The stalk domain and the glycosylation status of the activating natural killer cell receptor NKp30 are important for ligand binding.

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Background: NKp30 is a major activating receptor of natural killer (NK) cells.

Results: The stalk domain of NKp30 increases ligand-binding affinity, which is modulated by glycosylation of the ectodomain of NKp30.

Conclusion: The stalk domain and the glycosylation status of NKp30 are critical for NK cell killing.

Significance: This is the first hint for a novel mode of receptor regulation.

SUMMARY

The natural cytotoxicity receptors (NCRs) are a unique set of activating proteins expressed mainly on the surface of natural killer (NK) cells. The human NCR family is comprised of the three type I membrane proteins NKp30, NKp44, and NKp46. Especially NKp30 is critical for the cytotoxicity of NK cells against different targets, including tumor, virus-infected, and immature dendritic cells. Although the crystal structure of NKp30 was recently solved (Li Y, et al. (2011) J Exp Med 208:703-714; Joyce MG, et al. (2011) Proc Natl Acad Sci USA 108:6223-6228), a key question, how NKp30 recognizes several non-related ligands, remains unclear. Therefore, we investigated the parameters which impact on ligand recognition of NKp30. Based on various NKp30::hIgG1-Fc-fusion proteins, which were optimized for minimal background binding to cellular Fc-gamma receptors, we identified the flexible stalk region of NKp30 as an important, but so far neglected module, for ligand recognition and related signaling of the corresponding full-length receptor proteins. Moreover, we found that the ectodomain of NKp30 is N-linked glycosylated at three different sites. Mutational analyses revealed differential binding affinities and signaling capacities of mono-, di- or tri-glycosylated NKp30, suggesting that the degree of glycosylation could provide a switch to modulate the ligand-binding properties of NKp30 and NK cell cytotoxicity.
NK cells are tightly regulated by a dynamic balance of signals from several agonistic and antagonistic cell surface receptors to prevent destruction of healthy cells while maintaining recognition and efficient killing of multiple adverse targets (1,6). NK activating receptors recognize viral and frequently induced cellular ligands, while most inhibitory receptors recognize self-major histocompatibility class I (MHC I) molecules (7). The major activating receptors on human NK cells include NKG2D and the natural cytotoxicity receptors (NCRs) NKp30, NKp44, and NKp46 (7). The expression of an insufficient amount of NCRs results in resistance of leukemia cells to NK cell cytotoxicity in patients with acute myeloid leukemia (AML) (8), demonstrating their importance for immunosurveillance of tumor cells. Notably, NKp30 and NKp46 are found on all NK cells, whereas NKp44 represents a marker for activated NK cells (6,9). The density of NCRs on the cell surface correlates with the degree of NK cell cytotoxicity against various tumors (10,11). Interestingly, the ligands of NCRs are expressed in many tissues including healthy cells and tumor cells (10); therefore, their expression level is critical for the ability of NK cells to destroy target cells (8,12-15). The human cytomegalovirus (HCMV) tegument protein pp65 (16), the BCL2-associated athanogene 6 (BAG-6, also known as BAT3) (17,18) and a structural homolog of B7 (B7-H6) (19) were shown as ligands for NKp30, indicating ligand promiscuity of NKp30. While ligation of pp65 leads to inhibition of NK cell cytotoxicity as part of an immune-escape strategy (16), engagement of BAG-6 or B7-H6 on tumor cells mediates target cell killing (17,19). Moreover, binding of NKp30 to BAG-6 on dendritic cells (DCs) leads to DC activation and killing of immature tolerogenic DCs (iDCs) as part of a quality control mechanism (18,20,21). Controversial data exist for potential binding of NKp30 to heparan sulfate/heparin molecules (22,23). NKp30, NKp44 and NKp46 recognize viral hemagglutinins (HA) (24-27) and binding of HA to NKp44 and NKp46 consequently promotes NK cell killing of virus-infected cells (24-27). Recently, the proliferating cell nuclear antigen (PCNA) was discovered as a novel inhibitory ligand for NKp44 (28).

NKp30 comprises an extracellular ligand binding domain, a transmembrane domain (TMD) that recruits the signaling adaptor protein CD3ζ, and a short cytoplasmic tail (6,12). The N-terminal part of the ectodomain of NKp30 comprises an Ig-like fold (ligand-binding domain, LBD) (29,30), which is connected to the TMD by a flexible stalk domain.

Within the current study, we identified the so far neglected stalk domain of NKp30 as an essential element for ligand binding. Moreover, we demonstrate that NKp30 is N-linked glycosylated at three consensus sequence motifs. Detailed studies reveal that differential glycosylation impacts on the ligand binding properties of NKp30 and might be a switch to modulate NK cell cytotoxicity.

**EXPERIMENTAL PROCEDURES**


**Cells** – Human chronic myeloid leukemia (CML) cells (K-562, CCL-243), human embryonic kidney cells (293, CRL-1573; 293T/17, CRL-11268), African green monkey kidney cells (COS-7, CRL-1651), and Chinese hamster ovarian cells (CHO K1, CCL-61) were purchased from American Type Culture Collection (ATCC). Murine pro B cells (Ba/F3, ACC 300) were purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ). Human melanoma cells (MelJuSo) were kindly provided by E. Wiertz (31). Ba/F3 cells transduced with B7-H6 (Ba/F3-B7-H6) or the empty vector (Ba/F3-mock) were provided by C. Watzel (IfADo, Germany) and A5 cells were kindly provided by A. Diefenbach (32).

**Recombinant Ig proteins** – To generate NKp30-Ig fusion proteins, the pFUSE-hlgG1-Fc2 vector (InvivoGen) was used. Two single (L118E (E) and N180Q (Q)) and one double mutation (L118E/N180Q (EQ), amino acid positions refer to accession number P01857) were introduced into the hlgG1-Fc part by site-directed mutagenesis. To generate 30LBD-Ig, 30Stalk-Ig and Ifnar2-Ig.
fusion proteins, the ectodomain-encoding gene segment of NKp30 (accession number NM_147130.2; 30LBD-Ig: residues 19-128; 30Stalk-Ig: residues 19-143) and Ifnar2 (accession number X89814.1; Ifnar2-Ig residues 28-239) were amplified by PCR from cDNA (NKp30 (GenScript), the Ifnar2 DNA was kindly provided by J. Piehler (University of Osnabrück, Germany)) and cloned into pFUSE-hIgG1-Fc2 vector variants. The pFUSE-hIgG1-FcEQ vector with a 30Stalk insert was used as template to produce an NKp30-Ig fusion construct containing a length-matched glycine-serine-(GS)-linker instead of the stalk domain (30GS-Ig) and three C-terminally truncated 30Stalk-Ig variants (30Stalk-Ig Δ3 (aa 19-140), 30Stalk-Ig Δ7 (aa 19-136), 30Stalk-Ig Δ11 (aa 19-132)). The pFUSE-hIgG1-FcQ vector with a 30Stalk insert was used as template to produce glycosylation-deficient mutants of the 30Stalk-Ig protein by site directed mutagenesis (three single (N42Q, N68Q, N121Q), three double (N42Q/N68Q, N42Q/121Q, N68Q/N121Q), and one triple amino acid mutation (N42Q/N68Q/N121Q)).

Generation of reporter cell lines – A5 cells are a CD4-positive T cell hybridoma expressing the 14.3.d TCR β chain (I-Eβ, HA110-119, Vα4.2, Vβ8.2) derived from 14.3.d TCR-transgenic mice (32). A5 cells were stably transfected with a reporter construct driving GFP expression under the control of three NF-AT binding sites found in the promoter region of the IL2 gene (A5-GFP) (32-35). A5-GFP cells were retrovirally transduced with various NKp30 receptor constructs. Two days after transduction, NKp30 expression was determined by staining with an antibody specific for NKp30 on a flow cytometer. As a control, A5-GFP cells were transfected with empty retrovirus (mock). NKp30-expressing cells were purified using flow cytometry-based cell sorting (MoFlo Astrios, Beckman Coulter). A5-GFP cells and the various NKp30 transductants were maintained in the presence of 0.5 mg/ml hygromycin. The NKp30 receptor associates with CD3ζ expressed by A5-GFP cells. Cognate interaction between the NKp30 receptor and its ligand induces activation of the NF-AT promoter resulting in GFP expression.

Immunofluorescence staining – Cells were cultured on glass slides for 48 h, blocked with 3% (w/v) BSA, and incubated with Ig fusion protein (50 µg/ml). After immunostaining (anti-human-IgG-Fc-DyLight 488, 7.5 µg/ml) cells were fixed with acetone/methanol (1:1 (v/v)) and stained with To-Pro-3 (1 µM, Invitrogen) prior to microscopy (DM IRBE CLSM instrument, Leica).

Flow cytometry – Adherent cells were detached (accutase, PAA), blocked with 5% (w/v) BSA and incubated with Ig fusion protein (50 µg/ml). After immunostaining (anti-human-IgG-Fc-DyLight 488, 7.5 µg/ml) cells were fixed with acetone/methanol (1:1 (v/v)) and stained with To-Pro-3 (11 µM, Invitrogen) prior to microscopy (DM IRBE CLSM instrument, Leica).

ELISA assays – 96-well ELISA plates (Greiner) were coated with recombinant BAG-6 protein (1

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Recombinant NKp30 receptor variants – To generate NKp30 receptor constructs, the LeGO-iZ vector was used. The LeGO-iZ vector consists of the LeGO-iG2 backbone (34) were the GFP is exchanged for a zeocin resistance gene. The full-length gene segment of human NKp30 (accession number NM_147130.2) fused to a C-terminal deca-histidine tag (30FL-his) was amplified by PCR from codon usage optimized cDNA (Genscript) and cloned into the LeGO-iZ vector. The LeGO-iZ vector with 30FL-his insert was used as template to produce a stalk domain truncated (30LBD-his) and a stalk domain GS-linker substituted (30GS-his) NKp30 construct (truncated/substituted residues: 129-135) by overlapping size extension PCR, as well as to produce glycosylation-deficient mutants of the 30FL-his insert by site directed mutagenesis (three single (N42Q, N68Q, N121Q), three double (N42Q/N68Q, N42Q/121Q, N68Q/N121Q), and one triple amino acid mutation (N42Q/N68Q/N121Q)).
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μg/well) blocked with 5% (w/v) BSA, and incubated with graded amounts of Ig fusion proteins (0-10 μg/well). The amount of bound Ig fusion proteins was quantified after immunodetection (anti-human-IgG-Fc) and visualization with TMB substrate in a microtiter plate reader (λ = 450 nm). K_D and B_max values were determined by fitting the curves to a 1:1 Langmuir binding model using the Prism 5 software (GraphPad).

Surface plasmon resonance (SPR) — The interaction of B7-H6-Ig and NKp30-Ig variants was assessed by SPR using the ProteOn XPR36 protein interaction array system (BioRad). Typically, 6,000 – 11,000 resonance units of NKp30-Ig variants were immobilized on a GLC sensor chip by random amine coupling. Different analyte concentrations of B7-H6-Ig were injected sequentially over the microfluidic cells immobilized with NKp30-Ig variants or buffer as a blank. The data were analyzed using ProteOn Manager 3.1.0 software (BioRad). K_D values were determined from bivalent analyte analysis, after correction for the interspot data.

Signaling reporter assays — A5-GFP effector (E) cells were mixed with 50,000 B/aF3-B7-H6 target cells (T) at E:T ratios of 2:1, 1:1 and 0.5:1. After 16 h of co-incubation at 37°C, cells were stained with a CD4-specific antibody, and GFP expression of CD4^+ A5 cells was determined on a flow cytometer. As a positive control, A5 cells were incubated for 16 h in the presence of 50 ng/ml PMA and 750 ng/ml ionomycin.

RESULTS

Optimized human NKp30-Ig fusion proteins with reduced binding to Fc receptors — Bivalent fusion proteins of the ectodomain of NK cell receptors with the IgG1-Fc part of human immunoglobulins (hlgG1-Fc) are a valuable tool to study receptor-ligand interactions in vitro (16,24,36). However, as a major drawback, these constructs display an inherent binding activity to the Fcγ receptor (FcγR) on target cells via their Ig domains and thus limited potential to investigate the actual receptor-ligand interaction. In order to overcome this limitation, we have mutated leucine 118 to glutamate (L118E, FeE) and removed a glycosylation acceptor site (mutation of asparagine 180 to glutamine, N180Q, FeQ) within hlgG1-Fc, both being essential for FcγR binding (37-40). Fusion proteins of the ectodomain of NKp30 and the novel hlgG1-Fc variants were generated and affinity-purified to homogeneity on Protein A (2 mg of pure protein from 10^8 cells) after secretion into the culture medium of 293T cells (Fig. 1A). For reference, the ectodomain of the human interferon receptor subunit Ifnar2 was fused to the hlgG1-Fc variants as well and produced accordingly. All of the Ig fusion proteins form disulfide-linked homodimers as shown by reducing and non-reducing SDS-PAGE and corresponding Western blot analyses. The fusion proteins with an FeQ mutation display a lower apparent molecular mass than their wild-type (wt) counterparts, demonstrating deficiency in glycosylation due to removal of the glycosylation targeting site. The ligand binding properties of the various Ig fusion proteins were assayed by flow cytometry on K-562 cells, which express a cellular ligand of NKp30 (19,24,41) and high levels of FcγR (42,43) (Fig. 1B). As detailed above, human NKp30 fused to a wt hlgG1-Fc (NKp30-Ig Fc wt) binds only slightly stronger to the cell surface of K-562 cells when compared to the analogous Ifnar2 reference construct (Ifnar2-1g Fc wt) or the IgG1 isotype control (it). By contrast, fusion proteins with the mutated hlgG1-Fc variants (FcE, FeQ, FeEQ) displayed reduced background binding, thus enabling the exclusive investigation of NKp30 binding to its cellular ligands. Interestingly, the double mutant (FcEQ) shows about the same reduction in background binding than the single mutants (FcQ, FeE) suggesting sufficiency of the individual mutations. For reference, the optimized Ig fusion constructs were tested for binding to COS-7 cells, which express ligands of NKp30 (41) but no human FcγR. The determined binding patterns resemble those observed for K-562 cells, demonstrating quantitative background reduction (Fig. 1B). Based on these results, the optimized NKp30-Ig variants are validated tools for the investigation of NKp30-dependent ligand binding, and the optimized hlgG1-Fc scaffolds were used to generate further NKp30-Ig fusion proteins throughout the current study.

The stalk domain of NKp30 impacts on ligand binding — Part of the ectodomain of NKp30 (amino acids 19-128) adopts an Ig-like fold (29,30) providing a binding pocket for B7-H6 at its membrane-distal face (30). Although, the stalk domain connecting the closed fold of the LBD of NKp30 with the TMD region was not resolved in
the crystal structure, its N-terminal end was defined. The border between the stalk domain and the TMD was predicted using TMpred (44) thus defining a stretch of 15 amino acids which comprise the stalk domain (amino acids 129-143). In order to investigate whether the stalk domain impacts on ligand binding of NKp30, we generated Ig fusion proteins containing either the LBD alone (30LBD-Ig) or the complete ectodomain of NKp30 including the stalk domain (30Stalk-Ig). These proteins were purified to define the stalk domain (amino acids 129-143). Ligand binding of the 30LBD-Ig and 30Stalk-Ig constructs were evaluated in cell decoration experiments by immunofluorescence microscopy and flow cytometry (FACS, Fig. 2D/E). Strikingly, the 30Stalk-Ig construct binds stronger to ligands on target cells than the 30LBD-Ig construct, demonstrating a significant contribution of the stalk domain of NKp30 to ligand binding. This effect was confirmed on a panel of several cell lines of different tissue and species origin (Fig. 2F, Tab. S1, Fig. S1). Importantly, none of the constructs bound to the surface of cells, which do not express NKp30-ligands, demonstrating ligand-dependent binding (Fig. 2G, Fig. S1). Notably, expression of the NKp30 ligand B7-H6 in the NKp30-ligand negative cell line Ba/F3 (Ba/F3-B7-H6) leads to NKp30 specific cell decoration confirming a significant contribution of the stalk domain of NKp30 to ligand binding (Fig. 2G). Since the ligand(s) recognized in cell decoration experiments on tumor cells are ill-defined, we have established an ELISA-based assay to study the molecular details of NKp30 binding to its ligand BAG-6 found on tumor cells and DCs (17,18). Additionally, the interaction of NKp30 and its ligand B7-H6 was analyzed in molecular detail using surface plasmon resonance (SPR). All of the NKp30-Ig fusion proteins bound specifically to recombinant BAG-6 and B7-H6 proteins demonstrating correct assembly and folding (Fig. 2H, Fig. 3A/B). In accordance with results from the cell decoration experiments (see above), the equilibrium binding constant (K_D) of the 30Stalk-Ig construct (BAG-6: 48 ± 5 nM; B7-H6: 76 ± 20 nM) was significantly lower than that of the 30LBD-Ig construct (BAG-6: 126 ± 14 nM; B7-H6: 149 nM ± 19 nM) confirming the importance of the stalk domain for ligand binding of NKp30.

**The integrity of the stalk domain is important for ligand binding of NKp30** – In order to further characterize the contribution of the stalk domain to ligand binding by NKp30, we generated an NKp30-Ig fusion construct containing a length-matched glycine-serine-(GS)-linker instead of the stalk domain (30GS-Ig). Ligand binding of the 30GS-Ig protein was investigated by FACS on several cell lines of different tissue and species origin. Notably, the GS-linker could not substitute for the intrinsic stalk domain of NKp30 as binding of the 30GS-Ig construct is comparable to the NKp30 construct without stalk domain (Fig. 4A, Fig. S2). Surprisingly, the 30GS-Ig construct showed an intermediate phenotype for the Ba/F3-B7-H6 cells when compared to the 30LBD-Ig and 30Stalk-Ig constructs (Fig. 4B, Fig. S2). In order to validate this observation, we determined the K_D of the 30GS-Ig construct for binding to BAG-6 by ELISA (Fig. 4C) and to B7-H6 by SPR (Fig. 3C). The determined K_D of the 30GS-Ig construct (BAG-6: 86 ± 7 nM; B7-H6: 114 nM ± 9 nM) was found in between of those for the 30Stalk-Ig (BAG-6: 48 ± 5 nM; B7-H6: 76 ± 20 nM) and the 30LBD-Ig construct (BAG-6: 126 ± 14 nM; B7-H6: 149 nM ± 19 nM), confirming the results from the cell decoration experiments with Ba/F3-B7-H6 cells (see above). Based on these data, we conclude that the amino acid composition of the stalk domain is important for ligand recognition and that the stalk domain contributes directly to ligand binding rather than acting solely as a flexible spacer for the LBD.

To determine the minimal functional requirements of the stalk domain, we generated 30Stalk-Ig proteins C-terminally truncated by 3 (30Stalk-Ig Δ3), 7 (30Stalk-Ig Δ7) or 11 (30Stalk-Ig Δ11) amino acids. Ligand binding of these constructs was analyzed by FACS on several cell lines of different tissue and species origin. Surprisingly, all of the truncated NKp30-Ig constructs lost the superior ligand binding...
properties of the 30Stalk-Ig construct as demonstrated by a binding phenotype comparable to the NKp30 construct without the stalk domain (Fig. 5A, Fig. S3A). Notably, this effect is less pronounced for cell decoration experiments with Ba/F3-B7-H6 (Fig. 5B, Fig. S3A), correlating with the \( K_D \) value determination of the 30Stalk-Ig truncation variants for binding to B7-H6 (SPR) (30Stalk-Ig \( \Delta 3 \), 132 \( \pm \) 36 nM; 30Stalk-Ig \( \Delta 7 \), 133 \( \pm \) 36 nM; 30Stalk-Ig \( \Delta 11 \), 137 \( \pm \) 27 nM) (Fig. 5C, Fig. 3D-F). Importantly, there is no non-specific binding of B7-H6 during surface plasmon resonance measurements (Fig. 2N/O). Moreover, binding of B7-H6 during surface plasmon resonance measurements (Fig. 5B, Fig. S3A), correlating with pronounced for cell decoration experiments with the \( K_D \) value determination of the 30Stalk-Ig mutants is a consequence of a length. Most importantly, impaired ligand binding domain with respect to sequence composition and length. Interestingly, successive deletion of potential acceptor sites for N-linked glycosylation went along with a stepwise reduction of the apparent molecular mass of the 30Stalk-Ig mutants (Fig. 6B/C). Moreover, PNGaseF treatment shifted the apparent molecular mass of all 30Stalk-Ig mutants to that of the glycosylation-deficient triple mutant (Fig. 6C).

To investigate the ligand binding properties of the differentially glycosylated 30Stalk-Ig mutants, they were analyzed by immunofluorescence microscopy and FACS in cell decoration experiments with NKp30 ligand-positive cell lines of different tissue and species origin. Specific staining was found for all mutants with varying intensity (Fig. 7, Fig. S5). No altered binding phenotype could be detected for K-562 cells. Interestingly, all di-glycosylated and some mono-glycosylated (N68Q, N121Q) mutants showed improved ligand binding to 293T, COS-7 and MelJuSo cells when compared to the 30Stalk-Ig wt construct. By contrast, the triple mutant of the 30Stalk-Ig construct, devoid of N-linked glycans, displays the same binding phenotype when compared to 30Stalk-Ig wt protein (Fig. 7B, Fig. 7D-C).
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S5). These results correlate with the different affinities ($K_D$) of the 30Stalk-Ig mutants for binding to BAG-6 as measured by ELISA (Tab. 1, Fig. S6). Notably, the $B_{max}$ values of the constructs for binding to BAG-6 were preserved. Surprisingly, on Ba/F3-B7-H6 cells all mono-, despite of glycosylation mutant N121Q, and di-glycosylated as well as the triple-mutant, show a reduced ligand binding phenotype when compared to the 30Stalk-Ig wt construct (Fig. 7C, Fig. S5). These results correlate with the different affinities ($K_D$) of the 30Stalk-Ig mutants for binding to B7-H6 as measured by SPR (Tab. 2, Fig. 3A/G-M). Notably, binding of NKp30 to B7-H6 is abrogated by destroying glycosylation site N42.

The stalk domain and the glycosylation status of NKp30 impacts on intracellular signaling – Since NKp30 possesses no signaling motive within its short cytoplasmic tail, NKp30 associates with the signaling adaptor protein CD3ζ via an opposing charge contact in the TMD. To analyze, if the observed binding phenotype of the various NKp30 Ig-fusion constructs directly correlates with the signaling capacity of corresponding full-length receptors in a cellular context, we expressed the various NKp30 receptor variants in a CD3ζ reporter cell line. In this cell line, CD3ζ signaling can be monitored by induced GFP expression.

To determine the influence of the stalk domain of NKp30 on CD3ζ signaling, we generated variants of the NKp30 receptor (30FL-his) without the stalk domain (30LBD-his) or with a length-matched glycine-serine-(GS)-linker instead of the stalk domain (30GS-his). The relative NKp30 surface expression was quantified by flow cytometry, demonstrating similar expression levels of the constructs and most importantly, successful targeting to the plasma membrane (Fig. 9A). Notably, the triple mutant (N42Q/N68Q/N121Q) was not detectable. Incubation of reporter cell lines expressing glycosylation variants of NKp30 with Ba/F3-B7-H6 cells revealed that all di-glycosylated mutants, as well as the mono-glycosylated mutants N42Q and N68Q show a reduced signaling capacity when compared to 30FL-his (Fig. 9B). For reference, stimulation with PMA/Ionomycin leads to robust CD3ζ signaling for all NKp30 receptor cell lines (Fig. 9B). In accordance with binding studies of NKp30 Ig-fusion proteins to Ba/F3-B7-H6 cells, glycosylation at residues N42 and N68 is critical for NKp30 function, whereas glycosylation at N121 has little influence on ligand binding and signaling of NKp30 (Fig. 9C/B). In conclusion, these data demonstrate that the ectodomain of NKp30 is differentially N-linked glycosylated at all three consensus motifs within the LBD in the plasmamembrane of living cells. The glycosylation status of NKp30 influences its ligand binding affinity and signaling capacity. Therefore, the glycosylation status of NKp30 may be a way to tune ligand recognition and NKp30-dependent NK cell cytotoxicity. In this context, N42 and N68 play a major role.

DISCUSSION

NK cell cytotoxicity against tumor cells and the cross-talk of NK cells with DCs depends largely on NKp30. However, little is known about the molecular determinants of ligand recognition. Previous studies were performed with Ig fusion
proteins of a partial ectodomain of NKp30 (16,24,41). These constructs display an inherent binding activity to the FcγR on target cells via their Ig domains and thus limited potential to investigate the actual receptor-ligand interaction. To overcome this limitation, we generated a novel set of NKp30-Ig fusion proteins with minimal binding affinity to the FcγR. Based on these proteins, we identified the so far neglected stalk domain of NKp30 as an important module of the ectodomain of NKp30 for engagement of cellular ligands. We demonstrate that the integrity of the entire stalk domain with respect to sequence and length is essential for ligand binding and subsequent intracellular signaling. In addition, we show that N-linked glycosylation of the ectodomain of NKp30 impacts on the affinity of ligand binding and subsequent CD3ζ signaling as well. Therefore, the glycosylation status of NKp30 might be way to tune ligand recognition and NKp30-dependent NK cell cytotoxicity.

Productive interaction of an NK cell with a target cell involves the formation of a stable and highly organized immunological synapse bridging an intercellular cleft of roughly 8 nm (as deduced from the length of an NKG2D-MICA pair) (45,46). Therefore, we hypothesized that the stalk domain of NKp30 might act as a flexible spacer for the LBD reminiscent of Ly49A (47). Strikingly, substitution of the stalk domain with a flexible length-matched GS-linker or C-terminal truncation by only three amino acids led to a significant reduction in ligand binding affinity and a loss of CD3ζ signaling. These data demonstrate that the stalk domain contributes directly to ligand binding rather than acting as a spacer for the LBD. This type of contribution is different from that described previously for the two other NCRs NKp44 and NKp46. In case of NKp44, sialic acid moieties attached to the stalk domain of NKp44 bind to influenza hemagglutinin and other viral hemagglutinin-neuraminidase proteins (25,48,49). For NKp46 it was shown that the O-glycosylated threonine at position 225 within the stalk of this receptor is critical for its binding to viral hemagglutinins (50).

The stalk domain was not resolved in the X-ray crystal structure of an NKp30-B7-H6 complex (30), which indicates flexibility and might argue against a direct contribution of the stalk domain to the B7-H6 binding interface. However, the stalk domain might contribute in a different way to ligand binding when it is connected to the TMD instead of being a “flexible C-terminal tail” of the soluble LBD. The stalk domain is rich in hydrophobic amino acids, which might favor the formation of NKp30 oligomers leading to increased apparent affinity for the ligand due to increased avidity. Although NK cell killing is not exclusively dependent on NKp30, this idea could at least partially explain the previous observation that some cell lines are killed by NK cells but fail to bind NKp30-Ig proteins devoid of a functional stalk domain (24,36).

Protein function can be modulated by posttranslational modifications such as glycosylation, which differs among tissues and cell types (51). In this context, we and others could show alterations in ligand binding of NKp30 from different expression hosts ((52), Fig. S7). These findings are supported by in vivo data from the endometrial epithelium demonstrating increased expression of differentially glycosylated variants of NKp30 (53). Along this line, it is already shown that depending on the NKp30 isoform distinct signals are transmitted (54).

Based on our results, NKp30 is N-linked glycosylated at all three consensus motifs within the LBD. Most importantly, these in vitro data were confirmed in vivo on primary NK cells by a mass spectrometry based approach (Fig. S8). Therefore, it is not surprising that the NKp30 variant devoid of all glycosylation targeting sites for N-linked glycosylation (N42Q/N68Q/N121Q) was not expressed in our CD3ζ reporter system. Moreover, this result argues for the presence of differentially glycosylated variants of NKp30 in NK cells rather than the existence of functional non-glycosylated variants.

Although, the glycosylation acceptor sites are located outside of the B7-H6 binding pocket of NKp30 (30), we show that differential glycosylation of NKp30 impacts on the ligand binding affinity of NKp30, since engagement of NKp30 and B7-H6 is dependent on glycosylation of N42. Moreover, glycosylation of N42 and N68 is essential for efficient intracellular signaling. In the X-ray crystal structure of the NKp30-B7-H6 complex (30), N42 is located at the opposite side of the B7-H6 binding pocket of the LBD. Interestingly, comparison of the unbound (29) and B7-H6-bound structures of NKp30 (30) reveals that N68 is situated underneath the B7-H6 binding pocket within a sequence stretch that undergoes
structural rearrangements upon ligand binding. These data suggest that glycosylation of N42 and/or N68 might contribute to subtle conformational changes of NKp30 and therefore shape the ligand binding pocket. Notably, for binding of NKp30 to its cognate ligand BAG-6 glycosylation of N68 was critical, whereas glycosylation at N42 and N121 had less impact. Notably, both available crystal structures of human NKp30 (29,30) are derived from purified NKp30 protein after heterologous expression in inclusion bodies of *E. coli* and subsequent refolding. Therefore, these NKp30 proteins are not glycosylated. Rearrangement of the binding pocket might be of particular importance in the light of the broad spectrum of non-related ligands, which are recognized by NKp30 when compared to the pool of structurally related ligands of NKG2D. To date, the *in vivo* glycosylation pattern and composition of N-linked glycans within NKp30 in the plasma membrane of NK cells is unknown; mainly due to limited availability of NK cell derived homogeneously glycosylated NKp30 and the technically demanding analytic process. Another layer of complexity is given by the observation that NKp30 derived from a polyclonal NK cell population appeared as a broad band (30-45 kDa) in reducing SDS-PAGE (12), which might reflect pools of differentially glycosylated NKp30 on the level of individual cells or the entire population of NK cells. One future perspective to overcome these limitations towards the determination of the native glycosylation pattern of NKp30 might be the use of NKp30-Ig fusion proteins derived from stably transduced NK-92 cells.

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FOOTNOTES

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7The abbreviations used are: NK, natural killer cell; NCR, natural cytotoxicity receptors; DCs, dendritic cells; MHC I, major histocompatibility class I; AML, acute myeloid leukemia; HCMV, human cytomegalovirus; iDCs, immature tolerogenic; TMD, transmembrane domain; HA, hemagglutinins; PCNA, proliferating cell nuclear antigen.

FIGURE LEGENDS

**FIGURE 1.** Optimized human NKp30-Ig fusion proteins with reduced binding to FcγR on tumor cells. (A) Non-reducing (−DTT) and reducing (±DTT) SDS-PAGE (coomassie-stained) and Western blot (anti-hIgG-Fc) of NKp30- and Ifnar2-ectodomains fused to hIgG1-Fc variants (wt and mutations (N180Q (FcQ), L118E (FcE), and L118E/N180Q (FcEQ)). (B) Flow cytometry of K-562 and COS-7 cells decorated with anti-human-IgG-Fc-DyLight 488 (solid gray), human isotype control (IC, gray line), 30Stalk-Ig variants (black line) and Ifnar2-Ig variants (dashed line).

**FIGURE 2.** Importance of the stalk domain of NKp30 for ligand binding. Reducing SDS-PAGE (coomassie-stained) and Western blot (anti-hlgG-Fc) of aliquots (input, ft. flow through, washes, eluates) from purification of 30LBD-Ig (without stalk, (A)) and 30Stalk-Ig (entire ectodomain, (B)) proteins. (C)
Non-reducing (-DTT) and reducing (+DTT) SDS-PAGE (coomassie-stained) and Western-blot (anti-hlgG-Fc; anti-NKp30) of purified 30LBD-Ig, 30Stalk-Ig and Ifnar2-Ig proteins. (D/E) 293T and COS-7 cells were decorated with 30LBD-Ig and 30Stalk-Ig proteins and analyzed by immunofluorescence microscopy (Ig fusion protein, green; To-Pro-3, blue) and FACS (30LBD-Ig, dashed line; 30Stalk-Ig, black line; Ifnar2-Ig, solid gray). A representative measurement is shown as histogram (middle), and the median fluorescence intensities (MFI, divided by the MFI of Ifnar2-Ig background staining) of ten independent measurements are plotted (right). (F/G) Same FACS experiment as D/E with CHO and K-562 cells and Ba/F3 cells either transduced with the NKp30 ligand B7-H6 (Ba/F3-B7-H6) or as control with GFP (Ba/F3-mock). (H) Binding of graded amounts of 30LBD-Ig (open circles) and 30Stalk-Ig (black circles) proteins to recombinant BAG-6 by ELISA. Data were corrected for Ifnar2-Ig background binding and fitted to a 1.1 Langmuir binding model to determine K_D and B_max values. A representative of three independent experiments is shown.

FIGURE 3. Equilibrium binding of NKp30 variants and B7-H6. Surface plasmon resonance (SPR) sensograms for the interaction for B7-H6-Ig fusion protein with immobilized NKp30-Ig fusion variants and Ifnar2-Ig as control. (A) 30Stalk-Ig wt (8850 RU)/B7-H6-Ig (250, 100, 50, 25, 10 and 0 nM). (B) 30LBD-Ig (9700 RU)/B7-H6-Ig (250, 100, 50, 25, 10 and 0 nM). (C) 30GS-Ig (10000 RU)/B7-H6-Ig (250, 100, 50, 25 and 0 nM). (D) 30Stalk-Ig Δ3 (8300 RU)/B7-H6-Ig (250, 100, 50, 25 and 0 nM). (E) 30Stalk-Ig Δ7 (7800 RU)/B7-H6-Ig (250, 100, 50, 25 and 0 nM). (F) 30Stalk-Ig Δ11 (6800 RU)/B7-H6-Ig (250, 100, 50, 25 and 0 nM). (G) 30Stalk-Ig N42Q (7676 RU)/B7-H6-Ig (1500, 1250, 1000, 500, 250 and 0 nM). (H) 30Stalk-Ig N68Q (10100 RU)/B7-H6-Ig (250, 100, 50, 25, 10 nM and 0 nM). (I) 30Stalk-Ig N121Q (10100 RU)/B7-H6-Ig (250, 100, 50, 25, 10 nM and 0 nM). (J) 30Stalk-Ig N42Q/N68Q (7800 RU)/B7-H6-Ig (250, 100, 50, 25, 10 nM and 0 nM). (K) 30Stalk-Ig N42Q/N121Q (9800 RU)/B7-H6-Ig (1500, 1250, 1000, 500, 250 and 0 nM). (L) 30Stalk-Ig N68Q/N121Q (10800 RU)/B7-H6-Ig (1500, 1250, 1000, 500, 250 and 0 nM). The gray lines represents the data corrected for the interspot data and were fitted to a bivalent binding model (colored lines) to determine K_D values. A representative of at least three independent experiments is shown. (O) Representative interspot data of 30Stalk-Ig wt/B7-H6-Ig demonstrating no non-specific binding to the surface.

FIGURE 4. Substitution of the stalk domain reduces the ligand binding affinity of NKp30. (A/B) 293T, COS-7, K-562 and MelJuSo cells (A) as well as Ba/F3 cells either transduced with the NKp30 ligand B7-H6 (Ba/F3-B7-H6) or as control with GFP (Ba/F3-mock) (B) were decorated with 30LBD-Ig, 30GS-Ig (stalk substituted by GS-linker), and 30Stalk-Ig proteins and analyzed by FACS. MFI ratios were determined as described in Fig. 2D/E (A/B) and normalized to 30Stalk-Ig (A). The MFI ratios of seven or more independent experiments are plotted. (C) BAG-6 ELISA with 30LBD-Ig (open circles), 30GS-Ig (open squares), and 30Stalk-Ig (black circles) proteins (compare Fig. 2G). A representative of three independent experiments is shown.

FIGURE 5. The stalk domain of NKp30 is sensitive to C-terminal truncation. (A/B) 293T, COS-7, K-562 and MelJuSo cells (A) as well as Ba/F3 cells either transduced with the NKp30 ligand B7-H6 (Ba/F3-B7-H6) or as control with GFP (Ba/F3-mock) (B) were decorated with 30LBD-Ig, 30Stalk-Ig variants C-terminally truncated by 3 (30Stalk-Ig Δ3), 7 (30Stalk-Ig Δ7) or 11 (30Stalk-Ig Δ11) amino acids or 30Stalk-Ig and analyzed by FACS (compare Fig. 4). (C) BAG-6 ELISA with 30LBD-Ig, 30Stalk-Ig Δ3, 30Stalk-Ig Δ7, 30Stalk-Ig Δ11 or 30Stalk-Ig protein. Data were fitted according to FIGURE 2G and shown as a Scatchard plot to visualize the different K_D but identical B_max values of the individual constructs. A representative of three independent experiments is shown.

FIGURE 6. The ectodomain of NKp30 is N-linked glycosylated at three sites. (A) Reducing SDS-PAGE (coomassie-stained) and Western blot (anti-hlgG-Fc) analysis of deglycosylated 30LBD-Ig and 30Stalk-Ig. (B) Non-reducing SDS-PAGE (coomassie-stained) and Western blot (anti-hlgG-Fc) analysis
of purified 30Stalk-Ig variants (wt, three single (N42Q, N68Q, N121Q), three double (N42Q/N68Q, N42Q/121Q, N68Q/N121Q), and one triple amino acid mutation (N42Q/N68Q/N121Q)). Asterisk indicates traces of serum albumin in the sample. (C) Reducing SDS-PAGE (coomassie-stained) and Western blot (anti-hIgG-Fc) analysis of deglycosylated 30Stalk-Ig variants. PNGaseF (removal of N-linked glycans); enzyme mix (removal of N- and O-linked glycans; protein deglycosylation mix, P6039S, New England Biolabs).

**FIGURE 7. The glycosylation status of NKp30 influences ligand binding.** (A) COS-7 cells were decorated with 30Stalk-Ig variants (wt, three single (N42Q, N68Q, N121Q), three double (N42Q/N68Q, N42Q/121Q, N68Q/N121Q), and one triple amino acid mutation (N42Q/N68Q/N121Q)) and analyzed by immunofluorescence microscopy (Ig fusion protein, green; To-Pro-3, blue). (B/C) Various cell lines (B) as well as Ba/F3 cells either transduced with the NKp30 ligand B7-H6 (Ba/F3-B7-H6) or as control with GFP (Ba/F3-mock) (C) were decorated with the 30Stalk-Ig variants and analyzed by FACS (compare Fig. 4).

**FIGURE 8. Importance of the stalk domain for NKp30 intracellular signaling.** (A) A5 reporter cells were retrovirally transduced with 30FL-his (full-length NKp30 with entire ectodomain), 30LBD-his (full-length NKp30 without stalk), or 30GS-his (full-length NKp30 stalk substituted by GS-linker) constructs or a control construct (mock). The indicated A5 transductants were sorted for NKp30 expression and NKp30 expression levels were determined by staining with an NKp30 antibody. Numbers represent mean fluorescence intensity. (B) The indicated NKp30 reporter cell lines were mixed with Ba/F3 target cells expressing the NKp30 ligand B7-H6 (Ba/F3-B7-H6) at the 1:1 effector:target ratios or stimulated with PMA and ionomycin. As a negative control, the NKp30 reporter cell lines were incubated without target cells. After 16 hours of co-culture, reporter gene expression was determined by analyzing GFP expression in CD4^+^ A5 cells. Numbers in histograms represent percentage of GFP-positive cells. (C) The indicated NKp30 reporter cell lines were mixed with Ba/F3 target cells expressing the NKp30 ligand B7-H6 (Ba/F3-B7-H6) at three effector:target ratios. After 16 hours of co-culture, reporter gene expression was determined by analyzing GFP expression in CD4^+^ A5 cells. Percentage of GFP-positive cells at the indicated reporter cell to target cell ratios are plotted as average of four independent experiments.

**FIGURE 9. The glycosylation status of NKp30 influences intracellular signaling.** (A) A5 reporter cells were retrovirally transduced with 30FL-his constructs (wt, three single (N42Q, N68Q, N121Q), three double (N42Q/N68Q, N42Q/121Q, N68Q/N121Q), and one triple amino acid mutation (N42Q/N68Q/N121Q)) or a control construct (mock). The indicated A5 transductants were sorted for NKp30 expression and NKp30 expression levels were determined by staining with an NKp30 antibody. Numbers represent mean fluorescence intensity. (B) The indicated NKp30 reporter cell lines were mixed with Ba/F3 target cells expressing the NKp30 ligand B7-H6 (Ba/F3-B7-H6) at the 1:1 effector:target ratios or stimulated with PMA and ionomycin. As a negative control, the NKp30 reporter cell lines were incubated without target cells. After 16 hours of co-culture, reporter gene expression was determined by analyzing GFP expression in CD4^+^ A5 cells. Numbers in histograms represent percentage of GFP-positive cells. (C) The indicated NKp30 reporter cell lines were mixed with Ba/F3 target cells expressing the NKp30 ligand B7-H6 (Ba/F3-B7-H6) at three effector:target ratios. After 16 hours of co-culture, reporter gene expression was determined by analyzing GFP expression in CD4^+^ A5 cells. Percentage of GFP-positive cells at the indicated reporter cell to target cell ratios are plotted as average of four independent experiments.

**TABLE LEGENDS**

**TABLE 1. Equilibrium binding constants (K_D) of 30Stalk-Ig variants to BAG-6 as determined by ELISA.** For experimental details refer to Fig. S6.
TABLE 2. Equilibrium binding constants ($K_D$) of 30Stalk-Ig variants to B7-H6 as determined by SPR. For experimental details refer to Fig. 3.
Figure 3

A. 30Stalk-Ig

B. 30LBD-Ig

C. 30GS-Ig

D. 30Stalk-Ig ∆3

E. 30Stalk-Ig ∆7

F. 30Stalk-Ig ∆11

G. 30Stalk-Ig N42Q

H. 30Stalk-Ig N68Q

I. 30Stalk-Ig N121Q

J. 30Stalk-Ig N42Q/N68Q

K. 30Stalk-Ig N42Q/N121Q

L. 30Stalk-Ig N68Q/N121Q

M. 30Stalk-Ig N42Q/N68Q/N121Q

N. 1fmar2-Ig

O. interspot data

Key:
- Response [RU]
- Time [s]

Kinetic Parameters:
- $K_c = 76 \pm 20$ nM
- $K_c = 149 \pm 19$ nM
- $K_c = 114 \pm 9$ nM
- $K_c = 132 \pm 36$ nM
- $K_c = 133 \pm 36$ nM
- $K_c = 83 \pm 19$ nM
- $K_c = 325 \pm 60$ nM
- $K_c = 472 \pm 125$ nM

Note: The diagrams show the binding kinetics for different ligands and mutants, with each panel representing a specific condition and its corresponding $K_c$ value.
Figure 4

A

B

C

Stalk domain-dependent ligand binding of NKp30
Figure 5

A

![Graph showing MFI ratio (%) for different samples](image)

B

![Graph showing MFI ratio (%) for different samples](image)

C

![Graph showing binding curves](image)
Figure 6

A  30LBD-Ig  30Stalk-Ig

<table>
<thead>
<tr>
<th>[kDa]</th>
<th>WT</th>
<th>N2Q</th>
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B  PNGaseF

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C  PNGaseF

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Figure 7

A

B

C

Stalk domain-dependent ligand binding of NKp30
Figure 8

A

expression level

122

7252

1292

1455

NKp30

B

negative control

Ba/F3-B7-H6

PMA/ionomycin

2.26

1.72

99.3

1.28

62.80

98.3

0.76

10.50

99.5

0.77

3.13

99.6

GFP

C

Ba/F3-B7-H6

mock

30FL-his

30LBD-his

30GS-his

E:T ratio

0

20

40

60

80

0

20

40

60

80

2:1

1:1

0.5:1
Figure 9

A
expression level

B
negative control
Ba/F3-B7-H6
PMA/ionomycin

C
Ba/F3-B7-H6

mock

30FL-his
wt

30FL-his
N42Q

30FL-his
N88Q

30FL-his
N121Q

30FL-his
N42Q/N88Q

30FL-his
N42Q/N121Q

30FL-his
N88Q/N121Q

GFP-positive effector cells [%]
Table 1

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<td>N68Q</td>
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<tr>
<td>N121Q</td>
<td>83 ± 19</td>
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<tr>
<td>N42Q/N68Q</td>
<td>&gt; 10 µM</td>
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<tr>
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<tr>
<td>N68Q/N121Q</td>
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Stalk domain-dependent ligand binding of NKp30