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"In vitro" studies on galectin-3 in human natural killer cells

Alvaro Brittoli[a], Silvia Fallarini[a], Hao Zhang[b], Roland J. Pieters[b], Grazia Lombardi[a,*]

[a] Department of Pharmaceutical Sciences, University of "Piemonte Orientale, A. Avogadro", Largo Donegani 2, 28100 Novara, Italy
[b] Department of Medicinal Chemistry and Chemical Biology, Utrecht University, 3508 TB Utrecht, Netherlands

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ABSTRACT

Galectin-3 (Gal-3) is a β-galactoside binding protein able to modulate both innate and adaptive immune responses. First identified in macrophages, Gal-3 has been studied widely in many mammalian immune cells, but scarcely in natural killer (NK) cells.

The aim of this study was to analyze Gal-3 in human NK cells, isolated from peripheral blood mononuclear cells.

Both PCR and RT-PCR analysis showed that resting human NK cells express Gal-3 mRNA, which can be modulated upon cytokine stimulation (100 U/ml IL–2 + 20 ng/ml IL-15) for different period of time (1–24 h). Western blot, cytofluorimetry, and confocal microscopy analysis clearly demonstrated that the Gal-3 gene can translate into the corresponding protein. From our results, resting NK cells, isolated from different healthy donors, can express high or low basal levels of Gal-3. In NK cells, Gal-3 was always intracellularly detected at both cytoplasm and nucleus levels, while never at the membrane surface, and its localization resulted independent from the cellular activation status. In addition, the intracellular Gal-3 can co-localize with perforin in exocytic vesicles.

Cell treatment with a thiodigalactoside-based Gal-3 inhibitor (1–30 μM) slightly increased the number of degranulating NK cells, while it significantly increased the percentage of cells releasing high amounts of cytotoxic granules (+ 36 ± 3% *vs.* inhibitor-untreated cells at 30 μM Gal-3).

In conclusion, our results demonstrate that human resting NK cells express Gal-3 at both gene and protein levels and that the Gal-3 expression can be modulated upon cytokine stimulation. In the same cells, Gal-3 always localizes intracellularly and functionally correlates with the degree of NK cell degranulation.

1. Introduction

Galectin-3 (Gal-3) belongs to the galectin family of glycan binding proteins, which share similar binding affinities for β-galactosides and a highly conserved amino acid sequence in the carbohydrate recognition domain (CRD), responsible for glycan binding [1]. In vertebrates, among all galectins discovered so far, Gal-3 is the only one "chimera-type" containing a CRD fused to a non-lectin collagen-like sequence, formed by Pro-Gly-Tyr tandem repeats and an amino-terminal region [2].

First identified in macrophages [3], Gal-3 is ubiquitously expressed in many immune cells, playing a key role in modulating both innate and adaptive immune responses [4–6]. In particular, Gal-3 has been found in monocytes/macrophages, dendritic cells (DCs), mast cells, eosinophils, neutrophils, basophils, T and B cells [7,4], and the level of relative expression seems to be tightly regulated by the cellular activation state. Gal-3, for example, is not detectable in resting B and T cells, but it can be induced following IL-4 stimulation or CD40 cross-linking in B cells [8], and by TCR engagement, or mitogen exposure, in T cells [9].

Depending on the cell type Gal-3 can exhibit a diverse range of subcellular localization, being found in the cytoplasm [10], in the nucleus [11], or in both compartments with a distribution that changes with cellular proliferation, differentiation, and development [12].

Gal-3 lacks a signal sequence for transferring through the endoplasmic reticulum and Golgi system (the classical secretory pathway) and it follows a non classical secretory pathway [13,14]. In particular, Gal-3 can be secreted via exosomes [15] with the N-terminal domain of

Abbreviations: (Gal-3), Galectin-3; (CRD), Carbohydrate recognition domain; (DCs), Dendritic cells; (NK), Natural killer; (IFN)-γ, Interferon; (LAK), Lymphokine-activated killer cells; (FACS), Flow cytometry; (MFI), Median fluorescence intensity; (ESCRT), Endocytic trafficking complexes; (FBS), Fetal bovine serum; (PBS), Phosphate-buffered saline; (BSA), Bovine serum albumin; (RT), Room temperature; (HRP), Horseradish peroxidase; (ECL), Enzyme linked chemiluminescence; (PFA), Paraformaldehyde; (rh), Recombinant human

* Corresponding author.
E-mail addresses: alvaro.brittoli@uniupo.it (A. Brittoli), silvia.fallarini@uniupo.it (S. Fallarini), H.Zhang2@uu.nl (H. Zhang), R.J.Pieters@uu.nl (R.J. Pieters), grazia.lombardi@uniupo.it (G. Lombardi).

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the protein responsible for locating Gal-3 into these structures [13].

In immune cells, trough different protein–protein interactions, Gal-3 can control several physiological and pathological processes, ranging from cell adhesion, activation, differentiation and survival to apoptosis, chemotaxis and cytokine secretion [16].

Concerning the expression and the function of Gal-3 in natural killer (NK) cells, literature data, although very scarce, suggest the importance of this protein on NK cell-mediated toxicity. Gal-3 knock-out mice, in fact, have a low amount of cells with altered cytotoxicity, if compared to wild type animals [17,18].

NK cells are a major player in innate immune responses, exerting direct cytotoxic activities against pathogen-infected or tumor cells with an activity similar to that of $CD8^+$ cytotoxic T lymphocytes [19].

To fulfill their direct toxicity on specific targets, NK cells do not express clonal antigen receptors, produced by somatic recombination, instead they are equipped with a wide array of germline-encoded activating and inhibitory receptors [20], which can recognize both soluble ligands, such as cytokines and cell surface antigens, released/expressed by cells under pathological conditions. The integration of the positive and negative signals, generated by both activating and inhibiting receptors, shape the type of NK cell responses [21].

NK cells can also influence other immune cell functions through the production of various pro-inflammatory cytokines (i.e., interferon [IFN]-γ) [22,23], and several chemokines (i.e., CCL3, CCL4, CCL5, and CXCL8) [20].

Similarly, cytokine released by surrounding cells and/or present in the microenvironment can promote or dampen NK cell activity. In particular, IL-2 and IL-15 represent two important cytokines able to tightly regulate NK cell functions. Both, in fact, are able to transform resting NK cells into lymphokine-activated killer cells (LAK) with effector cytotoxic functions against cancer cells, which otherwise are resistant to NK cell-mediated toxicity [24,25].

Here, we have examined the expression and localization of Gal-3 in human resting and activated NK cells. Collectively, our results demonstrate that these cells express both Gal-3 mRNA and protein, whose expression levels are highly variable among donors. In addition, we show that Gal-3 protein localizes in different subcellular compartments and often co-localizes with perforin in exocytic vesicles.

Furthermore, Gal-3 expression functionally correlates with the degree of exocytic vesicles released during degranulation.

2. Materials and methods

2.1. Human NK cell isolation and cell cultures

Human NK cells were isolated from buffy coats of healthy donors ($n \geq 6$), after their informed consensus, as previously described [26]. After cell separation through negative selection (Miltenyi Biotec, Calderara di Reno, Italy), the percentage of $CD3^-CD56^+$ cells was routinely analyzed by FACS (S3 flow cytometer, Biorad, Segrate, Italy) using FITC anti-human CD56 (NCAM) Antibody [Clone: MEM-188 (BioLegend, San Diego, CA, USA)], PerCP anti-human CD3 [Clone: BW264/56 (Miltenyi Biotec)] and always resulted ≥97%. Approximately 6×10^6 NK cells were routinely obtained from each patient.

Human NK cells, human erythroleukemia K562 cells (a generous gift from Prof. M.C. Mingari, Department of Experimental Medicine, IRCCS AOU San Martino-IST, Genova, Italy), human monocytic leukemia THP-1 cells (ATCC TIB-202; American Type culture Collection, Manassas, VA, USA) and lymphoblastic leukemia Jurkat cells (ATCC TIB-152) were cultured (1×10^6 cells/ml) in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Carlo Erba, Cornaredo, Italy), 2 mM L-glutamine, 100 μg/ml kanamycin, 1 mM sodium pyruvate e 1% MEM amino acid solution (Sigma-Aldrich, Milan, Italy) in a humidified incubator at 5% CO_2 and 37 °C. Recombinant human (rh) IL-2 (100 U/ml) and rh IL-15 (20 ng/ml) (PeproTech, London, UK) were added, if required, to the NK cells only at the beginning of the culture.

Table 1
Oligonucleotide primers used for PCR and real-time PCR.

Template	Primers
Galectin-3	forward 5′-GCAGACAATTTTTCGCTCCATG-3′ reverse 5′-CTGTTGTTCTCATTGAAGCGTG-3′
GAPDH	forward 5′-GGTCGGAGTCAACAACGGATTTGG-3′ reverse 5′-ACCACCCTGTTGCTGTAGCCA-3′
S18	forward 5′-TGCGAGTACTCAACACCAACA-3′ reverse 5′-CTGCTTTCCTCAACACCACA-3′

2.2. PCR and real-time PCR

Total RNA was isolated from 5×10^5/ml unstimulated/stimulated NK, THP-1 (positive control) and Jurkat (negative control) cells using the RNeasy Mini Kit (Qiagen, Hilden, Germany), according to manufacturer's instructions, and reverse transcribed into cDNA [27].

For PCR amplification, 3 μL of cDNA were added to GoTaq Flexi DNA Polymerase (Promega, Madison, WI, USA) in 25 μL reactions, containing 0.5 μM of forward and reverse primers. Amplification products were visualized on 1% agarose gel, containing 1 μg/ml ethidium bromide (Sigma–Aldrich). For real-time PCR, cDNA samples were equally diluted for subsequent PCR amplification with Maxima SYBR Green qPCR Master Mix (Fermentas, Milan, Italy) and a final volume of 20 μL, which contained 1 μL of template cDNA, was used. Real-time PCR analysis were performed in triplicate for each sample in a CFX96 Real-Time PCR system (Bio Rad). The level of 18S RNA was measured and used for normalization of the target gene abundance. The primer sequences used for PCR and real-time PCR are listed in Table 1.

2.3. Western blot

Proteins were extracted from 1×10^6 human unstimulated/stimulated (24 h) NK cells, THP-1 (taken as positive control for Gal-3 expression), K562 (taken as positive control for Gal-1 expression), and Jurkat (negative control) cells [28].

Equal amounts of proteins (35 μg) were separated on 12% polyacrylamide gel and electro-blotted on nitrocellulose membrane. The membranes were blocked and then incubated with the following primary antibodies: rabbit anti-human Gal-3 antibody [Clone: EP2275Y (Abcam, Cambridge, UK); 1:5000], mouse anti-human Gal-1 antibody [Clone: C-8 (Santa Cruz Biotechnology, Heidelberg, Germany); 1:500], or mouse anti-human β-Actin antibody [Clone: AC15 (Sigma-Aldrich); 1:5000] with gentle shaking overnight at 4 °C or for 1 h at room temperature (RT) for β-Actin. The blots were, then, incubated with a specific anti-rabbit horseradish peroxidase (HRP) labelled secondary antibody (Ge Healthcare Life Sciences; 1:5000) or a specific anti-mouse HRP labelled secondary antibody (Sigma–Aldrich; 1:5000) for 1 h at RT.

Proteins were visualized with an enzyme linked chemiluminescence (ECL) detection kit, according to the manufacturer's instructions (Ge Healthcare Life Sciences). The protein signals were detected using an enhanced chemiluminescence system (Biorad). Band intensities were quantified by the computer program Image Lab from Biorad.

All samples were analyzed independently and the final increase in Gal-3 protein was expressed as mean ± SEM of at least six independent experiments.

2.4. Flow cytometry

5×10^5/ml human NK cells were seeded in 24 multiwell plates and unstimulated/stimulated (24 h) with IL-2 (100 U/ml) and IL-15 (20 ng/ml). THP-1 (positive control) and Jurkat (negative control) cells were always included in the experiments. Cells were harvested and washed