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LETTER TO THE EDITOR

Targeting of C-type lectin-like receptor 2 or P2Y12 for the prevention of platelet activation by immunotherapeutic CpG oligodeoxynucleotides: reply

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See also Delierneux C, Donis N, Servais L, Wéra O, Lecut C, Vandereyken M, Musumeci L, Rahmouni S, Schneider J, Eble JA, Lancellotti P, Oury C. Targeting of C-type lectin-like receptor 2 or P2Y12 for the prevention of platelet activation by immunotherapeutic CpG oligodeoxynucleotides. *J Thromb Haemost* 2017; **15**: 983–97 and Flierl U, Nero TL, Lim B, Andrews RK, Parker MW, Gardiner EE, Peter K. Targeting of C-type lectin-like receptor 2 or P2Y12 for the prevention of platelet activation by immunotherapeutic CpG oligodeoxynucleotides: comment. *J Thromb Haemost* 2017; https://doi.org/10.1111/jth.13877.

In a recent issue of the *Journal of Thrombosis and Haemostasis*, we published a study demonstrating that phosphorothioate (PS)-modified CpG oligodeoxynucleotides (PS-CpG ODNs) types A, B and C activate platelets via CLEC-2 [1]. This study followed an initial article by Flierl *et al.* showing that PS-CpG ODN type C exerts a platelet activating effect through GPVI [2]. In a Letter to the Editor, Flierl *et al.* now present new computational modeling data confirming that PS-CpG ODN type C can bind to CLEC-2 monomers or homodimers, or even to two homodimers, and would therefore be able to cause receptor clustering on the platelet surface. Then, by using random PS-ODNs of different size, the authors determined the minimum number of nucleotides required for binding to platelets, $\alpha_{IIb}\beta3$ integrin activation or P-selectin exposure. They found that six PS-modified nucleotides are needed to obtain significant ODN binding to platelets, whereas the induction of significant integrin activation and P-selectin requested at least 10-mer and 18-mer, respectively.

These data do not fit with our observation that type A CpG ODNs, with only eight PS-modified nucleotides, potently activate human washed platelets, and induce their immediate aggregation, whereas type C CpG ODN, with a full 22-mer PS backbone, only weakly bind to human washed platelets, hardly activate them, and do not induce aggregation in washed platelet suspensions [1]. This is even more surprising because we showed that human washed platelets aggregate in response to type B CpG ODN that contain 24 PS nucleotides.

We believe that not only size, but also sequences, matter when considering CpG ODN-induced platelet activation.

First, as a result of binding of poly(G) motifs to scavenger receptors on the cell surface, CpG ODN type A that contain these motifs are expected to display an increase in cellular uptake efficiency [3]. This is consistent with our data obtained with human washed platelets, showing more efficient uptake of type A CpG ODN than type B (FITC-CpG ODN binding to platelets after 30 min incubation: type A = 25219 ± 2317; type B = 2143 ± 743; values = means MFI ± SD).

Second, it has been reported that the differential effects of types A, B and C CpG ODN on immune cells very much rely on higher-order tertiary structures that form in a sequence-dependent manner. For instance, the greater ability of type A CpG ODN to induce IFN-α production by plasmacytoid dendritic cells (pDC) than type B CpG ODN has been related to the higher-order structure of the A type [4,5]. Indeed, because of the presence of a palindromic sequence at the center of the ODN and poly (G) at its 5′ and 3′ end, type A CpG ODN spontaneously self-assembles to higher-order tertiary structures (G-tetrads), forming nanometer-size particles [5]. Such higher-order structures are not observed in type B CpG ODNs

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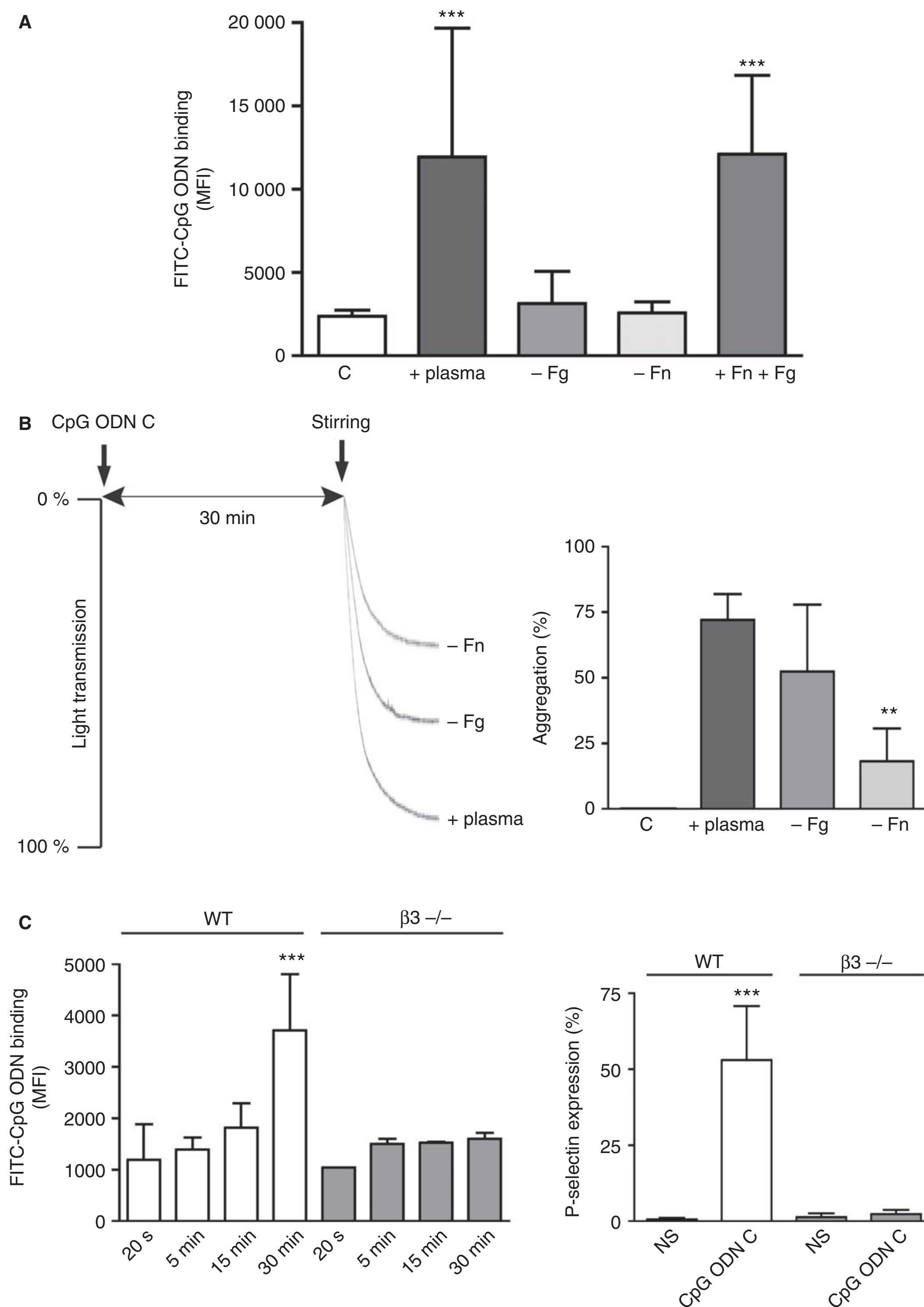

Fig. 1. Fibronectin, and to a lesser extent fibrinogen, contribute to CpG ODN type C-induced platelet activation. (A) Flow cytometric analysis of FITC-conjugated CpG ODN C uptake by human washed platelets without (C) or in the presence of normal plasma, Fn- or Fg-depleted plasma, or of a mixture of purified Fn and Fg (250 μg mL^{-1}) (***P < 0.001 vs. C, n = 4–8). (B) Amplitude of aggregation induced by CpG ODN type C (10 μM) in human washed platelet suspensions supplemented with normal plasma, fibronectin (Fn)- or fibrinogen (Fg)-depleted plasma (**P < 0.01 vs. normal plasma, n = 5). (C) Flow cytometric analysis of CpG ODN C (10 μM) induced P-selectin exposure (left panel) or of FITC-conjugated CpG ODN C uptake (right panel) performed on washed platelets isolated from control mice (WT) or from β3$^{-/-}$ mice (n = 3) (***P < 0.001 vs. 20 s or NS). NS = non-stimulated.

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harboring a linear structure. Type C CpG ODNs form a duplex, because of the palindromic sequence at the 3′ end, which may explain their ability to induce IFN-α secretion [6]. The duplex formation of type C CpG ODN has not been taken into account in the model of Flierl. It would thus be interesting to model the binding of type C CpG ODN duplexes or of type A G-tetrads to GPVI and CLEC-2 and determine the impact of ODN duplexing or multiplexing on clustering of these platelet surface receptors.

Third, interactions of CpG ODN with proteins could also influence their platelet activating effect. Indeed, it has been shown that type A CpG ODN achieve much of the INF-α effect through interaction with CXCL16 expressed on pDC, which enhances the uptake of type A, but not of type B, CpG ODNs [7]. The possibility that cofactors may take part in the recognition of one or more CpG ODN types by platelets has not been investigated yet.

Our own recent work indicates that fibrinogen (Fg) and fibronectin (Fn) might contribute to CpG ODN type C-induced platelet activation. We indeed observed that addition of plasma to washed platelets significantly increased CpG ODN C uptake (Fig. 1A), which could not be achieved by using plasma depleted in Fn or in Fg. Moreover, the addition of purified Fn and Fg to washed platelet suspensions increased CpG ODN C uptake similarly to normal plasma. Upon supplementation with normal plasma, CpG ODN type C efficiently induced the aggregation of washed platelets, whereas this platelet response was much weaker in the presence of Fn-depleted plasma (Fig. 1B). Altogether, these results suggest that CpG ODN type C might interact with Fg and Fn, two major plasma proteins, which may promote ODN uptake and subsequent platelet activation. On the platelet surface, the $\alpha_{IIb}\beta_3$ integrin represents the main common receptor for Fg and Fn, whereas fibronectin can also bind to $\alpha_5\beta_1$, GPIb/V/IX and GPVI. We thus wanted to assess the role of $\alpha_{IIb}\beta_3$ integrin in CpG ODN C-induced platelet activation. We found that the $\alpha_{IIb}\beta_3$ integrin antagonist, tirofiban, significantly reduced CpG ODN C uptake and CpG ODN C-induced P-selectin exposure on the platelet surface by $44 \pm 17\%$ and $50 \pm 14\%$, respectively ($P < 0.01$). Furthermore, CpG ODN uptake and P-selectin exposure were abrogated in β3-deficient mouse platelets (Fig. 1C).

Because plasma Fn can bind PS-modified DNA [8] and can be taken up by platelets via $\alpha_{IIb}\beta3$ integrin [9], this mechanism could also contribute to CpG ODN uptake, in addition to CLEC-2. Furthermore, because plasma Fn deposits rapidly onto the injured vessel wall, prior to platelets [10], the interaction of CpG ODN with deposited fibronectin might enhance its interaction with platelets, and promote subsequent thrombus formation.

In conclusion, the new set of data support the involvement of multiple platelet receptors in CpG ODN platelet activating effects. We observed some sequence-specific features of platelet responses to PS-modified CpG ODNs types A, B and C that could be useful for future development of safe therapeutic CpG ODNs. However, further investigations are needed in order to further delineate these sequence specificities, and to address the importance of higher-order structures, as well as of possible interactions with cofactors, for the effects of CpG ODN on platelets.

Material and methods

Reagents

All oligodeoxynucleotides with phosphorothioate modification were from Invivogen (Toulouse, France). Type A (2216), B (2006) and C (2395) CpG ODN were used. Human fibrinogen was from Haematologic Technologies Inc. (Essex Junction, VT, USA) and human fibronectin from CoaChrom Diagnostica (Maria Enzersdorf, Austria). Fibrinogen- and fibronectin-deficient human plasma were from Stago (Asnières-sur-Seine, France) and Innovative Research (Novi, MI, USA), respectively.

Mice

β3 knockout mice and their littermates were provided by Jochen Schneider. All mice were kept under specific pathogen-free conditions. Eight to 12-week-old male mice were used in all experiments. All animal care and experimental procedures were performed as recommended by the European Community Guidelines (directive 2010/63/UE) and approved by the Ethical Committee of the University of Liège.

Preparation of human and mouse washed platelets

Human and mouse washed platelets were prepared from freshly drawn ACD-anticoagulated blood according to standard procedures [1]. All procedures on human blood were approved by our institutional review committee (Comité d'Ethique Hospitalo-Facultaire Universitaire de Liège). The healthy volunteers gave written informed consent.

Platelet aggregation analyses

Light transmission was recorded during platelet aggregation induced by CpG ODNs on a Chrono-Log Lumi-Aggregometer (Kordia, Leiden, the Netherlands).

Flow cytometry

Platelet degranulation was assessed with flow cytometry by measuring the expression of P-selectin (PE-conjugated anti-mouse CD62P, BD Biosciences, Erembodegem, Belgium). In binding experiments, FITC-conjugated ODNs were incubated with platelets for increasing amounts of time. Samples were analyzed on a FACSVerse flow

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cytometer using the FACSuite software (BD Biosciences, Erembodegem, Belgium).

Statistics

Data are presented as mean ± SD of at least three independent experiments. Three or more groups were compared using one-way ANOVA and the Bonferroni multiple comparison test. A *P*-value < 0.05 was considered statistically significant. Calculations were performed using GraphPad-Prism (GraphPad Software, Inc., La Jolla, CA, USA).

Addendum

C. Oury and P. Lancellotti contributed to the conception and design of the work. C. Delierneux, N. Donis, L. Servais, O. Wera, and C. Oury performed experiments and acquired data. C. Delierneux, P. Lancellotti, and C. Oury were involved in the analysis and interpretation of the data. C. Oury supervised the experiments and analyses. C. Oury and C. Delierneux wrote the manuscript.

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Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

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