Galectin-9 regulates serum amyloid A-induced inflammasome activation in human neutrophils

Jumpei Temmoku

Fukushima Kenritsu Ika Daigaku

Yuya Fujita

Fukushima Kenritsu Ika Daigaku

Haruki Matsumoto

Fukushima Kenritsu Ika Daigaku

Naoki Matsuoka

Fukushima Kenritsu Ika Daigaku

Tomoyuki i Asano

Fukushima Kenritsu Ika Daigaku

Shuzo Sato

Fukushima Kenritsu Ika Daigaku

Makiko Furuya

Fukushima Kenritsu Ika Daigaku

Eiji Suzuki

Fukushima Kenritsu Ika Daigaku

Hiroshi Watanabe

Fukushima Kenritsu Ika Daigaku

Kiyoshi Migita (✉ migita@fmu.ac.jp)

Fukushima Daigaku

Research note

Keywords: galectin-9, IL-1 β , inflammasome, nod-like receptor (NLR) Family Pyrin Domain Containing 3, serum amyloid A, T cell immunoglobulin domain and mucin-3

Posted Date: December 31st, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-136825/v1>

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Abstract

Objective

The Nod-like receptor (NLR) Family Pyrin Domain Containing 3 (NLRP3) inflammasome plays roles in host defense and the development of autoinflammation. Galectin-9 (Gal-9), one of the β -galactoside binding lectins, plays important regulatory roles in autoimmune diseases. T cell immunoglobulin and mucin-domain containing molecule 3 (TIM-3)/Gal-9 inhibitory interaction has been proposed in innate immune system. We investigate the role of Galectin-9 (Gal-9) on serum amyloid A (SAA)-induced inflammasome activation and IL-1 β processing by human neutrophils.

Results

SAA stimulation induced the release of cleavage of IL-1 β (p17) from neutrophils suggesting that SAA induces the inflammasome activation and subsequent processing of pro-IL-1 β . ELISA data demonstrated that SAA stimulation also induced cleaved caspase-1 (p20) secretion from human neutrophils, and this release was suppressed by Gal-9 pretreatment. Gal-9 pretreatment diminished the SAA-induced cleaved IL-1 β secretion, however, did not affect SAA-induced pro-IL-1 β secretion from neutrophils. Furthermore, Gal-9 pretreatment suppressed SAA-induced intracellular accumulation of cleaved IL-1 β , suggesting that Gal-9 functions as a negative regulator of SAA-induced inflammasome activation and may be a potential therapeutic target for the treatment of autoinflammatory disorders.

Introduction

The intracellular multi-protein complex known as the inflammasome contributes to innate immune responses and host defense [1]. However, excessive inflammasome activation can promote numerous inflammatory disorders, most notably, autoinflammatory diseases [2]. The Nod-like receptor protein 3 (NLRP3) inflammasome is multiprotein assembly composed of NLRP3, apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) and caspase-1, which serves as a platform that regulate the mature interleukin-1 β (IL-1 β) production. [3].

The galectin family, which are characterized by the presence of conserved carbohydrate-recognition domain (CRD) that binds galactose, have emerged as important regulators of immune function [4]. However, their role in innate immunity, including inflammasome activation process, remains poorly understood. Galectin-9 (Gal-9) is a prominent member of the galectin family [5] and it interacts with various ligands, including T cell immunoglobulin mucin 3 (TIM-3), which is a cell surface molecule that is widely expressed on both innate and adaptive immune cells [6]. With respect to adaptive immunity, Gal-9 promotes apoptosis of T helper type 1 (Th1) cells via its interactions with TIM-3 [7], and plays immunomodulatory roles [8]. Gal-9 has been shown to be expressed in the inflamed tissues of autoimmune or inflammatory disorders [9]. In general, Gal-9 is thought to be induced during the activated innate immune response and downregulate the innate immune cells. For example, Gal-9 engagement

impairs the function of NK cells, including cytotoxicity and cytokine function [10]. Furthermore, exogenous Gal-9 was shown to have beneficial effects in mouse models of autoimmunity [11]. Recently, we found high serum levels of Gal-9 that correlated positively with disease activity in patients with Adult Still's disease (ASD), in which inflammasome activation is believed to play a central role in disease pathogenesis [12]. However, the immediate impact of Gal-9 with respect to innate immune responses, including inflammasome activation, has not yet been fully elucidated. In this study we examined the impact of exogenous Gal-9 and its role in modulating NLRP3 inflammasome activation in innate immune cells

Materials And Methods

Reagents

Recombinant human Galectin-9 and anti-human TIM-3 antibody were purchased from R&D Systems (Minneapolis, MN, USA). Recombinant human SAA was purchased from Peprotech (Rocky Hills, NJ). Anti-pro-IL- β Polyclonal antibody (MBS 125139) was purchased from MyBioSource (San Diego, CA USA). Anti-cleaved-IL- β (p17, D3A3Z) antibody was purchased from Cell Signaling Technology (CST, Danvers, USA). Anti-NLRP-3 antibody was purchased from MERCK MILLIPORE (Billerica, MA USA).

Neutrophils isolation

Venous peripheral blood were obtained from Japanese healthy subjects (6 males, 1 females, mean age of 35.2 ± 7.7 years). Written informed consent for blood donation was obtained from each individuals. The blood was layered on a Polymorphprep TM (Axis-Shield, Oslo, Norway) cushion and neutrophils were purified using density sedimentation according to the manufacturer's instructions. To determine the effects of Gal-9 on SAA-induced IL-1 β production in neutrophils, freshly isolated neutrophils were incubated with Gal-9 for 1hr and stimulated with physiological concentrations of SAA.

ELISA analysis

IL-1 β and caspase-1 (p20) amounts in cell-free neutrophils-conditioned media were measured by enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis) according to the manufacturers' protocols.

Cell lysis and immunoblot analysis

Freshly isolated neutrophils were stimulated with SAA for indicated periods and the cells were washed by PBS and added RIPA Lysis Buffer (Sigma-Aldrich) supplemented with proteinases inhibitor cocktail on ice. The cell lysates were centrifuged at 10,000 g for 10 minutes at 4 °C and collect the supernatant. An equivalent amount (30 μ g) were subjected to 12% SDS-PAGE and electrotransferred onto polyvinylidene fluoride membranes, which were blocked for 1 h at room temperature with 5% bovine serum albumin. The membrane was incubated with primary antibodies against human NLRP3, pro-IL-1 β , cleaved IL-1 β and β -actin and then incubated with secondary antibodies at room temperature, followed by visualization using

ECL reagent (Amersham, Little Chalfont, UK). Immunoblot detection was achieved by LAS-3000 Imaging System (Fuji Film, Tokyo Japan).

Statistical analysis

Differences between groups were examined for statistical significance using Student t-test. *P* values less than 0.05 were considered statistically significance.

Results

SAA induced cleaved IL-1 β secretion from neutrophils

In these studies, we employed serum amyloid A (SAA) to activate the NLRP3 inflammasome and to induce IL-1 β secretion. Previous studies revealed that human innate immune cells secreted IL-1 β in response to SAA stimulation in an NLRP3-dependent manner [13]. Consistent with these findings, we found that addition of SAA triggered the secretion of IL-1 β from human neutrophils (Fig. 1A). We also confirmed that SAA-stimulation resulted in the release of cleaved IL-1 β which was detected in neutrophil-conditioned media (Fig. 1B).

Gal-9 inhibited SAA-induced cleaved IL-1 β secretion from neutrophils.

To examine the impact of Gal-9 on human neutrophils, we first determined whether TIM-3, the major ligand for Gal-9, could be detected on neutrophils. We found that isolated neutrophils constitutively expressed TIM-3 and that its expression was not modulated in response to SAA stimulation (data not shown). To evaluate the effect of Gal-9 on inflammasome activation, neutrophils were pre-treated with exogenous Gal-9 and stimulated with a physiological concentration (5 μ g/ml) of SAA for 16 hr. ELISA analysis showed that pretreatment with Gal-9 did not affect the SAA-induced IL-1 β release from human neutrophils (Fig. 2A). Once activated by NLRP3 inflammasome complex, pro-caspase-1 is processed into p20 and p10 subunits and p20 subunit can be secreted from activated cells [14]. Therefore, the neutrophils-conditioned supernatants were analyzed for the detection of cleaved caspase-1 by an ELISA specific for caspase-1 (p20). We found that pretreatment with Gal-9 resulted in dose-dependent inhibition of caspase-1 (p20) release from SAA-stimulated human neutrophils (Fig. 2B). By combining analysis using anti-pro-IL-1 β and anti-cleaved IL-1 β (p17) antibodies, we evaluated the processing of pro-IL-1 β to the cleaved IL-1 β (p17) using the same neutrophils-conditioned media. As shown in Fig. 3A, stimulation with SAA induced the release of both pro-IL-1 β and cleaved IL-1 β from human neutrophils. Interestingly, Gal-9 pretreatment had no impact on the release of pro-IL-1 β from SAA-stimulated neutrophils, although it resulted in markedly diminished release of cleaved IL-1 β under the same conditions. Taken together, these results indicated that administration of Gal-9 negatively regulated the processing of pro-IL-1 β to cleaved IL-1 β in SAA-stimulated neutrophils.

Gal-9 inhibited SAA-induced intracellular accumulation of cleaved IL-1 β in neutrophils.

To determine whether Gal-9 pretreatment has an influence on the intracellular accumulation of cleaved IL-1 β , we evaluated pro-IL-1 β processing by immunoblotting using neutrophil lysates. Immunoblot analysis confirmed that SAA-stimulation induced the intracellular accumulation of both pro-IL-1 β and cleaved IL-1 β (p17); Gal-9 pretreatment had no impact on SAA-induced intracellular accumulation of pro-IL-1 β . However, pretreatment with Gal-9 negatively regulated the induction of intracellular IL-1 β cleavage in SAA-stimulated neutrophils (Fig. 3B). Finally, we examined the impact of Gal-9 on NLRP3 protein expression in SAA-stimulated neutrophils. SAA stimulation induced the expression of NLRP3 in neutrophils and Gal-9 pretreatment had no impact on this response (Fig. 3C).

Discussion

Among the innate immunity, neutrophils represent one of the important factors that initiate autoinflammation [15]. Uncontrolled neutrophil activation can result in both localized and systemic inflammation and associated tissue damage [15]. Galectins are established regulators of both innate and adaptive immune responses [16]. However, due to their complex tissue distribution and expression patterns, the role of galectins with respect to innate immune responses remains not completely understood. Inflammasome is mainly involved in the processing of pro-IL-1 β to active IL-1 β [17]. In this study we investigated the role of Gal-9 in inflammasome activation processes. Our results showed that IL-1 β is released from human neutrophils in response to SAA stimulation. SAA stimulation also resulted in the release of cleaved IL-1 β ; these results suggested that pro-IL-1 β is processed in response to inflammasome activation. Gal-9 prevented SAA-induced pro-IL-1 β processing and likewise suppressed the release of mature IL-1 β . We identified Gal-9 as a negative regulator of NLRP3 inflammasome, which plays an important role in autoinflammation.

The mechanisms by which Gal-9 suppresses the release of cleaved-IL-1 β have yet not be identified. Galectins interact with a broad range of receptors and proteolytic processes [18] and it is likely that Gal-9 may modulated the functions of immune cells.

Gal-9 has an important role in regulating adaptive immunity through its interactions with TIM-3, where it regulates Th1 and Treg expansion. [19] Since Gal-9 was expressed in innate immune cells [20], Gal-9 is likely to participate in multiple roles with respect to regulation of innate immunity. TIM-3 is presumed to be a ligand for Gal-9 and has been associated with Gal-9-mediated inhibitory functions [20]. More recently, TIM-3 has been shown to act as a negative regulator of the NLRP3 inflammasome by dampening NF- κ B response and NLRP-3 expression in peritoneal macrophages [21]. However, our results indicated that Gal-9 pretreatment had no impact on SAA-induced NLRP3 expression in neutrophils. These findings suggest that exogenous Gal-9 inhibited SAA-induced inflammasome cascade by affecting the activation step not priming step.

Although the role of Gal-9 in inflammation has yet to be fully characterized, our data suggest that the interactions between Gal-9 and neutrophils are critical factors during the inflammasome activation processes. It is also possible that Gal-9 exerts the inhibitory effects on immune responses not only

directly but also via the induction of other regulator populations [22]. For example, administration of Gal-9 resulted in the induction of naive T cell differentiation to Tregs and suppressed the activity and differentiation of Th17 cells [23]. Gal-9 also suppresses the production of TNF- α and IL-1 β , and supports the production of IL-10 by immune complex-activated macrophages [23]. Similarly, treatment with Gal-9 resulted in diminished levels of pro-inflammatory cytokines and serum C5a in the joints observed in mice subjected to anti-CII monoclonal antibody (mAb)-induced arthritis (CAIA) [24]. More recently, Gal-9 has shown to abolish B cell receptor (BCR) signaling by reducing BCR mobility through inducing nanocluster formation between Gal-9 and BCR. [25]. Galectins are group of mammalian lectins characterized by a high affinity for β -galactosides and a highly conserved carbohydrate recognition domain (CRD) [26]. The galectin lattice appears to modulate receptor signaling by influencing glycoprotein compartmentalization [27]. It is possible that Gal-9-induced modifications of membrane proteins may have an influence on the inflammasome activation processes. As galectins possess the multivalent adaptor protein-like functions in both extracellular and intracellular [28], future studies will be required to elucidate the relationship between Gal-9 and inflammasome.

Our study revealed that Gal-9 has the capacity to regulate amyloid-induced inflammasome activation and to suppress pro-IL-1 β processing in human neutrophils. Taken together, these results suggest that the Gal-9 pathway represents a potential target for novel therapies designed to regulate inflammasome-mediated disorders.

Limitations

This study has limitations. The direct evidence for Gal-9-mediated inhibition of NLRP3 inflammasome assembly and activation processes has not been presented. The mechanism through which Gal-9 contributes to the inhibition of pro-IL-1 β processing was not clarified. It is meaningful to perform immunoprecipitation analysis in the future to verify that Gal-9 treatment indeed inhibits the assembly of the NLRP3 inflammasome in future studies.

Abbreviations

ASD

Adult Still's disease

Gal-9

Galectin-9

NLRP-3

Nod-like receptor (NLR) Family Pyrin Domain Containing 3

SAA

serum amyloid A

TIM-3

T cell immunoglobulin and mucin-domain containing molecule 3

Declarations

Ethical Approval and Consent to participate

Ethical approval for this study (No. 29282) was provided by the Ethics Committee of Fukushima Medical University and written informed consent was obtained from each individual. All methods were carried out in accordance with relevant guidelines and regulations under Declaration section.

Consent for publication

Not applicable

Availability of data and materials

All data generated or analysed during this study are included in this published article.

Competing interests

KM has received research grants from Chugai, Pfizer, Eli Lilly and AbbVie.

Rest of the authors declares that they have no competing interests

Funding

The study was supported by the Japan Grant-in-Aid for Scientific Research (20K08777). JT, YF, NM, MF, TA, SS, HM, ES, HW, KM carried out the molecular biochemical studies, participated in the sequence alignment and drafted the manuscript. JT, YF, KM carried out the genetic assays. JT, KM participated in the sequence alignment and drafted the manuscript. JT, KM participated in the design of the study, performed the statistical analysis. All authors discussed the results and commented on the manuscript.

Acknowledgements

We are grateful to Ms Kanno Sayaka for her technical assistance in this study.

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Figures

Figure 1

A SAA induces IL-1 β secretion from neutrophils Neutrophils (2×10^6 /ml) were incubated with the indicated concentrations of SAA for 16 hr and supernatants were analyzed for IL-1 β production by ELISA. Values represent the mean $\pm$ SD of two independent experiments. * compared to unstimulated neutrophils. B SAA induces cleaved IL-1 β secretion from neutrophils Neutrophils (2×10^6 /ml) were incubated with the indicated concentrations of SAA for 16 hr and supernatants were analyzed by immunoblot for the

presence of cleaved IL-1 β (p17). Three experiments were performed using different neutrophils and a representative result is shown.

Figure 2

Effects of Galectin-9 on IL-1 β and cleaved caspase-1 (p20) secretion from SAA-stimulated neutrophils. Neutrophils were pretreated with the indicated concentrations of Galectin-9 for 1 hr and stimulated with SAA (5 μ g/ml) for 16 hr and supernatants were analyzed for IL-1 β (A) or cleaved caspase-1 (p20; B) by ELISA. Values represent the mean $\pm$ SD of two independent experiments. * compared to SAA-stimulated neutrophils.

Figure 3

A Effects of Galectin-9 on pro-IL-1 β or cleaved IL-1 β secretion from SAA-stimulated neutrophils. Neutrophils were pretreated or untreated with the indicated concentrations of galectin-9 for 1 hr. After pretreatment, the cells were stimulated with of SAA (5 $\mu\text{g/ml}$) for 16 h and supernatants were analyzed by immunoblot for the presence of pro-IL-1 β and of cleaved IL-1 β (p17). Three experiments were performed using different neutrophils and a representative result is shown. **B** IL-1 β immunoblot analysis using the

cellular lysates of SAA-stimulated neutrophils Neutrophils were pretreated or untreated with the indicated concentrations of galectin-9 for 1 hr. After pretreatment, the cells were stimulated with of SAA (5µg/ml) for 16 hr. Cellular lysates were analyzed by immunoblotting with either antibodies that recognize pro-IL-1β or cleaved IL-1β (p17). β-actin was the loading control. Three experiments were performed using different neutrophils and a representative result is shown. C Effects of Galectin-9 on NLRP3 expression in SAA-stimulated neutrophils Neutrophils were pretreated or untreated with the indicated concentrations of galectin-9 for 1 hr. After pretreatment, the cells were stimulated with of SAA (5µg/ml) for 16 h. Cellular lysates were analyzed by immunoblotting using anti-NLRP3. β-actin was the loading control. Three experiments were performed using different neutrophils and a representative result is shown.

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