Immunology Letters 194 (2018) 4–12

Contents lists available at ScienceDirect

Immunology Letters

journal homepage: www.elsevier.com/locate/immlet

"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

ARTICLE INFO

Keywords:
Glycan binding proteins
Lectins
Innate immune cells
NK cell activation

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).

https://doi.org/10.1016/j.imlet.2017.12.004
Received 27 July 2017; Received in revised form 12 October 2017; Accepted 11 December 2017
Available online 14 December 2017

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

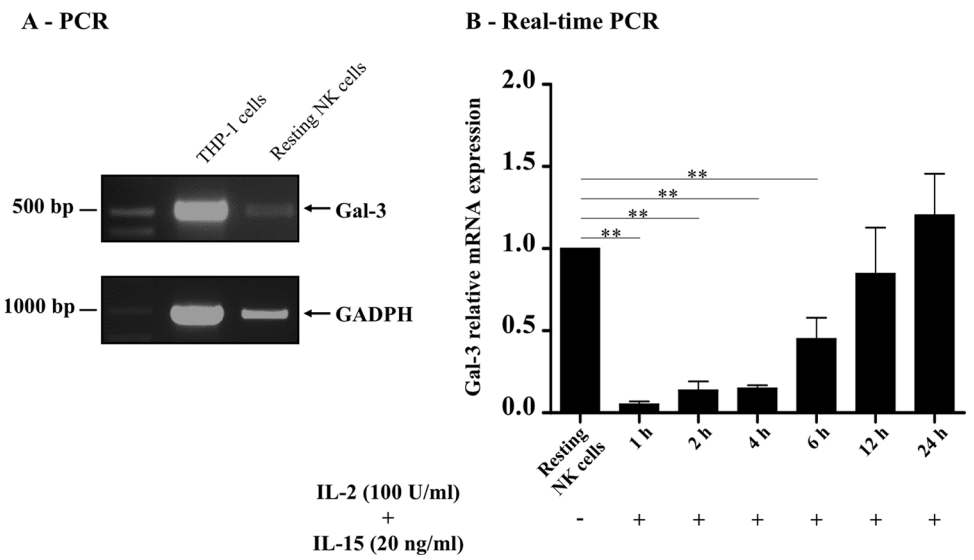

Fig. 1. Gal-3 mRNA is expressed in human NK cells and its expression is modulated by cell activation. (A) Gal-3 mRNA expression was analyzed by PCR in NK and THP-1 (positive control) cells. The total extracted mRNA was reverse transcribed to cDNA and Gal-3 cDNA amplified using gene-specific primers (see Table 1). PCR products were electrophoresed in 1% agarose gel and PCR fragments sized using a 50–10,000 bp ladder. The expected fragment size for Gal-3 is 500 bp. GAPDH was amplified as a loading control with a fragment size of 1000 bp. (B) Time course of Gal-3 gene expression in activated NK cells measured by real-time PCR. Total mRNA was extracted from NK cells unstimulated/stimulated with IL-2 (100 U/ml) and IL-15 (20 ng/ml) for 1–24 h, converted to cDNA, and analyzed by real-time PCR for Gal-3 expression. The mRNA levels are expressed relative to 18S mRNA, and the value of the resting cells was set at 1. The results represent the mean ± SEM of at least six independent experiments. $^{**}P \leq 0.01$ vs. unstimulated NK cells.

with a FACS wash solution (0,5% bovine serum albumin [BSA]-supplemented phosphate-buffered saline [PBS] and 1% FBS). For the intracellular staining, the samples were fixed with cold paraformaldehyde (4% PFA) for 15 min at RT and permeabilized with 100% cold acetone for 10 min at −20 °C. All samples were, then, incubated with a blocking solution (1% BSA-supplemented PBS and 2% FBS) for 30 min at RT. Afterwards, the samples were incubated for 30 min with the following primary antibodies: anti-human Gal-3 antibody [Clone: EPR2774 (Abcam); 1:65] or isotype control [Clone: EPR25A (Abcam); 1:65]. After washing, the cells were labelled with the Alexa 555-conjugated secondary antibody (Abcam; 1:1000). Samples were acquired and analyzed using a S3 flow cytometer (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.

2.5. Confocal microscopy

5×10^5/ml human NK cells, 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 harvested, washed with PBS and fixed with cold 4% PFA for 15 min at RT. For intracellular staining, the cells were permeabilized with 100% cold acetone for 10 min at −20 °C; for extracellular staining, the permeabilization occurred before nuclear staining. Fixed cells were blocked with blocking solution for 1 h at RT, incubated with anti-human Gal-3 [Clone: EP2275Y (Abcam); 1:65] or isotype primary antibody [Clone: EPR25A (Abcam); 1:65] for 30 min at RT and subsequently with Alexa 555-conjugated secondary antibody (Abcam; 1:1000) for 30 min at RT. To localize Gal-3 in exocytic vesicles NK cells were also stained with FITC-conjugated anti-perforin antibody [Clone: HCD56 (BioLegend)] (positive control) for 15 min at RT. After washing, nuclei were counterstained with To-pro-3 iodide (Thermo Fisher Scientific; 1:500) for 15 min at RT and cells centrifuged onto cytospin slides at 1200 rpm for 5 min (Shandon Cytospin 4, Thermo Fisher Scientific). Prolong Gold anti-fade kit (Thermo Fisher Scientific) was used as mounting fluid on the cells. Images were collected using a Leica TCS SP2 confocal microscope with 63X oil immersion objective and by sequential scanning. Images were acquired at a scanning speed of 400 Hz, 1024 × 1024 pixel resolution and a line average of 4. A 488 nm laser was used for IgG-FITC, 543 nm for IgG-Alexa 555 and 633 nm for To-Pro 3-iodide.

2.6. NK cell degranulation assay

NK cell degranulation was measured as previously described [29], by determining the expression of CD107a, the lysosome-associated membrane protein-1 (LAMP-1) in absence/presence of a thiodigalactoside-based Gal-3 inhibitor [30]. This inhibitor (Bis-{3-deoxy-3-[4-(phenyl)-1H-1,2,3-triazol-1-yl]-β-D-galactopyranosyl} sulfane) was a generous gift by Prof. Roland J. Pieters (Department of Medicinal Chemistry and Chemical Biology, Utrecht University, Netherlands). For the experiments, a stock solution in 40% DMSO was prepared and diluted in RPMI-1640 before cell treatment (DMSO concentration did not exceed 0.1% v/v). NK cells unstimulated/stimulated (24 h) with IL-2 (100 U/ml) and IL-15 (20 ng/ml), were untreated/treated with increasing concentrations (1–30 μM) of the Gal-3 inhibitor for 1 h at 37 °C. To induce NK cell degranulation, the cells were incubated with K562 cells (target cells) at an effector/tumor cell ratio of 1:1 for 1 h at 37 °C in presence of anti-CD107a-Phycoerythrin (PE) (Miltenyi Biotec). Monensin (BD GolgiStop™ reagent, BD Biosciences, Milan, Italy) was added and cells incubated for 3 h at 37 °C. Cells were washed, stained using FITC anti-human CD56 (NCAM) Antibody [Clone: MEM-188 (BioLegend)], and CD107a expression on $CD56^+$ cells evaluated by FACS [26]. To detect spontaneous NK cell degranulation, a negative control (NK cells without K562 target cells) was always included, while to measure the basal response, NK cells were stimulated only with IL-2 (100 U/ml) and IL-15 (20 ng/ml) (positive controls). All control samples were treated with 0.1% DMSO.

2.7. Statistical analysis

Results were expressed as means ± SEM of at least four independent experiments always run in triplicate. Statistical significance was evaluated by the one-way ANOVA followed by Student's *t*-test for unpaired populations (Graph Pad Software, Inc., San Diego, USA). Differences were considered statistically significant when $P \leq 0.05$.

3. Results

3.1. Galectin-3 gene expression in human resting NK cells

To determine whether human NK cells express the gene coding for Gal-3 we first performed PCR studies on resting NK cells. Human THP-1 cells were always taken as positive control. Our results clearly demonstrate that human resting NK cells express Gal-3 mRNA, as shown in Fig. 1A.

3.2. Galectin-3 mRNA in human activated NK cells

Since the expression of *Gal-3* in a variety of immune cells can be modulated by cellular activations and environmental stimuli [31,8,32,33], we measured the levels of Gal-3 mRNA in NK cells unstimulated/stimulated with IL-2 (100 U/mL) and IL-15 (20 ng/ml) for different periods of time (1–24 h) by real-time PCR. Fig. 1 B shows that the expression of Gal-3 mRNA significantly ($P \leq 0.01$) decrease (–92 ± 2% in comparison to resting NK cells) after 1 h of cytokine stimulation, and this decrease lasts for at least 6 h. Afterwards (12–24 h), the mRNA expression level progressively restore to the basal level.

The overall data demonstrate that human NK cells can regulate the gene expression of Gal-3 following specific stimuli for an appropriate period of time.

3.3. Galectin-3 protein in human activated NK cells

Then, to determine whether the modulation of Gal-3 mRNA levels, measured in activated NK cells (see Results, 3.2), correlates with changes at protein levels, the same cells were analyzed by western blot. The estimated total Gal-3 protein levels were always normalized against the corresponding β-Actin level and compared to those measured in resting cells.

As shown in Fig. 2, we determined resting NK cells with high (Fig. 2A) or low (Fig. 2B) basal level of Gal-3 protein. This variability was consistently measured in all experiments and seems related to the individual donor analyzed. Following cytokine stimulation for 24 h the Gal-3 levels resulted significantly ($P \leq 0.05$) increased in all samples analyzed (+116 ± 32% in comparison to resting NK cells) (Fig. 2C).

In THP-1 cells (positive control) Gal-3 protein was always detectable, while no expression was determined in Jurkat cells (negative control).

These data indicate that upon IL-2 and IL-15 stimulation human NK cells can increase the level of Gal-3 protein.

3.4. Galectin-3 protein localization in human NK cells

While being predominantly a cytosolic protein, Gal-3 can be detected also at cellular surface [34]. To study the Gal-3 localization in human NK cells the Gal-3 levels were measured in resting cells by flow cytometry (FACS). Median fluorescence intensity (MFI) measurements confirm previous data from the western blot analysis (see Results, 3.3): resting NK cells express high or low basal levels of Gal-3 protein, as clearly shown by the distinct separation of each histogram from the isotype control (Fig. 3A). These data confirm that human NK cells can express different levels of Gal-3 depending on the individual donor analyzed.

IL-2 (100 U/mL) and IL-15 (20 ng/ml) stimulation for 24 h, significantly ($P \leq 0.01$) increased (+30 ± 9% in comparison to resting cells) (Fig. 3B) the levels of Gal-3 expression in NK cells.

Then, to specifically localize this lectin NK cells were incubated with the primary antibody before (extracellular) or after (intracellular) the permeabilization step (see Methods, 2.5). In all samples analyzed, Gal-3 did not localize at the membrane cell surface, neither in resting nor in activated cells, indicating that human NK cells do not express this protein at the membrane level. In THP-1 cells, taken as positive control, Gal-3 was both extracellular and intracellular detectable (Fig. 3A and 4A), while no expression was determined in Jurkat cells (negative control).

Since Gal-3 differently localize depending on the immune cell type [7,35,33], to investigate the subcellular localization of Gal-3 in human NK cells immunofluorescence staining studies were performed in cells, unstimulated/stimulated with IL-2 (100 U/mL) and IL-15 (20 ng/ml) for 24 h (Fig. 4A), before (extracellular) or after (intracellular) the permeabilization step. At the membrane level, confocal microscopy analysis revealed an extracellular diffuse staining in THP-1 cells (positive control: Fig. 4Aa), while all NK cell samples analyzed, either resting or activated (Fig. 4Ab and 4Ac, respectively), resulted negative. These data demonstrated that human NK cells do not express membrane-bound Gal-3 protein, confirming the previous results obtained by FACS. Conversely, all NK cell samples resulted positive for the intracellular staining (Fig. 4Ae-f and 4Ah-i), where Gal-3 was visualized as scattered dots within the cells. Interestingly, we observed positive (Fig. 4Af and h, close arrows) and negative (Fig. 4Af and h, open arrows) cells in both resting (Fig. 4Ae and f) and activated (Fig. 4Ah and i) samples from the same individual. In positive cells, the intracellular localization of Gal-3 resulted heterogeneous, as some cells mainly localize the protein in cytoplasm and nucleus (Fig. 4Ba and b, open arrows), while others showed fluorescent dots only in cytoplasm (Fig. 4Ba

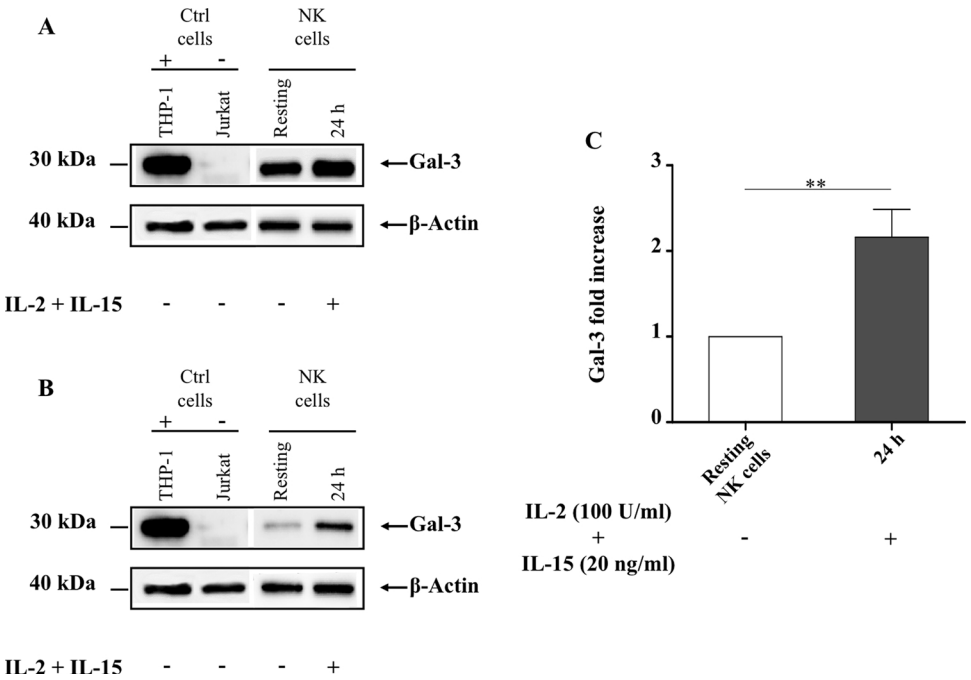

Fig. 2. Gal-3 protein is expressed in human NK cells and its expression increases upon cell activation. Gal-3 protein expression was evaluated by western blot analysis in NK cells, unstimulated/stimulated (24 h) with IL-2 (100 U/ml) and IL-15 (20 ng/ml), THP-1 (positive control) and Jurkat (negative control) cells. Total cellular proteins (35 µg) were resolved by 12% polyacrylamide gel and immunoblotted with specific anti-human galectin-3 antibody. β-Actin was used as internal control. Left panels are representative of typical immunoblottings from NK cells expressing high (A) or low (B) basal levels of Gal-3. The western blot signals were densitometrically analyzed by Image Lab software and results expressed as fold increase of Gal-3 signal in stimulated cells compared to unstimulated cells set at 1 (C). The results represent the mean ± SEM of at least six independent experiments. $^{**}P \leq 0.01$ vs. unstimulated cells.

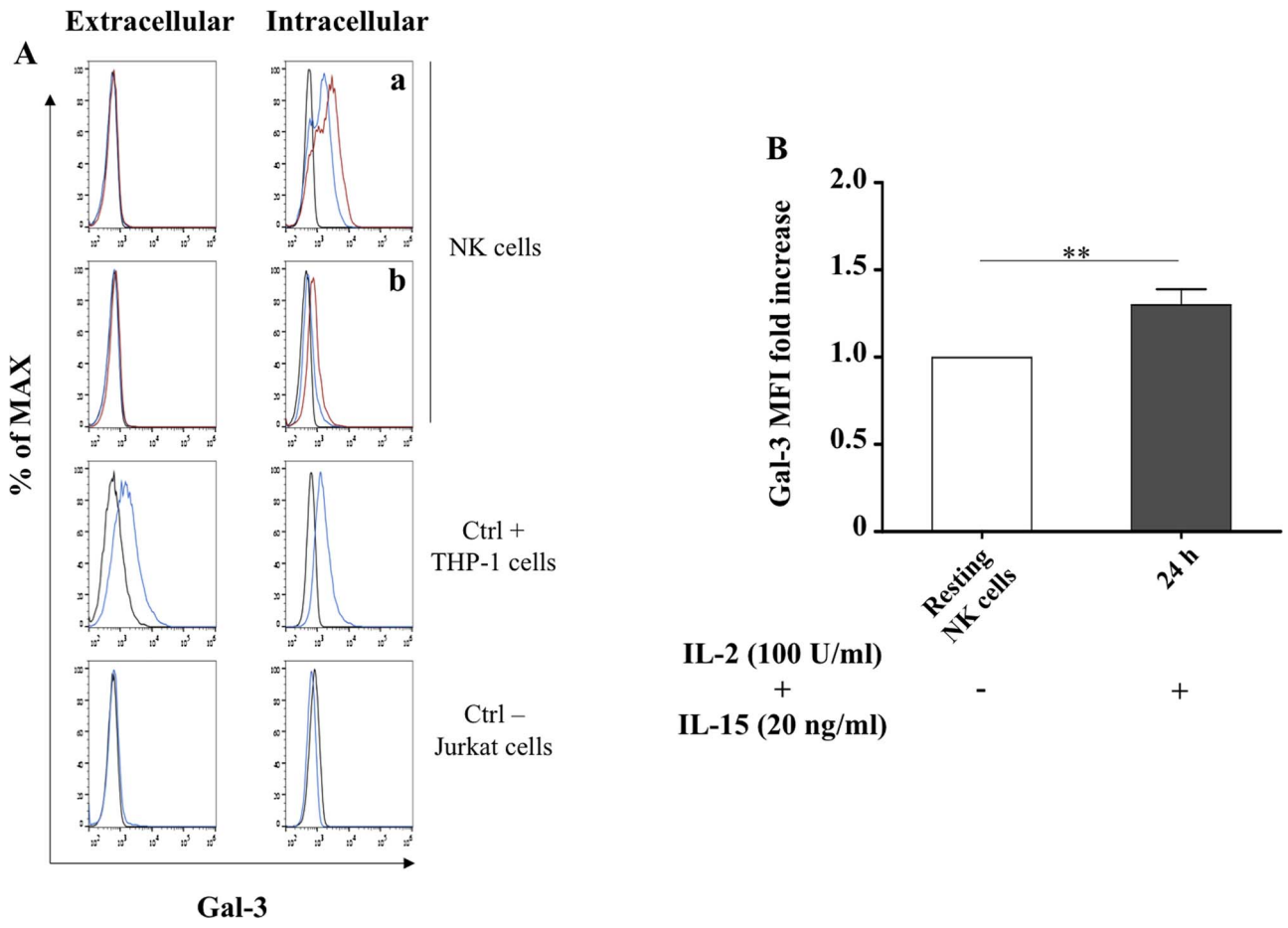

Fig. 3. Gal-3 is intracellularly expressed in human NK cells and its expression increases upon cell activation. NK cells, unstimulated/stimulated for 24 h with IL-2 (100 U/ml) and IL-15 (20 ng/ml), THP-1 (positive control) and Jurkat (negative control) cells were harvested, stained with anti-human Gal-3 and analyzed by FACS. (A) Representative histograms of extracellular (left panels) and intracellular (right panels) Gal-3 expression in human NK with high (a) and low basal (b) Gal-3 expression, THP-1 and Jurkat cells. Resting cells are shown as blue histograms, stimulated NK cells as red, while the isotype controls as black histograms. (B) Mean Fluorescence Intensity (MFI) levels of Gal-3 in human NK cells expressed as fold increase in stimulated cells compared to unstimulated cells set at 1. The results represent the mean ± SEM of at least six independent experiments. $^{**}P \leq 0.01$ vs. unstimulated cells. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

and b, close arrows). These results were independent from the cellular activation status. Moreover, overlapping the perforin and Gal-3 stains (yellow dots; Fig. 4Bc), we observed that Gal-3 can occur within perforin in exocytic vesicles. Finally, the analysis of positive cells confirm the high variability of Gal-3 expression in NK cells: some samples having high levels (Fig. 4Ae and h), while others low levels of Gal-3 (Fig. 4Af and i). Jurkat cells, taken as negative control, resulted always unstained (Fig. 4Ag).

3.5. Galectin-3 functional studies in human NK cells

NK-cell toxicity is mainly mediated by exocytosis of cytosolic vesicles, containing perforin, granzymes, and other lytic proteins [22]. Since Gal-3 expression can be upregulated upon cytokine stimulation and accumulated within vesicles (see Results, 3.4), to determine whether these data have functional correlates, NK cell responses were studied in the absence/presence of a thiodigalactoside-based Gal-3 inhibitor (Kd = 44 nM) (see Materials, 2.6) in a typical degranulation assay (see Methods, 2.6).

As shown in Fig. 5Aa (representative contour plots), unstimulated NK cells represent a small percentage of degranulating cells (dashed gate); among these cells only a little percentage releases a high number of granules (solid gate). Cytokine stimulation (Fig. 5Ab) induced an increase in NK cell degranulation (dashed gate) and the number of cells expressing high levels of CD107a (solid gate) resulted larger than in resting cells. When cytokine-stimulated cells were treated with increasing concentrations (1–30 μM) of the Gal-3 inhibitor, the number of degranulating cells slightly increased (data not shown). The maximum increase was measured at 30 μM Gal-3 inhibitor (+15 ± 1% vs. inhibitor-untreated cells, Fig. 5Ac, dashed gate). Of note, a significant ($P \leq 0.05$) concentration-dependent increase was determined (Fig. 5B) in NK cells releasing high amount of cytotoxic granules (high level of CD107a expression, Fig. 5A, solid gates).

The maximum increase was measured at 30 μM Gal-3 inhibitor (+ 36 ± 3% vs. inhibitor-untreated cells at 30 μM; Fig. 5B and Fig. 5Ac, solid gate).

No changes in the CD107a levels were observed in resting NK cells incubated with K562 cells, even in the presence of 30 μM Gal-3 inhibitor (data not shown).

Finally, since the Gal-3 inhibitor we used has binding affinity also for the Gal-1 lectin with similar Kd values (49 *vs.* 44 nM) [36] we determined whether unstimulated/stimulated NK cells express Gal-1. Results show that human NK cells do not express Gal-1 protein at least in our experimental conditions (Fig. 5C), suggesting that the results we obtained are related to Gal-3 inhibition.

The overall data demonstrate that Gal-3 functionally correlates with NK cell degranulation.

4. Discussion

In this study we demonstrated, for the first time, that: i) human resting NK cells express Gal-3 at both gene and protein levels; ii) resting

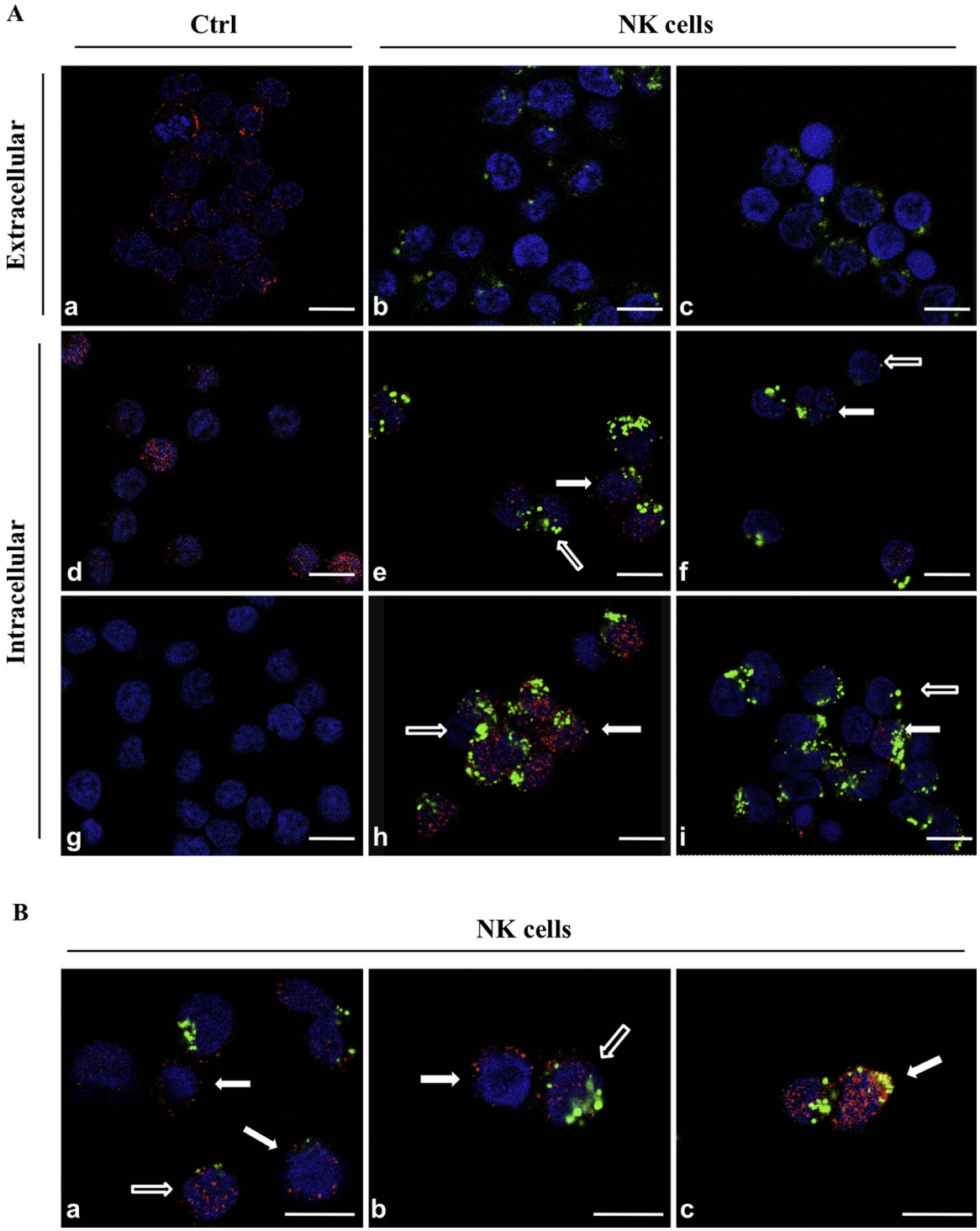

Fig. 4. Confocal microscopy analysis of cytoplasmic and nuclear Gal-3 subcellular distribution in human NK cells. (A) NK cells, unstimulated/stimulated for 24 h with IL-2 (100 U/ml) and IL-15 (20 ng/ml), THP-1 (positive control) and Jurkat (negative control) cells were harvested, stained with anti-human Gal-3, and analyzed by confocal microscopy. Representative images of Gal-3 expression in: THP-1 cells (positive control; a and d), Jurkat cells (negative control; g), resting NK cells (b, e and f), stimulated NK cells (c, h and i). Gal-3 is shown as red dots, perforin as green dots. Nuclei are shown in blue. Scale bar, 10 μm. (B). Representative images of Gal-3 localization in the nucleus and/or in the cytoplasm (a-b) or co-localization of Gal-3 with perforin (c). Gal-3 is shown as red, perforin as green dots. Yellow dots represent co-localization of Gal-3 with perforin. Nuclei are shown in blue. Scale bar, 10 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

NK cells can express high or low basal levels of Gal-3 protein, depending on the individual donor analyzed; iii) upon cytokine stimulation, NK cells can translate the basal Gal-3 mRNA into protein; iv) Gal-3 localizes in both cytoplasm and nucleus, while it was not detected at the cell membrane surface, neither in resting nor stimulated cells; v) Gal-3 can occur within perforin in exocytic vesicles; vi) Gal-3 inhibition leads to an increase in NK cell degranulation.

Several galectins can regulate the homeostasis and functions of immune cells, influencing both innate and adaptive immune responses [4–6]. In NK cells, particularly, the expression of Gal-1 has been described in the third trimester of normal pregnancy [37], while that of Gal-3 remains to be investigated. Scanty literature data focus only on

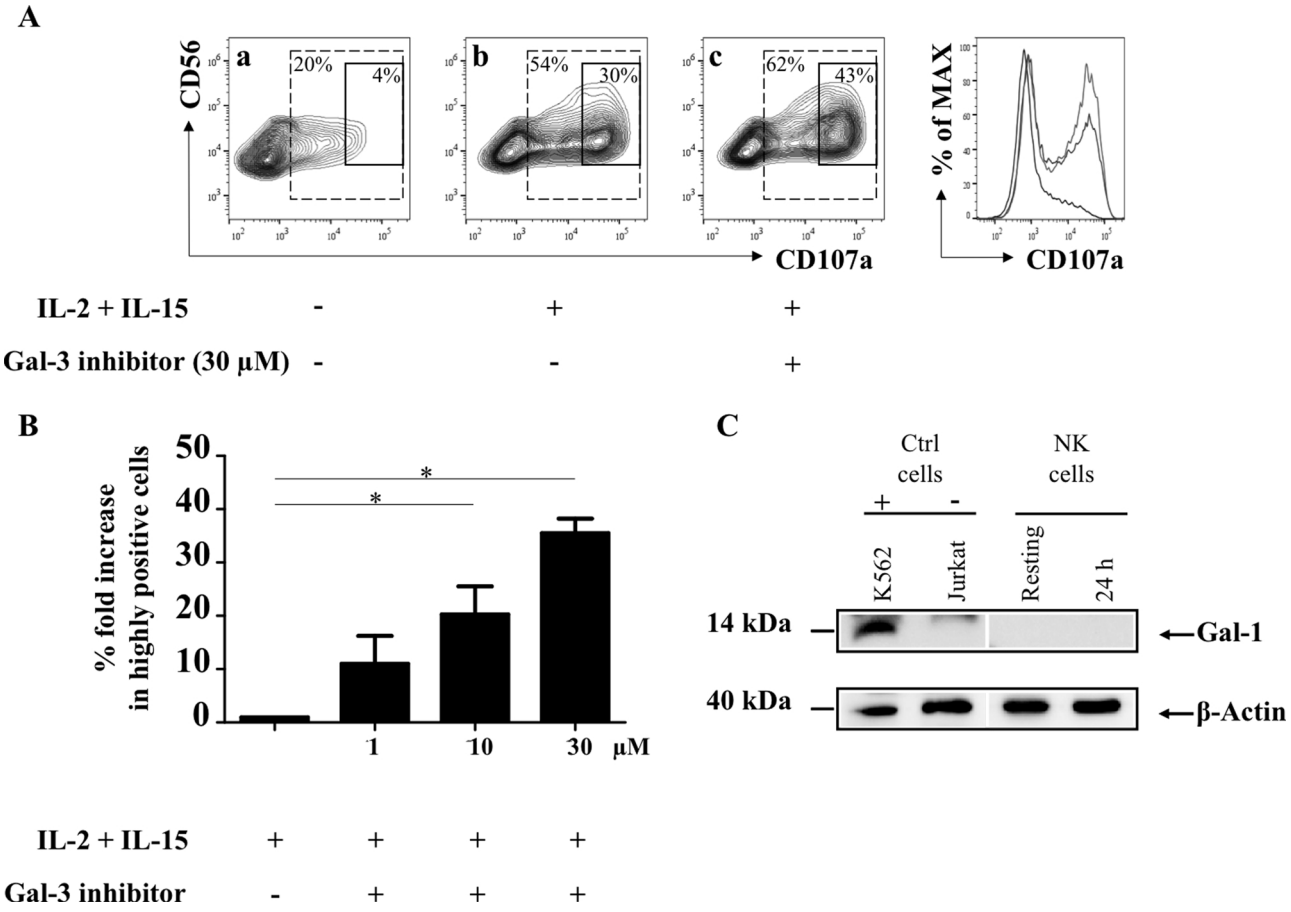

Fig. 5. Gal-3 inhibition increases the degree of degranulation in human activated NK cells. A classical degranulation assay was used to measure NK cell activity (see Methods, 2.6). (A) The first three panels are representative contour plots of resting and cytokine-stimulated NK cells in the absence/presence of the 30 µM Gal-3 inhibitor (dashed gates represent the percentage of total CD56/CD107a positive cells, while solid gates those highly positive) incubated (4 h) with K562 cells. The fourth panel on the right represents overlapped histograms of CD107a expression in resting (black histogram, 5Aa), cytokine-stimulated (blue histogram, 5Ab), cytokine-stimulated plus Gal-3 inhibitor (red histogram, 5Ac) NK cells incubated (4 h) with K562 cells. (B) Percentage of highly positive CD107a cells in activated NK cells untreated/treated with increasing concentrations (1–30 µM) of the Gal-3 inhibitor. The percentage increases in CD107a highly positive cells are calculated *vs* the value of inhibitor-untreated cells set at 1. The results represent the mean ± SEM of at least four independent experiments. *$P \leq 0.05$ *vs.* inhibitor-untreated cells. (C) Representative Gal-1 protein expression evaluated by western blot analysis in NK cells, unstimulated/stimulated (24 h) with IL-2 (100 U/ml) and IL-15 (20 ng/ml), K562 (positive control) and Jurkat (negative control) cells. Total cellular proteins (35 µg) were resolved by 12% polyacrylamide gel and immunoblotted with specific anti-human galectin-1 antibody. β-Actin was used as internal control. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

profiles of gene expression in mouse NK cells [38] and on counter-receptor isolation for Gal-3 from tissues of the murine utero-placental complex [39]. Using Gal-$3^{(-/-)}$ transgenic mice, finally, a dysregulation of NK cell activity has been observed [17,18], when compared to the wild type mice. If this effect is due to the absence of Gal-3 in NK cells or mediated by its absence in other cells remain to be clarified.

To fill the research gap in this field we, here, decided to characterize Gal-3 in human NK cells.

We, first, concentrated on human resting NK cells, because a downregulation of the transcription of genes involved in cellular quiescence and an upregulation of genes associated with cell proliferation [40] can occur in activated NK cells.

From our results, resting NK cells express Gal-3 at both mRNA and protein levels with a highly variable relative expression, depending on the individual donor analyzed. In particular, NK cells, expressing low or high levels of Gal-3 protein, were identified. In our opinion, this is not a surprising result because NK cells are a highly heterogeneous population. These cells not only can be roughly categorized in two major subsets ($CD56^{dim}$ and $CD56^{bright}$), based on different expression level of surface CD56 molecule [21], but they can also exert effector functions through a wide array of activating or inhibitory receptors (see Introduction, 1), which are finely controlled [20,41]. NK cells can be thus considered as plastic cells, that can adapt their responsiveness and different receptor pattern, according to environment stimuli, aging, and diseases [20,42,43]. Consistently, Lavin et al. [44], analyzing the distribution of NK cells in lung tumor lesions, showed that the specific microenvironment can shape NK cell protein expression leading to cells that are unique to tumors.

Concerning our results, a possible explanation for different levels of Gal-3 basal expression among donors may be the presence in some individuals of latent chronic stimulations of the immune systems or functional changes linked to the natural process of aging [45].

Collectively, our data complete those on Gal-3 expression on mammalian immune cells; Gal-3 has been, indeed, previously demonstrated in macrophages [10], DCs [35], neutrophils [46], monocytes [10], eosinophils [47], mast cells and basophils [7], but not in human NK cells.

IL-2 and IL-15 have been here used for activating NK cells, because these two cytokines, more than any others, are implicated in cell survival and cytotoxic function [24]. Following cytokine stimulation, the intracellular level of Gal-3 mRNA resulted significantly reduced at 1–6 h, while it returned to the basal levels after 12–24 h. A two fold increase in Gal-3 protein was also measured after 24 h cytokine stimulation, compared to unstimulated cells (see Figs. 1 and 2). In the present study we did not investigated the molecular mechanisms underlying these events; experiments are in progress in our laboratory to

clarify this point. Our results are consistent, however, with other transcriptional/post-transcriptional gene regulations described for NK cells, and here we may speculate a similar scenario. Resting murine cells can be “pre-armed” with a high amount of specific mRNA that upon cell activation rapidly translates into protein, allowing a rapid production of effector proteins without new gene transcription and with minimal or absent change in mRNA levels [48]. In addition, human NK cells, have a number of genes, involved in cytolytic functions, which are already expressed and that rapidly downregulate upon cytokine stimulation [40,49]. Of note, NK cells can transcribe/retain at nuclear level specific mRNA, such as for IFN-γ, which upon stimulation can be transported into the cytoplasm, translated into the corresponding protein, and rapidly degraded. Such type of mechanism allows not only a fast mRNA translation, but also a tight regulation of the amount of intracellular protein [50,51].

mRNA stability and turnover is an important aspect of post-transcriptional regulation.

Many different sequences within the mRNA together with several proteins able to interact with these sequences are responsible for mRNA stabilization/destabilization, determining the transcript longevity and consequently the expression/abundance of specific proteins [52,53]. On the other hand, we cannot rule out the possibility that Gal-3 mRNA is unstable and degrade after cytokine stimulation in human NK cells.

Gal-3 cellular localization seems to be highly heterogeneous: the protein has the ability to space from nucleus to cytoplasm/cellular surface and to be released into extracellular compartments [34]. Depending on localization Gal-3 can modulate cellular homeostasis, organogenesis, apoptosis, immune responses, cytokine secretion, cell migration, tumor invasion and metastasis [16]. A direct interaction of tumor-secreted Gal-3 with the NK cell surface activating receptor NKp30, having inhibitory activities on cytotoxicity, has been specifically reported [54].

In our cells, we never detected Gal-3 at cell surface membrane, even after cytokine stimulation, while in THP-1, a monocytic cell line, taken as positive control, we found Gal-3 also bound to the plasmatic membrane. This result is in agreement with data from Dabelic et al. [55] on THP-1 cells and from van Stijn et al. [35] on monocytes.

All samples analyzed were, on the contrary, positive for the intracellular localization of Gal-3 with different levels of protein expression, depending on the individual donor. Gal-3 was always detected in each sample analyzed, being found in the nucleus and/or in the cytoplasm in a heterogeneous manner.

From our results, Gal-3 co-localize with perforin in exocytic vesicles, which represent one of the main mechanisms underlying NK cell functions. Gal-3 could, thus, directly participate to NK cell degranulation. When Gal-3 function was inhibited by a thiodigalactoside-based Gal-3 inhibitor [30] a significant increase in the degree of granules released by NK cells was measured (see Fig. 5B). This result suggest that the level of intracellular Gal-3 expression into NK cells may modulate the mobilization of secretory vesicles for releasing their cytotoxic contents.

Previously, Chen et al. (2009) [56] demonstrated that endogenous Gal-3 negatively regulates T-cell activation by TCR down-regulation through the interaction with Alix. Alix protein, in turn, can interact with the endocytic trafficking complexes (ESCRT), involved in exosome biogenesis and secretion [57], as well as with other intracellular regulators, involved in signal transduction [58]. Similarly, endogenous Gal-3 might negatively regulate the release of cytotoxic granules from activated NK cells through the modulation of Alix [59]. The Gal-3 upregulation may be, consequently, a physiological mechanism specifically designed to protect healthy cells against toxic compounds released in the surrounding microenvironment. Our observations support also previous data from Radosavljevic et al. [17], showing that splenic NK cells from Gal-3$^{(-/-)}$ transgenic mice have higher cytotoxic activities than wild type animals.

The function of intracellular Gal-3 in NK cells remains, however, to be fully characterized. In addition to the above mentioned hypothesis it could: i) exert anti-apoptotic activities by binding to molecules involved in apoptosis (i.e., Bcl-2, CD95 (APO-1/Fas), Nucling, Alix/AIP1, Synexin) [16]; ii) regulate cell proliferation, differentiation, and survival through the modulation of specific signal pathways (i.e., K-Ras/MEK, PI3 K/Akt and Wnt [60,61]; iii) control the dynamics of membrane domains and subsequent signaling events, when localized at the inner leaflet of plasma membrane [62].

Similarly, the nuclear Gal-3, associated with a ribonucleoprotein complex, could: i) act as a pre-mRNA splicing factor; ii) operate as a regulator of gene transcription, promoting the stabilization/transactivation of several transcription factors (i.e., CREB, Sp1, TTF-1, β-catenin) [16,63].

The signal transduction cascades, initiated by IL-2/IL-5 stimulation, resulting in Gal-3 upregulation should be investigated. The mechanism underlying Gal-3 gene regulation is still poorly understood [16]: Jack/STAT [24], NF-kB [64], or the Ras/MEKK1/MKK1-dependent/AP-1 signal transduction pathway [65] may control the Gal-3 expression as in other cells, but this will be the topic of future research.

In conclusion, the present study provides a clear demonstration of Gal-3 expression in human NK cells. The presence of this lectin in resting cells supports its roles on NK cell homeostasis, while the protein upregulation after cytokine stimulation sustains roles in cell function. Finally, the Gal-3 co-localization with perforin in granules, as well as the increase in NK cell degranulation following Gal-3 inhibition suggests functional roles in NK cytotoxicity.

Conflicts of interest

None.

Funding

This work was supported by Ministry of Instruction, University and Research, Italy [PRIN 2015–2018, cod. 2015RNWJAM_003].

Acknowledgments

We thank Dr. Antonella Vallario, Department of Pharmaceutical Sciences, Univ. ‘Piemonte Orientale’, Novara, Italy, for flow cytometry and technical assistance.

References

[1] P. Argüeso, N. Panjwani, Focus on molecules: galectin-3, Exp. Eye Res. 92 (2011) 2–3.
[2] A. Fortuna-Costa, A.M. Gomes, E.O. Kozlowski, M.P. Stelling, M.S.G. Pavão, Extracellular galectin-3 in tumour progression and metastasis, Front. Oncol. 4 (2014) 138.
[3] M.K. Ho, T.A. Springer, Mac-2, a novel 32, 000 Mr mouse macrophage subpopulation-specific antigen defined by monoclonal antibodies, J. Immunol. 128 (1982) 1221–1228.
[4] H.Y. Chen, F.T. Liu, R.Y. Yang, Roles of galectin-3 in immune responses, Arch. Immunol. Ther. Exp. (Warsz) 53 (2005) 497–504.
[5] F.T. Liu, Regulatory roles of galectins in the immune response, Int. Arch. Allergy Immunol. 136 (2005) 385–400.
[6] G.A. Rabinovich, F.T. Liu, M. Hirashima, A. Anderson, An emerging role for galectins in tuning the immune response: lessons from experimental models of inflammatory disease, autoimmunity and cancer, Scand. J. Immunol. 66 (2007) 143–158.
[7] S.S. Craig, P. Krishnaswamy, A.M. Irani, C.L. Kepley, F.T. Liu, L.B. Schwartz, Immunoelectron microscopic localization of galectin-3 an IgE binding protein, in human mast cells and basophils, Anat. Rec. 242 (1995) 211–219.
[8] E.V. Acosta-Rodríguez, C.L. Montes, C.C. Motrán, E.I. Zuniga, F.T. Liu, G.A. Rabinovich, A. Gruppi, Galectin-3 mediates IL-4-induced survival and differentiation of B cells: functional cross-talk and implications during Trypanosoma cruzi infection, J. Immunol. 172 (2004) 493–502.
[9] H.G. Joo, P.S. Goedegebuure, N. Sadanaga, M. Nagoshi, W. von Bernstorff, T.J. Eberlein, Expression and function of galectin-3, a beta-galactoside-binding protein in activated T lymphocytes, J. Leukoc. Biol. 69 (2001) 555–564.
[10] F.T. Liu, D.K. Hsu, R.I. Zuberi, I. Kuwabara, E.Y. Chi, W.R. Henderson, Expression and function of galectin-3 a beta-galactoside-binding lectin, in human monocytes

and macrophages, Am. J. Pathol. 147 (1995) 1016–1028.
[11] I.K. Moutsatsos, M. Wade, M. Schindler, J.L. Wang, Endogenous lectins from cultured cells: nuclear localization of carbohydrate-binding protein 35 in proliferating 3T3 fibroblasts, Proc. Natl. Acad. Sci. U. S. A. 84 (1987) 6452–6456.
[12] A.U. Newlaczyl, L.G. Yu, Galectin-3 –A jack-of-all-trades in cancer, Cancer Lett. 313 (2011) 123–128.
[13] R.P. Menon, R.C. Hughes, Determinants in the N-terminal domains of galectin-3 for secretion by a novel pathway circumventing the endoplasmic reticulum-golgi complex, Eur. J. Biochem. 264 (1999) 569–576.
[14] P.P. Ruvolo, Galectin 3 as a guardian of the tumor microenvironment, Biochim. Biophys. Acta 1863 (2016) 427–437.
[15] B. Mehul, R.C. Hughes, Plasma membrane targetting, vesicular budding and release of galectin 3 from the cytoplasm of mammalian cells during secretion, J. Cell Sci. 110 (1997) 1169–1178.
[16] J. Dumic, S. Dabelic, M. Flögel, Galectin-3: an open-ended story, Biochim. Biophys. Acta 1760 (2006) 616–635.
[17] G. Radosavljevic, I. Jovanovic, I. Majstorovic, M. Mitrovic, V.J. Lisnic, N. Arsenijevic, S. Jonjic, M.L. Lukic, Deletion of galectin-3 in the host attenuates metastasis of murine melanoma by modulating tumor adhesion and NK cell activity, Clin. Exp. Metastasis 28 (2011) 451–462.
[18] A.D. Chaudhari, R.P. Gude, R.D. Kalraiya, S.V. Chiplunkar, Endogenous galectin-3 expression levels modulate immune responses in galectin-3 transgenic mice, Mol. Immunol. 68 (2015) 300–311.
[19] J.C. Sun, L.L. Lanier, NK cell development, homeostasis and function: parallels with $CD8^+$ T cells, Nat. Rev. Immunol. 11 (2011) 645–657.
[20] E. Vivier, D.H. Raulet, A. Moretta, M.A. Caligiuri, L. Zitvogel, L.L. Lanier, W.M. Yokoyama, S. Ugolini, Innate or adaptive immunity? The example of natural killer cells, Science 331 (2011) 44–49.
[21] L. Moretta, E. Montaldo, P. Vacca, G. Del Zotto, F. Moretta, P. Merli, F. Locatelli, M.C. Mingari, Human natural killer cells: origin, receptors, function, and clinical applications, Int. Arch. Allergy Immunol. 164 (2014) 253–264.
[22] M.A. Caligiuri, Human natural killer cells, Blood 112 (2008) 461–469.
[23] A. Moretta, E. Marcenaro, S. Parolini, G. Ferlazzo, L. Moretta, NK cells at the interface between innate and adaptive immunity, Cell Death Differ. 15 (2008) 226–233.
[24] B. Becknell, M.A. Caligiuri, Interleukin-2 interleukin-15, and their roles in human natural killer cells, Adv. Immunol. 86 (2005) 209–239.
[25] N.W. Zwirner, C.I. Domaica, Cytokine regulation of natural killer cell effector functions, Biofactors 36 (2010) 274–288.
[26] S. Fallarini, A. Brittoli, M. Fiore, G. Lombardi, O. Renaudet, B. Richichi, C. Nativi, Immunological characterization of a rigid α-Tn mimetic on murine iNKT and human NK cells, Glycoconj. J. 34 (2017) 553–562.
[27] M. Manuelli, S. Fallarini, G. Lombardi, C. Sangregorio, C. Nativi, B. Richichi, Iron oxide superparamagnetic nanoparticles conjugated with a conformationally blocked α-Tn antigen mimetic for macrophage activation, Nanoscale 6 (2014) 7643–7655.
[28] S. Fallarini, T. Paoletti, N. Orsi Battaglini, G. Lombardi, Invariant NKT cells increase drug-induced osteosarcoma cell death, Br. J. Pharmacol. 167 (2012) 1533–1549.
[29] E. Aktas, U.C. Kucuksezer, S. Bilgic, G. Erten, G. Deniz, Relationship between CD107a, expression and cytotoxic activity, Cell. Immunol. 254 (2009) 149–154.
[30] H. van Hattum, H.M. Branderhorst, E.E. Moret, U.J. Nilsson, H. Leffler, R.J. Pieters, Tuning the preference of thiodigalactoside- and lactosamine-based ligands to galectin-3 over galectin-1, J. Med. Chem. 56 (2013) 1350–1354.
[31] A.B. Dietz, P.A. Bulur, G.J. Knutson, R. Matasić, S. Vuk-Pavlović, Maturation of human monocyte-derived dendritic cells studied by microarray hybridization, Biochem. Biophys. Res. Commun. 275 (2000) 731–738.
[32] D.K. Hsu, H.Y. Chen, F.T. Liu, Galectin-3 regulates T-cell functions, Immunol. Rev. 230 (2009) 114–127.
[33] R. Novak, S. Dabelic, J. Dumic, Galectin-1 and galectin-3 expression profiles in classically and alternatively activated human macrophages, Biochim. Biophys. Acta 1820 (2012) 1383–1390.
[34] A. Krześlak, A. Lipińska, Galectin-3 as a multifunctional protein, Cell Mol. Biol. Lett. 9 (2004) 305–328.
[35] C.M. van Stijn, M. van den Broek, R. van de Weerd, M. Visser, I. Taşdelen, B. Tefsen, I. van Die, Regulation of expression and secretion of galectin-3 in human monocyte-derived dendritic cells, Mol. Immunol. 46 (2009) 3292–3299.
[36] T. Delaine, P. Collins, A. MacKinnon, G. Sharma, J. Stegmayr, V.K. Rajput, S. Mandal, I. Cumpstey, A. Larumbe, B.A. Salameh, B. Kahl-Knutsson, H. van Hattum, M. van Scherpenzeel, R.J. Pieters, T. Sethi, H. Schambye, S. Oredsson, H. Leffler, H. Blanchard, U.J. Nilsson, Galectin-3-binding glycomimetics that strongly reduce bleomycin-induced lung fibrosis and modulate intracellular glycan recognition, Chembiochem 17 (2016) 1759–1770.
[37] A. Molvarec, S.M. Blois, B. Stenczer, G. Toldi, I. Tirado-Gonzalez, M. Ito, T. Shima, S. Yoneda, B. Vásárhelyi, J. Rigó Jr., S. Saito, Peripheral blood galectin-1-expressing T and natural killer cells in normal pregnancy and preeclampsia, Clin. Immunol. 139 (2011) 48–56.
[38] H.S. Kang, E.M. Kim, S. Lee, S.R. Yoon, T. Kawamura, Y.C. Lee, S. Kim, P.K. Myung, S.M. Wang, I. Choi, Stage-dependent gene expression profiles during natural killer cell development, Genomics 86 (2005) 551–565.
[39] S. Crider-Pirkle, P. Billingsley, C. Faust, D.M. Hardy, V. Lee, H. Weitlauf, Cubilin, a binding partner for galectin-3 in the murine utero-placental complex, J. Biol. Chem. 277 (2002) 15904–15912.
[40] K. Dybkaer, J. Iqbal, G. Zhou, H. Geng, L. Xiao, A. Schmitz, F. d'Amore, W.C. Chan, Genome wide transcriptional analysis of resting and IL2 activated human natural killer cells: gene expression signatures indicative of novel molecular signaling pathways, BMC Genomics 8 (2007) 230.
[41] A. Thielens, E. Vivier, F. Romagné, NK cell MHC class I specific receptors (KIR): from biology to clinical intervention, Curr. Opin. Immunol. 24 (2012) 239–245.
[42] J. Hazeldine, J.M. Lord, The impact of ageing on natural killer cell function and potential consequences for health in older adults, Ageing Res. Rev. 12 (2013) 1069–1078.
[43] O.N. Terra Junior, G.C. Maldonado, G.R. Alfradique, V.C. Lisboa, A. Arnóbio, D.B. de Lima, H.R. Diamond, M.H. de Souza, Study of natural cytotoxicity receptors in patients with HIV/AIDS and cancer: a cross-sectional study, Sci. World J. (2016) 2085871.
[44] Y. Lavin, S. Kobayashi, A. Leader, E.D. Amir, N. Elefant, C. Bigenwald, R. Remark, R. Sweeney, C.D. Becker, J.H. Levine, K. Meinhof, A. Chow, S. Kim-Shulze, A. Wolf, C. Medaglia, H. Li, J.A. Rytlewski, R.O. Emerson, A. Solovyov, B.D. Greenbaum, C. Sanders, M. Vignali, M.B. Beasley, R. Flores, S. Gnjatic, D. Pe'er, A. Rahman, I. Amit, M. Merad, Innate immune landscape in early lung adenocarcinoma by paired single-cell analyses, Cell 169 (2017) 750–765.
[45] S. Weilner, V. Keider, M. Winter, E. Harreither, B. Salzer, F. Weiss, E. Schraml, P. Messner, P. Pietschmann, F. Hildner, C. Gabriel, H. Redl, R. Grillari-Voglauer, J. Grillari, Vesicular Galectin-3 levels decrease with donor age and contribute to the reduced osteo-inductive potential of human plasma derived extracellular vesicles, Aging (Albany NY) 8 (2016) 16–33.
[46] S.Y. Wu, J.H. Huang, W.Y. Chen, Y.C. Chan, C.H. Lin, Y.C. Chen, F.T. Liu, B.A. Wu-Hsieh, Cell intrinsic galectin-3 attenuates neutrophil ROS-dependent killing of candida by modulating CR3 downstream syk activation, Front. Immunol. 8 (2017) 48.
[47] S.P. Rao, Z. Wang, R.I. Zuberi, L. Sikora, N.S. Bahaie, B.L. Zuraw, F.T. Liu, P. Sriramarao, Galectin-3 functions as an adhesion molecule to support eosinophil rolling and adhesion under conditions of flow, J. Immunol. 179 (2007) 7800–7807.
[48] T.A. Fehniger, S.F. Cai, X. Cao, A.J. Bredemeyer, R.M. Presti, A.R. French, T.J. Ley, Acquisition of murine NK cell cytotoxicity requires the translation of a pre-existing pool of granzyme B and perforin mRNAs, Immunity 26 (2007) 798–811.
[49] A.Y. Mah, M.A. Cooper, Metabolic Regulation of Natural Killer Cell IFN-γ Production, Crit. Rev. Immunol. 36 (2016) 131–147.
[50] T.D. Kim, J.Y. Park, I. Choi, Post-transcriptional regulation of NK cell activation, Immune Netw. 9 (2009) 115–121.
[51] D.L. Hodge, A. Martinez, J.G. Julias, L.S. Taylor, H.A. Young, Regulation of nuclear gamma interferon gene expression by interleukin 12 (IL-12) and IL-2 represents a novel form of posttranscriptional control, Mol. Cell Biol. 22 (2002) 1742–1753.
[52] F. Mignone, C. Gissi, S. Liuni, G. Pesole, Untranslated regions of mRNAs, Genome Biol. 3 (2002) (REVIEWS0004).
[53] W. Eberhardt, A. Doller, el.-S. Akool, J. Pfeilschifter, Modulation of mRNA stability as a novel therapeutic approach, Pharmacol. Ther. 114 (2007) 56–73.
[54] W. Wang, H. Guo, J. Geng, X. Zheng, H. Wei, R. Sun, Z. Tian, Tumor-released Galectin-3, a soluble inhibitory ligand of human NKp30, plays an important role in tumor escape from NK cell attack, J. Biol. Chem 289 (2014) 33311–33319.
[55] S. Dabelic, R. Novak, S.S. Goreta, J. Dumic, Galectin-3 expression in response to LPS, immunomodulatory drugs and exogenously added galectin-3 in monocyte-like THP-1 cells, In Vitro Cell Dev. Biol. Anim. 48 (2012) 518–527.
[56] H.Y. Chen, A. Fermin, S. Vardhana, I.C. Weng, K.F. Lo, E.Y. Chang, E. Maverakis, R.Y. Yang, D.K. Hsu, M.L. Dustin, F.T. Liu, Galectin-3 negatively regulates TCR-mediated CD4+ T-cell activation at the immunological synapse, Proc. Natl. Acad. Sci. U. S. A. 106 (2009) 14496–14501.
[57] J.H. Hurley, G. Odorizzi, Get on the exosome bus with ALIX, Nat. Cell. Biol. 14 (2012) 654–655.
[58] M. Colombo, C. Moita, G. van Niel, J. Kowal, J. Vigneron, P. Benaroch, N. Manel, L.F. Moita, C. Théry, G. Raposo, Analysis of ESCRT functions in exosome biogenesis, composition and secretion highlights the heterogeneity of extracellular vesicles, J. Cell Sci. 126 (2013) 5553–5565.
[59] W. Liu, D.K. Hsu, H.Y. Chen, R.Y. Yang, K.L. Carraway 3rd, R.R. Isseroff, F.T. Liu, Galectin-3 regulates intracellular trafficking of EGFR through Alix and promotes keratinocyte migration, J. Invest. Dermatol. 132 (2012) 2828–2837.
[60] R. Shalom-Feuerstein, S.J. Plowman, B. Rotblat, N. Ariotti, T. Tian, J.F. Hancock, Y. Kloog, K-ras nanoclustering is subverted by overexpression of the scaffold protein galectin-3, Cancer Res. 68 (2008) 6608–6616.
[61] S. Song, N. Mazurek, C. Liu, Y. Sun, Q.Q. Ding, K. Liu, M.C. Hung, R.S. Bresalier, sGalectin-3 mediates nuclear beta-catenin accumulation and Wnt signaling in human colon cancer cells by regulation of glycogen synthase kinase-3beta activity, Cancer Res. 69 (2009) 1343–1349.
[62] D. Compagno, F.M. Jaworski, L. Gentilini, G. Contrufo, I. González Pérez, M.T. Elola, N. Pregi, G.A. Rabinovich, D.J. Laderach, Galectins: major signaling modulators inside and outside the cell, Curr. Mol. Med. 14 (2014) 630–651.
[63] T. Funasaka, A. Raz, P. Nangia-Makker, Nuclear transport of galectin-3 and its therapeutic implications, Semin. Cancer Biol. 27 (2014) 30–38.
[64] D.K. Hsu, S.R. Hammes, I. Kuwabara, W.C. Greene, F.T. Liu, Human, lymphotropic virus-I infection of human T lymphocytes induces expression of the beta-galacto-side-binding lectin, galectin-3, Am. J. Pathol. 148 (1996) 1661.
[65] K. Kim, E.P. Mayer, M. Nachtigal, Galectin-3 expression in macrophages is signaled by Ras/MAP kinase pathway and up-regulated by modified lipoproteins, Biochim. Biophys. Acta 1641 (2003) 13–23.