Leibniz-Gemeinschaft

Final report

Project:
Defining Natural Killer Cell reactivity by quantitative analysis of activating and inhibitory receptor signal integration

Leibniz-Institute: IfADo
Reference number: SAW-2013-IfADo-2
Project period: 01.07.2013 – 31.12.2016
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Executive summary

Natural Killer (NK) cells are an important component of the innate immune system and many immune reactions against viral infections and tumors are critically dependent on the activity of these cells. NK cells are regulated via an interplay of activating and inhibitory cell surface receptors. In this project we aimed to better understand this regulation on a molecular level and to get a quantitative insight into how much receptor engagement is necessary to activate NK cells. Using a synthetic biology approach where we rebuilt human NK cell signaling pathways in a Drosophila cell line, we found that ITAM-based activating receptors can result in SLP-76 phosphorylation independently of the activity of Src-family kinases. This was dependent on the kinase Syk, which is expressed in NK cells and B cells. However, the related kinase ZAP-70, which is expressed in NK cells and T cells did require the activity of Src-family kinases to phosphorylate SLP-76 in response to ITAM-receptor engagement. We also developed a novel technology, where we used DNA-directed immobilization (DDI) technology to immobilize ligands for NK cell receptors on glass surfaces in a quantitative and highly organized fashion. These surfaces could then be used to stimulate NK cells and to elicit NK cell responses such as degranulation and cytokine production. Using these surfaces we found that the engagement of specific surface receptors induces a stop-signal in NK cells and induces the accumulation of these cells. In a further development of this technology we used DNA-origami structures to control for the organization of the NK cell ligands on a nanometer-scale. Using this technology we could show that not only the ligand quantity determines the strength of an NK cell response, but that also the nano-scale organization of the ligands has an influence on NK cell reactivity. These exciting results will be further explored in a follow-up project. In a last step we also developed three-dimensional surfaces in the form of latex beads for the controlled stimulation of NK cells. We could load these beads with defined amounts of ligands for NK cell receptors and could determine how much ligand was necessary to stimulate NK cell responses via the different activating receptors. Also this work will be continued past the funding period of this grant.

Research Question and Aims

The immune system is essential for the protection of the host against a variety of infectious agents and the growth of transformed cells. Natural Killer (NK) cells are at the junction of the innate and the adaptive immune response and serve a very important role in host defense (Colonna et al., 2011; Vivier et al., 2008). NK cell deficiencies in humans result in overwhelming fatal infections during early childhood (Orange, 2006) and low NK cell activity is linked to an increased risk of developing cancer (Imai et al., 2000), demonstrating the importance of these cells. NK cell effector functions include cellular cytotoxicity against virally infected or malignantly transformed cells and the secretion of chemokines and cytokines, such as IFNγ and TNF-α. Many immune reactions against infections are critically dependent on NK cells - not only because of their ability to kill infected cells, but also because of their important capacity to regulate adaptive immune responses (Andoniou et al., 2008).

NK cells are regulated by a redundant array of activating and inhibitory cell surface receptors (Watzl and Long, 2010). Many inhibitory NK cell receptors are specific for self-MHC class I and are important to ensure the self-tolerance of NK cells. Loss of MHC class I upon viral infection or malignant transformation can therefore result in the so called 'missing-self' reactivity of NK cells. NK cell activation is mediated by a variety of different surface receptors that can recognize specific ligands on transformed or infected cells. Engagement of activating NK receptors by their ligands results in receptor clustering in the immunological synapse – the contact area between the NK cell and the target cell. It is believed that this change in receptor proximity and localization is sufficient to induce signaling, ultimately leading to target cell lysis and the production of cytokines and chemokines.

In order to better understand the regulation of NK cells on a molecular level, this project aimed to (1) determine how positive and negative signals are integrated on a molecular level and to (2) get a quantitative and qualitative understanding of how the different ligands for activating receptors regulate NK cell reactivity. To answer these questions, we worked on three different work packages (WPs):

WP1: At which signaling point do activating and inhibitory signals intersect?

WP2: What is the minimal system to re-create the NK cell signaling network?

WP3: How much activating ligand is necessary for NK cell stimulation and regulation and what are the spatial constraints?

Results and Discussion

WP1: At which signaling point do activating and inhibitory signals intersect?
Based on our finding that Vav-1 is a direct target of SHP-1 when bound to an inhibitory receptor (Stebbins et al., 2003), we proposed the hypothesis described in Figure 1A. Upon target cell encounter, activating NK cell receptors or integrins such as LFA-1 may be engaged by their ligand on the target cell. This will lead to an early and actin-independent phosphorylation of Vav-1. Through the activation of small G-proteins such as Rac-1 or Cdc42 this will induce the re-organization of the actin cytoskeleton. Actin polymerization is necessary for the recruitment of activating receptors into the contact area, which can subsequently be engaged by their ligands resulting in more Vav-1 phosphorylation and creating a positive feed-back loop. Finally, this will lead to the formation of the immunological synapse, full NK cell activation and target cell lysis. Co-engagement of inhibitory receptors will result in SHP-1-mediated de-phosphorylation of Vav-1. This will effectively block actin-dependent clustering of activating receptors, their phosphorylation, formation of the immunological synapse and ultimately control NK cell activation. In support of this hypothesis we could demonstrate that inhibitory receptor engagement can indeed interfere with the phosphorylation of activating receptors and with their recruitment to the immunological synapse and to membrane micro-domains (Endt et al., 2007; Watzl and Long, 2003). We furthermore developed a mathematical model to describe this process (Mesecke et al., 2011) and which also supported our hypothesis.

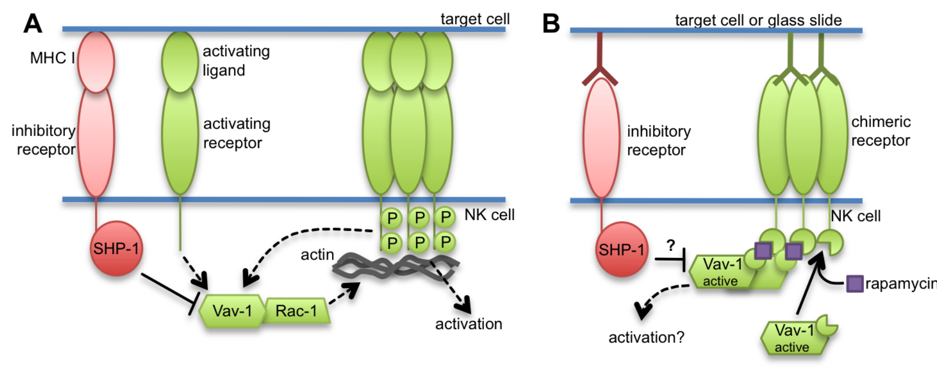

Figure 1
(A) Hypothesis for the integration of positive and negative signals in NK cells at the level of Vav-1. (B) Schematic representation of the experimental system used in WP1. See text for more details.

While these data support our hypothesis, they cannot directly address the question if Vav-1 is the first and only integration point of positive and negative signaling. We therefore wanted to directly test how positive and negative signals are integrated on a molecular level by bypassing the system with membrane recruitable constitutively active signaling molecules (Fig. 1B). This approach is based on the ability of rapamycin to induce protein hetero-dimerization by binding with high affinity to FKBP12 and FRAP through the latter's 11 kDa FRB domain (Castellano and Chavrier, 2000). We constructed a chimeric CD4 membrane receptor that includes an FKBP domain in its cytoplasmic tail and expressed it in the NK cell line NKL, which we had extensively characterized in our previous biochemical and mathematical approaches (Endt et al., 2007; Mesecke et al., 2011). We further generated signaling molecules fused to a FRB domain. These included wild-type and constitutively active mutants of Vav-1, Rac-1, or Cdc42 (Castellano and Chavrier, 2000). These signaling molecules were then co-expressed with the FKBP containing chimeric receptor in the NK cell line NKL. Addition of rapamycin resulted in the recruitment of the signaling molecule to the chimeric receptor (Fig. 2).

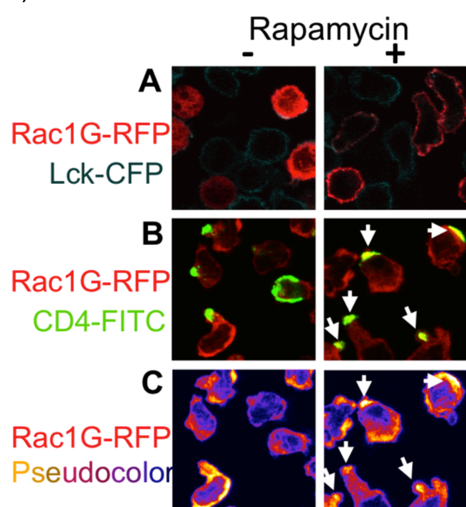

Figure 2
Inducible recruitment of Rac1G to the membrane. (A) In the absence of Rapamycin (left panels) Rac1G is evenly distributed in the cytosol of the transfected NKL cells. Addition of Rapamycin (right panels) induces the recruitment to the Lck-CFP plasma membrane anchor. (B, C) To enable recruitment to sub-regions of the plasma membrane, we utilized a chimeric CD4 receptor (green), which was clustered by antibody-mediated cross-linking. Addition of Rapamycin (right panels) then induces recruitment of Rac1G (shown in pseudocolors for better visibility in C) to the clustered CD4 receptor (see arrows).

Unfortunately, we encountered some technical difficulties in this part of the project. The expression of the FKBP containing chimeric receptor was not stable in the NKL cells. We tried other cell lines such as NK92 cells, however, also there the expression of the chimeric receptor was lost over time. This made the co-transfection with the signaling molecules challenging, as the expression of the chimeric receptor was often lost or too low once we had selected the transfected cells for the expression of the FRB-containing signaling molecules. Therefore, it took much longer than expected to generate and to analyze the transfected cells described in the application. We used three different FKBP-containing molecules. First, a membrane anchor, where FKBP was fused to an SH4 domain, targeting the construct to the inner leaflet of the plasma membrane. Then, a chimeric CD4 receptor where FKBP was replacing the intracellular part of CD4, and finally a chimeric antigen receptor. This receptor was constructed of a single-chain antibody recognizing CD19 (obtained from Dr. W. Wels, Georg Speyer Haus, Frankfurt), which was fused to a transmembrane region and an intracellular FKBP domain. Upon analysis of the double transfected cells we could demonstrate that the

hetero-dimerization system worked as expected. Addition of Rapamycin induced the recruitment of the FRB-tagged signaling molecule to the FKBP containing chimeric molecule. However, recruitment of constitutively active Vav-1 or Rac-1 to the membrane or to a (clustered) transmembrane receptor did not result in detectable NK cell activation (measured by CD107a externalization, Ca2+ flux, actin reorganization or MAPK phosphorylation). Therefore, we speculated that additional signals were necessary for full NK cell activation. To test this, we used NKL cells expressing the anti-CD19 chimeric antigen receptor. We observed some clustering of the receptor upon contact with CD19-expressing target cells (such as 721.221). Addition of rapamycin induced the recruitment of FRB-containing signaling molecules. However, this recruitment had no influence on the NK cell activation and the killing of the CD19-expressing target cells. There are several possible explanations for this unexpected result. (1) With our retroviral transduction method of human NK cell lines we routinely only obtain a few percent of cells expressing the transduced gene. This requires the selection of stably transfected cells in order to have a sufficient number of cells for our analysis. In case of cells expressing constitutively active mutants of Vav-1 or Rac-1 the selection may have resulted in adaptations, which counteracted the activity of the signaling molecules. We are currently working on different technologies for the genetic modification of NK cells in order to get higher transfection efficiencies, thereby allowing for transient transfections. (2) While we did saw some clustering of the chimeric anti-CD19 receptor upon target cell contact, this clustering was not very efficient. Therefore, we may not have reached sufficiently high local concentrations of the recruited signaling molecules to observe an effect. (3) The simple recruitment of constitutive active Vav-1 or Rac-1 to the immunological synapse is not sufficient to induce detectable levels of NK cell activation. While this explanation would be scientifically interesting, as it would point to other important signaling events at the immunological synapse, we would first need to exclude the other possible explanations. While we worked on improving our technology, we could not solve these problems within the funding period of the project. As we could not induce detectable NK cell activation via our approach, we could not test if and how signals of inhibitory receptors could interfere with this activation as we had initially planned.

WP2: What is the minimal system to re-create the NK cell signaling network?
In WP2 we followed an alternative approach to investigate the signaling pathways of activating and inhibitory NK cell receptors. By rebuilding the receptor proximal signaling pathways in a synthetic biology approach, we aimed to get a detailed understanding about the signaling requirements for the different NK cell receptors and to identify critical components. Therefore, we used Drosophila S2 cells in which many foreign proteins can be simultaneously expressed. Using this system we could successfully rebuild a signaling pathway from an ITAM (Immunoreceptor Tyrosine-based Activation Motif)-containing receptor to the phosphorylation of the adapter protein SLP-76. In short, we could show that SLP-76 is directly phosphorylated at tyrosines Y113 and Y128 by the kinase SYK in the presence of ITAM-containing adapters such as CD3ζ, DAP12 or FcεRγ. This phosphorylation was dependent on at least one functional ITAM and a functional SH2-domain within SYK. Inhibition of Src-kinases by inhibitors PP1 and PP2 did not reduce SLP-76 phosphorylation in S2 cells, suggesting an ITAM and SYK dependent, but Src-kinase independent signaling pathway. This direct ITAM-SYK-SLP-76 signaling pathway therefore differs from previously described ITAM signaling. However, the SYK family kinase ZAP70 required the additional co-expression of the Src-family kinases Fyn or Lck to efficiently phosphorylate SLP-76 in S2 cells. This difference in Src-family kinase dependency of SYK versus ZAP70-mediated ITAM-based signaling was further demonstrated in human lymphocytes. ITAM signaling in ZAP70 expressing T cells was dependent on the activity of Src-family kinases. In contrast, Src-family kinases were partially dispensable for ITAM signaling in SYK expressing B cells or in NK cells, which express SYK and ZAP70. This demonstrates that SYK can signal using a Src-kinase independent ITAM-based signaling pathway, which may be involved in calibrating the threshold for lymphocyte activation. As this work has been successfully published in a leading immunological open access journal, we refer to this publication for more details (Fasbender et al., 2017).

During the course of our investigation we also had to realize the limitations of the S2-cell system. When trying to rebuild the 2B4-signaling pathway we found that the simple co-expression of the 2B4 receptor together with a Src-family kinase such as Fyn resulted in full phosphorylation of the 2B4 receptor. This was independent of any receptor cross-linking or the expression of adapters such as SAP or EAT-2. Also the co-expression of CD45 and Csk, which regulate the activity of Src-family kinases, could not prevent the constitutive phosphorylation of 2B4. One explanation for this could be the simple overexpression of the transfected proteins in the S2 cells. However, the surface expression of 2B4 was comparable to the one found on NK cells. An alternative explanation is that in NK cells the membrane organization or other proteins shield the tyrosines within the cytoplasmic tail of 2B4 from kinases. Only engagement of the receptor releases this shielding and allows access of kinases to the receptor. While this would be an interesting and novel regulatory mechanism, we could not formally prove this hypothesis during the funding period.

WP3: How much activating ligand is necessary for NK cell stimulation and regulation and what are the spatial constraints?

Many studies have identified different ligands for activating NK cell receptors on tumor tissues or on virally infected cells. However, in most cases it is unclear if these ligands will be sufficient to stimulate NK cell reactivity. In order to understand, predict and also to manipulate NK cell responses against infected or transformed cells we aimed to get a quantitative understanding about how much ligand is necessary to efficiently stimulate NK cells. Therefore, we used a reductionist approach using microstructured protein arrays (Sacca and Niemeyer, 2011) to address this essential question. The controlled lateral separation of active biomolecules by microstructured protein arrays has been proven to be a powerful tool to investigate ligand-induced lateral clustering processes at the molecular level. In collaboration with Prof. Christof Niemeyer (KIT Karlsruhe) we used the DDI (DNA-directed immobilization) technology to produce surface-bound arrays of proteins. The Niemeyer group developed the respective techniques to control both density and localization of ligands on solid surfaces by taking advantage of oligonucleotide capture arrays, produced with sub-micrometer resolution by dip-pen nanolithography. These arrays were then functionalized by means of the robust and highly modular DDI system. Using this technology we immobilized antibodies directed against different activating NK cell receptors on glass slides. Incubation of NK cells on these arrays induced NK cell activation as determined by degranulation (CD107a externalization), Ca^{2+} flux and the production of cytokines. We established technologies to titrate the amount of different ligands on the protein array and observed that this influences NK cell reactivity in a quantitative manner. As these results have been published (Garrecht et al., 2016), we refer to the publication for more details.

While we could quantify the amount of ligands on the DDI protein arrays, it was difficult to directly relate this to the reactivity of the NK cells. Therefore, we decided to develop a three dimensional system of artificial target cells using cell sized (10 μm) latex beads. These beads were loaded with different amounts of antibodies directed against activating NK cell receptors. We could quantify the exact amount of antibodies on the surface of the beads. We then incubated human NK cells with these different beads and determined their degranulation by measuring the amount of CD107a externalization. This analysis showed a clear dose-dependent response of the NK cells, which differed depending on which activating receptor was engaged (Fig. 3). For engagement of NKp44 or CD16 less than 10.000 antibody epitopes per bead were sufficient to induce half-maximal NK cell activation. For NKp46 about 10.000 antibody epitopes and for NKp30 or NKG2D about 15.000 antibody epitopes were necessary for half-maximal NK cell activation, whereas 2B4 required 65.000 antibody epitopes to induce half-maximal NK cell activation. This clearly demonstrates that activating NK cell receptors differ in their ability to stimulate NK cells. More details of these experiments can be found in the following master thesis: “Quantitative Untersuchungen zur Reaktivität von NK Zellen mithilfe von Antikörper beladenen Latex Beads”, Masterarbeit an der Fakultät für Chemie und Chemische Biologie der TU Dortmund, 2016.

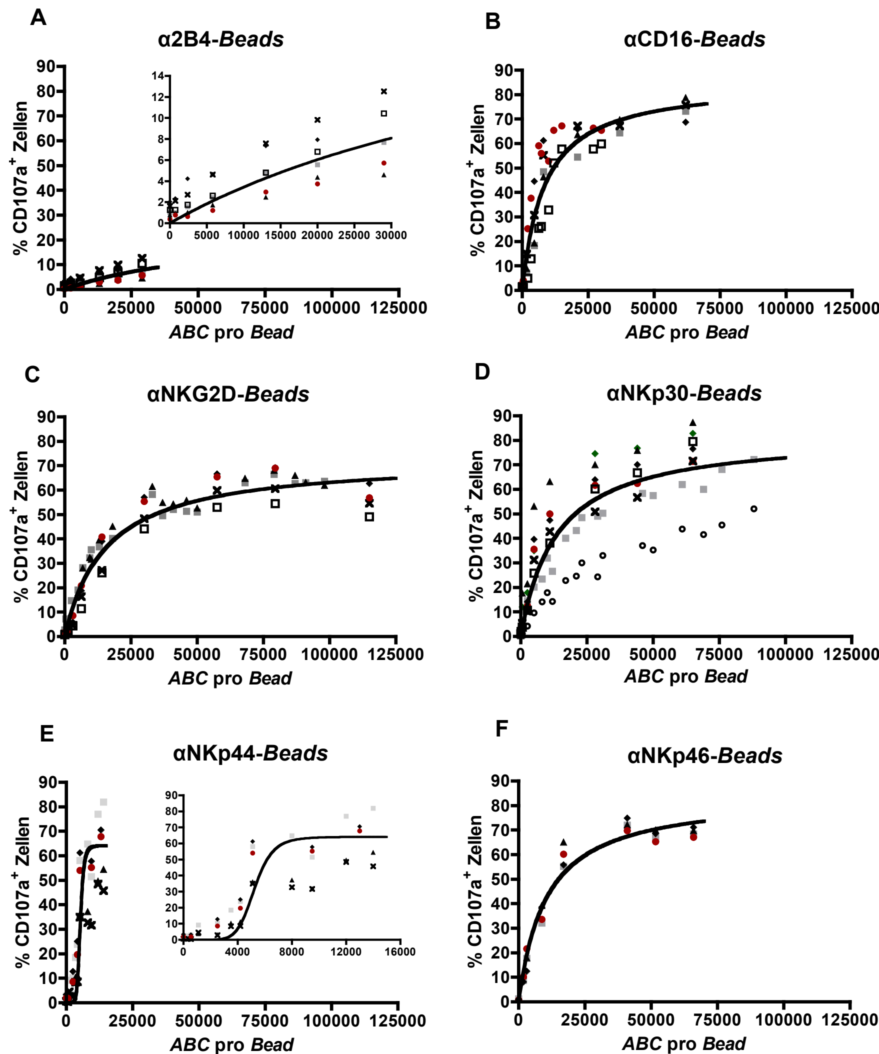

Figure 3: Degranulation of cultured human NK cells (every symbol represents cells of a different donor) in response to α2B4-, αCD16-, αNKG2D-, αNKp30-, αNKp44-, or αNKp46-loaded beads. (A-D+F) The response curve for every donor was fitted as a hyperbolic function to the following formula: ($y = B_{max} \cdot \frac{x}{K_D+x}$). A medium response curve was determined from the response curves of the different donors. (E) The response curve for every donor was fitted to the following formula and a medium curve from all response curves was determined ($y = \frac{B_{max}}{(1+\left(\frac{x}{K_D}\right)^c)}$).$y = \frac{B_{max}}{(1+\left(\frac{x}{K_D}\right)^c)}$

When working with the DDI arrays, we noticed that NK cells preferentially accumulated in areas where their activating receptors were stimulated (Fig. 4A, B). This was not simply caused by antibody-antigen interactions, as antibodies engaging MHC class I did not show this phenotype (Fig. 4C). Therefore, we speculated that the engagement of activating receptors such as CD16, NKp46, NKp44, NKG2D or 2B4 induces a STOP signal in NK cells, similar to what had been shown in T cells (Dustin, 2004). This stop signal likely involves the integrin LFA-1, as we saw even better adherence of the NK cells to the DDI arrays when we co-immobilized the LFA-1 ligand ICAM-1 (Fig. 4D, E).

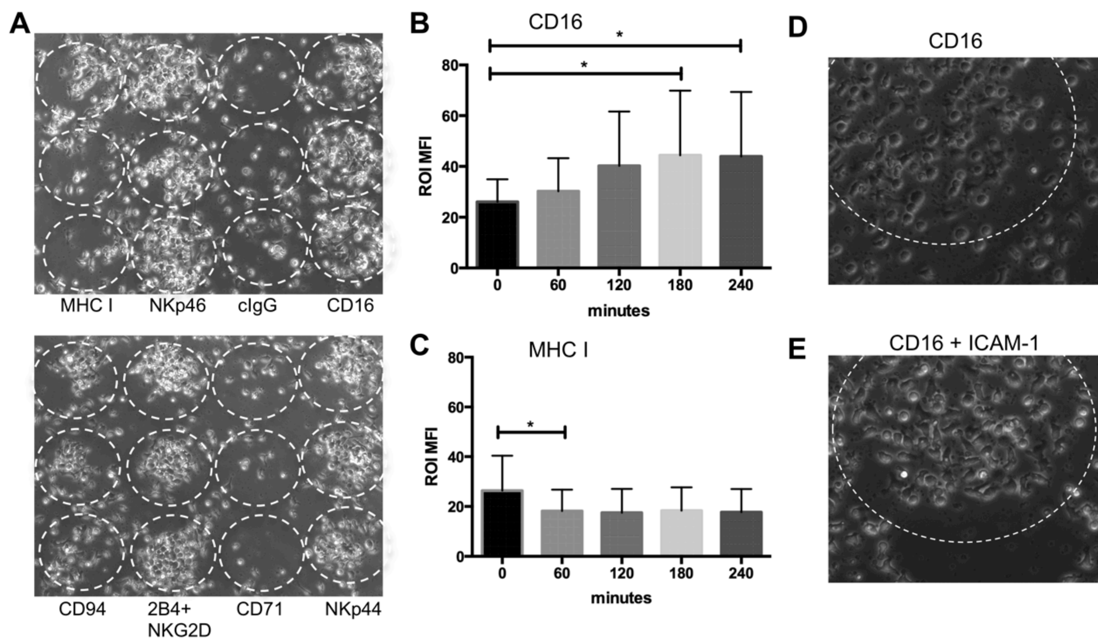

Figure 4: Stop signaling in NK cells. (A) Arrays with constant ratios of 4 different capture oligomers aF1, aF5, aF9 and aF10 were functionalized with different biotinylated antibodies against activating (CD16, NKp46, NKp44, 2B4/NKG2D), inhibitory (CD94, HLA) NK cell receptors, CD71 (binds to transferrin) or MOPC21 (non-binding) control antibody. The accumulation of cultured NK cells was visualized after 3 hours incubation time with fluorenscence microscopy (20x magnification). The areas where the antibodies were immobilized are indicated by circles. NK cells preferentially accumulate on spots where antibodies against activating NK cell receptors were present (NKp46, CD16, 2B4+NKG2D, NKp44). (B, C) quantification of NK cell accumulation on anti-CD16 and anti-MHC-1 spots. (D, E) flattening and tight adherence of NK cells on spots where ICAM-1 was co-immobilized together with antibodies against CD16.

During the funding period our collaboration partner Prof. Niemeyer advanced the development of the DDI technology to incorporate DNA-origami structures in order to manipulate the organization of protein ligands on a nanometer scale (Angelin et al., 2015). We made use of this new technology in our project to investigate how the nanoscale organization of ligands for activating NK cell receptors influences NK cell activation. We used different DNA origami structures to which we could immobilize 4, 5, 8 or 12 antibodies against CD16 (Fig. 5A, B). The origami nanostructures were designed to immobilize the antibodies either in a close cluster or spaced further apart. When incubating cultured human NK cells in these arrays, we observed a dose-dependent NK cell activation as determined by degranulation (CD107a externalization). However, we also observed that the same amount of antibodies was more potent in activating NK cells when they were presented in a clustered organization (Fig. 5C). Interestingly, when using freshly isolated NK cells we observed the opposite effect where antibodies presented in a clustered organization were less effective in stimulating NK cells compared to the same amount of antibodies presented in a more spaced arrangement (Fig. 5D). This clearly indicates that the nanocale organization of ligands for activating NK cell receptors has an impact on receptor engagement and subsequent NK cell activation.
We are continuing to investigate the interesting finding of the STOP signaling and the relevance of the nanoscale organization of ligands for activating NK cell receptors for NK cell activation. The results shown above will be the basis for an application for DFG funding to continue this exciting project. As our investigations are mostly exploring a basic understanding of NK cell regulation, the results of this project are most likely not of immediate economic relevance.

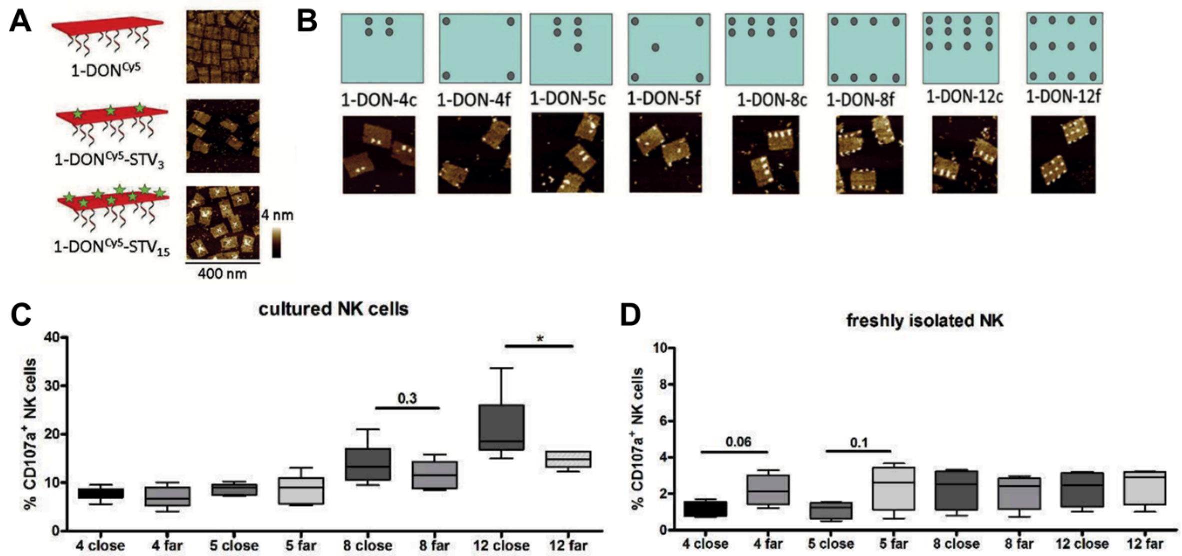

Figure 5: Nanostructure dependent strength of NK cell activation. (A) Schematic illustration of DON designs with respective AFM images. (B) AFM images (250x250 nm^2 scans) of nanostructural features of MOSAIC Cy3-labeled 1-DON constructs with different amounts and arrangements of streptavidin for the immobilization of biotinylated antibodies. (C, D) Degranulation of (C) cultured or (D) freshly isolated human NK cells stimulated with αCD16-Biotin antibodies immobilized on the indicated DNA origamis. The results (means and SD) of 6 (cultured NK cells) and 4 (freshly isolated NK cells) experiments are shown.

Publications

The following thesis were published within the framework of this project:

Master thesis:

- “DNA-vermittelte Immobilisierung von Proteinen auf funktionalisierten Oberflächen", Masterarbeit, Karlsruher Institut für Technologie, September 2014.
- “Quantitative Untersuchungen zur Reaktivität von NK Zellen mithilfe von Antikörper beladenen Latex Beads”, Masterarbeit an der Fakultät für Chemie und Chemische Biologie der TU Dortmund, 2016.

PhD thesis:

- „NK cell regulation“, Dissertation, Fakultät für Chemie und Chemische Biologie der TU Dortmund, 2017
- “Herstellung und Anwendungen von DNA-strukturierten Oberflächen", geplante Dissertation, Karlsruher Institut für Technologie, 2018

The following publications were published as a result of this project and the SAW funding is cited in the acknowledgements:

Fasbender, F., Claus, M., Wingert, S., Sandusky, M., and Watzl, C. (2017). Differential Requirements for Src-Family Kinases in SYK or ZAP70-Mediated SLP-76 Phosphorylation in Lymphocytes. Frontiers in Immunology 8.

Urlaub, D., Hofer, K., Muller, M.L., and Watzl, C. (2017). LFA-1 Activation in NK Cells and Their Subsets: Influence of Receptors, Maturation, and Cytokine Stimulation. J Immunol 198, 1944-1951.

Claus, M., Dychus, N., Ebel, M., Damaschke, J., Maydych, V., Wolf, O.T., Kleinsorge, T., and Watzl, C. (2016). Measuring the immune system: a comprehensive approach for the analysis of immune functions in humans. Arch Toxicol 90, 2481-2495.

Garrecht, R., Meyer, R., Duppach, J., Reipschlager, S., Watzl, C., and Niemeyer, C.M. (2016). Designed DNA Surfaces for in Vitro Modulation of Natural Killer Cells. Chembiochem 17, 486-492.

Merkt, W., Claus, M., Blank, N., Hundemer, M., Cerwenka, A., Lorenz, H.M., and Watzl, C. (2016). Active but not inactive granulomatosis with polyangiitis is associated with decreased and phenotypically and functionally altered CD56(dim) natural killer cells. Arthritis Res Ther 18, 204.

Merkt, W., Lorenz, H.M., and Watzl, C. (2016). Rituximab induces phenotypical and functional changes of NK cells in a non-malignant experimental setting. Arthritis Res Ther 18, 206.

Urlaub, D., Bhat, R., Messmer, B., and Watzl, C. (2016). Co-Activation of Cultured Human Natural Killer Cells: Enhanced Function and Decreased Inhibition. J Toxicol Environ Health A 79, 1078-1084.

Watzl, C. (2014). How to trigger a killer: modulation of natural killer cell reactivity on many levels. Adv Immunol 124, 137-170.

Watzl, C., Urlaub, D., Fasbender, F., and Claus, M. (2014). Natural killer cell regulation - beyond the receptors. F1000Prime Rep 6, 87.

References

Andoniou, C.E., Coudert, J.D., and Degli-Esposti, M.A. (2008). Killers and beyond: NK-cell-mediated control of immune responses. Eur J Immunol *38*, 2938-2942.

Angelin, A., Weigel, S., Garrecht, R., Meyer, R., Bauer, J., Kumar, R.K., Hirtz, M., and Niemeyer, C.M. (2015). Multiscale Origami Structures as Interface for Cells. Angew Chem Int Ed Engl *54*, 15813-15817.

Castellano, F., and Chavrier, P. (2000). Inducible membrane recruitment of small GTP-binding proteins by rapamycin-based system in living cells. Methods Enzymol *325*, 285-295.

Colonna, M., Jonjic, S., and Watzl, C. (2011). Natural killer cells: fighting viruses and much more. Nature Immunology *12*, 107-110.

Dustin, M.L. (2004). Stop and go traffic to tune T cell responses. Immunity *21*, 305-314.

Endt, J., McCann, F.E., Almeida, C.R., Urlaub, D., Leung, R., Pende, D., Davis, D.M., and Watzl, C. (2007). Inhibitory receptor signals suppress ligation-induced recruitment of NKG2D to GM1-rich membrane domains at the human NK cell immune synapse. J Immunol *178*, 5606-5611.

Fasbender, F., Claus, M., Wingert, S., Sandusky, M., and Watzl, C. (2017). Differential Requirements for Src-Family Kinases in SYK or ZAP70-Mediated SLP-76 Phosphorylation in Lymphocytes. Frontiers in Immunology *8*.

Garrecht, R., Meyer, R., Duppach, J., Reipschlager, S., Watzl, C., and Niemeyer, C.M. (2016). Designed DNA Surfaces for in Vitro Modulation of Natural Killer Cells. Chembiochem *17*, 486-492.

Imai, K., Matsuyama, S., Miyake, S., Suga, K., and Nakachi, K. (2000). Natural cytotoxic activity of peripheral-blood lymphocytes and cancer incidence: an 11-year follow-up study of a general population. Lancet *356*, 1795-1799.

Mesecke, S., Urlaub, D., Busch, H., Eils, R., and Watzl, C. (2011). Integration of activating and inhibitory receptor signaling by regulated phosphorylation of Vav1 in immune cells. Sci Signal *4*, ra36.

Orange, J.S. (2006). Human natural killer cell deficiencies. Curr Opin Allergy Clin Immunol *6*, 399-409.

Sacca, B., and Niemeyer, C.M. (2011). Functionalization of DNA nanostructures with proteins. Chem Soc Rev *40*, 5910-5921.

Stebbins, C.C., Watzl, C., Billadeau, D.D., Leibson, P.J., Burshtyn, D.N., and Long, E.O. (2003). Vav1 dephosphorylation by the tyrosine phosphatase SHP-1 as a mechanism for inhibition of cellular cytotoxicity. Mol Cell Biol *23*, 6291-6299.

Vivier, E., Tomasello, E., Baratin, M., Walzer, T., and Ugolini, S. (2008). Functions of natural killer cells. Nat Immunol *9*, 503-510.

Watzl, C., and Long, E.O. (2003). Natural killer cell inhibitory receptors block actin cytoskeleton-dependent recruitment of 2B4 (CD244) to lipid rafts. J Exp Med *197*, 77-85.

Watzl, C., and Long, E.O. (2010). Signal transduction during activation and inhibition of natural killer cells. Curr Protoc Immunol *Chapter 11*, Unit 11 19B.