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High-level expression and purification of soluble form of human natural killer cell receptor NKR-P1 in HEK293S GnTI⁻ cells

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ABSTRACT

Human natural killer receptor protein 1 (NKR-P1, CD161, gene *klrb1*) is a C-type lectin-like receptor of natural killer (NK) cells responsible for recognition of its cognate protein ligand lectin-like transcript 1 (LLT1). NKR-P1 is the single human orthologue of the prototypical rodent NKR-P1 receptors. Naturally, human NKR-P1 is expressed on the surface of NK cells, where it serves as inhibitory receptor; and on T and NKT cells functioning as co-stimulatory receptor promoting secretion of IFNγ. Most notably, it is expressed on Th17 and Tc17 lymphocytes where presumably promotes targeting into LLT1 expressing immunologically privileged niches. We tested effect of different protein tags (SUMO, TRX, GST, MsyB) on expression of soluble NKR-P1 in *E. coli*. Then we optimized the expression construct of soluble NKR-P1 by preparing a library of expression constructs in pOPING vector containing the extracellular lectin-like domain with different length of the putative *N*-terminal stalk region and tested its expression in Sf9 and HEK293 cells. Finally, a high-level expression of soluble NKR-P1 was achieved by stable expression in suspension-adapted HEK293S GnTI⁻ cells utilizing pOPINGTTneo expression vector. Purified soluble NKR-P1 is homogeneous, deglycosylatable, crystallizable and monomeric in solution, as shown by size-exclusion chromatography, multi-angle light scattering and analytical ultracentrifugation.

1. Introduction

Natural killer (NK) cells are large granular lymphocytes described to be on the functional borderline of innate and adaptive immunity [1,2]. They are mainly recognized for their singular ability to provide defence against viral infection and tumour development without prior antigen sensitization [3], but they also contribute to the regulation of the adaptive system via secretion of cytokines [1] and are even able to form antigen specific immunologic memory [4–6]. NK cell activity is controlled by a fine balance of signals from its variety of inhibitory and activating receptors [3] that engage a broad range of health and disease markers in the accepted "missing-self" and "induced-self" modes of recognition, respectively [7–11].

NK cell receptors are divided into the immunoglobulin-like [12] and the C-type lectin-like (CTL) structural classes [13,14]. C-type lectins bind calcium and carbohydrates; however, CTL receptors recognize protein ligands instead, despite the fact that they are homologous to C-type lectins [15,16]. The NKR-P1 receptor family, encoded in the Natural Killer Cell (NKC) gene complex (human chromosome 12), encompasses the prototypical NK cell receptors belonging to the CTL class [15]. Unlike many CTL NK receptors that are recognizing MHC class I glycoproteins [14,17,18], NKR-P1 receptors interact with a genetically and structurally highly related ligands from *clec2* gene subfamily [13].

Human NKR-P1 (CD161, gene *klrb1*) was identified in 1994 as a human orthologue of rodent NKR-P1 receptors [19] and up to now remains the only described human NKR-P1 receptor. However, human NK receptors from the *klrf* subfamily – i.e. NKp65 [20] and NKp80 [21] share distinct similarity to NKR-P1 and were proposed to represent activating counterparts of human NKR-P1 [13,22].

Apart from NK cells, human NKR-P1 was found to be expressed on NKT cells [23] and subpopulations of T lymphocytes [24]. Most

Abbreviations: CTL, C-type lectin-like; GnTI⁻, *N*-acetylglucosaminyltransferase I negative; HEK, human embryonic kidney; LLT1, lectin-like transcript 1; lPEI, linear polyethylenimine; MALS, multi-angle light scattering; NK, natural killer; SEC, size-exclusion chromatography.

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notably, human NKR-P1 is present on regulatory T cells [25] and is currently recognized to be a marker for all Th17 cells [26]. It was found also on some Tc17 cells [27] which are being more and more implicated in autoimmune diseases like multiple sclerosis [28], rheumatoid arthritis [29] and Crohn's disease [30]. It was proposed that NKR-P1 could play a role in targeting of these lymphocytes and promote transendothelial extravasation into immunologically privileged niches [26,31–34].

Under homeostasis NKR-P1 functions as inhibitory receptor of NK cells [19,35,36] and co-stimulatory receptor of NKT and T cells [35,37] promoting secretion of IFNγ. However, it was also described that the inhibitory function of human NKR-P1 is in an undesirable way exploited by glioblastomas [38] and B-cell Non-Hodgkin's lymphomas [39] which overexpress the NKR-P1 physiological ligand – lectin-like transcript 1 (LLT1, gene *clec2d*) [35,36,40,41] and thus escape immune response.

From a protein point of view, human NKR-P1 shares common CTL receptor features. It was identified as a homodimeric type II. transmembrane glycoprotein lacking O-linked glycosylation [19]. Its short intracellular portion contains an immunoreceptor tyrosine-based inhibitory motif that is noncanonical for the presence of alanine residue in the −2 position relative to the tyrosine residue [42]. A transmembrane helix is followed by 25 residues long stalk region that presumably functions as a flexible linker providing a scaffold for cystine homodimerization and a *C*-terminal CTL domain that itself contains 6 conserved cysteine residues stabilizing this domain by formation of three intramolecular disulphide bridges [14].

Although there have been recently promising results for refolding of murine NKR-P1 receptors from inclusion bodies produced in *E. coli* [43,44], so far only an unsuccessful renaturation of the human orthologue have been reported [45]. Mammalian cell lines were used previously to express full-length human NKR-P1 receptor or its extracellular part in low-scale for immunological studies [35,36,40,41] or surface plasmon resonance measurements [45], respectively. Here we present an optimization of the human NKR-P1 ectodomain expression in different expression systems and finally an utilization of HEK293S GnTI$^-$ cells [46] for generation of stably transfected cell line that provides a high yield of soluble human NKR-P1 ectodomain usable for structural studies.

2. Material and methods

2.1. Vectors and NKR-P1 library cloning

A cDNA clone (GenBank accession no. BC114516) containing the entire coding sequence of *klrb1* gene was obtained from Source BioScience (GenomeCUBE IRCMp5012E0732D). A library of NKR-P1 stalk region deletion expression vectors was constructed by In-Fusion cloning at the Oxford Protein Production Facility (OPPF; Oxford, UK) as described before [47,48]. Primers used for amplification of the selected NKR-P1 constructs from the cDNA clone contained In-Fusion overlaps at the 5′ of the specific forward and the reverse primers as described in supplementary data, Table S1. For transient expression in HEK293 cells, NKR-P1 ectodomain (G90-S225) was amplified from the cDNA clone using 5′–AAAAAAACCGGTGGTCTCTTAAACTGCCCAATATATTG–3′ and 5′–AAAAAAGGTACCAGAGTCAGGATACACTTTATTTCTCAC–3′ and using *Age*I and *Kpn*I sites subcloned into pTT28 expression plasmid (kindly provided by Dr. Yves Durocher; a derivative of pTT5 [49] containing *N*-terminal secretion leader and *C*-terminal His_8-tag sequence, thus leaving ITG- and -GTKHHHHHHHHG at expressed protein *N*-and *C*-termini).

2.2. Small-scale NKR-P1 expression tests

Expression of the library of stalk region deletion constructs was performed at OPPF following standard OPPF protocols for high-throughput expression testing in *E. coli* [50] and Sf9 cells [51] as described before.

Briefly, for prokaryotic expression 150 μl of overnight cultures grown from selected colony of *E. coli* Rosetta2(DE3) pLysS or B834(DE3) strains (both Novagen) transformed with the given expression plasmid were used for inoculation of 3 ml of Power Broth (Molecular Dimensions) and Overnight Express Instant TB Medium (TBONEX; Novagen) with an appropriate antibiotic in 24-well deep well blocks. The blocks were shaken at 37 °C until an average OD_{595} reached ca 0.5. The Power Broth cultures were cooled to 20 °C and expression was induced by addition of IPTG to 1 mM final concentration and left to produce overnight. The TBONEX cultures were cooled to 25 °C and left to produce for 20 h.

Bacterial cultures were centrifuged and frozen until analysis. Defrosted cell pellets were resuspended in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, 1% v/v Tween 20, pH 8.0) supplemented with lysozyme and DNAse I, incubated for 30 min and the lysates were cleared by centrifugation in deep-well block (6000 × g, 30 min, 4 °C). Expression levels were analysed by Coomassie stained reducing SDS-PAGE from soluble fraction of cell lysates [50].

For insect cell expression, to generate a P0 virus stock Sf9 cells were co-transfected with linearized bacmid DNA (Bac10:KO_{1629} [52]) and pOPIN vector from the stalk region deletion library as a transfer vector. For small-scale expression tests 3 ml of 1 × 10^6 Sf9 cells/ml in 24-well deep blocks were infected with 3 and 30 μl of P1 virus stock and left to produce at 27 °C for 72 h. For scale up, 800 ml of Sf9 production culture in shaken Thompson flask was infected with 800 μl of P2 virus stock. Media were harvested and purified after 7 days.

For mammalian expression tests, 4 μg of the given expression plasmid and 10 μl of Lipofectamine 2000 (Invitrogen, USA) were each diluted into 25 μl of Freestyle F17 media (Invitrogen, USA), incubated for 5 min, mixed and incubated for 10 min again before addition to 2 × 10^6 HEKT293T cells grown in 1 ml of Freestyle F17 medium on a shaken 24-well culture plate (Corning, USA) at 37 °C, 5% CO_2. After 4 h cell cultures were diluted with 1 ml of EX-CELL293 serum-free medium (Sigma, USA) and left to produce for 72 h.

Expression tests from insect and mammalian cell cultures were analysed by enriching the secreted products by IMAC on Ni-NTA magnetic beads (Qiagen and Biotool, USA) from 1 ml of the production media and either analysed by SDS-PAGE or by Western blot and immunodetection with primary mouse PentaHis anti-His-tag monoclonal antibody (Qiagen, USA) and secondary HRP or AP conjugated anti-mouse IgG antibody (R&D Systems, USA; Sigma, USA).

2.3. Transient NKR-P1 expression in HEK293T cells

HEK293 cell lines were grown in suspension as described in Ref. [41] in mixture of equal volumes of EX-CELL293 and Freestyle F17 media in shaken square-shaped glass bottles within humidified 37 °C, 5% CO2 incubator. For transient expression of soluble NKR-P1 ectodomain, 400 μg of the pTT28 expression plasmid were diluted in PBS, filter-sterilized and 25 kDa linear polyethylenimine (Polysciences, USA) was added in 1:3 (w/w) ratio to 4 ml final volume, the mixture was shaken and incubated for 5 min. Meanwhile, 400 × 10^6 HEK293T cells were centrifuged and resuspended in 200 ml of Freestyle F17 and immediately transfected. Following 4 h incubation, the culture was diluted with 200 ml of EX-CELL293. 5–7 days post-transfection culture medium was harvested by