INTRODUCTION

Natural Killer (NK) cells are classified as lymphocytes of the innate immune system because they are capable of directly killing virus-infected or transformed cells without prior priming. Therefore their responses need to be tightly regulated to allow proper distinction between diseased cells and neighboring healthy tissues. To ensure this, NK cell activation is shaped by the balance of signals induced from inhibitory and activating germline-encoded receptors expressed on their surface. Integration of signals from cell surface receptors takes place across a dynamic structured interface – the immune synapse (Davis et al., 1999). Assembly of the immune synapse occurs in sequential stages of receptor–ligand organization, cytoskeletal rearrangements, and cellular polarization, from initial adhesion to an appropriate response such as killing a target cell by the release of cytolytic granules (Orange et al., 2003).

The formation of the immune synapse is a critical event in NK cell behavior; and hence it is important to determine the where and when protein–protein interactions occur to regulate NK cell behavior; and hence it is important to determine the where and when protein–protein interactions occur to regulate events occurring while NK cells decide an appropriate response. This is broadly the gap in much of contemporary cell biology – we know which protein–protein interactions are important but have little understanding as to how all these interactions play out in space and time to give rise to complex cell behaviors, such as making a decision about the state of health of another cell. To understand how this works in detail requires knowing where and when protein–protein interactions occur to regulate NK cell behavior; and hence it is important to determine the.

Natural Killer (NK) cell responses are shaped by the integration of signals transduced from multiple activating and inhibitory receptors at their surface. Biochemical and genetic approaches have identified most of the key proteins involved in signal integration but a major challenge remains in understanding how the spatial and temporal dynamics of their interactions lead to NK cells responding appropriately when encountering ligands on target cells. Well over a decade of research using fluorescence microscopy has revealed much about the architecture of the NK cell immune synapse – the structured interface between NK cells and target cells – and how it varies when inhibition or activation is the outcome of signal integration. However, key questions – such as the proximity of individual activating and inhibitory receptors – have remained unanswered because the resolution of optical microscopy has been insufficient, being limited by diffraction. Recent developments in fluorescence microscopy have broken this limit, seeding new opportunities for studying the nanometer-scale organization of the NK cell immune synapse. Here, we discuss how these new technologies, super-resolution imaging and other novel light-based methods, can illuminate our understanding of NK cell biology.

Keywords: NK cells, immune synapse, signal integration, super-resolution imaging, microclusters, nanoclusters

NK cells are activated through surface receptors that can recognize signs of disease on other cells using different strategies. Receptors such as natural-killer group 2 member D (NK2D) recognize ligands that are encoded in our genome and can be expressed or up-regulated on diseased or infected cells – as treatments recognize ligands that are encoded in our genome and can be expressed or up-regulated on diseased or infected cells – as treatments Recognize ligands that are encoded in our genome and can be expressed or up-regulated on diseased or infected cells – as treatments Recognize ligands that are encoded in our genome and can be expressed or up-regulated on diseased or infected cells – as treatments Recognize ligands that are encoded in our genome and can be expressed or up-regulated on diseased or infected cells – as treatments Recognize ligands that are encoded in our genome and can be expressed or up-regulated on diseased or infected cells – as treatments

Natural Killer (NK) cells are classified as lymphocytes of the innate immune system because they are capable of directly killing virus-infected or transformed cells without prior priming. Therefore their responses need to be tightly regulated to allow proper distinction between diseased cells and neighboring healthy tissues. To ensure this, NK cell activation is shaped by the balance of signals induced from inhibitory and activating germline-encoded receptors expressed on their surface. Integration of signals from cell surface receptors takes place across a dynamic structured interface – the immune synapse (Davis et al., 1999). Assembly of the immune synapse occurs in sequential stages of receptor–ligand organization, cytoskeletal rearrangements, and cellular polarization, from initial adhesion to an appropriate response such as killing a target cell by the release of cytolytic granules (Orange et al., 2003).

The formation of the immune synapse is a critical event in NK cell behavior; and hence it is important to determine the where and when protein–protein interactions occur to regulate events occurring while NK cells decide an appropriate response. This is broadly the gap in much of contemporary cell biology – we know which protein–protein interactions are important but have little understanding as to how all these interactions play out in space and time to give rise to complex cell behaviors, such as making a decision about the state of health of another cell. To understand how this works in detail requires knowing where and when protein–protein interactions occur to regulate NK cell behavior; and hence it is important to determine the.

Natural Killer (NK) cell responses are shaped by the integration of signals transduced from multiple activating and inhibitory receptors at their surface. Biochemical and genetic approaches have identified most of the key proteins involved in signal integration but a major challenge remains in understanding how the spatial and temporal dynamics of their interactions lead to NK cells responding appropriately when encountering ligands on target cells. Well over a decade of research using fluorescence microscopy has revealed much about the architecture of the NK cell immune synapse – the structured interface between NK cells and target cells – and how it varies when inhibition or activation is the outcome of signal integration. However, key questions – such as the proximity of individual activating and inhibitory receptors – have remained unanswered because the resolution of optical microscopy has been insufficient, being limited by diffraction. Recent developments in fluorescence microscopy have broken this limit, seeding new opportunities for studying the nanometer-scale organization of the NK cell immune synapse. Here, we discuss how these new technologies, super-resolution imaging and other novel light-based methods, can illuminate our understanding of NK cell biology.

Keywords: NK cells, immune synapse, signal integration, super-resolution imaging, microclusters, nanoclusters

NK cells are activated through surface receptors that can recognize signs of disease on other cells using different strategies. Receptors such as natural-killer group 2 member D (NK2D) recognize ligands that are encoded in our genome and can be expressed or up-regulated on diseased or infected cells – as treatments recognize ligands that are encoded in our genome and can be expressed or up-regulated on diseased or infected cells – as treatments Recognize ligands that are encoded in our genome and can be expressed or up-regulated on diseased or infected cells – as treatments Recognize ligands that are encoded in our genome and can be expressed or up-regulated on diseased or infected cells – as treatments Recognize ligands that are encoded in our genome and can be expressed or up-regulated on diseased or infected cells – as treatments Recognize ligands that are encoded in our genome and can be expressed or up-regulated on diseased or infected cells – as treatments

Natural Killer (NK) cells are classified as lymphocytes of the innate immune system because they are capable of directly killing virus-infected or transformed cells without prior priming. Therefore their responses need to be tightly regulated to allow proper distinction between diseased cells and neighboring healthy tissues. To ensure this, NK cell activation is shaped by the balance of signals induced from inhibitory and activating germline-encoded receptors expressed on their surface. Integration of signals from cell surface receptors takes place across a dynamic structured interface – the immune synapse (Davis et al., 1999). Assembly of the immune synapse occurs in sequential stages of receptor–ligand organization, cytoskeletal rearrangements, and cellular polarization, from initial adhesion to an appropriate response such as killing a target cell by the release of cytolytic granules (Orange et al., 2003).

The formation of the immune synapse is a critical event in NK cell behavior; and hence it is important to determine the where and when protein–protein interactions occur to regulate events occurring while NK cells decide an appropriate response. This is broadly the gap in much of contemporary cell biology – we know which protein–protein interactions are important but have little understanding as to how all these interactions play out in space and time to give rise to complex cell behaviors, such as making a decision about the state of health of another cell. To understand how this works in detail requires knowing where and when protein–protein interactions occur to regulate NK cell behavior; and hence it is important to determine the.
In the initial studies describing T cell and NK cell immune synapses, imaging at the micrometer-scale revealed that proteins at the interface between interacting cells segregated into distinguishable supramolecular clusters (Monks et al., 1999; Davis et al., 1999; Graikou et al., 1999; Vuš et al., 2001). In the T cell synapse, the T cell receptor (TCR) was enriched in the central region, the central supramolecular activating cluster (cSMAC), where it colocalized with Lck and PKC signaling molecules, segregated from the adhesion molecule LFA-1 which formed a ring around the center named the peripheral SMAC (pSMAC). Similarly, in the cytolytic NK cell synapse, lytic granules accumulated at the cSMAC, while adhesion molecules segregated into a pSMAC (Orange et al., 2003). These ‘prototypical’ types of organization are sometimes referred to as mature immune synapses.

The process of an immune cell meeting another cell and then moving on without effector functions being elicited should not necessarily be considered a non-event because, at least for NK cells, a structured interface does still assemble – a so-called inhibitory synapse – and specific signals trigger inhibition to allow the NK cell to move on (Davis et al., 1999). At the inhibitory synapse, proteins organize in such a way that KIR and associated components accumulate in central clusters segregated from LFA-1 at the periphery (Schlenützer et al., 2008). Intriguingly, segregation of KIR from integrins is determined by the density of interacting class I MHC protein on target cells (Almeida and Davis, 2006), indicating a link between the function of KIR – to assess levels of class I MHC expression – and its supramolecular organization.

The mechanisms by which proteins organize at the immune synapse are thought to involve the actin cytoskeleton, although whether by direct tethering to sub-synaptic actin filaments or confinement within an actin “picket-fence” meshwork remains unclear. It has also been proposed that proteins would segregate according to their localization into cholesterol-enriched lipid raft domains, in which activating receptors are concentrated and inhibitory receptors and phosphatases such as CD45 are excluded (Leupin et al., 2000). The polarization of lipid rafts to the immune synapse has been implicated in NK cell activation and has been modeled in microclusters at the cell periphery that then moved centripetally (Abeyweera et al., 2011), forming a ring around a central secretory domain through which release of lytic granules occurred (Liu et al., 2012). Assembly of these microclusters of CD16, as visualized through their interaction with fluorescent Fc portions of antibodies in supported lipid bilayers, formed at the periphery of the contact and moved toward the center of the synapse (Liu et al., 2012). Assembly of these microclusters was impaired when inhibitory receptor CD94/NKG2A was co-engaged, indicating their formation was already an outcome of

### HIGH-RESOLUTION IMAGING OF IMMUNE SYNASPS

One of the high-resolution techniques introduced early-on to visualize membrane-proximal events occurring at the immune synapse was total internal reflection fluorescence (TIRF) microscopy. This allows imaging of processes happening at the interface between a cell and a coverslip with strongly decreased background fluorescence (Table 1). Cells could be imaged when plated on activating surfaces, such as slides coated with antibodies or protein ligands, or protein-rich planar lipid bilayers. Particularly useful is that the protein composition of bilayers and coated slides can be precisely controlled – allowing quantitative studies regarding numbers of ligands used to stimulate immune cells. The application of TIRF microscopy to studies of immune synapses made it possible to observe proteins organize into discrete assemblies on a micrometer-to-sub-micrometer-scale, named microclusters, where (at least some) signaling takes place. Microclusters of TCR were detected and shown to be involved in signaling while migrating from the periphery of the synapse toward the center (Yokosuka and Saito, 2010) and this movement was dependent on cytoskeletal processes. Indeed, retrograde flow of actin occurring during synapse formation can carry microclusters of TCR in T cells and activating receptors in NK cells from the cell periphery toward the center (Varma et al., 2006; Kainzka et al., 2007; Abeyweera et al., 2011). Interestingly, inhibitory NK cell receptors, KIR specifically, accumulate at the synapse largely independently of cytoskeletal processes (Davis et al., 1999; Standeven et al., 2004), likely moving to the synapse by passive diffusion and accumulating there by adhesion within the synaptic cleft.

Inhibitory NK cell KIR proteins also organize into microclusters (Oddos et al., 2008; Abeyweera et al., 2011). Phosphorylation of KIR2DL1 was visualized using fluorescence lifetime imaging (FLIM) to detect Förster resonance energy transfer (FRET; Treanor et al., 2006). This enabled detection of discrete signaling microclusters of KIR2DL1 at the synapse and provided the first indication that NK cell signaling may occur within small protein assemblies. Subsequently, the use of optical tweezers with confocal microscopy to study T and NK cell synapses revealed the presence of micro-scale clusters of protein at the contact between two cells rather than at the interface between one cell and a cover-slip (Oddos et al., 2008). An increased resolution was achieved by avoiding the need for an ex vivo 3D reconstruction from multiple planes imaged by confocal microscopy by reorienting conjugates so that the immune synapse lies horizontally in the imaging plane (Table 1). This study established that microclusters of KIR2DL1 within the inhibitory NK cell synapse moved from the synapse periphery to the center (Oddos et al., 2008).

At the lytic synapse activating receptor NKG2D also accumulated in microclusters at the cell periphery that then moved centripetally (Abeyweera et al., 2011), forming a ring around a central secretory domain through which release of lytic granules occurred (Liu et al., 2009; Brown et al., 2011). Similarly, microclusters of CD16, as visualized through their interaction with fluorescent Fc portions of antibodies in supported lipid bilayers, formed at the periphery of the contact and moved toward the center of the synapse (Liu et al., 2012). Assembly of these microclusters was impaired when inhibitory receptor CD94/NKG2A was co-engaged, indicating their formation was already an outcome of
| Technique                          | Principle                                                                 | Advantages                                                                 | Limitations                                                                 |
|-----------------------------------|---------------------------------------------------------------------------|-----------------------------------------------------------------------------|----------------------------------------------------------------------------|
| Total internal reflection fluorescence (TIRF) | The excitation beam is directed onto the sample at a critical angle and is reflected off the coverslip-sample interface, generating an evanescent wave in which fluorophores are excited | - High sectioning strength (about 100 nm into the sample)  
- Minimal background noise from intracellular out-of-focus regions | - Limited to regions at or near the cell surface  
- Remains subject to the diffraction limit |
| Optical tweezers with confocal microscopy | Manipulation of live cells in all dimensions using a tightly focused laser beam that can trap particles, conjugates are oriented so that synapse is aligned to the imaging plane | - En face observation of the immune synapse at high speed and high resolution  
- Promotion of formation of specific cell conjugates | - Remains subject to the diffraction limit |
| Structured illumination microscopy (SIM) | Full-field illumination of the sample with spatially structured light generates images with high spatial frequency information that are reconstructed into a super-resolution image | - 3D resolution improvement  
- Possible to image deep within a cell  
- Conventional dyes can be used (if sufficiently photostable)  
- Straightforward sample preparation | - At best two-fold improvement in resolution  
- Complex post-acquisition processing of data (introduction of artefacts needs to be controlled)  
- Long acquisition and processing times |
| Stimulated emission depletion (STED) microscopy | The sample is scanned by two overlapping concentric laser beams to minimize the volume of detection: the first laser excites the fluorophores, the second laser of longer wavelength drives the fluorophores into the ground state by stimulated emission depletion | - Fast acquisition and potential for live cell imaging  
- Potential for extremely high resolution (5 nm)  
- Great depth penetration and potential for 3D imaging  
- No need for post-acquisition data processing (minimal artefacts) | - Problems with photobleaching in biological samples  
- Only some conventional dyes and fluorescent proteins can be used  
- Long acquisition time to collect sufficient photons |
| Stochastic optical reconstruction microscopy (STORM) | Individual fluorophores are stochastically excited, localized and bleached. A super-resolution image is reconstructed from individual localizations across thousands of frames | - Potential for very high resolution (10–20 nm) and single-molecule data  
- 3D resolution improvement 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 labeled antibody  
- Limited to fixed cells |
| Photoactivated localization microscopy (PALM) | Principle is similar to STORM, but relies on genetically encoded photoswitchable fluorescent proteins | - Potential for very high resolution (10–20 nm) and single-molecule data  
- 3D resolution improvement possible when combined with TIRF or a cylindrical lens  
- 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 |
| Fluorescence correlation spectroscopy (FCS) | Correlation analysis of fluctuations in fluorescence intensity within a small confocal volume reveals information about diffusion, concentration, and dynamics of molecules | - Conventional dyes and fluorescent proteins can be used  
- Used with confocal or multi-photon microscopy  
- Single-molecule sensitivity | - Indirect measurements  
- Complex curve fitting  
- Long acquisition times |

Table summarizing the main high- and super-resolution technologies mentioned in the main text and comparing some of their major advantages and limitations.
signal integration. Inhibition was partially mediated by phospho-
rylation of Crk, a small adaptor protein that with other proteins
forms a cytoskeleton scaffold complex, which regulates actin rear-
rangements (Peterson and Long, 2008). Upon phosphorylation,
Crk dissociated from the scaffold complex, disrupting F-actin at
the center of the synapse, thereby preventing movement of CD16
microclusters and transduction of activating signals (Liu et al.,
2012). Interestingly, engagement of CD94/NKG2A in the absence of
co-engagement of other receptors was sufficient to induce Crk
phosphorylation. This suggests a dual function for ITIM-bearing
receptors, whereby inhibitory signaling can be induced even in the
absence of activation in order to remove physical constraints on
activating receptors and “license” cells for subsequent engagement
of activating receptors (Liu et al., 2012).

**USING PHOTOCHEMISTRY TO PROBE THE IMMUNE SYNPASE**

Peptides can be made to include a photocleavable group protecting
a functionally important site for recognition that can be cleaved
by light to yield the native active version, thus enabling the time-
resolved study of fast biological processes. DeMond et al. (2006)
exploited a caged antigenic peptide presented by class II MHC
protein to selectively activate CD4+ T cells on a supported lipid
bipolar in a spatially and temporally controlled way, demonstrating
the strategy for examining the arrangements of the T cell immune
synapse. Independently, Huse and colleagues developed a photo-
cleavable peptide to study the temporal and spatial aspects of T
cell signaling and protein organization at the synapse (Huse et al.,
2007; Quan et al., 2009, 2011).

Huse and colleagues also extended this approach to study NK
cells (Abeyweera et al., 2011). It had been established previously
that large residues in the P8 position of peptides in HLA-C can
mask the binding site for KIR (Raijegopalan and Long, 1997; Boy-
ington et al., 2005). By introducing a large photocleavable moiety
on this residue, peptide–MHC complexes are not recognized by
KIR until irradiation with UV light. Such a photolabile peptide, in
complex with HLA-Cw6, was introduced into lipid bilayers con-
taining activating ligand ULBP3 and integrin ICAM-1, and used to
precisely control the timing of inhibition via KIR2DL2 (Abey-
weera et al., 2011). Inhibition was induced at various time points
after NK cells contacted the bilayer, to study how early inhibitory
signaling needs to be initiated to impair the process of activation.
Interestingly, the authors observed that initiation of inhibition up
to 15 min after the NK cells landed was still able to reverse acti-
vation. These results suggest that a cell is never fully committed
to killing and is continuously integrating signals induced by sur-
facing receptors. This is consistent with earlier imaging using slides
coated with micro-patterned stripes of activating and inhibitory
ligands, which also demonstrated that a continued dominance of
local activating signals is necessary for a stimulatory NK cell
response (Calley et al., 2009).

**NANOMETER-SCALE ORGANIZATION OF THE IMMUNE SYNPASE REVEALED BY SUPER-RESOLUTION MICROSCOPY**

Many different super-resolution techniques capable of nanometer-
scale resolution have now been demonstrated. The principles
behind some of these techniques along with their main advantages
and limitations are summarized in Table 1. Although their appli-
cation to immune cell biology is very new, they have already
led to some important discoveries about the nanometer-scale
organization of proteins at immune synapses. Super-resolution
images obtained using structured illumination microscopy (SIM)
revealed how the cortical actin mesh – which underlies all cell sur-
face membranes – is remodeled upon NK cell activation (Brown
et al., 2011). Observing actin at the synapse with such resolution
revealed that the periodicity of the actin mesh is increased to
create holes sized to fit lytic granules. Independently, and pub-
lished at the same time, another super-resolution microscopy
technique stimulated emission depletion (STED) microscopy also
revealed an opening of the actin mesh in domains within the
synapse center (Bak et al., 2011). Together, these findings refute
the earlier dogma that actin was entirely cleared from the cen-
ter of the sub-synaptic area in order to allow release of lytic
granules.

Super-resolution imaging can examine far more than just actin
and immunologists have begun to exploit its potential for imag-
nering the nanometer-scale organization of receptors and ligands at
synapses – with most studies so far focused on T cell synapses.
Photoactivated localization microscopy (PALM) has been applied
by several groups (Lillemoe et al., 2010; Owen et al., 2010; Sher-
man et al., 2011; Williamson et al., 2011), focused on visualizing
the distribution and interaction of TCR and membrane-proximal
signaling proteins and adaptors, such as LAT, during T cell acti-
vation. One study found TCR and LAT to be pre-clustered in
quiescent T cells (Lillemoe et al., 2010). Upon activation of T
cells, these small aggregates – or protein islands as the authors
termed them – concatenated but surprisingly, instead of over-
lapping, TCR and LAT remained in distinct domains that became
juxtaposed to each other. In a subsequent study using PALM, Sher-
man et al. (2011) also observed LAT in pre-existing nanoclusters
in resting T cells. The authors suggest that by using PALM, what
was previously detected as microclusters can now be visualized as
a group of smaller subunits – nanoclusters, containing only few
molecules. Two-color PALM extended these findings to demon-
strate that TCR and ZAP-70 nanoclusters colocalized after TCR
ligation. However, clusters of TCR were largely segregated from
LAT clusters, with sparse regions of overlap potentially represent-
ing “hot spots” where LAT phosphorylation is triggered by ZAP-70
(Sherman et al., 2011). These findings highlight the importance of
super-resolution imaging to disentangle contradictory models
for immune receptor signaling dynamics. With the knowledge
we now have, it is clear that proteins known to interact often
transiently meet via the movements of nanometer-scale protein
clusters to facilitate signaling – a process somewhat distinct from
the textbook-level version of events involving a linear cascade of
single protein–protein interactions.

Using TIRF microscopy as well as optical tweezers coupled
with confocal microscopy, motile sub-synaptic vesicles containing
LAT were seen to repeatedly move between clusters of SLP-76, where
they decreased motility, highlighting that both vesicular LAT and
surface clusters of LAT can play a role in TCR signaling (Parbhoo
et al., 2010). A subsequent study combining PALM, live PALM, and
stochastic optical reconstruction microscopy (STORM) indicated
that TCR signaling may depend only on LAT molecules associated
with sub-synaptic intracellular vesicles rather than LAT clusters already present in the plasma membrane (Williamson et al., 2011). Together, these two studies have led to the proposal of a new model in which vesicular traffic to and from the membrane is important for the recruitment and disassembly of signaling complexes – another refinement to the textbook-level description of events following TCR ligation.

Clearly, much can be gained by the application of super-resolution imaging to the study of NK cell signaling – to detect small changes in protein localization, nanocluster size distribution and molecular density within clusters during the process of target cell recognition. Indeed, the importance of the nanoscale organization of receptors was highlighted in a recent study using fluorescence correlation spectroscopy (FCS) using murine NK cell models (Gaia et al., 2011). By recording the transit time of cell surface proteins moving through differently sized volumes illuminated by laser light and comparing to freely diffusing molecules, Gaia et al. (2011) presented striking evidence that NK cell receptors were differentially restricted in their movement at the cell surface. Specifically, the authors proposed that activating receptors were compartmentalized by the organization of the plasma membrane, while inhibitory receptors were confined by the actin meshwork. Surprisingly, at the surface of hyporesponsive, i.e. incompetent, NK cells, activating and inhibitory receptors were found to be constrained by the actin meshwork. This, along with previous work and recent advances in the field, suggests that receptor clustering at NK cell immune synapses may be important to how NK cell tolerance is achieved (Guia et al., 2011). The importance of the nanometer-scale organization of proteins in T and NK cell membranes for appropriate signal integration was established the importance of the nanometer-scale organization of proteins in T and NK cell membranes for appropriate signal integration. An important paradigm emerging from the studies described here is that interactions between receptors and signaling proteins are controlled in part by the dynamics of supramolecular clusters rather than by isolated protein–protein interactions as once imagined. In the near future, super-resolution imaging techniques will reveal much about the spatial and temporal dynamics that underlie NK cells making appropriate decisions during immune surveillance of target cells.

**ACKNOWLEDGMENTS**

We thank S.-P. Cordoba and A. Aucher for critical reading of the manuscript.

**REFERENCES**

Bolton, S. C., Moir, A. S., Schuck, P., Brooks, A. G., and Sun, P. D. (2005). Crystal structure of an NK cell natural killer cell immune synapse. Nature 435, 537–543.

Brown, A. C. N., Oddo, S., Dohic, I. M., Alakoskela, J.-M., Patton, R. M., Ziemann, P., et al. (2011). Remodelling of cortical actin where lytic granules dock at natural killer cell immune synapses revealed by super-resolution microscopy. PLoS Biol. 9, e1001152. doi:10.1371/journal.pbio.1001152

Burroughs, N. J., Köhler, K., Milewicz, V., Davis, M. M., Van der Most, P. A., and Dinzl, D. M. (2011). Boltzmann energy-based image analysis demonstrates that extracellular domain size differences explain protein segregation at immune synapses. PLoS Comput. Biol. 7, e1001726. doi:10.1371/journal.pcbi.1001726

Burdett, D. N., Schulze, A. M., Wagemann, N., Reijngoud, S., Berrada, K., Yu, T., et al. (1996). Recruitment of tyrosine phosphatase FCP by the killer cell inhibitory receptor. Immunity 4, 77–85.

Chakraborti, K., Wiesman, D., Brown, M. H., Gould, K., and Van der Merwe, P. A. (2004). Cell receptor triggering is critically dependent on the dimensions of its-peptide-MHC ligand. Nature 435, 575–582.

Chowdhuri, K., Wiseman, D., Brown, M. H., Gould, K., and Van der Merwe, P. A. (2000). T-cell receptor triggering in a reconstituted system: a molecular machine controlling T cell activation. Science 285, 223–227.

Gaia, S., Joger, B. N., Pintok, S., Mallis, S., Trombik, T., Fentei, A., et al. (2011). Confinement of activating receptors at the plasma membrane controls natural killer cell tolerance. Sci. Signal. 4, ra21.

Hose, M., Klaen, L. O., Gervin, A. T., Furq, J. M., Li, Q.-J., Kubanac, M. S., et al. (2005). Spatial and temporal dynamics of T cell receptor signaling with a photoactivatable agonist. J. Immunol. 176, 5606–5611.

Grakoui, A., Bromley, S. K., Sumen, C., Dava, M. M., Shae, S. A., Allen, P. M., et al. (1999). The immunological synapse: a molecular machine controlling T cell activation. Science 285, 217–223.

Guia, S., Joger, B. N., Pintok, S., Mallis, S., Trombik, T., Fentei, A., et al. (2011). Confinement of activating receptors at the plasma membrane controls natural killer cell tolerance. Sci. Signal. 4, ra21.

Hose, M., Klaen, L. O., Gervin, A. T., Furq, J. M., Li, Q.-J., Kubanac, M. S., et al. (2005). Spatial and temporal dynamics of T cell receptor signaling with a photoactivatable agonist. J. Immunol. 176, 5606–5611.

James, J. R., and Vale, R. D. (2012). Bio-physical mechanisms of T-cell receptor triggering in a reconstituted system. Nature 487, 64–69.

Koscoff, R., Ljunggren, H. G., Forssell, G., and Kozodoy, H. (1996). Selective inhibition of H-2-deficient lymphoma variants suggests alternative immune defence strategy. Nature 385, 675–678.

Köhler, K., Xiong, S., Brezove, I., Mehrabi, M., Eismann, P., Harris, A., et al. (2010). Matched sets of activating and inhibitory receptor/ligand pairs are required for optimal signal integration by human natural killer cells. PLoS ONE 5, e10574. doi:10.1371/journal.pone.0010574

Lefrançois, O., Zaru, R., Lacroix, T., Müller, S., and Váncsa, S. (2008). Localization of CD45 from the T-cell receptor signaling area in antigen-stimulated T lymphocytes. Curr. Biol. 18, 277–281.

Liddle, M. B., Mörk, M., Aftimos, M. B., Forstner, M. B., Hopp, J. B., Groves, J. T., and Davis, M. M. (2010). TCR and LAT are expressed on separate protein islands on T cell membranes and concentrate during activation. Nat. Immunol. 11, 93–98.

Liu, D., Byronson, Y. T., Mackel, T., Vautier-Usamis, G., Davis, M. L., and Long, E. O. (2009). Integrin-dependent organization and bidirectional vesicular traffic at cytotoxic immune synapses. Immunity 31, 99–109.

**PLoS Comput. Biol.** 2012 | Volume 8 | Article 308 — page 5 — #5

“fimmu-03-00308” — 2012/10/3 — 10:38 — page 5 — #5
Pageon, S. V., Rudnicka and Davis. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in any medium, provided that the original authors and source are credited and subject to any copyright notices concerning any third-party graphics etc.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 18 August 2012; accepted: 9 October 2012; published online: 19 October 2012.

Copyright © 2012 Pageon, Rudnicka and Davis. This article was submitted to frontiers in NK cell Biology, a specialty of Frontiers in Immunology.

Published: 10 September 2012; accepted: 9 October 2012; paper pending publication: 10 September 2012; accepted: 17 September 2012; published online: 9 October 2012.

Clontech: Pagew S, Budayrick D and Davis DB (2012) Illuminating the dynamics of signal integration in natural killer cells. Front Immunol 3:308. doi: 10.3389/fimmu.2012.00308

This article was submitted to Frontiers in NK Cell Biology, a specialty of Frontiers in Immunology.

Copyright © 2012 Pageon, Budayrick and Davis. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in any medium, provided that the original authors and source are credited and subject to any copyright notices concerning any third-party graphics etc.