

NLRP10 A Potential Contributor to the Anti-Inflammatory Effects of IVIg in Sepsis

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Abstract

Intravenous immunoglobulin (IVIg) is used to treat a number of inflammatory diseases including sepsis. The objective of this study was to evaluate whether NLRP10, postulated as a negative regulator of inflammasome activation, can play a role in the anti-inflammatory effects observed in the treatment of severe sepsis with IVIg. Human peripheral blood monocytes were isolated and treated either with LPS or IVIg, or alternatively treated first with LPS followed by addition of IVIg. The induction of IL-1 β and IL-18 was used as an index of inflammasome activation. Treatment of resting monocytes with IVIg resulted in a significant increase in the expression of NLRP10. LPS but not IVIg resulted in a significant increase in both IL-1 β and IL-18 protein release, consistent with inflammasome activation. Furthermore, addition of IVIg to LPS-treated monocytes resulted in a significant increase in the expression of NLRP10 which was associated with a complete inhibition of both IL-1 β and IL-18 increased expression. These data suggest that the IVIg-mediated induction of NLRP10 expression in monocytes contributes to the control of inflammation during sepsis and thus highlight a complementary mechanism by which IVIg exerts their anti-inflammatory effects in sepsis.

Keywords: NLRP10; IVIg; Sepsis; Inflammasome; IL-18; IL-1 β

Introduction

Intravenous immunoglobulin (IVIg) is a highly purified preparation of polyspecific immunoglobulin G obtained from pooled plasma of several thousand healthy donors and is currently used to treat a number of inflammatory and autoimmune diseases and as an adjunctive therapy in sepsis [1-3]. Indeed, administration of IVIg significantly reduces mortality in critically ill adult patients with severe sepsis and septic shock [4,5]. The beneficial effects of IVIg in infectious diseases have been largely attributed to the presence of antibodies reacting with bacterial cell components, toxins and cytokines [6,7]. However, the precise mechanism behind the inhibitory effect of IVIg in severe sepsis and particularly on monocyte functions remains unclear.

Recently, it has been reported that IVIg suppresses the activity of the NOD-like receptors, leucine-rich repeats and pyrin domain-containing (NLRP) 1 and 3 inflammasome, leading to prevention of neural death in ischemic stroke [8]. NLRP are a family of cytosolic proteins that play an important role in inflammation and immunity [9]. NLRP detect specific pathogen-associated molecules or endogenous damage signals and initiate the innate immune response. NLRP have been reported to form a large, multiprotein inflammasome complex that subsequently leads to the processing and secretion of pro-inflammatory cytokines interleukin-1 β (IL-1 β) and IL-18 [10]. Interestingly, a subfamily of NLRP that includes NLRP10 has been shown to have anti-inflammatory activities. Indeed, NLRP10 was previously proposed as a negative regulator of NF- κ B, cell death, IL-18 and IL-1 β release [11,12]. These results have been supported by NLRP10 over-expression studies in *Nlrp10* knock-in mice and in *in vitro* studies. *Nlrp10* knock-in mice were found to be resistant to LPS-induced endotoxic shock, due to a decreased release of inflammatory cytokines [12]. This was consistent with the observation that cells from these animals secreted reduced amounts of IL-1 β following infection with *Salmonella* or TLR7 stimulation [11,12]. In the present work, we evaluated whether modulation of NLRP10 expression was associated with the anti-inflammatory effects observed in the treatment of severe sepsis with IVIg.

Materials and Methods

Preparation of human mononuclear cells

This study has been approved by the Héma-Québec's Research Ethics Committee and all participants in this study have signed an informed consent. Leukoreduction system (LRS) chambers from Trima Accel™ collection systems (Gambro BCT, Lakewood, CO) were obtained after routine apheresis. Leukocytes were recovered from LRS chambers, as previously described [13] and used to prepare peripheral blood mononuclear cells (PBMCs). PBMC were isolated using Ficoll-Paque density gradient following the manufacturer's instructions (GE Healthcare, Baie d'Urfé, Canada).

Monocyte isolation and culture

PBMC were washed thrice with PBS containing 2 g/L of glucose (PBS-glucose) and then suspended in PBS containing 2 mM EDTA. Monocytes were purified using the EasySep™ Human Monocyte Enrichment Kit (Stem Cell Technologies, Vancouver, Canada). To assess purity, cells were labeled with an anti-CD14-FITC conjugate (BD Bioscience, San Diego, CA, USA) and analyzed by flow cytometry. CD14+ monocytes purity was $\geq 95\%$. The purified monocytes were seeded in the wells of a 6-well microplate in RPMI 1640 medium supplemented with 10% FBS, 2 mM L-glutamine, 1X penicillin-streptomycin, 1 mM sodium pyruvate and allow to adhere for 4 hours before stimulation with 1 μ g/mL of LPS (Sigma-Aldrich, St. Louis, MO)

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during 3 hours. Cells were then washed twice with PBS and cultured in the presence or not of 15 mg/mL of IVIg (Grifols Canada Ltd., Mississauga, Canada) or human serum albumin (HSA, Grifols Canada Ltd.) for 24, 48 and 72 hours for protein expression by flow cytometry.

ELISA

Culture supernatants of monocytes incubated for 48 hours in the different experimental conditions were collected and immediately stored frozen (-80°C) until use. The expression of IL-1 β and IL-18 was evaluated by ELISA (Human IL-18 ELISA kit, R&D system, Minneapolis, MN) according to the manufacturer's instructions. The SoftMax Pro Data Acquisition and Analysis Software (Molecular device; Sunnyvale, CA, USA) were used to generate a standard curve. The cytokine concentration was determined using the standard curve and the optical densities of IL-1 β and IL-18.

Flow cytometry

Monocytes were detached from the microwells using TrypLE™ Express recombinant enzyme (Life Technologies Inc.). To avoid unwanted binding of antibodies to human Fc γ R and to allow permeabilization, cells were incubated for 10 min at 4°C in PBS containing 2% FcR-block reagent (Miltenyi Biotec, Cambridge, MA) and 0.05% saponin prior to labeling. Then, unconjugated rabbit antibodies directed against NLRP10 (Sigma-Aldrich, St-Louis, MO) was added and incubated for 1 hour followed by washing in PBS containing 0.05% saponin. Alexa Fluor 488-conjugated goat anti-rabbit IgG (Life technologies) were then added. After another wash step with PBS, the expression of NLRP10 was measured on a Partec Cyflow ML flow cytometer (Partec North America, Inc., Swedesboro, NJ). The data was analyzed using FCS express 4 software (De NovoSoftware, Los Angeles, CA).

Statistical analyses

All statistical analyses were performed with the Graph-Pad InStat software (GraphPad Software Inc., La Jolla, CA). Comparison of three or more means was done using one-way analysis of variance (ANOVA) with the appropriate post-test (Tukey-Kramer Multiple Comparisons Test). Values of $P < 0.05$ were considered to indicate statistical significance.

Results

IVIg modulates NLRP10 expression

To determine whether IVIg could have anti-inflammatory effects in sepsis, we used LPS-stimulated human monocytes as an *in vitro* model. Monocytes were first stimulated with 1 μ g/mL of LPS during 3 hours. Cells were then washed twice with PBS and cultured in the presence or not of 15 mg/mL of IVIg or the same concentration of human serum albumin (HSA, used as a protein control) for 24 h, 48 h and 72 h before being recovered and used for flow cytometry analysis of the expression of NLRP10 in LPS-activated (Figure 1A) or resting (Figure 1B) monocytes, treated or not with IVIg. Our results first show that only a small proportion of resting monocytes (about 15%, Figure 1A and 1B) express NLRP10. Activation of monocytes by LPS did not result in an increased expression of NLRP10 at all time points studied (Figure 1A). Similarly, the addition of IVIg or HSA did not affect NLRP10 expression at 24 hours. In contrast, the same analysis done at 48 and 72 hours revealed a significant increase in the proportion of cells expressing NLRP10 (>3 fold, $P < 0.001$) in both LPS-activated (Figure 1A) and resting monocytes (Figure 1B) cultured in the presence of

IVIg but not with HSA. The absence of NLRP10 modulation by HSA indicated that the effect was specific to IVIg and not due to the high concentration of protein present in the assay.

IVIg suppresses the inflammasome activation

To determine whether the increased NLRP10 expression at 48 hours correlated with a decreased inflammasome activation, we measured the secretion of IL-1 β and IL-18 as readout of inflammation. Monocytes were first stimulated with 1 μ g/mL of LPS during 3 hours to activate the inflammasome. Cells were then washed twice with PBS and cultured during 48 hours in the presence or not of 15 mg/mL of IVIg. Culture supernatants were then collected and used for the quantification of both IL-1 β and IL-18 protein expression by ELISA. Our results revealed that stimulation of resting monocytes with LPS resulted in a significant increase in the secretion of both IL-1 β and IL-18 (>2 fold; Figure 2A and 2B) consistent with inflammasome activation, as reported in several previously published studies [11,14,15]. This increase was reduced to baseline level in IVIg-supplemented cultures and mirrors the significant increase in the expression of NLRP10 at 48 hours (Figure 1A and 1B).

Discussion

The current study revealed that IVIg inhibited the expression of IL-1 β and IL-18, two cytokines associated with inflammasome activation and that this effect was associated with an increased expression of NLRP10 on the surface of monocytes. This latter effect was specific to IVIg since it was not observed in the presence of a similar concentration of HSA.

Inflammasomes are key signalling platforms that detect pathogenic microorganisms and activate the highly pro-inflammatory cytokines IL-1 β and IL-18 [16]. Indeed, cytokine overproduction is observed in septic patients and can be produced in experimental animal models with the injection of a high dose of LPS leading to pathological symptoms resembling those of the septic patient [17]. NLRP10 (also known as PYNOD, NALP10, PAN5 and NOD8) is the only NLR lacking the putative ligand-binding leucine-rich-repeat domain, and has been postulated to be a negative regulator of the inflammasome [11,12]. It has been shown previously that IVIg inhibition of IL-1 β and IL-18 secretion may involve the suppression of the classical NLRP1 and NLRP3 inflammasome or/and a rapid inhibition of caspase-1 and NF- κ B activation leading to a reduced IL-1 β and IL-18 expression [8,11]. Our results provide an additional mechanism by which IVIg can reduce the secretion of inflammatory cytokines, by upregulating the expression of NLRP10 on activated monocytes. Although other studies have described an essential role of NLRP10 in immune defense [18,19], our study rather supports an anti-inflammatory function for NLRP10.

Interestingly, we observed that the treatment of resting monocytes with IVIg also resulted in a significant increase in the expression of NLRP10. These data suggest that under both inflammatory and non-inflammatory conditions, IVIg promotes the acquisition of an anti-inflammatory phenotype prone to limit the inflammatory response. This hypothesis is supported by a recent finding by Padet et al. who showed that IVIg induces anti-inflammatory monocytes with a CD80 (low) PD-L1 (high) phenotype [20]. In addition, Imamura et al. previously showed that the increased expression of NLRP10 in mouse was associated with an inhibition of pro-inflammatory cytokine synthesis [12]. Taken together, our data provides new insights into the mechanisms by which IVIg mediates its anti-inflammatory effects.

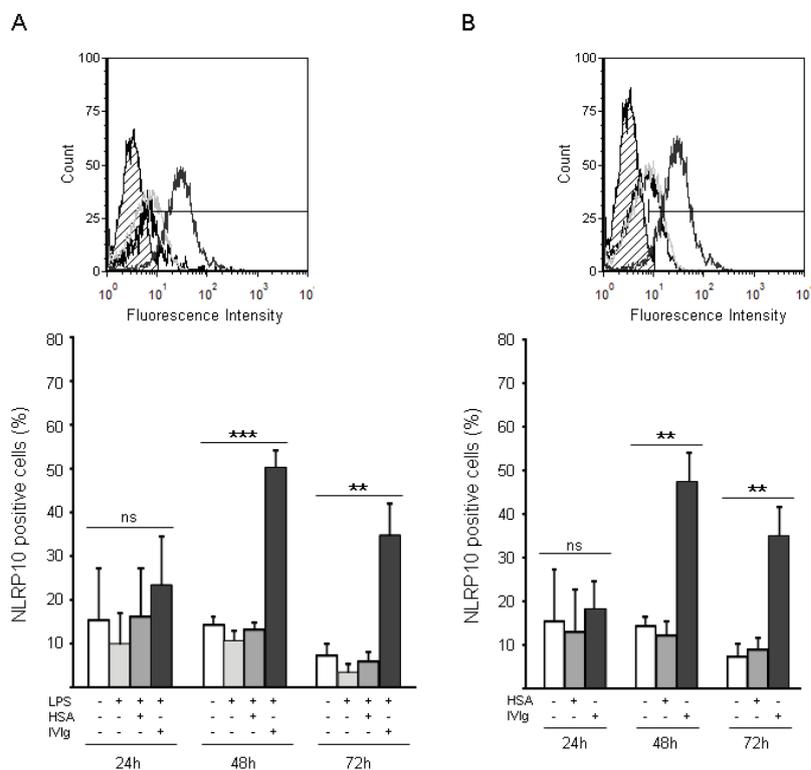


Figure 1: Effect of IVIg on the expression of NLRP10. Monocytes were pretreated (A) or not (B) during 3 hours with LPS (1 μ g/mL) followed by washing and cultured in the presence of IVIg or HSA (15 mg/mL) for 24, 48 and 72 h. The intracellular expression of NLRP10 was analyzed by flow cytometry. Representative histograms (at 48 h) are shown (hatched, isotype control; light grey, untreated monocytes; black, HSA-treated monocytes; dark grey, IVIg-treated resting monocytes) and in B (hatched, isotype control; light grey, untreated monocytes; black, LPS-treated monocytes; dark grey, IVIg-treated LPS activated monocytes). Data are presented as mean \pm SEM of 8 independent experiments. ** P <0.01; *** P <0.001; ns: not significant.

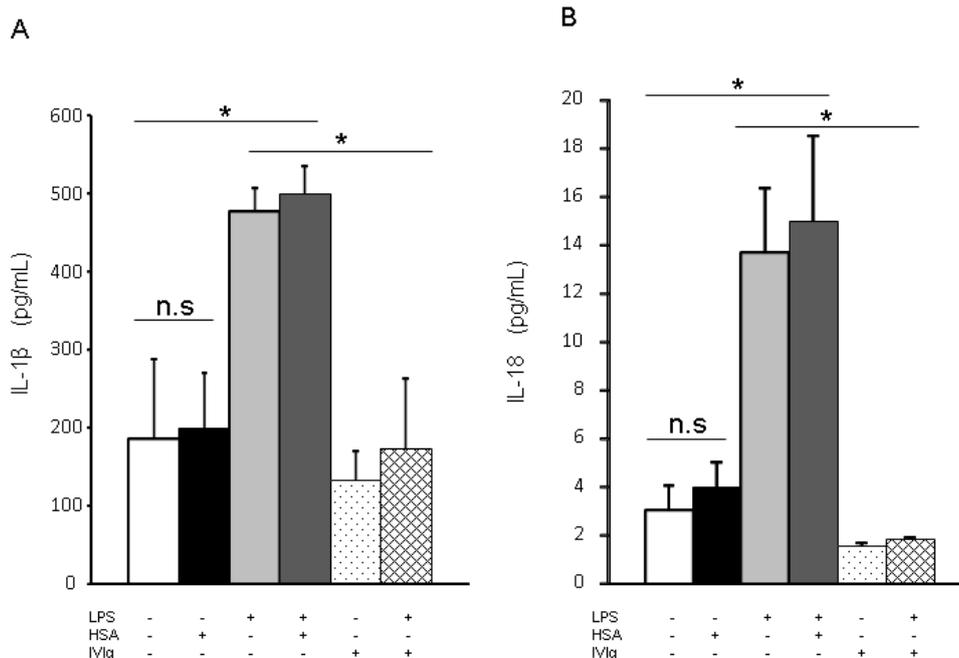


Figure 2: Effect of IVIg on the inflammasome activation. Monocytes were pretreated during 3 hours with LPS (1 μ g/mL) followed by washing and culture in the presence of IVIg or HSA (15 mg/mL) for 48 h. Culture supernatants were collected and used to measure the concentration of (A) IL-1 β and (B) IL-18 by ELISA. Data are presented as mean \pm SEM of 8 independent experiments. * P <0.05; *** P <0.001.

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