Scale bars represent 15 μm .

In a control experiment, the cells were treated with different concentrations of Lat A (0, 2, 5, 10 and 20 μM) to determine the appropriate concentration, i.e. the lowest concentration at which actin polymerisation is disrupted. Following the incubation with Lat A, the cells were fixed and stained with phalloidin conjugated to a fluorescent dye (Atto647N) on PLL-coated slides and then imaged by confocal microscopy. Phalloidin is a toxin that binds filamentous actin with high affinity and is frequently used as a probe to visualise actin. The results presented in Figure 3.27 confirmed that Lat A has a detrimental effect on the integrity of the actin cytoskeleton, as shown by the reduction in the number of cells with intact actin structures. The effect of Lat A at a concentration of 2 μM was already very striking, with more than 90% of cells being affected, and hence this concentration was chosen for treating cells in subsequent experiments.

YTS-KIR2DL1-tdEosFP cells were treated with 2 μM of Lat A, fixed on EB6-coated slides and imaged by PALM ($n = 61$). Treatment with this drug did not affect the nanoscale organisation of KIR2DL1 as compared to samples on EB6-coated slides without Lat A. There was no significant difference in the number of molecules detected at the interface or in the cluster properties, as shown in Figure 3.28. These results indicate that the reorganisation of KIR2DL1 induced by EB6 occurs independently of actin polymerisation.

3.4.4 Activation of NK cells can induce reorganisation of KIR2DL1

In order to test whether there is a link between different receptors expressed at the cell surface, it was important to investigate whether the ligation of other receptors could also influence the nanoscale organisation of KIR2DL1. Specifically, the aim was to study whether activating the NK cells via different receptors could change the distribution of KIR2DL1 at the membrane contacting the coated slide.

Activation of NKL cells via ligation of NKG2D

NKL-KIR2DL1-tdEosFP cells were imaged by PALM on slides coated with anti-NKG2D antibody. NKG2D is an activating NK cell receptor expressed by NKL cells. Surface expression of NKG2D on NKL and NKL-KIR2DL1-tdEosFP cells was confirmed by flow cytometry, whereas NKG2D was not

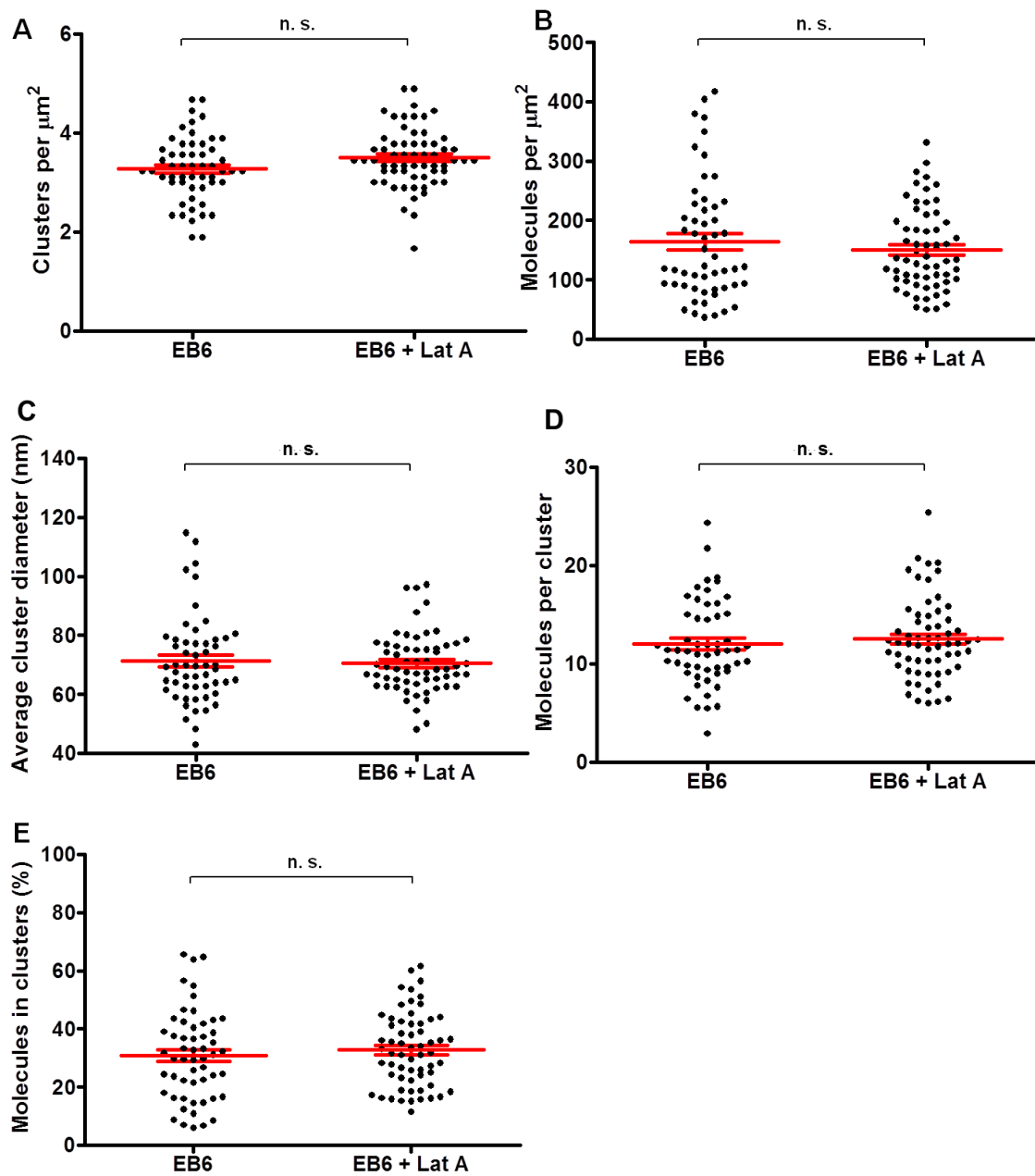


Figure 3.28: Actin polymerisation is not required for the reorganisation of KIR2DL1 induced by receptor ligation. YTS-KIR2DL1-tdEosFP cells were treated with Lat A then imaged by PALM on slides coated with anti-KIR2DL1 antibody. Cluster analysis of regions from the PALM images generates information about **(A)** the cluster density, **(B)** the molecular density, **(C)** the average cluster diameter, **(D)** the number of molecules per cluster, and **(E)** the percentage of molecules contained in clusters. Significance was determined using the student's t-test. No changes were seen in the organisation of KIR2DL1 when compared to untreated cells imaged on slides coated with anti-KIR2DL1 antibody.

detected on YTS cells (Figure 3.29). NK cell activation can be triggered through the NKG2D receptor alone, without the need for co-stimulation of other receptors or adhesion molecules, when IL-2 is present in the medium (Bryceson et al., 2006, 2009; Culley et al., 2009).

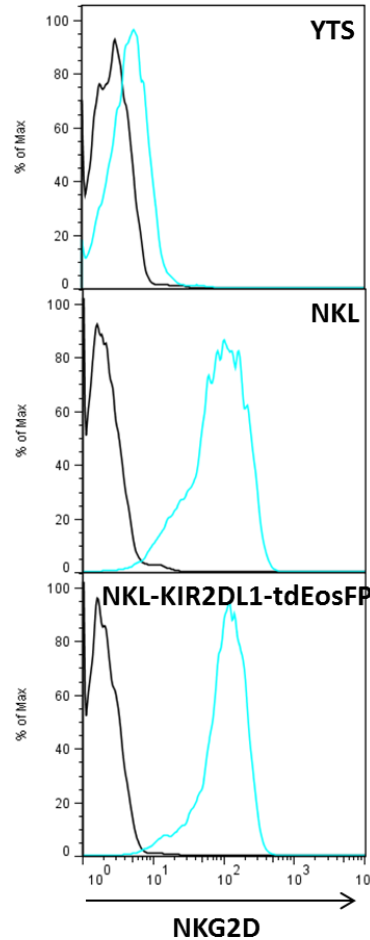


Figure 3.29: Surface expression of NKG2D on NK cells. YTS, NKL and NKL-KIR2DL1-tdEosFP cells were analysed by flow cytometry for surface expression of NKG2D. Specific staining (blue) is compared to isotype control (black).

The cluster organisation of KIR2DL1 in NKL-KIR2DL1-tdEosFP cells was examined when the cells were activated on anti-NKG2D antibody (Figure 3.30). The total molecular density of KIR2DL1 at the interface with the slide significantly increased compared to cells imaged on an isotype-control antibody, going from 113.2 ± 6.4 molecules per μm^2 in resting cells ($n = 55$) to 202.2 ± 9.2 molecules per μm^2 in activated cells ($n = 50$; $p < 0.0001$; Figure 3.30B). The cluster density was also significantly increased in activated cells, with 4.72 ± 0.08 clusters per μm^2 in activated cells versus 4.00 ± 0.09 clusters per μm^2 in resting cells ($p < 0.0001$; Figure 3.30A). The average cluster diameter was significantly reduced upon activation with anti-NKG2D antibody, from 85.5

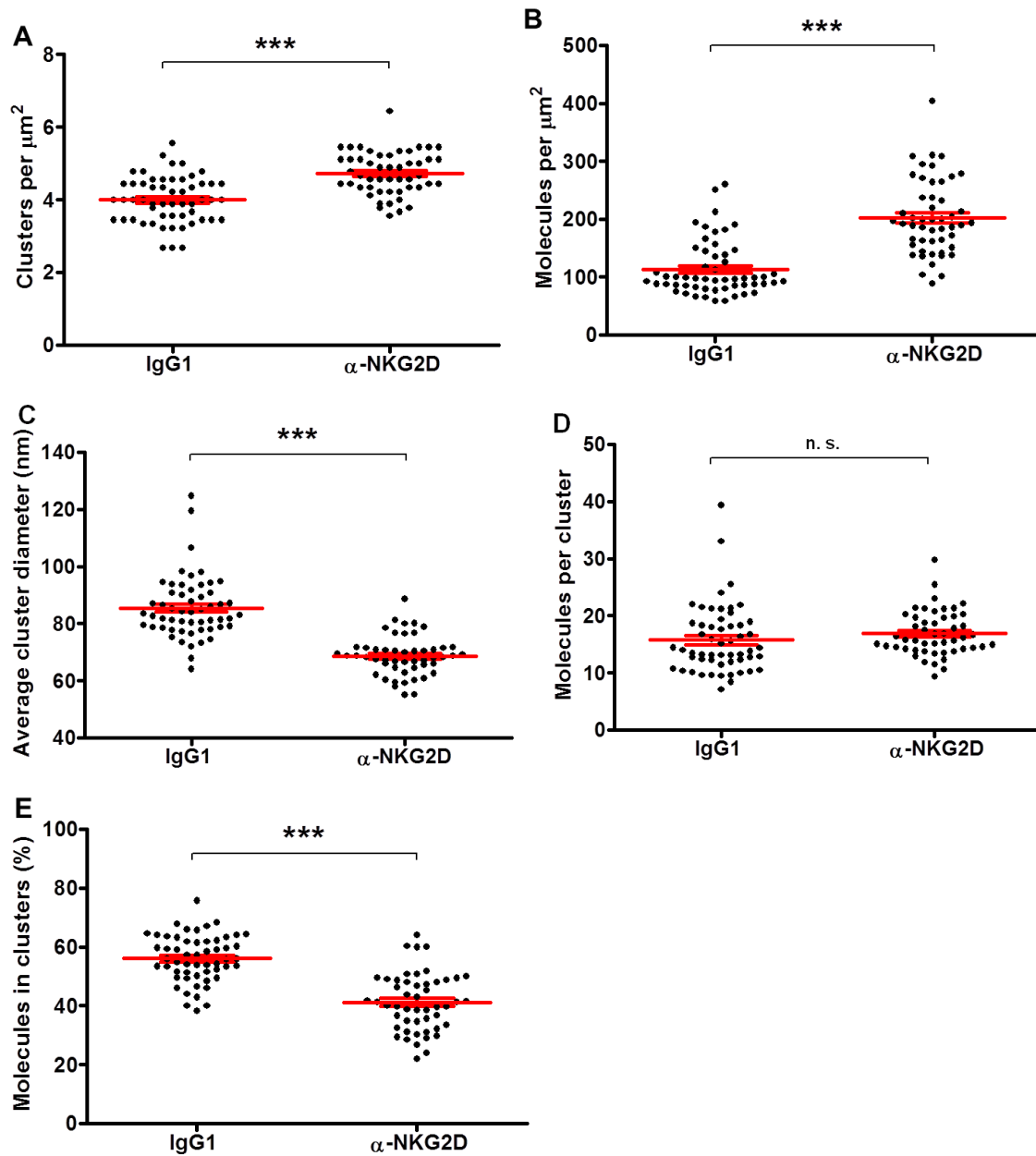


Figure 3.30: The nanoscale organisation of KIR2DL1 is changed upon activation of NKL-KIR2DL1-tdEosFP cells via NKG2D. NKL-KIR2DL1-tdEosFP cells were imaged by PALM on slides coated with anti-NKG2D antibody and compared to cells imaged on slides coated with isotype control IgG1 antibody. Cluster analysis of regions from the PALM images generates information about **(A)** the cluster density, **(B)** the molecular density, **(C)** the average cluster diameter, **(D)** the number of molecules per cluster, and **(E)** the percentage of molecules contained in clusters. Significance was determined using the student's t-test.

± 1.46 nm in non-stimulated cells to 68.6 ± 0.97 nm in activated cells ($p < 0.0001$; Figure 3.30C). However, the number of molecules per cluster was not significantly affected by activation ($p = 0.2581$; Figure 3.30D), although the percentage of molecules contributing to clusters decreased (from $56.1\% \pm 1.07$ in resting cells to $41.2\% \pm 1.38$ in activated cells; $p < 0.0001$; Figure 3.31E). These results show that activation of NK cells via NKG2D can change the nanoscale organisation of KIR2DL1 in the cell membrane at the interface with the antibody-coated slide.

Activation of YTS cells via co-ligation of CD28 and LFA-1

Another type of NK cell activation was also tested. YTS cells can be activated by co-ligating CD28 and CD11a (also known as LFA-1) (Azuma et al., 1992). To confirm that co-ligation of CD28 and CD11a is sufficient to activate YTS and YTS-KIR2DL1-tdEosFP cells, these cells were fixed and stained with phalloidin, then imaged by confocal microscopy. The cells extended lamellipodia out across the surface of the slide and exhibited a spreading phenotype typical of activated NK cells on slides coated with anti-CD28 and anti-CD11a antibodies, but did not undergo a spreading response on slides coated with an isotype-matched control antibody (Figure 3.31). These results demonstrate that co-ligation of CD28 and CD11a is indeed sufficient to induce activation in the YTS cell line.

YTS-KIR2DL1-tdEosFP cells were activated on slides coated with anti-CD28 and anti-Cd11a antibodies and imaged by PALM. Surprisingly, activation of NK cells through this combination of receptors ($n = 47$) did not induce any changes in the organisation of KIR2DL1 compared to cells imaged on slides coated with an isotype-control antibody ($n = 39$; Figure 3.32).

Taken together, these results indicate that ligation of some specific NK cell receptors can modify the organisation and clustering of KIR2DL1 molecules in the membrane contacting the surface, whereas the ligation of other receptors has no significant influence on the distribution of KIR2DL1.

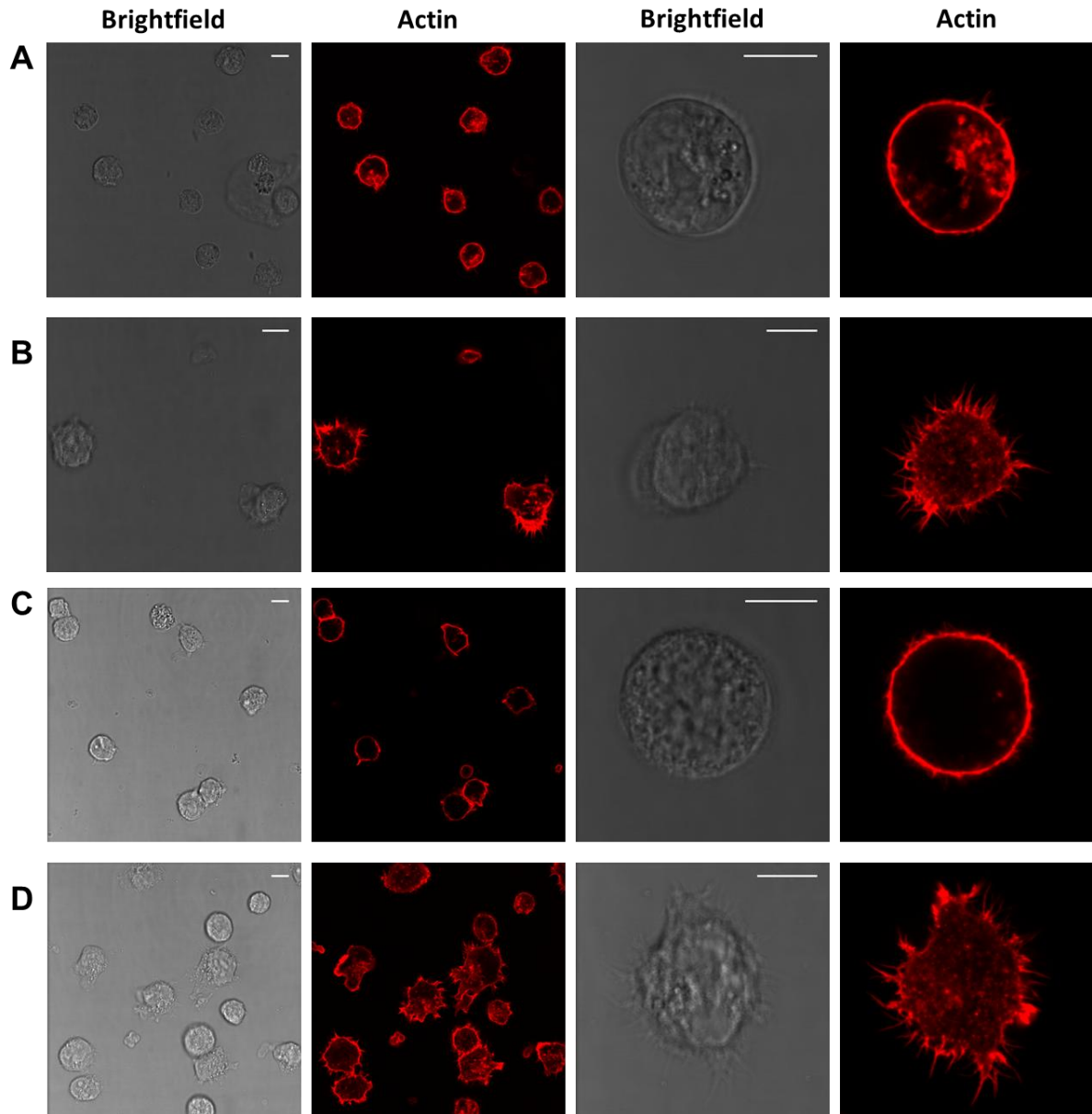


Figure 3.31: YTS and YTS-KIR2DL1-tdEosFP cells get activated on slides coated with anti-CD28 and anti-CD11a antibodies. (A&B) YTS cells were fixed and stained with phalloidin-Atto647N then imaged by confocal microscopy on slides coated with isotype control IgG1 antibody **(A)** or slides coated with anti-CD28 and anti-CD11a antibodies **(B)**. **(C&D)** YTS-KIR2DL1-tdEosFP cells were fixed and stained with phalloidin-Atto647N then imaged by confocal microscopy on slides coated with isotype control IgG1 antibody **(C)** or slides coated with anti-CD28 and anti-CD11a antibodies **(D)**. A representative field of view containing several cells is shown for each experiment (left) and a representative cell is shown on the right. Images are representation of **(A)** n=33, **(B)** n=14, **(C)** n=27 and **(D)** n=45. Scale bars represent 10 μ m.

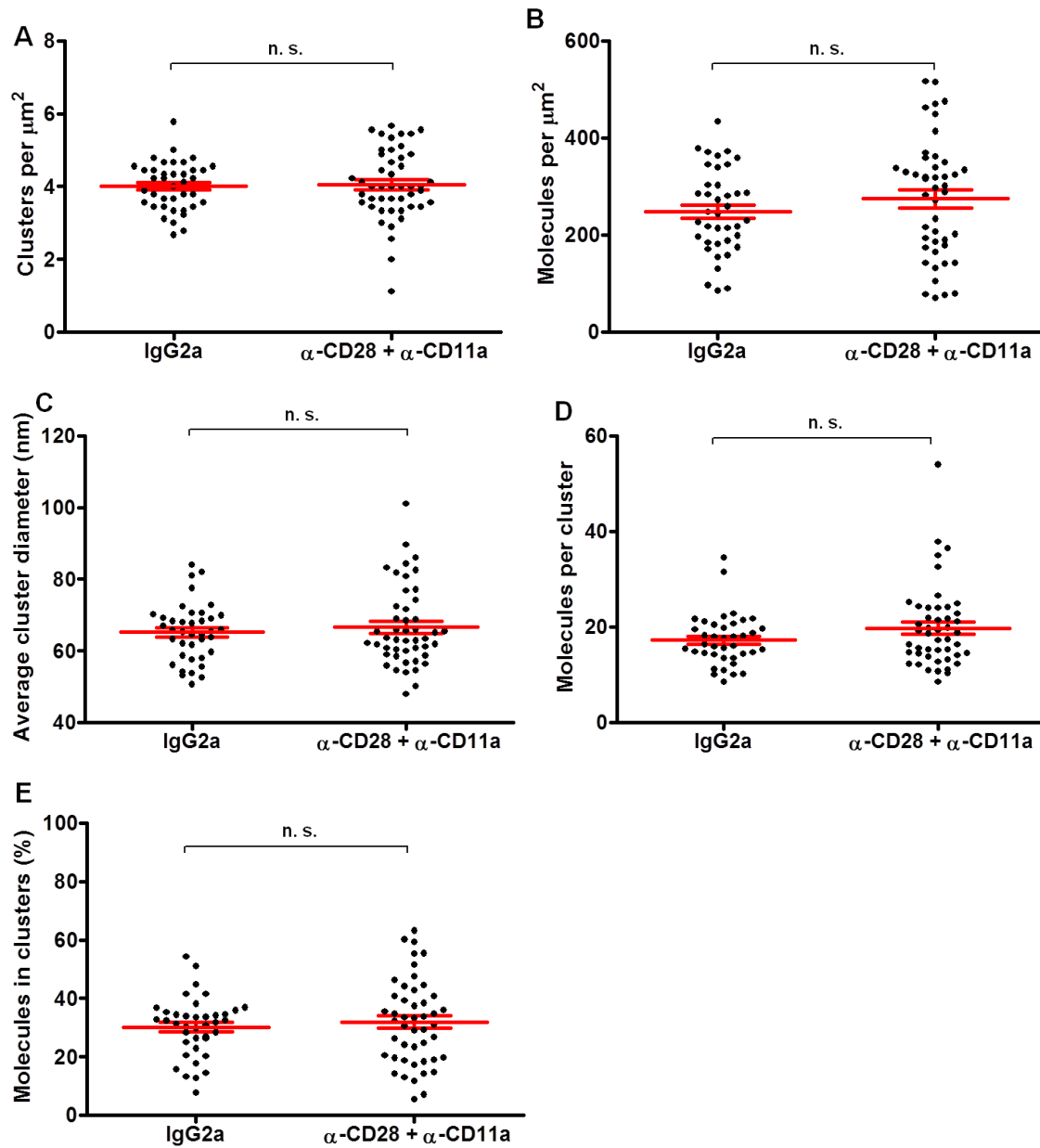


Figure 3.32: No changes in KIR2DL1 clustering occur when YTS-KIR2DL1-tdEosFP cells are activated via co-ligation of CD28 and CD11a. YTS-KIR2DL1-tdEosFP cells were imaged by PALM on slides coated with anti-CD28 and anti-CD11a antibodies. Cluster analysis of regions from the PALM images generates information about **(A)** the cluster density, **(B)** the molecular density, **(C)** the average cluster diameter, **(D)** the number of molecules per cluster, and **(E)** the percentage of molecules contained in clusters. Significance was determined using the student's t-test.

3.4.5 Ligation of CD94/NKG2A causes reorganisation of KIR2DL1

After looking at the effect of engaging activating receptors on the organisation of KIR2DL1, the aim was to determine whether engaging another inhibitory receptor on the same cell could induce changes in the clustering of KIR2DL1. The CD94/NKG2A heterodimer is an inhibitory complex that also recognises MHC class I molecules (Braud et al., 1998). The surface expression of CD94 on NKL and NKL-KIR2DL1-tdEosFP cells was checked by flow cytometry (Figure 3.33). These results confirm that NKL and NKL0KIR2DL1-tdEosFP do express CD94/NKG2A on their surface.

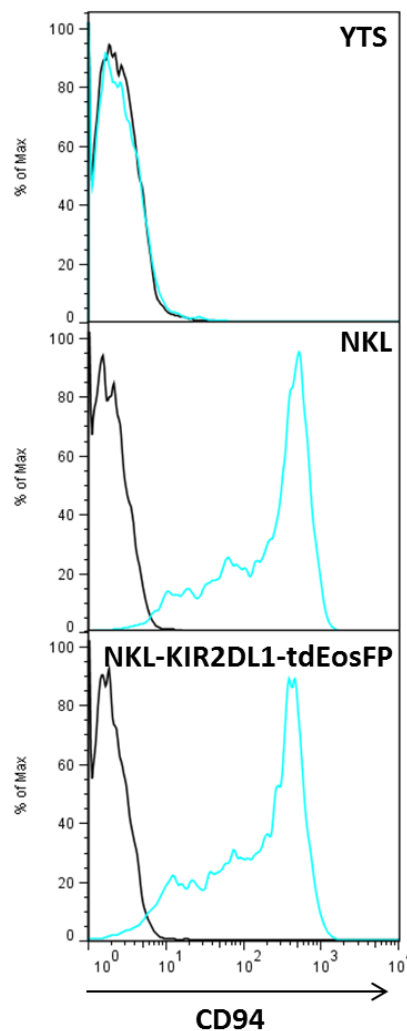


Figure 3.33: Surface expression of CD94 on NK cells. YTS, NKL and NKL-KIR2DL1-tdEosFP cells were analysed by flow cytometry for surface expression of CD94. Specific staining (blue) is compared to isotype control (black).

NKL-KIR2DL1-tdEosFP cells were imaged by PALM on slides coated with monoclonal anti-CD94 antibody in order to ligate the CD94/NKG2A complex (Figure 3.34). The molecular density of KIR2DL1 at the interface with the antibody-coated slides increased by more than 50%, from 113.2 ± 6.4 molecules per μm^2 in resting cells ($n = 55$) to 177.4 ± 6.9 molecules per μm^2 in cells on slides coated with anti-CD94 antibody ($n = 54$; $p < 0.0001$; Figure 3.34B). The density of clusters also significantly increased, with 4.79 ± 0.08 clusters per μm^2 in cells on slides coated with anti-CD94 antibody versus 4.00 ± 0.09 clusters per μm^2 in resting cells ($p < 0.0001$; Figure 3.34A). The average diameter of clusters was reduced by 20%, from 85.5 ± 1.5 nm in resting cells to 68.6 ± 0.9 nm in cells on slides coated with anti-CD94 antibody ($p < 0.0001$; Figure 3.34C), whereas the number of molecules per cluster was not affected (15.7 ± 0.8 molecules per cluster in resting cells compared to 15.0 ± 0.5 molecules per cluster in cells on slides coated with anti-CD94 antibody; $p = 0.4387$; Figure 3.34D). However, the percentage of molecules contributing to clusters was significantly decreased from $56.1\% \pm 1.1$ in resting cells to $41.1\% \pm 1.3$ in cells on slides coated with anti-CD94 antibody ($p < 0.0001$; Figure 3.34E). These results demonstrate that the organisation of KIR2DL1 was significantly altered when CD94/NKG2A was engaged.

These experiments were carried out by coating slides with antibody at a concentration of $3 \mu\text{g}.\text{ml}^{-1}$. NKL-KIR2DL1-tdEosFP cells were also imaged by PALM on slides coated with a higher concentration of anti-CD94 antibody ($10 \mu\text{g}.\text{ml}^{-1}$) in order to detect whether there would be a different effect on the organisation of KIR2DL1. At higher concentrations, the organisation of KIR2DL1 was also affected, generally following the trends described above (Figure 3.35). The most notable difference was that, at higher concentrations, the number of molecules per cluster was significantly increased compared to the controls ($p < 0.0001$).

These results suggest that when the CD94/NKG2A inhibitory receptor complex is engaged, the nanoscale organisation of KIR2DL1 is affected. Indeed, a higher number of KIR2DL1 molecules and KIR2DL1 clusters are detected at the interface with the slides, and the clusters are smaller and denser than in control samples.

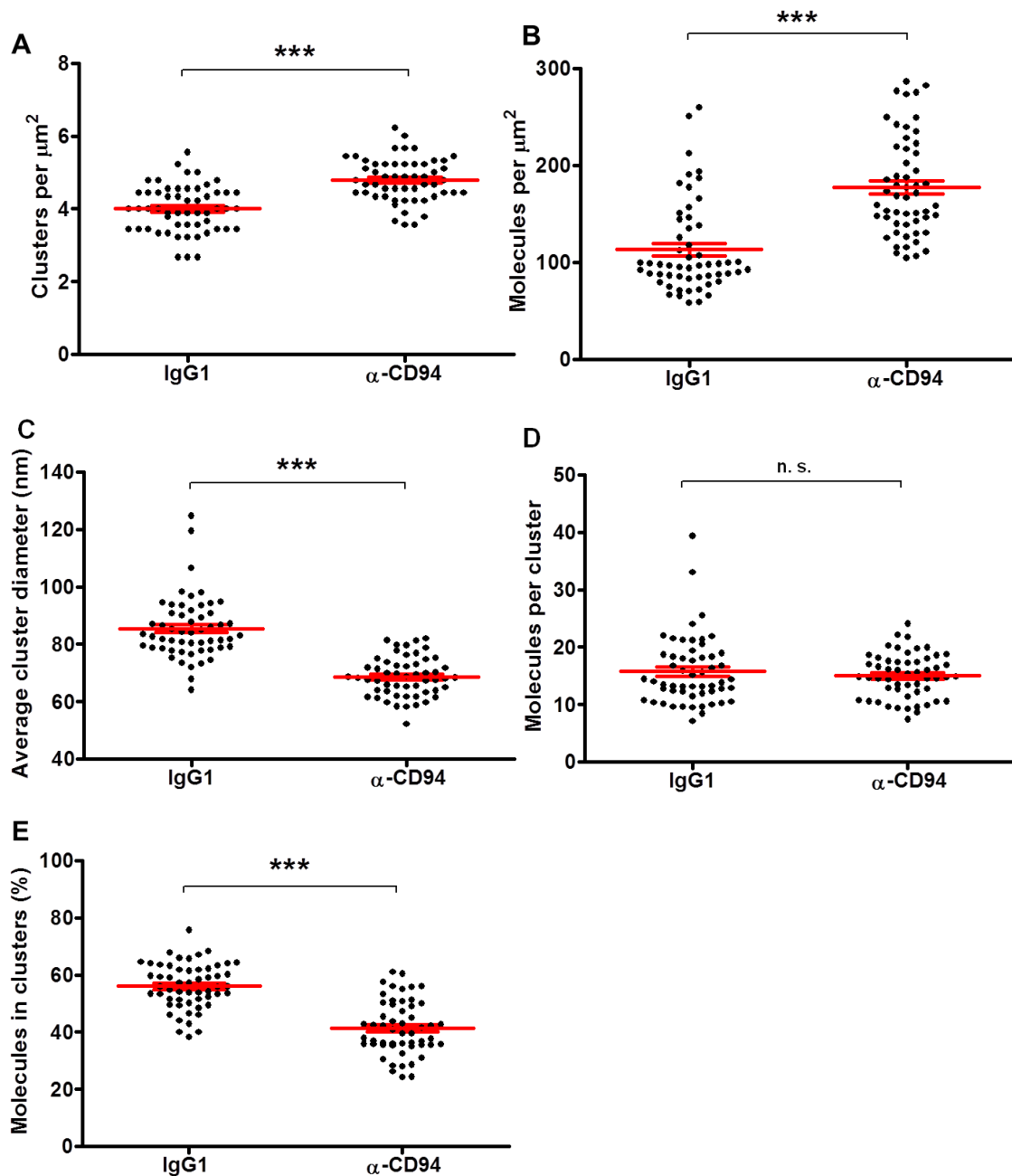


Figure 3.34: The nanoscale organisation of KIR2DL1 is changed upon engagement of a different inhibitory receptor. NKL-KIR2DL1-tdEosFP cells were imaged by PALM on slides coated with anti-CD94 antibody and compared to cells imaged on slides coated with isotype control IgG1 antibody. Cluster analysis of regions from the PALM images generates information about (A) the cluster density, (B) the molecular density, (C) the average cluster diameter, (D) the number of molecules per cluster, and (E) the percentage of molecules contained in clusters. Significance was determined using the student's t-test.

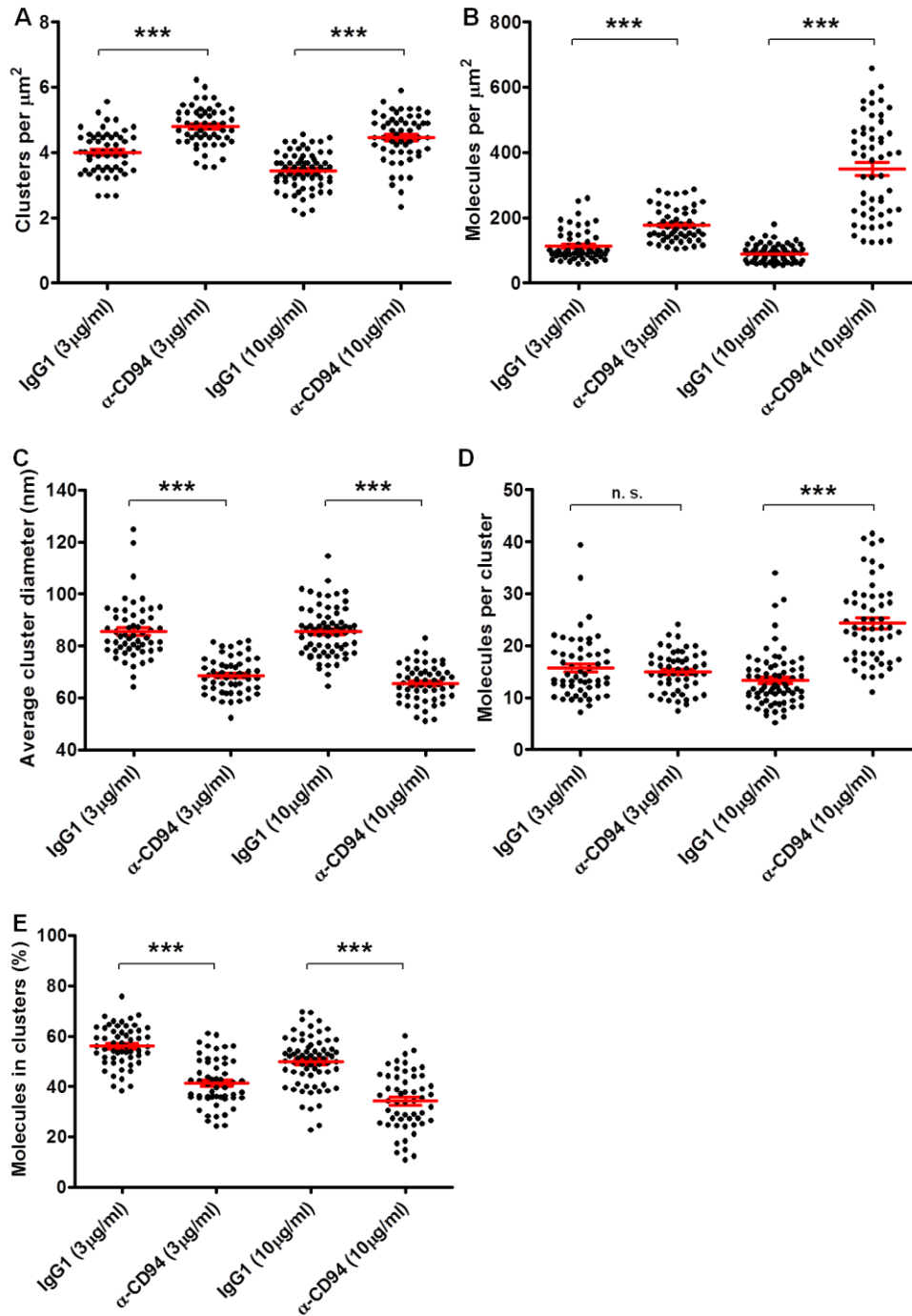


Figure 3.35: Higher concentrations of anti-CD94 antibody induce similar changes in KIR2DL1 organisation. NKL-KIR2DL1-tdEosFP cells were imaged by PALM on slides coated with different concentrations of anti-CD94 antibody and compared to cells imaged on slides coated with isotype control IgG1 antibody. Cluster analysis of regions from the PALM images generates information about **(A)** the cluster density, **(B)** the molecular density, **(C)** the average cluster diameter, **(D)** the number of molecules per cluster, and **(E)** the percentage of molecules contained in clusters. Significance was determined using the student's t-test.

3.5 Discussion

3.5.1 Summary of results

In this study, the main objective was to investigate the organisation of the inhibitory receptor KIR2DL1 in the membrane of NK cells at the nanoscale level, using the super-resolution imaging technique PALM and quantitative cluster mapping. In particular, the main results from this investigation are as follows:

- A protocol for single-molecule PALM imaging and cluster analysis was set up and optimised
- Molecules of the inhibitory NK cell receptor KIR2DL1 were found to be organised in pre-existing nanoclusters in the plasma membrane of resting, unstimulated NK cells
- KIR2DL1 nanoclusters were smaller and denser when KIR2DL1 was ligated by monoclonal antibody
- The nanoscale organisation of KIR2DL1 molecules triggered by ligation of KIR2DL1 was not dependent on actin polymerisation
- The ligation of an activating receptor (NKG2D) could influence the nanoscale organisation of KIR2DL1 molecules
- The ligation of a different inhibitory receptor (CD94/NKG2A) also affected the nanoscale organisation of the inhibitory receptor KIR2DL1.

To summarise, the super-resolution single-molecule localisation technique known as PALM was successfully utilised to study the molecular distribution of KIR2DL1 within the membrane of NK cells. The results obtained in this chapter demonstrate that the ligation of other receptors can influence the nanoscale organisation of KIR2DL1, leading to intriguing new questions about the mechanisms that may be involved in regulating this organisation.

3.5.2 Technical discussion

When performing PALM microscopy of membrane proteins tagged to photoswitchable fluorescent proteins, several technical issues arise, both concerning PALM acquisition and data analysis.

PALM acquisition

As originally described, PALM was afflicted by very slow acquisition times exceeding several hours (Betzig et al., 2006) and was not considered a serious candidate for live cell imaging. However, technical developments and the use of brighter photoswitchable fluorescent proteins (such as tdEosFP) have significantly reduced PALM acquisition times. In this study, a single PALM dataset, consisting of 20,000 frames at a frame rate of 9.3 frames per second, was collected in 36 minutes, although faster acquisition times have been reported. For live cell PALM, acquisition speeds can be improved by increasing the imaging laser power and decreasing the exposure time. However, some detail is sacrificed for speed as fewer frames are used to reconstruct each image (e.g. 3,000 frames versus 20,000 frames). Live cell PALM is very useful as it can reveal important information on cellular processes, such as the dynamics of LAT clusters in activated T cells (Williamson et al., 2011). Overall, combining super-resolution images of protein organisation at a fixed point in time using fixed cells with live cell PALM imaging of protein dynamics is a powerful way of deciphering the interactions and mechanisms governing immune cell signalling. In addition, PALM has been used for single-particle tracking of specific proteins in live cells (Manley et al., 2008).

Another issue encountered with PALM is the fact that highly specialised samples are required. For instance, PALM generally requires two-dimensional samples, such as cells on a slide combined with TIRF microscopy, otherwise the gain in resolution would be cancelled out by background fluorescence emanating from out-of-focus planes. For the scope of this study, where the aim was to investigate the organisation of a membrane receptor in different conditions, this meant that the cells were imaged on glass slides coated with different antibodies, rather than in a more physiological setting. However, this study provides the first super-resolution description of the organisation of receptors in NK cells. The details emerging from these experiments are very informative and provide an important basis for further investigation. In the future, progress with three-dimensional super-resolution techniques such as interferometric PALM (iPALM) (Shtengel et

al., 2009) will enable the investigation of molecular localisation and interactions at sub-diffraction-limit resolution in all three dimensions.

PALM image reconstruction

Following data acquisition, the extensive data processing required for the reconstruction of a super-resolution image needs to be performed carefully to discriminate background noise from real signal without eliminating any important subsets of molecules. Minor linear adjustments that are generally acceptable in conventional fluorescence microscopy are not applicable to single-molecule super-resolution techniques providing images of mapped points. Applying threshold criteria to a PALM data table will result in the loss of a discrete subset of data from the table and the discarded data may represent a structure of interest. The consideration of processing settings and parameters should therefore not be undertaken lightly. The main parameters that promote the robustness of the analysis are the SNR and the FWHM. Choosing incorrect parameters can impose radical changes on the data processing outcomes and the subsequent data analysis and interpretation. As described previously, the values entered for SNR and FWHM in the reconstruction software were not arbitrarily chosen but carefully calculated from measurements performed on each dataset prior to data processing.

Cluster analysis

From the analysis of PALM images of KIR2DL1 by spatial point pattern analysis, it is clear that a high proportion of this plasma membrane receptor is arranged into clusters. These clusters are fairly consistent in shape and size, being circular with a diameter of approximately 60-120 nm.

However, certain parameters emerging from the cluster analysis are highly dependent on the acquisition. For instance, the total number of molecules that are detected depends on the sample – mainly the labelling density and the number of molecules that were already bleached before the beginning of the acquisition. Therefore, the absolute values are not robust indicators of KIR2DL1 density within the sample, but instead reveal information about the number of molecules that are detected within the sample. Even though high n numbers were used in this study to even out the variability between samples, the values were used for relative studies where they were compared

between different conditions. For this application, the cluster analysis programme that was used was ideal, as it provided many important parameters of cluster organisation that could be compared.

Interestingly, a new PALM analysis method was recently developed that is not based on absolute protein numbers but combines pair correlation analysis with PALM (PC-PALM) (Sengupta et al., 2011). This approach uses an algorithm that enables quantification of several parameters of spatial organisation of a protein, including the presence of protein clusters, the size of clusters, their density patterns and abundance in the membrane, without relying on the determination of absolute values. However, in the present investigation, the parameters obtained from the cluster analysis were carefully compared between conditions and provided important information on the changes that could be induced in KIR2DL1 organisation at the nanoscale level.

tdEosFP

An important consideration in the interpretation of PALM data is the presence of clustering artefacts. Phenomena such as protein oligomerisation or clustering can only be properly identified if the same molecule is not being counted twice. An improved, brighter and more photostable variant of mEos called mEos2 was recently developed (McKinney et al., 2009) and has outcompeted other EosFP variants in PALM microscopy. However, even when using this promising photoswitchable protein, clustering artefacts were observed due to multiple reactivations of the fluorophore (Annibale et al., 2010). Indeed, a non-negligible fraction of mEos2 molecules (approximately 50% in a typical experiment) were seen to reactivate upon illumination with UV light, leading to an overestimation of the number of molecules (Annibale et al., 2010). Instead of entering a permanent bleached state, these molecules go into a long-lived dark state and fluorescence can be re-induced by UV light, hinting at similarities between mEos2 and the reversibly photoswitchable protein Dronpa, which also exhibits over-counting artefacts (Habuchi et al., 2005; Flors et al., 2007). These fluorophores undergo a transition to a non-fluorescent dark state but resume their fluorescence after a certain duration (t_d). If a subsequent fluorescence signal is detected in the same spatial spot within t_d , it can be attributed to the same molecule. In PALM experiments using this type of fluorophore, this photoblinking effect has to be taken into account. In the case of mEos2, it has been shown that using constant irradiation with low-level powers (a few W.cm^{-2}) of the activating (UV) wavelength instead of pulses during PALM acquisition can significantly reduce the off-time duration

(t_d), therefore decreasing the chances of counting the same molecule multiple times (Annibale et al., 2010). In addition, allowing for longer single-molecule dark times during data analysis reduces the risk of re-sampling (Annibale et al., 2011). An analysis method based on pair correlation analysis can be used to estimate over-counting due to reactivation and eliminate artefacts resulting from re-sampling (Veatch et al., 2012).

Although tdEosFP has not directly been shown to exhibit cycles of reactivation, it is structurally related to mEos2; they share the same structural basis for photoconversion and it would therefore not be surprising that tdEosFP molecules do not all directly reach the bleached state. In this study, PALM acquisitions were carried out with continuous illumination of UV light instead of pulsed light as originally described (Betzig et al., 2006). Hopefully this will have reduced t_d and restricted the reactivation of molecules.

An interesting way of determining the effect of using tdEosFP on the outcome of the cluster analysis would be to compare the cluster distribution of KIR2DL1 tagged to tdEosFP with the distribution of KIR2DL1 as detected with an anti-KIR2DL1 antibody. Very recently, new antibodies that can be used in PALM imaging have been developed by a company called Abberior. Antibodies are conjugated to caged fluorescent dyes, which can be cleaved to yield their fluorescent form upon illumination with UV light (Abberior, 2011).

3.5.3 Nanoscale organisation of KIR2DL1 and role in signalling

Pre-existing nanoclusters of KIR2DL1 in resting NK cells

It is interesting to observe that KIR2DL1 molecules form pre-existing protein clusters. This phenomenon has been previously described for several T cell proteins involved in signalling, including TCR and LAT (Lillemeier et al., 2010; Sherman et al., 2011; Williamson et al., 2011), but was not known for KIR2DL1 or other NK cell receptors. Electron microscopy has revealed varying degrees of pre-clustering of other plasma membrane-associated proteins, such as the Ras family proteins (Prior et al., 2003; Tian et al., 2007; Henis et al., 2009). It has been suggested that pre-clustering of signalling proteins may improve the efficiency and robustness of cellular signalling by regulating the probability of interactions occurring between signalling molecules (Cebecauer et al., 2010). This is in contrast to membrane proteins that have a more homogeneous

distribution, such as vesicular stomatitis virus G protein (VSVG) (Manley et al., 2008). KIR2DL1 molecules were organised into nanoclusters in the membrane of both YTS and NKL cells, suggesting this might be a common organisation in all NK cells. To confirm this, it would be interesting to investigate the organisation of KIR2DL1 molecules in the membrane of primary human NK cells from peripheral blood by using Abberior antibodies.

KIR2DL1 clusters are smaller and denser upon KIR2DL1 ligation

Ligation of KIR2DL1 resulted in an increased number of KIR2DL1 molecules detected in the cell membrane at the interface with the antibody-coated slide. This finding is consistent with the fact that KIR2DL1 molecules accumulate at an inhibitory synapse (Davis et al., 1999). This result indicates that ligating KIR2DL1 with monoclonal antibody can recruit KIR2DL1 molecules to the interface. Surprisingly, the number of clusters was not increased. The percentage of KIR2DL1 molecules contained in clusters decreased, which is consistent with the increase of total KIR2DL1 protein at the interface. This increase of total KIR2DL1 led to an increase of molecules that are not in clusters, but also to an increase in the number of molecules per cluster. The average diameter of clusters was significantly reduced. These results were clearly evident in YTS cells, but there was no significant difference in the number of KIR2DL1 molecules per cluster in NKL cells.

The ligation of KIR2DL1 therefore induced the formation of smaller clusters with an increased number of molecules per cluster, compared to pre-existing clusters detected in resting NK cells. This rearrangement of the nanoscale organisation of KIR2DL1 upon ligation may reflect an important physiological event regulating inhibitory signalling. It is possible that upon receptor ligation, KIR2DL1 molecules are brought closer together for efficient signalling. Indeed, there might be a threshold of KIR2DL1 density within clusters that needs to be reached for inhibitory signalling to be initiated. This hypothesis could be tested by coating the slides with increasing concentrations of EB6 antibody and observing whether different patterns of KIR2DL1 clustering occur at different concentrations and whether there are any step changes in the organisation of KIR2DL1. This hypothesis might explain how receptor patterns at the immune synapse can transmit information about the density of ligands on the target cell.

The mechanism governing the reorganisation of KIR2DL1 molecules upon receptor ligation remains unclear. After treatment with Lat A, an inhibitor of actin polymerisation, the nanoscale organisation of KIR2DL1 molecules on EB6-coated slides remained the same. This finding implies that the packing of KIR2DL1 molecules into clusters is not dependent on the integrity of the actin cytoskeleton. Other mechanisms must be involved in the formation of KIR2DL1 nanoclusters and the reorganisation of KIR2DL1 molecules upon receptor ligation, which will need to be investigated in the future.

The nanoscale organisation of KIR2DL1 is affected by the ligation of different receptors

The effects of ligating different receptors on the nanoscale organisation of KIR2DL1 were assessed by PALM imaging and cluster analysis, and the changes in molecular distribution of KIR2DL1 in the different conditions are summarised in Table 3.2.

Condition	Molecular density (molecules/ μm^2)	Cluster density (clusters/ μm^2)	N° molecules per cluster	Cluster diameter	% molecules in clusters
α -KIR2DL1 (EB6)	↑ ↑	- -	↑ -	↓ ↓	↓ ↓
α -KIR2DL1 (EB6) + Lat A	↑	-	↑	↓	↓
α -NKG2D	↑	↑	-	↓	↓
α -CD28/ α -CD11a	-	-	-	-	-
α -CD94	↑ (↑)	↑ (↑)	- (↑)	↓ (↓)	↓ (↓)

Table 3.2: Summary of the changes in the nanoscale organisation of KIR2DL1 in different conditions, as detected by PALM microscopy and quantitative analysis. Experiments were performed either with the YTS cell line (black) or the NKL cell line (brown). Different parameters describing the nanoscale organisation of KIR2DL1 were compared to control samples on slides coated with an isotype control antibody. ↑ indicates an increase; ↓ indicates a decrease; - indicates no significant change. The brackets in the bottom row represent results obtained with a higher concentration of anti-CD94 antibody on the slides.

Ligation of an activating receptor changes the nanoscale organisation of KIR2DL1

The main finding of this investigation was that ligating other receptors can have an impact on the nanoscale organisation of KIR2DL1. The ligation of the activating receptor NKG2D in NKL cells resulted in a rearrangement of KIR2DL1 molecules that did not reflect the organisation induced by EB6 antibody. Unexpectedly, the total number of KIR2DL1 molecules recruited to the interface was found to have increased compared to resting cells on control surfaces, although the cause for this remains unknown. Even though the clusters had a smaller diameter, the number of molecules per cluster was not changed, but the total number of clusters was increased. It is intriguing to consider the mechanisms that might be underlying the fact that ligation of NKG2D can significantly influence the distribution of KIR2DL1 molecules. These mechanisms might include (i) a direct physical association between the two receptors, (ii) an indirect signalling pathway induced by NKG2D ligation, or (iii) other mechanisms such as an active involvement of the cytoskeleton. NKG2D and KIR2DL1 might be physically associated within the cell membrane, perhaps by direct physical interaction or by colocalisation within lipid rafts. More information could be obtained about the link between KIR2DL1 and NKG2D by simultaneously co-ligating the two receptors using different ratios of antibody and observing the changes in KIR2DL1 clustering and how these are linked to the cellular response. The organisation of KIR2DL1 might be influenced by signalling events triggered by NKG2D ligation, including perhaps a role for the cytoskeleton. The mechanisms linking NKG2D ligation and KIR2DL1 organisation could be explored by performing experiments with lipid raft inhibitors (e.g. methyl- β -cyclodextrin) or actin polymerisation inhibitors (such as Lat A or Cytochalasin D).

Interestingly, co-ligation of the activating receptor CD28 and the adhesion molecule CD11a on YTS cells, which results in cell activation, did not affect the nanoscale organisation of KIR2DL1. This result suggests that there is no direct association between CD28 and KIR2DL1 and may reflect a different mechanism of signal integration when activation is mediated by NKG2D or by CD28. Ligation of NKG2D alone is sufficient to activate cells (Culley et al., 2009). Therefore, it may be useful for the distribution of an inhibitory receptor to be closely linked to NKG2D, should it be necessary to initiate inhibitory signalling. In the case of CD28 however, co-ligation of the adhesion molecule CD11a is needed (Azuma et al., 1992), which represents an additional requirement for cell activation as well as direct receptor ligation. It is possible that in this case, a

direct link with the inhibitory receptor KIR2DL1 is not required, since an additional checkpoint for activation is already present.

Although ligating different activating receptors led to different outcomes, these experiments were also carried out in different cell lines. In order to clarify whether the differences were mainly due to the specific receptor that was being ligated or the cell type, in the future the effect of ligating these different receptors should be investigated in primary human NK cells.

Ligation of another inhibitory receptor changes the nanoscale organisation of KIR2DL1

The ligation of the inhibitory complex CD94/NKG2A in NKL cells resulted in a rearrangement of the nanoscale distribution of KIR2DL1 that was similar to that induced by ligation of NKG2D. This finding suggests that the CD94/NKG2A complex also has a direct influence on the organisation of KIR2DL1, either by direct physical interaction or mediated by signalling. When a higher concentration of anti-CD94 antibody was used ($10 \mu\text{g}.\text{ml}^{-1}$ compared to $3 \mu\text{g}.\text{ml}^{-1}$), the number of molecules per cluster also increased. This result might imply that when CD94/NKG2A ligands are present at higher densities on the target cell, KIR2DL1 clusters get more densely populated, which in turn might result in KIR2DL1-mediated signalling. This mechanism could be used by the cell to enhance the inhibitory response when the density of CD94/NKG2A ligands exceeds a certain threshold, or when both types of ligand are present. Similarly to NKG2D, the link between KIR2DL1 and CD94/NKG2A might be probed by further experiments involving inhibitors or co-ligation of receptors.

It is interesting to note that the ligation of both NKG2D and CD94/NKG2A can influence the organisation of KIR2DL1 molecules, whereas the ligation of CD28 cannot. This leads to the speculation that this type of interaction with KIR2DL1 might be specific to members of the NKG2 family, which are structurally related. If the link with KIR2DL1 is based on a physical interaction, this might be a mechanism that keeps KIR2DL1 in close proximity in order to inhibit activating signals or enhance inhibitory signals. Further investigations will be required to explore the integration of all these events, as the mechanisms underlying signal integration in NK cells are important for maintaining tightly regulated immune responses. Deciphering the ways in which different receptors are organised and how they can influence each other will be crucial for describing the spatio-temporal dynamics of signal integration.

3.5.4 Future directions

This investigation on the nanoscale distribution of KIR2DL1 has revealed that KIR2DL1 forms nanoclusters that can be altered upon receptor ligation and can be influenced by other receptors. The findings described in this chapter give rise to interesting questions for the future, which will need to be addressed using more advanced technologies.

Supported lipid bilayer

In order to study the organisation of proteins at the NK cell immune synapse in a more physiological setting, PALM microscopy should be combined with the use of planar lipid bilayers on a glass slide to mimic the membrane of a target cell. Artificial model lipid bilayers were first deposited onto solid supports in the early 1980s by McConnell and colleagues (Hafeman et al., 1981; Balakrishnan et al., 1982; Brian and McConnell, 1984; Tamm and McConnell, 1985). Since then, supported lipid bilayers have been used for many purposes, including the investigation of events occurring at immunological synapses, mainly in T cell-APC interactions (McConnell et al., 1986; Grakoui et al., 1999; Dustin et al., 1998).

Membrane proteins, such as ligands and adhesion molecules, rather than antibodies, could be inserted into the lipid bilayer to image the physiological interaction between an NK cell and another membrane, reflecting events occurring at the immune synapse. An important advantage of using a bilayer rests on the fact that proteins can retain their motility when inserted into a lipid bilayer, rather than being immobilised on a slide like antibodies. By titrating different concentrations of ligand into the bilayer, it will become apparent whether different patterns of KIR2DL1 organisation will form depending on ligand density on the opposing membrane and thus whether the nanoscale organisation of a receptor can transmit information on the density of its ligand, thereby influencing the cell's response. Different combinations of ligands and adhesion molecules could be used to visualise the distribution of KIR2DL1 in different conditions, such as activating versus inhibitory synapses.

Multi-colour PALM

With the appropriate lasers and microscope set-up, it is possible to image two or more types of proteins at the nanoscale level using multi-colour PALM (Shroff et al., 2007). This is achieved by choosing compatible combinations of fluorescent proteins. For two-colour PALM, this includes the pairs Dronpa/tdEosFP and photoswitchable cyan fluorescent protein (PS-CFP)/tdEosFP (Shroff et al., 2007). Two-colour PALM imaging can reveal how two different proteins localise relative to each other during important cellular processes. Proteins that appear colocalised by conventional confocal fluorescence microscopy are sometimes detected in separate non-overlapping nanoclusters once observed at the super-resolution level (Shroff et al., 2007; Sherman et al., 2011; Williamson et al., 2011).

Two-colour PALM experiments would improve our understanding of the integration of signals at the NK cell immune synapse. For example, imaging the nanoscale organisation of the inhibitory receptor KIR2DL1 and the activating receptor NKG2D simultaneously would show whether these receptors co-localise within the same protein islands or form distinct clusters. These experiments could help explain how the ligation of NKG2D can influence the organisation of KIR2DL1. Imaging KIR2DL1 and downstream signalling proteins such as SHP-1 would also be exciting, shedding light on spatial aspects of the inhibitory signalling pathway. This would be important for understanding exactly where proteins are located during signalling and where the inhibitory and activating signalling pathways interact at the nanoscale level.

Live-cell PALM

Nanoscale protein dynamics can be probed using live-cell PALM (Shroff et al., 2008). Live-cell PALM has successfully been used to study the dynamics of LAT protein clusters in activated T cells (Williamson et al., 2011). This study focussed on the nanoscale organisation of KIR2DL1 at a fixed point in time. Live-cell PALM experiments, using a supported lipid bilayer, could reveal important information about the dynamics of KIR2DL1 clusters within the immune synapse. Importantly, it was previously shown that KIR2DL1 patterns at the immune synapse are not fixed but change over time at the micrometer scale (Carlin et al., 2001; Almeida and Davis, 2006; Oddos et al., 2008). It would be interesting to observe the changes occurring over time at the nanoscale level and how these changes relate to downstream signalling events. This approach would provide new temporal insights

into signal integration, such as the order of signalling events and the timing of interactions between protein clusters.

Imaging NK cell signal integration

To image the sites where signalling events, as determined by phosphorylation, are occurring, the common method has been to use phospho-specific antibodies in fixed samples. Antibodies are available that bind any phosphorylated tyrosine residue, although more specific antibodies against given phosphorylated residues from a specific protein have also been developed. By using a generic anti-phosphotyrosine monoclonal antibody and by combining fluorescence lifetime imaging microscopy (FLIM) and Förster resonance energy transfer (FRET), Davis and colleagues were able to visualise KIR2DL1 phosphorylation at the inhibitory immune synapse (Treanor et al., 2006). As mentioned previously, they found that phosphorylated KIR2DL1 was spatially confined to distinct microclusters within the aggregate of KIR2DL1 at the immune synapse (Treanor et al., 2006). It would be interesting to see how the distribution of phosphorylated KIR2DL1 correlates with that of total KIR2DL1 at the nanometer level. This could be achieved by PALM using Abberior-conjugated antibodies against phospho-tyrosine or by dSTORM using antibodies conjugated to standard dyes. By using specific antibodies against KIR2DL1 phosphorylation and DAP10 phosphorylation for example, it would be possible to investigate the phosphorylation patterns of inhibitory and activating signalling at the same time and observe how these patterns change in different conditions, such as activating and inhibitory synapses, which might reveal crucial spatial information about how activating and inhibitory signals are integrated at the NK cell immune synapse.

In conclusion, super-resolution imaging by PALM has revealed new information about the molecular distribution of KIR2DL1 at the immune synapse and given rise to many exciting questions of biological relevance regarding the integration of activating and inhibitory signals at the NK cell immune synapse. Indeed, questions regarding the biological significance of pre-existing KIR2DL1 clusters, the mechanisms governing the organisation of KIR2DL1 and its role in signalling require further investigation. In addition, the nanoscale organisation of other receptors and signalling proteins relative to KIR2DL1, the dynamics of protein clusters and the localisation of specific signalling events at the nanometer scale would provide important insights into signal integration. In the future, technical progress should be geared towards both improving spatio-temporal resolution and achieving greater physiological relevance in order to address these questions.

Chapter 4 - Imaging the dynamics of KIR2DL1 using a photoswitchable fluorescent protein

4.1 Introduction

In the previous chapter, the organisation of the inhibitory receptor KIR2DL1 at the immune synapse was investigated at the nanometer scale level. Here, the focus is geared towards studying the dynamics of KIR2DL1 within the NK cell membrane at the micrometer scale.

4.1.1 Receptor dynamics

Membrane proteins are involved in many cellular processes, including immune recognition and synapse formation. It is therefore very important to characterise these proteins, not only in terms of their expression levels, structural properties or distribution within the membrane, but also with respect to their dynamics and interactions in the context of living cells. For immune cell receptors, such as those expressed on the surface of NK cells, a deeper understanding of factors regulating receptor movement and synapse formation could potentiate the development of drugs to control recognition of target cells.

NK cells can simultaneously bind susceptible and MHC class I-protected target cells, with selective killing of the susceptible target cells, indicating that inhibitory signals do not act globally, but are spatially restricted towards the resistant target cells (Eriksson et al., 1999). The NK cell inhibitory receptor KIR2DL1 is known to be recruited to the contact site between an NK cell and a resistant target cell, which is known as the inhibitory immune synapse (Davis et al., 1999). The ability of inhibitory receptors to enrich at the point of cell contact is critical for their function. Indeed, when cells were treated with an anti-KIR2DL1 antibody (EB6) that blocks the binding site for HLA-Cw4, KIR2DL1 and HLA-Cw4 did not accumulate at the site of contact between NK cells and target cells (Davis et al., 1999). In cytotoxicity assays, treatment with the same antibody resulted in NK cells being able to lyse HLA-Cw4⁺ target cells (Bottino et al., 1994).

A lot of work has focussed on investigating the molecular organisation of receptors and proteins at the immune synapse, showing that KIR2DL1 can organise itself into different patterns at the synapse: homogeneous, ring-shaped, and multifocal distributions have all been observed (Carlin et al., 2001; Almeida and Davis, 2006). KIR2DL1 recognises HLA-C ligands on the target cell and the kinetics of KIR2DL1-HLA-C interactions have been investigated (Valés-Gómez et al., 1998). The binding between soluble HLA-Cw6 and KIR2DL1 exhibits extremely rapid association and dissociation rates ($K_{on} > 200,000 \text{ M}^{-1} \cdot \text{s}^{-1}$; $K_{off} > 2 \text{ s}^{-1}$) (Valés-Gómez et al., 1998). The kinetics of this interaction are much faster than the binding of TCR with its ligands and are more similar to the interactions of adhesion molecules with their ligands. The authors postulate that in a cell-cell interaction, at physiological temperatures, the KIR2DL1-HLA-C interaction would occur even more rapidly since the molecules are brought close together and are anchored in the cell membrane in the right orientation (Valés-Gómez et al., 1998).

The kinetics of KIR2DL1 movement within the NK cell membrane and the dynamics of KIR2DL1 recruitment to the immune synapse have not been studied in great detail. However, in order to obtain a better understanding of events occurring during synapse formation and target cell recognition, kinetic information about the behaviour of the proteins involved is required.

4.1.2 Methods for studying protein dynamics in live cells

Proteins are always on the move, and cellular functions are regulated by processes that are dynamic both in space and in time. In recent years, numerous new technologies using fluorescent proteins have emerged to unravel the details of protein dynamics (Lippincott-Schwartz et al., 2003; Miyawaki, 2011). These novel imaging techniques can be used to quantitatively describe the dynamics and kinetic properties of proteins in living cells and are mainly based on photobleaching or photoactivation.

Photobleaching methods

Photobleaching is the light-induced alteration of a fluorophore that permanently extinguishes its fluorescence. Several imaging methods that exploit photobleaching have been developed to study protein dynamics.

In fluorescence recovery after photobleaching (FRAP), fluorophores are irreversibly bleached within a small region of interest using high intensity laser light. Over time, the fluorescence within this region is recovered as unbleached fluorophores from outside the region replace the population of bleached molecules. By analysing the rate of fluorescence recovery within the bleached region, FRAP can provide valuable information about the mobility and diffusion of proteins within the cell (Axelrod et al., 1976; Jacobson et al., 1976).

In fluorescence loss in photobleaching (FLIP), a fluorescent cell is repeatedly photobleached within a specific small region, while the cell is repeatedly imaged. This technique is used to monitor the continuity of cellular compartments. Regions of the cell that are connected to the bleached area will gradually lose fluorescence due to the lateral movement of mobile fluorophores into the bleached area; whereas fluorescence will be unaffected in regions that are not connected (Cole et al., 1996; Zaal et al., 1999; Phair and Misteli, 2000).

Fluorescence localisation after photobleaching (FLAP) is a related technique, in which the protein of interest carries two fluorophores: one that will be photobleached and the other that acts as a reference label. The first type of fluorophore is bleached within a localised region of the cell and FLAP allows tracking of the bleached molecules as well as the unbleached ones. Basic image processing consisting of simple image differencing reveals the distribution of the two populations of molecules and enables the study of protein dynamics in living cells (Dunn et al., 2002).

One of the main limitations when using photobleaching techniques to study protein dynamics is that photobleaching requires long and intense illumination in a specific region of the cell to obtain full photobleaching. In addition, these methods generally use indirect ways to track protein movements.

Photoactivation techniques

Photoactivation is the light-induced activation of an inert molecule to an active fluorescent state. Photobleaching techniques have recently been complemented by photoactivation experiments, based on the use of caged fluorophores and photoactivatable or photoswitchable fluorescent proteins.

Photoactivation of caged compounds involves the light-induced release of a 'caging' group from a 'caged' compound; the fluorophore does not fluoresce until the caging group is cleaved and the movement of the fluorophore can then be tracked based on its fluorescence signal (Politz, 1999).

Since 2002, numerous genetically encoded photoactivatable and photoswitchable fluorescent proteins have been generated. Examples include photoactivatable GFP (PA-GFP) (Patterson and Lippincott-Schwartz, 2002), the Kaede protein (Ando et al., 2002), and more recently EosFP and its variants (Wiedenmann et al., 2004; Nienhaus et al., 2005, 2006). In a typical photoactivation experiment, a region of a cell is locally irradiated with a short pulse of activating light. Only the fluorophores in this region are photoactivated. The ability to selectively switch on molecules in space makes photoactivatable and photoswitchable proteins excellent tools for exploring the behaviour of proteins within live cells. Photoactivation is a fast process and partial photoactivation is sufficient to generate an instantaneous source of fluorescence. This technique is therefore very useful for studying fast and slow processes. Another important advantage of this method is that the distribution of both populations can be visualised after photoswitching (the unswitched molecules in green and the photoswitched molecules in red). In this investigation, the photoswitchable fluorescent protein tdEosFP was tagged to the inhibitory NK cell receptor KIR2DL1 and used to monitor the movement of KIR2DL1 in the membrane of NK cells.

4.1.3 Aims

Previous work has demonstrated that the inhibitory NK cell receptor KIR2DL1 is recruited to the immune synapse and accumulates there (Davis et al., 1999; Carlin et al., 2001). However, the diffusion of KIR2DL1 proteins in the NK cell membrane and the dynamics of the recruitment of KIR2DL1 to the immune synapse have not been studied in great detail.

Therefore, the main goal of this project was to further investigate how KIR2DL1 molecules are moving in the membrane of an NK cell in the absence or presence of target cells. More specifically, the aims were as follows:

- (i) To establish a microscopy-based method to follow the movements of a protein in a cell's membrane
- (ii) To visualise the diffusion of KIR2DL1 molecules at the surface of NK cells and calculate the diffusion coefficient of KIR2DL1

- (iii) To follow the movements of KIR2DL1 molecules upon formation of an inhibitory immune synapse
- (iv) To observe KIR2DL1 movements in the presence of multiple immune synapses.

4.2 Cell system

To study the dynamics of the inhibitory NK cell receptor KIR2DL1, YTS cells expressing KIR2DL1 fused to the photoswitchable protein tdEosFP were imaged by confocal microscopy. The cells were transfected to express KIR2DL1-tdEosFP as described in the previous chapter.

To first investigate the diffusion of KIR2DL1 in the membrane of NK cells, YTS-KIR2DL1-tdEosFP cells were imaged by confocal microscopy in the absence of target cells. A small portion of the membrane was selected and within this region tdEosFP molecules were photoconverted from green to red fluorescence by illumination with the UV laser. Each cell was imaged for 20 minutes after photoswitching to follow the movement of the KIR2DL1-tdEosFP fusion protein in the membrane of the cell. YTS-KIR2DL1-tdEosFP cells were also imaged in the presence of target cells (221-Cw4) in order to study the dynamics of KIR2DL1 when an immunological synapse has formed, as shown in Figure 4.1.

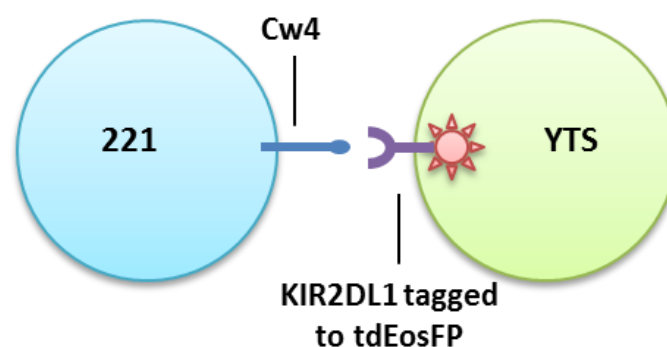


Figure 4.1: Schematic diagram depicting the cell system used for studying KIR2DL1 dynamics. The NK cell line YTS transfected to express KIR2DL1 fused to tdEosFP was imaged by confocal microscopy in the presence of target cells, which were 221 cells expressing HLA-Cw4.

221 cells previously transfected to express the MHC class I molecule HLA-Cw4 that is recognised by KIR2DL1 (Biaassoni et al., 1995) were analysed by flow cytometry to confirm expression of surface MHC class I (Figure 4.2). 221-Cw4 cells were indeed found to express high levels of MHC class I surface protein.

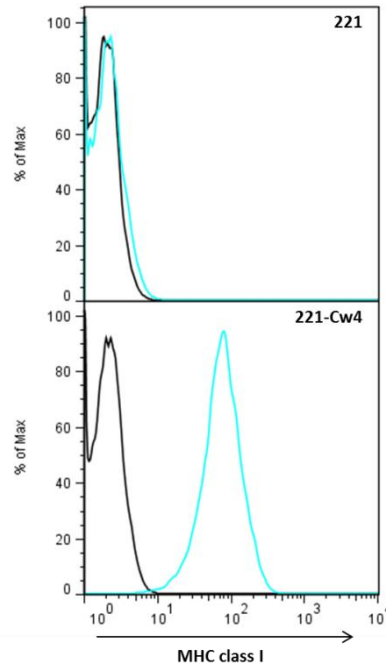


Figure 4.2: Surface expression of MHC class I on 221 and 221-Cw4 cells. 221 and 221-Cw4 cells were analysed by flow cytometry for surface expression of MHC class I. Specific staining (blue) is compared to isotype control (black).

When YTS-KIR2DL1-tdEosFP and 221-Cw4 cells were incubated together, they formed inhibitory synapses in which the fluorescent KIR2DL1 protein was seen to accumulate (Figure 4.3).

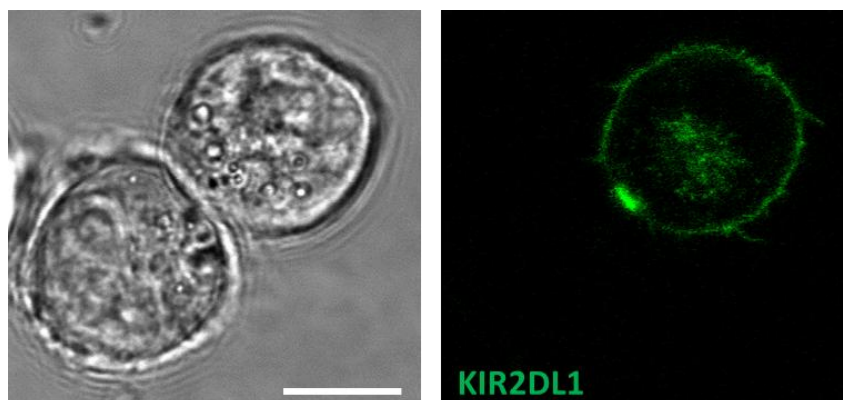


Figure 4.3: KIR2DL1 accumulates at the inhibitory immune synapse. YTS-KIR2DL1-tdEosFP cells were imaged by confocal microscopy in the presence of 221-Cw4 cells. Scale bar represents 10 μ m.

As shown in the previous chapter, it was possible to photoswitch a cell expressing KIR2DL1-tdEosFP from emitting green fluorescence to emitting red fluorescence (Figure 3.13). In this study, a specific region of the cell was chosen and tdEosFP molecules were selectively photoswitched in this region only, as demonstrated in a preliminary imaging experiment (Figure 4.4). After photoswitching, the cells could be imaged by confocal microscopy to visualise how the red fluorescence was moving around the cell membrane.

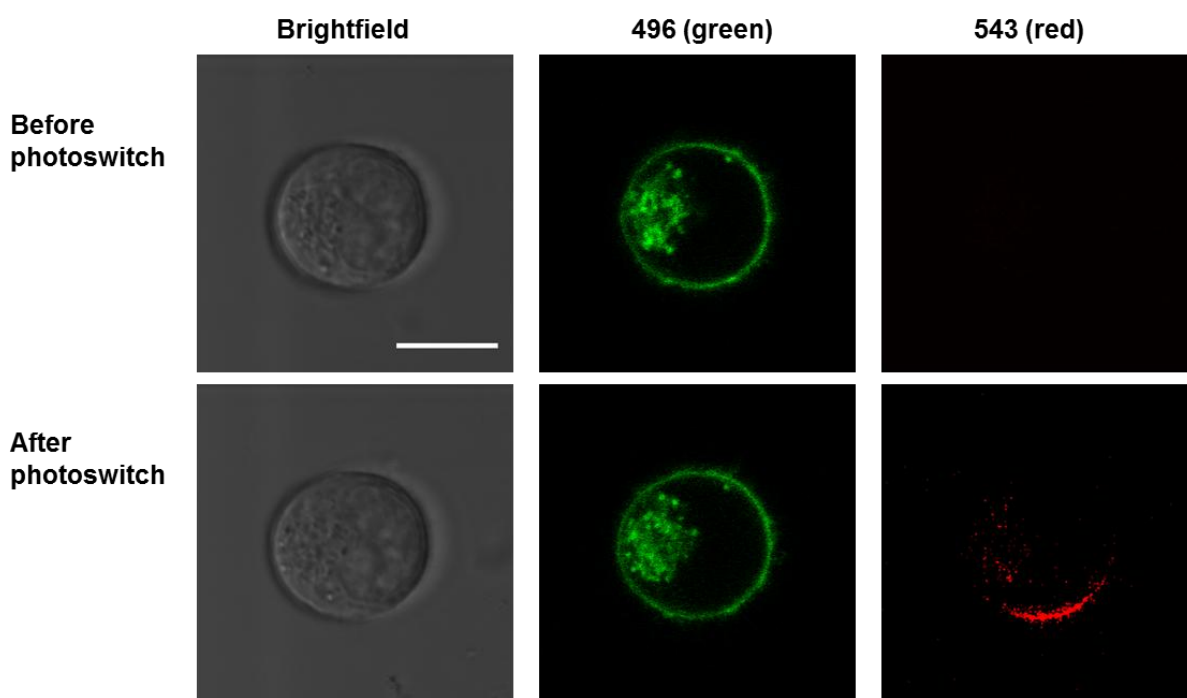


Figure 4.4: Photoswitching a specific region of the membrane from green to red fluorescence. YTS-KIR2DL1-tdEosFP cells were imaged by confocal microscopy. As described in Materials and Methods, a specific region of the cell membrane could be selected and targeted with UV light. The tdEosFP molecules within this region were seen to switch from green to red fluorescence. Scale bar represents 10 μm .

Two control experiments were performed to verify that photobleaching or background signal would not interfere with the quantification of the red fluorescence. In the first experiment, YTS-KIR2DL1-tdEosFP cells were imaged in the red fluorescence channel without photoswitching to detect any variations in background (Figure 4.5A). This graph shows that the level of background fluorescence in the red channel was very low and remained stable over the course of 20 minutes of imaging. In the second experiment, whole YTS-KIR2DL1-tdEosFP cells were photoconverted from green to red fluorescence and fluorescence from the red channel was recorded for 15 minutes (Figure 4.5B). The results show that over the course of 15 minutes of imaging, even though there were slight variations in the signal, there was no overall significant drop in the red signal, indicating that the effect of

photobleaching is negligible. Taken together, these control experiments suggest that photobleaching and background signal would not have a strong effect on the quantification of the red fluorescence over 15 minutes of imaging and that any significant changes observed in the data would be due to real biological differences.

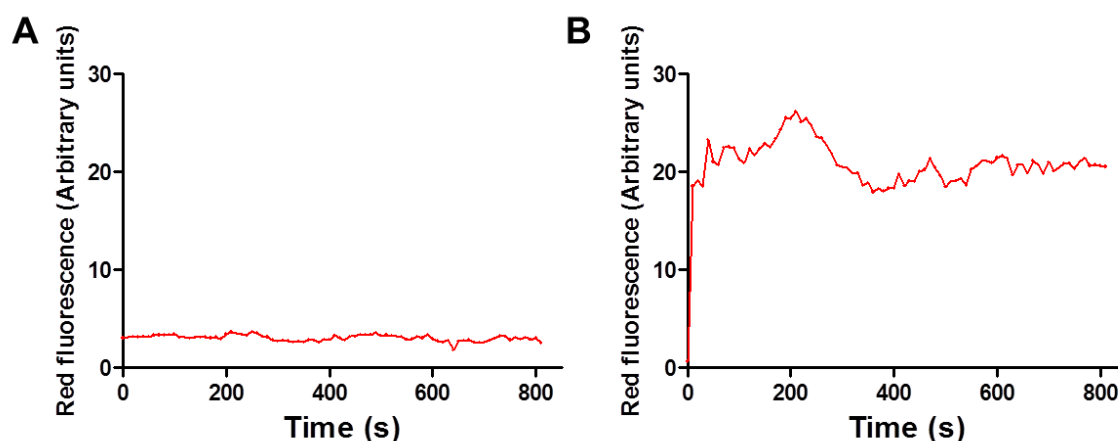


Figure 4.5: Control experiments to assess red background fluorescence and photobleaching. (A) Assessing red background fluorescence in the absence of photoswitching. The graph shows red fluorescence measured from a whole YTS-KIR2DL1-tdEosFP cell that was imaged by confocal microscopy without photoswitching. **(B)** Assessing photobleaching. Red fluorescence was measured from a whole YTS-KIR2DL1-tdEosFP cell that was photoswitched by UV light.

4.3 Results

4.3.1 Diffusion of KIR2DL1 in the NK cell membrane

YTS-KIR2DL1-tdEosFP cells were studied using this microscopy-based method in order to quantify the diffusion rate of KIR2DL1 molecules in the membrane of these cells. A small region of the cell membrane was selected and the tdEosFP molecules in this region were photoswitched. The cell was then imaged for a further 20 minutes, during which time the red fluorescence was seen to diffuse around the cell, until it was uniformly distributed in the cell membrane (Figure 4.6A). This procedure was repeated for ten YTS-KIR2DL1-tdEosFP cells. For each cell, the fluorescence both at the site of photoswitching and in the region opposite the site of photoswitching was quantified and analysed as described in the Materials and Methods, and as shown in Figure 4.6B. The data were normalised to allow a comparison between cells.

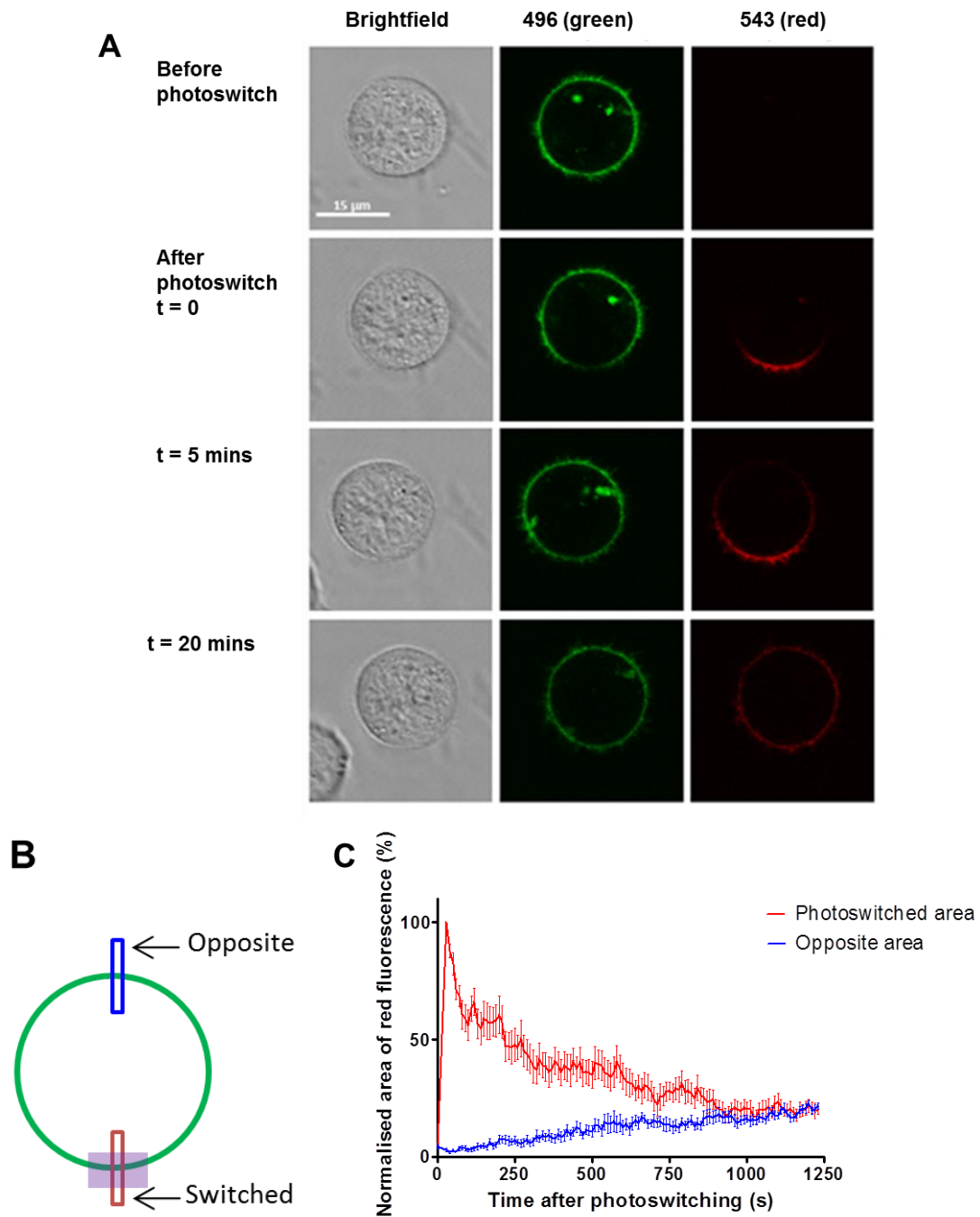


Figure 4.6: Experimental analysis of KIR2DL1 diffusion in the NK cell membrane. (A) Representative images of a YTS-KIR2DL1-tdEosFP cell, where a specific region of the cell membrane was targeted with UV light to photoconvert the tdEosFP molecules within this region. The cell was imaged by confocal microscopy for 20 minutes and KIR2DL1 can be seen to diffuse around the cell. Scale bar represents 15 μm . (B) Schematic diagram showing the strategy for measuring the diffusion of the receptor. The red fluorescence was measured in the photoswitched region and in the region directly opposite, as indicated by the boxes. The purple rectangle represents the region that was photoswitched. (C) Graphical representation of red fluorescence over time in both regions. A threshold was applied to the images to remove background fluorescence. Data was normalised so that the initial measurement of red fluorescence in the photoswitched region was 100%. $n = 10$ cells from 3 independent experiments. Error bars represent $\pm$ SEM.

Figure 4.6C shows an exponential decrease in the amount of red fluorescence at the site of photoswitching, coupled with a gradual increase of red fluorescence in the region opposite the site of photoswitching. Importantly, the red fluorescence within the cell membrane appears to reach equilibrium in the two sites of measurement, as demonstrated by the two curves meeting on the graph after approximately 1000 seconds (16-17 minutes). These results show that KIR2DL1 molecules do indeed diffuse around the cell membrane.

For the quantification of the diffusion rate of KIR2DL1 molecules in the cell membrane, a collaboration was established with Robert Endres and Gerardo Aquino in the Division of Molecular Biosciences (Imperial College), who worked on the mathematical modelling described in the Materials and Methods. To summarise briefly, a mathematical model was established that describes diffusion around a sphere, or in this case a cell, which is assumed to be spherical in shape. This model can describe diffusion over the whole area of a sphere using only two parameters: the diffusion coefficient and a normalisation coefficient. This equation was fit to the experimental data obtained from the analysis of ten cells to extract the diffusion coefficient of KIR2DL1 molecules around the cell (Figure 4.7).

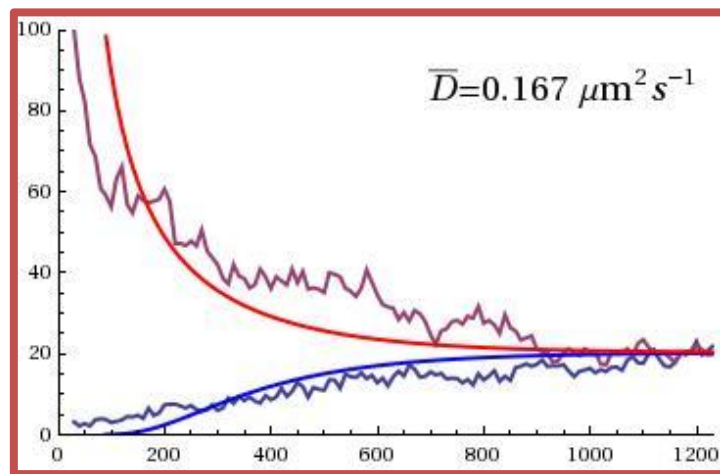


Figure 4.7: Mathematical determination of KIR2DL1 diffusion in the NK cell membrane. The diffusion coefficient of KIR2DL1, D , was determined by fitting the spherical diffusion equation to the experimental data (as described in Materials and Methods). Graph from Gerardo Aquino.

Using this model, the diffusion coefficient of KIR2DL1 was found to be $0.167 \pm 0.077 \mu\text{m}^2\cdot\text{s}^{-1}$. The coefficient of determination R^2 , which quantifies the “goodness of fit” of a predictive model, had a value of 0.90, indicating that the experimental data and the model simulations were well correlated.

4.3.2 KIR2DL1 is continuously trafficked to the immune synapse

To study how KIR2DL1 molecules behave in the presence of target cells and during the formation of immune synapses, YTS-KIR2DL1-tdEosFP cells were imaged in the presence of 221-Cw4 target cells. When incubated together, the cells formed inhibitory immune synapses where KIR2DL1 accumulated at the site of contact between an effector cell and a target cell. Conjugates where KIR2DL1 had accumulated were chosen for further investigation. In these conjugates, tdEosFP molecules were photoswitched only in the region directly opposite the synapse (Figure 4.8B).

Interestingly, photoswitched KIR2DL1-tdEosFP molecules continuously travelled to the immune synapse and accumulated there (Figures 4.8A & C), whereas the red fluorescence detected in the region opposite the synapse was steadily decreasing (Figure 4.8C). These results were striking, because even though a significant proportion of KIR2DL1 molecules were already located within the immune synapse, the photoswitched molecules were continuing to accumulate there anyway, suggesting that the recruitment of KIR2DL1 to the immune synapse is a continuous and dynamic process.

Further mathematical models were established within the framework of the collaboration with Robert Endres and Gerardo Aquino. One of the models describes a scenario in which a force is present, which physically traps the receptors within the synapse. Simulations using this model provide curves that follow the trends described in the experimental setting (Figure 4.9). Qualitatively, these simulations suggest that this type of trapping mechanism might explain the fact that KIR2DL1 is continuously accumulating at the immune synapse, without the requirement for additional mechanisms.

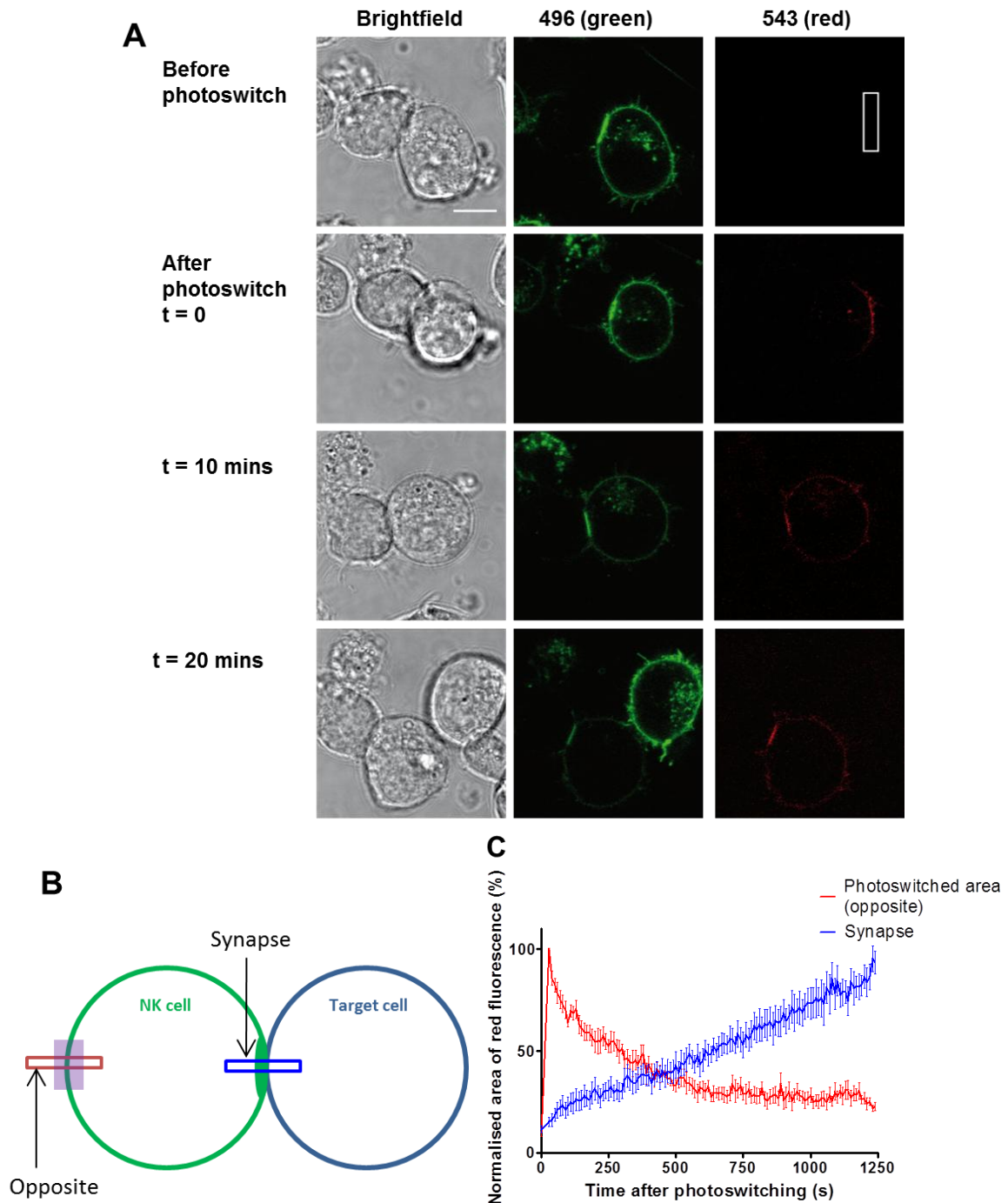


Figure 4.8: KIR2DL1 is continuously trafficked to the immune synapse. (A) Representative images of a conjugate formed between a YTS-KIR2DL1-tdEosFP cell and a 221-Cw4, where the region of the cell membrane directly opposite to the synapse was targeted with UV light to photoswitch the tdEosFP molecules within this region. The cells were imaged by confocal microscopy for 20 minutes and KIR2DL1 movement was monitored. Scale bar represents 10 μ m. **(B)** Schematic diagram showing the strategy for monitoring the movement of the receptor. The red fluorescence was measured in the photoswitched region (opposite the synapse) and in the synapse region, as indicated by the boxes. The purple rectangle represents the region that was photoswitched. **(C)** Graphical representation of red fluorescence over time in both regions. n = 10 cells from 4 independent experiments. Error bars represent $\pm$ SEM.

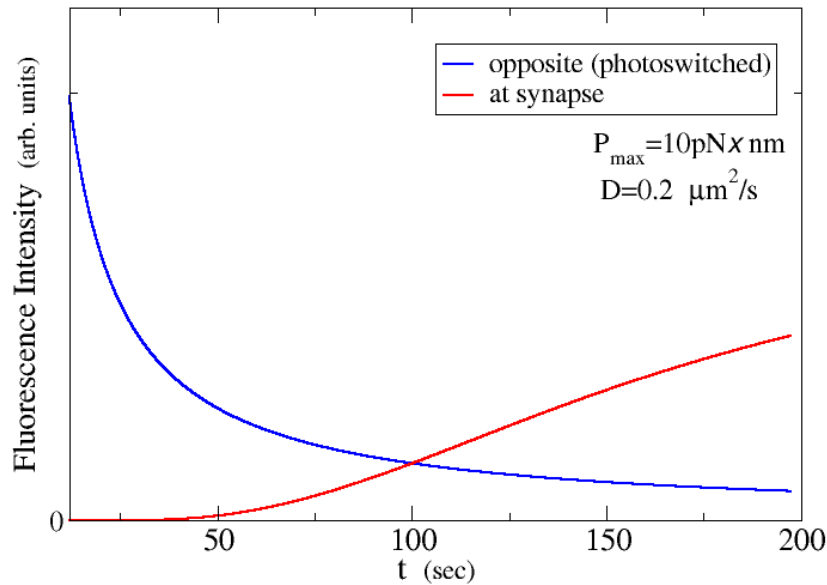


Figure 4.9: Mathematical modelling of KIR2DL1 diffusion towards the synapse in the presence of a target cell. Simulation generated from a mathematical model described in Materials and Methods, whereby a force is present which physically traps the receptors within the synapse. Graph from Gerardo Aquino.

4.3.3 KIR2DL1 remains stable at the immune synapse

In the next set of experiments, YTS-KIR2DL1-tdEosFP cells were again imaged whilst in contact with 221-Cw4 cells, but only the tdEosFP molecules within the synapse were photoswitched from green to red fluorescence (Figure 4.10). The cells were then imaged for 10 to 15 minutes to follow the movements of KIR2DL1 molecules. This procedure was repeated for ten cells. Strikingly, in all cells the red fluorescence was seen to remain within the synapse (Figure 4.10C), although there was a slight decrease in the fluorescence, which mirrored the results found in the control experiment testing for photobleaching effects (Figure 4.5B). In parallel, the fluorescence detected in the opposite area remained very low and stable throughout (Figure 4.10C). Taken together, these results show that receptors that are found within the synapse do not freely diffuse out of the synapse and around the cell membrane, but remain ‘trapped’ within the synapse.

The two mathematical models described in Materials and Methods were applied to this scenario. The simulations obtained from both models, model 1 (where the diffusion of receptors within the synapse is lower than the diffusion in the rest of the cell) and model 2 (where a physical force traps the receptors inside the synapse), followed the trends described above, where the fluorescence

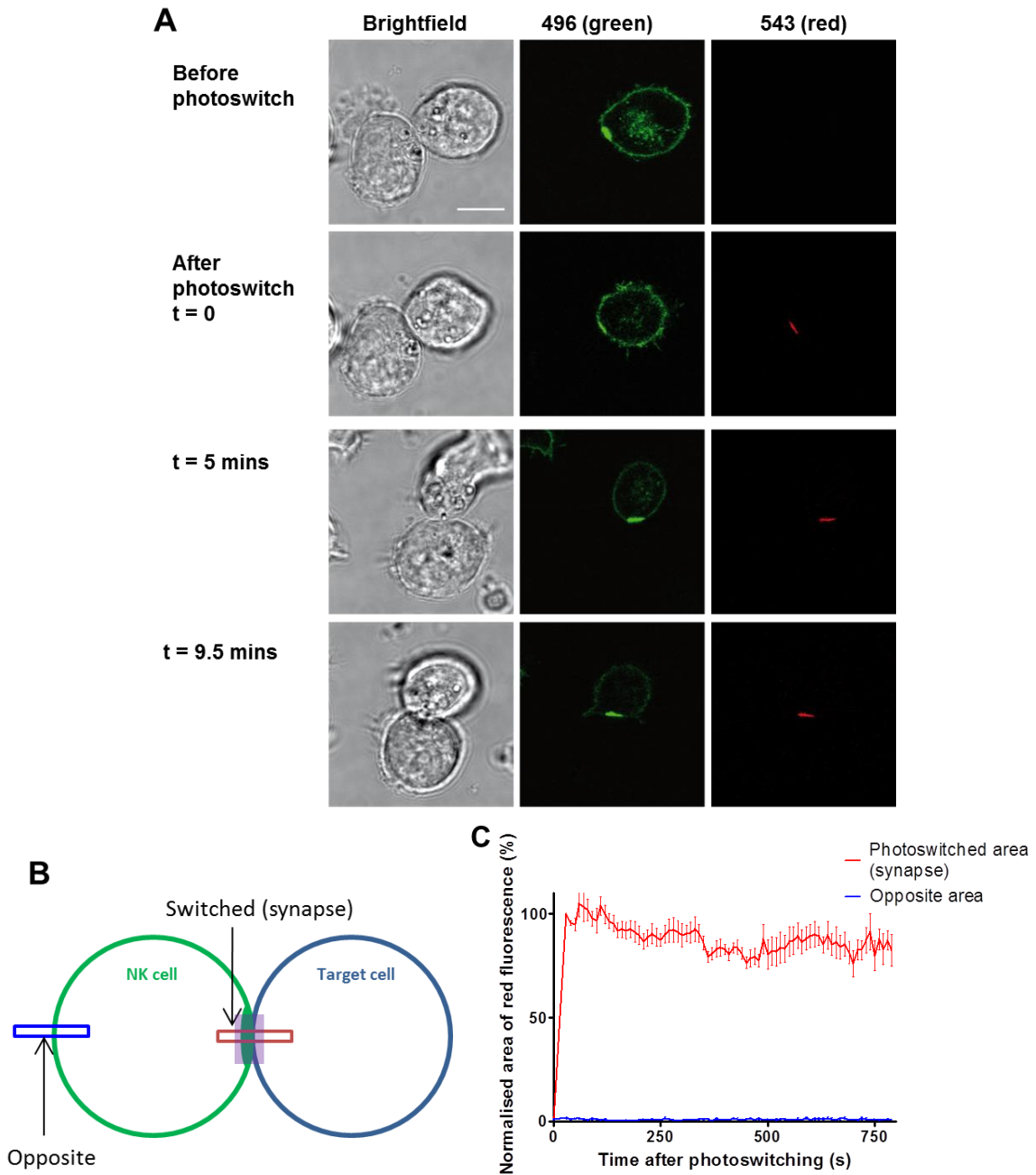


Figure 4.10: KIR2DL1 remains stable at the immune synapse. (A) Representative images of a conjugate formed between a YTS-KIR2DL1-tdEosFP cell and a 221-Cw4 cell, where the synapse region was targeted with UV light to photoswitch the tdEosFP molecules within the synapse. The cells were imaged by confocal microscopy for 10-15 minutes and KIR2DL1 movement was monitored. Scale bar represents 10 μ m. **(B)** Schematic diagram showing the strategy for monitoring the movement of the receptor. The red fluorescence was measured in the photoswitched region (the synapse) and in the opposite region, as indicated by the boxes. The purple rectangle represents the region that was photoswitched. **(C)** Graphical representation of red fluorescence over time in both regions. $n = 10$ cells from 4 independent experiments. Error bars represent $\pm$ SEM.

within the synapse remained stable, whereas the fluorescence in the opposite region remained very low (Figure 4.11). These models can therefore qualitatively explain the experimental results obtained here. Taken together, these results suggest that once KIR2DL1 molecules have been recruited to the immune synapse, they are physically constrained to remain within the synapse.

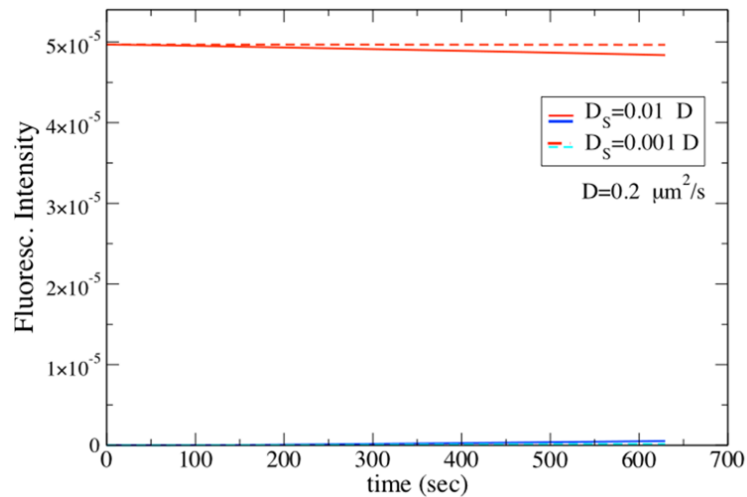


Figure 4.11: Mathematical modelling of KIR2DL1 movement at the synapse in the presence of a target cell. Simulation generated from a mathematical model described in Materials and Methods, where the diffusion of receptors within the synapse is lower than the diffusion in the rest of the cell. Graph from Gerardo Aquino.

4.3.4 Trafficking of KIR2DL1 between synapses

NK cells can form synapses with multiple target cells, resulting in the appropriate cellular response for each target cell (Eriksson et al., 1999). YTS-KIR2DL1-tdEosFP cells incubated with 221-Cw4 cells were occasionally seen to form two or more synapses with target cells simultaneously. The question arose whether KIR2DL1 would remain within one synapse or whether it would be able to move from one synapse to the other in the context of multiple synapses with similar target cells.

YTS-KIR2DL1-tdEosFP cells were therefore imaged in the presence of 221-Cw4 cells. When NK cells with multiple synapses were found (usually displaying two synapses), the tdEosFP molecules in one of the synapses were photoconverted and the cells would then be imaged for approximately 20 minutes. It quickly became obvious that there was a gradual accumulation of red fluorescence detected within the second synapse (Figure 4.12). This phenomenon was observed in at least seven different experiments. These results suggest that KIR2DL1 receptors that are ‘trapped’ within one

synapse are actually free to move to a different synapse formed by the same effector cell with another target cell.

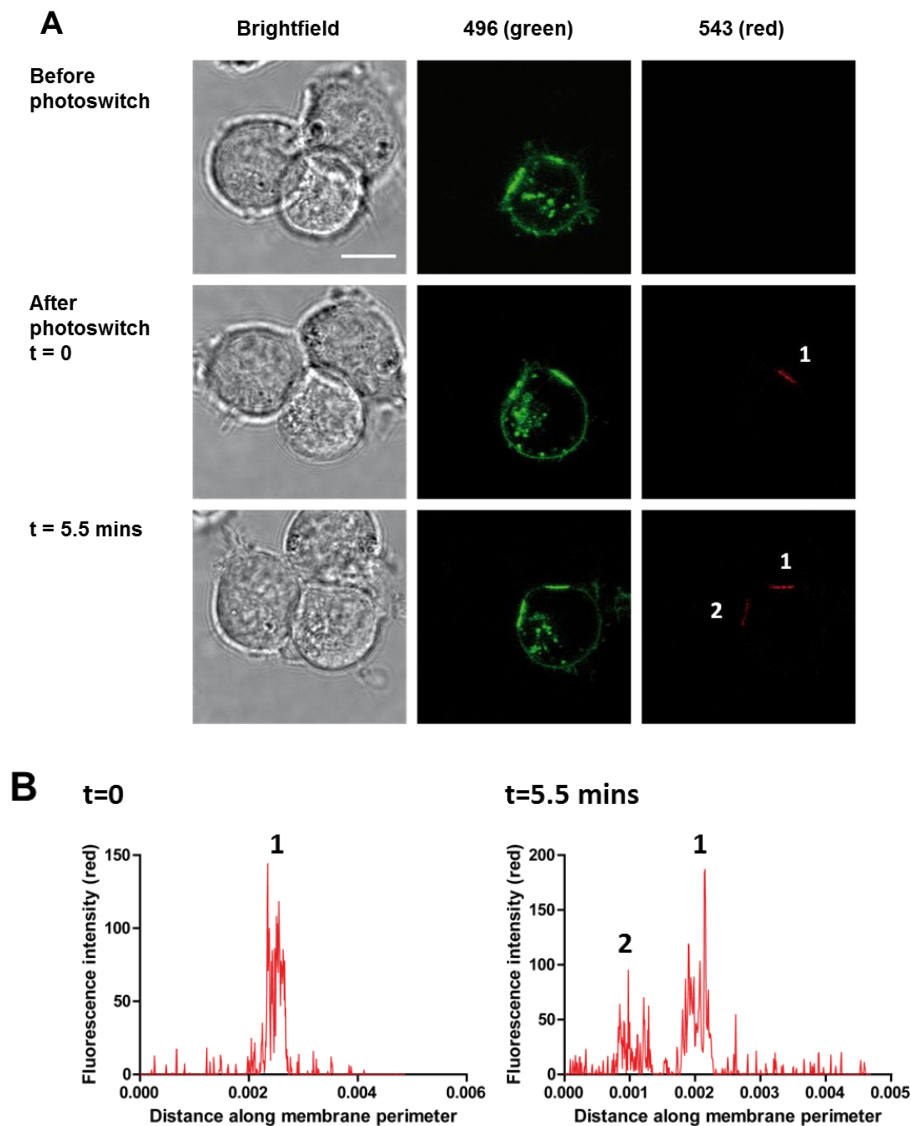


Figure 4.12: KIR2DL1 can traffic between synapses. (A) Representative images of a YTS-KIR2DL1-tdEosFP cell forming inhibitory immune synapses with two 221-Cw4 cells, where one of two synapses was targeted with UV light to photoconvert the tdEosFP molecules within this synapse. The cells were imaged by confocal microscopy for 10-15 minutes and KIR2DL1 movement was monitored. Scale bar represents 10 μm . (B) The red fluorescence was measured around the periphery of the NK cell, just after photoswitching ($t = 0$) where one fluorescence peak is visible (corresponding to the photoswitched synapse), and again later on ($t = 5.5$ mins) where a second fluorescence peak is appearing, showing KIR2DL1 accumulating at the second synapse. Data is representative of $n = 7$.

4.4 Discussion

4.4.1 Summary of results

In this investigation, the main aim was to study the microscale dynamics of the inhibitory receptor KIR2DL1 in the NK cell membrane in single cells as well as in NK cells forming inhibitory synapses with target cells. In particular, the main achievements of this project are as follows:

- A microscopy-based method was established to visualise the dynamics of KIR2DL1 in NK cell membranes by exploiting the properties of a photoswitchable fluorescent protein
- The diffusion of KIR2DL1 was imaged in the membrane of single non-conjugated NK cells and the diffusion coefficient of KIR2DL1 in the NK cell membrane was found to be $0.167 \pm 0.077 \mu\text{m}^2 \cdot \text{s}^{-1}$
- It was found that KIR2DL1 molecules are continuously trafficked to the immune synapse formed between NK cells and target cells
- By photoswitching molecules within the synapse, it was shown that KIR2DL1 molecules remain stable at the immune synapse
- When multiple synapses were formed between a single NK cell and multiple target cells, KIR2DL1 molecules were seen to traffic from one synapse to another.

Together, these results demonstrate that an imaging method based on photoswitchable fluorophores could be exploited to derive new information about the movements and dynamics of the inhibitory NK cell receptor KIR2DL1.

4.4.2 Dynamics of KIR2DL1 in the NK cell membrane

Diffusion of KIR2DL1 in the NK cell membrane

The diffusion of KIR2DL1 receptors within the membrane of NK cells was analysed by quantifying the red fluorescence in different membrane regions over time after photoswitching. From these quantifications, the diffusion coefficient of KIR2DL1 could be calculated, which reflects the mean squared displacement explored by a protein through a random walk over time (Lippincott-Schwartz et al., 2001). The diffusion coefficient of membrane proteins is dependent on the viscosity of the environment, the hydrodynamic radius of the particle, and its interaction with other molecules or cellular components such as the cytoskeleton. For example, the diffusion coefficient is decreased when proteins associate with lipid rafts or protein complexes (Kusumi et al., 2005).

The diffusion coefficient of KIR2DL1-tdEosFP in the NK cell membrane is $0.167 \pm 0.077 \mu\text{m}^2.\text{s}^{-1}$, which is comparable to the values obtained for other plasma membrane receptors fused to fluorescent proteins, including immune proteins such as the TCR and HLA-Cw6 (shown in Table 4.1). These results indicate that in the absence of receptor ligation, KIR2DL1 can move relatively freely within the plasma membrane of an NK cell.

Molecule	Diffusion coefficient ($\mu\text{m}^2.\text{s}^{-1}$)	Reference
E-cadherin-GFP	0.03-0.04	(Adams et al., 1998)
Luteinising hormone receptor-GFP	0.16	(Horvat et al., 1999)
GFP-aquaporin	0.009	(Umenishi et al., 2000)
TCR-GFP	0.12	(Favier et al., 2001)
CD94/NKG2A-GFP	0.067	(Sanni et al., 2004)
HLA-Cw6-GFP	0.32	(Yang et al., 2010)
ULBP1	0.122	(Martinez et al., 2011)
KIR2DL1-tdEosFP	0.167	From this study

Table 4.1: Diffusion coefficients of plasma membrane proteins.

It needs to be noted that the diffusion of a free protein might differ slightly from that of the same protein fused to a fluorescent protein. Fusion with a fluorescent protein will have an effect on the

protein size and may affect its binding affinity to other molecules. In this study, the KIR2DL1-tdEosFP fusion construct was shown to be functional, as it was capable of inducing inhibitory signalling within NK cells, and therefore its functional binding properties seem unaffected. However, the change in size might indeed affect the diffusion rate. The overexpression of the construct may also influence normal cellular processes and produce artefactual movement of the transfected protein construct, although it was properly recruited to the immune synapse.

KIR2DL1 molecules are continuously recruited to the immune synapse

NK cells form inhibitory immune synapses when in contact with resistant, MHC class I-expressing target cells. It is known that KIR2DL1 accumulates at an inhibitory synapse (Davis et al., 1999). In this study, KIR2DL1-tdEosFP molecules were photoswitched from green to red fluorescence in the region directly opposite the synapse. Surprisingly, these molecules were not only diffusing around the cell, but continued to accumulate at the immune synapse. These results suggest that even when an inhibitory synapse has already formed, KIR2DL1 molecules from the entire plasma membrane are continuously recruited into the synapse. The immune synapse is therefore a dynamic structure, with new molecules being recruited over time following its formation. Dominance of the inhibitory signal at an NK cell immune synapse has been shown to be achieved once the concentration of KIR2DL1 at the contact site exceeds a certain threshold (Borszcz et al., 2003). It would be interesting to determine whether at some time-point the red fluorescence level would reach a plateau, indicating that the synapse is somehow 'saturated' with KIR2DL1 molecules.

KIR2DL1 molecules remain 'trapped' within the synapse

To determine whether the newly recruited KIR2DL1 molecules were displacing other KIR2DL1 proteins from the synapse, KIR2DL1-tdEosFP molecules that were present within the synapse were photoconverted from green to red fluorescence. Strikingly, the red fluorescence remained stable and constant at the immune synapse over time, and the fluorescence in the membrane region opposite the synapse stayed very low. These results fitted well with the model that KIR2DL1 receptors were physically trapped within the immune synapse once they had been recruited there.

The model allows for a small proportion of receptors to be able to diffuse out of the synapse, although the resolution of the microscope did not permit visualisation of this effect.

It has been suggested that enrichment of inhibitory receptors to the immune synapse is not sufficient for efficient inhibitory signalling, but that immobilisation of receptors at the site of contact, as described here, is a requirement for a sustained localised inhibitory signal within cells that are continuously poised to kill the target cells that they encounter (Sanni et al., 2004).

In the future, it would be interesting to investigate which forces may be acting on KIR2DL1 receptors to sequester them within the synapse. The kinetic parameters of KIR2DL1-HLA-C binding are extremely rapid, with very fast association and dissociation rates (Valés-Gómez et al., 1998). As a consequence, the authors suggested that the limiting factor in this receptor-ligand interaction is the availability of HLA-C on the target cell (Valés-Gómez et al., 1998). It is therefore a possibility that if the concentration of HLA-C on the target cell is such that an inhibitory synapse is formed, this density is sufficient to maintain high levels of association between KIR2DL1 receptors and their ligands, thereby restraining them within the synapse. Any receptors that are not associating and dissociating from their ligands due to lack of availability might diffuse out of the synapse. However, the reduced mobility of receptors at the synapse might result from a more complex process.

Trafficking of KIR2DL1 between synapses

When an NK cell formed synapses with two target cells simultaneously, KIR2DL1-tdEosFP molecules from one synapse were able to traffic to the other synapse. This was a surprising result, since the previous experiments showed that KIR2DL1 receptors seem to remain trapped within a synapse once they have been recruited there. These results lead to new questions about the underlying mechanisms that regulate the recruitment of KIR2DL1 to an immune synapse. Some important mechanisms must be involved that are able to overcome the constraints keeping KIR2DL1 molecules within one synapse to allow them to travel to a second synapse. It is possible that some active processes linked to the cytoskeleton or signalling events are involved, although the enrichment of KIR2DL1 at the synapse has previously been shown to be independent of signalling (Fassett et al., 2001) and does not require an intact cytoskeleton, lipid rafts or ATP (Davis et al., 1999; Lou et al., 2000; Fassett et al., 2001).

It is unclear what path KIR2DL1 molecules followed to traffic from one synapse to the other: through the plasma membrane or via endocytic pathways. It seems that KIR2DL1 receptors were leaving the first synapse at low densities, since no signal was detected anywhere else in the cell or in other parts of the plasma membrane. The density of molecules was only high enough for detection once they started accumulating in the second synapse.

The movement of KIR2DL1-tdEosFP molecules from one synapse to another was rapid and in all cases occurred within a few minutes. Since inhibitory signalling needs to interfere with activating signals, it is clear that the response of inhibitory receptors must be rapid and predominant. In the case of multiple synapses, it is possible that KIR2DL1 receptors that are already involved in signalling at one synapse are recruited to another synapse to enhance and accelerate the response to another target cell. It would be interesting to investigate whether the receptors that traffic between synapses are already involved in signalling at the first synapse and can therefore enhance signalling in the second synapse by being already phosphorylated, or whether they are free to leave the first synapse to enrich at the second synapse because they were not involved in signalling at the first synapse.

4.4.3 Future directions

The results obtained in this chapter have shown that KIR2DL1 molecules are free to diffuse in the NK cell membrane. Upon formation of an inhibitory immune synapse, KIR2DL1 receptors are continuously trafficked to the site of contact, where they are trapped, although they can traffic between synapses formed by the same NK cell with multiple target cells. These results raise interesting new questions that future experiments should aim to address.

Although it has been shown that the formation of inhibitory synapses is largely actin-independent (Davis et al., 1999; Carlin et al., 2001), there is evidence that blocking actin polymerisation inhibits initial clustering of KIR2DL1 at the immune synapse, although over longer times enrichment of KIR2DL1 occurs independently of actin remodelling (Standeven et al., 2004). These studies suggest that even though actin is not necessary for KIR2DL1 accumulation at the immune synapse, the actin cytoskeleton might play a role in the regulation of KIR2DL1 recruitment. In order to test whether the lateral mobility of KIR2DL1 is controlled by the actin cytoskeleton during synapse formation or during trafficking between synapses, the same experiments as described here could be repeated using inhibitors of actin polymerisation, such as Latrunculin A or Cytochalasin D.

It is thought that on T cells, ligation of the TCR complex enhances its endocytosis and degradation as a means to regulate cell activation (Alcover and Alarcón, 2000). The trafficking and endocytosis of many immune receptors have been investigated, such as immunoglobulin Fc receptors (Amigorena and Bonnerot, 1999), MHC class I molecules (Caplan et al., 2002) and others. As far as NK cells are concerned, several activating receptors have been shown to be down-regulated by internalisation following ligation by soluble or membrane-bound ligands, including NKG2D (Roda-Navarro and Reyburn, 2009), CD16 (Cecchetti et al., 2007) and 2B4 (Sandusky et al., 2006). Less is known about endocytosis and intracellular trafficking of inhibitory receptors. The inhibitory CD94/NKG2A complex has been shown to continuously recycle between the cell surface and intracellular compartments, independently of inhibitory signalling (Borrego et al., 2002). However, conflicting results have been reported regarding down-regulation or internalisation of KIR receptors. For example, KIR2DL2 was found to be down-modulated after incubation of NK clones with target cells expressing the appropriate ligands (Huard et al., 2001), whereas crosslinking KIR2DL1 on a cell line using a specific monoclonal antibody did not result in down-regulation of the receptor (Sandusky et al., 2006). It seems that it would be detrimental for inhibitory receptors such as KIR2DL1 to be down-regulated, as there is a requirement that they be constantly expressed at the cell surface and exposed to ligands expressed by surrounding cells to prevent undesirable activation. It would therefore be desirable for a continuous supply of inhibitory receptors to remain at the cell surface, which could be achieved by *de novo* synthesis, rapid recycling, or a combination of both (Masilamani et al., 2009). To determine whether endocytosis is involved in the regulation of KIR2DL1 recruitment to inhibitory immune synapses, experiments could be carried out using endocytosis inhibitors, such as Dynasore, to examine how the dynamics of KIR2DL1 are affected.

The technique used here to study the dynamics of KIR2DL1 has some important advantages, as it is based on a physiological system of direct cell-cell interactions and visualises the movement of KIR2DL1 in a real synapse formed between two cells, rather than a 'mimic' of a synapse on a slide. However, the resolution remains limited and it would be interesting to combine this technique with high-resolution imaging methods. For example, the movement of KIR2DL1 within the synapse could directly be imaged at improved resolution by manipulating the cell-cell conjugate using optical tweezers such that the synapse would be in the horizontal plane of the microscope. In a slightly less physiological setting, live PALM/TIRF microscopy could be utilised to track individual KIR2DL1 molecules at super-resolution within a 'synapse' formed on an antibody-coated glass slide or a supported lipid bilayer. The diffusion coefficient of KIR2DL1 molecules within the synapse could then be compared to the diffusion coefficient of KIR2DL1 in the plasma membrane, as determined in this study.

In conclusion, imaging the dynamics of KIR2DL1 by exploiting a photoswitchable fluorescent protein has revealed new aspects of KIR2DL1 recruitment to the synapse and has shown for the first time that there can be trafficking of KIR2DL1 molecules between inhibitory synapses. These results have given rise to exciting new questions regarding the mechanisms regulating KIR2DL1 dynamics and recruitment. In the future, combining this technique with high- and super-resolution imaging techniques will provide a more detailed understanding of KIR2DL1 dynamics.

Chapter 5 - Imaging NK cell inhibition with a photocleavable peptide system

5.1 Introduction

One of the primary goals in the field of NK cell signalling is to relate cellular function to the integration of activating and inhibitory signals both in space and time. A variety of new tools are emerging to study the dynamics and the timing of signalling events within cells. In this chapter, the use of photocleavable peptides to study the timing of inhibitory signals in NK cells will be explored.

5.1.1 Photocleavable peptides

Caged compounds

The behaviour and function of proteins have generally been studied by genetic and biochemical approaches, with for example site-directed mutagenesis, interfering RNA, inhibitors and small molecules proving to be useful tools to study biological events. More recently, methodologies borrowed from the field of chemistry have led to the development of light-activatable ('caged') compounds, including peptides and proteins (McCray and Trentham, 1989; Adams and Tsien, 1993; Lawrence, 2005). Caged compounds contain a photolabile protecting group ('caging' group) on functionally important regions, rendering the compounds inactive. The photocleavable group can be cleaved by light to yield the native active compound. A caged compound can therefore be 'switched on' with a short burst of light wherever and whenever the investigator so desires.

In this way, photolysis of caged compounds is a technique that enables the time-resolved study of fast biological processes. For example, caged glutamate was used to study the giant squid neuronal synapse (Corrie et al., 1993) and caged chemoeffectors were used to investigate bacterial chemotaxis (Khan et al., 1993). Various photosensitive caging groups have been used, although the majority of caged compounds consist of ortho-nitrobenzyl compounds (Lawrence, 2005), which disintegrate after UV irradiation.

Applications to immune cell biology

More recently, this technique has been successfully applied to the study of signalling dynamics involved in T cell activation. DeMond and colleagues used 6-nitroveratryloxycarbonyl (NVOC) as a protecting group for an antigenic peptide (moth cytochrome c, or MCC) presented by MHC and recognised by murine T cells (DeMond et al., 2006). This caged antigenic peptide was used to selectively activate T cells on a supported lipid bilayer in a spatially and temporally controlled way using cleavage by near-UV light, providing a novel strategy for examining the arrangements of the T cell immune synapse (DeMond et al., 2006).

In more detailed studies, Huse and co-workers prepared an alternative synthetic MCC peptide bearing a photosensitive nitrophenylethylurethane (NPE) moiety with similar photocleavable properties (Huse et al., 2007; Quann et al., 2009, 2011). UV irradiation of TCR-peptide-MHC complexes enabled temporal analysis of T cell activation dynamics. They found that phosphorylation of LAT occurred after 4 seconds, calcium flux after 6.5 seconds, and cytoskeletal polarisation was observed within 2 minutes (Huse et al., 2007). By selectively illuminating specific regions of the cell, this approach also provided spatial information on dynamic events occurring within T cells: the polarisation of the MTOC initiated by T cell activation was driven by localised accumulation of diacylglycerol (Quann et al., 2009) and the polarity of T cells towards the immune synapse required the recruitment of PKC proteins to the immune synapse in an ordered cascade (Quann et al., 2011).

Huse and his group also recently developed a photochemical approach for stimulation of inhibition in NK cells (Abeyweera et al., 2011). They prepared supported lipid bilayers containing photocleavable peptide-MHC complexes that were not recognised by the inhibitory receptor KIR2DL2 until irradiation with UV light. NK cells formed stable activating synapses on these surfaces, but photostimulation of KIR2DL2 resulted in the formation of inhibitory receptor microclusters, suppression of new activating receptor microclusters, reorganisation of the actin cytoskeleton and retraction of the cells from the surface (Abeyweera et al., 2011).

These studies demonstrate the potential of photocleavable peptides to contribute to our understanding of immune cell signalling and cytoskeletal dynamics.

5.1.2 Photocleavable peptide strategy for studying NK cell inhibition

Despite recent advances in our knowledge of NK cell signalling, much work remains to be done on understanding the timing and dynamics of how inhibitory signals act to neutralise ongoing activating signalling. For example, it remains unclear how long NK cells may remain sensitive to inhibitory stimulation before becoming committed to a killing response. The exact sequence of events following the initiation of inhibitory signalling also remains unclear, due to the difficulties in separating activating and inhibitory signalling pathways in time.

Photocleavable peptide strategy

In this project, the objective was to set up a cell system exploiting the properties of a photocleavable peptide to study the timing of NK cell inhibition occurring at an immune synapse between an NK cell and a target cell, as opposed to the response of an NK cell on a supported lipid bilayer.

NK cell-mediated lysis of normal cells is avoided by inhibitory receptors on NK cells that recognise MHC class I molecules on target cells. MHC class I molecules consist of a heavy chain, β_2 -microglobulin (β_2m), and an intracellularly derived short peptide. Peptides are involved in the generation of stable, properly folded MHC class I molecules at the cell surface (Townsend et al., 1989).

The aim is to generate a photocleavable peptide that can be presented by MHC class I molecules on the target cell, but which blocks binding to the inhibitory receptor KIR2DL1. The lack of an inhibitory response would lead to activation of the NK cell and lysis of the target cell. However, if the peptide is illuminated and hence cleaved by UV light, the interaction between MHC class I and KIR2DL1 would be restored, resulting in stimulation of KIR2DL1 and initiation of NK cell inhibition (Figure 5.1). In this way, initiation of inhibitory signalling could be controlled both temporally and spatially. Using this system, it would then be possible to directly visualise the behaviour of fluorescently-tagged receptors and signalling molecules and monitor changes in effector responses following controlled inhibition. The system used in this project involves specific recognition of the MHC class I protein HLA-Cw4 by the inhibitory receptor KIR2DL1.

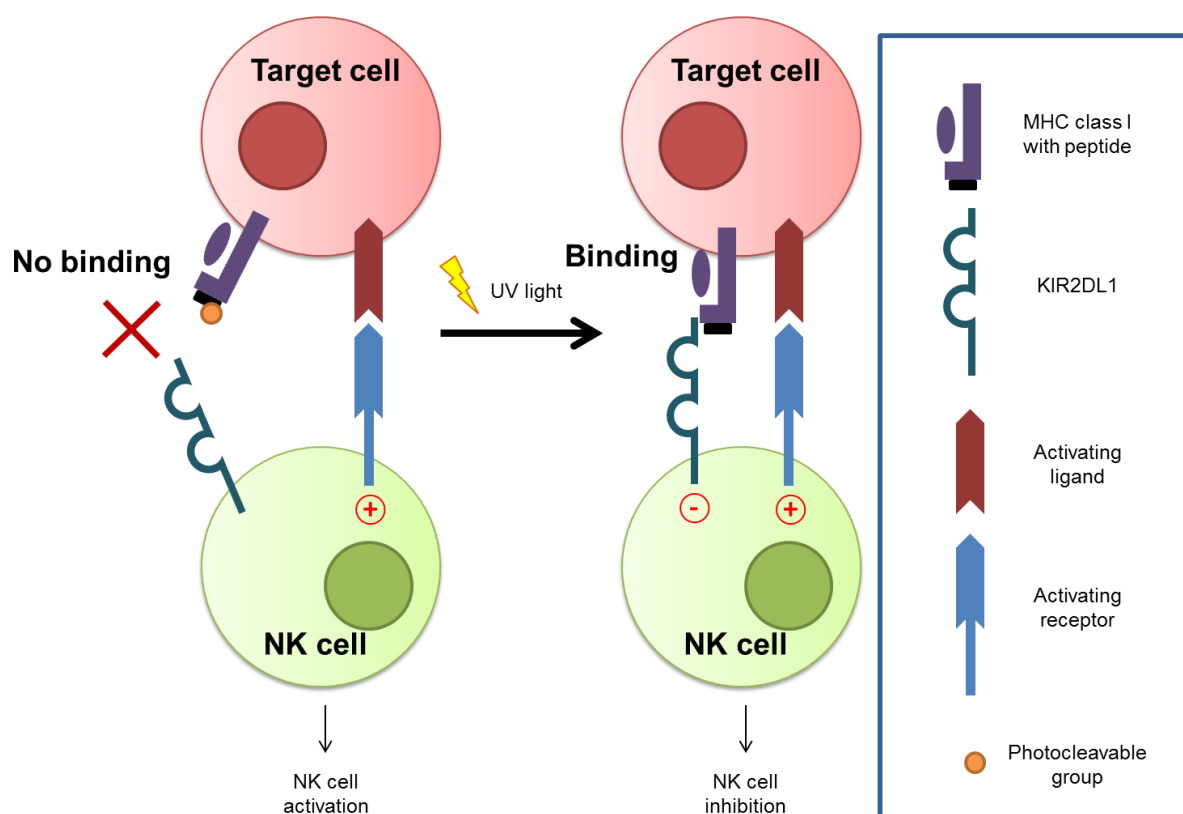


Figure 5.1: Schematic diagram depicting the photocleavable peptide strategy for controlling NK cell inhibition. The inhibitory receptor KIR2DL1 cannot bind the peptide-MHC class I complex on the target cell when the peptide contains a caging group and therefore the NK cell is activated. Upon exposure to UV light, the caging group is cleaved, yielding the native peptide, restoring binding between KIR2DL1 and peptide-MHC which results in NK cell inhibition. Using this strategy, NK cell inhibition can be temporally, and potentially also spatially, initiated at will.

Design of the photocleavable peptide

Although KIR receptors bind MHC class I molecules that present a peptide in their peptide-binding groove, it has been shown that recognition of 'empty' HLA-C molecules without bound peptide was sufficient for the inhibition of NK cell-mediated lysis (Falk et al., 1995; Mandelboim et al., 1996). However, there has been evidence for a degree of peptide selectivity in the recognition of HLA-C molecules by NK clones expressing inhibitory KIR receptors (Sidney et al., 1995; Mandelboim et al., 1996; Rajagopalan and Long, 1997) and for peptides playing a crucial role in KIR recognition (Rajagopalan and Long, 1997; Mandelboim et al., 1997; Fan et al., 2001). HLA-C molecules associate

with specific peptides, generally with a preferred length of nine amino acids and containing allele-specific motifs (Falk et al., 1993).

QYDDAVYKL is the original consensus peptide that binds HLA-Cw4 (Sidney et al., 1995). The HLA-Cw4 anchor motifs are tyrosine, valine and leucine at positions 2, 6 and 9, respectively (Falk et al., 1993; Sidney et al., 1995).

The N-terminal glutamine residue of the QYDDAVYKL could spontaneously cyclise to form pyroglutamate, which might interfere with experiments. Therefore, the sequence was modified by using an N-terminal serine instead of glutamine in the native peptide. SYDDAVYKL has been previously tested by others and shares the properties of QYDDAVYKL concerning affinity for HLA-Cw4 and binding to KIR2DL1 (Rajagopalan and Long, 1997). When SYDDAVYKL was bound to HLA-Cw4 on target cells, the specific lysis of these cells by an NK clone expressing KIR2DL1 was reduced to the same extent as with QYDDAVYKL compared to cells without peptide (Rajagopalan and Long, 1997), suggesting that the SYDDAVYKL peptide is able to induce protection from lysis by NK clones as efficiently as the original consensus peptide QYDDAVYKL.

In this project, the aim was to modify the HLA-Cw4-specific peptide SYDDAVYKL so that its ability to bind the inhibitory NK cell receptor KIR2DL1 could be controlled using UV light. Previous studies have indicated that residues at positions 7 and 8 in the peptide (tyrosine and lysine, respectively) are critical for enabling the binding of KIR2DL1 to HLA-Cw4 and initiating subsequent inhibitory signals (Rajagopalan and Long, 1997; Betser-Cohen et al., 2006). Indeed, the presence of a negatively charged residue such as aspartate or glutamate instead of the positively charged lysine at position 8 of the peptide did not affect the stabilisation of HLA-Cw4 at the cell surface but abrogated the binding of KIR2DL1 (Rajagopalan and Long, 1997). In addition, phosphorylation of the lysine in position 8 of the peptide completely blocked the interaction with KIR2DL1 and abolished HLA-Cw4-mediated inhibition (Betser-Cohen et al., 2006). These results are in agreement with the solved crystal structure of the HLA-Cw4/KIR2DL1 complex, where the direct contact between the peptide and HLA-Cw4/KIR2DL1 was limited to the C-terminal region of the presented peptide (Fan et al., 2001).

These studies suggest that the binding of KIR2DL1 to HLA-Cw4 is very sensitive to the nature of the side chain at position 8 in the bound peptide. It was therefore decided that the photocleavable peptide should be designed with a caging group on the lysine residue at position 8.

5.1.3 Aims

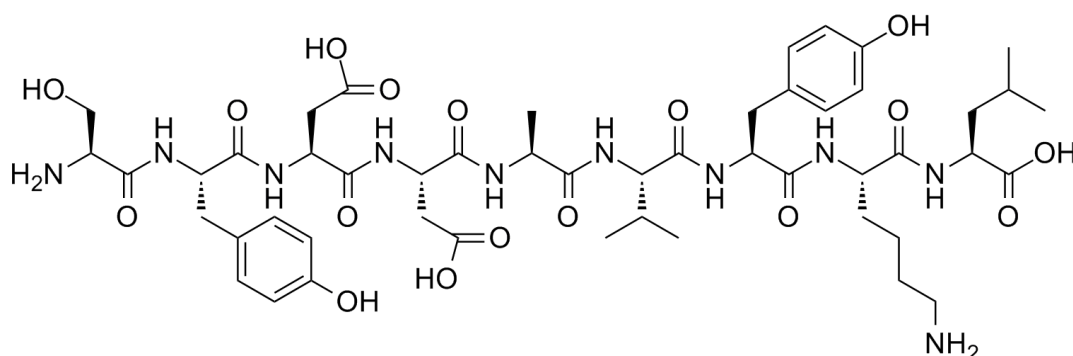
Deciphering the details of signal integration and the dynamics of NK cell recognition remains a major challenge in NK cell research (Bryceson and Long, 2008). Therefore, the overall aim of this project was to set up an imaging system using a photocleavable peptide. This system could then be used to study the timing and dynamics of NK cell signal integration at the immune synapse formed between an NK cell and a target cell, as well as the timing of an NK cell's decision to kill or not to kill a cell it is in contact with. The specific aims in this chapter were as follows:

- (i) To design and synthesise an appropriate photocleavable peptide
- (ii) To successfully cleave the photocleavable peptide to yield the native sequence
- (iii) To set up a cellular system in which the inhibition of NK cells can be controlled using the photocleavable peptide
- (iv) To carry out preliminary imaging experiments visualising a functional outcome, demonstrating that NK cell inhibition can be controlled using the photocleavable peptide.

5.2 Peptides

5.2.1 Control peptide

The HLA-Cw4-specific peptide SYDDAVYKL was used as a control peptide for use in subsequent experiments (Figure 5.2). This peptide was synthesised by solid-phase peptide synthesis, as described in Materials and Methods. After synthesis, the peptide was analysed and purified by LC-MS (Figure 5.3). The data from the LC-MS analysis following purification clearly demonstrate that the peptide was pure (i.e. there was a single peak corresponding to a single compound in the solution) and the mass of the compound (1074) corresponded to the expected theoretical mass of the peptide (1073.15). These results show that the control peptide was successfully synthesised and purified.



Chemical Formula: $C_{49}H_{72}N_{10}O_{17}$

Exact Mass: 1072.51

Molecular Weight: 1073.15

Figure 5.2: Chemical structure of control peptide SYDDAVYKL.

5.2.2 Attempts at synthesising a photocleavable peptide

In the first instance, SYDDAVYKL was going to be synthesised with an α -carboxy-2-nitrobenzyl (CNB) photocleavable group on the lysine found in position 8 of the peptide. This type of caging group was introduced by Walker and colleagues (Walker et al., 1986) and has previously been successfully used for biological applications (Milburn et al., 1989; Wieboldt et al., 1994; Aarhus et al., 1995; Grewer et al., 2000).

Synthesis of a CNB-caged SYDDAVYKL peptide was carried out in collaboration with David Trentham and John Offer (NIMR). The chemical structure of the desired peptide is shown in Figure 5.4.

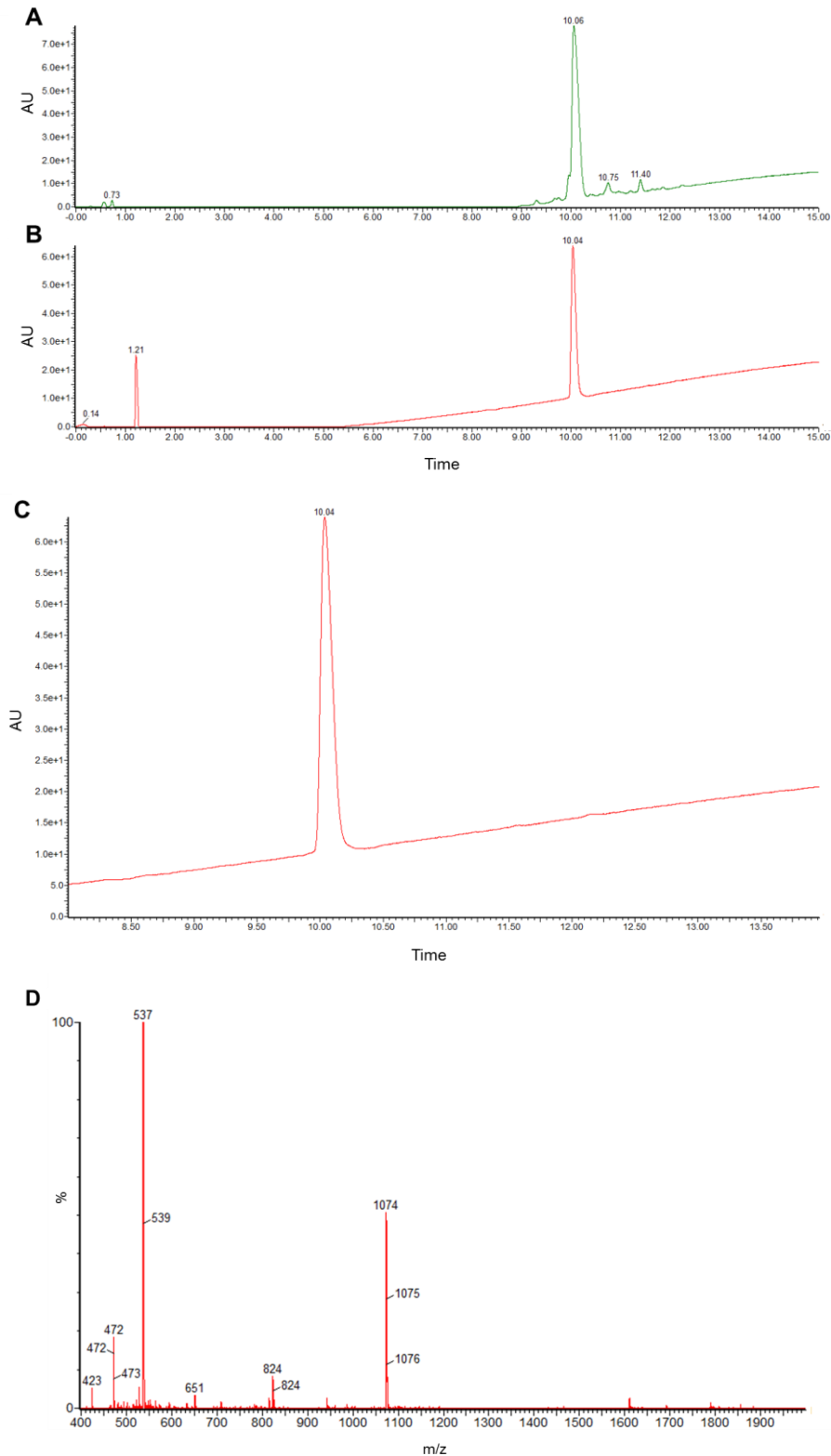


Figure 5.3: LC-MS analysis of the control peptide SYDDAVYKL. (A) Chromatogram of the peptide before purification (AU = arbitrary units.) **(B)** Chromatogram of the peptide after purification. **(C)** Enlarged view of the chromatogram of the peptide after purification. All subsequent chromatograms in this report will be shown in the enlarged mode. **(D)** Mass spectrum corresponding to the peak visible in the chromatogram of the control peptide (m/z = mass/charge ratio).

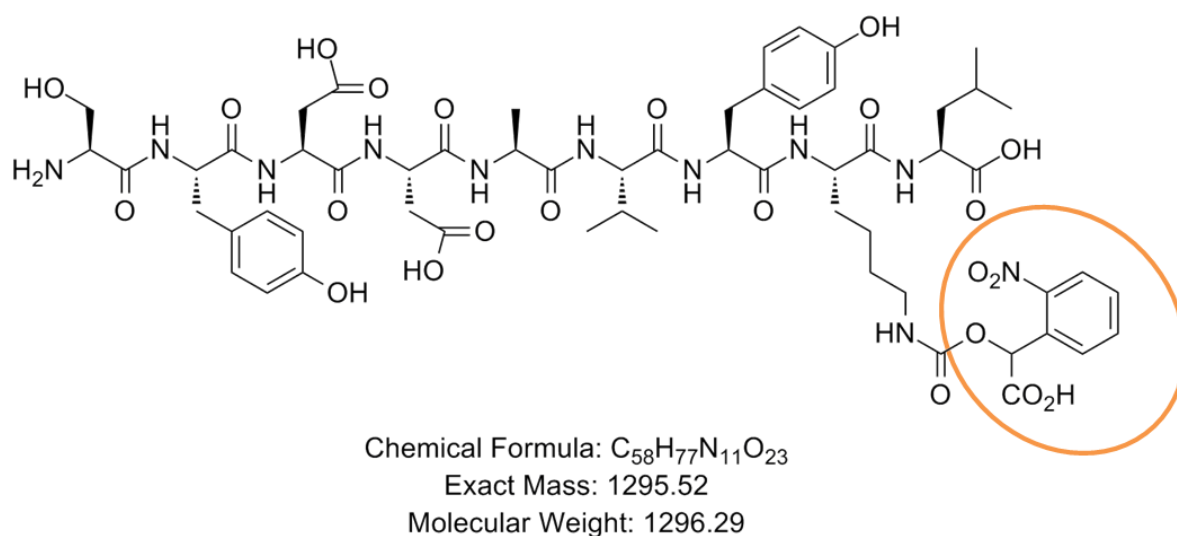


Figure 5.4: Chemical structure of the CNB-caged SYDDAVYKL peptide. The CNB caging group is circled in orange.

The compound that was obtained was purified and analysed by flash chromatography. Using this method, a compound of appropriate absorption was detected by UV spectroscopy (Figure 5.5A). In addition, the final compound was analysed using the ninhydrin assay to verify that the free amine on the lysine of the peptide had indeed reacted (Figure 5.5B). As shown in Figure 5.5B, the non-caged peptide on resin had a high maximum absorption (λ_{max}), indicating that the ninhydrin had reacted with the free amine to form a blue colour. In contrast, the caged peptide on resin had a much lower λ_{max} , indicating that there was little free amine on the peptide, thus the amine had reacted with the caging group.

These results suggest that the addition of the CNB caging group on the peptide was successful. Unfortunately, due to time restrictions and technical issues (including insufficient yield of final photocleavable peptide), the synthesis of this peptide had to be abandoned. A new strategy was devised in which a photocleavable peptide would be acquired commercially.

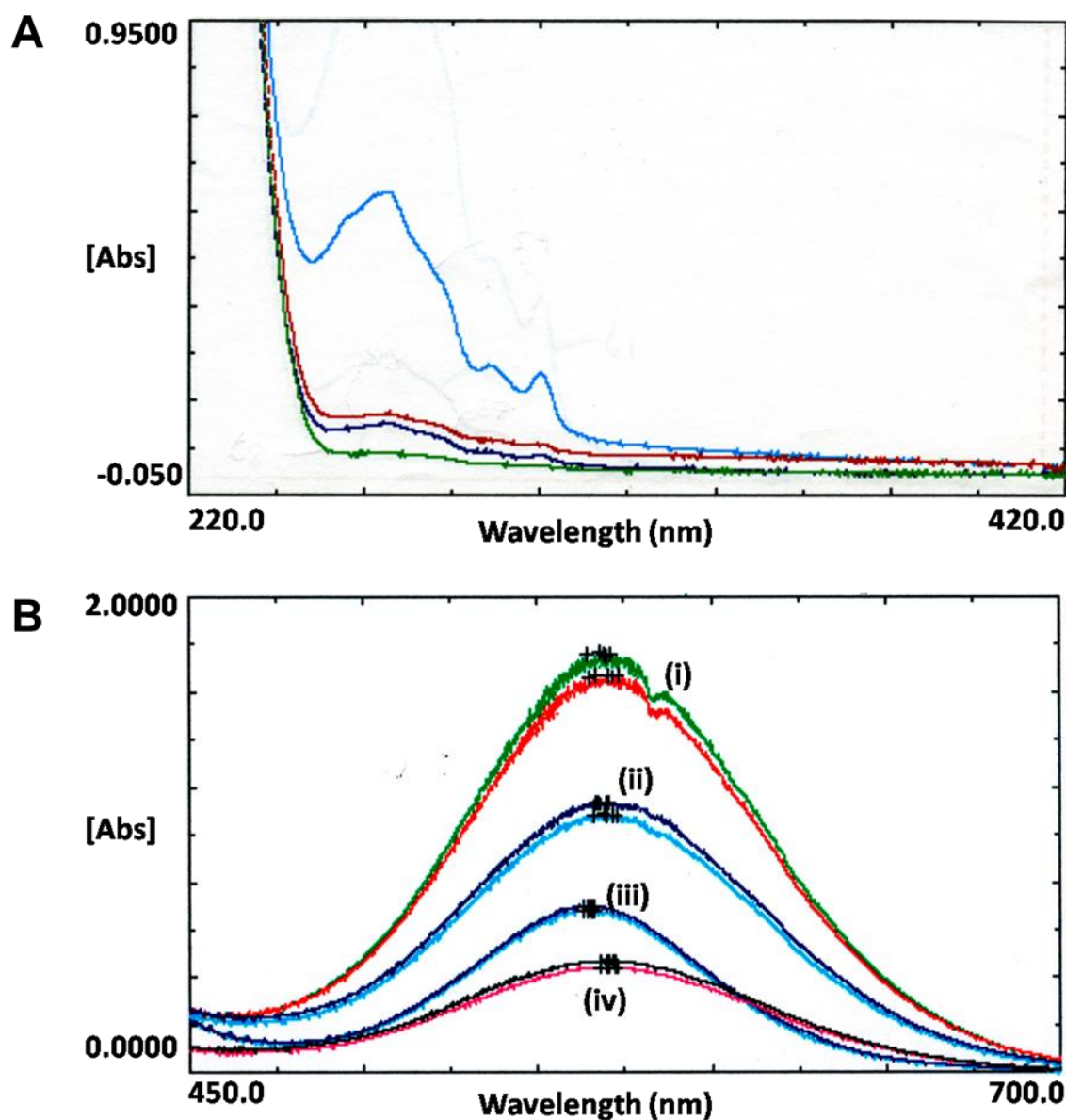


Figure 5.5: Analysis of the CNB-caged photocleavable peptide. (A) UV spectrum of four representative elution fractions from the flash chromatography purification of the caged peptide on resin. The final product was found in certain fractions (e.g. light blue) which were pooled for further purification and characterisation, but not in other fractions (e.g. red, dark blue, green). (B) UV spectra from a ninhydrin assay performed in duplicate on non-caged peptide on resin (i), L-glutamate standard (ii), caged peptide on resin (iii) and control solution (iv).

5.2.3 Designing and buying a photocleavable peptide

A photocleavable peptide with the sequence SYDDAVYKL and a CmnCbz caging group on the lysine residue was purchased from Cambridge Research Biochemicals. This caging group was chosen for several reasons: (i) it was commercially available for caging peptides, (ii) it has been used previously for biological applications (Kuner et al., 2008) and (iii) after illumination, photolysis occurs quickly (Papageorgiou and Corrie, 1997). The chemical structure of the CmnCbz-caged photocleavable peptide is shown in Figure 5.6.

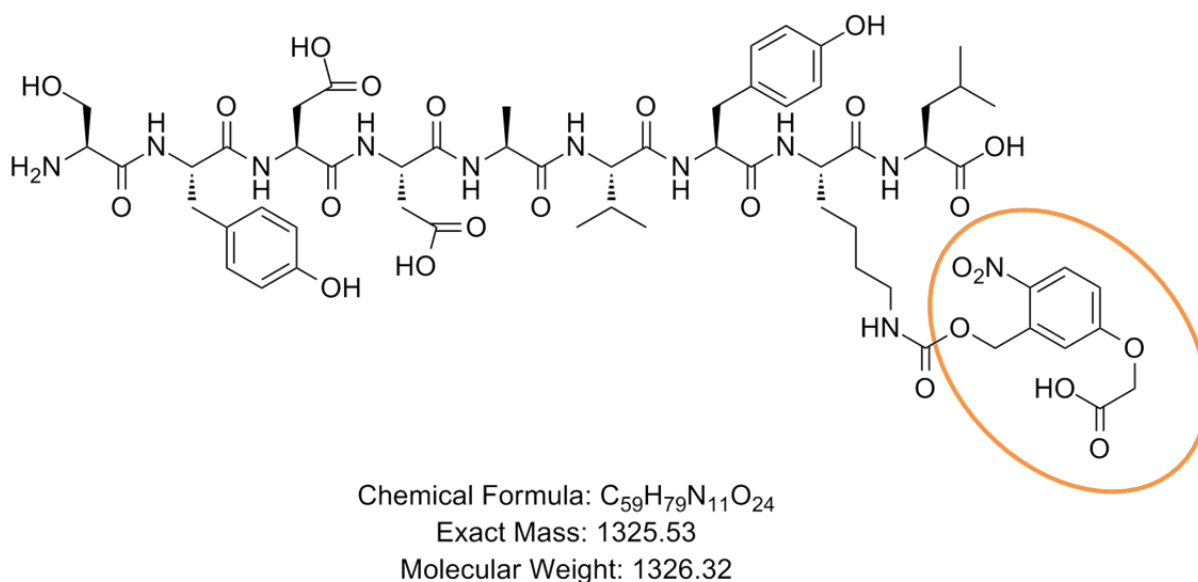


Figure 5.6: Chemical structure of the CmnCbz-caged SYDDAVYKL peptide. The CmnCbz caging group is circled in orange.

The photocleavable peptide was analysed by LC-MS (Figure 5.7). This analysis confirmed that the peptide was pure as there was a single peak detected in the solution, and the mass of the compound (1325) corresponded to the expected theoretical mass of the peptide (1326.32). These results suggest that the photocleavable peptide obtained commercially was correct.

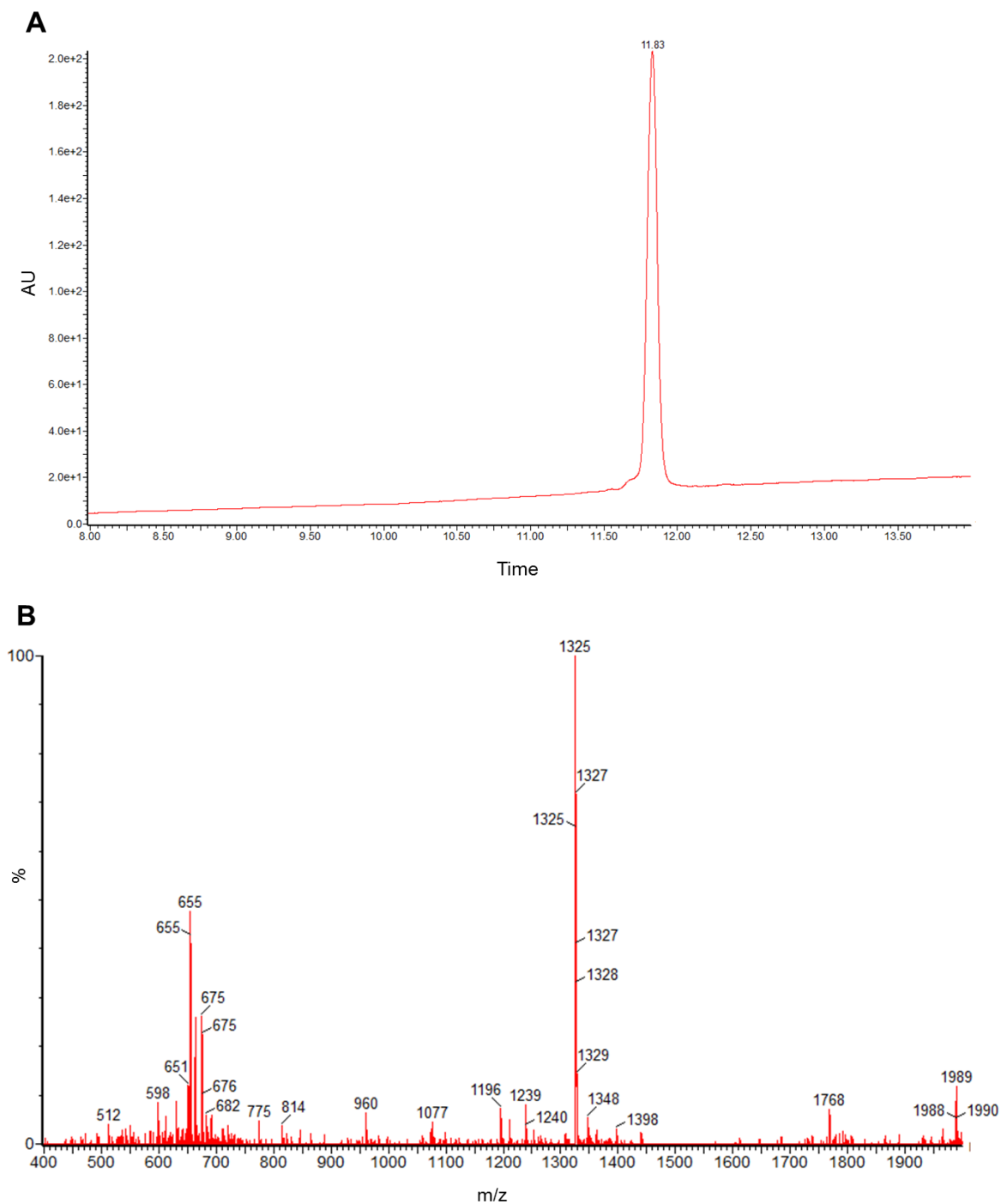


Figure 5.7: LC-MS analysis of the CmnCbz-caged photocleavable peptide. (A) Chromatogram of the commercially obtained peptide (AU = arbitrary units.) **(B)** Mass spectrum corresponding to the peak visible in the chromatogram of the photocleavable peptide (m/z = mass/charge ratio).

A calibration curve was established from three independent experiments using six different concentrations of the photocleavable peptide (Figure 5.8). The slope of this curve could then be used to quantify the concentration of photocleavable peptide in any solution analysed by LC-MS.

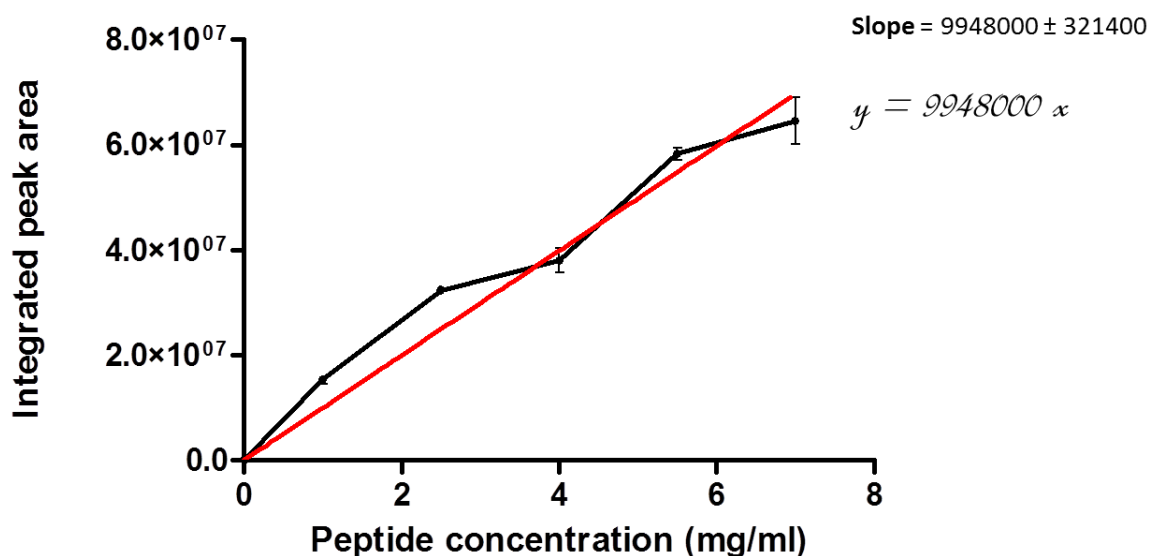


Figure 5.8: LC-MS calibration curve for the CmnCbz-caged photocleavable peptide. A calibration curve of the photocleavable peptide was obtained using different concentrations of peptide analysed by LC-MS. $n = 3$ independent experiments; error bars represent SEM.

5.2.4 Comparison of control and photocleavable peptides

The control and photocleavable peptides were analysed by LC-MS and their spectra were compared (Figures 5.9A & B). The two peptides came up in two separate peaks with different elution times and could easily be distinguished. To confirm that the native peptide and the photocleavable peptide can also be distinguished when they are both in the same solution, the two peptides were mixed together at equal concentrations and the solution was analysed by LC-MS (Figure 5.9C). The chromatogram clearly demonstrates the presence of two peaks. The mass spectrometry data from each peak (Figures 5.9D & E respectively) confirms that the first peak corresponds to the control peptide (with a mass of 1074) and the second peak corresponds to the photocleavable peptide (with a mass of 1326).

These results demonstrate that the native peptide and the photocleavable peptide can be distinguished when present in the same solution using LC-MS. This technique can therefore be used to assess whether subjecting the photocleavable peptide to various light sources can yield the native

peptide in the same solution. The calibration curve shown above was then used to quantify the concentration of photocleavable peptide remaining in the solution after photolysis and hence calculate the fraction of peptide that was successfully cleaved and thus obtain the cleaving efficiency of that specific light source.

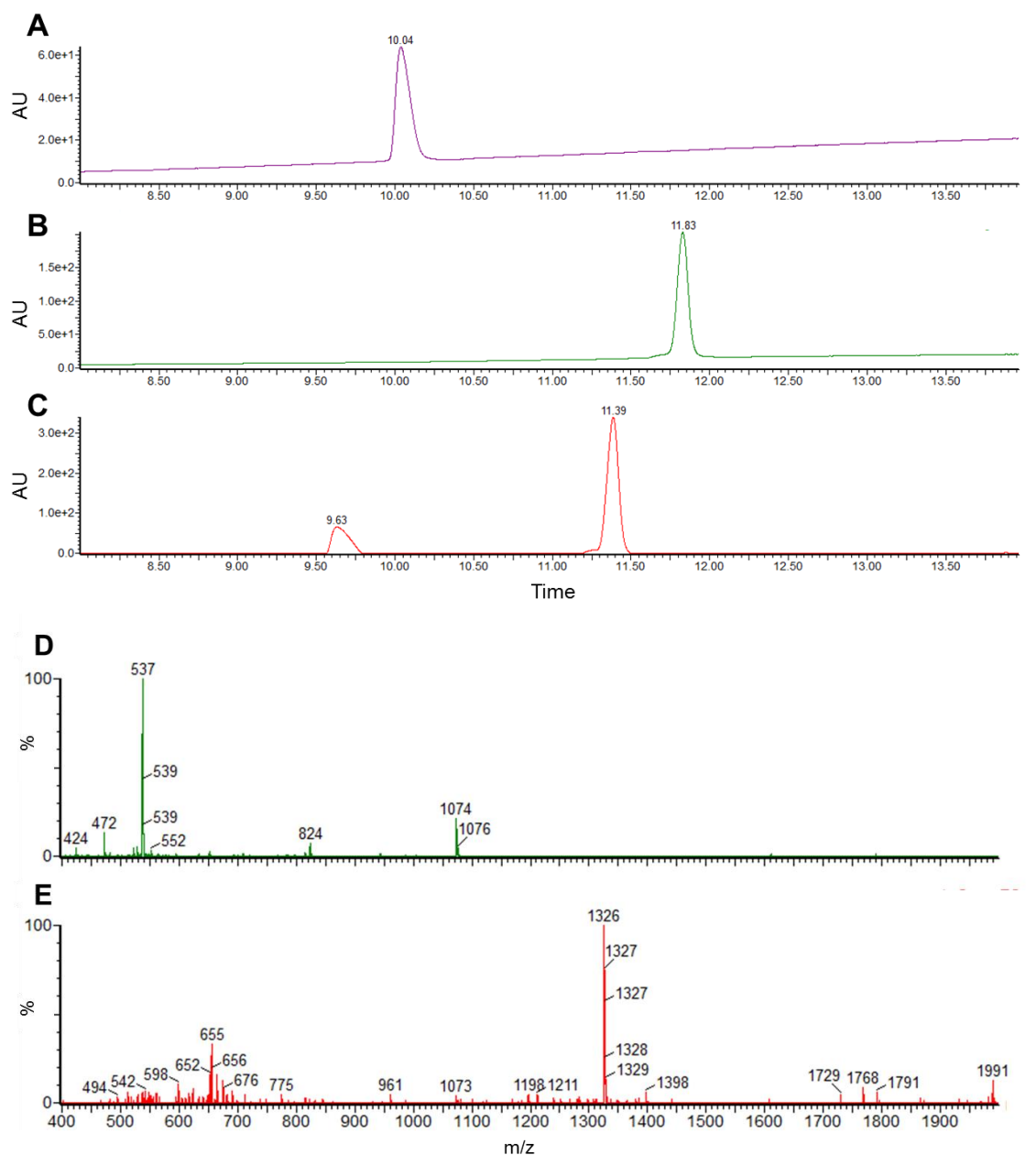


Figure 5.9: LC-MS analysis of control peptide and CmnCbz-caged photocleavable peptide. (A) Chromatogram of the control peptide (AU = arbitrary units.) (B) Chromatogram of the photocleavable peptide. (C) Chromatogram of a solution containing both control and photocleavable peptides. (D) & (E) Mass spectra corresponding to the two peaks visible in the chromatogram of the control-photocleavable peptide mixed sample (m/z = mass/charge ratio). (D) Mass spectrum of the peak corresponding to the control peptide. (E) Mass spectrum of the peak corresponding to the photocleavable peptide.

5.2.5 Photolysis of the photocleavable peptide

The CmnCbz caging group can be cleaved by UV light around 350 nm. After illumination with UV light, photolysis of the CmnCbz caging group proceeds in two steps (Figure 5.10) (Papageorgiou and Corrie, 1997). The first step is very fast and occurs within 1 millisecond. During this step, absorption of a photon of UV light rapidly causes most of the cage to dissociate from the peptide. In the second step, a slower, spontaneous decarboxylation removes the rest of the cage and generates free CO₂. The whole process is complete within 15 milliseconds (Papageorgiou and Corrie, 1997) and the cleaved peptide should be identical to the non-caged native peptide. The peptide should therefore be in an active conformation within a few milliseconds after illumination with UV light.

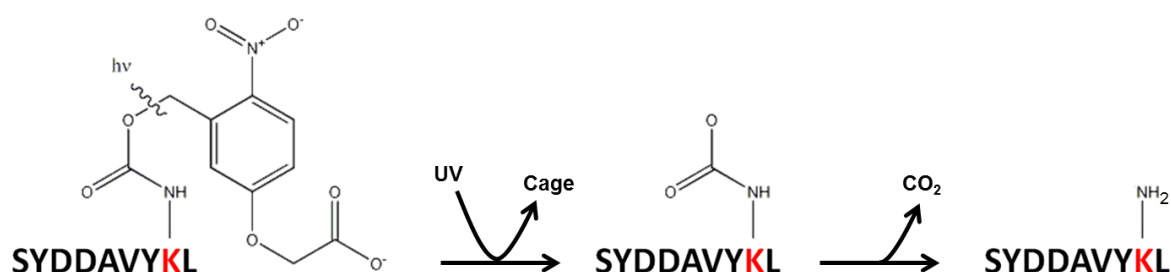


Figure 5.10: Photochemistry of the CmnCbz-caged SYDDAVYKL peptide. Absorption of a photon of UV light rapidly removes most of the caging group, whereas a slower spontaneous decarboxylation step removes the rest, generating free CO₂.

Photolysis of the CmnCbz-caged peptide was tested using different light sources and assessed by LC-MS. The different light sources available to test were: an X-Cite light source, an HBO lamp, an Nd YAG pulsed laser and a mercury discharge UV lamp. These light sources are described in more detail in the Materials and Methods section.

X-Cite light source

The photocleavable peptide was illuminated with UV light peaking at 350 nm from the X-Cite light source for different durations. The final products were analysed by LC-MS. In the diode array chromatograms the peak corresponding to the photocleavable peptide was clearly visible, although there was no second peak corresponding to the native peptide (Figure 5.11A). The positive ion

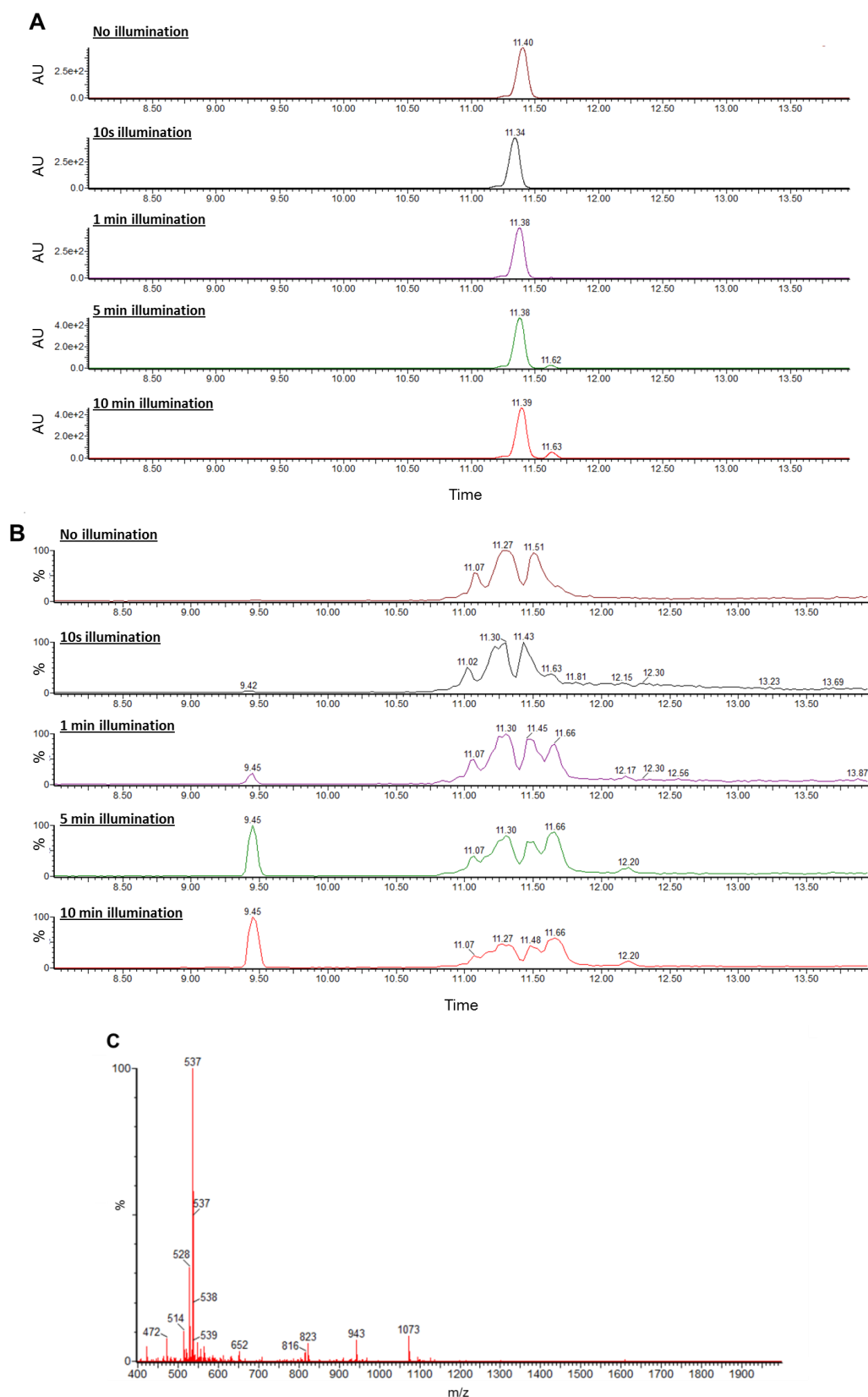


Figure 5.11: Cleaving the peptide with an X-Cite light source. (A) Diode array chromatogram of the cleaved peptide solution (AU = arbitrary units.) **(B)** Positive ion chromatogram of the cleaved peptide solution. **(C)** Mass spectrum of the peak corresponding to the 'new' product, which has the same mass as the native peptide (m/z = mass/charge ratio).

chromatograms (Figure 5.11B) revealed a small peak corresponding to the native peptide (as confirmed by the mass spectrum; Figure 5.11C) in the samples illuminated for 10 seconds or longer. In the control sample that was not illuminated, only the peak corresponding to the photocleavable peptide was visible, as expected. Quantification of the peak corresponding to the photocleavable peptide revealed that after 10 minutes of illumination with the X-Cite lamp, only 10% of the photocleavable peptide had been cleaved.

HBO Lamp light source

Photolysis of the photocleavable peptide was also tested using an HBO lamp source peaking at 360 nm. The photocleavable peptide was illuminated for 5 minutes and the product was analysed by LC-MS. The diode array chromatogram shows that in addition to the peak corresponding to the photocleavable peptide, there is a peak corresponding to the native peptide (Figure 5.12A). Here again, the mass spectrum confirms the mass of the peptide (Figure 5.12B). In the control sample that was not illuminated, only one peak corresponding to the non-cleaved photocleavable peptide was visible. After 5 minutes of illumination with the HBO lamp, 52% of the peptide was cleaved.

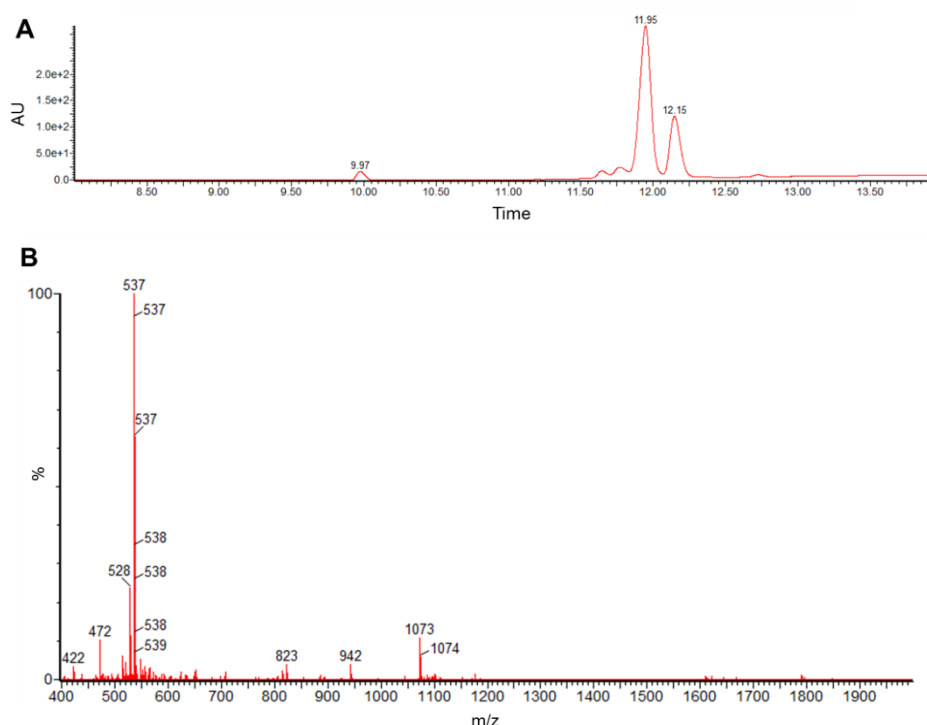


Figure 5.12: Cleaving the peptide with an HBO lamp. (A) Chromatogram of the cleaved peptide solution (AU = arbitrary units.) (B) Mass spectrum of the peak corresponding to the 'new' product, which has the same mass as the native peptide (m/z = mass/charge ratio).

Nd YAG pulsed laser

The photocleavable peptide was also illuminated with UV light emanating from an Nd YAG pulsed laser that was frequency tripled to peak at 355 nm. The sample was irradiated over increasing time scales. After each illumination, the sample was analysed by UV spectrophotometry and compared to the control and photocleavable peptides that had not been illuminated. The control and photocleavable peptides can clearly be distinguished from each other by UV spectrophotometry, as can be seen by the presence of an additional absorbance peak in the 320 nm range in the spectrum of the photocleavable peptide (Figure 5.13A). As the duration of illumination increased, the absorbance spectra of the irradiated samples gradually lost the additional peak that was characteristic of the photocleavable peptide and looked more similar to the spectrum of the control peptide (Figure 5.13B). These results suggest that the photocleavable peptide was being successfully cleaved using this light source. The final product obtained after 26 minutes of illumination was also analysed by LC-MS (Figure 5.13C). A clear peak corresponding to the native peptide is visible in the diode array chromatogram. Quantification of the peak corresponding to the photocleavable peptide revealed that 82% of the photocleavable peptide had been cleaved after 26 minutes of illumination with the Nd YAG pulsed laser.

Mercury discharge UV lamp

Finally, the photolysis of the photocleavable peptide was tested using a Mercury discharge UV lamp. The photocleavable peptide was illuminated for 30 minutes under the lamp and the sample was analysed by LC-MS (Figure 5.14). In addition to the peak corresponding to the photocleavable peptide, a second peak corresponding to the native peptide was clearly visible in the diode array chromatogram. Quantification of the peak corresponding to the photocleavable peptide showed that after 30 minutes of illumination under the Mercury discharge lamp, 46% of the peptide had been cleaved.

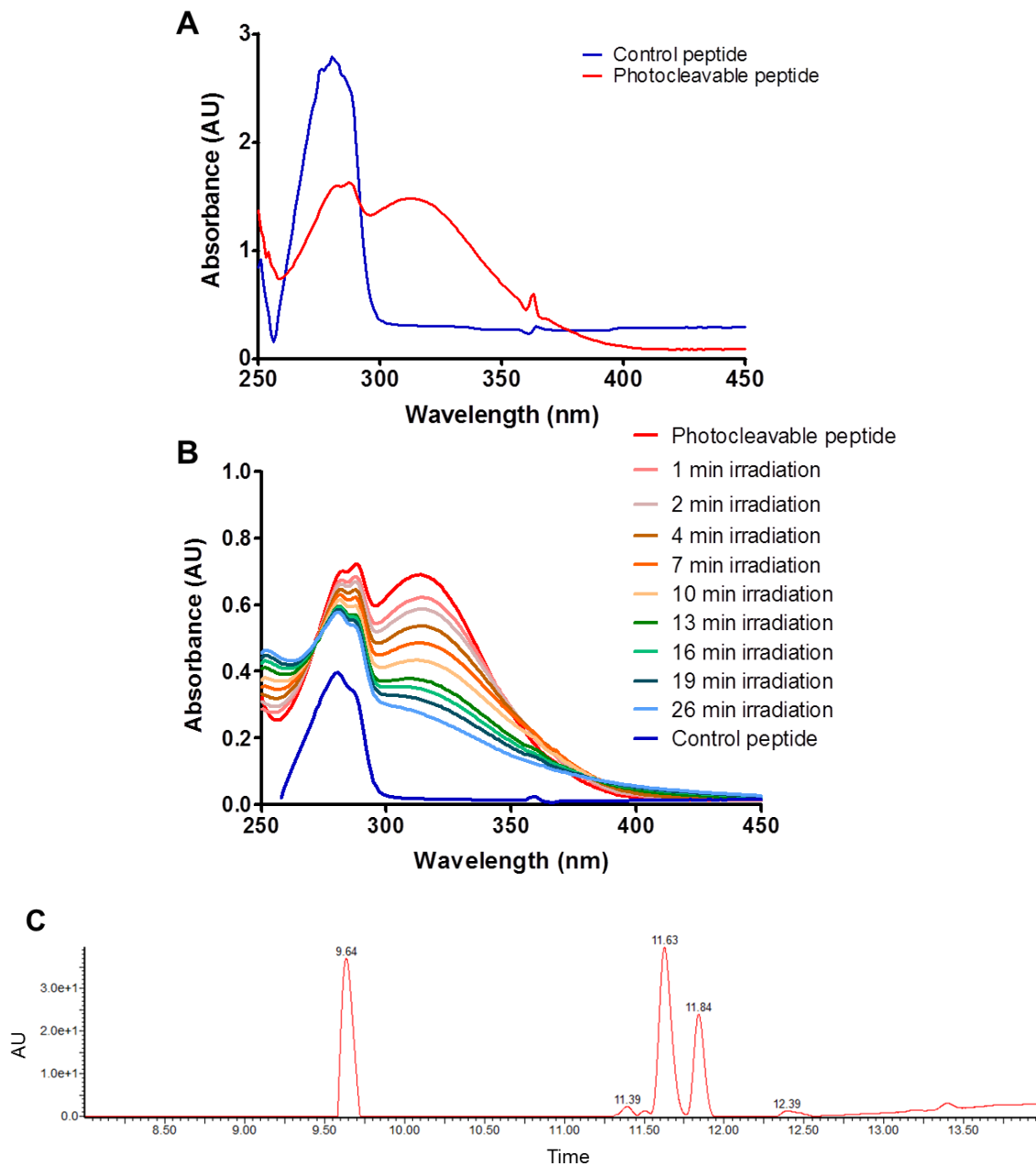


Figure 5.13: Cleaving the peptide with an Nd YAG pulsed laser. (A) UV spectra of the control and photocleavable peptides. **(B)** UV spectra of the photocleavable peptide subjected to increasing durations of illumination. **(C)** Chromatogram of the cleaved peptide solution (AU = arbitrary units).

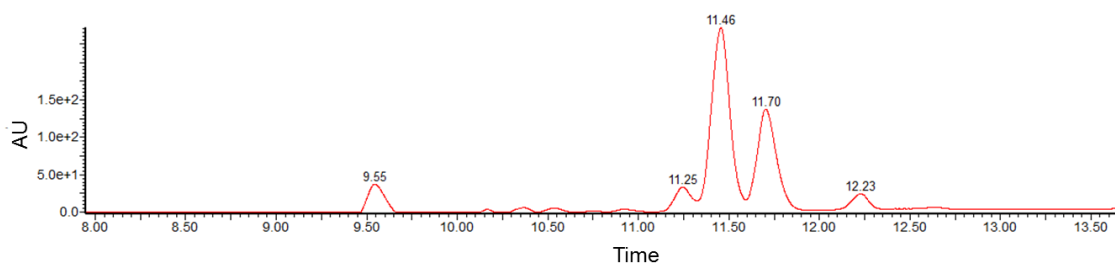


Figure 5.14: Cleaving the peptide with a Mercury discharge UV lamp. Chromatogram of the cleaved peptide solution (AU = arbitrary units).

The photolysis results are summarised in Table 5.1. When choosing the appropriate light source for cleaving the peptide in subsequent experiments, the mobility and accessibility of the light source were also taken into account. Therefore, although it was not the light source with the highest cleaving efficiency, the Mercury discharge UV lamp was chosen because it is mobile and yielded a good cleaving efficiency. In subsequent experiments using cleaved peptide, the photocleavable peptide was illuminated with UV light from the Mercury discharge lamp for 30 minutes.

Light source	Cleaving efficiency (%)	Access to sample for live cell imaging
X-Cite Light Source (350 nm peak)	10% (10 minutes)	Would need to build a special chamber for live cell imaging
HBO Lamp (360 nm peak)	52% (5 minutes)	Suitable for live cell imaging, but not confocal microscopy
Nd YAG pulsed laser (355 nm peak)	82% (26 minutes)	Not suitable for imaging
Mercury discharge UV lamp (365.5 nm peak)	46% (30 minutes)	Mobile

Table 5.1: Cleaving efficiencies of the different light sources tested for cleaving the photocleavable peptide. The four light sources tested for cleaving the peptide are summarised here, with their respective cleaving efficiencies and some additional information regarding their accessibility.

5.3 Cell systems

In order to successfully exploit the photocleavable peptide in this project, an appropriate cellular system is required with an appropriate target cell-effector cell combination. Ideal target cells for this system would express at their surface only MHC class I molecules that are presenting the peptide of interest. To achieve this, the main strategy was to use cells with low levels of endogenous MHC class I surface expression (due to mutations, or as a result of acid treatment, as detailed below) and to stabilise HLA-Cw4 molecules at the cell surface by incubating the cells with exogenous peptide.

The ideal effector cells would be NK cells that are able to lyse these cells in the absence of peptide, but are inhibited in the presence of control peptide.

Three target cell lines were tested in this context, as described in the following three sections. Various NK cell lines, primary NK cells and NK clones were tested as effector cells with these different target cells.

5.3.1 RMA-S- β_2m -Cw4 cells as target cells

RMA-S cells

The murine RMA-S cell line is an MHC class I-deficient cell line derived from a mouse T cell lymphoma variant (Ljunggren and Kärre, 1985; Townsend et al., 1989). Efficient intracellular transport of most MHC class I complexes to the cell membrane depends on the association of the MHC heavy chains with β_2m and specific peptides. Peptides derived from the cytosol are transported into the endoplasmic reticulum (ER) or *cis*-Golgi via transporter molecules called TAP proteins (Transporter associated with Antigen Processing), where they can bind and stabilise nascent MHC class I molecules (Monaco, 1992; Kleijmeer et al., 1992). TAP-deficient cells such as RMA-S cells are unable to stably express MHC class I proteins at the cell surface. Therefore, they express low levels of endogenous surface MHC class I and are unable to process and present whole antigen to T cells (Townsend et al., 1989).

RMA-S cells were analysed by flow cytometry for surface expression of human MHC class I using W6/32 antibody, which reacts with the heavy chain- β_2m complex (Barnstable et al., 1978a). As

expected, RMA-S cells did not express human MHC class I at their surface (Figure 5.15). The human 221 cell line was used as a negative control and 221 cells transfected to express HLA-Cw6 (221-Cw6) were used as a positive control.

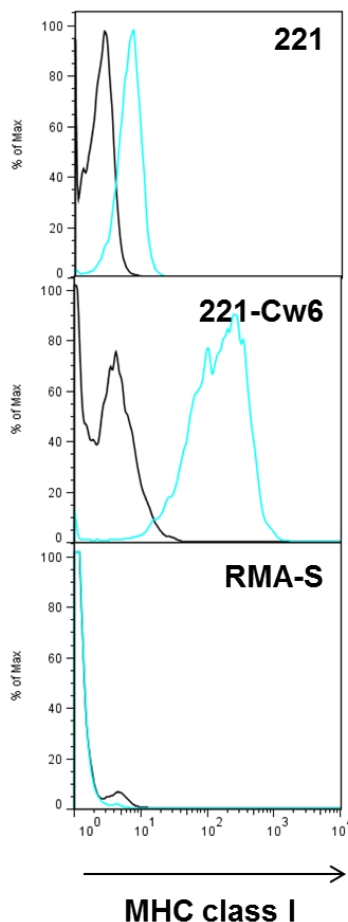


Figure 5.15: Surface expression of MHC class I on RMA-S cells. RMA-S cells were analysed by flow cytometry for surface expression of MHC class I as detected by staining with W6/32 antibody. Specific staining (blue) is compared to isotype control (black). 221 and 221-Cw6 cells were used as negative and positive controls respectively.

RMA-S cells cannot stabilise HLA-C at their surface in the absence of human β_2m

RMA-S cells were transfected to express the human MHC class I molecules HLA-Cw3 or HLA-Cw4 fused to GFP, using linearised DNA constructs (Figure 5.16) to facilitate efficient transfection. The transfected cells were enriched by cell sorting based on GFP expression (Figure 5.17).

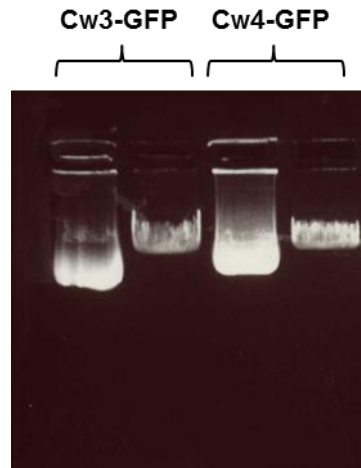


Figure 5.16: Gel electrophoresis of linearised DNA plasmids. The plasmids encoding Cw3-GFP and Cw4-GFP were linearised before being used to transfect RMA-S cells. For each plasmid, the band on the left shows the uncut DNA and the band on the right shows the linearised DNA.

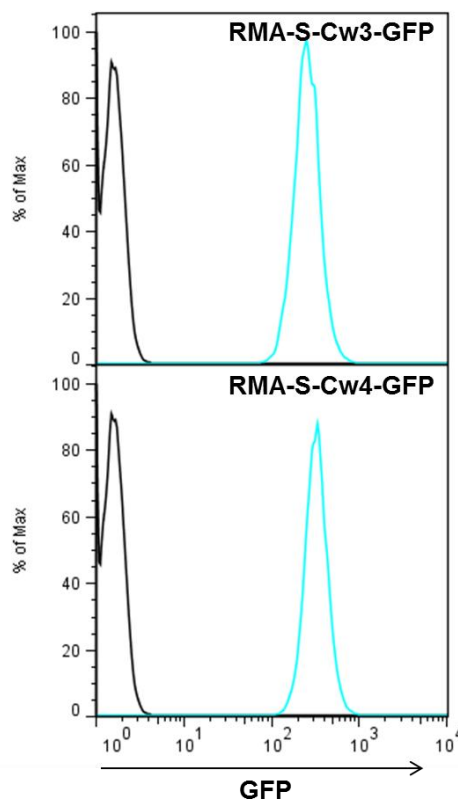


Figure 5.17: GFP fluorescence on transfected RMA-S-Cw3-GFP and RMA-S-Cw4-GFP cells. RMA-S cells were transfected to express either Cw3-GFP or Cw4-GFP and enriched by FACS. The stable transfected cell lines were analysed by flow cytometry for GFP fluorescence (blue). Untransfected RMA-S cells were used as a negative control (black).

In the next experiment, the aim was to determine whether the presence of exogenous peptide could stabilise HLA-Cw4 at the surface of RMA-S-Cw4-GFP cells, using RMA-S-Cw3-GFP cells as a negative control because the peptide binds specifically to HLA-Cw4 and cannot stabilise HLA-Cw3. RMA-S-Cw4-GFP cells were incubated with peptide at 37°C, stained with W6/32 antibody and analysed by flow cytometry. These peptide loading experiments carried out with the control peptide SYDDAVYKL resulted in no shift in MHC class I staining in peptide-loaded cells compared to non-peptide-loaded cells (Figure 5.18), indicating that the peptide was not able to stabilise the MHC class I molecules at the cell surface.

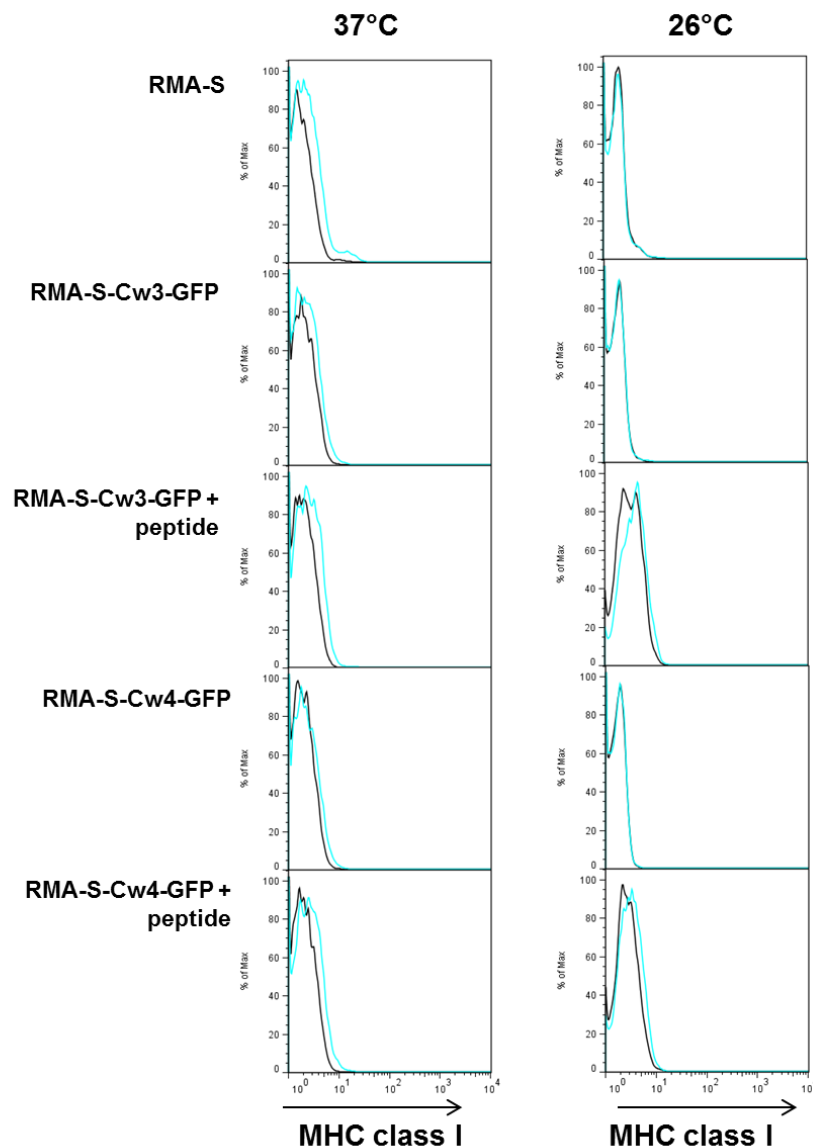


Figure 5.18: Peptide loading experiments with RMA-S-Cw3-GFP and RMA-S-Cw4-GFP cells. RMA-S-Cw3-GFP and RMA-S-Cw4-GFP cells were loaded with the control peptide SYDDAVYKL (at 37°C or at 26°C) and analysed by flow cytometry for increased stabilisation of surface MHC class I proteins compared to non-peptide-loaded cells. Specific staining (blue) is compared to isotype control (black).

Previous work has shown that the stabilisation of MHC class I proteins at the surface of TAP-deficient cells is most effective at 25-26°C, or in the presence of exogenous peptides or β_2m (Townsend et al., 1989; Ljunggren et al., 1990; Anderson et al., 1993). However, incubating RMA-S-Cw4-GFP cells with peptide at 26°C did not result in increased stability of human MHC class I surface expression (Figure 5.18). These results suggest that the addition of exogenous peptide alone cannot stabilise human MHC class I at the surface of RMA-S cells. One possible explanation could be that mouse cells cannot express human MHC class I molecules at the cell surface in the absence of human β_2m . This might be because mouse β_2m weakly associates with certain human MHC alleles (Rein et al., 1987; Chamberlain et al., 1988), even though human β_2m can rapidly associate with both human and mouse MHC class I heavy chains (Schmidt et al., 1981; Hochman et al., 1988). Therefore, RMA-S cells transfected to express human β_2m (RMA-S- β_2m cells), as well as RMA-S cells transfected to co-express human β_2m and HLA-Cw4 (RMA-S- β_2m -Cw4) were used in subsequent peptide loading experiments.

RMA-S- β_2m -Cw4 cells can stabilise surface HLA-C in the presence of peptide at 26°C

The next experiments were carried out to determine whether the presence of peptide could increase the expression of MHC class I at the surface of cells expressing both human β_2m and HLA-Cw4. RMA-S- β_2m -Cw4 cells were unable to increase MHC class I surface stability when incubated with the peptide (SYDDAVYKL) at 37°C, even though the peptide is specific to HLA-Cw4 (Rajagopalan and Long, 1997). However, when peptide loading experiments were carried out at 26°C, human MHC class I surface expression as detected with W6/32 antibody was increased on peptide-loaded RMA-S- β_2m -Cw4 cells compared to non-peptide-loaded cells (Figure 5.19). These results confirmed previous work showing that stabilisation of HLA-Cw4 at the surface of RMA-S cells is only possible with exogenous peptide when the cells co-express human β_2m and are incubated at 26°C (Rajagopalan and Long, 1997).

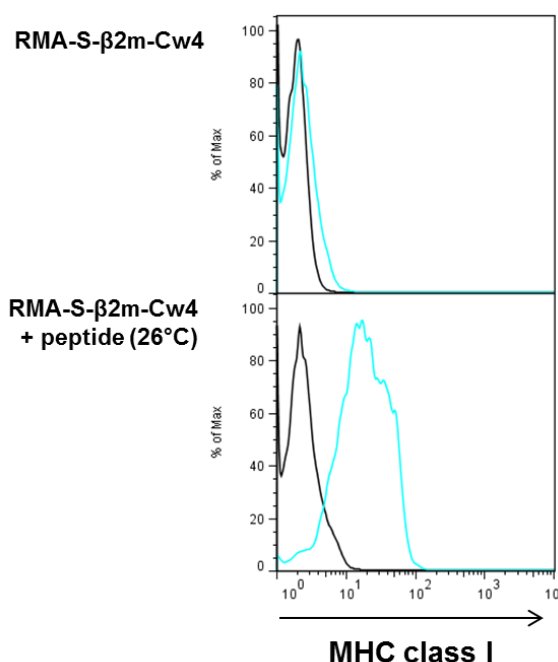


Figure 5.19: Peptide loading experiments with RMA-S- β_2 m-Cw4 cells. RMA-S- β_2 m-Cw4 cells were loaded with the control peptide SYDDAVYKL at 26°C and analysed by flow cytometry for increased stabilisation of surface MHC class I proteins compared to non-peptide-loaded cells. Specific staining (blue) is compared to isotype control (black).

RMA-S- β_2 m cells can be killed by specific NK clones

Cytotoxicity assays were carried out using RMA-S cells as target cells in order to find effector cells that are able to efficiently kill RMA-S cells. RMA-S cells were tested for susceptibility to lysis by various NK cell lines (YTS, YTS-KIR2DL1-GFP, NKL, NK-92C1) and by human primary NK cells isolated from blood. However, RMA-S cells were not killed by any of the NK cells tested in these experiments (Figure 5.20).

Once it was established that RMA-S- β_2 m cells would be more appropriate for further experiments, RMA-S- β_2 m and RMA-S- β_2 m-Cw4 cells were also tested for susceptibility to lysis by NK cells. RMA-S- β_2 m cells were not killed by the NK cell lines YTS or NKL (Figures 5.21A & B), nor by primary human NK cells from peripheral blood (Figure 5.21C). However, it is known that the expression of activating and inhibitory NK receptors on peripheral blood NK cells is variable and expression of any given receptor is restricted to distinct subsets of NK cells. This could explain why the whole population of primary NK cells only lysed a very small proportion (less than 10%) of RMA-S- β_2 m cells. Thus, NK clones that express distinct receptor repertoires were grown from human peripheral blood NK cells,

expanded and screened for killing RMA-S- β_2 m-Cw4 cells. In the representative experiment shown here (Figure 5.21D), 40 NK clones obtained from the same donor were screened for killing RMA-S- β_2 m-Cw4 cells, of which six were found to have the ability to efficiently lyse RMA-S- β_2 m-Cw4 cells. These results indicate that NK clones obtained from human peripheral blood can be used as effector cells with RMA-S- β_2 m-Cw4 cells as target cells.

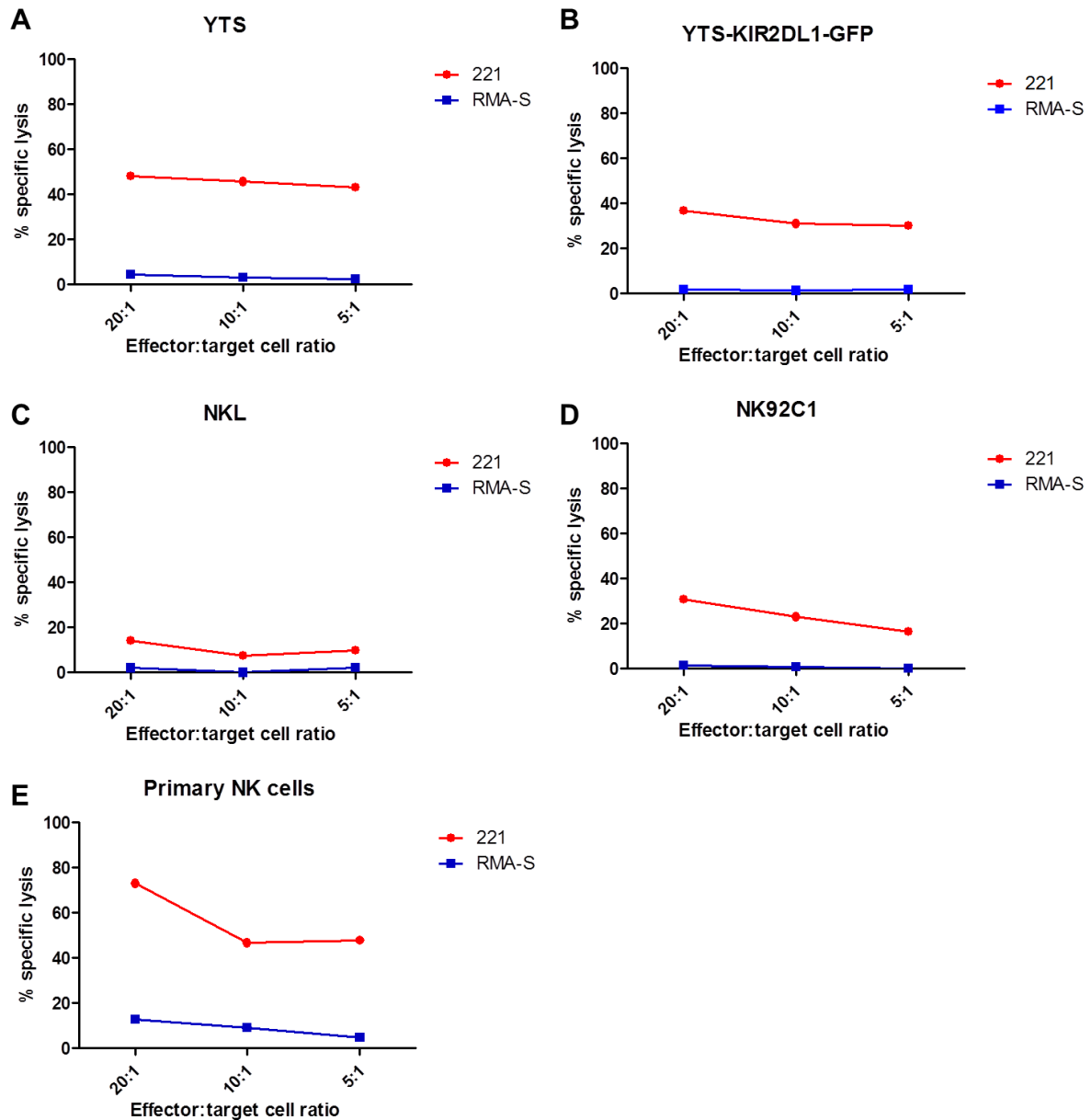


Figure 5.20: Susceptibility of RMA-S cells to lysis by NK cells. RMA-S were tested for susceptibility to lysis by the NK cell lines YTS (A), YTS-KIR2DL1-GFP (B), NKL (C), and NK-92C1 (D) and by primary NK cells isolated from human blood (E). The 221 cell line was used as a positive control. Data shown are representative of two independent experiments per assay.

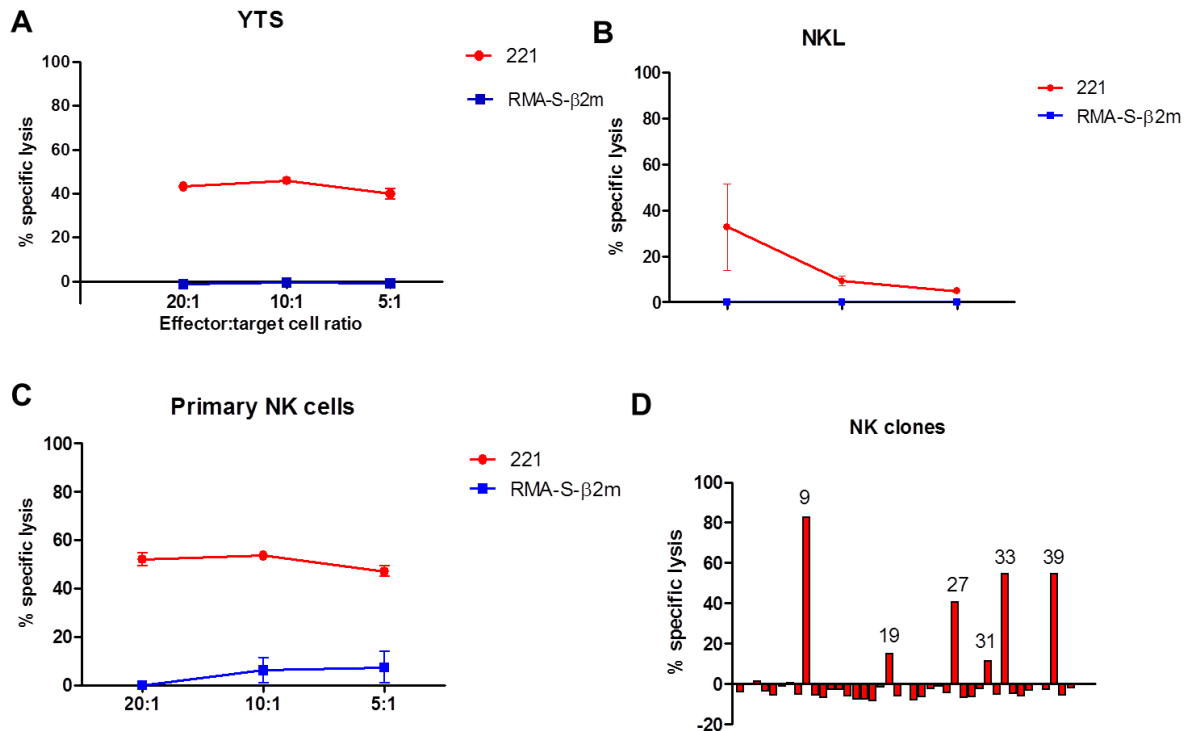


Figure 5.21: Susceptibility of RMA-S- β ₂m cells to lysis by NK cells. RMA-S- β ₂m were tested for susceptibility to lysis by the NK cell lines YTS (A), and NKL (B), and by primary NK cells isolated from human blood (C). The 221 cell line was used as a positive control. (D) NK clones were grown and screened for killing of RMA-S- β ₂m cells. Data shown are representative of at least two independent experiments per assay.

Peptide loading on RMA-S- β ₂m-Cw4 cells can decrease NK clone-mediated killing

Stabilisation of HLA-Cw4 at the cell surface by peptide loading at 26°C should theoretically allow an efficient interaction between HLA-Cw4 on target cells and KIR2DL1 on NK cells and hence result in an inhibition of NK cell killing. RMA-S- β ₂m-Cw4 cells were loaded with control peptide (SYDDAVYKL) at 26°C and tested for susceptibility to lysis by NK clones that in a previous experiment had been found to efficiently kill RMA-S- β ₂m-Cw4 cells. In the example shown here, 45 clones from the same donor were screened, four of which were found to efficiently lyse RMA-S- β ₂m-Cw4 cells (Figure 5.22A). For each of these four NK clones, the percentage of specific lysis of RMA-S- β ₂m-Cw4 cells incubated with peptide was lower than for RMA-S- β ₂m-Cw4 without peptide (Figure 5.22B). However, only one clone showed a really convincing inhibition (from 26.2% to 8.2% specific lysis). These data nonetheless suggest that loading RMA-S- β ₂m-Cw4 cells with specific peptide at 26°C can inhibit lysis by NK clones.

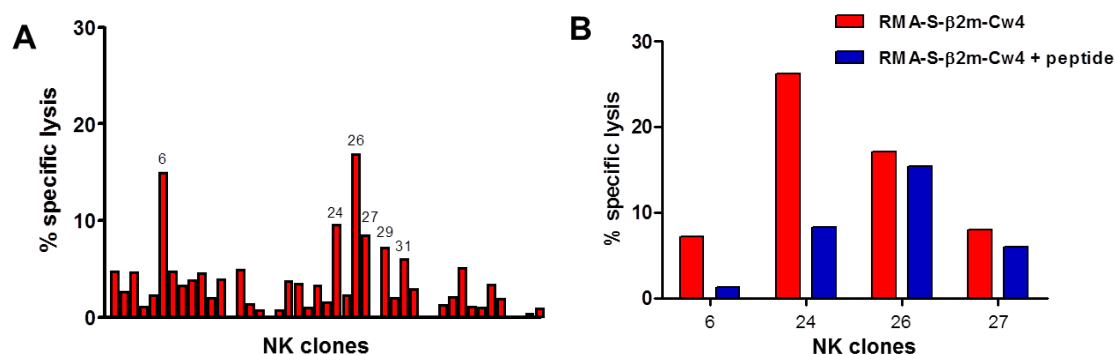


Figure 5.22: Peptide loading on RMA-S-β₂m-Cw4 cells can decrease NK clone-mediated killing. RMA-S-β₂m-Cw4 cells were loaded with the control peptide SYDDAVYKL at 26°C. NK clones that were found to be able to kill RMA-S-β₂m-Cw4 cells in **(A)** were then tested for specific killing of peptide-loaded RMA-S-β₂m-Cw4 cells compared to non-peptide-loaded cells **(B)**.

RMA-S-β₂m-Cw4 cells as target cells

The ultimate aim of this project is to set up an imaging system where the photocleavable peptide can be cleaved at will to control inhibition in NK cells whilst imaging target-effector conjugates under the microscope. Peptide loading experiments were successful with RMA-S-β₂m-Cw4 cells, but further experiments using effector cells are only possible with NK clones. However, for each imaging experiment it would be an extremely time-consuming and arduous process to get suitable clones: growing NK clones, selecting clones that kill RMA-S-β₂m-Cw4 cells effectively, then selecting clones that are efficiently inhibited in the presence of peptide, before finally moving on to difficult imaging experiments. Importantly, the response of NK clones is very variable and donor-dependent. In addition, RMA-S-β₂m-Cw4 cells are murine cells and perhaps not the most physiological setting for studying interactions between human MHC and human KIR2DL1. Therefore, alternative target cell systems based on human cell lines were explored and are described in the following sections.

5.3.2 Acid-stripped 221-Cw4 cells as target cells

221-Cw4 cells

The MHC class I-negative human B cell line 221 expresses very low levels of HLA-A, -B and -C and can be transfected to express MHC class I molecules that are efficiently expressed at the cell surface by association with endogenous β_2m (Shimizu et al., 1988). 221 cells stably expressing HLA-Cw4 (221-Cw4) were available in the lab. These cells express stable MHC class I at their surface. However, in this situation, the peptides contained in the grooves of the HLA-Cw4 molecules at the cell surface are derived from the intracellular environment. For experiments with a specific peptide, it is desirable to generate HLA-Cw4 proteins that are completely or partially empty of peptides before loading the cells with the appropriate peptide. Acid treatment of 221-Cw4 cells can release peptides from the binding groove of HLA-Cw4 and initiates the dissociation of β_2m and the heavy chain from the MHC complex (Rock et al., 1991). As a result, empty HLA-Cw4 molecules are free to be bound by exogenously added peptide.

Effector cells

The human NK cell line YTS is known to efficiently kill 221 cells. Cytotoxicity assays were carried out using YTS cells and YTS cells expressing KIR2DL1 (YTS-KIR2DL1 cells). YTS and YTS-KIR2DL1 were able to efficiently lyse 221 cells, whereas specific lysis by YTS-KIR2DL1 cells was significantly reduced with 221-Cw4 as target cells (Figure 5.23). These results suggest that the interaction between KIR2DL1 on the effector cells and HLA-Cw4 on the 221-Cw4 cells can inhibit lysis of the target cells.

Peptide stabilisation of MHC class I on acid-stripped 221-Cw4 cells

221-Cw4 cells were treated with acid to 'empty' the HLA-Cw4 proteins at the cell surface and were then incubated at 26°C without peptide, or in the presence of either control peptide (SYDDAVYKL) or CmnCbz-caged peptide. The susceptibility of these cells to lysis by YTS-KIR2DL1 effector cells was tested in a classical cytotoxicity assay. Acid-stripped 221-Cw4 cells were efficiently killed by YTS-

KIR2DL1 cells, whereas a strong inhibition of NK cell cytotoxicity was observed when the acid-stripped 221-Cw4 cells were incubated with control peptide (Figure 5.24A). These results confirm

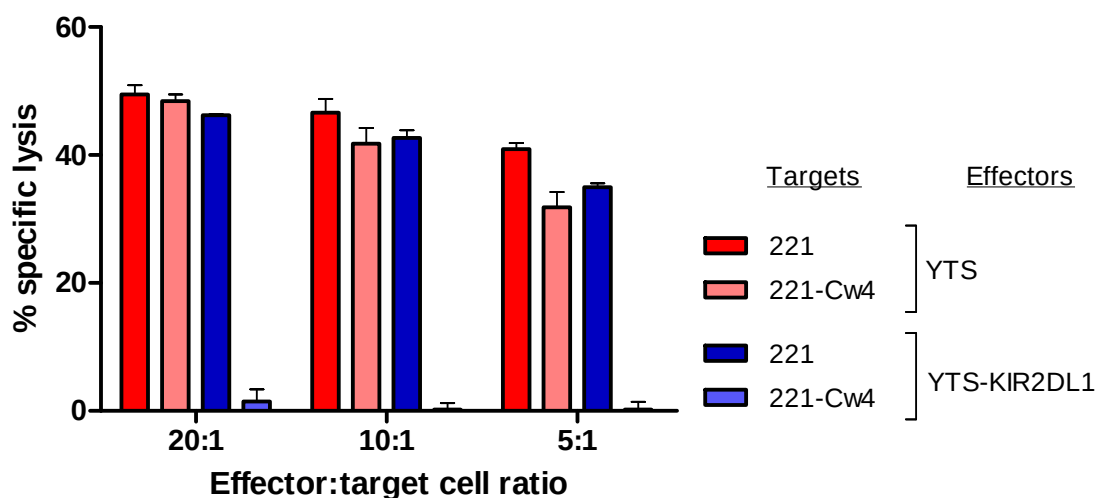


Figure 5.23: KIR2DL1 is functional in YTS-KIR2DL1 cells. YTS and YTS-KIR2DL1 cells were tested in cytotoxicity assays with 221 and 221-Cw4 target cells. The interaction between KIR2DL1 and Cw4 resulted in inhibition of NK cell cytotoxicity, showing that KIR2DL1 is functional in the YTS-KIR2DL1 cells, as published previously (Cohen et al., 1999).

that the acid treatment of 221-Cw4 cells removed peptide from the HLA-Cw4 complex and that this complex was dissociated, resulting in KIR2DL1 being unable to bind to its ligand and transmit inhibitory signals within the NK cell; once the control peptide was added however, HLA-Cw4 molecules were stabilised at the cell surface and could efficiently bind KIR2DL1 to induce inhibitory signalling inside the YTS-KIR2DL1 cells. When acid-stripped 221-Cw4 cells were incubated with the photocleavable peptide, they were once again efficiently lysed by YTS-KIR2DL1 cells (Figure 5.24A). In this situation, the peptide was binding HLA-Cw4 proteins but the caging group on the peptide was blocking the interaction with KIR2DL1, so that no inhibition was achieved. These experiments were repeated with YTS cells expressing KIR2DL1 fused to tdEosFP (used in Chapters 3 and 4) as effector cells and similar results were obtained (Figure 5.24B).

Acid-stripped 221-Cw4 cells as target cells

The inhibition of NK cells was successfully achieved in cytotoxicity assays using acid-stripped 221-Cw4 cells loaded with control peptide as target cells. This inhibition was reversed when acid-stripped

221-Cw4 cells were incubated with photocleavable peptide, as expected. This cellular system therefore appears to be a potential candidate for studying the inhibition of NK cells using the photocleavable peptide. However, the acid treatment is quite harsh and could have unwanted side-effects. Therefore, another human cell line was investigated as a potential target cell line for this project.

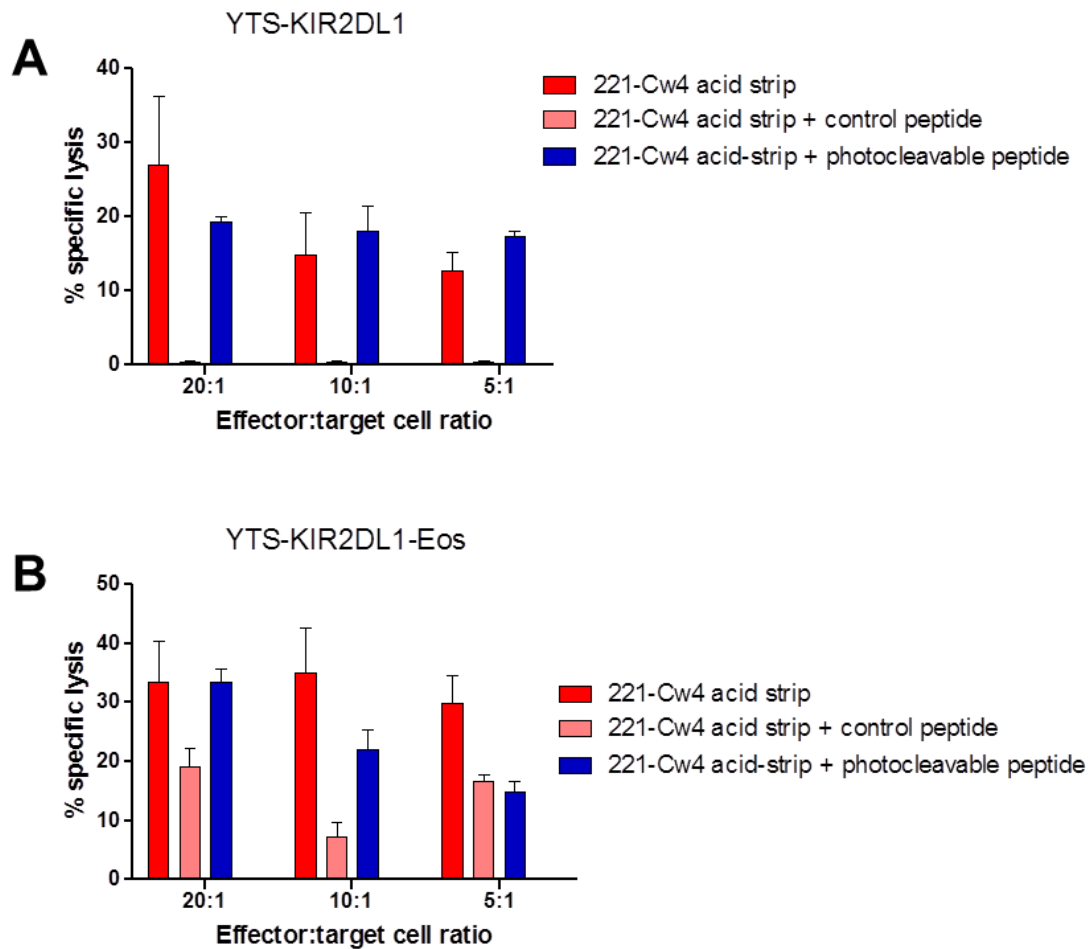


Figure 5.24: Incubation of acid-stripped 221-Cw4 cells with photocleavable peptide blocks KIR2DL1-mediated NK cell inhibition. (A) After acid treatment, 221-Cw4 cells were incubated either without peptide, with control peptide or photocleavable peptide at 26°C and tested for susceptibility to lysis by YTS-KIR2DL1 cells. Data are representative of 6 out of 8 independent experiments. **(B)** The same experiment was carried out with YTS-KIR2DL1-tdEosFP cells as effector cells. Data are representative of 2 independent experiments.

5.3.3 T2-Cw4-GFP cells as target cells

T2 cells

The human T2 cell line is a TAP-deficient cell line that cannot efficiently express MHC class I molecules at its surface (Salter et al., 1985; Kelly et al., 1992; Anderson et al., 1993). Like RMA-S cells, T2 cells express very low levels of endogenous surface MHC class I molecules and are unable to process and present antigen at their surface (Alexander et al., 1989; Hosken and Bevan, 1990).

T2 cells were analysed by flow cytometry for surface expression of human MHC class I using W6/32 antibody. Surprisingly, T2 cells exhibited high levels of MHC class I surface expression (Figure 5.25). Although in T2 cells, MHC class I molecules are retained in the ER and are unable to present endogenous peptides, T2 cells are known to express low levels of HLA-A2, -B51 and -Cw1 at their surface (Boyle et al., 2004). The staining for MHC class I was much higher than expected, but could be due to the presence of these MHC class I molecules at the surface of T2 cells.

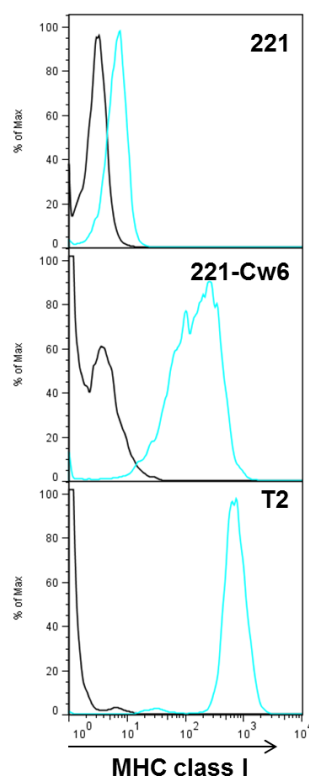


Figure 5.25: Surface expression of MHC class I on T2 cells. T2 cells were analysed by flow cytometry for surface expression of MHC class I as detected by staining with W6/32 antibody. Specific staining (blue) is compared to isotype control (black). 221 and 221-Cw6 cells were used as negative and positive controls, respectively.

The susceptibility of T2 cells to lysis by various NK cell lines and human primary NK cells was tested. T2 cells were efficiently killed by YTS and YTS-KIR2DL1 cells (Figures 5.26A & B), but were not killed by the NKL and NK92-C1 cell lines (Figures 5.26C & D). T2 cells were also efficiently killed by primary NK cells (Figure 5.26E). These results demonstrate that YTS-KIR2DL1 and cells could be used as effector cells in subsequent experiments.

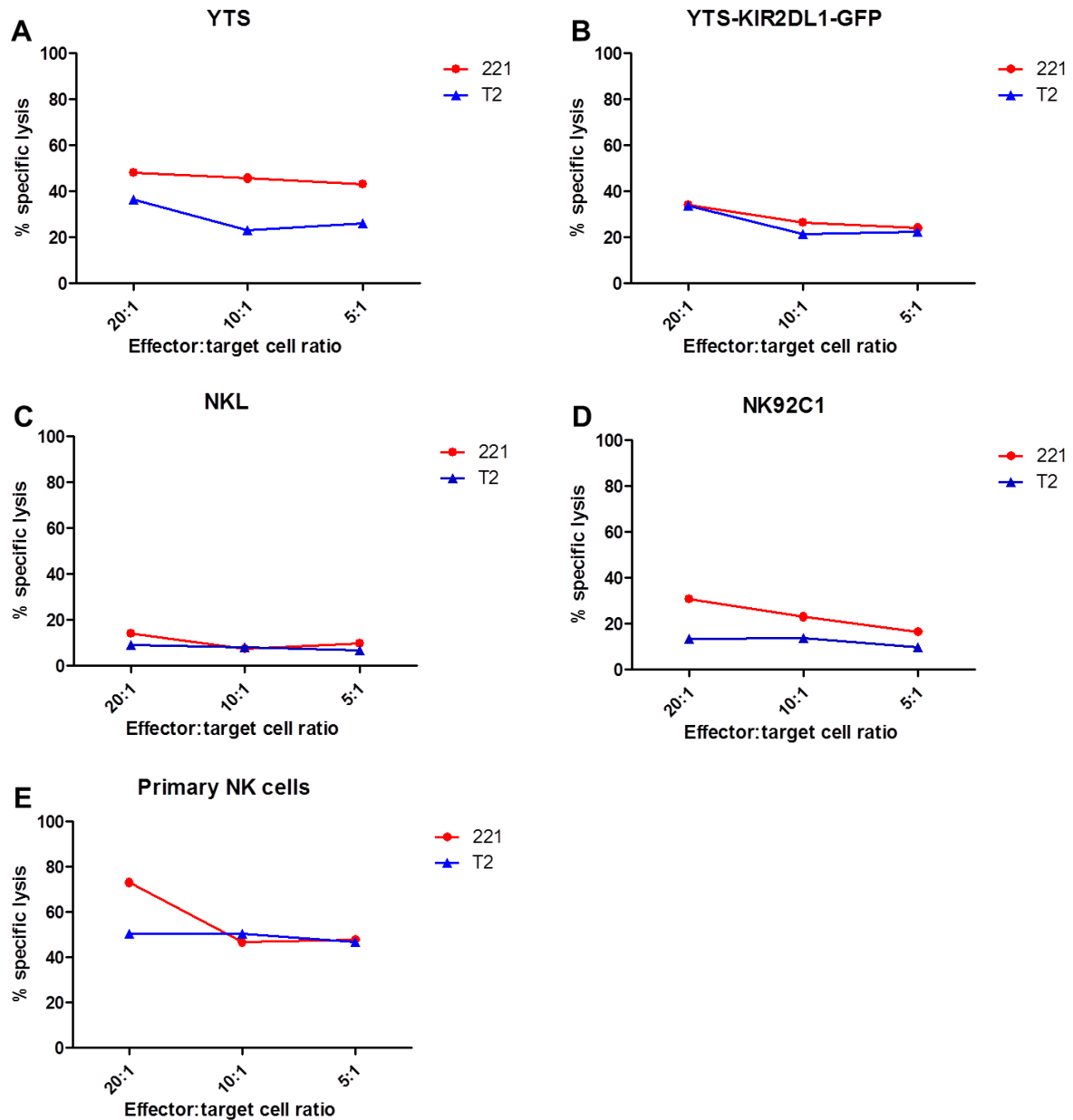


Figure 5.26: Susceptibility of T2 cells to lysis by NK cells. T2 were tested for susceptibility to lysis by the NK cell lines YTS (A), YTS-KIR2DL1-GFP (B), NKL (C), and NK-92C1 (D) and by primary NK cells isolated from human blood (E). The 221 cell line was used as a positive control. Data shown are representative of two independent experiments per assay.

T2-Cw4-GFP cells

T2 cells were transfected to express HLA-Cw4 fused to GFP and were subcloned into 96-well plates. Transfected clones were analysed by flow cytometry and GFP-positive clones were expanded and enriched by FACS. In this way, a population of stably transfected T2-Cw4-GFP cells was obtained, as confirmed by flow cytometry (Figure 5.27).

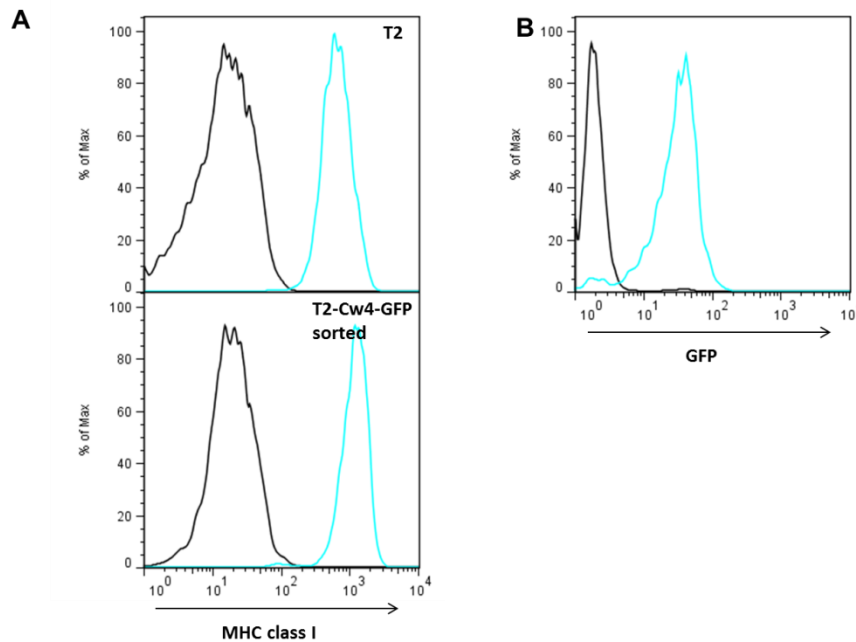


Figure 5.27: Enrichment of T2 cells transfected to express Cw4-GFP. (A) The enriched population of transfected T2 cells was analysed by flow cytometry for surface expression of MHC class I as detected by staining with W6/32 antibody. Specific staining (blue) is compared to isotype control (black). Untransfected T2 cells were used as a negative control. **(B)** The sorted population of T2-Cw4-GFP cells was also analysed by flow cytometry for GFP fluorescence (blue). Untransfected T2 cells were used as a negative control (black).

One of the main activating receptors through which YTS cells recognise target cells is CD28 (Chen et al., 2006). CD28, first identified as a T cell co-stimulatory receptor before being implicated in NK cell cytotoxicity, recognises human B7 molecules, CD80 and CD86. T2 and T2-Cw4-GFP cells were analysed by flow cytometry for surface expression of the CD28 ligand CD80. Both T2 and T2-Cw4-GFP cells were found to express high levels of CD80 compared to the negative control, the murine cell line P815 (Figure 5.28). In this target-effector combination, with T2-Cw4-GFP cells as targets and YTS-KIR2DL1 cells as effectors, the activating (CD80-CD28) and inhibitory (Cw4-KIR2DL1) interactions are known, making it easier to manipulate and understand this cell system.

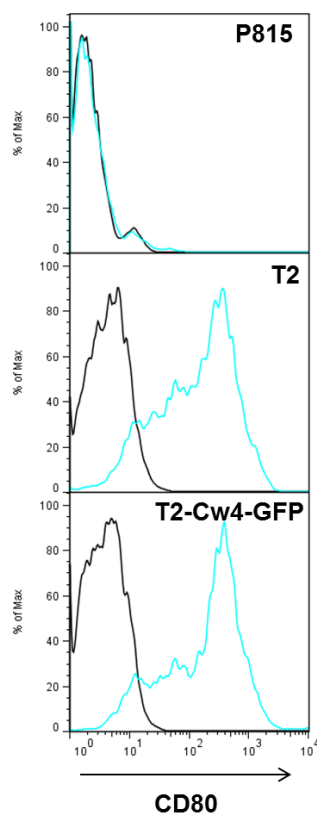


Figure 5.28: Surface expression of CD80 on T2 cells. T2 and T2-Cw4-GFP cells were analysed by flow cytometry for surface expression of MHC class I as detected by staining with α -CD80 antibody. Specific staining (blue) is compared to isotype control (black). Murine P815 cells were used as a negative control.

Loading T2-Cw4-GFP cells with peptides can alter their susceptibility to NK cell lysis

T2-Cw4-GFP cells were incubated at 26°C without peptide or with control peptide or photocleavable peptide. T2-Cw4-GFP cells incubated without peptide were efficiently killed by YTS-KIR2DL1 cells, but in the presence of control peptide target cell lysis was inhibited (Figure 5.29). In addition, in the presence of photocleavable peptide with its caging group blocking interaction with KIR2DL1, T2-Cw4-GFP cells were efficiently killed by YTS-KIR2DL1, at a level comparable to the killing seen without peptide (Figure 5.29). By loading T2-Cw4-GFP cells with the control or the photocleavable peptide, it is possible to control the inhibition of YTS-KIR2DL1 cells.

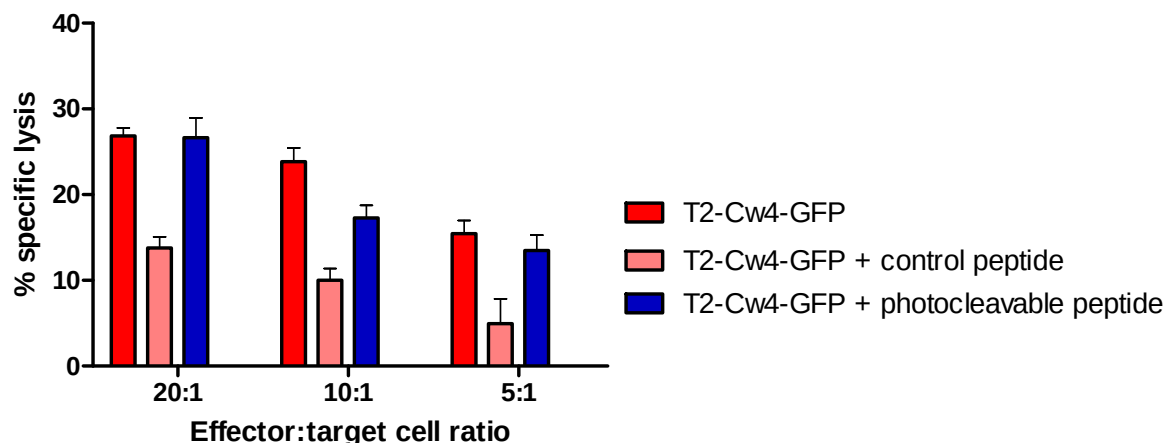


Figure 5.29: Incubation of T2-Cw4-GFP cells with photocleavable peptide blocks KIR2DL1-mediated NK cell inhibition. T2-Cw4-GFP cells were incubated either without peptide, with control peptide or photocleavable peptide at 26°C and tested for susceptibility to lysis by YTS-KIR2DL1 cells. Data are representative of 4 independent experiments.

Cleaving the peptide restores inhibition in YTS-KIR2DL1 cells

As shown previously, the photocleavable peptide could be efficiently cleaved using different light sources. Here, the photocleavable peptide was pre-cleaved using the Mercury discharge UV lamp. T2-Cw4-GFP cells were incubated at 26°C in the presence of cleaved peptide and tested in a cytotoxicity assay. When the cells were incubated with the cleaved peptide, inhibition of target cell lysis by YTS-KIR2DL1 cells was observed to similar levels than with the control peptide, whereas once again with the non-cleaved photocleavable peptide, T2-Cw4-GFP cells were efficiently killed (Figure 5.30). This effect was seen at all three effector to target cell ratios that were tested. These results show that two opposite results can be obtained with the photocleavable peptide depending on whether it is cleaved or not. When the photocleavable peptide had not been cleaved, the interaction between Cw4 and KIR2DL1 was blocked, whereas when the peptide had been cleaved to yield the native peptide, the interaction could occur and inhibitory signals were induced within the NK cell. This experiment shows for the first time that cleaving the photocleavable peptide can change the response of NK cells towards the same target cells.

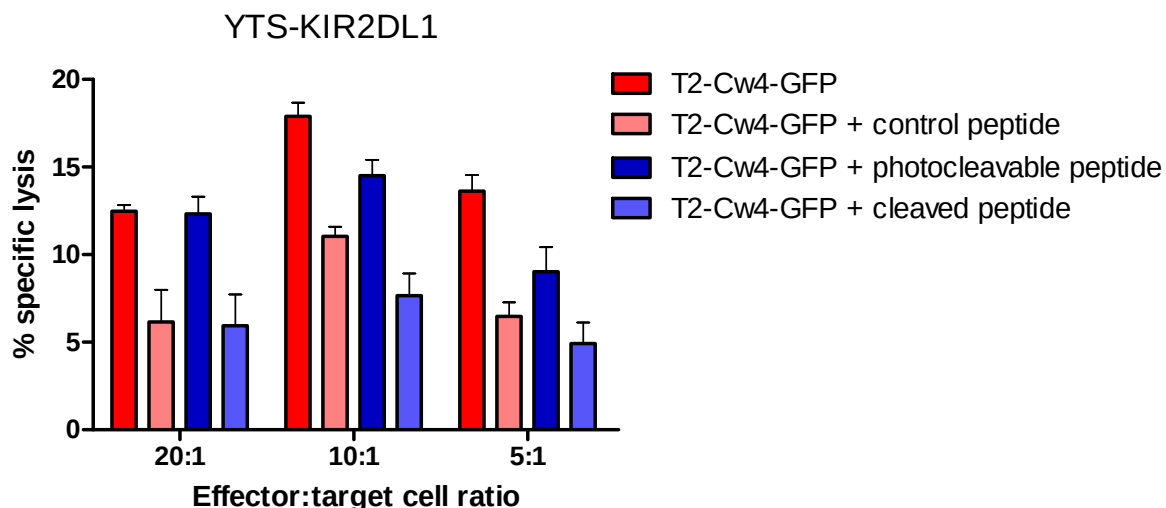


Figure 5.30: Incubation of T2-Cw4-GFP cells with cleaved photocleavable peptide restores KIR2DL1-mediated NK cell inhibition. T2-Cw4-GFP cells were incubated either without peptide, with control peptide, photocleavable peptide or pre-cleaved photocleavable peptide at 26°C and tested for susceptibility to lysis by YTS-KIR2DL1 cells. Data are representative of 3 independent experiments.

T2-Cw4-GFP cells as target cells

The experiments described above demonstrate that, using the photocleavable peptide, the interactions between T2-Cw4-GFP and YTS-KIR2DL1 cells can be controlled and shifted towards activation or inhibition of the NK cells. This cellular system seems extremely promising for further investigation. Of the three target cell types tested, it is the most physiological, since T2 cells are a human cell line, and the experiments do not require any harsh treatment of the cells, such as the acid treatment required with the 221-Cw4 cells.

5.4 Preliminary imaging experiment

A preliminary imaging experiment was carried out to determine whether the T2-Cw4-GFP and YTS-KIR2DL1 cell system was viable for imaging studies with the photocleavable peptide. T2 cells had been transfected to express fluorescent HLA-Cw4 and therefore the position of HLA-Cw4 in the target cell membrane could be visualised by fluorescence confocal microscopy. T2-Cw4-GFP and YTS-

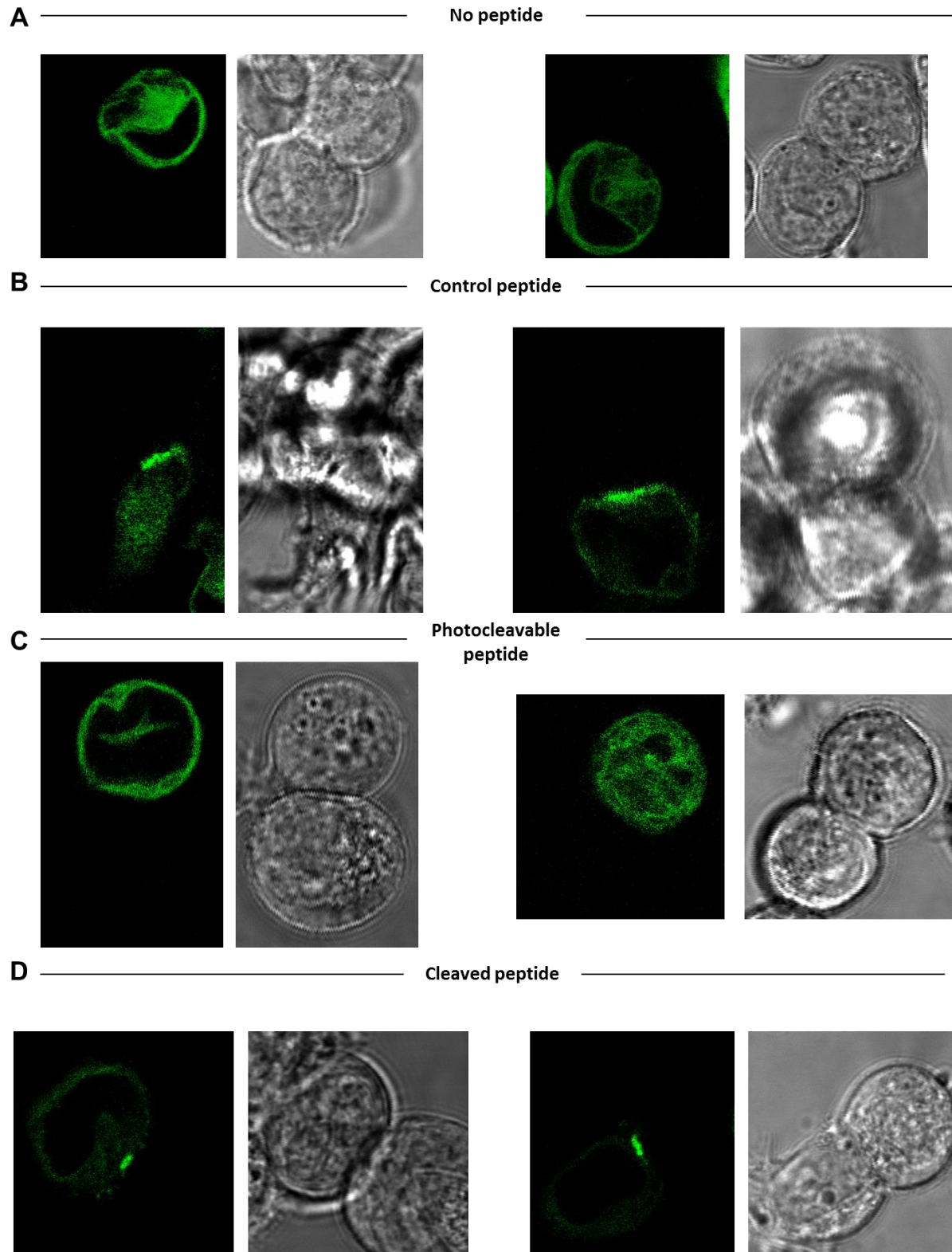


Figure 5.31: Incubation of T2-Cw4-GFP with photocleavable peptide blocks Cw4 recruitment to the synapse. T2-Cw4-GFP cells were incubated either **(A)** without peptide, **(B)** with control peptide, **(C)** with photocleavable peptide or **(D)** with pre-cleaved photocleavable peptide at 26°C, then incubated with YTS-KIR2DL1 cells and imaged by confocal microscopy.

KIR2DL1 cells were incubated together at 26°C, either without peptide, or in the presence of control peptide, photocleavable peptide or pre-cleaved photocleavable peptide. The cell conjugates were then imaged by confocal microscopy.

In the absence of peptide, HLA-Cw4-GFP was uniformly distributed around the cell (Figure 5.31A), most probably dissociated from β_2m due to the absence of stabilising peptide. When T2-Cw4-GFP cells were incubated with YTS-KIR2DL1 in the presence of control peptide, Cw4-GFP was seen to accumulate at the immune synapse (Figure 5.31B), as expected when an efficient interaction occurs between HLA-Cw4 and KIR2DL1. However, this was not seen in all conjugates, but in only approximately 10% of conjugates (2 out of total 22 conjugates imaged). In the presence of photocleavable peptide, there was no accumulation of HLA-Cw4 at the synapse, but it was uniformly distributed around the cell membrane (Figure 5.31C), as no interaction between HLA-Cw4 and KIR2DL1 could occur. Finally, when the cells were incubated with pre-cleaved photocleavable peptide, an accumulation of HLA-Cw4 at the immune synapse was observed in a small proportion of conjugates (Figure 5.31D), again in approximately 10% of cases (3 out of 29 conjugates imaged). These results indicate that the cellular system developed in this project is viable for studying the timing of inhibitory signalling in NK cells using a photocleavable peptide.

5.5 Discussion

5.5.1 Summary of results

In this project, the main goal was to set up a cellular system in which NK cell inhibition could be controlled by exploiting the properties of photocleavable peptides. In particular, the results were as follows:

- A control peptide with the sequence SYDDAVYKL, which specifically binds and stabilises HLA-Cw4 molecules at the cell surface, was successfully synthesised and purified; and a photocleavable peptide, with the same sequence and with a photolabile caging group able to block KIR2DL1 binding, was designed and commercially obtained
- An analytical method based on liquid chromatography-mass spectrometry (LC-MS) was successfully developed to be able to distinguish between the control and photocleavable

peptides present in a solution and to quantify the concentration of photocleavable peptide in a given solution; in addition, the photocleavable peptide was cleaved by different UV light sources and the cleaving efficiency of each method was calculated based on the LC-MS measurements

- Three potential cell systems were investigated for controlling NK cell inhibition using the photocleavable peptide, with different target cells: RMA-S- β_2m -Cw4 cells, acid-stripped 221-Cw4 cells and T2-Cw4-GFP cells
- The best cellular system proved to be T2-Cw4-GFP cells as target cells and YTS-KIR2DL1 cells as effector cells; in this system, incubation with the photocleavable peptide blocked NK cell inhibition and resulted in lysis of the target cells, whereas incubation with pre-cleaved peptide restored the interaction with KIR2DL1 and inhibition of the NK cells
- Preliminary imaging experiments with T2-Cw4-GFP and YTS-KIR2DL1 cells without peptide or in the presence of control, photocleavable or cleaved peptide demonstrated that the interaction of HLA-Cw4 and KIR2DL1 as well as the accumulation of HLA-Cw4 at the immune synapse can be controlled depending on which peptide is present.

Taken together, these results indicate that a successful system was set up in which the inhibition of NK cells can be controlled using a photocleavable peptide.

5.5.2 Photocleavable peptide approach for studying NK cell signalling

The basis of signal integration in NK cells has remained largely unexplained. In this project, a photochemical approach was used to set up a system by which the stimulation of activating and inhibitory signals can be temporally separated to observe the interactions occurring between them.

A peptide that specifically binds to the groove of HLA-Cw4 was synthesised as a control peptide for this study. A photocleavable peptide was designed, based on the sequence of the control peptide and with a photosensitive caging group, aimed at blocking the interaction between HLA-Cw4 and KIR2DL1. This peptide was successfully cleaved by different UV light sources, yielding the native peptide. However, cleaving efficiencies were not very high and a more powerful and focussed laser source should be utilised in the future.

In order for target cells to selectively present the peptide of interest within their MHC class I molecules, cells were required that exhibited 'empty' MHC class I molecules at their surface or MHC class I molecules that were not stable at the cell surface in the absence of exogenous peptides. Several target cell lines matching these criteria were transfected to express HLA-Cw4 and tested to determine the optimal cell system: two TAP-deficient cell lines (murine RMA-S and human T2 cells), which are unable to stabilise MHC class I molecules at their surface, and a human cell line (221) that was treated with acid to dissociate the peptide-MHC class I complexes at their cell surface.

Both the control and photocleavable peptides were able to stabilise HLA-Cw4 at the cell surface at 26°C, as was expected since the anchor motifs within the peptide that provide specificity to HLA-Cw4 were not affected by the caging group. This peptide-mediated stabilisation of HLA-Cw4 required the presence of human β_2m within the cells and incubation at 26°C rather than the physiological temperature of 37°C, as previously shown.

Incubation of target cells with control peptide protected them from lysis by NK cells, as expected. When the same cells were pre-incubated with the photocleavable peptide, the interaction between HLA-Cw4 on the target cells and KIR2DL1 on the effector cells was blocked, therefore abolishing KIR2DL1-mediated inhibitory signalling and leading to increased lysis of target cells by NK cells. When instead, the target cells were incubated with pre-cleaved peptide, the interaction was restored and the NK cells were inhibited. These results clearly demonstrate the potential of using this photocleavable peptide approach to control NK cell inhibition.

Target and effector cells were also imaged by confocal microscopy following incubation without peptide, or with control, photocleavable or pre-cleaved peptide. In the scenarios where the interaction between HLA-Cw4 and KIR2DL1 was able to occur (i.e. with control and pre-cleaved peptide), an inhibitory synapse characterised by the accumulation of fluorescent HLA-Cw4 to the site of contact was formed in approximately 10% of conjugates. In the situations where no binding was occurring between HLA-Cw4 and KIR2DL1 (i.e. without peptide to stabilise HLA-Cw4 at the cell surface, or with the photocleavable peptide, which blocks the binding), no accumulation of HLA-Cw4 was observed at the interface between the two cells.

These results are promising for the future application of this system in imaging experiments. The cell conjugates that are being imaged will be subjected to brief pulses of UV illumination to cleave the photocleavable peptide at the desired moment in time following the beginning of the intercellular interaction.

The photostimulation of KIR2DL1 during ongoing activating signals may not be a perfect model of the simultaneous signalling occurring within NK cells, but provides a nonetheless biologically relevant framework for studying NK cell signalling in more detail. Many interesting questions remain unanswered that this approach, based on the use of photocleavable peptides, will hopefully address.

5.5.3 Future directions

Photochemistry has provided a way to study intracellular signalling with subcellular spatial resolution and millisecond temporal resolution, based on the use of photocleavable compounds. Combining imaging experiments with the use of a photocleavable peptide to control NK cell inhibition will be a useful tool for studying events occurring at the NK cell immune synapse and for understanding the mechanisms underlying NK cell inhibition in more detail.

Timing of inhibition

Initial experiments carried out with the photocleavable peptide should monitor simple readouts of the cell's response. For example, the accumulation of HLA-Cw4-GFP can be used as an indicator of the formation of an inhibitory synapse. By irradiating the conjugates at different times following initial cell-cell contact, important temporal information about an NK cell's commitment towards killing will be established.

Other cellular readouts within the NK cell, which do not require transfections, would be calcium fluxes and lysosome movements. Previous studies have described a rapid rise in intracellular calcium levels in NK cells within two minutes after contact with susceptible target cells (Honda and Miyazaki, 1996), a phenomenon also observed in T cells. These calcium fluxes are commonly used as an indicator of immune cell activation and can be detected by loading cells with a calcium-indicator dye such as Fluo-4AM. It would be interesting to monitor calcium fluxes in NK cells after initiating inhibitory signalling at different times after cell-cell contact. In addition, NK cell activation culminates in the polarisation and secretion of lytic granules towards the target cell (Lieberman, 2003), causing target cell death. A fluorescent probe called LysoTracker is commonly used as a marker of lytic granules, by enabling the tracking of acidic organelles in live cells. Visualising lysosomes in the NK

cell would allow to directly observe the effect of initiating inhibition at different time-points at the level of the cell's response.

This approach could also be used to study the dynamics of cytoskeletal components as the balance of signals is tipped towards activation or inhibition. Activating and inhibitory signals seem to integrate by controlling cytoskeletal processes, since signalling by inhibitory KIR receptors leads to dephosphorylation of Vav-1, a crucial molecule involved in actin reorganisation at the immune synapse (Stebbins et al., 2003). It would be interesting to see whether KIR2DL1-mediated inhibitory signals could interfere with actin or MTOC polarisation once these events have already started occurring within the NK cell in response to activating signals.

Timing of inhibitory signalling events

Imaging fluorescently-labelled signalling molecules involved in the NK cell inhibitory signalling cascade during photostimulation experiments could be useful for determining the timing of signalling events, such as the rate of recruitment of SHP-1. This technique would provide accurate measurements of signalling kinetics, because by using photostimulation the signalling response can be temporally isolated from other events such as cell adhesion and synapse formation (Huse, 2010). To probe the molecular processes occurring at the immune synapse in more detail, cell conjugates could be manipulated using optical tweezers such that the immune synapse is parallel to the focal plane of the microscope, providing an *en face* view of the synapse. Upon initiation of inhibitory signalling by photostimulation, the formation and dynamics of protein microclusters could directly be observed at higher resolution. It would be interesting to specifically compare the organisation of protein microclusters at the synapse, the proximity of activating and inhibitory receptors, the recruitment of membrane-proximal signalling proteins and how these events relate to downstream functional effects. Experiments such as these have the potential to reveal new details of synapse reorganisation triggered by inhibitory signalling as well as the timing of these processes.

Spatio-temporal integration of signals

The photocleavable peptide could also be used to spatially control NK cell inhibition. During an imaging experiment, a source of focussed UV light could be used to irradiate a small micron-scale

region of the immune synapse, creating a small zone where KIR2DL1-mediated signalling is initiated. The downstream events occurring within the NK cell could then be recorded. This approach would provide information about the importance of spatial localisation of signalling triggers for optimal downstream responses. This strategy could be used to study conjugates both from the side view as in traditional confocal microscopy or *en face* using optical tweezers. Different parts of the cell membrane in general or specific regions within the immune synapse could be targeted by localised photostimulation, revealing spatial information about signal integration both at the level of the whole cell and within the synapse.

To conclude, a cell system based on a photocleavable peptide has been set up with which stimulation of KIR2DL1-mediated inhibition can be controlled. This approach holds great potential for addressing important questions regarding the integration of activating and inhibitory signals at the NK cell immune synapse that culminates in the appropriate effector response. In the future, this technique will be used to determine the dynamics of activating and inhibitory signalling events and how these pathways interact in time and in space.

Chapter 6 - Conclusions

6.1 Overview

NK cells are lymphocytes that play a crucial role in the body's surveillance against pathogens and tumours. NK cell effector functions are regulated by a balance of activating and inhibitory signals that are integrated at the site of contact between an NK cell and a target cell, which is known as the immune synapse. It has been suggested previously that the organisation of proteins at the NK cell immune synapse relates to the functional outcome of the interaction. Recent advances in fluorescent microscopy techniques and the development of new approaches based on the photophysical and photochemical properties of molecules provide novel tools for studying the localisation, dynamics, interactions and kinetics of proteins within cells. By exploiting some of these novel imaging techniques, this investigation has revealed interesting new aspects of NK cell signalling, in particular with regards to the organisation and dynamics of the inhibitory receptor KIR2DL1.

The results of this study are discussed in detail in the relevant chapters. To summarise briefly, this investigation has:

- studied the nanoscale organisation of the inhibitory receptor KIR2DL1 using the super-resolution imaging technique called PALM (Chapter 3)
- studied the micrometer-scale dynamics of KIR2DL1 using a photoswitchable fluorescent protein (Chapter 4)
- developed a photostimulation strategy for studying the kinetics of signalling within NK cells with high temporal resolution (Chapter 5).

6.2 A complex view of inhibitory signalling in NK cells

The presence of inhibitory receptors on NK cells is crucial for regulating their activation to avoid the killing of healthy cells. The inhibitory receptor KIR2DL1 has been shown to accumulate at the interface between NK cells and target cells expressing cognate ligand (Davis et al., 1999). The

organisation of KIR2DL1 within this so-called ‘inhibitory synapse’ has been described at the micrometer level and has been shown to be dynamic (Davis et al., 1999; Carlin et al., 2001; Almeida and Davis, 2006; Oddos et al., 2008), with KIR2DL1 microclusters moving within the synapse over time (Oddos et al., 2008). In this investigation, KIR2DL1 was studied at different spatial and temporal scales: the nanometer-scale organisation of KIR2DL1 was studied using PALM, and the micrometer-scale dynamics of KIR2DL1 recruitment to the immune synapse were studied by tracking the movement of KIR2DL1 molecules in NK cell-target cell conjugates. In addition, a cellular system was set up that can be used to investigate the timing of NK cell inhibition mediated by KIR2DL1. Within the scope of this thesis, the aim was to set up such a system, although the overarching goal is to exploit this approach in order to decipher the precise timing and sequence of events in the integration of activating and inhibitory signals at the immune synapse.

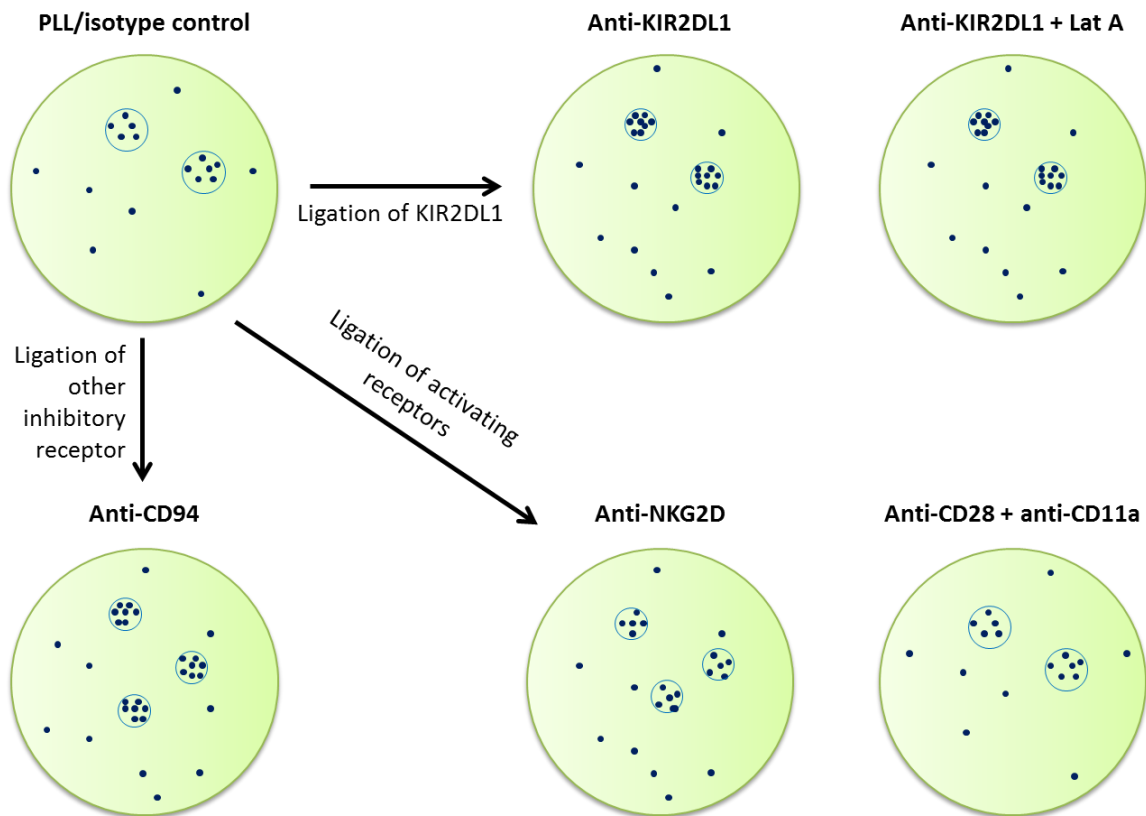


Figure 6.1: Summary of the changes in the nanoscale organisation of KIR2DL1 in different conditions, as detected by PALM microscopy and quantitative analysis. Schematic representation of the differences found in molecular density, cluster density, cluster size and number of KIR2DL1 molecules per cluster, compared to cells imaged on slides coated with PLL or an isotype control antibody. The green circles are representations of the surface of NK cells at the interface with the glass slide. Blue circles represent the periphery of KIR2DL1 clusters. Dark blue dots represent individual KIR2DL1 molecules.

The super-resolution imaging described in this report has revealed important new information about the nanometer scale organisation of KIR2DL1 at the membrane and how this distribution changes in different conditions. The results are schematically summarised in Figure 6.1.

This investigation has demonstrated that KIR2DL1 molecules arrange into nanometer-scale clusters in the NK cell membrane and that these clusters become smaller and denser upon receptor ligation. Interestingly, this re-organisation was not dependent on actin polymerisation, even though previous reports suggest that the rate of recruitment of KIR2DL1 to the synapse is affected by inhibitors of actin polymerisation (Standeven et al., 2004). Most importantly, the nanoscale organisation of KIR2DL1 was influenced by the ligation of other receptors expressed on the same cell. To our knowledge, this is the first time that such a phenomenon has been observed, although the underlying molecular mechanisms remain undefined and an attractive area of research for the future. The proximity of inhibitory and activating receptors within the synapse might be crucial for efficient signal integration within the cell and this will be investigated in more detail in the future. The results from this investigation underline the importance of visualising the interactions occurring between receptors, signalling molecules and other cellular components such as the cytoskeleton at the super-resolution level in order to decipher the fundamental molecular processes involved in signalling.

The dynamics of the recruitment of KIR2DL1 to the inhibitory synapse have not previously been studied in great detail. By tracking the movement of KIR2DL1 by fusing it to a photoswitchable fluorescent protein, this study has shown that KIR2DL1 molecules are continuously trafficked to the synapse, where they accumulate, even when an inhibitory synapse seems to be already fully formed with a substantial accumulation of KIR2DL1 already present at the synapse. The recruitment of new KIR2DL1 molecules to the synapse might serve to enhance the inhibitory signal. Importantly, this investigation showed for the first time that KIR2DL1 molecules are able to traffic between multiple synapses formed by the same NK cell with several target cells. These results are schematically summarised in Figure 6.2. It would be important to relate these findings to the nanoscale distribution of KIR2DL1 within the synapse and the interactions occurring between KIR2DL1 and other receptors, which might influence the behaviour of KIR2DL1. Indeed, studying the behaviour of KIR2DL1 within the synapse, possibly by live PALM, would provide greater knowledge about the mechanisms that may hold KIR2DL1 'trapped' within a synapse, but enable them to travel from one synapse to another.

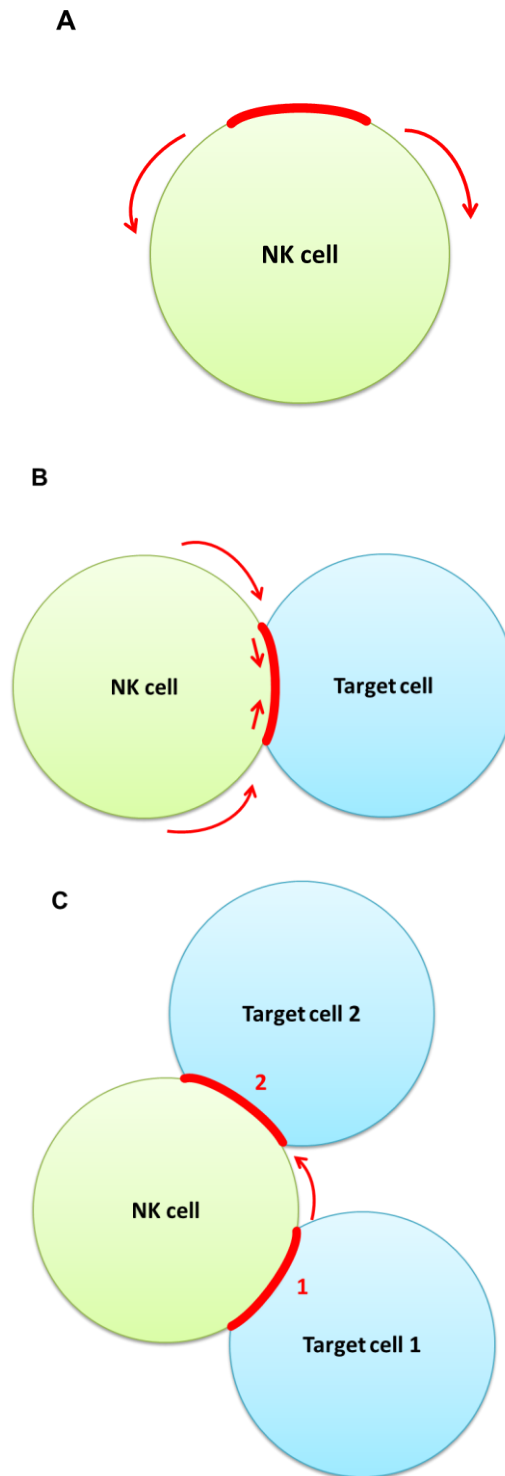


Figure 6.2: The dynamics of KIR2DL1 in the NK cell membrane. (A) KIR2DL1 molecules freely diffuse around the cell membrane in a single non-conjugated NK cell. (B) When an NK cell forms an inhibitory immune synapse with a target cell, KIR2DL1 molecules are continuously recruited to the immune synapse, where they remain trapped. (C) When an NK cell forms multiple inhibitory immune synapses with two or more target cells, KIR2DL1 molecules that have accumulated at one synapse are able to traffic to a second synapse.

6.3 Closing remarks

If NK cell receptors can influence each other and their distribution, and can traffic between multiple synapses, as shown in this thesis, events underlying NK cell signalling seem to be even more complex than previously anticipated. The only way to fully comprehend these events will have to be by further exploiting the three approaches described in this thesis, perhaps in combination with other super- or high-resolution imaging techniques, highlighting the necessity of multidisciplinary approaches for solving important biological problems.

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Appendices

Appendix 1: Cluster analysis programme.

MATLAB code for the cluster analysis programme (written by Dylan Owen).

The code itself is written in black (with parameters in purple and loops in blue); user annotations are in green. Orange square brackets represent a section of code that was excluded here – this section contained numerical information for the colour scale used in the colour map (and filled several pages with numbers).

```
% Set user parameters
areax=1500; % x length of cropped side in nm from centre coordinate
areay=1500; % y length of cropped side in nm from centre coordinate
maxcol=250; %Enter maximum value of colour scale here
binarychange=100; %enter value of highest clustering level to be coloured
black in binary image
clusscale=50; % cluster scale to examine

%read in coordinates table and define variables
[FileNameC,PathNameC] = uigetfile({'*.txt*';'*..*'}, 'Choose your Coordinates
file');
cd(PathNameC);
coordinates=dlmread(FileNameC, '\t', 0,1);
index=[0];
black=64/maxcol*binarychange;
a=zeros(black,3);
b=ones((64-black),3);
c=vertcat(a,b);
areax2=areax+100;
areay2=areay+100;

%read in data and set to batch process
for a=1:100;
    str=num2str(a);
    b=strcat(str, '.xlsx');
    [datac]=xlsread(b, 1, 'A2:O1000000');
    coordinates2=coordinates;
    sheet=find(coordinates2(:,1)~=a);
    coordinates2(sheet,:)=[];
    howmany=size(coordinates2);

%crop the data into regions and save to excel
for n=1:howmany(1);
    data2=datac;
    data4=[];
    xcrops=find(data2(:,5)<coordinates2(n,2)-areax2);
    data2(xcrops,:)=[];
    xcrop1=find(data2(:,5)>coordinates2(n,2)+areax2);
    data2(xcrop1,:)=[];
    ycrops=find(data2(:,6)>coordinates2(n,3)+areay2);
    data2(ycrops,:)=[];
    ycrop1=find(data2(:,6)<coordinates2(n,3)-areay2);
    data2(ycrop1,:)=[];
    datas=datac;
```

```

xcrops=find(datas(:,5)<coordinates2(n,2)-areax);
datas(xcrops,:)=[];
xcropl=find(datas(:,5)>coordinates2(n,2)+areax);
datas(xcropl,:)=[];
ycrops=find(datas(:,6)>coordinates2(n,3)+areay);
datas(ycrops,:)=[];
ycropl=find(datas(:,6)<coordinates2(n,3)-areay);
datas(ycropl,:)=[];
index=index+1;
xlswrite('regions',datas,index,'A1');

%calculate cluster values
numparticles=size(data2);
for p=1:numparticles(1);
    newtabl=[];
    for m=1:numparticles(1);
        xone=data2(p,5); yone=data2(p,6); xtwo=data2(m,5);
        ytwo=data2(m,6);
        distance=((xone-xtwo)*(xone-xtwo))+((yone-ytwo)*(yone-
        ytwo));
        if distance<=clusscale*clusscale;
            newtabl=vertcat(newtabl,data2(m,:));
            Kfunc=size(newtabl);
            Kfuncans=Kfunc(1)-1;
        end
    end
    Kfuncans=((2*areax2)*(2*areay2)*Kfuncans/(numparticles(1)-
    1)/(pi))^0.5;
    oneliner=horzcat(data2(p,:),Kfuncans);
    data4=vertcat(data4,oneliner);
end

%calculate cluster map
temp1=data4(:,5:6);
temp2=data4(:,16);
data=horzcat(temp1,temp2);
tx=(coordinates(index,2)-areax):10:(coordinates(index,2)+areax);
ty=((coordinates(index,3))-areay):10:((coordinates(index,3))+areay);
[XI, YI]=meshgrid(tx, ty);
ZI=griddata (data(:,1), data(:,2), data(:,3), XI, YI, 'v4');

%generate the plots
figure1 = figure('Color',[1 1 1]);
axes('Parent',figure1,'Layer','top',
'YTick',zeros(1,0),'XTick',zeros(1,0),'DataAspectRatio', [1,1,1],'CLim',[0
maxcol]);
box('on');hold('all');
contourf(XI,YI,ZI,100,'LineColor','none','Fill','on');
plot3(data(:,1), data(:,2),
data(:,3),'Marker','.', 'MarkerSize',4, 'LineStyle','none','Color',[0 0 0]),
hold off;
print('-dpng','-r300',strcat(num2str(index), 'colourmap.png'));
hgsave(strcat(num2str(index), 'cmap.fig'));
figure2 = figure('Color',[1 1 1],'Colormap',[c]);
axes('Parent',figure2,'Layer','top',
'YTick',zeros(1,0),'XTick',zeros(1,0),'DataAspectRatio', [1,1,1],'CLim',[0
maxcol]);
box('on');
hold('all');
contourf(XI,YI,ZI,100,'LineColor','none','Fill','on');
print('-dpng','-r300',strcat(num2str(index), 'threshold.png'));

```

```

figure3 = figure('Color',[1 1 1],'Colormap',[0 0 0;[...;...;...];1 1
1]);
axes('Parent',figure3,'Layer','top',
'YTick',zeros(1,0),'XTick',zeros(1,0),'DataAspectRatio',[1,1,1],'CLim',[0
maxcol]);
box('on');
hold('all');
contourf(XI,YI,ZI,100,'LineColor','none','Fill','on');
print('-dpng','-r300',strcat(num2str(index),'stics.png'));
hold off;
close(figure1);
close(figure2);
close(figure3);
xcrops=find(data4(:,5)<coordinates2(n,2)-areax);
data4(xcrops,:)=[];
xcrop1=find(data4(:,5)>coordinates2(n,2)+areax);
data4(xcrop1,:)=[];
ycrops=find(data4(:,6)>coordinates2(n,3)+areay);
data4(ycrops,:)=[];
ycrop1=find(data4(:,6)<coordinates2(n,3)-areay);
data4(ycrop1,:)=[];
abovethresh=sum(data4(:,16)>binarychange);
numtotal=size(datas);
numtotal2=numtotal(1);
percentabove=abovethresh/numtotal2*100;
xlswrite('regions', abovethresh,index,'Q1');
xlswrite('regions', numtotal2,index,'Q2');
xlswrite('regions', percentabove,index,'Q3');
end
end;

```

Appendix 2: Screening anti-KIR2DL1 antibody supernatants.

Several anti-KIR2DL1 antibody supernatants obtained from Stipan Jonjić were screened by confocal microscopy and flow cytometry and compared to the commercial anti-KIR2DL1 antibody EB6. Data shown here were obtained using supernatant 03, which was found to be the best supernatant and was used for experiments in this thesis. NKL cells were used as a negative control and NKL-KIR2DL1-GFP were used as a positive control known to express KIR2DL1. **(A)** NKL and NKL-KIR2DL1-GDP cells were fixed and stained either with anti-KIR2DL1 supernatant or with commercial antibody. These cells were then imaged by confocal microscopy. **(B)** NKL and NKL-KIR2DL1-GFP were stained either with anti-KIR2DL1 supernatant or with commercial antibody and analysed by flow cytometry.

