The Inter-Relationship of Platelets with Interleukin-1 β-Mediated Inflammation in Humans

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Abstract

Background Inflammation and coagulation are key processes in cardiovascular diseases (CVDs). The Canakinumab Anti-inflammatory Thrombosis Outcome Study trial affirmed the importance of inflammation in CVD by showing that inhibition of the interleukin (IL)-1β pathway prevents recurrent CVD. A bi-directional relationship exists between inflammation and coagulation, but the precise interaction of platelets and IL-1β-mediated inflammation is incompletely understood. We aimed to determine the inter-relationship between platelets and inflammation—and especially IL-1β—in a cohort of healthy volunteers.

Methods We used data from the 500-Human Functional Genomics cohort, which consists of approximately 500 Caucasian, healthy individuals. We determined associations of plasma levels of IL-1β and other inflammatory proteins with platelet number and reactivity, the association of platelet reactivity with ex vivo cytokine production as well as the impact of genetic variations through a genome-wide association study (GWAS).

Results Platelets were associated with IL-1β on different levels. First, platelet number was positively associated with plasma IL-1β concentrations ($p = 8.9 \times 10^{-9}$) and inversely with concentrations of α-1-anti-trypsin ($p = 1.04 \times 10^{-18}$), which is a known antagonist of IL-1β. Second, platelet degranulation capacity, as determined by agonist-induced P-selectin expression, was associated with ex vivo IL-1β and IL-6 production. Third, several platelet single-nucleotide polymorphisms (SNPs) were associated with

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cytokine production and there was a significant platelet SNP enrichment in specific biological important pathways. Finally, platelet SNPs were enriched among SNPs earlier identified in GWAS studies in blood-related diseases and immune-mediated diseases. **Conclusion** This comprehensive assessment of factors associated with platelet number and reactivity reinforces the important inter-relationship of platelets and IL-1β-mediated inflammation.

Introduction

A wealth of pre-clinical and clinical data implicates inflammation as an important driver of ischaemic cardiovascular diseases (CVDs). Until recently, clinical data demonstrating a beneficial effect of inhibiting inflammation were lacking. This has definitely changed with the publication of the results of the Canakinumab Anti-inflammatory Thrombosis Outcome Study (CANTOS) trial, showing that the therapeutic monoclonal interleukin-1β (IL-1β) antibody canakinumab significantly reduced the risk for recurrent cardiovascular events in high-risk patients who have sustained a prior myocardial infarction.[1] The pro-inflammatory cytokine IL-1β is a master regulator of inflammation and fever,[2] and the CANTOS trial affirmed the importance of the IL-1 pathway in atherogenesis.[3] Nonetheless, the precise processes via which IL-1β neutralization improves cardiovascular outcome remain unclear. Possible mechanisms include local effects in the atherosclerotic plaque but also systemic actions of IL-1β on platelets and leukocyte mobilization from the bone marrow.

Platelets are increasingly recognized as important effector cells of inflammation and atherosclerosis.[4,5] Platelets have a multitude of surface receptors that mediate not only haemostatic, but also inflammatory responses. Platelet activation induces the release of granules that contain a diverse array of chemokines, cytokines and growth factors.[6] Despite being anucleated cells, platelets were suggested to harbour a complex ribonucleic acid (RNA) transcriptome and functional splicing and translational machinery for the synthesis of proteins,[7] including IL-1β.[8,9]

There is considerable variation between individuals in platelet count and platelet reactivity to stimuli. Interestingly, recent data from population-based studies suggest that even variation in platelet count in the range of normality determines future cardiovascular events and mortality.[10] Variation in platelet count and reactivity is determined by both genetic and non-genetic factors, including inflammation.[11,12] For example, administration of recombinant IL-1β to patients with gastrointestinal cancer or mice increases platelet count.[13,14]

Several experimental studies have investigated the role of platelets in inflammation and vice versa, but data in humans are limited. We therefore studied the inter-relationship between platelets and inflammation, and especially IL-1β, in the 500 Functional Genomics (500FG) cohort, which consists of approximately 500 healthy individuals of Western-European genetic background that is part of the Human Functional Genomics Project (www.humanfunctionalgenomics.org)[15]. This cohort has the advantage that confounding effects by underlying illness and/or use of drugs are minimal. We first determined the associations of platelet and leukocyte number and platelet reactivity with plasma levels of IL-1β and other inflammatory proteins. Next, we determined associations between platelet reactivity and ex vivo cytokine production as well as the impact of genetic variations through a genome-wide association study (GWAS).[15]

Methods

500 Functional Genomics Study

Cohort description: The 500FG study was approved by the Ethical Committee of Radboud University Medical Center (NL42561.091.12, 2012/550). A total of 534 healthy individuals, aged > 18 years and of Western-European descent, were sampled between July 2013 and December 2014 at the Radboud University Medical Center, The Netherlands.[15–18] Exclusion criteria were pregnancy/breastfeeding, chronic or acute disease at the time of sampling and use of any medication in the last month before the study.

Measurements of platelet function: Venous blood was collected in citrated tubes (3.2% sodium citrate; Becton Dickinson, United States) and handled gently to prevent pre-activation. Platelet reactivity was determined by a whole blood flow cytometry assay within 30 minutes as described earlier.[19] In short, the platelet membrane expression of the α-granule protein P-selectin and the binding of fibrinogen to the activated integrin $\alpha_{IIb}\beta_3$ were measured in unstimulated whole blood samples and after ex vivo stimulation by seven increasing concentrations of adenosine diphosphate (ADP, 7.8–125 μM, Sigma-Aldrich, United States) or cross-linked collagen-related peptide (CRP-XL, 9–625 ng/mL, kind gift from Prof. Dr. R. Farndale, Cambridge, United Kingdom). The following antibodies were used: phycoerythrin -labelled anti-CD62P (P-selectin; Bio-Legend, San Diego, California, United States), fluorescein isothiocyanate-labelled anti-fibrinogen (DAKO Ltd., High Wycombe, United Kingdom) and PC7-labelled anti-CD61 (platelet identification marker; Beckman Coulter, France). Samples were analysed using a FC500 flow cytometer and analysed using the CXP software (Beckman Coulter, France). ADP- and CRP-XL-induced platelet P-selectin (termed as APR and CPR) and platelet-fibrinogen reactivity to either ADP or CRP-XL (termed as AFR and CFR) were determined by calculating the area under the curve from the median fluorescence intensity (MFI) of CD62P or fibrinogen on CD61-positive events generated from the

unstimulated platelets and seven increasing concentrations of ADP or CRP-XL. Plasma concentrations of the platelet α-granule protein, β-thromboglobulin (β-TG), a plasma soluble marker for platelet activation, were measured in one batch using enzyme-linked immunosorbent assay (ELISA) as previously described from platelet-poor plasma.[20]

Stimulation of whole blood and peripheral blood mononuclear cells (PBMCs): Full details of these experiments have been detailed elsewhere.[16] In short, whole blood or PBMCs were co-incubated with the different stimuli for 48 hours (whole blood), 24 hours or 7 days (both PBMCs) at 37°C and 5% CO_2. Supernatants were collected and stored at a temperature of –20°C. Cytokine concentrations were measured in the supernatant using ELISA.

Measurements of plasma cytokines, acute phase proteins and adipokines: Ethylenediaminetetraacetic acid (EDTA) plasma samples were thawed at room temperature, mixed with an equal volume of specified diluent, centrifuged at 900 × *g* for 12 minutes at 4°C. The diluted plasma supernatants were measured using the Simple Plex cartridges run on the Ella platform (ProteinSimple, San Jose, California, United States) following the manufacturer's instructions. The first step of sample preparation was to perform ultracentrifugation on each sample to remove all particulates and platelets from the plasma. The circulating mediators high sensitive C-reactive protein, leptin, adiponectin and α-1 antitrypsin (AAT) were measured in EDTA plasma using the R&D Systems DuoSet ELISA kits following the manufacture's standard protocol.

In Vitro *Validation* Experiments

PBMCs and isolated washed platelets were obtained and experiments were performed as previously described.[21] In short, PBMCs (3×10^6 cells/mL) isolated from buffy coat (Sanquin Bloodbank, Nijmegen, The Netherlands) were co-cultured for 24 hours at 37°C and 5% CO_2 with either washed platelets in a PBMC: platelet ratio of 1:150 or RPMI in the presence of one of the following stimuli: lipopolysaccharides (LPS) (6 ng/mL; *Escherichia coli* serotype 055:B5; Sigma-Aldrich), *Candida albicans* yeast (1×10^6 organisms/mL; UC820), *Staphylococcus aureus* (1×10^6 organisms/mL; ATCC29213) or *E. coli* (1×10^6 organisms/mL; ATCC 35218). A Transwell system was used to physically separate platelets and PBMC in some experiments. Platelet-rich plasma (PRP) was isolated from whole blood and was pre-treated with recombinant IL-6 (100 ng/mL; eBioscience), recombinant IL-1β (100 ng/mL; R&D Systems), recombinant interferon (IFN)-γ (100 ng/mL; Boehringer Ingelheim Limited), recombinant IL-18 (10 ng/mL; R&D Systems) or AAT (10 µg/mL; Alpha Therapeutic Corporation) for 120 minutes in 37°C and 5% CO_2. Platelet reactivity to different platelet agonists was subsequently determined by flow cytometry as described above.

Genotyping and Imputation

Genotyping in our cohort was performed using the commercially available single-nucleotide polymorphism (SNP) chip, Illumina HumanOmniExpressExome-8 v1.0., and was imputed to obtain genotypes at approximately 7 million SNPs. The strands and variant identifiers were aligned to the reference Genome of The Netherlands (GoNL)[22] dataset using Genotype Harmonizer.[23] The data were phased using SHAPEIT2 v2.r644[24] using the GoNL as a reference panel. Finally, these data were imputed using IMPUTE2[25] with the GoNL as the reference panel.[26]

Statistical Analysis

Associations for each measurements of platelet count and platelet function in connection with the acute reactive proteins, adipokines, circulating cytokines and whole blood/PBMC cytokine responses were analysed using the Spearman's correlation coefficient and the statistical significance was based on the false discovery rate (threshold $\alpha \leq 0.1$), unless stated otherwise. Analyses were performed using SPSS version 22.

Results

The Impact of Non-Genetic Host Factors on Platelets Parameters

Characteristics of the cohort have been previously described.[16–18] The median (interquartile range [IQR]) age was 23 years (21–27 years) and 56% were female. The median body mass index (BMI) was 22.3 (20.7–24.4) and 83.1% had a BMI ≤ 25. Forty-eight per cent of women used an oral contraceptive and 13.2% of subjects regularly used tobacco.

Representative platelet reactivity curves, the distribution of these platelet responses and flow cytometry gating strategy are shown in ►**Supplementary Figs. S1** and **S2** (available in the online version). Platelet reactivity is expressed as ADP-induced P-selectin expression (APR, available in $n = 489$ volunteers) and fibrinogen-binding (AFR, $n = 396$) and CRP-XL-stimulated P-selectin expression and fibrinogen binding (CPR and CFR, both $n = 302$). ►**Fig. 1** illustrates the correlations between platelet parameters. Whereas no correlation was found between unstimulated P-selectin and fibrinogen binding, the four readouts of platelet reactivity correlated well with each other, with the strongest correlations between those in response to the same platelet agonist. There was a positive correlation between plasma concentrations of β-TG and platelet count, and this was the reason to normalize β-TG levels for platelet mass in further analyses.

We did not find significant associations between platelet reactivity measures or β-TG levels with intrinsic, non-genetic host factors such as age, gender, BMI, oral contraceptive usage and smoking. Women had a significantly higher platelet count than men with median (IQR) values of 280 (218–352) × 10^9/L versus 251 (201–319) × 10^9/L ($p = 0.002$), an observation previously reported by Biino et al.[27]

Associations of Inflammatory Molecules with Platelet Parameters and Leukocyte Count

We used a novel, high-sensitive cytokine assay capable of measuring very low circulating cytokine concentrations.[28] Plasma was ultracentrifuged before measurement to remove platelet remnants and other particulates. ►**Fig. 2A** shows associations between these cytokines and platelet parameters.

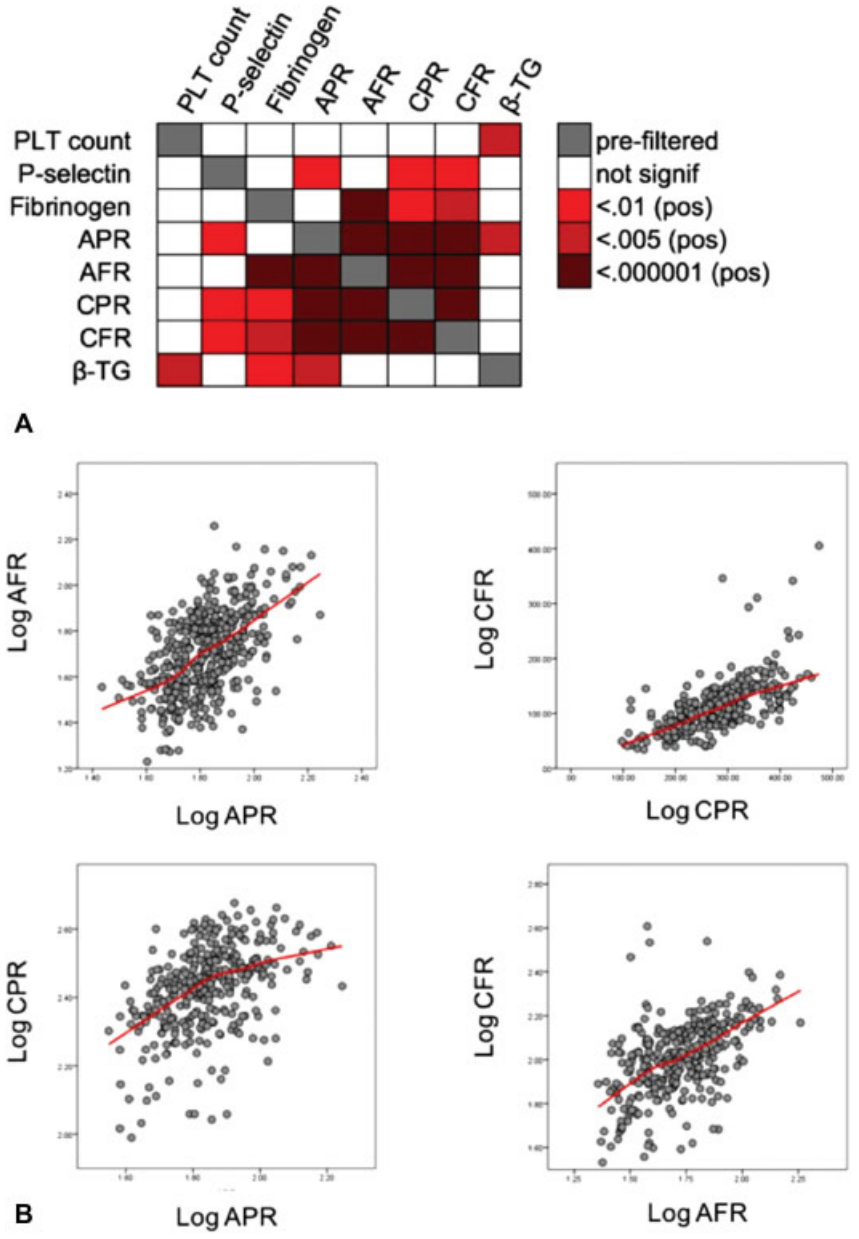

Fig. 1 Correlations between platelet parameters. Data of adenosine diphosphate (ADP)-induced P-selectin expression (APR, available in $n = 489$ volunteers) and fibrinogen binding (AFR, $n = 396$) and cross-linked collagen-related peptide (CRP-XL) stimulated P-selectin expression and fibrinogen binding (CPR and CFR, both $n = 302$) were shown. (**A**) *p*-Values of the correlations of platelet and coagulation parameters, corrected for multiple testing using the false discovery rate (FDR) method. The colour legend indicates the range of *p*-values. (**B**) Scatterplots of highly significant correlations from panel (**A**). The red line shows the LOESS fit to the data. Abbreviations: PLT count, platelet count; P-selectin, unstimulated P-selectin expression; Fibrinogen, unstimulated fibrinogen expression; β-TG, plasma concentration of β-thromboglobulin.

The strongest correlation was between plasma IL-1β concentrations and platelet count (Spearman's $R = 0.29$; uncorrected $p = 8.9 \times 10^{-9}$) and this correlation remained significant after correction for sex, age and use of oral contraceptives using a linear regression model. When volunteers were stratified in tertiles according to their platelet number, those in the highest tertile had a significantly higher median (IQR) IL-1β concentrations than those in the lowest tertile (0.21 pg/mL, 0.1–0.53 pg/mL, vs. 0.09 pg/mL, 0.06–0.16 pg/mL; $p < 0.0001$). Plasma IL-1β were also associated with leukocyte counts ($R = 0.24$; $p = 1.3 \times 10^{-6}$), including neutrophil ($R = 0.23$; $p = 8.9 \times 10^{-7}$) and monocyte ($R = 0.23$; $p = 2 \times 10^{-6}$) count.

Similar to IL-1β, platelets were suggested to harbour IL-18 transcripts and synthesize and secrete IL-18 upon activation. They also contain the IL-18 antagonist IL-18 binding protein (IL-18BP).[29] We found an inverse correlation between platelet number and IL-18BP, as well as a positive association with vascular endothelial growth factor A (VEGF-A), which is also stored in α-granules.[30] In contrast, when plasma cytokine concentrations were related to platelet reactivity, only few weak correlations were present: IL-10 had a negative

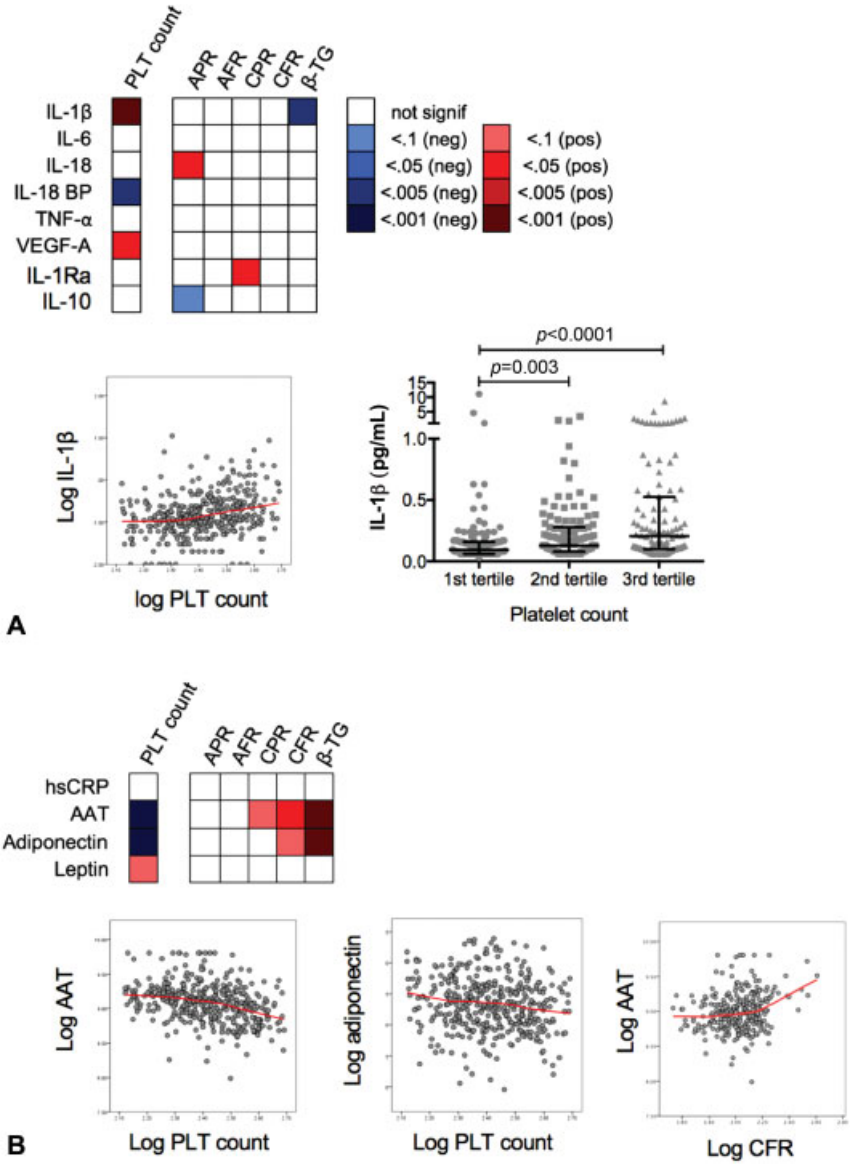

Fig. 2 Correlations of circulating inflammatory mediators with platelet parameters. (**A**) *p*-Values of correlations of circulating cytokines with platelet parameters (top panel); scatterplot of the correlation between interleukin (IL)-1β and platelet counts (lower left panel); IL-1β levels in the different platelet count tertiles (lower right panel). (**B**) *p*-Values (false discovery rate [FDR] corrected) of correlations of acute phase proteins (high sensitive C-reactive protein [hsCRP] and α1-anti-trypsin [AAT]) and adipokines (leptin and adiponectin) with platelet parameters as well as scatterplot of the most significant correlations. The colour legend indicates the range of *p*-values. The red line shows the LOESS fit to the data. Abbreviations: PLT count, platelet count; P-selectin, unstimulated P-selectin expression; Fibrinogen, unstimulated platelet-fibrinogen binding; APR, ADP-induced P-selectin expression; AFR, ADP-induced platelet-fibrinogen binding; CPR, CRP-XL-induced P-selectin expression; CFR, CRP-XL-induced platelet-fibrinogen binding; β-TG, β-thromboglobulin/10^6 platelets.

association with APR, IL-18 a positive association with APR and IL-1Ra a positive association with CPR. However, after correction for multiple testing, none of these associations remained statistically significant.

When concentrations of the acute phase proteins C-reactive protein and AAT and the adipokines leptin and adiponectin were related to platelet number and function (►**Fig. 2B**), strong negative associations were found between platelet count and AAT ($R = -0.398$; $p = 1.04 \times 10^{-18}$) as well as adiponectin ($R = -0.178$; $p = 1.41 \times 10^{-4}$). Both AAT and adiponectin have anti-inflammatory effects and AAT is well known to inhibit IL-1β,[31] whereas it stimulates IL-1Ra release.[32–34] Indeed, we found that both AAT and adiponectin correlated inversely with IL-1β concentrations ($R = -0.348$; $p = 4.7 \times 10^{-13}$ and $R = -0.146$; $p = 0.003$, respectively), but not with IL-1Ra. AAT and adiponectin concentrations were also associated with a higher platelet reactivity to CRP-XL. C-reactive protein concentrations were not associated with any of the platelet parameters. None of the cytokines, acute phase proteins or adipokines correlated with baseline unstimulated P-selectin expression on platelets or platelet–fibrinogen binding.

As platelet counts were higher in women, we analysed the associations between platelet counts and the inflammatory

molecules separately in males and females. The negative correlations between platelet count and IL-18BP remained in males ($p = 0.01$) and females ($p = 0.09$) despite not being statistically significant for the latter. The negative correlation of platelet count with VEGF-A remained only in females ($p = 0.02$). Plasma levels of cytokines and acute phase proteins in females and males were comparable except for VEGF-A and IL-1Ra levels, both higher in women ($p = 0.001$ and $p = 0.004$, respectively).

Platelet Reactivity is Associated with Cytokine Production Capacity

We next studied the relationship between platelets and the capacity of immune cells to produce IL-1β and other cytokines. We measured the production of monocyte derived (IL-1β and IL-6) and lymphocyte-derived cytokines (IFN-γ) in culture supernatants after ex vivo stimulation of whole blood and PBMCs with the following five stimuli: two purified synthetic microbial ligands: LPS or Pam3CSK4; two bacterial stimuli: *E. coli* and *S. aureus*; and one fungal stimulus: *C. albicans* yeast. Relating platelets to cytokine production by isolated PBMCs is possible because PBMC isolates still contain considerable numbers of platelets.[21]

Platelet P-selectin reactivity to ADP (APR), which corresponds with the release of platelet α-granules, was related to IL-1β and IL-6 production to multiple stimuli, whereas CPR was inversely associated with IFN-γ production in whole blood (►**Fig. 3A** and **B**). Interestingly, no such associations were found for fibrinogen reactivity, which corresponds more with platelet aggregation, whereas unstimulated fibrinogen binding to $\alpha_{IIb}\beta_3$ even had few negative associations with cytokine production. Strikingly, while unstimulated P-selectin expression had positive association with IL-6 response to LPS, platelet integrin $\alpha_{II}b\beta_3$ activity was negatively associated with LPS-induced cytokine responses (►**Fig. 3A**). These observations were validated in a set of in vitro experiments. In line with the observations from our cohort, addition of platelets decreased IFN-γ production in response to *C. albicans* and *S. aureus*, whereas IL-1β and IL-6 responses to all stimuli were significantly increased. Physical separation of PBMC and platelets using a Transwell system reversed these cytokine-modulating effects of platelets (►**Fig. 3C**). Conversely, exposure of PRP to recombinant IL-1β, IL-6, IFN-γ (►**Supplementary Fig. S3**, available in the online version) and IL-18 (►**Supplementary Fig. S4**, available in the online version) neither increased P-selectin expression or fibrinogen binding, nor reactivity to ADP or CRP-XL. The exception was AAT, which increased P-selectin expression (►**Supplementary Fig. S4**, available in the online version).

Genetic Architecture of Platelet Parameters and Relation with Cytokine Production

We next tested whether we could also demonstrate a relationship between platelets and inflammation at the genetic level. Genetic association studies have identified several SNPs that modify platelet number and reactivity.[11,35,36] Our genome-wide association analysis did not identify significant hits for the above-mentioned measurements in our cohort. We therefore first performed a pathway analysis using genes in the vicinity of the top platelet quantitative trait locus (QTLs) that had the strongest association with platelet parameters in our cohort (adjusted $p = 0.0009$). The results indicate that ErbB1, integrin β1 receptors, adenosine 5′-diphosphate–ribosylation factor 6 (Arf6), insulin-like growth factor-1 (IGF-1) and platelet-derived growth factor receptors-β (PDGFR-β) signalling pathways were identified to be significantly enriched (►**Fig. 4**). These are important biological pathways, including roles in inflammation, and we have identified in an unbiased way the association with platelets.

We then examined platelet QTLs that were reported in the earlier studies. A list of 133 QTLs was extracted from the literature, including those implicated in platelet number,[37–40] platelet reactivity[11,35,41–44] and mean platelet volume (MPV).[37,40,45] Even though the latter parameter was not measured in our cohort, it was included as platelet size is related to the number of integrins on the platelet membrane[11,37] and genetic variance in MPV is also associated with platelet reactivity.[40] Seventy-four QTLs were detected in our cohort and 24 (36%) of these showed at least one suggestive association with platelet number, platelet reactivity or β-TG (►**Fig. 5**). The strongest associations with platelet reactivity were with QTLs in the genes encoding the surface proteins glycoprotein VI (collagen receptor; *GP6*), ADP receptor P2Y1 (*P2RY1*), platelet endothelial aggregation receptor 1 (*PEAR1*), in the gene *JMJD1C* and in the *IL1F10/IL1RN* region.

Next, we tested whether the 24 top platelet QTLs (threshold $p = 5 \times 10^{-2}$) that influence platelet function in our cohort also impacted IL-1β, IL-6 and IFN-γ cytokine responses in whole blood and PBMC. A sizeable number of these genetic variants were indeed associated with cytokine production: 15 SNPs showed at least marginal association ($p < 0.05$) with one or more cytokine responses, and 5 (rs10761741, rs6734238, rs3794153, rs3091242 and rs11602954) had more than 5 associations (►**Fig. 6A**). QTLs in the genes of the surface receptors *GP6* and *P2YR1* and in *PEAR1 or JMJD1C*, which all had strong associations with platelet reactivity, had either no or only suggestive association with cytokine production. Interestingly, strong, negative associations with cytokine responses was found for rs6734238, a genetic variant located downstream of *IL1F10* and upstream of *IL1RN*, which encodes the anti-inflammatory cytokine IL-1Ra.[46] This QTL also correlated inversely with AFR in our cohort.

Lastly, we tested for associations in the opposite direction, that is, whether several QTLs that we recently found in the same cohort to be related with cytokine responses[47] were associated with platelet responses (►**Fig. 6B**). Only few associations were found. Again, a QTL in a locus near *IL1RN* (rs4496335) was inversely related with AFR, whereas rs10908219, which is located near the genes for *fibroblast growth factor* (*FGF*)*-19*, *FGF4* and *FGF3* were positively associated with ADP- and CRP-XL-induced $\alpha_{IIb}\beta_3$ integrin activation. Finally, given the association of platelet count with plasma IL-1β concentrations, we assessed whether the IL-1β pathway was enriched for platelet genes. However, no shared SNPs were found for platelet count and IL-1β.

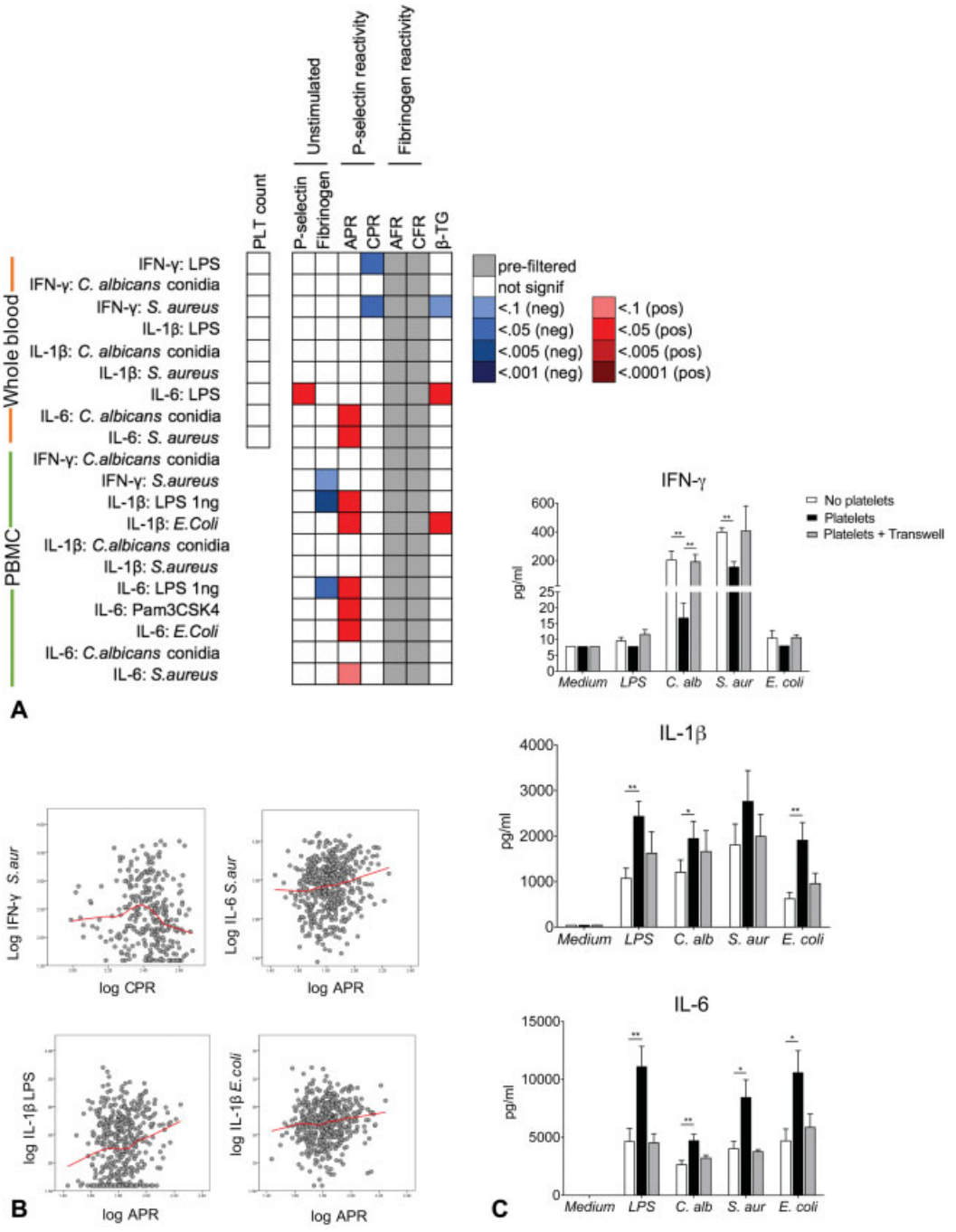

Fig. 3 Associations of platelets with cytokine responses. (**A**) *p*-Values (false discovery rate [FDR] corrected) of the correlations of whole blood and peripheral blood mononuclear cells (PBMCs) cytokine responses with platelet parameters. The colour legend indicates the range of *p*-values. (**B**) Scatterplots of the highly significant correlations of whole blood cytokine responses from panel A. The red line shows the LOESS fit to the data. (**C**) PBMCs (3×10^6 cells/mL) were co-cultured with either washed platelets or RPMI and the following stimuli for 24 hours: lipopolysaccharides (LPS) (6 ng/mL), *C. albicans* yeast (1×10^6 organisms/mL), *S. aureus* (1×10^6 organisms/ml) and *E. coli* (1×10^6 organisms/mL) with or without physical separation between platelets and PBMC using a Transwell system. Interleukin (IL)-1β, IL-6 and interferon (IFN)-γ concentrations were determined in the culture supernatant. Platelets were added in a ratio of 150 platelets for every PBMC. Presented data are means with standard error of the mean (SEM) from 6 healthy donors. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$. *C. alb*, *Candida albicans*; S. aur, *Staphylococcus aureus*; PLT count, platelet count; P-selectin, unstimulated P-selectin expression; Fibrinogen, unstimulated platelet-fibrinogen binding; APR, ADP-induced P-selectin expression; CPR, CRP-XL-induced P-selectin expression; β-TG, β-thromboglobulin/10^6 platelets.

Platelet SNPs Overlap with SNPs Associated with Immune-Mediated Diseases

Finally, we intersected our platelet QTLs with the information of GWASs for various diseases available in the literature. We extracted GWAS SNPs from the National Human Genome Research Institute GWAS catalog[48] and binned them into eight categories on the basis of their association with different human phenotypes (cancer, immune-mediated diseases, infectious disease, heart-related traits, blood-related traits, metabolic traits, height and type 2 diabetes-related traits).

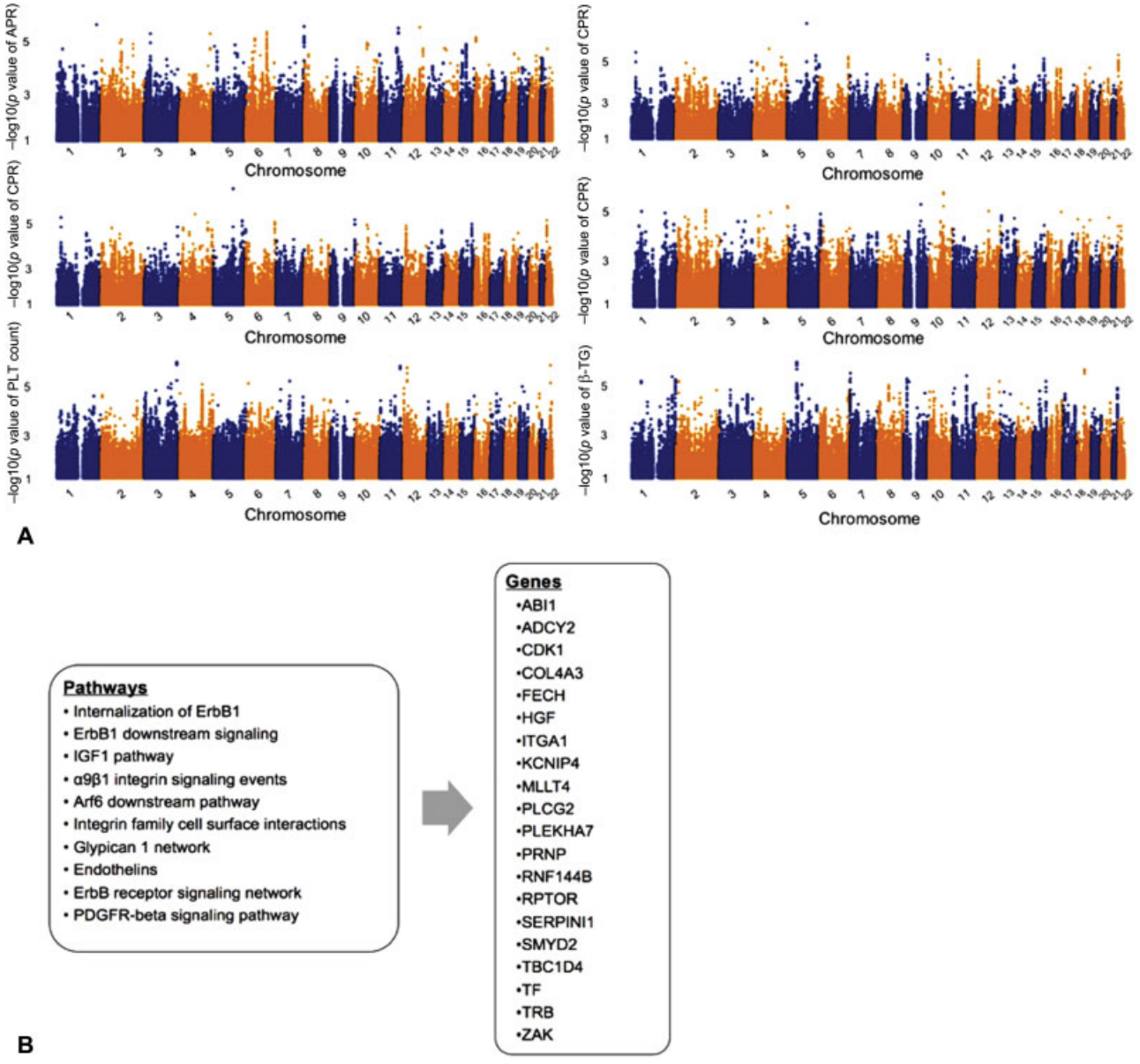

Fig. 4 Genome-wide quantitative trait locus (QTL) mapping for platelet phenotypes. (**A**) Manhattan plots showing the genome-wide QTL mapping results for APR, CRP, AFR, CFR, platelet count and β-TG. The vertical axis in the Manhattan plots indicates (−log10 transformed) observed *p*-values. QTLs are considered as reaching genome-wide significance at the level of $p = 5 \times 10^{-8}$. (**B**) Lists of pathways and genes enriched for platelet QTLs.

Next, we identified all QTLs that were associated with platelets at $p < 1 \times 10^{-4}$ and tested whether they are also GWAS QTLs or their proxies ($r^2 > 0.8$). Platelet QTLs are enriched among SNPs earlier identified in GWASs in blood- and immune-mediated diseases, suggesting the role for platelets in the pathogenesis of those diseases (►**Fig. 7**). In contrast, no enrichment was found for heart-related diseases or infections.

Discussion

In this study, we report a comprehensive assessment on the functional and genomic architecture of platelet number and reactivity, and the relation with inflammation and cytokine production in a healthy human population. We report that IL-1β plasma levels are associated with platelet number and that platelet reactivity is associated with inflammatory markers and IL-1β and IL-6 production capacity. We also found that several crucial signalling routes (e.g. ErbB1, PDGFR-β and IGF-1 pathways) are enriched for platelet SNPs, and that SNPs that influence platelet number and function are enriched in GWASs on haematologic and immune-mediated diseases.

Platelets are long known to play an important role in inflammation, including the onset and progression of atherosclerosis.[5] The results of the CANTOS trial now offer important new evidence for a key role of IL-1β in atherogenesis and cardiovascular events. Our study shows for the first time in a large cohort of healthy, predominantly young adults that platelets and IL-1β are related on different levels: platelets numbers are associated with IL-1β, but not with IL-6 or

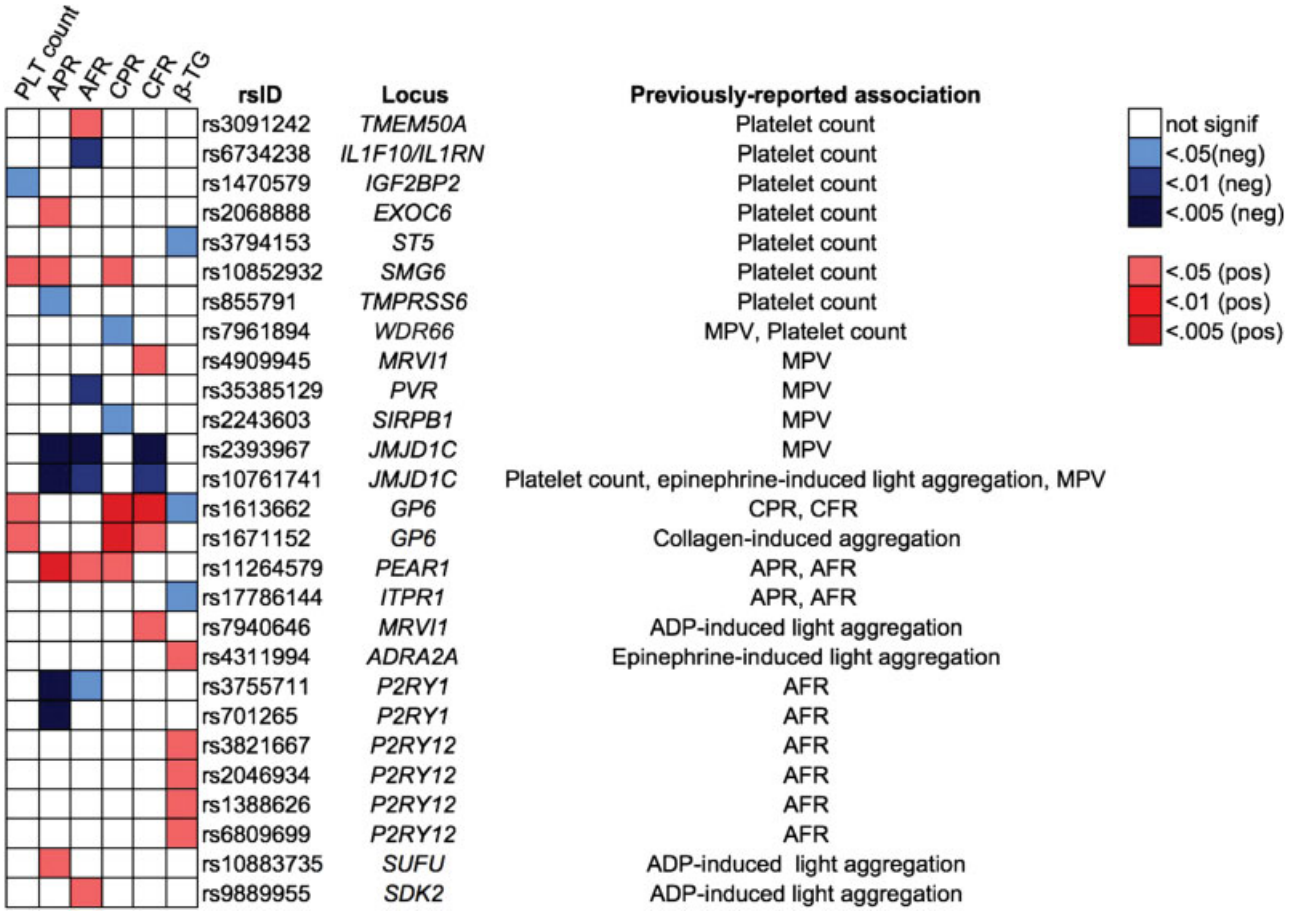

Fig. 5 Associations between known platelet single-nucleotide polymorphisms (SNPs) from the literature and platelet parameters in our cohort. The colour legend indicates the range of *p*-values. *p*-Values were obtained from a linear regression model of platelet parameters on genotype data. APR, ADP-induced P-selectin expression; AFR, ADP-induced platelet-fibrinogen binding; CPR, collagen-induced P-selectin expression; CFR, collagen-induced platelet-fibrinogen binding. APR, AFR, CPR and CFR were data obtained from whole blood flow cytometry. MPV, mean platelet volume.

tumor necrosis factor-α, and platelet degranulation capacity was associated with IL-1β cytokine production capacity of the leukocytes. The latter may especially be important locally in the atherosclerotic plaque. These findings reinforce the notion that platelets are integrated with the function of the immune system,[4,49] and that not only the thrombotic but also the inflammatory function of platelets is important in atherothrombosis. Similarly, they suggest that part of the results of CANTOS may be due to effects of IL-1β inhibition on platelets production, but not on platelet reactivity. The administration of human recombinant IL-1β was previously demonstrated to increase platelet numbers in patients with gastrointestinal cancer[13] and in mice.[14] Interestingly, thrombocytopaenia was significantly more common in patients in the canakinumab groups in the CANTOS trial compared with the placebo group. Our findings in healthy volunteers therefore support that IL-1β is a regulator of platelet number.[50] Platelets were also reported to have the capacity to synthesize and secrete IL-1β, the latter primarily within micro-particles.[8,9,51] Due to their high circulating number (150–400.000/μL blood), platelets may as such be a relevant source for IL-1β. This creates a feedback loop whereby IL-1β induces thrombopoiesis which in turn leads to more platelet-derived IL-1β. The strong inverse correlation of AAT with platelet number can be explained by the fact that AAT inhibits the expression of IL-1β and other pro-inflammatory cytokines.[31]

Platelets themselves do not contain mature IL-1β but are capable of its release through signal-dependent splicing of intra-platelet IL-1β pre-messenger RNA (mRNA). Although measurements of platelet-derived IL-1β were not performed in our 500FG study, evidences were available from several other studies. IL-1β synthesis and inflammasome assembly in platelets are initiated in response to pro-coagulant or pathogen-associated stimuli.[52,53] Signal-dependent splicing of IL-1β pre-mRNA in platelets is regulated by cdc-like Kinase 1 (CLK1)[51,54] and depends on activation by thrombin or other classic agonists in the presence of fibrinogen and requires outside-in signalling from integrin activation.[9,51] CLK1-mediated IL-1β RNA splicing also occurs after Toll-like receptor (TLR)-2- and TLR-4-mediated platelet activation by Pam3Cys or LPS, respectively.[51,54] Inflammasomes are also one source of IL-1β. Furman et al showed that the nucleotide-derived metabolites adenine and N4-acetylcytidine, which were detectable in the blood of older individuals with constitutive expression of IL-1β, primed and activated

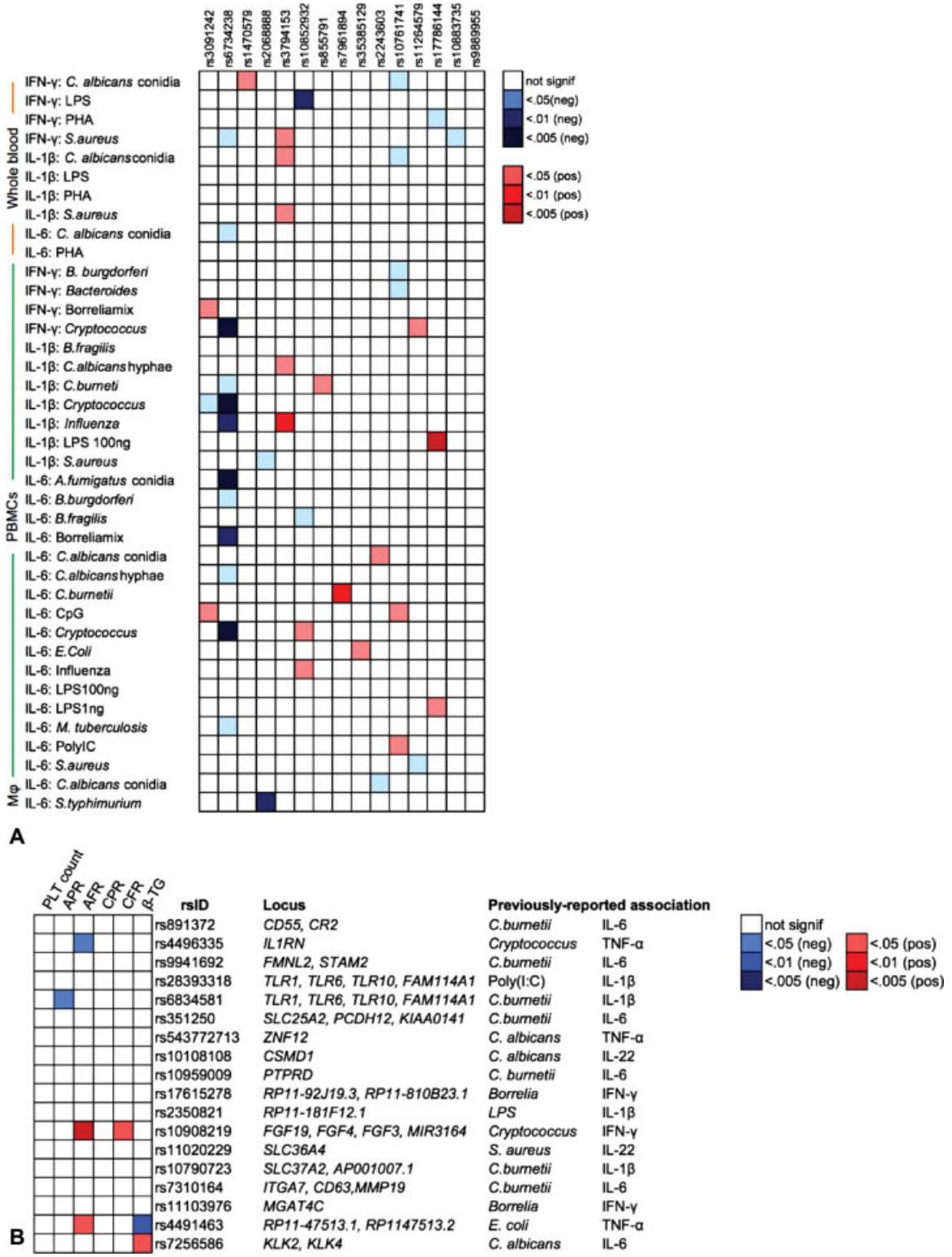

Fig. 6 Single-nucleotide polymorphisms (SNPs) associated with overlapping platelet traits and cytokine production capacity in whole blood and peripheral blood mononuclear cells (PBMCs). (**A**) *p*-Values of the associations of cytokine responses with platelet SNPs; *p*-Values determined from linear regression analysis of cytokine responses on genotype data. (**B**) *p*-Values of the associations of platelet parameters with cytokine SNPs. *p*-Values were from linear regression analysis of platelet parameters on genotype data. The colour legend indicates the range of *p*-Values.

the NOD-like receptor family CARD domain-containing protein 4 inflammasome, induced the production of IL-1β, activated platelets and neutrophils and elevated blood pressure in mice.[55] Additionally, dengue virus infection was reported to lead to the assembly of nucleotide-binding domain leucine-rich repeat containing protein (NLRP3) inflammasomes, activation of caspase-1 and caspase-1-dependent IL-1β secretion. This study also indicated that

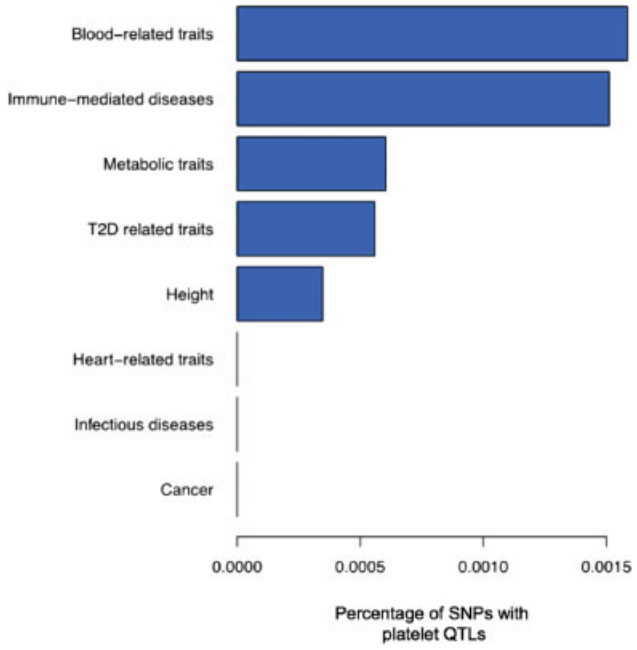

Fig. 7 Platelet quantitative trait locus (QTLs) overlap with single-nucleotide polymorphisms (SNPs) associated with immune-mediated diseases. The percentage of SNPs associated with each category of disease that also qualified as suggestive platelet QTLs.

platelet-derived IL-1β is released in micro-particles through mechanisms dependent on mitochondrial reactive oxygen species-triggered NLRP3 inflammasomes.[8]

The effects of IL-1β on the bone marrow were not restricted to platelets, as plasma IL-1β levels were also strongly associated with leukocyte counts, including neutrophils and monocytes. Similar to thrombocytopaenia, leukopenia was also more common in the canakinumab-treated groups in the CANTOS trial. Leukocytes are also key cells in the aetiology of atherosclerosis and cardiovascular events, and inhibition of IL-1β-mediated leukocyte mobilization from the bone marrow may further explain the results of canakinumab.

Whereas platelet degranulation capacity, as measured by P-selectin reactivity, was positively associated with ex vivo IL-1β and IL-6 production, these cytokines had little effect on platelet reactivity. This largely one-directional relationship whereby more reactive platelets boost IL-1β and IL-6 production is supported by earlier studies that, even though platelets contain a functional IL-1 receptor, IL-1β and LPS act as atypical platelet activators.[53] In contrast, others have found that IL-1β increases platelet aggregation.[50] IFN-γ inhibits IL-1β production,[56] which may be a possible explanation for the opposite associations between platelet reactivity with primarily monocyte-derived (IL-1β and IL-6) and T-lymphocyte-derived (IFN-γ) cytokines. Platelet micro-particles may also impair differentiation of regulatory T-cells into IL-17 and IFN-γ–producing cells, and as such inhibit IFN-γ production.[57] In contrast to the positive associations of platelet degranulation (P-selectin) capacity with cytokine production, we speculate that platelet integrin $\alpha_{IIb}\beta_3$ activity inhibits cytokine responses. The fact that platelets can attenuate cytokine responses to LPS has been reported recently.[58]

Platelet degranulation may be inter-linked with cytokine production in different ways: (1) the ligation of platelet P-selectin with monocyte P-selectin glycoprotein ligand-1 triggers nuclear translocation of nuclear factor-kappa B (NF-κB) and activation of the NF-κB-dependent inflammatory genes in monocytes,[59] as observed in our current and prior experiments using TLR4 ligand[21] (2) platelet phagocytosis by PBMCs may lead to concomitant surface TLR internalization or platelet microRNA-dependent cytokine down-regulation in leukocytes[21,58] (3) platelets contain a large array of granule-stored chemokines and cytokines, and when stimulated, platelets also produce inflammatory molecules de novo[9,51]; and (4) genetic overlap exists in pathways involved in platelet activation and cytokine production, for example, *JMJD1C*. Interestingly, whereas genetic variation in surface platelet receptors (such as P2Y12 and GPVI) had no effect on cytokine production, two QTLs in a locus near *IL1RN* (rs6734238 and rs4496335) were inversely related to ADP-induced fibrinogen reactivity.

The GWAS also enabled us to show enrichment of platelet QTLs in different biologically important pathways, including the ErbB1, PDGFR-β, Arf6,[60] IGF1[61] and integrin β1 receptors pathways. ErbB1 and PDGFR are important regulators of cell proliferation, survival, differentiation and migration.[62,63] The other pathways had been more directly associated with platelets: Arf6 protein was recently reported to control platelet spreading and clot retraction via integrin $\alpha_{IIb}\beta_3$ trafficking[60]; the integrin β1 signalling is required for platelet granule secretion and hemostasis[64]; and IGF-1, released from the α-granules upon platelet activation, potentiates platelet aggregation.[61] Some of these pathways are also important in inflammation and the importance of platelets in these pathways is thus independently confirmed by our study using this unbiased approach. In addition, there was a strong enrichment of platelet QTLs in GWASs identifying SNPs that impact haematologic and immune-mediated disorders. The latter is not surprising given the role of platelets in inflammation and immune-mediated illnesses. For example, platelets have been implicated in the pathogenesis of rheumatoid arthritis.[65,66] There was much less enrichment of platelet QTLs in the SNP dataset identified in GWASs to increase susceptibility and control to a selection of infections. These infections, however, included hepatitis B, hepatitis C, human immunodeficiency virus, smallpox, leprosy and malaria, and platelets are less likely to play a role in the pathophysiology of these specific infections.

Finally, of the non-genetic factors, the limited effect of gender and age on platelet function suggests that variability of platelet function based solely on age or sex is not a major factor in the known difference of platelet-dependent pathologies in these groups. Interestingly, smoking or BMI also did not have a major impact on platelets in these predominantly young, healthy individuals. Although smokers were previously reported to have increased platelet aggregation response, this was only found transiently after smoking[67] and/or in those with underlying coronary artery disease.[68]

Previous studies also did not find any association between obesity and platelet activation.[69]

Our study has different limitations. First, measurements of CRP-XL-induced platelet reactivity were only available in 302 individuals. Second, the associations presented here do not necessarily imply causality, although many of the significant associations, such as platelet number and IL-1β, are supported by data from earlier pre-clinical studies. Finally, whereas the enrolment of young, healthy volunteers without co-morbidities is a particular strength of our studies, it may not reflect the common and important relations between platelets and inflammatory parameters as may be observed in a diseased setting.

In conclusion, we comprehensively describe the pattern of interactions of the number and function of platelets with host factors, genetic variation and inflammation and immune responses. We demonstrate the association between platelets and IL-1β-mediated inflammation as well as circulating leukocyte numbers, thereby adding important novel insights in how IL-1β neutralization may protect against cardiovascular events.

What is known about this topic?

- Platelets are key cells in inflammation and host defence.
- Interleukin (IL)-1β inhibition was recently shown to prevent against recurrent cardiovascular events.

What does this paper add?

- IL-1β plasma levels are strongly associated with platelet number in healthy human individuals, but not with platelet reactivity.
- Platelet reactivity is associated with IL-1β and IL-6 production capacity.
- Several crucial signalling routes in inflammation are enriched for platelet SNPs.

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Conflict of Interest
None.

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