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Expression and function of IL-1R8 (TIR8/SIGIRR): a regulatory member of the IL-1 receptor family in platelets

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Aims Platelets express functional interleukin-1 receptor-1 (IL-1R1) as well as a repertoire of toll-like receptors (TLRs) involved in platelet activation, platelet–leucocyte reciprocal activation, and immunopathology. IL-1R8, also known as single Ig IL-1-related receptor (SIGIRR) or TIR8, is a member of the IL-1R family that negatively regulates responses to IL-1R family members and TLRs. In the present study, we addressed the expression of IL-1R8 in platelets and megakaryocytes and its role in the control of platelet activation during inflammatory conditions and thromboembolism.

Methods and results Here, we show by flow cytometry analysis, western blot, confocal microscopy, and quantitative real-time polymerase chain reaction that IL-1R8 is expressed on human and mouse platelets at high levels and on megakaryocytes. IL-1R8-deficient mice show normal levels of circulating platelets. Homotypic and heterotypic (platelet–neutrophil) aggregation triggered by Adenosine DiPhosphate (ADP) and IL-1 or lipopolysaccharide (LPS) was increased in IL-1R8-deficient platelets. IL-1R8-deficient mice showed increased soluble P-selectin levels and increased platelet–neutrophil aggregates after systemic LPS administration. Commensal flora depletion and IL-1R1 deficiency abated platelet hyperactivity and the increased platelet/neutrophil aggregation observed in *Il1r8*$^{-/-}$ mice *in vitro* and *in vivo*, suggesting a key role of IL-1R8 in regulating platelet TLR and IL-1R1 function. In a mouse model of platelet-dependent pulmonary thromboembolism induced by ADP administration, IL-1R8-deficient mice showed an increased frequency of blood vessel complete obstruction.

Conclusion These results show that platelets, which have a large repertoire of TLRs and IL-1 receptors, express high levels of IL-1R8, which plays a non-redundant function as a regulator of thrombocyte activity *in vitro* and *in vivo*.

Keywords Inflammation • Platelets • Thromboembolism • Sepsis • Immunity

1. Introduction

Several lines of evidence indicate that platelets are more than a component of blood haemostasis and coagulation, playing an important role in immunity and inflammation.[1,2] Platelets express functional IL-1R1, which is responsible for IL-1β induction and thus for activation of an autocrine stimulatory loop.[3] In addition, platelets have been reported to express a wide repertoire of toll-like receptors (TLRs), from TLR1 to TLR9, some of which are involved in platelet activation, platelet–leucocyte reciprocal activation, immunopathology,[4] and in platelet-dependent anti-microbial activity.[5–7] In particular, platelet TLR4 contributes to thrombocytopenia through neutrophil-dependent platelet sequestration in the lung, increased production of TNFα and IL-1β in response to lipopolysaccharide (LPS),[7–10] neutrophil activation and degranulation, generation of neutrophil extracellular traps (NETs), and bacteria ensnaring and trapping.[5] Furthermore, LPS

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induces rapid splicing of platelet IL-1β pre-RNA, followed by translation, and secretion of mature IL-1β, through a signalling pathway involving Akt and JNK activation.[11]

IL-1R8, also known as single Ig IL-1-related receptor (SIGIRR) or TIR8,[12] is a member of the IL-1 receptor family, characterized by the presence of a conserved TIR domain, which does not elicit a conventional signal.[13,14] IL-1R8 is widely expressed in several cell types, including epithelial cells and leucocyte populations such as monocytes, B cells, and dendritic cells.[13,15,16] *In vitro* and *in vivo* studies with transfectants and gene-targeted mice have demonstrated that IL-1R8 inhibits signalling by IL-1R family members and TLRs and tunes NFκB and mTOR activation.[17,18] In addition, IL-1R8 is an essential part of the receptor complex of IL-37, an anti-inflammatory member of the IL-1 family interacting with IL-18Rα.[19,20] IL-1R8 plays non-redundant roles by negatively regulating inflammatory responses, as well as T cell polarization, in several pathological conditions, including intestinal inflammation and colitis-associated cancer,[21,22] infections,[23] autoimmune diseases,[24] kidney ischaemia/reperfusion injury, and allotransplantation.[25,26]

Here, we report that human and murine platelets and megakaryocytes express IL-1R8, and this molecule plays a key role in tuning platelet activation during inflammation and thromboembolism in mice.

2. Methods

2.1 Cell culture

MEG-01 cells, established from the bone marrow of a patient with Philadelphia chromosome–positive chronic myelogenous leukaemia,[27] and DAMI cells established from the blood of a patient with megakaryoblastic leukaemia,[28] were from american type culture collection. MEG-01 and DAMI cells were grown in RPMI medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, and 100 U/mL of streptomycin. To exclude mature megakaryocyte-like cells from the analysis, only non-adherent cells were used. When specified, DAMI and MEG-01 were stimulated with LPS 100 ng/mL for 30 min at 37°C.

2.2 Mice

IL-1R8-deficient (*Il1r8*$^{-/-}$) mice were generated as described previously.[21] Mice used in this study were 8–12 weeks old and backcrossed for 11 generations on the C57BL/6J background. Wild-type controls (*Il1r8*$^{+/+}$) were C57BL/6J mice obtained from Charles River Laboratories (Lyon, France). IL-1R1-deficient (*Il1r*$^{-/-}$) mice were from The Jackson Laboratory. IL-1R8/IL-1R1-double-deficient (*Il1r8*$^{-/-}$/*Il1r1*$^{-/-}$) mice were obtained by crossing *Il1r8*$^{-/-}$ and *Il1r1*$^{-/-}$ mice. Mice were housed in a specific pathogen-free (SPF) animal facility of the Istituto Clinico Humanitas in individually ventilated cages.

Procedures involving animals and their care conformed to institutional guidelines in compliance with national (D.L. N.116, G.U., suppl. 40, 18-2-1992; D.L. N. 26, G.U. 4-3-2014) and international law and policies (EEC Council Directive 2010/63/EU, OJ L 276/33, 22-09-2010; NIH Guide for the Care and Use of Laboratory Animals, US National Research Council-2011). The protocol was approved by the Italian Ministry of Health (Protocol 43/2012-B). All efforts were made to minimize the number of animals used and their suffering.

2.3 Platelet and leucocyte preparation

Detailed procedures of human and murine platelet preparation and *in vitro* stimulation are reported in Supplementary material online, Methods and Ref. [29]

2.4 Immunofluorescence and immunohistochemistry

Platelets, MEG-01, and DAMI cells were fixed with 4% paraformaldehyde, adhered to microscope slides coated with 0.05 mg/mL poly-L-lysine, washed with phosphate-buffered saline (PBS), permeabilized with PBS/Tween 0.1%, incubated with PBS/bovine serum albumin 1% for 15 min, and then stained with different primary antibodies: anti-IL-1R8/SIGIRR (1:50, R&D Systems), anti-CD63 (1:50, BioLegend), anti-PF4 (1:50, Abcam), and Phalloidin (1:100, Invitrogen) for 1 h at room temperature (RT). After washing with PBS, the cells were incubated with the appropriate secondary antibodies (-Alexa-488, -Alexa-594, or -Alexa-647, 1:1000, Molecular Probes). Nuclei were counterstained with Hoechst 33258 (0.1 μg/mL). Slides were mounted with ProLong (Molecular Probes). Negative controls included cells incubated with the secondary antibodies alone or with appropriate isotype primary antibodies.

Acquisition of images was made by confocal microscopy Fluoview FV1000 (Olympus, Tokyo, Japan) with an oil immersion objective (60× 1.4 NA Plan-Apochromat; Olympus) using laser excitation at 405, 488, 543, and 647 nm. Images were processed with Adobe Photoshop 9.0.2.

Human and murine IL-1R8 immunohistochemistry was performed as described in Supplementary material online, Methods, using a biotinylated goat anti-IL-1R8/SIGIRR polyclonal antibody (1:50, R&D Systems).

Lung sections of mice undergoing thromboembolism were processed and stained with a polyclonal rabbit anti-fibrinogen antibody (Dako) in order to discriminate vessels occluded by fibrin clots. The percentage of occluded vessels was calculated by counting at least 50 vessels per lung.

2.5 Flow cytometry analysis

IL-1R8, TLR4, IL-1R1, and IL-18Rα cell surface staining on human washed platelets, DAMI, MEG-01, and leucocytes was performed in the dark with the appropriate saturating concentrations of the following unconjugated antibodies and relative isotype controls: biotinylated goat anti-human IL-1R8/SIGIRR (R&D Systems), biotinylated normal goat IgG (R&D Systems), purified mouse anti-human CD284 (TLR4) and purified mouse IgG2a (both eBioscience), purified goat anti-human IL-1R1 and purified normal goat IgG (both R&D Systems), and purified mouse anti-human IL-18Rα (eBioscience). Detection of primary antibodies was performed using Alexa-647 conjugated streptavidin or Alexa-647 conjugated goat anti-mouse or rabbit anti-goat secondary antibodies (Molecular Probes, Invitrogen).

The modulation of IL-1R8 expression on platelet membrane was analysed by resuspending 10^6 cells in Tyrode's buffer and stimulating them at RT for 15 min with recombinant IL-18, IL-1β (both from R&D Systems), LPS 0127/B8, Adenosine DiPhosphate (ADP), or thrombin (both from Sigma-Aldrich).

Leucocyte subpopulations were analysed using the following conjugated monoclonal antibodies: CD14 (M5E2), CD15 (MMA), CD3 (UCHT1), and CD19 (HIB19) (all from BD Pharmingen).

Stained cells were analysed with fluorescence-activated cell sorting (FACS) LSRFortessa (four lasers) or FACSCanto II (three lasers) flow cytometers (BD Bioscience). Diva software (BD Pharmingen) and Flow-jo (Tree Star) were used for data acquisition and analysis, respectively.

2.6 Real-time polymerase chain reaction

RNA extraction from platelets (from two to three donors or a pool of eight mice), DAMI, MEG-01 cells and mouse stools, processing, analysis, and primers used are reported in Supplementary material online, Methods.

2.7 Western blotting

Murine and human platelet pellets were resuspended and homogenized in 2 mL of cold lysis buffer [50 mM Tris–HCl pH 7.5, 150 mM NaCl, 4% NP40, 2% TritonX100, 1% Zwitterion 3.14, 5 mM EDTA, supplemented with a protease inhibitor cocktail (Sigma-Aldrich) and 1 mM phenylmethylsulfonyl fluoride], left for 30 min on ice, and finally centrifuged for 15 min at 16 100*g* at 4°C. Fifty-nanogram aliquots of total proteins were loaded on 10% SDS–PAGE gels (30% acrylamide/bis solution; Biorad) and western blotted on nitrocellulose membrane (Biorad). Immunodetection was carried out using goat anti-human IL-1R8/SIGIRR polyclonal antibody (R&D Systems) (0.1 μg/mL in PBS–Tween 0.1% with 5% skim milk,

Sigma-Aldrich) overnight at 4°C. A HorseRadish Peroxidase (HRP)-conjugated, anti-goat IgG secondary antibody (Sigma-Aldrich) was used (1:2000 dilution; 1 h at RT). Positive bands were detected using a chemiluminescent HRP substrate (Millipore) as specified by the manufacturer.

2.8 Enzyme-linked immunosorbent assay

Soluble P-selectin was measured in plasma using a commercially available Duo Set ELISA kit (R&D System). IL-1β was measured in the supernatant of washed platelets, isolated as described in Supplementary material online, Methods, upon *in vitro* stimulation (ADP 20 μM or LPS 1 μg/mL for 15 min at 37°C), using a commercially available immunoassay (R&D System).

2.9 Platelet homo- and heterotypic aggregation

Murine platelet aggregation was analysed by light transmittance aggregometry (ChronoLog Optical Aggregometer Mascia Brunelli, Milan, Italy). Washed platelets resuspended in modified Tyrode's buffer containing 30% murine poor platelet plasma from *Il1r8*$^{+/+}$ or *Il1r8*$^{-/-}$ (in order to provide fibrinogen in the assay) were stirred at 37°C for 1 min and then stimulated with two different doses of ADP and collagen. For thrombin aggregation, washed platelets were resuspended in Tyrode's buffer plus $CaCl_4$ 2 mM. All the aggregation curves were recorded for 6 min.

To analyse heterotypic aggregate formation between murine platelets and neutrophils *in vitro*, whole blood was stimulated with LPS 1 μg/mL,[30,31] IL-1β 25 ng/mL, or IL-18 100 ng/mL for 15 min at RT and stained with rat anti-mouse CD41 (BD Pharmingen). After ammonium-chloride-potassium (ACK) lysis of red blood cells heterotypic aggregates were evaluated analysing CD41 expression levels [expressed as mean fluorescence intensity (MFI)] in morphologically gated (SSChigh/FSChigh) neutrophils.[32] For *in vivo* heterotypic aggregation studies, mice were treated intraperitoneally (i.p.) with 100 μg/kg LPS, blood was collected 1 h later and treated as described above. Heterotypic aggregates between *Il1r8*$^{+/+}$ or *Il1r8*$^{-/-}$ bone marrow neutrophils and *Il1r8*$^{+/+}$ or *Il1r8*$^{-/-}$ platelets were induced by centrifugation of neutrophils and platelets (1:100 ratio) at 1300 rpm for 5 min at RT and subsequent incubation for 3 min at 37°C and analysed by flow cytometry upon fixation as described above.

2.10 Commensal flora depletion

Mice were treated every day for 5 weeks with an antibiotic cocktail (ABX), administered by oral gavage and composed by ampicillin (Pfizer) 10 mg/mL, vancomycin (PharmaTech Italia) 10 mg/mL, metronidazol (Società Prodotti Antibiotici) 5 mg/mL, and neomycin (Sigma-Aldrich) 10 mg/mL. Control group mice were treated with drinking water. Gavage volume of 10 mL/kg body weight was delivered with a flexible plastic feeding tube without prior sedation of mice. Commensal bacteria depletion was confirmed by real-time polymerase chain reaction (RT–PCR) analysis of rRNA 16S extracted from lysates of stools collected from antibiotic-treated and antibiotic-untreated mice.

2.11 Pulmonary thromboembolism

Mice were anaesthetized with ketamine (80 mg/kg) and xylazine (10 mg/kg). After exposure of the left jugular vein, a cannula was inserted and secured to the vein. Eighty microlitres of 2 mM ADP was administered in 1 min at constant flow rate using a peristaltic pump. After 5 min from the ADP injection, mice were sacrificed, and the lungs were collected for histological analysis.

2.12 Patients and controls

A prospective study, which complies with the Declaration of Helsinki, was conducted at Humanitas Clinical and Research Institute after approval from the Ethical Review Board. Thirty-five patients who met previously defined criteria for Systemic Inflammatory Response Syndrome (SIRS) or sepsis[33] were enrolled. In particular, SIRS was considered to be present when patients had more than one of the following clinical findings: body temperature, >38 or <36°C; heart rate, >90/min; hyperventilation evidenced by a respiratory rate of 20/min or a $PaCO_2$ of <32 mmHg; and a white blood cell count of >12 000 or <4000 cells/μL. We defined sepsis to be the clinical syndrome defined by the presence of both an infection and a systemic inflammatory response.[33] Patients' characteristics are reported in *Table 1*. Patients aged over 80 years and suffering from chronic infectious diseases were excluded. Seventeen healthy subjects matched for age and gender were recruited among the hospital staff. All participants gave written informed consent. IL-1R8 expression on platelets was analysed in 35 patients (age range, 20–79) and 17 controls (age range, 24–64), whereas IL-1R8 expression in monocytes was analysed in the last 27 patients (age range, 20–79) and 13 controls (age range, 25–64) enrolled.

Table 1 Characteristics of patients included in the study

	N (%)
	35
Males	15 (43%)
Age[a]	62 ± 15
SEPSIS	26
SIRS	9
Site of origin	
Respiratory system	10 (29%)
Urinary system	8 (23%)
Gut	4 (11%)
Skin	1 (3%)
Liver or gall bladder	4 (11%)
Cancer	3 (9%)
Undetermined	5 (14%)

[a]Mean ± SD.

2.13 Microparticle isolation and flow cytometric analysis

Detailed procedures of human microparticle (MP) isolation and the following flow cytometric analysis are reported in Supplementary material online, Methods.

2.14 Statistical analysis

Data are expressed as mean ± SEM. Student's *t*-test or one-way ANOVA for comparison of the mean was used to compare groups. Tukey's *post hoc* test was applied to correct multiple comparisons. Two tailed *P* values <0.05 were considered statistically significant. Two to five experiments were performed as specified. Statistics were calculated with GraphPad Prism version4, GraphPad Software.

3. Results

3.1 Human platelets and megakaryocytic cells express IL-1R8

Platelets express a wide repertoire of members of the IL-1R and TLR families. This prompted us to investigate the expression of IL-1R8 on resting human platelets and on megakaryocytic cell lines (DAMI and MEG-01). Flow cytometry analysis showed that human platelets expressed higher IL-1R8 surface levels when compared with the other leucocyte populations analysed (*Figure 1A*). IL-1R8 expression in platelets was confirmed by western blot analysis (*Figure 1B*) and confocal

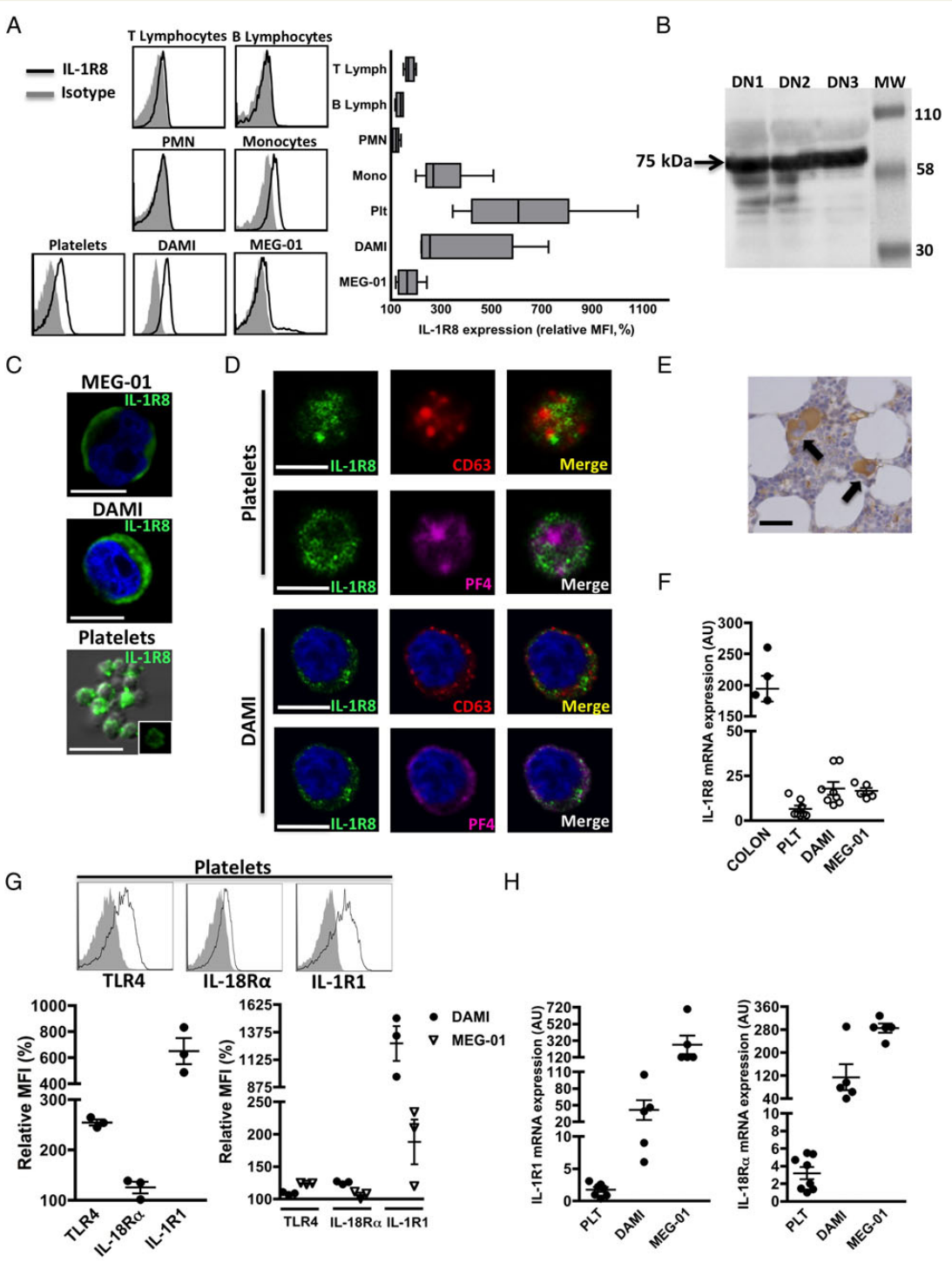

Figure 1 IL-1R8 is expressed by human platelets and megakaryocytic cell lines. (*A*) Flow cytometric analysis of IL-1R8 surface expression on primary leucocytes, platelets, and megakaryocytic cell lines. The right panel shows box plots from 6 to 16 healthy donors or the mean of 3 independent observations for megakaryocytic cell lines. Results are reported as the percentage of the relative MFI. (*B*) Western blot analysis of IL-1R8 expression in human platelets from three healthy donors (DN). One representative western blot out of four is shown. (*C*) Confocal microscopy analysis of IL-1R8 expression in human platelets and megakaryocytic cell lines (DAMI and MEG-01). IL-1R8 (green), DAPI (blue), bars 10 μm. Representative images out of 10–30 analysed regions of interest (ROI). (D) Confocal microscopy qualitative colocalization analysis between IL-1R8 (green) and CD63 (red) or PF4 (purple) performed on human platelets or DAMI cell line. Bars: 5 μm for platelets and 10 μm for DAMI cells. Representative images out of 20–30 analysed ROI. (*E*) Immunohistochemical analysis of IL-1R8 expression in human bone marrow megakaryocytes (arrows). Bar: 50 μm. (*F*) RT–PCR analysis of IL-1R8 mRNA expression. Colon epithelial cells were used as positive control. Data shown are from eight healthy donors or five to eight independent observations for megakaryocytic cell lines. (*G*) Flow cytometric analysis of TLR4, IL-18R, and IL-1R1 expression on platelets (left) and megakaryocytic cell lines (right). Data shown are from three healthy donors (left) or three independent observations (right) and are reported as percentage of the relative MFI. (*H*) IL-1R1 and IL-18Rα mRNA expression by RT–PCR in platelets, DAMI, and MEG-01 cells. Data shown are from eight healthy donors and five independent observations for DAMI and MEG-01 cell lines. Error bars represent the SEM.

microscopy (*Figure 1C*). Similarly, the DAMI and MEG-01 cell lines expressed IL-1R8 as shown by flow cytometry and confocal microscopy (*Figure 1A* and *C*). Confocal microscopy showed that IL-1R8 was also weakly localized in $PF4^+$ α granules and in $CD63^+$ granules of both platelets and DAMI (*Figure 1D*). Immunohistochemical analysis of bone marrow sections from healthy donors confirmed IL-1R8 expression in megakaryocytes (*Figure 1E*). Finally, RT–PCR analysis showed that both platelets and megakaryocytic cells expressed IL-1R8 mRNA, suggesting that the protein is newly synthesized by platelets in addition to deriving from megakaryocytes (*Figure 1F*).

IL-1R family members are widely expressed on haematopoietic cells[34]; however, only IL-1R1 has been shown in platelets so far.[3,35,36] In an effort to better characterize the expression of IL-1R family members in platelets and megakaryocytes, we next examined the expression of IL-18Rα on resting human platelets and on DAMI and MEG-01 megakaryocytic cell lines. Flow cytometry analysis showed that platelets and megakaryocytic cell lines expressed detectable IL-18Rα, IL-1R1, and TLR4 (*Figure 1G*). Similar to IL-1R1, IL-18Rα mRNA was detected at high levels in megakaryocytic cells, in particular in MEG-01, and at a lower extent in platelets (*Figure 1H*).

3.2 Murine IL-1R8 is involved in modulating platelet activation

We next explored by confocal microscopy and RT–PCR whether IL-1R8 was expressed in mouse platelets. As shown in *Figure 2A* and *B*, murine platelets expressed IL-1R8 protein and mRNA. In addition, mouse megakaryocytes expressed IL-1R8 protein, as shown by immunohistochemistry of the spleen (*Figure 2C*). Murine platelets expressed also IL-18Rα mRNA to levels comparable with IL-1R1 and TLR4 mRNA (*Figure 2D*).

The functional role of IL-1R8 in murine platelets was investigated taking advantage of $Il1r8^{+/+}$ and $Il1r8^{-/-}$ deficient mice. The analysis of platelet absolute number between age- and gender-matched $Il1r8^{-/-}$ and $Il1r8^{+/+}$ mice revealed no significant difference (*Figure 3A*). However, in basal conditions, $Il1r8^{-/-}$ platelets showed significantly higher levels of P-selectin (CD62P) and active α2bβ3 surface expression than $Il1r8^{+/+}$ platelets (*Figure 3B* and *C*, $P = 0.041$ and 0.002). In addition, soluble P-selectin plasma levels were higher in $Il1r8^{-/-}$ mice than in $Il1r8^{+/+}$ mice (70.96 ± 5.6 vs. 56.4 ± 1.8 ng/mL, $P = 0.034$; *Figure 3D*). Moreover, upon *in vitro* treatment with pro-thrombotic stimuli, $Il1r8^{-/-}$ platelets showed significantly higher expression levels of active α2bβ3 than $Il1r8^{+/+}$ platelets (ADP 20 μM: $P = 0.045$; thrombin 0.1 U/mL: $P = 0.013$; *Figure 3E*, right panel). Finally, *in vitro* aggregation experiments performed at two different concentrations of ADP showed significantly higher aggregation amplitude in $Il1r8^{-/-}$ platelets than in $Il1r8^{+/+}$ platelets (ADP 1 μM: $P = 0.001$; ADP 20 μM: $P < 0.0001$; *Figure 3F*). Moreover, significantly higher area under the curve (AUC) and slope, and lower lag time were observed with $Il1r8^{-/-}$ platelets (see Supplementary material online, *Table S1*). Upon stimulation with collagen, minor differences were observed in terms of AUC and lag time, whereas no differences were observed upon stimulation with thrombin (see Supplementary material online, *Figure S1A* and *B*; *Table 1*).

Leucocyte–platelet aggregate formation is a correlate of platelet activation and potentially involved in immunopathology as well as in defence.[1] To address the involvement of IL-1R8 in heterotypic aggregation, we analysed leucocyte–platelet aggregation *in vitro* under homeostatic and inflammatory conditions. As shown in *Figure 3G*, in basal conditions, IL-1R8 deficiency was associated to higher platelet/neutrophil aggregate formation ($CD41^+$ neutrophils; $P = 0.029$). In addition, the percentage of $Il1r8^{-/-}$ platelet/neutrophil aggregates induced *in vitro* by LPS, IL-1β, or IL-18 was significantly higher than the percentage of $Il1r8^{+/+}$ aggregates ($P = 0.04$, 0.0003, and 0.01, respectively; *Figure 3H*). To discriminate the role played by IL-1R8 expressed by neutrophils or platelets in aggregate formation, we next examined the formation of mixed aggregates between $Il1r8^{+/+}$ or $Il1r8^{-/-}$ neutrophils and $Il1r8^{+/+}$ or $Il1r8^{-/-}$ platelets. FACS analysis showed significantly higher $CD41^+$ levels in both $Il1r8^{-/-}$ platelet/$Il1r8^{+/+}$ neutrophil and $Il1r8^{-/-}$ platelet/$Il1r8^{-/-}$ neutrophil aggregates than in $Il1r8^{+/+}$ platelet/$Il1r8^{+/+}$ neutrophil and $Il1r8^{+/+}$ platelet/$Il1r8^{-/-}$ neutrophil aggregates ($P = 0.0056$ and 0.0001, respectively; *Figure 3I*), indicating a major role played by platelet IL-1R8 in heterotypic aggregation.

Altogether these results suggest that IL-1R8 deficiency is associated to platelet hyperactivity and increased hetero-aggregate formation both in basal and inflammatory conditions.

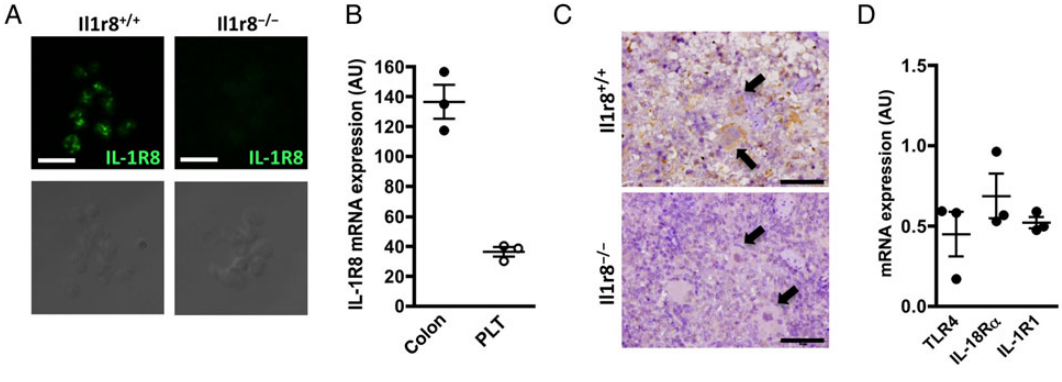

Figure 2 IL-1R8 is expressed in murine platelets and megakaryocytes. (*A*) Immunofluorescence analysis of IL-1R8 expression on murine platelets. $Il1r8^{-/-}$ platelets were used as negative control. Representative images out of 10 analysed ROI. (*B*) RT–PCR of IL-1R8 mRNA expression in murine platelets. Colon was used as positive control. Data shown are from three independent observations. (*C*) Immunohistochemical analysis of IL-1R8 expression in splenic megakaryocytes (arrows). Bottom panel: $Il1r8^{-/-}$ spleen was used as negative control. Bar 50 μm. (*D*) TLR4, IL-18R, and IL-1R1 mRNA expression by RT–PCR in murine platelets. Data shown are from three independent observations. Error bars represent the SEM.

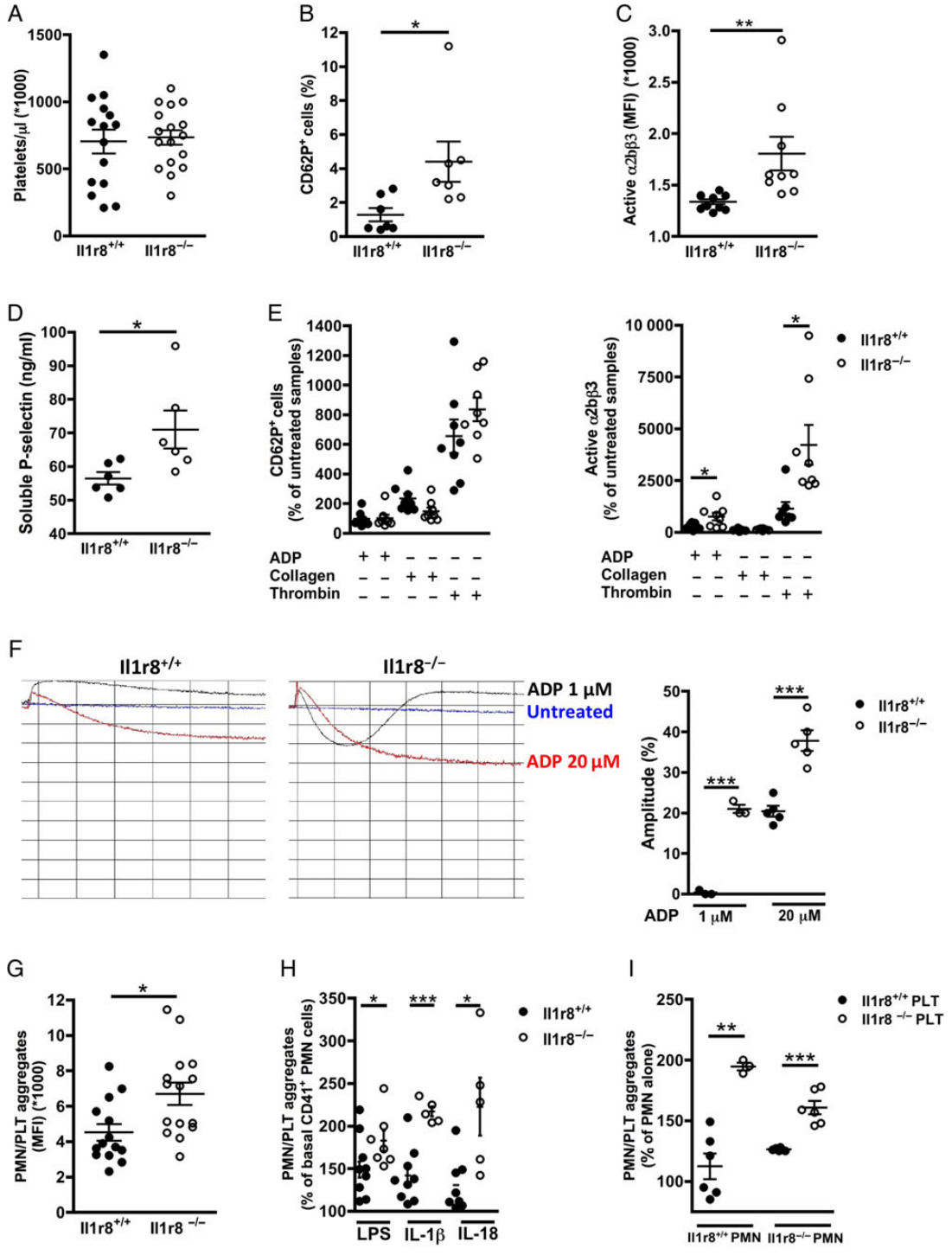

Figure 3 IL-1R8 deficiency is associated with increased platelet activation. (*A*) Platelet absolute count in *Il1r8*$^{+/+}$ ($n = 15$) and *Il1r8*$^{-/-}$ ($n = 17$) mice in healthy conditions. (*B* and *C*) Flow cytometric analysis of P-selectin (CD62P) (*B*) and active α2bβ3 (*C*) membrane expression in platelets from healthy *Il1r8*$^{+/+}$ and *Il1r8*$^{-/-}$ mice ($n = 7–10$). Data are reported as percentage of positive cells (*B*) or as MFI (*C*). (*D*) Soluble P-selectin plasma levels in healthy *Il1r8*$^{+/+}$ and *Il1r8*$^{-/-}$ mice ($n = 6$). (*E*) Flow cytometry analysis of P-selectin (CD62P) (left panel) and active α2bβ3 (right panel) surface expression in *Il1r8*$^{+/+}$ and *Il1r8*$^{-/-}$ platelets collected from seven to eight mice *in vitro* stimulated with ADP (20 μM), collagen (20 μg/mL), and thrombin (0.1 U/mL). Data are reported as the percentage of basal CD62P or α2bβ3 expression on platelet surface. (*F*) ADP (1 and 20 μM)-induced aggregation of washed *Il1r8*$^{+/+}$ and *Il1r8*$^{-/-}$ platelets. Results shown in the right panel are from three to five independent observations where both *Il1r8*$^{+/+}$ and *Il1r8*$^{-/-}$ platelets were pooled from six to eight mice. (*G* and *H*) Flow cytometry analysis of *Il1r8*$^{+/+}$ or *Il1r8*$^{-/-}$ neutrophil/platelet (PMN/PLT) aggregates (CD41^{+} neutrophils) in basal conditions (*G*) or after stimulation *in vitro* with LPS (1 μg/mL), IL-1β (25 ng/mL), or IL-18 (100 ng/mL). Results are expressed as MFI (*G*) or the percentage of basal CD41 expression on neutrophil cells (*H*). Data shown are from 14 to 15 mice (*G*) or from 5 to 12 mice (*H*). (*I*) *In vitro* hetero-aggregate formation between *Il1r8*$^{+/+}$ or *Il1r8*$^{-/-}$ neutrophils and *Il1r8*$^{+/+}$ or *Il1r8*$^{-/-}$ platelets. Results are expressed as the percentage of CD41 expression in neutrophil alone. Data shown are from three to six independent observations. Student's *t*-test (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). Error bars represent the SEM.

3.3 Role of microbiota and IL-1 in hyperactivity of IL-1R8-deficient platelets

In order to investigate the molecular mechanisms responsible of hyperactivity of IL-1R8-deficient platelets, we first investigated the role of TLR-dependent platelet activation by microbiota-derived microbial moieties. As shown in *Figure 4A* and *B*, the depletion of gut microbiota significantly reduced the levels of soluble P-selectin ($P < 0.05$) and platelet/neutrophil hetero-aggregates ($P < 0.05$) in *Il1r8*$^{-/-}$ mice and abolished the difference between *Il1r8*$^{+/+}$ and *Il1r8*$^{-/-}$ mice. Furthermore, IL-1β plasma levels significantly decreased in *Il1r8*$^{-/-}$ mice upon flora depletion ($P < 0.01$; *Figure 4C*).

Since it has been recently demonstrated that IL-1β is a platelet agonist that induces its own expression and amplifies platelet activation by LPS,[3] we next analysed the role of IL-1 in the phenotype observed. First, we observed that *Il1r8*$^{-/-}$ platelets released higher levels of IL-1β *in vitro* both in basal conditions ($P = 0.038$) and upon 15 min activation with ADP or LPS ($P = 0.012$ and 0.001, respectively; *Figure 4D*) and that IL-1R1 expression was not affected by IL-1R8 deficiency (not shown). Finally, we analysed platelet P-selectin surface expression and platelet/neutrophil aggregation in *Il1r8*/*Il1r1*-double-deficient mice *in vitro*. As shown in *Figure 4E* and *F*, the deficiency of IL-1R1 abrogated the increase of platelet P-selectin surface expression and platelet/neutrophil aggregation observed in *Il1r8*$^{-/-}$ mice compared with *Il1r8*$^{+/+}$ mice ($P < 0.01$ and <0.001, respectively). Altogether these data suggest that microbial TLR ligands are involved in platelet hyper activation in IL-1R8-deficient mice and that IL-1R8-dependent negative regulation of platelet IL-1R1 function has a direct role in tuning platelet activation.

3.4 Lack of IL-1R8 affects platelet activation during systemic inflammation

A systemic LPS-induced inflammation model was used to assess the role of IL-1R8 during *in vivo* platelet activation. Mice were injected with 100 μg/kg LPS i.p., and blood was collected 1 h later. As shown in *Figure 5A* and *B*, upon LPS treatment, higher levels of soluble P-selectin and platelet/neutrophil hetero-aggregates were detected in *Il1r8*$^{-/-}$ than in *Il1r8*$^{+/+}$ mice ($P = 0.0042$ and <0.01, for P-selectin and hetero-aggregates, respectively). In addition, the percentage of

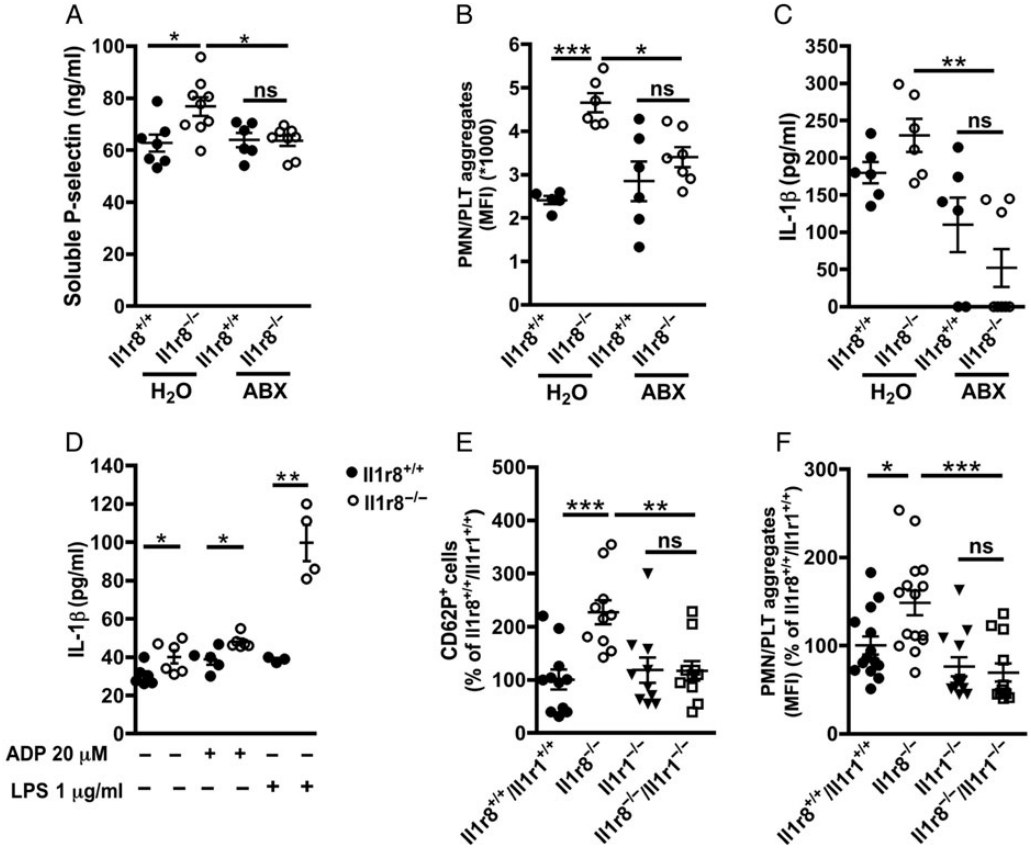

Figure 4 Mechanisms involved in IL-1R8-dependent negative control of platelet activity. (*A* and *B*) Soluble P-selectin plasma levels (*A*) and flow cytometric analysis of neutrophil/platelet (PMN/PLT) aggregates (CD41$^+$ neutrophils) (*B*) in *Il1r8*$^{+/+}$ and *Il1r8*$^{-/-}$ mice in microbiota-depleted (ABX) or control mice (H_2O). (*C*) IL-1β plasma levels in *Il1r8*$^{+/+}$ and *Il1r8*$^{-/-}$ microbiota-depleted mice. (*A*–*C*) Data show the results from five to seven *Il1r8*$^{+/+}$ and six to nine *Il1r8*$^{-/-}$ mice per treatment. (*D*) IL-1β secretion from *Il1r8*$^{+/+}$ and *Il1r8*$^{-/-}$ platelets in resting conditions and after *in vitro* stimulation with ADP or LPS for 15 min at 37°C. Data shown are from three to six independent observations where both *Il1r8*$^{+/+}$ and *Il1r8*$^{-/-}$ platelets were pooled from three or four mice. (*E*) Flow cytometric analysis of P-selectin (CD62P) membrane expression on platelets and (*F*) neutrophil/platelet aggregates (CD41$^+$ neutrophils) in basal conditions in *Il1r8*$^{+/+}$/*Il1r1*$^{+/+}$, *Il1r8*$^{-/-}$, *Il1r1*$^{-/-}$, or *Il1r8*$^{-/-}$/*Il1r1*$^{-/-}$ ($n = 9$–15) mice. Data are reported as the percentage of increase compared with *Il1r8*$^{+/+}$/*Il1r1*$^{+/+}$ samples (*E* and *F*). Student's *t*-test or one-way ANOVA test (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). Error bars represent the SEM.

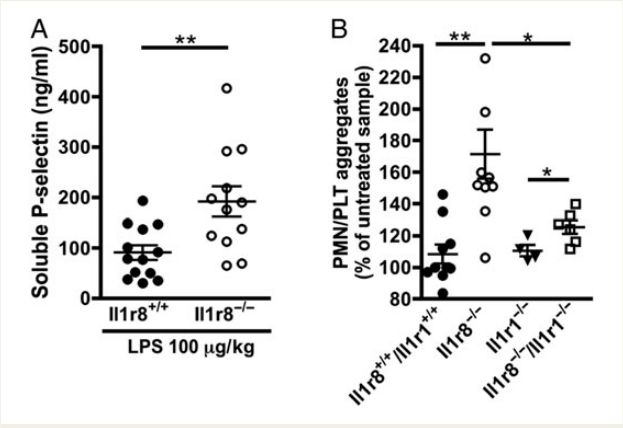

Figure 5 Relevance of platelet IL-1R8 expression during LPS-induced inflammation. (*A*) Soluble P-selectin plasma levels in LPS-treated $Il1r8^{+/+}$ and $Il1r8^{-/-}$ mice ($n = 12–13$). (*B*) Flow cytometry analysis of neutrophil/platelet aggregates ($CD41^{+}$ neutrophils) in LPS-treated $Il1r8^{+/+}/Il1r1^{+/+}$, $Il1r8^{-/-}$, $Il1r1^{-/-}$, and $Il1r8^{-/-}/Il1r1^{-/-}$ mice ($n = 4–10$). Results are expressed as the percentage of increase compared with untreated samples. Student's *t*-test or one-way ANOVA test ($^{*}P < 0.05$, $^{**}P < 0.01$). Error bars represent the SEM.

increase of soluble P-selectin and platelet/neutrophil aggregates after LPS injection compared with the basal level was significantly higher in $Il1r8^{-/-}$ mice than in $Il1r8^{+/+}$ mice (soluble P-selectin: 156 ± 22% in $Il1r8^{+/+}$ mice, 312 ± 56% in $Il1r8^{-/-}$ mice, $P = 0.015$; platelet/neutrophil aggregates: 118 ± 7% in $Il1r8^{+/+}$ mice, 194 ± 29% in $Il1r8^{-/-}$ mice, $P = 0.037$).

To assess whether the regulatory function of IL-1R8 targeted IL-1R1 during systemic inflammation, double-deficient mice were used. As shown in *Figure 5B*, IL-1R8/IL-1R1-double-deficient mice showed a drastic reduction of LPS-induced heterotypic aggregation compared with IL-1R1-competent/IL-1R8-incompetent mice ($P < 0.05$). Thus, the regulatory action of IL-1R8 is largely targeted on IL-1R1. However, IL-1R8/IL-1R1-double-deficient mice showed a small increase of LPS-induced heterotypic aggregation compared with IL-1R1-deficient mice, suggesting that IL-1R8 tunes the function of other members of the IL-1R and TLR families.

These results indicate that IL-1R8 is involved in tuning platelet activation and platelet/neutrophil aggregation in inflammatory conditions.

3.5 *In vivo* relevance

Next, a platelet-dependent, ADP-induced thromboembolism model[37] was used to evaluate the relevance of IL-1R8-mediated regulation in pathological conditions. Histological analysis of lung sections and immunohistochemical analysis for fibrin showed a significantly higher percentage of vessels occluded by fibrin clots in IL-1R8-deficient mice ($P < 0.0001$; *Figure 6A* and *B*). Furthermore, systemic levels of soluble P-selectin were significantly higher in $Il1r8^{-/-}$ mice after ADP-induced pulmonary thromboembolism than in wild-type mice (329 ± 59 ng/mL in $Il1r8^{-/-}$ vs. 65 ± 6.7 ng/mL in $Il1r8^{+/+}$, $P < 0.0001$; *Figure 6C*). These results indicate that $Il1r8^{-/-}$ mice had more severe thrombotic lesions in the lung and increased platelet activation, suggesting increased susceptibility to ADP-induced pulmonary thromboembolism.

3.6 IL-1R8 expression is downmodulated in platelets from SIRS/septic patients

In *Figure 5*, we showed that IL-1R8 deficiency is associated with platelet dysfunction in LPS-induced inflammatory conditions. We thus addressed whether IL-1R8 expression was modified in human pathological conditions associated to platelet dysfunction, in particular in SIRS/sepsis patients.[38] Using a small cohort of patients, classified as SIRS or sepsis patients (according to criteria defined in [33]), we observed a significant reduction of IL-1R8 surface expression in platelets isolated from patients at diagnosis compared with healthy age-matched donors ($P < 0.0001$; *Figure 7A*). In contrast, IL-1R8 surface expression on monocytes did not differ in sepsis patients and healthy donors, suggesting that the regulation of IL-1R8 expression in this condition is restricted to platelets. Moreover, IL-1R8 surface expression in platelets was significantly lower in septic patients compared with SIRS patients ($P = 0.05$; *Figure 7B*), suggesting a correlation between IL-1R8 platelet expression and the severity of the inflammatory disease. The down-regulation of IL-1R8 expression on platelets of SIRS/septic patients compared with healthy donors was confirmed by immunofluorescence analysis, as shown in *Figure 7C*.

In agreement with these data, flow cytometry and immunofluorescence analysis showed that IL-1R8 expression in platelets and megakaryocytic cell lines DAMI and MEG-01 was significantly down-regulated by the treatment with LPS *in vitro* (*Figure 7D–F*). Along the same line, platelets collected from LPS-treated wild-type mice showed a marked decrease in IL-1R8 expression (see Supplementary material online, *Figure S2*).

Since LPS induces platelet-derived MP release,[11,39] we hypothesized shedding of IL-1R8 from the cells through platelet MP formation. Flow cytometry analysis showed that MPs isolated from plasma of septic patients expressed a significant higher level of IL-1R8 on their surface compared with MPs collected from healthy donors ($P = 0.047$; *Figure 7G*). These data were supported by *in vitro* experiments showing a seven-fold increase in IL-1R8 expression levels in MPs released from platelets upon stimulation with LPS (*Figure 7H*). Altogether these results suggest an active shedding of this receptor from platelets through MP formation during inflammatory conditions, which could be involved in IL-1R8 downmodulation observed on platelets from septic patients.

4. Discussion

The expression of functional IL-1R and TLRs by platelets is one of the molecular mechanisms underlying the contribution of platelets to inflammatory and innate immune responses. In this study, we investigated whether platelets express IL-1R8, a negative regulator of IL-1 receptor family and TLR signalling. The results presented here show that both human and murine platelets express IL-1R8 on the membrane and to a lower extent, in intracellular compartments. In addition, megakaryocytes and megakaryocytic cell lines express IL-1R8.

In mice, IL-1R8 deficiency was associated with normal platelet numbers, but increased platelet homotypic and heterotypic aggregation in response to ADP, LPS, IL-1, or IL-18 and increased susceptibility to platelet-dependent thromboembolism.

Platelets express a variety of molecules involved in immune responses, including cytokines (for instance IL-1β), chemokines, and pattern recognition molecules (for instance TLRs). These molecules are key mediators of the interactions between platelets and other cell types, such as endothelial cells or leucocytes, in particular

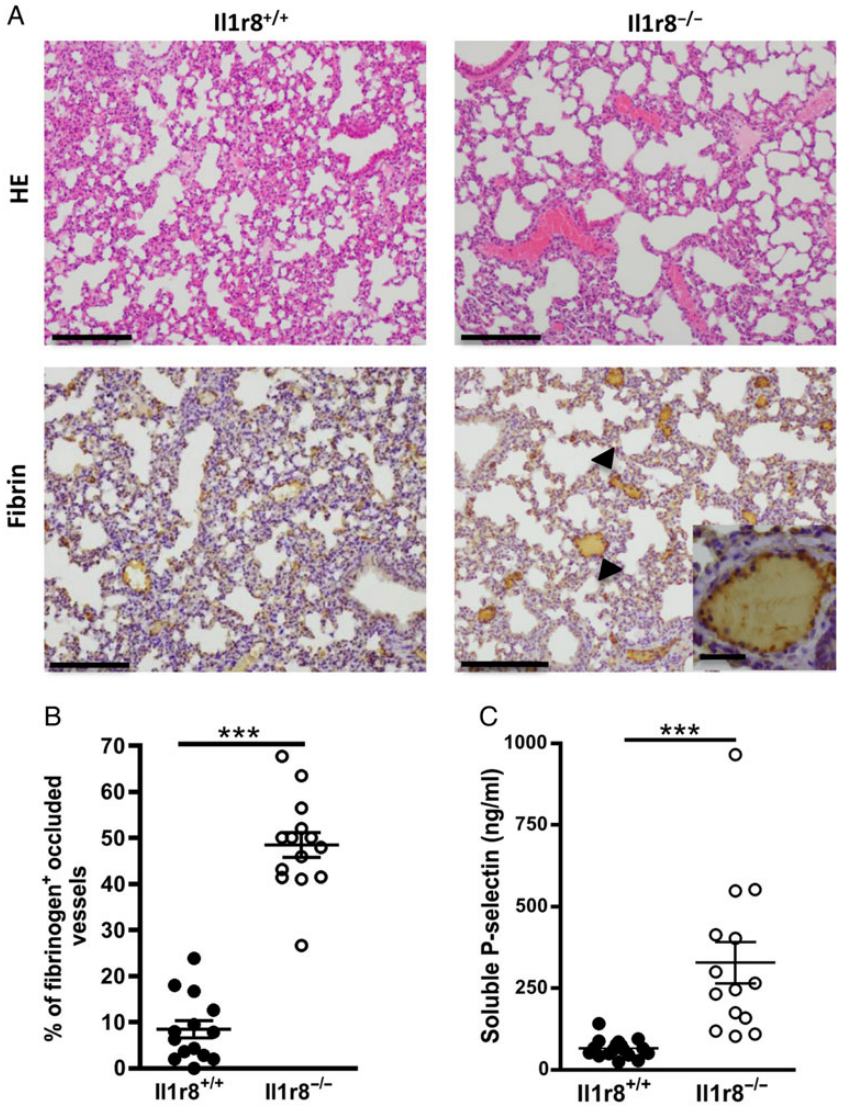

Figure 6 Involvement of IL-1R8 in thromboembolism. (*A*) Histological analysis (top panels) and fibrin immunohistochemical analysis (bottom panels) of *Il1r8*$^{+/+}$ and *Il1r8*$^{-/-}$ lungs in a murine model of ADP-induced thromboembolism. Arrowheads: Vessels occluded by fibrin clots. Inset: Representative picture of a vessel occluded by a fibrin clot. Bar: 100 μm (left panels) and 20 μm (inset). (*B*) Quantitative analysis of vessels showing fibrin clots on total vessels. Fifty fields/lung were analysed. (*C*) Soluble P-selectin plasma levels in *Il1r8*$^{+/+}$ and *Il1r8*$^{-/-}$ mice undergoing thromboembolism. Data shown are from 14 to 17 mice. Student's *t*-test (***$P < 0.001$). Error bars represent the SEM.

neutrophils.[1,2] In contrast to TLRs, whose functional expression on platelets is generally recognized,[4] the expression of IL-1 receptors on platelets has been the object of limited attention. Two studies reported platelet responsiveness to IL-1 in terms of activation (α2bβ3 and P-selectin expression) and showed abnormal IL-1 responsiveness or IL-1R1 expression in platelets from preeclampsia or Inflammatory Bowel Disease patients, compared with healthy donors.[35,36] More recently, it has been confirmed that platelets express IL-1R1 and respond to IL-1β by activating an autocrine signalling loop leading to increased IL-1β production which amplifies platelet activation by LPS.[3] The results presented here show that IL-1R8 is included in the armamentarium of molecules involved in the inflammation-related functions of human and murine thrombocytes. In addition, we show the expression of IL-18Rα in human and murine platelets, suggesting that expression and function of IL-1R family members in platelets are wider than previously recognized.

Platelets are involved in leucocyte adhesion to the endothelium and in pro-inflammatory activation of both leucocytes and endothelium. In addition, activated neutrophils contribute to platelet recruitment through P-selectin and CD11b/CD18 and produce tissue factor. Thus, the reciprocal activation of platelets and neutrophils, which promotes immune defence, also results in a pro-thrombotic status.[1] This is of particular importance in sepsis, a condition associated to thrombocytopenia and platelet activation and sequestration in lung and liver microvasculature.[38,40] The results presented here, showing that IL-1R8 deficiency is associated to increased platelet heterotypic aggregation with neutrophils in basal conditions and after treatment with LPS, IL-1, or IL-18 *in vitro* and/or *in vivo*, indicate that IL-1R8 is a negative

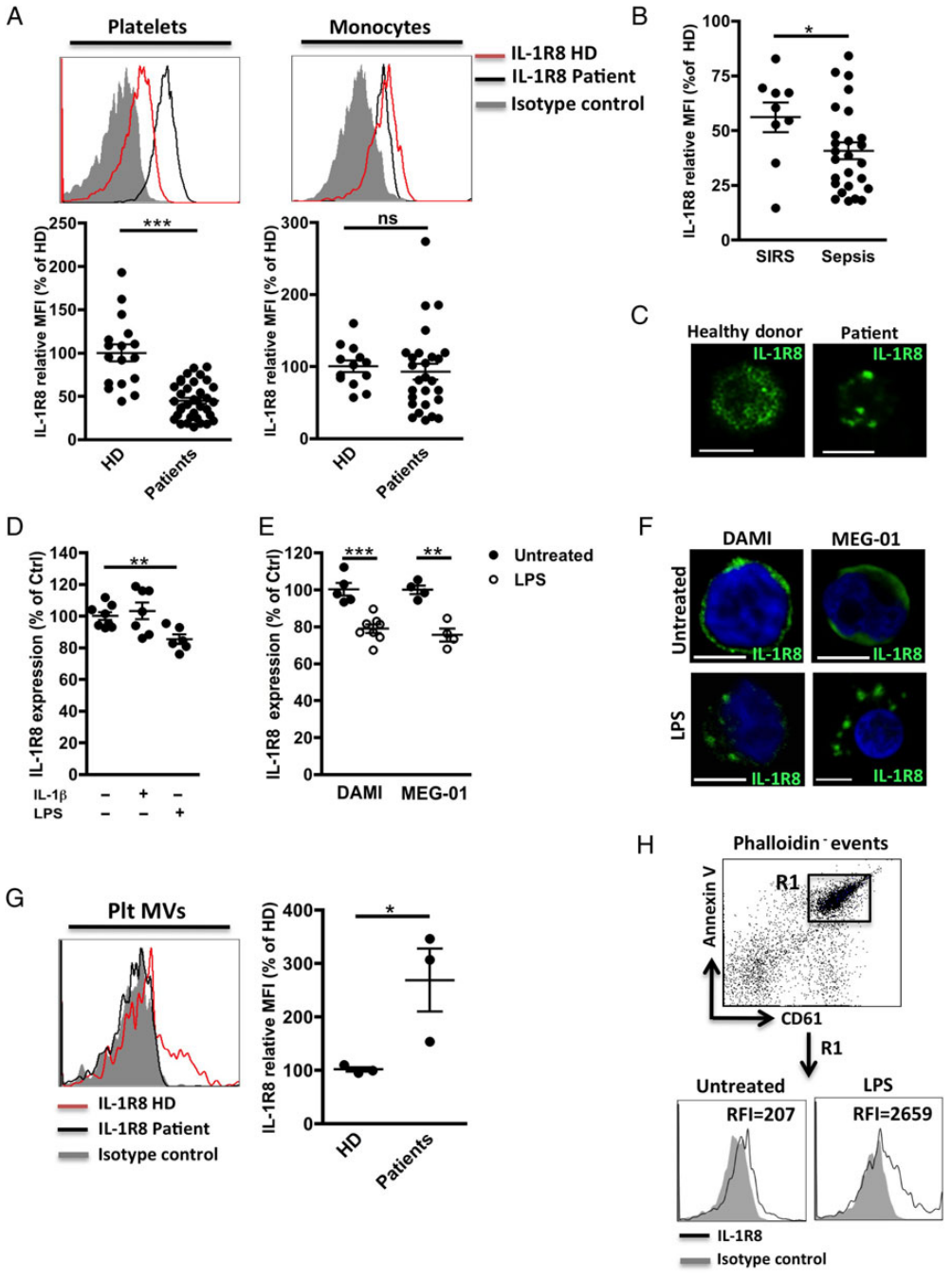

Figure 7 Downmodulation of IL-1R8 surface expression in platelets and megakaryocytic cell lines in inflammatory conditions. (*A*) Flow cytometric analysis of IL-1R8 expression in platelets and monocytes from healthy donors and age- and gender-matched SIRS/sepsis patients. Flow cytometry histograms show isotype control (grey fill), IL-1R8 surface expression in a representative healthy donor (black line), and in a representative SIRS/sepsis patient (red line). (*B*) Flow cytometric analysis of IL-1R8 expression in platelets from ($n = 9$) SIRS and ($n = 26$) sepsis patients. (*A* and *B*) Results are reported as the percentage of the relative MFI referred to healthy donors. Student's *t*-test ($^*P < 0.05$, $^{***}P < 0.0001$). (*C*) Confocal analysis of IL-1R8 expression in platelets from healthy donors and SIRS patients. Representative images from one out of two different patients and healthy donors analysed. Scale bar 5 μm. (*D* and *E*) Flow cytometric analysis of IL-1R8 surface changes on platelets treated with IL-1β (12.5 ng/mL) or LPS (100 ng/mL) (*D*) and DAMI and MEG-01 cells treated with LPS (100 ng/mL) for 30 min (*E*). Data, expressed as the percentage of untreated samples, are from four to eight independent observations. Student's *t*-test (*E* and *G*) or one-way ANOVA test (*D*) ($^{**}P < 0.01$, $^{***}P < 0.001$). (*F*) Confocal microscopy analysis of IL-1R8 expression in megakaryocytic cell lines (DAMI and MEG-01) untreated or treated with LPS (100 ng/mL) for 30 min. IL-1R8 (green), DAPI (blue), bars 10 μm. Representative images out of 10–30 analysed ROI. (*G*) Flow cytometric analysis of IL-1R8 expression on platelet-derived MPs obtained from plasma. Left panel: Flow cytometry histogram showing isotype control (grey fill), IL-1R8 surface expression in a representative healthy donor (black line), and in a representative septic patient (red line). Right panel: Results are reported as the percentage of the relative MFI (RFI). $n = 3$ healthy donors and 3 septic patients. Student's *t*-test ($^*P < 0.05$). (*H*) Upper panel: Gate strategy of MPs released from washed platelets stimulated *in vitro* with LPS (100 ng/mL). Lower panel: Flow cytometric histograms show isotype control (grey fill) and IL-1R8 surface expression (black line). One representative experiment out of two is shown. Error bars represent the SEM.

regulator of platelet heterotypic aggregation associated to inflammatory conditions and endotoxemia.

IL-1R8 deficiency is associated to platelet hyperactivity in basal conditions and upon *in vitro* stimulation with pro-thrombotic stimuli (ADP and thrombin), independently of stimulation with TLR or IL-1R family ligands. The higher levels of IL-1β observed in IL-1R8-deficient platelets in basal condition and upon *in vitro* stimulation, and the results obtained in IL-1R8/IL-1R1-double-deficient mice suggest that IL-1R1-dependent signalling plays a major and direct contribution to platelet hyperactivity observed in IL-1R8-deficient mice, in line with results obtained in other inflammatory models or cell types.[23,41] In addition, results obtained in microbiota-depleted mice suggest the involvement of microbial TLR ligands in platelet hyperactivity observed in IL-1R8-deficient mice. Indeed, since IL-1R1 deficiency reduced but did not abrogate the difference between IL-1R8-competent and IL-1R8-deficient mice after endotoxin injection, deregulated IL-1R1 signalling in the absence of IL-1R8 is not sufficient to explain the observed phenotype, and additional molecular mechanisms, likely TLR4-dependent, are involved. Platelets release IL-1 following activation or synthetize it upon inflammatory stimulation by microbial moieties, including LPS, and IL-1β itself.[3,42,43] In this context, IL-1R8 might contribute to dampening both LPS-dependent activation of platelets and the IL-1β-dependent autocrine stimulatory loop.[3]

The relevance *in vivo* of platelet hyperactivity associated to IL-1R8 deficiency was demonstrated in a model of thromboembolism induced by ADP. In this sterile condition, stimuli potentially involved in setting IL-1R8-deficient platelets in a pro-thrombotic status are IL-1α and IL-1β derived from activated platelets themselves, acting in a paracrine manner.[3] The higher number of vessels occluded by fibrin clots and higher plasma levels of soluble P-selectin suggest a more severe thrombotic process in the absence of IL-1R8.

The results obtained in mice showing that in LPS-induced inflammatory conditions, IL-1R8 deficiency was associated with platelet activation and platelet/neutrophil aggregation, prompted us to analyse IL-1R8 expression in human pathological conditions associated to platelet dysfunction, in particular in SIRS/sepsis.[38,44,45] In a preliminary study in a small cohort of patients with severe inflammatory conditions, IL-1R8 expression was significantly reduced in platelets of patients compared with platelets from healthy donors and down-regulation correlated with the severity of the disease. These results were supported by *in vitro* studies showing that in resting platelets and megakaryocytic cells, IL-1R8 was expressed on the cell surface and marginally in endosomal compartments, and upon stimulation with LPS, it was rapidly downmodulated. Furthermore, IL-1R8 expression in platelet-derived MPs isolated from septic patients was significantly higher compared with MPs from healthy donors. Along the same line, platelet–MPs expressed higher levels of IL-1R8 after platelet activation *in vitro* by LPS. These results suggest that the rapid down-regulation of platelet IL-1R8 in inflammatory conditions may be due to release through MPs.

Further studies will be necessary to confirm these results in larger cohorts of SIRS/sepsis patients and to address whether the modulation of IL-1R8 expression in platelets and the following release in MPs might represent a potential pathogenetic mechanism contributing to platelet dysfunction associated to severe inflammatory conditions.

Thus, the results obtained in this study demonstrate that platelets, which have a large repertoire of TLRs and IL-1 receptor family members (IL-1R1 and as shown here IL-18R), express high levels of IL-1R8, which plays a non-redundant role as regulator of thrombocyte function *in vitro* and *in vivo*.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

Conflict of interest: none declared.

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