Neuropeptides xxx (2016) xxx–xxx

Contents lists available at ScienceDirect

Neuropeptides

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

The neuropeptide galanin modulates natural killer cell function

Andreas Koller [a], Rodolfo Bianchini [a], Sandra Schlager [b], Christian Münz [c], Barbara Kofler [a,*], Silke Wiesmayr [d]

[a] Laura Bassi Centre of Expertise THERAPEP, Research Program for Receptor Biochemistry and Tumor Metabolism, Department of Pediatrics, Paracelsus Medical University, Müllner Hauptstraße 48, 5020 Salzburg, Austria
[b] Department of Blood Group Serology and Transfusion Medicine, Paracelsus Medical University, Müllner Hauptstraße 48, 5020 Salzburg, Austria
[c] Viral Immunobiology, Institute of Experimental Immunology, University of Zürich, Rämistraße 71, 8006 Zürich, Switzerland
[d] Department of Neonatology, Paracelsus Medical University, Müllner Hauptstraße 48, 5020 Salzburg, Austria

ARTICLE INFO

Article history:
Received 24 August 2016
Accepted 1 November 2016
Available online xxxx

Keywords:
Galanin
Immunomodulation
Interleukin 12
Interleukin 18
Interferon gamma
Natural killer cells

ABSTRACT

Natural killer (NK) cells are part of the innate immune system and combat pathogens and tumors by secreting pro-inflammatory cytokines like interferon gamma (IFN-γ) and by their cytotoxic action. Galanin is a neuropeptide also expressed in peripheral tissue where it impacts several physiological functions, including inflammation. The effects of galanin are mediated via three receptors, GAL_{1-3}. Since other neuropeptides have been shown to regulate NK cell activity, we investigated the potential of galanin to modulate human NK cell function.

NK cells were isolated from human peripheral blood mononuclear cells. mRNA expression was analyzed by qRT-PCR. The dynamic mass redistribution of NK cells upon regulatory peptide stimulation was determined by label-free biochip technology. IFN-γ producing NK cells were identified by flow cytometry analysis and IFN-γ secretion was measured by ELISA. NK cell cytotoxicity was analyzed by flow cytometry via CD107a mobilization.

NK cells were found to express the receptor GAL_2 but not GAL_1, GAL_3 or galanin. Galanin per se did not affect the dynamic mass redistribution of NK cells, but significantly enhanced the response of NK cells to IL-18. Galanin significantly modulated the IFN-γ production of the $CD56^{bright}$ NK cell population upon IL-12 and IL-18 stimulation. Furthermore, galanin significantly modulated the IL-12 and IL-18 stimulated IFN-γ secretion. NK cell cytotoxicity was not modulated by galanin treatment.

Galanin can be classified as an immunomodulatory peptide as it is able to sensitize NK cells toward specific cytokines.

1. Introduction

The immune system defends the organism against invading pathogens by a variety of innate and adaptive immune pathways. A crosstalk between the neuroendocrine and the immune system constitutes an important part of how that protective immune response is mediated (Butts and Sternberg, 2008). This communication relies on a common biochemical interaction in which neuropeptides are of great relevance (Holzer et al., 2012). However, an imbalance of pro- and anti-inflammatory neuropeptides can also compromise host immune homeostasis (Souza-Moreira et al., 2011). Neuropeptides help to regulate the action of cytokines mainly by modulating the level of transcription factors in the target cells (Souza-Moreira et al., 2011).

Natural killer (NK) cells represent a particular subset of lymphocytes of the innate immune system. NK cells have the ability to both lyse target cells and produce immunoregulatory cytokines in the very early phase of inflammation (Robertson and Ritz, 1990). Phenotypically they are characterized by expression of CD56 and lack of expression of CD3 (Butts and Sternberg, 2008). Based on the cell-surface density of CD56, two distinct populations of human NK cells can be identified: cytokine-producing $CD56^{bright}$ and cytototoxic $CD56^{dim}$ cells (Cooper et al., 2001; Lanier et al., 1986, 1989).

A number of neuropeptides have already been reported to exert effects on NK cells, among which substance P (SP) is the most investigated (Evans et al., 2008; Feistritzer et al., 2003; Fu et al., 2011; Lang et al., 2003; Monaco-Shawver et al., 2011). Its immunoregulatory effects on the functions of NK cells seem to be dose-dependent, resulting in either increased or decreased NK cell cytotoxicity. SP thus exerts bi-phasic control on the activation of NK cells in physiological or pathological conditions, which underscores its importance in immune cell regulation (Fu et al., 2011; Lang et al., 2003). Even in chronic diseases such as human immunodeficiency virus (HIV) infection, SP has a significant influence on NK cell function (Evans et al., 2008).

The neuropeptide galanin shows a widespread distribution in both the central and peripheral nervous systems (Lang et al., 2015). Its role in non-neuronal cells is poorly understood, nevertheless it has been demonstrated that, during inflammation, peripheral tissues (e.g. the

* Corresponding author at: Laura Bassi Centre of Expertise THERAPEP, Research Program for Receptor Biochemistry and Tumor Metabolism, Department of Pediatrics, Paracelsus Medical University, Müllner Hauptstrasse 48, 5020 Salzburg, Austria.
E-mail address: B.Kofler@salk.at (B. Kofler).

http://dx.doi.org/10.1016/j.npep.2016.11.002
0143-4179/

skin) produce and release galanin (Bauer et al., 2008; Kofler et al., 2004; Xu et al., 2000). Immune cells as well express galanin, and increased expression levels have been observed in several animal models of inflammation (Ji et al., 1995; Lang and Kofler, 2011).

Recently we demonstrated that galanin acts as an immune modulator of cytokine potency in human peripheral polymorphonuclear neutrophils (PMNs) and murine bone marrow PMNs (Locker et al., 2015).

Galanin exerts its action via three known galanin receptors (GAL_1, GAL_2 and GAL_3) which belong to the superfamily of G-protein-coupled transmembrane receptors (Floren et al., 2000). It has been shown that the expression of galanin receptors is upregulated during acute inflammation in peripheral tissues (Hempson et al., 2010; Matkowskyj et al., 2000; Saban et al., 2002).

Although the importance of galanin on the innate immune system has been emphasized (Lang and Kofler, 2011), the effect of galanin on NK cells has not yet been investigated. Therefore, the aim of this study was to analyze the expression and functional significance of the galanin system in NK cells.

2. Materials and methods

2.1. Isolation of human NK cells and monocytes

After informed consent was obtained from healthy donors, in accordance with the Helsinki Declaration and following the guidelines of the Salzburg State Ethics Research Committee, peripheral mononuclear cells (PBMCs) were isolated from human buffy coats. Buffy coats are concentrated leukocytes and platelets obtained from whole blood. Whole blood was centrifuged at 4160 ×*g* for 20 min at 22 °C and afterwards the leukocyte band was separated from most of the erythrocytes and plasma. Buffy coats were pre-selected based on the sedimentation rate with a cut-off of 5% sedimentation after 1 h, and the ratio of neutrophils, lymphocytes, monocytes, eosinophils and basophils determined with a Sysmex XS-800i (Sysmex, Kobe, Japan) system. A Ficoll-Paque 1.077 g/l (GE Healthcare, Chicago, IL, USA) gradient was used for PBMC extraction. NK cells were separated from PBMCs with a human NK cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) following the manufacturer's instructions. After separation, NK cell purity was at least 95%. NK cells were maintained in RPMI medium (Sigma-Aldrich, St. Luis, MO, USA) containing 10% fetal bovine serum (FBS) (Biochrom, Berlin, Germany), 2 mM GlutaMAX (Gibco), 10 mM HEPES (Sigma-Aldrich, St. Luis, MO, USA) and 1× penicillin-streptomycin-amphotericin b mixture (Lonza, Basel, Switzerland). Monocytes were isolated from PBMCs using the MACS Pan Monocyte Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany) following the manufacturer's protocol. For differentiation, monocytes were cultured for 6 days in Xvivo 10 (Lonza, Basel, Switzerland) containing 1× penicillin-streptomycin-amphotericin b mixture and 100 ng/ml granulocyte macrophage colony-stimulating factor (GM-SCF), leading to a M1 phenotype called monocyte-derived macrophage M1 (MDM1) (Rey-Giraud et al., 2012).

2.2. Quantitative real-time PCR

RNA of NK cells, monocytes and MDM1 was isolated using TRI-Reagent (Molecular Research Center, Cincinnati, OH, USA) following the manufacturer's protocol. Maxima Reverse Transcriptase (Thermo Scientific, Pittsburgh, PA, USA) and random hexamer primers were used for cDNA synthesis according to the manufacturer's instructions. To quantify galanin and galanin receptor expression, iQ SYBR green supermix (Quanta BioSciences, Gaithersburg, MD, USA) was used. Forward and reverse primers were synthesized by Microsynth (Balgach, Switzerland), with the respective target sites separated by at least one intron (galanin: fwd 5′- CTTCTCGCCTCCC TCCTC-3′, rev 5′-TGTCGCTGAATGACCTGTG-3′; GAL_1: fwd 5′-TCT GCTTCTGCTATGCCAAGG-3′, rev 5′-AGAGATGGATGATGTGGTGCG-3′; GAL_2: fwd 5′-GCCGACCTGTGTTTCATCC-3′, rev 5′-GGAGTGCAGCGGGT AG-3′; GAL_3: fwd 5′-TTACGCTGGCTGCTGTCTCC-3′, rev 5′-CGGTGCCG TAGTAGCTGAGGTA-3′, Ribosomal protein L 27 (RPL27): fwd 5′-GCTGGAATTGACCGCTACC-3′, rev 5′-TCTCTGAAGACATCCTTATTGACG-3′). The amplification reaction was performed in duplicates for 45 cycles (97 °C for 15 s, 63 °C for 30 s and 72 °C for 10 s). The relative expression of the genes was determined by the delta delta threshold cycle (Ct) $[2^{-\text{(Ct of the gene of interest – Ct of the housekeeping gene RPL27)}}]$.

2.3. Evaluation of GAL_2 surface expression by flow cytometry

Isolated PBMCs (2×10^6 cells) were resuspended in 80 μl flow cytometry staining buffer solution (eBioscience, San Diego, CA, USA) and 20 μl human Fc receptor binding inhibitor solution (eBioscience, San Diego, CA, USA) were added for 20 min at 4 °C. PBMCs were centrifuged with 300 ×*g* for 5 min at 4 °C and resuspended in 80 μl flow cytometry staining buffer solution. GAL_2 antibody (AP01317PU-N; Acris, Herford, Germany) was added in a dilution of 1:50 and incubated 20 min at 4 °C. Cells were centrifuged at 300 ×*g* for 5 min at 4 °C and resuspended in 190 μl flow cytometry staining buffer solution and incubated with 10 μg/ml anti-rabbit Alexa fluor 488 antibody (Life Technologies, Carlsbad, CA, USA) and 10 μl of anti-CD56 (PE-Cy7) (BioLegend, San Diego, CA, USA, clone: HCD56) for 20 min at 4 °C. PBMCs were centrifuged at 300 ×*g* for 5 min at 4 °C and resuspended in 500 μl flow cytometry staining buffer solution. Cell staining was excited with a blue laser (488 nm) and the emission of Alexa Fluor 488 (519 nm) and PE-Cy7 (774 nm) was detected via FL1 and FL5 channel by a flow cytometer (Cytomics FC 500 flow cytometer; Beckman Coulter, Brea, CA, USA) and analyzed using Flowing Software 2.5.1.

2.4. Measurement of dynamic mass redistribution (DMR)

NK cells were resuspended in HBSS +/+ (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) containing 20 mM HEPES and seeded (6×10^4 cells/well) in an EnSpire fibronectin-coated 384-well cell assay plate (PerkinElmer, Waltham, MA, USA). The cells were left for 1 h to attach to the coating and to acclimate to the assay temperature (26–29 °C) of the EnSpire machine (PerkinElmer, Waltham, MA, USA). Polarized light passes through the bottom of a biosensor 384 well plate and the light is reflected constantly. The receptor activation leads to a shift in wavelength of the reflected light indicating a redistribution of cellular constituents (Schroder et al., 2010). The DMR baseline of untreated cells was measured via EnSpire label-free biosensor technology. The baseline was set by 4 measurements in an interval of 30 s. The cells were then treated with galanin (GL Biochem, Shanghai, China) (13 nM–10 μM), IL-12 (PeproTech, Rocky Hill, NJ, USA) (13 pg/ml – 10 ng/ml) and IL-18 (R&D Systems, Minneapolis, MN, USA) (41 pg/ml – 30 ng/ml) alone or in combination with a fixed galanin concentration (0.05 μM, 0.1 μM and 1 μM). The change in DMR was measured in triplicates every 2 min for 2 h. The peak of the DMR (at ~1.5 h) was used for generating a dose–response curve and calculating the half maximal effective concentration (EC_{50}).

2.5. IFN-γ flow cytometry

NK cells were seeded (3×10^5 cells in 100 μl RPMI medium) in 96 v-well plates (Greiner Bio-One, Frickenhause, Germany) and treated with IL-12 (10 ng/ml), IL-18 (1.5 ng/ml) and different concentrations of galanin (0.1 μM, 1 μM and 10 μM) for 4 h. To block cytokine secretion, cells were incubated with 5 μg/ml Brefeldin A (BioLegend, San Diego, CA, USA). Cells were centrifuged with 300 ×g for 5 min at 4 °C and resuspended in 100 μl flow cytometry staining buffer solution and stained with 5 μl anti-CD56 (PE-Cy7) (BioLegend, San Diego, CA, USA, clone: HCD56) for 20 min at 4 °C. NK cells were pelleted with 300 ×*g* for 5 min at 4 °C. Cells were resuspended and fixed with 200 μl 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA, USA) overnight

Please cite this article as: Koller, A., et al., The neuropeptide galanin modulates natural killer cell function, Neuropeptides (2016), http://dx.doi.org/10.1016/j.npep.2016.11.002