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Proteome Analysis of Distinct Developmental Stages of Human Natural Killer Cells

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Running title: Proteome of distinct $CD56^+$ NK cell subsets

ABBREVIATIONS

CD56	NK cell marker, Neural Cell adhesion molecule (NCAM 1)
CD57	Senescence marker in T and NK cells (HNK-1 or Leu-7)
CMV	Cytomegalovirus
CPDA-1	Anti-coagulant , containing citric acid, sodium citrate, monobasic sodium phosphate and dextrose
CTLs	Cytotoxic T lymphocytes
FDR	False Discovery Rate
HLA	Self-Human Leukocyte Antigen
HSC	Hematopoietic Stem Cell
iTRAQ™	isobaric tags for relative and absolute quantification in mass spectrometry
KIR	Killer immunoglobulin-related receptors in NK cells
LC-MS/MS	Liquid-chromatography coupled with peptide sequencing (mass spectrometry)
MAD	Median Absolute Deviation from the median
NK	Natural Killer
NKIS	NK cell immune synapse
RF	Regulation factor

SUMMARY

The recent Natural Killer (NK) cell maturation model postulates that $CD34^{+}$ hematopoietic stem cells (HSC) first develop into $CD56^{bright}$ NK cells, then into $CD56^{dim}CD57^{-}$ and finally into terminally maturated $CD56^{dim}CD57^{+}$. The molecular mechanisms of human NK cell differentiation and maturation however are incompletely characterized. Here we present a proteome analysis of distinct developmental stages of human primary NK cells, isolated from healthy human blood donors. Peptide sequencing was used to comparatively analyze $CD56^{bright}$ NK cells *versus* $CD56^{dim}$ NK cells and $CD56^{dim}CD57^{-}$ NK cells *versus* $CD56^{dim}CD57^{+}$ NK cells and revealed distinct protein signatures for all of these subsets. Quantitative data for about 3400 proteins were obtained and support the current differentiation model. Furthermore, 11 donor-independently, but developmental stage specifically regulated proteins so far un-described in NK cells were revealed, which may contribute to NK cell development and may elucidate a molecular source for NK cell effector functions.

Among those proteins, S100A4 (Calvasculin) and S100A6 (Calcyclin) were selected to study their dynamic sub-cellular localization. Upon activation of human primary NK cells, both proteins are recruited into the immune synapse (NKIS), where they co-localize with Myosin IIa.

INTRODUCTION

Natural Killer (NK) cells are large granular lymphocytes that provide a first innate immune defense. They are able to kill virus-infected and transformed cells and furthermore release cytokines and chemokines to activate adaptive immune cells [1, 2].

The balance of signals from activating and inhibitory NK cell surface receptors tightly regulates NK cell activity. Activated NK cells release lytic granules through a process called degranulation. Therefore, NK cell cytotoxicity requires the formation of the F-actin-rich NKIS and the transport of Perforin-containing lytic granules to the NKIS. Furthermore, this process requires granule-associated MYH9 protein (non-muscle Myosin IIa) mediating the interaction of granules with F-actin at the NKIS [3–5], leading to lytic granule exocytosis. Whereas related phenotypes and functional properties are well characterized, the underlying regulatory protein network mediating differentiation, cytokine release and cytotoxicity, is still incomplete.

NK cells are defined by the expression of the surface molecule CD56 (NCAM1) and the absence of the T cell receptor (TCR) associated protein CD3 and can be further subdivided into subsets [6, 7]. CD56 expressing cells originate from $CD34^+$ HSCs. Notably, the commitment to the NK lineage includes discrete steps from HSC to cells, expressing high CD56 levels ($CD56^{bright}$) [8, 9], which act immune regulatory by the release of various cytokines. NK cells with low CD56-expression ($CD56^{dim}$) predominantly constitute cytotoxic responses [10, 11]. Contact of CD56 (NCAM1) with fibroblasts [12] and neutrophils [13] supports the differentiation process from $CD56^{bright}$ to $CD56^{dim}$ NK cells. The progression of early differentiation steps is proven by telomere length investigation [14] and early presence in blood after HSC transplantation (HSCT) [14, 15]. Indeed, $CD56^{dim}$ NK cells are able to change their phenotypic properties, which can be correlated with continued differentiation throughout their whole lifespan [15–18]. CD57 was determined to be a senescence marker in T cells [19]. Recent studies determined $CD57^+$ NK cells as a fully mature NK cell subset among the $CD56^{dim}$ NK cell population ($CD56^{dim}CD57^+$ and $CD56^{dim}CD57^-$).

Furthermore, the NK cell differentiation process is characterized by a reversible loss of NKG2A in parallel with an irreversible acquisition of KIRs and CD57 [15].

Furthermore, $CD57^+$ NK cells are characterized by a specialized phenotype that includes increased CD16- and Perforin-expression, reduced responsiveness to cytokines and decreased proliferation capacity. CD57 is mostly studied in the context of NK cell education that runs in parallel but un-coupled from NK cell differentiation [15, 17, 18]. The NK cell education process encompasses the acquisition of activating and inhibitory surface receptors, like KIRs, which in turn interact with HLA class I ligands, e.g. during viral infections [19, 20]. $CD57^+$ NK cells can recognize Cytomegalovirus (CMV) and developed memory effects towards this virus [21, 22]. Likewise, an expansion of the $CD57^+$ NK cell population is observed during Hantavirus [24], Chikungunya virus [25] and HIV-1 [16], but not during HSV-2 [26] infections. Recently, Lanier and colleagues showed that IL-12 is indispensable for NK cell expansion and the generation of memory NK cells during MCMV infection [27]. Up to now the molecular network underlying $CD57^+$ phenotypes are mostly characterized by FACS techniques and microarray analyses on the systemic level [15, 17, 18].

Mass spectrometry can identify and quantify proteins in a global and unbiased manner, and thereby certainly contribute to the elucidation of developmental processes and the acquisition of specialized functions. Proteomic studies on human NK cells are accomplished mainly at the level of NK cell lines, due to the scarcity of primary NK cell subsets. Cytotoxicity, but not development, was studied in NK-92 and YTS NK cells by 2D-PAGE and peptide sequencing approaches pending on activating signals [28–32]. At least one proteome study investigates *ex vivo* expanded primary human NK cells, and focuses on the characterization of kinases, involved in NK cell activation [33]. Attempts to unravel the basics of NK cell development in mice were successful [34] but not completely transferable to the human NK cell system due to a different set of surface receptors. Hence, several studies have contributed to our understanding of the role of

surface receptors in different developmental stages, but studies targeting the regulation of intracellular proteins are still missing.

In this study we characterized distinct developmental stages of primary human NK cells by proteomics. To get better insight into the molecular mechanisms of the NK cell differentiation process we comparatively analyzed freshly isolated primary $CD56^{bright}$, $CD56^{dim}$, $CD56^{dim}CD57^{-}$ and $CD56^{dim}CD57^{+}$ NK cells by iTRAQ™-based LC-MS/MS. We obtained relative quantitative data for more than 3400 proteins and observed a specific $CD56^{+}$ NK cell core proteome. The obtained proteomic data strongly supports the current differentiation model of NK cells by highlighting strong distinctions between $CD56^{bright}$ and $CD56^{dim}$ NK cells and high similarity among $CD57^{-}$ and $CD57^{+}$ NK cells. In addition to a significant set of anticipated and well-known proteins involved in NK cell development and effector functions, we detected also 11 novel protein candidates so far un-described in NK cells. The expression patterns of S100A4 (UniProt accession name S10A4) and S100A6 (UniProt accession name S10A6), both belong to the family of S100 calcium binding proteins and contain two EF-hand domains, correlated with the developmental stages of cytotoxic NK cell subsets. Both proteins were recruited into the NKIS, following NK cell activation.

EXPERIMENTAL PROCEDURES

Monoclonal antibodies and reagents

For fluorescence-activated cell sorting (FACS), anti-CD56 (clone AF12-7H3, mouse IgG1, Miltenyi Biotec), anti-CD3 (clone HIT3a, mouse IgG1 κ, BD Bioscience) and anti-CD57 (clone TB03, mouse IgM, Miltenyi Biotec) mouse monoclonal antibodies (mAbs) were used. The following reagents were used: sodium chloride, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and Triton X-100 (Sigma), Mini Complete Protease Inhibitor Cocktail Tablets (Roche), Benzonase (Merck), Trypsin (Promega) and the iTRAQ™ Reagent Multiplex Kit (Applied Biosystems). Organic solvents, such as ethanol, methanol, and acetonitrile (ACN), were

obtained from J. T. Baker Inc. For co-localization studies: anti-Perforin (mouse, deltaG9, BD Pharmingen); anti-CD107a (mouse, H4A3, BD Pharmingen); anti-CD107a (rabbit, 24170, Abcam); Myosin IIa (non-muscle, rabbit, Sigma Aldrich); S10A4 (directed against S100A4; mouse, NJ4F3, Abcam) and S10A6 (directed against S100A6; mouse, CACY-100, GeneTex) were used.

Human NK cells

This study was conducted in accordance with the rules of the Regional Ethics Committee of Lower Saxony, Germany and the declaration of Helsinki. Buffy coats from blood donations of healthy human volunteers who provided informed consent were obtained from the Institute for Clinical Transfusion Medicine, Klinikum Braunschweig, Germany. Blood donors' health is rigorously checked before being admitted for blood donation. This process included a national standardized questionnaire with health questions, an interview with a medical doctor and standardized lab tests for a) infections HIV1/2, HBV, HCV, Syphilis (serology and/or nucleic acid testing) and b) hematological cell counts.

Buffy Coats were produced from whole blood donations on day 1 by using the Top & Bottom Extraction Bag System (Polymed Medical Devices™, Triple Blood Bag System, No. 7300; containing CPDA-1. Peripheral blood mononuclear cells (PBMCs) were isolated from these buffy coat products by Biocoll density gradient centrifugation (Biochrome AG) on day 2. PBMCs were cultured overnight in RPMI 1640 medium (GIBCO) supplemented with 10% fetal bovine serum (FBS) gold (PAA Laboratories), 2 mM L-glutamine, 50 units/ml penicillin and 50 µg/ml streptomycin (all Invitrogen) at 37°C in a humide 7.5% CO_2 atmosphere.

FACS sorting

PBMCs were incubated with fluorochrome-conjugated anti-CD3, anti-CD56 and anti-CD57 monoclonal antibodies (mAbs) for 15 minutes at 4°C on day 3. $CD3^-CD56^{dim}$ and $CD3^-CD56^{bright}$

NK cells as well as $CD3^-CD56^{dim}CD57^+$ and $CD3^-CD56^{dim}CD57^-$ NK cells were isolated by FACS using a FACSAria II flow cytometer (BD Biosciences; Bionozzle size: 70 µm; system pressure: 70 PSI; flow rate 30,000 events/sec; laser: 561 nm with 50 mWatt for PE; 640 nm with 60 mWatt for APC; detection: APC 670/14, PE 585/15; 488 nm with 100 mW for FITC; detection with bandpass filters for PE 585/15, APC 670/15 and FITC 525/50). The purity and viability of the NK cell subsets were assessed by flow cytometry. In all experiments, the purity of the isolated NK cell subsets ($CD3^-CD56^{bright}$/$CD56^{dim}$; $CD3^-CD56^{dim}CD57^+$/$CD57^-$) was higher than 96 %.

Cell lysis, protein digestion and iTRAQ™ modification of peptides

$CD3^-CD56^{dim}$/ $CD3^-CD56^{bright}$ ($CD56^{dim}$ *versus* $CD56^{bright}$) and $CD56^{dim}CD57^+$/ $CD56^{dim}CD57^-$ ($CD57^+$ *versus* $CD57^-$) NK cells were each isolated from five individual human donors and mass spectrometry data were obtained and processed for each of the 10 donors individually. In minimum, 0.9 $x10^6$ NK cells were used for each MS experiment. NK cells were lysed in ice-cold lysis buffer (50 mM HEPES pH 7.5, 150 mM NaCl, protease inhibitor cocktail supplemented with EDTA, 1% Triton-X100) on day 3. Protein concentrations were determined using a NanoDrop spectrophotometer (ND-1000, Peqlab, Biotechnology GmbH, Erlangen). Proteins were extracted from lysates by chloroform/methanol precipitation as described previously [35], and re-dissolved in dissolution buffer (50mM TEAB). Equal amounts of protein from each NK cell subset was digested with sequencing grade modified trypsin from Promega, as recommended in a ratio of 1:50 at 37 °C on day 3 overnight. Subsequently, digestion was completed by adding a further 1 µg of trypsin for 2 h to each sample. Labeling of tryptic peptides with isobaric iTRAQ™ reagents was performed on day 4, according to the manufacturer's guidelines (Applied Biosystems). Peptides derived from $CD56^{dim}$ NK cells were labeled with iTRAQ™ reagent 117, those derived from $CD56^{bright}$ NK cells were labeled with iTRAQ™ reagent 115 Da. For quantitative MS analyses of peptides from $CD3^-CD56^{dim}CD57^+$ ($CD57^+$) NK cells, iTRAQ™ label 114 was used, and iTRAQ™ 116 for $CD3^-CD56^{dim}CD57^-$ ($CD57^-$) NK cell peptides (SUPPLEMENTAL TABLE 1;

SUPPLEMENT). Peptides of two different NK cell subsets ($CD56^{bright}$ and $CD56^{dim}$ or $CD57^{-}$ and $CD57^{+}$ NK cells) were combined (1:1 ratio), vacuum dried, dissolved in 0.2% TFA and desalted on self-packed LiChroprep RP-18 (Merck) SPE columns.

Strong cation exchange chromatography (SCX)

The combined iTRAQ™-labeled peptide samples were further sub-fractionated by Strong Cation Exchange Chromatography (SCX) on day 4 to support representative and comprehensive protein identification by LC-MS/MS. Peptides were dissolved in SCX buffer (0.065% formic acid, 25% ACN) and fractionated on a Mono SPC1.6/5 column connected to an Ettan micro-LC system (both GE Healthcare), and separated at a flow rate of 150µl/min for 30 min with a linear gradient from 0% to 35% SCX buffer supplemented with 0.5M potassium chloride. Fractions were collected by a microfraction collector, every minute (SunCollect). Peptide elution was monitored by an UV detector at 214 nm. Peptide-containing fractions were vacuum-dried, desalted by RP-C_{18} chromatography ZipTip pipette tips (Millipore) and analyzed separately by LC-MS/MS.

LC-MS/MS measurement and protein identification

LC-MS/MS analyses were performed with an UltiMate 3000 RSLCnano LC system (Thermo Scientific) connected to an LTQ Orbitrap Velos Fourier transform mass spectrometer (Thermo Scientific). Peptides were applied to a C_{18} pre-column (3-µm, Acclaim, 75 µm x 20 mm, Dionex) and washed with 0.1% TFA for 3 min at a flow rate of6 µl/min. Subsequently, peptides were separated on a C_{18} analytical column (3- µm, Acclaim PepMap RSLC, 75 µm x 25 cm, Dionex) at 350 µl/min *via* a linear 120-min 3.7% - 31.3 % B gradient with UPLC solvent A (0.1% formic acid in water) and UPLC solvent B (0.1% formic acid in 80% ACN). The LC system was operated with Chromeleon Software (version 6.8, Dionex). The effluent from the column was electro-sprayed (Pico Tip Emitter Needles, New Objectives) into the mass spectrometer. The mass spectrometer

was controlled by Xcalibur software (version 2.1, Thermo Scientific) and operated in the data-dependent mode allowing the automatic selection of doubly and triply charged peptides and their subsequent fragmentation. Peptide fragmentation was carried out using High Collision Dissociation (HCD) settings, with Collision Energy (CE) 44 optimized for iTRAQ™-labeled peptides. MS/MS raw data from all SCX fractions, corresponding to one experiment, were visualized by Xcalibur software (.raw-file) and merged for Mascot Daemon-aided searches (Mascot version V2.3.02, Matrix Science) against UniProtKB/Swiss-Prot protein database (release 2011_03; with 525,997 entries; taxonomy: *Homo sapiens* with 20,226 entries). The following search parameters were used: enzyme, trypsin; maximum missed cleavages, 1; fixed modifications, iTRAQ™ 4-plex (K), iTRAQ™ (N terminus), Methylthio (C); variable modifications, oxidation (M); peptide ion mass tolerance, 10 ppm; MS/MS tolerance, 0,2 Da. Mascot result files (.dat files) were uploaded into the software Scaffold (Version Scaffold_3_00_01, Proteome Software Inc.), which was used for inspection of MS/MS data-based identification of peptides/proteins, to support protein quantifications and to prepare statistical validation and comparison of donor specific datasets, respectively. Only proteins with a minimal protein identification probability of 99% and identified by at least two unique peptides showing a minimal peptide identification probability of 95% and a Mascot (peptide) Score of at least 30 were accepted in this survey. Mascot-aided decoy searches were performed against the randomized Uniprot/Swiss-Prot protein database Mascot version V2.3.02, Matrix Science) against UniProtKB/Swiss-Prot protein database (release 2011_03; with 525,997 entries; taxonomy: *Homo sapiens* with 20,226 entries) and protein false discovery rates (FDRs) were calculated by Scaffold on the basis of FDR = FP (false positive) / (FP + TP (true positives)). On average we determined FDRs always lower than 0.9% (with significance level 0.05 in Scaffold). All MS-data associated with this manuscript are published in the PROteomics IDEntification Database (PRIDE) (www.ebi.ac.uk/pride/).

Protein quantification

The Scaffold software (Version Scaffold_3_00_01, Proteome Software Inc.) was used for relative quantification of protein expression in NK cell subsets. Mascot result files (.dat files) were uploaded into Scaffold and all identified proteins as defined by the criteria described above were considered for quantitative data analyses. For quantification, only unique peptides were used. Scaffold provided normalized log_2-regulation factors (RFs), basing on the iTRAQ™-label intensity of individual peptides, respectively. Log_2 regulation factors represent the relative abundance of a protein in $CD56^{dim}$ compared to $CD56^{bright}$ or $CD57^{+}$ compared to $CD57^{-}$ NK cells (RF=$CD56^{dim}$/$CD56^{bright}$; $CD57^{+}$/$CD57^{-}$). Regulation factors calculated by Scaffold were exported to Excel (Microsoft Office 2007) and statistically analyzed.

Statistical data analysis

The aim of the statistical evaluation was to distinguish random fluctuations of protein regulation factors (RF) or potential donor variations from statistically significant and conserved regulations within the NK cell datasets. To identify significant protein regulations the variation of the regulation factors was statistically estimated. In our statistical model we assume that the log_2-regulation factors (RF) of each protein follow a normal distribution (5 x log_2 RFs per protein due to 5 analyzed donors per NK cell subset comparison) with different expected values, but with the same standard deviation σ_0. It is necessary to estimate σ_0 in order to distinguish significant (donor-dependent) deviations from no regulation. The limited number of replicates (n=5 donors in each of both comparative NK subset analyses) required an estimation of the standard deviation by taking RFs of all proteins into account. In our model we used the mean of the MADs

(Median Absolute Deviation from the median) of all proteins as an estimator for the standard deviation of a normal distribution (with a correction for small sample sizes). The MAD was corrected by the factor of 1.4826 (determined by Monte Carlo simulation) according to the number of *k*=5 datasets (donors) to obtain an unbiased and robust estimator for the standard deviation σ_0. In this way we obtain many estimates for the MAD with a large variance. But averaging this larger number of not very reliable estimates leads to a reliable estimate of the MAD.

Based on the previous estimation we are able to construct a hypothesis test for the identification of significantly regulated proteins. Thereby, the hypothesis of the test encompasses that a protein is considered to be significantly regulated if its mean regulation deviates significantly from 0. The function Φ represents the cumulative distribution function of the standard normal distribution. As previously shown, we propose a strict test [36], where *α* is the significance level of the test (here *α*= 5%; before FDR correction) and where the absolute value of the $\log_2$-regulation exceeds a given threshold c_α in at least *m* (*m*=3, 4, 5) out of the *k*=5 replicates. n_m is the number of proteins which have been measured in at least m replicates. We used this value for the FDR correction, since we cannot apply the test to proteins that have been measured in less than *m* replicates. Taken all the parameters together we obtained the value for the threshold as follows:

$$c_\alpha = \sigma_0 \cdot \Phi^{-1}\left(1 - \left(\frac{\alpha}{2 \cdot n_m \cdot \binom{k}{m}}\right)^{\frac{1}{m}}\right)$$

These thresholds (for m=5, m=4 and m=3 donors) were used to define a set of significantly and donor-independently regulated proteins (36 proteins).

Microscopy of fixed NK cells

NK cells were freshly isolated from PBMCs of healthy donors by negative selection using the NK cell Isolation Kit (Miltenyi Biotec). Target cells (K562- leukemia cells)were allowed to settle overnight on poly-L-lysine coated coverslips (Sigma Aldrich). Pure NK cells (≥ 96%), were added at a ratio of 1:1 and incubated for 5, 10, 15, 20 and 30 min, at 37°C and 7.5% CO_2. Cells were fixed for 20 min with 2% Para-formaldehyde (Sigma) in sterile PBS, permeabilized for 3 min in 0.5% Saponin (Sigma) in PBS and 1h blocked with 1% BSA in PBS, supplemented with 0.05% Tween-20 (Roth). Cells were stained [3] with anti-Perforin, anti-CD107a, anti-S100A4 and anti-S100A6 mabs. Secondary antibody staining was performed with goat anti-mouse IgG, conjugated with Alexa 594 and goat anti-rabbit IgG, conjugated with Alexa 488) in 1:400 dilution supplemented with DAPI (1:1000). Imaging was performed on an Axiovert 135 microscope with HBO lamp and CCD camera. Images were analyzed by MetaMorph software, version 7.5.3.0 (Analytical Technologies). Five independent experiments were performed using pure NK cells and 20-30 images were acquired per coverslip in one experiment. Image analysis was performed with ImageJ (version 1.44p).

RESULTS

The proteome of primary human $CD56^+$ NK cells

Comparative proteome studies of two distinct developmental steps, from $CD56^{bright}$ to $CD56^{dim}$ and from $CD57^-$ to $CD57^+$, each from five healthy human blood donors were performed.

Peripheral blood mononuclear cells (PBMCs) were freshly isolated from buffy coats of clinically approved healthy blood donors. These PBMCs were separated to generate the different

developmental stages of primary human $CD56^+$ NK cells. The used gating strategy for distinct $CD56^+$ NK cell subsets is depicted in FIGURE 1A. PBMCs were gated on forward scatter area (FSC) *versus* side scatter area (SSC), which revealed the lymphocyte population. These were further gated on FSC area versus FSC height and SSC area versus SSC height to exclude doublets and aggregates (not shown). $CD3^-CD56^+$ NK cells were gated on $CD56^{dim}$ and $CD56^{bright}$ subsets. The challenge here was to overcome the scarcity of $CD56^{bright}$ NK cells, which account for only 5-10% of all NK cells. Therefore, $6x10^8$ PBMCs were sorted for $CD56^+$ NK cell subsets to obtain about $1x10^6$ $CD56^{bright}$ NK cells from each donor, sufficient material to study the first developmental step from $CD56^{bright}$ to $CD56^{dim}$. Similarly, $CD56^{dim}$ NK cells from further five healthy human blood donors (D6-D10; SUPPLEMENTAL TABLE 1; SUPPLEMENT; SUPPLEMENTAL TABLE 2; SUPPLEMENT) were isolated separately and sub-divided into $CD57^-$ and $CD57^+$ NK cell subsets to study the second developmental step.

$CD56^{bright}$ NK cells were used to normalize (i) the cell numbers of subsets analyzed by proteomics and (ii) the obtained protein regulatory data. Proteins were extracted directly from the freshly sorted primary NK cell subsets and digested with trypsin (FIGURE 1B). Peptides originating from the distinct subsets were then labeled differentially by iTRAQ™, combined and further sub-fractionated by strong cation exchange chromatography (SCX) to reduce ion suppression effects and to improve protein sequence coverage. Fifteen SCX fractions were analyzed for each donor by peptide sequencing (LC-MS/MS). In total, 415203 fragmentation experiments of peptide ions were performed and searched against human entries of the UniProt Database. Considering result tables from all subsets revealed the expression of 3412 proteins in $CD56^+$ primary NK cells. 3345 of those proteins were identified commonly in both comparative proteome approaches (FIGURE 2A). Only 13 proteins were exclusively detected in the $CD56^{dim/bright}$ subsets and 54 in $CD57^{+/-}$ NK cell subsets (SUPPLEMENTAL TABLE 3; SUPPLEMENT). Among these uniquely identified proteins, several signaling components, like UB2Q2 (Ubiquitin-conjugating enzyme E2 Q2; $CD56^{dim/bright}$), PTN9 (Tyrosine-protein

phosphatase non-receptor type 9; $CD57^{+/-}$), P52K (52 kDa repressor of the inhibitor of the protein kinase; $CD57^{+/-}$), SLAP2 (Src-like-adapter 2; $CD57^{+/-}$), RN167 (E3 ubiquitin-protein ligase RNF167; $CD57^{+/-}$), CPPED (Calcineurin-like phosphoesterase domain-containing protein 1; $CD57^{+/-}$) and the C-X-C chemokine receptor type 2 CXCR2 ($CD57^{+/-}$) were detected. These proteins may possibly represent novel markers for the corresponding NK cell subsets, but a multiplicity was identified only in one donor (8 of 13 proteins in $CD56^{dim/bright}$ and 37 of 54 proteins in $CD57^{+/-}$ NK cell subsets, whereas 4 proteins showed RFs above the statistical thresholds, namely GTPB6 (Putative GTP-binding protein 6; $CD56^{dim/bright}$; $Log_2RF=-0.8$); ZN574 (Zinc finger protein 574; $CD57^{+/-}$; $Log^2RF=0.7$); CLN5 (Ceroid-lipofuscinosis neuronal protein 5; $CD57^{+/-}$; $Log^2RF=0.8$) and AMPN (Aminopeptidase N; $CD57^{+/-}$; $Log^2RF=0.8$). All uniquely identified proteins are highlighted with an * in SUPPLEMENTAL TABLE 4 and 5). This minor difference regarding the number of proteins identified by our two-dimensional proteome strategy indicates that NK cell development does not depend on the presence but on the regulation of certain proteins. By evaluating individual donors, the comparison of $CD56^{dim}$ *versus* $CD56^{bright}$ NK cell subsets revealed 2941 common proteins, whereof 40% (1162) could be detected in all investigated donors. Corresponding numbers for the MS analyses of $CD57^{+}$ *versus* $CD57^{-}$ NK cells were 3224 and 1427 (44%) (SUPPLEMENTAL FIGURE 1; SUPPLEMENT). The 2941 identified proteins of the $CD56^{dim}$*versus* $CD56^{bright}$ MS analysis are listed in SUPPLEMENTAL TABLE 4 (Supplemental excel file: CD56dimversusCD56bright.xls) and the detected proteins of the $CD57^{+}$ *versus* $CD57^{-}$ NK cell subset analysis (3224) are summarized in SUPPLEMENTAL TABLE 5 (Supplemental excel file: CD57+versusCD57-.xls), including their Mascot Protein Scores, number of unique peptides, log_2 regulation factors and protein coverage for each individual donor investigation and as median value, including the 5 investigated donors.

All identified proteins were analyzed using GeneGo™, a tool which gives an overview about preferentially covered pathways. This bioinformatics analysis gave directly insight into the functionality of the investigated $CD56^{+}$ NK cell proteomes and revealed predominant expression

of two main biological processes: cytoskeleton re-arrangements and immune response pathways. Thereby, we observed striking similarities in pathway coverage between $CD56^{dim/bright}$ compared to $CD57^{+/-}$ NK cells, as indicated before (FIGURE 2A). Also numerous NK cell signaling pathways were found highly represented by the identified proteins, i.e. CD16 signaling (identified proteins covered 50% of the entire pathway); formation of the immunological synapse (56%) and Granzyme A signaling (86%) (FIGURE 2 B). Hence, the GeneGo™ analysis confirmed a functionally consistent protein set in $CD56^{+}$ NK cells subsets and underlines the presence of a putative core proteome.

Evaluating NK cell specific protein expression

Donor and subset specific peptide samples were labeled differentially with iTRAQ™ reagents for relative quantification in addition to peptide sequencing. Thus, each individual MS fragment ion spectrum provides quantitative information in parallel to the identified peptide sequence. The ratios of iTRAQ™ reporter intensities were used to determine the relative protein abundances in the different $CD56^{+}$ NK cell subsets.1D-LC-MS/MS experiments were performed with 1% of each sample to confirm the completeness of peptide labeling. Next, manual inspection of the quantitative data using Scaffold uncovered a set of well-described NK cell-specific proteins. For example, expression of CD56 (NCAM1) and Perforin (PERF) is fully in accordance with the literature: CD56 expression declines from $CD56^{bright}$ to $CD56^{dim}$ NK cells, whereas Perforin abundance increases simultaneously (FIGURE 3 A and B) [37]. Furthermore, a stable CD56 expression in $CD57^{+}$ and $CD57^{-}$ NK cells was recently described [15]. MS data of this study indeed confirm non-regulated CD56 expression profiles and in parallel revealed a slight increase in Perforin abundance in $CD57^{+}$ NK cells. Thus, abundances of well-described NK cell proteins are in perfect accordance with the literature (SUPPLEMENTAL TABLE 6; SUPPLEMENT) [15–17, 37]. Regarding to NK cell differentiation the level of cytotoxicity related proteins, like Perforin, multiple

Granzymes and LAMP1/3 increases from $CD56^{bright}$ to $CD56^{dim}$ NK cells and induction of Perforin, as well as KIR expression coincides with CD57 appearance [15, 37].

Characterizing donor-specific variations

Although the blood donor volunteers were clinically approved to be healthy, we paid particular attention to determine potential individual variations.

A global inspection of regulatory data from each donor was performed by box plot analysis. Protein regulation factors in assessed donors were symmetric and normally distributed within the box plots, which indicates data robustness and only minor donor variations in both separate comparative analyses of $CD56^{dim/bright}$ and $CD57^{+/-}$ NK cell data. A wide regulation range from +3.8 to -4.0 ($log_2RF=log_2$ regulation factor) was detected in $CD56^{dim/bright}$ NK cells and only minor regulation differences in $CD57^{+/-}$ NK cells (+2.3 to -2.3; FIGURE 4A, right hand panel).

Next, regulatory information were sorted at the protein level and inspected by heat maps (FIGURE 4B). Generally, the expression profiles indicate a remarkable ratio of proteins similarly regulated in each set of five donor samples. As already indicated by the box plot analyses, a higher total number and more pronounced protein regulations were observed in $CD56^{dim/bright}$ NK cells compared to $CD57^{+/-}$ NK cells. Only slight donor variations could be detected for donor 3 of the $CD56^{dim}$ and $CD56^{bright}$ NK cell subsets and donor 8 of the $CD57^{+/-}$ NK cell subsets. Hence, the heat map analyses generally confirmed reproducible quantification and highly conserved protein regulation signatures within both sets of donors. Individual variations are limited and thus likely not compromise the definition of conserved regulated proteins. However, their identification might be of interest as clinical markers. Thus, we characterized strongly deviating proteins, which occur donor-dependently, through MAD analysis. Proteins with a MAD higher than 0.3, were considered to show donor-dependent protein regulations. The vast majority of proteins

exhibited remarkable consistent protein. However, 13 proteins were found with MAD values higher than 0.3 and therewith donor-dependent protein deviations. 10 of those cases were detected in $CD56^{bright/dim}$ and 3 in $CD57^{+/-}$ NK cells. The 13 candidates include GRAH (Granzyme H) and GNLY (Granulysin), which were already previously described to be donor-dependently regulated in human primary lymphocytes [38, 39] (SUPPLEMENTAL FIGURE 2, SUPPLEMENT). Another strongly deviating candidate is CD44, whose regulation occurs infection-dependently in mice and steers human postnatal thymocyte development [40, 41]. CD63 (Granulophysin) is well characterized in NK cells and was shown to be differentially regulated in decidual human primary NK cell subsets [42]. CATW (Cathepsin W) is exclusively expressed in $CD8^+$ T cells and NK cells. This protein may mediate cytolytic activity, but is not essentially involved in cytotoxicity [43]. Unfortunately, donor-dependent expression patterns of CD44, CD63, CATW and the other proteins with high MAD values have not been accessed to this date in primary human NK cells.

In conclusion, donor dependent variations were observed to a minor degree (13 proteins among 3412) in this study. Regulations of well-described NK-specific proteins serve without exception as proof-of-concept data. A broader dynamic range of regulations coinciding with the step from $CD56^{bright}$ to $CD56^{dim}$, compared to the subsequent step from $CD57^-$ to $CD57^+$, confirmed the recently suggested differentiation model. On the other hand, regulations observed for proteins so far not described in this context likely contribute to the biology of distinct NK cell subsets, including NK cell effector functions, status of differentiation and responsiveness.

Subset-specific protein regulation in $CD56^+$ NK cell development

After investigating donor variation, particular attention was paid to the detection of the most significant and subset-specific protein regulations. Therefore, a statistical model was developed to detect the most pronounced and conserved protein regulations [36]. Standard deviations were calculated based on the assumption that log_2-regulation factors of each protein follow a normal distribution, taking the limited number of 5 donors into account. The 3412 identified proteins were used to estimate the standard deviation and this served as a base to determine statistical

significance thresholds depending on the number of donors. Notably, the thresholds considering only 3 donors (0.83 for $CD56^{dim/bright}$ and 0.51 for $CD57^{+/-}$ NK cells) did not vary significantly from thresholds calculated for 4 or 5 donors ($log_2RF_{CD56dim/bright}$: 0.809 and 0.745 and $log_2RF_{CD57+/-}$: 0.503 and 0.467; see SUPPLEMENTAL TABLE 8; SUPPLEMENT). Hence, the thresholds 0.83 for comparing $CD56^{dim}$ *versus* $CD56^{bright}$ NK cells and 0.51 for $CD57^{+}$ *versus* $CD57^{-}$ NK cells were applied to extract subset-specific protein regulations. Although the threshold for $CD56^{dim/bright}$ NK cell subsets was significantly higher than in $CD57^{+/-}$ NK cells, a list of 31 proteins was generated (TABLE 1), instead of 5 proteins in $CD57^{+/-}$ NK cell subsets (TABLE 2).

None of these 36 proteins belongs to the group of donor-dependently regulated candidates with MAD values over 0.3. Thus, the regulation of these 36 proteins can be directly related to the distinct developmental NK cell stages. They can be grouped into the following categories: (1) NK signaling; (2) Cytoskeletal dynamics; (3) Differentiation and (4) Cytotoxicity. Top-regulated novel $CD56^{+}$ NK cell protein candidates (11 proteins; depicted in bold; with $-1.0<log_2RF>1.0$, FIGURE 5) are comparatively and subset-specifically summarized together with six key components of NK cell biology in FIGURE 5. Among those, the expression of the AK1C3 (Aldo-keto reductase family 1 member C3) protein was highest in $CD56^{dim}$ NK cells together with Perforin and declined within the last step of differentiation into $CD57^{+}$ NK cells. Expression of NHRF1 (Na^{+}/H^{+} exchange regulatory cofactor NHE-RF1), SH2K1 (SH3 domain-containing kinase-binding protein 1), EFHD2 (Swiprosin), ANXA2 (Annexin A2) and PTCA (Protein tyrosin phosphatase receptor type C-associated protein) increased during the first differentiation step from $CD56^{bright}$ into $CD56^{dim}$ NK cells and was stagnating during the last step from $CD57^{-}$ to $CD57^{+}$ NK cells.

However, we also detected proteins highly abundant in $CD56^{bright}$ NK cells, with a decline in expression during all following differentiation steps (from $CD56^{dim}$ to $CD57^{-}$ and finally to $CD57^{+}$ NK cells), e.g. FLNB (FilaminB), LMNA (PrelaminA/C) and COTL1 (Coactosin-like protein), which indicates a pivotal role especially in this early NK cell subset. In total 3 proteins showed continuous increase in expression during both differentiation steps, namely S100A4

(Calvasculin), S100A6 (Calcyclin) and LEG1 (Galectin-1). LEG1 was previously described in NK cells, but without NK cell subset-specific regulation. Of note, all three proteins were shown previously to be involved in the thymocyte differentiation process [52].

S100A4 and S100A6 are recruited to the NKIS and co-localize with Myosin IIa

NK-cell cytotoxicity depends on the formation of the F-actin-rich NKIS and the Myosin IIa (MYH9) mediated transport of Perforin-containing lytic granules to the NKIS [3–5]. In this study, we detected MYH9 expression highest in mature $CD56^{dim}$ ($log_2RF=0.7$), but reduced in terminally maturated $CD57^{+}$ NK cells ($log_2RF=0.1$), confirming its relevance for cytotoxic NK effector functions. Like MYH9, other subset-specific proteins exhibit a similar expression profile, e.g. S100A4 and S100A6. Notably, MYH9 was previously shown to interact with S100A4 in murine fibroblasts (NIH3T3) and human endothelial cells (CSML-0 and -100) [44, 45]. Therefore, both proteins (S100A4 and S100A6) were selected for subcellular localization studies that were performed using cytotoxic $CD56^{dim}$ (with high Perforin expression levels) NK cells to analyze S100A4 and S100A6 contribution to the immunological synapse of NK cells. NK activation was performed by co-incubation with their natural targets - K562 cells that we conducted in a time-resolved manner for 5, 10, 15, 20 and 30 minutes. The induced formation of the NKIS was documented by fluorescence microscopy. Staining for Perforin together with CD107a [46] and MYH9 [3] allowed following the dynamics of formation and recruitment of lytic granules to the interphase between NK and target cell. All three proteins localized time-dependently into the NKIS, thus favoring this generally accepted model system for further functional characterization of novel protein candidates (FIGURE 6 and FIGURE 7, left panel). Intensity of co-localization was determined with the help of 3D density surface plots, generated by ImageJ.

Microscopic investigation revealed time-resolved co-localization events of the here characterized NK cell proteins S100A4 and S100A6 together with MYH9. First co-localization between S100A4 and MYH9 was observed already after 5 minutes, formation of complete complexes after 10

minutes that peaked after 15 minutes, when specific recruitment and intense accumulation of this complex to the NKIS was observed (FIGURE 6). Instead, peak accumulation of the S100A6 and MYH9 complex occurred already after 10 minutes (FIGURE 7). The disassembly of this complex then starts immediately after 15 minutes whereas the S100A4 and MYH9 interactions lasted longer and started to disintegrate after 20 minutes and were apparent for further 10 minutes until full disintegration and detachment of the NK cell from the target cell.

To summarize, complex formation of S100A6 and MYH9 occurred very quickly, but between S100A4 and MYH9 much more intense and over a longer period of time. Hence, the microscopic investigation on the NK cell subset specifically regulated proteins S100A4 and S100A6, revealed distinct localization of both proteins at the NKIS after NK cell activation, followed by intense co-localization together with MYH9.

DISCUSSION

Research on NK cell development has rapidly emerged during the past few years, starting with the introduction of the 4-step NK cell differentiation model, leading to fully active $CD56^{bright}$ cells [9], their conversion into $CD56^{dim}$ cells [12, 13] and their final complementation into terminally differentiated $CD56^{dim}CD57^{+}$ NK cells [15,17,18].

NK cell subset analyses mostly relied on FACS-based approaches and microarray analyses to determine subset specific protein expression from *ex vivo* expanded primary NK cells [11, 15, 37]. As a complementation of these approaches, mass spectrometry can provide an unbiased and systematic overview about protein expression and regulation in the distinct developmental stages. Here, we provide the first insight into the proteome of primary $CD56^{+}$ NK cells of distinct developmental stages isolated from healthy human blood donors. We investigated the proteomes of $CD56^{bright}$ *versus* $CD56^{dim}$ and $CD57^{-}$ *versus* $CD57^{+}$ NK cell subsets. Quantitative data were determined for 3400 proteins and describe already known as well as novel functional

aspects of NK cell subsets. Notably, regulatory data of well-characterized NK cell proteins were in accordance with gene expression data from previous studies [16, 37].

$CD56^{bright}$ cells characterize an immune regulatory NK cell subset able to release various pro-inflammatory cytokines for activating, modulating and recruitment of adaptive immune cells [11]. $CD56^{dim}$ NK cells also release cytokines, but their main biological function encompasses the detection of transformed and virus infected target cells, followed by target cell lysis [47]. This cytotoxic capacity requires expression of cytolytic components, like Granzymes, Perforin and Granulysin, as well as vesicle forming and transport related proteins (CD107a, Rab proteins, WASP), also involved in endocytosis. Our proteome approach covered a significant portion of known cytolytic and vesicle-forming components, which were consistently, detected less abundantly in $CD56^{bright}$ NK cells but significantly higher amounts in $CD56^{dim}$ cells that present the mature and prototypic cytotoxic NK subset.

Recently, it was reported that CD57 expression occurs independently of, but simultaneously with KIR acquisition and thus NK cell differentiation proceeds un-coupled, but in parallel to NK cell education. Our proteome results confirmed this observation, since KIR2DS2, KIR3DL2 and KIR3DL1 were solely detected regulated in the $CD57^{+}$ NK cell subsets (SUPPLEMENTAL TABLE 6; SUPPLEMENT). This study also provides evidence for the specialization of terminally mature $CD57^{+}$ NK cells, because expression of KIRs and broad coverage of CD16 signaling-associated components (50% of the whole pathway) were determined only for this NK cell subset. It has been shown previously, that the intensity of NK cell cytotoxicity occurs donor-dependently [48, 49]. Interestingly, donors of this study were clinically approved as healthy but statistical proteome analyses revealed slight but significant variations that might serve as markers to better understand these donor-variations. While the majority of identified regulations (TABLE 1 and TABLE 2) were found conserved and widely donor-independent, a limited number of protein regulation showed a MAD value higher than 0.3 (in summary 13 proteins). Among them we detected 9 proteins with significant regulations, crossing the statistical threshold

(SUPPLEMENTAL TABLE 7; SUPPLEMENT). Four of them, GRAH (Granzyme H), CATW (Cathepsin W) and CD63 (LAMP-3), are well-characterized NK cell specific proteins involved in cytotoxicity and antigen processing, whereas CD44 is also known as marker for early T cell development in the thymus. The other three, protein kinase C (PACN1), SH2 domain containing protein 1A (SH21A) and MAP kinase 3 (MK03), are generally involved in NK cell signaling but were characterized as regulated in distinct developmental stages here for the first time. Unfortunately, the function of FETUA (Alpha-2HS glycoprotein) and CLIC3 (Chloride intracellular channel protein 3), showing most pronounced regulation, remain unknown in NK cells.

Particular attention was paid to determine statistically conserved NK subset-specific proteins. Thirty six proteins were detected by this approach and functionally categorized. LEG1 (Galectin-1), S100A4 (Calvasculin) and S100A6 (Calcyclin) occurred with pronounced increasing expression profiles both during the first and the second maturation step, indicating their general relevance in NK cell biology. LEG1 was described previously as significantly expressed and secreted by activated T_{reg} cells from mice and men [50, 51]. Furthermore, it was shown that LEG1, S100A4 and S100A6 promote thymocyte differentiation [52]. S100A4 and S100A6 belong to the calcium-binding family of S100 proteins and are generally involved in cell cycle progression and differentiation. For S100A4 a direct interaction with MYH9 was determined at the leading edge of migrating cells, which naturally contains a high calcium concentration [45]. Since MYH9 is also known to play an important role by mediating the interaction of cytotoxic granules with F-actin for further transport to NKIS [3–5], we were wondering whether S100A4 and S100A6 are involved in these processes. Time-resolved localization studies actually revealed the recruitment of S100A4 and S100A6 together with MYH9 to the NKIS in cytotoxic $CD56^{dim}$ NK cells, with peak levels after 10 and 15 minutes (see FIGURE 6 and FIGURE 7). These findings confirm their involvement in NKIS-mediated responses, although their direct functional contribution remains at this time un-characterized. S100A4 can regulate MYH9 activity [45] and might be an indirect mediator of cytotoxic granule transport.

Further candidates were detected regulated mostly at individual developmental stages, and their functional characterization will likely help to unravel the unique capabilities of distinct NK subsets: The AK1C3 (Aldo-ketoreductase family 1 member C3) converts steroid aldehydes and ketones into alcohols and may play a role in controlling cell growth and/or differentiation. AK1C3 was previously shown to be expressed in NK cells and was also suggested as marker for "healthy and normal" NK cells [53]. This proteomic study revealed a subset-specific expression profile for AK1C3 that was detected most abundant in cytotoxic $CD56^{dim}$ NK cells. This suggests AK1C3 as marker for fully functional and cytotoxic NK cells and a role in NK cell effector functions.

On the other hand, FLNB (Filamin B) was exclusively identified in $CD56^{bright}$ NK cells in high amounts. Kanters and colleagues identified the actin cross-linking molecule Filamin B as a novel binding-partner for intracellular adhesion molecule-1 (ICAM-1) and confirmed the ICAM-driven transendothelial migration of leucocytes [54]. Thus, Filamin B may be an important mediator of transendothelial migration of $CD56^{bright}$ NK cells from secondary lymphoid tissues (SLO) to peripheral blood during maturation.

In conclusion, results of this study fully support the recent NK cell differentiation model and revealed a limited number of novel donor-independent and subset-specific protein regulations. The systematic inspection of distinct primary NK development stages thereby helps to prioritize candidates such as LEG1, AK1C3 and Filamin B for perspective functional studies. S100A4 and S100A6 most likely are of general importance in NKIS-mediated responses. Following the example of this time-resolved localization studies, single molecule high-resolution imaging may guide us likely to the next molecular hallmarks of NK cell biology.

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FIGURE LEGENDS

FIGURE 1. Isolation of distinct NK cell subsets and MS workflow. (A) Sorting procedure for NK cell subset isolation. Dot plot A1 depicts the PBMC population with gate on lymphocytes (blue). Gating on $CD3^{-}CD56^{+}$ NK cells, with separate gates on $CD56^{dim}$ and $CD56^{bright}$ NK cells within the lymphocyte population is depicted in dot plot 2. Further discrimination of the $CD56^{dim}$ NK cell population into $CD57^{+}$ and $CD57^{-}$ NK cell subsets is shown in dot plot 3. (B) Experimental design of MS-based proteomic analysis of NK cell subsets. NK cell subsets were

sorted from healthy human blood donor PBMCs to produce specific $CD56^{pos}$ NK cell subsets. Isolated NK cells were prepared for MS analysis and differentially labeled with iTRAQ™ for further quantification. Labeled peptides were sequenced by nanoLC-MS/MS. Database searches supported protein identification and comparing iTRAQ™ reporter intensities revealed protein regulation factors. Statistical evaluation was performed on the generated datasets for quality control and to filter out conserved donor-independent protein regulations.

FIGURE 2. GeneGo™-based signaling pathway annotations of proteins identified in $CD56^+$ NK cell subsets. (A) The Venn diagram depicts the numbers of identified proteins in $CD56^+$ NK cells subsets. 3345 common proteins were identified in $CD56^{dim/bright}$ and $CD57^{+/-}$ NK cells. 13 proteins were unique in $CD56^{dim/bright}$ NK cells and 54 in $CD57^{+/-}$ NK cells. (B) Datasets (all proteins, n=5, median) were loaded into GeneGo™. This program assigns proteins to certain pathways by computing a statistical relevance value (negative log p-value). The negative log p-value depicts the ratio of proteins identified in this study and proteins known to be present within this pathway. Hierarchical order was defined by $CD57^-$ NK cell subsets. Numbers depict the order within the $CD56^{bright/dim}$ GeneGo™ analysis.

FIGURE 3. MS/MS spectra of peptides with corresponding iTRAQ™ reporter intensities identified in $CD56^+$ NK cell subsets. Sorted NK cells were lysed, and trypsin-digested peptides were tagged with iTRAQ™-label 115 (for $CD56^{bright}$ NK cell subset) and 117 (for $CD56^{dim}$ NK cell subset), or with 114 (for $CD57^+$ NK cell subset) and 116 (for $CD57^-$ NK cell subset). Subset peptides ($CD56^{dim}$ *versus* $CD56^{bright}$, $CD57^+$ *versus* $CD57^-$) were combined in 1:1 ratio and sequenced via nanoLC-MS/MS. Peptide fragmentation detected b (red)- and y (blue)-ions. Peptide sequences were derived from these ions through defined amino acid masses. Through Mascot-based database searches in UniProt/Swiss-Prot, peptide sequences were assigned to the proteins CD56 (A) and Perforin (B). Quality of this assignment depends on high peptide Mascot Scores. iTRAQ™ reporters occur in low mass regions of the spectrum (green). By comparing normalized reporter intensities (115-ref against 117 and 114 against 116-ref), peptide abundances were quantified and log_2-regulation factors (RFs) were generated. The RFs of all identified peptides of one protein represent the corresponding protein regulation factor.

FIGURE 4. Statistical evaluation of $CD56^+$ NK cell subset data. (A) Box plots of protein regulation factors in $CD56^{dim/bright}$ and $CD57^{+/-}$ NK cell subsets. The box of each graph displays 50% of all determined protein regulation factors, the box height depicts their spreading and the black line within the boxes indicates the median. The restricting lines (high and low Whisker) display 1.5x height of the box. All protein regulation factors above or beneath the Whiskers are categorized as strongly regulated and are shown as light dots. The symmetric distribution of protein regulations in all assessed donors with slight fluctuations is visible. Regulation ranges differ strongly within $CD56^{dim/bright}$ (between +3.8 and -4) and only slightly within $CD57^{+/-}$ NK cells (between +2.3 and -2.3), corresponding to their individual stage of differentiation. (B) Heat maps of log_2-protein regulation factors determined by iTRAQ™-based LC-MS/MS from primary human $CD56^+$ NK cell subsets. Heat maps were generated by loading NK cell subset-specific lists of proteins, identified in all 5 assessed human blood donors by iTRAQTM-based LC-MS/MS, into the statistical program R (1162 in $CD56^{dim/bright}$ and 1427 within $CD57^{+/-}$ NK cells). Colored boxes in one row depict the regulation of one protein in five individual blood donors. One row depicts

protein regulations in one blood donor. The histogram above displays the distribution of protein regulation factors and the corresponding color code.

FIGURE 5. Changes in protein expression during distinct stages of NK cell differentiation. Selected proteins are shown together with their corresponding regulation intensity in $CD56^{bright/dim}$ and in $CD57^{-/+}$ NK cells. Proteins with previously un-described regulation in NK cells are shown in bold. Regulation intensity is depicted with the following symbols: +++ log_2RF over 1.5; ++ log_2RF over 1.0; + log_2RF over 0.5; / unregulated (in between -0.5 and 0.5); - less than -0.5; -- less than -1.0; --- less than -1.5.

FIGURE 6. Time-resolved co-localization studies of S100A4 and MYH9 in activated NK cells. NK cells were incubated and activated with K562 target cells ('T' for target cell and 'NK' for NK cell) for 5, 10, 15, 20 and 30 minutes. First two rows show proof of concept co-localization events between Perforin (red) and CD107a (green), and between Perforin (red) and MYH9 (green). Time-resolved activation of NK cells and stain for S100A4 (red) and MYH9 (green) and DNA (DAPI, blue) with appropriate antibodies revealed remarkable time-dependent formation of co-localization events at the NKIS. Anti S100A4 was stained with goat anti-mouse IgG (Alexa 594) and anti-MYH9 (Myosin IIA) with goat anti-rabbit IgG (Alexa 488) supplemented with DAPI (1:1000). Imaging was performed on an inverted microscope (Axiovert 100TV; Carl Zeiss, Jena, Germany) microscope using standard epifluorescence illumination (light source HXP120, Zeiss) and 63×/NA1.4 or 100×/NA1.4 plan-apochromatic objectives. Imaging was performed at room temperature with immersion oil. Images were acquired with a back-illuminated, cooled charge-coupled-device camera (CoolSNAP HQ2, Photometrics, Tucson, AZ, USA) driven by Metamorph software (Version 7.5.3.0; Molecular Devices Corp., Downingtown, PA, USA). Five independent experiments were performed and 20-30 images were acquired per coverslip in each experiment. Image analysis was performed with ImageJ (version 1.44p).

FIGURE 7. Time-resolved co-localization studies of S100A6 and MYH9 in activated NK cells. NK cells were incubated and activated with K562 target cells ('T' for target cell and 'NK' for NK cell) for 5, 10, 15, 20 and 30 minutes. First two rows show proof of concept co-localization events between Perforin (red) and CD107a (green), and between Perforin (red) and MYH9 (green). Time-resolved activation of NK cells and stain for S100A6 (red) and MYH9 (green) and DNA (DAPI, blue) with appropriate antibodies revealed remarkable time-dependent formation of co-localization events at the NKIS. Anti S100A6 was stained with goat anti-mouse IgG (Alexa 594) and anti-MYH9 (Myosin IIA) with goat anti-rabbit IgG (Alexa 488) supplemented with DAPI (1:1000). Imaging was performed on an inverted microscope (Axiovert 100TV; Carl Zeiss, Jena, Germany) microscope using standard epifluorescence illumination (light source HXP120, Zeiss) and 63×/NA1.4 or 100×/NA1.4 plan-apochromatic objectives. Imaging was performed at room temperature with immersion oil. Images were acquired with a back-illuminated, cooled charge-coupled-device camera (CoolSNAP HQ2, Photometrics, Tucson, AZ, USA) driven by Metamorph software (Version 7.5.3.0; Molecular Devices Corp., Downingtown, PA, USA). Five independent experiments were performed and 20-30 images were acquired per coverslip in each experiment. Image analysis was performed with ImageJ (version 1.44p).

TABLES

TABLE 1. Threshold-determined (+/- 0.83, n=3) list of 31 significantly regulated proteins in CD56$^{dim/bright}$ NK cell subsets. Included are UniProt accession names and numbers, as well as protein names. The median Mascot Score = median (n=5) value of the peptide Mascot Scores sum per protein, median log_2-RF = median of the log_2-protein regulation factors of 5 approached donors, MAD value of the log_2-regulation factors and Median Protein Coverage in [%]. Functions are transferred from UniProt and were integrated into the following categories: (1) NK signaling; (2) Cytoskeletal dynamics; (3) Differentiation and (4) Cytotoxicity with corresponding references.

UniProt Acession	niProt Accession	Protein name	ian Mascot Score	Median log_2-RF	MAD	Median Protein Coverage [%]	Function	category	Reference
AK1C3	P42330	Aldo-keto reductase family 1 member C3	286	1.9	0.3	14	Converts aldehydes and ketones into alcohols, involved in hormone system, marker for “normal NK cells”	1	Choi et al., 2004
PERF	P14222	Perforin-1	1145	1.9	0.1	24	Key role in cytolysis, forms large pores, facilitates the uptake of cytotoxic Granzymes	4	Thiery et al., 2011
PKHF1	Q96S99	Pleckstrin homology domain-containing family F member 1	129	1.5	0.2	7.9	May induce apoptosis through lysosomal-mitochondrial pathway, initiates permeabilization of lysosomal membrane, results in release of PDCD8	4	Chen et al., 2005
S10A6	P06703	Calcyclin	182	1.5	0.2	17	Calcium sensor, plays a role in reorganization of actin cytoskeleton/motility	2	Nedjadi et al., 2009
GRAP2	O75791	GRB2-related adapter protein 2	138	1.4	0.25	7.45	Interacts with SLP-76 to regulate NF-AT activation, binds to tyrosine-phosphorylated shc.	1	Qiu et al., 1998
S10A4	P26447	Calvasculin	961	1.3	0.1	36	Involved in thymocyte differentiation together with S10A6 and Galactin 1 (LEG1), regulates MYH9 acticvity	3	Jeon et al., 2009
CAN2	P17655	Calpain-2 catalytic subunit	338	1	0.1	9.4	Involved in cytoskeletal remodeling and signal transduction, regulates lamellopodia dynamics	2	Potter et al., 1998 Franco et al., 2004

TABLE 2. Threshold-determined (+/- 0.51, n=3) list of 5 significantly regulated proteins within the CD57$^{+/-}$ NK cell subsets. Included are UniProt accession names and numbers, as well as protein names. The median Mascot Score = median (n=5) value of the peptide Mascot Scores sum per protein, median log_2-RF = median of the log_2-protein regulation factors of 5 approached donors, MAD value of the log_2-regulation factors, Median Protein Coverage in [%]. Functions are transferred from UniProt and were integrated into the following categories: (1) NK signaling; (2) Cytoskeletal dynamics; (3) Differentiation and (4) Cytotoxicity with corresponding references.

iProtAccession name	niProtAccesion number	Protein name	Median Mascot score	Log_2-RF Median	MAD	Median Protein Coverage [%]	Function	category	Reference
GRAK	P49863	Granzyme K	242	-1.7	0.3	6	Cytotoxic molecule in $CD56^{bright}$ NK cells, promotes T cell killing to shape adaptive immune responses	4	Jiang et al., 2011
F169A	Q9Y6X4	Protein FAM169A	153	0.6	0.1	3.3	Unknown	-	-
LEG1	P09382	Galectin-1	328	0.6	0.1	45	Regulates apoptosis, differentiation and proliferation	3	Molvarec et al., 2011
S10A4	P26447	Calvasculin	261	0.6	0.3	36	Involved in thymocyte differentiation together with S10A6 and Galactin 1 (LEG1), regulates MYH9 acticvity	3	Jeon et al., 2009
COTL1	Q14019	Coactosin-like protein	212	-1.6	0.2	11	Binds to F-actin in a calcium-independent manner, regulator of Leukotrine metabolism	1	Rakonjac et al., 2006

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AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: MS, SK, MvH, LJ. Sorting of NK cell subsets: LG. Performed the MS-experiments: MS, UL. Performed statistical evaluation: FK. Performed microscopic analysis: MS, BB, MvH. Analyzed the data: MS and UL. Wrote the paper: MS SK LJ.

CONFLICT OF INTEREST DISCLOSURES

The authors Maxi Scheiter, Ulrike Lau, Marco van Ham, Björn Bulitta, Lothar Gröbe, Frank Klawonn, Sebastian König and Lothar Jänsch declare their affiliation to Helmholtz-Zentrum fuer Infektionsforschung GmbH. This does not alter the authors' adherence to all the MCP policies on sharing data and materials.