Platelet α -Granules modulate the inflammatory response under systemic lipopolysaccharide injection in mice

Sofiane Tariket^{*,†}, Jose A Guerrero^{‡,§}, Olivier Garraud^{*, ¶}, Cedric Ghevaert^{‡,§}, Fabrice Cognasse^{*,†}

* Université de Lyon, GIMAP-EA3064, Saint-Etienne, France

† Établissement Français du Sang Auvergne-Rhône-Alpes, Saint-Etienne, France

‡ Department of Haematology, University of Cambridge and NHS Blood and Transplant, Long Road, Cambridge, CB2 0PT, UK

§ National Health Service Blood and Transplant, Cambridge Biomedical Campus, Cambridge, UK.

¶ Institut National de la Transfusion Sanguine, Paris, France

* **Address for correspondence and reprint requests:** Dr. Fabrice Cognasse, Etablissement Français du Sang, Auvergne-Rhône-Alpes & Université de Lyon, GIMAP-EA3064, Faculté de Médecine, 10 rue de la Marandière - 42270 St Priest en Jarez, France. Tel.: +33 4 77 42 14 67; Fax: +33 4 77 42 14 86; E-mail address: fabrice.cognasse@univ-st-etienne.fr

Running head: Platelet and inflammation

Key words: α -granule, CD40L, Inflammation, Nbeal2, Platelet

Conflicts of Interest: None of the authors declare any conflicts of interest.

Word count: 1969; **Abstract:** 209; **References:** 6; **Figures:** 2; **Tables:** 1;

This work was supported by grants from the French Blood Establishment (Grant APR), France; the Agence Nationale de la Sécurité et du Médicament et des Produits de Santé (ANSM - AAP-2012-011, Reference 2012S055); the French “Agence Nationale de la

Recherche" (ANR-12-JSV1-0012-01); and the Association "Les Amis de Rémi" Savigneux, France.

Abstract

Background: Beyond their role in haemostasis and thrombosis, platelets are also important mediators of inflammation by the release of hundreds of factors stored in their α -granules. Mutations in *Nbeal2* cause gray platelet syndrome (GPS) characterized by the lack of platelet α -granules. This study aims to evaluate the immunological (pro-inflammatory) effects of platelet α -granules.

Methods and Materials: We performed experiment using *Nbeal2*^{-/-} mice, the mouse model of GPS. Systemic inflammation was induced by intravenous injection of lipopolysaccharide (LPS). Inflammatory response was assessed by quantification of inflammatory soluble factors and platelet biological response modifiers.

Results: The lack of *Nbeal2* (in *Nbeal2*^{-/-} mice, compared to controls) significantly reduced the recruitment of circulating neutrophils and monocytes. Moreover, after LPS injection, there was a significant increase in neutrophil and monocyte counts in control animals, compared to *Nbeal2*^{-/-} mice. The control of inflammation, evaluated by the production of anti-inflammatory cytokines, appeared to be greater in *Nbeal2*^{-/-} mice compared with controls. Conversely, the production of certain inflammatory-soluble mediators known to characterize normal platelet secretion, such as sCD40L, was decreased under experimental inflammation in *Nbeal2*^{-/-} mice.

Conclusions: These results show that α -granules play a direct role in platelet-mediated inflammation balance, confirming the need to further investigate platelet-associated inflammatory pathophysiology and inflammatory adverse events related to blood transfusion.

Introduction

Platelets are enucleated secretory cells that circulate in blood and are mostly characterized by their role in haemostasis and thrombosis.¹ However, platelets have also been recognized as mediators of inflammation and immunity.² Platelets and their progenitors, megakaryocytes (MKs), express surface receptors that can initiate/propagate inflammatory responses such as "Toll-like" receptor- 4 (TLR-4), TLR-2 and TLR-9,³ sialic acid-binding immunoglobulin-type lectin (Siglec) receptors and specifically Siglec-7,⁴ and numerous cytokine and chemokine receptors that are essential in cell migration and communication.⁵ Therefore, the role of platelets in inflammation is not limited to the expression of the multiple aforementioned receptors that primarily sense pathogens, relying also on inflammatory soluble factors stored in the α -granules and released upon secretion,⁶ such as CD62P, CD40L, platelet factor 4 (PF4), MIP-1 α , RANTES and IL-1. The majority of soluble factors stored in α -granules are proinflammatory,⁷ however, many studies also suggest platelets have an anti-inflammatory role. Indeed, higher levels of tumor necrosis factor- α (TNF- α), IL-6 and interferon- γ (IFN- γ) have been observed in sera and plasma in the absence of platelets.^{8,9} Moreover, the transfusion inflammatory reaction in the vascular compartment involves endothelial cell activation due to cell-cell interactions and inflammatory soluble factors infused with the blood products. Furthermore, platelets can regulate the inflammatory response of blood neutrophils by influencing the expression of macrophage receptor-1 (Mac-1) on their surface, notably through platelet integrin GPIb-IX exposure.⁹ The balance between the pro- and anti-inflammatory factors stored in the α -granules and the platelet response through the TLR and chemokine receptors should thus determine the overall role of platelets in inflammation.

Gray platelet syndrome (GPS) is a rare platelet bleeding disorder caused by loss of function mutations in *NBEAL2* and characterized by a low platelet count, a lack of platelet α -granules and early myelofibrosis. Deletion of its murine ortholog, *Nbeal2*, recapitulates all major features of GPS. Mouse *Nbeal2*^{-/-} platelets have a significant decrease, or an almost complete absence, in α -granule proteins such as PF4, vWF and P-selectin¹⁰⁻¹³ with a potential imbalance of inflammation. In fact, *Nbeal2*^{-/-} mice are more vulnerable to bacterial (*Staphylococcus aureus*) and viral (murine cytomegalovirus) infection.¹⁴ The objective was therefore to investigate the effect of platelet α -granule content on the amplification or

down-regulation of LPS-induced systemic inflammation in *Nbeal2*^{-/-} mice, to reveal the pro- and anti-inflammatory balance.

Material and methods

Mice

Adult, male, C57BL6 mice between 8 and 13 weeks old were used. For each experiment, a minimum of 6 mice were used. The mice were randomly distributed into different groups (PBS wild type [WT] vs. LPS WT vs. PBS *Nbeal2*^{-/-} vs. LPS *Nbeal2*^{-/-}). This research was performed under the Animals (Scientific Procedures) Act 1986 Amendment Regulations 2012, following an ethical review by the University of Cambridge Animal Welfare and Ethical Review Body (AWERB).

LPS Challenge

Mice were intravenously (*i.v.*) injected with LPS extracted from *Escherichia coli* (0111) (Sigma Aldrich, Saint-Louis, USA) used at 10 mg/kg, or with PBS (control). Mice were monitored for 5 hours. The surviving mice were then euthanized in a carbon dioxide chamber and blood was collected in acid citrate-dextrose (ACD) (Sigma Aldrich, Saint-Louis, USA).

Cell Counting

Blood was collected in ACD solution. Full blood counts were measured using a “scil Vet abc” instrument (scil Vet abc, Montpellier, France). Plasma was obtained by centrifugation and frozen at -80°C until used.

Inflammatory Soluble Factors and Platelet Biological Response Modifiers Immunoassay

The mouse biological response modifier (BRM) magnetic 16-plex kit (IL-1 β , bFGF, IL-10, IL-13, IL-6, IL-12, IL-17, GM-CSF, IL-5, IL-1 α , IFN- γ , TNF- α , IL-2, IP-10, MIG and IL-4) (ThermoFisher, Waltham, USA), magnetic 5-plex kit (KC, MCP-1, MIP-1 α , RANTES and VEGF) and magnetic simplex kit (sCD40L) (Merck Millipore, Billerica, USA) were used according to the manufacturers' instructions. The reading was taken through the Luminex 200 (Luminex, Austin, USA).

Statistical Tests

Statistical analyses were performed using GraphPad Prism 5 software (GraphPad, San Diego, USA). P-values were calculated using a 2-tailed unpaired T test, a one-way ANOVA and a

Bonferroni post-hoc test when the Kolmogorov-Smirnov normality test was passed. P-values were calculated using a 2-tailed Mann-Whitney test, a Kruskal-Wallis test, a one-way ANOVA and Dunn's post-hoc test when the Kolmogorov-Smirnov normality test failed. A p-value was considered significant when it was < 0.05 for all tests. Using *, † and # < 0.05; **, †† and ## < 0.01; ***, ††† and ### < 0.001 symbols.

Results and Discussion

Inflammatory Cell Recruitment Changes Dependent upon NBEAL2 Protein Expression

Firstly, the circulating cell count revealed a significant 3- and 4- fold increase in the neutrophil and monocyte counts respectively, in *Nbeal2*^{-/-} mice compared with control mice. The effect of LPS intravenous injection was then assessed demonstrating that LPS injection significantly increased the number of monocytes and reduced the number of platelets and circulating lymphocytes in both WT and *Nbeal2*^{-/-} mice. Reciprocally, the number of neutrophils significantly increased in WT mice after LPS injection, but not in *Nbeal2*^{-/-} mice (**Fig 1, A, B**). The fold change of the cell populations before and after LPS injection revealed a significant increase in the neutrophil and monocyte counts in control animals compared with *Nbeal2*^{-/-} mice (**Fig 1, C**). These results show that *Nbeal2*^{-/-} mice mobilize leukocytes (neutrophils and monocytes) less efficiently than WT mice following LPS-induced systemic inflammation. A likely explanation for this is that *Nbeal2*^{-/-} mice have a limited capacity for secreting pro-inflammatory mediators capable of synergizing at the systemic level. Indeed, it has already been shown that platelets, by the release of a multitude of soluble mediators contained in the α-granules, play an essential role in attracting and capturing leukocytes.⁷

An Anti-Inflammatory Pattern is Revealed in *Nbeal2*^{-/-} Mice after LPS Injection

The purpose was to evaluate the influence of platelet secretion on inflammation evaluated through cytokine/chemokine production from inflammatory cells. The concentrations of 15 cytokines/chemokines were therefore measured with pro- and anti-inflammatory function, in WT and *Nbeal2*^{-/-} mice challenged systematically by LPS. Under the baseline condition (*i.e.*, PBS injection), levels of IL-10, an anti-inflammatory BRM,¹⁵ were only higher in WT mice compared with *Nbeal2*^{-/-} mice. Systemic LPS injection induced a significant increase in 10 pro-inflammatory soluble factors in both WT and *Nbeal2*^{-/-} mice, including IL-6, IL-12, GM-

CSF, IL-5, IL-1 α , IFN- γ , TNF- α , IL-2, IP-10 and MIG. Only the concentration of IL-17 was higher in *Nbeal2*^{-/-} mice than in control mice under the effects of LPS-induced inflammation, but this difference was not significant. Higher levels of anti-inflammatory BRMs (IL-10, ¹⁵ IL-13, and IL-4¹⁶) were only observed in *Nbeal2*^{-/-} mice after systemic LPS injection (**Table 1**), which correlated with their fold increase (**Fig 2, A**). In summary, under systemic inflammatory conditions, *Nbeal2*^{-/-} mice show a more sustained anti-inflammatory response normally hidden or limited in WT animals. These results could therefore support the possibility that the secretion of platelet α -granule products can change the secretory pattern of inflammatory cells and balance pro- and anti-inflammatory BRMs and, overall, inflammation.

Circulating Blood Cells Compensate for the Decrease in Pro-inflammatory Platelet-released factors in *Nbeal2*^{-/-} Mice, Except for sCD40L.

Platelets and other blood and vascular cells secrete BRMs such as IL-1 β , KC, MCP-1, MIP-1 α , RANTES, VEGF and sCD40L under physiological conditions and, to a greater degree, upon stimulation. While sustained production of IL-1 β ,¹⁷ KC,¹⁸ MCP-1,¹⁹ MIP-1 α ,²⁰ RANTES¹⁹ and VEGF²¹ is a common feature of platelets and other cells, sCD40L is mostly secreted by platelets.²² It was therefore investigated whether the lack of platelet secretion, particularly for these seven soluble factors, could be compensated by other blood cells in *Nbeal2*^{-/-} mice. Concentrations of IL-1 β , KC, MCP-1, MIP-1 α , RANTES and VEGF, measured in blood plasma, were significantly increased after stimulation induced by LPS injection in both wild type and *Nbeal2*^{-/-} mice (**Table 1**). These results indicate that other inflammatory blood cells have a potential compensatory capacity for secretion. However, sCD40L followed a different pattern as observed by a 2.5-fold increase in its levels in PBS-injected *Nbeal2*^{-/-} mice when compared to control animals (**Fig 2, A and Table 1**). sCD40L follows a pattern previously observed with other factors released by platelets, such as PF4 and BTG, which have been found at higher levels in plasma of patients with gray platelet syndrome.²³ Additionally, CD40L expression might be greater in *Nbeal2*^{-/-} megakaryocytes than in WT cells, as has been reported for other pro-inflammatory chemokines.¹⁰ We furthermore cannot exclude the possibility that the expression of CD40L in cells other than platelets may influence its serum levels. The increased concentration of sCD40L may therefore be the consequence of a significant expression of certain proteins by the megakaryocytes or a lack of clearance of the plasma. Lastly, this raised concentration of sCD40L, evidenced in non-stimulated *Nbeal2*^{-/-}

mice, is likely to partially explain the high baseline count of circulating neutrophils and monocytes (**Fig 1, A, B**). Indeed, CD40L, and its agonist form sCD40L, typically participate in leukocyte recruitment notably by binding leukocyte CD40 and Mac-1 receptors ²⁴. Under inflammation induced through LPS intravenous injection, sCD40L levels increased in WT mice in contrast to *Nbeal2*^{-/-} mice (**Fig 2, B**), which seems to correlate with the limitation of neutrophil and monocyte recruitment in control (**Fig 1, C**). The secretion of certain platelet BRMs,²⁵ such as sCD62P, vWF, PF4, and fibrinogen, is indeed reduced in *Nbeal2*^{-/-} mice, ¹⁰⁻¹³ which may contribute to the increased concentration of anti-inflammatory factors observed in these mice (**Fig 2, A**). In this study, a comparable pro-inflammatory cytokine/chemokine secretion pattern was observed in *Nbeal2*-gene deficiency and wild type conditions, except for sCD40L secretion. Nevertheless, the anti-inflammatory profile was greater when mice lacked α -granules and were stimulated with intravenous LPS.

These observations are consistent with a recent study showing that the lack of NBEAL2 protein expression in mice increases their vulnerability to bacterial infection.¹⁴ Sowerby et al. demonstrated that abnormalities exist in the *Nbeal2*-deficient immune system, especially with respect to neutrophil and NK cell function. Our results suggest that other inflammatory blood cells may be involved in compensatory secretion. Because the circulating cell count indicated non-significant modulation of lymphocyte concentration in *Nbeal2*^{-/-} mice, in comparison with control mice, we hypothesize that activating lymphocytes may exhibit such compensatory secretion. We cannot exclude a possible role of nonimmune cells, such as endothelial cells, in the release of immunomodulatory factors.²⁶

Platelets concentrates are known to provide a higher rate of adverse recipients reactions than other blood components. Cytokine accumulation during platelets storage may result in increased risk of transfusion reactions. Our understanding of the molecular mechanisms of secretion and the genetic regulation of granule biogenesis still remains incomplete. In the present report, we expand our understanding of how platelets balance of anti- and pro-inflammatory factors to regulate inflammation. In an elegant approach, Deppermann et al. ^{11,12} show that a deficiency in platelet α - and dense granules results in impaired vascular integrity in the course of thrombo-inflammatory brain infarction but not in experimental inflammation of the skin or lung. This indicates that the mechanisms by which platelets maintain vascular integrity differ between inflamed organs. Moreover, these results

demonstrate that platelet granule release safeguards hemostasis during stroke injury. Platelet-specific mediators released from platelets (especially from α -granules) can themselves promote inflammation. Such platelet-specific mediators are able to regulate endothelial activation and migration to sites of vascular lesions. Platelets have the potential to modulate vascular permeability and integrity. In the context of transfusion, adverse reactions depend not only on the blood products but also on patients' genetic backgrounds and underlying pathological conditions. Further investigation is needed to understand the extent to which platelet components affect patients and their inflammatory status, and future studies using platelet-specific knockouts of α -granule-derived mediators will enhance our understanding of the role of platelets in adverse events related to blood transfusion.

Acknowledgments

We are grateful to MA Eyraud, CA Arthaud and Astrid Meneveaux for their contributions to the original data. We would also like to thank the facility technical staff of the University of Saint-Etienne PLEXAN. Thanks to Biomedical Excellence for Safer Transfusion (BEST) collaborative for fruitful discussion about platelet and inflammation.

Author contributions

ST, GJA, FC and OG conceived the study hypothesis, analysed the data and wrote the paper. ST and GJA designed the protocol, trained personnel, collected samples, performed the experiments and statistical analyses. GC participated in all steps of the process and reviewed the manuscript.

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		PBS WT	PBS <i>Nbeal2</i> ^{-/-}	LPS WT	LPS <i>Nbeal2</i> ^{-/-}	p-value symbol
Pro inflammation properties	<i>bFGF</i>	144.29 ± 55.04	90.61 ± 19.93	104.67 ± 19.75	164.71 ± 66.61	
	<i>IL-18</i>	64.86 ± 17.30	62.05 ± 17.42	290.35 ± 40.82	293.78 ± 64.20	*** †††
	<i>IL-6</i>	57.50 ± 20.57	62.66 ± 57.32	41752.00 ± 15259.62	41295.70 ± 19058.35	*** †
	<i>IL-12</i>	63.19 ± 8.98	73.74 ± 23.88	742.91 ± 266.31	746.19 ± 437.51	** ††
	<i>IL-17</i>	2.62 ± 1.65	1.45 ± 1.10	17.26 ± 12.22	24.39 ± 17.05	**
	<i>GM-CSF</i>	12.23 ± 5.47	4.90 ± 0.31	81.25 ± 18.74	74.17 ± 18.57	*** †
	<i>IL-5</i>	59.02 ± 36.87	12.82 ± 3.37	417.85 ± 71.15	271.65 ± 77.84	* ††
	<i>IL-1α</i>	1.67 ± 0.38	1.40 ± 0.23	20.60 ± 14.60	19.28 ± 9.30	*** †
	<i>IFN-γ</i>	52.46 ± 28.58	22.92 ± 18.98	866.40 ± 496.46	761.59 ± 511.26	** †
	<i>TNF-α</i>	15.80 ± 9.77	7.51 ± 5.66	410.75 ± 63.25	403.07 ± 162.51	*** †
	<i>IL-2</i>	11.33 ± 2.81	7.06 ± 1.86	35.04 ± 5.67	33.40 ± 4.27	*** †††
	<i>IP-10</i>	39.43 ± 13.98	36.14 ± 6.25	8911.97 ± 2241.85	9673.37 ± 4528.40	*** †††
	<i>MIG</i>	157.17 ± 30.59	123.60 ± 38.18	101899.66 ± 32534.36	100400.95 ± 35539.99	*** †††
	<i>KC</i>	140.09 ± 78.44	292.41 ± 230.32	12455.28 ± 418.95	13141.09 ± 2113.74	** ††
	<i>MCP-1</i>	38.39 ± 11.31	70.24 ± 35.87	13718.63 ± 2553.77	18277.49 ± 3738.30	** †
	<i>MIP-1α</i>	59.62 ± 38.13	36.32 ± 4.31	1279.17 ± 745.14	1154.02 ± 500.22	** †
	<i>RANTES</i>	23.42 ± 6.19	52.85 ± 25.47	6961.41 ± 4197.66	9727.42 ± 5734.19	** ††
	<i>VEGF</i>	3.60 ± 0.77	3.38 ± 0.73	10.90 ± 3.90	10.36 ± 2.93	** ††
	<i>sCD40L</i>	138.27 ± 38.84	331.12 ± 108.81	322,14 ± 88,72	293.33 ± 74.19	† #
Anti-inflammatory properties	<i>IL-10</i>	351.20 ± 185.54	65.74 ± 24.46	431.90 ± 142.41	490.79 ± 293.82	** #
	<i>IL-13</i>	61.89 ± 18.39	45.81 ± 13.68	85.07 ± 23.62	91.93 ± 20.80	**
	<i>IL-4</i>	51.55 ± 21.95	20.21 ± 4.36	75.91 ± 11.47	91.32 ± 29.51	***

Table 1: Value Representation of the Soluble Factor Evaluations

Value quantification for each soluble factor in plasma was determined through a multiplex assay for each group of mice. The unit is pg/ml. Data are presented as means ± SEM (n = 6 – 9). *p < 0.05; **p < 0.01 and ***p < 0.001 represent differences between *Nbeal2*^{-/-} groups. ; †p < 0.05; ††p < 0.01 and †††p < 0.001 represent differences between wild type groups. ; #p

< 0.05; ##p < 0.01 and ###p < 0.001 represent differences between PBS groups. One-way ANOVA revealed no statistical difference between LPS groups.

Figure legends

Figure 1: Evaluation of Blood Cell Count

Blood cell counts **(A)** and Mean platelet volume were evaluated for each group of mice **(B)**. The fold increase in numbers of cells after LPS injection for each condition was determined **(C)**. Data are presented as means (n = 6–9). *p < 0.05, **p < 0.01, and ***p < 0.001 represent differences between *Nbeal2*^{-/-} groups. †p < 0.05, ††p < 0.01, and †††p < 0.001 represent differences between wild type groups. #p < 0.05, ##p < 0.01, and ###p < 0.001 represent differences between PBS groups. §p < 0.05, §§p < 0.01, and §§§p < 0.001 represent differences between LPS groups.

Figure 2: Evaluation of Pro-Inflammatory Pattern for Mouse Soluble Factors and Platelet Soluble Factor Assay

Evaluation of pro-inflammatory pattern for mouse soluble factors: The fold increases in IL-10, IL-13, IL-17 and IL-4 concentrations (with respect to the corresponding control group mean) for LPS WT and *Nbeal2*^{-/-} mice were compared **(A)**. Platelet soluble factor assay: The fold increases in sCD40L concentration (with respect to the corresponding control group mean) for LPS WT and *Nbeal2*^{-/-} mice were compared **(B)**. Data are presented as means (n = 6–9). §p < 0.05, §§p < 0.01, and §§§p < 0.001 represent differences between LPS groups. No significant difference between *Nbeal2*^{-/-} groups, between wild type groups, or between PBS groups was observed.

Figure 1.

A

B

C

Figure 2.

A

B