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### ► To cite this version:

Marie Wiatr, Nicolas Merle, Idris Boudhabhay, Victoria Poillerat, Sofia Rossini, et al.. Anti-inflammatory activity of intravenous immunoglobulin through scavenging of heme. *Molecular Immunology*, 2019, 111, pp.205-208. 10.1016/j.molimm.2019.04.020 . hal-02127299

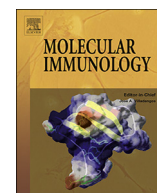
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Submitted on 13 May 2019

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## Short communication

## Anti-inflammatory activity of intravenous immunoglobulin through scavenging of heme

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## ARTICLE INFO

## Keywords:

Intravenous immunoglobulin (IVIg)

Heme

Complement system

Immunomodulation

IgG

Endothelial cells

Hemolysis

## ABSTRACT

Therapeutic intravenous immunoglobulin preparations (IVIg) are used for treatment of wide range of auto-immune and inflammatory diseases. Versatile mechanisms have been reported to contribute to the immunomodulatory effects of IVIg. Here we demonstrate that IVIg has a strong potential to inhibit pro-inflammatory effect of extracellular heme. Indeed, the presence of immunoglobulins reduced the potential of heme to activate the complement system on the surface of human endothelial cells. Since extracellular heme is considered as one of the principal pathogenic factors in hemolytic disorders, its therapeutic scavenging by IVIg may have significant clinical repercussions.

### 1. Introduction

Intravenous therapeutic immunoglobulin (IVIg) represents a pool of IgG isolated from plasma of thousands of healthy individuals. The large number of distinct antigen binding specificities of antibodies in IVIg recapitulates the immune diversity at a population level. In addition to its primary use as a replacement therapy in different types of immune deficiencies, IVIg has also found wide clinical application as an anti-inflammatory agent in various inflammatory and autoimmune diseases (Galeotti et al., 2015; Kazatchkine and Kaveri, 2001; Schwab and Nimmerjahn, 2013). Plethora of mechanisms has been attributed to the therapeutic effects of IVIg in autoimmune and inflammatory disorders. IVIg can exert anti-inflammatory effects via the constant immunoglobulin fragment of IgG molecules through interaction with Fc receptors (FcR), expressed on immune cells (Schwab and Nimmerjahn, 2013). Many anti-inflammatory effects ascribed to IVIg depend also on binding to self-antigens. These Fab-dependent effects include neutralization of pro-inflammatory cytokines and scavenging of complement components, interaction with activating or inhibitory receptors on the immune cells, blocking the variable regions of pathogenic auto-antibodies, etc (Basta and Dalakas, 1994; Basta et al., 2003; Galeotti et al., 2017; Kaveri, 2012; Kazatchkine and Kaveri, 2001; Svetlicky et al., 2013; von Gunten et al., 2014). IVIg has also been proposed to replace pathogenic antibodies by interference with interactions with

the neonatal Fc receptor (FcRn) and hence the circulatory half-life of endogenous IgG (Akilesh et al., 2004). In a pathological context, the simultaneous involvement of different anti-inflammatory mechanisms is most probably responsible for the overall immunomodulatory effect of IVIg. Here we provide evidence for a novel mechanism through which IVIg may exerts anti-inflammatory effects – scavenging of endogenous low-molecular-weight pro-inflammatory mediators.

### 2. Material and methods

#### 2.1. Preparation of heme stock solutions

Stock solution of heme was prepared by dilution of hemin (Frontier Scientific) in 0.05 M NaOH to final concentration of 10 mM, followed by addition of 25 fold molar excess of H<sub>2</sub>O<sub>2</sub>. Catalytic degradation of H<sub>2</sub>O<sub>2</sub> by heme was monitored by formation of gas bubbles (O<sub>2</sub>) and it was completed for 10 min. The oxidized species of heme were designated as heme-ox. The preparation of heme-ox was always performed before experiment. The stock solution was stored on ice in dark.

#### 2.2. Intravenous immunoglobulin

Therapeutic immunoglobulin preparation, IVIG (Endobulin, Baxter) at 80 mg/ml was dialyzed exhaustively against PBS and stored at

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– 20 °C until use.

### 2.3. Cell experiments

HUVEC (Lonza) used for these experiments were cultured in 24 wells (approximately  $4.5 \times 10^6$  per wells), pre-coated with bovine gelatin 1% (Sigma), in complete Medium 199 (Gibco), supplemented with 20% decompartmented Fetal Calf Serum (FCS), 1% glutamine, 0.1% heparin, 10% HEPES, and 1% Penicillin/Streptomycin cocktail. ECGS growth factors and EGM2 complete medium (Gibco), were added at 0.5% and 20%, respectively, of the final volume. For all experiments the normal human serum (purchased from EFS, Paris, ethical authorization N°12/EFS/079), diluted three-folds in M199 medium without FCS was added to the wells for 30 min at 37 °C.

After reaching confluence, cells were washed in PBS ( $\text{Ca}^{2+}$ ;  $\text{Mg}^{2+}$ ). To test the capacity of heme-ox to activate complement, HUVEC were exposed or not to 100  $\mu\text{M}$  heme-ox for 30 min, washed and incubated with sera from 6 different healthy donors.

To test the capacity of IVIG to inhibit heme-ox induced complement deposits, HUVEC were incubated or not with 15 mg/ml (100  $\mu\text{M}$ ) IVIG diluted in the M199 medium but without FCS. After 30 min of pre-incubation, increased concentration from 6.25 to 100  $\mu\text{M}$  of heme-ox, were added directly to the medium. Cells were incubated for 30 min, at 37 °C, washed, and exposed to normal human serum.

In order to test whether IVIG has a direct impact on heme or influences the endothelial cells, IVIG was incubated with the cells for 30 min, washed or not and heme-ox or medium was added to the system, followed by normal human serum as above. Alternatively, heme was added to the cells, washed and IVIG was added afterwards, followed by serum.

### 2.4. Flow cytometry

Following treatments with heme and immunoglobulins, cells were washed 2 times in PBS, and a cocktail of PBS + 5%EDTA + 5 mg/ml Lidocaïne (Sigma-Aldrich) was added to the wells for 30 min at 4 °C with gentle shaking for non-enzymatic detachment to preserve the C3 activation fragments, deposited on the surface. Cells were labeled with monoclonal murine anti-human C3c antibody (Quidel Corporation), recognizing an epitope common to C3b and iC3b, followed by anti-mouse IgG (H + L)-PE (BD Pharmingen). After staining the cells were washed in FACS buffer, fixed in PBS + 0.5% paraformaldehyde solution and analyzed by in flow cytometry (BD LSRII). Data analyses were done on FLOW JO software (LLC).

### 2.5. Real-time kinetic measurements

Kinetics of the interaction of IVIg with heme-ox was studied by using surface plasmon resonance-based optical biosensor system (Biacore 2000, GE Healthcare, Uppsala, Sweden). IVIG was covalently immobilized on the surface of CM5 sensor chip (Biacore, GE Healthcare) using amine-coupling kit (Biacore, GE Healthcare). Briefly, the immunoglobulin preparation was diluted to final concentration of 50  $\mu\text{g}/\text{ml}$  in 5 mM maleate with pH 4 and injected over pre-activated sensor surface. For activation of the sensor surface amine-coupling kit was used as recommended by manufacturer (Biacore). Unconjugated activated carboxyl groups on the sensor surfaces were saturated by injection of 1 M ethanolamine.HCl. A control surface was prepared by activation and subsequent deactivation of the carboxyl groups on the chip surface. The signal of the control surface was always subtracted from the signal of the protein-coated surfaces.

The running buffer HBS-EP (10 mM HEPES pH 7.2; 150 mM NaCl; 3 mM EDTA, and 0.005% Tween 20) was filtered through 0.22  $\mu\text{m}$  filter and degassed under vacuum, and was used during the immobilization and the binding analyses. All kinetic measurements were performed at 25 °C. The flow rate of the running buffer was set at 30  $\mu\text{l}/\text{min}$ .

Dilutions of heme-ox (from stock solution of 13.8 mM in 0.05 N NaOH) were prepared in HBS-EP immediately prior each injection. Heme-ox concentrations of 5, 2.5, 1.25, 6.25, 3.125, 1.56 and 0.78  $\mu\text{M}$  were consequently injected simultaneously over control and IVIg-immobilized sensor surfaces. The association and dissociation were followed each for 5 min. Regeneration of the sensor surface was done by injection of 0.15 M solution of imidazole. The evaluation of the kinetic data was by BIAevaluation version 4.1.1 Software (Biacore).

### 2.6. Absorbance spectroscopy

We used absorbance spectroscopy for study the interaction of IVIg with heme-ox in solution. The absorbance spectra were recorded by using Agilent Cary 300 UV-vis spectrophotometer (Agilent Technologies). IVIg was diluted to final concentration 10  $\mu\text{M}$  in PBS and titrated with increasing concentrations of heme-ox (0, 2.5, 5, 10, 20, 40, and 80  $\mu\text{M}$ ). Reaction volume was 1 mL. Aliquots of heme-ox stock solution were added both to cuvette containing IVIg and to a reference cuvette, containing only PBS. After addition of each heme-ox aliquot, the absorbance spectra in the wavelength range 300–700 were recorded. The spectral resolution was 1 nm and the bandwidth was set at 2 nm. All measurements were done in quartz cuvette with optical path of 1 cm.

## 3. Results and discussion

Heme (ferroprotoporphyrin IX) has a dualistic nature. When compartmentalized inside the cells, it serves as an indispensable cofactor for aerobic life. However, when liberated in the extracellular space or in plasma, heme displays high pro-oxidative and pro-inflammatory potentials (Soares and Bozza, 2016). Indeed, free heme can interact with Toll like receptor 4 (TLR4), activate inflammasome, activate complement system, and oxidize lipids. As a result of these interactions, heme triggers pro-inflammatory programs in different cell types – neutrophils, monocytes, endothelial cells, etc. These effects classify heme as a typical damage associated molecular pattern (DAMP) or alarmin (Soares and Bozza, 2016). Extracellular heme exerts also potent effects on coagulation and complement system (Roumenina et al., 2016).

Liberation of heme occurs in a diverse set of pathological conditions such as mutations in hemoglobin (sickle cell disease, b-thalassemia), mutations in complement regulators (atypical hemolytic uremic syndrome, paroxysmal nocturnal hemoglobinuria), infections (malaria), trauma (rhabdomyolysis), ischemia reperfusion, hemolytic transfusion reactions, autoimmune diseases (hemolytic anemia), tissue damage, etc. Numerous studies have linked the inflammation accompanying the above listed pathological situations with the pro-inflammatory effect of extracellular heme (Soares and Bozza, 2016).

Our previous studies have revealed that IVIG contains a fraction of antibodies that can bind heme (Dimitrov et al., 2007). Based on this observation, we hypothesized that, in the context of pathological heme release and when natural hemoglobin or heme scavenging plasma proteins (haptoglobin and hemopexin) are overwhelmed, IVIG may exert anti-inflammatory potential through scavenging the free extracellular heme. Previously, we demonstrated that exposure of human endothelial cells to heme results in activation of the alternative complement pathway and deposition of C3 fragments (C3b and iC3b) at the cells surface (Frimat et al., 2013; Merle et al., 2018). This effect of heme is accompanied by a decreased expression of the negative complement regulators CD46 and CD55. Moreover, heme triggers the degranulation of Weibel-Palade bodies resulting in expression of P-selectin which could recruit C3b on the endothelial cell surface (Frimat et al., 2013; Merle et al., 2019; Morigi et al., 2011).

The overactivation of complement at the endothelial surface results in cell damage. Here, we used a model of complement activation on endothelial cells to investigate the anti-inflammatory activity of IVIG. Pre-incubation of human endothelial cells with oxidized form of heme

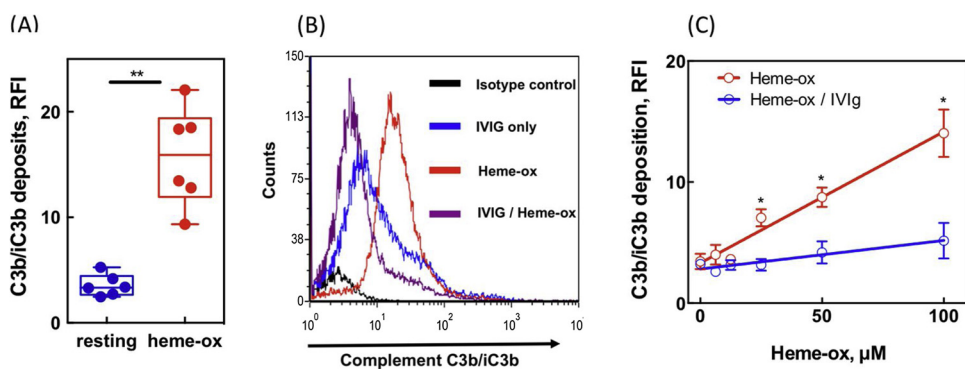


Fig. 1. Deposition of C3 activation fragments (C3b/iC3b) on the surface of human endothelial cells after treatment with oxidized heme in the absence or presence of IVIG. (A) Increased deposits on HUVEC, exposed to 100  $\mu\text{M}$  heme-ox and sera from 6 different healthy donors. (B) A representative histogram depicting membrane deposition of C3b/iC3b after treatment of HUVEC with 50  $\mu\text{M}$  of heme-ox in the presence or absence of 15 mg/ml of IVIG. After incubation with heme-ox and immunoglobulins, cells were exposed to normal human (AB) serum as a source of complement. The C3b was then detected by BD LSRII flow cytometer. (C) Heme-ox concentration-dependent deposition of C3b/iC3b on HUVEC for varying concentration of heme-ox in the presence of 15 mg/ml IVIG. Each point represents relative-fluorescence intensity for varying concentration of heme-ox in the presence of 15 mg/ml IVIG. Average RFI values from four independent repetitions  $\pm$  standard deviation are presented. Comparison between values at each concentration was performed by Mann-Whitney test (asterisk indicates  $p < 0.05$ ).

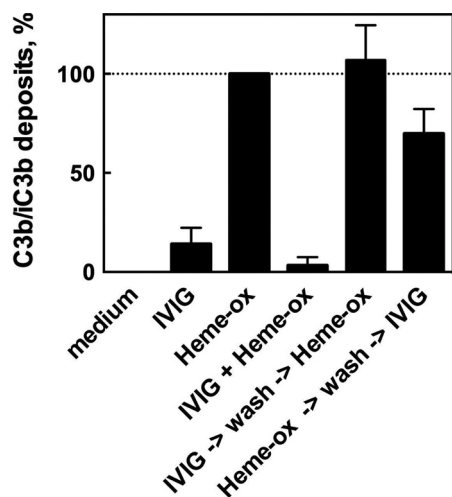
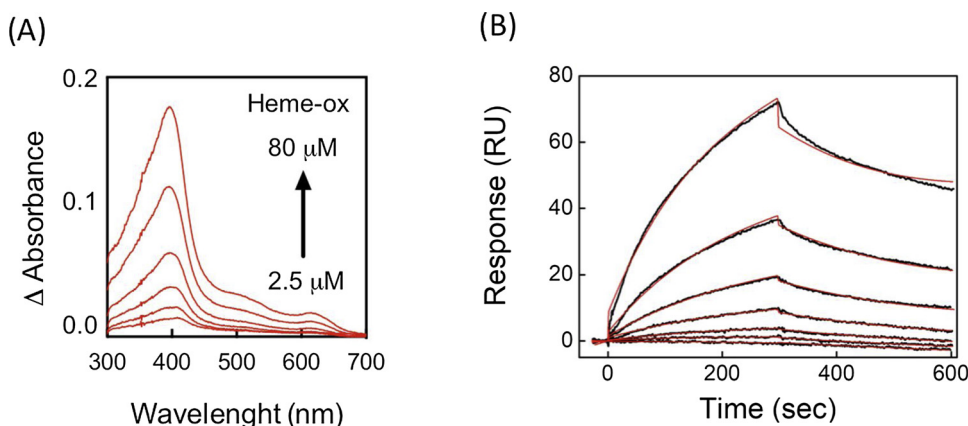


Fig. 2. IVIG protects endothelial cells from heme-mediated complement activation by direct scavenging. HUVEC were exposed to medium or IVIG (15 mg/ml) or Heme-ox (100  $\mu\text{M}$ ), washed or not with PBS, and incubated with medium or IVIG (15 mg/ml) or Heme-ox (100  $\mu\text{M}$ ). After these successive incubation steps, the cells were washed and incubated with normal serum and the deposition of C3b/iC3b was followed by flow cytometry. Data were normalized, taking RFI of the medium-exposed cells as 0 and the one of 100  $\mu\text{M}$  heme as 100%. Average  $\pm$  SD,  $n = 3$ .

(hemin pre-treated with 25 molar excess of  $\text{H}_2\text{O}_2$ , here referred to as heme-ox) resulted in increased C3 activation fragments deposition from sera from 6 different healthy donors, serving as a source of complement proteins (Fig. 1A). These deposits increased with increasing concentrations of heme-ox (Fig. 1). To evaluate the anti-inflammatory



effect of IVIG, we incubated endothelial cells with heme-ox in the presence of IVIG at concentration of 15 mg/ml. The presence of IVIG when cells were incubated with heme-ox, resulted in marked inhibition of deposition of complement C3 fragments on the cell surface (Fig. 1B,C). These effects were observed even at high doses (100  $\mu\text{M}$ ) of free heme-ox (Fig. 1). To exclude possible effects of IVIG on endothelial cells, that are independent of scavenging of heme, we first incubated the cells with IVIG in absence of heme-ox. In a second step IVIG was removed by washing and heme-ox was added. As illustrated on Fig. 2, in this experimental setting IVIG was not able to inhibit the ability of heme-ox to induce deposition of C3 fragments. This result ruled out indirect effect of IVIG on the endothelial cells. We can also exclude a potential direct effect of IVIG on complement proteins since addition of normal human sera was always performed after removal of free heme-ox and IVIG by washing. Instead we suggest that a fraction of IgG molecules within IVIG preparation bound to heme and prevented its effects on the endothelial cells in terms of alteration of the expression of P-selectin and complement regulators (Frimat et al., 2013; Merle et al., 2019).

To provide direct evidence that IVIG contain a fraction of antibodies that are able to bind heme-ox, we used absorbance spectroscopy. Titration of IVIG with increasing concentrations of heme-ox resulted in changes in the UV-vis absorbance spectrum of heme (Fig. 3A). The increased absorbance intensity and observed red shift of the Soret band in the spectrum of oxidized heme are consistent with binding to protein molecules. Further, the interaction of heme-ox with IVIG was confirmed by surface plasmon resonance-based biosensor assay (Fig. 3B). This assay allowed estimation of apparent affinity of binding of heme-ox to IVIG. The obtained value of  $K_D$  of  $4.7 \pm 0.5 \mu\text{M}$  indicated that IVIG binds heme-ox with moderated affinity. This can be explained by the fact that IVIG contains only a fraction of antibodies that are able to bind

Fig. 3. IVIG binds heme. (A) Differential absorbance spectra obtained after titration of 10  $\mu\text{M}$  of IVIG with increasing concentrations of heme-ox (2.5–80  $\mu\text{M}$ ). The differential spectra of heme-ox were generated after subtraction of spectrum of heme-ox in PBS from the spectrum at the corresponding concentration of heme-ox in the presence of IVIG. (B). Real-time interaction profiles obtained after injection of increasing concentrations of heme-ox (0.78–5  $\mu\text{M}$ ) over surface-immobilized IVIG. The profiles were obtained after subtraction of the response in the control flow cell. The black lines depict the experimental data; the red line depicts the global kinetics analyses fit. The measurements were performed at 25  $^\circ\text{C}$ .

heme. Moderate affinity of interaction, however, may not be an issue in clinical practice since therapeutic doses of IVIG are usually high and can result in administrated IgG concentrations exceeding 150  $\mu$ M.

These experiments indicate that IVIG can bind heme and prevent its deleterious effects on endothelial cells and on complement activation in hemolytic diseases. The scavenging of heme adds up to the previously established mechanisms explaining the beneficial effect of IVIG in hemolytic conditions such as sickle cell disease (Turhan et al., 2004) or hemolytic transfusion reactions (Pirenne and Yazdanbakhsh, 2018), including the inhibition of adhesion of neutrophils and sickled erythrocytes to the endothelium in a Mac-1 (complement receptor 3, CD11b/CD18)-dependent mechanism (Jang et al., 2012). On this basis, a clinical trial with IVIG was initiated for patients with sickle cell disease (#NCT01757418). C3b deposited in a heme-dependent manner on the endothelium, might interact with Mac-1 and facilitate the leukocytes adhesion and vaso-occlusion in sickle cell disease. In this context we hypothesize that scavenging of heme by IVIG may decrease complement activation and hence reduce the vaso-occlusion.

Taken together, these data suggest that IVIG exert anti-inflammatory effects by scavenging endogenous low-molecular-weight pro-inflammatory molecules. This hitherto undescribed anti-inflammatory effect of IVIG would be relevant in hemolytic conditions as well as in all conditions accompanied by severe inflammation and tissue damage. Further studies should be performed to underscore the therapeutic potential of IVIG as an inhibitor of the pro-inflammatory effects of extracellular heme.

#### Conflicts of interests

The authors declare that they do not have any conflicts of interests related to this study.

#### Acknowledgments

This work was supported by INSERM France and by grants from Agence Nationale de la Recherche (ANR-13-JCV1-006-01 to JDD and ANR-15-CE15-0001 to LTR) and European Research Council (ERC-StG-678905 to JDD).

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